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Effect of Grape Seed Extract on Human Norovirus GII.4 and Murine Norovirus 1 in Viral Suspensions, on Stainless Steel Discs, and in Lettuce Wash Water

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The anti-norovirus (anti-NoV) effect of grape seed extract (GSE) was examined by plaque assay for murine norovirus 1 (MNV-1), cell-binding reverse transcription-PCR for human NoV GII.4, and saliva-binding enzyme-linked immunosorbent assay for human NoV GII.4 P particles, with or without the presence of interfering substances (dried milk and lettuce extract). GSE at 0.2 and 2 mg/ml was shown to reduce the infectivity of MNV-1 (>3-log PFU/ml) and the specific binding ability of NoV GII.4 to Caco-2 cells (>1-log genomic copies/ml), as well as of its P particles to salivary human histo-blood group antigen receptors (optical density at 450 nm of >0.8). These effects were decreased as increasing concentrations of dried milk (0.02 and 0.2%) or lettuce extract were added. Under an electron microscope, human NoV GII.4 virus-like particles showed inflation and deformation after treatment with GSE. Under conditions that simulated applications in the food industry, the anti-NoV effect of GSE using MNV-1 as a target organism was shown to be limited in surface disinfection (<1-log PFU/ml, analyzed in accordance with EN 13697:2001). However, a 1.5- to 2-log PFU/ml reduction in MNV-1 infectivity was noted when 2 mg of GSE/ml was used to sanitize water in the washing bath of fresh-cut lettuce, and this occurred regardless of the chemical oxygen demand (0 to 1,500 mg/ml) of the processing water.

The microbial safety of foods and control of food-borne pathogens in the food supply chain is a major concern to food industries, consumers, as well as regulatory agencies. Although synthetic food preservatives are authorized and widely used in many countries, the exploration of natural sources for antimicrobial components which are effective, as well as generally perceived to consumers to be more safe and thus more acceptable, has become a new trend worldwide (20). As reviewed by Negi (23), plant extracts, which contain innumerable constituents, are valuable sources of new and biologically active molecules possessing antimicrobial properties. However, most of the time, antimicrobial activity is solely tested against bacteria, yeast, and molds. In contrast, reports on the antiviral effects of plant extracts are rather limited (16, 26, 31).

Noroviruses (NoVs), belonging to the family *Caliciviridae*, cause a majority of the outbreaks and sporadic cases of human acute gastroenteritis worldwide (15, 35). In 2011, it was estimated that 58% of the 9.4 million episodes of food-borne illnesses in the United States each year were caused by NoVs (24). In the European Union, food-borne viruses (mainly NoVs) were identified to be the second most frequently detected causative agent in food-borne outbreaks in 2010, accounting for 15% of the reported outbreaks (6). Human NoVs are transmitted mainly through the fecal-oral route, with infected food handlers being identified as the major sources for food-borne NoV transmission, along with shellfish harvested from contaminated aquaculture areas and fresh produce being contaminated via irrigation water (surface water or untreated wastewater) (2). Thus, it is important to find a promising NoV disinfectant that is safe, environment friendly, and preferably inexpensive for use in the food industry.

Grape seed extract (GSE), which can be produced in large quantities at low cost from the by-product of the wine and grape juice industries, is a rich source of proanthocyanidins, a class of phenolic compounds that take the form of oligomers or polymers of polyhydroxy flavan-3-ol units, such as (+)-catechin and (−)-epicatechin (13). These components have been shown to have multiple functions affecting human health such as being neuroprotective, cardioprotective, anti-ulcer, and anticarcinogenic (11, 39). Furthermore, due to its lack of toxicity and its antimicrobial and antioxidant activities (13, 38), GSE has been identified as a natural and economical food preservative (21). Recently, it was reported that GSE could effectively reduce the infectivities of human NoV surrogates (feline calicivirus [FCV] F9, murine norovirus 1 [MNV-1], and bacteriophage MS2), coxsackievirus, and hepatitis A virus (HAV) (12, 29). However, the mechanism of GSE against food-borne viruses, as well as its application in food industries, has not been explored.

Virus-like particles (VLPs) of human NoVs, which are expressed in baculovirus-infected insect cells (14), have been shown to have the same morphological, antigenic, and glycan-binding properties as those of authentic viruses found in human feces (10, 22). Furthermore, the protruding (P) domain of the major structural protein of NoV capsid VP1 forms subviral particles, the P particles, when the protein is expressed in *Escherichia coli*. The P domain forms the outermost surface of the capsid and contains all elements required for viral capsid binding to host carbohydrate

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receptors (33). Both VLPs and P particles may be used to study the reaction of human NoVs toward GSE treatment, indicating the change of their morphology and binding abilities to specific receptors.

