Journal of Food Protection, Vol. 64, No. 2, 2001, Pages 189–194

Novel Quantitative Assays for Estimating the Antimicrobial Activity of Fresh Garlic Juice†‡

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MS 00-110: Received 3 April 2000/Accepted 14 September 2000

ABSTRACT

Novel agar diffusion and broth dilution assays were developed for quantitatively estimating the antimicrobial activity of fresh garlic juice. Bacteria found to be inhibited by garlic juice in agar diffusion assay included two gram-positive and five gram-negative species. *Leuconostoc mesenteroides* was not inhibited. *Escherichia coli* B-103 (HB101, with pJH101, ampicillin resistant, 100 μg ml^{-1}) was inhibited and chosen as the standard culture for quantitative assays. The agar diffusion assay was based on the slope ratio method, where the slope of dose response for garlic juice was divided by the slope of dose response for methylmethane thiosulfonate ($MMTSO_2$). Juice from fresh garlic varied in activity between 1.76 and 2.31 μg of $MMTSO_2$ per mg of garlic juice. The activity of juice decreased during 11 months of storage of garlic cloves at 5°C from 2.31 to less than 0.1 μg of $MMTSO_2$ per mg of juice. The broth dilution assay also used the *E. coli* B-103 culture, which permitted selective enumeration of this bacterium when 100 μg ml^{-1} of ampicillin was incorporated into the enumerating agar. Selective enumeration was essential since the garlic juice was not sterile and, thus, contained natural flora. Growth of *E. coli* was unaffected by 0.1%, delayed by 0.25%, and completely inhibited at 0.5 and 2% garlic juice in broth during 24 h of incubation at 37°C. The minimum inhibition concentration of garlic juice by broth dilution assay was, thus, estimated to be 0.5%, which is equivalent to 3.46 μg of $MMTSO_2$ per mg of garlic juice by the agar diffusion assay.

Many studies have been performed concerning the antimicrobial activity of plant extracts and their essential oils. Measuring, testing, and evaluating the antimicrobial activity of plant extracts and oils can be difficult because of their volatility, insolubility in water, and presence of multiple inhibitory compounds. The test method, growth medium, test microorganism, and plant extract, or its essential oil, are important factors when testing the antimicrobial activity *(14)*. Each factor is subject to a great deal of variation *(9)*. However, to evaluate the reported data, it is necessary to consider the methods used to test for antimicrobial activity.

Garlic (*Allium sativum*) has been extensively studied for its antimicrobial properties. The principal antimicrobial compound of garlic has been reported to be allicin (diallyl thiosulfinate) *(4, 5)*, which is one of several sulfur compounds present. It is known that the major antimicrobial compounds of garlic are not present in intact garlic cloves *(4)*. Stoll and Seebeck *(24)* reported that intact garlic cloves contain 0.24% *S*-allylcysteine *S*-oxide (alliin) by fresh weight, and an enzyme, allinase, converts alliin into allicin. Ziegler and Sticher *(29)* reported that alliin content in five different fresh garlic samples varied between 0.09 and 1.15% as fresh weight. Freeman and Whenham *(12)* showed that the cysteine sulfoxide fraction of garlic consists of 85% alliin, along with 2% *S*-propyl cysteine sulfoxide and 13% *S*-methyl cysteine sulfoxide. Action of allinase on the mixture of these sulfoxides forms allyl methanethiosulfinate, methyl methanethiosulfinate, and other mixed or symmetrical thiosulfinates found in garlic extracts, in addition to allicin. On injury of garlic tissue cells, allinase (cysteine sulfoxide lyase) comes in contact with alliin and forms allicin *(16)*. Allicin is a very unstable compound that immediately degrades into the strong-smelling constituents of garlic oil; it represents about 0.4% (wt/wt) fresh garlic *(13)*. Brodnitz et al. *(3)* reported that allicin underwent complete decomposition at 20°C after 20 h, giving diallyl disulfide (66%), diallyl trisulfide (9%), diallyl sulfide (14%), and SO_2. Yu et al. *(28)* characterized the effects of temperature and pH on the stability and formation of volatile sulfur compounds in garlic.

