

CHARACTERIZATION OF ANTIVIRAL ACTIVITY OF GREEN TEA EXTRACT AND APPLICATIONS FOR IMPROVING FOOD SAFETY



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Instituto de Agroquímica
y Tecnología de Alimentos

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January 2021

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Programa doctoral:
Ciencias de la alimentación

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INFORMAN

Que el presente trabajo de investigación titulado “**CHARACTERIZATION OF ANTIVIRAL ACTIVITY OF GREEN TEA EXTRACT AND APPLICATIONS FOR IMPROVING FOOD SAFETY**” ha sido realizado bajo nuestra dirección por D^a Irene Falcó Ferrando, licenciada en Ciencia y Tecnología de los Alimentos, para optar al título de Doctora.

En Valencia, enero de 2021

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La graduada en Ciencia y Tecnología de los Alimentos, Dña. Irene Falcó Ferrando, ha realizado bajo su dirección el trabajo que lleva por título “*Characterization of antiviral activity of green tea extract and applications for improving food safety*”. El trabajo ha dado lugar a siete artículos en los que la participación de la doctoranda ha sido decisiva para su elaboración, firmando como primera autora en seis de ellos y compartiendo autoría en otro. Dichas publicaciones no han sido utilizadas en otras tesis y están publicadas con el factor de impacto que se indica a continuación:

1. **Effect of green tea extract on enteric viruses and its application as natural sanitizer.** Food microbiology (2017), 66, 156. Índice de impacto JCR (2017): 4,090 Q1. Food Science & Technology.
2. **Fostering the antiviral activity of green tea extract for sanitizing purposes through controlled storage conditions.** Food Control (2018), 84, 485-492. Índice de impacto JCR (2018): 4,248 Q1. Food Science & Technology.
3. **Effect of epigallocatechin gallate at different pH conditions on enteric viruses.** LWT-Food Science and Technology (2017), 81, 250-257. Índice de impacto JCR (2017): 3,129 Q1. Food Science & Technology.

4. **Antiviral activity of aged green tea extract in model food systems and under gastric conditions.** International Journal of Food Microbiology (2019), 292, 101-106. Índice de impacto JCR (2019): 4,187 Q1. Food Science & Technology.
5. **Green tea extract assisted low-temperature pasteurization to inactivate enteric viruses in juices.** International Journal of Food Microbiology (2020), 334, 108809. Índice de impacto JCR (2020): 4,187 Q1. Food Science & Technology.
6. **On the use of carrageenan matrices for the development of antiviral edible coatings of interest in berries.** Food Hydrocolloids (2019), 92, 74-85. Índice de impacto JCR (2019): 7,053 Q1. Food Science & Technology.
7. **Antiviral activity of alginate-oleic acid based coatings incorporating green tea extract on strawberries and raspberries.** Food Hydrocolloids (2019), 87, 611-618. Índice de impacto JCR (2019): 7,053 Q1. Food Science & Technology.

Autorizan la presentación de la Tesis Doctoral para optar al Grado de Doctor en Ciencias de la Alimentación. Y para que conste a los efectos oportunos,

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Effect of green tea extract on enteric viruses and its application as natural sanitizer

Author: W. Randazzo, I. Falcó, R. Aznar, G. Sánchez

Publication: Food Microbiology

Publisher: Elsevier

Date: September 2017

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Fostering the antiviral activity of green tea extract for sanitizing purposes through controlled storage conditions

Author: Irene Falcó, Walter Randazzo, Laura G. Gómez-Mascaraque, Rosa Aznar, Amparo López-Rubio, Gloria Sánchez

Publication: Food Control

Publisher: Elsevier

Date: February 2018

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

Author: Irene Falcó, Walter Randazzo, Laura Gómez-Mascaraque, Rosa Aznar, Amparo López-Rubio, Gloria Sánchez

Publication: LWT - Food Science and Technology

Publisher: Elsevier

Date: August 2017

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

Author: Irene Falcó, Walter Randazzo, Jesús Rodríguez-Díaz, Roberto Gozalbo-Rovira, Daniel Luque, Rosa Aznar, Gloria Sánchez

Publication: International Journal of Food Microbiology

Publisher: Elsevier

Date: 2 March 2019

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

Author: Irene Falcó, Azahara Díaz-Reolid, Walter Randazzo, Gloria Sánchez

Publication: International Journal of Food Microbiology

Publisher: Elsevier

Date: 2 December 2020

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On the use of carrageenan matrices for the development of antiviral edible coatings of interest in berries

Author: Irene Falcó, Walter Randazzo, Gloria Sánchez, Amparo López-Rubio, María José Fabra

Publication: Food Hydrocolloids

Publisher: Elsevier

Date: July 2019

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Antiviral activity of alginate-oleic acid based coatings incorporating green tea extract on strawberries and raspberries


Author: Irene Falcó, Patricia L. Flores-Meraz, Walter Randazzo, Gloria Sánchez, Amparo López-Rubio, María José Fabra

Publication: Food Hydrocolloids

Publisher: Elsevier

Date: February 2019

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A mi hermano 

AGRADECIMIENTOS

Más de 5 años han pasado desde que aquella estudiante de grado pisó por primera vez el IATA sin saber muy bien con qué y con quien se iba a encontrar, nada le hacía pensar que un golpe de suerte le esperaba tras la puerta del laboratorio 306.

Mi primer agradecimiento es para **Gloria**, mi directora de tesis, por confiar en esa niña recién titulada y que poco sabía de este mundo, por contar conmigo para empezar a formar un equipo desde cero, por toda la formación recibida, la paciencia cuando los ensayos no salían o las células se contaminaban; por delegar en mí, una de las líneas más bonitas de la biología, cultivo celular. Gracias por enseñarme el lado bonito de la ciencia y las personas que forman parte de ella. Pero mis agradecimientos van más allá del ámbito laboral; gracias por todo el cariño, la ayuda y la confianza tanto dentro como fuera del laboratorio, por hacerlo todo tan fácil, por los abrazos y las palabras cargadas de energía en los momentos más necesarios, no sé dónde me llevará el camino de la ciencia, pero ojalá me permita seguir disfrutando y aprendiendo a tu lado durante mucho tiempo.

A todo el **laboratorio 306**. Esta etapa tiene un comienzo y un final junto a **Walter**, gracias por haber llevado la co-dirección de esta tesis, tus consejos y ayuda, por hacer que el GTE cruce fronteras poniendo aún más en valor mi trabajo. A **Alba** y **Enric**, tan diferentes, pero tan complementarios. Alba, contigo he aprendido a no molestar a la gente antes de su segundo café mañanero, a hablar como Ralph Wiggum y a sonreírnos con la mirada siendo actrices por unos segundos, a conocer cada rincón de Scopus y Web of Science, entre otras muchas cosas. Enric, creador de memes

por excelencia y conocedor de los últimos éxitos bailables que no salen de nuestras cabezas; siempre me ha sorprendido que alguien con la mesa tan desordenada tenga las cosas tan claras en su cabeza. Gracias a los dos por igual, por aparecer en el momento que más os necesitaba, por las horas de risas, cotilleos y confidencias, ¡siempre nos quedará Don Pa! A **Agustín**, por todo tu conocimiento burocrático y que de tantas dudas nos ha sacado, pero sobre todo por tu serenidad, tu paciencia y tu amabilidad, eres una suerte para todo el 306. A mis minions, **Azahara**, **Andrea** e **Inés**, sois la pieza que completa el puzle, es un placer veros trabajar juntas.

Más allá de la puerta del 306... **Mamen**, humildad y pasión, ojalá algún día llegue a ser la científica y la persona en la que he visto evolucionar y convertirte. **Robi**, mi casualidad más bonita, mi fuente de paz y tranquilidad, mi primer recurso cuando todo se desmorona, aun estando lejos te tengo cerca. **Lore**, mi punto de conexión con la realidad. Desde aquel día en el que nos vimos en el pasillo del sótano supe que te tendría siempre cerca, nunca podré agradecerte todo lo que has hecho por mí. **Dulce**, energía, sencillez, calidez... me apasiona escuchar todas tus aventuras, tus mil y una historias en las que andas metida. Me alegro por tus logros como si fueran míos, te mereces todo lo bueno que te pase. Algo me dice que nos quedan muchas cosas bonitas por vivir. Por todos los **M&M's**, **kit-kats** y **Kinder buenos**, las horas bajo de la cabina, las papeleras compartidas, las conversaciones eternas, las sonrisas, las risas y alguna que otra lágrima... gracias por hacerlo todo mucho más bonito y divertido.

A Hayden, Valeria, Andrea, Vicen, María José, Amparo, Mert, Alberto, Pilar, las chicas del 114, Emmanuel... a todas y cada una de las personas que de alguna u otra forma van a formar parte de mis recuerdos.

Fuera de este mundo de científicos locos, a **Jessica, Gloria, María A., Lucia, Pola, Gomis, Alba, Borrull, Elena, Folch** y **Elia**, aún recuerdo cuando con 17 años nos dijeron que disfrutáramos de las amistades del colegio porque todo eso se perdería en la siguiente etapa... 15 años después seguimos juntas. Gracias por todos los momentos de desconexión que tanto he necesitado. El tiempo con vosotras es un regalo.

A **Chris**, quien comparte mi día a día. Gracias por estar siempre a mi lado, apoyándome y dándome alas para crecer. No sabemos qué bueno y qué malo nos tiene la vida preparado, lo que sí sabemos es que lo afrontaremos de la mano. *I wolf you.*

A **Kala**, mi compañía incondicional. Ojalá fueras eterna.

A quienes jamás encontraré la forma perfecta de agradecer, a mis **padres**, sin ellos ninguna de estas líneas estaría escrita. Gracias por darme absolutamente todo, por creer en mí y hacer que me convierta en la mujer que hoy en día soy. Mis esfuerzos y logros son también los vuestros. Si he llegado hasta este punto es gracias a vosotros. Lo habéis hecho y lo estáis haciendo perfecto.

Por último, agradecer a mi persona favorita, al protagonista indiscutible de mis días, a mi **hermano**, mi ejemplo de lucha y superación constante. Gracias por mostrarme el valor de la vida. Seguiremos siempre juntos. Esta tesis es por y para ti.

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ABBREVIATIONS

AGE	Acute gastroenteritis
APP	Atmospheric pressure plasma
CPE	Cytopathic effect
DMEM	Dulbecco's Modified Eagle's Medium
ECDC	European centre for disease control and prevention
ECG	Epicatechin gallate
EFSA	European Food Safety Authority
EGCG	Epigallocatechin gallate
EtOH	Ethanol
FBS	Fetal bovine serum
FCS	Fetal calf serum
FCV	Feline Calicivirus
FDA	Food and Drug administration
FFD	Film-forming dispersions
GRAS	Generally recognised as safe
GSE	Grape seed extract
GTE	Green tea extract
HAdV	Human Adenovirus
HAsV	Human Astrovirus
HAV	Hepatitis A virus
HBGAs	Histo-blood group antigens

HEV	Hepatitis E virus
ISC	In situ capture
Log	Logarithm
MAP	Modified atmosphere packaging
MBs	Magnetic Beads
MNV	Murine Norovirus
NTD	No decreasing titers
ON	Overnight
PAA	Peroxyacetic acid
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
PGM	Porcine gastric mucine
qPCR	Real time-PCR
RH	Relative humidity
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
RT-PCR	Reverse transcription-PCR
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SSF	Simulated salivary fluid

TCID ₅₀	50% Tissue culture infectious dose
TEM	Transmission electron microscopy
TV	Tulane virus
UDL	Undetectable limit
US	United States
UV	Ultraviolet light
VLP	Virus-like particle
WHO	World Health Organization
WWTP	waste water treatment plant

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SUMMARY

Nowadays, foodborne viral outbreaks are a growing concern for food safety authorities. A total of 448 foodborne outbreaks caused by enteric viruses were reported by the European Food Safety Authority (EFSA) in 2019. A wide variety of viruses may be transmitted by food, nevertheless, the most relevant reported viruses are human noroviruses, hepatitis A virus (HAV) and more recently hepatitis E virus (HEV). These viruses are some of the main health risks associated with food consumption having a high impact on food safety and being responsible for diverse pathologies in consumers (from moderate gastroenteritis to more serious pathologies such as hepatitis, meningitis or encephalitis). In particular human noroviruses cause gastroenteritis, supposing the leading causes of foodborne illnesses in industrialized countries. Based only on clinical manifestations, it is not easy to differentiate if a pathogenic bacterium or a virus is the causative agent of the gastroenteritis, hence, microbiological laboratory tests are needed to identify them.

Moreover, HAV is considered as a re-emerging pathogen and is responsible for about half the total number of human hepatitis infections diagnosed worldwide. In the case of HEV, the first confirmed cases were reported in 1983,

it should be noted that it was not until 2018 when the EFSA declared HEV as an emerging virus detailing the health risks associated with the virus.

Generally, the fecal-oral-route is the primary way of transmission of human enteric viruses, and food-handlers, equipment and contaminated surfaces (fomites) could represent a reservoir of cross-contamination viral particles which become sources of secondary transmission, especially in food related environments. Presence of human enteric viruses can occur in food which has been directly contaminated with faecal material or contaminated water. The main foodstuffs involved in foodborne infections are mollusc bivalves, vegetables and salads, berries, and ready-to-eat food which have been contaminated during their production or along the supply chain by improper handling after their preparation or cooking. Due to their low infectious dose, estimated to be 10-100 viral particles, and to their high stability and resistance to food manufacturing processes, the development of alternative strategies for the viral decontamination of food has been recently promoted by public authorities. Also as a consequence of the increasing number of foodborne outbreaks, international organisms, such as the World Health Organization (WHO) or the EFSA, propose studies on the efficacy of food manufacturing processes for virus inactivation.

To this end, phytochemical compounds (especially plant extracts) are gaining an increasing interest among consumers, researchers and food industries mainly because (i) they are suitable for food applications since most of them are Generally Recognized as Safe (GRAS); (ii) they represent natural and cheap alternatives to chemically synthesized antimicrobials; (iii) the growing “green consumerism” trend stimulates the use of natural products and ingredients. Even if the antibacterial and antifungal activities of many natural compounds have been extensively investigated, data on their antiviral food applications are somewhat limited.

Beyond the high acceptance, plant extracts or their derivatives represent an economically sustainable source of potential antiviral compounds due to the abundance of raw materials and the low cost of their production. So far, many natural compounds of plant origin and some animal-derived compounds (i.e. chitosan) have been screened against human enteric virus mainly by using *in vitro* cell-culture assays while only few studies assessed their antiviral activity on food application i.e. grape seed extract (GSE) and carvacrol. Overall, results showed inactivation rates varying at different extent depending on the experimental set up since factors such as the extract chemical composition, relative concentrations of the active compounds, the virus strain tested, the food matrix, temperature, humidity and contact-time deeply affect the final

findings making difficult direct comparisons. Moreover, results showed that proteins and fat decreased the efficacy of bioactives in food by protecting viral particles from their action.

To this end, the present doctoral thesis has focused on the effect of green tea extract (GTE) against human enteric viruses, and their surrogates, and its potential application in food products or food contact surfaces to enhance food safety.

GTE is a derivative of cultivated evergreen tea plant (*Camellia sinensis* L.) of the family *Theaceae*. GTE, rich in polyphenolic and proanthocyanidin compounds, is a popular plant extract that has been used for its health benefits including anti-inflammatory, antioxidant, and anticarcinogenic properties. GTE also demonstrated inhibitory properties against a wide range of foodborne pathogens, and therefore it has been widely used as an ingredient in multiple hurdle approaches to enhance food quality and safety considering that it has a GRAS status approved by Food and Drug Administration (FDA).

The low infectious dose of most human enteric viruses, together with their high stability in the environment, make them extremely infectious and highly transmissible. As non-enveloped viruses, human enteric viruses tend to be more resistant than foodborne bacteria to inactivation caused by common food

manufacturing processes. Overall, mild food manufacturing processes show only marginal effects on the viral concentration, but when processes are combined, the synergistic effects may enhance the level of human enteric virus inactivation. Therefore, the combination of the effect of natural compounds, as GTE, together with food manufacturing processes could be an interesting future approach for the inactivation of human enteric viruses finally reducing the risk of infection to consumer.

In this regard, antiviral edible coatings are a promising area to explore due to the risk of disease transmission by food contaminated with enteric viruses. Furthermore, edible coatings with antiviral properties could provide an added value, as they could be specifically designed to inactivate viruses, which are normally more resistant than bacteria. For instance, they could play an important role in raw and minimally processed fruits and vegetables which are typically sold to the consumer in a ready-to-eat form and, thus, they do not usually contain preservatives or antimicrobial substances and they are not normally heat processed prior to consumption.

At first, the antiviral activity and characterization of GTE was evaluated on murine norovirus (MNV), a cultivable human norovirus surrogate, and HAV at different temperatures, exposure times and pH conditions. Initially, GTE at 0.5

or 5 mg/ml were mixed with virus suspension and incubated 2 h at 37 °C at different pHs ranging from 5.5 to 8.5. Treated and un-treated viral suspensions were titrated by cell culture assays. Results showed that GTE affected both viruses depending on pH with higher reductions observed at neutral and alkaline conditions. Secondly, different concentrations of GTE (0.5 and 5 mg/ml) were mixed with viral suspensions and incubated for 2 or 16 h at 4, 25 and 37 °C at pH 7.2. A concentration-, temperature- and exposure time-dependent response was showed by GTE in suspension tests, in which complete inactivation was achieved after overnight (ON) exposure at 37 °C for both viruses and also at 25 °C for HAV. In following assays, the aim was to evaluate the antiviral activity of GTE at 25 °C as a function of pH and storage time and to correlate it with changes in its chemical composition as a consequence of degradation and epimerization reactions during its storage at different conditions (process referred as ageing). The obtained results confirmed that freshly prepared GTE was very effective in inactivating MNV and HAV at neutral and alkaline pH but was ineffective at pH 5.5 when experiments were performed at 25 °C. Additionally, the storage of the GTE solutions for 24 h (aged-GTE) at various pH conditions significantly increased its antiviral activity. HPLC/MS analyses demonstrated that the enhanced antiviral activity was related to changes in the chemical composition resulting from the formation of catechin derivatives during

storage. Among other, epigallocatechin gallate (EGCG) accounted for about 40-50% of GTE composition.

Furthermore, human norovirus inactivation by GTE was indirectly assessed by testing aged-GTE on virus-like particles (VLPs) and on fecal norovirus suspensions. VLPs are morphologically and antigenically similar to the native infectious viruses and have been previously used to determine the antiviral activity of other natural compounds. Norovirus VLPs were treated with aged-GTE and analyzed by a porcine gastric mucine (PGM)-ELISA binding assay and by transmission electron microscopy (TEM), and besides, human norovirus suspensions by an *in situ capture*-RT-qPCR method. Results displayed a significant reduction close to 50% in the viral binding capacity to PGM. TEM showed that treatment with aged-GTE at 0.5 mg/ml dramatically decreased the number of VLPs while aged-GTE at 5 mg/ml completely degraded VLPs. Further experiments were performed with fecal norovirus suspensions by an *in situ capture*-RT-qPCR method. Results demonstrated that aged-GTE at 0.5 mg/ml reduced the binding of human norovirus to PGM by approximately 65%, while aged-GTE at 5 mg/ml and heating completely prevented norovirus suspension binding.

With the aim of getting more information about potential virus infectivity, a viability-RT-qPCR procedure was applied on fecal human norovirus suspensions and HAV. Briefly, EGCG-treated virus and untreated virus were added to PMAxx and incubated in the dark for 10 min at 150 rpm. Thereafter, samples were photoactivated for 15 min. Then, viral RNA was extracted and analyzed by RT-qPCR. EGCG at 2.5 mg/ml only affected the infectious HAV titer, with infectivity reduced below the detection limit. Despite this high effect on HAV infectivity, no effects were observed in any of the genome copy numbers calculated by RT-qPCR alone or PMAxx-RT-qPCR. These results suggested that viral capsids suffer slight alterations that render them non-infectious without much affecting capsid integrity through which PMAxx can get inside.

With the aim to apply GTE to enhance food safety, the potential application of GTE was evaluated in different scenarios. 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. Thus, the stability of GTE was evaluated in simulated gastric conditions (salivary, gastric and intestinal) to shed light on its capacity as a therapeutical antiviral compound to fight human enteric viruses inside the organism. Initially, simulated salivary fluid (SSF; pH 7.0), simulated gastric fluid (SGF; pH 3.0) and simulated intestinal fluid (SIF;

pH 7.0) were prepared and added with 10 mg/ml of GTE. Stocks of MNV and HAV with titers ca. 5 log TCID₅₀/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 at 37 °C during 2 min for SSF and 2 h for SGF and SIF. Treatments were neutralized by adding DMEM containing 10% FCS. Results indicated that 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 °C 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 °C, 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 °C, 2 h). These results are consistent with the inactivation rates previously reported for grape seed extract (GSE) and blueberry proanthocyanidins (PAC-B) in SIF. GSE prepared in SIF reduced MNV and HAV titers by 1.7 and 1.4 log, respectively, while PAC-B prepared in SIF reduced MNV infectivity to undetectable levels. 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.

Moreover, aged-GTE was incorporated into different beverages (i.e. orange and apple juice, horchata and milk). As described before, MNV and HAV suspensions (ca. 4 log TCID₅₀/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. Samples were incubated at 37 °C ON at concentrations of 2.5 and 5 mg/ml of aged-GTE.

Several factors could be responsible for the observed decrease in the antiviral efficacy of aged-GTE in beverages, such as the interaction of the active compounds or the viruses with food matrices or their component, especially protein, fat or sugar. 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. 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 HAV by 1.7 log. When aged-GTE was prepared in milk, its effectiveness significantly decreased, with only 1 log reduction of HAV and MNV infectivity. These results are in agreement with previous studies, where B-PAC and GSE retained their antiviral activity in apple juice, although their antiviral effect decreased in milk. For instance, aged- GTE (5 mg/ml) prepared in milk

reduced MNV titers by 1.0 log after 24 h at 37 °C, while B-PAC at the same experimental conditions (5 mg/ml, 24 h, 37 °C) decreased MNV titers by 0.8 log. For HAV, aged-GTE at 5 mg/ml reduced HAV titers by 1.2 log in milk while similar inactivation rates (0.8 log) were reported for GSE at 4 mg/ml tested under the same experimental conditions. Although horchata contains 2.2% fat, 5 mg/ml aged-GTE in horchata reduced MNV and HAV infectivity by ca. 2 log, resulting in a potential carrier of natural antivirals.

Epidemiological investigations suggest that unpasteurized and inadequate heat-treated juices, contaminated fruits, contaminated water and environmental contamination are the responsible of foodborne outbreaks along with person to person transmission. Following the principles of hurdle technology, next assays tested a multiple simultaneous preservation approach based on the use of aged-GTE and mild heat treatments to assess the inactivation kinetics of MNV and HAV in artificially contaminated mix fruit juices (J1 - containing strawberry, carrot, beetroot and apple at pH 4.10, and J2 - apple juice, pH 3.75)

By combining mild heat treatments at 40, 50 or 63 °C with aged-GTE (3.3 mg/ml), MNV titers were lower than those resulting from the thermal treatment alone. No synergistic effect was observed when aged-GTE was added to juices

and treated at 40 °C for 30 min indicating a protective effect of juice compounds. As commented before, similar protective effects have been observed when aged-GTE was prepared in milk. However, a statistically significant synergistic effect was observed when aged-GTE (3.3 mg/ml) and heat treatments were combined, resulting in undetectable infectious MNV particles for treatments at 50 and 63 °C for J2 and at 63 °C for J1.

In line with the results obtained by applying aged-GTE at 5 mg/ml, no significant differences were observed when HAV in PBS was treated with aged-GTE (3.3 mg/ml) and combined with mild-heat treatments. 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 TCID₅₀/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 HAV infectivity by 1.37, 0.62, and 1.44 log TCID₅₀/ml, 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.

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 than the thermal treatment applied alone.

Subsequent assays were on track to elucidate the antiviral behaviour of GTE as natural sanitizers for food contact surfaces and washing solution for vegetables. To date, different non-porous (aluminium, china, glazed tile, glass, latex, plastic, polystyrene and stainless steel) and porous (cloth, different types of papers and cotton cloth) surfaces have been indicated as suitable for harbouring enteric viruses. In the framework of this thesis, GTE was further evaluated as food-contact surface sanitizer based on ISO 13697:2001 standard. Since 2019, a specific standard for viruses has been issued: the European Standard 16777:2019 that describes a test method and the minimum requirements to assess the virucidal activity of chemical disinfectants that form a homogeneous physically stable preparation when diluted in hard water or in water, in case of ready to use products. This European Standard applies in areas and situations where disinfection is indicated to preserve human health.

Briefly, MNV and HAV stocks were diluted 1:1 with the interfering substance, 0.3 and 3 g/l bovine serum albumin (BSA) in PBS pH 7.2, for clean and dirty working conditions, respectively. Then 50 µl of resulting inoculum (5-6 log TCID₅₀/ml) were spotted into the middle of a clean and disinfected stainless

steel and glass discs and dried at room temperature for about 15 min, and verified by visual inspection. Afterward, 100 µl of two different concentrations of GTE and aged-GTE (5 and 10 mg/ml) prepared on hard water were spotted on the inoculated discs, followed by incubation at RT for 15 or 30 min for GTE and 5 and 15 min for aged-GTE. Then, the effect of GTE was stopped with a neutralizer (DMEM supplemented with 10% FCS) and the viruses were recovered and titrated by cell culture assays.

Results on HAV reported less than 1 log reduction when freshly GTE was applied for 15 min. On the contrary, GTE, when applied in dirty conditions, was more effective against HAV than MNV. Moreover, a complete inactivation (below the recovery limit) of HAV was achieved after 30 min of contact with 10 mg/ml of GTE for both materials tested in both cleanness conditions. In dirty conditions, GTE at 10 mg/ml for 30 min reduced MNV infectivity by 2.09 and 1.64 log in stainless steel and glass discs, respectively, while after 15 min of contact time, a marginal reduction (0.42 and 0.33 log, stainless steel and glass discs) was obtained. Moreover, no remarkable differences were reported between virus inactivation in stainless steel or glass surfaces.

When aged-GTE at 5 mg/ml was applied in clean conditions on stainless steel surfaces, infectious titers of MNV decreased by 0.75 and 1.75 log after 5 and

15 min of exposure, respectively, while for HAV, reductions of 0.79 and 1.92 log were recorded. On glass surfaces, no significant reduction in MNV and HAV infectivity was observed after 5 min treatment, while aged-GTE reduced MNV titers by 1.27 log and HAV titers by 1.54 log after 15 min treatment. The results of the surfaces tests proved the efficacy of GTE on food contact surfaces showing better performance compared to others natural compounds tested as sanitizers, such as GSE. In fact, others studies reported marginal reduction (<1 log PFU/ml) for MNV in stainless steel surfaces treated with 2 mg/ml of GSE for 10 min in clean conditions. Our results showed a significant decrease (>1.5 log TCID₅₀/ml) of MNV titers on stainless steel and glass surfaces treated with 10 mg/ml of GTE for 30 min while complete inactivation was reported for HAV in glass and stainless steel surfaces either in clean and dirty conditions. Usually, reduced antiviral effects have been reported when a compound was tested in dirty conditions mainly due to its binding and masking effects of the organic load (such as protein) interfering with the effectiveness of treatment. Results are partially in accordance with those masking effects, since MNV showed slightly greater reductions when applied on clean surfaces than on dirty ones. On the contrary, HAV reduction was not affected by the presence of protein in the surface tests.

Comparing studies on the application of GTE and aged-GTE, an increment of the antiviral activity was observed when aged-GTE used. GTE solution prepared 24 h before its use incurs to degradation and epimerization of its compounds, thus by modulating its storage conditions (ageing), the antiviral activity of this natural compound can be fostered as demonstrated not only *in vitro*, but also in natural disinfectant formulations for environmental surfaces.

In the fresh-cut vegetable industry, leafy greens present a high risk of cross-contamination, and chlorine, the most common disinfectant, has been already limited in some European countries due to the formation of chemical by-products. Therefore, following such rising need for alternative disinfection solutions, in the framework of this thesis, GTE was assayed as sanitation treatment for fresh lettuce and spinach leaves.

Determination of the virucidal activity of freshly prepared GTE washing solution for produce was performed by adapting procedures previously described. Briefly, locally purchased fresh lettuce (*Lactuca sativa* L.) and spinach (*Spinacia oleracea*) were cut in pieces and decontaminated with UV light in a biosafety cabinet under laminar flow for 15 min prior to virus inoculation. Then, viral suspensions at two concentration levels (approx. 5 and 6 log TCID₅₀/ml) of MNV or HAV were seeded separately by distributing 50 µl over spots onto the

vegetable surface. Inoculated samples were air dried in a laminar flow hood for about 15 min. Thereafter, 100 µl of PBS or a GTE solution at 5 or 10 mg/ml was added for 15 or 30 min to inoculated vegetable samples. The action of GTE was stopped with a neutralizer (DMEM supplemented with 10% FCS) and the viruses were recovered and titrated. Results showed a significant reduction of MNV infectivity (1.38 and 1.80, respectively) on lettuce and spinach exposed to GTE at 10 mg/ml for 30 min when the virus was inoculated at low titer. Lower reductions were obtained with GTE treatment at 5 mg/ml. Treatments with GTE at 10 mg/ml showed 2.59 log reduction in spinach inoculated with higher viral titer. Regarding HAV, reductions below detectable limits were obtained for lettuce and spinach inoculated with low virus titers and treated with GTE at 10 mg/ml for 30 min. Whereas, high viral titers were reduced by 0.79 and 1.37 in lettuce and spinach, respectively.

Assessment of natural compounds applications in vegetable sanitation is scarce, so far only carvacrol and GSE have been evaluated as natural sanitizers against enteric virus contamination. Treating lettuce with carvacrol at 1% for 30 min reduced MNV titers by 1.8 log. Similarly, reported a marginal reduction (<0.8 log) for MNV and 1.23 and 1.29 log reductions for HAV, respectively on lettuce and pepper, after 1 min treatment with 1 mg/ml of GSE.

There is an increasing interest in the application of edible coatings and films in the food industry, because of the increasing consumer demand for safe, stable foods, and the consciousness of the undesirable environmental effects of non-biodegradable packaging. Edible coatings are particularly interesting in food preservation since they have demonstrated the ability of improving food quality and safety by controlling water vapour, gases and aroma transfer in food systems.

Incorporation of antimicrobials has also become popular to prepare active films and coatings since the growth of spoilage or pathogenic microorganisms is one of the major problems affecting food quality and safety. Extensive research has been conducted to include natural antimicrobials in the film or coating such as essential oils, bacteriocins and phenolic compounds. However, although their bactericide and fungicide properties have been broadly investigated, little information is available in the literature about how biopolymers could act as carriers of antiviral compounds and how they interact with edible film or coating's components.

Therefore, the last sets of experiments of this thesis related to GTE were its incorporation into edible films and coatings with the goal of controlling virus

contamination in berries. Initially, edible films were prepared by adding GTE into alginate films. For this, pieces of each edible film (25 ± 5 mg) were sterilized with UV light in a biosafety cabinet under laminar flow for 15 min and then mixed with 500 μ l of MNV and HAV suspensions diluted in PBS pH7.2 (ca. 5 logs TCID₅₀/ml). The samples were incubated ON at 37 °C in a shaker (180 rpm). Then, the effect of the active compound was neutralized with DMEM supplemented with 10% FCS. Results showed that alginate films containing 0.75 g GTE extract/g alginate decreased MNV titers by 1.92 log TCID₅₀/ml. Films containing 0.50 g extract/g alginate, reduced MNV titers by 2.00 log TCID₅₀/ml. HAV titers decreased by 1.92 log when treated with 0.75 g GTE extract/g alginate, while 1.25 log reduction were recorded at lower GTE concentrations. The obtained results indicated that active films exert lower antiviral activity than the pure extract, suggesting that a fraction of the GTE interact with film's components, preventing a complete release of the active compounds.

Different edible coatings based on different carrageenans and incorporating GTE were also developed as an innovative strategy to guarantee the food safety of blueberries and raspberries. Edible coatings were applied to raspberries and blueberries, and the antiviral activity tested against MNV and HAV at refrigerated (10 °C) and ambient conditions (25 °C). Blueberries and raspberries were exposed to UV for 15 min on a sterile plate in a laminar flow hood to

reduce the microbial load. Then 50 µl of MNV and HAV suspensions were inoculated on the berry surfaces and let dry for 1 h at room temperature under a laminar flow hood. Then each berry was immersed for 2 min into carrageenan-based coating, after drying, samples were incubated ON at 10 and 25 °C. Samples incubated at 10 °C were also stored for 4 days. On each sampling day, individual berry samples were placed in a tube containing 5 ml of DMEM supplemented with 10% FBS and shaken for 2 min at 180 rpm to release viral particles from the surface. Berries were removed from the DMEM suspension, and then viruses were recovered and titrated. Control samples were coated berries without GTE.

In general, the effect of the type of the carrageenan on MNV and HAV infectivity in coated raspberries and blueberries was higher at 25 °C although a similar trend was also observed at lower temperatures. As observed, MNV infectivity in coated fresh blueberries stored ON at 25 °C was reduced under the detection limit for two carrageenan films containing GTE. Lower reductions were reported in coated raspberries where MNV titers were significantly ($p < 0.05$) reduced. This effect can be ascribed to the different roughness of the fruit surface, which can affect the recovery of viruses during the assay.

There was noticeable less reduction of HAV titers in coated berries. For instance, HAV titers in blueberries were reduced by nearly 3 log after ON incubation at 25 °C for carrageenan coatings containing GTE. At refrigerated temperatures, higher efficacy of coatings was observed in raspberries than blueberries.

Overall, the results obtained in this thesis support the conclusion that GTE is a natural and inexpensive option to improve viral food safety in different types of applications.

RESUMEN

En la actualidad los brotes causados por virus de transmisión alimentaria, o virus entéricos, están siendo foco de interés por parte de las autoridades sanitarias debido a su aumento. A modo de ejemplo, la Autoridad Europea de Seguridad Alimentaria (EFSA) declaró un total de 448 brotes causados por virus entéricos en el año 2019. Se han descrito una gran variedad de virus que pueden ser transmitidos a través del consumo de alimentos contaminados, sin embargo, los más relevantes son los norovirus humanos, el virus de la hepatitis A (VHA) y más recientemente el virus de la hepatitis E (VHE). Estos virus suponen uno de los principales riesgos asociados al consumo de alimentos teniendo un alto impacto en la seguridad alimentaria y siendo responsables de diversas patologías en los consumidores (desde gastroenteritis leves hasta patologías más serias como pueden ser hepatitis, meningitis o encefalitis). En concreto, los norovirus humanos son causantes de gastroenteritis, siendo responsables etiológicos de la mayoría de brotes de transmisión alimentaria en países industrializados. En base a las manifestaciones clínicas, no es fácil diferenciar si la causa de una gastroenteritis proviene de un patógeno bacteriano o vírico, por lo que son necesarios ensayos microbiológicos para su identificación.

Además, el VHA está considerado como un patógeno reemergente responsable de la mitad de casos de hepatitis diagnosticadas a nivel mundial. En el caso del VHE, el primer caso confirmado fue notificado en 1983 pero no fue hasta 2018 cuando la EFSA lo declaró como virus emergente remarcando su riesgo para la salud.

Los virus entéricos son transmitidos principalmente por la vía fecal-oral y, por tanto, pueden estar potencialmente presentes en alimentos que hayan sufrido contaminación directa con materia fecal, o a través de manipuladores de alimentos, aguas contaminadas o superficies (fómites) de contacto alimentario que pueden representar un reservorio de contaminación cruzada de partículas virales, convirtiéndose en fuente secundaria de transmisión, especialmente en ambientes relacionados con la alimentación. Los principales alimentos involucrados con brotes de infecciones víricas transmitidas por alimentos son moluscos bivalvos, vegetales y ensaladas, frutos tipo baya y alimentos preparados y listos para el consumo, debido a una falta de higiene durante la preparación o manipulación de este tipo de alimentos. Debido a la dosis infecciosa tan baja que presentan, alrededor de 10-100 partículas víricas, y su gran estabilidad y resistencia a los tratamientos de conservación utilizados por la industria alimentaria, el desarrollo de estrategias alternativas para la descontaminación de virus en alimentos está ganando importancia por parte de

las autoridades. También, como consecuencia del incremento de brotes por el consumo de alimentos, organismos internacionales como la Organización Mundial de la Salud (OMS) o la EFSA, proponen evaluar la eficacia de los procesos de conservación para el control de virus de transmisión alimentaria.

Para garantizar la calidad de los alimentos, alargar su vida útil y dar respuesta a una demanda cada vez mayor de productos más naturales, la industria alimentaria busca alternativas al uso de aditivos químicos. Con este fin los compuestos fitoquímicos (especialmente los extractos de plantas) están viendo incrementado su interés por parte tanto de consumidores como de investigadores e industrias alimentarias especialmente porque (i) son aptos para su aplicación en alimentos puesto que se consideran productos generalmente reconocidos como seguros (GRAS, del inglés Generally Recognized As Safe); (ii) representan una alternativa natural y barata frente a los antimicrobianos químicos sintetizados; (iii) y, debido a un aumento en la tendencia del "consume verde", el uso de estos productos naturales e ingredientes se ha visto incrementado. Aunque la actividad bacteriana y antifúngica de muchos compuestos naturales se ha investigado ampliamente, el estudio sobre su capacidad antiviral en aplicaciones alimentarias es escasa.

Más allá de su alta aceptación por parte de los consumidores, los extractos de plantas o sus derivados representan una alternativa natural y barata frente a los aditivos químicos para su uso como compuestos naturales virucidas.

Así pues, muchos compuestos naturales originales de plantas o derivados de compuestos animales (por ejemplo, el quitosano) se han estudiado frente a virus entéricos humanos usando ensayos *in vitro* de cultivo celular, aunque sólo un número limitado de ellos se han estudiado en aplicaciones alimentarias, un ejemplo es el extracto de semilla de uva (GSE, del inglés grape seed extract) y el carvacrol. En general, los resultados con estos compuestos muestran diferentes niveles de inactivación en función de las condiciones experimentales aplicadas, tales como la composición del extracto, las concentraciones relativas de los compuestos activos, la cepa de virus ensayada, la matriz alimentaria, temperatura, humedad y tiempo de contacto, haciendo difícil la comparación de resultados de diferentes estudios. Por otro lado, se ha demostrado que la presencia de proteínas o grasas disminuye la eficacia de los compuestos bioactivos en los alimentos, protegiendo así a las partículas víricas frente a su acción.

Por todo ello, esta tesis doctoral se ha centrado en el estudio del efecto del extracto de té verde (GTE, del inglés green tea extract) frente a virus entéricos

humanos, así como frente modelos cultivables, y su potencial uso en aplicaciones alimentarias con la finalidad de mejorar la seguridad alimentaria.

El GTE es un derivado de la planta de té (*Camellia sinensis L.*) perteneciente a la familia de las teáceas (*Theaceae*). El GTE, rico en polifenoles y proantocianidinas, es un extracto que popularmente se ha utilizado por sus beneficios en la salud, atribuyéndole entre otras propiedades, las de antiinflamatorio, antioxidante y anticancerígeno. También ha sido demostrada su capacidad de inhibición frente a una gran variedad de patógenos alimentarios y por tanto ha sido utilizado como ingrediente para mejorar de la calidad y seguridad alimentaria teniendo en cuenta su clasificación como compuesto GRAS por parte de la administración de alimentos y medicamentos (FDA).

La baja dosis infectiva de la mayoría de los virus entéricos, junto con su alta estabilidad ambiental, hace que sean extremadamente infecciosos y con una alta tasa de transmisibilidad. Debido a que se tratan de virus sin envuelta lipídica, los virus entéricos, en general, resultan ser más resistentes que las bacterias a los procesos de conservación habitualmente aplicados por parte de la industria alimentaria. En general, procesos no muy agresivos muestran leves efectos en la carga vírica pero cuando estos se combinan entre ellos, los efectos sinérgicos parecen mejorar la inactivación de los virus entéricos. Por lo tanto,

la combinación de compuestos naturales como el GTE, junto con tratamientos de conservación tradicionales, puede suponer una medida interesante de aplicación para conseguir reducciones en la concentración de virus infecciosos en alimentos con la finalidad de minimizar el riesgo de infección en los consumidores sin verse perjudicadas las propiedades organolépticas del producto.

Desde este punto de vista, los recubrimientos antivirales comestibles son un área interesante de investigación ya que pueden reducir la contaminación viral en alimentos. Además, los recubrimientos con propiedades antivirales pueden tener un valor añadido debido a que pueden ser específicamente diseñados para la inactivación de virus, de hecho, juegan un papel fundamental en frutas y verduras crudas o mínimamente procesadas, ya que se suelen vender listas para su consumo sin la adición de conservantes o sustancias antimicrobianas y sin haber pasado por un proceso térmico antes de su consumo.

En el marco de esta tesis, inicialmente, se caracterizó y se evaluó la capacidad antiviral del GTE frente al norovirus murino (MNV), un modelo cultivable de norovirus humanos, y al VHA a diferentes temperaturas, tiempos de exposición y condiciones de pH. Primero, el GTE a concentraciones de 0,5 y 5 mg/ml se mezclaron con las suspensiones de virus y se incubaron durante 2

h a 37 °C en rangos de pH de 5,5 a 8,5. Tanto las suspensiones víricas tratadas como las sin tratar (controles) se cuantificaron mediante ensayos de cultivo celular. Los resultados mostraron que el GTE afectó la infectividad de ambos virus en función del pH, observándose mayores reducciones a pH neutros y alcalinos. Seguidamente, el GTE a las mismas concentraciones mezcladas con las suspensiones de virus se incubaron durante 2 y 16 h (ON, del inglés overnight) a 4, 25 y 37 °C a pH 7,2, observándose actividad antiviral en función de la concentración, temperatura y el tiempo de exposición, observándose la reducción total de la infectividad tras tratamientos ON a 37 °C para ambos virus, y a 25 °C para VHA. En ensayos posteriores, el objetivo fue evaluar la actividad antiviral del GTE a 25 °C en función del pH y tiempo de almacenamiento y correlacionarlo con cambios en su composición química como consecuencia de reacciones de degradación y epimerización durante el periodo de almacenamiento a diferentes condiciones (proceso referido como envejecimiento). Los resultados concluyeron que el GTE recién preparado era muy efectivo frente a MNV y VHA a pH neutros y alcalinos pero muy ineficiente a pH 5,5 cuando los ensayos se realizaron a 25 °C, resultados similares a los que se observaron a 37 °C. Sin embargo, el almacenamiento de la solución de GTE durante 24 h (GTE envejecido) a diferentes condiciones de pH, incrementaron significativamente la actividad antiviral del extracto. También, mediante el análisis por HPLC/MS se demostró un incremento de la actividad

antiviral debido a cambios en la composición química a causa de la formación de derivados de catequinas durante el periodo de almacenamiento. Entre otras, después del almacenamiento del GTE, la epigalocatequina galato (EGCG) resultó ser el compuesto más abundante, suponiendo entre un 40-50% de la composición total del GTE.

