Synergistic Inhibitory Effect of Citral with Selected Phenolics against Zygosaccharomyces bailii

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ABSTRACT

Antifungal susceptibilities of Zygosaccharomyces bailii to individual and binary mixtures of citral with selected phenolics were evaluated to identify synergistic combinations. Individual effects of citral, vanillin, thymol, carvacrol, and eugenol concentrations and combined effects of citral with the other phenolic compounds on the growth of Z. bailii were evaluated in potato dextrose agar, adjusted with sucrose to a water activity of 0.99 or 0.95, and hydrochloric acid to pH 4.5 or 3.5. MICs for individual and binary antimicrobial mixtures were identified and then transformed to fractional inhibitory concentrations. Inhibitory concentrations of citral and vanillin were higher than 650 ppm, whereas for thymol, eugenol, and carvacrol, concentrations were lower than 250 ppm for several of the studied water activity–pH conditions. Combining citral with the other phenolic compounds, fractional inhibitory concentration (FIC) and FICindex varied from 0.216 to 0.582. FICindex demonstrated synergistic effects on Z. bailii inhibition when citral was used in combination with vanillin, thymol, carvacrol, or eugenol. Therefore, the relative amount of antimicrobials could be greatly reduced.

Nowadays, consumers demand high-quality foods that are more natural, minimally processed, and free of preservative while remaining safe and having an adequate shelf life (1, 19). These demands, along with tighter legislation regarding current preservatives, have promoted the search for “naturally occurring” antimicrobials for use by the food industry (13). In this search, a wide range of compounds from plants is being studied (19, 20). Major components with antimicrobial activity found in plants, herbs, and spices are phenolic compounds (e.g., eugenol, thymol, carvacrol, vanillin), terpenes (e.g., citral), aliphatic alcohols, aldehydes, ketones, acids, and isoflavonoids (1, 19, 25).

Food spoilage by yeasts and filamentous fungi is a prime issue for the food industry. Zygosaccharomyces bailii is frequently implicated in spoilage of high-acid and high-sugar foods and can develop resistance to sorbate and benzoate (11, 24, 31, 34). For Z. bailii inoculated in a salsa mayonnaise (pH 3.74, water activity $a_w$ 0.946) 0.3% potassium sorbate was not enough to inhibit growth for 28 days at ambient temperature (37). For foods in which shelf stability relies either on acidity, weak acid preservatives, or their combination, the tolerance for Z. bailii counts must be less than the threshold level of detection (26).

High-quality products with satisfactory shelf life rely in many cases on the incorporation of antimicrobials. Therefore, antimicrobial mixtures need to be studied and evaluated (1, 19). Combinations of common antimicrobial agents are routinely used in the food industry. However, interactions among traditionally used preservatives are poorly understood (10, 22). There is a need for a better understanding of the effects of antimicrobial mixtures on microbial inhibition, including combinations of naturally occurring antimicrobials. The use of antimicrobial mixtures theoretically provides an increased antimicrobial action. It is thought that combined agents act on different microbial metabolic elements, which theoretically results in improved microbial control over the use of one antimicrobial agent alone (10, 22).

When an antimicrobial mixture is used, three effects can occur: synergistic, antagonistic, or additive (10, 22). Synergism refers to an enhancement of antimicrobial activity for a compound because of the presence of a second compound. Antagonism occurs when antimicrobial effectiveness of a compound is reduced in the presence of the second compound. Additive effects occur when antimicrobial effectiveness of a compound is neither reduced nor enhanced in the presence of the second compound (22, 23).

The objective of this work was to evaluate, at selected $a_w$ and pH values, the susceptibility of Z. bailii to individual and binary antimicrobial mixtures of citral with a phenolic compound such as vanillin, thymol, carvacrol, or eugenol, to identify synergistic combinations.

MATERIALS AND METHODS

Microorganism and culture preparation. Z. bailii isolated from a papaya sugared product (LMUDLA-0042) was obtained from Universidad de las Américas Food Microbiology Laboratory and maintained on potato dextrose agar (Merck, Merck-México, Naucalpan, Mexico) slants at 4°C. Inocula were prepared by growing cells for 48 h in malt extract broth (Merck, Merck-México) under agitation at 27 ± 1°C.
TABLE 1. Zygosaccharomyces bailii growth (G) or no growth (NG) response after 12 days of incubation in potato dextrose agar formulated with binary mixtures of citral and vanillin at selected  aw and pH values

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3.5  1,000  —  —  NG  NG  NG  NG  NG  NG  NG  NG
900  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG
800  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG
700  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG
600  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG
500  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG
400  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG
300  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG
200  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG
100  —  —  NG  NG  NG  NG  NG  G   G   NG  NG  NG

0.95 1,000  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG
900  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG  NG
800  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG  NG
700  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG  NG
600  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG  NG
500  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG  NG
400  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG  NG
300  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG  NG
200  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG  NG
100  —  —  NG  NG  NG  NG  NG  G   G   NG  NG  NG

3.5 1,000  —  —  —  NG  NG  NG  NG  NG  NG  NG  NG
900  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG  NG
800  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG  NG
700  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG  NG
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300  —  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG
200  —  —  —  NG  NG  NG  NG  NG  NG  NG  NG  NG
100  —  —  —  NG  NG  NG  NG  NG  G   G   NG  NG  NG

a —, not tested.