Willis *(27)* reported that 5×10^{-4} M allicin inhibited several sulfhydryl metabolic enzymes, but few nonsulfhydryl enzymes and some enzymes, such as triosephosphate dehydrogenases, were inhibited by 5×10^{-5} M allicin. Willis *(27)* also observed that inhibition of sulfhydryl enzymes was associated with the presence of the -SO-S- grouping (not -SO-, -S-S-, or -S- groups). Barone and Tansey *(2)* reported that allicin, the major anticandidal component of garlic extract, disrupted microbial cell metabolism primarily by inactivation of -SH proteins by oxidation of thiols to

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‡ Paper number FSR005 of the Journal Series of the Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624.

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disulfides. Furthermore, Barone and Tansey *(2)* reported that the antimicrobial activity of allicin was nonselective and hypothesized that allicin interfered with electron flow through the sulfhydryl groups within the cell wall, which results in an "uncoupling" of cell division from cell metabolism, which, in turn, could lead to an increase in mycelial form in dimorphic yeasts. Data reported by Conner *(8)* indicate that key metabolic enzymes of several foodborne yeasts are likely inhibited in this manner.

Garlic and its extracts have been shown to inhibit numerous other microorganisms, including *Bacillus cereus (23), Staphylococcus aureus (18), Lactobacillus plantarum (17), Clostridium botulinum* type B (but not type E) *(10), E. coli (15), Salmonella* Typhi, *Bacillus subtilis, Pseudomonas pyocyaneus (1), Candida albicans (26),* and a variety of yeastlike fungi *(19).*

Garlic and numerous spices have been proposed as natural food preservatives. For such purposes, it is desirable to have a quantitative estimate of the antimicrobial activity of the plant material or extract. Rees et al. *(22)* found quantitative ranges of MIC of garlic for various bacteria and yeasts of 0.8 to 40 mg ml^{-1} of garlic.

The objective of this study was to develop novel agar diffusion and broth dilution assays for the quantitative estimation of the antimicrobial activity of fresh garlic extracts. The novelties introduced in the study involved validation of methylmethane thiosulfonate ($MMTSO_2$) as a standard compound in the agar diffusion assay and the use of a genetically marked culture of *E. coli* (ampicillin resistant) to facilitate selective enumeration of this bacterium in broths containing nonsterile extracts of garlic. The use of $MMTSO_2$, a stable, readily obtainable compound, in the slope ratio assay establishes a universally applicable standard for comparison of garlic samples with varying activity. The slope ratio assay quantifies activity in units of μg of $MMTSO_2$ per mg of garlic juice, which avoids the ambiguity of units based solely on volume or weight for garlic samples.

MATERIALS AND METHODS

Garlic. Samples of freshly peeled garlic were provided by Christopher Ranch (Gilroy, Calif.). The garlic was stored at 5°C until use, and antimicrobial activity was measured throughout 11 months. In addition, garlic samples were obtained from local grocery stores for comparative purposes.

Methyl methanethiosulfinate (MMTSO) and $MMTSO_2$. MMTSO was synthesized by the oxidation of dimethyl disulfide with peracetic acid *(20).* It was purified by vacuum distillation at 2 mm Hg, and the fraction boiling at 65°C was collected *(7).* Purified MMTSO was frozen and stored at −85°C. $MMTSO_2$ was purchased from Sigma Chemical Company (St. Louis, Mo.).

Bacterial cultures and inocula. *E. coli* B-103 (HB101, with pJH101, ampicillin resistant, 100 μg ml^{-1}), *Salmonella* Typhimurium B-38 (LT2), *Enterobacter aerogenes* B-148 (ATCC 29940), *Enterobacter agglomerans* ATCC 2115, *Pseudomonas fluorescens* B-14 (MD13), *S. aureus* B-105 (KUS13), *Listeria monocytogenes* B67 (F5069, from C. Donnelly), and *Leuconostoc mesenteroides* LA-108 (ATCC 13146) were obtained from the stock cultures of our laboratory. The cultures were stored at −85°C in LB broth (Difco Laboratories, Detroit, Mich.) and contained 16% glycerol. All strains were grown on tryptic soy agar (Difco) containing 2% glucose (TSAG) and stored at 5°C. Before each use, a single colony of each test culture was transferred to 5 ml of tryptic soy broth (Difco) containing 2% glucose (TSBG) and then incubated at 37°C for 18 h. All microorganisms tested grew sufficiently to produce turbidity in TSBG or a visible lawn on TSAG plates within 24 h. The *E. coli* B-103 culture used in bioassays was grown overnight at 37°C in TSBG, harvested by centrifugation (10 min, 4,000 × *g*; Sorvall GSA rotor, Dupont, Wilmington, Del.), and washed twice in sterile 0.85% saline.

