

**INHIBITORY EFFECTS OF FLAVOR COMPOUNDS ON
STAPHYLOCOCCUS AUREUS WRRC B124¹**

ABSTRACT

Acetanisole (4'-methoxyacetophenone) (AC), benzaldehyde (BE), cinnamaldehyde (CI), diacetyl (DI), phenylpropionaldehyde (PH), and pyruvaldehyde (PY) were tested against growth of S. aureus WRRC B124 in brain heart infusion broth. Activity was assessed in the presence and absence of oxygen at 12, 19 and 37C, and in combination with mild (20 min at 50 or 60C) heat treatments. The carbonyl compounds limited S. aureus growth at minimal inhibitory concentrations (MIC) of ≤ 0.5 –8.0 mM. After 4 h at 37C, a 2 to 3-log₁₀ CFU/ml population reduction was observed with cultures containing 8.0 mM PH, PY, DI or CI. Activity was O₂-tension independent, with CI (0.5 mM), DI(2.0 mM) and PY(2.0 mM) being most active. The MIC for CI was temperature independent, while PY was most effective at 19C, and PH and DI at 12C. Mild heat treatment of carbonyl-supplemented samples reduced previously observed MICs. At 60C, for example, the MICs for AC and BE, 4.0 and 8.0 mM respectively, were both reduced to ≤ 0.5 mM. The decimal reduction times for S. aureus exposed to both UV-light and 8 mM flavor compounds were 3.3 and 4.3 s for CI and DI, respectively. However, the other compounds were not as effective in the presence of UV since the decimal reduction times ranged from 7.7 to 9.0 s. The carbonyl compounds tested were effective antistaphylococcal agents and their use in combination with thermal processing may serve as a new approach to control S. aureus growth and other gram-positive foodborne pathogens.

INTRODUCTION

Naturally occurring inhibitors of foodborne pathogens are being sought by the food industry, and a variety of approved carbonyl-containing food flavors have recently been shown to have antitoxigenic activity. Little work seems to be directed toward *Staphylococcus aureus* which continues to be a major cause of foodborne disease in the United States. The organism is a gram-positive, facultative coccus that is well-adapted to warm-blooded animals. Various strains of this organism are etiologic agents of several disease syndromes, including conjunctivitis, impetigo, mastitis, pneumonia, toxic shock syndrome, wound infections and gastroenterocolitis (Baird-Parker 1990). Staphylococci are found in commercial food products that are of animal origin or those that are handled by humans (Baird-Parker 1990; Bean and Griffin 1990; Genigeorgis 1989).

Staphylococcal gastroenteritis is caused by the ingestion of food that contains preformed *S. aureus* enterotoxin, or in some instances, by growth of the organism in the intestinal tract (Baird-Parker 1990; Genigeorgis 1989). Up to 16% of all foodborne gastroenteritis outbreaks and cases reported from 1973-1987 implicated *S. aureus* (Bean and Griffin 1990). Improper holding temperatures and poor personal hygiene were the two major underlying causes, with pork products the leading vehicle of transmission (Bean and Griffin 1990). Estimated U.S. foodservice and food processing costs attributed to staphylococcal food poisoning incidences during 1978 to 1982 were \$1.9 and \$1.4 million, respectively (Todd 1989).

Although several naturally occurring plant components have demonstrated to have an antimicrobial nature (Bowles and Miller 1993a,b; Chung *et al.* 1990; Grzybowski *et al.* 1990, 1991), and in some instances to act as antistaphylococcal agents (Chung *et al.* 1990, 1993; Kawano *et al.* 1992; Knobloch *et al.* 1989), little has been done to exploit their inhibitory properties to improve the microbial safety of foods or to enhance product shelf-life. Broad-spectrum antibacterial activity against 25 staphylococcal strains has been reported for a variety of Japanese green teas (bancha, sencha, gyokuro, hojicha and matcha) (Kawano *et al.* 1992), 0.1% *Thymus vulgaris* or 0.5% *Salvia officinalis* extracts (Grzybowski *et al.* 1991; 1990), Matsuke mushrooms (Kawano *et al.* 1992), and for certain Mediterranean plant extracts (*Inula dysenterical*, *Echinochloa crus-galli*, and *Artemisia caerulescens*) (March *et al.* 1991).

