Antimicrobial Properties of Garlic Oil against Human Enteric Bacteria: Evaluation of Methodologies and Comparisons with Garlic Oil Sulfides and Garlic Powder

Z. M. Ross, E. A. O’Gara, D. J. Hill, H. V. Sleightholme and D. J. Maslin


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Garlic (*Allium sativum*) has traditional dietary and medicinal applications as an anti-infective agent (11, 17). In vitro evidence of the antimicrobial activity of fresh and freeze-dried garlic extracts against many bacteria (5, 16), fungi (1), and viruses (20) supports these applications.

Early steps involved in identifying the active constituents of garlic were the discovery that the compound allicin (allyl 2-propene thiosulfinate) is formed when garlic cloves are crushed (5, 6, 7) and that its formation depends upon the action of the enzyme alliinase of the bundle sheath cells upon the alliin of mesophyll cells (19). Methyl and allyl sulfide derivatives of allicin are formed by the steam distillation of mashed garlic (6, 7) and that its formation depends upon the action of the enzyme alliinase of the bundle sheath cells upon the alliin of mesophyll cells (19). Methyl and allyl sulfide derivatives of allicin are formed by the steam distillation of mashed garlic (13) to produce garlic oil (GO), which is used in many medicinal garlic products.

The classic studies of Cavallito and coworkers (5, 6, 7) attributed the antibacterial properties of garlic clove homogenates to allicin. These properties were confirmed against *Escherichia coli* and *Staphylococcus aureus* for garlic clove homogenates plus related garlic compounds and commercial supplements (9). In Cavallito’s studies, antimicrobial activity was found neither with aqueous garlic extracts lacking allicin nor following the addition of GO or diallyl sulfides (5) and no allyl sulfides were found in freshly prepared aqueous garlic clove extracts (6). Also, an early gas chromatographic study (4) indicated that the relatively rapid decomposition of allicin present in aqueous garlic extracts (14) involved transformation mainly to diallyl sulfides. For these reasons it was concluded that GO and its constituent sulfides lack antimicrobial activity. However, high-performance liquid chromatography (HPLC) applied to aqueous garlic extracts stored at room temperature for 24 h revealed that allicin transformation (14) was not to sulfides. Also, the use of agar plate methods (5), which provide free exposure of garlic compounds to the air and require good water solubility of test substances, might explain why antimicrobial activity was not detected for GO, which is both volatile and hydrophobic. The early conclusion that GO is not antimicrobial was influential, and investigations of the antimicrobial properties of GO and its sulfides have been few (1, 8). GO volatilization losses were avoided in a recent study (15) which revealed that GO and its major diallyl sulfides, as well as aqueous garlic extract (reconstituted freeze-dried garlic powder [GP]) and its major thiosulfinate product, allicin, each possess significant activity against *Helicobacter pylori*. The MIC of GO was found to lie between those of the most abundant GO constituents, diallyl disulfide (DADS) and diallyl trisulfide (DATS). Comparable findings for *S. aureus* and *E. coli* (17) also suggested that GO and GO sulfides have considerable antibacterial potency. Methyl sulfides of GO have not previously been studied.

The influence of test environments upon the activity of antimicrobial agents is well-recognized (3, 10), but possible influences of different culture media upon the antimicrobial activities of garlic components are little investigated. It is known that allicin reacts with cysteine (13) as do major diallyl sulfides of GO (DADS and DATS) but not diallyl sulfide (DAS) under simulated physiological conditions (13). Such reactions have potential importance for the interpretation of in vitro tests...
involving peptone-rich growth media and for the antimicrobial activity of garlic components in the gut.

In summary, the methodology of early experiments might explain the negative antimicrobial reputation of GO, while more recent findings indicate substantial activity against the few bacterial species that have been tested. Reevaluation of early methods and of the antibacterial properties of GO and GO sulfides with appropriate methodologies is therefore merited. The increasing incidence of bacterial food poisoning and evidence for considerable sensitivity of *H. pylori* to GO (15) indicated enteric bacteria as a suitable subject for this reevaluation.

