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Characteristic of Antibiotic Resistance of Foodborne Pathogens Adapted to Garlic, *Allium sativum* L.

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Abstract Antibiotic resistance of foodborne pathogens adapted to garlic (*Allium sativum* Linn.) was determined in order to understand the relationship between antibiotic resistance and garlic. The Gram (-) strains of *Escherichia coli* and *Salmonella typhimurium* and the Gram (+) strains of *Bacillus cereus* and *Staphylococcus aureus* were subcultured consecutively in a garlic broth, and the surviving colonies on the agar were selected as the adapted strains. Minimal inhibitory concentrations (MIC) for 15 antibiotics on the adapted strains were determined on Muller-Hinton Infusion agar. Adaptation to 1.3%(v/v) garlic juice increased MIC for vancomycin, aminoglycoside, and erythromycin on *B. cereus*, and for ampicillin and erythromycin on *E. coli* O157:H7. MIC of aminoglycosides, chloramphenicol, and vancomycin on the adapted *S. aureus* increased. The adapted *S. typhimurium* was more resistant to penicillin and vancomycin than the non-treated strain. The adapted *S. typhimurium* and *S. aureus* lost their antibiotic resistance in non-garlic stress conditions. However, the adapted *B. cereus* was still resistant to erythromycin and vancomycin, and the adapted *E. coli* was also resistant to erythromycin. Antibacterial garlic might increase the antibiotic resistance of *E. coli*, *B. cereus*, *S. aureus*, and *S. typhimurium* and this resistance can continue even without the stress of garlic. Therefore, garlic as a food seasoning could influence the resistance of such pathogens to these antibiotics temporarily or permanently.

Keywords: garlic, adaptation, *E.coli*, *S. typhimurium*, *B. cereus*, *S.aureus*, antibiotics resistance

Introduction

Garlic (*Allium sativum* Linn.) is used as a natural seasoning to improve taste and flavour. Large quantities of garlic, cooked or raw, are consumed in Korean dishes. One example is *kimchi* which contains about 1.4%(w/w) garlic (1). In recent years, garlic has been reported to have anticancer (2), blood pressure-lowering (3), antithrombosis (4), cholesterol-lowering, and anti-aging activities (5, 6) so it has come to be regarded as a very valuable food. Garlic also acts as a food preservative, and it inhibits the growth of foodborne and clinical pathogens (7-12). One of the principal antibacterial agents in garlic is allicin (diallyl thiosulfinate), a major thiosulfinate compound (13) which is formed when garlic is crushed or cut by the action of the enzyme allinase on alliin (*S*-allyl-cysteine sulfoxide). Another antibacterial agent is methyl methanethio-sulfinates which is formed from *S*-methyl-L-cysteine sulfoxide by the action of allinase (8). More than 30 kinds of low molecular weight sulfide compounds such as diallyl-monosulfide, disulfides, oligosulfides, vinyldithiins, and ajoenes have also been reported (15). Diallyl disulfide and diallyl sulfide comprise 75% of these compounds (16, 17). The antibacterial activities of garlic have a broad spectrum as assessed by their growth inhibition of *Escherichia coli* O157:H7, *Salmonella typhi*, *Staphylococcus aureus*, and *Shigella dysenteriae* (11, 14). However, a Gram-negative strain such as *E. coli* O157:H7 is more sensitive than Gram-positive strain.

Antibiotics have been powerful agents for disease treatment and prevention for a very long time. However, recently their overuse and misuse have increased rapidly so that resistant strains are appearing. Some people worried that the emergence of such strains could ultimately threaten human life. Resistant strains seem to appear more when the antibiotic dose was low, at less than therapeutic doses, and with prolonged usage. These factors optimally select for bacterial resistance (18). One report described that most antibiotic resistance in fecal flora came from uncooked food contaminated with antibiotic-resistant bacteria such as carrots, celery, lettuce, cucumbers, peppers, and tomatoes (19). There are many unanswered questions related to the emergence of resistant strains such as whether the selective factor is misuse or hospital-style antibacterial chemicals in the home (20).

