SPICE OILS AS NATURAL ANTIMICROBIAL AGENTS OF FOOD-BORNE MICROORGANISMS

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AN ABSTRACT OF THE THESIS OF

Jose W. Rodriguez for the Master of Science

in Biology presented on May 1989

Title: Spice Oils as Natural Antimicrobial Agents of Food-Borne Microorganisms

Abstract approved: R J Sokansanj

In order to search for new substitutes for sodium nitrite in foods, this research investigated the antimicrobial activity of garlic, clove, onion, and oregano oils against two Gram-positive cocci, Bacillus cereus and Staphylococcus aureus, and one Gram-negative bacillus, Salmonella typhimurium. Garlic oil proved to have the highest antimicrobial activity among the four spice oils using a growth inhibition test of the bacteria on tryptic soy agar (TSA) plates. The minimum inhibitory concentration (MIC) of garlic oil was determined using a growth inhibition test of the bacteria on cooked meat (Difco) and rat chow (Purina) broth. The MIC of garlic oil was 400 ppm. The pH and the composition of the media influenced the antimicrobial activity of the spice oils. The toxic effects of orally administrated garlic oil (400 ppm) was also evaluated on albino rats. After feeding the rats with garlic oil for one month, the rats lost 15% of their normal weight and no other visual toxic effects were observed. Garlic oil proved to be an effective natural antimicrobial agent against food-borne microorganisms, but requires further evaluations.

R. J. Sobieski

Approved for Major Department

James L. Wolfe

Approved for Graduate Council

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INTRODUCTION

Sodium nitrite has been added to foods for many years. It was initially used in meats as a food additive in 1925 after Haldane (1901) found that the pink color in curing meats resulted from the reaction of nitrite with the meat pigments. Sodium nitrite was first used as a food preservative when Steinke and Foster (1951) demonstrated that nitrite was an effective antibotulinal agent. Pivnick (1967) used sliced, processed meats in vacuum packages that had been inoculated with spores of Clostridium botulinum to demonstrate that at any given temperature, delay in toxinogenesis and spoilage was directly related to the concentration of nitrite.

The use of nitrite in meat results in the occurrence of very low levels of nitrosamines, which at higher levels have been shown to be carcinogenic in laboratory animals. Mirvish (1975) demonstrated that acute liver damage and lung adenomas were produced when rats and mice were fed amines and nitrite.

Extensive surveys of foods for nitrosamines have been conducted by several groups around the world. A survey of cured meat products available in the United States was published by the Nitrite Safety Council (1980). Some representative values for nitrosamines in foods are presented in Table I.

In addition to preformed nitrosamines found in some foods, humans are also exposed to nitrosamines through normal physiological processes. Ohshima and Bartsch (1981) demonstrated that nitrosamines can be formed in the human stomach through the reaction between amines

Table I. Representative Nitrosamine Content of Several Foods.

Food	No. positive/ No. analysed	Content range (ug/kg)	Country	Reference
Fried bacon	22/22	7-139	U.S.	Harvey, 1976
Fried bacon	33/56	* nd-200	U.K.	Gough, 1977
Cured meats	25/64	nd-8.6	U.K.	Gough, 1977
Beers	27/29	nd-5	Japan	Kawabata, 1980
Cured meats	72/81	nd-1	Japan	Maki, 1980
Meat products	127/395	0.5->5	Germany	Spiegelhalder, 1980
Cheese	49/209	0.5-5	Germany	Spiegelhalder, 1980
Beer	142/215	nd-68	Germany	Spiegelhalder, 1980
Cured meats	77/118	nd-55	Canada	Sen, 1980
Milk products	11/26	nd-0.7	Canada	Sen, 1980

*nd= not detected.

Table II. Nitrate concentration in vegetables and grains as purchased.*

	Replicates n	Average umol/100g
Vegetables		
Beans (green)	4	273 ± 176
Broccoli	3	526 ± 479
Cabbage	2	1084 ± 303
Carrot	23	256 ± 216
Cauliflower	1	73
Celery	1	5000
Pepper	5	169 ± 214
Potato	16	205 ± 137
Radish	1	3571
Spinach	6	3840 ± 1292
Grain products		
Beverages		
Beer	1	10
Bread		
Oat	1	10
Wheat	1	13
Grains		
Puffed rice	1	3
Wheat flour	1	15
Water		4500

* Adapted from Schuster (1987).

and nitrite. The nitrite can be derived from two sources, consumption of food which contains nitrite and conversion of nitrate to nitrite in the body. Nitrate can be derived from normal nitrogen metabolism and from the diet. Table II presents some values for nitrate levels in vegetables and grains products. Dietary nitrate is quickly absorbed into the blood after ingestion. It is then secreted and concentrated in the saliva where microorganisms in the mouth convert it to nitrite. The nitrite is swallowed and results in the formation of nitrosamines in the stomach (Spiegelhalder et al., 1976).

Vitamin C has been added to meats undergoing curing that contain sodium nitrite because vitamin C can block the formation of nitrosamines. Sen (1974) indicated that the addition of ascorbic acid can completely inhibit the formation of nitrosamines. However, Wang (1987) demonstrated that ascorbic acid cannot reduce the nitrosamine formation in acid or neutral solutions, and in addition, ascorbic acid cannot affect the nitrosamines after they have already formed.

Subsequently, the United States Department of Agriculture and the Food and Drug Administration (1978) lowered the acceptable level of sodium nitrite in meat and poultry products. In 1986 (USDA, 1986), the levels of sodium nitrite were lowered again as presented in Table III. The actual levels being used by the industry are proprietary, but cannot exceed USDA maxima.

In view of possible but unquantified risk resulting from the use of nitrite as a curing agent, the search for alternatives and alternative approches to the use of nitrite is desired. However, no

Table III. Summary of USDA Action on Nitrate and Nitrite in Foods.*

Law/Regulation	Agency	Provision
Federal Meat Inspection Act of 1906	USDA	Inspection of meat and meat products
Nitrate sanctioned in meats, 1908	USDA	Use of nitrate (saltpeter) detailed
Nitrite sanctioned in meats, 1925	USDA	Meat curing regulations detailed
Nitrite and nitrate in bacon, 1978	USDA	Lowers nitrite, eliminates nitrates, requires ascorbate
Nitrite levels in bacon, 1986	USDA	Allows 100 or 400 ppm of sodium nitrite in some circumstances

USDA = United States Dept. of Agriculture
* Adapted from USDA (1986).

new agent or combination of agents can be subtituted for nitrite in our food as preservatives until adequate testing has ensured that the new agent or agents do not present a hazard to human health.

The use of spices as inhibitors of microbial growth has been studied previously by Beuchat (1976) who demonstrated that thyme and oregano oils were able to inhibit the growth of Vibrio parahaemoliticus. Blank (1985) showed the sporostatic effect of spices by treating Bacillus subtilis spores with clove oil. Hall (1986) reported that extracts of mace effectively inhibited C. botulinum toxin production in turkey frankfurter slurries. Also, it was shown that garlic extract could inhibit the outgrowth of C. perfringens spores (Mantis et al., 1979).

Spices can also inhibit the growth of fungi. Conner and Beuchat (1984) used 14 different species of yeast and treated them with 30 different spices, and they observed that onion and garlic oils had the highest fungistatic activity. Sharma (1979) demonstrated that onion extracts could inhibit the growth of the aflatoxin-producing fungi, Asperigillus flavus and A. parasiticus.

The observed high, anti-bacterial and anti-fungal activities of spices are due to components found in their essential oils (Hitokoto et al., 1980). The antimicrobial components of garlic, oregano, onion, and clove are presented in Table IV. Wills (1956) showed that allicin, the antimicrobial component of garlic, had the capability of inhibiting several metabolic enzymes of microorganisms. Barone and Tansey (1977) also demonstrated that allicin was able to disrupt microbial cell

Table IV. Antimicrobial compounds found in the essential oils of spices.

Spice	Antimicrobial compound	Reference
Garlic	Allicin	Cavallito, 1944
Oregano	Carvacrol	Fenaroli, 1971
Onion	Lacrimatory factor	Bandyopadhyay, 1973
Clove	Eugenol	Azzouz, 1982

metabolism by inactivation of sulfhydryl proteins by oxidation of thiols to disulphides.

In order to find new alternatives for the subtitution of sodium nitrite, this research was designed to investigate the effectiveness of garlic, onion, clove, and oregano oils as inhibitors to food-borne microorganisms such as Staphylococcus aureus, Salmonella typhimurium, and Bacillus cereus. S. aureus is a Gram-positive coccus organism that when it multiplies in foods produces an enterotoxin which is the agent that causes nausea, vomiting, and diarrhea (Banwell and Sherr et al., 1973). The toxin produced by this bacterium is highly resistant to heat, cold, and chemicals (Bergdoll et al., 1974). S. typhimurium is a Gram-negative bacillus that once ingested in foods can multiply rapidly in the intestine causing headache, diarrhea, abdominal discomfort, and vomiting (Gunn and Markakis et al., 1978). B. cereus is an aerobic spore forming, Gram-positive bacillus. The spores formed by this organism are resistant to heat, cold, and drying. If food containing this bacterium is ingested, it can cause a gastrointestinal illness characterized by abdominal cramps, nausea, vomiting, and diarrhea (Terranova, 1978; Gilbert et al., 1979).

These three bacteria do not change the organoleptic qualities of the food, so it is hard to detect the presence of these organisms. These organisms are found most often in meat products, poultry products, fish, gravies, soups, rice, and potato salads (Midura, 1970; New and Notes, 1973; CDC, 1975; Lai-King and Stiles, 1978; Giannella et al., 1979). Because it is very difficult to control food poisoning by

these bacteria, prevention becomes a matter of stopping the growth of these organisms or preventing their entry into the food products.

In order to test the effectiveness of spice oils as a nitrite substitute against the three microorganisms, in vitro and in vivo evaluations were designed. The in vitro tests consisted of several variations. The first test was a growth inhibition test of the bacteria on tryptic soy agar (TSA) media containing the spice oils, and at the same time, the influence of pH on the antimicrobial activity of the spice oils was also determined. The spice oil with the highest antimicrobial activity was selected for the second in vitro test. Here, a growth inhibition test of the bacteria by the "ideal" spice oil in a complex organic environment of either cooked meat bacterial media or purina rat chow compared these results with the previous in vitro findings.

In order to determine possible toxic hazard effects of the "ideal" spice oil in rats, an in vivo test was designed which consisted of including the "ideal" spice oil in the diet of laboratory rats. The diet was given to rats for one month. Alterations of the growth of the rats were determined and compared with the in vitro findings.

MATERIALS AND METHODS

Essential Oils

Garlic, onion, clove, and oregano essential oils were kindly provided by Fritzsche, Dodge, and Olcott Inc., New York, NY.

Cultures

Staphylococcus aureus, Salmonella typhimurium, and Bacillus cereus were obtained from the Division of Biological Sciences stock culture collection.

Culture Standardization and Growth Curves

The cultures of Staphylococcus aureus, Salmonella typhimurium, and Bacillus cereus were standardized as described below. First, each microorganism was grown on a TSA slant for 15 hr at 37 C. Two loops of this growth were used to inoculate two milliliters of fresh tryptic soy broth (TSB). The TSB was then diluted to 10^{-6}. Twenty milliliters of fresh TSB were inoculated with 0.1 ml of the 10^{-6} dilution. The 20 ml was incubated in a rotary water bath shaker overnight (12-15 hr) at 37 C. After this incubation, the optical density at 425 nm of the overnight culture was taken. The overnight culture (2.0 ml) was used to inoculate 18 ml of fresh TSB and then incubated in a rotary water bath shaker at 37 C. The optical density (425 nm) of this culture was taken every 30 min. and 0.1 ml of this culture was diluted to 10^{-7} to allow plate counts. Plate counts of dilutions 10^{-5}, 10^{-6}, and 10^{-7} were used to determine colony forming units (CFU). The time, optical density, and the colony forming units per milliliter (CFU/ml)

for each microorganism were recorded and plotted to produce a growth curve for each microorganism. The growth curve showed how many CFU/ml there were at a specific optical density (Appendix 1).

