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Antilisterial effects of gravinol-s grape seed extract at low levels in aqueous media and its potential application as a produce wash.

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Abstract
Grape seed extract (GSE) is a rich source of proanthocyanidins, a class of natural antioxidants reported to have wide-ranging bioactivity as anti-inflammatory, anticarcinogenic, and antimicrobial agents. The ability of GSE to rapidly inactivate Listeria monocytogenes in vitro and the generally recognized as safe status of GSE make this extract an attractive candidate for control of Listeria in or on foods. Previously, GSE has been used at relatively high concentrations (1%) in complex food matrices and in combination with other antimicrobials. We sought to characterize the antilisterial effects of a commercial GSE preparation (Gravinol-S) alone at much lower concentrations (0.00015 to 0.125%) in aqueous solution and to test its possible use as an antimicrobial wash for fresh produce surfaces. Based on broth microdilution tests, the MICs of GSE against L. monocytogenes Scott A and Listeria innocua ATCC 33090 were as low as 50 and 78 mg ml21, respectively. GSE was evaluated in 0.85% saline against live cells of L. innocua via flow cytometry, using propidium iodide as a probe for membrane integrity. At sub-MICs and after only 2 min of exposure, treatment with GSE caused rapid permeabilization and clumping of L. innocua, results that we confirmed for L. monocytogenes using fluorescence microscopy and Live/Dead staining. At higher concentrations (0.125%), GSE reduced viable cell counts for L. monocytogenes by approximately 2 log units within 2 min on tomato surfaces. These results suggest the potential for GSE as a natural control of Listeria spp. on low-complexity foods such as tomatoes.

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Antilisterial Effects of Gravinol-S Grape Seed Extract at Low Levels in Aqueous Media and Its Potential Application as a Produce Wash

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ABSTRACT

Grape seed extract (GSE) is a rich source of proanthocyanidins, a class of natural antioxidants reported to have wide-ranging bioactivity as anti-inflammatory, anticarcinogenic, and antimicrobial agents. The ability of GSE to rapidly inactivate *Listeria monocytogenes* in vitro and the generally recognized as safe status of GSE make this extract an attractive candidate for control of *Listeria* in or on foods. Previously, GSE has been used at relatively high concentrations (1%) in complex food matrices and in combination with other antimicrobials. We sought to characterize the antilisterial effects of a commercial GSE preparation (Gravinol-S) alone at much lower concentrations (0.00015 to 0.125%) in aqueous solution and to test its possible use as an antimicrobial wash for fresh produce surfaces. Based on broth microdilution tests, the MICs of GSE against *L. monocytogenes* Scott A and *Listeria innocua* ATCC 33090 were as low as 50 and 78 μg ml^{-1}, respectively. GSE was evaluated in 0.85% saline against live cells of *L. innocua* via flow cytometry, using propidium iodide as a probe for membrane integrity. At sub-MICs and after only 2 min of exposure, treatment with GSE caused rapid permeabilization and clumping of *L. innocua,* results that we confirmed for *L. monocytogenes* using fluorescence microscopy and Live/Dead staining. At higher concentrations (0.125%), GSE reduced viable cell counts for *L. monocytogenes* by approximately 2 log units within 2 min on tomato surfaces. These results suggest the potential for GSE as a natural control of *Listeria* spp. on low-complexity foods such as tomatoes.

Listeria monocytogenes is an environmentally ubiquitous pathogen found in soil and water and on decaying vegetable matter. Infection with *L. monocytogenes* can cause listeriosis, a rare but serious disease with a mortality rate of almost 30% *(19)*. Populations at risk for contracting listeriosis include pregnant women, fetuses or neonates, and individuals with compromised immune systems, such as HIV-infected individuals or those undergoing cancer chemotherapy *(19)*. Although *L. monocytogenes* is ubiquitous in the environment, nearly all outbreaks of listeriosis can be traced to consumption of contaminated foods. *L. monocytogenes* may be present in foods ranging from raw and processed meats to fruits and vegetables, fish and seafood, and dairy products such as milk, soft cheeses, and ice cream. Although relatively high numbers of pathogen cells may be required to cause disease *(19),* the prevalence of *L. monocytogenes* in foods and food processing environments requires the development and effective use of antimicrobial interventions capable of addressing this pathogen.

Consumer demand for fresher foods containing fewer synthetic preservatives has driven the development of more ''natural'' antimicrobial treatments to improve food safety, extend shelf life, and improve the quality of foods *(23)*. Compounds of interest for this purpose include chitosan, lysozyme, antimicrobial peptides, and plant compounds such as essential oils and other phenolic-rich materials *(23)*. Recently, grape seed extract (GSE), a by-product of the wine and grape juice processing industries, has emerged as a value-added source of food-grade plant phenolics with promising and wide-ranging bioactive properties *(3, 17)*. Activities attributed to GSE include anticancer, anti-inflammatory, antinociceptive (analgesic), antioxidant, and antimicrobial effects *(8, 17)*. GSE is commercially available from a number of manufacturers and has generally recognized as safe status. These attributes make it attractive for use as a functional ingredient in foods. GSE has a chemical preservative effect in foods such as cooked meats, leading to improved color and longer shelf life of these products *(2)*. The antimicrobial properties of GSE have been evaluated against *L. monocytogenes, Salmonella* Typhimurium, *Staphylococcus aureus, Bacillus cereus, Enterobacter* (*Cronobacter*) *sakazakii, Escherichia coli* O157:H7, *Aeromonas hydrophila,* and other foodborne pathogens, both in vitro and to a limited extent, in foods *(1, 3, 10, 20–22)*. The antilisterial activity of GSE seems to be particularly promising, with multilog reductions in viable cell counts reported after only a few minutes of exposure to GSE in vitro *(20)*. However, the utility of GSE alone in complex food systems such ground beef and turkey frankfurters appears to be limited *(2, 21)*. For example, in cooked beef treated with 1% GSE and held at 4°C, *L. monocytogenes*