We examined here the effect of GSE on NoVs by plaque assay for MNV-1, cell-binding reverse transcriptase PCR (RT-PCR) for human NoV GII.4, and saliva-binding enzyme-linked immunosorbent assay (ELISA) for human NoV GII.4 P particles with or without the presence of interfering substances (dried milk and lettuce extract). VLPs of human NoV GII.4 before and after treatment of GSE were examined by transmission electron microscopy (TEM). We also studied the efficacy of GSE in reducing viral loads on stainless steel surfaces and in fresh-cut lettuce wash water using MNV-1 as the model.

MATERIALS AND METHODS

Viruses, viral surrogates, and cells. Cells of the murine macrophage cell line RAW 264.7 (ATCC TIB-71; kindly provided by H. W. Virgin, Washington University School of Medicine, St. Louis, MO) were maintained in complete Dulbecco modified Eagle medium (DMEM) and grown at 37°C under a 5% CO_2 atmosphere. Complete DMEM consisted of DMEM (Lonza, Walkersville, MD) containing 10% low-endotoxin fetal bovine serum (HyClone, Logan, UT), 100 U of penicillin/ml, 100 μg of streptomycin (Lonza)/ml, 10 mM HEPES (Lonza), and 2 mM L-glutamine (Lonza).

Cells of the human enterocytic cell line Caco-2 (ECACC 86010202) were cultured in Eagle minimum essential medium with Earle's salts (EMEM; Lonza) supplemented with 10% low-endotoxin fetal bovine serum (HyClone), 100 U of penicillin/ml, 100 μg of streptomycin (Lonza)/ml, and 2 mM L-glutamine (Lonza) and grown at 37°C under a 5% CO_2 atmosphere.

RAW 264.7 cells were infected with MNV-1.CW1, passage 7, at a multiplicity of infection (MOI) of 0.05 (MNV-1 to cells) for 2 days. After two freeze-thaw cycles, low-speed centrifugation was used to remove cellular debris from the virus suspension, as described by Wobus et al. (36). The lysate containing suspended MNV-1 was stored in aliquots at −75°C.

Fecal suspensions of human NoV GII.4 were kindly provided by the National Institute for Public Health and the Environment (RIVM, Bilthoven, Netherlands).

VLPs and P particles of human NoV GII.4 (Hu/NoV/GII.4/VA387/1998/US, GenBank accession number AY038600) were prepared as described previously by Tan and Jiang (33).

Anti-NoV activity of GSE. The Leucoselect GSE was kindly provided by Indena USA, Inc. (Seattle, WA). The components of this product were determined to be between 95.0 and 105.0% proanthocyanidins by gel permeation chromatography and between 13.0 and 19.0% catechin and epicatechin by high-performance liquid chromatography. GSE was dissolved in phosphate-buffered saline (PBS; pH 7.5) to obtain concentrations of 0.4 and 4 mg/ml and then filtered through a 0.2-μm-pore-size filter.

Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) was purchased from a local market in Cincinnati, OH. Lettuce leaves (10 g) were cut into small pieces (<1 cm^3 per piece). A volume of 10 ml of PBS was added, a suspension was obtained manually by the use of a glass homogenizer and centrifuged at 3,000 × *g* for 5 min at 4°C. The supernatant was collected as the lettuce extract.

The MNV-1 lysate, a fecal suspension of human NoV GII.4, and P particles of human NoV GII.4 (0.01 mg/ml) were diluted at 1:100 using PBS, dried milk (Blotto; 0.02 and 0.2%), and lettuce extract, respectively. The VLPs (1 mg/ml) of human NoV GII.4 were diluted to 1:100 using PBS.

Each GSE solution (0.5 ml of each) was mixed with an equal volume of each diluted MNV-1, NoV GII.4 virus suspension, P particles, or VLPs (0.5 ml of each), followed by incubation at 37°C for 1 h. After incubation, the samples were detected with the assays described below on the same day.

Quantification of MNV-1 infectivity. The titer of MNV-1 (PFU/ml) was determined by plaque assay as described by Wobus et al. (36). Briefly, RAW 264.7 cells were seeded into six-well plates at a density of 2×10^6 viable cells per well. On the following day, 10-fold dilutions of samples of unknown virus titer were prepared in complete DMEM, and 1 ml per sample dilution was plated onto two wells at 0.5 ml per well. The plates were incubated for 1 h at room temperature and manually rocked every 15 min before aspirating the inoculum and overlaying the cells with 1.5% SeaPlaque agarose (Cambrex, Rockland, ME) in EMEM supplemented with 10% low-endotoxin fetal bovine serum, 1% HEPES, 1% penicillin-streptomycin, and 2% glutamine (complete EMEM) per well. The plates were incubated at 37°C and 5% CO_2 for 2 days. To visualize plaques, cells were stained with 1.5% SeaKem agarose in complete EMEM containing 1% neutral red (Sigma-Aldrich, St. Louis, MO) per well for 6 h.

