Food Environ Virol (2016) 8:125–132
DOI 10.1007/s12560-016-9235-7

ORIGINAL PAPER

Efficacy of Cinnamaldehyde Against Enteric Viruses and Its Activity After Incorporation Into Biodegradable Multilayer Systems of Interest in Food Packaging

M. J. Fabra[1] · J. L. Castro-Mayorga[1] · W. Randazzo[2,3] · J. M. Lagarón[1] · A. López-Rubio[1] · R. Aznar[3,4] · G. Sánchez[3,4]

Received: 7 January 2016 / Accepted: 14 March 2016 / Published online: 23 March 2016

Abstract Cinnamaldehyde (CNMA), an organic compound that gives cinnamon its flavor and odor, was investigated for its virucidal activity on norovirus surrogates, murine norovirus (MNV) and feline calicivirus (FCV), and hepatitis A virus (HAV). Initially, different concentrations of CNMA (0.1, 0.5 and 1 %) were individually mixed with each virus at titers of *ca.* 6–7 $\log_{10}$ $TCID_{50}$/ml and incubated 2 h at 4 and 37 °C. CNMA was effective in reducing the titers of norovirus surrogates in a dose-dependent manner after 2 h at 37 °C, while HAV titers were reduced by 1 $\log_{10}$ after treatment with 1 % of CNMA. When incubation time was extended, HAV titers were reduced by 3.4 and 2.7 $\log_{10}$ after overnight incubation at 37 °C with 1 and 0.5 % of CNMA, respectively. Moreover, this paper analyzed, for the first time, the antiviral activity of adding an active electrospun interlayer based on zein and CNMA to a polyhydroxybutyrate packaging material (PHB) in a multilayer form. Biodegradable multilayer systems prepared with 2.60 mg/cm^2 (~9.7 %) of CNMA completely inactivated FCV according to ISO 22196:2011, while MNV titers were reduced by 2.75 $\log_{10}$. When the developed multilayer films were evaluated after one month of preparation or at 25 °C, the antiviral activity was reduced as compared to freshly prepared multilayer films evaluated at 37 °C. The results show the excellent potential of this system for food contact applications as well as for active packaging technologies in order to maintain or extend food quality and safety.

Keywords Enteric viruses · Cinnamaldehyde · Active packaging · Multilayer structures

Introduction

Enteric viruses are viruses that are primarily transmitted by the fecal–oral route, either by person-to-person contact or by ingestion of contaminated food or water. For most food products, handling is often the source of contamination, while shellfish is most commonly contaminated by fecally polluted water in the harvesting area.

Moreover, enteric viruses, in particular human norovirus (NoV), are the leading causes of foodborne illnesses in industrialized countries (Anonymous 2013; EFSA and ECDC 2015), while hepatitis A virus (HAV) has recently been considered as a re-emerging foodborne public health threat in Europe due to the number of foodborne outbreaks associated to imported foods (Sprenger 2014).

Cinnamaldehyde (CNMA) is the major component in cassia and cinnamon bark oils. CNMA is Generally Recognized As Safe (GRAS) by the Flavoring Extract Manufacturers' Association and is approved for food use (21 CFR 182.60) by the Food and Drug Administration (FDA) to impart a cinnamon flavor in numerous foods.

Furthermore, CNMA is known to have anti-inflammatory (Youn et al. 2008), antioxidant, and antimicrobial

✉ G. Sánchez
gloriasanchez@iata.csic.es

1 Food Preservation and Food Quality Department, Institute of Agrochemistry and Food Technology (IATA-CSIC), Avda, Agustin Escardino 7, 46980 Paterna, Valencia, Spain

2 Department of Agricultural and Forest Science, University of Palermo, Palermo, Italy

3 Biotechnology Department, IATA-CSIC, Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

4 Microbiology and Ecology Department, University of Valencia, Avda. Dr. Moliner, 50, 46100 Burjassot, Valencia, Spain

properties (reviewed by Patel 2015). Although bactericidal efficacy of CNMA is well established, current knowledge of the antiviral efficacy is limited and requires investigation (Liu et al. 2009).