Agar Screening Test

The four essential oils were screened for their ability to inhibit growth of these three microorganisms using tryptic soy agar (TSA) medium. The medium was prepared at different pHs (5.0, 6.0, 7.0, and 8.0) by using 1N HCL and 1N NaOH. Oils were diluted in 95 % ethanol, the final percent of ethanol was 1 %. Dilutions of spice oils were dispersed in the media to give final concentrations of 10, 25, 50, 100, and 200 ppm (Conner and Beuchat et al., 1984). After sterilization, TSA plates were poured to a thickness of 5-6 mm and allowed to dry at room temperature for 24 hr or more. There were two controls in this experiment, one containing no added ethanol or essential oil and the other containing only the diluent, ethanol (1 %).

Following the standardization of the cultures, each TSA plate was inoculated with 0.1 ml of standardized S. aureus, S. typhimurium, and B. cereus suspension. Each plate was inoculated with approximately 10^8 CFU. After inoculation, the plates were incubated at 37 C for 24 to 72 hrs. The CFU/ml on each plate was determined after 24, 48, and 72 hr. At each pH, the CFU/ml on the control plates (ethanol and no ethanol) was then compared with the CFU/ml on the plates containing different amounts of each essential oil in order to calculate the percent of inhibition. Having calculated the percent of inhibition for each spice

oil against the three bacteria, the effect of pH on the antimicrobial activity of each spice oil and the minimum inhibitory concentration (MIC) of each spice oil was determined. The screening tests were repeated three times per species.

Complex Food Influence on Previously Determined Spice Oil MIC

Cooked meat media and purina rat chow were used to evaluate the influence that complexities of food may have on the antimicrobial effect of garlic oil. The cooked meat medium was prepared at different pHs (6.0, 7.0, and 8.0) by using a phosphate buffer containing 0.067 M potassium phosphate and 0.067 M sodium phosphate. The purina rat chow was kept at its natural pH of 6.5. Both media were distributed into tubes and sterilized. The oil with the highest antimicrobial activity was then diluted as previouly described in 95 % ethanol and dispersed into the media to give final concentrations of 200, 250, 300, 350, and 400 ppm. There were two controls, one contained no ethanol or essential oil and the other contained only ethanol (1 %).

After addition of the oil, the tubes containing a total of 10 ml were inoculated with 0.1 ml of 10^9 CFU/ml standardized Staphylococcus aureus, Salmonella typhimurium, and Bacillus cereus suspension. The tubes were incubated statically at 37 C in a water bath for 120 hr. The bacterial growth was determined by taking the optical density (425 nm) of each tube after 24, 72, and 120 hr and comparing to the concentration of spice oil in that tube. These tests were repeated three times.

Experimental Animals

Ten male, 40 day old albino rats of similar weight were obtained from the Emporia State University animal facility. They were maintained in the animal facility at Emporia State University under standard USDA regulations.

Food Preparation

Thirty microliters of the oil to be tested were diluted in 3.0 ml of 95 % ethanol to give 400 ppm of the spice oil and 1 % ethanol. The three milliliters were then incorporated into 75 g of purina rat chow and mixed to ensure uniform distribution. A control diet was also prepared having 1 % ethanol without any oil extract. Both diets were dried at 60 C for 8 hr.

Toxicity Test

The ten rats were distributed into 2 groups. They were housed and fed 15 g of food per day. One group was treated as a control and fed only ethanol treated purina chow diet. The other group was fed with purina rat chow plus the oil extract. Feeding was continued for a period of 4 weeks. The animals were weighed before and after the feeding in order to determine if the spice oil altered their growth. The weight gain was recorded at the end of four weeks, and the difference between the means of the control and experimental group was analysed via a t-test.

RESULTS

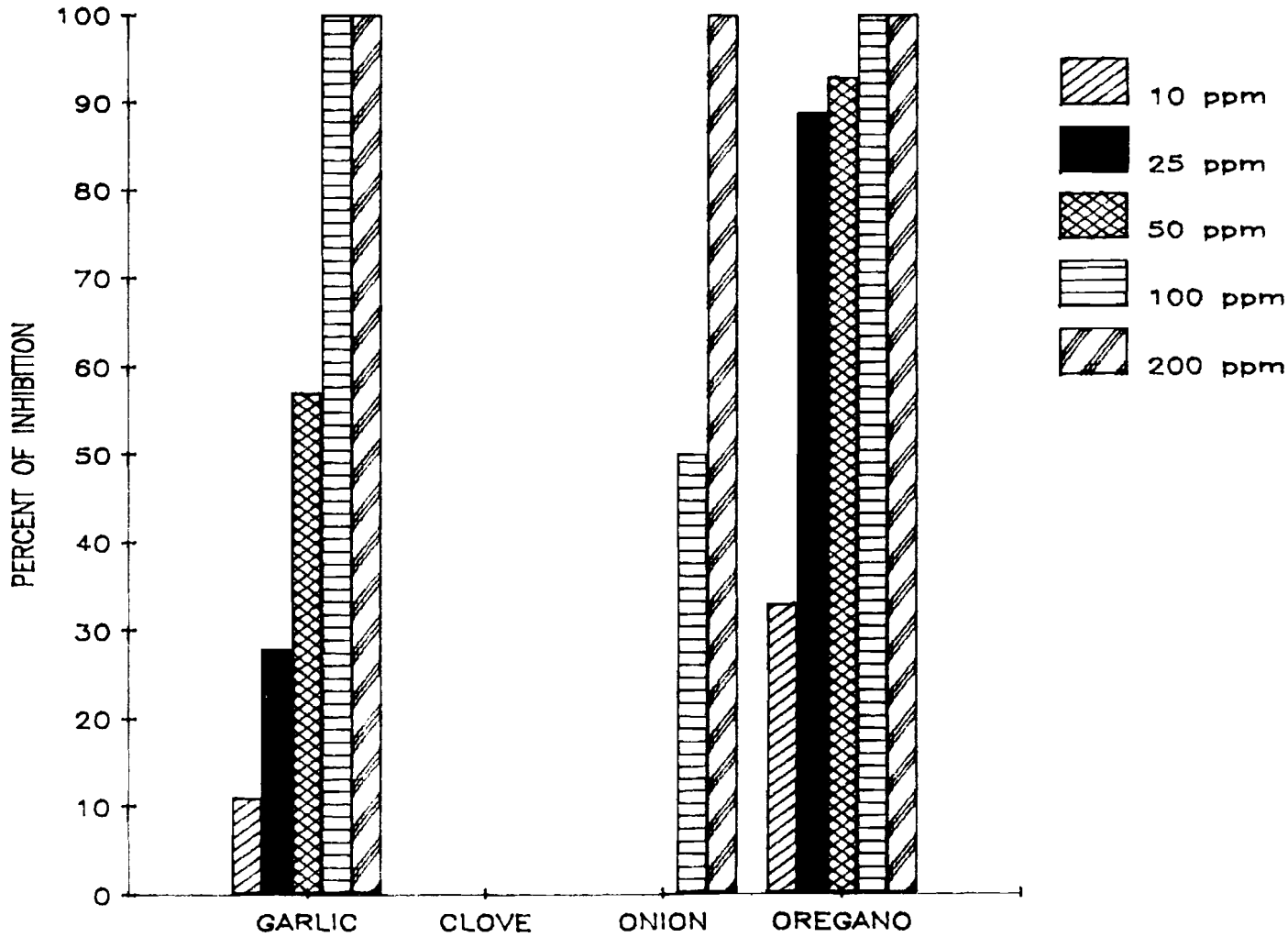
Agar Screening Test

The results of the tests that were designed to examine the effects of garlic, onion, clove, and oregano essential oils on the growth of the test microorganisms at pH 5.0, 6.0, 7.0, and 8.0 are illustrated in Figures 1 through 10. There were two controls in this experiment one containing only 1 % ethanol and the other containing no ethanol or essential oil. The results showed that 1 % ethanol does not affect the growth of the three microorganisms as compared with the other control. Therefore, the antimicrobial effect shown in each figure is only due to the presence of the essential oil.

Figures 1 to 4 present the effect of the four spice oils at 10, 25, 50, 100, and 200 ppm on the growth of S. aureus. Figure 1 shows that at pH 5.0, oregano oil was the most effective in inhibiting the growth of S. aureus followed by garlic oil. Onion, oregano, and garlic oils at 200 ppm completely prevented bacterial growth. However, at 100 ppm only garlic and oregano oils inhibited the growth of S. aureus. Onion oil lost 1/2 of its effectiveness at 100 ppm. Clove extract showed no growth inhibition against this Gram-positive organism even at the highest level, 200 ppm.

Figure 2 presents the growth inhibition of S. aureus by the four spice oils at pH 6.0. Both onion and oregano oils showed less effectiveness against S. aureus at pH 6.0. The antimicrobial activity of these two spice oils was reduced by about 50 % due to the pH shift from 5 to 6. Contrary to onion and oregano oils, garlic oil showed

Fig. 1. Effects of essential oils on growth of S. aureus at pH 5.0


PERCENT OF INHIBITION
100
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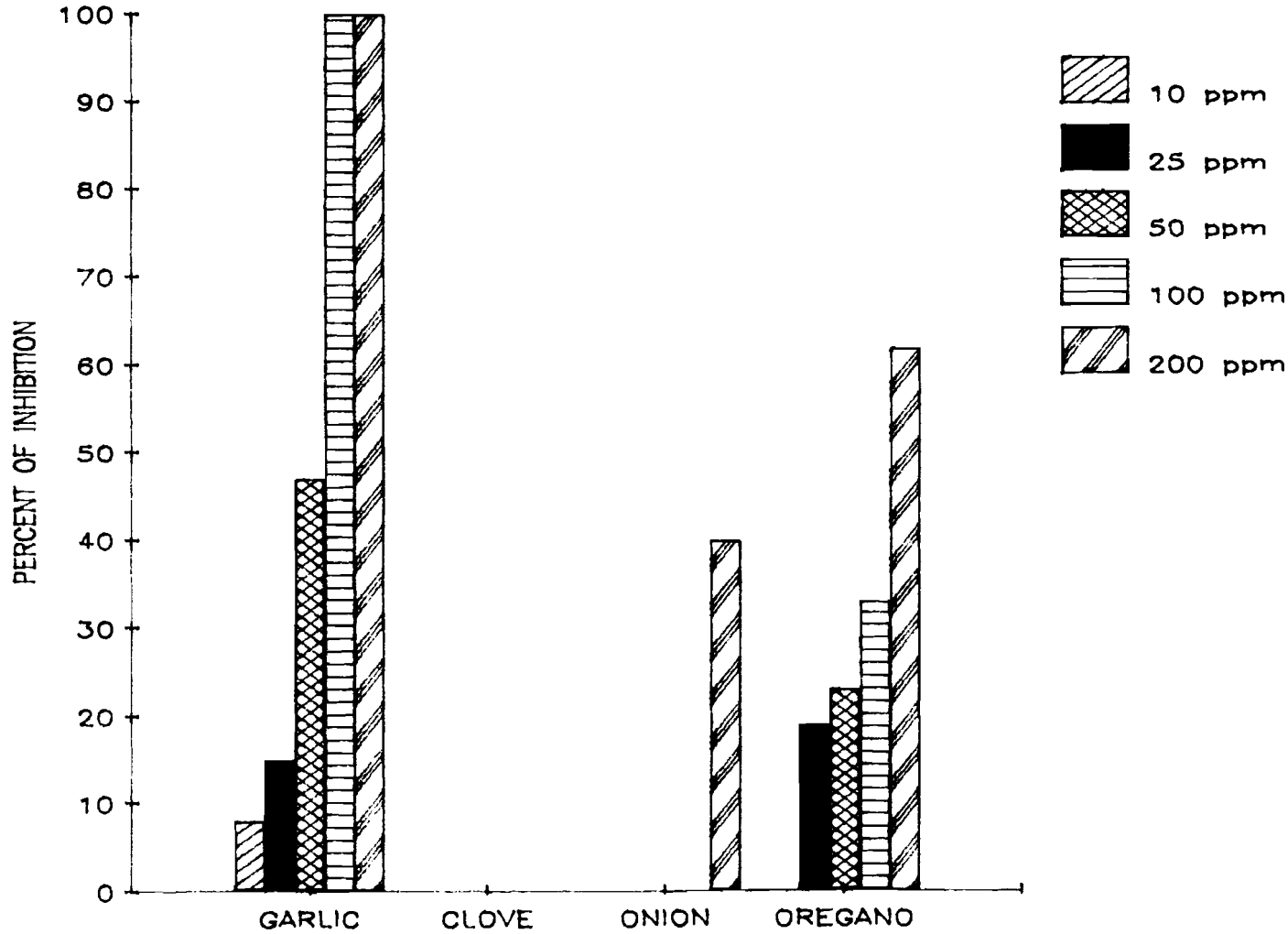
GARLIC
CLOVE
ONION
OREGANO
10 ppm
25 ppm
50 ppm
100 ppm
200 ppm