As well as a valuable food, garlic is also recommended as an antibiotic and is a natural preservative in place of synthetic preservatives to protect food from putrefying organisms and pathogens. This trend came from distrust of the nutritional and physiological effects of synthetic additives and preservatives.

There have been some studies of the potential risk of unlimited intake of herbal therapies (21, 22). However, more studies are needed on the cellular immunity, the side-effects for each disease, the resistance to antibacterial activity, and the antibiotic resistance due to garlic consumption. Therefore, antibiotic resistance for adapted strains of foodborne pathogens under stress of garlic was analyzed and the risk of increased antibiotic resistance was evaluated.

Methods and Materials

Strains The foodborne pathogens tested were *E. coli* O157:H7 505B, *E. coli* O157:H7 932, *B. cereus* KCCM 40935, *S. typhimurium* ATCC12103, and *S. aureus*

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KCCM12023. Each strain was activated in tryptic soy broth (TSB) (Difco laboratories, Detroit, MI, USA) for 24 hr at 37°C from the stock culture at -70°C, and subcultured three times consecutively for the experiment.

Preparation of the garlic juice The husked garlic was purchased from local markets, and washed before preparation with running tap water to remove any remaining husk or damaged surface. The garlic was mixed with an equal weight of water, pulverized with a blender, and then stored at 4°C for 48 hr to allow the crude garlic juice to be separated from the cake under gravity. The crude juice was filtered through cheese cloth, centrifuged at 22,000×g, and then sterilized with a 0.22 μm membrane filter. The garlic juice extract was stored at 4°C until needed.

Determination of garlic juice concentration and adaptation of the pathogens To decide how much garlic juice should be added to the TSB broth for pathogen adaptation, the doses of garlic juice that caused 50, 90, and 99% lethality (LD_{50}, LD_{90}, and LD_{99}, respectively) were determined. The strains were inoculated in Muller Hinton Infusion (MHI) broth (Difco Laboratories) with and without various amounts of garlic juice, and cultivated for 48 hr at 37°C. The cultures were serially diluted, spread and cultivated on MHI agar for 24 hr at 37°C after which cell viability was assessed and compared to establish the LD_{50}, LD_{90}, and LD_{99} of the garlic juice.

The strains were aerobically subcultured daily during eight days in new TSB broth with the LD_{50}, LD_{90}, and LD_{99} of garlic juice at 37°C. The 4th and the 8th subcultures of the adapted strains were used to determine the minimal inhibitory concentration (MIC) of 15 antibiotics. The adapted strains were subcultured a further four days without garlic juice (non-reverted strains), and these strains together with pathogen strains without garlic treatment were used as controls.

Antibiotic resistance of the adapted strains The antibiotics used to determine the antibiotic resistance of the adapted strains were penicillin, ampicillin, amoxicillin, cephalothin, neomycin, kanamycin, streptomycin, paromomycin, nalidixic acid, vancomycin, erythromycin, tetracycline, rifampicin, chloramphenicol, and lincomycin (Sigma, St. Louis, MO, USA). Each antibiotic was dissolved in the recommended solvent and stored at -20°C. The agar dilution method (21) was used to determine the antibiotic resistance and MHI was used for the medium to which the antibiotic solution corresponding to each concentration was added. MIC was determined when its strain did not grow on the media with each concentration of antibiotics.

Results and Discussion

Lethality of garlic juice against foodborne pathogens Four genus pathogens of *E. coli* and *S. typhimurium* as examples of Gram (-) strains, and *B. cereus* and *S. aureus* as examples of Gram (+) strains were selected and the lethality of garlic juice was determined for each strain.