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counts increased from 4.86 to 6.8 log CFU g^{-1} after 9 days of storage *(2)*. Similar results were obtained for turkey frankfurters dipped in 1% GSE, inoculated with *L. monocytogenes,* and held at 4°C for up to 28 days. In these experiments, GSE-treated samples yielded final cell counts comparable to those of the no-antimicrobial control *(21)*. Although GSE was ineffective by itself in these complex systems, the combination of 1% GSE and 6,400 IU of nisin was useful for control of *L. monocytogenes* in turkey frankfurters, with complete inhibition after 21 days *(21)*. These data suggest the possible use of GSE as a hurdle element in multicomponent antimicrobial systems applied to complex foods. Although GSE alone may not be appropriate for use in complex foods such as meats, it may still have value as an antilisterial treatment for low-complexity foods, including produce.

Like other plant phenolic compounds or extracts rich in these compounds, the antimicrobial properties of GSE, its primary targets, and its mode of action are poorly understood. GSE is a rich source of proanthocyanidins (oligomers or polymers of flavan-3-ols such as (+)-catechin and (−)-epicatechin); therefore, its antimicrobial properties are often attributed to generic phenolic activities such as enzyme inactivation, protein denaturation, and alteration or destruction of the cell membrane *(23)*. As complex natural mixtures, extracts such as GSE may not have a single mode of action but may act simultaneously on multiple cellular targets. The purpose of this study was to evaluate the antilisterial activity of relatively low concentrations of aqueous GSE using multiple tools, including culture-based methods, fluorescence microscopy, and flow cytometry, in an effort to gain further understanding of the antilisterial effects of this natural plant material. We also investigated the practical application of low-concentration aqueous GSE as an antimicrobial wash for tomatoes, a low-complexity food that has been implicated in transmission of *L. monocytogenes (14)*.

MATERIALS AND METHODS

Cultures and growth conditions. *L. monocytogenes* NADC-2045 (Scott A) was obtained from the culture collection of the Microbial Food Safety Laboratory (Iowa State University, Ames). *Listeria innocua* ATCC 33090 was from the American Type Culture Collection (Manassas, VA). Stock cultures were stored at −80°C in brain heart infusion (BHI) broth (Difco, Becton Dickinson, Sparks, MD) supplemented with 10% glycerol. At least two passages (18 to 22 h at 35°C) of thawed stock cultures in 10 ml of BHI broth were made to prepare working cultures. For MIC and time course plating experiments, *L. monocytogenes* NADC-2045 was grown in borosilicate glass screw-cap tubes containing 10 ml of BHI broth. After 22 h, the cells were harvested by centrifugation (2,000 × *g* for 5 min) using a Spectrafuge 16 M centrifuge (Labnet International, Woodbridge, NJ) and washed once in 0.1% peptone water (PW). Pelleted cells were suspended in fresh 0.1% PW to give a final viable cell level of approximately 1.0×10^9 CFU ml^{-1}, which was determined by plating onto tryptic soy agar supplemented with 0.6% yeast extract (TSAYE). For fluorescence microscopy (*L. monocytogenes*) and flow cytometry experiments (*L. innocua*), strains were cultured for 18 to 22 h at 30°C, and cells were harvested by centrifugation (2,000 × *g* for 5 min) and washed once in 0.85% saline before use.

GSE. The antimicrobial used in this study was a commercial preparation of GSE (Gravinol-S, Kikkoman Corporation, Tokyo, Japan). For MIC and plating experiments, a stock solution of GSE (25 mg ml^{-1}) was prepared in distilled water containing 10% (vol/vol) ethanol (to increase solubility of the extract) and then filter sterilized (0.2-μm-pore-size filter) before use in experiments. All appropriate controls were included to examine the effects of ethanol exposure at the highest concentration used (0.5% final ethanol concentration). To separate potentially confounding pH effects from other biological activities, we also measured the pH of a series of GSE solutions (1.5 to 1,250 μg ml^{-1}) made in 0.85% saline or phospate-buffered saline (PBS).

Total phenolic content of GSE. The concentration of phenolics in the GSE was determined using the method described by Waterman and Mole *(24)*. This assay is based on reduction of iron from the ferric to the ferrous state by phenolic compounds, with concomitant formation of the Prussian blue complex $Fe_4[Fe(CN)_6]_3$, which is detected colorimetrically *(9)*. A 1% solution of GSE in water-ethanol was prepared as previously stated. Formation of the Prussian blue complex was measured by absorbance at 720 nm, and total phenolic content was expressed as the number of catechin equivalents present in the 1% GSE sample. The total phenolic content of the sample was calculated as the number of catechin equivalents × 100.