Cell-binding RT-PCR. The cell-binding RT-PCR for human NoVs GII.4 was performed as described by Li et al. (18). Briefly, Caco-2 cells were seeded into 24-well plates at a density of 5×10^5 viable cells per well, incubated for at least 7 days postconfluence, and used as differentiated Caco-2 cells. The treated samples were plated (100 μl per well), incubated for 1 h at 4°C, and rocked manually every 15 min. The inocula were removed after 1 h of incubation, and the cells were washed three times using PBS. The first washing step was performed by adding 0.5 ml of PBS and by rocking the plates manually. Second, the liquid was removed, 0.5 ml of PBS was added again, and the cells were scraped off. The suspensions were vortexed and centrifuged at 6,000 × *g* for 5 min (Eppendorf 5417C), and the supernatant was removed. Third, the pellets were resuspended in PBS, vortexed, and centrifuged again. The final pellets were resuspended in PBS (100 μl per sample), and the viral RNA was extracted by using an RNeasy minikit (Qiagen, Hilden, Germany) according to the RNA cleanup protocol.

The RT-PCR assay was performed as previously described by Stals et al. (25) for human NoVs GII.4. For the reverse transcription, a prereaction mix consisting of 4 μl of RNA and 0.5 μg of random hexamers (Invitrogen, Carlsbad, CA) in a final volume of 5 μl was heated at 95°C for 2 min and then cooled on ice. This first prereaction mix was then mixed with a second prereaction mixture of 15 μl to obtain a final 20 μl of reverse transcription mix containing 0.5 μg of random hexamers (Invitrogen), 1 μl of ImProm-IITM RT (Promega, Madison, WI), 5 mM $MgCl_2$ (Promega), 1× ImProm-IITM reaction buffer (Promega), 0.1 mM deoxynucleoside triphosphates (Invitrogen), and RNA. Reverse transcription was carried out in a GeneAmp PCR System 2400 (Perkin-Elmer, Waltham, MA) using the following temperature profile: 22°C for 10 min, 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min. All cDNA was stored at −20°C.

The real-time PCR was carried out in a 25-μl reaction mix containing 4 μl of template DNA, 500 nM concentrations of each forward primer, 900 nM concentrations of each reverse primer, 100 nM concentrations of each probe, and 12.5 μl of TaqMan Universal PCR master mix (Applied Biosystems). Real-time quantification was performed on the ABI 7300 system (Applied Biosystems) with the following temperature profile: 50°C for 2 min, 95°C for 10 min, and then 50 cycles of 95°C for 15 s and 60°C for 1 min. To obtain representative positive control standards, plasmids containing primer-probe binding sites were used for the quantifications (25). Tenfold serial dilutions ranging from 10^6 to 10 copies of plasmids per reaction were used to prepare the standard curves.

Human NoV GII.4 P particle saliva-binding ELISA. A saliva-binding ELISA was performed as described by Huang et al. (10) with a few modifications. Briefly, saliva samples of type B (5 to 10 ml), the collection and determination of which was defined previously by Huang et al. (10), were boiled at 100°C for 10 min and centrifuged at 10,000 × *g* for 5 min. The supernatant (100 μl) was diluted by PBS (1:500) and was used to coat 96-well microtiter plates (100 μl/well) at 4°C overnight. Uncoated wells were included as blank controls. After blocking with 5% nonfat dried milk

(Blotto, 200 μl/well), incubated P particle solutions with or without food extracts were added (0.1 μg/ml, 100 μl/well). The bound capsid proteins were detected with hyperimmune guinea pig anti-NoV antisera (1:3,300) and by adding horseradish peroxidase-conjugated goat anti-guinea pig immunoglobulin G (1:2,000; ICN, Aurora, OH). Horseradish peroxidase activity was detected with a TMB (3,3′,5,5′-tetramethylbenzidine) kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and the signal intensities (i.e., the optical density at 450 nm [OD_{450}]) were read with an enzyme immunoassay spectrum reader (Tecan, Durham, NC).

As described by Huang et al. (10), the guinea pig anti-NoV antiserum was a mixture of the hyperimmune sera of guinea pigs cross-immunized with recombinant VLPs both from human NoV genogroups I and II: Norwalk virus (M87661), C59 (AF435807), VA115 (AY038598), VA387 (AY038600), MxV (U22498), GrV (AJ004864), HV (U07611), VA207 (AY038599), and MOH (AF397156). The antiserum recognized the authentic virions, VLPs, and P particles over a wide range of human NoV strains.