Due to the beneficial properties of CNMA, its use for food applications is a growing field of interest, either in washing solutions or incorporated into packaging materials (Burt 2004). In this sense, the antimicrobial activity of CNMA incorporated into alginate (Raybaudi-Massilia et al. 2008), polycaprolactone (Martínez-Abad et al. et al. 2013b), polypropylene (Gutiérrez et al. 2009), polyethylene-co-vinyl acetate (Nostro et al. 2012), polylactic acid (Qin et al. 2015), chitosan (Peng and Li 2014), starch (De Souza et al. 2014), and apple-based edible films (Ravishankar et al. 2009) has extensively been evaluated as an alternative to the modified atmosphere packaging and addition of preservatives. However, the addition of essential oils or its active biomolecules to food or packaging materials at concentrations that resist the conventional thermal treatments may also affect the organoleptic properties of the food product (Vergis et al. 2015).

Because none of the currently available pure biodegradable polymer material exhibits all the desired mechanical and barrier properties required for every conceivable food packaging application, complex multilayer films are suggested as suitable systems to tune the performance of biopolymers. In this sense, Fabra et al. (2013) successfully developed an innovative route based on the electrospinning processing to produce biodegradable multilayer food packaging structures based on polyhydroxybutyrate (PHB) outer layers with an electrospun zein interlayer which showed enhanced barrier and mechanical properties. Electrospinning is a simple, versatile, and efficient method to produce high-performance polymeric fibers with diameters ranging from the micro to the nanoscale. This technique relies on the application of electrostatic forces to draw polymer solutions or melts into ultrathin fibers, which can be deposited as fibrous mats for many potential applications (Huang et al. 2003). Taking into account that many active agents are thermally sensitive and, thus, cannot be directly incorporated during typical processing methods used for biopolymeric materials, the electrospinning process already described could be of interest to develop new active packaging systems with the additional advantage of simultaneously producing interlayers with encapsulation performance.

Pioneering studies demonstrated the potential of natural compounds (e.g., grape seed extract (GSE) and carvacrol) to control enteric viruses in food applications (Sánchez et al. 2015; Su and D' Souza 2013) and the potential of antimicrobial packaging for virus inactivation (Bright et al. 2009; Martínez-Abad et al. 2013a). Therefore, to expand the scope of active packaging and antimicrobial surfaces, the effect of CNMA on the infectivity of HAV and two norovirus surrogates, murine norovirus (MNV) and feline calicivirus (FCV) was evaluated. Furthermore, CNMA was encapsulated within the electrospun zein interlayer conferring the active character to the multilayer packaging structures. It is presumed that the virucidal activity of this compound (which was applied within the multilayers) could be preserved by the encapsulation process induced by the electrospinning technique here applied to produce the electrospun interlayer. Thus, the efficacy of these multilayer structures containing CNMA in reducing viral loads was also analyzed.

Materials and Methods

Virus Cultivation and Infectivity

Feline calicivirus (F9 strain, ATCC VR-782) was cultured in CRFK (ATCC CCL-94) cells. Murine norovirus (MNV-1 strain; kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA) was propagated and assayed in RAW 264.7 (kindly provided by Prof. H. W. Virgin). The HM-175/18f strain of HAV (ATCC VR-1402) was propagated and assayed in FRhK-4 cells (kindly provided by Prof. Albert Bosch, University of Barcelona). Semi-purified viruses were obtained following three cycles of freeze-thawing infected cells and centrifugation at 660 × *g* for 30 min. The supernatant was stored at -80 °C until use. Infectious viruses were enumerated by determining the 50 % tissue culture infectious dose ($TCID_{50}$) with eight wells per dilution and 20 µl of inoculum per well using the Spearman-Karber method (Pintó et al. 1994).

CNMA Cytotoxicity on Cell Monolayers

Different concentrations of CNMA (3-Phenylprop-2-enal; ≥95 % purity; Sigma Aldrich) were added to 96-well cell culture plates containing a monolayer of RAW 264.7, CRFK, and FRhK-4 cells and incubated 2 h at 37 °C under 5 % CO_2. Thereafter, cells were added with 150 µl of DMEM supplemented with 2 % of fecal calf serum (FCS) and incubated further for 2 to 10 days. Cytotoxicity effects were determined by both visual inspection under the optical microscope and Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Antiviral Activity of CNMA on Virus Suspensions

Each CNMA solution diluted in 50 % ethanol was mixed with an equal volume of each virus suspension (6-7 $\log_{10}$ $TCID_{50}$/ml) and further incubated at 4 or 37 °C in a water-bath shaker at 150 rpm for 2 or 16 h (ON). Then, infectious

viruses were enumerated by cell culture assays as described above. Positive controls were virus suspensions added with ethanol in amounts corresponding to the highest quantity present. Each treatment was done in triplicate. Antiviral activity of CNMA was estimated by comparing the number of infectious viruses on suspensions without CNMA and on the CNMA-treated virus suspensions.