MIC of GSE. MICs of GSE against *L. monocytogenes* NADC-2045 and *L. innocua* ATCC 33090 were determined using the Bioscreen C Microbiology Reader (Growth Curves, Piscataway, NJ), a combined incubator and automated turbidimeter. To minimize interference with optical density (OD) readings from the inherent coloration of GSE, OD data were collected using the instrument's wideband setting (420 to 580 nm). Interference from turbidity caused by GSE was minimized by using Mueller-Hinton (MH) broth instead of BHI broth. Final concentrations for GSE in MH broth ranged from 3 to 1,250 μg/ml^{-1} (0.0003 to 0.125%) and were obtained by serially diluting the stock solution of Gravinol-S (100 mg ml^{-1}) according to the CLSI (formerly NCCLS) methodology for preparing solutions of antimicrobial agents for use in broth microdilution susceptibility tests *(18)*. Each well had a final volume of 200 μl and contained a total of 10^5 CFU of the test organism, and triplicate wells were used for all treatments. Cultures were incubated at 35°C for 24 h, and OD measurements were taken every 15 min, with shaking before each reading. The MIC of the Gravinol-S GSE for *L. monocytogenes* or *L. innocua* was defined as the lowest concentration that completely inhibited growth after 24 h (<0.05 OD unit increase). Controls included MH alone and MH plus 0.5% (vol/vol) ethanol. To facilitate direct comparisons of growth curves, all treatments were normalized to an arbitrary level of 0.3 OD units, minimizing the contribution to OD of GSE added to the growth medium. Differences from this normalized OD level were added or subtracted from values obtained at all time points.

Time course plating. The bactericidal activity of GSE at different concentrations was determined as a function of time with a time course plating assay. Washed cells of *L. monocytogenes* NADC-2045 were suspended in 0.85% saline or PBS in some experiments to obtain approximately 10^9 CFU ml^{-1}. Aliquots of 100 μl of this cell suspension were placed into separate 1.5-ml microcentrifuge tubes, pelleted, and resuspended in 100 μl of 0.85% saline solution containing GSE to yield final concentrations of 30, 50, 100, or 1,250 μg ml^{-1} (0.003, 0.005, 0.01, or 0.125%). Tubes were incubated at 25°C statically. At 2, 4, and 10 min, the samples were pelleted to remove excess GSE (2,000 × *g* for 5 min), resuspended

in 1 ml of 0.1% PW, serially diluted (1:10) in 0.1% PW, and plated on TSAYE. Plates were incubated at 35°C for 24 h, and bacterial colonies were counted. Three independent replications of the experiment were conducted. To investigate the potentially protective effects of complex organic or buffered environments, initial time course experiments also were carried out in MH broth and PBS, respectively.

Flow cytometry. Flow cytometry experiments were performed in an effort to obtain information on the antilisterial activities of low concentrations of GSE over very short time scales. Because live cell cytometry of *L. monocytogenes* represented an aerosol hazard, we used the physiologically similar *L. innocua* ATCC 33090 for these experiments. Cells of *L. innocua* were prepared and washed in 0.85% saline as previously described. Aliquots of 100 μl (~10^8 cells) of washed cells were pelleted via centrifugation (2,000 × *g* for 5 min). The supernatant was discarded, and the cells were resuspended in a small amount of the residual supernatant to ensure an even slurry of individual cells. Cells were then resuspended in 1 ml of fresh 0.85% saline containing GSE at concentrations of 1.5, 15, 30, 50, or 100 μg ml^{-1} (0.00015 to 0.01%), with 0.3% as the highest concentration of ethanol in this series. A separate control for exposure to this concentration of ethanol was included. Cells were exposed to GSE in 0.85% saline for up to 10 min at 25°C, and samples were taken at 2-, 4-, 8-, and 10-min intervals. Once sampled, cells were quickly (2 min) harvested by centrifugation (2,000 × *g*), washed once in fresh saline without added GSE, resuspended in a final volume of 250 μl saline, and submitted to fluorescent staining.

The membrane integrity probe propidium iodide (PI; component B from the L13152 Live/Dead *Bac*Light Kit, Invitrogen Corporation, Carlsbad, CA), was prepared by dissolving the contents of one applicator in 5 ml of filtered distilled water (0.2-μm-pore-size filter) to form a 2× working solution. Two hundred fifty microliters of this working stock was added to control or GSE-treated cell suspensions, mixed, incubated in the dark for 15 min, and submitted to cytometric analysis using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA). For each sample, data on cell scatter and PI fluorescence (488 nm excitation and 670 nm long-pass emission) were collected for 20,000 events at a flow rate of 10 μl min^{-1}. Controls included live cells with or without PI, cells treated with GSE for 10 min without PI staining, both stained and unstained isopropanol-killed cells, and a mixture of equal parts of isopropanol-killed and live cells.

Fluorescence microscopy. For microscopy experiments, fluorescent staining of *L. monocytogenes* NADC-2045 was performed using the Live/Dead *Bac*Light kit. These experiments were carried out in saline suspension because the manufacturer's instructions state that phosphate-containing buffers may interfere with staining efficiency. Ten-microliter portions (~10^7 cells) of saline-washed cells were spread over an ~1-cm^2 area on poly-L-lysine–coated microscope slides, placed in a BSL-2 biosafety hood, and air-dried to facilitate cell attachment to slide surfaces. One hundred microliters of saline containing the GSE at concentrations of 1.5, 15, 30, 50, 100, and 1,250 μg ml^{-1} (0.00015 to 0.125%) was applied to the cells on the surface of the slide, completely covering the area containing the cells. Cells were exposed to GSE for 10 min, the antimicrobial overlay was discarded, and the slides were washed gently with 100 μl of fresh saline to remove excess GSE. Ten microliters of the Live/Dead stain were applied to the sample, and the sample was sealed with a coverslip. Samples were viewed after 15 min of staining in the dark and again after 30 min. A control for the Live/Dead stain was prepared from a mixture of equal parts of live and isopropanol-killed cells and stained as described above. This mixture also served as a control for potential staining artifacts stemming from sample processing, such as the on-slide drying step.