The main objectives of this study were, therefore, to examine the influences of test environment on antimicrobial activities of GO and evaluate these activities upon a wide range of enteric bacteria, including pathogens. Additional objectives were comparison of antimicrobial activities of GO and GP upon enteric bacteria and assessment of the antimicrobial effects of diallyl and methyl sulfides of GO.

GO and GP were supplied and stored as before (15). A mixture of diallyl sulfides (supplied as DADS and named DASS) was obtained from Fluka Chemicals (Gillingham, United Kingdom). Dimethyl trisulfide (DMT) and dimethyl disulfide (DMD) were obtained from Pfaltz & Bauer (Waterbury, Conn.).

Garlic components were analyzed by HPLC analysis with established methods (12, 14) using an LKB Bromma HPLC System with a 2152 Controller, 2150 HPLC Pump, 2158 UV-CORD SD (set at 254 nm), and a 2220 Recording Integrator. Separations were achieved using a Spherisorb ODS-2 column with previously described methods (15).

The compositions of GO and GP were similar to those of samples utilized previously (15). DMT was 99% pure and DASS included diallyl polysulfides at levels (33% DADS, 16.5% DATS, and 17.5% diallyl tetrasulfide [DATTS], balance DAS) broadly comparable to those of GO.

Cultures were from the University of Wolverhampton microbiological culture collection (mainly NCTC or NCIMB strains) or, in the case of clinical enteric isolates, from local hospitals (Queen Elizabeth Hospital, Birmingham, United Kingdom; West Park Hospital, Wolverhampton, United Kingdom). Confirmatory identification procedures were routinely applied to the latter. Freeze-dried cell cultures were revived by culture onto suitable agar media. *Lactobacillus acidophilus* was routinely cultivated on Mann Rogosa Sharpe Agar (MRS) (Oxoid, Basingstoke, United Kingdom). Campylobacter Selective Agar (CSA) (Oxoid) was used for *Campylobacter spp.*, and all other bacteria were cultured on Tryptone Soya Agar (TSA) (Oxoid, Basingstoke, United Kingdom). *L. acidophilus* and *Bacteroides fragilis* were incubated at 37°C for 48 h in an anaerobic cabinet (Forma Scientific, Marietta, Ohio) under anaerobic conditions (10% H2, 10% CO2, 80% N2). *Campylobacter spp.* were incubated at 37°C for 48 h under microaerophilic conditions generated using Oxoid CampyGen sachets in gas jars (Oxoid). All other bacteria were incubated at 37°C for 24 to 48 h, under aerobic conditions. Broth cultures were prepared by inoculating the respective liquid growth medium Mann Rogosa Sharpe broth (MRSB) (Oxoid) or Tryptone Soya broth (TSB) (Difco) and incubated at 37°C for 24 to 48 h under appropriate conditions.

Enumeration of viable cells was achieved by serial 10-fold dilutions in 1/4-strength Ringers solution (Oxoid) and spread-plating of 0.1 ml of diluted samples onto the appropriate agar medium. Colonies were counted after incubation for 24 to 48 h at 37°C.

TSA (Difco) plates used in antimicrobial assays poured to a depth of approximately 7 mm were surface inoculated by spreading 0.1 ml of culture over their surface and were allowed to air dry before storage at 4°C for 1 h. All tests using TSA plates involved incubation at 37°C for 48 h. Implementation of the agar radial-diffusion technique involved adding neat GO or GO diluted and emulsified in TSB to pre-cut 4-mm-diameter wells. Where direct contact of GO with the bacterial lawn occurred, bacterial growth was inhibited but no measurable zones of inhibition of growth for the several species of enteric bacteria tested (data not shown) were obtained. These results indicated that studies relying upon diffusion in agar were unsuitable for testing GO, presumably because of its hydrophobic nature. When a bacterial lawn was inverted over a 100-μl drop of undiluted GO placed inside the lid of a glass petri dish, growth inhibition was observed on the part of the lawn immediately adjacent to the GO, which apparently resulted from air diffusion of GO vapor. This indicated both antimicrobial activity by GO and likely underestimation of such activity in agar plate studies because of volatilization loss.