System preparation. The effects of aw (0.99 or 0.95) and pH (4.5 or 3.5) on the susceptibility of Z. bailii to individual and binary mixtures of citral with vanillin, thymol, carvacrol, or eugenol were evaluated. Potato dextrose agar systems were prepared with sucrose to reach aw 0.99 or 0.95, sterilized for 15 min at 121°C, cooled, and acidified with hydrochloric acid to the desired pH (4.5 or 3.5). The amounts of sucrose and hydrochloric acid needed in each case were determined previously (17–19). The sterile acidified agar was aseptically divided and the necessary amount of citral, vanillin, thymol, carvacrol, eugenol, or combinations (0, 10, 20, 30, 40, 50, 100, 150, up to 1,500 ppm) was added. Compounds tested (Sigma Chemical Co., St. Louis, Mo.) were mechanically incorporated with a vortex shaker under sterile conditions. Agar solutions were poured into sterile petri dishes. Resulting combinations of antimicrobials, pH, and aw were tested in triplicate.

Binary combinations were made with the MIC of each antimicrobial as the maximum concentration in the mixture. Checkerboard arrays (22) were used to evaluate antimicrobial concentration effects. Different combinations of antimicrobial concentrations were tested depending on type of agents in the mixture and environmental conditions (aw and pH). Combinations tested included citral with vanillin, thymol, carvacrol, or eugenol. As an example, Table 1 presents the array of combinations tested for the vanillin-citral mixture.

Inoculation and incubation. Petri dishes of each system were inoculated in a spiral plater (Autoplate 4000, Spiral Biotech,
TABLE 2. MICs of antimicrobials against Z. bailii inoculated in potato dextrose agar formulated at selected a w and pH values

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>pH 4.5</th>
<th>pH 3.5</th>
<th>pH 4.5</th>
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<tr>
<td>Vanillin</td>
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<td>50</td>
</tr>
<tr>
<td>Citral</td>
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<td>1,100</td>
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<tr>
<td>Eugenol</td>
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<td>100</td>
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</table>

* Defined as concentrations that inhibit yeast growth for at least 12 days at 27°C.

Bethesda, Md.) with 50 μl of inocula (1.0 to 5.0 × 10⁶ CFU/ml). Growth controls without antimicrobials were prepared and inoculated as above. Three plates of every tested system were maintained without inoculation for a w and pH measurements. Inoculated plates and controls were incubated for a maximum of 12 days at 27 ± 1°C in hermetically closed plastic containers to avoid dehydration. Enough headspace was left in the containers to avoid anoxic conditions. Periodically, inoculated plates were removed briefly for observation. If growth was observed, plates were counted and discarded; the rest was immediately reincubated.

**Inhibitory concentrations.** MIC for individual antimicrobials, as well as for binary mixtures of antimicrobics, was defined as the minimum required concentration of those tested that inhibited yeast growth in the three replicates. Inhibition was determined if clean plates (without any colonies) were observed and were corroborated with the spiral counting software (Casba 4, Spiral Biotech), after scanning the inoculated plate, to have a yeast count lower than 20 CFU/ml (lowest value that can be detected). MIC data were transformed to fractional inhibitory concentration (FIC), as defined by Davidson and Parish (10) for binary mixtures.

\[
\text{FIC}_A = \frac{(\text{MIC}_{\text{compound A in the presence of B}})}{(\text{MIC}_{\text{compound A alone}})}
\]

\[
\text{FIC}_B = \frac{(\text{MIC}_{\text{compound B in the presence of A}})}{(\text{MIC}_{\text{compound B alone}})}
\]

\[
\text{FIC}_{\text{Index}} = \text{FIC}_A + \text{FIC}_B
\]

**a w and pH measurements.** The a w was measured with a Decagon CX-1 (Decagon Devices, Inc., Pullman, Wash.), calibrated and operated according to the procedure described by López-Malo et al. (21). pH was determined with a Beckman pH meter (model 50, Beckman Instruments, Inc., Fullerton, Calif.). Every measurement was made in triplicate. pH and a w of the potato dextrose agar systems without inoculation, determined at the beginning and at the end of incubation, demonstrated that the desired values remained constant under incubation conditions.

