Journal of Food Protection, Vol. 73, No. 5, 2010, Pages 888–894

In Vitro Evaluation of the Antimicrobial Activity of Eugenol, Limonene, and Citrus Extract against Bacteria and Yeasts, Representative of the Spoiling Microflora of Fruit Juices

ANTONIO BEVILACQUA,[1,2] MARIA ROSARIA CORBO,[1,2] AND MILENA SINIGAGLIA[1,2]*

[1]*Department of Food Science, Faculty of Agricultural Science, and* [2]*Istituto per la Ricerca e le Applicazioni Biotecnologiche per la Sicurezza e la Valorizzazione dei Prodotti Tipici e di Qualità (BIOAGROMED), Foggia University, Via Napoli 25, 71122 Foggia, Italy*

MS 09-276: Received 24 June 2009/Accepted 23 November 2009

ABSTRACT

This article reports on the investigation on the bioactivity of eugenol, limonene, and citrus extract against three bacteria (*Lactobacillus plantarum, Lactobacillus brevis,* and *Bacillus coagulans*) and three yeasts (*Saccharomyces bayanus, Pichia membranifaciens,* and *Rhodotorula bacarum*), representing the spoilage microflora of fruit juices. The experiments were performed with laboratory media by using a microdilution method. Data were fitted using the Gompertz equation, and the kinetic parameters were used to evaluate the MIC and the dose-dependent effect (at suboptimal doses for each essential oil). Citrus extract was the most effective essential oil, and the results suggested the following susceptibility hierarchy, from the most sensitive microorganism to the most resistant one (values in parentheses represent MICs): *S. bayanus* (2 ppm) > *R. bacarum* (3 ppm) > *P. membranifaciens* (5 ppm) > *B. coagulans* (cells, 20 ppm) > *L. brevis* (40 ppm) > *L. plantarum* (>40 ppm).

Traditional juice pasteurization is usually designed to inactivate pectin methylesterase (PME) and reduce the levels of spoilage microorganisms *(36)*; however, it is well known that thermal treatments adversely affect perceived quality including fresh-like flavor *(36)*. Nonpasteurized juices have some desirable characteristics in terms of vitamin C concentration, color, and overall quality but have a short shelf life due to microbial and enzymatic spoilage *(36)*. Some promising approaches have been proposed including such nonthermal strategies as high hydrostatic and homogenization pressure, pulsed electric fields, and ultrasound as well as the addition of essential oils and other natural compounds *(7, 11)*.

Essential oils (EOs) are aromatic oil liquids, extracted from plant materials; they possess antiviral, antibacterial, antimycotic, antitoxigenic, antiparasitic, and insecticidal properties, and their use as food grade additives is allowed in the United States (21 CFR 182.60) *(33)* and European Union countries *(7)*. An EO can contain more than 60 individual components; the major component (usually regarded as the active compound) constitutes up to 85% of the EO. Although other components are present in only trace amounts, they may also have a critical role in antibacterial activity, by producing a synergistic effect *(7)*.

In this article we focus on the use of two active components, eugenol and limonene, and citrus extract. Eugenol is the most important component of clove oil (ca. 85%) *(16)*; it is active against a wide range of bacteria (e.g., *Bacillus* spp., *Alicyclobacillus acidoterrestris, Enterobacter aerogenes, Micrococcus* and *Staphylococcus* spp., *Helicobacter pylori, Listeria monocytogenes, Escherichia coli,* and *Salmonella* Typhimurium) *(1, 4, 7, 20, 24),* and yeasts and molds (*Saccharomyces cerevisiae, Lenzites betulina, Laetiporus sulphureus, Penicillium expansum, Botrytis cinerea, Monilia fructigena,* and *Phlyctema vagabunda*) *(2, 37)*. Wendakoon and Sakaguchi *(35)* suggested that the hydroxyl group of eugenol could bind to proteins and prevent enzyme activity, in addition to causing cell wall deterioration and cell lysis *(30)*. The effect of eugenol against fungi was quite different; in fact, Yen and Chang *(37)* suggested that eugenol altered both the surface and the structure of the fungal cell wall and accelerated fungal death by scavenging free radicals produced by the fungus.

D-Limonene, an aliphatic monoterpene widely distributed in many oils, especially in citrus extract, is used as a flavoring agent for cosmetics, soap, and many other products *(8, 17, 32)*. Citrus oil was used as a medicine in ancient times by the Greeks and Romans; at present, many authors have demonstrated its bioactivity against bacteria, yeasts, and molds. Thus, they have proposed its addition in food (e.g., fish, meat, chicken, dairy products, fruit and vegetables, and confectionary goods) as an ingredient or as a volatile compound in the headspace of the package *(18)*.

Juice spoilage primarily results from the growth of yeasts, molds, and acid-tolerant bacteria *(14, 26, 31, 36)*. *Pichia, Candida, Saccharomyces,* and *Rhodotorula* are the yeasts generally isolated from spoiled juices *(20, 31)*; in particular, *Saccharomyces* and *Pichia* are responsible for juice spoilage, as a consequence of ethanol production from sugars or film formation onto the surface *(28)*.

Acid-tolerant bacteria able to grow in juices include lactic acid (*Lactobacillus* and *Leuconostoc* spp.) and acetic

* Author for correspondence. Tel: +39 0881-589233; Fax: +39 0881 589231; E-mail: m.sinigaglia@unifg.it.