Growth curves for each pathogen in TSB broth at 37°C were established in order to determine their specific appropriate subculturing schedule. The starting points of the stationary phase were five, seven, and nine hours for *E. coli*, *S. typhimurium*, *B. cereus*, and *S. aureus* respectively (data not shown). The growth of each pathogen was delayed when garlic juice was present in the culture broth. However, the stationary phase showed at least on 24 hr in the TSB broth so that a cultivating schedules of one subculture every 24 hr was used.

The LDs of garlic for the various pathogens were shown in Table 2. Garlic juice caused 50% lethality (LD_{50}) of two types of *E. coli*, O157:H7 932 and O157:H7 505B, and *B. cereus* at 1.3%(v/v) in TSB broth, 90% lethality (LD_{90}) at 1.6%(v/v), and 99% lethality (LD_{99}) at 1.9%(v/v). The lethal doses of garlic juice against *S. typhimurium* were LD_{50} at 20%(v/v), LD_{90} at 21%(v/v), and LD_{99} at 23%(v/v) and against *S. aureus* were LD_{50} at 12%(v/v), LD_{90} at 14%(v/v), and LD_{99} at 17%(v/v).

LD of the garlic juice varied greatly, from 1.3 to 20%(v/v), according to the pathogens. The antibacterial activity of garlic has been reported to be due to allicin, *S*-methyl-L-cysteine sulfoxide and the other sulfide compo-nents. The principal agent, allicin, has been shown to be inactivated four hours after being crushed (24). Al-Delaimy and Ali (25) reported that 4%(w/v) fresh garlic extract inhibited the growth of *S. aureus*. *E. coli*, and *S. typhi*. Banerjee and Sarkar (24) reported that *E. coli* and *B. cereus* seem to be more sensitive to garlic juice than *Salmonella* and *S. aureus*, however, they suggested that MIC seemed to be a relative value, not absolute. Our results showed a similar sensitivity to that seen in other studies, and the LD or MIC seemed to vary according to the pathogen. In our experiment, the garlic juice was used in 48 hr after preparation so the allicin in the juice might not be active or very weakly effective.

Table 1. Antibiotics to be used for determination of minimal inhibitory concentration (MIC) of the adapted foodborne pathogens to the garlic juice

Classification	Antibiotic
β-Lactam	Penicillin, ampicillin, amoxicillin, cephalothin
Aminoglycoside	Neomycin, streptomycin, kanamycin, paromomycin
G(+) spectrum	Erythromycin, vancomycin
G(-) spectrum	Nalidixic acid, rifampicin
Broad spectrum	Chlorampenicol, tetracycline
Anaerobic spectrum	Lincomycin

Table 2. Lethal concentrations of garlic juice in tryptic soy broth (%, v/v) on various pathogens

Pathogen	LD_{50}	LD_{90}	LD_{99}
E. coli O157:H7 932	1.3	1.6	1.9
E. coli O157:H7 505B	1.3	1.6	1.9
S. typhimurium	20	21	23
B. cereus	1.3	1.6	1.9
S. aureus	12	14	17

Antibiotic resistance pattern of *E. coli* and *S. typhimurium* adapted to garlic juice Resistance to 15 antibiotics was determined for non-adapted pathogen strains, for adapted strains at the 4th and 8th consecutive subculture, and for non-reverted strains. The adapted strains from the 4th culture were subcultured again four times without the garlic juice, and designated as the non-reverted strains.

The resistance of the adapted *E. coli* O157:H7 932 to ampicillin, streptomycin, kanamycin, and erythromycin increased according to the subculture times in comparison to non-adapted strains (Fig. 1). MIC of ampicillin for the non-adapted strain was 8 μg/mL, whereas the strains adapted to the LD_{50} and LD_{99} of garlic juice had a MIC of 128 μg/mL, a 16-fold increase. However, the resistance of the non-reverted strains returned to 8 μg/mL (data not shown). The MIC of kanamycin was 4 μg/mL for the non-adapted strains and 8 mg/mL for the adapted strains. The MICs of streptomycin and erythromycin for the adapted strains increased four-fold in comparison to the non-adapted strains, and the non-reverted strains returned to the MIC for the non-adapted strains. Only the MIC of erythromycin did not return to the MIC for the non-treated strains (Table 3).