Fig. 2. Effects of essential oils on growth of S. aureus at pH 6.0


PERCENT OF INHIBITION
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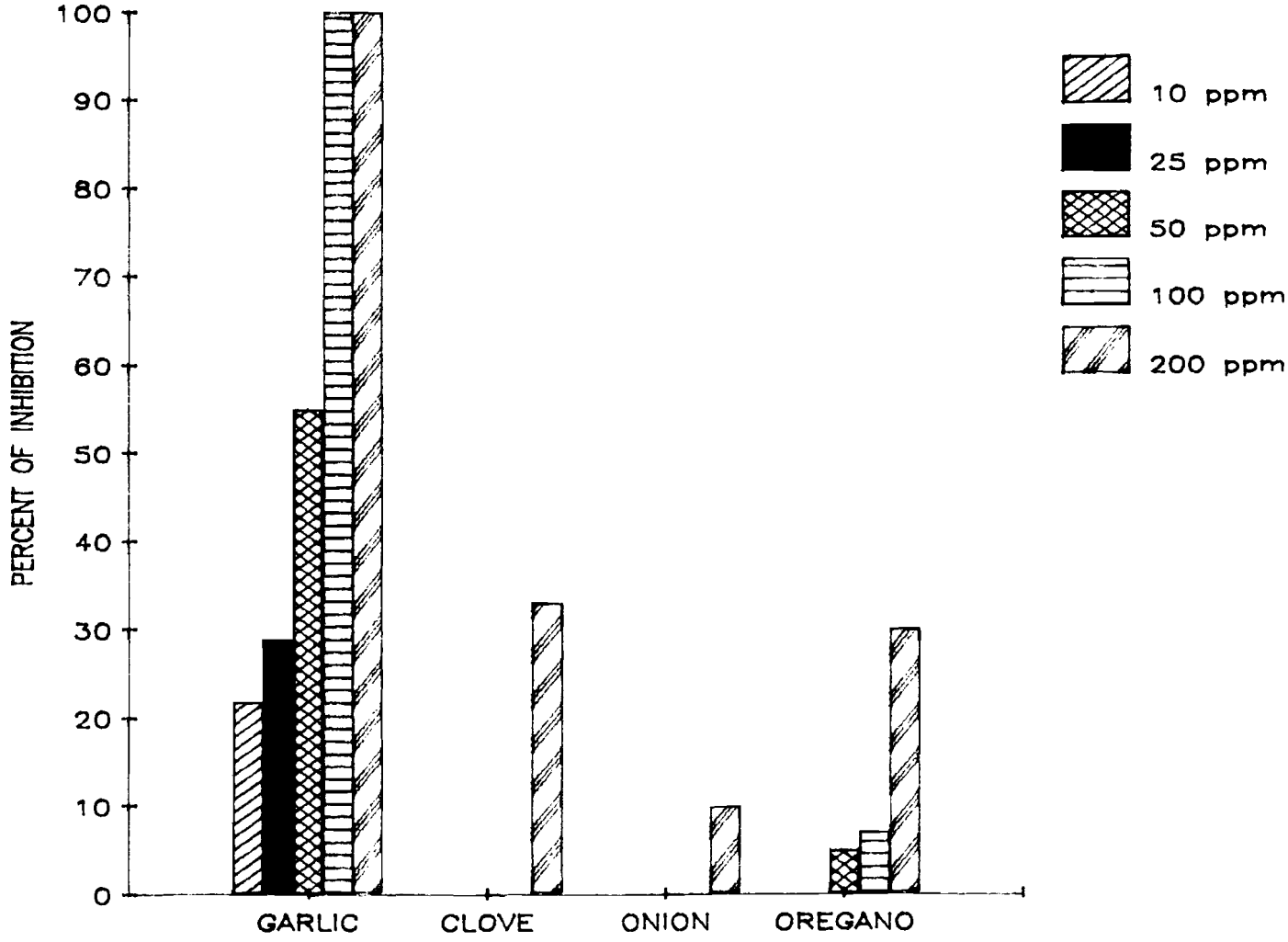
GARLIC
CLOVE
ONION
OREGANO
10 ppm
25 ppm
50 ppm
100 ppm
200 ppm

Fig. 3. Effects of essential oils on growth of S. aureus at pH 7.0.


PERCENT OF INHIBITION
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GARLIC
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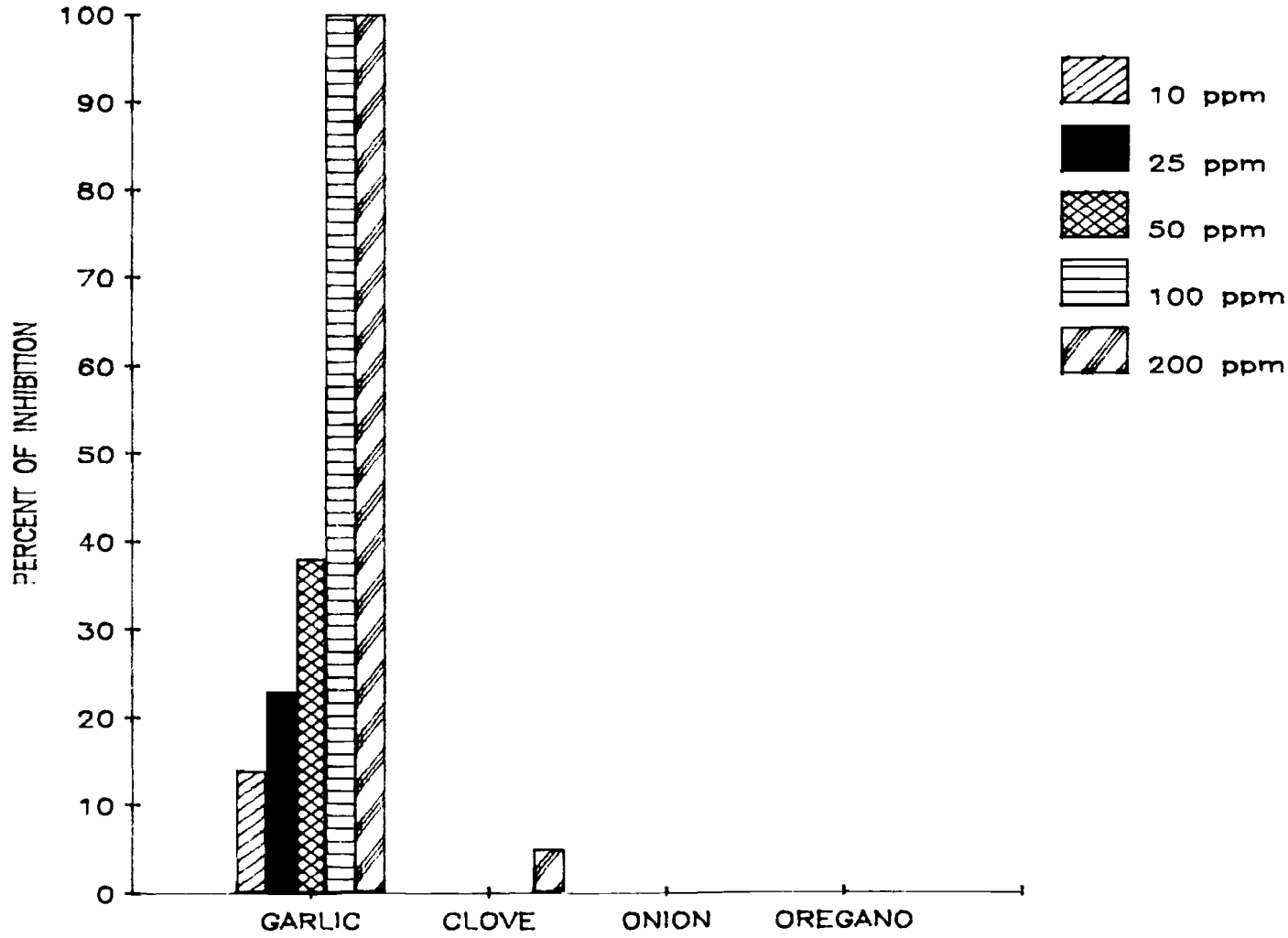
ONION
OREGANO
10 ppm
25 ppm
50 ppm
100 ppm
200 ppm

