THE INHIBITION OF *CLOSTRIDIUM BOTULINUM* GROWTH AND TOXIN PRODUCTION BY ESSENTIAL OILS OF SPICES

by

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(Abstract)

The essential oils of clove, thyme, black pepper, pimenta, origanum, garlic, onion, and cinnamon were evaluated for their effect on germination, outgrowth, growth, and toxin production of C. botulinum strains in microbiological media. The oils of clove, thyme, origanum, and cinnamon were studied for their mechanism of inhibition of C. botulinum 67B. The most effective oil, in combination with sodium nitrite at different levels was further tested against the growth and toxin production of C. botulinum (mixed types) in a meat model system.

Among all the spice oils, origanum and pimenta were the most effective in inhibiting six strains of types A, B, and E of C. botulinum in prereduced PY medium. These oils at a concentration of 200 ppm completely inhibited C. botulinum growth. Garlic, onion and black pepper exhibited the lowest inhibitory activity towards the growth of C. botulinum strains. Strains of type A were more sensitive to the inhibitory action of the oils than those of types B and E.

The inhibition of germination of C. botulinum by the eight spice oils indicated that garlic oil was the most potent inhibitor. Oils of pimenta, and clove were the least

effective in inhibiting germination. The inhibitory effect of the oils was shown to be reversible.

The oils appeared to have no significant effect on the outgrowth of the germinated spores. Nevertheless, the oils were highly active in inhibiting vegetative growth (cell division). Black pepper, clove, cinnamon, and origanum were the strongest inhibitors of vegetative growth. Yet, the oils had no direct effect on toxin production. The delay in toxin production caused by the oils was attributed to the effect of the oils on growth rather than on toxin production.

Origanum oil acted synergistically with sodium nitrite in inhibiting the growth of C. botulinum in a microbiological medium. In vacuum-packaged comminuted pork, origanum oil at 400 ppm, in combination with 50-100 ppm of sodium nitrite, significantly delayed the growth and toxin production of C. botulinum (mixed types). The probability of growth and toxin production of C. botulinum (p) in the vacuum packaged comminuted pork was calculated with Hauschild 's formula. The results showed that sodium nitrite significantly lowered p values, whereas origanum oil had very low effect on p values.

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TABLE OF CONTENTS

page

I. INTRODUCTION 1

II. REVIEW OF LITERATURE 4

A. Spices and Essential oils of spices 4

1. Introduction 4

2. Antimicrobial properties of spices and their extracts 8

a. Effect on molds and yeasts. 9

b. Effect on lactic acid bacteria 13

c. Effect on food spoilage and food poisoning bacteria 16

3. Antioxidant properties of spices and their extracts 22

B. Clostridium botulinum 25

1. Introduction 25

2. Sequence of events during formation of vegetative cells from dormant spores . . 28

a. Activation 28

b. Germination 30

c. Outgrowth 32

C. Nitrite and meat preservation 33

1. Introduction 33

2. Function 33

a. Color and flavor development 33

b. Antioxidative properties 35

c. Antimicrobial properties 36
3. Factors affecting the antimicrobial action of nitrite 39
a. Number of spores and level of nitrite 39
b. NaCl and pH 39
c. Incubation temperature and heat processing 40
d. Isoascorbate, EDTA, cysteine, polyphosphate 40
e. Microbial interaction 41
4. Nitrosamines, formation, risk, elimination 41
5. Alternatives to the use of nitrites in cured meats 44
III. MATERIALS AND METHODS 51
A. Essential oils of spices 51
B. Preparation of spores of C. botulinum . . . 51
C. Effect of spice oils on growth of C. botulinum 52
D. Effect of spice oils on germination of C. botulinum 54
E. Effect of spice oils on C. botulinum 67B. . 54
1. Effect on germination of C. botulinum 67B (optical density method) 54
2. Duration of germination sensitivity. . . 56

3. The reversibility of inhibition of spore germination 57
4. Effect of spice oils on outgrowth of C. botulinum 67B 58
5. Effect of spice oils on vegetative growth (cell division) 59
F. Effect of spice oils on toxin production. . 60
1. Preparation of medium 60
2. Preparation of spore mixture 60
3. Toxin production . . . 60
4. Toxin extraction and mouse bioassay. . . 61
G. Effect of combination of sodium nitrite and origanum oil on growth of C. botulinum (mixed types) 62
1. In TYG 62
2. In meat 63
IV. RESULTS AND DISCUSSION 66
A. Effect of spice oils on growth of C. botulinum 66
B. Effect of spice oils on germination of C. botulinum by microculture method 77
C. Effect of spice oils on C. botulinum 67B. . 87
1. Effect of spice oils on germination (optical density method) 87
2. Duration of germination sensitivity to spice oils 92

3. Reversibility of inhibition of germination of C. botulinum 67B. 94
4. Effect of spice oils on outgrowth and vegetative growth of C. botulinum 67B. . 99
D. Effect of spice oils on toxin production 103
F. Effect of origanum oil and sodium nitrite on C. botulinum growth. 106
1. In bacteriological medium. 106
2. In meat. 109
V. SUMMARY AND CONCLUSIONS. 125
VI. REFERENCES 129
VITA 147

LIST OF FIGURES

Figure		page
1	Chemical structures of some antimicrobial substances in spice oils.	21
2	Inhibition of C. botulinum 33A growth by spice oils in PY medium	67
3	Inhibition of C. botulinum 10755A growth by spice oils in PY medium	68
4	Inhibition of C. botulinum 40B growth by spice oil in PY medium	70
5	Inhibition of C. botulinum 67B growth by spice oil in PY medium	71
6	Inhibition of C. botulinum 1623E growth by spice oil in PY medium	72
7	Inhibition of C. botulinum 2157E growth by spice oil in PY medium	74
8	Germination of C. botulinum 33A in the presence of spice oils	78
9	Germination of C. botulinum 10755A in the presence of spice oils	80
10	Germination of C. botulinum 40B in the presence of spice oils	78
11	Germination of C. botulinum 67B in the presence of spice oils	79
12	Germination of C. botulinum 1623E in the presence of spice oils	80

13 Effect of spice oils clove (A), thyme (B), black pepper (C), and pimenta (D) on germination of C. botulinum 67B suspended in GO medium 88

14 Effect of spice oils origanum (A), garlic (B), onion (C), and cinnamon (D) on germination of C. botulinum 67B suspended in GO medium 90

15 The influence of the time of addition of cinnamon oil on inhibition of germination of C. botulinum 67B spores suspended in GO medium 93

16 The reversibility of clove oil induced inhibition of C. botulinum 67B spore germination 95

17 The reversibility of thyme oil induced inhibition of C. botulinum 67B spore germination 96

18 The reversibility of origanum oil induced inhibition of C. botulinum 67B spore germination 97

19 The reversibility of cinnamon oil induced inhibition of C. botulinum 67B spore germination 98

20 Effect of spice oils on growth (measured as growth ratios) of germinated spores of C. botulinum 67B in TYG medium 100

21 Effect of spice oils on growth (measured as growth ratios) of vegetative cells of C. botulinum 67B in TYG medium 102

22 Effect of various levels of sodium nitrite (A) and origanum oil (B) on probability (p) of C. botulinum outgrowth and toxigenesis in vacuum packaged comminuted pork. The packages were inoculated with 300 spores per g, and were incubated at 27°C for 1 week 119

23 Effect of various levels of sodium nitrite (A) and origanum oil (B) on probability (p) of C. botulinum outgrowth and toxigenesis in vacuum packaged comminuted pork. The packages were inoculated with 300 spores per g, and were incubated at 27°C for 2 weeks 120

24 Effect of various levels of sodium nitrite (A) and origanum oil (B) on probability (p) of C. botulinum outgrowth and toxigenesis in vacuum packaged comminuted pork. The packages were inoculated with 3000 spores per g, and were incubated at 27°C for 1 week 121

25 Effect of various levels of sodium nitrite (A) and origanum oil (B) on probability (p) of C. botulinum outgrowth and toxigenesis in vacuum packaged comminuted pork. The packages were inoculated with 3000 spores per g and

incubated at 27°C for 2 weeks 123

LIST OF TABLES

Table		page
1	Summary of some important characteristics of selected spice oils	7
2	Summary of growth characteristics of C. botulinum type A and E	34
3	Possible nitrite alternatives for the inhibition of C. botulinum	46
4	Means of % inhibition of growth of C. botulinum (A, B, and E) caused by spice oils (10, 50, 100, 150, and 200 ppm)	75
5.	Inhibitory effect of some spice oils on different microorganisms.	76
6	Means of % inhibition of germination of C. botulinum (A, B, and E) by spice oils (10, 50, 100, 150, and 200 ppm)	85
7	Effect of spice oils on toxin production by C. botulinum (mixed types 300 spores per ml) in prereduced medium TYG incubated at 32°C	104
8	Effect of origanum oil on growth of C. botulinum (A, B, E) in TYG medium containing different concentrations of sodium nitrite	107
9	Effect of origanum oil on growth of C. botulinum (A, B, and E) in TYG medium containing different concentrations of	

sodium nitrite which was further autoclaved for 5 min at 121°C 108

10 Proximate composition and total microbial count of pork 111

11 Effect of origanum oil and nitrite in different combinations on swell formation and toxin production by C. botulinum (mixed types A, B, and E) in packaged comminuted pork inoculated with 300 spores per gram and incubated at 27°C 112

12 Effect of origanum oil and nitrite in different combinations on swell formation and toxin production by C. botulinum (mixed types A, B, and E) in packaged comminuted pork inoculated with 3000 spores per gram and incubated at 27°C 113

13 Effect of origanum oil with sodium nitrite on means of days for appearance of swelling in vacuum packaged pork inoculated with 300 per g of spores of C. botulinum (A, B, and E) 114

14 Effect of origanum oil with sodium nitrite on means of days for appearance of swelling in vacuum packaged pork inoculated with 3000 per g of spores of C. botulinum (A, B, and E) 117

I. INTRODUCTION

Clostridium botulinum is an anaerobic, gram positive bacterium. The strains of C. botulinum are divided into types A, B, C, D, E, F ,and G based on their production of antigenically specific neurotoxins (Sugiyama, 1980). Outbreaks of botulism in humans are generally caused by strains A, B, and E or occasionally F. (Smith, 1977).

C. botulinum has been found in the soil of each of the continents, in the sediments and in the intestinal contents of birds and mammals (Smith, 1977). All foods come in contact with the soil either directly or indirectly via airborne dust; thus all foods have the potential to be contaminated with spores of C. botulinum (Lechowich et al. 1978).

The anaerobic condition in canned foods provides a good environment for growth and toxin production by C. botulinum. Feldman et al. (1981) has pointed out that canned or home processed foods account for 92% of the foodborne outbreaks of botulism. All low acid canned foods are protected from C. botulinum health hazard by heat processing (commercial sterilization). This process is designed to leave less than one surviving spore present in one can containing 1×10^{11} spores.

Cured meat products (bacon, sausage, wieners, luncheon meat, and canned hams), which amounted to 25% of all meat

products in the United States in 1979, are protected from the toxicity of C. botulinum by sodium nitrite; 120-200 ppm of sodium nitrite is allowed in these products by the United States Department of Agriculture (USDA) (NAS, 1982). Commercial sterilization is not used with cured meats because the process renders cured meats aesthetically unacceptable (Lechowich et al, 1978).

In recent years, concern that nitrite itself as well as nitrosamines might be carcinogenic has led to investigations for safer preservatives. Several hundred compounds have been tested but no single alternative has been identified as a replacement of all the useful functions of nitrite (Brown, 1973).

Spices were recognized by the Egyptians over 3000 years ago as having preservative possibilities (Farrell, 1985). Many publications have shown the effects of spices and their extracts on various microorganisms, bacteria, molds and yeasts (Huhtanen, 1980; Azzouz, 1981; Conner Beuchat, 1984a). Limited studies have indicated the antibotulinal effect of spices or extracts of spices; in addition, most of the spices possess antioxidant properties which allow for a partial replacement of nitrites.

The essential oils of spices are considered to contain all the antimicrobial factors of spices (Farrell, 1985). The oils are generally recognized as safe (GRAS) in foods (Furia and Bellanca, 1971). In the present study, essential oils of

clove, thyme, black pepper, pimenta, origanum, garlic, onion, and cinnamon were evaluated for their effect on growth (spore-to-cell transition), germination, outgrowth, vegetative growth, and toxin production of C. botulinum. Tests were conducted to show the duration of the sensitivity of the spores to the inhibitory action of the oils. Also, the reversibility of the inhibition of germination by the oils was studied to postulate a mechanism for the mode of action of the oils on germination. Based on the results of the tests mentioned, origanum oil was selected to be tested for its antibotulinal effect in combinations with low concentrations of sodium nitrite in a bacteriological medium and in vacuum packaged meat.

The objective of the study was to demonstrate the effect of the oils of spices on different activities of C. botulinum strains for the possibility of partial replacement of sodium nitrite in cured-meat products with the oils. Thus, the reduced exposure of humans to carcinogenic nitrosamines is considered an attainable goal.

II. REVIEW OF LITERATURE

A. Spices and Essential Oils of Spices

1. Introduction

Over the centuries, man has learned to distinguish between edible and inedible plants, either by trial and error or by watching animals and birds. There are references that people used spices with foods (meat) and for medicines about 5000 years ago. The use of spices and their derivatives by the Egyptians and Babylonians is well-known. There are numerous historical records written by the historian Herodotus (484-424 B.C.), Hippocrates (477-360 B.C.), the father of modern medicine, Theophrastus (372-287 B.C.), considered by many to be the father of Botany, Pliny the Elder (62-110 A.D.), the greatest writer of the Vespasions region; each one contributed to the intriguing, if not always true, story of spices.

In the middle ages, Arabs and then, the merchants of Venice, controlled the trading of spices for a long time. The desire to discover the shortest route to the Indies to reduce the price of spices was possibly one of the reasons for Columbus', Vasco da Gama's , and Magellan's voyages.

In the United States, by the eighteenth century, sailing from New England to the East Indies began, and a small seaport town of Salem, Massachusetts, became the center of the new world spice trade. Today, the center of

the world-wide spice trade is focused in the Wall Street area of New York City (Farrell,1985).

According to the International Organization for Standardization (ISO, 1968, 1972) the terms "spices" and "condiments" apply to "the natural vegetable products or mixtures thereof, without any extraneous matter, that are used for flavoring, seasoning, and imparting aroma to foods, the term applies to the product in whole form or in the ground form."

The United States Food and Drug Administration, in Title 21 of the Code of Federal Regulations, defines spices as: "any aromatic vegetable substance in the whole, broken or ground form, except for those substances which have been traditionally regarded as foods, such as onions, garlic, and celery; whose significant function in food is seasoning rather than nutritional; that is true to name; from which no portion of any volatile oil or other flavoring principle has been removed" (Farrell,1985). Botanically, spices are parts of plants; they are fruits, e.g. black pepper; seeds:celery; rhizomes or roots: ginger; leaves: thyme; bark: cinnamon; flowers: cloves; and bulbs: onion and garlic (Pruthi, 1980).

The aroma and flavoring properties of all spices are attributed to their essential oil contents. The term "essential oil" is defined as volatile oils obtained by (1) water distillation, (2) steam distillation, (3) enzymatic action followed by steam distillation, and (4) water and

steam distillation. (Pruthi, 1980). Guenther (1948) reported that the chemical compositions of the majority of the essential oils can be classified into four categories: 1. Terpenes, related to isoprenes; 2. Straight chain compounds, not containing any side branches; 3. Benzene derivatives; 4. Miscellaneous (compounds specific to certain spices, e.g., allyl isothiocyanate in mustard oil, allyl sulfide in garlic oil).

There are many publications concerning the composition of essential oils of different plants including spices. In his book, Masada (1976) describes the composition of the essential oils of 64 plants; the essential oils were obtained by steam distillation, and analyzed by using gas chromatography and mass spectrometry. Formacek and Kubeczka (1982) published a study on the chemical analyses of twenty-one essential oils. The analyses were performed by using gas chromatography and C^{13} NMR. Data are presented in Table 1 on some important aspects of the essential oils used in the research reported in this dissertation.

The essential oils of the spices are added to several different foods. These foods include non-alcoholic beverages, ice cream, alcoholic beverages, candies, baked goods, gelatins and puddings, chewing gum, condiments, pickles, spiced fruits, and meats (Furia and Bellanca, 1971).

Table 1. Summary of some important characteristics of selected spice oils[a].

Spice	Botanical parts[b]	% Essential oils	Main components
Clove	Leaves, buds stems	15-18	Eugenol , Eugenol acetate
Thyme	The entire flowering part	Ø.5-1.2	Thymol , Carvacrol
Black Pepper	Berries	1-2.6	α-Pinene β-Pinene
Pimenta	Leaves, berries	3.3-4.3	Eugenol
Origanum	Flowering tops	< 1	Thymol, carvacrol
Garlic	Bulbs	Ø.1-Ø.2	Allyl propyl disulfide
Onion	Bulbs	Ø.Ø2	d-n-propyl disulfide
Cinnamon	Bark and leaves	Ø.5-1	Cinnamic aldehyde

[a] Compiled from Furia and Bellanca (1971).
[b] Botanical parts containing the essential oil.