TEM. The treated VLPs of human NoV GII.4 were fixed in copper grids using 1% ammonium molybdate as the staining solution. Specimens were examined under an EM10 C2 microscope (Zeiss, Germany) at 80 kV at a magnification of ×80,000.

Surface disinfection test using stainless steel discs. The surface disinfection test was performed based on EN 13697:2001 (5).

Stainless steel discs (20 mm in diameter) were incubated in a 5% (vol/vol) Decon 90-solution (Decon Laboratories, Ltd., Hove, England) for 1 h. Afterward, the discs were rinsed twice with distilled water for 10 s and then placed in 70% isopropanol (Sigma-Aldrich, Steinheim, Germany) for 15 min. Finally, the discs were dried by evaporation in sterile petri dishes.

As specified by EN 13697:2001 (5), 0.3 g of bovine serum albumin (BSA)/liter was used to stimulate the protein in clean working conditions (surface disinfection), and 3 g/liter was used for dirty conditions. Since the initial titer of the MNV-1 lysate was comparatively lower, and the protein concentration of MNV-1 lysate was determined to be 3.8 g/liter by a Bradford protein assay, the MNV-1 inocula were prepared by 10-fold dilution of the MNV-1 lysate using PBS to simulate the clean conditions and using a BSA solution (3 g/liter in PBS) to simulate the dirty conditions.

The virus inoculum of MNV-1 (50 μl) was pipetted into the middle of a clean and disinfected stainless steel disc and dried at 37°C for 30 min. GSE was dissolved in hard water (5) to obtain concentrations of 0, 0.2, and 2 mg/ml, filtered through a 0.2-μm-pore-size filter, and used within 2 h. A volume of 100 μl of the test solution was pipetted on the inoculated discs, followed by incubation at room temperature for 10 min. As a positive control, the inoculated disc received 100 μl of hard water instead of a GSE solution.

The discs representing the positive control and treated discs were transferred into 25-ml sterile containers, which were previously filled with 10 ml of neutralizer (complete DMEM) to stop the inactivating activity and 5 g of glass beads (0.25 to 0.50 mm in diameter) to increase virus recovery by mechanical abrasion. After a neutralization of 5 min (including a shaking of 1 min), the neutralized treated virus solution (positive and treated samples) was stored at −75°C until the titers of MNV-1 were determined according to the plaque assay described above.

Water disinfection test using model fresh-cut lettuce wash water. The process wash water with high chemical oxygen demand (COD) was prepared as described by López-Gálvez et al. (19). Iceberg lettuce (*Lactuca sativa* L.) was purchased from a local market in Belgium. The outer leaves were manually removed and discarded, and the internal leaves were cut into 3-cm pieces. Afterward, 67 g of lettuce was placed into a stomacher bag with a filter compartment (full filter blender bag, 190 by 300 mm, FBAG-04; Novolab, Geraardebergen, Belgium), 200 ml of tap water was added, and the mixture was homogenized for 2 min in a stomacher (Tekmar 80; Seward, United Kingdom). Finally, the filtrate was removed from the stomacher bag and diluted at 1:10 by tap water. The COD values were measured immediately by using a Nanocolor COD 1500 (Macherey-Nagel GmbH, Duren, Germany). The process wash water were further diluted with tap water to obtain COD values of 500, 800, and 1,500 mg/liter, respectively, and stored at 4°C.

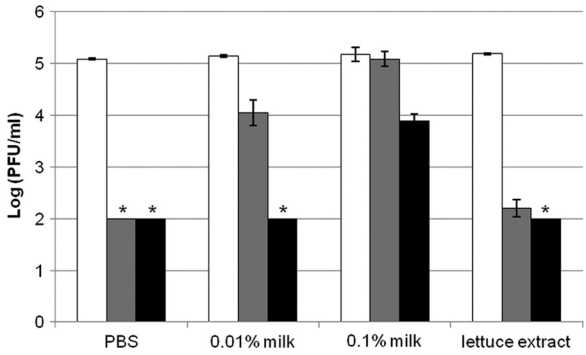

FIG 1 Infectivity of MNV-1 detected by plaque assay before (white bars) and after GSE treatment (0.2 mg/ml [gray bars] or 2 mg/ml [black bars]) in different solutions (*x* axis). Each column represents the average of triplicates, and each error bar indicates the data range. *, lower than the detection limit.

On the following day of the COD measurement (the COD values of the process wash water were determined to be stable after storage for 1 day), tap water and process wash water with different COD values (500, 800, and 1,500 mg/liter) were inoculated with MNV-1 lysate (0.5 liter of water with 5 ml of virus lysate), and GSE powder was added to the inoculated water to obtain concentrations of 0, 0.2, and 2 mg/ml. After incubation on a shaking platform (KS130; IKA, Germany) rotating at 200 rpm for 1 h at 7°C, the treated virus solutions were 10-fold serial diluted using complete DMEM, and plaque assays were performed the same day.