Fig. 4. Effects of essential oils on growth of S. aureus at pH 8.0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10
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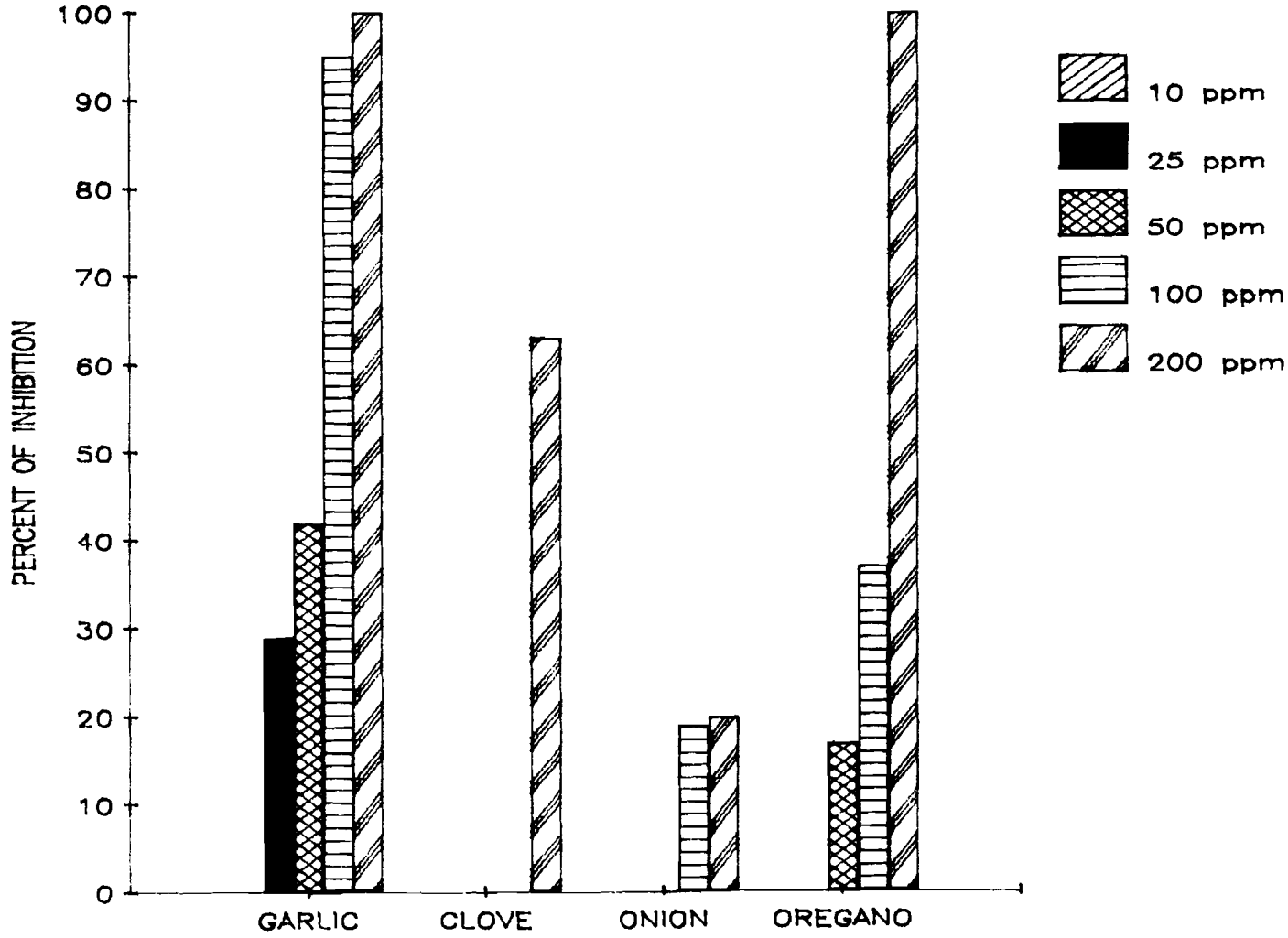
GARLIC
CLOVE
ONION
OREGANO
10 ppm
25 ppm
50 ppm
100 ppm
200 ppm

similar results to those seen at pH 5.0. The pH did not affect the antimicrobial effect of garlic oil. Clove oil was again unable to inhibit the growth of S. aureus. Figure 3 shows the antimicrobial action of the four spice oils on the growth of S. aureus at pH 7.0. At this pH, the antimicrobial activity of onion and oregano oils was reduced again to approximately 1/3 the effectiveness seen at pH 5.0. Garlic oil again did not show any changes in its antimicrobial activity. Clove oil, for the first time, showed a 33 % inhibition of the bacterial growth at 200 ppm. Figure 4 shows that at pH 8.0, garlic oil was the only spice oil that could inhibit the growth of S. aureus. With the exception of garlic, it appears that a pH shift from 5 to 8 is detrimental to the inhibitory action of these spice oils against S. aureus.

Figures 5 to 7 present the antimicrobial effect of the spice oils on the growth of S. typhimurium at pH 5.0, 6.0, 7.0, and 8.0. Figure 5 shows that at pH 5.0, garlic and oregano oils could inhibit 100 % of the growth of S. typhimurium at 200 ppm. Onion oil was not as effective against S. typhimurium at pH 5.0 as it was against S. aureus at the same pH. Clove oil, however, inhibited 63 % of the bacterial growth at 200 ppm. Clove oil was more effective against S. typhimurium than it was against S. aureus at pH 5.0. Figure 6 shows the growth inhibition of S. typhimurium by these spice oils at pH 6.0. In contrast to S. aureus, none of the spice oils completely inhibited the growth of S. typhimurium. Curiously, the percent of growth inhibition by garlic oil at pH 6.0 did not change even though the concentration of

Fig. 5. Effects of essential oils on growth of S. typhimurium at pH 5.0


PERCENT OF INHIBITION
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GARLIC
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OREGANO
10 ppm
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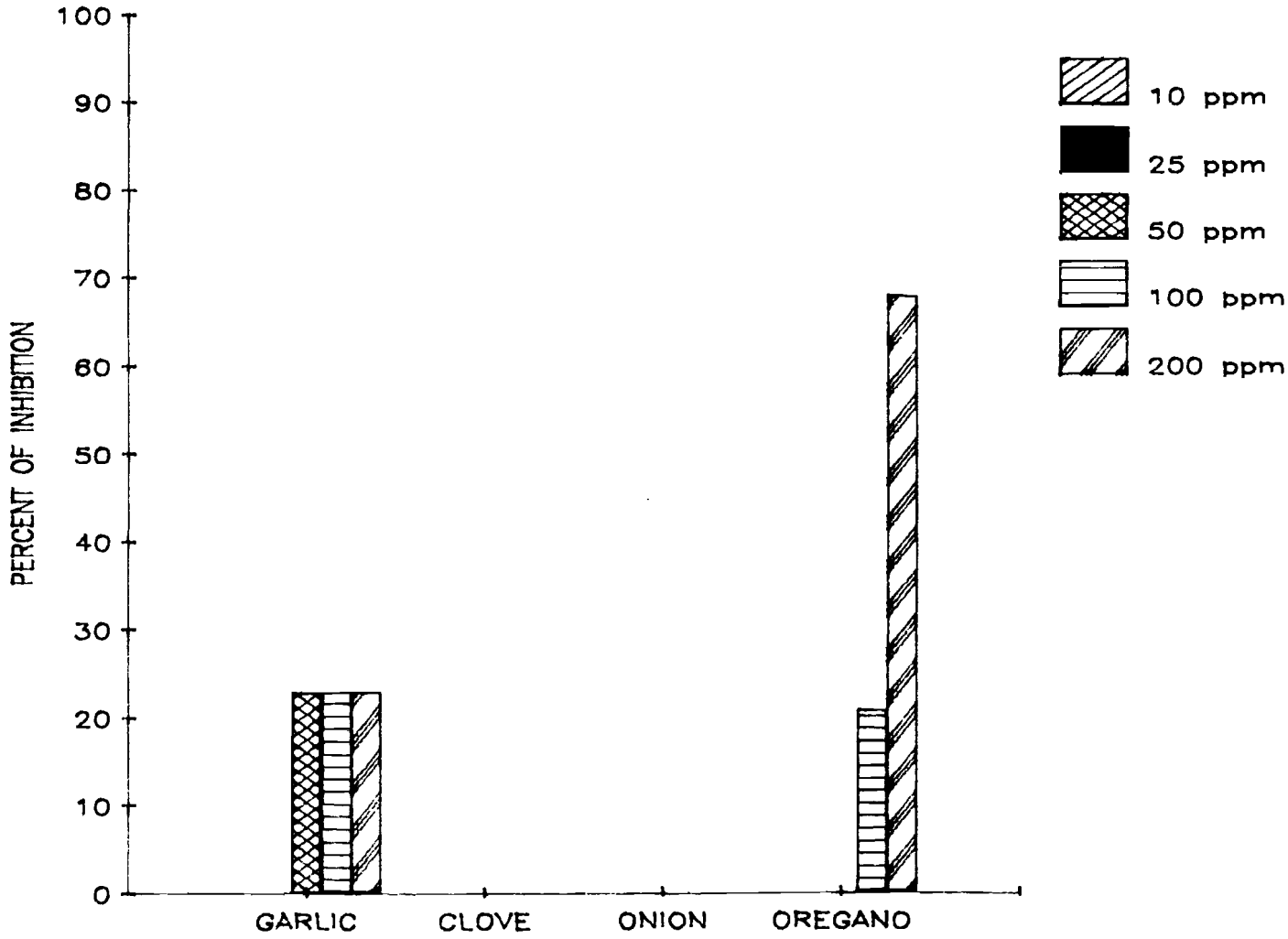
Fig. 6. Effects of essential oils on growth of S. typhimurium at pH 6.0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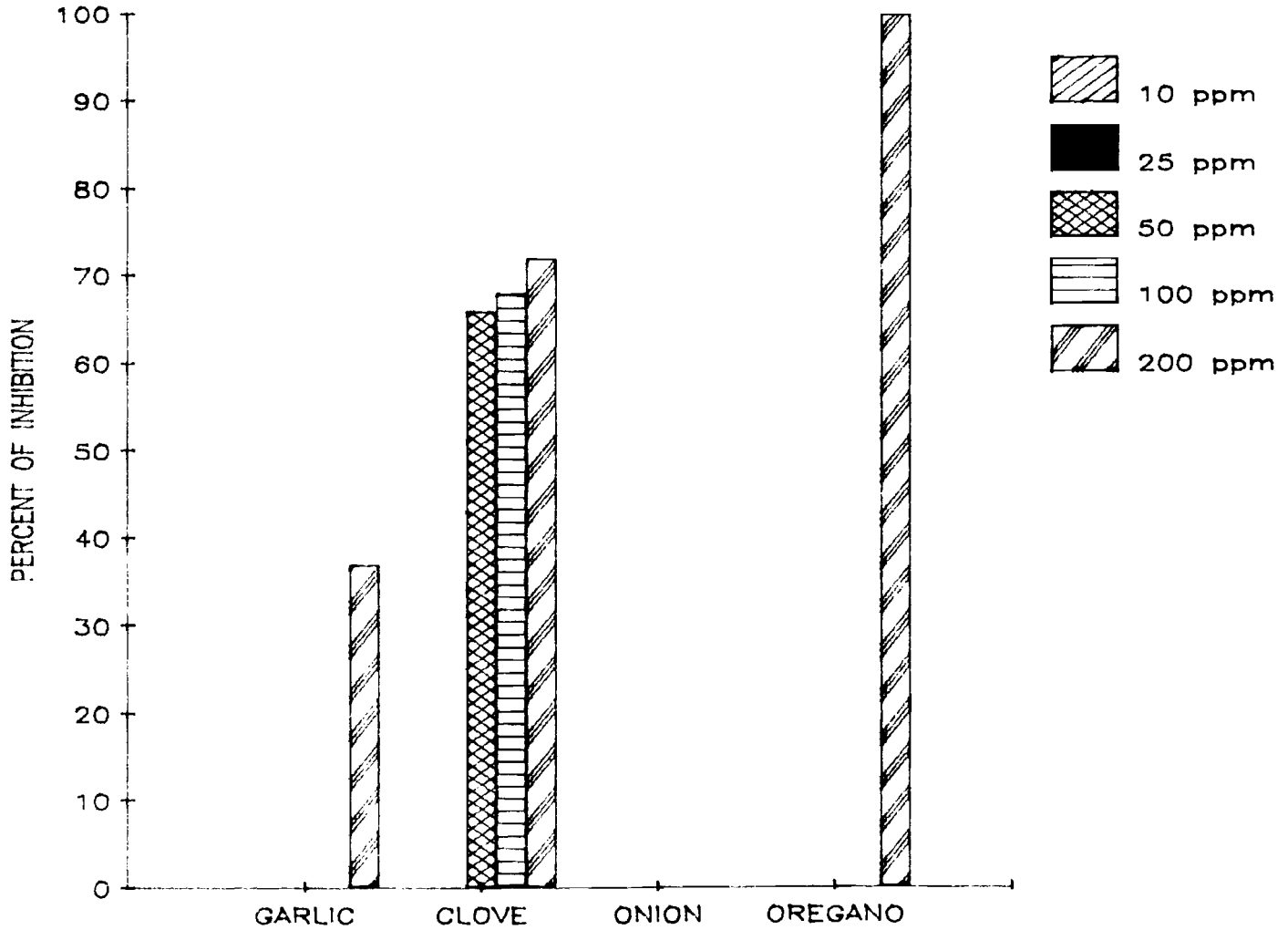
PERCENT OF INHIBITION
GARLIC
CLOVE
ONION
OREGANO
10 ppm
25 ppm
50 ppm
100 ppm
200 ppm

Fig. 7. Effects of essential oils on growth of *S. typhimurium* at pH 8.0.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GARLIC
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OREGANO
10 ppm
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50 ppm
100 ppm
200 ppm

the oil ranged from 50 to 200 ppm.