Bacterial numeration. Viable cell numbers were counted as CFU ml^{-1} by a spiral plater (Autoplate 3000, Spiral Biotech, Bethesda, Md.) on TSAG plates incubated aerobically at 37°C for 24 h.

Preparation of garlic juice. Samples of garlic cloves (50 g) were extracted by an electrical centrifuge-type juice extractor (type 4290, Braun Inc., Frankfurt, Germany) at 5°C and the juice filtered in a sterile stomacher bag (Model 7×12, Spiral Biotech). Aliquots (1 ml) of garlic juice were transferred into microfuge tubes on ice and centrifuged at 13,000 × *g* for 15 min in a microcentrifuge (Model 5415 Eppendorf, Brinkmann Instruments Co., Westbury, N.Y.). The clear, viscous supernatant was recentrifuged and held on ice before use (within 30 min).

Agar diffusion assay. A modification of the agar diffusion assay technique of Tramer and Fowler *(25)* was used. Autoclaved liquid TSAG was immediately placed in a 55°C water bath. When cooled, 30-ml aliquots of the medium were aseptically poured into square, plastic petri dishes (9 by 9 cm) and allowed to solidify and cool to room temperature. Then, the excess moisture on the surface of the plates was evaporated in an incubator (30°C) for 48 h. Nine wells were punched in the solidified medium using a sterile glass tube (6 mm diameter). The disks of agar were removed from the plate using a vacuum device. Stock solutions of MMTSO and $MMTSO_2$ (5,000 μg/ml), as well as garlic juice samples, were diluted to the desired concentrations, and 250 μl of the appropriate dilution and 250 μl of sterile liquid (45 to 55°C) TSAG were mixed. Aliquots (50 μl) of the mixture were immediately added into the wells in the agar plates, in triplicate, and allowed to solidify. A soft agar overlay consisting of 10^6 CFU/ml of the appropriate test culture and 5 ml of the liquid TSAG (as above but with 0.75% agar) was poured over the surface of the plates. Plates were then incubated for 24 h at 37°C, and the diameters of the inhibition zones were measured using a vernier caliper. Mean diameters of triplicate inhibition zones were calculated.

For quantitative assay of garlic juice antimicrobial activity by agar diffusion, the statistical slope ratio method of Finney *(11)* was used. In this method, dose-response relations for a standard compound and unknown are determined. By transforming the data to establish a linear dose-response relation, a ratio of the slopes for standard and unknown samples is computed. In the assay used in this article, this ratio is calculated as:

$$\text{slope ratio} = \frac{\text{unknown}}{\text{standard}} = \frac{\dfrac{\text{mm zone}}{\text{mg garlic}}}{\dfrac{\text{mm zone}}{\mu\text{g } MMTSO_2}} = \frac{\mu\text{g } MMTSO_2}{\text{mg garlic}} \quad (1)$$

In this way, the antimicrobial activity of the garlic sample is ex-

TABLE 1. *The antimicrobial activity of fresh garlic juice against selected bacteria*[a]

Bacteria used	Diameter of inhibition zone (mm) of organism[b]								
	0.5	1.0	2.0	2.5	5.0	6.25	7.5	8.75	10.0
S. aureus	15.70	17.73	23.58	25.28	29.58	31.42	32.45	33.63	35.63
E. agglomerans	12.16	14.61	15.28	18.60	20.80	21.53	22.43	24.50	25.16
E. aerogenes	11.26	13.15	14.83	16.13	19.42	20.56	20.88	21.63	21.80
E. coli	9.73	11.56	13.85	18.51	20.48	21.28	22.08	23.30	23.53
Salmonella Typhimurium	8.48	9.51	13.50	16.31	18.60	19.23	20.50	21.76	22.31
L. monocytogenes	8.23	10.36	12.83	15.92	20.58	21.50	22.68	24.15	24.03
P. fluorescens	—[c]	—	8.73	10.26	14.25	16.03	16.83	17.83	18.88
L. mesenteroides	—	—	—	—	—	—	—	—	—

[a] Data are the concentration of garlic juice (mg/well).
[b] Mean value of three well determinations, each from a different plate.
[c] No inhibition was observed.

pressed in terms of $MMTSO_2$ equivalents. In determining the dose-response relation reported herein, each point graphed was the mean of three replicate determinations.

