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Short communication

Efficacy of vanillin and geraniol in reducing *Escherichia coli O157:H7* on strawberry juice

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ABSTRACT

In the present study, vanillin (Van) and geraniol (Ger) were studied as potential food biopreservatives. Minimal Inhibitory Concentration (MIC) was obtained *in vitro* using four different pathogens as indicators. Studies *in vivo* in strawberry juice inoculated with *Escherichia coli* O157:H7 were conducted to evaluate the pathogen survival. Biopreservatives were applied at 1 and 2 MIC concentration. Samples were stored at 5 °C during 14 days, in order to study the effects of the treatments against the inoculated pathogen. At day 0, untreated sample showed counts of 5.4 log CFU/mL. Samples treated with Van2MIC, Ger1MIC, Ger2MIC showed a significant control on *E. coli* population at day 0 (immediately before application of the compounds), with counts of 3.0–3.2 log. Furthermore, from day 3 until the end of storage, those samples showed *E. coli* counts below the detection limit. More studies should be made in order to investigate the impact of vanillin and geraniol application on sensory and nutritional characteristics of strawberry juice, and the effects on its native microflora and spoilage microorganisms.

1. Introduction

Despite of the inherent acidity of unpasteurized fruit juices, which is lethal to most bacterial species likely to contaminate such products, several outbreaks of infections caused by *Escherichia coli* O157:H7 have occurred caused by the consumption of unpasteurized fruit juices (Keller & Miller, 2006). In reported outbreaks of diseases involving fruit juices, *E. coli* O157:H7 and enterotoxigenic *E. coli* strains were reported to be most common causative agent, followed closely by *Salmonella* spp. (Vojdani, Beuchat, & Tauxe, 2008).

Therefore, there has been an increasing interest in the search of novel technologies that can avoid the application of thermal treatments, where the application of naturally occurring antimicrobials is gaining popularity (Vasantha Rupasinghe, Boulter-Bitzer, Ahn, & Odumeru, 2006). Little is known about the resistance mechanisms of microorganisms against these naturally occurring antimicrobial compounds, although many, such as vanillin, benzaldehyde, ferulic acid, estragole, guaiacol and eugenol, contain aromatic structures similar to the ones found in classical preservatives such as benzoic acid (Vasantha Rupasinghe et al., 2006).

Vanillin (4-hydroxy-3-methoxybenzaldehyde, Fig. 1a) is the predominant phytochemical that occurs in vanilla beans, and it is known to be antimycotic and bacteriostatic (Fitzgerald et al., 2004). Like many other low-molecular weight phenolic compounds, vanillin displays antioxidant and antimicrobial properties and hence has the potential for use as a food preservative (Davidson & Naidu, 2000).

Another natural occurring antimicrobial is geraniol, an acyclic monoterpene alcohol with the chemical $C_{10}H_{18}O$ (3,7-dimethylocta-trans-2,6-dien-1-ol, Fig. 1b). It is a common constituent of several essential oils, emitted from the flowers of many species and it is present in vegetative tissues of many herbs (Chen & Viljoen, 2010). Researchers have shown geraniol to be an effective insect repellent (Barnard & Xue, 2004) and its potential as an antimicrobial agent has been highlighted in several studies (Chen & Viljoen, 2010).

The reports on antimicrobial properties of vanillin and geraniol are limited, and little is known about their *in vivo* effects on food produce. Even though *in vitro* results usually differ from the ones obtained with *in vivo* applications, a study is necessary to establish *in vitro* quantitative parameters to consequently apply the natural

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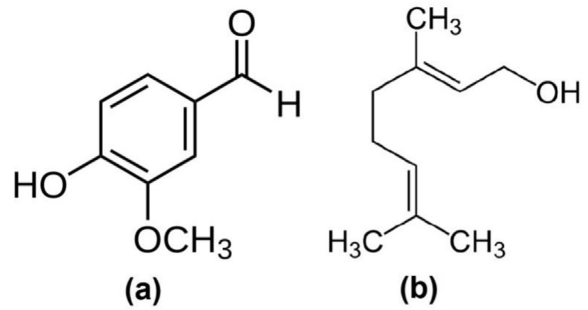

Fig. 1. Chemical structures of (a) vanillin and (b) geraniol.

agents in the actual product. Therefore, the objectives of the present study were: (a) to evaluate the *in vitro* antimicrobial activity of vanillin and geraniol against typical food pathogens; and (b) to apply the selected natural compounds *in vivo* in strawberry juice inoculated with *E. coli* O157:H7.