Asimismo, la eficacia del GTE envejecido se evaluó de forma indirecta frente a norovirus humanos mediante ensayos con partículas pseudovíricas (VLPs, del inglés virus-like particles) y en suspensiones fecales de norovirus humanos. Las VLPs son partículas con carácter tanto morfológico como antigénico similar a los virus infecciosos y han sido utilizadas en trabajos previos para determinar la capacidad antiviral de otros compuestos naturales. En esta tesis doctoral, las VLPs fueron tratadas con GTE envejecido y analizadas mediante ensayos de unión a mucina porcina gástrica mediante ELISA y microscopio electrónico de transmisión. Por otro lado, las suspensiones fecales de norovirus se analizaron mediante *in situ capture*-RT-qPCR. En estos ensayos se observó una reducción significativa cercana al 50% en la capacidad de unión de las VLPs a la mucina gástrica, mientras que la observación por microscopía electrónica mostró que los tratamientos con 0,5 mg/ml de GTE envejecido redujeron significativamente el número de VLPs mientras que con una concentración de 5 mg/ml las VLPs se degradaron completamente. Las suspensiones fecales de norovirus tratadas con

GTE envejecido a 0,5 mg/ml y evaluadas mediante *in situ capture*-RT-qPCR mostraron una reducción del 65% de la unión a la mucina gástrica, mientras que las suspensiones tratadas a 5 mg/ml o tratadas térmicamente a 99 °C (muestra control) la unión a la mucina se inhibía completamente.

Con el propósito de obtener más información sobre la posible infectividad de los virus, se llevaron a cabo ensayos de PCR de viabilidad en suspensiones de norovirus y VHA. De manera resumida, suspensiones de virus tratados y no tratado con EGCG se trataron con PMAxx y se incubaron durante 10 min a 150 rpm en oscuridad, posteriormente las muestras se fotoactivaron durante 15 min y finalmente el ARN viral fue extraído y analizado mediante RT-qPCR. La EGCG a concentración de 2,5 mg/ml resultó afectar solo a la infectividad del VHA analizado mediante cultivo celular, reduciendo la infectividad del VHA por debajo del límite de detección. Las mismas suspensiones, analizadas en paralelo mediante PCR de viabilidad no mostraron diferencias significativas en el número de copias detectadas comparando con los resultados obtenidos mediante el uso exclusivo de RT-qPCR. Estos resultados sugieren que el tratamiento con EGCG induce ligeros cambios conformacionales en cápside viral que afecta a la capacidad infecciosa del virus, pero que no permiten la entrada del PMAxx.

Se evaluó la estabilidad del GTE en condiciones gástricas simuladas (saliva, gástrica e intestinal) para dilucidar su eficacia como compuesto antiviral terapéutico frente a virus entéricos después de la ingesta

Con el fin de utilizar el GTE como virucida natural, se evaluó su uso en diferentes aplicaciones alimentarias. Uno de los factores a tener en cuenta cuando se pretende utilizar compuestos naturales como antivirales es conocer su estabilidad bajo las condiciones que suponen su consumo y su paso a través del tracto gastrointestinal. Para ello, la estabilidad del GTE fue evaluada en condiciones gástricas simuladas (salivares, gástricas e intestinales) para dilucidar su eficacia como compuesto antiviral terapéutico frente a virus entéricos después de la ingesta. Inicialmente, fluidos salivares (pH 7,0), gástricos (pH 3,0) e intestinales (pH 7,0) (SSF, SGF y SIF del inglés simulated salivary, gastric o intestinal fluid, respectivamente) fueron preparados *in vitro*, mezclados y almacenados durante 24 h con 10 mg/ml de GTE. Suspensiones de MNV y VHA con títulos iniciales de aproximadamente 5 log TCID₅₀/ml fueron mezcladas a proporciones iguales con las suspensiones previamente preparadas consiguiendo una concentración final de GTE envejecido de 5 mg/ml. La muestra SSF se incubó durante 2 min a 37 °C, mientras que las de SGF y SIF se incubaron a la misma temperatura, pero durante 2 h. Una vez pasado el tiempo de incubación las reacciones se neutralizaron con DMEM suplementado

al 10% de suero fetal bovino (FBS). Los resultados analizados indicaron que el GTE envejecido en SSF redujo la concentración de MNV en 0,7 órdenes logarítmicos mientras que para VHA se redujo en 1,5. En soluciones de SGF se observaron reducción de 3,1 y 2 órdenes logarítmicos mientras que para SIF se redujeron por debajo del límite de detección y en 2 órdenes logarítmicos para MNV y VHA, respectivamente. Estos resultados concuerdan con estudios previos con GSE y proantocianidinas de arándanos (PAC-B, del inglés blueberry proanthocyanidins) en SIF, donde el GSE redujo las concentraciones de MNV y VHA en 1,7 y 1,4 órdenes logarítmicos, respectivamente, mientras que las soluciones de PAC-B inactivaron por completo al MNV. Por otro lado, el GTE preparado en SGF redujo en menor medida la infectividad del MNV comparado con SIF. Una de las posibles razones podría ser por el hecho de que el GTE envejecido es muy efectivo inactivando a MNV a pH neutros y alcalinos, pero esta efectividad se pierde a pH de 5,5, correlacionado con la formación de derivados de las catequinas.

Del mismo modo, el GTE envejecido fue añadido a diferentes bebidas como zumos de naranja o manzana, horchata y leche. Como se describe anteriormente, suspensiones de MNV y VHA se mezclaron a iguales volúmenes con las diferentes bebidas, zumo de naranja (pH 2,6), de manzana (pH 3,8), y horchata de chufa (una bebida local típica compuesta de un 83,5% de agua,

10% de azúcar y un 2,2% de grasa; pH 6,8) comprados en una tienda local, y leche reducida en grasa (2%). Las muestras se incubaron a 37 °C ON a concentraciones de 2,5 y 5 mg/ml de GTE envejecido.

Diversos factores pueden ser los causantes de la pérdida de efectividad del GTE en bebidas, como la interacción de los compuestos activos o de los virus con las matrices alimentarias o sus componentes, especialmente con las proteínas, las grasas o los azúcares. El zumo de manzana (rico en hidratos de carbono) y la leche (rico en proteínas y grasas) han sido utilizados como modelos alimentarios en algunos estudios para evaluar la eficacia de antivirales naturales. En este trabajo, cuando el GTE envejecido (5 mg/ml) se preparó en zumo de manzana la infectividad del MNV se redujo por debajo del límite de detección mientras que la del VHA se redujo en 1,7 órdenes logarítmicos. Cuando el GTE se preparó en leche, la eficacia se vio reducida, observándose sólo reducciones de 1 orden logarítmico para ambos virus. Estos resultados están en línea con estudios realizados anteriormente con PAC-B y GSE. De hecho, el GTE envejecido preparado en leche mostró prácticamente la misma reducción (0,8 órdenes logarítmicos) frente al MNV que trabajos previos utilizando PAC-B bajo las mismas condiciones experimentales (5 mg/ml, 24 h, 37 °C). En cuanto a VHA, el GTE envejecido consiguió reducciones de 1,2 órdenes logarítmicos en leche, datos similares a los que se observaron para el

GSE a 4 mg/ml. A pesar del contenido en grasa de 2,2% de la horchata, la infectividad del MNV y VHA se redujo aproximadamente 2 órdenes logarítmicos lo que podría servir como un buen alimento terapéutico antiviral con la adición del GTE.

Distintos estudios epidemiológicos sugieren que el consumo de bebidas, agua o frutas no pasteurizadas o tratadas inadecuadamente está relacionado con brotes virales de transmisión alimentaria. Por esto, siguiendo los principios de las tecnologías barrera, los siguientes ensayos se focalizaron en analizar la actividad antiviral simultánea de diferentes procesos de conservación, en este caso, el uso combinado del GTE envejecido y tratamientos térmicos moderados (40, 50 y 63 °C) para evaluar la cinética de inactivación del MNV y VHA en zumos de frutas artificialmente contaminados (J1 – compuesto por fresa, zanahoria, remolacha y manzana, pH 4,10, y J2 – zumo de manzana, pH 3,75).

En PBS, el tratamiento combinado de GTE envejecido (3,33 mg/ml) y tratamiento térmico a 40, 50 y 63 °C resultó en mayor nivel de inactivación frente al MNV que cuando sólo se aplicaron los tratamientos térmicos. Este efecto sinérgico no se observó tras el tratamiento de zumos a 40 °C, indicando un posible efecto protector de los zumos sobre las partículas virales. Sin embargo, la infectividad del MNV se redujo por debajo del límite de detección

tras tratamientos a 50 y 63 °C para J2 y a 63 °C para. La infectividad del VHA no se vio afectada significativamente tras tratamientos térmicos y adición de GTE en J1, sin embargo, en J2 el tratamiento térmico sin GTE reducía la infectividad del virus en 1,37, 0,62 y 1,44 órdenes logarítmicos a 40, 50, y 63 °C, respectivamente, seguramente debido al efecto combinado de alguno de los ingredientes o el pH bajo. La adición de GTE envejecido a los zumos no mejoró el efecto antiviral de las temperaturas contra VHA.

Los resultados obtenidos de este trabajo mostraron que la incorporación de GTE envejecido podría ser una buena opción para mejorar la seguridad alimentaria en zumos tratados a temperaturas moderadas, exhibiendo una actividad antiviral mayor frente al MNV que sólo los tratamientos térmicos.

Los siguientes ensayos evaluaron el potencial del GTE como higienizante natural para su aplicación en superficies de contacto alimentario o como solución higienizante de vegetales de cuarta gama. Hasta la fecha, la presencia de virus entéricos ha sido descrita en diferentes superficies porosas (tejido, diferentes tipos de papel y algodón) y no porosas (aluminio, porcelana, vidrio, látex, plástico, poliestireno y acero inoxidable). En el contexto de esta tesis, el GTE fue evaluado como higienizante natural para la higienización de superficies de contacto alimentario siguiendo el procedimiento descrito en la norma ISO

13697:2001. Cabe destacar que, en 2019, se publicó una norma análoga para la evaluación frente a virus: la norma europea 16777:2019 que describe un método y los requerimientos mínimos para la evaluación de la actividad antiviral de desinfectantes químicos que forman una solución homogénea cuando se diluyen en agua dura. Esta norma europea se aplica en áreas y situaciones donde la desinfección es necesaria para preservar la salud de las personas.

Para estos estudios suspensiones de MNV y VHA fueron diluidas en una ratio 1:1 con suero de albumina bovina (BSA) como sustancia interferente para simular condiciones limpias (0,3 g/l) y condiciones sucias (3 g/l). La suspensión viral (50 µl) se depositó en la superficie, de los materiales de acero inoxidable y vidrio dejando que secan a temperatura ambiente durante aproximadamente 15 min. Después, 100 µl una suspensión de GTE (recién preparada y envejecido a 5 y 10 mg/ml) preparadas en agua dura se depositaron sobre los materiales previamente inoculados. La solución higienizante se dejó actuar durante 15 y 30 min para las soluciones de GTE recién preparado y 5 y 15 min para el GTE envejecido. Finalmente, el efecto del GTE se bloqueó neutralizando la reacción con DMEM suplementado al 10% con FBS. Finalmente se recuperó el virus de la superficie y se cuantificó ensayos en cultivo celular. Para el VHA se obtuvieron reducciones de menos de 1 orden logarítmico cuando se aplicó el GTE recién preparado durante 15 min en

condiciones limpias. Por el contrario, el GTE aplicado en condiciones sucias resultó ser más efectivo frente a ambos virus. Además, la infectividad del VHA se redujo completamente tras 30 min de contacto con una solución higienizante de GTE envejecido a 10 mg/ml para ambos materiales y ambas condiciones de limpieza. En condiciones sucias, GTE a 1 mg/ml y 30 min de contacto la infectividad del MNV se redujo en 2,09 y 1,64 órdenes logarítmicos en acero y vidrio, respectivamente. Cuando el GTE envejecido (5 mg/ml) se aplicó en condiciones limpias en acero inoxidable, los títulos infecciosos de MNV se redujeron en 0,75 y 1,75 órdenes logarítmicos después de 5 y 15 min de exposición, respectivamente. Para VHA las reducciones bajo las mismas condiciones resultaron similares, 0,79 y 1,92 órdenes logarítmicos. En superficies de vidrio, no se observaron reducciones significativas para ninguno de los dos virus después de 5 min de contacto, mientras que tras 15 min de contacto se observaron reducciones de 1,57 y 1,54 órdenes logarítmicos para MNV y VHA.

Los resultados de estos ensayos demostraron la eficacia del GTE como higienizante natural para su aplicación en superficies de contacto alimentario mostrando una mejor actividad comparándolo con otros compuestos naturales estudiados bajo condiciones similares, como el GSE. De hecho, anteriores estudios reportaron leves reducciones (<1 orden logarítmico PFU/ml) para MNV

en superficies de acero tratados con 2 mg/ml de GSE durante 10 min en condiciones limpias. Los resultados obtenidos en los ensayos realizados en esta tesis mostraron reducciones significativas para MNV (>1,5 órdenes logarítmicos TCID₅₀/ml) tanto en acero como en vidrio tras la aplicación de GTE a 10 mg/ml durante 30 min y la inactivación completa para VHA en ambas superficies y condiciones de limpieza. En general, se aprecia un detrimento del efecto antiviral cuando un compuesto es ensayado en condiciones sucias, principalmente debido a su unión con la materia orgánica (como las proteínas) interfiriendo en la efectividad del tratamiento. Este efecto se observó para los ensayos frente a MNV, donde el GTE fue menos efectivo en condiciones sucias. Por el contrario, en el caso de VHA la efectividad del GTE no se vio afectada por la presencia de materia orgánica en las superficies tratadas.

Si se comparan los estudios realizados entre el GTE recién preparado y el GTE envejecido, se observa que la actividad antiviral del extracto se ve incrementada en el segundo caso. Como se ha comentado anteriormente, la solución de GTE preparada 24 h antes de su uso lleva consigo la degradación y epimerización de algunos de sus compuestos, por lo que modulando el tiempo de almacenamiento del GTE antes de su uso se puede mejorar su actividad antiviral como se ha demostrado no solo *in vitro*, sino también en la formulación de desinfectante natural para aplicación en superficies de contacto alimentario.

En la industria de IV gama, los vegetales de hoja verde presentan un alto riesgo de sufrir contaminación cruzada, hasta no hace mucho el cloro ha sido el desinfectante más comúnmente utilizado, pero debido a la formación de trialometanos, en algunos países europeos se está limitado su uso. Por ello, viendo la necesidad de soluciones alternativas de desinfección, en esta tesis, se ha querido ensayar el GTE como solución de lavado en hojas de lechuga y espinacas.

Mediante la metodología descrita anteriormente, se determinó la capacidad viricida del GTE recién preparado con el fin de aplicarlo como solución de lavado. De manera resumida, hojas frescas de lechuga (*Lactuca sativa* L.) y espinaca (*Spinacia oleracea*) se cortaron en piezas y se higienizaron con luz UV en una cabina de bioseguridad de flujo laminar durante 15 min. Seguidamente se inocularon suspensiones de 50 µl de MNV y VHA (a dos niveles de inoculación), por separado, sobre las superficies de los vegetales. Las muestras inoculadas se dejaron secar aproximadamente 15 min, para luego depositar 100 µl de GTE a diferentes concentraciones (5 y 10 mg/ml) durante 15 y 30 min. La reacción se paró recuperando la muestra con DMEM suplementado al 10% con FBS como neutralizante para finalmente cuantificar las muestras mediante cultivo celular. Los resultados mostraron reducciones significativas de la infectividad del MNV observándose reducciones de 1,38 y 1,80 órdenes logarítmicos tras el lavado

de lechuga y espinacas con soluciones de GTE a 10 mg/ml durante 30 min. Menores reducciones se observaron cuando se aplicó el GTE a una menor concentración. Cuando los vegetales se inocularon con concentraciones de MNV más elevada, se observó que la infectividad del MNV se reducía en 2,59 órdenes logarítmicos tras el lavado con GTE. En relación a VHA, la infectividad se redujo por debajo del límite de detección en inóculos bajos, tratados con GTE a 10 mg/ml de durante 30 min para ambos vegetales. Mientras que cuando el inóculo inicial fue alto, se observaron reducciones de 0,79 y 1,37 órdenes logarítmicos tras el lavado de lechuga y espinacas.

Los estudios sobre el uso de higienizantes naturales para el lavado de vegetales son escasos; hasta la fecha sólo el carvacrol y el GSE han sido evaluados como higienizantes naturales frente a virus entéricos. El carvacrol a 10 mg/ml mostró tener actividad frente MNV tras el lavado de lechugas durante 30 min, reduciendo la infectividad del MNV en 1,8 órdenes logarítmicos. Sin embargo, el GSE no fue muy eficaz como higienizante natural frente al MNV, mientras que si lo fue para el VHA donde se observaron reducciones de 1,23 y 1,29 en lavados de lechuga y pimiento.

Otra aplicación muy novedosa por parte de la industria alimentaria es el uso de materiales y recubrimientos comestibles, debido a la demanda por parte de

los consumidores en adquirir alimentos más saludables y seguros y a la vez respetuosos con el medio ambiente evitando los efectos nocivos que llevan consigo los envases no biodegradables. En particular, los recubrimientos comestibles son interesantes desde el punto de vista alimentario ya que han demostrado mejorar la calidad y seguridad alimentaria controlando la transferencia de agua, gases o aromas a los alimentos.

La incorporación de antimicrobianos a estos recubrimientos ha tenido una gran aceptación dando lugar a envases bioactivos capaces de contrarlar el crecimiento de microorganismos alterantes y patógenos, siendo estos factores críticos que afectan a la calidad y seguridad de los alimentos.

Muchos esfuerzos se han dedicado al desarrollo de materiales y recubrimientos donde se han incorporado compuestos naturales como aceites esenciales, bacteriocinas o compuestos fenólicos. Sin embargo, aunque las propiedades bactericidas y fungicidas de estos compuestos han sido ampliamente investigadas, todavía es escasa la información relativa al desarrollo de materiales o biopolímeros complementados con compuestos antivirales naturales y su interacción con los alimentos.

Por ello, los últimos trabajos realizados en el marco de esta tesis se llevaron a cabo con el fin de elucidar si la incorporación del GTE en films y recubrimientos comestibles podría ser una buena estrategia para el control de la contaminación vírica en frutos rojos. Inicialmente, el GTE fue incorporado a films de alginato (0,5 o 0,75 g GTE/g alginato). Estos films se cortaron en trozos (25 ± 5 mg) que fueron higienizados en cabina de flujo laminar expuestos a luz UV durante 15 min. Posteriormente, las muestras se sumergieron en 500 μ l de MNV y VHA, incubándolas a 37 °C ON en agitación (180 rpm). Los resultados mostraron que los films redujeron la infectividad del MNV en 1,92 órdenes logarítmicos. Mientras que los films que contenían una menor concentración de GTE reducían la infectividad del MNV 1,25 órdenes logarítmicos. Los resultados obtenidos indicaron que la actividad que mostraron los films era menor a la del extracto puro, sugiriendo que el GTE interactúa con los componentes del film impidiendo la completa liberación del compuesto activo.

Por otro lado, se desarrollaron recubrimientos comestibles con diferentes carragenatos y GTE como una estrategia innovadora para garantizar la seguridad en frutos como arándanos y frambuesas. Los recubrimientos fueron aplicados en dichos frutos, previamente inoculados con suspensiones de MNV y VHA e almacenados a temperatura de refrigeración (10 °C) y ambiente (25 °C). Los arándanos y las frambuesas se expusieron previamente a luz UV durante

15 min en una cabina de flujo laminar para minimizar la carga microbiana. Posteriormente, se depositaron 50 μ l de cada suspensión vírica sobre la superficie de cada fruto dejándolas secar durante aproximadamente 1 h a temperatura ambiente. Seguidamente cada fruto se sumergió durante 2 min en los diferentes recubrimientos. Las muestras se almacenaron durante ON a 25 °C, y ON o 4 días a 10 °C. Transcurrido el periodo de incubación cada una de las muestras se sumergió en 5 ml de DMEM (suplementado al 10% de FBS) y se agitó a 180 rpm durante 2 min para recuperar las partículas virales de las superficies de los frutos. Finalmente, los frutos se retiraron de la suspensión de DMEM y se cuantificó los virus recuperados mediante cultivo celular.

En general, el efecto antiviral de tres carragenatos evaluados, tanto para MNV como para VHA en ambos frutos, fue mayor a 25 °C, aunque se observó una tendencia similar a temperaturas de refrigeración. Los resultados mostraron que la infectividad del MNV se redujo por debajo del límite de detección en arándanos tratados ON a 25 °C con los tres recubrimientos de carragenatos y GTE. La efectividad de los recubrimientos fue mucho menor en frambuesas bajo las mismas condiciones experimentales. Este efecto puede deberse a la diferencia estructural de la superficie de los frutos, lo que puede afectar a la recuperación de los virus de la superficie de los mismos. Los recubrimientos de carragenatos resultaron también muy efectivos frente a VHA, especialmente en

arándanos, donde tras el almacenamiento ON a 25 °C se observaron reducciones de la infectividad superiores a 3 órdenes logarítmicos. En condiciones de refrigeración, se observó una mayor eficacia en frambuesas que en arándanos.

Finalmente, en base a los resultados de la presente tesis, se puede concluir que el GTE es una opción natural y de bajo coste capaz de mejorar la seguridad alimentaria frente a virus entéricos en diferentes aplicaciones alimentarias.

1. INTRODUCTION

INTRODUCTION

In recent years, all over the world, human enteric viruses have been referred to as one of the most important causative agents of foodborne diseases (ECDC, 2019; Harrison and DiCaprio, 2018). A wide variety of pathogenic viruses can be transmitted through the consumption of contaminated water or food, among them human norovirus, sapovirus, astrovirus, rotavirus and adenovirus are responsible for gastroenteritis, which appears abruptly with diarrhea and vomiting and sometimes is accompanied by fever profile and abdominal cramps (Table 1). Based only on clinical manifestations, it is not easy to differentiate if a pathogenic bacterium or a virus is the causative agent of the gastroenteritis, hence, microbiological laboratory tests are needed to identify them. Severe gastroenteritis caused by human enteric viruses usually results in 2 – 5 days of treatment focused on maintaining good hydration of the patient (Glass et al., 2009). On the other hand, hepatitis A virus (HAV) is the most frequent etiological causative agent of acute hepatitis associated with water or food consumption (Solé et al., 2011). HAV infection can be completely asymptomatic, as is usual in children under 5 years of age, or it can cause acute hepatitis that occurs frequently in adults and has two stages of development: a pre-jaundice stage and a jaundice stage, requiring hospitalization in some cases (Arguedas and Fallon, 2004). Another faecal-oral transmitted hepatitis virus is the hepatitis

E virus (HEV). HEV is an emerging foodborne pathogen that causes 44,000 deaths every year, according to the World Health Organization (WHO) (Kupferschmidt, 2016). Depending on the genotypes, the routes of transmission can be either faecal-oral, usually by consumption of contaminated drinking water or animal meat or direct contact with infected animals which typically occurs in under-developed countries (Sooryanarain and Meng, 2019).

Human enteric viruses are mainly transmitted through the faecal-oral route; as contaminated people are capable of excreting high numbers of viral particles in their feces. This makes transmission and infection easy (Daniels et al., 2009). Contamination by food handlers or cross-contamination through contaminated surfaces is mainly associated with ready-to-eat products, such as salads, sandwiches, or bakery items, which are prepared or handled raw, or after the foods have been already cooked. Contamination can also occur during production, which is the case for shellfish usually harvested from waters contaminated by the discharge of sewage, or leafy greens and berries contaminated in the fields by pickers or through polluted irrigation waters. These pose a higher health risk because both items are frequently eaten raw (Schmid et al., 2009; Shin et al., 2019).

Globally, foodborne hazards cause approximately 600 million illnesses annually, with the human norovirus being responsible for 120 million cases attributed to water and food (WHO, 2015). In 2018, 10.3% of total foodborne

outbreaks in the European Union were caused by human enteric virus infections (EFSA, 2019). Enteric viruses are the most common etiologic agents identified in produce-associated outbreaks (54%) in the US, frequently linked with food-handling issues (Bennett et al., 2018). Norovirus was described as one of the most causative agents in developed countries. A total of 448 foodborne outbreaks caused by enteric virus were reported by the European Food Safety Authority (EFSA), with 389 associated with norovirus and only 56 and 3 with HAV and HEV, respectively (EFSA, 2019). Although the first confirmed cases were reported in 1983, it should be noted that it was not until 2018 when the EFSA declared HEV as an emerging virus detailing the health risks associated with the virus (Harrison and DiCaprio, 2018; Nelson et al., 2019). Recently, the presence of HEV has been described in different animal meat (cow, donkey) and in raw sheep's milk (Demirci et al., 2019; EFSA, 2019).

Enteric viruses present a natural stability to environmental conditions that offer them longer survival periods under extreme conditions, finally providing resistance against inactivation processes that are commonly used in the food industry or in wastewater treatment plants (WWTPs). Moreover, only a very low infectious dose (from 10 to 1000 viral particles) is needed to produce a norovirus infection (Teunis et al., 2008). This characteristic, together with their resistance to desiccation and to different chemical products, seems to be one the most important factors contributing to maintenance of their infectivity on inner

surfaces (fomites) and in food products, with the final result of host infection (Kuusi et al., 2002).

Table 1. Characteristics of common foodborne enteric viruses (Carter, 2005)

Virus	Virus family (Genus)	Food vehicle	Associate illness
Aichi virus	<i>Picornaviridae</i> (<i>Kobuvirus</i>)	Shellfish	Gastroenteritis
Hepatitis A virus	<i>Picornaviridae</i> (<i>Hepatovirus</i>)	Leafy green vegetables	Hepatitis, mild in the young
Hepatitis E virus	<i>Hepeviridae</i> (<i>Orthohepevirus</i>)	Water and pork meat	Hepatitis, severe in pregnancy
Human norovirus	<i>Caliciviridae</i> (<i>Norovirus</i>)	Shellfish, water, berries	Vomiting, diarrhea, nausea, cramps, chills, dehydration
Human sapovirus	<i>Caliciviridae</i> (<i>Sapovirus</i>)	Shellfish	Explosive projective vomiting in older children/young adults
Rotavirus	<i>Reoviridae</i> (<i>Rotavirus</i>)	Often water	Diarrhea-common in the young, incidence with age but increases in the elderly

1.1. THE MOST RELEVANT ENTERIC VIRUSES

1.1.1. Human norovirus

Human norovirus belongs to the *Caliciviridae* family under the *Norovirus* genus. Structurally they are non-enveloped viruses with a capsid of 28-35 nm diameter and a positive-sense, single-stranded RNA genome of about 7.4–7.7 kb (Greening and Cannon, 2016). Human norovirus are classified in ten

genogroups, which GI, GII, GIV, GVIII and GX cause infection in humans, being GI and GII prevail in clinical cases (Chhabra et al. 2019)

Norovirus are recognized as one of the most important causes of foodborne infections and acute gastroenteritis (AGE). Since they are very ubiquitous and are transmitted by the faecal-oral route, the high number of viral particles that infected people are able to spread due to diarrhoeal and vomiting episodes, represents the main source of contamination, especially in places where space is reduced such as in nursing homes, hotels or cruise ships. Infected food handlers are also identified as a major source for foodborne norovirus outbreaks together with the consumption of fresh fruits, molluscan shellfish (mainly oysters), and leafy greens (Figure 1). Moreover, it is possible to find the presence of norovirus in all kinds of foodstuffs due to cross-contamination or improper food-handling in every step of the supply chain during production and distribution. Since the Norwalk-like virus was reported in 1972 as the first viral source of human diarrhoeal outbreak (Kapikian et al., 1972), noroviruses have been considered as one of the common causative agents of food and waterborne gastroenteritis. Even though the number of outbreaks associated with the human norovirus has increased, its actual prevalence is still a challenge to estimate (Atmar et al., 2015). It is believed that human norovirus outbreaks are generally under-reported because of the intrinsic nature of the gastrointestinal disease they cause, which rarely requires hospitalization or

specific medical care except in the case of elderly or immuno-compromised patients (Atmar et al., 2013). Once the infection occurs, clinic manifestations appear after 12 to 48 hours and the illness usually lasts 1 to 3 days (Teunis et al., 2008). Diarrhoea, vomiting, nausea, fever, headache and stomachache are the most common symptoms and it is estimated that 18% of gastrointestinal disorders worldwide are due to human norovirus infections (Atmar et al., 2015).

In the United States, a recent report from the Centre for Disease Control and Prevention (CDC) reported 140 foodborne outbreaks, while in Europe, 389 outbreaks were reported by the EFSA in 2019. All of these were related with norovirus (CDC et al., 2019; EFSA, 2019), which represents a total of 12.599 illnesses that resulted in 259 hospitalizations (CDC et al., 2019; EFSA, 2019). Last year, in the European Union, noroviruses were reported as the causative agents in at least 7.6% of foodborne outbreaks, resulting in an increase of 29.9% with respect to 2017. The most important food vehicles were water, fish and fishery products, mixed food and buffet meals with 1.089, 736, 735 and 399 human cases reported, respectively. Fish and its products have shown a substantial increase in recent years, as has water albeit to a lower degree, while mixed food and buffet meals have remained stable over this period (EFSA, 2019).

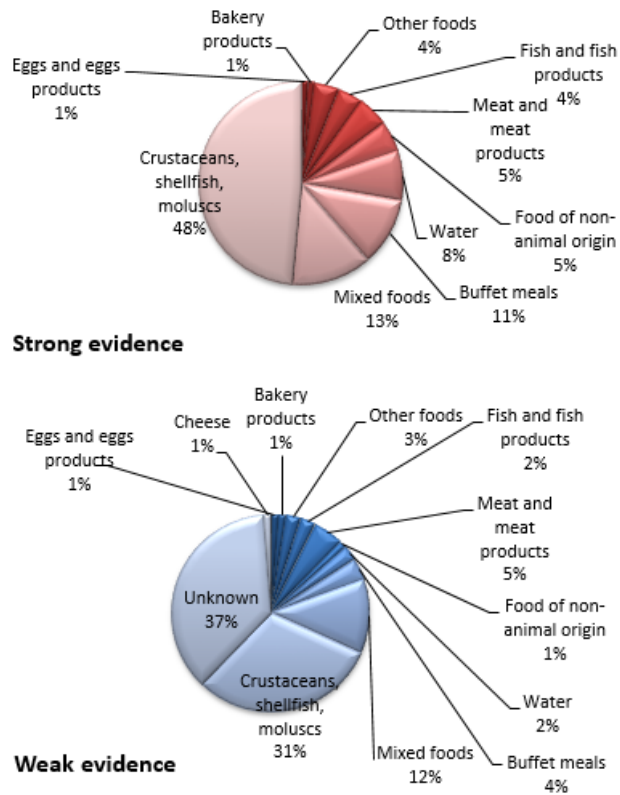


Figure 1. Food vehicles in strong and weak evidence associated to the human norovirus foodborne outbreaks in the EU, 2018 (EFSA, 2019)

It is not surprising that the most common reported sites of exposure are restaurants, pubs, street vendors and take-aways, followed by canteen or catering services for workplaces, schools, ships, and hospitals, since these settings provide food and water shared between many people and thus outbreaks are more easily investigated.

The stability of human norovirus

The stability and persistence of human noroviruses have been mainly assessed by using cultivable animal surrogates since human noroviruses can not be easily cultivated routinely (Duizer et al., 2004). Although norovirus replication has been tested in human intestinal enteroids (Costantini et al., 2018; Ettayebi et al., 2016), up to now it was believed that the lack of cell lines with good specific receptors and co-receptors for human norovirus could be the cause for the failure to replicate them *in vitro* which has created an important barrier in the study of these viruses. Thus, cultivable surrogates such as murine norovirus (MNV), feline calicivirus (FCV) or Tulane virus (TV) have been extensively used to indirectly assess human norovirus infectivity (Ailavadi et al., 2019; Cromeans et al., 2014; Joshi et al., 2015).

Cannon et al. (2006) investigated the thermal stability of human norovirus with norovirus surrogate studies, reporting the necessity of 3.5 min, 25 seconds and 10 seconds at 56°C, 63°C, and 72°C, respectively, to reach reductions by 1 log for MNV.

Some examples of norovirus stability in food matrices, dependent on temperature and time, have been observed in Kimchi or sauerkraut, a fermented food made from cabbage and brine and these were assayed against norovirus and MNV, respectively. In Kimchi norovirus titers were reduced by 1.31 and 1.66 log for 4°C and 10°C, respectively after 28 days of storage. A

similar trend was observed in sauerkraut with MNV titers decreasing by almost 1 log after 90 days at 4°C (Gagné et al., 2015; Lee et al., 2017).

Overall, the genotype and status (virus aggregation or virus bound to organic matter), temperature, ionic strength, microbial antagonism or chemical constituents are some of the factors that finally influence the environmental viral persistence (Knight et al., 2016). Strong evidence suggests that human noroviruses are very stable and can maintain their infectivity in the environment and food for several months.

There is evidence that norovirus outbreaks are usually more common during the cooler months, so temperature is one of the most important factors affecting their stability and persistence. Moreover, adsorption to particulate material increases the survival of norovirus in environmental water resulting in sediments becoming reservoirs of viral particles, which eventually greatly contribute to the contamination of coastal areas (Burke et al., 2019). Environmental water and sediments act as reservoirs of viral particles due to their adsorption to particulate material. This increases the survival of human noroviruses, making these waters and sediments an important source of contamination for coastal and shellfish harvesting areas (Rzeżutka and Cook, 2004). In addition, human noroviruses have been detected in influent and effluent sewage and represent a risk, not only for farm and wastewater workers, but also for consumers given the re-use of contaminated water for agriculture (Randazzo et al., 2016). Due

to the environmental stability that noroviruses exhibit, their prevalence in certain foods is comprehensible, as Figure 1 shows.

1.1.2. Hepatitis A virus

HAV causes the well-known infection called hepatitis A, disease that causes inflammation of the liver and affects its function. This virus belongs to the *Hepatovirus* genus within the *Picornaviridae* family (King et al., 2011). An icosahedral capsid about 27-32 nm in diameter contains its genetic material. This genetic material is composed of a single-stranded, positive-sense, linear RNA of about 7.5 kb in length. HAV displays two infectious forms; the naked form which are non-enveloped virions, and quasi-enveloped virions from infected cells in non-lytically form (Feinstone, 2019; Lemon et al., 2018). The genome has a single open reading frame (ORF) where a polyprotein of around 250 kDa is encoded. The gene expression and binding proteins require a maturation of the proteins during and after the translational proteolysis from the polyprotein. HAV is classified in six genotypes based on the analysis VP1-2A region. Genotypes I, II and III, have human origin and are divided into subtypes A and B, while genotypes IV, V and VI cause infections in simians (Desbois et al., 2010).

Similarly to norovirus, HAV is primarily transmitted by the faecal-oral route with the consumption of contaminated food and water being the most common

vehicles of viral spreading even though physical contact (such as oral-anal sex) with an infectious person has also been reported (Ndumbi et al., 2018). Once ingested through the consumption of contaminated water or food (Jacobsen, 2018), HAV goes through the stomach, small intestine and large intestine. Then, particles circulate until reaching the liver where they infect hepatocytes, replicate and are released into bile, enabling their excretion in feces (Asher et al., 1995; Sui et al., 2006).

Tiredness, sudden nausea and vomiting, stomachache (especially in the area near the liver), loss of appetite, fever, headache and jaundice are symptoms of the disease (Arguedas and Fallon, 2004). Illness persists for a few weeks to several months. The condition is less acute in children than in adults, because in the former, it often causes subclinical or asymptomatic infections. The existence of an asymptomatic period makes infection easier to transmit because infected people are able to excrete 10^6 to 10^{11} viral particles of HAV per gram of feces (Pintó et al., 2012).

In 2019 the European Centre for Disease Prevention and Control (ECDC) reported more than 10.000 cases of HAV where 82% resulted in hospitalizations (<http://atlas.ecdc.europa.eu/public/index.aspx> (accessed 11.2.20)). The most relevant source of contamination was from restaurants, pubs, street vendors and take aways. In recent years, the incidence of implicated food from non-animal origin has risen to 50% (Figure 2). This food group consists of fruits and

vegetables and their juices, herbs and spices, sweets and chocolates. After norovirus, HAV is ranked second on the list of causative pathogens with 6.8% of all outbreaks involving hospitalization reported last year (EFSA, 2019).

Outbreaks of hepatitis A due to person-to-person transmission usually happen when contact, between infected and susceptible people, occurs over extended periods with close personal contact. This transmission is facilitated due to the prolonged shedding of HAV before and after the onset of symptoms. There is evidence of transmission of HAV in communities where there is a larger amount of persons susceptible to infection, lack of hygiene, and sharing of objects in close places.

The contamination risk of vegetables and berries by HAV can occur during pre- or post-harvest and distribution. Frequently, these products grow near the ground where agricultural products are deposited increasing the contact with contaminated biosolids or sewage-polluted irrigation water (Randazzo and Sánchez, 2020). Waterborne outbreaks commonly occur by drinking contaminated water or swimming in lakes, ponds, wetlands or puddles (de Paula et al., 2003; De Paula et al., 2007).

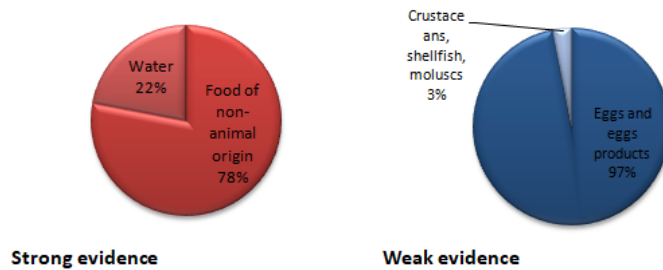


Figure 2 Food vehicles in strong and weak evidence associated to hepatitis A virus foodborne outbreaks in the EU, 2018 (EFSA, 2019)

The stability of the hepatitis A virus

As other enteric viruses, HAV is a highly stable virus, displaying great tolerance against different chemical, biological, and physical factors (Sánchez and Bosch, 2016). Several studies have demonstrated the resistance of HAV to temperatures. It is only in part inactivated when treated at 60°C or remains stable when stored at -20°C to -70°C for years. HAV is remarkably resistant in acidic environments keeping its infectivity at pH of 1.0 for 2h; also in dry atmospheres it is capable of maintaining its infectivity in different foods and waters (Baert et al., 2009; Bidawid et al., 2001; Rzeżutka and Cook, 2004; Sánchez, 2013).

A unique codon which provides a very stable capsid to the virus is involved in the resistance of HAV: it allows a more stable molecular structure of the viral particle which prevents direct competition with the host cell system (Costafreda et al., 2014). Because of this, it is known that HAV exhibits high stability in the

environment, being infectious for long periods, including in foodstuffs (Sánchez, 2013) which increases the risk of contamination between the purchase of products and their consumption. For example, Shieh et al. (2009), detected HAV in fresh spinach leaves after 42 days at 5°C with 6.75% of the original titer remaining.

1.1.3. Hepatitis E virus

The HEV is an emerging foodborne pathogen that belongs to the *Hepeviridae* family, a small (27–34 nm), positive-sense, single-stranded RNA virus of 27-34 nm and 7.2 kb in size (Kenney and Meng, 2019; Sooryanarain and Meng, 2019). HEV circulates in the blood as a quasi-enveloped form but it is excreted as non-enveloped virions in faeces (Feng et al., 2014; Yin et al., 2016). Different genotypes classify HEV: G1 and G2 are specific to humans while G3, G4 and G7 infect humans as well as animals and have been isolated in various animals, especially pigs (Sooryanarain and Meng, 2019; Van Der Poel, 2014). Different HEV genotypes have different geographical distribution, in particular, in Central America, Asia and Africa, the oral-faecal route is the main route of transmission of G1 and G2 by consumption of contaminated water. In comparison, G3 and G4 are associated with the consumption of animal meat or direct contact with infected animals and is endemic in under-developed countries (Khuroo et al., 2016; Nelson et al., 2019; Sooryanarain and Meng,

2019). It was not until 1983 when HEV was described as a novel agent of hepatitis disease. Before this, different epidemics of HEV were reported retrospectively in New Delhi, China, Nepal or Mexico (Khuroo, 2011; Meister et al., 2019; Viswanathan, 2013).

Although people infected by HEV are ordinarily asymptomatic, itching or jaundice are the characteristic manifestations of hepatitis E. Also, infections can lead to vomiting or myalgia (Kupferschmidt, 2016). It is when infection occurs in immunocompromised patients that the infection can be chronic or end in fulminant hepatitis. Mortality rates of 25% are associated with HEV positive pregnant women ending in hepatic failure (Clemente-Casares et al., 2016; Pérez-Gracia et al., 2017). Usually, there is no need for medication of subclinical patients, while antiviral treatments are considered for use in persons with risk factors (Dalton et al., 2018).