Application of GSE as an antimicrobial wash for fresh produce. To determine the efficacy of GSE as an antimicrobial treatment for fresh produce, a proof-of-concept experiment was conducted using spot-inoculated tomatoes. *L. monocytogenes* NADC-2045 cells were washed in 0.1% PW, and 100 μl was used to spike Roma tomatoes at approximately 10^6 CFU g^{-1}. The inoculum was allowed to adhere to tomato surfaces for 4 to 5 h. Whole tomatoes were treated at 25°C inside plastic bags with GSE in distilled water at 100, 625, or 1,250 μg ml^{-1} (0.001, 0.065, or 0.125%) for 2 min as a dip (1:1, wt/vol, sample-antimicrobial solution). After exposure, the antimicrobial solution was discarded and samples were washed with 45 ml of 0.1% PW for 30 s using a Pul100 Pulsifier to detach cells from tomato surfaces. Serial dilutions were made in 0.1% PW, plated onto modified Oxford agar plates, and incubated at 35°C for 24 h, after which colonies were counted. Three independent replications of the experiment were conducted, and a distilled water control was used to account for simple mechanical removal of cells via washing.

Statistical analysis. For the time course plating and antimicrobial produce challenge tests, data from three independent replications were subjected to statistical analysis. A one-way analysis of variance was performed using the data analysis tools in Microsoft Excel 2007 (Microsoft, Redmond, WA). Pairwise comparisons between samples were conducted using the *t* test with the significance level set at 0.95 ($\alpha < 0.05$).

RESULTS AND DISCUSSION

For many natural antimicrobials, including GSE, their efficacy in complex food systems is lower than that in simpler in vitro systems *(23)*. This difference could reflect the intrinsic reactivity of the antimicrobial fractions of GSE, which might also bind to components of the food system, making these fractions less available for interaction with bacterial cells *(23)*. For some natural antimicrobials, temperature may also be a critical parameter; some compounds could precipitate from solution at colder temperatures and thus be ineffective in refrigerated foods. Although lowered activity in complex foods and/or at low temperature appears to be a fundamental problem for many natural antimicrobials, some compounds or extracts, such as GSE, might still be valuable as food safety interventions when their limitations are recognized and they are applied to appropriate food systems. We therefore sought to assess the utility of GSE alone and at relatively low concentrations via time course plating in simple aqueous media and in a low-complexity food system using the GSE as an antimicrobial produce wash. For the food system study, we used tomatoes as an example of raw or ready-to-eat produce that has been implicated in transmission of *L. monocytogenes (14)*. In support of this applied work, we used both flow cytometry and fluorescence microscopy to evaluate the physiological effects of low-concentration GSE exposure (concentrations of 0.00015 to 0.125% [1.5 to 1,250 μg ml^{-1}]) on *Listeria* spp. in vitro.

Chemical properties of GSE: total phenolic content and pH. The total phenolic concentration in GSE could not

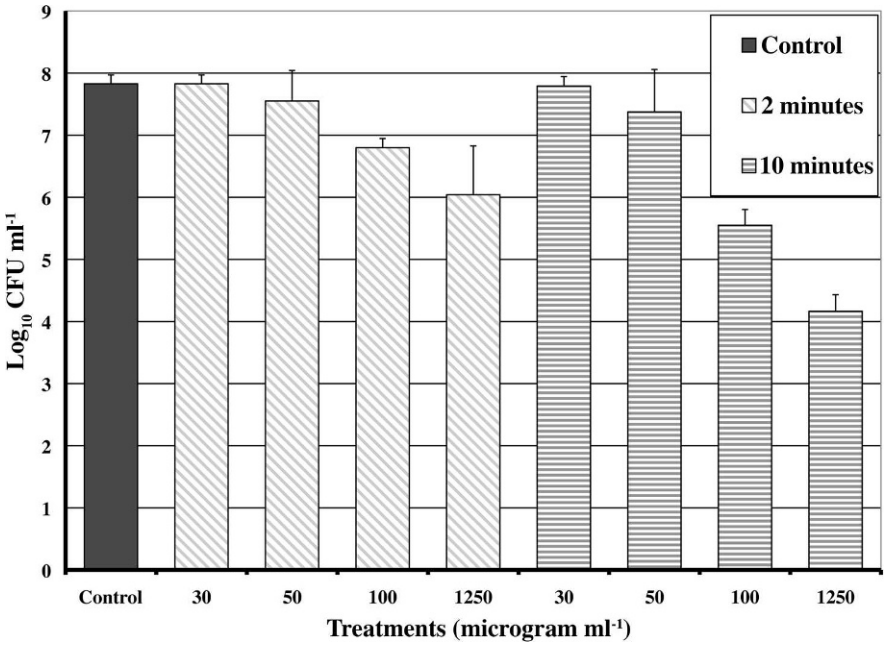

FIGURE 1. *Antimicrobial effects of different concentrations of GSE on* Listeria monocytogenes *Scott A at 2 and 10 min.* L. monocytogenes *cultures of* 10^8 *CFU* ml^{-1} *were pelleted and resuspended in 100 μl of 0.85% saline containing 30, 50, 100, or 1,250 μg* ml^{-1} *GSE. After 2 or 10 min of static exposure to the antimicrobial at 25°C, cells were pelleted, the supernatant was discarded, and the pellets were resuspended in 1 ml of 0.1% PW to reduce activity from residual GSE. Significant reductions* ($P < 0.05$) *in initial numbers were achieved at concentrations of 100 and 1,250 μg* ml^{-1}*, including a 3.6-log reduction following 10 min of exposure to 1,250 μg* ml^{-1} *GSE.*