A 10% (wt/vol) GO suspension in TSB formed by shaking was initially homogeneous, but upon standing, GO (density = 1.037 g/ml) coalesced at the bottom of the vessel within 20 min. The clear upper fraction obtained failed in MIC tests against *Enterobacter aerogenes*. In contrast, samples collected from the turbid lower fraction yielded a MIC of 0.68 mg/ml, comparable to that of homogeneous 10% (wt/vol) GO suspensions sampled immediately after shaking.

To assist dispersal of GO, detergents were incorporated into the TSB medium, and MICs against *E. aerogenes* were determined. Dimethyl sulfoxide, dimethyl formamide, Tween 80, Triton X-100, and bile salts (Sigma-Aldrich Company Ltd, Poole, United Kingdom) at concentrations below their MICs (25% [vol/vol], 12.5% [vol/vol], 25% [vol/vol], 25% [vol/vol], 12.5% [vol/vol], respectively) all produced significant dispersal of GO. However, no influence upon MICs was observed, so detergents were not routinely used.

To produce a homogeneous emulsion without detergent, sterile GO, or GO sulfides, were first diluted 50-fold as 0.5 ml was added into 9.5 ml of the appropriate broth in a 25-ml Universal bottle. The bottle was tightly sealed, and the mixture was shaken vigorously for 3 min just before use.

In tests of incubation conditions, lower MICs and higher sulfide values were observed at 24 h (data not shown) under static conditions than those observed after shaking. Under static conditions, MICs did not differ when metal caps were compared with tight-sealing ground glass stoppers. Therefore, static incubation of broths was utilized routinely to minimize volatilization, and tubes with metal caps were used for convenience and low cost and to ensure a gaseous environment suitable for microbial growth.

MICs were determined by incubation at 37°C for up to 72 h of serial twofold dilutions of garlic substances in 5 ml of broth in 16-ml test tubes (for *Campylobacter spp.*, 2-ml broth in bijoux bottles). MIC determinations were carried out in trip-
dilutions of GO or component sulfides, were prepared in 50-ml
volumes of 20 ml. The tubes were inoculated with 40
overnight culture of \textit{E. aerogenes}, producing initial viable cell
counts of 10^6 CFU/ml, capped with aluminum foil, and
incubated aerobically at 37°C for 24 h. Periodically, tubes were
aspirated to homogenize the culture, and then 0.5-ml samples
were removed for viable count determinations. Exposure to
GO (Fig. 1A) and GP (Fig. 1B) of \textit{E. aerogenes} grown in TSB
resulted in concentration-dependent capacities to kill or inhibit
population growth. This pattern of effect replicates that of
other time studies that we have performed upon other micro-
organisms with garlic components (reference 15 and unpub-
lished observations).

With GO, an initial lag phase of 30 to 60 min occurred at
concentrations of 0.34 to 2.75 mg/ml. Longer lag phases were
observed with GP and were greater at lower concentrations
(e.g., lag phases with GO were 30 min for 50 mg/ml and 400
min for 6.25 mg/ml). Lag phases may reflect delay in uptake of
garlic molecules and/or of toxic effect upon metabolic pro-
cesses between the sensitivities of clinical and culture collection
isolates tested. No obvious differ-
ences in the activity of \textit{GO} were observed, although with GO the latter tended to greater
sensitivity than pathogens. However, with the human enteric
pathogens \textit{Campylobacter jejuni}, \textit{Campylobacter coli}, and \textit{Campylobacter lari}
MICs varied widely (0.02 to 5.5 mg/ml) between different
species and mostly within a 10-fold GO concentration range
for different isolates of the same species. No obvious differ-
ences between the sensitivities of \textit{GO} and \textit{GP} indicated growth inhibition of all the
bacteria tested. Incubation of MIC tubes for 48 h gave either
no growth or colonies on TSA, which were confirmed by plating out onto TSA.