Antibiotic resistance to ampicillin and erythromycin for the adapted *E. coli* O157:H7 505B increased as shown in Fig. 2. MIC of ampicillin increased 16-fold and the MIC of erythromycin increased four-fold, which were similar to the values seen for *E. coli* O157:H7 932.

Antibiotic resistance of the adapted *S. typhimurium* ATCC 12103 seemed to be higher than the adapted *E .coli*

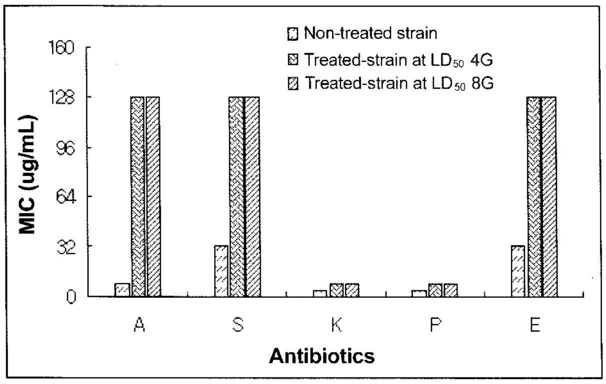

Fig. 1. Changes in antibiotic resistance for the adapted *E. coli* O157: H7 932 at different garlic doses. A, ampicillin; S, streptomycin; K, kanamycin; P, paromomycin; E, erythromycin. Non-treated strain, culture without garlic juice; Treated-strain at LD_{50}, strain was subcultured four (4G) and eight times (8G) with each lethal dose (LD_{50}).

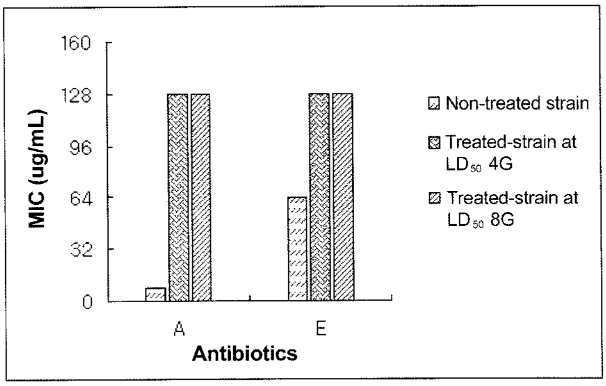

Fig. 2. Changes in antibiotic resistance for the adapted E. coli O157:H7 505B at different garlic doses. A, ampicillin; E, erythromycin. Non-treated strain, culture without garlic juice; Treated-strain at LD_{50}, strain was subcultured four (4G) and eight times (8G) with each lethal dose (LD_{50}).

Table 3. Comparison of minimum inhibitory concentration of antibiotics for non-reverted pathogens

Strain	Antibiotics	Non-treated[1)] strain (μg/mL)	Non-reverted[2)] strain (μg/mL)
E. coli O157:H7 932	Streptomycin	32	16
	Kanamycin	4	8
	Paromomycin	4	8
	Erythromycin	32	128
E. coli O157 : H7 505B	Ampicillin	8	64
	Erythromycin	64	128
B. cereus KCCM40935	Ampicillin	64	4
	Vancomycin	64	128
	Erythromycin	0.5>	128
S. typhimurium ATCC12023	Cephalothin	8	16
	Streptomycin	16	32
	Nalidixic acid	2	4
S. aureus ATCC 12023	Neomycin	2	4
	Paromomycin	4	8
	Vancomycin	8	16

[1)]Non-treated strain = the culture without garlic juice.
[2)]Non-reverted strain = the treated strain at the 4th culture with the LD_{50} of garlic juice was subcultured a further four times without garlic juice.