In this study, four aromatic (phenylpropionaldehyde, acetanisole, cinnamaldehyde, benzaldehyde) and two aliphatic (pyruvaldehyde, 2,3-butanedione) flavor carbonyl compounds were tested for inhibitory activity (antistaphylococcal) against *S. aureus* WRRRC B124. The efficacy of these compounds as antistaphylococcal food additives to improve the microbial safety

of some foods was defined by assessing: (1) bacteriostatic/bacteriocidal activities at various incubation temperatures; (2) O₂-tension effects; and (3) bacteriostatic activity when used in combination with mild-heat treatments or ultraviolet-light.

MATERIALS AND METHODS

Bacterial Strain

Coagulase-positive *Staphylococcus aureus* WRRRC B124 was obtained from the USDA/ARS Midwest Area Regional Research Center (Peoria, IL). Species identification was confirmed on the basis of catalase activity, coagulase or thermonuclease production, and anaerobic utilization of glucose and mannitol (Bennett 1984). Although isolated from a foodborne intoxication incident, *S. aureus* WRRRC B124 was found to be enterotoxin A, B, C and D-negative using a SET-RPLA Staphylococcal Enterotoxin Test Kit (OXOID, Hampshire, England). Brain heart infusion broth (BHI; pH 7.4 ± 0.2) or agar (BHIA; pH 7.4 ± 0.2) (Difco Laboratories, Detroit, MI) was the test medium used throughout the study. Twenty-four h prior to all analyses, 0.1 ml of a *S. aureus* WRRRC B124 stock culture was transferred to 9.9-ml BHI broth and incubated aerobically without shaking for 24 ± 2 h at 37C. One-tenth ml of the 24 ± 2 h culture was diluted in 9.9 ml of 0.1% peptone-H₂O (pH 7.2 ± 0.2) for antimicrobial testing. An average inoculum concentration of 4.0-log₁₀ CFU/ml was used in all analyses.

Test Compounds

Phenylpropionaldehyde (PH) (95%), pyruvaldehyde (PY) (40%), diacetyl (2,3-butanedione) (DI) (99%), cinnamaldehyde (CI) (99%), acetanisole (AC) (4'-methoxyacetophenone) (99%) and benzaldehyde (BE) (99%) were purchased from either Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO) chemical companies. Stock solutions were prepared in 33%-reagent grade ethanol and were stored as suggested by the supplier.

Antimicrobial Test

Determination of Inhibitory Activity. A quantitative broth dilution method was used to determine the effect of various flavor compounds on *S. aureus* WRRRC B124 (Bowles and Miller 1993a,b). Test compounds were

serially diluted (0, 63, 31, 16, 8, 4, 2, 1 and 0.5 mM) in replicate sets of 16 × 150 mm test tubes that contained 1.0 ml BHI broth (final pH: 6.8 to 7.4), and inoculated with 0.1 ml of a 24-h *S. aureus* WRRC B124 culture that was diluted in 0.1% peptone-H₂O. The tubes were incubated aerobically or anaerobically, without shaking, 24 ± 2 h at 12, 19 or 37C, and then examined visually for turbidity. Treatments at 12C were incubated an additional 12 to 24 h beyond the initial incubation period or until unsupplemented controls were visually turbid. Inhibition was based on 24-h *S. aureus* WRRC B124 growth in unsupplemented BHI broth, and growth response of the organism in a series of EtOH-supplemented BHI broth tubes (0.016-16.5%). The ethanol concentrations tested, represented the final EtOH content of flavor-supplemented treatments. The BBL GasPak Anaerobic Systems (Becton Dickinson Microbiological Systems, Cockeysville, MD) was used for anaerobic incubation.