Minimal inhibitory concentrations (MICs) were determined at 48 h.
Minimal inhibitory concentrations (MICs) were determined at 72 h.
Each MIC determination was performed in triplicate per bacterial isolate. Range values refer to MICs determined for the different enteric isolates tested.

**TABLE 1. MIC determinations of GO and GP against human enteric pathogens and commensals**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>GO assay</th>
<th>GP assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of strains tested</td>
<td>24 h</td>
</tr>
<tr>
<td><strong>Human enteric pathogens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Bacillus cereus}</td>
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<tr>
<td>\textit{Escherichia coli O55}</td>
<td>1</td>
<td>5.5</td>
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<tr>
<td>\textit{Escherichia coli O128}</td>
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<tr>
<td>\textit{Escherichia coli O112}</td>
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<td>2.75</td>
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<tr>
<td>\textit{Shigella boydi}</td>
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<td>1.37</td>
</tr>
<tr>
<td>\textit{Shigella flexneri}</td>
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<td>1.37</td>
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<td>2.75</td>
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<td>0.02</td>
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<tr>
<td>\textit{Vibrio para-haemolyticus}</td>
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<td>\textit{Yersinia enterocolitica}</td>
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<td>\textit{Listeria monocytogenes}</td>
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<td>0.02</td>
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<tr>
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<td>5.5</td>
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<td>\textit{Campylobacter coli}</td>
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<td>\textit{Campylobacter lari}</td>
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<td><strong>Commensal enteric bacteria</strong></td>
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<tr>
<td>\textit{Bacteroides fragilis}</td>
<td>2</td>
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<tr>
<td>\textit{Bacillus subtilis}</td>
<td>3</td>
<td>0.17</td>
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<tr>
<td>\textit{Enterobacter aerogenes}</td>
<td>1</td>
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<td>\textit{Enterococcus faecalis}</td>
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<tr>
<td>\textit{Escherichia coli}</td>
<td>13</td>
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<td>\textit{Klebsiella aerogenes}</td>
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<td>0.04</td>
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<td>\textit{Proteus vulgaris}</td>
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<td>1.37</td>
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<tr>
<td>\textit{Lactobacillus acidophilus}</td>
<td>5</td>
<td>0.17–2.75</td>
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<tr>
<td>\textit{Streptococcus faecalis}</td>
<td>2</td>
<td>0.34</td>
</tr>
<tr>
<td>\textit{Streptococcus mutans}</td>
<td>1</td>
<td>0.08</td>
</tr>
<tr>
<td>\textit{Streptococcus pyogenes}</td>
<td>1</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* Each MIC determination was performed in triplicate per bacterial isolate. Range values refer to MICs determined for the different enteric isolates tested.

**a** Enteric isolates.

**b** MICs were determined at 48 h.

**c** MICs were determined at 72 h.

No. of strains
tested
24 h 48 h
24 h 48 h

**TABLE 1. MIC determinations of GO and GP against human enteric pathogens and commensals**

lactic, and consistent values were obtained for each bacterial isolate. Killing effects were confirmed by plating out onto TSA.

For a range of gram-positive and gram-negative enteric bacteria, including food-borne pathogens, MICs (Table 1) obtained for GO and GP indicated growth inhibition of all the bacteria tested. Incubation of MIC tubes for 48 h gave either the same or around twofold the 24-h MICs.
cesses. However, at the higher concentrations used (5.5 to 22 mg/ml), and at lower concentrations in nutrient-deficient media (Fig. 1C), a potential for rapid bactericidal action of GO was indicated by complete loss of viability during the first 2 h. Comparable bactericidal effects were observed with GP concentrations of 6.25 to 50 mg/ml. At concentrations of 0.34 to 1.375 mg/ml, declines in viability were temporary and recovery of growth occurred. The latter ensued after shorter delays, at higher rates, and to greater levels the lower the GO concentration. This recovery of viability was not observed with GP.