The regression coefficients for the slope ratio assays were computed by SAS (SAS Institute, Cary, N.C.) using the General Linear Models method. The model we used was a separate-slopes model; this included all eight batches of garlic and the standard compound $MMTSO_2$, fitted to a common intercept. The coefficient of determination was 0.97, indicating a good fit to the model. In the instances where there were no responses to the extract, i.e., no zone of inhibition, these data were not included in the statistical calculations since activity was absent or too low to be measured.

Enumeration of viable *E. coli* B-103. A 5-ml TSBG culture of *E. coli* B-103 was incubated at 37°C for 18 h, harvested by centrifugation, and resuspended in an equal volume of TSBG. Dilutions of garlic juice extract (0 to 20 mg/ml, as indicated in the text) were prepared in TSBG, and the *E. coli* B-103 culture was added to yield a final concentration of 10^6 CFU/ml. Aliquots of 100 μl were removed after 24 h and plated on TSAG plates containing 100 μg/ml of ampicillin.

A nonlinear dose-response relation (polynomial) between the logarithm of the viable cell numbers (CFU ml^{-1}) and the concentration of garlic juice was found to be appropriate for the assay. A computer graphic software program (EXCEL 5.0) was used to generate and fit the growth inhibition curve of *E. coli* B-103 by garlic juice. The dose was the concentration of garlic juice in each tube, whereas response was defined as the logarithm of the viable cell numbers (CFU ml^{-1}). The following polynomial function was used to fit the relation between dose (mg) and response (CFU ml^{-1}):

$$y = f(x) = a + bx + cx^2 + dx^3 + ex^4 \quad (2)$$

where y (response) is a function of x (dose, mg).

Broth dilution assays. A stock solution of 500 mg ml^{-1} of garlic juice (nonsterile) was diluted to appropriate concentrations (0 to 20 mg ml^{-1} of garlic juice) with 10 ml of sterile TSBG in 16- by 150-mm glass culture tubes with caps. Then, about 10^6 CFU ml^{-1} of *E. coli* B-103 was added and incubated at 37°C for 24 h. Cell counts were determined by spiral plating on TSAG containing 100 μg/ml of ampicillin. The MIC was estimated as the concentration of garlic juice, which resulted in no change in cell numbers at 24 h. The MIC values for MMTSO and $MMTSO_2$ (using 10 to 100 μg/ml) were determined similarly, except incubation was continued for 96 h before plating. The lowest concentrations of the compounds that completely inhibited visible growth (turbidity) were defined as the MIC. The percentage of the growth inhibition was also calculated as shown below:

$$\% \text{ inhibition} = \frac{(\text{CFU ml}_{\text{control}}^{-1}) - (\text{CFU ml}_{\text{treatment}}^{-1}) \times 100}{(\text{CFU ml}_{\text{control}}^{-1})} \quad (3)$$

where the control is the growth of the test organism without garlic and the treatment is the sample when garlic was added.

The effects of atmosphere and temperature on antimicrobial activity of MMTSO, $MMTSO_2$, and garlic juice. The agar diffusion assay (above) was used to determine the effect of oxygen on the potential antimicrobial activity of MMTSO, $MMTSO_2$, and garlic juice. After overlaying the test microorganism on TSAG plates, supplemented and control plates were incubated aerobically and under reduced O_2 conditions in gas-pak anaerobic jars. The diameter of the inhibition zones was measured after 24 h of growth using a vernier caliper. To compare the units of antimicrobial activity of MMTSO, $MMTSO_2$, and garlic juice, equation 1 was used.