Inhibition by Combinations of Flavor Compounds and Mild Heat Treatment. Combinations of mild heat treatments and carbonyl compounds were tested to determine whether the previously observed antistaphylococcal inhibitory concentrations of the flavor compounds were reduced. Test compounds were serially diluted and inoculated as previously described and allowed to stand at 25C until a total time of 30 min was reached to normalize pre-heat treatment exposure times among treatments. Unsupplemented, EtOH, and nonheat-treated controls were included for each of the compounds tested. The test tubes were then transferred to a 50 or 60C water bath (Exacal, Model EX-251HT, NesLab Instruments Inc., Newington, NH) for 20 min, and rapidly cooled in an ice-water bath. All tubes were subsequently incubated aerobically without shaking for 24 h at 37C, and then examined visually for turbidity.

Rate of *S. aureus* WRRC B124 Inactivation at Various Incubation Temperatures. The antistaphylococcal activity of PH, PY, DI, CI, AC and BE was defined further by determining the rate of *S. aureus* WRRC B124 population density reduction. A single concentration, at which each of the flavor compounds was previously shown to be antistaphylococcal, was tested. Bellico 150-ml trypsinizing flasks containing 25 ml BHI broth were supplemented with 200 µl of a 1.0 M stock solution (8.0 mM final conc.) (Final pH: 7.2 ± 0.2), and inoculated with 0.1 ml of a 24-h *S. aureus* WRRC B124 culture. The trypsinizing flasks were incubated at 37, 19 or 12C on a model G-26 New Brunswick shaker (120 rpm) for 24 ± 2 h. Aliquots were removed at 0, 4, 7 and 24 h, diluted in 0.1% peptone-H₂O, plated in duplicate on BHI agar using a Spiral Systems Model D plating instrument (Spiral Systems Instruments, Inc., Cincinnati, OH) and incubated aerobically at 37C for 24 h. Colonies were enumerated using a Spiral Systems Model 500A bacterial colony counter (Exotech Inc., Gathersburg, MD), then converted into bacterial counts with

Spiral Biotech CASBATM II BEN software (Spiral Systems Instruments, Inc., Bethesda, MD). The flavor compounds were rated according to their overall rate of *S. aureus* WRRC B124 inactivation, and the time required to achieve a decimal reduction (D_{90}) in population density.

Effect of Flavor Compounds on *S. aureus* WRRC B124 Ultraviolet-Light Sensitivity. The ultraviolet light sensitivity of *S. aureus* B124 was tested on BHI agar that was supplemented with 8.0 mM PH, PY, DI, CI AC or BE. A 24 ± 2 h *S. aureus* WRRC B124 culture was diluted in 0.1% peptone-H₂O and spiral plated as previously described onto flavor compound-supplemented BHI agar plates (Final pH: 7.2 ± 0.2). Unsupplemented, EtOH, and nonUV-treated controls were included for each of the compounds tested. All plates were allowed to air dry for 30 min at 25C and then exposed at 25C in the dark for 0, 5, 10, 15, or 20 s to a 253.7 nm germicidal lamp in a Forma Biological Safety Cabinet (Marietta, OH). To prevent photoreactivation, plates were wrapped in aluminum foil immediately after exposure, and incubated aerobically 24 ± 2 h at 37C. Colonies were enumerated using a Spiral Systems Model 500A bacterial colony counter, then converted into bacterial counts with Spiral Biotech CASBATM II BEN software (Bethesda, MD). Increased sensitivity was assessed by comparing the population density of flavor supplemented treatments to those of unsupplemented, EtOH, and non-UV-treated supplemented controls.

RESULTS AND DISCUSSION

The flavor carbonyl compounds tested were found to be effective inhibitors of *Staphylococcus aureus* WRRC B124 in BHI broth at 37C (Table 1). Cinnamaldehyde was the most active compound tested, with < 0.5 mM required to inhibit *S. aureus* B124 (Table 1). At 2.0 mM, pyruvaldehyde or diacetyl was the next most active, followed by 4.0 mM phenylpropionaldehyde or acetanisole, and 8.0 mM benzaldehyde. When tested under aerobic or anaerobic conditions at 37C, no difference was observed in the antistaphylococcal inhibitory concentrations of each compound (data not shown).