be measured directly because of the natural pigmentation of GSE, which interfered with the spectrophotometric assay used. Therefore, a less pigmented 1% solution of GSE was used for this assay. The total phenolic content of the 1% solution of Gravinol-S was 0.95 catechin equivalents. This result coincides with the manufacturer's claim of ''up to 95% total polyphenols'' in the full-strength extract. To determine whether the observed antimicrobial activities may be attributable to plant acids potentially present in GSE, we measured the pH of a series of GSE solutions made in 0.85% saline: 1.5 μg ml^{-1} (pH 6.77), 15 μg ml^{-1} (pH 6.36), 30 μg ml^{-1} (pH 5.98), 50 μg ml^{-1} (pH 5.54), 100 μg ml^{-1} (pH 5.39), and 1,250 μg ml^{-1} (pH 4.73). These results indicate that the commercial GSE we evaluated contains acidic compounds that may lower the pH of the test system in a dose-related fashion. Tartaric acid is the major organic acid present in GSE, and such organic acids have been suggested to play a large role in GSE activities against *E. coli* O157:H7 and *E. sakazakii (10, 11)*. However, Rhodes et al. *(20)* found that adjusting the pH of grape seed polymeric phenolics from 3.5 to 7.0 did not affect the antimicrobial activity of these phenolics against *L. monocytogenes,* and Mayer et al. *(16)* fractionated GSE via chromatography and found the highest antimicrobial activities in fractions containing proanthocyanidins and their gallate esters. *L. monocytogenes* is acid tolerant, as demonstrated in previous studies in which cells held for 2 h at an external pH of 3.0 were able to maintain a constant internal pH of ≥5 and then recover fully and retain viability when returned to an external pH environment of 6.0 *(7)*. In contrast, the lowest pH (pH 4.73) in our saline GSE series was for the 1,250 μg ml^{-1} concentration, and cells were exposed to these conditions for only brief periods (2 to 10 min).

MIC of GSE. The MICs of Gravinol-S were determined for both *L. monocytogenes* NADC-2045 (Scott A) and *L. innocua* ATCC 33090. Initial MIC determinations using *L. monocytogenes* alone were made in BHI broth at 600 nm. However, the intrinsic turbidity caused by GSE in this medium and to a lesser degree the coloration of GSE led us to seek alternate conditions for MIC determination. We used MH broth and the wideband (420 to 580 nm) setting on the Bioscreen C reader. Under these conditions, spikes in OD caused by interference from visible sediment (a problem with the BHI method) were minimized and MICs were clear. MH-based MICs of GSE were 50 and 78 μg ml^{-1} against *L. monocytogenes* and *L. innocua,* respectively. These values are lower than MICs reported by others against *L. monocytogenes,* although a different strain, a different inoculum level, a different GSE preparation, and a different method (plating versus broth microdilution) were used, all of which can affect MICs *(1)*.

Time course plating in 0.85% saline. Initial plating studies using agitated cell suspensions in 0.85% saline produced large (~6-log) reductions in cell viability within 10 min with 1,250 μg ml^{-1} GSE *(25)*. These results are consistent with those reported by other researchers for *L. monocytogenes* and other pathogens *(10, 11, 20)*. However, after reviewing the literature, we hypothesized that residual activity of GSE might contribute to an overestimation of the antimicrobial effects of GSE. Therefore, we modified our sampling procedure to include a washing step so that excess GSE could be removed before plating. This method was used in all subsequent work. Using this approach, we obtained a maximum kill of only 3.6 log units within the same 10-min period, suggesting that some of the apparent activity of GSE observed without washing might be attributable to residual activity. Figure 1 shows our results for exposure of *L. monocytogenes* to a range of GSE concentrations in 0.85% saline during static incubation for 2 or 10 min. We sought to examine the activity of GSE in aqueous solution over relatively short time frames because practical application of GSE on food or food contact