Data analysis. The samples were tested in triplicate. Each error bar represents the data range. Statistical analyses were performed by one-way analysis of variance (the Tukey's test was used as a post hoc test) with SPSS 19.0 for Windows (SPSS, Inc., Chicago, IL). Significant differences were considered when P was <0.05.

RESULTS

Effect of GSE on the infectivity of MNV-1. The infectivity of MNV-1 detected by plaque assay before and after the treatment of GSE in different solutions was shown in Fig. 1. When diluted in PBS, the infectivity of MNV-1 was reduced to a level lower than the detection limit (reduction of >3-log PFU/ml) by a treatment with 0.2 mg of GSE/ml. With the presence of 0.01% dried milk, a similar effect was not obtained until the dose of GSE was increased to 2 mg/ml. As the concentration of the interfering dried milk in the solution was increased to 0.1%, 2 mg of GSE/ml only induced a reduction of MNV-1 infectivity by 1-log PFU/ml. When the MNV-1 was diluted in lettuce extract, a reduction of 3-log PFU/ml was obtained by 0.2 mg of GSE/ml, and further reduction (>3-log PFU/ml) was obtained using 2 mg of GSE/ml.

Effect of GSE on the binding ability of human NoVs GII.4. The genomic copies of human NoVs GII.4 detected by cell-binding RT-PCR before and after treatment with GSE in different solutions are shown in Fig. 2. The binding levels of NoVs GII.4 to differentiated Caco-2 cells were reduced by treatment with GSE significantly ($P < 0.05$) in a dose-dependent manner, except in the presence of 0.1% dried milk, where no significant reduction ($P > 0.05$) was observed after treatment with 0.2 mg of GSE/ml.

Effect of GSE on the binding ability of human NoVs GII.4 P particles. As a further confirmation of the cell-binding RT-PCR, the binding ability of human NoVs GII.4 P particles was tested by saliva-binding ELISA after treatment with GSE (Fig. 3). Similar to

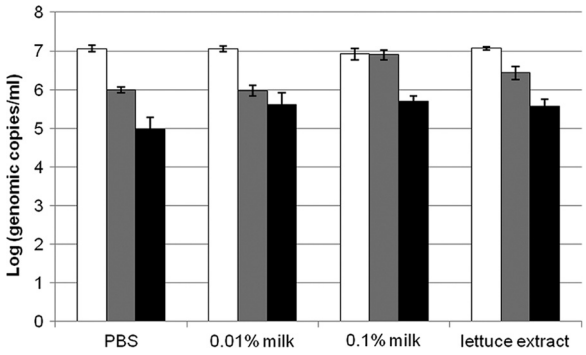

FIG 2 Genomic copies of human NoVs GII.4 detected by cell-binding RT-PCR before (white bars) and after GSE treatment (0.2 mg/ml [gray bars] or 2 mg/ml [black bars]) in different solutions (*x* axis). Each column represents the average of triplicates, and each error bar indicates the data range.

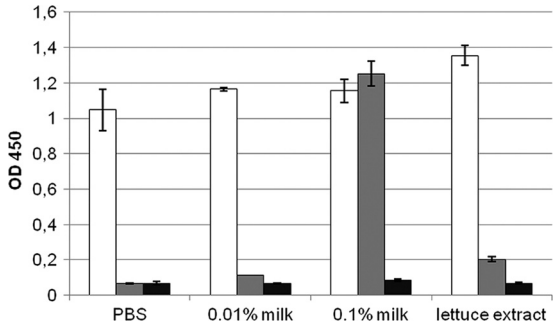

FIG 3 OD_{450} values of human NoVs GII.4 P particles detected by saliva-binding ELISA before (white bars) and after GSE treatment (0.2 mg/ml [gray bars] or 2 mg/ml [black bars]) in different solutions (*x* axis). Each column represents the average of triplicates, and each error bar indicates the data range.

the results of cell-binding RT-PCR, the binding signal (OD_{450}) was reduced from a level higher than 1 to a level lower than 0.2 after treatment with GSE at 0.2 and 2 mg/ml, except that in the presence of 0.1% dried milk no reduction was observed after treatment with 0.2 mg of GSE/ml.