Temperature effects were determined using 50-g samples of peeled and refrigerated garlic cloves, which were extracted at 5°C using the juice extractor as described above. Fresh garlic juice was immediately frozen at −20°C and held for 1, 2, 3, 7, 21, and 28 days. Samples were thawed and prepared for agar diffusion assay. To determine the effect of heating on the biological activity of garlic juice, 10 ml of fresh garlic juice and 50 g of garlic cloves were heated at 121°C for 5 min. An unheated garlic sample was used as a control. The antimicrobial activity of samples was then determined by the agar diffusion assay.

Statistical analysis. Statistical inferences (polynomial curve fitting, linear regressions for slope ratio assays, analysis of variance) were computed with the General Linear Models procedure of SAS (SAS Institute, Cary, N.C.) or with Excel 5.0 (Microsoft Corporation, Redmond, Wash.) for equation 2.

RESULTS AND DISCUSSION

Antimicrobial activity by agar diffusion. Eight bacterial species were screened for their sensitivity to fresh garlic juice by agar diffusion assay (Table 1). Of these spe-

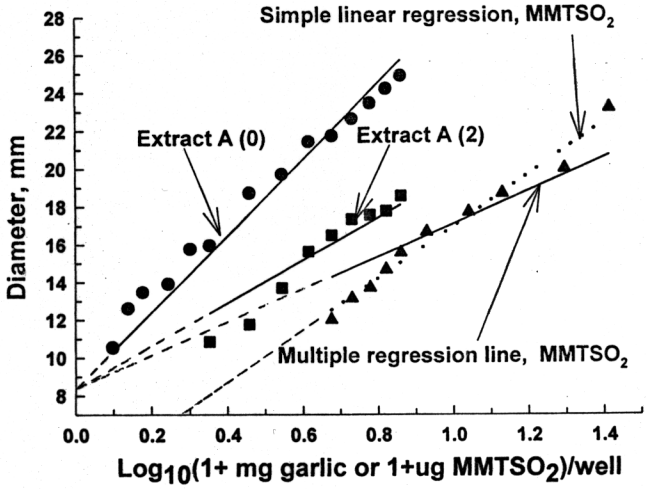

FIGURE 1. *Dose-response relations for $MMTSO_2$ and fresh garlic juice by agar diffusion assay. The dose responses of garlic sample A stored for 0 and 2 months at 5°C (see Table 2) are illustrated. The dose was the amount of $MMTSO_2$ or garlic juice per well. The dose was transformed to provide linearity when plotted against the response (i.e., inhibition zone). Actual amounts graphed included 7.5 to 50 μg/well for $MMTSO_2$ and 0 to 12.5 mg/well for garlic juice.* E. coli *B-103 was the assay bacterium.*

TABLE 2. *Antimicrobial activity of garlic samples against* E. coli *B-103 by agar diffusion*

Sample[a]	Storage before extraction (months at 5°C)	Antimicrobial activity (μg/mg of extract)	
		Slope ratio	Single point
A	0	2.31 A[b]	1.61
	2	1.30 C	1.00
	7	0.43 D	0.34
	11	−0.13 E	0.12
B	0	2.04 A	2.06
C	0	1.76 B	1.44
D	0	1.93 AB	1.96
E	0	1.97 A	1.69

[a] Sample A was peeled garlic shipped and held under refrigeration for the time indicated before extraction. On extraction, the juice was stored at −20°C until assay. Sample A was provided by Christopher Ranch (Gilroy, Calif.). Samples B, C, D, and E were purchased as whole garlic from grocery stores in the Raleigh, N.C., area. They were then peeled, extracted, and assayed.

[b] Letters for antimicrobial activity designate statistically significant differences ($P \leq 0.05$) by orthogonal comparisons.

cies, only *L. mesenteroides* was not inhibited at the highest level of garlic juice tested (10 mg/well).

For quantitative estimations of antimicrobial activity of garlic juice, $MMTSO_2$ was used as a standard compound for comparative purposes, and *E. coli* B-103 was used as the test bacterium. MMTSO is reportedly one of the thiosulfinates formed by the enzyme allinase from S-methyl-L-cysteine sulfoxide in garlic extracts, in addition to the major thiosulfinate, allicin *(12)*. Allicin, the principal antimicrobial constituent of garlic in aqueous solutions, is unstable and not commercially available *(6)*. $MMTSO_2$ *(21)*, a thermal breakdown product of *S*-methyl-L-cysteine sulfoxide, was more stable in the assay systems investigated, as discussed below. Furthermore, $MMTSO_2$ and garlic juice gave the same relative responses by the agar diffusion assay under both aerobic and anaerobic conditions. MMTSO activity was lower under aerobic compared with anaerobic conditions for storage of the agar diffusion assay plates, apparently because of its instability under aerobic conditions. Although the inhibition zones on anaerobically grown plates were clear and sharp, aerobically grown plates had smaller zones with indistinct edges. *E. coli* grew faster on aerobic than anaerobic plates.