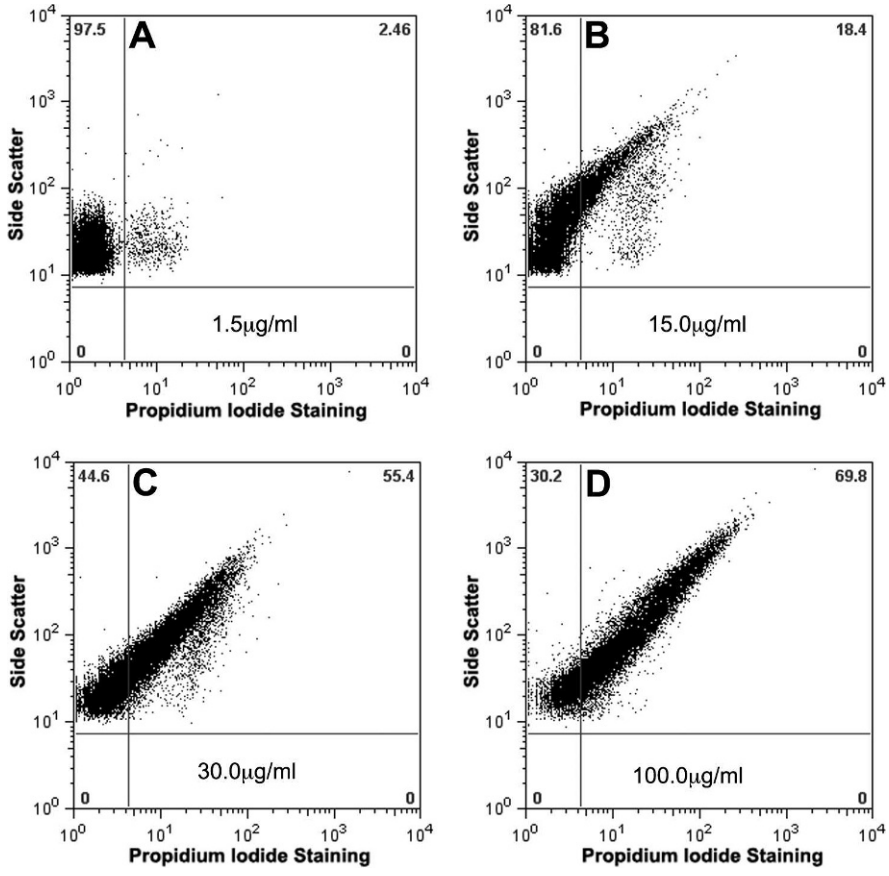

FIGURE 2. *Flow cytometric analysis of the effects of various GSE concentrations (in 0.85% saline) on live cells of* Listeria innocua *ATCC 33090. GSE was applied at 1.5, 15, 30, or 100 μg ml^{-1} (0.00015 to 0.01%) for 2 min and then analyzed by flow cytometry. These data highlight the rapid dose-dependent activity of GSE against* L. innocua, *showing the capacity of GSE to permeabilize* L. innocua *cells, even at the lowest concentration. Higher concentrations of GSE led to additional permeabilization and cell clumping.*

surfaces would require rapid activity to be useful to the food industry. The highest concentration of GSE used here (1,250 μg ml^{-1}) reduced viable counts of *L. monocytogenes* by ~2 log units after 2 min and by 3.6 log units after 10 min of exposure in saline solution, suggesting the potential for this concentration of GSE (or higher) as an antimicrobial treatment for low-complexity food systems such as produce. We also explored the potentially protective effects of buffered or more complex media. Time course plating in both saline and a buffered system (PBS) at a GSE concentration of 1,250 μg ml^{-1} resulted in similar inactivation for *L. monocytogenes* in both media after only minimal exposure (2 to 10 min; data not shown). The final pH for the PBS plus GSE system was 7.29 (versus 7.43 for the PBS control) and 4.80 for saline plus GSE. In the same experiment, MH broth conferred a protective effect at 2 and 10 min, but viable cell numbers in MH broth still declined by ~3 log units after 60 min of exposure to 1,250 μg ml^{-1} GSE (data not shown). Together with the findings of others, these data suggest that complex media is protective against GSE activity. Further, although simple pH effects cannot be ruled out as contributors to the overall antimicrobial activity of GSE, our results suggest that pH is not the only factor responsible for the inactivation we observed in nonbuffered systems, especially for the lower concentrations of GSE used where pH change was minimal.

Flow cytometry. Flow cytometry is used to analyze whole populations of cells on the basis of single cell characteristics, such as light scatter or reaction to externally applied stains *(4)*. Stains useful for cytometric assessment of antimicrobial activity include membrane integrity probes such as PI or fluorescent respiratory substrates such as 5-cyano-2,3-ditolyl tetrazolium chloride *(4)*. Valuable information on the physiological effects, such as lysis or clumping, of antimicrobials against live cell preparations can also be obtained from analysis of cell light scatter during or after exposure to antimicrobials. Flow cytometry can be especially useful for probing antimicrobial action over short time scales. In our work, plating experiments demonstrated the efficacy of GSE against *L. monocytogenes* but did not yield information on its possible mode of action. We used flow cytometry in an effort to answer questions about the physiological effects of GSE on *L. monocytogenes* as a function of both time and extract concentration. Because of safety concerns about potential aerosol generation during these live cell cytometry studies, we used *L. innocua* ATCC 33090 instead of *L. monocytogenes*. *L. innocua* is physiologically similar to *L. monocytogenes (13)* and has been used as a surrogate for this pathogen in various studies ranging from ripening of Camembert cheese *(15)* to the antimicrobial effects of ultrahigh pressure *(5)*. *L. innocua* ATCC 33090 was also reported by Rhodes et al. *(20)* to be similar to *L. monocytogenes* in its susceptibility to GSE, findings that we confirmed here via MIC determinations.

We hypothesized that use of lower concentrations of GSE would enable us to follow extract-induced physiological changes in *L. innocua* as a function of time, providing further insight into the antilisterial action of GSE. Therefore, we assayed lower concentrations of GSE (1.5 to 100 μg ml^{-1}) over a short time frame (2 to 10 min) via flow cytometry using PI as a probe for membrane integrity. Even with the relatively low concentrations of GSE and brief time intervals used here, GSE exerted clear and immediate