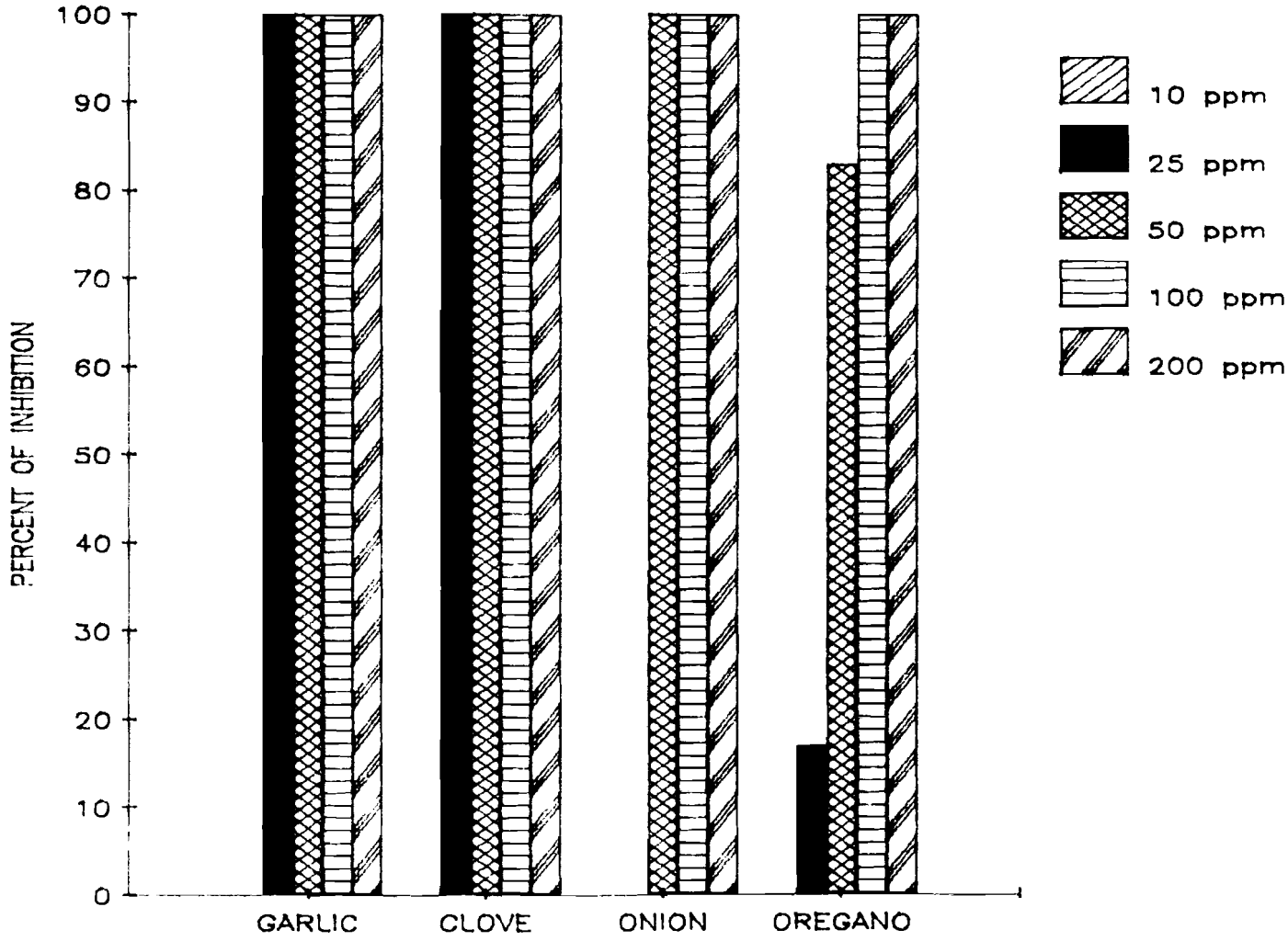
The results at pH 7.0 were not shown because it was found that at this pH none of the spice oils were able to inhibit the growth of S. typhimurium.

In Figure 7, the antimicrobial effect of the spice oils on the growth of S. typhimurium at pH 8.0 is shown. Oregano oil did not show any antimicrobial activity until the concentration of the oil was increased to 200 ppm. Lower levels of this oil were not inhibitory. Clove oil was more effective against S. typhimurium at pH 8.0 than at pH 5.0. Onion oil did not show antimicrobial activity at pH 8.0; however, it inhibited 20 % of the growth of S. typhimurium at pH 5.0.

Figures 8 to 10 present the antimicrobial effect of the spice oils on the growth of B. cereus at pH 6.0, 7.0, and 8.0 (The results at pH 5.0 were difficult to obtain because B. cereus did not grow well).

Figure 8 shows that at pH 6.0, the spice oils could inhibit the growth of B. cereus. It appears that B. cereus was the most sensitive to the antimicrobial effects of the spice oils among the three bacteria tested. None of the spice oils inhibited the growth of B. cereus at 10 ppm. However, garlic and clove oils inhibited 100 % the bacterial growth at 25, 50, 100, and 200 ppm. Onion oil did not show any antimicrobial activity until the concentration of the oil was increased to 50 ppm. This oil inhibited 100 % the bacterial growth at 100 and 200 ppm. Oregano oil showed less effectiveness against B. cereus than the other spice oils at pH 6.0. In Figure 9 the antimicrobial effect of the spice oils on the growth of B. cereus at pH 7.0 is shown. At

Fig. 8. Effects of essential oils on growth of *B. cereus* at pH 6.0.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OREGANO
10 ppm
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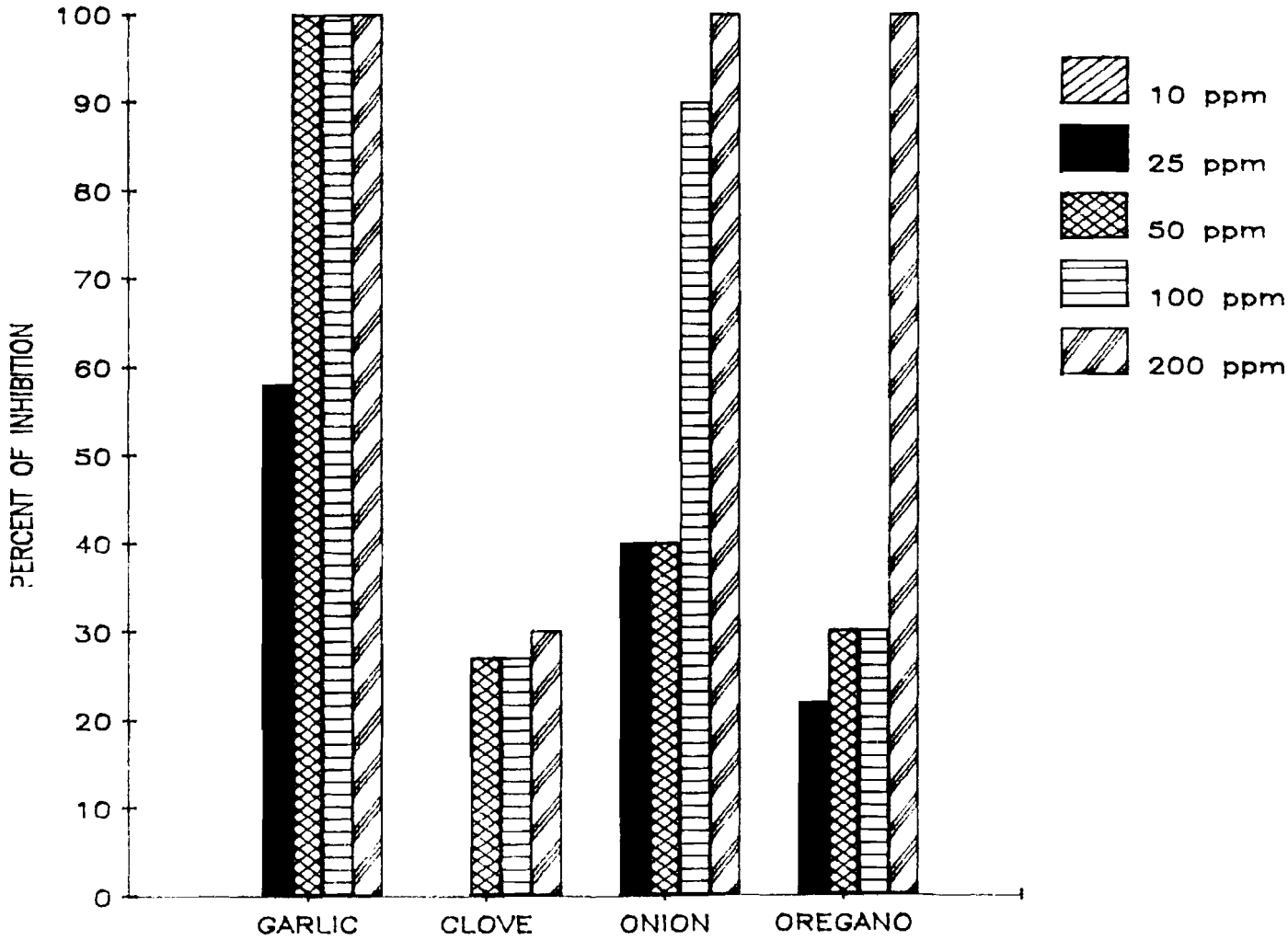
200 ppm

Fig. 9. Effects of essential oils on growth of *B. cereus* at pH 7.0.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OREGANO
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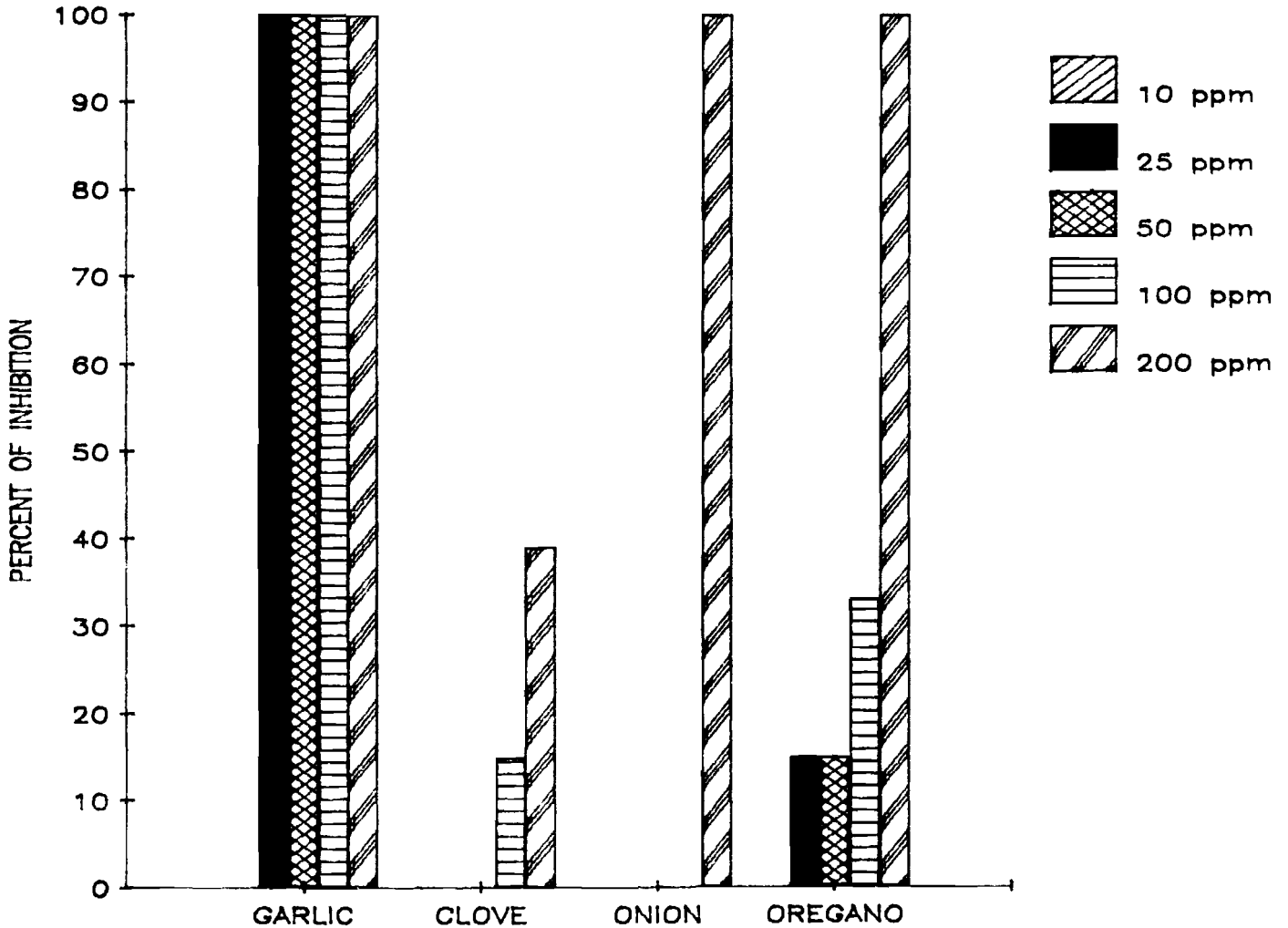
Fig. 10. Effects of essential oils on growth of *B. cereus* at pH 8.0.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100 ppm
200 ppm