The dose-response relations for fresh garlic juice and $MMTSO_2$ are illustrated in Figure 1. The antimicrobial activities of fresh juice from various garlic samples are given in Table 2, calculated by using the slope ratio method described in the "Materials and Methods" section. Sample A (stored for 0 months at 5°C) was used in Figure 1. The antimicrobial activity of this sample decreased during a 11-month storage period at 5°C (Table 2). Samples B, C, D, and E (obtained from local grocery stores) were lower in activity than sample A, but only sample C was significantly lower (≤0.05; Table 2).

For comparative purposes, activities from inhibition zones of the garlic extracts were interpreted directly from the simple linear regression plot of $MMTSO_2$ per well versus zone diameters (Fig. 1). Garlic extract dilutions giving zones near the middle of the regression line were used. Interestingly, the activities of the extracts from this single point method were similar to those calculated from the slope ratio method (Table 2). Both methods gave nearly the same order of activity; only samples D and E varied.

Antimicrobial activity by broth dilution. MMTSO and $MMTSO_2$ were tested for their antimicrobial activity by broth dilution, using the MIC technique. MMTSO and $MMTSO_2$ were equally inhibitory under anaerobic conditions. Aerobically, the MIC of $MMTSO_2$ remained constant at 30 μg/ml, both aerobically and anaerobically, throughout an incubation time of 96 h. The MIC of MMTSO was constant at 30 μg/ml of TSBG broth for 48 h but increased to 40 and 50 μg/ml after 72 and 96 h, respectively. Apparently MMTSO was less stable than $MMTSO_2$ on extended incubation, or the *E. coli* culture adapted to MMTSO during extended incubation. Because of its stability, $MMTSO_2$ is preferred as a standard to measure the antimicrobial activity of garlic juice, whether tested by agar diffusion or broth dilution.

The effect of garlic juice concentration (sample A, Table 2) on survival of *E. coli* B-103 after 24 h at 37°C in broth is illustrated in Figure 2. Conversely, the percentage of inhibition caused by the garlic juice also is illustrated. At 20 mg/ml of garlic juice, approximately 98% of the *E. coli* culture added was not viable after 24 h from an initial population of more than 10^8/ml. Based on the crossing point of the survival curve with the MIC line (the initial inoculum CFU ml^{-1}) and application of equation 1, the MIC for garlic juice by broth dilution is 1.5 mg/ml^{-1} of

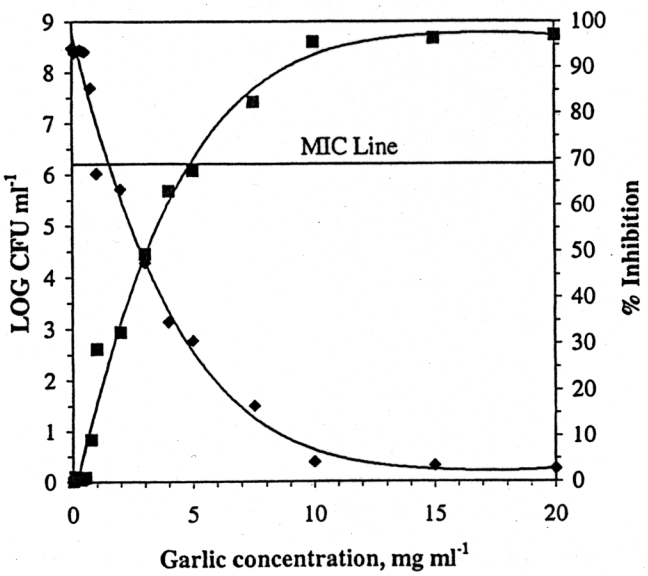

FIGURE 2. *Effect of fresh garlic juice concentration on the survival and percentage of inhibition of* E. coli *B-103 in TSBG after incubation at 37°C for 24 h. The horizontal line shows the initial CFU ml^{-1} of the inoculum. The MIC is the concentration of garlic juice (~1.5 mg ml^{-1}) that resulted in no change in CFU ml^{-1} after 24 h. ♦, log CFU ml^{-1}; ■, percentage of inhibition.*