TABLE 1. *Strains used in this study*

Strain	Culture conditions for the preparation of inocula[a]
L. plantarum DSMZ 2601	MRS broth, incubated at 30°C for 24 and 48 h
L. brevis DSMZ 20054	MRS broth, incubated at 30°C for 24 and 48 h
B. coagulans DSMZ 2308	MRS broth, incubated at 44°C for 24 and 48 h
P. membranifaciens DSMZ 70169	YPD broth, incubated at 25°C for 2 and 4 days
S. bayanus DSMZ 70547	YPD broth, incubated at 25°C for 24 and 48 h
R. bacarum DSMZ 70854	YPD broth, incubated at 25°C for 2 and 4 days

[a] All the microorganisms were grown under static conditions and passaged two times in the broth. The duration of the incubation is expressed as a sum of two terms; e.g., *L. plantarum* was incubated for 24 h (first culture) and then 48 h (second culture). YPD broth corresponds to the substrate no. 393 of DSMZ *(13)*.

acid bacteria (*Acetobacter* and *Gluconobacter* spp.), *A. acidoterrestris (6), Propionibacterium cyclohexanicum (34),* and *Bacillus coagulans (22).*

In this study we investigated the effect of citrus extract on three yeasts, *Saccharomyces bayanus, Pichia membranifaciens,* and *Rhodotorula bacarum,* and three bacteria, *Lactobacillus plantarum, Lactobacillus brevis,* and *B. coagulans*, by determining the MIC and the effect of the oil at suboptimal doses through the dose-response effect (DDE). These results were compared with those from limonene, which is one of the major constituents of citrus oil, and eugenol, a phenolic compound showing a strong bioactivity against a wide range of microorganisms.

MATERIALS AND METHODS

Strains. Three bacteria, *L. plantarum, L. brevis,* and *B. coagulans,* and three yeasts, *P. membranifaciens, S. bayanus,* and *R. bacarum,* were purchased from a public collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Yeasts were received as actively growing cultures and maintained on yeast extract–peptone–dextrose YPD *(13)* slants at 4°C and transferred monthly. The bacteria, which were received as lyophilized cultures, were grown as suggested (lactobacilli were grown in de Man Rogosa Sharpe [MRS] broth, Oxoid, Milan, Italy, and *B. coagulans* was grown in Nutrient broth [Oxoid]) and then stored at −20°C in MRS broth containing 33% of sterile glycerol (J. T. Baker, Milan, Italy).

Before each experiment, the strains were grown as reported in Table 1; then, the cultures were centrifuged at 1,000 × *g* for 10 min, washed in a saline solution (0.9% NaCl), and serially diluted to 5 log CFU/ml. The *B. coagulans* assays were performed with vegetative cells only.

Antimicrobials. Eugenol [2-methoxy-4-(2-propenyl) phenol] (MP Biomedicals, Aurora, OH), D-limonene (4-isopropenyl-1-methyl-1-1 cyclohexene) (MP Biomedicals), and citrus extract (Biocitro^{LIQUID}) (Quinabra, Probena, Spain) were used throughout this study. The supplier reported the following composition for citrus extract: ascorbic acid and ascorbates (vitamin C), linked with citrus bioflavonoids, 4.00 to 7.20%; hydrated glycerin linked with other traces of citrus polyphenols, carbohydrates, bioflavoproteins, pectin, citrus sugars, citric acid, 30.80 to 36.60%; water, 6.00 to 11.00%; stabilizer and inert carrier, 50.00%.

Chemical analyses revealed that the concentration of ascorbic acid and citrus bioflavonoid was ca. 56,000 ppm; among the bioflavonoids, naringin was present at a minimum of 6,500 ppm. Finally, the limonene content was 30,000 to 50,000 ppm. Stock solutions of eugenol (9,000 to 18,000 ppm), limonene (18,000 to 30,000 ppm), and citrus extract (100 to 4,000 ppm) were freshly prepared in ethanol-water (1:1) and distilled water, respectively, and filtered through a membrane (0.22-μm pore size) before each experiment.

Antimicrobial activity assays. The antimicrobial activity of eugenol, limonene, and citrus extract was evaluated by the microdilution method. Aliquots of MRS broth for lactobacilli and *B. coagulans* and YPD broth for yeasts, containing 300 to 600 ppm of eugenol, 600 to 1,000 ppm of limonene, or 1 to 40 ppm of citrus extract, were separately inoculated to contain ca. 3 log CFU of the strains per ml as shown in Table 2.

A control sample (inoculated broth) was also prepared for each microorganism and active compound (Table 2).

The samples were incubated at the optimal temperature for each microorganism (Table 1) for 7 days, and microbial growth was evaluated by measuring absorbance at 600 nm (UV-Visible Beckman DU 640 spectrophotometer, Beckman, Inc., Fullerton, CA).

Data modeling. The analyses were performed in triplicate on three independent batches ($n = 3$); data were submitted to one-way analysis of variance and Tukey's test through the software Statistica for Windows (Statsoft, Tulsa, OK) ($P < 0.05$).

Absorbance data were also modeled using the Gompertz equation, as modified by Zwietering et al. *(38)*:

$$y = k + A \times \exp\left\{-\exp\left[(\mu_{max} \times 2.7182) \times \frac{(\lambda - t)}{A} + 1\right]\right\}$$

where y is the absorbance (dependent variable), k is the initial level of the dependent variable, A is the maximum absorbance value attained at the stationary phase, μ_{max} is the maximal growth rate (change in absorbance at 600 nm per hour), λ is the delay time for the microbial response *(15),* and t is the time (in hours).