this pH, the antimicrobial activity of the spice oils was reduced as seen with the other two bacteria. The antimicrobial activity of clove oil was severely reduced at pH 7.0 when compared to its pH 6.0 results. Figure 10 shows the growth inhibition of B. cereus by the spice oils at pH 8.0. The spice oils lost their antimicrobial activity at this pH with the exception of garlic. Garlic oil still inhibited 100 % the growth of B. cereus at 25, 50, 100, and 200 ppm at pH 8.0. These results were similar to those at pH 5.0. Tables V and VI summarize the results of these tests. Garlic oil was chosen for the additional tests because of its overall effectiveness against the three microorganisms. Out of 11 combinations of microbe types and pH, garlic oil gave 100 % inhibition in 8 of those combinations. In addition, according with the overall results, the minimum inhibitory concentration (MIC) of garlic oil was 200 ppm.

Food Influence on Spice Oil

This experiment was conducted in order to determine if the previouly determined MIC of garlic oil is influenced in a more complex environment. Two different media were used for this evaluation. One was commercial, bacteriological cooked meat medium (Difco), and the other was commercial rat chow (Purina). The growth of B. cereus and S. aureus was completely inhibited in cooked meat at 200 ppm (Table VII). However, S. typhimurium was only partially inhibited at this concentration. Therefore, the concentration of garlic oil was increased to determine if higher concentrations would influence this

Table V. Percent of Growth Inhibition of *S. aureus*, *S. typhimurium*, and *B. cereus* by Spice Oils at pH 5.0 and 6.0.

		Concentration of Spice (ppm)									
		pH 5.0					pH 6.0				
Microorganism	Spice	10	25	50	100	200	10	25	50	100	200
S. aureus	Garlic	11	28	57	100	100	8	15	47	100	100
	Clove	0	0	0	0	0	0	0	0	0	0
	Onion	0	0	0	50	100	0	0	0	0	40
	Oregano	33	89	93	100	100	0	19	23	33	62
S. typhimurium	Garlic	0	29	42	95	100	0	0	23	23	23
	Clove	0	0	0	0	63	0	0	0	0	0
	Onion	0	0	0	19	20	0	0	0	0	0
	Oregano	0	0	17	37	100	0	0	0	21	68
*B. cereus**	Garlic	-	-	-	-	-	0	100	100	100	100
	Clove	-	-	-	-	-	0	100	100	100	100
	Onion	-	-	-	-	-	0	0	100	100	100
	Oregano	-	-	-	-	-	0	17	83	100	100

* The results at pH 5.0 were difficult to obtain because *B. cereus* did not grow well.

Table VI. Percent of Growth Inhibition of *S. aureus*, *S. typhimurium*, and *B. cereus* by Spice Oils at pH 7.0 and 8.0.

		Concentration of Spice (ppm)									
		pH 7.0					pH 8.0				
Microorganism	Spice	10	25	50	100	200	10	25	50	100	200
S. aureus	Garlic	22	29	55	100	100	14	23	38	100	100
	Clove	0	0	0	0	33	0	0	0	0	5
	Onion	0	0	0	0	10	0	0	0	0	0
	Oregano	0	0	5	7	30	0	0	0	0	0
S. typhimurium	Garlic	0	0	0	0	0	0	0	0	0	37
	Clove	0	0	0	0	0	0	0	66	68	72
	Onion	0	0	0	0	0	0	0	0	0	0
	Oregano	0	0	0	0	0	0	0	0	0	100
B. cereus	Garlic	0	58	100	100	100	0	100	100	100	100
	Clove	0	0	27	27	30	0	0	0	15	39
	Onion	0	40	40	90	100	0	0	0	0	100
	Oregano	0	22	30	30	100	0	15	15	33	100

microbe in a food or food-like environment. S. typhimurium was completely inhibited at a concentration of garlic oil of 400 ppm (Table VII).

Comparing these results with those in the agar screening test, the MIC of garlic oil tested in the absence of particulate food was lower than the MIC of garlic oil in its presence. It appears that the presence of proteins, fat, fiber, and other complex nutrients influence the antimicrobial effect of garlic. This experiment also demonstrated that the pH of the "food" did not affect the antimicrobial activity of garlic oil against the test microorganisms.

Table VIII shows the growth inhibition of the three microorganisms by garlic oil in purina rat chow as the experimental medium. The MIC of garlic oil was similar to the MIC determined by using cooked meat medium. According to the results of this test, the minimal inhibitory concentration of garlic oil was found to be 400 ppm.

Toxicity Test

After having determined that garlic oil was the most "ideal" antimicrobial agent, it was incorporated into purina rat chow in order to determine if any toxic effects occurred using healthy laboratory rats. Each rat in both the control and garlic treated groups ate all 15 g of diet given to them daily. The results are presented in Table IX. After one month feeding garlic oil, there was a significant 15 % decrease in the weight of the rats fed with garlic oil diet as compared to the control. The statistical analysis (t-test) is presented in

Table VII. Inhibition of growth of food-borne microorganisms by garlic oil in cooked meat.*

pH	Microorganism	Optical density (425 A) Control	200 ppm	300 ppm	400 ppm
6.0	B. cereus	26.0	00.0	00.0	00.0
	S. aureus	56.0	00.0	00.0	00.0
	S. typhimurium	32.0	25.0	22.0	00.0
7.0	B. cereus	21.0	00.0	00.0	00.0
	S. aureus	52.0	00.0	00.0	00.0
	S. typhimurium	34.0	29.0	17.0	00.0
8.0	B. cereus	10.0	00.0	00.0	00.0
	S. aureus	23.0	00.0	00.0	00.0
	S. typhimurium	20.0	13.0	10.0	00.0

* Cultures were examined after 5 days of incubation at 37 C.

Table VIII. Growth inhibition of food-borne microorganisms by garlic oil in rat chow.*

Microorganism	Optical density (425 A)		
	Control	300 ppm	400 ppm
B. cereus	06.0	00.0	00.0
S. aureus	14.0	00.0	00.0
S. typhimurium	10.0	08.0	00.0

* Cultures were examined for 5 days of incubation at 37 C purina rat chow (pH 6.5).

Table IX. Effect of feeding garlic on the growth of albino rats.*

	Initial weight (g)		Weight after four weeks (g)	
Rat	Control	Garlic oil	Control	Garlic oil
1	97.30	112.40	274.30	216.10
2	96.60	96.10	265.60	214.00
3	92.80	92.10	256.60	209.10
4	90.50	85.30	224.40	206.80
5	88.20	79.20	209.80	200.30
Average	93.08	93.02	246.14	209.26
Percent of weight loss	--	--	--	14.98

* The amount of garlic given daily to each rat was 400 ppm.

Table X, and it shows that the mean of the control group is significantly different from the mean of the experimental group. There were no deaths or other visible changes in the treated group. Both groups appeared to have healthy looking coats, activity levels and behavior.

Table X. Statistical analysis (t-test) of the toxicity test

Group	Final weight		Initial weight		Y	Y-Y	(Y-Y)
Control	244.3	-	97.3	=	177.0	23.94	573.12
	265.6	-	96.6	=	169.0	15.94	254.08
	256.6	-	92.8	=	163.8	10.74	115.35
	224.4	-	90.5	=	133.9	-19.16	367.11
	209.8	-	88.2	=	121.6	-31.46	989.73
Experimental	216.1	-	112.4	=	103.7	-12.54	157.25
	214.0	-	96.1	=	117.9	1.66	2.76
	209.1	-	92.1	=	117.0	0.76	0.58
	206.8	-	85.3	=	121.5	5.26	27.67
	200.3	-	79.2	=	121.1	4.86	23.62

$\bar{Y}_1 = 153.06$ $\quad n = 51$

$\bar{Y}_2 = 116.24$ $\quad df = 2(n-1) = 2(4) = 8$

$$S_1^2 = \frac{\sum (Y-Y1)^2}{n-1} = \frac{2299.39}{4} = 574.85$$

$$S_2^2 = \frac{\sum (Y-Y2)^2}{n-1} = \frac{211.87}{4} = 52.97$$

$$t = \frac{(\bar{Y}1-\bar{Y}2)}{\sqrt{1/n\ (S_1^2 + S_2^2)}} = \frac{153.06-116.24}{\sqrt{1/5\ (574.85+52.97)}} = \frac{36.82}{11.20} = 3.28$$

Critical value: $t_{0.05\ [8]} = 2.31$

t_s is higher than the critical value; therefore, the means are significantly different.

DISCUSSION

This study demonstrated that spice oils have the ability to inhibit the growth of microorganisms, particularly microorganisms that are known to cause food-borne diseases. Not all spice oils have the same antimicrobial activity. For example, in the present study, garlic oil consistantly had the highest antimicrobial activity. It was a very effective inhibitor against both B. cereus and S. aureus which are Gram-positive organisms. At 200 ppm of garlic oil, both microorganisms were inhibited at pH 5.0, 6.0, 7.0, and 8.0 (Figures 1-4 and 8-10). It appears that the pH of the media did not affect the antimicrobial activity of garlic oil at 200 ppm. However, comparing the results of Figure 5 to those in Figure 1, it appears that S. typhimurium, which is a Gram-negative bacillus, was less sensitive to the antimicrobial effect of garlic oil than S. aureus, especially at an alkaline pH (Figure 7). Garlic oil completely inhibited the growth of S. typhimurium only at pH 5.0 at levels of 200 ppm (Figure 5). It appears that garlic oil was more effective against S. typhimurium at acidic pH than at alkaline pH which is similar to the findings with S. aureus. Only when the level of garlic oil was increased to 400 ppm, did it completely inhibit the growth of S. typhimurium at pH 6.0, 7.0, and 8.0 (Tables VII and VIII). The presence of food and food-like constituents (cooked meat and rat food) also influenced the antimicrobial activity of garlic oil (tables VII and VIII). The minimal inhibitory concentration (MIC) of garlic oil in rat food and cooked meat was found to be 400 ppm. Having determined the MIC of garlic oil in these foods,

one would be able to incorporate this amount of garlic oil into foods to prevent the growth of microorganisms. Hence, garlic oil appears to be a good substitute for nitrite in foods.