The stability of hepatitis E virus

HEV is considered a novel emerging virus and the lack of a robust cell culture system has limited the number of studies on its stability in the environment and in food (Cook and van der Poel, 2015). Despite this, various studies have been carried out to assess heat efficacy confirming the role of temperature in HEV inactivation. Emerson et al., (2005) demonstrated by cell culture and immunofluorescence assays, that 95% of HEV was inactivated after treatments at 56°C for 15 min and only 1% of infectious viral particles remained after 1h

exposure at the same temperature. Complete inactivation was achieved when treated at 66-70°C for 1h. Yunoki et al., (2008) reported no infectivity levels after 30 min at 60°C by RT-qPCR after incubation in A549 cell cultures for 7 days. Moreover, the author demonstrated the importance of the matrix, as HEV resuspended in 25% human albumin serum exhibited more resistance to inactivation needing 5h at 60°C to reduce 2.2 log.

HEV stability at different pH values is interesting to understand because of the importance of the use of pH values in food processing to prevent spoilage or extend shelf-life (Singh and Shalini, 2016). Recent studies have shown an extended stability of HEV when diluted in phosphate buffer saline (PBS) in a range of pH 2 to 9, with only decreasing titers of 0.6 log; besides, proteins of the capsid have demonstrated high stability at pH values of between 2.0 and 3.0, retaining viral infectivity of the particle. In contrast, infectivity at extreme pH values was reduced to 3 log or under detectable limits, for pH 1 or pH 10, respectively (Wolff et al., 2020; Zafrullah et al., 2004).

1.2. METHODS TO STUDY INFECTIOUS VIRUSES IN FOOD

In the field of food, detection of enteric viruses is more difficult than for bacteria as they replicate only in specific eukaryotic host cells and thus, their concentration does not rise previously to their detection. The assessment of infectivity is a major requirement in environmental and food virology. Traditionally, in faecal samples, transmission electron microscopy (TEM) was the main technique used to determine the presence of viruses. But a high concentration of viral particles is required in order to be detected by TEM, and the discrimination between infectious and non-infectious particles is not possible, so other methods are now preferred. Common techniques in use today can be classified into two broad groups: cell culture and molecular detection methods, and both have specifications and different variants. However, the low number of viral particles and the lack of a robust cell culture for some viruses have made it challenging to detect viruses by cell culture methods. Alternatively, molecular detection has emerged as a fast, sensitive, and specific alternative.

1.2.1 Cell culture methods

Cell culture methods are used to determine viral replication by observing, or not, cytopathic effects (CPE) (Figures 3 and 4) on specific host cells. When replication of viruses occurs without CPE, immunological techniques or

polymerase chain reactions (PCR) are needed to detect viral concentration after replication in cell cultures.

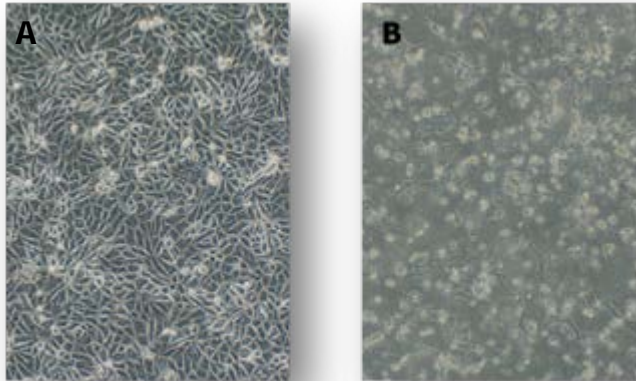


Figure 3. FRhK-4 cell line before (A) and after (B) HAV infection showing cytopathic effect.

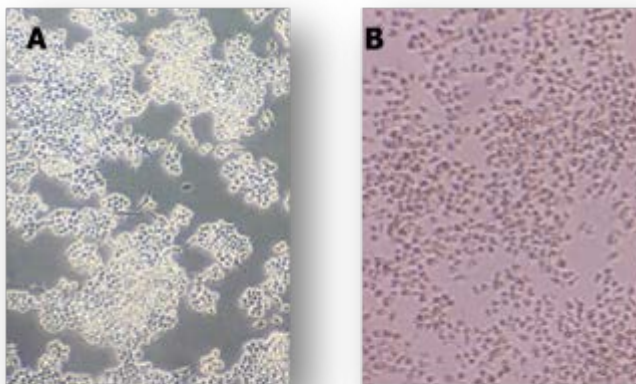


Figure 4. RAW 264.7 cell line before (A) and after (B) MNV infection showing cytopathic effect.

Cell culture methods have displayed some drawbacks that limit their application. For instance, they are labour-intensive and time-consuming requiring trained and experienced personnel. Moreover, some cell lines do not

show cytopathic effects , as is the case with human enteroids cells used to replicate human norovirus (Costantini et al., 2018; Estes et al., 2019), or they are not sensitive to wild type strain infections which also occurred with HAV. Because of these limitations, it is widely accepted to infer the viral infectivity by assaying cultivable surrogates. In the case of human norovirus, MNV, FCV, TV and porcine sapovirus have been extensively used as cultivable surrogates.

1.2.2 Molecular detection methods

Due to the lack of robust cell culture systems for certain enteric virus, molecular methods have grown in significance. Since the first description of a PCR assay by Saiki et al. (1985), PCR and real time PCR (qPCR), have been the most popular techniques for virus detection in contaminated food. Furthermore, standardized and approved methods have been implemented by authorities, like the ISO procedures 15216-1:2017 and ISO 15216-2:2019 for qualitative and quantifiable detection of norovirus and HAV in food and on food contact surfaces (Hennechart-Collette et al., 2015; Lowther et al., 2019). Although molecular techniques represent the greatest progress in the detection of virus in food and environmental samples, it is not without problems. The main disadvantage is the concentration, extraction and purification of target genetic material from complex food matrices because the presence of inhibitory substances is common (Hennechart-Collette et al., 2015; Lee et al., 2012; Suffredini et al.,

2014). Furthermore, potential cross-contamination implies the use of different controls (positive, negative and matrix controls) for the experiments to avoid false-positive results.

The increasing application of this technique has been remarkable from its first use in environmental samples and food samples such as in shellfish, lettuce, berries and hamburger meat among others (Atmar et al., 1995; Gouveaa et al., 1994; Laverick et al., 2004; Teixeira et al., 2020). However, this technique only detects genetic material regardless of damage to the viral particle. This renders the discrimination between infectious and non-infectious viral particles impossible (D'Souza, 2016; Freeman et al., 1999).

Since viral infectivity depends on the integrity of the capsid, advanced molecular techniques are being used as indirect approximations. These approaches assume that the detection of RNA from intact viral particles could be used to estimate infectivity.

Enzymatic pre-treatments

Given the concept that viruses with a damaged capsid lose their infectivity, enzymatic treatments have been developed to elucidate this question.

The enzymatic digestion with RNase/DNase prior to nucleic acid extraction, is a simple approach to remove amplification signals of exposed genomes and it has been successfully applied to detect potentially infectious viruses in

different water and food matrices (Escudero-Abarca et al., 2014; Nowak, 2011a, 2011b).

Similarly some authors used a proteinase K and RNase pre-treatment to differentiate between an intact virus and an inactivated virus (Moore et al., 2015). Nuanualsuwan and Cliver (2002) showed that HAV lose signal amplification after heat inactivation and hypochlorite treatment when both enzymes (RNase and proteinase K) were applied. Marti et al. (2017) also applied DNase and RNase linked to (RT)-qPCR in fresh lettuce, strawberries and green onions detecting intact particles of HAdV, norovirus and HAV, asserting the efficiency of this alternative method to detect undamaged RNA viruses and therefore, potential infectious viruses.

Intercalating dyes

A similar alternative to assess the viral viability is the use of intercalating dyes before the (RT)-qPCR technique. The intercalating dyes prevent amplification by trespassing the damaged or destroyed capsids (Sánchez et al., 2012) or membranes labelling the genetic material of microorganisms (Nogva et al., 2003). Hence, this method seems suitable to determine the integrity of viral capsids after treatment or food processing, and so can facilitate the extrapolation of infectivity status.

Two different categories of viability markers have been tested to this end: photoactivatable dyes that bond nucleic acids covalently during photoactivation

(propidium monoazide -PMA- or ethidium monoazide -EMA-); and metal compounds (i.e. platinum and palladium compounds). The latter compounds are cheaper than mozoazide dyes, and they do not need photoactivation to cross-link the nucleic acids, although only a few studies have tested them with viruses (Fraisse et al., 2018; Puente et al., 2020; Soejima and Iwatsuki, 2016).

Table 2. Application of intercalating dyes for the detection of potentially infectious enteric virus in food and water samples.

Matrix	Dye	virus	Food processing	Reference
Fermented sausages	PMA	AdV	No process	Quijada et al. (2016)
Clams		HAV	99°C 5 min	Moreno et al. (2015)
Lettuce	PMAxx	Norovirus	Chlorine dioxide (5.5ppm) 6 min Room temperature	López-Gálvez et al. (2018)
Parsley		HAV	99°C 5 min	Moreno et al. (2015)
Spinach	PMA / EMA	Norovirus	65, 75, 85, 95°C	Jeong et al. (2017)
			99°C 5 min	Randazzo et al. (2018b)
Water	PMA	HAV	Hypochlorite (100 ppm) 30 min Room temperature 70, 85, 99°C 5 min	Fuster et al. (2016)
	PMAxx	Norovirus	Chlorine dioxide (5.5ppm) 6 min Room temperature	López-Gálvez et al. (2018)
Berries	PMAxx	Norovirus	60, 72, 95°C	Chen et al. (2020)
	PtCl4	HAV	15 min	
Drinking water	PMA / EMA	AdV	4, 20, 37°C	Prevost et al. (2016)
			UV (253,7nm) Room temperature 50 ppm chlorine	
Cockles	PMAxx	HAV	99°C 5 min	Moreno et al. (2015)
Shellfish		Norovirus	No process	Randazzo et al. (2018b) Walter Randazzo et al., (2018b)

AdV: Adenovirus; HAV; Hepatitis A virus

Sanchez et al. (2012) concluded that PMA treatment performed better than RNase to estimate the infectivity of HAV after thermal treatments, decreasing viral titers by 2.4 log using RT-qPCR. Coudray-Meunier et al. (2013) applied PMA and EMA to evaluate the thermal inactivation of HAV and RV at different concentrations. The highest reductions were reported at 50 to 100 μ M for PMA, while only 20 μ M EMA was needed to observe the maximum RT-qPCR amplification decrease. Recently, PMAxx, an improved version of PMA, showed better performance for norovirus and HAV detection in food and water (Table 2). In conclusion, optimization of viability RT-qPCR is needed and depends on the marker, tested virus, matrix and procedure (D'Souza, 2016).

Porcine gastric mucin binding

What enables norovirus (and norovirus surrogates) to attach to intestinal cells and saliva, is the presence of human histo-blood group antigens (HBGAs), which are necessary for infection (Tan and Jiang, 2005). It has been reported that HBGAs are present in porcine gastric mucine (PGM) (Tian et al., 2008), a fact that has led to several studies of the effectiveness of antiviral treatments or the discrimination between infectious or non-infectious virus (Dancho et al., 2012; Knight et al., 2013).

As for the viability treatments commented earlier, PGM binding works with the idea that only particles with intact viral capsid are able to bind to HBGAs present in PGM, and therefore potentially infectious virus can be detected using

RT-qPCR. Damaged or destroyed ones are discarded after washings in the procedure (Li et al., 2011).

Usually, the PGM binding method has been used in combination with magnetic beads (MBs). As described by Li et al. (2013), MBs are firstly washed and then put in contact with PGM to cover the MBs surfaces and put both in conjugation (PGM-MBs). Samples are incubated together with PGM-MBs in agitation to allow the binding of the integral capsid to HBGAs, then PGM-MBs are separated from the liquid using a magnetic bead attractor. Finally, PGM-MBs are washed and virus with intact capsids are detected using RT-qPCR elucidating the infectious capacity of the sample. This approach has been applied to detect enteric viruses in different food matrices (Table 3) and determine the survival of enteric virus in food.

In order to solve the PMG-MBs drawback of magnetic bead loss, and therefore viral particles during the collection and transfer of samples to other tubes, a modification of PMG-MBs was proposed by Wang et al. (2014). This method uses immunostrips coated with saliva or PGM replacing the magnetic beads. Then, isolated viruses with intact capsids are detected in downstream RT-qPCR. In this case, there is no need for separation steps and samples are processed all at once. This *in situ* capture RT-qPCR (ISC-RT-qPCR) method is easier and faster than PGM-MB, and is used to detect infectious viruses in food and environmental waters (Shan et al., 2016; Zhou et al., 2017).

Table 3. Viral recovery by PGM-MBs in different food matrices.

Food matrix	Virus	Rec (%)	Reference
Blueberries	Norovirus	0.4	Huang et al. (2016)
Chocolate	MNV	5.6	Nasheri et al. (2020)
	FCV	1.65	
Pistachios	MNV	21.3	
	FCV	18	
Raspberries		9.5	Huang et al. (2016)
Seaweed	Norovirus	47	Suresh et al. (2019)
Spinach		5.5	
Strawberries		0.05	DiCaprio et al. (2016)
	TV	0.1	
Strawberry puree	Norovirus	40	Huang et al. (2016)
	TV	0.9	DiCaprio et al. (2019)
Oysters	Norovirus	0.5	Ye et al. (2015)

Rec: Recovery; MNV: Murine norovirus; FCV: Feline Calicivirus; TV: Tulane virus.

1.3. VIRAL STABILITY IN THE ENVIRONMENT AND FOOD

The resistance and stability of human enteric viruses in the environment and different types of food products have been widely investigated. The time viruses retain their infectivity or in which concentrations, are essential questions to answer to understand their behaviour in depth. Depending on the place enteric viruses are found, in the environment or incorporated in food matrices, different concerns arise. So, the study of stability in the environment is focused on waters, soils, fomites, and contaminated hands. For viruses in foods, relevant factors to be considered are; whether the food is chilled, dried, or frozen; relative humidity; modified atmosphere; and acidification processes.

1.3.1 Environmental stability

Waters

Temperature, solids adhesion, UV (ultraviolet light) exposure and the presence of microorganisms contribute to virus stability in water through mechanisms such as protein denaturalization or damage in genetic material. The inactivation of viral particles exposed to high temperatures is mainly due to protein damage (Adeyemi et al., 2017; Nguyen et al., 2018).

As was commented earlier, solids adhesion increases the persistence of viruses in water. This fact is critical when it comes to sea water because inshore

contamination areas result in contamination of shellfish that are harvested there. Apart from solids, it is demonstrated that salinity has a variable effect on viruses; also, in the marine water environment, the presence of microorganisms is relevant in the inactivation of virus. This seems to be due to the proteolytic activity of the bacterial enzymes exhibiting viral RNA to digestion nucleases (Bosch et al., 1993).

Especially, temperature and sunlight (UV) are the most important factors to bear in mind when it comes to the stability of viruses in water in natural conditions. Recently, Zhu et al. (2020) registered data for long periods of temperature effect against MNV in surface water. The higher the temperature, the more inactivation was reported. 7 days were needed to observe a complete inactivation at 37⁰C, compared to 42 days when the temperature decreased to 22⁰C. Only 2.5 log of reduction was achieved when MNV stored at 4⁰C. HAV and HEV resulted in being more resistant than MNV. UV sensibility depends on the type of virus, with HAdV being the most persistent, and HAV more resistant than PV (Fenaux et al., 2019; Hijnen et al., 2006).

Sludges

Sludges are soils of organic matter produced from wastewater that can be used as fertilizers in agricultural practices. Sludges pose a health risk if used in direct contact with food due to the presence of microorganisms and their interaction with the soils (Schlindwein et al., 2010).

In the past, various outbreaks have been reported in which the main vector has been contaminated soils; this occurs especially in developing countries (Fraun et al., 2010). Crucial points to consider include: type, saturation, pH, water conductivity, and soluble organic material. Particularly when there is a higher concentration of clays, there is higher virus adsorption and, generally, it is this that blocks viral transfer to other matrices (Gerba et al., 1981). Usually, viral particles are negatively charged which leads to an attachment to positive charges of the soils. Conversely, in neutral or alkaline environments, viruses remain free. Different enteric viruses were found in sludges maintaining their concentration before other techniques such as UV were applied (Lizasoain et al., 2018). Inactivation of MNV and HAV at 4 and 20°C is demonstrated in different sludges at alkaline pH (Arraj et al., 2005; Sobsey et al., 1980; Wei et al., 2010).

Fomites

Cross-contaminations are hard to detect and so more difficult to trace and prevent: in the worst cases bad hygiene practice on food surfaces makes the dissemination of virus even easier (Fankem et al., 2014).

Fomites include a wide diversity of common surfaces where viruses can remain infectious in both public institutions and domestic areas for long periods of time and they constitute an important source of transmission. The activity of porous and non-porous surfaces, against enteric viruses, has been thoroughly

explored (Boone and Gerba, 2007). One of the most determining factors that affects virus survival seems to be resistance to desiccation. Viruses associated to foodborne outbreaks have shown title decays when subjected to desiccation processes (Abad et al., 2001). It is clear that after numerous studies (Table 4), virus persistence in fomites depends on the type of fomite; even in the same kind of fomite, virus stability varies depending on the type of surface (porous or non-porous), temperature, humidity, presence of interference substances, etc. (D'Souza et al., 2006; Greening and Cannon, 2016; Mattison et al., 2007). It has been shown that enteric virus can result infectious for up to 45 days on surfaces (Reynolds et al., 2005).

Norovirus surrogates, norovirus, HAV or HAsV, have been recovered from several porous and non-porous surfaces like stainless steel, porcelain, paper, rubber or wood (Abad et al., 2001; Rönnqvist and Maunula, 2016; Tuladhar et al., 2013), and environmental conditions also affect their persistence. Abad et al. (2001) showed decreasing titers of HAsV and HAV at 4°C and 20°C. For HAsV, 4 log of reduction was obtained on porcelain and paper surfaces after 60 and 90 days, respectively, at 4°C. At higher temperature, 20°C, the time of

Table 4. Survival, recovery and/or reduction of enteric viruses on different food contact surfaces.

Surfaces	Virus	°C	RH (%)	Detection time	Rec (%)	Red (Log)	References
Glass	FCV	4		57 days*			Doultree et al. (1999)
					4.5		Taku et al. (2002)
Stainless steel	Norovirus	25			18		Scherer et al. (2009)
	HAV					0.98	Rajiuddin et al. (2020)
	MNV					0.45	
Paper	HAV	21		21 days		1.45	Leblanc et al. (2019)
	MNV			3 days		2.91	
	HAV	4	90	>60 days*			Abad et al. (1994)
Wood	AdV						
	MNV	25	30	>30 days*			Kim et al. (2012)
Aluminum	RV	20	45-55	>60 days*			Abad et al. (1994)
Plastic	HAV	25				0.56	Rajiuddin et al. (2020)
	MNV		85-95	60 days*		1.15	
China	HAsV	4					Abad et al. (2001)
Fingerpads	MNV	26	40-45	1 min	57		Tuladhar et al. (2013)
	Norovirus			10 min	20		
				1 min	80		
				10 min	31		

Rec: Recovery; Red: Reduction; FCV: Feline Calicivirus; NoV: HAV: Hepatitis A virus; MNV: Murine norovirus; AdV: Adenovirus; RV: Rotavirus; HAsV: Astrovirus *Detected until that time.

reduction decreased considerably for both surfaces to 7 days. In contrast, HAV showed different behaviour in the same conditions and on the same surfaces with no decreasing titers after 7 days on porcelain and no more than 1.5 log on paper material. Similar results were obtained by D'Souza et al. (2006) for FCV on ceramic, porcelain and stainless steel. FCV appeared to be less resistant on a stainless steel surface, although infectious viral titer was reported after 7 days on the other two surfaces.

Contaminated hands

There is evidence that contaminated hands can spread the viruses to fomites and food products, especially in places where food is prepared and manipulated, thereby facilitating transmissions and infections (Rönngqvist and Maunula, 2016). However, the persistence of norovirus on hands is lower than on inanimate surfaces (Mbithi et al., 1992). In addition, humidity is considered an essential factor for viral stability on hands and the transfer to other contact surfaces; on the contrary, drying exerts an opposite effect (Tuladhar et al., 2013). Sattar et al. (2011) suggested the persistence of infectious norovirus on hands for 2 hours using surrogates. FCV and MNV lost their viability at a rate of 1.91% or 1.65% per minute, and 35% or 45% of virus load was detected after 40 minutes on hands, respectively. A similar study carried out by Mbithi et al. (1992) determined the survival of HAV over time on fingerpads. Four hours after

initial exposure, viral recovery was detectable to 30%, showing more persistence than norovirus surrogates.

- **Handwashing**

Hand sanitizers are crucial to prevent enteric virus outbreaks caused by viral dissemination mediated by hand. Several agents have been tested with excellent results against HAV, such as 70% ethanol (EtOH) which decreases infectious titers under detectable limits after washing hands and touching stainless steel disk (Mbithi et al., 1993). Comparable effects were obtained for MNV treated with a commercial product based in 70% EtOH. This reduced viral infection by 3 log more than handwashing alone (Tung et al., 2013).

1.3.2 Human enteric virus stability in food products

Chilled food products

Some processes used in the food industry to extend the shelf-life of minimally processed fruits and vegetables can contribute to control transmission and limit the survival of enteric viruses (Rzeżutka and Cook, 2004). Chilled storage is a typical process to minimize microbial growth, respiration, browning, senescence, and organoleptic loss in fresh food. Yet, many studies (Table 5) have determined that several enteric viruses and some surrogates survive at chilled temperatures (2 – 11°C) for long periods of food storage.

Frozen products

Previous studies confirmed that if active viral particles are present in food before freezing, they may remain infectious during frozen storage. It has been observed that low temperatures have limited ability to reduce enteric viruses (Li et al., 2015). Similarly, Harper et al. (2009), reported that low temperatures improve the survival of viruses. As an example, survival titers of HAV were detected in oysters and clams after -20°C storage (Webby et al., 2007).

Different foodborne outbreaks caused by enteric viruses have been described after the consumption of contaminated frozen food such as berries and shellfish (Bozkurt et al., 2020; Randazzo et al., 2018; Randazzo and Sánchez, 2020). In 2013 and 2014, Italy reported an HAV outbreak with 1.803 cases caused by the consumption of frozen strawberries (Scavia et al., 2017). A similar HAV outbreak was detected in Sweden caused by the consumption of frozen strawberries produced in Poland (Enkirch et al., 2018). At the same time, HAV and norovirus have been detected in frozen shellfish as the causal agent of individual cases of gastrointestinal illness in Australia, Chile, Spain or New Zealand, among others (Gyawali et al., 2019; Hassard et al., 2017; Loutreul et al., 2014; Webby et al., 2007)

Table 5. Viral reduction of human enteric viruses and surrogates in different food products under refrigerated temperatures.

Food product	Virus	Time (Days)	Temp (°C)	Red (Log)	Reference
Apples	Norovirus	7	11	0.2	Mormann et al.(2010)
Cabbage	PV	14	4	NDT	Kurdzial et al.(2001)
Carrot	HAV	4	6	UDL	Croci et al.(2002)
	RV	25	4	3.5	Badawy et al.(1985)
Fennel	HAV	7	6	UDL	Croci et al.(2002)
Green onions			16	10	1.15
		PV	14	4	NDT
Lettuce	HAV	9	6	2.46	Croci et al.(2002)
	Norovirus	10	7	1.78	Lamhoujeb et al.(2008)
	RV	30		2.00	Badawy et al.(1985)
	PV	15	4	0.09	Kurdzial et al.(2001)
Radish	RV	30		3.5	Badawy et al.(1985)
Spinach leaves	HAV		5.4	1.00	Shieh et al. (2009)
Strawberry	FCV	6	4	2.5	Mattison et al.(2007)
Mincemeat	Norovirus	2	6	NDT	Mormann et al.(2010)
Shellfish (mussels)	HAV	30	4	1.7	Hewitt and Greening (2004)
		28		NDT	
Turkey	Norovirus	10	7	1.5	Lamhoujeb et al.(2008)
Yogurt	PV	24		UDL	Strazynski et al.(2002)
Bottle water	HAV	360	4	0.68	Biziagos et al.(1988)
	PV			1.16	

Red: Reduction; PV: Poliovirus; HAV: Hepatitis A virus; RV: Rotavirus; FCV: Feline calicivirus; NDT: No decreasing titers; UDL: Undetectable limits

Modified atmosphere packaging

Modified atmosphere packaging (MAP) is a relatively novel process where the natural air inside of the package is replaced by a mixture of inert gases to protect sensory and organoleptic characteristics of the food, mostly raw or ready-to-eat products. The objective of MAPs is to extend the shelf-life of products by inhibiting microbial growth, although it does not affect enteric viruses, as they do not replicate in food products. This fact was supported by Bidawid et al. (2001), who demonstrated the survival of HAV under different modified atmospheres and in other conditions. Mattison et al. (2010) also investigated the prevalence of norovirus in ready-to-eat packaged leafy greens, reporting that 54% of the total tested samples (328) tested positive for norovirus by RT-PCR and only 1% for rotavirus.

1.4. EFFICACY OF METHODS COMMONLY USED IN THE FOOD INDUSTRY

Food products are susceptible to contamination with microorganisms implying a risk to consumers. Because of this, industry has been using different processes to guaranty the safety and also quality of food products. It must be noted that the majority of these processes are designed for bacterial inactivation, so information about their effectiveness on human enteric viruses is often lacking. Generally, food manufacturing processes are classified using 4 levels, negligible, low, medium, or high according to their capacity for viral inactivation with 1, 2, 3 or 4 log of titers reduction, respectively (Koopmans and Duizer, 2004).

1.4.1. Acid products

Natural or formulated food products depend on acidified conditions to keep microbial growth under control. Nevertheless, acid conditions do not seem to be enough to inactivate potential viral contamination in food. Different studies have shown different effects depending on the type of targeted virus. Different results were reported for the RV strain SA11 in pineapple juice at 28°C for 3 h (pH 3.60) or the RV strain DS1 in fruit juice at 4°C for 3 days (pH 3.01) with

reductions of 1.70 log and no significant decrease, respectively (Leong et al., 2008; O'Mahony et al., 2000).

At pH 1, HAV did not report decreased titers after 5h of exposure at 25°C or 1.5h at 37°C, however enterovirus 9 and PV could not be detected at the same pH after 2h of incubation under the same conditions (Scholz et al., 1989). Studies on food reported worsening norovirus titers using RT-qPCR of only 1.7 log after 24 days at 6°C at pH 5.0 to 5.5 in potato salad and no reductions in noodle salad at the same experimental conditions or in ketchup sauce after 58 days at pH 5.4 (Mormann et al., 2010).

1.4.2. Freeze-drying products

Dried products are common products in the food industry due to longer stability and industrial shelf-life. The freeze-drying process is considered one of the best methods to maintain the high quality of products using dehydration. Dehydration essentially reduces water activity in food products preserving them against the proliferation of microorganisms (Bhatta et al., 2020; Oyinloye and Yoon, 2020). Only a few studies exist on the detection and survival of enteric viruses in these products (Table 6).

Table 6. Antiviral effectiveness of freeze-drying process on enteric viruses.

Virus tested	Determination method	Food matrix	Freeze-drying time (h)	Reduction (Log)	Reference
HAV	Infectivity (PFU)	Strawberries	24	0.5	Zhang Yan et al. (2017)
				1.42	
	Infectivity (TCID ₅₀)	Basil	18	1.71	Butot et al. (2009)
		Raspberries		1.30	
		Parsley		1.24	
		Blackberries		1.79	
	Blueberries	1.42			
MNV	Infectivity (PFU)	Strawberries	24	1.80	Zhang Yan et al. (2017)
				1.47	
		Basil		2.06	
Norovirus	RT-qPCR	Raspberries	18	1.21	Butot et al. (2009)
		Parsley		3.52	
		Blackberries		1.71	
				1.71	
		Blueberries		2.67	

HAV: Hepatitis A virus; MNV: Murine norovirus.

1.4.3. Enteric virus inactivation by thermal processing

One of the most effective methods traditionally used in the food industry to preserve food safety is thermal processing. The effectiveness of thermal processing against bacteria and yeast is well-known but, up to now, few studies have explored how these processes affect enteric virus infectivity in food matrices. Viral inactivation is influenced by factors related to any food matrix (e.g. fat or protein content), the presence of organic matter and the level of contamination (Li et al., 2012).

Exposing viruses to medium temperatures for extended time periods (e.g., pasteurization) is more powerful in viral inactivation than using high temperatures in shorter periods (e.g., HTST). Nonetheless, to assure a total reduction of viral contamination, a temperature of at least 90°C inside food for 1.5 min is needed (Alimentarius, 2012).

However, the occurrence of foodborne outbreaks associated with consumption after lighter treatments such as boiling, frying, stewing, grilling... reveal that common cooking practices are insufficient for the inactivation of enteric viruses (McDonnell et al., 1997). In Table 7, levels of viral reductions dependent on temperature and time are shown for HAV and norovirus (and its surrogates) in different common foods.

Table 7. Effectiveness of different temperature range against enteric viruses on foods.

Temp (°C)	Virus	Food Matrix	Time (min)	Red (Log)	References	
40	HAV	Oysters	1.1	1.0	Lee et al. (2015)	
50		Mussels	54.1			
60		Shellfish		3.2	2.0	Cappelozza et al. (2012)
90			Manila clams	10.0		
100				8.0		
250		Mussels	5.0	>3.0	Croci et al. (2005)	
40			Pepper	432	1.0	Lee et al. (2015)
50			Spinach	34.4		
60				4.5		
75			Vegetables	Basil	2.5	2.0
	Chives					
	Mint					
	Parsley					
90		Strawberries	5.0	3.2	Rajiuddin et al. (2020)	
				4.7		
95		Basil	2.5	>3.0	Butot et al., (2009b)	
		Chives				

Red: Reduction; HAV: Hepatitis A virus; HEV: Hepatitis E virus; MNV; Murine norovirus; FCV: Feline calicivirus.

Cont. Table 7.

95			Mint	2.6	>3.0	Butot et al., (2009b)
			Parsley			
99		Vegetables	Raspberries		2.2	
			Blueberries	5.0	1.6	Chen et al. (2020b)
			Strawberries		>4.5	
120	HAV		Freeze-dried berries		>4.0	Butot et al., (2009b)
80			Milk	0.5		
			Cream		>5.0	Bidawid et al. (2000)
85		Dairy foods	Milk	0.7		
			Cream	1.2		
100.5			Milk	0.1	6.0	El-Senousy et al. (2020)
	HEV	Meat	Mincemeat	5.0	3.0	Imagawa et al. (2018)
70		Shellfish	Soft-shell clams	1.5	3.3	Sow et al. (2011)
				3.0	>5.5	
75	MNV	Vegetables	Raspberries	0.3	2.8	Baert et al. (2009)
80				0.2	>7.0	Bartsch et al. (2019)
50		Meat	Turkey	6.0	>1.0	Bozkurt et al. (2015)
72				0.5		

Red: Reduction; HAV: Hepatitis A virus; HEV: Hepatitis E virus; MNV; Murine norovirus; FCV: Feline calicivirus.

Cont. Table 7.

52				8.0			
54	TV	Vegetables	Strawberries	4.0	>1.0	Ailavadi et al. (2019)	
56				1.5			
			Raspberries	0.2	>7	Bartsch et al. (2019)	
80		Shellfish	Mussels	3.0	3	Croci et al. (2012)	
			Raspberries	0.2	3.5	Bartsch et al. (2019)	
	Norovirus	Vegetables			1.8		
99				Blueberries	5.0	1.9	Chen et al. (2020b)
				Strawberries		2.1	

Red: Reduction; HAV: Hepatitis A virus; HEV: Hepatitis E virus; MNV; Murine norovirus; FCV: Feline calicivirus

1.4.4 Enteric virus inactivation by non-thermal processing

Heat application in the food industry improves food safety and the shelf-life of products, but simultaneously it produces unsatisfactory changes in organoleptic and nutritional qualities. It is well-known that some vitamins degrade or food textures are altered by the effect of temperature (San Martin et al., 2002). The use of non-thermal technologies allows industry to guarantee the reduction of pathogens without deteriorating the organoleptic, sensorial, or nutritional food qualities. Due to consumers' demand for fresh and minimally processed food, an increasing interest of both food industries and researchers are driving the development of novel techniques, such as high hydrostatic pressure, high intensity pulse light or pulse electric field, active packaging, irradiation, and natural antimicrobial compounds, which are nowadays the most relevant non-thermal technologies.

High pressure processing

High pressure processing (HPP) technique consists of immersing products in low or non-compressible liquid (typically potable water). Once the vessel is completely closed, additional liquid is injected inside the chamber and the pressure increased reaching determined values, usually between 100–700 MPa. The energy consumption of this method is not much different from that used in thermal processing; it takes the same energy to reach 400 MPa or 30°C, and no extra energy is needed to keep the pressure while the process is working

(Cheftel, 1995; Farr, 1990). Independently of the characteristics of the product, HHP ensures a homogeneous treatment of the product (Hoover et al., 1989).

HPP in the food industry is widely used and accepted. Fruit and vegetable beverages, vegetable sauces, precooked meat, ready-to-eat products, or seafood are the principal foods treated by HPP. The physical and chemical effects on food are minimal compared to thermal processes, but some changes in conformational structures have been reported for proteins due to the denaturalization that occurs at room temperature with pressures of 100-200 MPa (Cheftel, 1995; Hoover et al., 1989; Kunugi and Tanaka, 2002).

There is a wide variety of operational conditions in HPP treatments that makes it difficult to compare the viral inactivation achieved in different studies. Inactivation is time-, pressure-, and to a major extent, temperature-dependent (Jurkiewicz et al., 1995). Grove et al. (2008) reported HAV reductions of 1 and 2 log TCID₅₀/mL after 600s at 300 or 400 MPa treatments, respectively, and under the limit of detection after 300s at 500 MPa. RV titers after 300 MPa at room temperature during 70s decline by 5 log TCID₅₀/mL while FCV exposed for 120s and 180s at the same pressure decreased to 3.6 TCID₅₀/mL and under the limit of detection, respectively. MNV was found to be more resistant than FCV, requiring 400 MPa or more for its complete inactivation (Khadre and Yousef, 2002; Kingsley et al., 2013). Leon et al. (2011) led a clinical human trial, in which volunteers ingested HPP treated oysters infected with human

GI.1. Volunteers who consumed oysters treated at 600 MPa pressure did not manifest infection, on the contrary, most who consumed oysters treated at 400 MPa showed clinical symptoms. These were, more accentuated in those who ingested oysters HPP treated at 25⁰C than at 6⁰C. Additionally, the effectiveness of HPP against TV was tested by DiCaprio et al. 2019 in strawberry puree, showing decreasing titers of more than 1 and 2 log when treated at 400MPa at 4⁰C for 2 min (DiCaprio et al., 2019).

Radiation

Radiation has been demonstrated to be an effective non-thermal process to ensure food safety (Farkas, 1998). UV, ionizing radiation, gamma radiation and electron beam (e-beam) irradiation are different radiation technologies applied to foods. On August 22, 2008, the US FDA published a final report that allowed and controlled the use of radiation in food production and processing after demonstrating that irradiation on fresh iceberg lettuce and spinach keeps their nutritional value and is safe for consumers (Food and Drug Administration, 2008).

UV is categorized as A, B or C levels depending on the wavelength ranges, 320-400 nm, 280-320 nm or 200-280 nm, respectively. FDA approved the UV-C level to control microorganism on food surfaces (Food and Drug Administration, 2008). Fino and Kniel (2008) applied UV to different fresh products and observed that UV at 240 mWs/cm² decreased HAV titers by 2.6

log in strawberries and more than 4.5 log in lettuce and green onions. MNV titers showed higher decays on fomites than in liquids due to the presence of organic matter when UV operated at 59 mWs/cm² (Jean et al., 2011).

Atmospheric pressure plasma

Atmospheric pressure plasma (APP) is a novel technique that uses a neutral ionized gas with a high amount of reactive species of molecules, protons and ions (Wan et al., 2009).

APP has been investigated on inoculated surfaces achieving reductions for norovirus, MNV, FCV or MS2 bacteriophage (Alshraideh et al., 2013). Bae et al. (2015) investigated the effect of APP against MNV on fresh meat reaching more than 2 log of viral reduction after 5 min of exposure while only 0.7 log was reported for TV in packaged lettuce (Min et al., 2016).

Washings

The presence of microbial load on the surface of fruit and vegetables could be reduced by washings with potable water, or 10 to 100-fold more if a sanitizer was used. The selection of the sanitizer or disinfectant to be used is crucial, primarily because it should be registered for its use in food (food grade) but also because its effectiveness can be affected by factors such as contact time, temperature, water composition, pH or organic charge (Table 8).

Table 8. Antiviral effect of different sanitizers against human norovirus and its surrogates in food.

Sanitizer	Virus	Food application	Conc. (ppm)	Time (min)	Red (Log)	References
Chlorine	FCV	Leafy vegetables	200	2	2.9	Allwood et al. (2004)
		Berries			-	UDL
	Norovirus	surfaces	33	1	1.4	Kingsley et al. (2014)
			189		4.1	
HAV	Strawberry	50	5	3.4	Zhou et al. (2017)	
		200		UDL		
Chlorine dioxide	FCV	Parsley	10	10	1.5	Butot et al. (2008)
	HAV	Raspberry			UDL	
Electrolyzed water	Norovirus	Suspension	250	1	4.8	Moorman et al. (2017)
		Stainless steel			0.4	
Ozone	MNV	Green onions	6	10	2.0	Hirneisen et al. (2013)
		Raspberry			3	1
	FCV	Water	6	4	6.0	Hirneisen et al. (2013)

Conc.: Concentration; Red: Reduction; FCV: Feline calicivirus; HAV: Hepatitis A virus; MNV: Murine norovirus; UDL: Undetectable limits.

Cont. Table 8.

Ozone	FCV	Lettuce	6	5	2.0	Hirneisen et al. (2013)
	HAV	Water	0.4		3.9	Hall and Sobsey (1993)
Peracetic (PAA)	Norovirus		195	-	<1.0	Kingsley et al. (2014)
	MNV	Lettuce	250		1.0	Baert et al. (2009)
			100	2	2,3	Fraisie et al. (2011)
	FCV				3,2	

Conc: Concentration; Red: Reduction; FCV: Feline calicivirus; HAV: Hepatitis A virus; MNV: Murine norovirus; UDL: Undetectable limits

Active packaging

Several guidelines list different measures to avoid food contamination or enhance food safety along the food chain (Alimentarius, 2012; WHO, 2015). Among them, the packaging industry is currently interested in a novel style of packaging that mixes materials with antimicrobial components to enhance food safety by controlling, reducing or inhibiting microbial load (Yildirim and Röcker, 2018).

The objective of food-packaging materials with antiviral activity is the inactivation of human enteric virus that is likely to be present in raw or partially cooked food. Human enteric viruses have been targeted in different food packaging applications with antiviral properties. Typically, compounds exerting antiviral activity are incorporated in the food packaging material. However, the final viral inactivation depends on food type, contact time, and temperature among others (Fabra et al., 2013; Martínez-Abad et al., 2013; Su and D'Souza, 2011). For example, silver applied in films showed an antiviral effect against norovirus surrogates, exerting reductions of 4.4 log for FVC after 24h of contact. Another aspect related to food packaging stems from the importance of consumer demand for green consumerism with minimally processed or fresh food. This demand has led to the use of edible films and coatings. The compounds included in such coatings are considered as Generally Recognised As Safe (GRAS) and can be eaten (Bakkali et al., 2008).

1.5. NATURAL COMPOUNDS

The search for new alternatives to traditional chemical and physical food decontamination is one of the main objectives of the WHO and food industry (WHO, 2013). To this end, and in addition to the demand from consumers for worthwhile and “green” alternatives to antimicrobial chemicals, a special interest has emerged in natural compounds; these display low toxicity and a lack of secondary effects as most of them are GRAS compounds (Burt, 2004).

The moderate costs for production and their abundance in raw materials make natural compounds an important source of antivirals and a great alternative to chemicals allowing them to be used as harmless formulations for food. While many *in vitro* studies have been performed with natural compounds in conjunction with human enteric viruses, only few assays have looked at their activity in food applications (Ryu et al., 2015).

For decades, several secondary metabolites that are present in plant extracts, otherwise known as phytochemicals, have been extensively studied because of their antimicrobial properties. Furthermore, their synergetic activity with many drugs to battle against multi drug-resistant pathogens has been reported (Ayaz et al., 2019; D'Souza, 2014). For these reasons, studies on natural compounds propose them as an alternative method to control enteric virus. Phytochemicals can be divided into different categories: organic acids,

essential oils (EOs), polypeptides, polyphenols, proanthocyanins, saponin and polysaccharides. In the last decade, antiviral studies have focused primarily on polyphenols and EOs (Battistini et al., 2019; Li et al., 2013; Zhang et al., 2012). For most of them, the antiviral action mechanisms are not fully understood, but in general terms, there is damage to different important structures involved in infection (viral capsid or cell membranes), which subsequently affects viral attachment to host cells (Chiang et al., 2003; Li et al., 2013). As summarized in Table 9, numerous natural compounds have been evaluated against enteric virus or surrogates.

Table 9. Natural compounds effectiveness against enteric virus.

Natural compound	Virus	Conc. (%)	Time/ Temperature	Inactivation (Log)	References
Carvacrol	FCV	0.5	2h/37°C	UDL	Sanchez et al. (2015)
		1.0			
	MNV	0.5			
		1.0			
		4.0			
HAV	0.5	1.0	Gilling et al. (2014)		
	1.0	1.0	Sanchez et al. (2015)		
Grape seed extract	FCV	0.1	5min/37°C	1.1	Joshi et al. (2015)
			10min/37°C	20	
	MNV		15min/37°C	19	
			24h/37°C	3.9	
	HAV		15min/37°C	1.1	
			24h/37°C	24	
Aloe vera extract	MNV	0.4	72h	>2.0	Ng et al. (2017)
Thyme			1h/3°C	0.5	Moussaoui (2013)
	Essential oils	HAV	2.0	2h/37°C	NDT
mint		1h/3°C		0.9	Moussaoui (2013)
		Oregano		2h/4°C	0.6
FCV				2h/37°C	3.8

Conc.: Concentration; FCV: Feline calicivirus; MNV: Murine norovirus; HAV: Hepatitis A virus; UDL: Undetectable limits; NTD: No decreasing titers.