MIC evaluation. The lowest concentration of each EO able to inhibit microbial growth for at least 7 days was considered to be the MIC.

TABLE 2. *Sample preparation*[a]

Sample	Amt of eugenol/ limonene (ml)	Amt of citrus extract (ml)
Broth	14.00	14.35
Stock solution	0.50	0.15
Inoculum	0.50	0.50

[a] The control samples contained 0.50 ml of ethanol-water solution or 0.15 ml of distilled water.

TABLE 3. *Gompertz parameters of* L. plantarum *and* L. brevis *in MRS with citrus extract added*

Organism and citrus extract concn (ppm)	Mean parameter value ± SE[a]			
	A	μ_{max}	λ (h)	R^2
L. plantarum DSMZ 2601				
Control	2.04 ± 0.03 A	0.23 ± 0.01 A	16.06 ± 0.48 A	0.998
5	2.03 ± 0.04 A	0.39 ± 0.04 B	19.80 ± 0.23 AB	0.998
10	2.07 ± 0.01 A	0.19 ± 0.01 A	28.33 ± 0.15 C	0.998
15	2.01 ± 0.00 A	0.11 ± 0.01 C	40.94 ± 0.13 D	0.998
20	2.19 ± 0.03 A	0.06 ± 0.00 D	52.17 ± 0.46 E	0.998
30	1.78 ± 0.01 B	0.06 ± 0.00 D	51.45 ± 0.22 E	0.998
40	1.36 ± 0.16 C	0.06 ± 0.01 D	80.00 ± 0.56 F	0.994
L. brevis DSMZ 20054				
Control	1.83 ± 0.04 A	0.40 ± 0.04 A	20.06 ± 0.21 A	0.996
5	1.80 ± 0.07 A	0.17 ± 0.03 B	20.11 ± 0.77 A	0.992
10	1.78 ± 0.04 A	0.09 ± 0.01 C	22.91 ± 0.67 A	0.998
15	1.81 ± 0.00 A	0.11 ± 0.00 C	38.81 ± 0.19 B	0.998
20	1.77 ± 0.01 A	0.08 ± 0.00 C	47.50 ± 0.10 C	0.998
30	1.00 ± 0.05 B	0.06 ± 0.01 CD	78.52 ± 1.75 D	0.980
40	—[b]	—	—	—

[a] *A*, maximum absorbance value attained in the stationary phase; μ_{max}, maximal growth rate (change in absorbance at 600 nm/h); λ, delay time for microbial response; R^2, regression coefficient. For each microorganism, values in a column followed by different letters are significantly different (one-way analysis of variance and Tukey's test, $P < 0.05$).

[b] —, no growth.

DDE. A first-order equation was used to find a correlation between the concentration of each EO and delayed growth (delay phase).

The equation reads as follows:

$$y = a + b \cdot x$$

where y is the delay time (in hours), a is the delay time in the control, x is the concentration of the antimicrobial, and b is the DDE, i.e., the increase in the delay time (delay phase) due to an increase in the concentration of EO.

RESULTS

Effects of eugenol, limonene, and citrus extract on the growth of bacteria. Table 3 reports the Gompertz parameters for *L. plantarum* and *L. brevis* in MRS containing citrus extract; the regression coefficients highlight the adequacy of the model. In particular, citrus extract decreased the population of *L. plantarum* in the stationary phase (parameter A) only at 30 and 40 ppm with these levels decreasing from 2.04 (control) to 1.78 and 1.36, respectively. A significant effect was also seen for the maximal growth rate and the delay time (λ); in particular, λ was ca. 16 h for the control and increased with increasing concentration of the EO in the broth up to 80 h when 40 ppm of citrus extract was added.

Citrus extract similarly affected the growth of *L. brevis*, with a decrease in parameter A only at 30 ppm and an increased delay time. However, the maximum concentration of citrus extract completely inhibited growth.

Citrus extract significantly affected *B. coagulans* (Fig. 1), with growth inhibition seen at a concentration of 20 ppm. Lower levels of citrus extract extended the delay time from 8.83 to 30.72 h (at 5 ppm of citrus extract) and ca. 63 h (at 10 to 15 ppm of citrus extract).

The other two compounds, eugenol and limonene, did not significantly affect the growth of lactobacilli or *B. coagulans*.

Effects of the antimicrobials on yeast growth. Growth of *P. membranifaciens* was unaffected by eugenol or limonene (data not shown); however, citrus extract was inhibitory (Fig. 2). In fact, citrus extract increased the delay time (ca. 14 h in the control) to 44.6 and 86 h at 3 and 4 ppm, respectively, with growth completely inhibited at 5 ppm.

The effectiveness of the three antimicrobials against *S. bayanus* is shown in Figure 3. Eugenol and limonene impacted yeast growth. In particular, eugenol (Fig. 3a) increased the delay time from 25.22 h (control) to 55.28 (at 400 ppm) and decreased slightly ($P < 0.05$) parameter *A*