The spices and their derivatives are used for their flavoring, antioxidant, preservative, physiological and medicinal properties. A comprehensive review of all these properties is presented in Pruthi (1980). Thus, the review in this dissertation will be limited to recent publications on the antimicrobial and antioxidant activities of spices and their derivatives.

2. Antimicrobial properties of spices and their extracts

Reports on the antimicrobial properties of spices in foods first appeared in the 1880s, describing activities of mustard, clove, and cinnamon, and their oils (Boyle, 1955). However, Bachmann (1916) reported that the levels of spices usually used in foods have limited antimicrobial action.

Today there is a renewed interest in the use of spices as antibacterials in foods. Shelef (1983) attributes this to three reasons: (1) The safety of synthetic food additives is questioned; therefore, there is a trend toward the use of natural substances of plant origin. (2) The reduction of salt and sugars in foods for dietary reasons tends to enhance the use of seasoning, which are low in sodium and calories. (3) There are reports that spices could reduce the risk of gastric cancer. Mei Xing (1982) compared the mortality rate due to gastric cancer among inhabitants of two provinces consuming different levels of garlic per day. He found that the inhabitants of the province who

usually eat fresh garlic daily, e.g. approx. 20 g/day had significantly lower mortality rate due to gastric cancer, and had a lower concentration of nitrite in their gastric juices than inhabitants of another province who rarely take garlic. Garlic was shown to promote inhibition of nitrate-reducing bacteria and reduce nitrite formation which may be considered as a protective factor against gastric cancer.

There have been numerous studies on the antimicrobial effects of spices and their derivatives in both laboratory media and foods. The studies can be divided into three sections.

a. Effect on molds and yeasts.

Molds and yeasts are the cause of spoilage of certain foods, such as the low pH and a_w foods (Banwart, 1981). Spices and their derivatives have shown considerable activity against these microorganisms. Bullerman (1974) showed that as little as 0.02% cinnamon in sucrose yeast extract broth, inhibited the growth and aflatoxin production by Aspergillus parasiticus. This result indicates that foods containing cinnamon may not readily support mold growth and / or aflatoxin production. Hartung et al. (1973) also showed that cinnamon in bread caused a decrease in growth and aflatoxin production by Aspergillus parasiticus as compared with breads with no cinnamon. This outcome points out the preservative quality of cinnamon, although the primary

function of cinnamon in these foods may not be preservative in nature.

Bullerman et al. (1977) tested the essential oils of cinnamon, clove, and the single components of the oils, cinnamic aldehyde, and eugenol against A. parasiticus. The results indicated that the essential oils were inhibitory at 200-250 ppm; for cinnamic aldehyde and eugenol, they were inhibitory at 150 and 125 ppm, respectively. Cinnamic aldehyde and eugenol are the major components of the essential oils of cinnamon and clove, respectively, and are considered the main antifungal constituent in these oils. The inhibitory effect of these substances were determined to be on growth rather than on toxin production.

Yamada and Azuma (1977) demonstrated that allicin, an extract of crushed garlic, inhibited growth of Cryptococcus, Trichophyton, Epedermophyton, Aspergillus, and Microsporium. The minimum inhibitory concentration was dependent on several factors: the method used, the incubation time, the inoculum size, the type of medium, and the pH of the medium. The compound was shown to be less effective on Aspergillus than on other molds. The effects were shown to be on both spore germination and hyphae growth.

Morozumi (1978) isolated and purified an antifungal substance from cinnamon. The substance, O-methoxy-cinnamaldehyde, caused complete inhibition of A. parasiticus and A. flavus at a concentration of 100 μg/ml,

whereas A. ochraceus and A. versicolor were inhibited at a higher concentration, 200 μg/ml.

Sharma et al. (1979) worked on the effect of various extracts of onion on growth of the molds A. flavus and A. parasiticus. An ether extract of onion showed higher antifungal activity than the onion oils and the ethyl acetate extract of onion. Gamma-irradiation, used to prevent sprouting, did not alter the inhibitory activities of the extracts.

Azzouz (1981) studied the affects of some spices on several toxigenic species of Aspergillus and Penicillium. The spices clove, cinnamon, mustard, allspice, garlic, and oregano inhibited all the molds studied at a concentration of 2% in yeast extract sucrose agar. A low concentration of clove and cinnamon (0.8%) prevented mycotoxin toxin production.

Hitokoto et al. (1980) studied the inhibitory activities of twenty-nine commercial powdered spices on growth and aflatoxin production by three species of Aspergillus. It appeared that of all the twenty-nine spices, only clove, star anise seeds and allspice prevented the growth of all the molds completely, whereas the rest inhibited only toxin production. Eugenol and thymol, extracts of clove and thyme, respectively, inhibited the growth of the molds at a concentration of 0.4 mg/ml; on the

other hand, anethol extracted from star anise seeds caused the same inhibition at a higher concentration, 2 mg/ml.

Buchanan and Shepherd (1981) worked on the antifungal effect of thymol; it caused the total inhibition of A. parasiticus at a concentration of Ø.5 mg/ml, which is close to the inhibitory concentration obtained by Hitokoto et al. (198Ø). The activity of thymol against aflatoxin production was equivalent to or less than that for growth, indicating that the inhibitory action was primarily on growth and indirectly on toxin production.

Azzoz and Bullerman (1982), in an extensive study, demonstrated antifungal activities of twenty-six substances, most of them spices. The results indicated that thyme, cinnamon, mustard, allspice, garlic, and oregano, at a concentration of 2% in potato dextrose agar, inhibited the growth of seven toxigenic molds completely for up to twenty-one days. When sorbate was combined with clove, an enhanced or possible synergistic effect was observed. Madhyastha and Bhat (1984) showed that piperine and pepper oil were inhibitory to growth and aflatoxin production in a dose-dependent manner. Pepper oil was more inhibitory than piperine.

The affect of spices on yeasts has also been studied. Conner and Beuchat (1984a) screened thirty-two essential oils for their inhibitory action on thirteen food spoilage and food industrial yeasts. Of all the oils, those of

allspice, cinnamon, clove, garlic, onion, oregano, savory, and thyme had the highest inhibitory action. Conner and Beuchat (1984b and 1985) reported on the recovery of heat-stressed yeasts in the presence of essential oils and oleoresins of spices. The essential oils, at concentrations of up to 200 ppm in recovery media did not interfere with colony formation by unheated cells. However, some oils, at concentrations as low as 25 ppm in recovery media, reduced populations of sublethally heat-stressed cells compared to populations recovered in media containing no test oils. This demostrates that the yeasts were either metabolically or structurally damaged as a result of exposure to elevated temperatures and that the essential oils prohibited repair of injury. The size of colonies and pigment production by heat-stressed yeasts in the presence of some essential oils were also affected. The essential oils may have an additive or synergistic effect on the thermal inactivation of the yeasts in foods.

b. Effect on lactic acid bacteria:

The use of starter cultures in meat products was introduced in the early forties. Bacterial starter cultures used in the production of dry sausages include lactic acid bacteria, belonging mainly to the genera Lactobacillus, Pediococcus, and Streptococcus (Nes and Skjelkvale, 1982). The reason for introducing these bacteria into meat products

is their ability to produce a constant and controlled acidification which inhibits the growth of undesirable microorganisms. Because of the contact between these bacteria with spices in fermented meats, it is important to know how the spices affect the growth of lactic acid bacteria.

Karaioannoglou et al. (1977) studied the effect of an aqueous extract of garlic on the growth of Lactobacillus plantarum in BHI broth. The extract was inhibitory at a concentration higher than 1 % , whereas at concentrations of 2% and 5% the extract was germicidal. The inhibitory effect of the extract was also dependent on growth conditions: pH, incubation temperature, and inoculation size.

Zaika and Kissinger (1979) reported on the effect of several spices on the growth of a starter culture composed of L. plantarum and P. cerevisiae. Clove was the only spice to show inhibitory action on the culture, while ginger, red pepper, mustard, mace, and cinnamon stimulated acid production by the starter culture, but did not affect bacterial population. In another study Zaika and Kissenger (1981) showed that the starter culture responded to the presence of oregano in a liquid medium in a concentration-dependent manner. The effects ranged from stimulatory at a low concentration to inhibitory at a higher concentration for both growth and acid production. The inhibitory effect

of oregano was removable by autoclaving and solvent extraction.

Nes and Skjelkvale (1982) studied the effect of oleoresins and natural spices on the fermentation abilities of commercial starter cultures. The results indicated that natural spices stimulated microbial fermentation of glucose to lactic acid in both a liquid medium and dry salami; however, the oleoresins of the same spices had no affect on the fermentation. The spices studied were black pepper, garlic, and a salami spice mixture.

Zaika et al. (1983) worked on the effects of four spices on the growth and acid production of L. plantarum and P. acidilactici in a liquid medium. The spices were bacteriostatic for a certain period. When the bacteriostatic effect was overcome, all four spices stimulated acid production. The inhibitory effects of the spices toward both microorganisms was oregano >> Rosemary = Sage >> Thyme. L. plantarum was more resistant than P. acidilactici. The results also showed that the cultures could be adapted to the spices and the adapted cells resisted higher concentrations of spices than nonadapted cells. Zaika and Kissinger (1984) observed a stimulatory effect of other spices (clove, cardamon, ginger, celery seeds, cinnamon, tumeric) on the bacterial acid production. The stimulatory action was attributed to the Mn content of the spices.

Spices with high Mn content showed a higher stimulatory effect on the acid production by the bacteria.

c. Effect on food spoilage and food poisoning bacteria

Johnson and Vaughn (1969) studied the action of garlic and onion powders on Salmonella typhimurium and E. coli. Both spice powders were bactericidal at concentrations of 1-5% in the medium. Concentrations of more than 5% increased the death rate of the bacteria. Julseth and Diebel (1974), in their studies with Salmonella spp., found slight inhibition by cassia, oregano, allspice, and onion at concentrations of 5% or higher.

Mori et al.(1974) demonstrated that the essential oils of celery, cinnamon, cumin, and dill could be used to reduce the slime spoilage on wieners; their effects were increased when combined with sorbic acid or lysozyme. Miyao (1975) reported that the shelf-life of sausage could be increased by dipping in a solution of 2% eugenol for 10 seconds at 10°C.

Beuchat (1976) detected antimicrobial effects of some dried spices and some essential oils of spices on the growth of Vibrio parahaemolyticus. Dried oregano and thyme were al. recommended that these oils not be used alone in meat industries as primary preservatives. Dankert et al. (1979) demonstrated the antibacterial activity of the juices of garlic, onion, and shallots on five gram negative and three gram positive bacteria. Garlic extract was inhibitory to all

the microorganisms, but onion and shallot had no effect on gram negative bacteria. Therefore, gram positive bacteria are more sensitive to the spice extracts than gram negative bacteria. Mantis et al. (1979) studied the combined effects of garlic extract concentration, pH , and temperature on the outgrowth of C. perfringens spores in culture media. One percent of garlic extract (homogenate of garlic with distilled water 1:2) at pH 7, and at a temperature 40°C prevented the outgrowth of inocula of 1×10^2 - 1×10^4 spores/ml. For a higher inoculum (1×10^5), 2% of the extract was needed to prevent the outgrowth of the spores.

Shelef et al. (1980) studied the sensitivity of 46 gram positive and gram negative bacteria towards the spices sage, rosemary, and allspice in media. The spices had a higher inhibitory activity on gram positive than on gram negative bacteria. Sage and rosemary at 0.3% in media produced inhibition of 21 gram positive bacteria, whereas, at a concentration of 0.5%, the two spices were bactericidal. Allspice was less effective on the inhibition of the bacteria than were sage and rosemary.

Martini (1980) studied the affect of garlic and onion on C. perfringens. Concentrations of 1-2% of the spices were required to inhibit the bacteria. Gamma ray treatment of the spices did not alter the spices inhibitory activity.

Nkanga and Uraih (1980) examined the effects of several spices on S. aureus in meat homogenate. Clove

was the most active of all the spices tested, with 1% (w/v) preventing growth of the bacterium for up to 18 h contact time. At higher concentrations, 10% (w/v), clove was bactericidal; it reduced the number of bacteria by 2 log cycles during 18 h of incubation.

Huhtanen (1980) investigated the action of alcoholic extracts of several spices on C. botulinum. According to their inhibitory behavior, the extracts were divided into three categories: the most active included mace and achiote; bay leaf, white and black pepper and nutmeg were active; least active were rosemary, clove, oreganium, and paprika.

Salzer (1982) described the effect of extracts of different spices on several microorganisms at the concentrations used in meat products (1:2000 and 1:1000). The results varied with the bacterial species, spice, and concentration. Pepper, mace, and nutmeg had the highest inhibitory effects, followed by ginger, caraway, and celery seeds.

Reddy et al. (1982) and Pierson and Reddy (1982) studied the antibotulinal activities of thymol (a major antibacterial agent in the essential oils of thyme and origanum). The compound was extremely active against C. botulinum (types A and B) at a concentration of 200 ppm in a microbiological medium. However, thymol exhibited low antibotulinal activity in meat products, even at a concentration of 1000 ppm.

Ueda et al. (1982a) tested alcoholic extracts of clove, thyme, sage, oregano, and rosemary on the growth of B. subtilis, B. cereus, and C. botulinum. The growth of C. botulinum was inhibited at a concentration of Ø.5% (based on dry weight of the spices) in media, whereas a lower concentration inhibited the growth of Bacillus spp. Single components of the extracts eugenol, iso-eugenol, D-borneol, and citronellol were inhibitory to C. botulinum at < Ø.Ø5%. Ueda et al. (1982b) reported on the action of ethanolic extracts of spices on a number of bacteria in media at different pH's. They observed that gram negative bacteria were more resistant than gram positive bacteria, and that the effects were more marked at pH 5 than at 7.

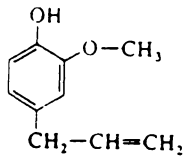
Sirnik and Gorisek (1983) showed the effects of nine spices on four gram negative bacteria. Cinnamon was determined to have the highest antibacterial activity. Lafont et al. (1984) studied the inhibitory activity of twenty-eight plant products on bacteria,and molds. Aqueous and chloroform extracts of several common spices were bacteriostatic against one or more species of bacteria and molds. Extracts of some of the spices showed genotoxic effects in at least one of the three bacterial tests.

Shelef et al. (1984) concluded that sage exhibited a higher inhibitory activity on food poisoning bacteria (S. aureus, B. cereus, and S. typhimurium) in media than in

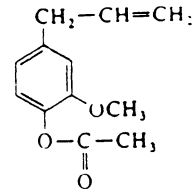
foods. The antimicrobial activity of sage was increased with the increase in its volatile fraction.

Hall and Maurer (1986) showed that ethanol extract of spices, mace, bay leaf, and nutmeg, at concentrations 31, 125, and 125 ppm, respectively, prevented toxin production by C. botulinum 62A in turkey frankfurter slurries. Savitri et al. (1986) studied the effect of several spices on gas production by C. perfringens in brain heart infusion broth. The results indicated that gas formation was totally inhibited by clove, cinnamon, and garlic. Ginger, pepper, and turmeric had some inhibitory action on gas formation, but autoclaving reduced inhibition by ginger and turmeric. The inhibition of gas formation was due to the inhibition of bacterial growth except in the case of garlic, where some change in the metabolic pathway may have been involved.

There has been considerable speculation on the mode of action of spices and their oils on microorganisms. Most of these studies refer to the role of the terpene fraction in the essential oils as the effective elements in the inhibitory process. However, few attempts have been made to isolate and identify the inhibitory components in the essential oils. Since many of the substances of the essential oils, such as eugenol and thymol, are phenolic in structure (Figure 1.), it is reasonable to assume that their mode of action is similar to that of phenols (Davidson et al., 1983).



Eugenol



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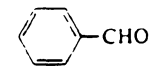
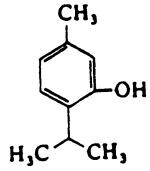
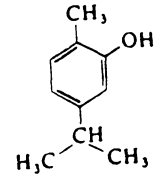


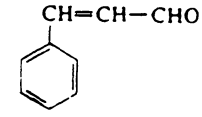
Benzaldehyde



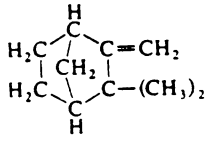
Thymol



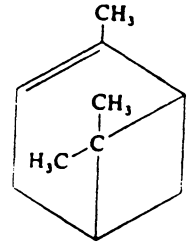
Carvacrol



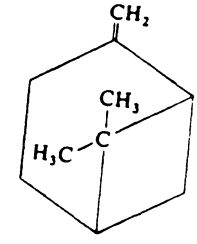
Cinnamaldehyde



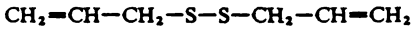
Camphene



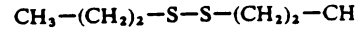
α-pinene



β-pinene



Allyl disulfide



Propyl disulfide

Figure 1. Chemical structures of some antimicrobial substances in spice oils.