Ethanol was used as the diluent for the flavor compounds tested, and final EtOH concentrations of the treatments tested ranged from 16.5 to 0.016%. EtOH concentrations of 6.0 to 7.0% have been reported to be antistaphylococcal, and intense cell wall morphological changes have been reported at EtOH concentrations of 5.0 to 6.5% (1085 to 1411 mM) (Ballesteros *et al.* 1993). In this study, *S. aureus* B124 growth was inhibited in unsupplemented controls that contained $\geq 8.25\%$ EtOH; however, the use of ethanol as a common diluent may have increased *S. aureus* WRRC B124 sensitivity to the flavor compounds tested.

TABLE 1.
EFFECT OF INCUBATION TEMPERATURE ON THE ANTISTAPHYLOCOCCAL
ACTIVITY OF VARIOUS FLAVOR COMPOUNDS

Test Compound	Inhibitory Concentration (mM) in BHI broth		
	Incubation Temperature (°C)		
	37	19	12
Phenylpropionaldehyde	4.0	4.0	1.0
Pyruvaldehyde	2.0	<0.5	2.0
Diacetyl	2.0	2.0	1.0
Cinnamaldehyde	<0.5	<0.5	<0.5
Acetanisoole	4.0	8.0	1.0
Benzaldehyde	8.0	2.0	2.0

Incubation temperature modulated the antistaphylococcal MIC of the flavor compounds (Table 1). At 12°C, the MIC of phenylpropionaldehyde, diacetyl or acetanisoole was reduced to 1.0 mM. When tested at 12 or 19°C, the MIC of benzaldehyde was reduced to 2.0 mM, while inhibitory activity of cinnamaldehyde was independent of the range of incubation temperatures tested. Pyruvaldehyde, a 3-carbon aliphatic dicarbonyl, was most active at 19°C, with < 0.5 mM required to inhibit *S. aureus* B124. In general, the aromatic carbonyls were most effective at either 19 or 12°C. While a number of carbonyl-containing compounds inhibit bacteria, information about the temperature-dependent antibacterial effectiveness of aldehydes and ketones is lacking. The observed temperature dependence of inhibition may be attributed to: (1) the physiological state of the organism; (2) lipid or other cell wall component modifications; or (3) altered flavor compound reactivity. Temperature has been reported to be a significant factor in acid-induced *S. aureus* 196E cell injury (Smith *et al.* 1984), and *S. aureus* growth and enterotoxin production in foods (Gomez-Lucia *et al.* 1990; Halpin-Dohnalek and Marth 1989). The staphylococcal growth-limiting properties of a fermented Nigerian spice-cereal drink "kunun zaki" were enhanced at 25 and 37°C (Onuorah *et al.* 1987). Although distinct flavor compound-specific changes in antistaphylococcal activity were observed in this study, structural/activity relationships were not apparent.

The antistaphylococcal MICs of the flavor compounds were reduced when tested in combinations with mild heat treatments (Table 2). A 20-min heat

treatment at 50C reduced the initially observed 4.0 mM MIC of both pyruvaldehyde and acetanisole, to 1.0 and ≤ 0.5 mM, respectively. Although, the MIC of cinnamaldehyde was consistent at each of the temperature combinations tested, antistaphylococcal activity of benzaldehyde was significantly increased when tested in combination with a 20-min heat treatment at 60C; 94% less benzaldehyde (< 0.5 mM) was inhibitory. While less dramatic MIC modulations occurred at 50C (20 min) for phenylpropionaldehyde or pyruvaldehyde; the antistaphylococcal MIC of both compounds was reduced to 1.0 mM.