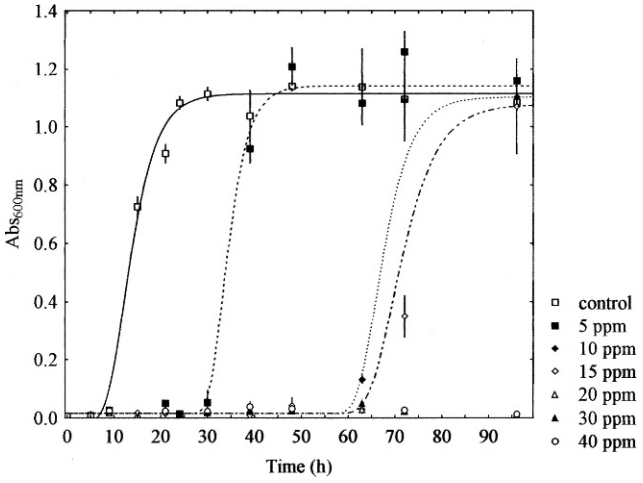

FIGURE 1. *Evolution of* B. coagulans *population in MRS broth with citrus extract added. Data are means ± standard deviations.*

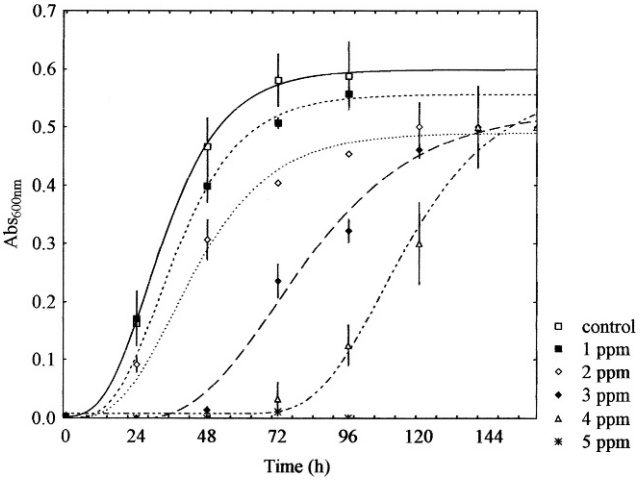

FIGURE 2. *Evolution of* P. membranifaciens *population in YPD broth with citrus extract added. Data are means ± standard deviations.*

(from 1.18 to 0.93 to 0.97). In addition to these effects, growth inhibition was seen when using 450 ppm of eugenol.

Limonene bioactivity (Fig. 3b) significantly prolonged the delay time for *S. bayanus* (from 26.09 h in the control to 56.51 h at 1,000 ppm) and slightly reduced parameter *A*. Finally, citrus extract (Fig. 3c) was strongly inhibitory, with 1 ppm of EO extending the delay phase to 72 h and decreasing the population level to 1. *S. bayanus* was completely inhibited at 2 ppm of EO.

Bioactivity of eugenol, limonene, and citrus extract against *R. bacarum* is reported in Table 4. These data are quite similar to those reported for *S. bayanus,* with *R. bacarum* inhibited by both eugenol and limonene. Moreover, citrus extract was inhibitory to the yeast population, with complete inhibition seen at 3 ppm.

MIC and DDE evaluation. MICs are reported in Table 5. Citrus extract yielded MICs of 5, 2, and 3 ppm for *P. membranifaciens, S. bayanus,* and *R. bacarum,* respectively, whereas higher MICs were seen for the bacteria (20 and 40 ppm for *B. coagulans* cells and *L. brevis,* respectively). The DDE values for citrus extract against *L. plantarum/L. brevis* and *B. coagulans* were 1.54 to 2.02 and 3.99 h, respectively (Fig. 4).

The DDE of citrus extract was evaluated only for *P. membranifaciens* (Fig. 5a) (13.21 h), due to the high susceptibility of *S. bayanus* and *R. bacarum*. The DDE for these two other yeasts was evaluated by using eugenol (0.07 and 0.03 h for *S. bayanus* and *R. bacarum,* respectively) (Fig. 5b) and limonene (0.03 h only for *S. bayanus*) (Fig. 5c).

