

Antimicrobial Action of Some Citrus Fruit Oils on Selected Food-Borne Bacteria

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The antimicrobial properties of essential oils, terpineol, and orange oil, in particular, varied according to the type of bacteria tested. Terpineol and other terpeneless fractions of citrus oils appeared to have greater inhibitory effect on food-borne bacteria than the other citrus oils or derivatives. Gram-positive bacteria were, in general, more sensitive to essential oils than gram-negative bacteria. Terpineol extended the shelf life of commercially pasteurized skim milk, low-fat milk, and whole milk for more than 56 days at 4 C. Orange oil extended the shelf life of skim milk and low-fat milk for the same period.

The use of antimicrobial agents to kill bacteria in processed food or at least to inhibit growth of bacteria could, in conjunction with the prevention of contamination and adequate detection methods, help control the wholesomeness of food.

As early as 1924, Schöbl (21) and Schöbl and Kusama (22) compared the disinfecting power of chaulmoogra oil with that of vegetable and animal oils. Maruzzella and co-workers (8-13) extensively studied the potential of essential oils as antimicrobial agents in perfumes and cosmetics or against wood pathogens.

Piacentini (18) reported that essential oils of bergamot, orange, and lemon are more antiseptic than phenol. Hahn and Appleman (4) studied the role of orange oil in the microbiological quality of frozen orange concentrate, whereas Murdock and Allen (14) showed that the addition of orange oil or *d*-limonene increases the preservative properties of sodium benzoate. Marth (7) reviewed the literature on the potential use of essential oils in the inhibition of *Salmonella*, whereas Nagy and Tengerdy (15, 16) and Oh et al. (17) studied their effect on rumen bacteria of sheep and deer. Pirie and Clayson (19) found that liquid seasonings containing emulsified essential oils have little or no antibacterial action, because partitioning between the oil and aqueous phases reduces the concentration of the antiseptic constituent of the essential oil.

Essential oils are used for flavoring in food and are generally accepted by the Food and Drug Administrations as additives in certain type of foods.

Gunther (3) reported that, because of the complexity of essential oils, no general statement can

be made as to their antimicrobial properties. Furthermore, Subba et al. (24) and Dupaigne (1) showed that the degree of inhibition of bacterial growth by citrus oils varies considerably with the bacteria tested.

According to Ingram et al. (5), an ideal antimicrobial agent in food should be effective, not only against food-poisoning and food-infecting species, but also against spoilage organisms.

This paper reports the antimicrobial properties of some citrus oils and derivatives on a number of common food-borne bacteria. Furthermore, the use of citrus oils in fluid milk products to increase their shelf life also will be discussed.

MATERIALS AND METHODS

Source of microorganisms. Most of our cultures were provided by G. Banwart, Market Quality Research Division, Agricultural Research Service, Beltsville, Md.

Stock cultures were maintained on Trypticase soy agar slants and transferred twice in Trypticase soy broth before use as inoculum. Incubation was at 35 to 37 C for 24 hr for all bacteria, except for food spoilage bacteria which were incubated at 20 C.

Source of essential oils and derivatives. *d*-Limonene (98%), cold pressed grapefruit, orange, lemon, and mandarin oils, and lemon, orange, and lime terpeneless oils were obtained from Green & Green, Inc., Houston, Tex. *d*-Limonene and geraniol were obtained from Eastman Organic Chemicals, Rochester, N.Y. Orange oil (USP; Citrus Valley Brand) was obtained from Dodge & Olcott, Inc., New York, N.Y., and terpineol (lilialcin) was obtained from Fisher Scientific Co., Pittsburgh, Pa.

Test for antimicrobial properties. All concentrations of essential oils refer to the final concentration in the

mixture of oil, nutrient broth, and bacterial suspension.

Essential oils were dispersed aseptically in sterile nutrient broth by sonic treatment for 10 min in a Sonifier Cell Disrupter (Branson Instruments, Inc., Melville, N.Y.) and then dispensed in 9.9-ml portions into screw-cap tubes. A 0.1-ml amount of a 24-hr culture was added to tubes containing the oil-nutrient broth mixture and to tubes with nutrient broth only; all were mixed with a Vortex mixer. This gave an initial inoculum of approximately 10^7 /ml. The tubes were incubated either at 35 to 37 C or at 20 C, depending on the type of bacteria tested, and enumerated on plate count agar (PCA), unless specified otherwise, after 24 and 48 hr of incubation. Plates were incubated for 24 to 48 hr at 37 C or for 48 to 72 hr at 20 C. The oil-nutrient broth mixture was also tested for sterility during the test period.

Calculation of antimicrobial property of essential oils. The effect of essential oils on growth was calculated as a percentage of growth inhibition by comparison between growth of bacteria in nutrient broth and growth of the same bacteria in nutrient broth and oil mixture. When the growth inhibition was 100%, we calculated a percentage of reduction of initial load by comparison between the original inoculum and the count in the nutrient broth and oil mixture.

RESULTS AND DISCUSSION

Inhibition in solid medium versus liquid medium.