Preparation of Multilayer PHB-Based Films

Preparation of PHB Films

PHB films were prepared by compression-molding. To this end, 3 g of PHB pellets (Biomerc ® 226, Germany) were compression-molded into films using a hot plate hydraulic press (Carver 4122, USA) at 175 °C and 2 MPa for 3 min.

Preparation of Electrospun Zein and Zein/CNMA Interlayers by Electrospinning

Zein ultrathin nanostructured interlayers were obtained as described by Fabra et al. (2013). Briefly, zein fibers were obtained from the electrospinning of 33 wt % of zein in an 80 % v/v ethanol/water solution prepared under magnetic stirring at 25 °C, using a voltage of 14 kV and a flow rate of 0.75 ml h^{-1}. The electrospinning apparatus was a Fluidnatek LE10 basic equipment by Bioinicia S.L., Paterna (Spain) that makes use of a single needle to electrospun the polymeric solution. The zein/CNMA interlayers were identically prepared but, in this case, CNMA (75 wt % respect to the protein weight) was previously incorporated and stirred for 30 min.

The zein and zein/CNMA ultrathin fibers were directly collected onto one side of the PHB films for 6 h to have 1.07 ± 0.08 g of the nanostructured layer. The amount of electrospun zein or zein/CNMA interlayer was calculated by weighing the PHB film before and after the collection of fibers. The mass fraction of the CNMA in the resulting multilayer system (Area = 176.72 cm^2) was very low ($x_{CNMA} = 0.097$) which means that 2.60 mg/cm^2 of CNMA (or ~9.7 %) were deposited.

Multilayer Assembly

Once the electrospun zein and zein/CNMA mats were collected onto the inner side of the PHB films, they were covered with another similarly prepared PHB film. The multilayer structures were then heated in a hot press (Carver 4122) at 160 °C during 2 min (without pressing) to promote fiber coalescence, hence becoming a very thin layer with controlled morphology, and also interlayer adhesion. Multilayer structures prepared with nanostructured zein interlayer will be named as control and those containing zein/CNMA interlayers will be called active multilayer films or CNMA multilayer films throughout the article. Films were stored in 100 % relative humidity desiccators at 24 ± 2 °C protected from light with aluminum wrapping before undergoing testing.

Determination of Virucidal Activity

To test the virucidal activity of active CNMA multilayer systems, a modification of the ISO 22196:2011 (Measurement of antibacterial activity on plastics and other non-porous surfaces) was used. Briefly, a suspension of viruses diluted in PBS buffer (4–6 $\log_{10}$ $TCID_{50}$/ml) was placed onto the test films of 3 × 3 cm and covered by an inert piece of Low-Density Polyethylene (LDPE) of 2.5 × 2.5 cm and 10 μm thickness. Samples were incubated at 37 or 25 °C overnight (ON) at 100 % relative humidity. Thereafter, the top film was lifted, and the virus droplet-exposed sides were recovered and 10-fold diluted with PBS. Lastly, the corresponding cell culture assays were performed to determine whether the multilayer films were effective in inactivating the viruses. A control film (without CNMA) was used as the negative control material.

Virucidal activity was calculated by comparing the number of infectious viruses on multilayer control films (without CNMA) and on the CNMA multilayer films. Each experimental condition was analyzed in triplicate.

Scanning Electron Microscopy (SEM)

SEM was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of 8–10 mm. After immersion in liquid nitrogen, cryo-fractured multilayer systems were sputtered with a gold–palladium mixture under vacuum and their morphology was subsequently examined using SEM.

Statistical Analysis

The significance of differences among the mean numbers of viruses determined after the various treatments was determined by Student's *t* test with a significance level of $p < 0.05$ (Microsoft Office Excel; Microsoft, Redmond, WA, USA).

Results

Determination of CNMA Toxicity

CNMA was found to be cytotoxic for the three cell lines at concentrations that exceeded 1 %. Therefore, this value was the maximum concentration of CNMA tested to evaluate its effect on MNV, FCV, and HAV.