Cont. Table 9.

Essential oils	Oregano	FCV	2.0	2h/4°C	0.3	Elizaquível et al. (2013)
		HAV		2h/37°C	0.1	Sánchez and Aznar (2015)
	Zataria	MNV	0.1	2h/4°C	0.3	Elizaquível et al. (2013)
		FCV		2h/37°C	1.0	
		HAV		2h/4°C	4.5	
		HAV		2h/37°C	NDT	
	clove	HAV	1.0	2h/37°C	0.4	Sánchez and Aznar (2015)
		MNV		2h/4°C	0.7	
		FCV		2h/37°C	0.8	
		FCV		2h/37°C	3.8	
		FCV		2h/4°C	0.3	
	lemon		0.5		2.8	
	Orange				2.1	
	Grapefruit	HAV	0.1	1h/25°C	2.9	Battistini et al. (2019)
	Rosemary		0.01		2.9	
Chitosan	MNV	0.7	3h/37°C	NDT	Davis et al. (2012)	
	FCV			>3.1		

Conc.: Concentration; FCV: Feline calicivirus; MNV: Murine norovirus; HAV: Hepatitis A virus; UDL: Undetectable limits; NTD: No decreasing titers.

1.5.1 Natural compound categories

Polyphenols

This group of phytochemicals is one of the most important due to its antioxidant, anticarcinogenic or neuroprotective properties, among others. Because of this, the trade in polyphenols has increased as functional food in the last decade (Li et al., 2013; Yilmaz and Toledo, 2004).

Foods such as cranberries, grapes or pomegranates are rich in polyphenols. Although the exact antiviral components which contribute to antiviral activity have not been identified, different studies have reported that cranberry and pomegranate juices (Lipson et al., 2007; Su et al., 2010a, 2010b) and grape seed extract (GSE) have antiviral activity against FCV, MNV and HAV (Su and D'Souza, 2011).

The mechanism of action discovered by Lipson et al., (2007) for cranberry polyphenols observed the prevention of virus replication inside the host cells. Nevertheless, Su et al., (2010a) reported changes in the viral capsids resulting in the failure of cell infection. About fifty Chinese plants were screened against human norovirus and results showed that antiviral activity was determined by the inhibition of norovirus-HBGA receptors bound by tannic acid (Zhang et al., 2012).

Essential oils and compounds thereof

EOs (and their derivatives) are aromatic compounds extracted from different plant parts. In the past, industry has used EOs as flavouring agents as well as natural antimicrobials to improve food safety (Burt, 2004; Chouhan et al., 2017).

Although there have not been so many studies looking at EOs and enteric viruses, published results suggest their use could be promising in the food sector. Carvacrol, lemongrass, allspice, mint oregano or thymol are some of these tested compounds (Elizaquível et al., 2013; Gilling et al., 2014; Kim et al., 2017; Kovač et al., 2012; Moussaoui, 2013; Sanchez et al., 2015). Significant reductions were shown by thyme, clove or allspice EO inhibiting replication by more than 3 log against MNV and FCV (Elizaquível et al., 2013; Damian H. Gilling et al., 2014; Sanchez et al., 2015). Discrepant results have been reported on the efficacy of EOs against HAV. For example, thymol was tested by Sanchez and Aznar (2015) without success, however Battistini et al., (2019) demonstrated the efficacy of lemon, grapefruit and rosemary cineole EOs on HAV, reducing viral titers by nearly 3 log.

The antiviral mechanism of EOs is dependent on the type, but generally, the action is on the capsid causing degradation and subsequently on the RNA, finally preventing the viral adsorption to host cells. Specifically, EOs denature

structural glycoproteins and proteins of the viral particle, that then completely lose their ability to infect (Damian H. Gilling et al., 2014; Reichling et al., 2009).

Polysaccharides

Polysaccharides are a group of bioactive compounds with huge structural diversity, some of them showing antiviral activity. For example, *Stevia rebaudiana*, one of the popular ingredients currently used by the food industry due to its sweetening characteristic with low calorific value, demonstrated antiviral activity against RV (Takahashi et al., 2001). Chitosan, derived from chitin, the second most abundant polysaccharide after cellulose (Akter et al., 2014), reduced FCV infectivity by more than 3 log (Amankwaah, 2013; Davis et al., 2012). Although not many natural compounds have been tested on HAV, three types of different carrageenans (iota-, lambda- and kappa-carrageenan) were assayed *in vitro* to assert their antiviral effect, resulting in up to 1.6 log reduction for iota-carrageenan at 500 mg/mL (Girond et al., 1991).

Organic acids

The use of citric acid is widespread in the food industry. Related to citric acid is citrate, which is used in food supplements and some medications. The effect of this compound on norovirus particles showed that epitopes were more accessible to antibodies making them more susceptible to inactivation (Koromyslova et al., 2015). Additionally, Hansman et al. (2012) proved that

citrate had the potential to inhibit the human norovirus interaction with HBGAs blocking host infection. This supports the idea that food rich in organic acids, such as citrus fruits, together with other bioactive compounds, may exert a stronger antiviral activity (Li et al., 2013).

1.5.2 Antiviral activity of natural compounds in food applications

Currently, only few studies deal with the use of natural compounds in food (Table 9). A direct application of different compounds has been tested on various types of food, such as jalapeño peppers, apple juice, milk, lettuce or oysters. This led to viral reductions for FCV, MNV and HAV when treated with GSE (Joshi et al., 2015; Su and D'Souza, 2013), carvacrol (Sanchez et al., 2015), and curcumin (Wu et al., 2015).

Natural compounds as sanitizers for produce

To date, chlorine has been used as the main sanitizer by the food industry, even though the European Union and the US are intending to ban its use because of the chemical residues produced (Van Haute et al., 2014, 2013). At this point, in the search for alternatives with lower risk for food and consumers, interest is being shown in the use of natural antiviral compounds in food processes, like washings. The antiviral action of carvacrol on inoculated lettuce

during washing was tested (Sanchez et al., 2015) and demonstrated reductions close to 2 log and under limit of detection for norovirus surrogates after 30 min of exposure using 1% of carvacrol. More effective was the use of GSE for peppers and lettuce washing decontamination with titers decreased by 5.02, 1.17 and 1.29 log for FCV, MNV and HAV, respectively, after 5 min (Su and D'Souza, 2013).

In spite of these promising applications of natural compounds, more studies still need to be carried out to specify treatment times and temperature conditions to adapt them to operational conditions.

Natural disinfectants for food-contact surfaces

As commented previously, cross-contamination through food contact surfaces is an important source of contamination of human enteric virus (Abad et al., 2001; Sánchez and Aznar, 2015). Food surfaces are susceptible to contamination through direct contact with body secretions, lack of hygiene from food handlers or aerosols generated by talking, sneezing, coughing or vomiting.

Nowadays, it is clear that chemical sanitizers exert great antiviral activity on food contact surfaces (Jean et al., 2011; Seymour and Appleton, 2001). Nevertheless, despite the importance of hygienic surfaces, there are no studies assessing the use of natural compounds as natural sanitizers except for GSE. Li

et al. (2012) reported decreasing titers for MNV after cleaning stainless steel surfaces with GSE at 2 mL/mg for 10 min.

Bio-Active Packaging

In recent years natural compounds have gained attention in this area beyond chemicals (Irkin and Esmer, 2015; Randazzo et al., 2018a) and various plant extracts have been incorporated into different matrices to evaluate their antiviral properties.

A novel aspect being developed in this scenario is the elaboration of edible coatings against pathogens. As mentioned earlier, food like berries, because they are consumed raw, are the principal sources of food contaminated by human enteric viruses (Butot et al., 2008). Thus, the incorporation of natural antimicrobial agents into edible coatings can be used to control virus contamination on fresh food products. Fabra et al. (2016) developed films incorporating cinnamaldehyde into a polyhydrobutyrate matrix effective against different enteric viruses. By adapting the ISO 22196:2011 the authors estimated reductions of 2.75 log for MNV and a complete inactivation for FCV, while HAV proved to be resistant. Amankwaah et al. (2013) studied the effect of GSE incorporated into chitosan films against different pathogens, showing reduced titers of 0.92, 1.89 and 2.27 log for MNV with films containing 5, 10 and 15% of GSE, respectively after 4h of exposure time. After 24h of contact, 1.90 and

3.26 log reductions were reached for the lowest concentrations tested and 15% of GSE inactivated MNV to undetectable levels.

2. OBJECTIVES

OBJECTIVES

The overall aim of this thesis is to evaluate the use of green tea extract as an innovative solution to improve viral food safety in food products and food applications. To achieve the core research objective, the following specific objectives have been defined:

1. *In vitro* characterization of the antiviral activity of green tea extract against human enteric viruses and surrogates at different concentrations, temperatures, storage time and pHs.
2. Indirect approximations to assess norovirus inactivation using green tea extract.
3. Kinetic characterization of the active compounds of green tea extract after its degradation over time and its effect against enteric viruses.
4. Evaluation of green tea extract stability and its antiviral activity under simulated gastric conditions.
5. Assessment of green tea extract antiviral activity in different food model systems and in combination with moderate temperatures.

6. Green tea extract evaluation as an antiviral natural sanitizer for food contact surfaces and fresh-cut vegetables.
7. Assessment of edible coatings incorporating green tea extract to control virus contamination in berries.

3. RESULTS

RESULTS

3.1. Characterization of the antiviral activity of green tea extract.

3.1.1. Article 1. *Effect of green tea extract on enteric viruses and its application as natural sanitizer.*



Effect of green tea extract on enteric viruses and its application as natural sanitizer



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ARTICLE INFO

Article history:

Received 24 November 2016

Received in revised form

25 April 2017

Accepted 28 April 2017

Available online 3 May 2017

ABSTRACT

In this work, the effect of green tea extract (GTE) was assessed against murine norovirus (MNV) and hepatitis A virus (HAV) at different temperatures, exposure times and pH conditions. Initially, GTE at 0.5 and 5 mg/ml were individually mixed with each virus at 5 log TCID₅₀/ml and incubated 2 h at 37 °C at different pHs (from 5.5 to 8.5). GTE affected both viruses depending on pH with higher reductions observed in alkaline conditions. Secondly, different concentrations of GTE (0.5 and 5 mg/ml) were mixed with viral suspensions and incubated for 2 or 16 h at 4, 25 and 37 °C at pH 7.2. A concentration-, temperature- and exposure time-dependent response was showed by GTE in suspension tests, where complete inactivation was achieved after overnight exposure at 37 °C for both viruses and also at 25 °C for HAV.

In addition, antiviral effect of GTE proved efficient in the surface disinfection tests since 1.5 log reduction and complete inactivation were recorded for MNV and HAV on stainless steel and glass surfaces treated with 10 mg/ml GTE for 30 min, analyzed in accordance with ISO 13697:2001. GTE was also evaluated as a natural disinfectant of produce, showing 10 mg/ml GTE reduced MNV and HAV titers in lettuce and spinach by more than 1.5 log after 30 min treatment.

The results show a potential of GTE as natural disinfectant able to limit enteric viral (cross-)contaminations conveyed by food and food-contact surfaces.

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1. Introduction

Foodborne viruses are recognized as the main causative agents among the reported outbreaks in Europe transmitted by food accounting for 20.4% of all outbreaks in 2014, and showing an increasing trend over the last 6-year period 2008–2014 (EFSA, 2015). A wide variety of viruses may be transmitted by food, nevertheless, the most frequently reported viruses are human noroviruses (NoV) causing gastroenteritis, and hepatitis A virus (HAV). Generally, the fecal-oral-route is the primary way of transmission of enteric viruses, and handlers, equipment and contaminated surfaces (fomites) could represent a reservoir of cross-contamination viral particles which become sources of secondary transmission, especially in food related environments (Abad et al.,

2001; Sánchez and Aznar, 2015).

The extremely low infectious dose (10–100 viral particles) (Teunis et al., 2008; Yezli and Otter, 2011), together with their resistance to desiccation and chemical inactivation (Cheesbrough et al., 2000; Kuusi et al., 2002), appear to be the major factors that make enteric viruses able to contaminate surfaces, persist and, finally, infect the host. To date, different non-porous (aluminum, china, glazed tile, glass, latex, plastic, polystyrene and stainless steel) and porous (cloth, different types of papers and cotton cloth) surfaces have been indicated as suitable for harboring enteric viruses (Abad et al., 2001; Boone and Gerba, 2007).

Currently the World Health Organization (WHO) is promoting the development of alternative methods for the decontamination of food (WHO, 2013). To this end, phytochemical compounds (especially plant extracts) are gaining an increasing interest among consumers, researchers and food industries mainly because (i) they are suitable for food applications since most of them are Generally Recognized as Safe (GRAS); (ii) the growing “green consumerism” trend stimulates the use of natural products; (iii) they represent natural and cheap alternatives to chemically synthesized

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antibacterials and antivirals (Burt, 2004). Even if the antibacterial and antifungal activities of many natural compounds have been extensively stated, reports on their antiviral food applications are somewhat limited.

Among natural extracts, green tea extract (GTE) is a derivative of cultivated evergreen tea plant (*Camellia sinensis* L.) of the family *Theaceae*. This polyphenolic and proanthocyanidin rich compound is a popular plant extract that has been widely used in various food and beverage applications due to its diverse health benefits including antioxidant, anti-inflammatory, and anticarcinogenic properties (Cooper et al., 2005a, b; Xia et al., 2010). GTE also demonstrated inhibitory properties against a wide range of food-borne pathogens (An et al., 2004; Gadang et al., 2008). For all these reasons, GTE has been used as a component in multiple hurdle approaches to enhance food safety and quality (Perumalla and Hettiarachchy, 2011).

Chemically, GTE mainly contains catechins, a group of flavonoids (Yilmaz, 2006) with antimicrobial properties on Gram-positive as well as Gram-negative bacteria (Gadang et al., 2008). Among the catechins constituting GTE, epigallocatechin-3-gallate (EGCG) and epicatechin gallate (ECG) showed the strongest antimicrobial (Shimamura et al., 2007) and antiviral (Dhiman, 2011; Xiao et al., 2008) activities, even when encapsulated within chitosan electro-sprayed microcapsules (Gómez-Mascaraque et al., 2016).

In the present work the effect of GTE on the infectivity of HAV and MNV, a cultivable human norovirus surrogate, was investigated. Furthermore, its application as disinfectant in vegetables and food-contact surfaces was also assessed.

2. Materials and methods

2.1. Virus propagation and cell lines

The cytopathogenic MNV-1 strain (kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA) and the HM-175/18f strain of HAV (ATCC VR-1402) were propagated and assayed in RAW 264.7 (kindly provided by Prof. H. W. Virgin) and FRhK-4 cells (kindly provided by Prof. A. Bosch, University of Barcelona, Spain), respectively. Virus stocks were subsequently produced from the same cells by centrifugation of infected cell lysates at $660\times g$ for 30 min. Infectious viruses were enumerated by determining the 50% tissue culture infectious dose (TCID₅₀) with eight wells per dilution and 20 μ l of inoculum per well (Sánchez et al., 2011).

2.2. GTE cytotoxicity on cell monolayers

A commercial green tea natural extract (GTE) (Naturex SA, France), listed as GRAS (21CFR or AAFCO), soluble in water, with an EGCG content of 40–50% was used in the present study. Firstly, different concentrations (0.5–10 mg/ml) of GTE were added to individual wells of confluent RAW 264.7 and FRhK-4 cells in 96-well plates and incubated 2 h under 5% CO₂. Thereafter cells were added with 150 μ l of DMEM supplemented with 2% of fetal calf serum (FCS) and further incubated for 2–15 days, depending on the cell line. Cytotoxicity effects were determined by visual inspection under the optical microscope.

2.3. Antiviral effect of GTE on MNV and HAV

Firstly, a preliminary assay was carried out to assess the influence of pH on GTE efficacy. To this end, about 5 log TCID₅₀/ml of MNV and HAV were treated for 2 h at 37 °C with GTE at 0.5 or 5 mg/ml prepared in PBS at different pHs (5.5, 6.5, 7.2, 8.0 and 8.5). Ten-fold dilutions of GTE-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. The decay of HAV and MNV titers was calculated as $\log_{10}(N_t/N_0)$, where N_0 is the infectious virus titer for untreated sample and N_t is the infectious virus titer for GTE-treated samples.

Then, to further explore the antiviral effect on MNV and HAV, GTE diluted in PBS at pH 7.2 was added to virus suspensions in DMEM with 2% FCS (ca. 6–7 log TCID₅₀/ml) at final concentrations of 0.5 and 5 mg/ml. Samples were further incubated at 37, 25 or 4 °C in a shaker (150 rpm) for 2 or 16 h (overnight) to mimic different environmental conditions. Experiments were performed in triplicate and infectious viruses were estimated as described above.

2.4. Surface disinfection tests on stainless steel and glass discs

GTE was further evaluated as food-contact surface sanitizer based on ISO 13697:2001 standard (ISO/TS 13697:2001). Briefly, MNV and HAV stocks were diluted 1:1 with the interfering substance, 0.3 and 3 g/l bovine serum albumin (BSA) in PBS pH 7.2, for clean and dirty working conditions, respectively. Then 50 μ l of resulting inocula (5–6 log TCID₅₀/ml) were spotted into the middle of a clean and disinfected stainless steel and glass discs (2 \times 2 cm) and dried at room temperature (RT) for about 15 min, and verified by visual inspection. Afterward, 100 μ l of two different concentrations of GTE (5 and 10 mg/ml) prepared on hard water (ISO/TS 13697:2001) were spotted on the inoculated discs, followed by incubation at RT for 15 or 30 min. Then, the effect of GTE was stopped with a neutralizer (DMEM supplemented with 10% FCS) and the viruses were recovered by continuous pipetting and titrated by cell culture assays (see paragraph 2.1). As a positive control sample, the inoculated discs were treated with 100 μ l of hard water instead of a GTE solution. The decay of virus titers was calculated as $\log_{10}(N_t/N_0)$, where N_0 is the infectious virus titer for inoculated discs treated with hard water and N_t is the infectious virus titer for GTE-treated surfaces.

2.5. Water disinfection test using fresh-cut vegetables

Determination of the virucidal activity of GTE wash was performed by adapting the procedure described by Su and D'Souza (2013a). Briefly, locally purchased fresh lettuce (*Lactuca sativa* L.) and spinach (*Spinacia oleracea*) were cut in pieces of 2 \times 2 cm and sterilized with UV light in a safety cabinet under laminar flow for 15 min prior to virus inoculation. Then, suspensions at two concentration levels (approx. 5 and 6 log TCID₅₀/ml) of MNV or HAV were seeded independently by distributing 50 μ l over spots onto the vegetable surface. Inoculated samples were air dried in a laminar flow hood for about 15 min. Thereafter, 100 μ l of PBS or a GTE solution at 5 or 10 mg/ml was added for 15 or 30 min to inoculated vegetable samples. The action of GTE was stopped with a neutralizer (DMEM supplemented with 2% FCS) and the viruses were recovered and titrated by cell culture assays (see paragraph 2.1).

2.6. Statistical analysis

Treatments were performed in triplicate. The post-hoc Tukey's method ($p < 0.05$) was used for pairwise comparison and to determine differences among the mean numbers of viruses determined after the various treatments (XLSTAT, Addinsoft SARL).

3. Results

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

The initial cytotoxicity assay showed that GTE diluted in PBS 7.2 was cytotoxic at concentrations that exceeded 5 mg/ml for RAW and FRhK cell lines. Thus, this was the highest concentration of GTE added to evaluate the effects against MNV and HAV suspensions. As showed in Fig. 1, both viruses were affected by GTE depending on pH conditions. In particular, GTE at 5 mg/ml reduced both MNV and HAV titers by more than 1 log TCID₅₀/ml after 2 h exposure at 37 °C in slight acidic solutions (pH 6.5). The same GTE concentration resulted in a complete inactivation (below the detection limit of 1.15 log TCID₅₀/ml) of both viruses at neutral (7.2) and slightly alkaline solutions (8 and 8.5). On the opposite, 0.5 mg/ml GTE showed generally poor inhibitions in the pH range tested, with slightly greater reductions detected at alkaline pHs (8 and 8.5).

The infectivity of MNV and HAV after GTE treatments at different temperatures and exposure times is reported in Table 1 and Table 2, respectively. In general, the exposure time deeply influenced the inactivation rates, with longer incubation times resulting in higher inactivation. For instance, GTE at 5 mg/ml incubated at 37 °C for 2 h reduced 2.42 and 1.08 log the infectivity of MNV and HAV, respectively, while poor inhibitions (<1 log) were achieved at lower temperatures. Moreover, overnight incubation of GTE at 5 mg/ml statistically decreased HAV infectivity at 4 °C and 25 °C (Table 2). In particular, after overnight exposure, complete inactivation was reported at 37 °C for both viruses, while HAV was completely inactivated also at 25 °C (Table 2).

As expected GTE at 5 mg/ml showed statistically higher inactivation rates compared to the lower concentrations for both viruses, and this was observed for all the suspension assays (Fig. 1, Tables 1 and 2).

3.2. Effect of GTE in surface disinfection using stainless steel and glass discs

Reductions in the infectious titers of MNV and HAV inoculated in stainless steel and glass, either in clean and dirty conditions, with or without GTE added as a surface sanitizer, are shown in Tables 3 and 4. In clean surfaces, significant reduction ($p < 0.05$) in MNV infectivity was observed in steel and glass discs for both GTE concentrations and exposure times (Table 3).

Infectious HAV titers, in the same experiment set, were reduced by less than 1 log when GTE was applied for 15 min. On the contrary, GTE, when applied in dirty conditions, was more effective against HAV than MNV. Moreover, a complete inactivation (below the recovery limit) of HAV was achieved after 30 min of contact with 10 mg/ml of GTE for both materials tested in both cleanliness conditions. In dirty conditions, GTE at 10 mg/ml for 30 min reduced MNV infectivity by 2.09 and 1.64 log in stainless steel and glass discs, respectively (Table 4), while after 15 min of contact time, only a marginal reduction (0.42 and 0.33 log, stainless steel and glass discs) was obtained. Moreover, no remarkable differences were reported between virus inactivation in stainless steel or glass surfaces.

3.3. Efficacy of GTE on fresh-cut vegetable surfaces

The reduction of MNV and HAV titers on inoculated lettuce and spinach leaves after 30 min treatment with GTE, at room temperature, are shown in Fig. 2. Sensorial parameters of vegetables were not affected (data not shown). When inoculated at low titer, significant reduction of MNV infectivity was observed (1.38 and 1.80, respectively) after treating lettuce and spinach with GTE at 10 mg/

ml for 30 min. Lower reductions were obtained in case of 5 mg/ml GTE treatment. Treatments with GTE at 10 mg/ml showed 2.59 log reduction in spinach inoculated with higher viral titer. Regarding HAV, reductions below detectable limits were obtained when lettuce and spinach inoculated with low virus titers were treated for 30 min with GTE at 10 mg/ml. Whereas, high viral titers were reduced by 0.79 and 1.37 in lettuce and spinach, respectively.

4. Discussion

Nowadays the rising incidence of viral foodborne outbreaks leads to an increasing interest on the enteric virus inactivation and natural compounds fulfil both food safety and health concerns. Even if many natural compounds have already been characterized for their antimicrobial activity, including their application in food, limited information is available for their antiviral properties (Li et al., 2013; Sánchez, 2015) and lesser if considering their use in food applications. For example, carvacrol was effective in reducing MNV and feline calicivirus (FCV) infectivity in lettuce and lettuce wash water (Sánchez et al., 2015). Similarly, cinnamaldehyde was recently incorporated into a novel multilayer system for food contact applications able to reduce both FCV and MNV titers (Fabra et al., 2016). Likewise, grape seed extract was effective reducing FCV, MNV and HAV infectivity on lettuce, jalapeño peppers, apple juice and milk (Joshi et al., 2015; Su and D'Souza, 2013a).

Studies on catechins, that constitute around the 40–50% of GTE, demonstrated their activity against human immunodeficiency virus, herpes simplex virus, and hepatitis B virus (Hsu, 2015; Nance et al., 2009). More recently FCV and MNV have been used to test the antiviral properties of catechins, resulting in a complete inhibition of FCV suspension (10^5 PFU/ml) after 6 h exposure with 3.13 mg/ml GTE fractions (Oh et al., 2013) and a complete inhibition of MNV suspension (6.45 TCID₅₀/ml) after overnight exposure with 2.5 mg/ml EGCG (Gómez-Mascaraque et al., 2016). Our results using GTE are in line with those previously reported, since the overnight exposure of MNV to GTE at 5 mg/ml resulted in a complete inactivation of the virus at 37 °C. As well, in a study focused on determining the antiviral effects of tannins on 12 different enveloped and non-enveloped viruses, Ueda et al. (2013) observed 4.1 and 1.7 log reduction for FCV and MNV, respectively, after only 3 min exposure at RT with 0.25% GTE. The different experiments set ups do not allow strict comparisons among these and our results, even if a clear dose-, temperature- and time-dependent antiviral effect of GTE (or EGCG, as main compound) was highlighted in all the studies. This trend has been observed for other natural antiviral compounds, such as curcumin (Randazzo et al., 2016), cinnamaldehyde (Fabra et al., 2016), different tannins and flavonoids (Su and D'Souza, 2013b; Ueda et al., 2013), many essential oils (Sánchez and Aznar, 2015) and plant extracts (Joshi et al., 2015; Su and D'Souza, 2013a).

Furthermore, a clear pH dependent antiviral effect was observed, since higher reductions were reported for GTE at basic pH. Similarly, Isaacs et al. (2011) reported a pH dependent effect of EGCG against herpes simplex virus, where no antiviral activity was recorded below pH 7.4. They indicated that EGCG derivatives provided an antiviral activity over a broader pH range than EGCG itself, especially due to the formation of EGCG dimers with two gallate groups. On the opposite, the grape seed extract (GSE), a natural extract rich in phenolic compounds, enhanced its antiviral effect in acid conditions against MNV and HAV (Joshi et al., 2015). These differences could be due to structural changes of compounds and/or by the formation of by-products, that, together with the synergistic effect of pH, finally result in a loss or a gain of antiviral effectiveness. Additionally, the different pH conditions at which GTE and GSE better inhibit enteric viruses could constitute an

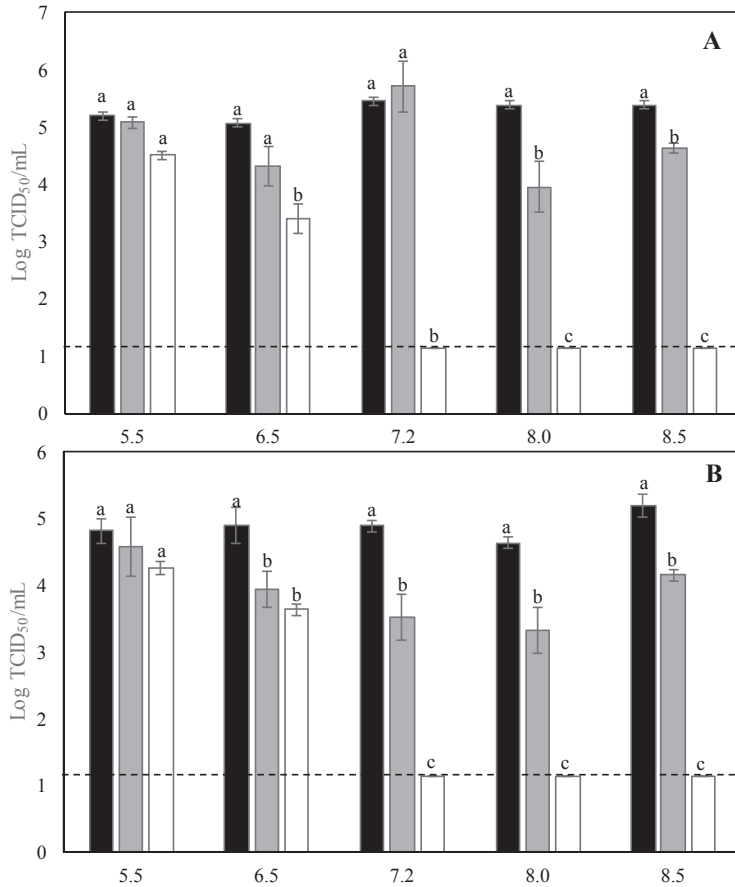


Fig. 1. Reduction of murine norovirus (MNV) (A) and hepatitis A virus (HAV) (B) titers (log TCID₅₀/ml) after 2 h treatment at 37 °C with GTE at different pHs. Each bar represents the average of triplicates. Within each column for each virus, different letters denote significant differences between treatments (P < 0.05). Black bars: Control; grey bars: 0.5 mg/ml GTE; white bars: 5 mg/ml GTE; dashed line depicts the detection limit.

Table 1
Effect of green tea extract (GTE) against murine norovirus (MNV) at different temperatures and exposure times.

Temperature	GTE (mg/ml)	Exposure time			
		2 h		Overnight	
		Recovered titer	Log reduction	Recovered titer	Log reduction
37 °C	0	6.74 ± 0.26a	–	4.87 ± 0.43a	–
	0.5	6.28 ± 0.31a	0.46	3.99 ± 0.14a	0.88
	5	4.32 ± 0.00b	2.42	<1.15b	>3.72
25 °C	0	6.28 ± 0.26a	–	6.32 ± 0.12a	–
	0.5	6.28 ± 0.07a	0.00	5.99 ± 0.26b	0.33
	5	5.32 ± 0.69a	0.96	4.57 ± 0.12c	1.75
4 °C	0	5.32 ± 0.25a	–	6.32 ± 0.36a	–
	0.5	5.32 ± 0.12a	0.00	5.95 ± 0.00a	0.37
	5	5.11 ± 0.40a	0.21	6.07 ± 0.00a	0.25

Each treatment was done in triplicate. Within each column for each temperature, different letters denote significant differences between treatments (P < 0.05).

advantage to be used to prepare a sanitizer solution effective in several environmental surroundings.

Additionally, we observed variability in antiviral activity of GTE at pH 7.2 (Table 1, Table 2 and Fig. 1). One plausible reason could

derive from the fact that the antiviral activity of GTE is most likely due to catechin derivatives rather than EGCG itself, given the evolution of these compounds at the various pH conditions (Falcó et al., 2017). Therefore more studies needs to be performed for further

Table 2
Effect of green tea extract (GTE) against hepatitis A virus (HAV) at different temperatures and exposure times.

Temperature	GTE (mg/ml)	Exposure time			
		2 h		Overnight	
		Recovered titer	Log reduction	Recovered titer	Log reduction
37 °C	0	6.49 ± 0.14a	–	5.39 ± 0.09a	–
	0.5	6.07 ± 0.12b	0.42	3.45 ± 0.12b	1.94
	5	5.41 ± 0.14c	1.08	<1.15	>4.24
25 °C	0	5.82 ± 0.00a	–	5.51 ± 0.26a	–
	0.5	5.62 ± 0.14a	0.20	3.28 ± 0.07b	2.23
	5	5.12 ± 0.79b	0.70	<1.15	>4.36
4 °C	0	6.32 ± 0.12a	–	6.15 ± 0.28a	–
	0.5	6.32 ± 0.12a	0.00	5.95 ± 0.12a	0.20
	5	6.07 ± 0.21a	0.25	4.99 ± 0.31b	1.16

Each treatment was done in triplicate. Within each column for each temperature, different letters denote significant differences between treatments ($P < 0.05$).

Table 3
Reduction of murine norovirus (MNV) and hepatitis A virus (HAV) titers (log TCID₅₀/ml) on clean surfaces after GTE treatments at room temperature.

Surface	Exposure time (min)	GTE (mg/ml)	MNV		HAV	
			Recovered titer	Log reduction	Recovered titer	Log reduction
Steel	15	0	6.12 ± 0.26a	–	4.70 ± 0.12a	–
		5	5.11 ± 0.07b	1.01	4.37 ± 0.07b	0.33
		10	4.70 ± 0.12c	1.42	3.95 ± 0.33c	0.75
	30	0	5.41 ± 0.19a	–	5.07 ± 0.53a	–
		5	3.76 ± 0.09b	1.68	2.99 ± 0.07b	2.08
		10	1.95 ± 0.12c	3.46	<1.15	>3.92
Glass	15	0	6.37 ± 0.31a	–	4.64 ± 0.38a	–
		5	4.64 ± 0.08b	1.73	4.62 ± 0.26a	0.02
		10	4.41 ± 0.56b	1.96	3.74 ± 0.07b	0.90
	30	0	6.57 ± 0.12a	–	3.95 ± 1.06a	–
		5	5.26 ± 0.08b	1.31	<1.15	>2.80
		10	4.78 ± 0.64b	1.79	<1.15	>2.80

Each treatment was done in triplicate. Within each column for each surface and exposure time, different letters denote significant differences between treatments ($P < 0.05$).

Table 4
Reduction of murine norovirus (MNV) and hepatitis A virus (HAV) titers (log TCID₅₀/ml) on dirty surfaces after treatment with GTE at room temperature.

Surface	Exposure time (min)	GTE (mg/ml)	MNV		HAV	
			Recovered titer	Log reduction	Recovered titer	Log reduction
Steel	15	0	4.95 ± 0.12a	–	4.43 ± 0.16a	–
		5	4.64 ± 0.26b	0.31	3.12 ± 0.26b	1.31
		10	4.53 ± 0.16b	0.42	3.20 ± 0.00b	1.23
	30	0	5.62 ± 0.26a	–	4.64 ± 0.09a	–
		5	3.51 ± 0.08b	2.11	3.53 ± 0.07b	1.10
		10	3.53 ± 0.26b	2.09	<1.15	>3.49
Glass	15	0	4.53 ± 0.38a	–	5.07 ± 0.35a	–
		5	4.45 ± 0.38a	0.08	3.87 ± 0.14b	1.21
		10	4.20 ± 0.00a	0.33	3.46**	1.61
	30	0	5.76 ± 0.26a	–	4.20 ± 0.00a	–
		5	4.01 ± 0.26b	1.75	<1.15	>3.05
		10	4.12 ± 0.07b	1.64	<1.15	>3.05

Each treatment was done in triplicate. Within each column for each surface and exposure time, different letters denote significant differences between treatments ($P < 0.05$). * One positive sample out of three; ** Two positive sample out of three.

developments.

The antiviral activity of GTE (2.42 and 1.08 log reductions for MNV and HAV, respectively, with 5 mg/ml GTE after 2 h at 37 °C) shows similar trends to the antiviral effects observed and reported for others natural compounds. For example, GSE at 2 mg/ml reduced MNV and HAV titers by 1.41 and 2.18 at the same experimental conditions (Joshi et al., 2015). Oregano essential oil at 2% reduced by 1.6 and 0.1 log MNV and HAV titers while 1% carvacrol was reported to decrease MNV titers from ~6 log TCID₅₀/ml to undetectable levels and HAV titers were reduced by almost 1 log (Sánchez et al., 2015; Sánchez and Aznar, 2015).

Moreover, in this study we observed greater reductions for HAV

than MNV in the suspension assays after overnight incubation and vegetable and surface assays. In line with these results, recently EGCG has shown to be more efficient on HAV than MNV at room temperature (Falcó et al., 2017). And similar pattern was reported by Su and D'Souza (2011) for the GSE, which also contains catechin, epicatechin and epicatechin-3-O-gallate (reviewed by Perumalla and Hettiarachchi, 2011).

In the fresh-cut vegetable industry, leafy greens present a high risk of cross-contamination (EFSA, 2015), and chlorine, the most popular food-grade disinfectant, has been already limited in some European countries due to the formation of chemical by-products (i. e. trihalomethanes) (Van Haute et al., 2013). Therefore,

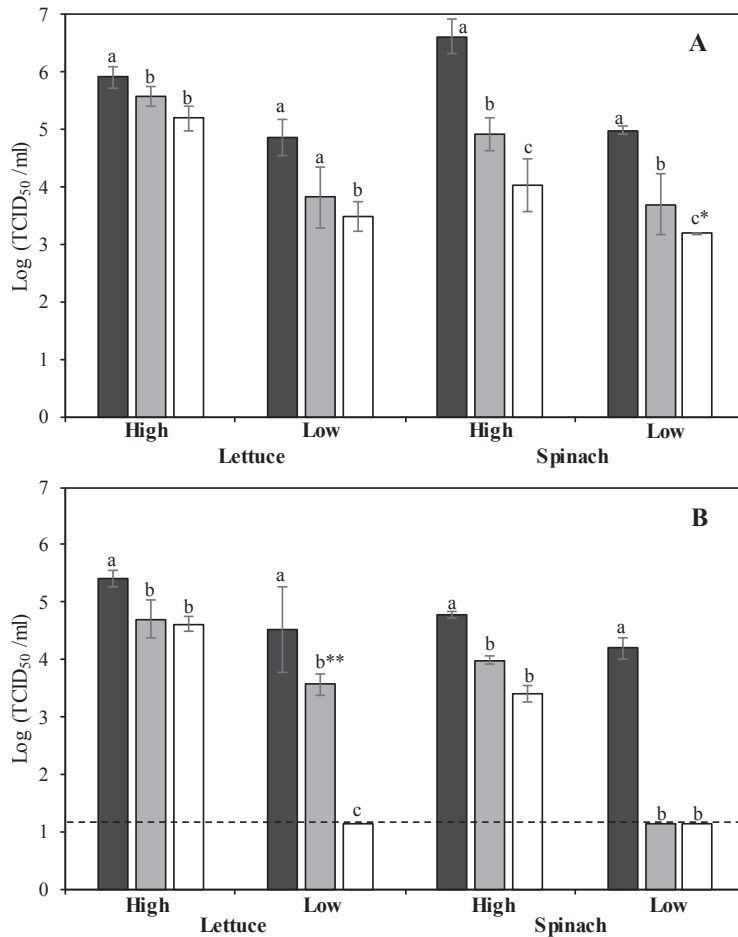


Fig. 2. Reduction of murine norovirus (MNV) (A) and hepatitis A virus (HAV) (B) titers (log TCID₅₀/ml) on lettuce and spinach surfaces after 30 min treatment with GTE at room temperature at two inoculation level.

Each bar represents the average of triplicates. Within each vegetable and each inoculation level, different letters denote significant differences between treatments ($P < 0.05$). *One positive sample out of three; **Two positive samples out of three.

Black bars: Control; grey bars: 5 mg/ml GTE; white bars: 10 mg/ml GTE; dashed line depicts the detection limit. High: high inoculation level; Low: low inoculation level.

following such rising need for alternative disinfection solutions we successfully assayed GTE as surface disinfectant in both vegetables and environmental surfaces. In the surface disinfection tests, efficacy of GTE on food contact surfaces showed better performance compared to others natural compounds tested as sanitizers, such as GSE. In fact, Li et al. (2012) reported only a marginal reduction (< 1 log PFU/ml) for MNV in stainless steel surfaces treated with 2 mg/ml of GSE for 10 min in clean conditions. Our results showed a significant decrease (> 1.5 log TCID₅₀/ml) of MNV titers on stainless steel and glass surfaces treated with 10 mg/ml of GTE for 30 min while complete inactivation was reported for HAV in clean and dirty glass and stainless steel surfaces.

Usually, reduced antiviral effects have been reported when a compound is tested in dirty conditions mainly due to its binding and masking effects of the organic load (such as protein) interfering with the effectiveness of treatment (Li et al., 2013). Our results are

partially in accordance with those masking effects, since MNV showed slightly greater reductions when applied on clean surfaces than on dirty ones. On the contrary, HAV reduction was not affected by the presence of protein in the surface tests.

In the vegetable sanitation tests, GTE treatments were also more effective for HAV than MNV. In particular, MNV was reduced by 2.59 log when treated with 10 mg/ml of GTE, while HAV titers decreased below the detection limit (> 4 log reduction). Higher reductions were observed in spinach than in lettuce for both viruses, possibly due to the different morphological characteristics of leaves.

Assessment of natural compounds applications in vegetable sanitation is scarce, so far only carvacrol and GSE have been evaluated as natural sanitizers against enteric virus contamination. Treating lettuce with carvacrol at 1% for 30 min reduced MNV titers by 1.8 log (Sánchez et al., 2015). Similarly, Su and D'Souza (2013a) reported a marginal reduction (< 0.8 log) for MNV and 1.23 and

1.29 log reductions for HAV, respectively on lettuce and pepper, after 1 min treatment with 1 mg/ml of GSE.

The antiviral mechanisms of GTE, as well as many natural antiviral compounds, have not yet been elucidated, but some conjectures could be done taking in consideration the effect of EGCG on virus (Friedman, 2007; Gómez-Mascaraque et al., 2016; Oh et al., 2013; Steinmann et al., 2013). Indeed, EGCG inhibits the infectivity of a diverse group of enveloped and non-enveloped viruses by interrupting viral attachment to cell membrane receptors through its high affinity but nonspecific binding to viral surface proteins. GTE could reduce MNV and HAV infectivity by a similar mechanism, or at least it could be considered as a synergism of inactivation. Screening of individual phenolic acid constituents of GTE that exhibit antiviral properties needs further investigation, as well as its application on food. In addition, delivering these antiviral compounds through advanced technologies such as electrospayed material for food packaging or through detergent formulations to be applied for hand and equipment disinfection, may provide promising results and a wide range of applications.

Acknowledgment

This work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO) (RYC-2012-09950) and the Spanish National Institute for Agriculture and Food Research and Technology (INIA) co-financed by the European Social Fund (Project RTA2014-00024-C03). GS was supported by the “Ramón y Cajal” Young Investigator. The authors thank María Cuenca for the technical support.

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3.1.2. Article 2. Fostering the antiviral activity of green tea extract for sanitizing purposes through controlled storage conditions.