O157:H7 as seen in Fig. 3. MICs of penicillin and vancomycin for the adapted *S. typhimurium* increased in comparison to the non-adapted strain, however, those of lincomycin and erythromycin decreased. The non-reverted strains had MIC values similar to the non-treated strains.

The chromosomal multiple antibiotic resistance (*mar*) operon has been reported to be essential for the adoption of a multiple antibiotic resistance phenotype in many species of bacteria (26). The *mar* operon regulates the expression of many genes that could contribute the regulon. The ubiquity of *mar* and *mar*-like operons/regulons has suggested that antibacterial substances could lead to mutations within the *mar* operon and might lead to broad spectrum resistance to antibacterial, biocide, and antibiotic compounds (27). Therefore, the pathogens adapted to garlic demonstrated antibiotic resistance and it is possible that some mutation at the *mar* operon/regulon might lead to permanent increased resistance to some antibiotics.

Antibiotic resistance pattern of *B. cereus* and *S. aureus* adapted to garlic juice Antibiotic resistance of *B. cereus* KCCM 40935 to the aminoglycoside family of neomycin, streptomycin, kanamycin, and paromomycin as well as that to vancomycin and erythromycin increased after garlic adaptation. There was no change in neomycin MIC for the adapted strains at the 4th subculture, however, MIC increased from 2 to 8 μg/mL for the adapted strains at the 8th subculture. MIC of streptomycin for the adapted strains increased four-fold from 16 to 64 μg/mL and there was no difference between the adapted strains at the 4th and 8th subcultures. MIC of kanamycin increased two-fold at the 4th subculture and 32-fold at the 8th subculture, which showed high resistance according to the number of subcultures. MIC of vancomycin increased two-fold from 64 to 128 μg/mL and MIC of erythromycin increased greatly from less than 0.5 to 128 μg/mL. MICs of vancomycin and erythromycin did not return to the values for the non-treated strains (Table 3).

MICs of neomycin, streptomycin, kanamycin, streptomycin, paromomycin, vancomycin, and chloramphenicol for the adapted *S. aureus* KCCM12023 increased. There was little difference in the β-lactam family of penicillin, ampicillin, and amoxicillin (data not shown). MICs of members of the aminoglycoside family such as neomycin, kanamycin, streptomycin, and paromomycin for the adapted strain increased according to the garlic juice concentration. MIC of chloramphenicol increased from 4 to 8 μg/mL. MIC of vancomycin increased from 8 to 16 μg/mL and the non-reverted strains did return to the MIC of the non-treated strain (Table 3).

In conclusion, the pathogens adapted to garlic juice at the LD_{50} and LD_{90} showed increased resistance to some antibiotics and very different patterns of antibiotic resistance. The adapted *S. typhimurium* and *S. aureus* recovered their antibiotic resistances after they were subcultured further without garlic juice. However, after subculturing without garlic juice, the adapted *B. cereus* still showed resistance to erythromycin and vancomycin, and the adapted *E. coli* showed resistance to erythromycin. The adapted *S. aureus* also showed persistent resistance to neomycin, paromomycin, and vancomycin. The antibacterial garlic seemed to increase the antibiotic resistances of *E. coli*, *B. cereus*, *S. aureus*, and *S. typhimurium* and this

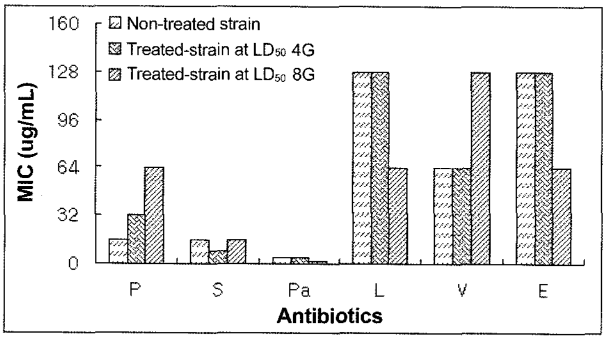

Fig. 3. Changes in antibiotic resistance for the adapted *S. typimurium* ATCC12023 at different garlic doses. P, penicillin; S, streptomycin; Pa, paromomycin; L, lincomycin; V, vancomycin; E, erythromycin. Non-treated strain, culture without garlic juice; Treated-strain at $LD_{50,}$ strain was subcultured four (4G) and eight times (8G) with each lethal dose (LD_{50}).