Effect of CNMA on Norovirus Surrogates and HAV

CNMA was found to be effective in reducing viral titers of norovirus surrogates and HAV depending on contact time and temperature. While incubation of CNMA at 4 °C for 2 h had no effect on the three viruses (Table 1), overnight incubation with CNMA at 0.5 and 1 % at 4 °C statistically decreased the titers of FCV ($p < 0.05$) (Table 2). Incubation of MNV and FCV with CNMA at concentrations of 0.5 and 1 % for 2 h at 37 °C significantly decreased ($p < 0.05$) the titers of the two norovirus surrogates while CNMA at 1 % decreased HAV titers by 1 $\log_{10}$ (Table 1).

When overnight incubations were performed at 37 °C, CNMA was found to be more effective on MNV and HAV. CNMA at 0.5 and 1 % reduced MNV titers to undetectable levels, while CNMA at 0.1 % reduced MNV titers by 1.7 $\log_{10}$. Furthermore, CNMA was effective in reducing the titers of HAV in a dose-dependent manner, where increasing concentrations of CNMA resulted in increased reduction in viral titers (Table 2). Efficacy on FCV was not established since the virus control was not surviving the experimental conditions (i.e., ON incubation at 37 °C).

Morphology of Multilayer Structures Based on PHB and Electrospun Zein/CNMA Interlayers

The cross section of the multilayer systems was analyzed by SEM and representative micrographs of each sample are displayed in Fig. 1. From these images, the interphase between the PHB outer layers and the interlayer was clearly observed in both control and active multilayer structures. As reported in previous works (Fabra et al. 2013, 2014), the developed multilayer systems showed laminar like structures in which the zein and zein/CNMA interlayers (*ca.* 32 μm) were thinner than the outer layers (*ca.* 78 μm) and presented a strong adhesion to the PHB matrices.

Antiviral Activity of Active Multilayer Films Following the ISO 22196:2011 at 37 °C and 100 % RH (Relative Humidity)

Initially, newly prepared active multilayer films were inoculated with norovirus surrogates and HAV adapting the ISO 22196:2011 and incubated at 37 °C and 100 % RH. After ON exposure, no infectious FCV were recovered when in contact with the active multilayer films while MNV and HAV titers decreased by 2.75 and 0.29 $\log_{10}$ $TCID_{50}$/ml, respectively (Table 3).

The effectiveness of active multilayer films were further evaluated after pre-conditioning films at 100 % RH and 25 °C during 1 month. Thereafter, virus suspensions were exposed to active multilayer films at 37 °C and 100 % RH following the ISO 22196:2011. Table 3 shows that efficacy of stored films slightly decreased, since MNV, FCV, and HAV titers decreased by 1.27, 2.66, and 0.02 $\log_{10}$ $TCID_{50}$/ml after ON contact with CNMA films.

Antiviral Activity of Active Multilayer Films Following the ISO 22196:2011 at 25 °C and 100 % RH

To have a further insight into the potential use of CNMA multilayer films, experiments were performed at 25 °C. When inoculated at high titers, no significant reduction

Table 1 Effect of cinnamaldehyde (CNMA) against murine norovirus (MNV), feline calicivirus (FCV), and hepatitis A virus (HAV) after 2 h of incubation at 4 or 37 °C

Virus	Treatment	Temperature			
		4 °C		37 °C	
	CNMA concentration (%)	$\log_{10}$ $TCID_{50}$/ml		$\log_{10}$ $TCID_{50}$/ml	
		Recovered titer	Reduction	Recovered titer	Reduction
MNV	0	7.07 ± 0.12A		6.57 ± 0.12A	
	0.1	6.45 ± 0.21B	0.62	6.07 ± 0.12A	0.50
	0.5	6.70 ± 0.17B	0.37	5.03 ± 0.07B	1.54
	1	6.57 ± 0.35B	0.50	4.32 ± 0.17B	2.25
FCV	0	7.16 ± 0.36A		7.53 ± 0.19A	
	0.1	7.15 ± 0.08A	0.01	4.24 ± 0.07C	3.29
	0.5	7.16 ± 0.19A	0.00	4.32 ± 0.17C	3.21
	1	7.07 ± 0.00A	0.09	3.37 ± 0.19B	4.16
HAV	0	6.41 ± 0.19A		6.45 ± 0.15A	
	0.1	6.41 ± 0.50A	0.00	6.43 ± 0.25A	0.02
	0.5	6.24 ± 0.14A	0.17	6.24 ± 0.50A	0.21
	1	6.20 ± 0.35A	0.21	5.41 ± 0.07B	1.04

Within each column for each virus, different letters denote significant differences between treatments ($p < 0.05$)