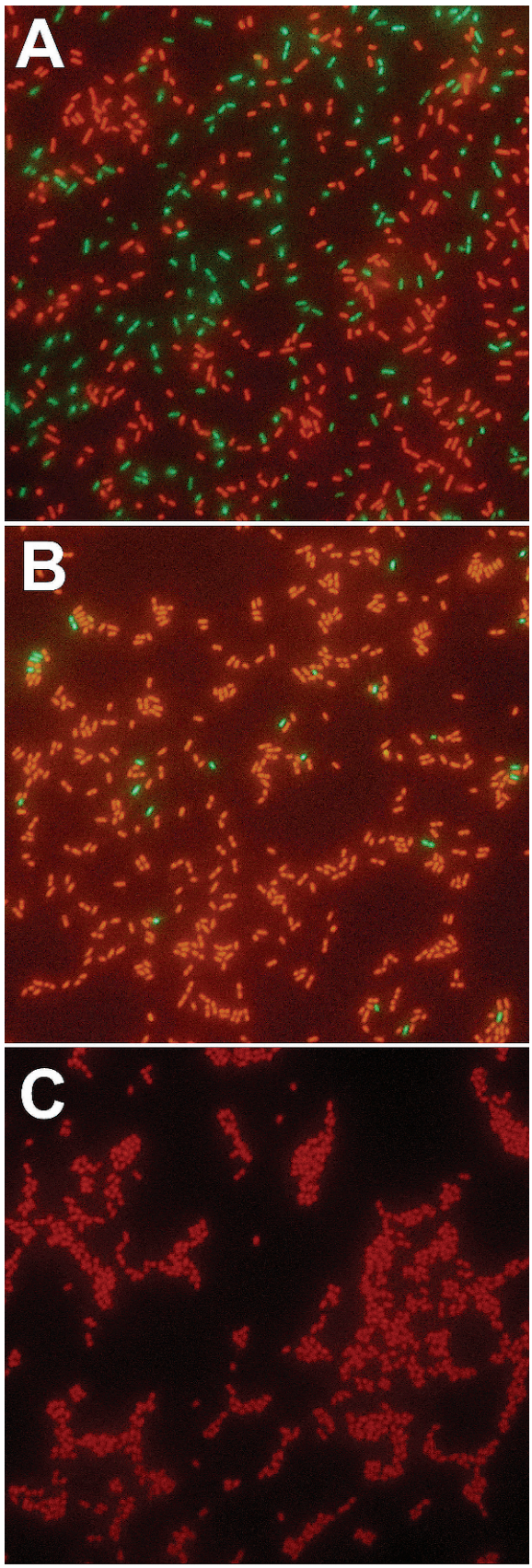

FIGURE 3. *Live/Dead staining of* L. monocytogenes *NADC-2045 (Scott A) treated with GSE in 0.85% saline. (A) A 50:50 mixture of live–isopropanol-killed cells. (B) Effects of 1.5 μg ml^{-1} GSE. The majority of cells were bright red (permeabilized or dead) after 10 min of exposure to GSE. (C) Further effects of 50 μg ml^{-1} GSE against* L. monocytogenes.

physiological effects on *L. innocua*. The extent and immediacy of the effects of GSE limited our ability to determine discrete time-sequenced steps that might occur during the inactivation of *Listeria* by this antimicrobial. However, an analysis of the effects of four different concentrations of GSE at the earliest time point investigated (2 min) provided valuable insight into the antilisterial action of GSE (Fig. 2). At the lowest concentration tested (1.5 μg ml^{-1}), a small subpopulation of membrane-compromised (PI-positive) cells emerged (Fig. 2A), highlighting the membrane permeabilizing effects of GSE even at this concentration. The light scatter properties of *L. innocua* cells were not affected at 1.5 μg ml^{-1} GSE compared with untreated controls (data not shown). However, a 10-fold increase in GSE concentration to 15.0 μg ml^{-1} had a large effect on the scatter properties of the cells, with the increased side scatter seen for this sample indicating cell clumping (Fig. 2B). For the GSE concentrations examined here, higher concentrations of GSE produced both increased clumping and increased PI staining. Specifically, PI-positive events (cell permeabilization or death) ranged from ~2.5% of the total population with 1.5 μg ml^{-1} to ~70% of the total population when 100 μg ml^{-1} was used (Fig. 2A through 2D). These observations for live *L. innocua* cultures were consistent with results obtained for *L. monocytogenes* using other methods, including time course plating and fluorescence microscopy. Together, these results indicate that GSE causes a rapid loss of cell integrity, indicated at lower GSE concentrations by permeabilization of the cell membrane and at higher concentrations by cell clumping and lysis. As used here, the culture-independent approaches of fluorescence microscopy and flow cytometry were effective in addressing possible artifacts; cell clumping could lead to an overestimation of the antibacterial activity GSE if treatments were evaluated by plating alone.

Fluorescence microscopy. To directly evaluate how GSE affects *L. monocytogenes,* we used a Live/Dead staining protocol on GSE-treated *L. monocytogenes* and viewed these results using fluorescence microscopy. Mixtures (~50:50) of live and isopropanol-killed *L. monocytogenes* stained as expected and were characterized by a mixed population of bright green cells (Syto 9 positive and PI negative), i.e., live cells, and bright red cells (PI positive), i.e., dead or permeabilized cells (Fig. 3A). At the lowest concentration of GSE examined here (1.5 μg ml^{-1}), the majority of cells were bright red (permeabilized or dead) after 10 min of exposure to GSE, with only a few green cells visible (Fig. 3B). Analysis of this same concentration of GSE at 2 min against *L. innocua* via flow cytometry indicated only 2.5% of the population was permeabilized (Fig. 1A). Assuming an equivalence between cytometric and microscopic assays and between responses of *L. innocua* and *L. monocytogenes* to GSE, these data suggest a large and rapid effect for GSE between the time points examined via flow cytometry (2 min) and fluorescence microscopy (10 min). Although we used poly-L-lysine–coated microscope slides in an effort to prevent or minimize GSE-mediated cell clumping, our microcopy results for *L. monocytogenes* were consistent