Fostering the antiviral activity of green tea extract for sanitizing purposes through controlled storage conditions



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ARTICLE INFO

Article history:

Received 3 July 2017

Received in revised form

24 August 2017

Accepted 30 August 2017

Available online 1 September 2017

Keywords:

Enteric viruses

Natural compounds

Food-contact surfaces

ABSTRACT

Food-contact surfaces is considered an important vehicle for the indirect transmission of foodborne viral diseases with enteric viruses, especially human norovirus (HuNoV) and hepatitis A virus (HAV). The aim of the present study was to evaluate the antiviral activity of green tea extract (GTE) at room temperature as a function of pH and storage time and to relate it with changes in composition as a consequence of degradation and epimerization reactions in the storage conditions. The obtained results revealed that freshly prepared GTE was very effective in inactivating murine norovirus (MNV) and HAV at neutral and alkaline pH but was ineffective at pH 5.5. Additionally, storage of the solutions for 24 h at various pH conditions significantly increased their antiviral activity. The reduction in MNV and HAV infectivity was related to the formation of catechin derivatives during storage, as demonstrated by HPLC/MS analysis.

In addition, the GTE prepared under the optimal conditions (24 h storage and pH 7.2) was applied at a concentration of 5 mg/mL for only 15 min on stainless steel and glass surfaces for sanitizing purposes, showing a reduction of more than 1.5 log of MNV and HAV infectivity. These findings indicate that GTE can be used as a natural disinfectant for decontamination of food contact surfaces, thus preventing the indirect transfer of enteric viruses to food or persons.

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1. Introduction

In the last decade an increased incidence of foodborne outbreaks have been attributed to human enteric viruses, most notably human norovirus (HuNoV) and hepatitis A virus (HAV). The World Health Organization (WHO) has recently estimated that HuNoV causes approximately 120 million illnesses and 35,000 deaths, while HAV causes 14 million cases and 28,000 deaths attributed to foodborne illness each year (WHO, 2013).

HuNoV and HAV are transmitted through the fecal-oral route, either by direct contact with a virus-infected person, or through ingestion of contaminated water or food (reviewed by Sánchez, 2015; de Graaf, van Beek, & Koopmans, 2016). Additionally, their low infectious dose, ranging from 10 to 100 (Teunis et al., 2008) together with their prolonged stability in the environment, make

HuNoV and HAV extremely infectious and highly transmissible through environmental surfaces (fomites).

All these factors justify the highest health risk associated to HuNoVs outbreaks in closed communities (e.g. nursing homes, hospitals, cruise ships, etc.) as exposure sites where common areas and facilities contribute to a rapid virus transmission (European Food Safety, European Centre for Disease, & Control, 2016; Hall, Wikswo, Pringle, Gould, & Parashar, 2014; Hedlund, Rubilar-Abreu, & Svensson, 2000).

In recent years, because of the great consumer awareness and concern regarding synthetic chemical additives or sanitizers, foods and food-contact surfaces treated with natural compounds have become very popular since they are considered safe, cheap, and pose no risk to both the environment and population (Li, Baert, & Uyttendaele, 2013). Many different natural compounds have been used as antimicrobials for food applications, either directly added into the product formulation, incorporated into the packaging material or used as a natural sanitizers (Irkin & Esmer, 2015). Although bactericidal efficacy of most of these natural compounds has been well established, current knowledge of the antiviral

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efficacy for food applications is limited and requires further investigation (reviewed by Li, Baert et al., 2013; Li, Lo et al., 2013).

So far several natural compounds have already been characterized for their antiviral activity (reviewed by Li, Baert et al., 2013; Li, Lo et al., 2013), however limited information is available for their use in food applications. For example, grape seed extract (GSE) was effective in reducing HuNoV surrogates, i.e. feline calicivirus (FCV) and murine norovirus (MNV), as well as HAV infectivity on lettuce, apple juice and milk (Joshi, Su, & D'Souza, 2015; Su & D'Souza, 2013). Likewise, carvacrol was effective in reducing MNV and FCV infectivity in lettuce and lettuce wash water (Sanchez, Aznar, & Sanchez, 2015). Recently, green tea extract (GTE) was proved very effective as a natural sanitizer to control viral contamination since 1.5 log reduction and complete inactivation were recorded for MNV and HAV on stainless steel and glass surfaces treated with 10 mg/mL GTE for 30 min (Randazzo, Falcó, Aznar, & Sanchez, 2017). Similarly, GTE demonstrated good effectiveness to decrease contamination with human adenoviruses on lettuces, strawberries and green onions (Martí, Ferrary-Américo, & Barardi, 2017).

GTE is a complex mix of various components; it is a source of polyphenols, especially flavonoids, being catechins the major substances which make up approximately 30% of the dry weight of the tea leaf (Yilmaz, 2006). Epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC) are the major catechins in GTE (Abdel-Rahman et al., 2011). Several studies confirmed that these catechins have anti-oxidative, thermogenic, anticarcinogenic, and anti-inflammatory effects depending on its concentration (Chacko, Thambi, Kuttan, & Nishigaki, 2010). Moreover, recent findings suggest that the antiviral activity of EGCG against HuNoV surrogates and HAV is due to the activity of catechin derivatives rather than EGCG itself (Falcó et al., 2017).

In this work, the antiviral activity of GTE over time was evaluated against MNV and HAV at 25 °C, using solutions freshly prepared at different pHs and after different times of storage. Moreover, HPLC analysis of the GTE solutions was performed to disclose if GTE derivatives would explain the differences in antiviral activity displayed. Finally, the optimum GTE solution was evaluated as a natural disinfectant for food-contact surfaces.

2. Materials and methods

2.1. Virus propagation and cell lines

MNV-1 was propagated and assayed in RAW 264.7 cells, both were kindly provided by Prof. H. W. Virgin (Washington University School of Medicine, USA). HAV (strain HM-175/18f) was purchased from ATCC (VR-1402), which was propagated and assayed in confluent FRhK-4 cells (kindly provided by Prof. A. Bosch, University of Barcelona, Spain). Semi-purified MNV and HAV viruses were harvested at 2 days and 12 days after infection, respectively, by three freeze-thaw cycles of infected cells followed by centrifugation at 660 × g for 30 min to remove cell debris. Infectious viruses were enumerated by determining the 50% tissue culture infectious dose (TCID₅₀) with eight wells per dilution and 20 µL of inoculum per well using the Spearman-Kärber method (Pinto, Diez, & Bosch, 1994).

2.2. Antiviral activity of GTE solution depending on aging

GTE (Naturex SA, France), listed as GRAS (21CFR or AAFCO), with an EGCG content of approximately 40–50% was used in this study. It was dissolved in PBS (pH 7.2) to obtain a concentration of 1 mg/

mL. Antiviral effect of GTE solutions was evaluated at time 0 (freshly prepared), after 24 h and seven days of storage. Each solution was mixed with an equal volume of HAV and MNV suspensions (ca. 5 log TCID₅₀/mL) getting a final concentration of GTE of 0.5 mg/mL, followed by incubation at 25 °C in a shaker for 2 h. Positive control was virus suspensions in PBS under the same experimental conditions. Each treatment was performed in triplicate. Confluent RAW 264.7 and FRhK-4 monolayers in 96-well plates were used to evaluate the effect of GTE as described above. Antiviral activity of GTE was estimated by comparing the number of infectious viruses on suspensions without GTE and on the GTE-treated virus suspensions. The decay of HAV and MNV titers was calculated as log₁₀ (N_x/N₀), where N₀ is the infectious virus titer for untreated samples and N_x is the infectious virus titer for GTE-treated samples.

2.3. Effect of GTE depending on aging and pH

In order to elucidate the effect of pH and aging on the antiviral activity of GTE, MNV and HAV suspensions were ten-fold diluted in PBS at different pHs (5.5, 7.2 and 8.5) and immediately incubated with a freshly prepared GTE solution at 0.5 and 5 mg/mL prepared in PBS at pH 5.5, 7.2 and 8.5. Moreover, GTE solutions at different pHs were stored for 24 h and further incubated with virus suspensions (1:1). Samples were incubated for 2 h at 25 °C in a shaker. Positive controls were virus suspensions in PBS under the same experimental conditions. Infectious viruses and effectiveness of the treatments were calculated as described above.

2.4. Time dependent effects of GTE

The effect of time over GTE solution was tested using a concentration of 10 mg/mL solution prepared by dissolving the supplied powder in PBS at pH 7.2 and stored for 24 h. This solution was then mixed with an equal volume of each virus to reach viral titers about 5 log TCID₅₀/mL and a final concentration of GTE of 5 mg/mL. Then samples were incubated in a water-bath shaker at 150 rpm at 25 °C for 15, 60, 120, 240 and 480 min. Positive controls were virus suspensions added with PBS at pH 7.2 under the same experimental conditions.

2.5. HPLC analysis

GTE solutions (5 mg/mL) in PBS at different pHs (i.e. 5.5, 7.2 and 8.5) were subjected to HPLC-MS analysis. Samples were analyzed after different storage periods: 0, 1 and 7 days. An Agilent 1290 HPLC system equipped with an Acquity UPLC BEH C18 column (Waters, 50 mm × 2.1 mm, 1.7 µm of particle size) was used, following the method described in Gómez-Mascaraque, Soler, and Lopez-Rubio (2016). The injection volume was 10 µL. Eluent A was water and eluent B methanol, both slightly acidified with 0.1% formic acid. 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). The parameter settings used were: ion spray voltage –4500 V, temperature 450 °C, curtain gas 30 psi, ion source gas 50 psi. Data were evaluated using the XIC manager in the PeakView™ software (version 2.2). The compounds detected were tentatively identified with the aid of the Phenol Explorer and Chemspider databases (<http://phenol-explorer.eu>, <http://www.chemspider.com>).

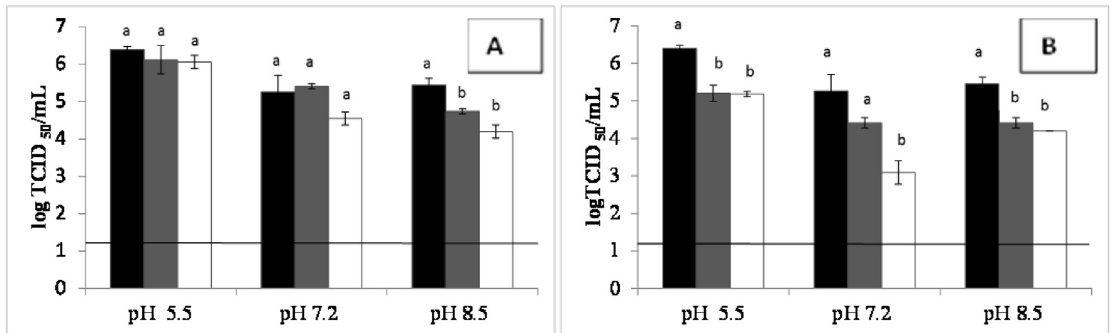


Fig. 1. Infectivity of murine norovirus (MNV) (log TCID₅₀/mL) after 2 h treatment at 25 °C with solutions of GTE freshly prepared (A) and after 24 h storage (B) at different pHs. Black: Untreated, grey: GTE 0.5 mg/mL, white: GTE 5 mg/mL. Each column represents the average of triplicates. Within each column for each pH, different letters denote significant differences between treatments ($P < 0.05$). Solid line depicts the detection limit.

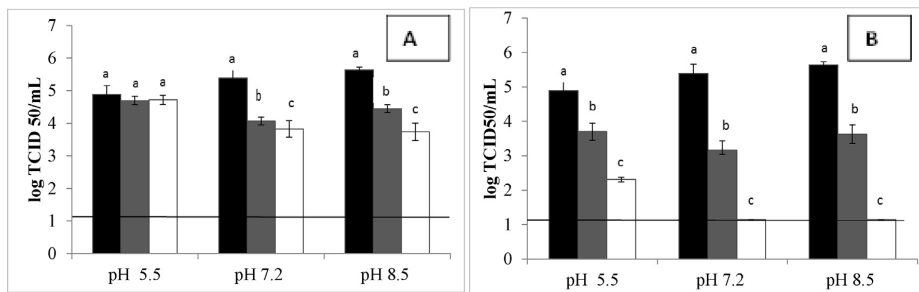


Fig. 2. Infectivity of hepatitis A virus (HAV) (log TCID₅₀/mL) after 2 h treatment at 25 °C with solutions of GTE freshly prepared (A) and after 24 h storage (B) at different pHs. Black: Untreated virus, grey: GTE 0.5 mg/mL, white: GTE 5 mg/mL. Each column represents the average of triplicates. Within each column for each pH, different letters denote significant differences between treatments ($P < 0.05$). Solid line depicts the detection limit.

Table 1

Green tea derived phenolic compounds identified in the HPLC/MS spectra of GTE.

RT ^a	m/z	Tentative identification
1.6	761 [M-H] ⁻	Theasinensin B
2.4/4.8	305 [M-H] ⁻	Epigallocatechin/gallocatechin
2.6/5.3	289 [M-H] ⁻	Epicatechin/catechin
4.8/5.8	169 [M-H] ⁻	Gallic acid
4.9/5.9	913 [M-H] ⁻	Theasinensin A
4.8	457 [M-H] ⁻ ; 915 [2M-H] ⁻	Epigallocatechin gallate
5.3	579 [M-H] ⁻	Kaempferol 3-O-xylosyl-glucoside
5.8	457 [M-H] ⁻ ; 479 [M + Na-2H] ⁻ ; 915 [2M-H] ⁻	Gallocatechin gallate
6.2	471 [M-H] ⁻ ; 493 [M + Na-2H] ⁻	Methylated EGCG
6.6/7.0	441 [M-H] ⁻	Epicatechin gallate/catechin gallate
6.6	883 [M-H] ⁻	P2
7.5	425 [M-H] ⁻	Epiafzechelin gallate
7.9/8.0	463 [M-H] ⁻	Quercetin galactoside
8.0	609 [M-H] ⁻	Theasinensin C/Prodelfinidin B4/Quercetin rutinoside
8.5	317 [M-H] ⁻	Myricetin
8.5/8.7	447 [M-H] ⁻	Kaempferol hexose/quercetin rhamnose
8.7	593 [M-H] ⁻	Kaempferol rutinoside
9.0/9.5	301 [M-H] ⁻	Quercetin
9.8	563 [M-H] ⁻	Theaflavin
10.3	285 [M-H] ⁻	Kaempferol

RT^a = retention time (min).

2.6. Surface disinfection tests on stainless steel and glass discs

Surface disinfection tests were performed adapting the ISO 13697:2001 standard (ISO/TS, 13697:2001) by adding 50 μ L of MNV

and HAV suspensions (around 5 log TCID₅₀/mL) on the stainless steel and glass discs (2 \times 2 cm) and let dry completely at room temperature (RT). Subsequently, 100 μ L of a solution of GTE (5 mg/mL, pH 7.2) prepared on hard water, according to the ISO

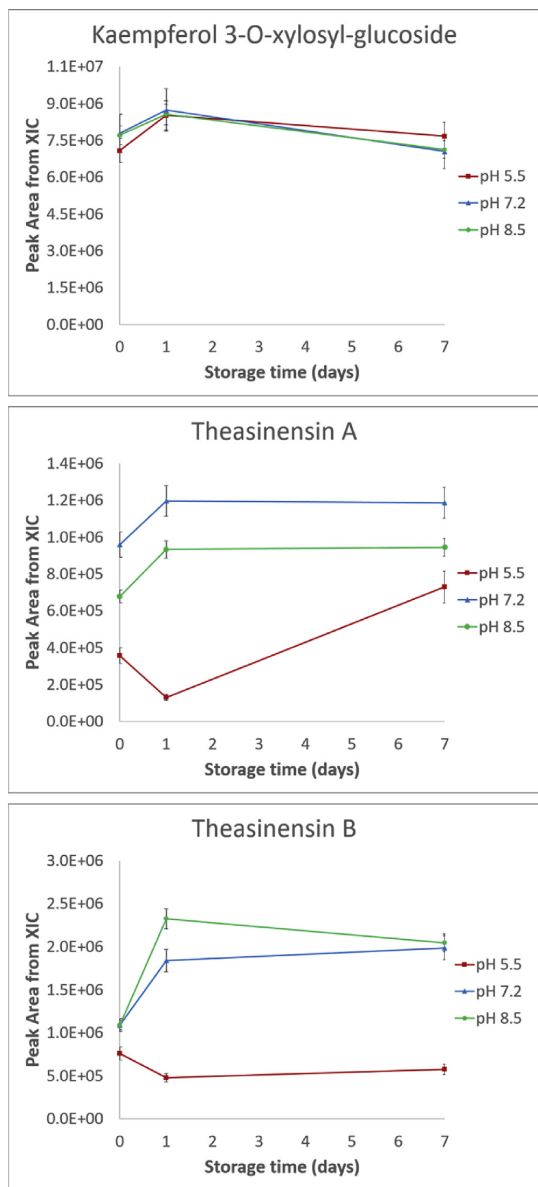


Fig. 3. Time- and pH-dependent changes in the concentration of kaempferol 3-O-xylosyl-glucoside, theasinensin A and theasinensin B in buffered solutions of the green tea extract. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

13697:2001, and stored for 24 h before the experiments were spotted on the inoculated discs, followed by incubation at RT for 5 or 15 min. Then the effect of GTE was neutralized by adding Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS). Positive controls were performed using discs treated with 100 μ L of hard water without GTE. HAV and MNV were recovered and titrated as described above. Infectious viruses

and effectiveness of the treatments were calculated as described above.

2.7. Data analysis

Results from three replicates of the treatments and controls were statistically analyzed 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. Antiviral activity of GTE solutions as a function of storage conditions

Initially, evolution of antiviral activity of GTE upon the time was evaluated. Titers of MNV and HAV before and after treatment with a freshly prepared GTE solution (time 0), after 24 h storage (time 1) or after 7 days storage (time 7) are shown in Fig. S1. GTE solution (0.5 mg/mL) decreased the titer of MNV by 0.23, 1.92 and 0.04 log while for HAV reductions were of 0.87, 2.02 and 0.69 with freshly prepared, after 24 h storage and 7 days storage, respectively.

Figs. 1 and 2 clearly show that the antiviral activity of GTE solutions was dependent on the pH of the solutions as well as on the storage time. In particular, GTE at 0.5 and 5 mg/mL significantly reduced MNV titers by 0.7 and 1.25 log after a 2 h exposure at 25 °C in slightly alkaline solutions (pH 8.5) (Fig. 1A). Similar reductions were reported after treatment with GTE stored for 24 h (Fig. 1B). On the opposite, freshly prepared GTE in neutral (pH 7.2) or acidic solutions (pH 5.5) showed no MNV inactivation while after 24 h storage, MNV titers were reduced by 1.19 and 1.21 log (at pH 5.5) and 0.85 and 2.17 log (at pH 7.2), when using 0.5 and 5 mg/mL of GTE solutions respectively (Fig. 1A and B).

For HAV, freshly prepared GTE at 0.5 and 5 mg/mL significantly reduced HAV titers by 1.30 and 1.50 log in neutral solutions (pH 7.2) and by 1.19 and 1.90 log in slightly alkaline solutions (pH 8.5), respectively (Fig. 2A). Greater reductions were obtained when the GTE solution was stored for 24 h (Fig. 2B). For instance, GTE at 5 mg/mL reduced HAV infectivity by 2.58 log in acidic solutions (pH 5.5) while HAV infectivity was reduced below the detection limit in neutral (pH 7.2) and slightly alkaline solutions (pH 8.5) (Fig. 2B).

3.2. HPLC analysis

Table 1 summarizes the main phenolic compounds found in the GTE solutions. These compounds were tentatively identified based on the values of m/z obtained from the mass spectra. The peak areas ascribed to each phenolic compound in the extracted-ion chromatograms (XIC) and, thus, their concentration was different depending on the pH as previously observed for EGCG solutions (Falcó et al., 2017) and on the incubation time. Figs. S2–S4 of the Supplementary Material depict these changes, which were the result of the degradation and epimerization of the tea-derived polyphenols. In general, a decrease in polyphenols concentration was observed upon storage, due to their degradation, with the greatest drops occurring within the first 24 h after preparation of the GTE solutions (Figs. S2–S4). However, three exceptions to this general trend were observed, which are shown in Fig. 3. The concentration of kaempferol 3-O-xylosyl-glucoside in the solutions slightly increased after 24 h of storage, irrespective of the pH conditions assayed. On the other hand, the concentration of theasinensin A and theasinensin B dramatically changed both with pH and time of storage, being considerably higher in alkaline than in acidic conditions and increasing with time.

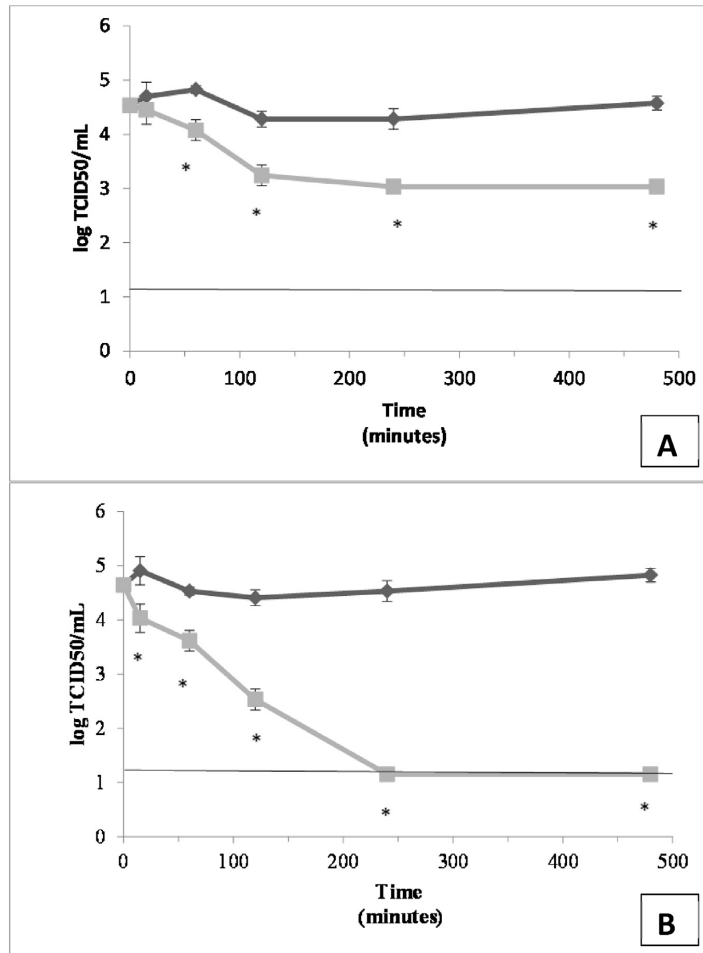


Fig. 4. Infectivity of murine norovirus (MNV) (A) and hepatitis A virus (HAV) (B) (log TCID₅₀/mL) after 0, 15, 60, 120 and 480 min treatment with GTE (5 mg/mL) at 25 °C. Black lines: untreated virus; grey lines: GTE-treated virus. Each point represents the average of triplicates. Asterisks denote significant differences between treatments ($P < 0.05$). Solid line depicts the detection limit.

3.3. Reduction of MNV and HAV titers by GTE over time

As the antiviral activity of GTE improved after 24 h of storage and at neutral or slightly alkaline pH conditions, the following experiments were performed with GTE solutions (pH 7.2) stored at RT for 24 h. Fig. 4 shows the reduction of MNV and HAV titers by GTE over the time. As it can be observed, after treatments with 5 mg/mL of GTE, MNV titers were significantly reduced by 0.25, 0.75, 1.04, 1.25 and 1.54 log after 15, 60, 120, 240 and 480 min of exposure, respectively. For HAV, under the same experimental conditions infectivity was reduced by 0.87, 0.92 and 1.88 log after 15, 60 and 120 min of exposure. Additionally, infectivity of HAV was reduced below the detection limit after 240 and 480 min of exposure.

3.4. Surface disinfection tests on stainless steel and glass discs

Fig. 5 shows titers of recovered MNV and HAV on stainless steel

and glass surfaces before, and after 5 and 15 min treatment with 5 mg/mL of a GTE solution (pH 7.2) stored at RT for 24 h. On stainless steel discs, titers of control samples were 5.45 ± 0.33 and 4.7 ± 0.13 log TCID₅₀/mL for MNV and HAV, respectively. GTE at 5 mg/mL reduced MNV infectivity by 0.75 and 1.75 log after 5 and 15 min of exposure, respectively, while for HAV reductions of 0.79 and 1.92 log were recorded. On glass discs, control titers were 5.39 ± 0.09 and 4.87 ± 0.07 log TCID₅₀/mL for MNV and HAV, respectively. No significant reduction ($P > 0.05$) in MNV and HAV infectivity was observed after 5 min treatment, while GTE reduced MNV titers by 1.27 log and HAV titers by 1.54 log after 15 min treatment.

4. Discussion

Human enteric viruses may persist on surfaces for long periods of time (Mormann, Heißenberg, Pfannebecker, & Becker, 2015). Additionally, transfer of human enteric viruses between several

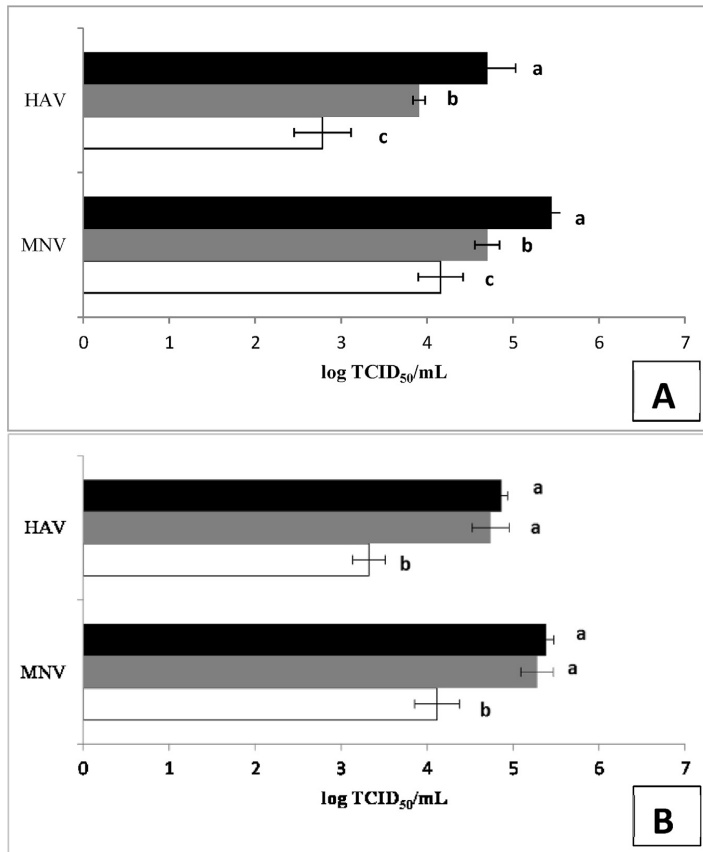


Fig. 5. Infectivity of murine norovirus (MNV) and hepatitis A virus (HAV) (log TCID₅₀/mL) before (black) and after 5 (grey) and 15 (white) min treatment with GTE (5 mg/mL) on stainless steel (A) and glass (B) discs. Each column represents the average of triplicates. Within each column for each virus, different letters denote significant differences between treatments ($P < 0.05$).

types of environmental and food surfaces have been extensively reported (Cliver, 1997; D'Souza et al., 2006; Escudero, Rawsthorne, Gensel, & Jaykus, 2012). In this context, prevention and hygiene measures should be taken to reduce the risk of cross-contamination and, thus, decrease the risk of viral pathogen transmission. Chemical compounds are commonly used for disinfecting surfaces in medical facilities. The efficacy of various chemical disinfectants and sanitizers (e.g. sodium hypochlorite, alcohols and quaternary ammonium compounds) against HAV, HuNoVs and HuNoV surrogates on different surface materials, primarily stainless steel, glass, and PVC, has been evaluated (Solomon, Fino, Wei, & Kniel, 2009). However, there are restrictions for their use for food-contact surfaces relevant to the food industry and restaurants, where other strategies such as the use of natural sanitizers are gaining attention. In this sense, GTE proved efficient in surface disinfection tests since 1.5 log reduction and complete inactivation were recorded for MNV and HAV on stainless steel and glass surfaces treated with 10 mg/mL GTE for 30 min at RT, analysed in accordance with ISO 13697:2001 (Randazzo et al., 2017). However, disinfection including 30 min contact time seems not feasible from a practical point of view. A possibility to improve the efficacy of GTE for surface disinfection is to increase the temperature to 37 °C (Randazzo et al.,

2017), but from a practical perspective neither seems to be feasible.

Therefore, the final aim of this study was to improve the antiviral activity of the GTE to be applied as a natural sanitizer. As the antiviral activity of EGCG, which constitutes around the 40–50% of GTE, has been attributed to EGCG derivatives, and given that degradation and epimerization reactions are known to take place in aqueous solutions (Falcó et al., 2017), the antiviral activity of GTE was evaluated after different solution preparation times and various pH conditions in order to promote degradation and epimerization reactions, thus increasing the amount of the potentially antiviral active compounds. The results of this study with freshly prepared GTE (0.5 mg/mL, pH 7.2 and 25 °C) showed comparable inactivation rates for MNV and HAV when compared to our earlier study (Randazzo et al., 2017), with less than 1.2 log reduction (Fig S1, 1 and 2). The present study clearly demonstrates that GTE was effective in reducing the titers of MNV and HAV in a time-preparation dependent manner, since 24 h storage of GTE solutions significantly enhanced its antiviral activity on HAV and MNV at 25 °C. For HAV, 24 h storage of GTE solutions rendered similar inactivation rates than freshly-prepared GTE evaluated at 37 °C (Falcó et al., 2017), thus highlighting the increase in effectivity upon storage of the solutions. Similar trends were observed regarding

pHs. As for GTE and EGCG at 37 °C (Falcó et al., 2017; Randazzo et al., 2017), antiviral activity of freshly-prepared GTE at 25 °C was effective in inactivating MNV and HAV at neutral and alkaline pHs but not at pH 5.5 (Figs. 1A and 2A). However, a marked improvement on the antiviral activity at pH 5.5, 7.2 and 8.5 was recorded after 24 h of storage of GTE solutions (Figs. 1B and 2B).

These results are in line with the compositional changes observed in the HPLC analysis as a consequence of the degradation and epimerization reactions of the tea-derived polyphenols in GTE, which are known to occur in aqueous solutions (Su, Leung, Huang, & Chen, 2003) with pH-dependent kinetics (Zimeri & Tong, 1999). All the main phenolic compounds found in the GTE solutions had been previously detected in tea leaves or tea-derived products (Li et al., 2013; Liu et al., 2016; Okello, Leylabi, & McDougall, 2012; Sang, Lee, Hou, Ho, & Yang, 2005; Suzuki et al., 2003; Wang, Zhou, & Jiang, 2008; Yoshino, Suzuki, Sasaki, Miyase, & Sano, 1999). While a general decrease in polyphenols concentration was observed upon storage of the GTE solutions, due to their degradation, the concentration of kaempferol 3-O-xylosyl-glucoside increased after 24 h storage, but decreased again when the solutions were stored for 7 days. These differences in the concentration of kaempferol 3-O-xylosyl-glucoside could partially explain the observed increase in the antiviral activity of the GTE solutions after 24 h storage, and subsequent decrease after longer storage periods (Fig. S1). However, no substantial differences were observed with the pH for this compound. On the other hand, the concentration of theasinensins A and B dramatically changed both with pH and time of storage, being considerably higher in alkaline than in acidic conditions and increasing with time. Similar results were obtained in a previous work in which a purified tea catechin, EGCG, exhibited increased antiviral activity against MNV and HAV at higher pHs, which could be correlated with an increase in EGCG derivatives, including theasinensin A (Falcó et al., 2017). Isaacs et al. (2011) also reported stronger antiviral activity of EGCG against herpes simplex virus at alkaline pH values, and observed a high antiviral activity of EGCG dimers regardless of the pH. This suggests that the presence of catechin dimers such as theasinensins A and B, in combination with kaempferol 3-O-xylosyl-glucoside, strongly contributed to the antiviral activity of the GTE used in this work against MNV and HAV. Results revealed that GTE has a pH dependent antiviral effect for both viruses at 25 °C, showing greater inactivation rates at neutral and slightly basic pHs, greatly enhanced upon storage of GTE solution for 24 h.

Studies evaluating natural compounds for virus inactivation within food service environments remain somewhat limited (Li, Baert et al., 2013; Li, Lo et al., 2013; Randazzo et al., 2017). In the surface disinfection tests, GTE treatments were more effective as previously reported (Randazzo et al., 2017) due to the fact that GTE solution was prepared 24 h before its use, allowing degradation and epimerization of compounds. Randazzo et al. (2017) reported only a marginal reduction for MNV and HAV in stainless steel surfaces treated with freshly-prepared GTE solution (5 mg/mL) for 15 min while on glass surfaces, while infectivity was reduced by 1.73 and 0.02 log TCID₅₀/mL for MNV and HAV, respectively. In the present study, a slight inactivation increase was recorded (1.75 and 1.92 log TCID₅₀/mL for MNV and HAV, respectively) on stainless steel treated with 5 mg/mL of GTE for 15 min (Fig. 5). Moreover, 24 h storage of the GTE solution improved its antiviral efficacy on HAV on glass surfaces, while similar inactivation rates were reported for MNV.

In conclusion, this work demonstrates that by modulating the pH and storage conditions of GTE solutions, the antiviral activity of this natural compound can be fostered. These findings could be of high relevance, not only to formulate natural disinfectants for environmental surfaces, but also to be applied as a natural washing

of fresh produce. For example, Polyphenon 60 from green tea was effective against human adenovirus but not for MNV in organic fresh products (Martí et al., 2017). Thus, aging of the solution may improve the efficacy of this natural sanitizer. However, as antiviral activity of GTE decreased after 7 days storage (Supplementary Material Fig. S1), encapsulation of GTE could be explored as an alternative for the formulation of GTE-based sanitizers (Gomez-Mascaraque, Sanchez, & Lopez-Rubio, 2016).

The findings from this study suggest that the enhanced antiviral activity of GTE is probably related to the change in composition and, more specifically, in the amount of catechin derivatives, as a function of pH and storage time, as observed from the HPLC/MS results. Therefore GTE may be a powerful natural tool for decontamination of food contact surfaces, preventing the indirect transfer of enteric viruses to food or persons. Moreover, these results open other possibilities like wiping with cleaning cloths (Gibson, Crandall, & Ricke, 2012), applying other cleaning regimens or use other natural compounds with potential synergistic antiviral activity to improve the efficacy of GTE as natural disinfectant.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO) (RYC-2012-09950 and AGL2015-63855-C2-1) 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. GS was supported by the “Ramon y Cajal” Young Investigator. Laura G. Gómez-Mascaraque is recipient of a predoctoral contract from the Spanish Ministry of Economy and Competitiveness (MINECO), Call 2013.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.foodcont.2017.08.037>.

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3.1.3 Article 3. *Effect of epigallocatechin gallate at different pH conditions on enteric viruses.*



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

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ARTICLE INFO

Article history:

Received 11 January 2017

Received in revised form

23 March 2017

Accepted 30 March 2017

Available online 31 March 2017

Keywords:

Catechins

Hepatitis A virus

Murine norovirus

Natural antimicrobials

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 food-borne viral reduction.

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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), gallicocatechin (GC), catechin (C) and epigallocatechin gallate (EGCG) have been found to be the main catechins present in GTE (Kajiji

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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 auto-oxidation 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, Elizaquivel, & 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 (TCID₅₀) with eight wells per dilution and 20 µl of inoculum per well using the Spearman-Kärber method (Pintó, Diez, & Bosch, 1994).

Faecal suspension of human norovirus genogroup II genotype 4 (GI.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 NaNO₃ (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 TCID₅₀/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 log₁₀ (N_x/N₀), where N₀ 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				25 °C				4 °C			
	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		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	6.49 ± 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

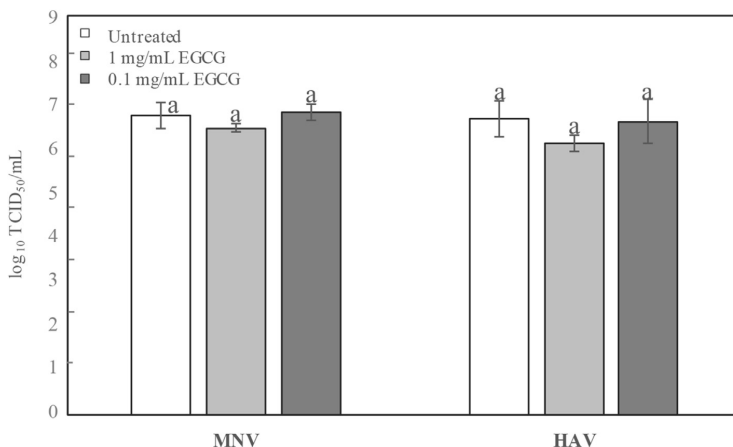


Fig. 1. Effect on murine norovirus (MNv) and hepatitis A virus (HAV) titers after pretreatment of RAW and FRhK-4 cells with EGCG at 0.1 and 1 mg/mL. Titers are the means ± standard deviations of results of three replicates.

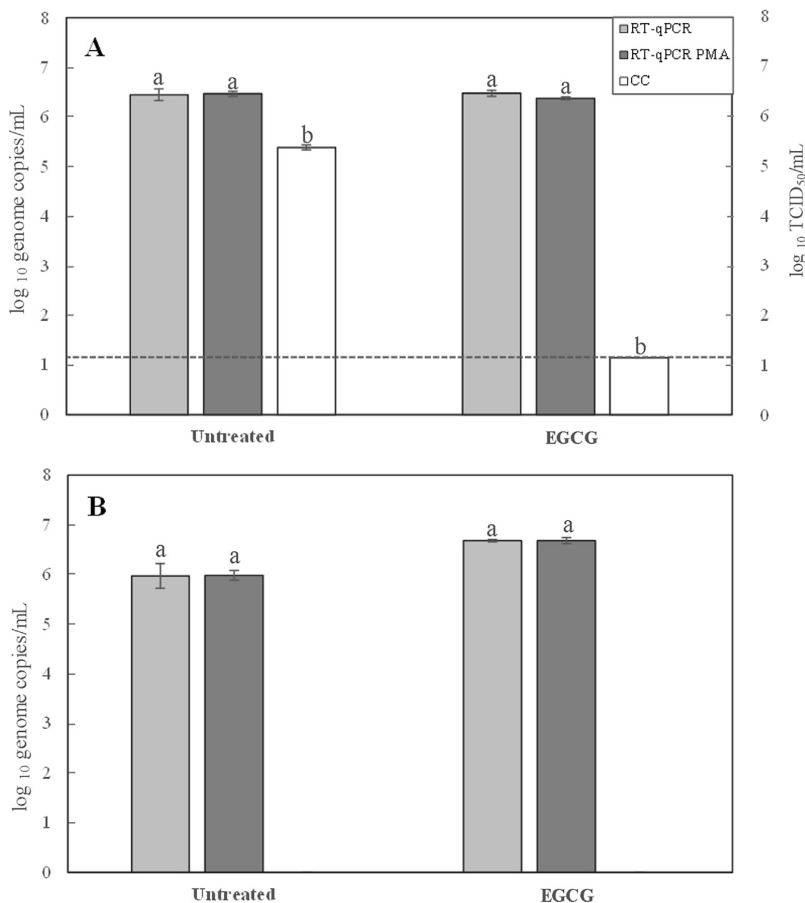


Fig. 2. Titers of HAV (A) after overnight treatment with 2.5 mg/mL EGCG as determined by infectivity (TCID₅₀) and by molecular methods (RT-qPCR alone and PMAxx-Triton-RT-qPCR) and NoV (B) by molecular methods (RT-qPCR alone and PMAxx-Triton-RT-qPCR). Dashed lines indicate the detection limit for the TCID₅₀ assay. Titers are the means ± standard deviations of results of three replicates.

PeakView™ 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 EGCG 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 log₁₀ 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-

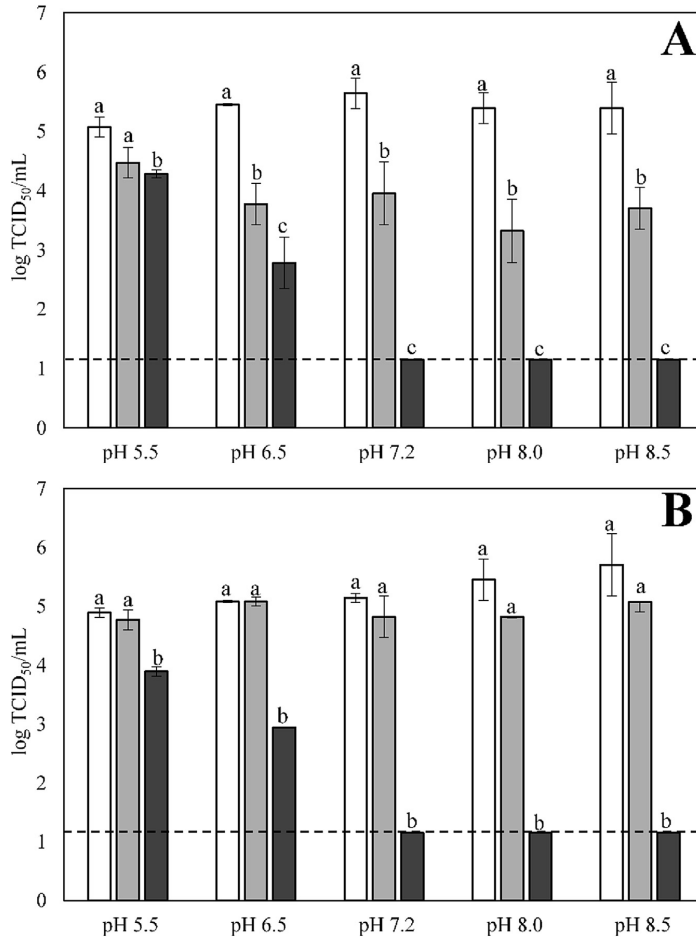


Fig. 3. Effect of pH on the activity of EGCG against MNV (A) and HAV (B). White bars indicate untreated samples, greys bars indicate 0.25 mg/mL EGCG and black bars 2.5 mg/mL EGCG. Dashed lines indicate the detection limit for the TCID₅₀/mL assay. Titers are the means \pm standard deviations of results 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 (Murriel-Galet, Cran, Bigger, Hernández-Muñoz, &

Gavara, 2015; Wrona, Cran, Nerin, & 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.