Effect of GSE on the morphology of human NoV GII.4 VLPs. Based on the results described above for plaque assay for MNV-1, cell-binding RT-PCR for human NoV GII.4, and saliva-binding ELISA for human NoV GII.4 P particles, we conclude that proteins (a major constituent of dried milk) are an interfering factor and inhibit the anti-NoV effect of GSE. Thus, in order to investigate whether the anti-NoV effect of GSE is due to the denaturation of viral capsid protein, the morphology of human NoV GII.4 VLPs before and after treatment of GSE was examined by TEM. For the untreated control, the VLPs of human NoV were small spherical structured particles with two sizes (larger particles between 30 and 38 nm and smaller particles between 18 and 20 nm; Fig. 4A), which is consistent with previous observations (8). After a treatment with GSE (0.2 mg/ml), the VLPs started to clump together. For most of the larger particles, obvious deformation and inflation was observed (Fig. 4B). At a GSE dose of 2 mg/ml, the spherically structured VLPs disappeared, and a large concentration of protein debris was observed (Fig. 4C). These results provide the most direct evidence that GSE could effectively damage the NoV capsid protein.

Effect of GSE in surface disinfection using stainless steel discs. Initially, the experimental setup of the surface disinfection test was evaluated. We found that the difference between the titers of the virus inoculum [(4.01 ± 0.09)-log PFU/ml] and the positive control [MNV-1 inoculated and recovered from surface without GSE treatment; (3.18 ± 0.21)-log PFU/ml] was not >1 log, which means that the treatments other than GSE treatment had no observable inactivating effects. A <0.2-log difference in virus titer was observed between the viral contents of an inoculated disc immersed in neutralizer supplemented with 100 μl of hard water [(3.18 ± 0.21)-log PFU/ml] and an inoculated disc immersed in neutralizer supplemented with 100 μl of GSE at 2 mg/ml [(3.24 ± 0.06)-log PFU/ml], suggesting complete neutralization.

The infectivity of MNV-1 detected by plaque assay before and after treatment with GSE on stainless steel surfaces is shown in Fig. 5. No significant reduction ($P > 0.05$) in MNV-1 infectivity in the surface disinfection test was observed except for the group in the clean condition treated with 2 mg of GSE/ml, but only a marginal reduction (0.56-log PFU/ml) was obtained.

Effect of GSE in water disinfection using model fresh-cut lettuce wash water. The infectivity of MNV-1, detected by plaque assay, suspended in lettuce wash water with different COD values, either with or without GSE added as a sanitizer with a potential antiviral effect is shown in Fig. 5. Significant reductions ($P < 0.05$) in MNV-1 infectivity were obtained by the addition of GSE in a dose-dependent manner in tap water as well as in lettuce wash water with different COD values. In tap water, 0.2 mg of GSE/ml induced an ~1-log PFU/ml reduction, and 2 mg of GSE/ml induced a reduction of >2-log PFU/ml. Although it should be noted that the inactivating effect GSE at 2 mg/ml was slightly decreased as the COD values increased, no significant difference ($P > 0.05$) was observed between groups in tap water and wash waters with COD values of 500, 800, or 1,500 mg/liter.

DISCUSSION

Due to the lack of suitable animal models and the inability to propagate in cell cultures for human NoVs (4), surrogates that share pathological and/or biological features with human NoVs have been used to study the inactivation of human NoVs (8, 9, 17). MNV-1 has been used since it is cultivable and belongs to the same genus as human NoVs (34). Su et al. (29) showed that MNV-1 was less susceptible to GSE treatment than were FCV and HAV and had a similar resistance with MS2. Consistent with previous studies (29), we demonstrated here that the commercial GSE could reduce the titer of MNV-1 as a surrogate of human NoV effectively (reduction of >3-log PFU/ml in PBS by a GSE treatment of 0.2 mg/ml).

Although MNV-1 is generally recognized as the most suitable surrogate for human NoVs, the applicability has always been questioned due to its different clinical manifestations, infected cell types, and host receptors (34). Therefore, in the present study, the anti-NoV effect of GSE was further explored by measuring the specific binding ability of human NoV. In 2011, a receptor-binding RT-PCR was developed to detect infectious human NoV using Caco-2 cells and porcine gastric mucin as receptors (18). The use of porcine gastric mucin as a binding receptor to discriminate infectious and noninfectious human NoV was further reported by Dancho et al. (3). However, Caco-2 cells were chosen to be used in the present study for two reasons. First, Caco-2 cells express H

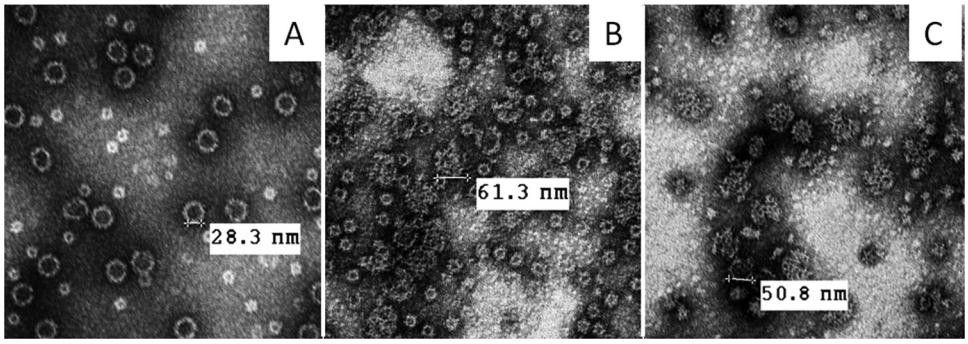

FIG 4 Electron micrographs of human NoVs GII.4 VLPs before (A) and after GSE treatment (0.2 mg/ml [B] or 2 mg/ml [C]).