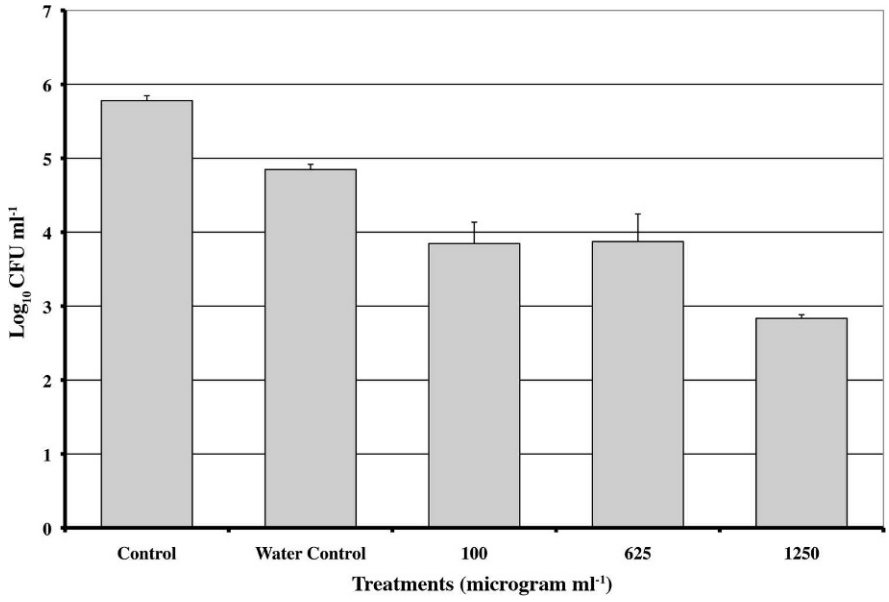

FIGURE 4. *Antimicrobial effects of three different concentrations of GSE on* Listeria monocytogenes *Scott A inoculated onto the surfaces of Roma tomatoes and exposed for 2 min. Whole Roma tomatoes weighing ~90 g each were spot inoculated at ~10^6 CFU g^{-1}, and the inoculum was allowed to attach to the surfaces for ~4 h in a laminar flow cabinet. Samples were then dipped in distilled water containing 100, 625, or 1,250 μg ml^{-1} GSE (1:1, wt/vol) inside sterile plastic bags for 2 min. Following exposure, the antimicrobial was discarded, 45 ml of diluent (0.1% PW) was added, and cells were detached using a Pulsifier Pul100, with a 30-s run time. Controls included samples that were dipped in distilled water without the antimicrobial to determine the extent of mechanical cell removal by liquid immersion. Significant reduction (*$P < 0.05$*) was achieved at the highest GSE concentration used (1,250 μg ml^{-1} or 0.125%) after only 2 min of exposure.*

with those of our cytometric analysis of *L. innocua,* with increasing concentrations of GSE leading to clumping (50 μg ml^{-1}; Fig. 3C) and ultimately cell lysis. At the highest concentration (1,250 μg ml^{-1}), we were able to find cellular ''ghosts'' via light microscopy, but these ghost cells were only dimly stained with PI, suggesting diffusive loss of nucleic acids from compromised and lysed cells (data not shown). Our microscopy samples were not static; when we revisited them after an additional 30 min of benchtop incubation, it was clear that GSE continued to exert antimicrobial activity against treated cells, suggesting that the gentle posttreatment rinse was not adequate to remove excess GSE. At lower concentrations of GSE (i.e., 15 μg ml^{-1}), cells that had previously been well spaced were later clumped and more brightly stained by PI. An example of clear cell clumping is shown in Figure 3C and some degree of clumping was also apparent at the 1.5 μg ml^{-1} concentration (Fig. 3B). At higher GSE concentrations (30 μg ml^{-1}), clumping and lysis were more apparent after the additional incubation period. A dose-related cell clumping effect for GSE was also recently observed in *Helicobacter pylori (6).* This common observation may ultimately help explain the mode of action for this natural extract.

The rapidity of GSE-mediated killing of *Listeria* spp. and our observation of the continued on-slide antimicrobial activity suggest that the active components in GSE may rapidly complex with target cells, where they remain bound and able to exert a continuing effect as a function of time. Rhodes et al. *(20)* suggested that cationic species present in GSE might interact with the negatively charged surfaces of *Listeria* spp., akin to the action of antimicrobial peptides. Kondo et al. *(12)* found that grape seed proanthocyanidins polymerize to form helical structures. One possible explanation for GSE activities may therefore include binding of and subsequent pore formation by cationic helical proanthocyanidin polymers, although such a theory must be tested experimentally.

Evaluation of low concentrations of GSE as a produce wash for tomatoes. To connect our basic findings on the physiological effects of GSE against *Listeria* spp. to the potential for practical use of GSE for food safety, we conducted an evaluation of GSE as a produce wash for Roma tomatoes artificially contaminated with *L. monocytogenes*. Tomatoes were spot inoculated with *L. monocytogenes* Scott A at ~10^6 CFU g^{-1}, the inoculum was allowed to dry for 4 to 5 h, and the tomatoes were subjected to 2-min dips in distilled water containing 100, 625, or 1,250 μg ml^{-1} GSE, as described in the ''Materials and Methods.'' Control treatments indicated a ~1-log reduction in *L. monocytogenes* due to simple mechanical washing, and 100 or 625 μg ml^{-1} GSE treatments yielded an additional reduction of ~1 log unit (Fig. 4). The highest concentration of GSE used (1,250 μg ml^{-1}) reduced *L. monocytogenes* by ~2 log units during the 2-min exposure, suggesting the practical utility of dips containing relatively low concentrations of GSE in aqueous solution for reduction of *L. monocytogenes* on fresh produce such as tomatoes.

In summary, our results indicate that GSE has a rapid antibacterial effect against *Listeria* spp. at relatively low (microgram or milligram) concentrations when tested against saline-suspended cells. Evidence collected indicates

that the active components of GSE affect cell integrity, causing gross ultrastructural damage including membrane permeabilization, cell clumping, and eventual cell lysis. Although this basic work on the physiological effects of low concentrations of GSE was conducted in 0.85% saline instead of a complex food system, our results for GSE in distilled water for inactivation of *L. monocytogenes* on tomatoes indicates that GSE-based antimicrobial wash solutions might offer an inexpensive, value-added approach to control of *Listeria* spp. on produce or in produce production environments. The effects of higher concentrations of GSE and/or additional antimicrobial hurdles in conjunction with GSE for inactivation of *L. monocytogenes* on fresh produce are potential areas for further research.

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