Antimicrobial properties of essential oils have been studied almost exclusively by the filter paper-disc method (8-13, 23, 25). Recently, Subba et al. (24) measured the bacteriostatic properties of citrus oils by incorporation of the oils in a solid agar medium and calculating the inhibition by comparison with the growth of bacteria in the same medium without added citrus

oil. We incorporated 1,000 μ liters of orange oil per liter in the plating agar and inoculated the agar with cultures of *Salmonella oranienburg*, *S. montevideo*, *S. typhimurium*, *S. heidelberg*, and *S. senftenberg* (775W). Calculation of percentage of inhibition of growth showed inhibition in the 40 to 50% range. When the orange oil was incorporated in nutrient broth and the mixture was inoculated with the same cultures as above and incubated for 24 hr at 37 C, inhibition of growth as measured by PCA was over 90%.

Concentrations of essential oil. Essential oils impart a flavor to food, the intensity of which increases with increases in concentration. Taste panel tests showed that a maximal concentration of 1,000 μ liters of orange oil per liter in skim milk was acceptable. Data not shown here indicated that increasing the concentration of orange oil from 1,000 to 10,000 μ liters per liter increased the growth inhibition effect of orange oil against *S. senftenberg* (775W) and *Escherichia coli* but not against *Staphylococcus aureus* and *Pseudomonas* species (no. 18) isolated from refrigerated pasteurized milk. Decreasing the concentration of orange oil from 1,000 to 10 μ liters per liter reduced its growth inhibition effect against these four bacteria. Terpeneol at 1,000 μ liters per liter completely inhibited the growth of all four bacteria and, with the exception of *Pseudomonas* species (no. 18), totally reduced the initial inoculum. Increasing the concentration of terpeneol to 10,000 μ liters per liter did not further affect the inhibition of growth or the reduction of initial inoculum. On the other hand, reduction of the concentration of terpeneol to 600 μ liters per liter

TABLE 1. Antimicrobial effect of 1,000 μ liters of citrus oils and derivatives per liter on four selected bacteria in nutrient broth^a

Citrus oils and derivatives	<i>Salmonella senftenberg</i> (775W)		<i>Escherichia coli</i>		<i>Staphylococcus aureus</i>		<i>Pseudomonas</i> species (no. 18)	
	Per cent inhibition	Per cent reduction	Per cent inhibition	Per cent reduction	Per cent inhibition	Per cent reduction	Per cent inhibition	Per cent reduction
Orange oil.....	93	0	93	0	100	87	87	0
Lemon oil.....	98	0	98	0	100	67	90	0
Grapefruit oil.....	65	0	58	0	100	82	84	0
Mandarin oil.....	98	0	96	0	100	98	87	0
Lime terpeneless.....	100	100	100	100	100	100	90	0
Orange terpeneless.....	100	86	100	97	100	100	92	0
Lemon terpeneless.....	100	78	100	97	100	100	93	0
<i>d</i> -limonene (Eastman) ^b	100	50	99	0	100	95	88	0
<i>d</i> -limonene (G & G) ^b	97	0	95	0	100	92	86	0
Terpeneol.....	100	100	100	100	100	100	100	0
Geraniol.....	100	100	100	100	100	100	99	0

^a Percent reduction of initial bacteria load was calculated when per cent growth inhibition was 100.

^b *d*-Limonene (Eastman) was purchased more than 4 years ago. *d*-Limonene (G & G) was obtained recently.

reduced drastically the growth inhibition of the four bacteria. A concentration of 1,000 μ liters of essential oils per 1 liter of solution was used in all our subsequent experiments.

Size of inoculum. Variations in the initial inoculum of from 10^4 to 10^8 bacteria of *S. senftenberg* (775W), *E. coli*, and *S. aureus* did not change the percentage inhibition of these cultures by 1,000 μ liters of orange oil per liter. With *Pseudomonas* species (no. 18), the inhibition of growth by orange oil increased from 30 to 90% when the inoculum was increased from 10^4 to 10^8 . The possibility of an optimal concentration of bacteria for a given amount of oil could explain the increase in growth inhibition. An initial inoculum of approximately 10^7 /ml was selected for the remainder of the study.

Effect of various essential oils and derivatives. Growth of *S. aureus* was completely inhibited by all of the citrus oils and derivatives tested, with the percentage of reduction of initial inoculum ranging from 100% for the terpenless citrus oils and terpenless derivatives to a low of 67% for lemon oil (Table 1). On the other hand, only terpineol reduced the initial inoculum of *Pseudomonas* species (no. 18). Inhibition of growth of *Pseudomonas* species (no. 18) ranged from 87% for orange oil to 100% for terpineol. The terpenless fractions of orange, lemon, and lime considerably reduced (86 to 100%) the initial inoculum of *S. senftenberg* (775W), *E. coli*, and *S. aureus*. The *d*-limonene that had been stored for more than 4 years showed greater activity against *S. senftenberg*; this confirms Zukerman's report (26) that oxidized *d*-limonene is more inhibitory than the freshly distilled product. However, in our study, artificially oxidized orange oil had no more inhibitory effect on bacteria than fresh orange oil.