At the intermediate GO concentration of 2.75 mg/ml, viability declined during the first 5 to 6 h and then levelled off, and HPLC analysis at this concentration showed that GO sulfide loss was greatest over this initial period when sampling-related agitation was greatest. Losses over 24 h were greatest for the smaller, lower-boiling-point sulfides (ca. 75% for DMT, 60% for DAS, 40% for DADS, and 20% for DATS and DATTS). Thus, volatilization is the most plausible explanation for these losses and could explain the recovery of E. aerogenes viability observed at concentrations of 0.34 to 2.75 mg of GO/ml. It could also explain why MICs were frequently higher at 48 h than at 24 h and why the MIC for E. aerogenes obtained in time studies was 2.75 mg/ml—considerably higher than the 0.68-mg/ml concentration obtained under static capped-tube conditions. With GP, the bactericidal concentration of 6.25 mg/ml obtained from the MIC study corresponded to that obtained in the time study, in which little evidence for recovery of viability was observed. This greater consistency of result probably results from the lesser volatility of the thiosulfinates of GP.

The time course study (Fig. 1C) of different microbial media indicates that medium composition is an important factor influencing antimicrobial activity of GO. Thus, at 2.75 mg of GO/ml, complete killing occurred within a few hours in each of SDW, MSM, and MSM plus glucose. In contrast, at the same GO concentration in TSB, after a steep initial drop, leveling-off of viability occurred, as was previously observed (Fig. 1A).

The role of tryptone in explaining this apparent protective effect of TSB was supported by positive correlation of E. aerogenes viability with levels of tryptone ranging from 0 to 15 g/liter of MSM (data not shown) in the presence of the potentially bactericidal concentration of 2.75 mg of GO/ml. Cysteine produced a similar effect as was shown by partial neutralization of the bactericidal effects of 2.75 mg of GO/ml in MSM in the presence of 10 mM cysteine and very substantial neutralization in the presence of 25 mM. As TSB and tryptones are likely sources of cysteine, protein components of other microbial growth matrices may produce similar reductions in antimicrobial effects of GP and GO. This means that the results of our own and previous studies of garlic materials may represent underestimates of their full antimicrobial potential.

MICs obtained for DMT and DASS were species dependent for a range of GO-sensitive bacteria (Table 2). The species most sensitive to GO were also those most sensitive to DMT and DASS (L. monocytogenes, Y. enterocolitica). For all five species tested, sensitivity to DMT was severalfold greater than that to DASS and similar or slightly greater to DMT than to

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**TABLE 2.** Mean 24-h MICs of a selection of GO sulfides against six enteric bacteria

<table>
<thead>
<tr>
<th>Enteric isolate</th>
<th>GO (mg/ml)</th>
<th>DASS (mg/ml)</th>
<th>DMT (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. aerogenes</td>
<td>0.68</td>
<td>25</td>
<td>0.31</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.68</td>
<td>25</td>
<td>0.31</td>
</tr>
<tr>
<td>S. enterica serovar Typhimurium</td>
<td>0.34</td>
<td>25</td>
<td>0.31</td>
</tr>
<tr>
<td>S. sonnei</td>
<td>2.75</td>
<td>10</td>
<td>0.02</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>0.02</td>
<td>0.63</td>
<td>0.04</td>
</tr>
<tr>
<td>Y. enterocolitica</td>
<td>0.02</td>
<td>0.63</td>
<td></td>
</tr>
</tbody>
</table>
GO. These similarities of ratio between MICs of DASS and DMT for each bacterium suggest a common antibacterial mode of action for GO sulfides.

The antimicrobial potency of DASS indicates that the major (i.e., diallyl) sulfide constituents of GO contribute significantly to the antimicrobial activity of GO. However, DASS exhibited MICs substantially higher than those of GO for all species tested so that diallyl sulfides do not fully explain GO activity. This result is consistent with the lower antimicrobial activities observed for diallyl sulfides separately when compared with GO (15, 17). These differences have been proposed to result from the synergistic activities (17, 18) of GO sulfides. However, our finding that DMT possesses high antimicrobial activity, and evidence that the antimicrobial potency of diallyl sulfides increases with each additional S atom (15, 17), raises the possibility of methyl sulfides and/or higher polysulfides explaining the high relative antimicrobial activity of GO.