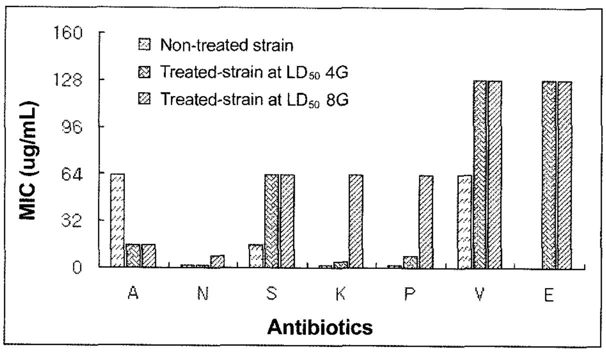

Fig. 4. Changes in antibiotic resistance for the adapted *B. cereus* KCCM40935 at different garlic doses. A, ampicillin; N, neomycin; S, streptomycin; K, kanamycin; P, paromomycin; V, vancomycin; E, erythromycin. Non-treated strain, culture without garlic juice; Treated-strain at $LD_{50,}$ strain was subcultured four (4G) and eight times (8G) with each lethal dose (LD_{50}).

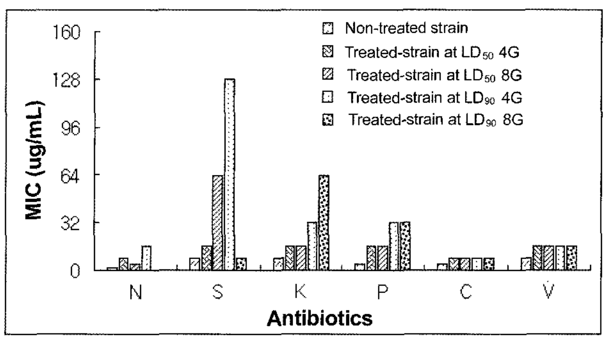

Fig. 5. Changes in antibiotic resistance for the adapted *S. aureus* KCCM12013 at different garlic doses. N, neomycin; S, streptomycin; K, kanamycin; P, paromomycin; C, chloramphenicol; V, vancomycin. Non-treated strain, culture without garlic juice; Treated-strain at LD_{50} and LD_{90}, strain were subcultured four (4G) and eight times (8G) with each lethal dose ($LD_{50,}$ LD_{90}).

resistance persisted in some cases even after the stress of garlic is removed.

Al-Delaimy and Ali (25) suggested a natural antimicrobial source, like garlic, could be an alternative to some well known antibiotics. However, everyday products with the antimicrobial agent would induce a response by bacteria and cause resistance to antibiotics. Hooton and Levy (27) also proposed that the use of the antibacterial in products be controlled. Recently, garlic has been recommended as a functional additive and food supplement due to its valuable physiological roles in humans. However, in this study the increased antibiotic resistance of foodborne pathogens under the stress of garlic was analyzed. Our results suggest that the high garlic consumption of Korean people might be related to a high antibiotic resistance of foodborne pathogens. Further research should be carried out into the effect of high consumption of an antibacterial agent like garlic in daily dishes on antibiotic resistance of intestinal bacteria. It may be necessary to analyze the daily consumption of garlic and to propose a safety guideline for the consumption of raw garlic and processed garlic products, especially for people who consume large quantities of garlic.

Acknowledgments

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