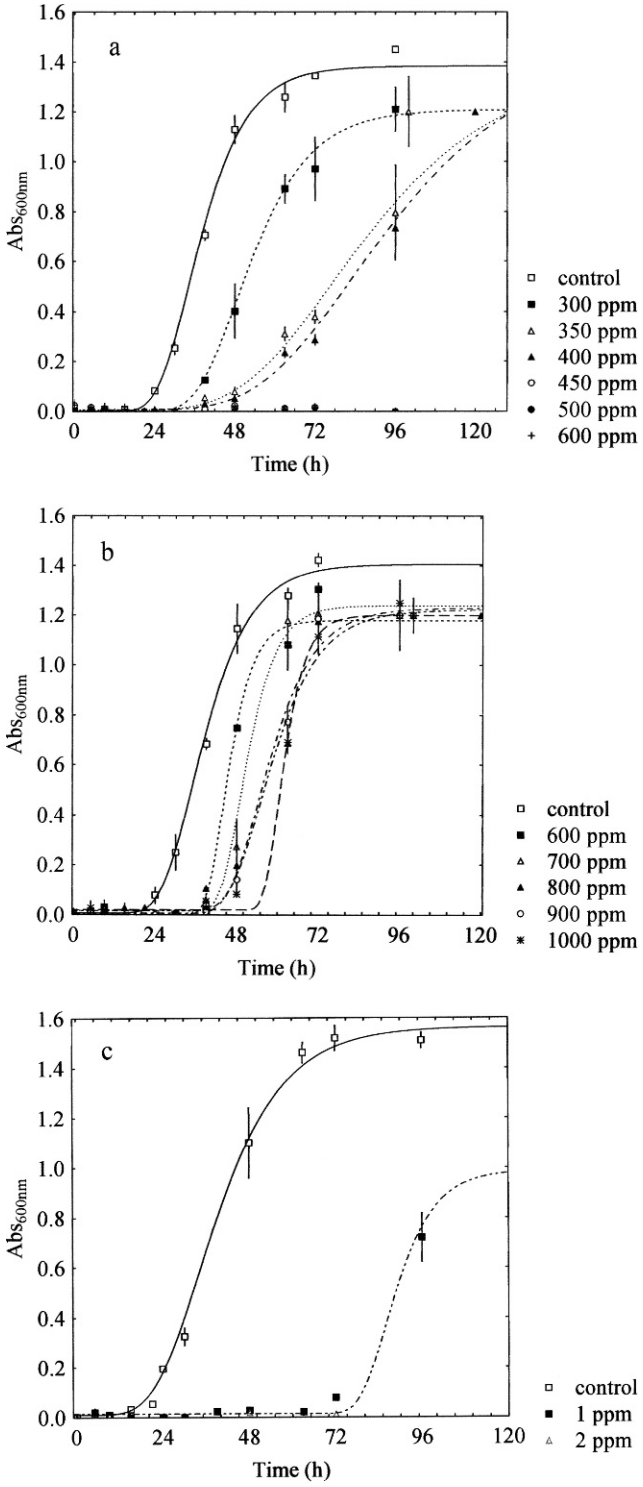

FIGURE 3. *Evolution of* S. bayanus *population in YPD broth with eugenol (a), limonene (b), and citrus extract (c) added. Data are means ± standard deviations.*

DISCUSSION

The antimicrobial activity of EOs, as well as the effectiveness of their active compounds, has been extensively investigated *(7, 11, 19, 25)*. Nowadays the bioactivity of EOs is generally attributed to phenolic compounds (phenols), which are soluble in the lipid layer of the membrane and alter membrane fluidity, along with causing leakage of intracellular constituents and dissipation of the transmembrane H^+ gradient *(7)*. Prashar et al. *(27),* who used Palmarosa oil (*Cymbopogon martinii*) to inhibit the growth of *S. cerevisiae,* found that the extract caused a change in the fatty acid composition of the membrane, with an increase of saturated fatty acids. In fact, Palmarosa oil increased $C_{16:0}$ and decreased both $C_{16:1}$ and $C_{18:0}$. In addition to these data, oregano and clove and their active

TABLE 4. *Gompertz parameters for* R. bacarum

Compound and concn (ppm)	Mean parameter value ± SE[a]			
	A	μ_{max}	λ (h)	R^2
Eugenol				
Control	0.62 ± 0.00 A	0.02 ± 0.00 A	43.58 ± 0.16 A	0.998
300	0.41 ± 0.01 B	0.05 ± 0.01 A	52.58 ± 2.00 B	0.998
350	0.40 ± 0.02 B	0.02 ± 0.00 A	49.32 ± 0.38 B	0.996
400	0.41 ± 0.02 B	0.02 ± 0.01 A	50.98 ± 0.02 B	0.996
500	0.39 ± 0.01 B	0.01 ± 0.00 A	52.82 ± 1.39 B	0.998
600	0.33 ± 0.00 C	0.05 ± 0.01 A	68.88 ± 0.44 C	0.998
Limonene				
Control	0.62 ± 0.00 A	0.02 ± 0.00 A	43.58 ± 0.16 A	0.998
600	0.51 ± 0.03 B	0.02 ± 0.01 A	61.47 ± 3.83 B	0.998
700	0.53 ± 0.01 B	0.02 ± 0.00 A	62.32 ± 1.12 B	0.994
800	0.53 ± 0.02 B	0.02 ± 0.00 A	63.01 ± 0.18 B	0.998
900	0.46 ± 0.03 B	0.02 ± 0.01 A	63.00 ± 1.73 B	0.978
1,000	0.47 ± 0.07 B	0.03 ± 0.01 A	63.02 ± 1.83 B	0.982
Citrus extract				
Control	0.68 ± 0.02 A	0.03 ± 0.02 A	40.00 ± 2.01 A	0.996
1	0.38 ± 0.05 B	0.03 ± 0.02 A	63.01 ± 1.21 B	0.993
2	0.33 ± 0.01 B	0.02 ± 0.01 A	63.05 ± 1.88 B	0.986
3	—[b]	—	—	—
4	—	—	—	—
5	—	—	—	—

[a] *A*, maximum absorbance value attained at the stationary phase; μ_{max}, maximal growth rate (change in absorbance at 600 nm/h); λ, delay time for microbial response; R^2, regression coefficient. For each antimicrobial, data in a column followed by different letters are significantly different (one-way analysis of variance and Tukey's test, $P < 0.05$).

[b] —, no growth.

components, thymol and eugenol, respectively, reportedly altered the yeast membrane and cell wall, resulting in cellular deformity *(3, 10).*

Two active compounds (eugenol and limonene), along with citrus extract, were used throughout this study against some bacteria and yeasts representative of juice microflora (lactobacilli, yeasts, and *B. coagulans*). The *B. coagulans* experiments were performed with vegetative cells only, as in a preliminary phase spores appeared to be more susceptible to some EOs (data not published), which is in agreement with the results reported by Chaibi et al. *(9)* for *Bacillus cereus* and *Clostridium botulinum.*

In this study neither eugenol nor limonene was effective against the bacteria or *P. membranifaciens,* and the compounds were only partially inhibitory to *S. bayanus* and *R. bacarum*. In contrast, citrus extract exerted a strong inhibitory effect, thus confirming the potential use of these EOs extracted from citrus as food grade preservatives. This possibility has been proposed by Fisher and Phillips *(18),* who regarded the EOs produced from citrus fruits as a ''possible answer for the use of essential oils in foods.''