TABLE 2.
INHIBITION BY COMBINATIONS OF FLAVOR COMPOUNDS AND MILD HEAT

Test Compound	Inhibitory Concentration (mM) in BHI broth at 37 C		
	25 C/20 min	50 C/20 min	60 C/20 min
Phenylpropionaldehyde	4.0	2.0	1.0
Pyruvaldehyde	2.0	1.0	1.0
Diacetyl	2.0	2.0	1.0
Cinnamaldehyde	< 0.5	< 0.5	< 0.5
Acetanisole	4.0	<0.5	< 0.5
Benzaldehyde	8.0	8.0	< 0.5

The rate of *S. aureus* B124 inactivation at various incubation temperatures is shown in Table 3. A decimal reduction in *S. aureus* population density was observed for cells held 6.9, 7.9 and 8.9 min at 12C, respectively, in the presence of 8.0 mM phenylpropionaldehyde, cinnamaldehyde, and diacetyl. At 19C, a decimal reduction was observed after 7.7 min in cinnamaldehyde-supplemented treatments, and after 7.6 min at 37C in those containing benzaldehyde. The respective inactivation rates of the flavor compounds were consistent with their individual MICs at various incubation temperatures. In general, *S. aureus* decline rate was fastest at 12 and 37C, with average decimal reduction times of 12 and 13 min, respectively. Of the carbonyls tested, cinnamaldehyde was most effective in reducing *S. aureus* numbers.

The UV-light susceptibility of *S. aureus* WRRRC B124 was increased when cells were irradiated on the surface of BHI agar supplemented with 8.0 mM of the various flavor compounds (Fig. 1). After a 20-s exposure, population

TABLE 3.
RATE OF *STAPHYLOCOCCUS AUREUS* B124 INACTIVATION AT VARIOUS
INCUBATION TEMPERATURES

Test Compound (8.0 mM)	Decimal Reduction Time ^a (min)			Population
	12 C	19 C	37 C	Variance ^b
Phenylpropionaldehyde	6.9	59.0	10.6	563.41
Pyruvaldehyde	17.9	10.3	19.2	15.41
Diacetyl	8.9	9.6	17.4	14.84
Cinnamaldehyde	7.9	7.7	10.7	1.88
Acetanisole	16.7	34.3	12.3	90.35
Benzaldehyde	12.0	10.8	7.6	2.99
Population Variance	18.14	356.81	16.42	

^a Average of 4 replicate treatments.

$$^b \sum (V_i - \text{avg})^2 / n$$

densities of unsupplemented cultures were unchanged, and 2.0- \log_{10} CFU/ml reduction observed for ethanol controls. According to the manufacturer of the biological hood used for UV-exposure in this study (Forma Scientific, Inc.), a 90% kill of *S. aureus* is achieved after 32.4-s (2600 $\mu\text{w-s/cm}^2$) exposure to 253.7 nm ultraviolet light. The decimal reduction time of *S. aureus* WRRRC B124 UV-inactivation at 25C was 3.3, 4.3, 7.7, 7.8, 8.1, and 9.0 s for cinnamaldehyde, diacetyl, acetanisole, benzaldehyde, pyruvaldehyde, and phenylpropionaldehyde, respectively. The rate of *S. aureus* WRRRC B124 population decline in phenylpropionaldehyde, pyruvaldehyde, acetanisole or benzaldehyde supplemented treatments was similar those of the 0.26% EtOH controls; however, population densities of diacetyl- or cinnamaldehyde-supplemented treatments were significantly less than those of unsupplemented or EtOH controls.

Although *S. aureus* growth and enterotoxin production may be controlled by low storage temperatures (5C), reduced water activity, competitive microflora, various acidulents, salts and other chemical agents, the organism is still a significant foodborne pathogen (Baird-Parker 1990; Bean and Griffin 1990; Palumbo and Smith 1984). The observed antistaphylococcal activity of the flavor compounds tested in this study are comparable to, and in some instances superior to that reported for other antibacterial food additives. *S. aureus*, *Escherichia coli* and *Pseudomonas fluorescens* were inhibited by 1.0 to 2.0 mM diacetyl in plate count agar at pH 6.0, with 30 mM of the aromatic carbonyls, 1,2-cyclohexanedione and phenylglyoxal required to inhibit nonlactic gram-positive bacteria (Jay *et al.* 1983). In addition, the naturally occurring