Most of the work on phenolic compounds has centered on their effect on cellular membranes of microorganisms. While studying L. plantarum, Juven et al. (1972) found that oleuropein (a phenolic glucoside isolated from green olives), caused the loss of labelled glutamate, potassium, and phosphate from the cells. Oleuropein had no effect on the rate of glycolysis in the cells, but reduced the ATP content in the cell. Further, they concluded that oleuropein resulted in an increase in the cell membrane permeability. While working with P. aeruginosa as a test organism, Bernheim (1972) proposed that phenols react primarily with the phospholipids in the cell membrane. Also, reports have shown that phenols at low concentrations selectively inhibit certain essential enzymes of bacteria (Prindle and Wright, 1977).

3. Antioxidant properties of spices and their extracts

The primary effect of the traditional use of spices as preservatives is bacteriostatic; nevertheless, there are many studies indicating that preservation is also enhanced by the antioxidant compounds present in the spices. Watanate and Ayano (1974) and Hirahara et al. (1974) found antioxidant activities in spices and in their aqueous and alcoholic extracts. Bishov et al. (1977) studied the antioxidant properties of some food ingredients including twenty spices, herbs, and some plant protein hydrolyzates. Of the substances tested, clove, cinnamon, sage, rosemary,

mace, oregano, allspice, and nutmeg showed the highest activity. Moreover, the spices showed synergistic effects when combined with BHA.

Cortanovacki et al. (1981) studied the antioxidant properties of seventeen spices and spice blends in lard and pork emulsion with peroxide and thiobarbituric acid values as indicators of oxidation. The results indicated that garlic was the only spice to act as an antioxidant in lard, whereas all the spices had antioxidant properties in the pork emulsion. Herrman (1981) conducted an experiment on the antioxidant activity of the alcoholic extracts of sixteen herbs and spices in lard. Under dark conditions, cloves, balm, and sage showed the greatest antioxidant activity. Under illumination, cloves retained antioxidant activity, but chlorophyll containing herbs showed prooxidant activity.

Huang et al. (1981) studied the antioxidative properties of nine spices (sweet basil, beefsteak plant, coiander, betel, ginger, red pepper, garlic, and mugwort) grown in Taiwan. Oleoresins prepared by means of methanol extraction and by means of methanol and subsequently n-hexane extraction were used in the study. The oleoresins were added to fresh lard at a concentration of 0.06%, and the test products incubated in an air-circulated oven at 90^{o}C. The time taken to a peroxide value (POV) of 20 meq/kg was recorded as the keeping time. All the spices except garlic were antioxidative in lard. Oleoresins extracted with

methanol were more active than ones extracted with methanol and subsequently with n-hexane. Further, Huang (1981) reported that oleoresins of rhizomes of ginger and red pepper had a higher antioxidant activity than BHT. Wu (1981) revealed the presence of a natural antioxidant in rosemary extracts with an activity comparable, or in some instances superior, to BHT. The antioxidant activity of rosemary extract was enhanced by the presence of ascorbic acid. The antioxidant was reported to be a mixture of several organic acids. Purification of the organic acids by liquid chromatography and subsequent identification showed that the most active single antioxidant compound in rosemary was carnosol.

Lee et al. (1982) investigated the antioxidant properties of twelve spices grown in Taiwan. The methods used by Huang et al. (1981) were followed in the preparation of the oleoresins and in studying the antioxidant activities of the oleoresins in lard. Among the twelve spices studied, the oleoresins extracted with methanol from rhizome of tumeric and rhizome of ginger had a higher antioxidant index than BHT. Barbut et al. (1985) showed that 20 ppm of oleoresin of rosemary was comparable to the commercial blend of BHA/BHT/Citric acid in suppressing lipid autooxidation in turkey breast sausages. Kuruppu et al. (1985) demonstrated that the irradiation and fumigation of spices have no effects on their antioxidative properties.

Lee et al. (1986) reported the presence of an antioxidant factor in ginger rhizome. The antioxidant was shown to be heat resistant. However, prolonged boiling caused reduction in the antioxidative activity (about 33% of activity was lost during two hours of boiling). The pH also was shown to affect the activity of the antioxidant; increasing the pH from 5 to 7 enhanced the antioxidant activity.

B. Clostridium botulinum

1. Introduction

Botulism has been recognized as a food-borne disease for more than 1000 years, and probably confronted mankind from the very beginning in attempts to preserve foods.

During the 18th century in Germany, the disease was regularly associated with smoked blood sausages. Because of this association, Muller in 1870 introduced the term "botulism" from the Latin word "Botulus" meaning "sausage". The disease, however, remained a mystery until 1897 when Van Ermergem isolated the causative organism, which he called Bacillus botulinus. The organism was renamed Clostridium botulinum in 1923 (Sperber, 1982).

The species now includes a heterogenous group of strains that are divided into types A through G on the basis of the antigenicity of their neurotoxins. Lynt et al. (1982) identified all the differences and similarities among the types of the bacterium. Types A, B, E, and F cause the

majority of human botulism outbreaks. Type G, the most recent type to be isolated, has not yet been found to be involved in any botulism outbreak (Lynt et al., 1984).

Smith (1977) divided types A, B, E, and F into two groups based on their proteolytic abilities. Group I includes all type A and the proteolytic strains of B and F, while group II includes the nonproteolytic strains of types B, and F, as well as all type E strains. The proteolytic and nonproteolytic strains of the same type produce toxins which are immunologically identical.

The toxins are proteins which are produced intracellularly as protoxins. These are liberated during autolysis of the vegetative cells and are activated by the proteolytic enzymes. The proteolytic types of C. botulinum activate their toxins, whereas the nonproteolytic types require exogenous enzymes such as trypsin (Sperber, 1982).

Baird-Parker (1971) reported that toxin production occurs under the same conditions which promote the growth of vegetative cells. Thus, most researchers report on the growth of C. botulinum by detection of toxin rather than the more classical indices of growth, such as turbidity increase and total counts. The bacterial production of toxins appears to be determined by the presence of specific bacteriophages, at least in some strains of types C and D, (Sugiyama, 1980).

Feldman (1981) divided botulism outbreaks into four

categories: food-borne, wound, infant, and vehicle undetermined. The most convincing proof that illness of a patient showing typical neurological signs and symptoms is botulism is by detection of botulinal toxins in serum (Hatheway and McCroskey, 1981). Demonstration of botulinal toxins in foods epidemiologically implicated in an outbreak is another confirmation of the illness.

The method most commonly used for toxin detection is mouse bioassay. The method is extremely sensitive; it can detect toxins as low as 10 picograms in the injected samples. However, nonspecific toxic reactions in mice may occur, rendering the bioassay inconclusive. Solberg et al. (1985) reported that in samples of fish products the presence of some gram negative microorganisms, inherent to fish, was responsible for the nonspecific reactions which caused the death of the mice. Treatments of the fish samples with bovine serum eliminated all factors causing the death of the mice except C. botulinum toxins.

Attempts have been made to develop rapid methods for toxin detection. The Enzyme Linked Immunosorbant Assay (ELISA) which depends on an antigen-antibody reaction, seems to be promising. The method is rapid and sensitive, and may be less expensive than the mouse bioassay test. The details of the procedure is described in Notermans et al. (1979).

The death rate among botulism victims in the United States has declined from approximately 70% in 1910-1919

to 12% in 1970-1979 , probably due primarily to the improvement in supportive and respiratory intensive care, and to prompt administration of antitoxins, (Morris and Hatheway, 1980).

Most often, by the time the diagnosis of botulism is made, the toxin has reached a substantial number of nerve terminals and has induced an irreversible blockage of actylcholine release, and causes a noticeable muscular paralysis. Therefore, treatment with antitoxins at this stage is not very helpful because the antitoxins react only with free, not bound, toxins. Moreover, antitoxins could cause adverse reactions in the patient (Morris, 1981).

2. Sequence of events during formation of vegetative cells from dormant spores:

Dormant spores pass through several stages during conversion to vegetative cells. These stages differ in their nutritional requirements. The biochemical reactions involved in these stages are different, as well. All aspects of the stages are well known. Gould and Hurst (1969) discuss this subject in detail; therefore, only a brief description is given below.

a. Activation

The first observation of the effect of activation (heat shock) on spores was recorded by Weizman (1919). Working on developing the technology of acetone fermentation by Clostridium acetobutylicum, he noticed that heat treatment

of the spores was required for a rapid process of fermentation. The exact reason for this was not known. Curren and Evans (1945) noticed an increase in the colony count of milk after heat treatment. They concluded that the treatment hastens the germination of spores of mesophilic aerobes, thus affecting the number of spores which will subsequently germinate and form colonies.

Activation is the first step in the conversion of a dormant spore to vegetative cells. It can be accomplished by different means, e.g. (1) sublethal heat treatment,(2) reduction of disulfide bonds, (3) exposure to pH extremes (4) abrasion, (5) aging, and (6) ionizing radiation (Cook and Pierson, 1983). Heat treatment is usually the method of choice because of its simplicity. Keynan et al. (1964) suggested that activation changes the tertiary structure of a protein responsible for the maintenance of the dormant state of the spores. Later, this suggestion was confirmed by the fact that all the means that cause activation of the spores affect the tertiary structure of the proteins (Keynan et al., 1965). The overall effect of the activation is on the germination rate, germination requirements, and permeability of the spores (Keynan and Evenchik, 1969). Cook and Pierson (1983) in their review showed that most studies suggest heat activation at 80^{o}C for 10-20 min for types A and B of C. botulinum, whereas heat activation at 60^{o}C for 10 min. is optimal for type E.

b. Germination

Germination has been throughly studied. Smoot and Pierson (1982) addressed all aspects of germination in detail. Keynan and Halvorson (1965) described the process of germination as the degradative processes which terminate the dormancy irreversibly. However, a more recent definition by Setlow (in Keynan,1978) describes germination "as the first minutes of the overall conversion of a dormant spore into a growing cell, and this time is further defined as that period of the process when all events occurring within the spore can be supported by endogenous resources." This definition, in contrast to the previous one, includes the synthesis of macromolecules as long as they are supported by endogenous resources.

Regardless of how one defines germination, it leads to a dramatic change in spore morphology. Gould (1970) described the sequence of events that occur during spore germination. These events include the loss of heat resistance, loss of chemical resistance, loss of calcium and dipicolinic acid, excretion of peptidoglycan, increase in stainability, loss of refractility, and decrease in optical density.

Certain chemicals are required by activated spores to start germination. The germinant requirements for clostridia include: L-alanine, glucose, bicarbonate, cysteine, lactate, and Ca-dipicolinate, (Gould, 1969 ; Ando

1973). Oxygen affects the germination of clostridia in a species-dependent manner.

The exact mechanism of the way the germinants initiate germination is not fully understood. Gould and Dring (1972) pointed out that there is a lapse between the time a spore is exposed to germination agents and the first visible changes that result from the degradation processes. The Trigger reaction, which is responsible for initiation of germination, occurs during this time lapse; therefore, it preceeds all visible changes. The time lapse has been called the "germination lag." All the reactions which may occur between the initiation of germination and the first visible changes has been called the "connecting reactions."

The nature of the trigger reaction has been the subject of much debate. While some believe in the metabolic nature of the trigger reaction, others think the opposite. Vary (1978), working with glucose as a germinant, proved that nonmetabolizable analogs to glucose initiate germination, which indicates that the germinants do not need be metabolized to induce germination.

Studies on L-alanine as a germinant also showed the allosteric nature of the trigger reaction. L-alanine was believed to convert to pyruvic acid, ammonia, and NADH by L-alanine dehydrogenase; NADH was thought to be important in germination. However, Freese et al.(1964) showed that mutants which lack the enzyme L-alanine dehydrogenase still

can germinate with L-alanine as a germinant. The receptor sites of the germinants are not known, however. Smoot and Pierson (1982) suggested spore protease or spore lytic enzymes as possible receptors for L-alanine (germinant).

c. Outgrowth

Germination and outgrowth are entirely distinct and separate processes. Spores usually germinate under conditions that do not support outgrowth (Spiegelman et al. 1969). Strange and Hunter (1969) reported that the requirements for germination are much simpler than those for outgrowth.

Outgrowth refers to the development of vegetative cells from germinated spores. During outgrowth, the germinated spores swell because of uptake of water and nutrients and elongate due to sheding of the coats, resulting in the emergence of vegetative cells (Cook and Pierson, 1983). The metabolic processes involved in outgrowth are RNA and protein synthesis (Kobayashi et al., 1965). DNA synthesis does not begin, however, until late in the outgrowth process (Yoshikaw, 1965).

After outgrowth the emerged cells will grow, if the requirements of cell growth are met. Generally the requirements for cell growth are close to those for outgrowth (Strange and Hunter, 1969). Many factors affect the growth of C. botulinum, such as the availability of nutrients, pH, Eh, a_w, temperature. The effect of all these factors are

well discussed in Sperber (1982). Table 2. , taken from Sperber (1982), shows in summary the effect of some factors on the growth of C. botulinum A and E.

C. Nitrite and meat preservation:

1. Introduction

Nitrite and nitrate have been used for centuries in curing salts to preserve meat. Binkerd and Kolari (1975) present a comprehensive review concerning the history of the use of these salts as food additives. Currently, nitrite (as sodium nitrite) is added to a wide variety of cured meats, and nitrate is used primarily in the curing of foods such as fermented sausages and dry-cured meats requiring production times. Nitrite and nitrate have been the subject of hundreds of publications. Some important aspects of nitrite are discussed below.

2. Function

a. Color and flavor development

The color of fresh meat is attributed to the presence of myoglobin and the differences in color depends on the oxidation state of the heme iron in the pigment. The purplish-red color of raw meats is due to the presence of myoglobin, the bright-red is due to oxymyoglobin, and the brown pigment is due to metmyoglobin (Fox, 1966). The

Table 2. Summary of growth characteristics of Clostridium botulinum types A and E

Characteristics	Type A	Type E
Heat-resistant spores	+	-
Minimum growth temp. (°C)	10	3.3
Minimum water activity	0.94	0.97
(% brine)	(10)	(5)
Minimum pH	4.8	4.8
Maximum Eh (mV)	+250	+250

From Sperber (1982)

mechanism of color development due to the nitrite in cured meats has been presented by Fox (1966). Certain biochemical reactions in meat reduce the nitrite to nitric oxide and the heme iron in myoglobin to a ferrous state. The reaction of myoglobin with nitric oxide results in the formation of nitric oxide myoglobin, which is a bright red pigment. Upon heating, the protein portion of the nitric oxide-myoglobin denatures and a relatively stable pigment is formed, called nitrosyl hemochrome.

Unlike color development, the mechanism of flavor development by nitrite is still obscure. Attempts to identify the volatile compounds produced during the cooking of cured meats have been unsuccessful because no compound or class of compounds has been shown to be responsible for the characteristic cured meat flavor (Pierson and Smoot, 1982)

Both the color and flavor of cured meats can be identified by sensory evaluation tests. Hustad et al. (1973) showed that as little as 25-40 ppm of nitrite in wieners is enough to give the typical color and flavor of cured meats. Similarly, Sales et al. (1980) indicated that panelists were able to detect the differences in color and flavor of turkey frankfurters containing 0 and 40 ppm of nitrite; yet they observed no differences between samples containing 40 and 100 ppm. MacDonald et al. (1980a) found that 50 ppm of nitrite provided the desirable cured color in ham.

b. Antioxidative properties

Nitrite has been shown to inhibit lipid oxidation in cooked meat and meat products. Sato and Hegarty (1971) reported on the reduction of TBA value by sodium nitrite in cooked ground beef stored at 4°C. MacDonald et al. (1980b) studied the effects of various concentrations of sodium nitrite (50, 200, and 500 mg/kg) on the oxidation of lipids in cured pork. The results indicated that nitrite caused reduction in TBA values. Sales et al. (1980) also observed a reduction in a rancid off flavor in turkey frankfurters by sodium nitrite.

The reactions that inhibit the oxidation of lipids by nitrite are not understood. Tarladgis (1961) suggested that nitrite affects rancidity in meat by the same reactions involved in the development of cured color. During these reactions the ferric iron in heme is reduced to ferrous iron. This reduction changes the iron from an active to an inactive catalyst for lipid oxidation. Some derivatives of nitrite in meat also have antioxidant properties. According to Kanner and Juven (1980), S-nitrosocysteine, a compound generated during the curing of meat, has been shown to act as an antioxidant in both an aqueous linoleate model system and ground cooked turkey meat.

c. Antimicrobial properties

The antimicrobial, specifically antibotulinal, activity of nitrite is well-known. Hauschild (1982) well summarized the results of most publications on the effect of

nitrite on C. botulinum.