Citrus extract provides a natural source for antioxidants and flavonoids (rutin, naringin, quercetin, and naringenin), as well as phenolic compounds (limonene, linalool, citral,

TABLE 5. *MICs of eugenol, limonene, and citrus extract*

Organism	MIC (ppm)		
	Eugenol	Limonene	Citrus extract
L. plantarum	>600	>1,000	>40
L. brevis	>600	>1,000	40
B. coagulans (cells)	>600	>1,000	20
P. membranifaciens	>600	>1,000	5
S. bayanus	450	>1,000	2
R. bacarum	>600	>1,000	3

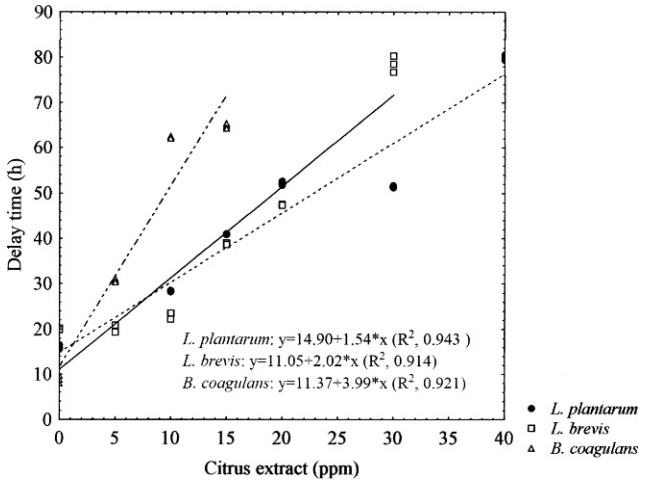

FIGURE 4. *Dose dependence of citrus extract against bacteria: concentration of the extract versus the delay time of* L. plantarum, L. brevis, *and* B. coagulans.

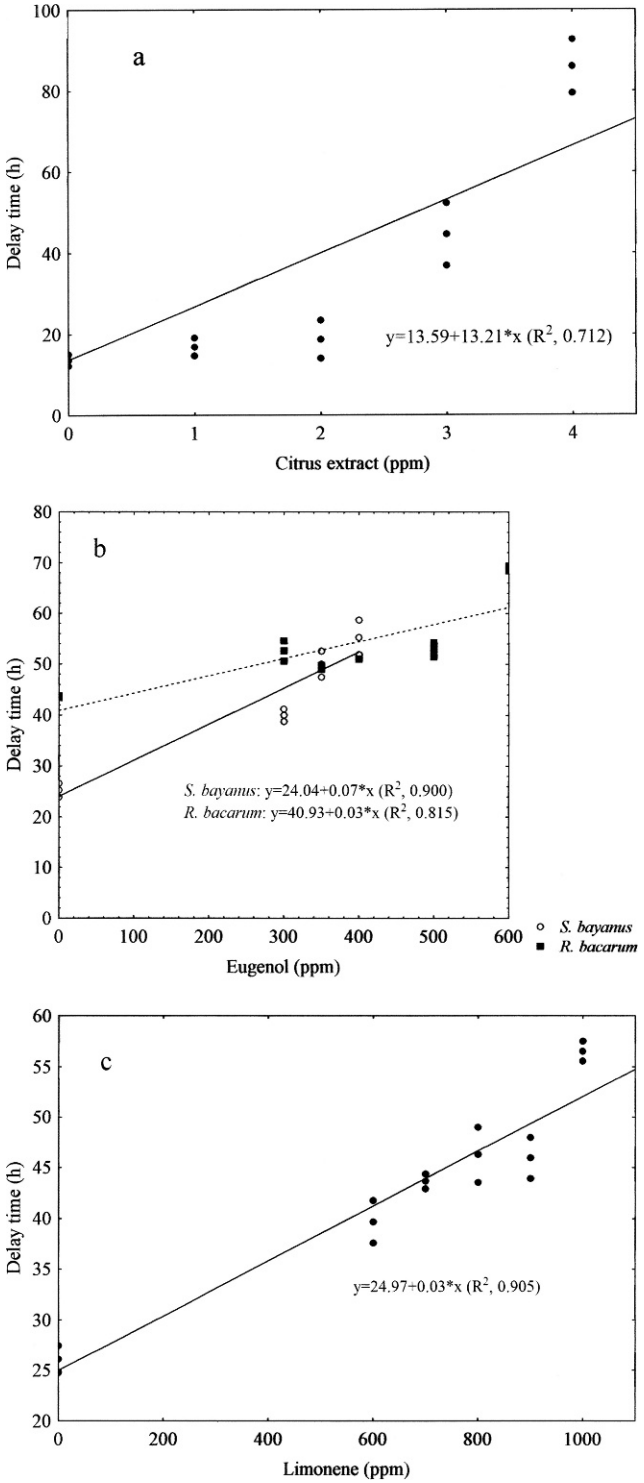

FIGURE 5. *Concentration of the antimicrobials versus delay time of yeasts: (a) citrus extract against* P. membranifaciens*; (b) eugenol against* S. bayanus *and* R. bacarum*; (c) limonene against* S. bayanus.

and other chemicals) *(18, 20),* and thus, it was suggested that its bioactivity could be the result of synergistic activity.