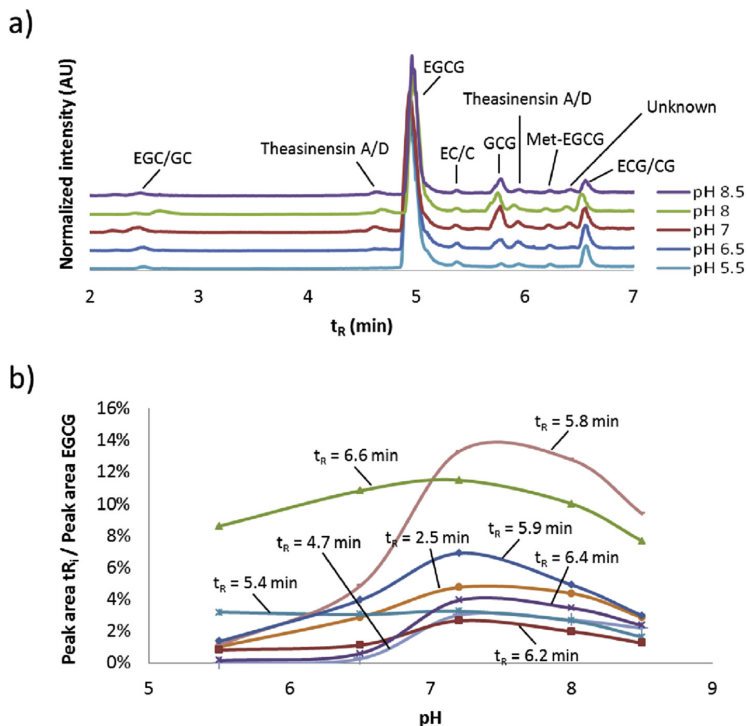


Fig. 4. Chromatograms of EGCG solutions after incubation at different pHs (a) and variation in the relative areas of the peaks at different retention times (t_R) with respect to EGCG ($t_R = 4.9$ min) (b).

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 TCID₅₀ 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] ⁻	EGC/GC

(*) Abbreviations: EGC: epigallocatechin; GC: galliccatechin; EGCG: epigallocatechin gallate; EC: epicatechin; C: catechin; GCG: galliccatechin 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 responsables 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 theasinensin 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.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO) (RYC-2012-09950 and AGL2015-63855-C2-1) 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. GS was supported by the “Ramón y Cajal” Young Investigator. Laura G. Gómez-Masaraque is recipient of a predoctoral contract from the Spanish Ministry of Economy and Competitiveness (MINECO), Call 2013.

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3.2. Evaluation of green tea extract capacity as therapeutical antiviral natural compound.

3.2.1. Article 4. *Antiviral activity of aged green tea extract in model food systems and under gastric conditions*



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

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ARTICLE INFO

Keywords:

Hepatitis A virus
Human norovirus
Simulated gastric fluid
Green tea extract
Food model systems

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 °C. 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 °C. 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.

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 toward 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,

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<https://doi.org/10.1016/j.ijfoodmicro.2018.12.019>

Received 25 July 2018; Received in revised form 30 October 2018; Accepted 19 December 2018

Available online 21 December 2018

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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 NaNO₃ (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 (TCID₅₀) in 96-well microtiter plates with eight wells per dilution and 20 µl of inoculum per well using the Spearman-Kärber 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 et al. (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 °C for 1 h and then incubated overnight (ON) at 4 °C. The following steps were performed at 37 °C. 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 1:2000. 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'-tetramethylbenzidine, Millipore-Sigma). Color development was stopped with 3 M H₂SO₄ 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 °C. 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 °C for 1 h and then incubated ON at 4 °C. Simultaneously, suspensions of HuNoV GI were mixed with aged-GTE at 0.5 and 5 mg/ml ON at 37 °C.

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 °C 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 °C for 1 h. HuNoV GI suspensions without aged-GTE treatment or treated at 99 °C 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 TCID₅₀/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 37 °C 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 N_x 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 TCID₅₀/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 °C during 2 min 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 analyzed 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 was 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.

3.3. 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 °C (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.

3.4. 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 37 °C 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.

3.5. 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 TCID₅₀/ml and the HAV titers were 4.7 ± 0.1 , 4.5 ± 0.5 , and 4.5 ± 0.0 log TCID₅₀/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 °C 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 °C, 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 °C, 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-Masquera et al., 2016; Randazzo et al., 2017). Moreover, a previous study demonstrated that storage of the GTE solutions for 24 h at 25 °C 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).

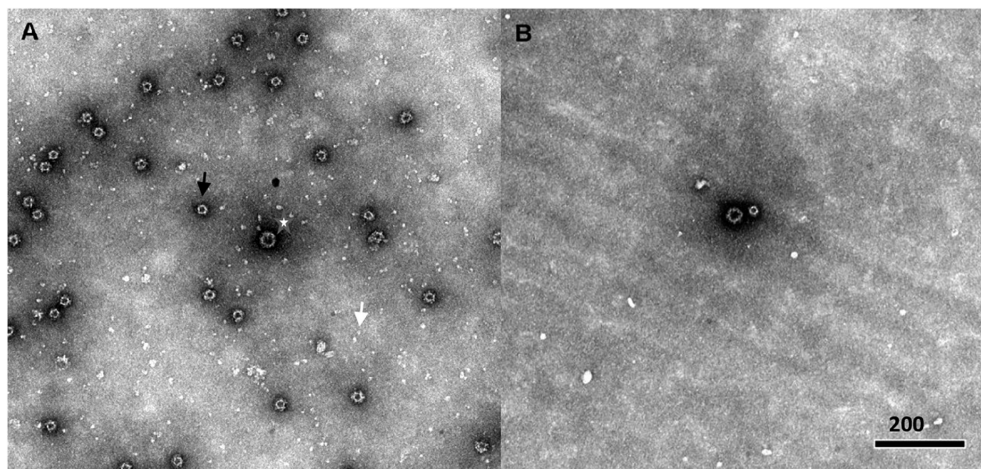


Fig. 1. Representative TEM field showing negatively stained untreated VLPs (A) and VLPs treated with aged-GTE (0.5 mg/ml) (B). The white star indicates a 40 nm putative T3 symmetry VLP. The black arrow points to a 23 nm putative T1 symmetry VLP. The white arrow points to VP1 capsomer. The scale bar indicates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

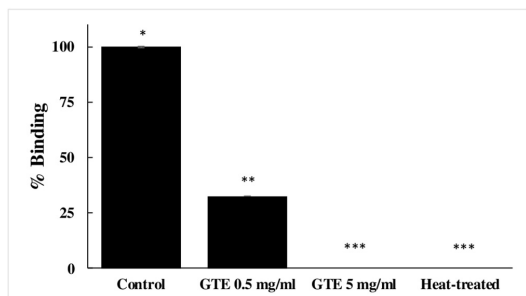


Fig. 2. Effect of aged-GTE on the binding of human norovirus GI to PGM analyzed by ISC-RT-qPCR. Each bar represents the average of triplicates. Asterisks show statistical differences ($P < 0.05$).

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 and coatings), the viral inactivation rate was reduced (Fabra et al., 2018; Falcó et al., 2019; 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

Table 1

Murine norovirus (MNV) and hepatitis A virus (HAV) titers (log TCID₅₀/ml) after treatments with aged-GTE prepared in different model food systems and incubated overnight at 37 °C. 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
	5	2.57 ± 0.22 _c	–	2.37 ± 0.29 _c	–
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 _d	–	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

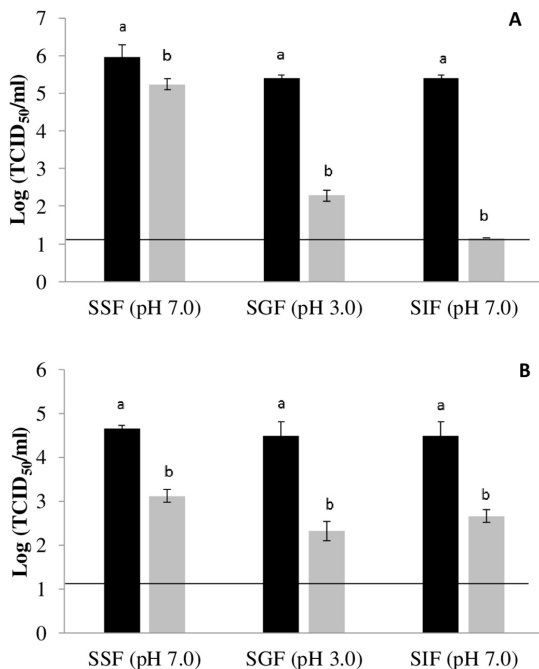


Fig. 3. Reduction of murine norovirus (MNV) (A) and hepatitis A virus (HAV) (B) titers (log TCID₅₀/ml) after treatments with aged-GTE (5 mg/ml) prepared in simulated salivary fluid (SSF; 2 min at 37 °C), simulated gastric fluid (SGF; 2 h at 37 °C) and simulated intestinal fluid (SIF, 2 h at 37 °C).

*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.

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 °C, while B-PAC at the same experimental conditions (5 mg/ml, 24 h, 37 °C) 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 horchata 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 Agricultural 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” Young Investigator Grants and WR by a postdoctoral fellowship from Generalitat Valenciana (APOSTD/2018/150).

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3.2.1. Article 5. *Green tea extract assisted low-temperature pasteurization to inactivate enteric viruses in juices.*



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

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ARTICLE INFO

Keywords:

Food safety
Norovirus
Hepatitis A virus
Natural antimicrobials
Hurdle technology

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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<https://doi.org/10.1016/j.ijfoodmicro.2020.108809>

Received 6 March 2020; Received in revised form 29 July 2020; Accepted 30 July 2020

Available online 31 July 2020

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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 2M NaNO₃ (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. 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 TCID₅₀/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 log₁₀ (N_x/N₀), where N₀ 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 TCID₅₀/ml) and HAV (ca. 6 log TCID₅₀/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

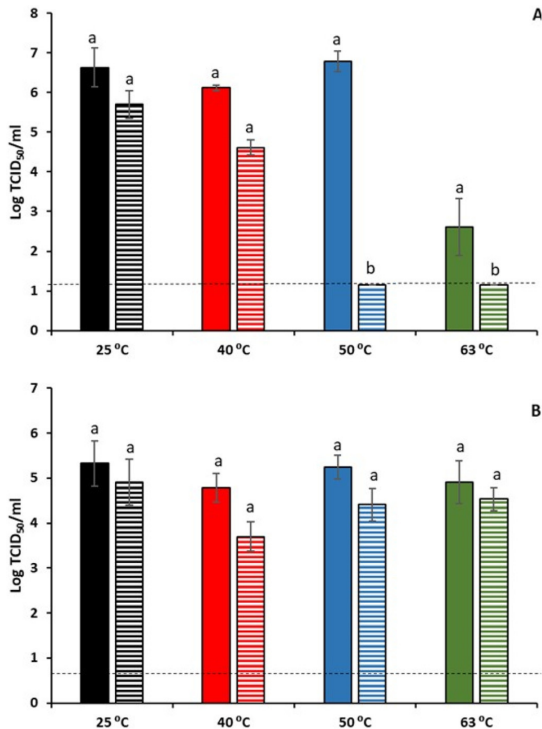


Fig. 1. Reduction of murine norovirus (MNV-1) (A) and hepatitis A virus (HAV) (B) titers (log TCID₅₀/ml) exposed to different mild heat treatments (30 min) alone (filled bars) and combined with aged-GTE (5 mg/ml) (stripped bars). Dashed line depicts the detection limit. Each column represents the average 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 TCID₅₀/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 GI, 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 TCID₅₀/ml) while a complete inactivation of the virus (> 5.82 log TCID₅₀/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

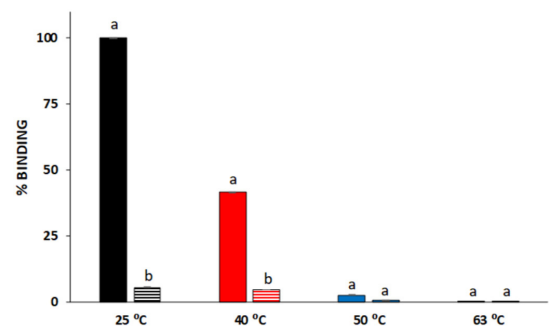


Fig. 2. Effect of different mild heat treatments alone (filled bars) and combined with aged-GTE (5 mg/ml) (stripped bars) on human norovirus GI. Norovirus GI binding ability to PGM was analyzed by ISC-RT-qPCR. Each bar represents the average of triplicates. Different letters denote significant differences 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 (Falcó 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 TCID₅₀/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 TCID₅₀/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 (Falcó 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 TCID₅₀/ml, and the increase of 10 °C in thermal regimen (to 50 °C) led to a reduction of 2.69 log TCID₅₀/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 TCID₅₀/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 TCID₅₀/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

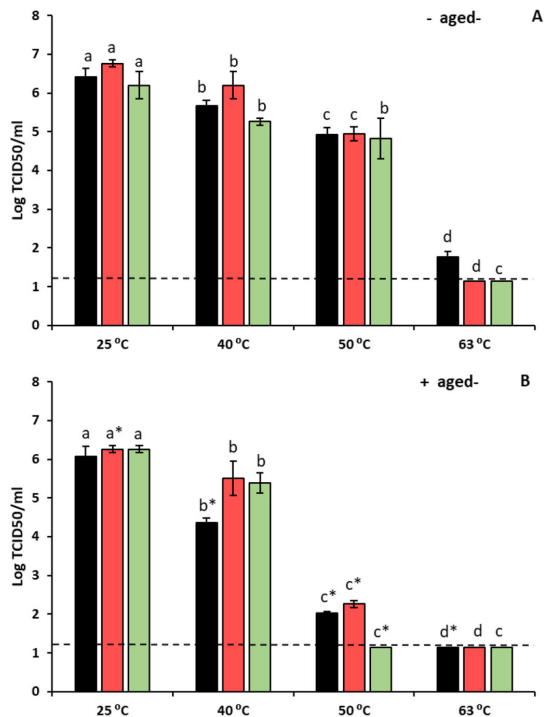


Fig. 3. Murine norovirus (MNV-1) titers (log TCID₅₀/ml) exposed to different mild heat treatments in PBS (black bars), commercial juice 1 (red bars) and commercial juice 2 (green bars) alone (A) and combined with aged-GTE (3.3 mg/ml) (B).

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 TCID₅₀/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

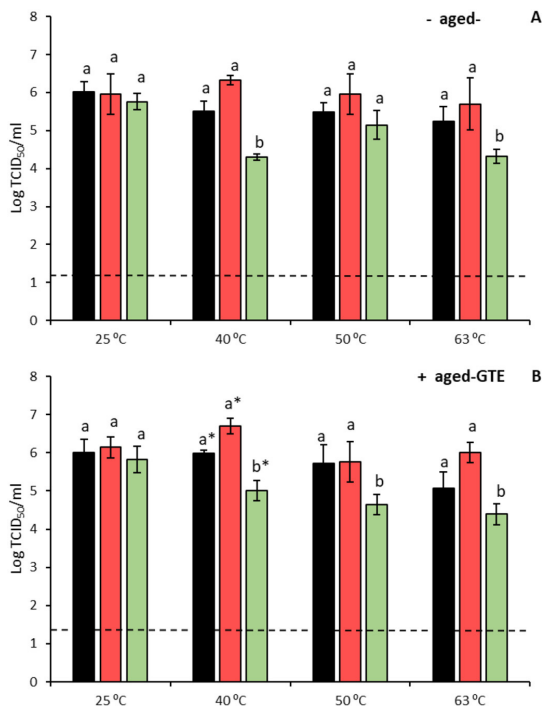


Fig. 4. Hepatitis A virus (HAV) titers (log TCID₅₀/ml) exposed to mild heat treatments in PBS (black bars), commercial juice 1 (red bars) and commercial juice 2 (green bars) alone (A) and combined with aged-GTE (3.3 mg/ml) (B). Dashed lines depict the detection limit. Each column represents the average 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.

Acknowledgements

This study was supported by the CSIC with the internal project 2017701088 and Spanish Ministry of Science and Innovation with the project PID2019-105509RJ-I00. W. Randazzo was supported by a postdoctoral fellowship from Generalitat Valenciana (APOSTD/2018/150).

Appendix A. Supplementary data

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

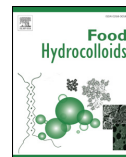
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3.3. Potential applications of green tea extract in food industry to improve food safety.

3.3.1. Article 6. *On the use of carrageenan matrices for the development of antiviral edible coatings of interest in berries.*



On the use of carrageenan matrices for the development of antiviral edible coatings of interest in berries

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ARTICLE INFO

Keywords:

Carrageenan
Antiviral
Active coatings
Food-borne pathogens
Berries

ABSTRACT

Different film-forming dispersions (FFD) based on κ -, ι - and λ - carrageenans and green tea extract (GTE) have been developed as an innovative strategy to guarantee the food safety of blueberries and raspberries. First, the FFD were characterized (surface tension and viscosity) and the physicochemical properties (water vapour permeability-WVP-, water sorption, contact angle, mechanical properties) of the stand-alone films were evaluated. Then, the FFD were applied to refrigerated-stored raspberries and blueberries, and the antiviral activity against murine norovirus (MNV), a cultivable norovirus surrogate, and hepatitis A virus (HAV) of coated fruits was determined at refrigerated (10 °C) and ambient conditions (25 °C). The type of carrageenan used significantly affected the WVP and the mechanical properties of the stand-alone films, being κ -carrageenan films more rigid and less permeable. The incorporation of GTE resulted in less ductile and deformable films and slightly more permeable than their counterparts prepared without the extract. All the coatings were effective in extending the shelf life of raspberries and blueberries under refrigeration, preserving their firmness to a greater extent and promoting better appearance. In general, FFD with similar viscosity (κ - and λ - carrageenans) showed higher antiviral activity as the gelling capacity of the carrageenan increased (κ - carrageenan) because of the formation of a more cohesive polymer matrix and the higher solid surface density (SSD) deposited onto the berry surfaces. Adding GTE enhanced the carrageenan antiviral activity at both refrigerated and ambient temperatures in blueberries and raspberries, being slightly more effective in the case of MNV.

1. Introduction

Over the last decade, the inclusion of fresh produce in the human diet has been steadily increasing due to heightened consumer awareness of the associated health benefits. Unfortunately, this increase in fresh produce consumption has been associated with a simultaneous increase in the incidence of foodborne illnesses. Despite accounting for the major causes of foodborne outbreaks in high-income countries, human enteric viruses have received comparatively less attention than other foodborne pathogens. In US, viruses are the most common etiologic agents identified in produce associated outbreaks (54%), frequently linked with food-handling errors (Bennett et al., 2018). Among them, berries have been identified as important vehicles for the transmission of foodborne viruses, such as human noroviruses and hepatitis A virus (HAV) (Lynch, Tauxe, & Hedberg, 2009). For example, since January 2018, of the 53 alert notifications involving viruses reported in the European Union's Rapid Alert System for Food and Feed (RASFF) database, 11 were associated with berries, namely strawberries,

raspberries, blueberries, blackberries, currants and cherries. Berries are usually picked for fresh consumption and are packed without washing because they are highly perishable, thus potential virus contamination coming from contaminated irrigation water or improper hygiene practices is unlikely to be removed (Maunula et al., 2013).

Given the lack of effective, realistic, and validated strategies to eliminate or reduce viral contamination in berries without significantly modifying the fruits' physicochemical and sensorial properties (Sánchez, 2015), the development of edible antiviral coatings has recently been postulated as an innovative strategy with the potential to guarantee the food safety of these products (Fabra, Falcó, Randazzo, Sánchez, & López-Rubio, 2018; Falcó et al., 2019; Randazzo, Fabra, Falcó, López-Rubio, & Sánchez, 2018).

Research interest in edible coatings based on natural polymers and food-grade additives has been increasing in recent years (Fang, Lin, Warner, & Ha, 2018; Poverenov et al., 2018; Saricaoglu, Tural, Gul, & Turhan, 2018). The natural polymers appropriate for this purpose include a variety of proteins, polysaccharides, and lipids that can be

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<https://doi.org/10.1016/j.foodhyd.2019.01.039>

Received 27 November 2018; Received in revised form 15 January 2019; Accepted 15 January 2019

Available online 25 January 2019

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combined to extend the shelf life and improve the safety of food products. These polymers can be customized to actively inhibit the growth of fungi, pathogenic bacteria, and, most recently, human enteric viruses (Randazzo et al., 2018). Food-grade additives generally recognized as safe (GRAS), including natural extracts, for example, green tea extract (GTE) and grape seed extract (GSE), essential oils, and the groups of chemical compounds (i.e. polyphenols) are excellent candidates for the development of active biopolymer-based coatings (D'Souza, 2014; Fabra et al., 2018; Li, Baert, & Uyttendaele, 2013; Randazzo et al., 2018; Ryu et al., 2015).

Many studies have been carried out to investigate the potential of marine polysaccharides extracted from algae (e.g., carrageenan, agar, and alginate) or crustaceans (e.g., chitin and chitosan) to be utilized as biopolymer matrices for the development of edible films and coatings (Fabra et al., 2018; Fang et al., 2018; Hajji, Younes, Affes, Boufi, & Nasri, 2018; Poverenov et al., 2018; Ramu, Shanmugam, & Bhat, 2018; Sapper, Wilcaso, Santamarina, Roselló, & Chiralt, 2018). Marine polysaccharides, especially those from seaweeds, are also attracting increasing attention in medicine and pharmaceuticals because they have been found to possess unique structures that exert virucidal effects by interfering with different stages of the viral infection process (Bouhhal et al., 2011; Chen & Huang, 2018; Shi et al., 2017; Wang, Wang, & Guan, 2012). An interesting group of marine polysaccharides are carrageenans, a family of linear sulfated polysaccharides that are extracted from red seaweeds and account for 30–75% of the algae's dry weight (Zhang et al., 2010). Carrageenans, sulfated D-galactans with high molecular weights, are composed of repeating disaccharide units with 3-linked- β -D-galactopyranose, 4-linked- β -galactopyranose, or 3,6-anhydro- β -galactopyranose (Funami et al., 2007; Vera, Castro, Gonzalez, & Moenne, 2011). The number and position of the sulfate groups on the repeating disaccharide units classify carrageenans into three major types: λ , ι , and κ .

A number of studies have found evidence for the antiviral activity of carrageenans, through a mechanism of neutralizing the positive charges present on cell surfaces with the negative charges from the sulfate groups, thus interfering with viruses' adsorption onto cells (Gomaa & Elshoubaky, 2016; Klimyte, Smith, Oreste, Lembo, & Dutch, 2016). However, although carrageenans have been widely used as polymer matrices (Fabra et al., 2009a,b; Farhan & Hani, 2017; Hambleton, Fabra, Debeaufort, Dury-Brun, & Voilley, 2009; Nur Fatin Nazurah, and Nur Hanani, 2017; Ramu et al., 2018; Sanchis et al., 2017; Thakur et al., 2016), their potential to assure the food safety by themselves has not yet been explored.

To the best of our knowledge, there is no existing literature on the formulation and characterization of carrageenan-based antiviral coatings. Furthermore, no data are present in the literature regarding the antiviral activity of κ -, ι -, and λ -carrageenans against human enteric viruses. Therefore, the main goals of this study were first to develop and characterize edible active antiviral coatings based on κ -, ι -, or λ -carrageenans and then to assess their antiviral efficacy when applied onto the surfaces of blueberries and raspberries at environment and refrigeration temperatures. The synergic antiviral effect of these polysaccharide matrices with GTE, a natural compound recently reported to possess antiviral properties (Fabra et al., 2018; Falcó et al., 2018), was also evaluated. In fact, several chemical constituents from the GTE have been previously identified as having antiviral capacity (Falcó et al., 2018, 2019). Furthermore, the morphology and physicochemical (mechanical, water vapour barrier, water sorption, contact angle) properties of the edible carrageenan–GTE film were analyzed to evaluate the applicability of these edible coatings.

2. Materials and methods

2.1. Materials

κ - ι - (specifically, sodium iota carrageenan) and λ - carrageenan

were kindly provided by Ceamsa (Pontevedra, España). GTE with high oxygen radical absorbance capacity (ORAC) (> 70% catechins content and > 50% EGCG content, measured by HPLC) was kindly donated by Naturex, S.A. (France). Blueberries (*Vaccinium corymbosum*) and raspberries (*Rubus idaeus* L.) were obtained from a local market, then immediately transported to the laboratory where berries were selected for uniformity of size and freedom from pathological and physiological defects for use in the experiments.

2.2. Preparation of film forming dispersions (FFD)

Six different film-forming dispersions (FFD) based on κ -, ι -, λ -carrageenan were prepared. FFD containing GTE were prepared as follows: each carrageenan (1% w/w) was directly dissolved in water at 40 °C and, once the carrageenan was completely dissolved, 0.7% (w/w) GTE was added to the aqueous dispersion and stirred until it was solubilized in the carrageenan-based matrices. Control κ -, ι - and λ - carrageenan films without GTE were also prepared for comparative purposes.

2.3. Preparation and characterization of stand-alone films

Stand-alone coatings (films) were obtained by casting. FFD were poured onto levelled Teflon casting plates of 15 cm in diameter and were dried at 30 °C and 45% relative humidity (RH) for 24 h. Films were prepared by pouring the amount of FFD that would provide 1 g of total solids, so as to keep the total solids content constant in the dry films (56 g/m²). Dried films were peeled off from the casting surface and preconditioned in desiccators at ~54% RH and 23 ± 2 °C, using an oversaturated salt solution of magnesium nitrate. Film thickness was measured in quintuplicate using a hand-held digital micrometer (Palmer-Comecta, Spain, ± 0.001 mm) and the average value was used in mechanical properties and water vapour permeability (WVP) calculations.

2.3.1. Scanning electron microscopy (SEM)

The microstructure of the films was visualized in a Hitachi SEM microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of 8–10 mm. The films (three samples per formulation) were frozen in liquid nitrogen and fractured immediately. Then, they were mounted on M4 Aluminium Specimen Mount and fixed on the support using double-side adhesive tape and, finally, a thin coating of palladium-gold was sprayed on their surface to explore the cross-section of the samples.

2.3.2. Mechanical properties

A Mecmesin MultiTest universal test machine (Landes Poli Ibérica, S.L., Barcelona, Spain) equipped with a 100-N static load cell was used to determine tensile strength (TS), elastic modulus (E) and elongation (EAB) properties, according to ASTM standard method D882-09 18 (ASTM, 2010a). E, TS and EAB properties were obtained from the stress-strain curves estimated from force-deformation data. After drying, three samples of each obtained film were selected for the tensile property measurements and they were equilibrated for four days at 54% relative humidity (RH) in a cabinet using magnesium nitrate saturated solution at 23 ± 2 °C. Prior to the test, the thickness of the samples was randomly measured at four points. At least eight replicates of each film formulation were tested. Equilibrated specimens were mounted in the film extension grips and stretched at 50 mm min⁻¹ until breaking. The experiments were carried out 54% RH and 24 °C.

2.3.3. Water vapour permeability (WVP)

WVP was determined gravimetrically at 23 ± 2 °C and 54–100% RH gradient, according to the ASTM E96/E96M-10 (ASTM 2010b) gravimetric method for hydrophilic films. Prior to the test, the thickness of the samples was randomly measured at five points. Payne

permeability cups of 3.5 cm in diameter (Elcometer SPRL, Hermelle/s Argenteau, Belgium) were filled with 5 mL of distilled water (100% RH) and then, film samples (35 mm diameter) were secured with the outwards-facing side in contact with the air during drying. The cups were placed in pre-equilibrated cabinets at 54% RH using magnesium nitrate saturated solution (Panreac Quimica, SA, Barcelona, Spain) and they were weighted periodically (± 0.00001 g) until the steady state was reached. The free film surface during film formation (air side) was exposed to the lowest relative humidity to simulate the actual application of the films in high water activity products when stored at intermediate relative humidity. Cups with aluminium samples were used as control samples to estimate solvent loss through the sealing. WVP was calculated as previously described by Fabra et al., 2012. Four replicates per formulation were made.

2.4. Challenge tests

2.4.1. Surface solid density (SSD)

Selected blueberries (*Vaccinium corymbosum*) and raspberries (*Rubus idaeus* L) were dipped in the FFD for 2 min and air-dried for 1 h at room temperature. The mean value of the coating was calculated in ten samples by quantifying the SSD, as described in Falcó et al., 2019 (Eq. (1)).

$$SSD = (M_{CA} \cdot X_s) / A_s \quad (1)$$

Where M_{CA} is the mass of coating solution adhered to the fruit surface, X_s is the mass fraction of solids present in the FFD and A_s is the average sample surface area. The average sample surface area (A_s) was estimated by considering blueberry and raspberry geometries as sphere and cone, respectively with a known height (measured in triplicate using a digital micrometer) and volume (measured with a pycnometer, by using water as reference liquid). Samples were weighed before and after coating, to determine the mass of coating solution adhered to the strawberry or raspberry surfaces (M_{CA}). The non-coated sample was used as a control.

2.4.2. Water vapour resistance (WVR), firmness and color properties

Coated and non-coated samples were stored on PET trays at 10 °C, where the pieces did not come into direct contact with each other. Physicochemical analysis of non-coated (control) and coated samples were performed during 7 and 14 days for raspberries and blueberries, respectively. The water vapour resistance (WVR) of raspberries and blueberries was determined in eight samples per FFD and in eight non-coated samples that were kept in desiccators containing an over-saturated magnesium nitrate solution to generate 54% RH. The desiccators were kept in an incubator Hot-Cold (Selecta, Barcelona, Spain) at 10 °C. WVR was calculated using a modified equation of Fick's First Law (Eq. (2)), as described by Avena-Bustillos, Krochta, Saltveit, Rojas-Villegas, & Saucedo-Pérez, 1994.

$$WVR = \frac{a_w - (\%HR/100) \times P_{wv}}{R \times T} \times \frac{A_s}{J} \quad (2)$$

where J is the slope of the weight loss curve in stationary conditions, A_s is the average sample surface area, a_w water activity of samples ($a_w - 0.99$), P_{wv} saturated water vapour pressure, T absolute temperature and R the universal constant of gases.

The firmness of berries was measured by using a TA-XTplus Texture Analyser (Stable Micro Systems, Surrey, UK), with a 50 N load cell, using a 2 mm diameter cylindrical probe. Berries were cut longitudinally and 80% compressed at a 0.2 mm/s deformation rate. Force and distance at the failure point were used as mechanical parameters. Measurements were carried out in four fruit (eight halves) per coating formulation and in four non-coated strawberries (eight halves).

The color parameters of berries were determined using a CM-3600d spectroradiometer (Minolta Co, Tokyo, Japan) with a 10 mm diameter window. The measurements were taken in four samples per FFD before

and after coating and at each time of storage. CIE-L*a*b* coordinates, hue (h^*_{ab}) and chroma (C^*_{ab}) were obtained from the reflection spectra of the samples using D65 illuminant/10° observer.

2.4.3. Antiviral test on berries

Murine norovirus (MNV-1) (kindly provided by Prof. H. W. Virgin, Washinton University School of Medicine, USA) and hepatitis A virus (HAV) strain HM-175/18f (purchased from ATCC VR-1402) were propagated and quantified in the cell line RAW 264.7 (also provided by Prof. H. W. Virgin) and in FRhK-4 (kindly provided by Prof. A. Bosch, University of Barcelona, Spain), respectively. Viruses were harvested and enumerated as described by Falcó et al., (2017).

Blueberries and raspberries were exposed to UV for 15 min on a sterile plate in a laminar flow hood to reduce the microbial load. Then 50 μ L of MNV and HAV suspensions (about ca. 5 log TCID₅₀/mL) were inoculated on the berry surfaces and let dry for 1 h at room temperature under a laminar flow hood. Then each berry was immersed for 2 min into carrageenan-based FFD, after drying, samples were incubated ON at 25 and 10 °C. Samples incubated at 10 °C were also stored for 4 days. On each sampling day, individual berry samples were placed in a tube containing 5 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS and shaken for 2 min at 180 rpm to release viral particles from the surface. Berries were removed from the DMEM suspension, and then viruses were recovered and titrated. Serial ten-fold dilutions were performed from the resultant virus suspension and confluent cell lines in 96-well plates were used to evaluate the antiviral effect of the coatings. Each treatment was performed in triplicate. Positive controls were uncoated berries and coated berries without GTE in its formulation under the same experimental conditions. 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 carrageenan coatings and N_x is the infectious virus titer for carrageenan-GTE coatings (Falcó et al., 2018).

2.5. Statistical analysis

Data of each test were statistically analyzed. The statistical analysis was carried out by means of IBM SPSS Statistics software (v.23) (IBM Corp., USA) through the analysis of variance (ANOVA). Comparison of the means was done employing the Tukey's Honestly Significant Difference (HSD) at the 95% confidence level. All data are presented as mean \pm standard deviation. Furthermore, data were analyzed using a Principal Component Analysis (PCA) to explain the total variance resulted in challenge tests. To this end, physicochemical data (SSD, WVR, F/D, and optical properties) were normalized and subjected to PCA. Only factors resulted to have Eigen-values higher than 1.00 were selected according to the Kaiser criterion (Jolliffe, 2011). Statistical data processing and graphical elaborations were achieved by using STATISTICA software version 7 (StatSoft Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. Properties of film-forming dispersions (FFD)

Table 1 summarizes the results of the surface tension test and the rheological analysis of the FFD under study. Surface tension is an important property for food coating solutions since it significantly affects both the suspension spreadability (the ability of the FFD to spread over a solid surface and adhere to it) and the integrity of the coating layer after drying (Mostafavi, Kadhodaee, Emadzadeh, & Koocheki, 2016). The presence of carrageenan reduced the surface tension of water (71 mN/m at 35 °C, Walstra, 2003) to 44–64 mN/m, being κ - and λ -carrageenan those with lower surface tension values probably due to the higher molecular weight of these carrageenans as compared to ι -carrageenan based FFD. Furthermore, the incorporation of GTE extract, with marked polar character, provoked a slight reduction of the surface tension of the carrageenan dispersions ($p < 0.05$), in

Table 1

Surface tension and rheological parameters of the FFD: Ostwald de Waale model parameters and apparent viscosity (η_{ap}) at 100 s^{-1} , at 35°C . Mean values (standard deviation).

FFD	μ (Pa × s)	n	κ (Pa × s)	η_{ap} (Pa × s) ($g = 100 \text{ s}^{-1}$)	Surface tension (mN/m)
$\kappa-$	–	0.584 (0.080) ^a	1.067 (0.613) ^a	0.146 (0.035) ^a	46.17 (2.92) ^a
$\kappa-$ GTE	–	0.681 (0.015) ^a	0.533 (0.078) ^a	0.122 (0.010) ^a	42.57 (1.71) ^a
$\iota-$	0.035 (0.002) ^a	–	–	–	63.70 (1.51) ^b
$\iota-$ GTE	0.036 (0.002) ^a	–	–	–	51.63(2.32) ^a
$\lambda-$	–	0.504 (0.007) ^a	1.436 (0.131) ^a	0.146 (0.008) ^a	48.87(1.08) ^a
$\lambda-$ GTE	–	0.513 (0.002) ^a	1.284 (0.011) ^a	0.136 (0.013) ^a	41.67 (2.12) ^b

Different letters in superscripts (a-b) indicate significant differences among the samples. n: flow index, κ : consistency index, η_{ap} : apparent viscosity at shear rate of 100 s^{-1} .

agreement with a partial substitution of the polysaccharide by polyphenolic molecules at the air-water interface which could contribute to reducing the surface tension. Similarly, Katsouli, Polychniatou, & Tzia, 2017 also reported a decrease in the net air/water surface tension of aqueous solutions by the presence polyphenols.

The average liquid film thickness (and the amount of liquid coating adhered to the surface on coated products) is directly related to viscosity and draining time of the FFD (Cisneros-Zevallos & Krochta, 2003). Complete flow curves presented in Fig. 1 show that ι -carrageenan dispersions behaved as Newtonians liquids with a linear relationship between the shear-stress (σ) and the shear-rate ($\dot{\gamma}$). However, a shear thinning behavior (pseudoplastic) was observed for κ - and λ -carrageenan dispersions, which from the viscosity values and effect on the surface tension of the solutions, seem to be higher molecular weight carbohydrates. The rheological data of pseudoplastic FFD were fitted to the Ostwald de Waale model. Table 1 gathers the viscosity values for the FFD with Newtonian behavior and, the flow (n) and consistency indexes (κ), together with the apparent viscosity (η_{ap}) values at a shear rate of 100 s^{-1} for the pseudoplastic FFD. The values of the correlation coefficient of the Ostwald de Waale model were in all cases around 0.99. The first clear observation was that $\kappa-$ and $\lambda-$ carrageenan made the FFD more viscous and more shear thinning ($n < 1$). When GTE was incorporated into the carrageenan-based solutions, the rheological behavior was very similar to that obtained for their counterparts prepared with pure κ -, ι - or λ -carrageenan.

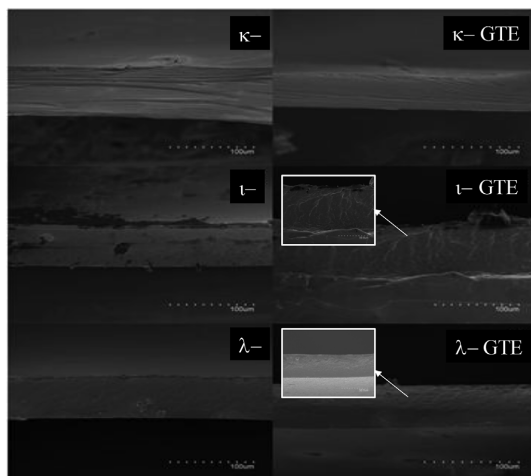


Fig. 2. SEM micrographs of the cryo-fractured section of the developed stand-alone coatings (scale marker 100 μm).

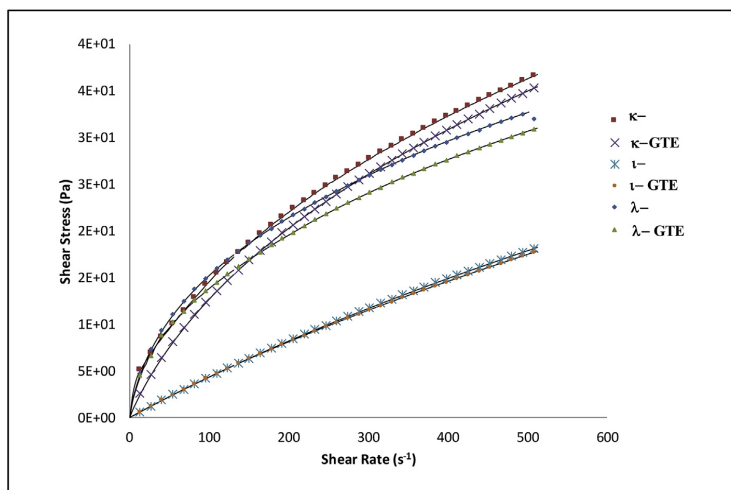


Fig. 1. Experimental (symbols) and predicted (lines) flow curves obtained by Ostwald de Waale model for the different FFD.

3.2. Properties of the stand-alone films

Physicochemical and functional properties of edible films, such as mechanical, water vapour/oxygen barrier and optical properties are directly related to their microstructure and affected by the interactions between film components and drying conditions (Fabra, Jiménez, Atarés, Talens, & Chiralt, 2009b). Fig. 2 displays representative images of the cross-sections of the films. On the microstructural level, the first thing to highlight is that κ -carrageenan films with and without GTE showed fragility probably due to the formation of a more cohesive structure than their counterparts ι - or λ -carrageenan and ascribed to the higher gelling capacity of κ -carrageenan. Therefore, despite of having a similar behavior in terms of surface tension and viscosity than the λ -carrageenan, the different sulfate content is known to affect the hydrogel network formed after drying (Campo, Kawano, Silva, & Carvalho, 2009) and, thus, to the final properties of the stand-alone films.

Generally, carrageenan and GTE are highly compatible as deduced from the SEM micrographs since no phase separation was observed. Therefore, interactions between carrageenan and GTE might favor the integration of the polyphenolic compounds in the polysaccharide matrices. In fact, polyphenols contain hydrophobic aromatic rings, which can interact with hydrophobic substrates (Fabra et al., 2018; Tamba et al., 2007) and, hydrophilic hydroxyl groups, which can interact with the carrageenan. These potential interactions between carrageenan and polyphenolic extracts via hydrogen bonding could be the responsible of the formation of a more fragile structure in GTE containing films as shown below in the analysis of the mechanical properties.

Optical properties (transparency and color parameters) are of great importance because they directly influence consumer acceptability. Fig. 3 shows the spectral distribution curves of the internal transmittance (T_i) of the developed films. Over the wavelength range considered, a similar pattern was observed for neat carrageenan films (without GTE), although λ -carrageenan films were less transparent. The incorporation of GTE, promoted a selective decrease in the T_i of the films between 400 and 550 nm due to the selective absorption of the red-brown components of GTE as well as the some additional light scattering brought about by the polyphenol extracts (with a different refractive index). Similar effects have been previously observed in chitosan-GTE (Siripatrawan & Harte, 2010), agar/carrageenan/GSE

Table 2

Color parameters of the developed films. Mean values (standard deviation).

Films	L*	C* _{ab}	h* _{ab}
κ -	75.2 (0.5)	13.1 (0.9)	82 (2)
κ - GTE	50.9 (1.2)	27.5 (1.0)	55 (2)
ι -	76.4 (0.4)	13.7 (0.5)	85 (1)
ι - GTE	41.9 (1.5)	20.1 (0.9)	57 (2)
λ -	58.0 (2.0)	20.4 (1.9)	69 (3)
λ - GTE	47.7 (1.6)	19.9 (2.0)	60 (3)

Different superscripts within a column indicate significant differences among formulations ($p < 0.05$).