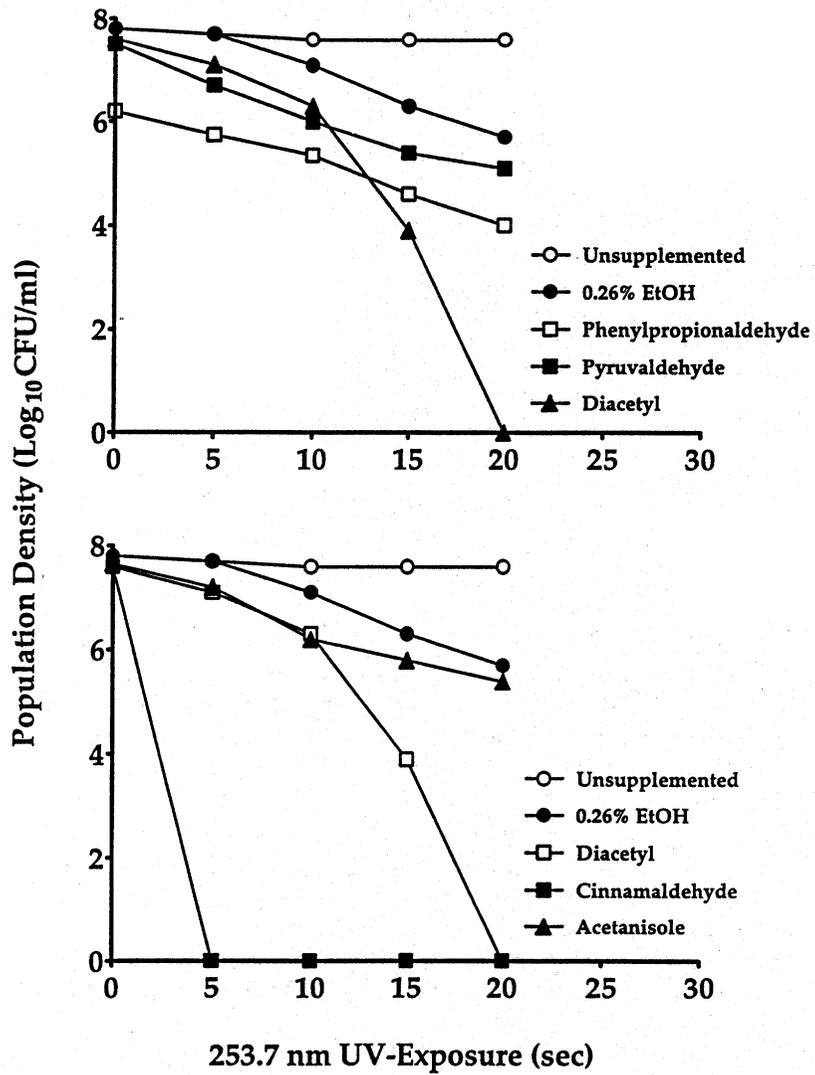


FIG. 1. MODULATED *STAPHYLOCOCCUS AUREUS* WRRc B124 ULTRAVIOLET LIGHT SENSITIVITY ON BRAIN HEART INFUSION AGAR SUPPLEMENTED WITH VARIOUS FLAVOR COMPOUNDS (8.0 mM)

milk volatile compounds, acetaldehyde, isobutyraldehyde and propionaldehyde were demonstrated by Bhalla *et al.* (1985) to be antistaphylococcal respectively, at 227, 138 and 172 mM.

CONCLUSIONS

Several flavor compounds approved as food additives were found to effectively control *Staphylococcus aureus* B124 growth. Relatively mild heat treatments enhance the antistaphylococcal activity of the compounds and *S. aureus* B124 was more sensitive to ultraviolet light in the presence of the carbonyl tested. As such, these compounds can be employed to control *S. aureus* growth and perhaps other gram-positive foodborne pathogens in foods, and thus improve their overall stability and microbial safety.

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