types 1 and 2 of human histo-blood group antigens (HBGAs) (1), which have been proven to bind to a wide range of human NoVs both from GI and GII (34). For NoV GII.4, which has been recognized as the predominant genotype of NoVs since mid-1990s worldwide, Yang et al. (37) investigated the binding patterns of a series of NoV GII.4 strains and demonstrated that all of the tested 10 strains had typical binding patterns in the saliva binding assay reacted strongly to H-1. Second, it was reported that coreceptors may be also involved in the binding of human NoVs to Caco-2 cells. In 2004, Tamura et al. (32) proved that NoV VLPs derived from two GI strains and three GII strains could all bind to differentiated Caco-2 cells effectively. It was suggested the cell surface glycosaminoglycans and H-type blood antigen both contributed to the binding of NoV GII VLPs to differentiated Caco-2 cells. In the present study, the binding levels of human NoVs GII.4 in PBS to differentiated Caco-2 cells were 1- and 2-log genomic copies/ml reduced by treatment with GSE at 0.2 and 2 mg/ml, respectively, as detected by cell-binding RT-PCR.

HBGAs, which are a group of complex carbohydrates present on the red blood cell surfaces, mucosal epithelia of the respiratory, genitourinary, and digestive tracts, as well as free oligosaccharides in biologic fluids such as saliva, intestinal contents, milk, and blood, have been shown to be the receptors of human NoVs (10, 34). On the NoV major structural protein capsid, the protruding (P) domain forms the outermost surface and contains all elements required for viral binding to host carbohydrate receptors. This protein forms subviral particles, the P particles, when the protein is expressed in *E. coli* (33). The saliva-binding ELISA, which could depict the receptor-binding interactions between salivary HBGAs and the P particles of human NoVs, was used to evaluate the effect of GSE on the specific binding ability of human NoV through a more microscopic perspective. When diluted in PBS, the binding signal (OD_{450}) of NoVs GII.4 P particles to the salivary carbohydrate coated on the ELISA plate was reduced from a level higher than 1 to a level lower than 0.2 only after a treatment with GSE at 0.2 mg/ml.

In order to investigate the interfering effect of food components to the anti-NoV effect of GSE, dried milk or lettuce extract was added to the PBS-diluted MNV-1 lysate, human NoV GII.4 fecal suspension, and human NoV GII.4 P particle solution, respectively, before treatment with GSE. According to our result, the interfering substances, especially dried milk, could reduce the anti-NoV effect of GSE to different extents, as evaluated by different methodologies. With the presence of 0.1% dried milk, no significant reduction of MNV-1 infectivity or human NoV GII.4 binding levels were obtained using a treatment with GSE of 0.2 mg/ml, which could induce reductions of >3-log PFU/ml for MNV-1 detected by plaque assay, 1-log genomic copies/ml for human NoV GII.4 detected by cell-binding RT-PCR, and >0.8 OD_{450} for NoV GII.4 P particles detected by saliva-binding ELISA, respectively, when diluted in PBS.

Since the presence of interfering protein (dried milk) has been shown to play a role in inhibiting the anti-NoV effect of GSE, the morphology of human NoV GII.4 VLPs before and after treatment of GSE was examined by TEM in order to investigate whether the anti-NoV effect of GSE is due to the denaturation of