There have been many attempts to establish the mechanism for the antimicrobial behavior of nitrite. Yarbrough et al. (1980) showed that nitrite attacks more than one site in the bacterial cells. They observed the inhibition of the enzyme aldolase and the active transport of proline in E. coli, but did not find any effect on the active transport of glucose via phosphoenol pyruvate:phosphotransferase system. Freese and Sheu (1973) showed that nitrite inhibited the active transport of serine in B. subtilis.

As an antibotulinal agent, Duncan and Foster (1968) revealed the ineffectiveness of nitrite on spore germination; hence the antibotulinal effect of nitrite is on outgrowth and / or growth. Tompkin (1978) suggested that nitrite inhibits C. botulinum through the reaction of nitrous oxide with an iron-containing compound such as ferredoxin; inhibition of ferredoxin interferes with the energy metabolism of the cells. Holly (1981) listed the following supportive observations for Tompkin's theory: (1) Addition of iron to meat containing nitrite reduces the inhibitory effect of nitrite. (2) Canned pork hearts, which contain twice as much iron as pork and ham, are significantly more susceptible to C. botulinum toxicity at the same nitrite concentration. (3) EDTA, polyphosphate, and isoascorbate enhance the antibotulinal activity of nitrite

by means of their ability to chelate iron, consequently preventing the repair of inactivated enzymes which contain iron as a cofactor.

Woods et al. (1981) also showed that nitrite affects ferredoxin activities. They reported on the inhibition of the phosphoroclastic system of C. sporogenes by nitrite. This system in clostridia is responsible for the cleavage of pyruvic acid to acetyl-CoA, CO_2, with the production of ATP and NADH. The inhibition of the system was evidenced from the accumulation of pyruvic acid and the reduction of ATP per cell. The mechanism of the inhibition was attributed to the inhibition of pyruvate:ferredoxin oxidoreductase, which is involved in the phosphoroclastic system of clostridia. More specifically, the inhibition was attributed to the reaction of nitric oxide, formed from nitrite, with the nonheme iron of ferredoxin. This is another supportive work for Tompkin's suggestion. Woods and Wood (1982) later confirmed the inhibition of the phosphoroclastic system by nitrite in C. botulinum.

One of the most interesting aspects of nitrite as an antibotulinal agent is its enhanced activity upon heating in microbiological media. Perigo et al. (1967) were the first to detect this behavior of nitrite. Later, it was called the Perigo-type factor by Chang et al. (1974). Huhtanen (1975) indicated that the formation of the factor was dependent on the composition of the media. Johnston et al. (1969) showed

that the Perigo-type factor in microbiological media can be neutralized by nonfat meat solid particles, which means that the factor has no value in meat preservation. Johnston and Loynes (1971) showed that the inhibitory factor in meat suspensions was different from the Perigo-type factor; the Perigo-type factor was nondialyzable, whereas the inhibitor formed in cooked meat suspensions was dialyzable. The overall mode of action of nitrite on C. botulinum in cured meats might be one of following (Pierson and Smoot, 1982):

(1) Enhance destruction of spores by heat.
(2) Increase in the germination of spores during the heat treatment followed by the heat destruction of the germinated spores.
(3) Inhibit germination and outgrowth of spores surviving the thermal process.
(4) React with some component(s) in the meat system to produce a more inhibitory compound.

3. Factors affecting the antibotulinal action of nitrite.

a. Number of spores and level of nitrite:

Antibotulinal activity increases with an increased nitrite input and decreases with an increase in the number of spores in the meat products. Hauschild (1982) showed that an increased nitrite level and a reduced spore load per package reduce the probability of outgrowth and toxigenesis of C. botulinum in vacuum-packaged and canned meat products.

b. NaCl and pH

An increased inhibitory action of nitrites in the presence of sodium chloride is well-known. A synergistic effect was reported for the combination of NaCl and $NaNO_3$ in the inhibition of vegetative cells of C. botulinum in culture media and a pork macerate broth (Baird-Parker and Baillie, 1974). pH is another important factor in the inhibitory action of nitrite. Roberts and Ingram (1966) reported an increase of about ten-fold in the antibotulinal activity of nitrite with a reduction of pH from 7 to 6.

c. Incubation temperature and heat processing

Higher temperatures for incubation of cured meats reduce the activity of nitrite as an antibotulinal agent. Possibly, this effect is due to the higher rate of nitrite depletion and/or to the higher temperatures which are more favorable for growth of C. botulinum (Christiansen et al., 1974). Pasteurization, the heat processing for cured meats, does not affect the susceptibility of spores to nitrite, but it eliminates certain competitors that germinating C. botulinum encounter in unheated products; higher temperatures injure the spores and make them more susceptible to nitrite and sodium chloride (Roberts and Ingram, 1966).

d. Isoascorbate, EDTA, cysteine, polyphosphate

All these chemicals increased the antibotulinal activity of nitrite. Chelating the iron in the meat products by these substances prevents the repair of enzymes inhibited

by nitrite. The activity of the inhibited enzymes depend on their iron, e.g. ferredoxins (Christiansen, 1980).

e. Microbial interactions

Other microorganisms that may be present in the product may directly or indirectly affect the growth of C. botulinum. For instance, it appears that enterococci have inhibitory action on C. botulinum; this effect is not related to the pH effect, although the mode of the action is not yet understood (Rieman, 1973). Details about all the factors which affect the antibotulinal activity of nitrite are available in Pierson and Smoot (1982), NAS (1981), and Christiansen (1980).

4. Nitrosamines, formation, risk, elimination.

A number of observations have led to a concern about the risk of using nitrite and nitrate as food additives in cured meats. The results of limited studies have shown that neither nitrite nor nitrate acts as a carcinogeic substance; however, they act as a precursor in the formation of nitrosamines, which are considered carcinogenic and teratogenic in laboratory animals,and mutagenic in microbial and mammalian test systems (NAS, 1981).

The mechanism of nitrosamine formation in vitro is well understood (Challes, 1981). Nitrite or nitrous acid in an aqueous acidic solution undergoes certain reactions that lead to the formation of nitrosonium ion (NO^+), which is usually combined with neucleophilic agents (NO_2, Cl^-,

SCN^-). The nitrosonium ion reacts with secondary and tertiary amines and amides leading to the formation of nitrosamines and nitrosamides, respectively.

There are numerous sources of nitrate and nitrite in a normal diet besides those attributable to cured meats. Estimates of the nitrate and nitrite intake from different sources for the U.S. population generally indicate that the average individual is exposed to nitrate primarily from vegetables 87%, fruits and juices 6%, water 3%, and cured meats 2%. Nitrite intake, on the other hand, is provided by cured meats 39%, baked goods and cereals 34% and vegetables 16%. The endogenous conversion of nitrate to nitrite is not considered in these estimates; about 50% of nitrate is converted to nitrite, in vivo, mostly by the action of normal bacteria in the mouth. When this conversion is taken into consideration, about 72% of nitrite comes from vegetables and less than 10% from cured meats (NAS, 1981).

Concerning reduced exposure to nitrate and nitrite, NAS (1981) recommended some modifications in the growth conditions of vegetables such as timing and level of nitrogen fertilizers, daytime temperature, time of harvesting, and storage temperature; all these affect the nitrite content of vegetables. NAS also made recommendations for nitrite reduction in cured meats; however the reduction may affect the protection against botulism. Miller (1980) reported the following:

> Based on the assumption that nitrite will be proven to be carcinogenic, it was estimated by FDA that the risk due to botulism associated with the removal of nitrite from cured meats approximately equalled the risk likely from cancer if nitrite is not removed from these products. This was interpreted to be between two to fifty deaths per year.

Another approach to reducing the risk of nitrite and nitrate involvement in nitrosamine formation is using inhibitors for reactions which lead to the formation of nitrosamines. Many compounds have been shown to be effective in this regard, e.g. ascorbic acid, dextrose, gallic acid, simple phenols, cysteine, glutathione, bisulfide, (NAS, 1982). Most of these inhibitors act by reducing the nitrosonating agent to either nitric oxide or nitrogen. Theiler et al. (1984) indicated that a combination of tocopherol, glucose, and liquid smoke reduced the nitrosamine formation by 80% in model systems as well as in a pilot plant manufactured bacon.

NAS (1981) also recommended more work on developing new and more accurate methods to detect nitrosamine compounds in foods. Nitrosamines can be divided into two groups: volatile and nonvolatile. The volatiles are nonpolar and have low molecular weight, whereas the nonvolatile are polar and have relatively high molecular weight. Usually,

the methods of detection of volatiles are easier to implement and more accurate than those for nonvolatile nitrosamines. Methods of detection of volatile nitrosamines involve the separation of the compounds by distillation, followed by an analysis with gas chromatography using a thermal energy detector (TED), which is very sensitive and specific for nitrosamines. AOAC methods can detect as low as 1 ppb of these compounds in foods. Two approaches are available for detection of nonvolatile nitrosamines: one is the conversion of the compounds to volatiles, which can be detected like volatile nitrosamines; the second is the separation of the compounds by HPLC, to be subsequently detected with TED (Scanlan and Reyes, 1985).

5. Alternatives to the use of nitrites in cured meats

Because of the concern over the safety of using nitrite in cured meats, several available or potentially alternative means of botulinal control have been studied. There are many publications discussing all aspects of the available alternatives. The following are some recent reviews on this subject: Sofos and Busta (1980), Holly (1981), NAS (1982), Pierson and Smoot (1982), Widdus and Busta (1982), Roberts and Gibson (1986).

Most of the alternatives to nitrite produce only one of the effects of nitrite, usually the antibotulinal effect. As discussed in the previous sections, the

concentration of nitrite required to fix color and give flavor to cured meats is between 40-50 ppm. Therefore, it is possible to achieve all the functions of nitrite in cured meats by combining low concentrations of nitrite with one of the alternative agents.

Most of the publications mentioned above divide the alternative agents into two categories: physical agents and chemical agents. The physical agents include radiation, heat treatment, low temperature storage, and dehydration. Although these means have proven effective, generally their uses are limited because of concerns about the stability and identity of the products, energy consumption, and requirements for new processing procedures and new manufacturing control programs (Sofos and Busta, 1980).

The chemical agents include a wide variety of substances: sodium chloride, ascorbate, isoascorbate, polyphosphate, sorbate, parabens, tochopherol, nisin, acids, and acidification by lactic acid bacteria. All these compounds are approved for use in foods by the FDA except for nisin and acidification by microorganisms, although both are in use in many other countries. Recent publications concerning the possible nitrite alternatives for the inhibition of C. botulinum are compiled in Table 3.

Table 3. Possible nitrite alternatives for the inhibition of C. botulinum

Additive	Conc.[a]	Medium	Type	Reference
Esters of P-hydroxybenzoic acid:				
Methyl	1200	M[b]	A	Robach and Pierson (1978)
	1000	M	A	Dymicky and Huhtanen (1979)
Ethyl	1000	M	A, B	Reddy et al. (1982)
	400	M	A	Dymicky and Huhtanen (1979)
	1000	M	A, B	Draughon et al. (1982)
	1000	Pork	A, B	Draughon (1982)
	1000	Pork	A, B	Pierson and Reddy (1982)
Propyl	200	M	A	Robach and Pierson (1978)
	1000	M	A, B	Reddy et al. (1982)
	400	M	A	Dymicky and Huhtanen (1979)
	1000	M	A, B	Draughon et al. (1982)
	1000	Pork	A, B	Pierson and Reddy (1982)
Butyl	200	M	A, B	Reddy et al. (1982)
	200	M	A	Dymicky and Huhtanen (1979)
	1000	M	A, B	Draughon et al. (1982)
	1000	Pork	A, B	Pierson and Reddy (1982)
Phenolic antioxidants:				
BHA	200	M	A, B	Reddy et al. (1982)
	25-50	M	A, B	Robach and Pierson (1979)
	100-200	Pork	A, B	Tompkin et al. (1978)
	1000	Pork	A, B	Pierson and Reddy (1982)
BHT	200	M	A, B	Reddy et al. (1982)
	200	M	A, B	Robach and Pierson (1979)
	1000	Pork	A, B	Pierson and Reddy (1982)

Table 3. (continued)

Additive	Conc.	Medium	Type	Reference
TBHQ	100-200	Pork	A, B	Tompkin et al. (1978)
	400	M	A, B	Reddy et al. (1982)
	1000	Pork	A, B	Pierson and Reddy (1982)
Esters of gallic acid:				
Propyl	1000	M	A, B	Reddy et al. (1982)
Butyl	1000	M	A, B	Reddy et al. (1982)
Isobutyl	800	M	A, B	Reddy et al. (1982)
	1000	Pork	A, B	Pierson and Reddy (1982)
p-Octyl	400	M	A, B	Reddy et al. (1982)
p-Dodecyl	200	M	A, B	Reddy et al. (1982)
Esters of Fumaric acid:				
Monomethyl	1250	Bacon	A, B	Huhtanen (1983)
	≥400	M	A	Dymicky et al. (1987)
Dimethyl	400	M	A	Dymicky et al. (1987)
	1250	Bacon	A, B	Huhtanen (1983)
Monoethyl	≥400	M	A	Dymicky et al. (1987)
	1250	Bacon	A, B	Huhtanen (1983)
Diethyl	400	M	A, B	Dymicky (1987)
Mono $C_{14}H_{29}$	1.3	M	A	Dymicky et al. (1987)
Di $C_{14}H_{29}$	≥40	M	A	Dymicky et al. (1987)
Fatty acids and esters:				
Short chain (C_1-C_7)	≥200	M	A	Dymicky and Trenchard (1982)
Medium chain (C_8-C_{15})	100	M	A	Dymicky and Trenchard (1982)
Long chain (>C_{16})	≥200	M	A	Dymicky and Trenchard (1982)
Monolaurin	5000	Meat slurry	A, B, E	Notermans and Dufrenne (1981)
Aliphatic alcohols:				
Methanol	30000	M	-[c]	Huhtanen (1980)
Ethanol	30000	M	-	Huhtanen (1980)
Propanol	15000	M	-	Huhtanen (1980)
Butanol	10000	m	-	Huhtanen (1980)
Octanol	200	M	-	Huhtanen (1980)
Decanol	25	M	-	Huhtanen (1980)
Aliphatic amines and aminodiamides	1.6-50	M	-	Huhtanen and

Table 3. (continued)

Additive	Conc.	Medium	Type	References
				Micich (1978)
Polyphosphates:				
Curaphos 700				
	3000	Pork	A, B	Roberts et al. (1981a, b, c)
	3000-5000	Pork	A, B	Jarvis et al. (1977)
Na-Hypophosphates				
	3000	Bacon	A, B	Pierson et al. (1981)
Na-Triphosphate				
	4000	Pork	A, B, E	Tompkin (1984)
Sorbate / Sorbic acid:				
K-Sorbate	2600	M	A, B	Blocher et al. (1982)
	2600	Bacon	A, B	Ivey et al. (1978)
	2600-5200	Canned chicken and turkey emulsion		
			A, B	Huhtanen and Feinberg (1980)
Sorbic acid				
	500-2000	Pork	A, B	Ivey and Robach (1978)
	1300	Bacon	A, B	Huhtanen et al. (1981)
	2000	M	A, B	Draughon et al. (1982)
	2000-4000	Canned chicken and turkey emulsion		
			A, B	Huhtanen and Feinberg (1980)
Acidification:				
By acetic or citric acid				
	pH $\leq$4.6	Shrimp and tomato puree	E	Post et al. (1985)
	pH 4.2-5.4	M	A,B,E	Tsang et al. (1985)
By lactic acid bacteria	-	Bacon	-	Tanaka et al. (1980)
Sodium chloride				
	3300-5000	Fish	E	Pelory et al. (1985)
	2.5-125	M	A, B, E	Scott and Taylor (1981a, b)
EDTA	730	Fish	A	Winarno et al. (1971)

[a] concentrations are in µ/ml unless otherwise specified.
[b] M refers to microbiological medium.
[c] - type of C. botulinum not specified.

As discussed previously, essential oils of spices appear to have antimicrobial activity against different types of microorganisms, bacteria, yeasts, and molds. Moreover, most of the oils possess antioxidative acitivities. Limited studies have shown effect of some spice oils on C. botulinum. However, the effect of the oils on specific stages of conversion of dormant spores to vegetative cells (germination, outgrowth and growth) is not understood. The sensitivity of different types of C. botulinum towards the spice oils is not known, either. In addition, the mode of action of the oils on C. botulinum activities and the interaction of the oils with other antibotulinal agents such as sodium nitrite have not been studied. Thus, the objectives of the study in this dissertation were to determine the effect of some spice oils (colve, thyme, black pepper, pimenta, origanum, garlic, onion and cinnamon) on germination, outgrowth, cell growth and toxin production of six strains of C. botulinum belonging to types A, B and E. Certain experiments were conducted to determine the mode of action of the oils on germination inhibition. The effect of origanum oil with different concentrations of sodium nitrite was studied in both a microbiological medium and vacuum packaged pork.

Some of the oils may also provide antibotulinal activity in foods in which C. botulinum is considered a health hazard. Thus, some of these oils may be used as

preservatives in cured meat products to reduce the level of sodium nitrite of cured meat products, which subsequently reduces exposure of humans to carcinogenic substances (nitrosamines and nitrosamides).