Table 2 Effect of cinnamaldehyde (CNMA) against murine norovirus (MNV), feline calicivirus (FCV), and hepatitis A virus (HAV) after overnight incubation at 4 or 37 °C

Virus	Treatment	Temperature			
		4 °C		37 °C	
	CNMA concentration (%)	$\log_{10}$ $TCID_{50}$/ml		$\log_{10}$ $TCID_{50}$/ml	
		Recovered titer	Reduction	Recovered titer	Reduction
MNV	0	6.41 ± 0.36A		4.91 ± 0.19A	
	0.1	6.40 ± 0.29A	0.01	3.24 ± 0.07B	1.67
	0.5	6.34 ± 0.19A	0.07	<1.82B	>3.09
	1	6.39 ± 0.09A	0.02	<1.15B	>3.76
FCV	0	6.91 ± 0.19A		No viruses recovered	
	0.1	6.78 ± 0.19A	0.13		
	0.5	<4.44B	>3.47		
	1	<1.15B	>5.76		
HAV	0	6.16 ± 0.19A		6.03 ± 0.94A	
	0.1	6.11 ± 0.07A	0.04	6.00 ± 0.19A	0.03
	0.5	6.15 ± 0.31A	0.01	3.32 ± 0.12C	2.71
	1	6.01 ± 0.08A	0.15	2.66 ± 0.07B	3.37

Within each column for each virus, different letters denote significant differences between treatments ($p < 0.05$)

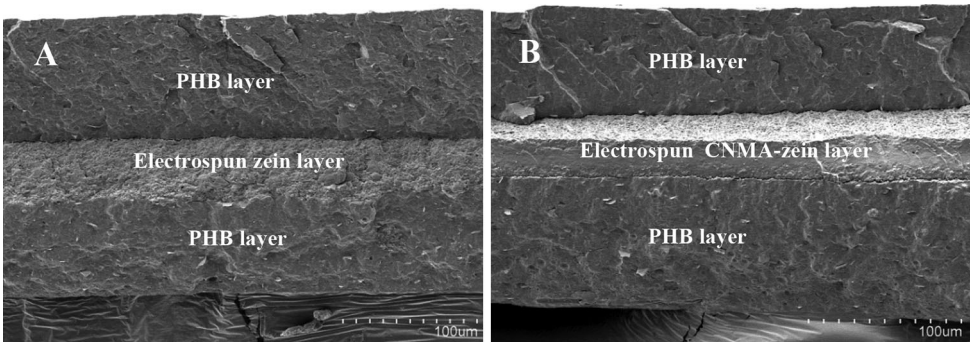

Fig. 1 SEM images of the cross sections from the PHB-multilayer systems: **a** control multilayer structure prepared with the electrospun zein interlayer and **b** active multilayer structure prepared with the electrospun zein/cinnamaldehyde interlayer (scale marker is 100 μm)

Table 3 Reduction of virus infectivity in contact with cinnamaldehyde (CNMA) multilayer films after production and after one month storage

Type of multilayer films	Storage time (days)	MNV ($\log_{10}$ $TCID_{50}$/ml)		FCV ($\log_{10}$ $TCID_{50}$/ml)		HAV ($\log_{10}$ $TCID_{50}$/ml)	
		Recovered titer	Reduction	Recovered titer	Reduction	Recovered titer	Reduction
Control	1	4.78 ± 0.31A		3.63 ± 0.19A		5.53 ± 0.38A	
CNMA multilayer films		2.03 ± 0.18B	2.75	<1.15B	>2.48	5.24 ± 0.23A	0.29
Control	30	5.03 ± 0.40A		5.91 ± 0.07A		5.45 ± 0.54A	
CNMA multilayer films		3.76 ± 0.08B	1.27	3.25 ± 0.00B	2.66	5.47 ± 0.25A	0.02

Antiviral effect of CNMA films on virus infectivity after overnight contact adapting the ISO 22196:2011 (37 °C and 100 % RH)

Mean values with different letters in the same column and same solution denote significant differences between treatments ($p < 0.05$)

($p < 0.05$) of MNV and HAV infectivity was observed, whereas 2.2 $\log_{10}$ reductions were recorded for FCV after ON contact with active multilayer films (Table 4). Moreover, when low titers of norovirus surrogates were exposed to the CNMA multilayer films, MNV and FCV titers were reduced by 1.3 and 2.4 $\log_{10}$ after ON contact with CNMA films at room temperature. For HAV, no differences ($p > 0.05$) in titers reduction were observed between HAV suspensions inoculated in control films or active multilayer films (Table 4).