Antimicrobial effects of orange oil and terpineol on pure cultures of bacteria: Salmonella group. Inhibition of growth by 1,000 μ liters of orange oil per liter ranged from 84% for *S. enteritidis* to 99% for *S. gallinarium* and *S. norwich*. Incubation for 48 hr showed no increase in inhibition over the 24-hr results. Orange oil did not reduce the initial bacterial load in any case. On the other hand, terpineol completely inhibited the growth of 24 serotypes of *Salmonella* tested and also totally reduced the initial inoculum.

Enterobacteriaceae other than Salmonella. Orange oil was bacteriostatic for all other tested *Enterobacteriaceae*. Inhibition of growth ranged from 88% against *Aerobacter aerogenes* to 100% against *Alcaligenes faecalis* and against 2 strains of the 12 *E. coli* tested. Orange oil also caused a reduction of the initial inoculum of those two strains of *E. coli* and completely killed the initial

inoculum of *A. faecalis*. Inhibition of growth in the *Proteus* group was approximately 95%.

As with the *Salmonella* group, terpineol completely inhibited the growth of all tested bacteria and totally reduced their initial inoculum with the exception of *Serratia marcescens*, against which the effect was 89% inhibition of growth.

Pseudomonas and gram-negative food spoilage organisms. Orange oil in all cases inhibited the growth of bacteria (10 to 100%); the initial inoculum of one strain of *P. fluorescens* was completely killed, whereas a 75% reduction occurred in the initial inoculum of a *P. aureginosa*. Terpineol inhibited the growth of bacteria in various degrees (94 to 100%). Terpineol was less effective in reducing the initial bacterial load of *Pseudomonas* species and *Achromobacter*, as well as that of most of the gram-negative spoilage psychrophilic organisms isolated from milk and dairy products, than against the *Enterobacteriaceae*.

Gram-positive bacteria. Orange oil completely inhibited the growth of various strains of *S. aureus*. The reduction of initial inoculum was more variable and ranged from 0 to 100%. Orange reduced the initial inoculum of *Bacillus subtilis* by 100% and *B. cereus* by 98%. Terpineol completely killed the initial inoculum of all tested gram-positive bacteria.

Use of essential oils to extend the shelf life of milk. Fruit-flavored milk products are not new. Gudnason et al. (2) indicated that their acceptability is good. Some, like Gudnason et al. (2), have used syrup and commercial fruit juice concentrate for their fruit-flavored milk; others like Kosikowski (6) have used fruits or fruit concentrate as flavoring for buttermilk.

We established earlier in this paper that essential oils such as orange oil or terpineol dispersed in a liquid medium inhibit the growth and even, in some cases, reduce the initial bacterial load of a variety of food-borne pathogens and spoilage organisms. The possibility of a new type of fluid milk product flavored with essential oil and with extended shelf life was tested.

Commercially pasteurized fluid milk products, purchased from retail outlets in the Beltsville, Md. area, were homogenized with terpineol to a final concentration of 1,000 μ liters per liter and stored in sterile containers for 52 days at 4 C. At regular intervals, the bacterial population was estimated by plating on PCA and incubating at 20 C for 72 hr. The effect of terpineol on fluid milk products stored at 4 C varied with the fat content of the product tested. For skim milk, the difference in viable count between milk with and without terpineol after 42 days of storage was 7 log cycles; for low butterfat milk (2% B.F.), the difference was 4 log cycles; for whole milk (3.5%

B.F.), the difference was 3 log cycles; finally, there were no differences in bacterial estimate for half cream-half milk (12% B.F.) and for chocolate milk.

When orange oil was mixed with various milk products to a final level of 1,000 μ liters per liter and then incubated at 4 C for more than 52 days, the differences in counts between the control and the samples were of a lower magnitude than those obtained with terpineol. For instance, no difference was obtained in counts for whole milk (3.5% B.F.). One or two log differences were obtained for skim milk or 2% B.F. fluid milk. As with terpineol, no difference was present in chocolate milk.

Taste panel evaluation of the milk products after 16 days of storage at 4 C indicated that the milk products with orange oil were favored over those without the oil. Whole milk and chocolate milk, with or without orange oil, curdled on the 28th day and were removed from the storage test. All the other milk products tested after 28 days were categorized as "spoiled" when stored without orange oil, whereas those with orange oil were judged "acceptable." After 60 days at 4 C, skim milk mixed with orange oil did not present characteristic signs of spoilage. After 73 days of storage, a slight odor described as "stale" or "unclean" was detected.

Terpineol, although more effective than orange oil in controlling bacterial population of commercially pasteurized milk, gave the milk a strong "medicinal" flavor which was not acceptable to our taste panel.

A large number of essential oils and derivatives were dispersed in skim milk and presented to our panel, who selected only orange, lemon, and grapefruit oils as "acceptable." Comments indicated that the traditional "chalky" taste of skim milk was improved by the addition of these oils.

The mechanism of the antimicrobial effect of essential oils is not known, although lipid solubility and surface activity of the oils at the surface of the bacteria have been implicated (15, 20).

The addition of citrus oils to milk, in addition to improving the shelf life, offers the possibility of introducing a wide range of new flavors to milk products.

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