Another topic of great interest is the different susceptibility of yeasts and bacteria to citrus extract; our data confirmed that yeasts are more susceptible, in agreement with the results of Marwah et al. *(23),* who reported a MIC of 7.8 μg/ml for the EO from *Plectranthus cylindraceus* against *Candida albicans,* whereas pathogens were inhibited at higher concentrations (31.3 μg/ml for *Staphylococcus aureus* and *Bacillus subtilis,* 62.5 μg/ml for *Klebsiella pneumoniae,* and 125 μg/ml for *E. coli, Pseudomonas aeruginosa,* and *Salmonella* serovar Choleraesuis). Similar results were reported by Falcone et al. *(15),* who investigated the effectiveness of thymol against *Bacillus* spp., *L. plantarum, Lactobacillus curvatus, Pichia subpelliculosa, Candida lusitaniae,* and *S. cerevisiae* and found higher susceptibility among yeasts than lactic acid bacteria.

A variety of methods have been developed to assess the bioactivity of citrus extract, limonene, and eugenol against various target organisms. The microdilution approach has been used extensively to evaluate the effects of some antimicrobials against a wide range of microorganisms, despite the limitation of a high detection threshold (ca. 6 log CFU/ml) *(12).* Although it has major limitations when attempting to detect low levels of pathogenic microorganisms, the turbidimetric assay is advantageous for spoilage microorganisms, as reported by Dalgaard and Koutsoumanis *(12).*

High numbers of spoilage microorganisms negatively impact food quality *(12)*; therefore, the estimation of the growth parameters from absorbance measurements could have practical implications. Focusing on the λ parameter (here labeled as the delay time), λ should not be regarded as a classical lag phase but as the time to achieve the threshold population of 6 log CFU/ml and should be used as a ''No Spoiling Time'' index *(5).* Based on these assumptions, the delay time is the time required to cause spoilage.

In conclusion, this article provides some useful details regarding the susceptibility and resistance of juice microflora to some EOs, suggesting that citrus extract could be a promising alternative to inhibit the spoilage microorganisms. Based on the MICs obtained, *S. bayanus* was the most susceptible (2 ppm) followed by *R. bacarum* (3 ppm), *P. membranifaciens* (5 ppm), *B. coagulans* (20 ppm), *L. brevis* (40 ppm), and *L. plantarum* (>40 ppm).

Due to the high resistance shown by *L. plantarum* and *L. brevis*, use of these lactic acid bacteria as potential target organisms should be considered for future challenge studies.

REFERENCES

1. Ali, K. S. M., A. A. Khan, I. Ahmed, M. Musaddiq, K. S. Ahmed, H. Polasa, L. Venkateswar Rao, C. M. Habibullah, L. A. Sechi, and N. Ahmed. 2005. Antimicrobial activities of eugenol and cinnamaldehyde against the human gastric pathogen *Helicobacter pylori*. *Ann. Clin. Microbiol. Antimicrob.* 4:20–26.
2. Amiri, A., R. Dugas, A. L. Pichot, and G. Bompeix. 2008. In vitro and in vivo activity of eugenol oil (*Eugenia caryophylata*) against four important postharvest apple pathogens. *Int. J. Food Microbiol.* 126:13–19.
3. Bennis, S., F. Chami, N. Chami, T. Bouchikhi, and A. Remmal. 2004. Surface alteration of *Saccharomyces cerevisiae* induced by thymol and eugenol. *Lett. Appl. Microbiol.* 38:454–458.
4. Bevilacqua, A., M. R. Corbo, and M. Sinigaglia. 2008. Inhibition of *Alicyclobacillus acidoterrestris* spores by natural compounds. *Int. J. Food Sci. Technol.* 43:1271–1275.
5. Bevilacqua, A., S. Ficelo, M. R. Corbo, and M. Sinigaglia. 2009. Bioactivity of grapefruit extract against *Pseudomonas* spp. *J. Process. Pres.* DOI: 10.1111/j.1745-4549-2008-00161.x.