2. Material and methods

2.1. Determination of MIC in vitro of natural compounds

The minimum inhibitory concentration (MIC) is the minimum concentration that produces a 90% reduction in the growth of microbial colonies. It is also defined as the lowest concentration of the corresponding compound that is able to inhibit visible growth of the microorganism (Hammer, Carson, & Riley, 1996). MIC of the selected natural compounds was determined using the Broth Microdilution Method.

Vanillin and geraniol antimicrobial activity was evaluated against two Gram positive bacteria: *Listeria monocytogenes* provided by CERELA (Centro de Referencia de Lactobacilos, Tucuman, Argentina) and *Staphylococcus aureus* ATCC 25923; and two Gram negative bacteria: *E. coli* O157H:7 ATCC 43895 and *Pseudomona aeruginosa* ATCC 27853.

The selected strains were maintained on tryptic soy broth (Britania, Argentina) at 4 °C. Before used, they were cultured in Brain Heart Infusion (BHI) (Britania, Argentina) for 24 h at 37 °C.

Assays were performed by triplicate in sterile 96-well microplates using Muller Hilton broth (MH) (Britania, Argentina) with the addition of the selected natural compounds. Vanillin (Sigma Aldrich) was previously dissolved in dimethyl sulfoxide (DMSO) and was used in a concentration range from 0.2 to 4.0 mg/mL. On the other hand, geraniol (Sigma Aldrich) was previously dissolved in tween 80 (Cicarelli, Argentina), and was used in concentrations from 0.2 to 2.4 μL mL^{-1}

Wells were filled with 200 μL of MH plus the corresponding natural compound and inoculated with each bacterial strain at a final bacterial density of 5.10^5 CFU mL^{-1} (which was achieved by adding 5 μL in each well of a 10^7 CFU mL^{-1} suspension of the indicator strain in MH). The microplates were incubated at 37 °C for 24 h.

Different controls were used in this study: a well filled with MH with no biopreservative and inoculated with each bacterial strain, in order to assess the growth of the pathogens; and wells filled with MH plus DMSO and MH plus tween 80, respectively, inoculated with each bacterial strain, in order to prove that neither of the solvents had antimicrobial effects by themselves.

2.2. Juice preparation

Strawberries (*Fragaria x ananassa*) were grown and harvested in Sierra de los Padres, Mar del Plata, Argentine. The strawberries were destemmed and the juices were prepared with a commercial juice extractor. Once juices were inoculated and the treatments were applied, the strawberry juices were stored in sterile polypropylene flasks at 5 °C for 14 days to assess the evolution of *E. coli* O157:H7.

2.3. Inoculation with E. coli O157:H7

2.3.1. Culture maintenance and inoculum preparation

E. coli O157:H7, ATCC 43895 was used. A stock culture was maintained in tryptic soy broth (Britania, Buenos Aires, Argentina) at 4 °C. Before use, *E. coli O157:H7* was cultured in brain heart infusion (BHI) broth (Britania) for 24 h at 37 °C. A 0.1 mL aliquot of the culture was transferred to 9.9 mL of BHI broth at two consecutive 24 h intervals followed by incubation at 37 °C before each experiment. A bacterial suspension was prepared by adding 10 mL of the *E. coli* culture to 90 mL of sterile peptonated water (0.1% w/v).

2.3.2. Inoculation of the samples

Samples were inoculated with *E. coli* O157:H7 before the application of the treatments, simulating an inadequate manipulation of the strawberries at postharvest. To carry this out, 100 μL of the bacterial suspension previously prepared were added to 10 mL of fresh strawberry juice to reach a final pathogen concentration of ~5 log colony-forming units (CFU) mL^{-1}. Control samples were untreated strawberry juice inoculated with *E. coli*.

After being treated, the strawberry juices were stored in refrigerated chambers at 5 °C for 14 days.