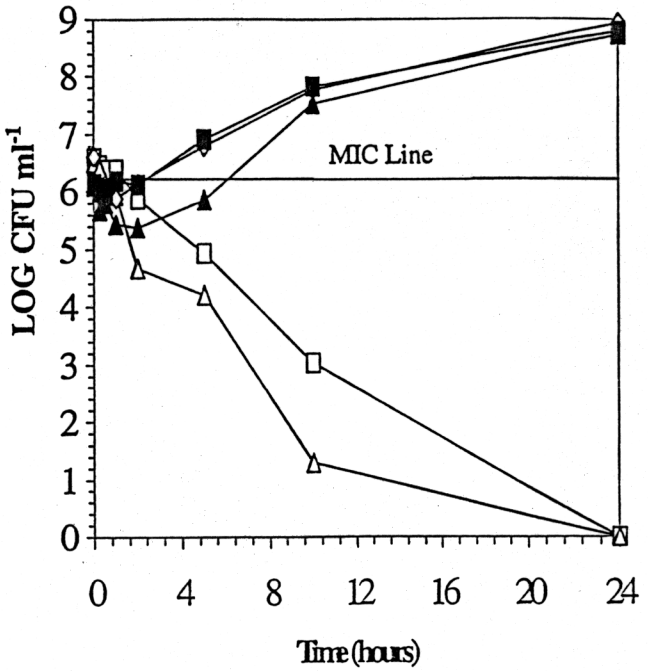

FIGURE 3. *Effect of fresh garlic juice on growth and survival of* E. coli *B-103 during incubation at 37°C during a 24-h period. Concentrations of garlic juice added to TSBG broth were as follows: ■, none (control); ◇, 0.1%; ▲, 0.25%; □, 0.5%; △, 2% or 0.1, 2.5, 5.0, and 20.0 mg/ml, respectively. The horizontal line shows the initial CFU ml^{-1} of the inoculum and was considered indicative of the MIC.*

garlic juice, which is equivalent to 3.46 μg of $MMTSO_2$, as determined by agar diffusion assay. In a related experiment, the effects of garlic juice concentrations up to 20 mg/ml on survival of *E. coli* B-103 were determined (Fig. 3). Viable cells were enumerated at various times during a 24-h incubation period at 37°C. In this case, both 5 mg/ml (0.5%) and 20 mg/ml (2.0%) of garlic juice reduced viable numbers to less than 10^2 during the 24-h incubation period. At 2.5 mg/ml (0.25%) of garlic juice, the viable count decreased during the first 2 h of incubation and then increased to numbers about 100-fold larger than the initial inoculum. Control and 1 mg/ml of garlic juice resulted in similar rapid growth of *E. coli* B-103.

Effect of temperature on the antimicrobial activity of garlic juice. In a study of the effect of temperature on the antimicrobial activity of garlic against *E. coli* B-103, heated (121°C, 5 min), unbroken garlic cloves and heated, fresh garlic juice lost their activity completely. It also was determined that the antimicrobial activity of fresh garlic juice did not change significantly when the fresh garlic juice was frozen at −20°C for 1 month (data not shown).

Comparison and utility of assay methods. Although agar diffusion and broth dilution methods for analysis of the antimicrobial activity of plant extracts have been previously described, unique features of assays described in this article offer advantages for the assay of garlic extracts. The use of $MMTSO_2$ as a standard in the slope ratio agar diffusion assay may help to reduce quantitative variabilities. The use of an ampicillin-resistant *E. coli* assay culture allows for exclusion of nonresistant microorganisms that may be natural contaminants. Such an antibiotic-resistant culture is particularly useful with viscous plant extracts such as garlic, which cannot be easily filter sterilized.

ACKNOWLEDGMENTS

This investigation was supported in part by a research grant from Pickle Packers International, Inc., St. Charles, Ill. We thank Mrs. Dean Toler for her excellent assistance in the preparation of the manuscript.

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