**RESULTS AND DISCUSSION**

Table 2 presents the MICs of tested antimicrobials at evaluated a w and pH values. MICs exhibit not as much a w and pH dependence as was reported for weak acid antimicrobials, such as sorbic, benzoic, or propionic (1, 19, 20). Among tested antimicrobials, citral exhibited the lowest activity against Z. bailii, followed by vanillin, whereas thymol and carvacrol were the most effective. MICs varied from 1,500 ppm of citral at a w 0.95 and pH 4.5 to 50 ppm of eugenol at a w 0.99 and pH 3.5 or carvacrol at a w 0.95 and both evaluated pHs.

Antimicrobial doses required to inhibit Z. bailii growth, reported as MIC (Table 2), represent the concentrations needed to inhibit yeast growth as a result of damaged cell activity, resulting in an inability to grow and form colonies. Conner and Beuchat (4) screened 32 essential oils from plant sources for inhibitory effects on yeasts and identified some phenolic compounds, eugenol and cinnamic aldehyde, as major constituents of the volatile oils of some spices with a strong inhibitory activity. Other phenolic compounds that have a wide antimicrobial spectrum include thymol and carvacrol (2, 3, 8, 16–18). Antimicrobials tested include vanillin, thymol, carvacrol, and eugenol, classified as phenolic compounds, as well as citral, which is chemically classified as a monoterpen. It has been reported (8) that antimicrobial activity depends on the chemical structure of the compound and on its concentration (Table 2).

For vanillin-citral mixtures (Table 1), only a few combinations resulted in Z. bailii growth. When citral was used in combination with thymol, carvacrol, or eugenol, the MIC of every antimicrobial in the mixture corresponded to the lowest tested (Table 3). Our results suggest that 200 ppm citral with 100 ppm vanillin, 20 ppm thymol, 20 ppm carvacrol, or 100 ppm eugenol were sufficient to inhibit Z. bailii growth at the a w and pH levels tested.

Binary mixtures of citral with vanillin, thymol, carvacrol, or eugenol were all synergistic with a FIC Index lower than 0.5. Synergistic effects were independent of a w and pH, at least in the tested range. The relative amount of every antimicrobial in the binary mixture was dramatically reduced (Table 3) compared with its individual MIC (Table 2), which could be attributed to different modes of action (of each antimicrobial) on Z. bailii cells. Some generally accepted mechanisms of antimicrobial interaction produce synergism: sequential inhibition of a common biochemical pathway, inhibition of protective enzymes, combinations of cell wall–active agents, and use of cell wall–active agents to enhance the uptake of other antimicrobials (12).

Although our intention was not to investigate the mode of action of the compounds tested, taking into consideration our results, antimicrobial action of the compounds tested could take place via different mechanisms, resulting in the observed synergistic effect. Several published results have shown that phenolic compounds are capable of inhibiting enzyme functions (4–6, 28, 35). They are also generally regarded as membrane-active compounds because of their hydrophobicity (8, 9, 15, 32). On the other hand, for citral and other terpenes such as geraniol, it has been reported that passive entry of the compound into the plasma membrane to initiate membrane disruption and accumulation in the plasma membrane results in cell growth inhibition (27), demonstrating that terpenes have the ability to disrupt or penetrate lipid structures (30, 36) and, because of their lipophilic nature, to diffuse into and damage cell membrane structures, partitioning from an aqueous phase into membrane structures (7, 27, 33).

Maintenance of an optimal degree of fluidity of mem-
brane lipids is important for normal function of cells (14, 27, 29), so in environments that affect membrane functions, enzyme activity, or both, we observed synergistic effects, especially when other factors such as pH and aw also contribute to yeast cell stress. Synergism resulting in inhibition of cell growth or cell death can be attributed to combined membrane effects such as increased bilayer disorder and ion leakage (27), caused by citral, and the interruption of enzymatic systems involved in structural component synthesis, energy production, or both because of the phenolics (vanillin, thymol, carvacrol, and eugenol).

The use of naturally derived preservatives at effective antimicrobial concentrations is limited by their associated flavors, which can alter food taste when they exceed acceptable sensory levels. Therefore, understanding how these natural antimicrobial agents act and affect microbial growth when used in combination can lead to new applications that improve quality of foods. Many phenolics and terpenes are currently used mainly as flavoring agents. The information derived from this study indicates the potential of natural antimicrobial mixtures as a viable alternative. Furthermore, the identified synergistic combinations could be used to reduce the amounts of antimicrobials needed to inhibit growth, diminishing consumer concerns regarding preservatives while reducing the effect on food sensory attributes and balancing antimicrobial efficacy and sensory acceptability.

ACKNOWLEDGMENTS

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### REFERENCES


### TABLE 3. FICs of citral and phenolic antimicrobial mixtures and FIC<sub>Index</sub> against Zygosaccharomyces bailii in potato dextrose agar formulated at selected aw and pH values

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* Defined as compounds that inhibit yeast growth for at least 12 days at 27°C.