Table 4 Effect of cinnamaldehyde (CNMA) multilayer films on norovirus surrogates (MNV and FCV) and HAV infectivity after overnight contact at 25 °C and 100 % RH

Virus	Treatment	High virus titer (log_{10} $TCID_{50}$/ml)		Low virus titer (log_{10} $TCID_{50}$/ml)	
		Recovered titer	Reduction	Recovered titer	Reduction
MNV	Control	5.82 ± 0.45A		4.91 ± 0.26A	
	CNMA multilayer films	5.20 ± 0.17A	0.62	3.57 ± 0.00B	1.34
FCV	Control	7.19 ± 0.35A		5.66 ± 0.22A	
	CNMA multilayer films	4.95 ± 0.79B	2.24	3.26 ± 0.26B	2.40
HAV	Control	5.57 ± 0.26A		4.78 ± 0.38A	
	CNMA multilayer films	5.54 ± 0.00A	0.03	4.28 ± 0.52A	0.50

Antiviral effect of CNMA films on virus infectivity after ON adapting the ISO 22196:2011 (25 °C and 100 % RH)

Mean values with different letters in the same column and same solution denote significant differences between treatments ($p < 0.05$)

Discussion

As a means of preventing contamination with foodborne pathogens and extending the shelf-life of foods, antimicrobial packaging is one of the most promising technologies in the food area. The incorporation of antimicrobial agents in food packaging can be used to control the microbiota, spoilage microorganisms, and even target specific foodborne pathogens to provide greater safety and to enhance food quality. Although there is an increasing awareness of the importance of foodborne diseases caused by enteric viruses, few studies have confronted the task of evaluating materials with antiviral activity against enteric viruses. In a recent innovative study, an active renewable packaging material with virucide properties was synthesized by the incorporation of silver ions into polylactide acid films. These films showed strong antiviral activity on FCV using the Japanese industrial standard (JIS Z 2801) (Martínez-Abad et al. 2013a). When films were applied to food samples, antiviral activity was very much dependent on the food type and temperature. Likewise, Bright and coworkers (Bright et al. 2009) evaluated the antiviral activity of active packaging, reporting that FCV titers were reduced by 5 log_{10} when in contact with plastic coupons impregnated with 10 % silver–copper zeolites.

An emerging application for antimicrobial packaging is the incorporation of active natural compounds. Natural additives have been proposed as potential alternatives to chemical additives since most of them are categorized as GRAS and due to increasing consumer demands for safe and "healthy" products.

Assessment of the effect of natural compounds on enteric viruses has mainly been evaluated on norovirus surrogates (reviewed by D'Souza 2014; Li et al. 2013; Ryu et al. 2015) and information about their efficacy on HAV is somewhat limited (reviewed by Aznar and Sánchez 2015). Moreover, studies on the use of natural compounds in food applications are very scarce. So far only carvacrol and grape seed extract (GSE) were reported as effective natural sanitizers against enteric viruses (Sánchez et al. 2015; Su and D'Souza 2013).

As CNMA films have shown great potential to control foodborne bacteria (De Souza et al. 2014; Martínez-Abad et al. 2013b; Peng and Li 2014; Qin et al. 2015; Raybaudi-Massilia et al. 2008), this paper reports the effect of CNMA on virus suspensions at two different temperatures and contact times. Results showed that incubation with increasing concentration of CNMA at 37 °C for 2 h increased the antiviral activity against norovirus surrogates. Furthermore, for the same conditions, CNMA treatment resulted in slight reductions on HAV infectivity with a maximum reduction of 1 log_{10} $TCID_{50}$/ml at the maximum concentration tested. Interestingly, when incubation time was extended, greater inactivation rates were reported, which may facilitate the final application in antimicrobial packaging or contact surfaces.