L* lightness, C*_{ab} Chroma, h*_{ab} hue.

(Kanmani & Rhim, 2014a, b) and alginate/lipid/GTE films (Fabra et al., 2018). Table 2 gathers the values of the color coordinates (L*, lightness; C_{ab}*, chrome; h_{ab}*, hue) of the different films. Due to the typical color of GTE, films with GTE were darker (lower L*), with a more saturated reddish color (higher C* and lower h*).

Table 3 shows the tensile parameters of the developed films: Young's modulus (E, MPa), tensile strength (TS, MPa) and elongation at break (EAB, %), which are closely related to the film microstructure. As it is well known, E represents the stiffness of the films, TS is the resistance to break and EAB refers to the stretching capacity. The analysis of tensile properties is interesting since edible films have to preserve their integrity during food manipulation until consumption. It is noticeable that κ -carrageenan films were much more rigid than ι - and λ -carrageenan, with a Young's modulus increased by more than twice. This different behavior can be mainly ascribed to a higher interaction between κ -carrageenan polymer chains (due to the lower sulfate content), which conferred them the gelling capacity and promoted different structural properties once they were dried. The tensile behavior of the films was also strongly affected by the incorporation of GTE, leading to stiffer films (higher E values) but less resistance to break (higher TS values) and stretchable (lower EAB values) than their counterparts prepared without GTE. This increase in stiffness and decrease in extensibility could be attributed to the carrageenan-polyphenols interactions, as it will be detailed below. Unexpectedly, κ -carrageenan/GTE films showed lower TS values than the corresponding κ -carrageenan prepared without GTE, although they were more rigid and less stretchable than neat κ -carrageenan films (without GTE), in

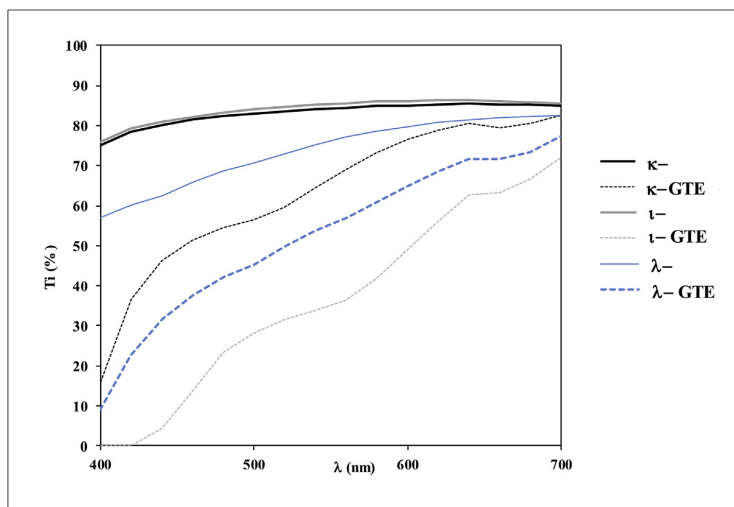


Fig. 3. Spectral distribution of internal transmittance (T_i) of the developed edible films.

Table 3

Mechanical properties, water vapour permeability, water vapour sorption and contact angle of the stand-alone coatings.

Films	E (MPa)	TS (MPa)	EAB (%)	WVP (Kg-/Pa·s·m ²) 10 ¹⁴	w _e (100%) (g water/100 g sample)	θ (°)
κ-	7532 (678) ^a	84.4 (17.6) ^a	2.0 (0.4) ^a	7.2 (0.5) ^a	88.4 (2.7) ^a	67.5 (5.5) ^a
κ- GTE	13370 (291) ^b	23.8 (6.9) ^b	0.4 (0.2) ^b	12.8 (1.5) ^c	64.7 (2.8) ^b	29.5 (1.5) ^b
ι-	3160 (144) ^c	41.8 (10.8) ^b	2.9 (0.6) ^b	9.3 (0.3) ^{ab}	127.8 (0.2) ^c	78.9(5.6) ^c
ι- GTE	7884 (330) ^a	83.9 (10.8) ^{cd}	2.0 (0.7) ^c	15.5 (4.0) ^{cd}	84.5 (5.3) ^d	27.0 (6.0) ^b
λ-	2772 (267) ^c	18.9 (9.0) ^c	1.2 (0.5) ^d	12.6(0.4) ^{bc}	131.2 (3.0) ^c	109.5 (7.6) ^d
λ- GTE	6649 (703) ^d	37.4 (4.0) ^{ad}	1.2 (0.3) ^d	17.3 (1.1) ^d	106.6 (1.6) ^e	37.0 (7.0) ^e

WVP: water vapour permeability. E: Elastic modulus. TS: Tensile strength. EAB: elongation at break. w_e: water vapour sorption. θ: contact angle values. Mean values (standard deviation).

Different superscripts within a column indicate significant differences among FFD/coating ($p < 0.05$).

agreement to that previously observed when ι- and λ- carrageenan films with and without GTE were compared.

Sivarooaban, Hettiarachchy, and Johnson (2008) have reported similar results for soy protein films containing GSE who also attributed this behavior (increased E and TS values and decreased EAB values) to the existing polyphenol-protein interactions. In fact, several works have demonstrated protein-polyphenol interactions (Gómez-Mascaraque, Llavata-Cabrero, Martínez-Sanz, Fabra, & López-Rubio, 2018; Jin et al., 2018; Liu et al., 2018; Ramos-Pineda, García-Estévez, Dueñas, & Escribano-Bailón, 2018). However, there is a controversy concerning the behavior of TS, based on the type of hydrocolloid and the concentration of polyphenolic extract. Giménez, López de Lacey, Pérez-Santín, López-Caballero, & Montero, 2013 pointed out that the decrease in TS due to the incorporation of polyphenolic compounds into agar-based films could be attributed to a decrease in the molecular interactions between agar molecules. In contrast, other studies have reported an increase in TS in different polymer matrices such as agar-gelatin, chitosan or soy protein that incorporate GTE or GSE in concentrations up to 50% in the FFD. They attributed this effect to the established interactions between polyphenolic compounds and polymer matrices (Siripatrawan & Harte, 2010; Sivarooaban et al., 2008). On the other hand, when the amount of extract was higher, it might not be interacting with the matrix, inducing the development of a heterogeneous structure and causing a decrease in TS (Bravin, Peressini, & Sensidoni, 2004).

Infrared spectroscopy was used to investigate potential interactions between GTE and carrageenan based matrices. ATR-FTIR spectra related to carrageenan-based films containing or not GTE are gathered in Fig. 4. In general, all the carrageenans considered presented a broad absorption band in the 1210–1260 cm⁻¹ spectral region which is ascribed to the S=O stretching vibration of the sulfated groups and it was correlated to the sulfate content of the samples: the intensity of the band decreased from the λ- carrageenan (higher intensity) to κ- carrageenan (lower intensity). The presence of the sulfate groups gave rise to characteristic bands with the frequency being dependent on the position of the sulfate ring within galactose and 3, 6-anhydrogalactose units (Pereira, Amado, Critchley, van de Velde, & Ribeiro-Claro, 2009). For instance, κ- and λ- carrageenan showed an absorption band at approximately 845–850 cm⁻¹ which is assigned to the sulfate group at the C₄ position in the D-galactose ring (C4-O-S stretching vibrations), and the band at around 805 cm⁻¹ resulting from the sulfate group at the C₂ position in the 3,6-anhydro-D-galactose ring (C₂-O-S bending vibrations) appeared in ι- carrageenan.

The broad absorption band in the region of 2900 and 3800 cm⁻¹ corresponds to hydroxyl group vibrational stretching and it is associated to OH bond in water or hydroxyl groups of the carrageenan. If the hydroxyl groups of the biopolymer matrices are largely in interaction with water, some water adsorption occurred under environmental conditions used. This is undoubtedly evidenced by the H–OH bending vibration at 1640 cm⁻¹, characteristic of physisorbed water.

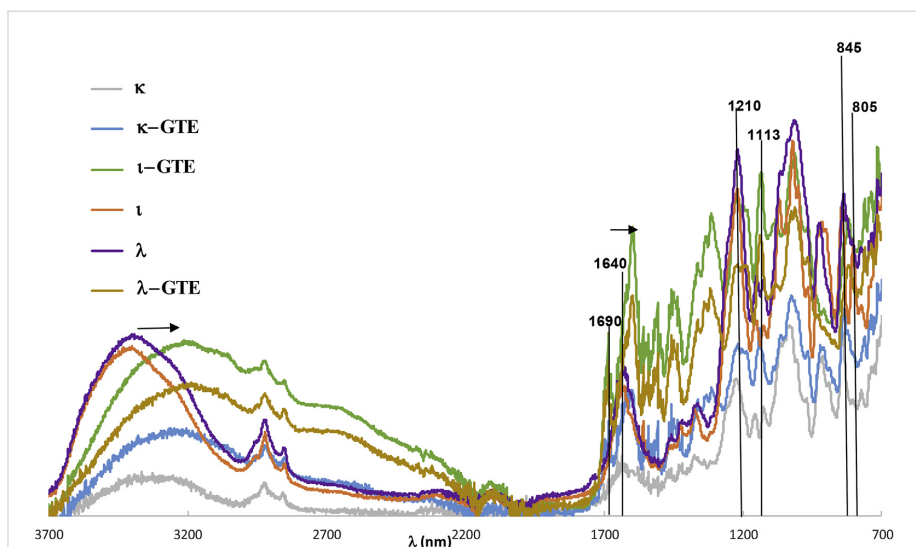


Fig. 4. ATR-FT-IR spectra of representative samples.

When incorporating GTE, the contribution of the –OH with a stretching vibration band at approximately 3400 cm^{-1} slightly shifted to lower wavenumbers, mainly in ν – and λ – carrageenan probably due to interactions with the hydroxyl groups of the GTE. Similarly, shifts in the bands associated with the physisorbed water region, mainly observed in ν – and λ – carrageenan-incorporating GTE can be ascribed to interactions that occur between GTE and carrageenan. These changes could also explain the lower water sorption found in GTE-containing films instead of their higher WVP values when they were compared to their counterparts without the extract. Furthermore, when GTE was incorporated in the carrageenan matrices, the band in the sulfate groups region of 1210 and 1260 cm^{-1} slightly shifted to lower wavenumbers. Similarly, a characteristic band at 1618 cm^{-1} , attributed to the aromatic ring quadrant and the band at 1097 cm^{-1} owed to the aromatic rings stretch of GTE (spectrum showed elsewhere, Robb, Geldart, Seelenbinder, & Brown, 2002) were slightly displaced in the GTE-containing films with respect to free GTE, being centered at 1690 cm^{-1} and 1113 cm^{-1} , respectively in the GTE-containing films.

Thus, the ATR-FTIR characterization allowed identifying the groups involved in GTE-carrageenan interactions, and confirming the assumptions from the physicochemical properties.

Barrier properties of edible films are usually described by their permeability values. Table 2 summarizes the measured water vapour permeability (WVP) for the developed films. The WVP values of the neat carrageenan-based films were lower than those previously reported for carrageenan cast films (Shojaee-Aliabadi et al., 2014; Rhim & Wang, 2014) since the films prepared in the present work did not incorporate plasticizers. It should be noted that the type of carrageenan affected the WVP values, observing a statistically significant increase ($p < 0.05$) as the number of sulfate groups increased in the carrageenan, that is, the WVP was lower in films formulated with κ – carrageenan than that obtained for ν –carrageenan based film and, they were also less permeable than films formulated with λ –carrageenan. This could be related to the gelling capacity of the carrageenan (it is well-known that weaker gels are obtained when the number of sulfate groups in the carrageenan molecules increases). Accordingly, the water sorption of κ – carrageenan films (Table 3) was lower than their counterparts ν – and λ – carrageenan films, suggesting that the diffusion of water molecules throughout the carrageenan-based matrices is dependent on the gel cohesion, being lower for κ – carrageenan-based films which had a greater gelling capacity than ν – and λ – carrageenan. It should be noted that, in film format (or dehydrated gel), the apparent diffusivity of water in carrageenan can be 100 times lower (Rondeau-Mouro, Zykwiniska, Durand, Doublier, & Buléon, 2004). This is due to the increase in stiffness (hardening) of the polymer network and the subsequent decrease in mobility. In fact, the hydration process involves interactions with the hydroxyl (–OH) and sulfate (–SO₃[−]) groups of the carrageenan, which can decrease the diffusion of water through the polymer matrix. In addition, the diffusion coefficient may vary with time. More probably, thickness could increase during the time of the experiment with the hydration shell and this could be a determining factor in the WVP.

All the carrageenan films incorporating GTE had significantly ($p < 0.05$) higher WVP, with a maximum permeability increase of 43%. Similar effects were previously observed in *Gelidium corneum*/nano-clay composite film containing GSE or thymol (Lim, Jang, & Song, 2010) and in rapeseed protein–gelatin film containing GSE (Jang, Shin, & Song, 2011). This increase in permeability was ascribed to a loss of cohesiveness of polymer matrices prepared with GSE or GTE contents up to 0.5 (w/v). Interestingly, the incorporation of GTE into the carrageenan matrices significantly reduced ($p < 0.05$) the water sorption of the films (Table 3). This effect can be ascribed, on the one hand, to the confinement of the polyphenolic compounds into the carrageenan matrices, interacting with the polymer chains through hydrogen bonds and limiting their interaction with water. In fact, this could also contribute to the formation of more rigid and less deformable films, as it

has been described above. On the other hand, the addition of GTE into the carrageenan matrices decreased the gelling capacity of the carrageenan, giving rise less cohesive networks and, thus, the sorbed water could easily diffuse through the biopolymer matrices to a greater extent than their counterparts prepared without GTE, promoting an increase in the WVP. Similar effects have been previously observed in tuna-fish gelatin with antioxidant extracts of two different murta ecotypes leaves (Gómez-Guillén, Ihl, Bifani, Silva, & Montero, 2007) and chitosan/GTE films (Peng, Wu, & Li, 2013).

The water contact angle values are normally used to estimate the degree of hydrophobicity of the material. Therefore, the wettability properties of the neat carrageenan films and those containing GTE were also determined by direct measurement of contact angles of a water drop deposited on the upper surface of samples and the results are listed in Table 3. The contact angle values (θ) are reported as a function of carrageenan type and GTE addition. As observed, the contact angle increased as the SO₃[−] increased, being higher for λ –carrageenan and lower for κ –carrageenan. The θ values were in the range of those found in the literature for κ –carrageenan) and ν –carrageenan, being 66° and 88° , respectively (Karbowski, Debeaufort, Champion, & Voilley, 2016; Rhim, 2012; Rhim & Wang, 2013). From Table 3, GTE addition provoked a significant decrease ($p < 0.05$) of the contact angle values, suggesting the more hydrophilic character of these films. This is explained by the increase of polar groups from the polyphenols, leading to an increase of the hydrophilic character of GTE-containing films. These results are in line with those reported by Moradi et al. (2012), who also noted a significant reduction in the contact angle values for chitosan-GTE films.

3.3. Challenge tests: application of edible coatings in blueberries and raspberries

Challenge test on coated blueberries and raspberries at refrigerated temperatures were carried out under conditions of *in vivo* storage, mimicking realistic scenarios of fresh fruit handling. The physicochemical quality and the antiviral effectiveness (for safety assurance of the fruits) of carrageenan-based edible coatings on berries under refrigeration were evaluated.

3.3.1. Surface solid density (SSD) and water vapour resistance (WVR)

SSD values and WVR of non-coated and coated blueberries and raspberries are shown in Table 4. SSD, which can be used as an estimation of coating thickness was significantly affected ($p < 0.05$) by the type of berry, type of carrageenan as well as the presence of GTE. The first clear observation is that the SSD was more than three-fold higher for raspberries than blueberries, which can be explained by the different roughness of the skin (reaching higher values for rougher skins such as raspberries).

The trend was broadly the same for raspberries and blueberries when comparing the different film forming dispersions (FFD).

Table 4

Surface solid density (SSD) and water vapour resistance (WVR) of non-coated and coated raspberries and blueberries. Mean value (standard deviation).

FFD	Raspberries		Blueberries	
	WVR (s/cm)	SDD (g/m ²)	WVR (s/cm)	SDD (g/m ²)
C	1.19 (0.36) ^a	–	1.96 (0.23) ^a	–
κ –	1.27 (0.19) ^a	7.08 (0.25) ^a	1.96 (0.23) ^a	1.85 (0.35) ^{ab}
κ – GTE	0.94 (0.15) ^a	10.05 (1.50) ^b	1.51 (0.30) ^a	4.41 (0.80) ^c
ν –	0.76 (0.26) ^a	4.56 (0.72) ^c	1.91 (0.28) ^a	1.08 (0.50) ^b
ν – GTE	1.32 (0.40) ^a	9.92 (0.81) ^d	1.73 (0.21) ^a	2.26 (0.65) ^d
λ –	0.86 (0.28) ^a	4.31 (0.70) ^c	1.73 (0.35) ^a	1.11 (0.81) ^b
λ – GTE	1.36 (0.33) ^a	9.72 (0.89) ^d	2.25 (0.43) ^a	2.42 (0.48) ^d

Different superscripts within a column indicate significant differences among FFD or coating ($p < 0.05$).

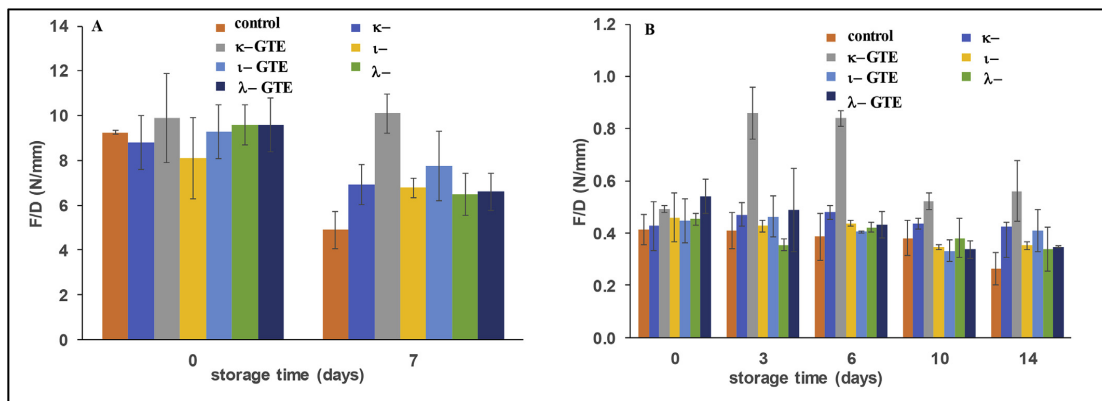


Fig. 5. Mechanical response (F/D) of non-coated and coated samples throughout at refrigerating conditions: (A) raspberries, (B) blueberries.

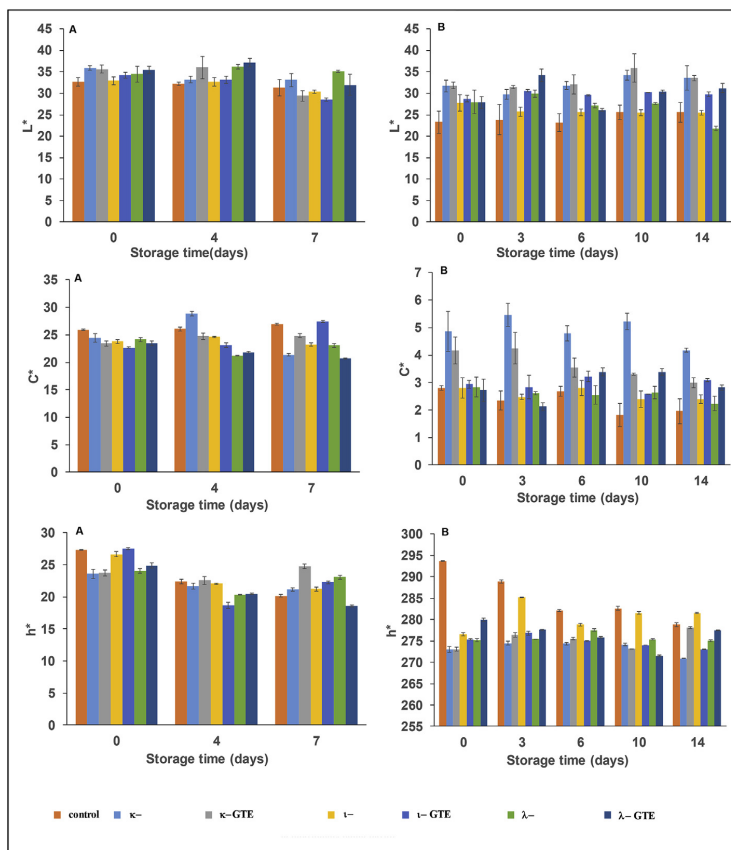


Fig. 6. Optical properties (L^* : luminosity, C_{ab}^* : Chroma and h_{ab}^* : hue) of non-coated and coated samples throughout at refrigerating conditions: (A) raspberries, (B) blueberries.

Regarding the FFD without GTE, it was observed that SSD significantly decreased ($p < 0.05$) when the amount of sulfate groups increased (lower gelling capacity) and the contact angle values of the corresponding stand-alone films increased (more hydrophobic films), being

lower in the case of $\iota-$ and $\lambda-$ -carrageenan and higher in $\kappa-$ -carrageenan coatings. It should be noted that the incorporation of GTE to the FFD, significantly increased ($p < 0.05$) the amount of SSD as compared to their counterparts prepared without GTE. This effect can be

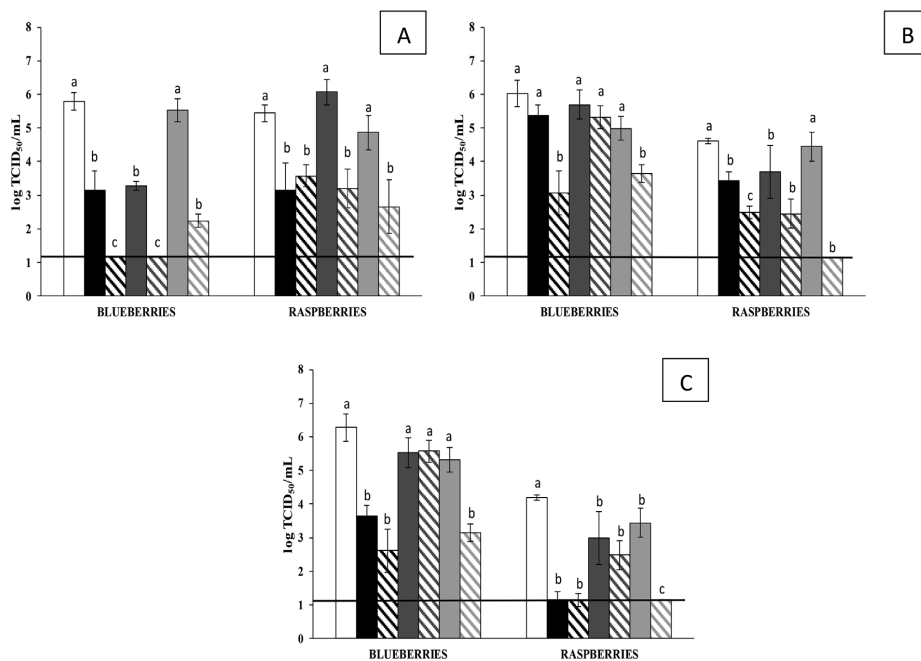


Fig. 7. Reduction of murine norovirus (MNV) titers (log TCID₅₀/mL) on blueberry and raspberry surfaces after treatment coatings at different temperatures and storage times.

*A: 25 °C/ON; B: 10 °C/ON; C: 10 °C/4 days.

**Each bar represents the average of triplicates. Within each column, different letters denote significant differences between treatments.

***White bars: control without coating; black bars: coating control κ; black-dashed bars: coating κ-GTE; dark grey bars: coating control ι; dark grey-dashed bars: coating ι-GTE; light grey bars: coating control λ; light grey-dashed bars: coating λ-GTE. Horizontal line depicts the detection limit.

ascribed to the lower contact angle and surface tension of these FFDs, which could favor a greater adhesion and spreadability of the coating to the solid surface. Therefore, the SSD is closely related to the hydrophilic character of the stand-alone films, the surface tension of the FFD and to the gelling capacity of each carrageenan. Furthermore, FFD with similar viscosity values were greater deposited onto the surface of raspberries and blueberries as the gelling capacity increased (κ-carrageenan) because of the formation of a more cohesive polymer matrix.

Coatings did not significantly improve the WVR of samples, in agreement with the high permeability values of carrageenan-based films, especially at high RH (100%) when the films are highly plasticized and barrier properties are greatly reduced (Fabra et al., 2009a).

3.3.2. Physicochemical properties

The physicochemical quality of the coated and uncoated samples was evaluated in terms of firmness and color appearance. Firstly, the ratio force-deformation at the break point (F/D) is shown in Fig. 5. The first clear observation is that, this parameter, which is related to the resistance to fracture of the product, did not change ($p < 0.05$) by the presence of the coating solutions, except in those coated with κ-carrageenan-GTE which showed even a two-fold increase for blueberries, in line with the greater rigidity of the corresponding stand-alone films (see Table 3). For raspberries, the F/D significantly decreased after one-week storage, except in the case of those coated with κ-carrageenan-GTE, indicating that this latter coating preserved the firmness of the raspberries to a greater extent. It should be noted that the decrease in the F/D parameter was significantly higher in non-coated raspberries than in coated ones. For blueberries, the F/D significantly decreased ($p < 0.05$) during cold storage in non-coated samples but these

changes were not significant ($p > 0.05$) in the coated samples, indicating that, in general, coatings had an important role in keeping the firmness of blueberries. However, the mechanical response of blueberries coated with κ-carrageenan-GTE also changed ($p < 0.05$) during cold storage, reaching values in the range of those obtained in non-coated and coated fresh samples ($t = 0$ days). These changes in the mechanical response of the samples can be attributed to changes in the structure of the cellular tissue during ripening and senescence (Chiralt et al., 2001).

Fig. 6 displays color parameters: luminosity (L^*), chroma (C_{ab}^*) and hue (h_{ab}^*) of blueberries and raspberries before and after coating applications. In general, non-significant differences were observed in raspberries although they became more red (lower h_{ab}^* values) during storage. Coated blueberries had a slightly more bluish hue (lower h_{ab}^* values) and a more vivid color (higher C_{ab}^* values) than the non-coated samples (Supplementary material S1), being this effect more pronounced in those coated with κ-carrageenan probably due to the higher SSD. During refrigerated storage, luminosity did not vary significantly ($p > 0.05$) neither in non-coated blueberries nor in coated ones. However, the C_{ab}^* values decreased in non-coated blueberries and this decrease was less marked in blueberries coated with the different FFD. The sample hue remained practically constant throughout the storage time and only significantly decreased in non-coated samples (blueberries became more bluish) between 11 and 14 storage days, reaching values in the range of coated samples.

Furthermore, a PCA was also carried out to evaluate the variance related to the physicochemical characteristics of coatings applied on blueberries and raspberries. Results of PCA analysis are represented as scatterplots showing the relationship between factors and samples

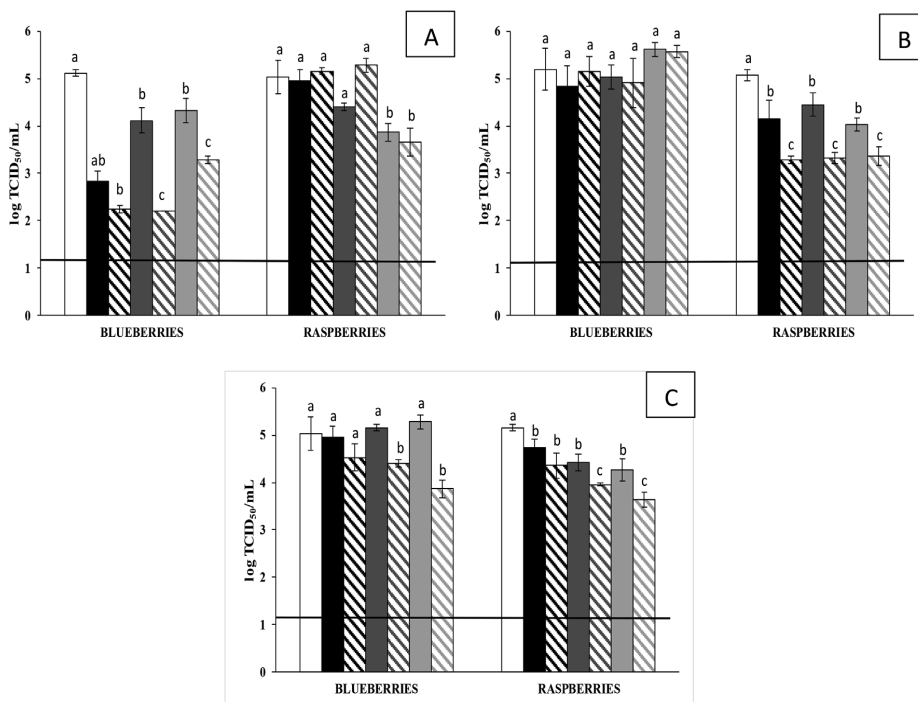


Fig. 8. Reduction of hepatitis A virus (HAV) titers (log TCID₅₀/mL) on blueberry and raspberry surfaces after treatment coatings at different temperatures and storage times.

*A: 25 °C/ON; B: 10 °C/ON; C: 10 °C/4 days.

**Each bar represents the average of triplicates. Within each column, different letters denote significant differences between treatments.

***White bars: Control without coating; black bars: coating control κ ; black-dashed bars: coating κ -GTE; dark grey bars: coating control ι ; dark grey-dashed bars: coating ι -GTE; light grey bars: coating control λ ; light grey-dashed bars: coating λ -GTE. Horizontal line depicts the detection limit.

(score plot, A) and variables (loading plot, B) for raspberries (Supplementary Material Fig. S2) and blueberries (Supplementary Material Fig. S3). For both coated food matrices, the first two Factors gained eigenvalues higher than 1, representing up to 82.3 and 81.0% of the total variance for raspberry' and blueberry 'coatings, respectively.

In raspberries (Supplementary Material Fig. S2), Factor1 was negatively correlated with all variables, except for L^* , while Factor2 was positively correlated with SSD and WVR and negatively with F/D, C_{ab} and h_{ab}^* . As a result, ι - and λ - carrageenans were correlated with L^* . On the contrary, in blueberries 'coatings, ι - and λ - carrageenans were positively correlated with variable h_{ab}^* , resulting plotted together in the lower-right quarter. The coatings with GTE resulted widely spread on the score plot, showing any discrimination clearly correlated with variables (Supplementary Material Fig. S3A).

In summary, the discrimination of samples based on the scatterplots highlighted differences among the samples that resulted in widely spaced points (Supplementary Material Figs. S2A and S3A). The PCA indicated a correlation among optical properties (L^* and h_{ab}^*) and ι - and λ - carrageenans, while a discrimination of samples based on the incorporation of GTE was not observed.

In conclusion, throughout storage, raspberries samples preserved their appearance although they became slightly redder and the coating did not have a notable effect on this development. Coated blueberries under refrigeration were slightly lighter and less bluish at the end of storage.

3.3.3. Antiviral activity

Carrageenan-based FFD, with and without GTE, were used to treat fresh raspberries and blueberries artificially inoculated with MNV and HAV and stored at 10 °C (ON and 4 days) and 25 °C (ON). In general, the effect of carrageenan type on the infectivity of MNV and HAV in fresh raspberries and blueberries after coating treatments was higher at 25 °C although a similar trend was observed at lower temperatures. As observed, FFD with similar viscosity (κ - and λ - carrageenan) showed higher antiviral activity as the gelling capacity of the carrageenan increased (κ - carrageenan) because of the formation of a more cohesive polymer matrix and the higher DSS deposited onto the fruit surfaces. In fact, the infectivity of MNV in fresh blueberries after coating treatments at 25 °C for ON incubation was reduced under the detection limit for κ - and ι -carrageenan films containing GTE and by approximately 3.54 log for λ -carrageenan coatings containing GTE (Fig. 7A). Additionally, under the same experimental conditions, κ - and ι -carrageenan coatings without GTE reduced by more than 2.5 log MNV infectivity. Surprisingly, despite of having higher DSS, lower reductions were reported in coated raspberries where MNV titers were significantly ($p < 0.05$) reduced by 2.25 and 2.79 log for ι - and λ -carrageenan coatings containing GTE, respectively. This effect can be ascribed to the different roughness of the fruit surface, which can affect the recovery of viruses during the assay. Furthermore, when blueberries were stored at 10 °C, great potential was showed obtaining reductions of 2.38 and 3.13 log after ON and 4 days storage while MNV infectivity was below the detection limit for both storage periods for raspberries coated with λ -carrageenan coatings containing GTE. Remarkable, levels of infectious

MNV in uncoated raspberries (Fig. 7B and C) were lower than in blueberries associated to the lower pH of the raspberry surface. Thus, the effect of coated-raspberries was, in some instances, below the detection limit most likely due to a synergistic effect of pH and GTE. Overall carrageenan coatings containing GTE showed higher antiviral activity against MNV than recently described alginate-oleic acid based coatings incorporated with GTE (Falcó et al., 2019) due to the higher SSD deposited onto the fruit surface and the intrinsic antiviral properties of the carrageenan-based matrix, as compared to the alginate matrices.

In addition, there was noticeable less reduction of HAV titers in coated berries (Fig. 8). For instance, HAV titers in blueberries were reduced by 2.88, 2.92 and 1.83 log after ON incubation at 25 °C for κ -, ι - and λ -carrageenan coatings containing GTE, respectively. However, only λ -carrageenan coatings prepared with GTE significantly reduced HAV infectivity by 1.37 log on coated raspberries. At refrigerated temperatures, higher efficacy of coatings was observed in raspberries than blueberries, with HAV titers by 1.79, 1.75 and 1.71 after an ON incubation for κ -, ι - and λ -carrageenan coatings containing GTE, respectively (Fig. 8B).

4. Conclusions

In this work, antiviral edible coatings based on κ -, ι - and λ -carrageenan and GTE have been successfully developed. Specifically, κ -, ι - and λ -carrageenan based coatings showed antiviral activity against MNV and HAV. A direct correlation between the surface tension of FFD, the contact angle of the corresponding stand-alone films, the gelling capacity of carrageenan, the SSD and the antiviral activity was observed. On the one hand, FFD with similar viscosity showed higher antiviral activity as the gelling capacity increased (κ -carrageenan) because of the formation of a more cohesive polymer matrix and the higher DSS deposited onto the raspberry or blueberry surfaces. On the other hand, when comparing FFD with and without GTE, lower contact angle and surface tension values (FFD containing GTE) favored a higher SSD onto the fruits, increasing the antiviral activity of the coatings, probably due to a synergistic effect of carrageenan and GTE. In general, carrageenan-based edible coatings did not promote significant changes in the physicochemical quality of raspberries and blueberries throughout refrigerated storage, although they provided better appearance. Nevertheless, they provided a better preservation of the fruits in terms of antiviral infectivity at refrigerated and ambient conditions, being accentuated by the presence of GTE. Overall, the antiviral activity varied depending on the formulation of coating forming solution, the tested virus, the type of berries and the storage conditions, thus, specific studies to optimize the formulation of antiviral coatings are needed for each specific food matrix.

Acknowledgments

M.J. Fabra is recipient of a Ramon y Cajal (RYC-2014-158) contract from the Spanish Ministry of Economy, Industry and Competitiveness. W. Randazzo was supported by a postdoctoral fellowship from Generalitat Valenciana (APOSTD/2018/150). We would like to thank Tina Lindemann and Nicolás Gil for technical assistance.

Appendix A. Supplementary data

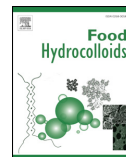
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodhyd.2019.01.039>.

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3.3.2 Article 7. *Antiviral activity of alginate-oleic acid based coatings incorporating green tea extract on strawberries and raspberries.*



Antiviral activity of alginate-oleic acid based coatings incorporating green tea extract on strawberries and raspberries

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ARTICLE INFO

Keywords:

Edible coatings
Foodborne viruses
Green tea extract
Alginate

ABSTRACT

Foodborne illnesses caused by the consumption of berries contaminated with human enteric viruses, namely human noroviruses (NoVs) and the hepatitis A virus (HAV), remain a significant food safety concern. The objective of this research was to investigate a food-grade edible coating composed of alginate/oleic acid and containing green tea extract (GTE) as an antiviral agent for the preservation of fresh strawberries and raspberries. Berries were stored at ambient (25 °C) temperature and refrigerated (10 °C) conditions. Initially, the effect of the pH of the film-forming dispersions (FFD) on their antioxidant and antiviral activity was analysed. Then, the physicochemical properties of edible alginate-oleic acid coatings containing GTE were studied, and finally, their antiviral efficacy when applied onto strawberries and raspberries at 10 and 25 °C was evaluated. The results showed that the antioxidant properties of the films were not pH-dependent, but the antiviral activity was higher at a pH 5.5. The infectivity of the murine norovirus (MNV), a human norovirus surrogate, and HAV in fresh strawberries after the coating treatments was reduced by approximately 1.5–2 log during the 4-days storage period at 10 °C as compared to the controls and a complete inactivation of both viruses was observed after overnight storage at 25 °C. However, the efficacy of the GTE-coatings was slightly reduced on the raspberries, probably due to the difference in the amount of coating that adhered to their surface (4.64 ± 0.23 g and 1.54 ± 0.15 g·cm⁻² for strawberries or raspberries, respectively). Therefore, this paper reports, for the first time, the potential of antiviral edible coatings to improve the safety of berries against foodborne pathogens.

1. Introduction

The global edible films and coatings market has recently become more interested in food preservation, and it is expected to grow in the future owing to the increased use of green strategies to minimize the use of chemical preservatives (Galus & Kadzińska, 2015; Hassan, Chatha, Hussain, Zia, & Akhtar, 2018). Edible films and coatings are based on natural polymers such as proteins, polysaccharides and/or lipids, which can improve food quality and safety by providing selective barriers to oxygen, moisture and aromas or by avoiding lipid oxidation (Hassan et al., 2018; Rezaei & Shahbazi, 2018; Umaraw & Verma, 2017). Furthermore, the natural antimicrobial and antioxidant agents incorporated into the edible coatings could also be suitable for active packaging development (Kaya et al., 2018; Musso, Salgado, & Mauri, 2017; Wang et al., 2017; Yuan, Chen, & Li, 2016), free from synthetic additives. These facts have boosted research in this area.

Active agents used for the formulation of edible films and coatings

need to be included in the approved list of food-grade additives or compounds generally recognized as safe (GRAS) by the pertinent regulations. However, although the bactericide, fungicide and antioxidant properties of different GRAS compounds have been broadly investigated, little information is available about how biopolymers could act as carriers of antiviral compounds (Fabra, Falcó, Randazzo, Sánchez, & López-Rubio, 2018; Randazzo, Fabra, Falcó, López-Rubio, & Sánchez, 2018), and to the best of our knowledge, there is no information about the application of antiviral edible coatings to foods.

Natural compounds such as polyphenols or essential oils having antiviral properties can be used for the development of edible films and coatings (Fabra et al., 2016; Randazzo et al., 2018). In particular, green tea extract (GTE) has demonstrated strong antiviral activity against foodborne pathogens such as hepatitis A virus (HAV) and murine norovirus (MNV), a human norovirus surrogate (Randazzo, Falcó, Aznar, & Sánchez, 2017). GTE is a polyphenol-rich extract obtained from the cultivated evergreen tea plant (*Camellia sinensis* L.) of the family

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<https://doi.org/10.1016/j.foodhyd.2018.08.055>

Received 9 July 2018; Received in revised form 30 August 2018; Accepted 31 August 2018

Available online 01 September 2018

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Theaceae. Catechins are the main polyphenols present in GTE and they include (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), and (–)-epigallocatechin-3-gallate (EGCG) (Chan, Soh, Tie, & Law, 2011; Pekal, Drozd, Biesaga, & Pyrzynska, 2012; Seeram et al., 2006). The main beneficial properties of GTE are usually ascribed to EGCG, which is the main catechin in green tea (~50%, Cabrera, Artacho, & Gimenez, 2006), with proven antioxidant, anticarcinogenic, anti-inflammatory and antimicrobial (bactericidal and virucidal) properties against a wide range of foodborne pathogens (Perumalla & Hettiarachchy, 2011). However, catechins are rather unstable at certain pH conditions and different tea-derived compounds can be formed as a consequence of degradation and epimerization reactions. In fact, Falcó et al. (2018) reported that the antiviral efficiency of GTE was pH-dependent, and that higher reductions against MNV and HAV were recorded for GTE at a pH of 7.2 probably due to the change in composition and, more specifically, in the amount of catechin derivatives. They found that the presence of catechin dimers such as theasinensins A and B, in combination with kaempferol 3-O-xylosylglucoside, seemed to contribute to the antiviral activity of the GTE at neutral pH.

The development of antiviral edible coatings could be of great interest, for instance, in relation to raw and minimally processed fruits and vegetables, which have been identified as important vehicles for foodborne virus transmission (Lynch, Tauxe, & Hedberg, 2009; Randazzo et al., 2018). In fact, there is increasing awareness of the significance of foodborne diseases caused by the consumption of berries contaminated with human enteric viruses (Scavia et al., 2017; Severi et al., 2015). For example, since January 2018, of the 48 alert notifications reported in the EU RASFF database that involved “viruses”, 9 were associated with berries, namely strawberries, raspberries, blueberries and blackberries. Berries are usually eaten raw and are not subjected to any industrial processes which facilitate virus inactivation or removal (Butot, Putallaz, & Sánchez, 2008). Thus, the incorporation of antimicrobial agents into edible coatings can be used to control viral contamination.