III. MATERIALS AND METHODS

A. Essential oils of spices

The essential oils of eight spices (pimenta leaf, clove leaf, thyme, black pepper, origanum spanish, onion, cinnamon leaf, and garlic) were obtained from Fritzsche, Dodge & Olcott Inc. (New York, NY). The oils were stored at 4°C until used. These oils were tested for their inhibitory activities against Clostridium botulinum types A, B, and E

B. Preparation of spores of Clostridium botulinum

The spores of three strains of C. botulinum (1623E, 2157E and 10755A) were obtained fron Anaerobic Laboratory, Virginia Polytechnic Institute and State University. The strains 33A and 40B were obtained from L. N. Christiansen, Research and Development Center, Oak brook, Ill. The strain 67B was obtained from D. B. Rowley, Natick, MA. The spores belonging to types A and B (33A, 10755A, 40B, 67B) were prepared according to the biphasic culture method of Anellis et al. (1972), whereas spores of type E (1623E, 2157E) were prepared by methods of Bruch (1968).

The spores of all the strains were harvested from the liquid phase according to the methods of Rowley and Feeherry (1970). The harvesting procedure included cetrifugation of the spores in the liquid phase at 4000 x g (5°C), and the washing of the pellet with distilled water. The centrifugation and washing were repeated from eight to ten times.

The remaining vegetative cells and debris were eliminated from the spore crop by using the methods set forth in Hawirko et al. (1976). In this procedure the spore crop was treated with an enzyme solution of 100 μg/ml trypsin, 10700 Baee units per mg protein (Sigma Chemical Company, St Louis, MO) and 200 μg/ml lysozyme, 41000 units per mg protein (Sigma Chemical Company, St Louis, MO) in phosphate buffer, pH 7.0) for 4 h at 37°C with continuous agitation. Then, the debris was removed by centrifugation at 1000 x g for 20 min, 4000 x g for 10 min, and 10,000 x g for 5 min. The cleaned spores were resuspended in sterile-distilled water. The concentration of the spores was adjusted so that with the addition of 0.2 ml of the spore suspension to 10 ml of distilled water, the absorbance at 600 nm would be about 0.3. The viable spore counts in the final crops of the spores were determined by using the roll tubes method of Pierson et al. (1974). The spores were stored in sterile vials at -20°C.

C. Effect of spice oils on growth of C. botulinum

Prereduced peptone yeast extract medium was prepared and dispensed using the anaerobic methods described in Holdeman et al. (1977). The medium (9.8 ml) was dispensed into roll tubes (18x142 mm, type 1; Bellco glass). The medium was autoclaved at 121°C for 15 min, and was maintained in a liquid state in a water-bath 55-60°C. The desired dilutions of spice oils were prepared in filter

sterilized 95% ethyl alcohol as described by Conner and Beuchat (1984a). The spores were heat-activated (80°C for 10 min for types A and B and 60°C for 10 min for type E).

The medium in roll tubes was inoculated with both activated spores and dilutions of spice oils. The activated spores were added to the tubes so that the colony count would be in the range of 300-400 colonies in the control tubes (tubes without spice oils). The spice oils were added to make the concentration of the oils in the tubes 0, 10, 50, 100, 150, and 200 ppm. Both the spice oils and the activated spores were inoculated using the VPI anaerobic system. The inoculated tubes were spun to solidify the medium around the tubes. The tubes were incubated at 35°C for three days. The number of colonies in the tubes was counted with the Darkfield Quebec Colony counter (American Optical Corporation).

The percent of inhibition of colony formation (growth) caused by each concentration of the spice oils was determined by comparing the number of colonies in the tubes with spice oils to the number of colonies in the control tubes.

The equation below was used to determine the percent of inhibition:

$$\% \text{ growth inhibition} = \left[1 - \frac{\text{Colony count in treatment tube}}{\text{Colony count in control tube}}\right] \times 100$$

D. Effect of spice oils on germination of C. botulinum

The essential oils of the eight spices were tested for their effect on the germination of spores of C. botulinum types A, B, and E by the microculture method. The GO medium used by Cook (1982), (supports the germination and outgrowth of C. botulinum spores), consisted of 1% Thiotone (BBL, Cockeysville, MD), 1% Yeast extract (BBL), 0.4% glucose, and 1.5% agar in 0.05 M sodium phosphate buffer pH 7.0 . The medium was dispensed (9.7 ml in per tube), and was autoclaved at 121°C for 15 min. The medium was maintained in a liquid state in a water-bath 55-60°C. A 0.1 ml of filter-sterlized solutions of sodium bicarbonate and chloramphenicol was added to the GO medium to make the concentrations of the two chemicals 30 mM and 10 µg/ml, respectively. Sodium bicarbonate enhances the germination rate of the spores, whereas chloramphenicol prevents the outgrowth of the germinated spores.

Microcultures were prepared by the methods of Duncan and Foster (1968). Approximately 0.5 ml of the GO medium, mixed well with spice oils (conc. 10, 50, 100, 150, 200 ppm), was layered on a sterile glass slide (75x25 mm). Approximately 20 µl of the activated spores was smeared onto the surface of a sterile glass cover-slip (18x18 mm). The

spores were allowed to dry on the surface of the cover-slip. Then, the cover-slip was inverted onto the hardened agar surface, with the edges of the cover-slip sealed with vasper. The microcultures were incubated at 32°C for six hrs, after which time the microcultures were examined under phase contrast microscopy. The germinated spores appeared dark, whereas the ungerminated spores appeared bright refractile. The number of germinated and ungerminated spores in ten microscopic fields (MF) were counted. The percent of germination was determined according to the following equations:

$$\% \text{ Germinated spores} = \frac{\text{\# of germinated spores in 10 MF}}{\text{Total \# of spores in 10 MF}}$$

$$\% \text{ Germination} = \left[\frac{X}{Y}\right] \times 100$$

where X refers to %germinated spores in microcultures prepared with certain type and concentration of spice oil, and Y represents %germinated spores in microcultures with no spice oil (control).

Dadd and Rumbelow (1986) used a similar method to determine the percent of germination of spores of Bacillus subtilis var niger.

E. Effect of spice oils on C. botulinum 67B

1. Effect on germination of C. botulinum 67B (optical

density method)

In this experiment GO medium without agar was prepared as previously described in section D. The medium was prepared anaerobically and dispensed into roll tubes (type 1, 18x142 mm; Bellco glass, Inc., Vineland, N. J.). Both chloramphenicol and sodium bicarbonate were added to the medium in the same concentrations and for the same purpose as mentioned in section D.

Spice oils (Ø.1 ml) were added to the medium in the tubes to make the concentration of the oils in the medium 1Ø, 5Ø, 1ØØ, 15Ø, and 2ØØ ppm. Then activated spores (Ø.3 ml) was added, and the initial O.D. was measured at 6ØØ nm.

The inoculated tubes were incubated at 35°C for two hrs. The O.D. was monitored every 3Ø min interval using a Spectronic 2Ø (Bausch & Lomb). The reduction in O.D. (% of initial value) was determined according to the following equation:

$$\% \text{ O.D. Reduction} = \left[\frac{\text{O.D. at time (x)}}{\text{O.D. at time (Ø)}}\right] \times 1ØØ$$

where x represents the O.D. at any time (3Ø-12Ø min).

The results were presented in plots of O.D. versus time (min).

2. Duration of germination sensitivity to spice oils

In this experiment the duration of sensitivity of germination of spores of C. botulinum 67B spores to oils of clove, thyme, origanum, and cinnamon were tested. The experiment was performed with GO medium containing chloramphenicol to prevent the outgrowth and sodium bicarbonate to increase the rate of spore germination. Activated spores of C. botulinum 67B were inoculated into the medium, and the tubes were incubated at 35°C. At intervals of 1, 5, 10, 15, and 30 min of incubation, two tubes were inoculated with 200 ppm of the spice oils. The fall in O.D. was monitored every 30 min at 600 nm. The purpose of this experiment was to determine if there is a stage of germination where the spores become insensitive to the spice oils.

3. The reversibility of inhibition of spore germination

This experiment was performed to determine if the inhibition of spore germination is reversible. The test was conducted with GO medium pH 7.0. Four spice oils (clove, thyme, origanum, and cinnamon) were tested in this experiment. Initially spores of C. botulinum 67B were suspended in GO medium containing 0 and 200 ppm of the spice oils. Four tubes were used for each concentration. The inoculated tubes were incubated at 35°C for two hrs. The O.D. of the medium in each tube was determined every 30 min.

After two hrs of incubation, the spores suspended in the medium with 200 ppm of the oils were separated from the medium by centrifugation at 4080 x g for 10 min at 4°C. The separated spores were resuspended in a fresh GO medium, with and without spice oils. The inoculated tubes were incubated for another two hrs at 35°C, and the fall in O.D. was monitored at 600 nm every 30 min. The results are plotted, O.D. versus time (min).

4. Effect of spice oils on outgrowth and growth of C. botulinum 67B

Spores of C. botulinum 67B were inoculated into GO medium containing chloramphenicol to allow germination but not outgrowth. The inoculated broth was incubated at 37°C for two hrs. Then, the spores were observed under phase contrast microscopy. More than 99% of the spores were germinated. The germinated spores were separated from the medium (GO) by centrifugation at 6000 x g for 10 min at 4°C. The germinated spores were diluted in 0.1% prereduced peptone water.

Separate tubes of TYG media were inoculated with 0.1 ml of dilutions of spice oils to make the final concentration of the oils 10, 50, 100, 150, and 200 ppm. Duplicate tubes were used for each concentration of the spice oils. The TYG media with spice oils were inoculated with 0.1 ml of appropriately diluted germinated spores for a concentration of 300 germinated spores per ml (the number of germinated

spores was determined by the roll tube method of Pierson et al., (1974). The broths were incubated for one week at 37°C. Growth of the germinated spores was monitored by measuring absorbancy at 600 nm with Baush & Lomb Spectronic 20.

The times for the test and control tubes to reach an O.D. of 0.35 was used to calculate growth ratios.

$$\text{Growth ratio} = \frac{\text{Time for control to O.D. = 0.35}}{\text{Time for treatment to O.D. = 0.35}}$$

These ratios were used to evaluate the effectiveness of the apice oils at all the concentrations used.

5. Effect of spice oils on vegetative growth (cell division)

Vegetative cells of C. botulinum 67B at late logarithmic phase were prepared according to the methods of Rhodehamel (1983). In this method, a loopful of a stock culture of C. botulinum 67B in chopped meat medium was transferred to TYG broth, incubated at 37°C for 12 hrs, and then observed microscopically for vegetative cells. All C. botulinum 67B were present as vegetative cells. The vegetative cells were diluted in prereduced 0.1% peptone water. An appropriate dilution of the vegetative cell culture (to a final concentration 300 cells per ml) was

added to TYG broth containing different concentrations of the spice oils. The TYG broths were incubated at 37°C for 1 week. Growth of the vegetative cells was monitored and growth ratios were calculated.

F. Effect of spice oils on toxin production

1. Preparation of medium

The basal prereduced growth medium (TYG) consisted of 1.0% thiotone (BBL, Cockeysville, MD), 1.0% yeast extract (BBL), 0.1% glucose, 0.05% cysteine hydrochloride, and 0.0001% resazurin. The TYG medium was prepared anaerobically using the methods of Holdeman et al. (1977). Prereduced TYG medium was dispensed into anaerobic culture tubes (18x142 mm, Bellco glass, Inc.; Vineland, N.J.), and autoclaved at 121°C for 15 min.

2. Preparation of spore mixture

The six strains of C. botulinum belonging to types A, B, and E were heat activated separately. The activated spores were mixed in equal proportion, and diluted in 0.1% prereduced peptone water. The number of viable spores in the dilutions were determined by the roll tube method of Pierson et al. (1974).

3. Toxin production

To the basal medium, TYG, 0.1 ml of the diluted spore mixture was added to make the concentration of the spores in the medium 300-400 spores per ml. Spice oils, 0.1 ml of

different dilutions, were added to the tubes to make the concentration of the spice oils in the tubes 0, 50, 100, and 200 ppm. The addition of both the dilutions of spores and spice oils were performed under the VPI anaerobic system. The inoculated tubes, five replicates for each concentration of the spice oils, were incubated at 32°C for seven days. The tubes were monitored for growth at one, three, five, and seven day intervals. The parameter for growth was increase in turbidity. The tubes showing an increase in turbidity were examined under the microscope for the appearance of vegetative cells, bacilli shaped. The tubes which were positive for growth were examined for toxin. In case of no growth appearance in any of the five replicate tubes at days of icubation (1, 3, 5, and 7), one of the negative tubes for growth was examined for toxin production.

4. Toxin extraction and mouse bioassay

The contents of the tubes were centrifuged at 34,000 x g for 10 min at 4°C. The supernatant fluid for each tube was divided into three portions, 2 ml each. One of the three portions was untreated; another portion was boiled for 10 min; the third portion was treated with the enzyme trypsin, 1:250 (Difco Laboratories, Detroit, MI). The trypsinization was conducted according to the modified method described in FDA (1978). In this procedure, a 1% solution of the trypsin was prepared in distilled water. Two ml of the enzyme

solution was added to 1.8 ml of the toxin extract. The mixture was incubated at 37°C for one hr. Trypsinization activated the toxins produced by nonproteolytic strains of type E.

The presence of C. botulinum toxin in the samples was detected by mouse bioassay using Swiss male mice (18-22 g weight). From each of the three portions prepared above, 0.5 ml was injected intraperitoneally into two mice. The mice were observed for up to three days for typical signs of botulinum toxicity.

G. Effect of sodium nitrite and origanum oil on growth of C. botulinum (mixed types)

1. In TYG

Filter-sterilized solutions of sodium nitrite and origanum oil were added to the medium in twenty-four different combinations, sodium nitrite in concentrations 0, 10, 20, 30, 40, and 50 ppm versus origanum oil in concentrations 0, 50, 100, and 200 ppm. Activated spores (mixed types) were also added to the medium in the tubes to a concentration of 300-400 per ml. The inoculated tubes were incubated at 32°C for twenty-one days. The tubes were examined for growth by the reading of the optical density of the medium at 600 nm. All tubes which showed an increase in optical density of 0.1 or above were considered positive for growth. Growth was also confirmed through the preparation of wet slides from tubes showing an increase in optical

density. The appearance of bacilli shaped vegetative cells under microscope confirmed the growth of C. botulinum.

A similar experiment was performed except that sodium nitrite was added to the medium, the mixture being heated for 5 min at 121°C. The purpose of this test was to see whether heating affects the inhibitory action of nitrite against C. botulinum or not.

2. In meat

Lean pork was obtained from the Meat Processing Laboratory, Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, VA. The meat was ground twice through 3/16 (5 mm) inch plates, and was formulated with 2.5% sodium chloride, Ø.5% dextrose, and Ø.Ø2% sodium isoascorbate. To this formula , sodium nitrite and origanum oil were added in sixteen different combinations. The final formula was mixed with a Stomacher (Tekmar-company; Cinncinati, OH). Then, the mixture was packed into ten different packages for each variable, Cryovac type, P 85Ø-S (W. R. Grace- Cyrovac, Simpsonville, S.C.), 4Ø gram in each package. The packages were vacuum sealed and cooked in a water-bath at 77°C to an internal temperature of 68.5°C. The time of cooking at 77°C for an internal temperature of 68.5°C was predetermined by use of thermocouples in the center of the packages. It was determined that the internal temperature reached 68.5°C in 1Ø min of cooking in the water-bath of 77°C. The packages

were chilled in an ice-bath to lower the temperature of the packages to 27°C in 5 min. Then, the packages were incubated at 27°C for up to sixty days. The packages were checked daily for the appearance of swelling.

The swollen samples were tested for the presence of C. botulinum toxin. The methods of Pierson and Reddy (1982) were used for extraction of toxin from the samples. The contents of the swollen packages were blended with 80 ml of phosphate gelatin buffer (0.05 M, 0.1% gelatin, pH 6.2) in a Stomacher for two min at room temperature. The homogenate was centrifuged at 34,000 x g for 10 min at 4°C. After centrifugation, the supernatant fluids were tested for the presence of botulinal toxin by the mouse bioassay test.

The probability of growth and toxin production in the meat packages was calculated using the formula suggested by Hauschild (1982):

$$p = (\ln n / q) / s$$

where p is probability of growth and toxin production in packages based on the presence of one spore per package; n is the number of packages in the test group, q is the number of nontoxic packages during a certain incubation time; and s is the number of challenge spores per package.

The presence of one spore per Kg of meat was considered for conversion of p (maximum incidence of C. botulinum spores in semipreserved meat in North America, Abrahamsson and Reimann, 1971). p values were multiplied by a factor (i), where i is the incidence of C. botulinum per package

based on the assumption of one spore per Kg of meat. The i value for the packages prepared in this research is Ø.Ø4, because 4Ø g of meat was filled into each package. The effect of origanum oil and sodium nitrite on the probability of C. botulinum growth and toxin production (p) in the packages were determined. The log 1/p values after one and two weeks of incubation of the packages were calculated and plotted versus concentrations of origanum oil and sodium nitrite.