Active multilayer packaging structures based on PHB and zein interlayers have been also developed and evaluated in terms of their virucidal activity. Multilayer structures based on PHB (outer layers) and electrospun zein interlayers have been previously demonstrated to be the most efficient form to constitute barrier materials (Fabra et al. 2013, 2014) of interest in food packaging. In this work, CNMA was encapsulated within the electrospun zein interlayer to confer them the active character. This proof-of-concept study should provide an innovative route to develop biodegradable and renewable active multilayer packaging systems with virucidal activity. The antiviral activity of CNMA multilayer films was evaluated adapting the ISO intended for the evaluation of antibacterial activity of plastics and other non-porous surfaces. Overall CNMA multilayer films showed great potential to inactivate norovirus surrogates while no virucidal effect was reported for HAV (Table 3). Changes in temperature resulted in significant differences among the effectiveness of CNMA multilayer systems, indicating that temperature is a major

factor influencing the release or effectiveness of the active compound. In line with these results, Martínez-Abad et al. (2013b) reported that higher temperatures resulted in an increased antibacterial effect. This phenomenon could be associated to the diffusion and evaporation rates of the CNMA being higher with increasing temperature.

Moreover, the CNMA multilayer films are promising for applications to reduce environmental contamination of food contact surfaces since they proved effective against norovirus surrogates after one month of production (Table 3).

Overall, this study showed that CNMA treatment, in suspensions and incorporated into biodegradable multilayer systems, caused greater reduction on norovirus surrogates than HAV. This behavior has also been observed for other natural compounds, such as carvacrol, thymol, oregano, and zataria essential oils (reviewed by Aznar and Sánchez 2015). In contrast, GSE was more effective against HAV than MNV (Su and D' Souza 2013). As GSE has the potential to be incorporated into edible films (Amankwaah 2013), future research should consider the combination of both compounds in order to improve the antiviral efficacy of biodegradable multilayer systems.

Acknowledgments This work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO) (RYC-2012-09950, AGL2015-63855-C2-1 and INIA Grant RTA2014-00024-C04-03). GS was supported by the "Ramón y Cajal" Young Investigator and MJF was recipient of a Juan de la Cierva contract from the MINECO. JLC-M was supported by the Departamento Administrativo de Ciencia, Tecnología e Innovación (Colciencias) of Colombian Government and WR by the "Student Mobility for Placement e SMP" Grant of the EU Life Learning Program. We thank G. Ramos for his technical assistance.

References

Amankwaah, C. (2013). *Incorporation of selected plant extracts into edible chitosan films and the effect on the antiviral, antibacterial and mechanical properties of the material*. PhD dissertation, The Ohio State University.

Anonymous. (2013). Surveillance for foodborne disease outbreaks—United States, 2009–2010. *Morbidity and Mortality Weekly Report, 62*, 41–47.

Aznar, R., & Sánchez, G. (2015). Evaluation of natural compounds of plant origin for inactivation of enteric viruses. *Food and Environmental Virology, 7*, 183–187.

Bright, K., Sicairos-Ruelas, E., Gundy, P., & Gerba, C. (2009). Assessment of the antiviral properties of zeolites containing metal ions. *Food and Environmental Virology, 1*, 37–41.

Burt, S. (2004). Essential oils: Their antibacterial properties and potential applications in foods—A review. *International Journal of Food Microbiology, 94*, 223–253.

De Souza, A. C., Dias, A. M. A., Sousa, H. C., & Tadini, C. C. (2014). Impregnation of CNMA into cassava starch biocomposite films using supercritical fluid technology for the development of food active packaging. *Carbohydrate Polymers, 102*, 830–837.

D'Souza, D. H. (2014). Phytocompounds for the control of human enteric viruses. *Current Opinion in Virology, 4*, 44–49.

European Food Safety Authority (EFSA), & European Centre for Disease Prevention and Control (ECDC). (2015). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2013. *EFSA Journal*, *13*(1), 3991, 162 pp. Retrieved Jan 8, 2016, from http://www.efsa.europa.eu/en/efsajournal/pub/3991.

Fabra, M. J., López-Rubio, A., & Lagaron, J. M. (2013). High barrier polyhydroxyalcanoate food packaging film by means of nanostructured electrospun interlayers of Zein Prolamine. *Food Hydrocolloids, 32*, 106–114.

Fabra, M. J., López-Rubio, A., & Lagaron, J. M. (2014). Nanostructured interlayers of zein to improve the barrier properties of high barrier polyhydroxyalkanoates and other polyesters. *Journal of Food Engineering, 127*, 1–9.

Gutiérrez, L., Escudero, A., Batlle, R., & Nerín, C. (2009). Effect of mixed antimicrobial agents and flavors in active packaging films. *Journal of Agricultural and Food Chemistry, 57*(18), 8564–8571.