As the considerable antimicrobial activity of GO and its sulfide constituents, several of which possess sulphydryl (SH) reactivity (2, 21), was reduced in the presence of SH groups, an SH-reactive mode of action like that proposed for allicin is suggested. The greater reactivity of allicin (13) than GO sulfides with cysteine is associated with its sulfur-bound oxygen atom (17) and could explain its stronger antimicrobial effect (15, 17).

The actions of GP reported here against a number of enteric bacteria are consistent with broad-spectrum antibacterial effects previously evidenced (17) for aqueous garlic bulb extracts. The MIC results for GP over 24 h (Table 1) were comparable to those reported earlier (16).

The potency of GO was substantially greater than that of GP (Table 3) on a weight-of-garlic-product basis. However, when allowing for the dilution of allin within the GP matrix and the consequent yield of allicin plus other thiosulfinates (1.6% of GP), the reverse was true for all species tested excepting for those most sensitive to GO (L. monocytogenes, B. fragilis). Thus, early findings (5) that the active constituents (i.e., allicin and other thiosulfinates) of garlic bulb extracts such as GP solution yield substantially greater antimicrobial activity than GO were confirmed. However, contrary to the early studies (5), which were negative for GO, our findings indicate that GO possesses substantial and broad-spectrum antibacterial activity against both gram-positive and -negative bacteria. For some isolates (Vibrio metschnikovii and L. monocytogenes), sensitivity to GO was as high as that recently reported for H. pylori (15). The far greater potency of GO on a unit weight of product basis reflects the much greater concentration of antibacterial substances present in GO than in water-soluble garlic bulb extracts such as GP. Whereas GP requires solubilization in a suitable aqueous medium (this excludes acidic gastric juice), so as to enable the enzyme-catalyzed formation of its active thiosulfinate components, the active components of GO are already present. Moreover, the observed reduction in the antimicrobial effect of GO by protein digestion products and cysteine, which are abundant in the gut, would probably be greater for GP-derived allicin, because of its higher cysteine affinity (13). These differences suggest that GO might have greater clinical potential than GP despite the usually greater antimicrobial potency of the latter. The potential of GO to combat human gut infection is supported by our initial observations of its significant antimicrobial effects within ex vivo samples of human digesta (data not shown).

In conclusion, the results suggest that earlier evaluation of the antimicrobial activity of GO sulfides using agar plate methods (5), which indicated negligible antimicrobial activity, failed to take account of the hydrophobic and volatile nature of GO. In contrast, our results show that GO exhibits considerable antimicrobial activity when tested in liquid cultures, especially when precautions to minimize volatilization losses are applied. Reduced antimicrobial activity in the presence of tryptone and cysteine suggests that GO activity may be underestimated in microbial culture media and reduced within the enteric environment and is compatible with evidence that the mode of action of garlic compounds depends upon SH reactions.

Both GO and GP revealed wide-spectrum antimicrobial activities. Molecule for molecule, these activities were greater with GP thiosulfinates than GO sulfides against most bacteria tested. However, the antibacterial activities of GO and GO sulfides were substantial against several pathogenic species, some of which are significant causes of food poisoning, so that further exploration of the potential of GO in enteric disease control is warranted.

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REFERENCES


| TABLE 3. Comparison of potencies of GO and GP by using 24-h MICs against selected isolates |
|---------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| GP/GO ratio of MICs | E. coli | Salmonella enterica serovar Typhimurium | L. monocytogenes | B. fragilis |
| GP:GO | 9.19 | 9.12 | 312.5 | 156.25 |
| GP:GO, allowing for GP thiosulfinate content | 0.15 | 0.15 | 5.11 | 2.56 |

a Expressed on a weight-of-garlic-product basis.

b Total thiosulfinate content of GP = 16.367 ± 0.964 mg/g, n = 3.

c Enteric isolate.