The proximate composition (moisture, fat, protein) of the meat preparation was determined by methods described in AOAC (198Ø). The aerobic plate count of the meat was determined by blending of 11 g of meat with 99 ml of Ø.1% peptone water. Appropriate dilutions were prepared in Ø.1% peptone water, and were pour plated in duplicate for each dilution with standard methods agar (BBL). The pour plates were counted after 48 h incubation at $32^{o}C$.

The meat was also examined for the presence of C. botulinum spores. Ø.1 ml from a $1Ø^{-1}$ dilution of meat in peptone water was inoculated into prereduced chopped meat glucose medium, described in Holdeman et al. (1977). The inoculated tubes were incubated at $35^{o}C$ for 48 hrs. The tubes which showed growth (turbidity) were tested for the presence of C. botulinum toxins by means of the mouse bioassay test, as in section F.

IV. RESULTS AND DISCUSSION

A. Effect of spice oils on growth of C. botulinum

The inhibition of growth was measured according to the ability of C. botulinum to form colonies in PY agar containing various concentrations of spice oils. Since spore inoculum was used, this represented the overall inhibition caused by the spice oils on all the stages of conversion of refractile spores to vegetative cells and cell growth.

Figure 2 shows the effect of spice oils on the growth of C. botulinum 33A. The spice oils clove, thyme, pimenta, origanum, and cinnamon at concentrations of 150 and 200 ppm caused 100% inhibition, whereas the inhibition caused by onion, garlic, and black pepper at the same concentrations ranged from 84-96%. At 10 ppm, inhibition ranged from 21% for black pepper to 37% for cinnamon. Ethanol (0.95%), at the same concentration used when adding spice oils to the medium, caused an inhibition of 13% , which was lower than the inhibition caused by the spice oils at the lowest concentration, 10 ppm (for all the spice oils).

The effect of spice oils on C. botulinum 10755A was also investigated (Figure 3). At a concentration of 200 ppm , the spice oils thyme, pimenta, origanum, onion, and cinnamon caused 100% inhibition. Clove, black pepper, and garlic, each caused over 94% inhibition at 200 ppm. At 10 ppm, the inhibition ranged from 7% for origanum to 26% for pimenta. Origanum was most effective in inhibiting strain

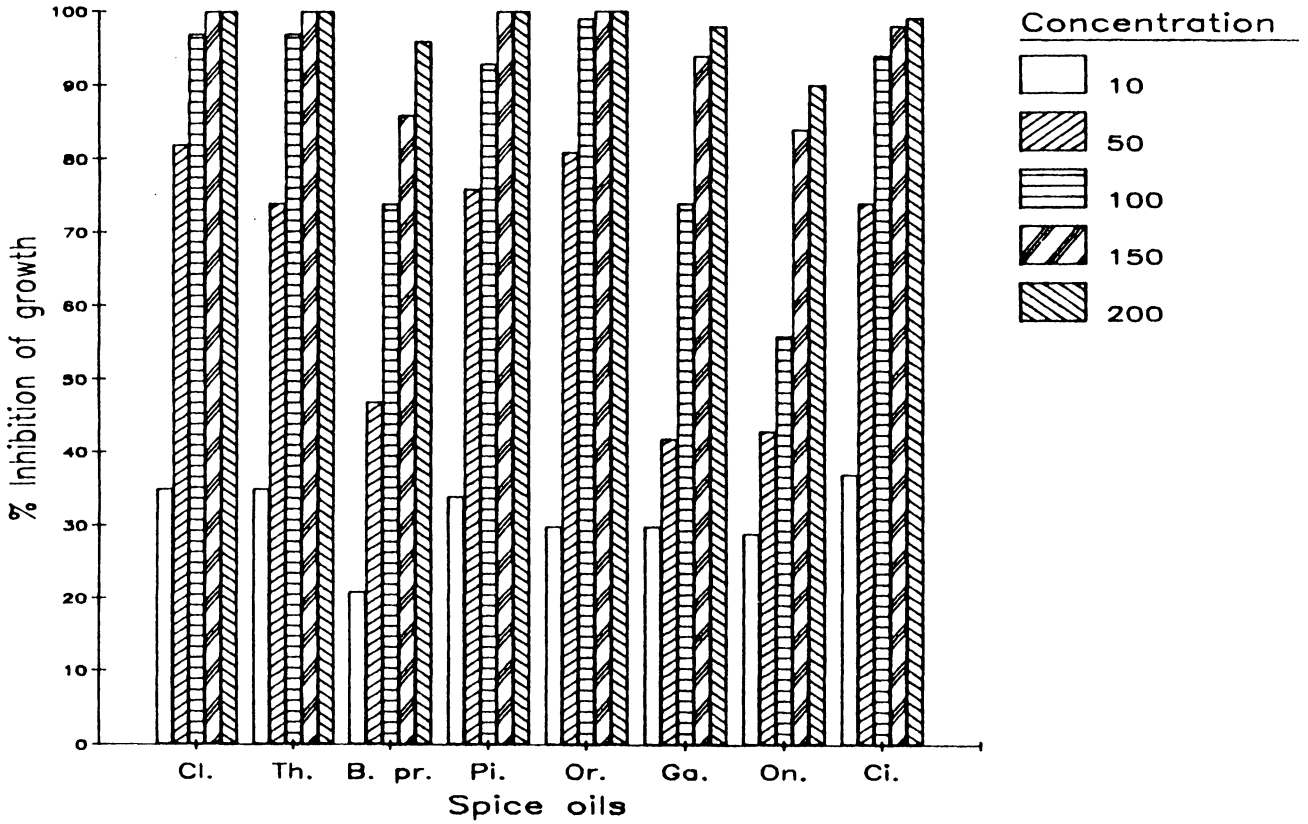


Figure 2. Growth inhibition of C. botulinum 33A by spice oils in PY medium. The abbreviations Cl., Th., B. pr., Pi., Or., Ga., On. and Ci. refer to clove, thyme, black pepper, pimenta, origanum, garlic, onion and cinnamon, respectively.

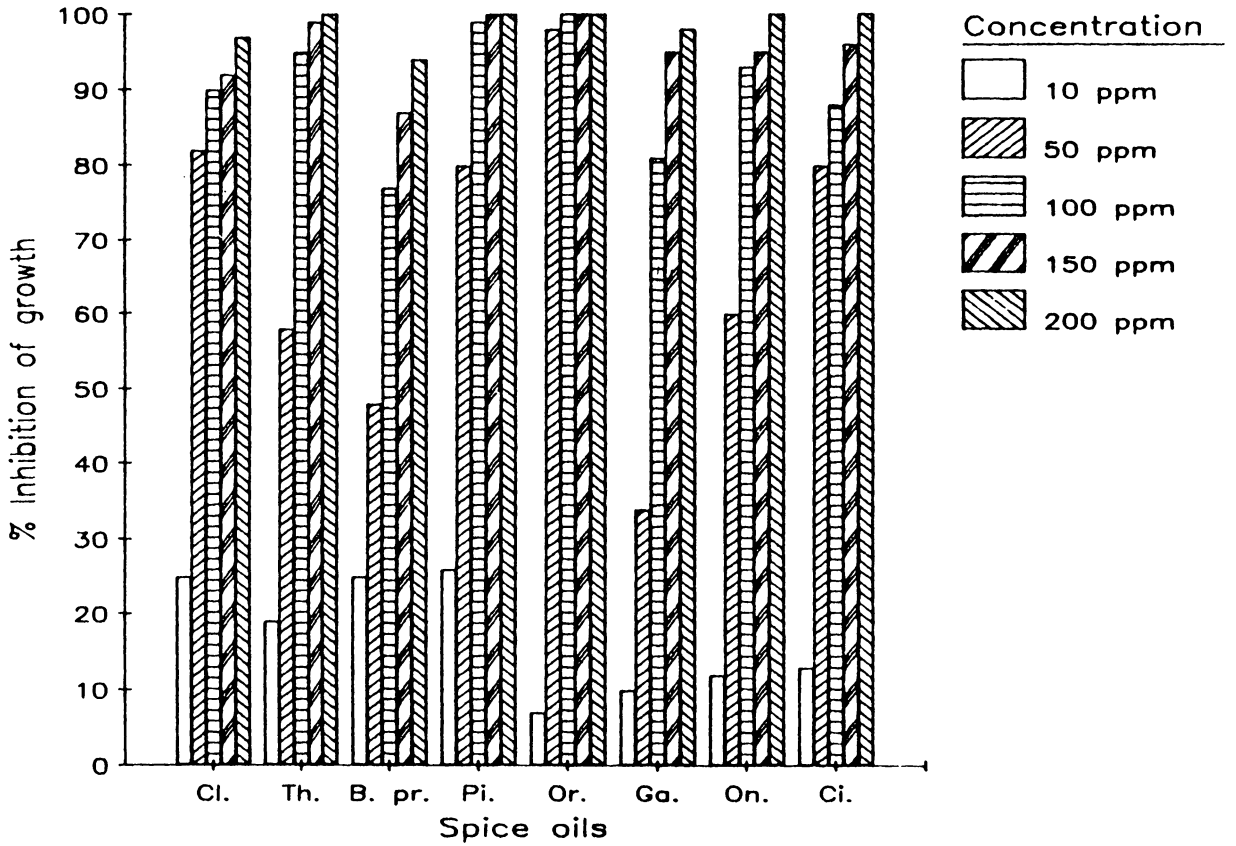


Figure 3. Growth inhibition of C. botulinum 10755A by spice oils in PY medium. See Figure 2 for the abbreviations.

10755A; it caused a total inhibition of colony formation at a concentration of 100 ppm and higher. Ethanol caused an inhibition of about 13%.

Pimenta and cinnamon were the most effective spice oils for inhibiting the growth of C. botulinum 40B (Figure 4). These oils caused a total inhibition of growth at 150 ppm and above. On the other hand, the oils of black pepper, garlic, onion, and clove were the least effective spice oils; they allowed some growth even at 200 ppm. At 10 ppm, the inhibition ranged between 10-32%. Black pepper and garlic at 10 ppm caused a lower level of inhibition than ethanol (0.95%).

The effect of the spice oils on the growth of C. botulinum 67B is presented in Figure 5. As with most of the C. botulinum strains, cinnamon was the most effective of the oils studied; it caused 100% inhibition of growth at concentrations of 150 and 200 ppm. The oils of origanum and clove followed cinnamon as effective inhibitory agents on growth. The pattern of increasing inhibition with increasing concentrations was obvious for all the oils. At the concentration of 10 ppm, the inhibition ranged from 0% caused by clove and pimenta to 32% caused by cinnamon. Inhibition by ethanol was 10%.

The inhibition of growth of C. botulinum 1623E by the spice oils is given in Figure 6. Origanum was the most effective oil, causing a total inhibition of growth at 150

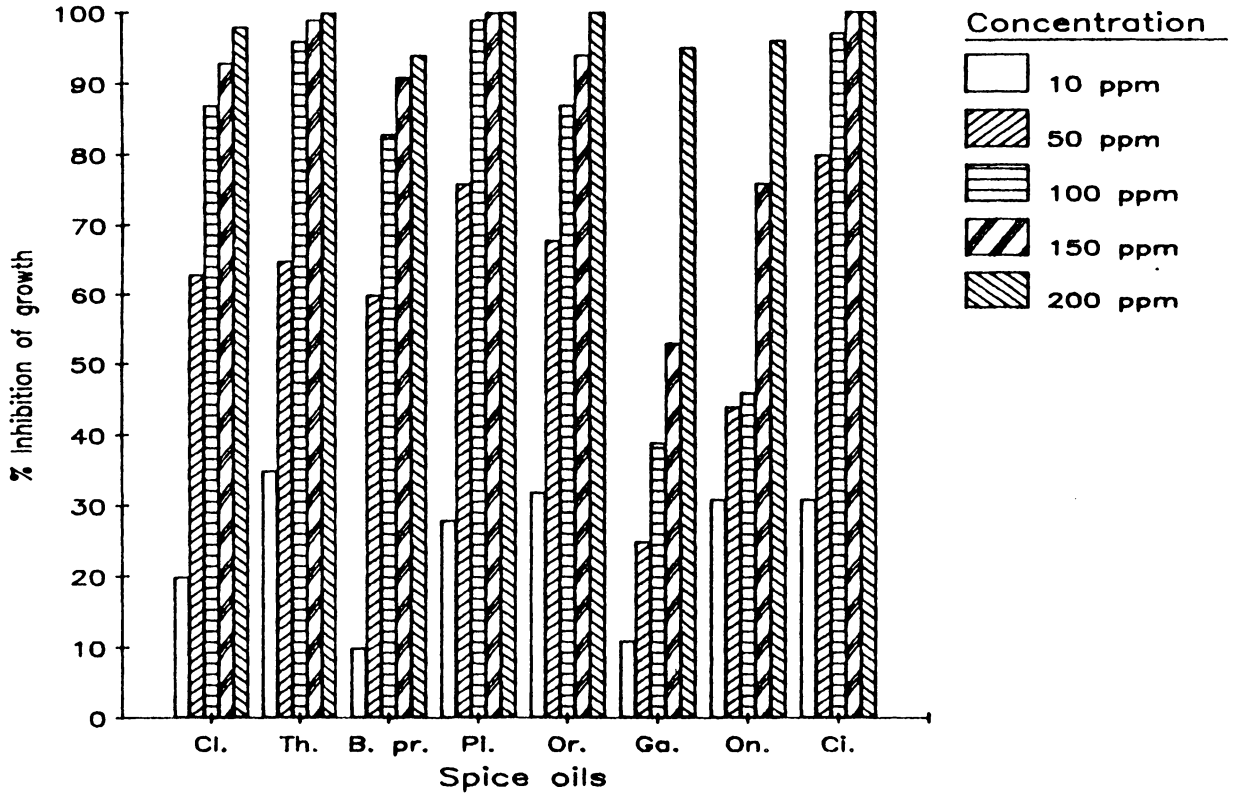


Figure 4. Growth inhibition of C. botulinum 40B by spice oils in PY medium. See Figure 2 for the abbreviations.

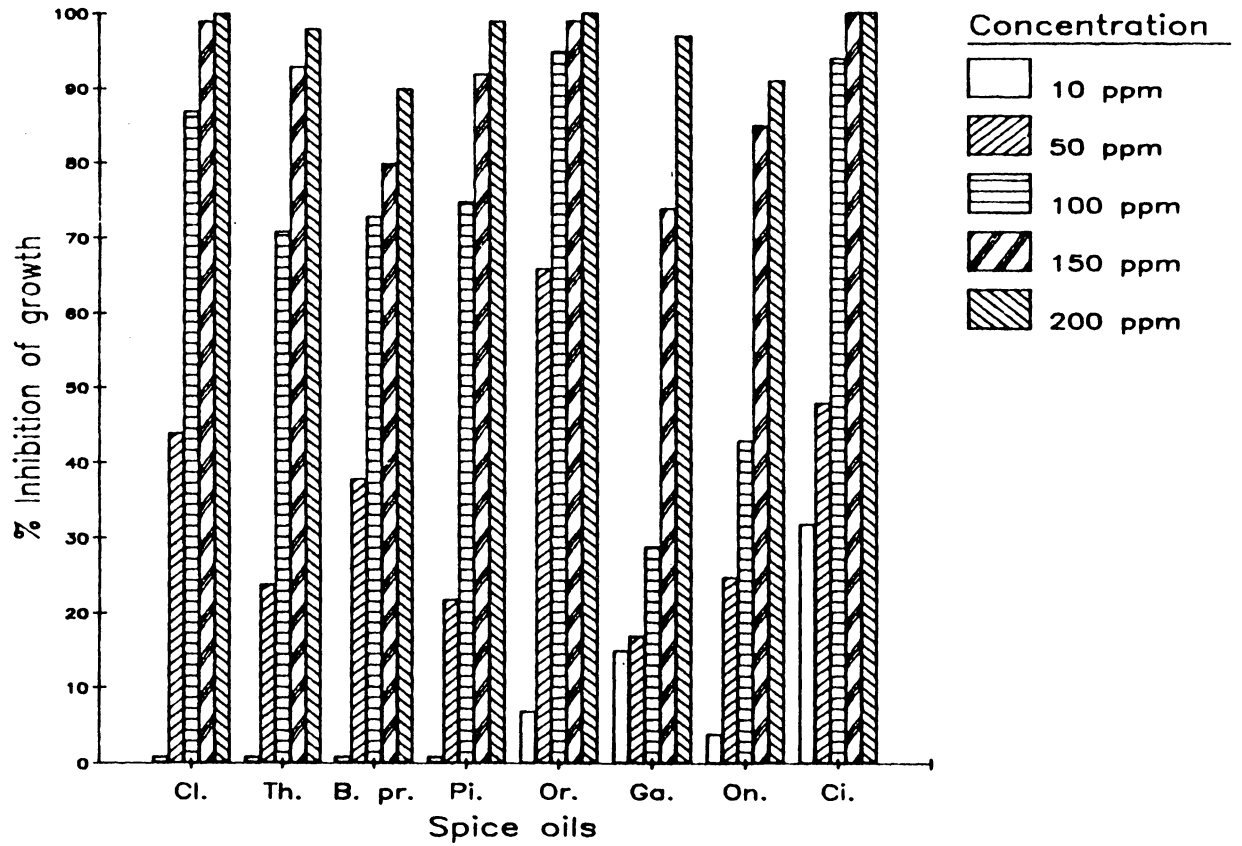


Figure 5. Growth inhibition of C. botulinum 67B by spice oils in PY medium. See Figure 2 for the abbreviations.