Cavallito (1944) was the first investigator who identified the compound that was responsible for the antimicrobial activity of garlic oil. He named this compound allicin, whose formula is CH2=CHCH2-SO-S-CH2CH=CH2. Block (1985) demonstrated that when garlic oil was kept at 0 C, allicin appeared as its precussor molecule called allin which showed no antimicrobial activity; however, when the temperature of the oil was increased to 25 C, an enzyme called allinase was activated and it converted allin into allicin. In addition, when the temperature of the oil was increased to 100 C, allicin was converted into a molecule called diallyldisulfide which does not have antimicrobial activity. It appears that the bactericidal effect of garlic oil depends on the temperature, and this fact will be an important factor that has to be considered if garlic oil is used as a food preservative.

The present study showed that garlic oil was effective against S. aureus and B. cereus which are Gram-positive organisms and S. typhimurium which is a Gram-negative organism. However, garlic oil was more effective against the Gram-positive organisms as compared to the Gram-negative organism. This may be due to the difference in cell wall structure between the two groups. The cell wall of Gram-negative organisms is more complex than the cell wall of the Gram-positive organisms (Laskin and Lechevalier et al., 1974), and this could make it more difficult for garlic oil (allicin) to enter the cytoplasm of the

bacteria.

Cavallito (1944) suggested a mechanism by which allicin acts as a bactericidal agent against bacteria. It can react with the amino group cysteine. The sulfhydryl (-SH) group was postulated by Hammett (1931) as a specific stimulator of cell multiplication. Allicin, therefore, may operate by destroying SH groups that are specific for cellular proliferation, thus inhibiting bacterial growth.

Cavallito (1946) also classified antimicrobics attacking sulfhydryl compounds into three groups depending on their specificity. Members of the first group are those that attack nearly all sulfhydryl compounds, and react rapidly with the SH group of cysteine and cysteine peptides. Allicin is a member of this group. The second, and third group which includes penicillin, only attack specific sulfhydryl groups. Wills (1956) showed that inhibition of sulfhydryl enzymes was associated with the presence of the sulfur group found in the chemical structure of allicin. He also demonstrated that allicin was able to dissolve into the lipid portion of lipoproteins of the cell membrane allowing allicin to inactivate the sulfhydryl enzymes. Consequently, cell metabolism, respiration, and multiplication would be disrupted, and this would result in inhibition of the bacterial growth. This would also protect foods from microbial spoilage or acting as a disease vector if allicin were used as a food preservative.

Buchman (1987) suggested that knowing the mechanism by which nitrite interferes with microbial growth would help discover other

compounds which mimic the same mechanism. Such compounds might be extremely important as new food preservatives or antibiotics. He demonstrated that nitrite inhibits microbial growth by inactivating membrane sulfhydryl groups, and suggested that the sulfhydryl groups of bacterial membranes constitute an antimicrobic target. In this respect, nitrite mimics the action of allicin. Allicin also inactivates membrane sulfhydryl groups. This present research supports the conclusion that allicin, a natural compound, would be a good subtitute for sodium nitrite in the preservation of foods.

One concern about food additives is their possible role as mutagens or carcinogens. Garlic has been found to be mutagenic in bacteria (Takemura and Shimizu et al., 1978). However, Abraham and Kesavan (1984) evaluted possible genotoxic effects following administration of 7.5 ml/kg body weight of garlic to mice and found that garlic did not enhance the frequency of micronucleated polychromatic erythrocytes. They concluded that garlic oil failed to be a carcinogen when tested in vivo using mammals. Therefore, garlic is an example of a substance that is mutagenic but not carcinogenic and appears to be an excellent candidate as a substitute for nitrite in foods.

The Food and Drug Administration (FDA, 1974) affirmed that garlic and garlic oil were generally recognized as safe. However, subsequent studies demonstrated that garlic oil caused serious side effects such as death, skin irritation, and organ injuries when it was consumed at 5 ml/kg body weight (Nakagawa et al., 1980). They reported that

administration of raw garlic juice at 5 ml/kg body weight to rats for 3 weeks caused 5 deaths out of 20 animals, and the remaining 15 animals had marked growth retardation due to severe stomach injury. Contrary to Nakagawa, Shashikanth (1986) reported that no deaths were seen in rats when garlic was administered for 4 weeks at 5 ml/kg body weight. He postulated that his contrary result was due to the protection given by ad libitum food availability and water which prevented the irritant action of garlic on epithelial tissue of the inner walls of the stomach.

Other studies have demonstrated that 2.5 ml/kg body weight of raw garlic can cause dermatitis due to an allergic response to its high sulfur content (Burks, 1954; Vanketel, 1978; Mitchell, 1980; Lybarger, 1982; Campolmi et al., 1980). Bleumink (1972) showed that the active sensitizer found in garlic was water-soluble, had a low molecular weight, and was heat labile. He indicated that domestic cooking for 15 min. and treatment with dilute acid inactivated the allergic reaction of this substance. Papageorgiou (1983) demonstrated that garlic was a sensitizer in both humans and animals. He identified the allergens of garlic which are diallyldisulfide, allylpropyldisulfide, and allicin. Allicin is responsible for inducing skin irritation at high levels, and thus has antimicrobial, allergenic, and direct irritant properties. Hence, it appears that garlic's adverse effects on humans if used as a nitrite substitute would need further testing, although its use in foods to be cooked, hot dogs for example, may be implemented with little concern.

In the present study, the toxicity of garlic oil was also questioned. Since previous studies used large doses of the spice in animals and this research focused on antimicrobial effects, food supplemented with garlic oil was tested in vivo. After feeding 5 rats with garlic oil (400 ppm) for one month, the rats had a significant 15 % lower weight gain than the matched controls (Table IX). There were no deaths or other notable changes between the two groups. The treated group had healthy looking fur and similar activity levels compared to the controls. Comparing the dose of garlic oil (0.03 ml/kg body weight) given to rats in the present study with the dose of garlic (5.0 - 2.5 ml/kg body weight) given to animals in the previous studies which demonstrated allergic effects and stomach injuries, it appears that 400 ppm of garlic oil in foods is considered safe. These results eliminate the possibility that garlic oil would be toxic in humans when added to foods because of the 1/100 fold reduction in its concentration compared to previously published studies. However, further studies would need to assess the 15 % weight loss in the experimental animals.

Adding garlic oil at low levels to food may not only be a good substitute for nitrite, but also it may help overcome several diseases. Garlic has exhibited numerous, remarkable, pharmacological properties when it is consumed at 0.5 - 0.25 ml/kg body weight. Augusti (1980) found that garlic lowered the levels of glucose in hyperglycemic rabbits. Lowering of serum cholesterol and antiatherogenic activities by garlic have also been demonstrated (Mathew, 1973; Augusti, 1974; Kritchevsky, 1975; Lau et al., 1987). Studies suggest that garlic may

decrease blood pressure of humans and animals by acting like prostaglandin E1 (Malik, 1981; Ruffin, 1983; Rashid et al., 1985). Garlic has great potential as an anticoagulant by inhibiting platelet aggregation and enhancing fibrinolytic activity (Makehia et al., 1979). Amonkar (1971) demonstrated that garlic has also larvicidal activities against Culex pipiens quinquefasciatus. Garlic has been shown to be effective in the dextoxification of heavy metals by binding to them, and it also binds to exogenous toxic food additives such as nitrite (Xing et al., 1982). Garlic has antitumor activities by inhibiting the enzymes that induce tumor growth (Wargovich et al., 1987); and Kandil (1987) reported that garlic can modulate the immune system by enhancing cytotoxic T-cells, natural killer cells, and macrophages.

The volatile sulfur compounds (allicin, ajoene, and diallyldisulfide), minerals (selenium, germanium, magnesium, zinc, and calcium), vitamins (thiamine, vitamin C and A), and aminoacids are generally considered to be responsible for most of garlic's pharmacological activities. Table XI presents the probable components contributing to each pharmacological activity.

In addition to these pharmacological effects, garlic has shown wide effectiveness against a number of opportunistic microbes that are found to be infecting patients with acquired immunodeficiency syndrome (AIDS). Garlic might have a strategic role to play in the AIDS pandemic since it is able to influence microbes such as Herpesvirus hominis type I (Tsai et al., 1985), cryptococcal (Fromtling and Bulmer et al., 1978), mycobacterial (Abbruzzese et al., 1987), and candial (Barone et al., 1977) organisms.

Table XI. The medical or pharmacological spectra of garlic.*

Pharmacological Activity	Probable Components Contributing to Activity
Anticoagulation	Ajoene
Antihypertensive	Selenium, germanium
Antimicrobial	
Antiparasitic	Allicin
Antibiotic	Allicin
Antimycotic	Allicin, ajoene
Antiviral	Allicin, ajoene
Hypolipemic	Diallyldisulfide
Detoxification of heavy metals	Selenium, germanium
Antitumor	Selenium, germanium, diallyldisulfide
Antioxidant	Selenium, germanium
Antiaging	Selenium, diallyldisulfide
Immune modulation	
Natural killer-cell activity and other kinds of cell-mediated immunity	Germanium, selenium zinc
Humoral immunity	Germanium, allicin
Complement activity	Magnesium, calcium

* Adapted from Abdullah, 1988.

consumers that desire the so-called "natural" foods. The problem will be to isolate, purify, stabilize and incorporate this natural antimicrobial into foods without adversaly affecting sensory and safety characteristics, and all of this should be done without increasing the cost for formulation and processing. In addition, garlic oil could be useful not only for the food industry, but also it could be useful for the field of medicine due to its pharmacological properties. Further investigations are needed in order to establish practical guidelines.

CONCLUSION

Garlic oil had the highest antimicrobial activity among the four spice oils tested. The MIC of garlic oil was found to be 400 ppm. The pH did not affect the antimicrobial activity of garlic at higher concentrations. The presence of proteins, fats, and fibers such as found in foods influenced the antimicrobial effect of garlic oil. Garlic oil was effective against S. aureus and B. cereus, as well as S. typhimurium, which was less sensitive. After feeding rats with 400 ppm of garlic oil for one month, the rats lost 15 % of their normal weight and no other visible toxic effects were observed.

This study suggests that garlic oil could be used as a preservative in foods such as meat products, gravies, sauces, baked goods, poultry products, salad dressings, or seafood products. Garlic may be a good substitute for sodium nitrite in foods. Further study using lower levels of sodium nitrite in conjunction with garlic oil should be pursued in foods challenged with old and new food-borne microorganisms such as Clostridium botulinum and Listeria monocytogenes to determine whether microbial growth inhibition or prevention of stored food deterioration occurs in an actual food system.