2.4. Application of biopreservatives to inoculated strawberry juice

Once the MIC *in vitro* was determined, in order to evaluate the antimicrobial effects of vanillin and geraniol *in vivo*, the natural antimicrobial agents were applied directly to the strawberry juice at two different concentrations for each natural compound: 1 and 2 MIC (Van1MIC and Van2MIC for vanillin, and Ger1MIC and Ger2-MIC for geraniol, respectively).

A juice with no treatment (untreated) was used as control sample.

2.5. Microbiological analysis

Viable *E. coli* counts were monitored as follows: 0.1 mL aliquot of each sample were spread on the surface of eosin methylene blue (EMB) agar plates and the colonies were counted after incubation at 37 °C for 24–48 h. EMB is a selective medium that allows the characterization of typical *E. coli* colonies; those that were dark centered, flat and with a metallic sheen were taken into account. Randomly, selected *E. coli* colonies were confirmed using an *E. coli* chromogenic test kit (Chromobrit, Britania). All culture media used were purchased from Britania.

Microbial counts were expressed as log CFU mL−1.

2.6. Statistical analysis

A completely randomized design was used. Three independent runs were performed. Data obtained was analyzed using R v. 2.12.2. (R Development Core Team, 2011). Results reported in this article are mean values accompanied by their standard errors (Kuehl, 2001). Analysis of variance ANOVA was performed and Tukey–Kramer comparison test was used to estimate significant differences between treatments ($p < 0.05$).

3. Results and discussion

3.1. In vitro antimicrobial activity of natural compounds against foodborne pathogens

Until a few years ago, fruit juices were not recognized as vehicles of foodborne illness because of their low pH and high organic acid levels (Mazzotta, 2000). However, several outbreaks associated with unpasteurized fruit juices have been reported in the last decade, and as a consequence, issues surrounding the safety of fruit products started to be an issue of concern (Anderson & Bailey, 2001).

Therefore, four different pathogens were carefully chosen to evaluate the antimicrobial capacity *in vitro* of the natural compounds. Two Gram positive bacteria: *L. monocytogenes* and *S. aureus*; and two Gram negative: *E. coli* O157H:7 and *P. aeruginosa*.

MIC values found in the *in vitro* study are shown in Table 1. Neither of the solvents used in this study (DMSO and tween 80) showed antimicrobial activity by themselves. Vanillin showed the same level of effectiveness against all the tested bacteria. However, geraniol showed less effectiveness against *P. aeruginosa* (MIC > 2.4 μL/mL), than *E. coli*, *L. monocytogenes* and *S. aureus*.

Vanillin MIC values found by Vasantha Rupasinghe et al. (2006) are in accordance with our findings. Vasantha Rupasinghe et al. (2006) studied antimicrobial activity of vanillin *in vitro* against *E. coli* and *P. aeruginosa*, among others food pathogens, and found that most microorganisms tested showed MIC values between 6 and 18 mM vanillin (between 0.9 and 2.7 mg/mL).

In accordance with our results Scortichini and Rossi (2008) studied the *in vitro* antimicrobial activity of geraniol towards seven strains of *Erwinia amylovora*, and all of the strains tested at 10^5 CFU/mL were inhibited for 24 h by geraniol in the range 0.6–1.5 μL/mL.

3.2. In vivo antimicrobial activity of natural compounds against E. coli O157:H7 inoculated in strawberry juice

In order to evaluate the effectiveness of the proposed treatments in the inactivation of *E. coli* O157:H7 in strawberry juice, samples were inoculated with the pathogen.

Fig. 2 shows the effect of vanillin treatment on *E. coli* survival throughout refrigerated storage at 5 °C. The initial count of *E. coli* on untreated sample was 5.4 log CFU/mL. Immediately after the application of the treatments (day 0), Van1MIC had no effect on *E. coli* counts, while the rest of the treatments (Van2MIC, Ger1MIC, Ger2MIC) showed significant reductions of 2.2–2.4 log.

At day 3, Van1MIC reached *E. coli* counts of 2.4 log. The rest of the treatments showed counts below the detection limit (<2 log), while the untreated sample showed counts of 5 log.

Until the end of the refrigerated storage, untreated sample showed a significant decrease in *E. coli* counts, reaching 3.7 log CFU/mL by day 14. Those reductions in *E. coli* observed in untreated strawberry juice along storage time could be due to effects such as storage temperature (5 °C) and competitive microflora (native flora). On the other hand, every treated sample (Van1MIC, Van2MIC, Ger1MIC, and Ger2MIC) maintained the counts below detection limit showing an important bactericidal effect of both natural antimicrobial agents tested on the control of the pathogen inoculated.