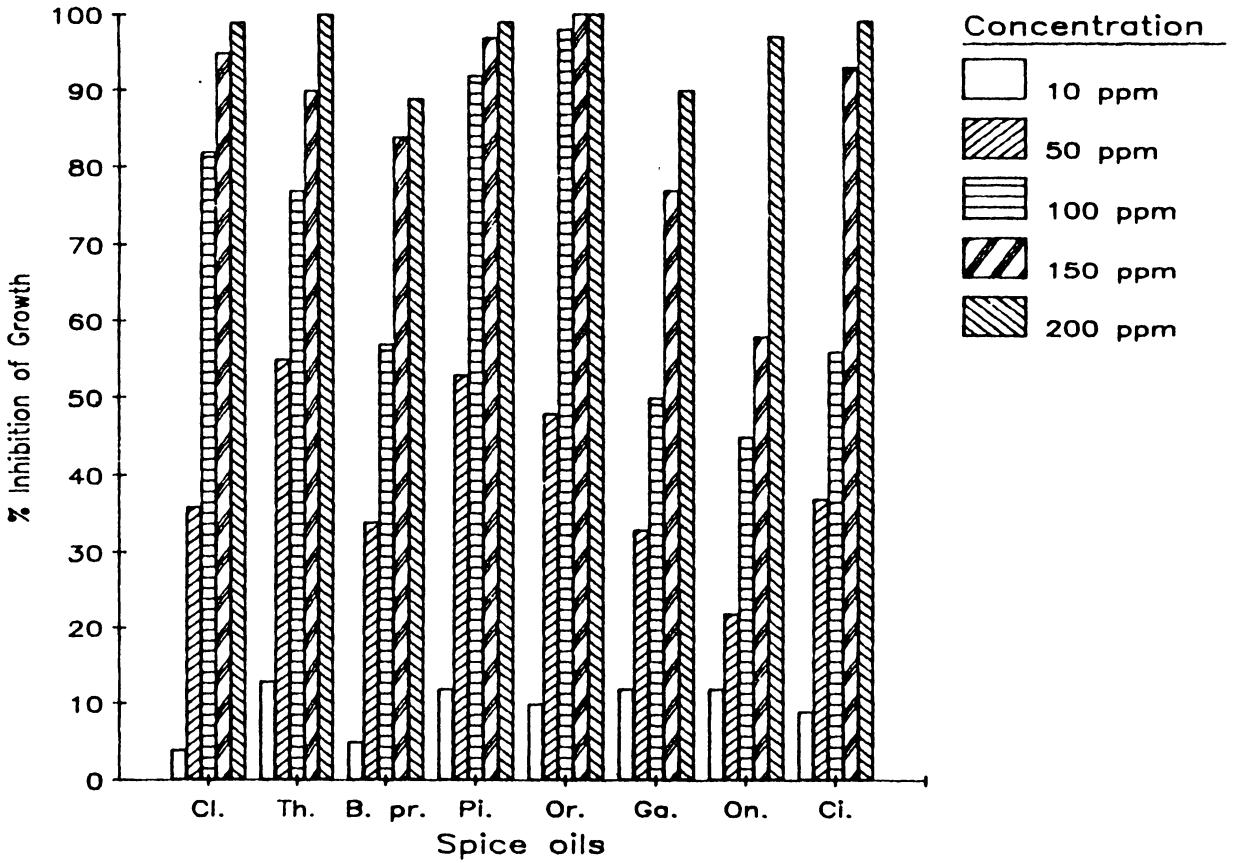


Figure 6. Growth inhibition of C. botulinum 1623E by spice oils in PY medium. See Figure 2 for the abbreviations.

ppm and higher. The oils thyme, clove, cinnamon, and pimenta at 200 ppm caused growth inhibition of 99% or higher. As with the previous strains, black pepper, garlic, and onion were the weakest oils; the total growth inhibition by these oils was not accomplished even at 200 ppm. The inhibition at 10 ppm of the oils ranged from 4% to 13%. Ethanol (95%) caused 9% growth inhibition.

The oils of origanum, clove, pimenta, and thyme were very effective in retarding the growth of C. botulinum 2157E (Figure 7). At concentrations of 150 and 200 ppm, these oils resulted in growth inhibition of over 99%. The oils of onion, garlic, and black pepper brought about lower inhibition levels than the other spice oils (94% and lower at 200 ppm). At 10 ppm, inhibition ranged between 12% (for onion) and 29% (for pimenta). As with the other strain of type E, ethanol caused an inhibition of 9%.

Based on Duncan's multiple range test, there were no significant differences in the effect of different oils at concentration of 200 ppm on growth of C. botulinum (Table 4). All the oils tested caused % growth inhibition of over 92. However, at lower concentrations (10-150 ppm), usually the oils of black pepper, onion and garlic had lower inhibitory effect than the rest of the oils. These results are comparable to the effect of some oils on other microorganisms (Table 5). However, the results are different from those reported by DeWit et al. (1979) in which they

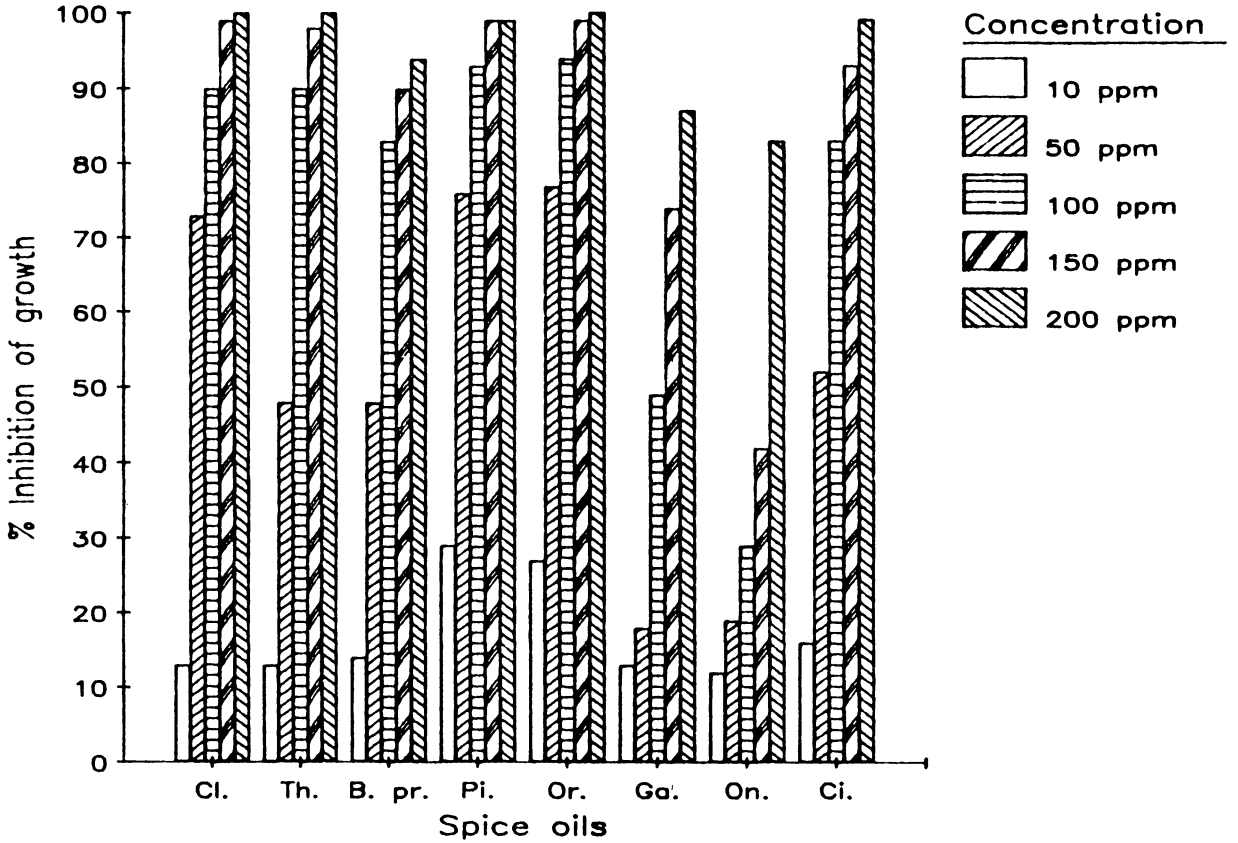


Figure 7. Growth inhibition of C. botulinum 2157E by spice oils in PY medium. See Figure 2 for the abbreviations.

Table 4. Means of % growth inhibition of C. botulinum (A, B and E strains) by spice oils in PY medium.

Spice oils	Concentration (ppm)				
	10	50	100	150	200
Origanum	18.8a,b[1]	73.0a	95.5a	98.6a	100.0a
Pimenta	21.6a,b	63.8b	91.8a,b	98.0a	99.5a
Clove	16.3a,b	63.3a	88.8a,b	96.3a	99.0a
Cinnamon	23.0a	61.8b,c	84.8b	96.6a	99.5a
Thyme	19.3a,b	54.0c,d	87.7a,b	96.5a	99.6a
B. pepper	12.6b	45.8d	74.5c	86.3b	92.8a
Onion	16.6a,b	35.5e	52.0d	73.3b,c	92.8a
Garlic	15.1a,b	28.2e	53.6d	77.8b,c	94.2a

[1] Means with different letters are significantly different ($p \leq 0.05$).

Table 5. Inhibitory effect of some spice oils on different microorganisms.

Miroorganism	Spice Oil	Conc.[a]	Reference
A. parasiticus	clove	200-250	Bullerman et al. (1977)
	cinnamon	200-250	" "
Yeasts (different genera)			
	garlic	25	Conner and Beuchat (1984a)
	onion	50	" "
	origanum	200	" "
	cinnamon	200	" "
	pimenta	150-200	" "
	clove	200	" "
	thyme	200	" "
Vibrio parahaemolyticus			
	origanum	100	Beuchat (1976)
	thyme	100	" "
Clostridium botulinum type A			
	garlic	1500	DeWit et al. (1979)
	onion	1500	" "

[a] Inhibitory concentration in ppm.

showed that a concentration of 1500 ppm of oils of each onion and garlic was sufficient to inhibit growth of C. botulinum type A. However, the experiment of DeWit et al. was performed in a meat system rather than a bacteriological medium.

Analysis of variance also showed that there were significant differences between the different strains of C. botulinum in their response to the oils ($p \leq 0.0001$). Strains of type A (33A and 10755A) were more sensitive to the inhibitory action of the oils than the other strains (types B and E). Similar results were reported by Dewit et al. (1979).

B. Effect of spice oils on germination of strains of C. botulinum by microculture method

Figures 8-12 show the effects of the spice oils on the germination of strains of C. botulinum. Garlic, onion, origanum, cinnamon and black pepper were the most effective spice oils on inhibiting germination of strain 33A (Figure 8). All these oils at concentrations of 100 ppm and above allowed less than 4% germination. Clove, thyme, and pimenta showed low activity towards inhibiting the germination of 33A strain; at 50 ppm these oils allowed 15-20% germination. At the lowest concentration (10 ppm), onion and garlic allowed the lowest percentage of germination, 18 and 21%, respectively. Ethanol allowed about 83% germination, which

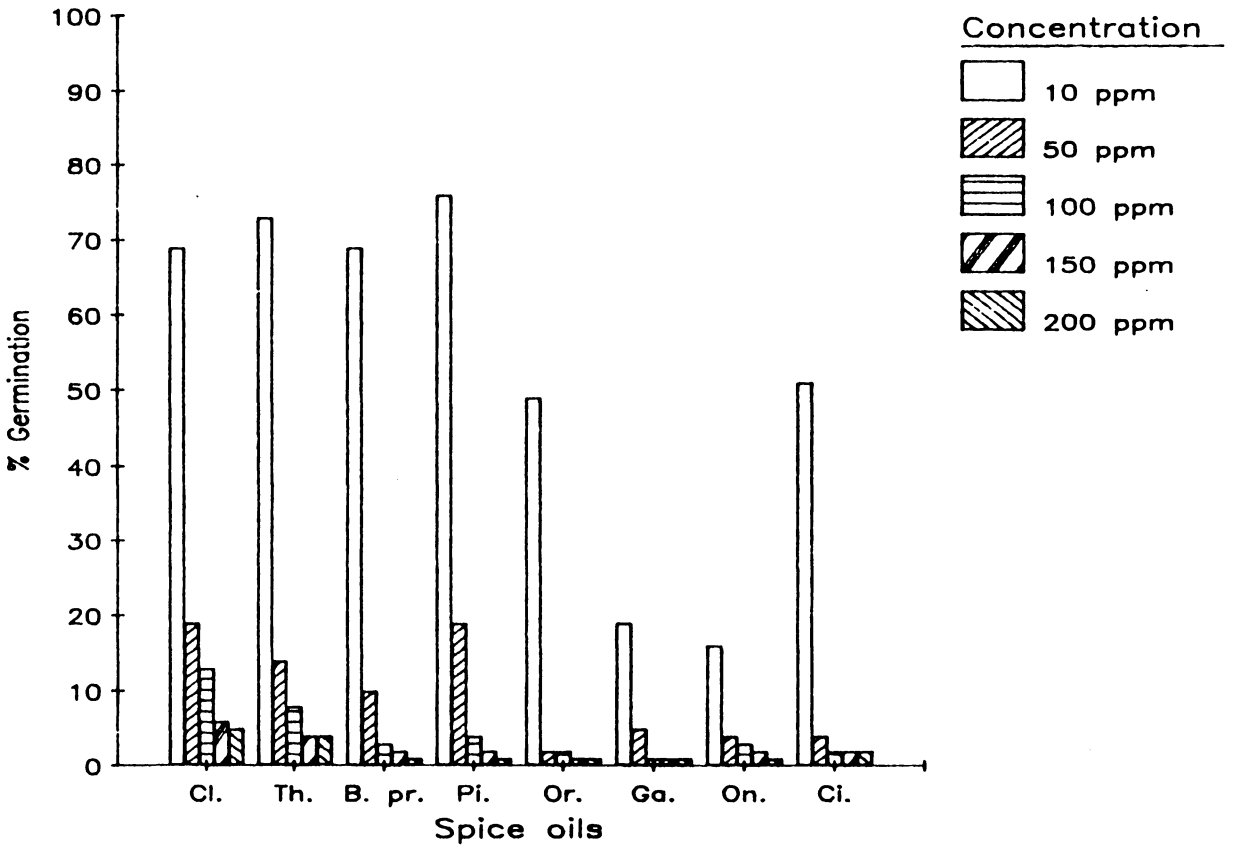


Figure 8. Germination of C. botulinum 33A in the presence of spice oils. See Figure 2 for the abbreviations.

8 was close to the germination percent in the presence of 10 ppm of pimenta.

Figure 9 shows the effects of the spice oils on the germination of the strain 10755A. All the spice oils at concentrations of 100 ppm and higher allowed a germination of less than 3%. Garlic, black pepper, and thyme were extremely active ; at 50 ppm, these oils allowed a germination of only 4% and lower. The effect of ethanol was lower than the effect of all the oils at 10 ppm. The percent of germination of this strain in the presence of 0.95% ethanol was 93%.

Onion and garlic oils provided the greatest inhibition of germination of strain 40B (Figure 10). These two oils at 10 ppm allowed a germination of 77 and 81%, respectively, compared to 90 percent and higher allowed by the rest of the oils at the same concentration. At concentrations of 100 ppm and above, all the oils allowed a germination of below 5%. Germination was less than 16% for all spice oils except pimenta at 50 ppm. Ethanol allowed 95% germination which was similar to the germination percentage in the presence 10 ppm clove oil.