Huang, Z. M., Zhang, Y. Z., Kotaki, M., & Ramakrishna, S. (2003). A review on polymer nanofibers by electrospinning and their applications in nanocomposites. *Composites Science and Technology, 63*, 2223–2253.

Li, D., Baert, L., & Uyttendaele, M. (2013). Inactivation of foodborne viruses using natural biochemical substances. *Food Microbiology, 35*(1), 1–9.

Liu, L., Wei, F. X., Qu, Z.-Y., Wang, S.-Q., Chen, G., Gao, H., et al. (2009). The antiadenovirus activities of cinnamaldehyde in vitro. *LabMedicine, 40*, 669–674.

Martínez-Abad, A., Ocio, M. J., Lagarón, J. M., & Sánchez, G. (2013a). Evaluation of silver-infused polylactide films for inactivation of Salmonella and feline calicivirus in vitro and on fresh-cut vegetables. *International Journal of Food Microbiology, 162*(1), 89–94.

Martínez-Abad, A., Sánchez, G., Fuster, V., Lagarón, J. M., & Ocio, M. J. (2013b). Antibacterial performance of solvent cast polycaprolactone (PCL) films containing essential oils. *Food Control, 34*, 214–220.

Nostro, A., Scaffaro, R., D'Arrigo, M., Botta, L., Filocamo, A., Marino, A., & Bisignano, G. (2012). Study on carvacrol and CNMA polymeric films: Mechanical properties, release kinetics and antibacterial and antibiofilm activities. *Applied Microbiology and Biotechnology, 96*(4), 1029–1038.

Patel, S. (2015). Plant essential oils and allied volatile fractions as multifunctional additives in meat and fish-based food products: A review. *Food Additives and Contaminants—Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 32*(7), 1049–1064.

Peng, Y., & Li, Y. (2014). Combined effects of two kinds of essential oils on physical, mechanical and structural properties of chitosan films. *Food Hydrocolloids*, *36*, 287–293.

Pintó, R. M., Diez, J. M., & Bosch, A. (1994). Use of the colonic carcinoma cell line CaCo-2 for in vivo amplification and detection of enteric viruses. *Journal of Medical Virology, 44*, 310–315.

Qin, Y., Liu, D., Wu, Y., Yuan, M., Li, L., & Yang, J. (2015). Effect of PLA/PCL/CNMA antimicrobial packaging on physicochemical and microbial quality of button mushroom (Agaricus bisporus). *Postharvest Biology and Technology, 99*, 73–79.

Ravishankar, S., Zhu, L., Olsen, C. W., McHugh, T. H., & Friedman, M. (2009). Edible apple film wraps containing plant antimicrobials inactivate foodborne pathogens on meat and poultry products. *Journal of Food Science, 74*(8), M440–M445.

Raybaudi-Massilia, R. M., Rojas-Graü, M. A., Mosqueda-Melgar, J., & Martín-Belloso, O. (2008). Comparative study on essential

oils incorporated into an alginate-based edible coating to assure the safety and quality of fresh-cut Fuji apples. *Journal of Food Protection, 71*(6), 1150–1161.

Ryu, S., You, H. J., Kim, Y. W., Lee, A., Ko, G. P., Lee, S. J., & Song, M. J. (2015). Inactivation of norovirus and surrogates by natural phytochemicals and bioactive substances. *Molecular Nutrition & Food Research, 59*(1), 65–74.

Sánchez, C., Aznar, R., & Sánchez, G. (2015). The effect of carvacrol on enteric viruses. *International Journal of Food Microbiology, 192*, 72–76.

Sprenger, M. (2014). *More can be done to stop 'silent disease' of hepatitis*. The Parliament Magazine394/395. Retrieved March 21, 2016, from http://viewer.zmags.com/publication/39b84223#/39b84223/9.

Su, X., & D' Souza, D. H. (2013). Grape seed extract for foodborne virus reduction on produce. *Food Microbiology, 34*, 1–6.

Vergis, J., Gokalakrishnan, P., Agarwal, R. K., & Kumar, A. (2015). Essential oils as natural food antimicrobial agents: A review. *Critical Reviews in Food Science and Nutrition, 55*(10), 1320–1323.

Youn, H. S., Lee, J. K., Choi, Y. J., Saitoh, S. I., Miyake, K., Hwang, D. H., & Lee, J. Y. (2008). CNMA suppresses toll-like receptor 4 activation mediated through the inhibition of receptor oligomerization. *Biochemical Pharmacology, 75*(2), 494–502.