Table 1
In vitro antimicrobial activity of vanillin and geraniol against Gram negative and Gram positive pathogenic bacteria: MIC values.

	E. coli	*L. monocytogenes*	*S. aureus*	*P. aeruginosa*
Vanillin	2.5 mg/mL	2.5 mg/mL	3 mg/mL	3 mg/mL
Geraniol	0.6 μL/mL	0.5 μL/mL	0.6 μL/mL	>2.4 μL/mL

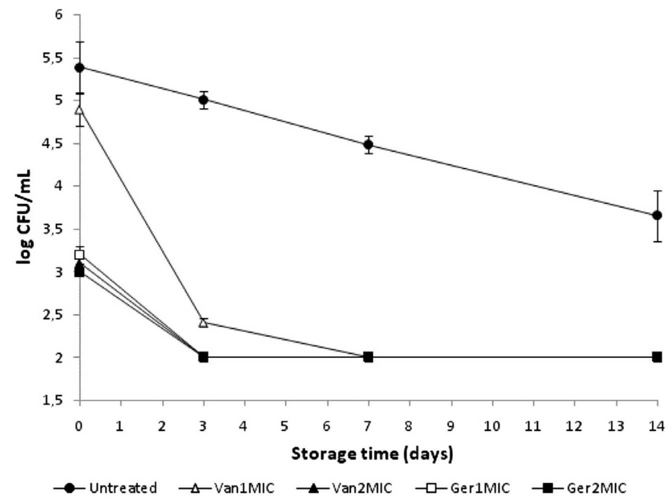

Fig. 2. Effect of vanillin and geraniol on *Escherichia coli O157:H7* in strawberry juice through refrigerated storage at 5 °C. Bars indicate standard deviations. (Van1MIC) vanillin application at 1 MIC; (Van2MIC) vanillin application at 2 MIC; (Ger1MIC) geraniol application at 1 MIC; (Ger2MIC) geraniol applicarion at 2 MIC.

In accordance with our results, Moon, Delaquisb, Toivonenb, and Stanich (2006) found significant reductions (5 log cycles) on *L. monocytogenes* and *E. coli* O157:H7 counts adding 6 mg/mL of vanillin to apple juice, after 24 h of storage at 4 or 15 °C. According to Fitzgerald et al. (2004), based on *in vitro* studies conducted using *E. coli*, *Lactobacillus plantarum*, and *Listeria innocua*, the inhibitory activity of vanillin resides primarily in its ability to detrimentally affect the integrity of the cytoplasmic membrane, with the resultant loss of ion gradient, pH homeostasis and inhibition of respiratory activity.

On the other hand, with respect to geraniol, few studies were found were this natural compound was tested *in vivo*. However, it was tested *in vitro* in several studies, and its antimicrobial activity has been associated with its ability to partition into membranes where it alters bilayer properties, making the membrane more fluid, increasing general permeability (Bard, Albrecht, Gupta, Guynn, & Stillwell, 1988) leading to potassium loss from within the yeast cells. The antimicrobial activity can be ascribed to the combined membrane effects such as increased bilayer disorder and ion leakage. These effects disturbed the osmotic balance of the cell through loss of ions, making its membrane associated proteins inefficient due to increased membrane disorder eventually leading to inhibition of cell growth or cell death (Dalleau, Cateau, Bergès, Berjeaud, & Imbert, 2008).

4. Conclusions

The results found are promising. Vanillin and geraniol antimicrobial activity was proven *in vitro*, against four different pathogens of great importance in the food industry. Both natural compounds studied showed great efficacy in reducing *E. coli* counts even when applied *in vivo* on inoculated strawberry juice (more than 2 log cycle reductions, compared to untreated sample). Furthermore, throughout storage both biopreservatives were able to reduce the pathogen counts below detected limit, showing an important bactericidal effect. Vanillin and geraniol are both GRAS and can easily replace the traditional thermal treatment in the beverage industry. Further studies are necessary, in order to evaluate the effects of vanillin and geraniol application in the sensory and nutritional parameters of the strawberry juice.

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