6. Bevilacqua, A., M. Sinigaglia, and M. R. Corbo. 2008. *Alicyclobacillus acidoterrestris*: new methods for inhibiting spore germination. *Int. J. Food Microbiol.* 125:103–110.
7. Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int. J. Food Microbiol.* 94: 223–253.
8. Cadwallader, K. R., R. J. Braddock, and M. E. Parish. 1992. Isolation of terpineol dehydratase from *Pseudomonas*. *J. Food Sci.* 57:241–248.
9. Chaibi, A., L. Ababouch, K. Belasri, S. Boucetta, and F. F. Busta. 1997. Inhibition of germination and vegetative growth of *Bacillus cereus* T and *Clostridium botulinum* 62A spores by essential oils. *Food Microbiol.* 14:161–174.
10. Chami, F., N. Chami, S. Bennis, T. Bouchikhi, and A. Remmal. 2005. Oregano and clove essential oils induce surface alteration of *Saccharomyces cerevisiae*. *Phytother. Res.* 19:405–408.
11. Corbo, M. R., A. Bevilacqua, D. Campaniello, D. D'Amato, B. Speranza, and M. Sinigaglia. 2009. Prolonging microbial shelf life of foods through the use of natural compounds and non-thermal approaches—a review. *Int. J. Food Sci. Technol.* 44:223–241.
12. Dalgaard, P., and K. Koutsoumanis. 2001. Comparison of maximum specific growth rate and lag times estimated from absorbance and viable count data by different mathematical models. *J. Microbiol. Methods* 43:183–196.
13. Deutsche Sammlung von Mikroorganismen und Zellkulturen. Available at: http://www.dsmz.de/yeasts. Accessed 27 March 2009.
14. Elzez-Martinez, P., J. Hescola-Hernandez, R. C. Soliva-Fortuny, and O. Martın-Belloso. 2005. Inactivation of *Lactobacillus brevis* in orange juice by high intensity pulsed electric fields. *Food Microbiol.* 22:311–319.
15. Falcone, P., B. Speranza, M. A. Del Nobile, M. R. Corbo, and M. Sinigaglia. 2005. A study on the antimicrobial activity of thymol intended as natural preservative. *J. Food Prot.* 68:1664–1670.
16. Farag, R. S., Z. Y. Daw, and S. H. Abo-Raya. 1989. Influence of some spice essential oils on *Aspergillus parasiticus* growth and production of aflatoxins in a synthetic medium. *J. Food Sci.* 54:74–76.
17. Filho, C. A., C. M. Silva, M. B. Quadri, and E. A. Macedo. 2003. Tracer diffusion coefficients of citral and D-limonene in supercritical carbon dioxide. *Fluid Phase Equilibr.* 204:65–73.
18. Fisher, K., and C. Phillips. 2008. Potential antimicrobial uses of essential oils in food: is citrus the answer? *Trends Food Sci. Technol.* 19:156–164.
19. Holley, R. A., and D. Patel. 2005. Improvement in shelf-life and safety of perishable foods by plant essential oil and smoke antimicrobials—a review. *Food Microbiol.* 22:273–292.
20. Kim, J., R. Marshall, and C. Wie. 1995. Antibacterial activity of some essential oil components against five foodborne pathogens. *J. Agric. Food Chem.* 43:2839–2845.
21. Las Heras-Vazquez, F. J., L. Mingorance-Cazorla, J. M. Clemente-Jimenez, and F. Rodriguez-Vico. 2003. Identification of yeast species from orange fruit and juice by RFLP and sequence analysis of the 5.8S rRNA gene and the two internal transcribed spacers. *FEMS Yeast Res.* 3:3–9.
22. Lucas, R., M. J. Grande, H. Abriouel, M. Maqueda, N. Ben Omar, E. Valdivia, M. Martínez-Cañamero, and A. Gálvez. 2006. Application of the broad-spectrum bacteriocin enterocin AS-48 to inhibit *Bacillus coagulans* in canned fruit and vegetable foods. *Food Chem. Toxicol.* 44:1774–1781.
23. Marwah, R. G., M. O. Fatope, M. L. Deadman, J. E. Ochei, and S. H. Al-Saidi. 2007. Antimicrobial activity and the major components of the essential oil of *Plectranthus cylindraceus*. *J. Appl. Microbiol.* 103:1220–1226.
24. Moleyar, V., and P. Narasimham. 1992. Antibacterial activity of essential oil components. *Int. J. Food Microbiol.* 16:337–342.
25. Nychas, G. J. E. (ed.). 1995. Natural antimicrobials from plants. Chapman & Hall, New York.
26. Parish, M. E. (ed.). 1988. Microbiological aspects of fresh squeezed citrus juice. *In* Ready to serve citrus juices and juice added beverages. Proceedings of the Food Industry Short Course. University of Florida, Gainesville.
27. Prashar, A., P. Hili, R. G. Veness, and C. S. Evans. 2003. Antimicrobial activity of palmarosa oil (*Cymbopogon martinii*) on *Saccharomyces cerevisiae*. *Phytochemistry* 63:569–575.
28. Rates, R. P., J. R. Morris, and P. G. Crandall. 2001. Principles and practices of small- and medium-scale fruit juice processing. Food and Agriculture Organization of the United Nations, Rome.
29. Rehman, Z. U. 2006. Citrus peel extract—a natural source of antioxidants. *Food Chem.* 99:450–454.
30. Thoroski, J., G. Blank, and C. Biliaderis. 1989. Eugenol induced inhibition of extracellular enzyme production by *Bacillus cereus*. *J. Food Prot.* 52:399–403.
31. Tournas, V. H., J. Heeres, and L. Burgess. 2006. Moulds and yeasts in fruit salads and fruit juices. *Food Microbiol.* 23:684–688.
32. Trytek, M., and J. Fiedurek. 2005. A novel psychrotrophic fungus, *Mortierella minutissima*, for D-limonene biotransformation. *Biotechnol. Lett.* 27:149–153.
33. U.S. Food and Drug Administration. Code of Federal Regulations, Title 21 (Food and Drugs), vol. 3. Available at: http://www.foodsafety.gov/. Accessed 27 March 2009.
34. Walker, M., and C. A. Phillips. 2008. The effect of preservatives on *Alicyclobacillus acidoterrestris* and *Propionibacterium cyclohexanicum* in fruit juice. *Food Control* 19:974–981.
35. Wendakoon, C. N., and M. Sakaguchi. 1995. Inhibition of amino acid decarboxylase activity of *Enterobacter aerogenes* by active components in spices. *J. Food Prot.* 58:280–283.
36. Winniczuk, P. P., and M. E. Parish. 1997. Minimum inhibitory concentrations on antimicrobials against micro-organisms related to citrus juice. *Food Microbiol.* 14:373–381.
37. Yen, T.-B., and S.-T. Chang. 2008. Synergistic effects of cinnamaldehyde in combination with eugenol against wood decay fungi. *Biores. Technol.* 99:232–236.
38. Zwietering, M. H., I. Jongenburger, F. M. Rombouts, and K. Van't Riet. 1990. Modeling of bacterial growth curve. *Appl. Environ. Microbiol.* 56:1875–1881.