Garlic oil contains nutritional value, and it is a naturally occurring compound, widely cultivated, cheap and safe, and used as a flavoring agent. Moreover, there are no reports on development of resistance in sensitive organisms against garlic, a common problem with antibiotics. Garlic's potential usage as a subtitute for sodium nitrite in foods would appear to be good, especially, for those

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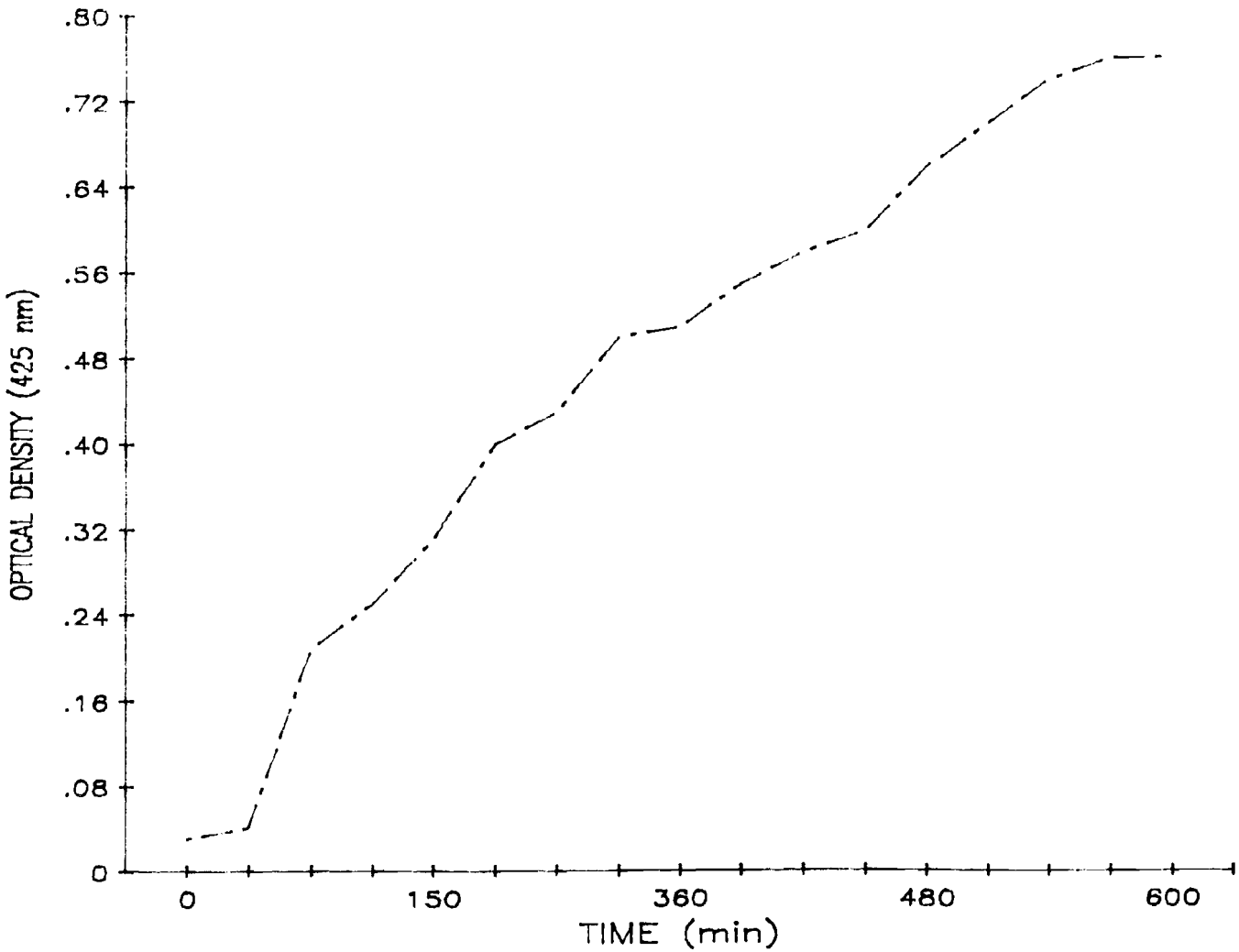
APPENDIX

Appendix 1

Figure 11. Growth curve of S. typhimurium determined after growing the bacterium for 10 hr. The optical density (425 nm) of the culture as well as plate count were recorded every 30 min. and used to determine the colony forming units of this bacterium at specific time. These data represent the average of three experiments.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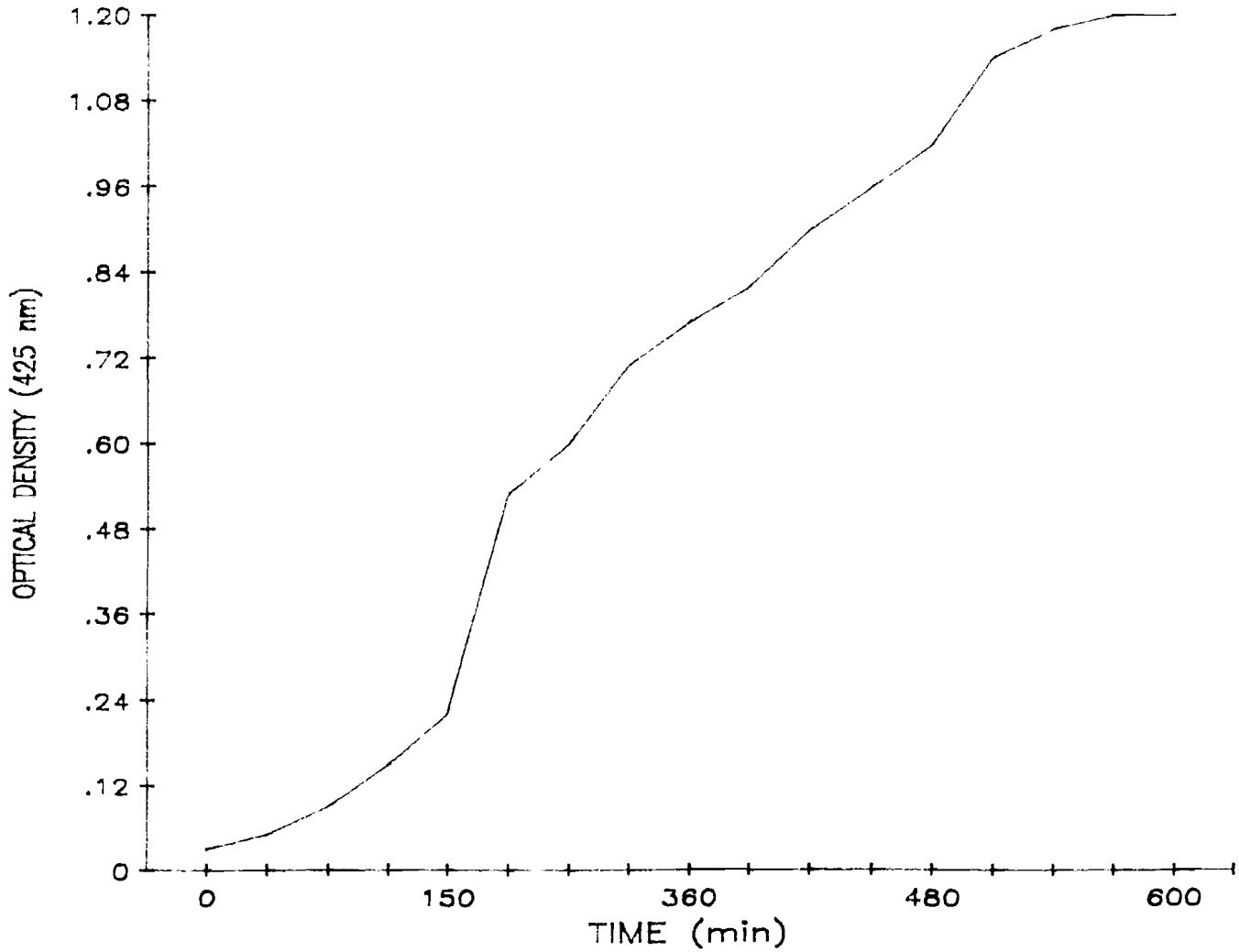
.56
.48
.40
.32
.24
.16
.08
0
OPTICAL DENSITY (425 nm)
0
150
360
480
600
TIME (min)

Figure 12. Growth curve of S. aureus determined after growing the bacterium for 10 hr. The optical density (425 nm) of the culture as well as plate count were recorded every 30 min. and used to determine the colony forming units of this bacterium at specific time. These data represent the average of three experiments.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OPTICAL DENSITY (425 nm)
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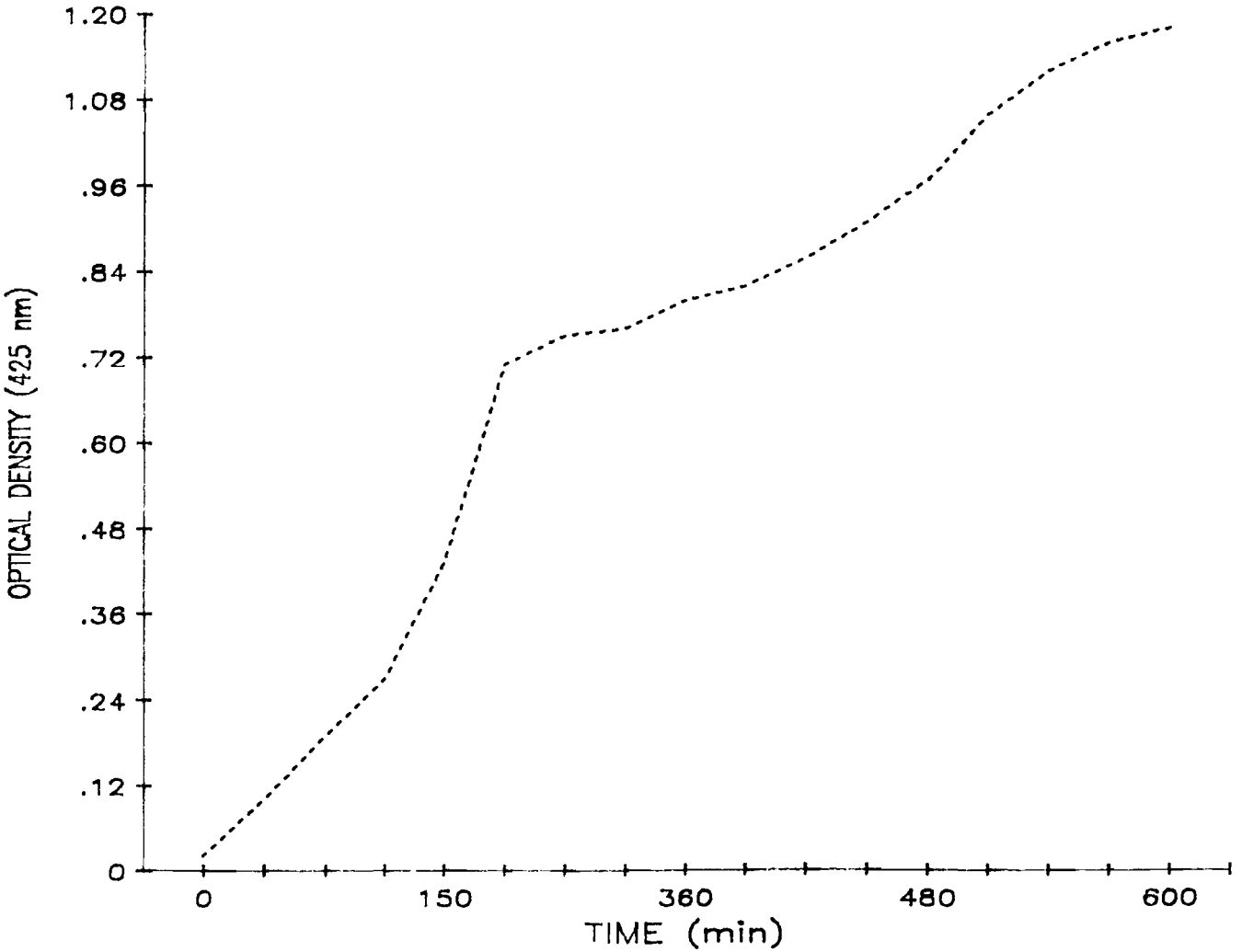
150
360
480
600
TIME (min)

Figure 13. Growth curve of B. cereus determined after growing the bacterium for 10 hr. The optical density (425 nm) of the culture as well as plate count were recorded every 30 min. and used to determine the colony forming units of this bacterium at specific time. These data represent the average of three experiments.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TIME (min)

Signature of Graduate Student

Signature of Major Advisor

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