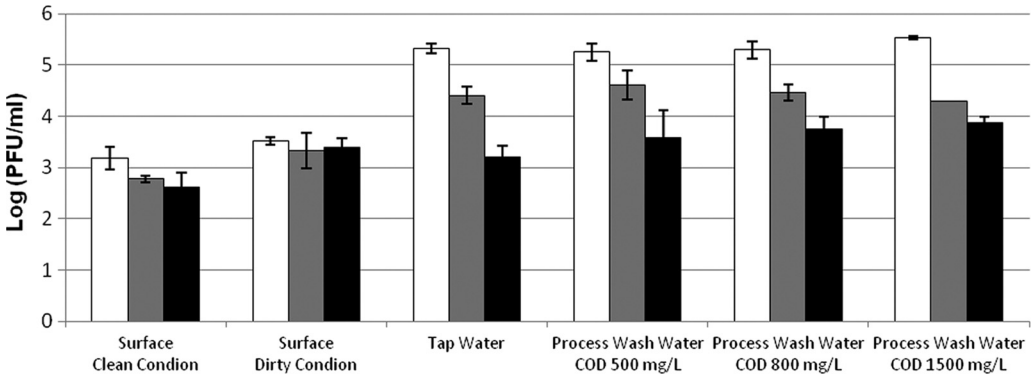

FIG 5 Infectivity of MNV-1 detected by plaque assay before (white bars) and after GSE treatment (0.2 mg/ml [gray bars] and 2 mg/ml [black bars]) in different practical scenarios (*x* axis). Each column represents the average of triplicates, and each error bar indicates the data range.

viral capsid protein. Observations under the microscope, after treatment with GSE (0.2 mg/ml) showed clumping of VLPs and obvious deformation and inflation. At the higher GSE dose of 2 mg/ml, the spherically structured VLPs disappeared, and a large concentration of protein debris was observed. This provides evidence that GSE could effectively damage the NoV capsid protein, which could reduce viral binding ability and infectivity accordingly. In 2004, Taguri et al. (30) evaluated the antibacterial activities of 10 different plant polyphenols and found that the sensitivity of bacteria to polyphenols depends on bacterial species and polyphenol structure. Thus, the structure-effect relationship between GSE and the NoV capsid protein should be studied in greater detail.

With regard to application in food safety enhancement, it should be noted that the anti-NoV effect of GSE as established in *in vitro* studies could be inhibited by food components, especially proteins. In the surface disinfection test, since the interfering protein concentrations were set as defined by EN 13697:2001 (0.3 g/liter for clean working conditions and 3 g/liter for dirty conditions) (5), no significant reduction ($P > 0.05$) in MNV-1 infectivity was obtained, except for the group in clean conditions treated with GSE at 2 mg/ml, in which only a marginal reduction (0.56-log PFU/ml) was observed. In the water sanitation test, the wash water of fresh-cut lettuce was prepared with different defined COD values, inoculated with MNV-1, and treated with GSE at a low temperature (<10°C) simulating the working environment in the factory. According to the results, GSE at a dose of 2 mg/ml shows promise for use as an effective disinfectant against NoV contamination in fresh-cut lettuce processing water (ca. 1.5- to 2-log PFU/ml reduction), while the COD value of the wash water was not found to play a significant role ($P > 0.05$). This result may be of great importance to the food industry since fresh produce has often been found to be related to NoV outbreaks (2, 7). The washing step at the fresh-cut processing company is considered to play an important role in the food pathogen transmission due to the cross-contamination via process water (19), and therefore disinfection of the process water is a critical step in reducing the viral load of fresh produce.

A few additional issues and/or limitations also need to be addressed. First, the cell-binding RT-PCR for human NoV GII.4 and the saliva-binding ELISA for NoV GII.4 P particles were performed with the same objective from macro- and microscopic perspectives; however, the results were expressed as genomic copy and OD_{450}, respectively, which are not exactly comparable values. Second, MNV-1 seemed to be more susceptible to human NoV GII.4, since with a GSE treatment of 0.2 mg/ml, a reduction of >3-log PFU/ml for MNV-1 could be detected by plaque assay, whereas reduction of only 1-log genomic copies/ml for human NoV GII.4 was detected by cell-binding RT-PCR. However, since the plaque assay determines the infectivity of MNV-1 and the cell-binding RT-PCR measures the binding ability of human NoV GII.4, it is possible that a proportion of GSE-treated human NoVs lost their infectivity but still retained their binding ability. Third, in the application study of GSE to the decontamination of food contact surfaces or the sanitation of processing water, only MNV-1 was tested but not with human NoVs due to the inaccessibility of a large quantity of clinical virus samples. In addition to this, the treatment time for GSE on MNV-1 in lettuce wash water used here was 1 h. Since the effect of plant polyphenols on NoV surrogates was reported to be time dependent within 1 h (27, 28), it should be noted that the effect of GSE on MNV-1 in lettuce wash water might be lower if the treatment time is shortened.

In conclusion, we examined the anti-NoV effect of GSE by investigating its effect on the infectivity of MNV-1 as a human NoV surrogate, the specific binding abilities of human NoV GII.4 and its P particles, and the morphology of NoV GII.4 VLPs under TEM. The mechanism of the anti-NoV effect was preliminarily determined as the denaturation of the viral capsid protein. Since this denaturation may also affect proteins other than the viral capsids, the presence of interfering substances should be taken into consideration if GSE is used as an antiviral component for application in a food-related setting such as the decontamination of food contact surfaces or the sanitation of processing water in the food industry.

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