To the best of our knowledge, there is no published work related to the antiviral properties of edible coatings applied to berries. Considering all these aspects, this work was designed to: (i) assess the effect of pH on the antiviral and antioxidant activity of films containing GTE; (ii) investigate the physicochemical properties of edible alginate-oleic acid films containing GTE; and (iii) evaluate the antiviral efficacy of the developed coatings when applied to strawberries and raspberries at 10 and 25 °C.

2. Materials and methods

2.1. Materials

Alginic acid sodium salt from brown Algae (medium viscosity), oleic acid (technical grade 90%) and Tween 80 were purchased from Sigma-Aldrich (Steinheim, Germany). GTE was kindly donated by Naturex, S.A. (France). Strawberries (*Fragaria x ananassa*) and raspberries (*Rubus idaeus* L.) were obtained from a local supermarket, then immediately transported to the laboratory where fruits were selected for uniformity of size and freedom from pathological and physiological defects for use in the experiments.

2.2. Preparation of film forming dispersions

Four different film-forming dispersions (FFD) based on alginic acid sodium salt were prepared: two with GTE at two different pHs (5.5 and 7.0) and two without the extract. FFD containing GTE were prepared as follows: alginic acid (A) sodium salt (1% w/w) was directly dissolved in water at room temperature (RT). Once it was completely dissolved, a controlled amount of GTE was added to obtain 1:0.7 alginate:extract ratio. At this point, for one of the formulations, the pH of the alginate-

GTE solution was modified until 7.0 by means of NaOH 1 M. The pH of the other alginate-GTE solution was not modified, being ~5.5. Then, 0.5% (w/w) oleic acid and 0.2% (w/w) Tween 80 were mixed, under magnetic stirring with the alginate-GTE solution at RT. Oleic acid and Tween 80 were used as a hydrophobic compound to improve the applicability of these coatings and as a surfactant to improve the stability of FFD, respectively. Finally, FFD were prepared by means of a rotor-stator homogenizer (D9, MICCRA GmbH, Müllheim, Deutschland) for 2 min at 13,500 rpm at RT. After homogenization, FFD were degassed at RT with a vacuum pump.

Control films without GTE were also prepared for comparative purposes.

Edible films' nomenclature was 'A' for control alginate film (without lipid), 'A-OA' for those containing oleic acid, 'A-OA-GTE x' for those containing GTE where 'x' refers to the pH.

2.3. Preparation and characterization of the stand-alone coatings

Stand-alone coatings (films) were prepared by weighing the amount of the FFD that would provide 1 g of total solids on Teflon casting plates of 15 cm in diameter, so as to keep the total solids content constant in the dry films (56 g/m²). The films were dried for approximately 24 h at 45% relative humidity (RH) and 30 °C on a levelled surface and, subsequently, they were peeled intact from the casting surface and conditioned in desiccators with an oversaturated salt solution of magnesium nitrate (~54% RH) at 23 ± 2 °C.

Film thickness was measured with a Palmer digital micrometer to the nearest 0.0025 mm at five random positions around the film used for WVP measurements and at four positions along the strips used for the mechanical properties. Average values of film thickness were used in all WVP and tensile property determinations which ranged between 94 and 115 µm.

2.3.1. Scanning electron microscopy (SEM)

The microstructure of the films was observed by means of a SEM (Hitachi S-4800). The films (three samples per formulation) were cryofractured by immersion in liquid nitrogen, and then mounted on M4 Aluminium Specimen Mount and fixed on the support using double-side adhesive tape to explore the cross-section of the samples. After gold-palladium coating, the images were captured using an accelerating voltage of 10 kV and a working distance of 10 mm.

2.3.2. Mechanical properties

A Mecmesin MultiTest universal test machine (Landes Poli Ibérica, S.L., Barcelona, Spain) equipped with a 100-N static load cell was used for the tensile testing, according to ASTM standard method D882-09 18 (ASTM, 2010a). The mechanical parameters: elastic modulus (E), tensile strength at break (TS) and elongation percentage at break (EAB) were obtained from the stress vs. strain curves. Equilibrated specimens were mounted in the film extension grips and stretched at 50 mm min⁻¹ until breaking. Eight replicates of each formulation were tested. The experiments were carried out 54% RH and 24 °C.

2.3.3. Water vapour permeability (WVP)

WVP was determined gravimetrically at 23 ± 2 °C and 54–100% RH gradient, according to the ASTM E96/E96M-10 (ASTM, 2010b) gravimetric method for hydrophilic films. Prior to the test, the thickness of the samples was randomly measured at five points. Payne permeability cups of 3.5 cm in diameter (Elcometer SPRL, Hermelle/s Argenteau, Belgium) were filled with 5 mL of distilled water (100% RH) and then, film samples (35 mm diameter) were secured with the outwards-facing side in contact with the air during drying. The cups were placed in pre-equilibrated cabinets at 54% RH using oversaturated solutions of Mg(NO₃)₂ (Panreac Quimica, SA, Barcelona, Spain) and they were weighted periodically (± 0.00001 g) until the steady state was reached. The free film surface during film formation (air side) was

exposed to the lowest relative humidity to simulate the actual application of the films in high water activity products when stored at intermediate relative humidity. Cups with aluminium samples were used as control samples to estimate solvent loss through the sealing. WVP was calculated as previously described by [Fabra, Talens, Gavara, & Chiralt, 2012](#). Four replicates per formulation were made.

2.3.4. Antioxidant activity

The Trolox Equivalent Antioxidant Capacity (TEAC) of the GTE and the films was determined using a modification of the original TEAC method ([Re et al., 1999](#)). Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid), was used as a standard of antioxidant capacity. Each film sample of 12 ± 2 mg was cut into small pieces and hydrated with 2 mL of distilled water for two hours, and then, 8 mL of methanol were added in order to favour the extraction of the natural extracts. The supernatant (film extract) obtained was analysed for ABTS^{•+} (2,2-azino-bis (3-4-ethylbenzothiazoline)-6-sulfonic acid) radical scavenging activity. To this end, 10 µL of the film extracts were added to 1 mL of the ABTS^{•+} solution, and absorbance at 734 nm was registered every minute for 6 min. For calibration, Trolox standards of different concentrations were prepared, and the same procedure was followed. The TEAC of the film samples was determined by comparing the corresponding percentage of absorbance reduction at 6 min with the Trolox concentration–response curve. All the determinations were carried out at least six times using a spectrophotometer (Beckman Coulter DU 730, England) and methanol: water solutions as the blank.

2.3.5. Determination of antiviral activity

MNV-1 was propagated and quantified in the murine macrophage cell line RAW 264.7 (both kindly provided by Prof. H.W. Virgin, Washington University School of Medicine, USA), while HAV strain HM-175/18f (ATCC VR-1402) in the FRhk-4 cells (kindly provided by Prof. A. Bosch, University of Barcelona, Spain).

Pieces of each edible film (25 ± 5 mg) were sterilized with UV light in a safety cabinet under laminar flow for 15 min each side and then placed into test microtubes containing 500 µL of MNV and HAV suspensions diluted in PBS pH 7.2 (ca. 5 logs TCID₅₀/mL). Samples were incubated overnight (ON) at 10, 25 or 37 °C and during 4 days at 10 °C in a shaker (180 rpm). Then, the effect of the active compound was neutralizing with complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Each treatment was performed in triplicate. Confluent RAW 264.7 and FRhk-4 monolayers in 96-well plates were used to evaluate the antiviral effect of the edible films. Positive controls were virus suspensions incubated with alginate films without GTE under the same experimental conditions. 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 alginate films and N_x is the infectious virus titer for alginate films containing GTE ([Falcó et al., 2018](#)).

2.3.6. HPLC analysis

GTE-containing films (5 mg film/mL) in PBS at pH 7.0 were subjected to HPLC-MS analysis. Samples were analysed after ON incubation in the PBS. An Agilent 1290 HPLC system equipped with an Acquity UPLC BEH C18 column (Waters, 50 mm × 2.1 mm, 1.7 mm of particle size) was used, following the method described in [Gomez-Mascaraque, Soler, and Lopez-Rubio \(2016\)](#). Data were evaluated using the XIC manager in the PeakView™ software (version 2.2). The compounds detected were tentatively identified with the aid of the Phenol Explorer and Chemspider databases (<http://phenol-explorer.eu> <http://www.chemspider.com>).

2.4. Challenge tests

Selected strawberries (*Fragaria x ananassa*) and raspberries (*Rubus idaeus* L) were dipped in the FFD for 2 min and air-dried for 1 h at RT.

The mean value of the coating was calculated in ten samples by quantifying the surface solid density (SSD) (Eq. (1)).

$$SSD = (M_{CA} \cdot X_s)/A_s \quad (1)$$

Where M_{CA} is the mass of coating solution adhered to the fruit surface, X_s is the mass fraction of solids present in the FFD and A_s is the average sample surface area. The average sample surface area (A_s) was estimated by considering strawberry and raspberry geometries as cones with a known height (measured in triplicate using a digital micrometer) and volume (measured with a pycnometer, by using water as reference liquid). Samples were weighed before and after coating, to determine the mass of coating solution adhered to the strawberry or raspberry surfaces (M_{CA}). The non-coated sample was used as a control.

The viscosity of the FFD used as a coating (A-OA and A-OA-GTE5.5) was measured using a rotational viscosity meter Visco Basic Plus L (Alpha Series) from Fungilab S.A. (Spain).

In order to evaluate the appearance of the coating, coated and uncoated berries were observed by means of Eclipse 90i Nikon microscope (Nikon corporation, Japan) equipped with 5-megapixels cooled digital color microphotography camera Nikon Digital Sight DS-5Mc. Strawberries were observed by illuminating either with visible or UV light, and acquiring the images along the complete visible spectrum without any filter or with a long pass emission UV-2A fluorescent filter block (Nikon corporation, Japan), respectively. Raspberries were observed by means of a band pass emission G-2E/C filter for red fluorescence (Nikon Corporation, Japan). Acquired images were analysed and processed by using Nis-Elements Br 3.2 Software (Nikon Corporation, Japan).

2.4.1. Antiviral test on berries

Berries were exposed to UV for 15 min on a sterile plate in a laminar flow hood. Thereafter, berries were inoculated by spotting 50 µL of MNV or HAV suspension and dried under continuously circulating laminar flow for 1 h at RT before application of the coating treatments. Each sample was dipped in the coating solution for 2 min. Berry samples were dried under the laminar flow hood and then stored for 24 h at 10 and 25 °C. Samples incubated at 10 °C were also stored during 4 days. On each sampling day, individual untreated and treated berry samples were placed in a tube containing 5 mL of DMEM supplemented with 10% FCS and shaken for 2 min at 180 rpm. Finally, berries were removed from the tube and serial dilutions were performed from the resultant virus suspension. Each treatment was performed in triplicate. Positive controls were uncoated berries and coated berries without GTE in its formulation under the same experimental conditions. Infectious viruses and effectiveness of the treatments were calculated as described above.

2.5. Statistical analysis

Data of each test were statistically analysed. The statistical analysis was carried out by means of IBM SPSS Statistics software (v.23) (IBM Corp., USA) through the analysis of variance (ANOVA). Comparison of the means was done employing the Tukey's Honestly Significant Difference (HSD) at the 95% confidence level. All data are presented as mean ± standard deviation.

3. Results and discussion

3.1. Effect of pH on the antioxidant and antiviral properties of films

In the first part of this work, the antioxidant and antiviral activity of the A-OA-GTE films were evaluated depending on the pH of the film-forming solution to explore if the effect reported in the pure extract by [Falcó et al., 2018](#) was also reproduced in the films. As clearly observed in [Table 1](#), the antioxidant activity of the films expressed, as mmol

Table 1
Antioxidant capacity (TEAC: trolox equivalent antioxidant capacity) of the developed active films at pH 5.5 and 7.0.

FFD/coating	TEAC (mM Trolox/g antioxidant in the film)
A-OA	–
A-OA-GTE 7.0	14.1 (0.6) ^a
A-OA-GTE 5.5	12.5 (1.1) ^a

Mean value (standard deviation). Different letters denote significant differences between samples. A-OA alginate film containing oleic acid; A-OA-GTE 7.0: alginate film containing oleic acid and GTE at pH 7.0; A-OA-GTE 5.5: alginate film containing oleic acid and GTE at pH 5.5.

Trolox per g antioxidant, was not pH-dependent which means that the pH did not alter the antioxidant properties of the extract when incorporated within the films. Thus, although epimerization and degradation reactions can be produced at neutral pH (Falcó et al., 2018), tea-derived compounds also have antioxidant properties (Gómez-Mascaraque, Lagarón, & López-Rubio, 2015). Furthermore, the antioxidant activity of the GTE was significantly higher (20.1 ± 0.41 mmol Trolox per g antioxidant, Fabra et al., 2018) than those reported in the literature for tea extracts (Magwebeba et al., 2016; Majchrzak, Mitter, & Elmadfa, 2004) and those obtained for the A-OA-GTE edible films. The lower antioxidant activity of the edible films as compared to the pure extract can be ascribed to the existing interactions between the polyphenols with compounds present in the films, preventing a complete release of the GTE from the film structure (Fabra et al., 2018). In fact, interactions between GTE and lipids have been previously reported (Fabra et al., 2018; Rashidinejad, Birch, Hindmarsh, & Everett, 2017, 2016; Tamba et al., 2007).

Tables 2 and 3 show the effect of edible films against MNV and HAV, respectively, at different temperatures and exposure times. Overall, A-OA-GTE5.5 films exhibited the most effective antiviral activity at 37 °C. In line with these results, higher reductions were reported for MNV after ON incubation at 37 °C, decreasing titers by 3.42 log TCID₅₀/mL and 5.76 log TCID₅₀/mL for A-OA-GTE 7.0 and 5.5, respectively. Statistically significant reductions ($p < 0.05$) on MNV infectivity were observed for both films at 25 °C, while no effects ($p > 0.05$) were reported at 10 °C (Table 2) since viruses usually persist better at lower temperatures than at higher temperatures. For HAV significant differences were reported for A-OA-GTE5.5 films after ON incubation at 25 and 37 °C (Table 3). In contrast, A-OA-GTE7.0 films had almost no effect against HAV after ON incubation at 10, 25 and 37 °C. These results differ from those reported by, Falcó et al., 2018 for pure GTE. They found that freshly prepared pure GTE was very effective in inactivating either MNV or HAV at neutral pH but was ineffective at pH 5.5 because of the change in GTE composition and, more specifically, in the amount of catechin derivatives (caused by degradation and epimerization reactions) that took place in aqueous solutions at pH 7.0. Thus, the differences in the antiviral efficiency between pure extract

Table 2
The effect of edible films on the infectivity of murine norovirus (MNV).

FFD/coating	Temperature							
	37 °C		25 °C		10 °C			
	Overnight		Overnight		Overnight		4 days	
	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction
A-OA	6.91 (0.31) ^a		6.24 (0.26) ^a		7.45 (0.25) ^a		7.32 (0.54) ^a	
A-OA-GTE 7.0	3.49 (0.14) ^b	3.42	4.49 (0.47) ^b	1.75	6.99 (0.26) ^a	0.46	6.62 (0.14) ^b	0.71
A-OA-GTE 5.5	1.15 (0.00) ^c	5.76	4.43 (0.19) ^b	1.71	7.53 (0.40) ^a	–0.08	6.53 (0.52) ^a	0.79

Mean value (standard deviation). Within each column for each temperature and time, different letters denote significant differences between treatments. A-OA alginate film containing oleic acid; A-OA-GTE 7.0: alginate film containing oleic acid and GTE at pH 7.0; A-OA-GTE 5.5: alginate film containing oleic acid and GTE at pH 5.5.

and GTE-containing films may be mainly attributed to the methodology followed for determining the antiviral activity. In the previous work, pure GTE extract was incubated in PBS under the same experimental conditions (pH in the original sample) (Falcó et al., 2018) whereas, in the present work, GTE-containing films were incubated with PBS at 7.0 after having been prepared at 5.5 or 7.0. Thus, one would hypothesize that during the ON incubation of A-OA-GTE 5.5 films, part of the GTE was released into the PBS (pH 7.0) becoming degraded as a consequence of the pH (as previously reported). In contrast, in the case of A-OA-GTE 7.0, the GTE was already degraded in the original FFD and the antiviral activity of the catechin derivative compounds formed during film-formation, decreased during storage, as demonstrated by Falcó et al., 2018 for pure GTE. To confirm this hypothesis, HPLC analysis of the developed active films at both pH conditions and after overnight incubation in PBS at pH 7.0 were carried out. Table S1 of the Supplementary Material summarizes the main phenolic compounds found in both films. These compounds were tentatively identified based on the m/z values obtained from the mass spectra. Fig. S1 of the Supplementary Material depicts the profiles of phenolic compounds found in both types of films. In general, a decrease in the amount and concentration of polyphenolic compounds was observed in the films prepared at pH 7.0, probably due to the degradation induced by the long-time exposure at this specific pH, as previously reported (Falcó et al., 2018). Unexpectedly, both theasinsensin A and B, which were previously described as potentially responsible of the increased virucidal activity in the pure extract (Falcó et al., 2018), did not appear neither in the A-OA-GTE 5.5 nor in A-OA-GTE 7.0 films after ON incubation at pH 7.0. This could be due to existing interactions between lipids and GTE as it has been previously reported in several works (Fabra et al., 2018; Tamba et al., 2007).

Interestingly, these results showed that alginate films containing GTE were more effective in reducing the titers of MNV and HAV than previous studies (Fabra et al., 2018) where films containing 0.75 g GTE/g alginate prepared at pH ~ 5.5 reduced MNV and HAV infectivity by 1.97 and 1.25 log after ON incubation at 37 °C. These differences can be ascribed to the different composition of the lipid fraction of the films, which seem to play a crucial role in the availability of the GTE to exert the antiviral activity.

Overall, edible films containing GTE at pH 5.5 showed higher antiviral efficiency than their counterparts prepared at pH 7.0, with higher activity on MNV than HAV.

Therefore, taking into account that the antioxidant properties were not pH-dependent and the antiviral activity was higher at pH 5.5, a deeper physicochemical characterization of the A-OA-GTE films prepared at pH 5.5 and the antiviral efficiency on raspberries and strawberries coated with A-OA-GTE5.5 were carried out.

3.2. Characterization of the developed edible films

The microstructure was characterized by SEM to examine the

Table 3

The effect of edible films on the infectivity of hepatitis A virus (HAV).

FFD/coating	Temperature											
	37 °C				25 °C				10 °C			
	Overnight		Overnight		Overnight		Overnight		4 days		4 days	
	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction	log TCID ₅₀ /mL	Reduction
A-OA	4.70 (0.45) ^a		4.53 (0.31) ^{ab}		6.07 (0.45) ^a		5.74 (0.72) ^a		4.87 (0.36) ^b		4.87 (0.36) ^b	0.87
A-OA-GTE 7.0	4.82 (0.25) ^a	-0.13	4.95 (0.22) ^a	-0.42	6.07 (0.66) ^a	0.00	5.74 (0.14) ^a	0.33	4.78 (0.59) ^b	0.96		
A-OA-GTE 5.5	3.33 (0.07) ^b	1.67	3.32 (0.13) ^b	1.21	5.74 (0.14) ^a	0.33	4.78 (0.59) ^b	0.96				

Mean values (standard deviation). Within each column for each temperature and time, different letters denote significant differences between treatments.

A-OA: alginate film containing oleic acid; A-OA-GTE 7.0: alginate film containing oleic acid and GTE at pH 7.0; A-OA-GTE 5.5: alginate film containing oleic acid and GTE at pH 5.5.

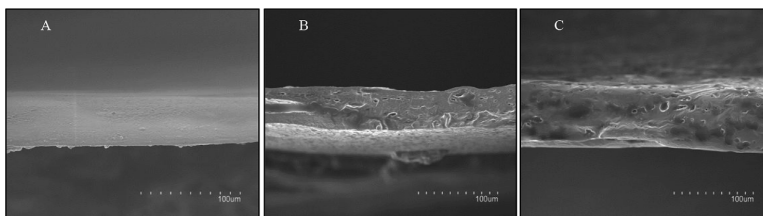


Fig. 1. SEM micrographs of the cryo-fractured section of the A (A), A-OA (B) and A-OA-GTE 5.5 (C) stand-alone coatings.

arrangement of the different compounds in the alginate matrix. Fig. 1 shows the cross-section SEM micrographs of the films where notable differences can be observed. Pure alginate films (Fig. 1A) exhibited a smooth appearance whereas the presence of OA (Fig. 1B) introduced discontinuities in the polysaccharide matrix. Films containing GTE (Fig. 1C) appeared similar to their counterparts prepared with OA but with a rougher aspect.

Water vapour is one of the main permeants studied in the application of edible films and coatings, because it may permeate from the internal or external environment through the coating, resulting in possible negative changes in product quality and shelf-life. Table 4 summarizes the measured WVP and water uptake values for the neat alginate films and those containing OA or OA-GTE. As previously reported by other authors (Ma, Hu, Wang, & Wang, 2016; Mohammad Amini, Razavi, & Zahedi, 2015), both WVP and water sorption were reduced by the addition of OA although a very limited reduction in the former was observed with respect to the pure alginate film due to the well-known plasticizing effect of this compound (Fabra, Talens, & Chiralt, 2010; Kowalczyk et al., 2016). Water sorption reduction can be

Table 4

Mechanical properties, water vapour permeability and water vapour sorption of the stand-alone coatings.

FFD/coating	E (MPa)	TS (MPa)	EAB (%)	We (g water/100 g sample) at 100% RH	WVP 10 ¹³ (Kg Pa ⁻¹ s ⁻¹ m ⁻²)
A	4524 (364) ^a	59 (5) ^a	1.5 (0.1) ^a	93 (1) ^a	2.45 (0.23) ^a
A-OA	883 (64) ^b	7.44 (0.24) ^b	0.8 (0.1) ^b	63 (6) ^b	1.87 (0.10) ^b
A-OA-GTE 5.5	1607 (76) ^c	11 (3) ^b	0.8 (0.2) ^{ab}	43 (3) ^c	2.00 (0.14) ^{ab}

WVP: water vapour permeability, E: Elastic modulus, TS: Tensile strength, EAB: elongation at break, We: water vapour sorption. Mean values (standard deviation).

Different superscripts within a column indicate significant differences among FFD/coating ($p < 0.05$).

ascribed to the fact that lipids correspond to a fraction of solids with small water uptake capacity (Fabra et al., 2010) and to the interactions between the polar groups of OA and the hydrophilic sites of the alginate matrix by means of hydrogen bonds, which could substitute the polymer-water interactions. Interestingly, GTE also contributed to reduce the water uptake capacity of A-OA films although its effect on the WVP was negligible. Thus, it seems that the polyphenolic compounds, which are mainly hydrophilic, when incorporated within the A-OA matrix may be actually confined and more restricted in terms of their interaction with water. In fact, this could also contribute to the formation of more rigid films as compared to the A-OA. GTE-lipid interactions have been previously described (Fabra et al., 2018) and could help to explain the reduction in the water uptake capacity without altering the WVP despite of the hydrophilic character of this extract. Similarly, interactions between GTE and milk fat globules surfaces (Rashidinejad, Birch, & Everett, 2016, 2017) and tea catechin (-)-epigallocatechin gallate with lipid membranes (Tamba et al., 2007) have been previously reported.

Table 4 summarizes the mechanical properties of the developed films. This analysis is interesting since an edible film or coating should be resistant in order to withstand manipulation and keep its integrity. As shown in Table 4, the addition of oleic acid induced a significant decrease in the elastic modulus and tensile strength at break, consistent with other proteins and polysaccharide-based films containing lipids (Fabra et al., 2010; Monedero, Fabra, Talens, & Chiralt, 2010). This fact can be ascribed to the presence of discontinuities in the polysaccharide matrix, which reduced the cohesion forces of the alginate network, thus implying a loss of mechanical resistance. In the case of GTE, a significant increase in the cohesive strength of the films occurred, thus increasing the hardness as compared to its counterpart prepared with OA. This seems to indicate that polyphenol extract could interact with the polysaccharide matrix or even with the OA, producing harder films and could also explain the above-mentioned lower antioxidant activity obtained in GTE-containing films as compared to the pure extract.

Both OA and GTE reduced the film stretchability, indicating that these components could interact to a certain extent with alginate, thus modifying the chain aggregation pattern in the biopolymer matrix. Both

Table 5

Surface solid density (SSD) in coated strawberries and raspberries and apparent viscosity values at 100 s^{-1} of FFD.

FFD/coating	SSD/(g cm ⁻²)		η_{app} (Pa s)
	Strawberries	Raspberries	
A-OA	4.13 (0.35) ^{a1}	1.70 (0.20) ^{a2}	281 (6) ^a
A-OA-GTE 5.5	4.64 (0.23) ^{a1}	1.54 (0.15) ^{a2}	230 (10) ^b

Mean value (standard deviation). SSD: Surface Solid Density, Different superscripts within a column indicate significant differences among FFD/coating ($p < 0.05$).

Different superscripts within a file indicate significant differences among the strawberries and raspberries ($p < 0.05$).

hydrophobic interactions and hydrogen bonds can occur between alginate and OA or GTE. Polar groups of OA and phenolic compounds present in the GTE will interact through hydrogen bond formation with the polar regions (-OH) of alginate chains. Similarly, hydrophobic interactions can occur between the hydrocarbon regions of the OA and the non-polar groups of GTE, as it has been demonstrated in a recent work (Fabra et al., 2018).

3.3. Challenge tests: application of edible coatings in strawberries and raspberries

Challenge tests on coated strawberries and raspberries at two different temperatures (10 and 25 °C) were carried out to ascertain the virucidal effectiveness of A-OA-GTE5.5 edible coatings containing 0.7 % wt. GTE on real food samples. Firstly, the surface solid density (SSD) which can be used as an estimation of coating thickness was calculated, being significantly higher in strawberries ($4.64 \pm 0.43 \text{ g}\cdot\text{cm}^{-2}$) than in raspberries ($1.54 \pm 0.15 \text{ g}\cdot\text{cm}^{-2}$). No significant effect of the apparent viscosity of the formulations with and without GTE were observed on the amount of FFD adhered to the samples, as reported by other authors (Villalobos-Carvajal, Hernández-Muñoz, Albers, & Chiralt, 2009), since strawberries and raspberries coated with GTE-containing films did not show significantly different SSD values with respect to those coated with A-OA solutions (see Table 5).

The overall appearance of the coatings on the fruit surface was qualitatively evaluated by means of optical microscopy and representative images of the samples are presented in Figs. 2 and 3. It should be mentioned that images were captured in different conditions for strawberry and raspberry due to the amount of the total solids adhered on the surface, which was nearly three times greater in the case of strawberries. This means that the microscope parameters established to get the relevant information of each sample were completely different, as it has been described in section 2.4. Specifically, the overall appearance of the strawberry's coating was clearly observed by coating only half of the strawberry and taking the rest as non-coated control. A detail of the coating is given in Fig. 2C where a continuous coating can be easily distinguished (see white arrows). However, it should be noted that a greater amount of coating was deposited in the seed hall due to the morphology (furrow-like structure) attained in these specific places of the strawberry surfaces, as it is shown in Fig. 2A and B.

Due to the lower amount of total solids adhered on the surface, differences between coated and uncoated raspberries were detected under fluorescence illumination using the red filter. As shown in Fig. 3, reduced fluorescence was observed in the uncoated samples (Fig. 3A and C) and, the fluorescence intensity detected on the coated raspberries (Fig. 3B and D) was higher at the surface of the cross-section images (Fig. 3D), indicating that the coating was mainly located on the surface.

Although it is outside the scope of this paper, it should be mentioned that the appearance of the coated strawberries and raspberries was completely different after 14 days of storage (Supplementary material Fig. S1), indicating that the presence polyphenols might also favour a delay in the growth of fungus in the berries.

GTE and control coatings were used to treat fresh strawberries and raspberries inoculated with MNV and HAV and stored at 10 °C (ON and 4 days) and 25 °C (ON). The infectivity of MNV and HAV in fresh strawberries after coating treatments was reduced by approximately 1.5 logs after ON storage at 10 °C. Additionally, more than 2.5 log reduction of MNV titers was observed after 4-days storage at 10 °C compared to the controls (Fig. 4). Complete inactivation for both viruses was observed after ON storage at 25 °C. The efficacy of GTE-coatings was slightly reduced on raspberries since MNV titers were reduced by 1.25 and 1.54 log at 10 °C, ON and 4 days storage, and by 1.96 after ON incubation at 25 °C (Fig. 5). These differences in the antiviral activity of

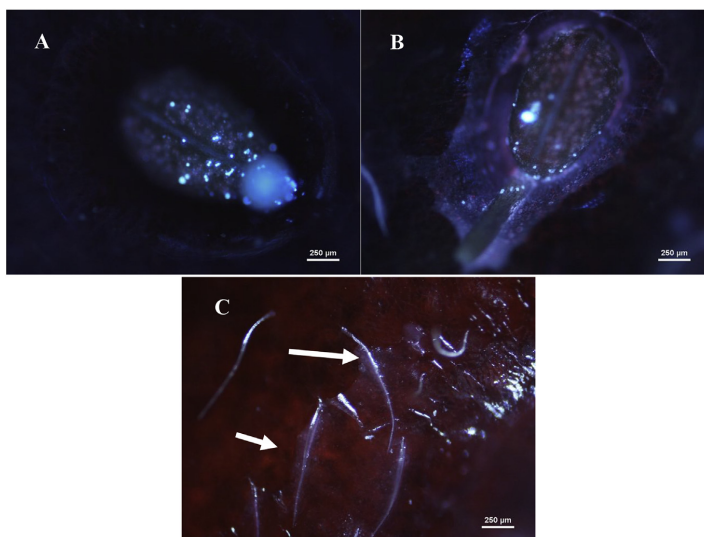


Fig. 2. Optical images of uncoated (A) and coated (B) strawberries. A detailed of the coating is also given (C).

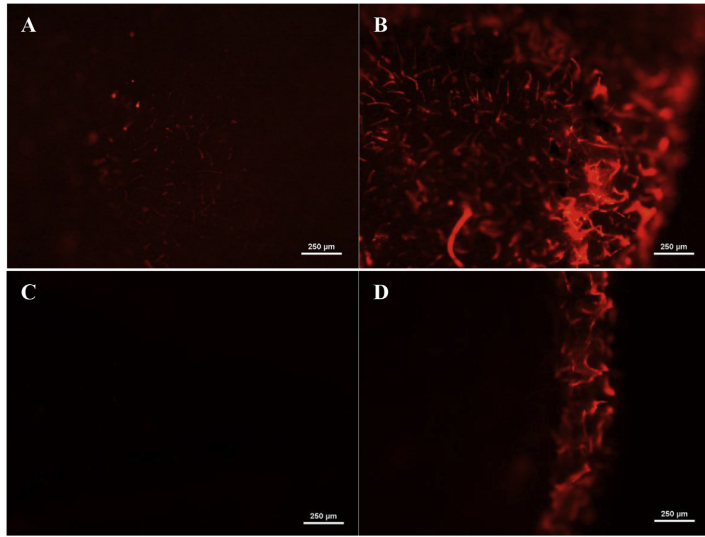


Fig. 3. Optical images of uncoated (A, C) and coated (B, D) raspberries: surface (A, B) and cross-section (C, D) images.

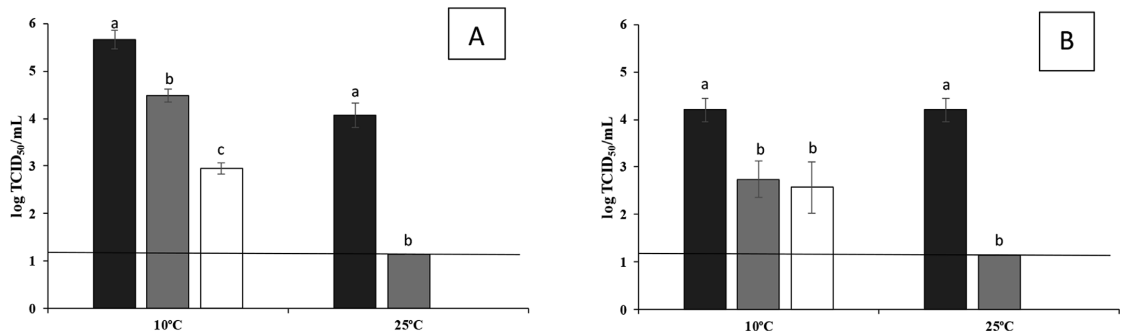


Fig. 4. Reduction of murine norovirus (MNV) (A) and hepatitis A virus (HAV) (B) titers (log TCID₅₀/mL) on strawberry surfaces after treatment with GTE-coatings at two different storage temperatures.

Each bar represents the average of triplicates. Within each column, different letters denote significant differences between treatments. Black bars: Control; grey bars: overnight storage period; white bars: 4-day storage period; dashed line depicts the detection limit.

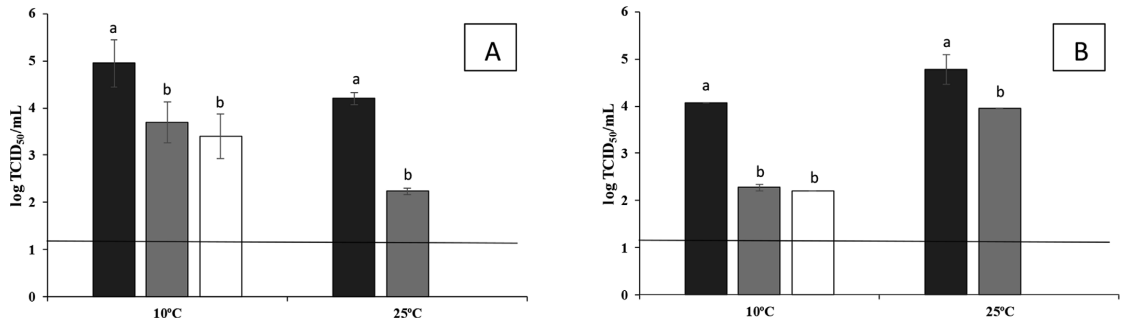


Fig. 5. Reduction of murine norovirus (MNV) (A) and hepatitis A virus (HAV) (B) titers (log TCID₅₀/mL) on raspberry surfaces after treatment with GTE-coatings at two different storage temperatures.

Each bar represents the average of triplicates. Within each column, different letters denote significant differences between treatments. Black bars: Control; grey bars: overnight storage periods; white bars: 4-day storage periods; dashed line depicts the detection limit.

the A-OA-GTE 5.5 coating can be ascribed to the amount of coating adhered (and thus, the amount of GTE) onto the surface of the berries, which was significantly lower in the case of raspberries (~1.47 or 0.49 g GTE·cm⁻² for strawberries and raspberries, respectively).

4. Conclusions

In conclusion, GTE was successfully incorporated into alginate-oleic acid film-forming dispersions at two different pH conditions (7.0 and 5.5). Results showed that the active antioxidant properties of the films were not pH-dependent and the antiviral activity of the films was reduced at basic pH (7.0). Active coating solution prepared at pH 5.5 was effective in controlling the infectivity of MNV and HAV at ambient and refrigerated conditions (25 and 10 °C) although the efficacy of GTE-coatings was greater in strawberries than in raspberries. This indicates that the amount of coating adhered onto the surface of the berries, and, more concretely, the amount of GTE played an important role. Thus, the active edible coatings prepared in this work exhibited potential antiviral properties to improve food safety of fresh strawberries and raspberries.

Acknowledgments

P. L. Flores-Meraz thanks CONACYT from the grant. M.J. Fabra is recipient of a Ramon y Cajal (RYC-2014-158) contract from the Spanish Ministry of Economy, Industry and Competitiveness. Authors would also like to thank J.M. Coll-Marqués for the optical microscopy service.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.foodhyd.2018.08.055>.

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4. CONCLUSIONS

CONCLUSIONS

From the results obtained during this work it can be concluded that:

1. Green tea extract (GTE) exhibited antiviral activity against murine norovirus (MNV) and hepatitis A virus (HAV) in a temperature-, pH-, storage time- and dose- dependent manner. The highest reductions observed were with an aged-green tea extract concentration of 5 mg/mL in neutral-alkaline conditions and at 37°C.
2. The viral infectivity reduction caused by GTE as related to the formation of catechin derivatives, mainly to epigallocatechin gallate that makes up around 40-50% of GTE composition, after storage as demonstrated by HPLC/MS analyses.
3. Norovirus virus-like particles and human norovirus suspensions treated with aged-GTE, analysed by PGM-binding ELISA and *in situ* capture RT-qPCR, respectively, showed a significant reduction in the binding ability to PGM close to 50%. However, results from viability RT-qPCR, using PMAXx as intercalating dye, indicate that epigallocatechin gallate does not dramatically affect viral capsid.

4. In order to use GTE as a natural antiviral, aged-GTE was effective in simulated gastric conditions, especially in intestinal fluid reducing MNV titers to undetectable limits and HAV by 2 log.
5. Aged- GTE incorporated in artificially contaminated beverages decreases infectious titers to undetectable limits for MNV in apple juice, and by more than 2 log for HAV in horchata. When food models were exposed to moderate heat treatments combined with aged-GTE, increased MNV inactivation was observed. However, no synergistic effect between treatments was observed for HAV.
6. GTE shows potential as a natural disinfectant being able to limit enteric viral cross contamination conveyed by vegetables and food contact surfaces, and completely reduced viral titers when aged-GTE was applied on surfaces for 30 min.
7. Adding aged-GTE enhanced the antiviral activity of carrageenans and alginate/oleic edible coatings in both refrigerated and ambient temperatures when applied on berries.

CONCLUSIONES

A partir de los resultados obtenidos durante la realización de este trabajo se concluye que:

1. El extracto de té verde (GTE) mostró actividad antiviral frente a norovirus murino y al virus de la hepatitis A (VHA) en función de la temperatura, pH, tiempo de almacenamiento y concentración. Los niveles de inactivación más elevados se obtuvieron tras el tratamiento de las suspensiones víricas con GTE envejecido a una concentración de 5 mg/mL, pH neutro-alcalino y temperatura de 37 °C.
2. Los análisis por HPLC/MS demostraron que la actividad antiviral del GTE envejecido está relacionada con la formación de productos derivados de las catequinas, principalmente con la formación de epigalocatequina galato, suponiendo entre un 40 y un 50% de su composición.
3. Mediante los ensayos ELISA de unión a PGM y de *in situ* capture RT-qPCR, partículas pseudovíricas de norovirus y suspensiones de norovirus humanos, respectivamente, mostraron una reducción significativa de casi el 50% en la capacidad de unión a la PGM. Sin embargo, los resultados de ensayos de viabilidad por RT-qPCR, usando PMAxx como marcador de

viabilidad, indicaron que la epigalocatequina galato no afecta de manera considerable a la cápside viral.

4. Con el fin de usar el GTE como un compuesto natural antiviral, su forma envejecida demostró gran efectividad en condiciones gástricas simuladas, especialmente en fluido intestinal, reduciendo las concentraciones de norovirus murino por debajo de los límites de detección y las del VHA en 2 órdenes logarítmicos.
5. El GTE envejecido añadido en zumos de manzana redujo la concentración de norovirus murino por debajo del límite de detección y en más de 2 órdenes logarítmicos la del VHA en horchata. Cuando se combinó el GTE envejecido con tratamientos térmicos moderados, la actividad antiviral del GTE mejoró los niveles de inactivación frente al norovirus murino. Sin embargo, no se observó ningún efecto sinérgico en los niveles de inactivación del VHA.
6. El GTE fue efectivo como desinfectante natural, limitando la contaminación de virus entéricos en vegetales y superficies de contacto alimentario y reduciendo completamente los niveles de virus en superficies tras 30 min de contacto.
7. Mediante la incorporación de GTE envejecido en recubrimientos comestibles de carragenatos y films de alginato/oleico aplicados sobre

frutos rojos se incrementó la actividad antiviral, tanto a temperatura ambiente como de refrigeración.

5. ANNEX

Table 10. Antiviral activity of diverse natural compounds evaluating during the current thesis.

Compound	Extraction procedure	Conc. (%)	Virus	Temp. (°C)	Incubation Time (h)	Red. (Log)	References
<i>Erodiun glaucophyllum</i>	Hydroethanolic conventional extraction	50	MNV	25	16	3.62	Abdelkebir et al. (2019)
				37		3.46	
			HAV	25		2.29	
				37		UDL	
			MNV	25		UDL	
				37		UDL	
<i>Posidonia Oceanica</i>	Ultrasound	0.5	MNV	25	16	2.6	Benito-González et al. (2019)
			FCV			3.4	
		0.05	MNV			0.7	
	FCV		2.7				
	Heating (90°C/2h)	0.5	MNV			2.1	
			FCV			3.1	
0.05		MNV	0.5				
	FCV	2.1					
<i>Zuccagnia punctata</i>	60% ethanol	0.5	MNV	37	16	2.5	Moreno et al. (2020)
0.05		UDL					

Conc.: Concentration; Red: Reduction; MNV: Murine norovirus; HAV: Hepatitis A virus; FCV: Feline calicivirus; UDL: under detection limit

Cont. Table 10.

<i>Larrea cuneifolia</i>		0.5				2.6	
<i>Larrea nitida</i>	60% ethanol	0.05		37		UDL	Moreno et al. (2020)
<i>Tetraglochin andina</i>						1.9	
		0.5	MNV		16	3.0	
				25		1.96	
Allyl-isothiocyanate	-			10		2.79	Sharif et al. (Under revision)
				37		3.25	
		0.1		25		0.75	
				10		1.58	
<i>Luma Apiculata</i> (leave extract)		0.5	MNV			UDL	
	30% ethanol	0.05			2	0.06	Carrasco-Sandoval et al., (Under revision)
		0.5	HAV			3.17	
		0.05				0.63	
<i>Persimmon</i> extract		0.5	MNV			UDL	
	Freeze-dry	0.05		37		1.25	
		0.5	HAV			NDT	
		0.05			16	0.42	Méndeza et al. (Under revision)
	Isopropanol	0.5	MNV			UDL	
		0.05				NDT	
	Mild temperatures	0.5				2.83	
		0.05	HAV			0.17	

Conc.: Concentration; Red: Reduction; MNV: Murine norovirus; HAV: Hepatitis A virus; FCV: Feline calicivirus; UDL: under detection limit.

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