The effects of the spice oils on germination of C. botulinum 67B are presented in Figure 11. Clove oil at 50 ppm allowed the highest percent of germination (68%), compared to 40% and below allowed by all the other oils at the same concentration. Garlic, black pepper, origanum, and

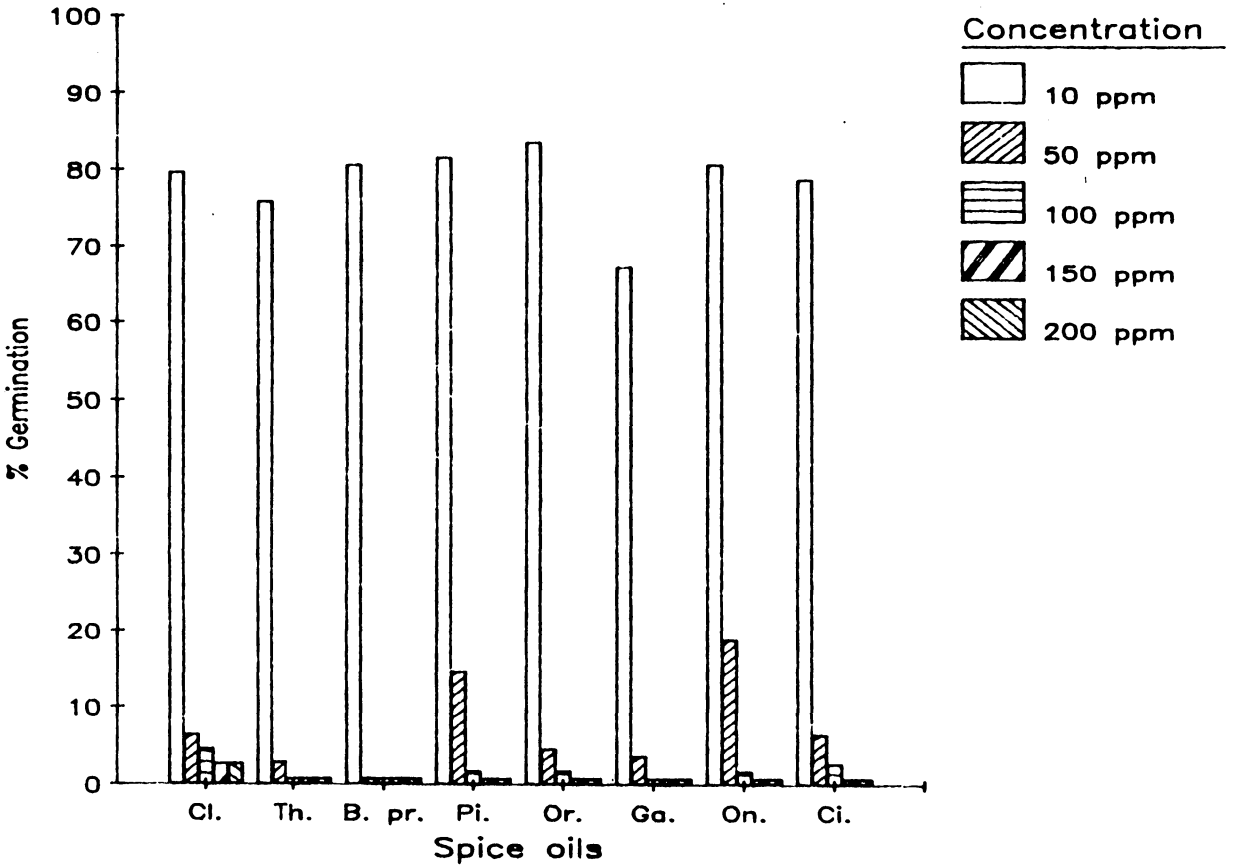


Figure 9. Germination of C. botulinum 10755A in the presence of spice oils. See Figure 2 for the abbreviations.

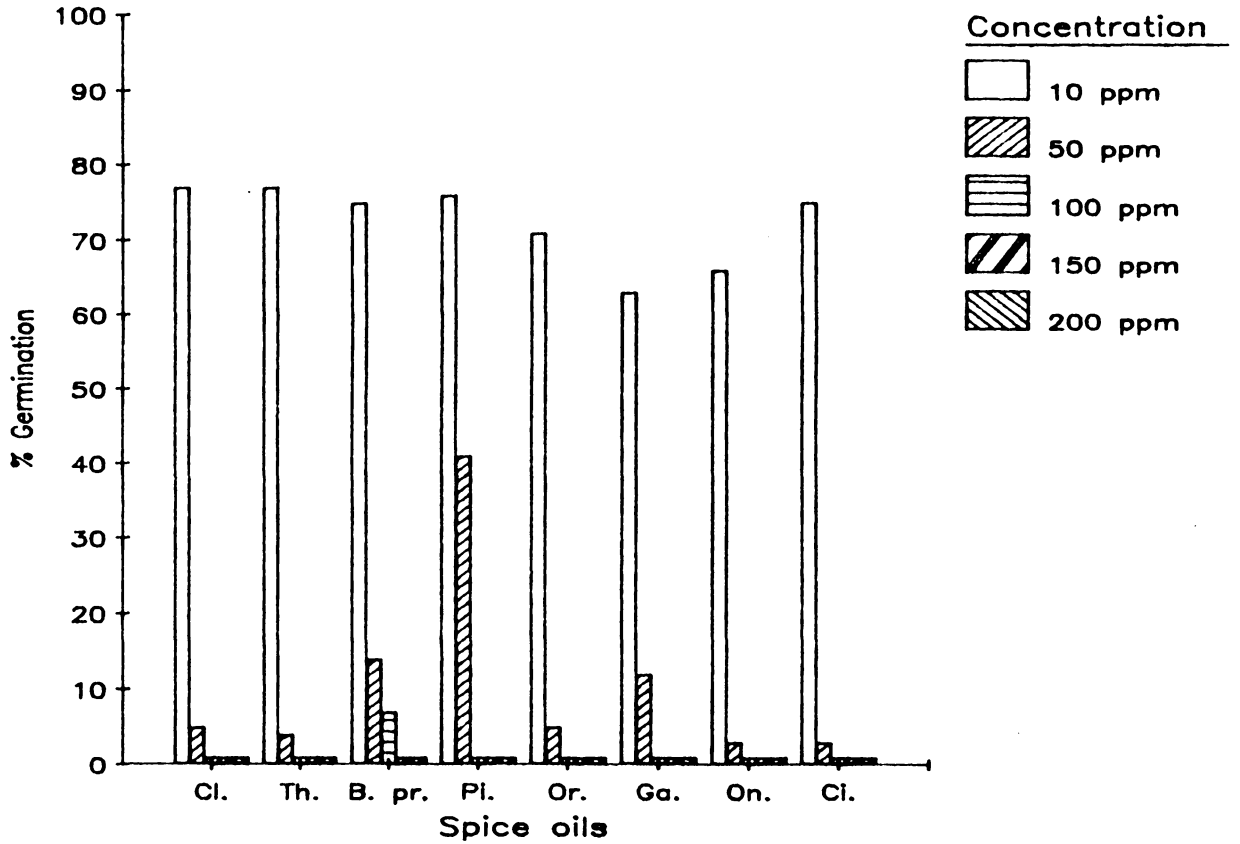


Figure 10. Germination of C. botulinum 40B in the presence of spice oils. See Figure 2 for the abbreviations.

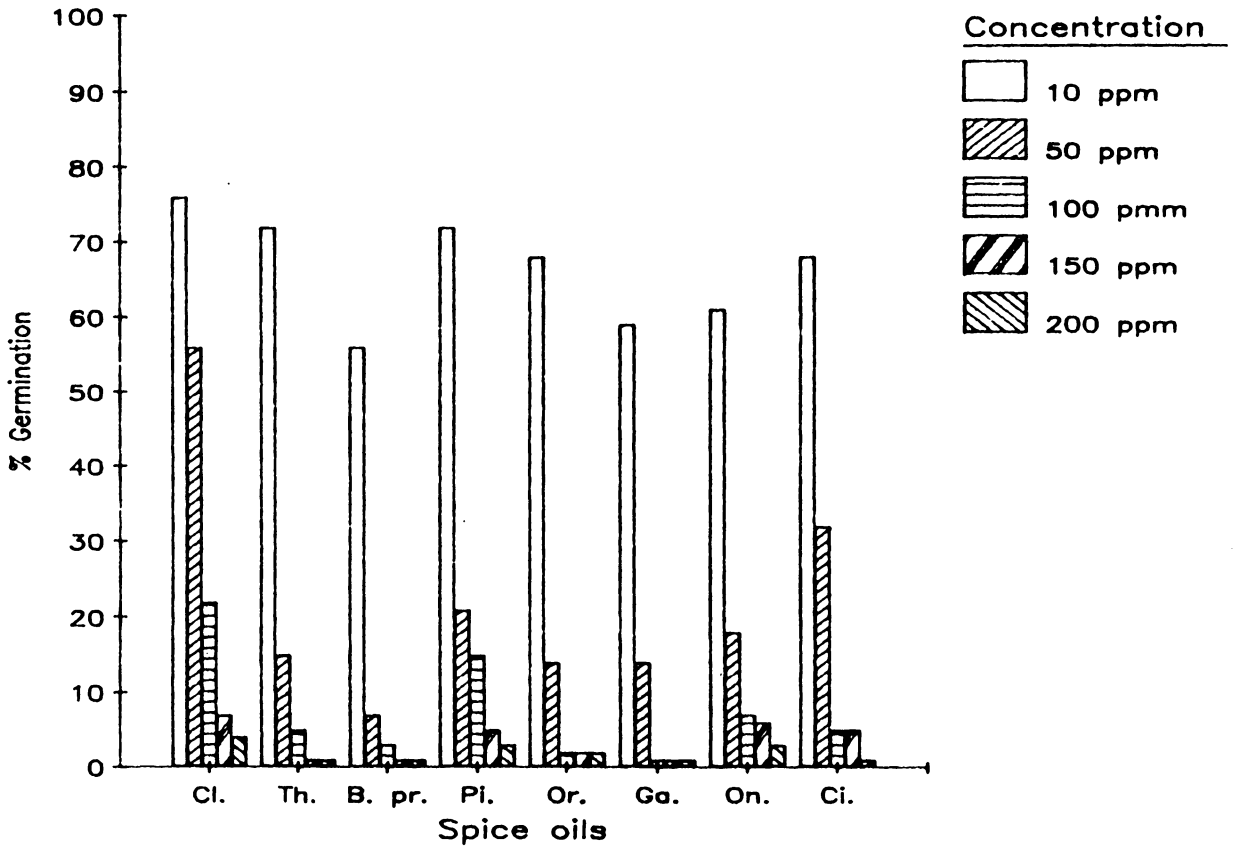


Figure 11. Germination of C. botulinum 67B in the presence of spice oils. See Figure 2 for the abbreviations.

thyme at concentrations of 150 and 200 ppm were the most effective spice oils in inhibiting germination. Ethanol allowed a 95% germination, which was higher than the germination percentage in the presence of the lowest concentration of the oils.

Figure 12 shows the effect of the oils on the strain 1623E. Pimenta and clove caused the lowest inhibition of germination of this strain. Germination in the presence of 50 ppm of the oils of clove and pimenta was 65 and 52 percent respectively, compared to a germination of less than 10 percent for the rest of the oils at the same concentration. At a concentration of 200 ppm , all the oils allowed a very low percentage of germination (1-2%). Ethanol allowed 97% germination , which reflects the resistance of this strain to ethanol.

The overall effect of the spice oils on the germination of different strains of C. botulinum spores is presented in Table 6. There were no significant differences in the effect of the oils at a concentration of 100 ppm and higher on germination of C. botulinum strains. All the oils at these concentrations caused % germination inhibition of over 90% At concentrations of 10 and 50 ppm, however, the oils of clove and pimenta had the least inhibitory effect on germination.

Nitrite and sorbate are two antimicrobials that have been studied extensively for their effectiveness in

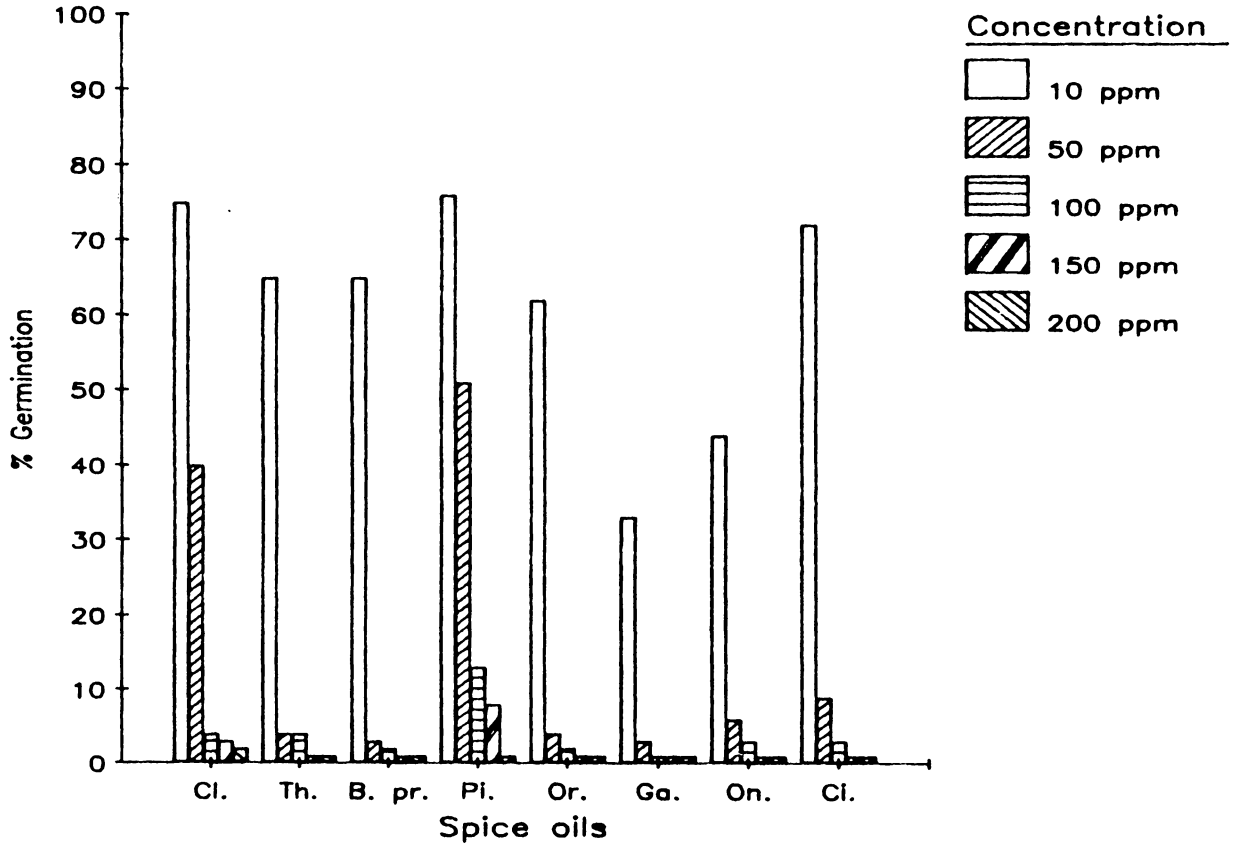


Figure 12. Germination of C. botulinum 1623E in the presence of spice oils. See Figure 2 for the abbreviations.

Table 6. Means of % germination inhibition of C. botulinum (A, B and E strains) by spice oils.

Spice oil	Concentration (ppm)				
	10	50	100	150	200
Origanum	27.2b,c[1]	93.8a	98.2a	98.8a	98.8a
B. pepper	24.8c,d	92.6a	96.6a	98.8a	99.0a
Garlic	47.4a	91.8a	99.0a	99.0a	99.0a
Thyme	21.0c,d	91.2a	96.2a	98.4a	98.4a
Onion	34.2b	89.2a	96.8a	97.6a	98.4a
Cinnamon	24.8c,d	88.2a	97.0a	98.0a	98.8a
Clove	17.8d	71.8b	90.4a	95.8a	97.0a
Pimenta	17.0d	67.8b	92.2a	96.4a	98.8a

1 Means with different letters are significantly different ($p \leq 0.05$).

inhibiting C. botulinum growth and toxin production. Except at very high (above 20000 ppm) concentration, nitrite has no effect on inhibiting C. botulinum spore germination. Sorbate inhibits spore germination at concentration above 1000 ppm; in contrast the spice oils were effective inhibitors of C. botulinum spore germination at concentration of 200 ppm; therefore, those oils may have application as inhibitors of C. botulinum in foods.

Comparing the effect of the oils on C. botulinum growth to germination (Table 4 and Table 6), it might appear that the percentage of inhibition of germination was higher than that of growth, especially with the oils of garlic, onion, and black pepper at concentrations of 50 and 100 ppm. Since germination is the first step for spores to develop into vegetative cells, it could possibly be concluded that the percent growth inhibition by any chemical or physical agent would be equal to or higher than the percent of germination inhibition. The reverse is true when coparing Tables 4 and 6. The apparent discrepency was probably due to the incubation time for the growth experiments (section A) and the germination experiments. The roll tubes in the growth experiment were incubated for three days, whereas the microculture slides were incubated for only six hrs. Incubation beyond 6 hrs may allow more extensive germination, thus growth was observed at several spice oils concentrations that appeared to inhibit germination.

C. Effect of spice oils on C. botulinum 67B

1. Effect of spice oils on germination (optical density method)

It is well-known that a drop in optical density (O.D.) occurs with the germination of bacterial spores. Depolymerization and excretion of spore constituents such as dipicolinic acid, calcium, manganese, and peptidoglycan during germination result in a loss of spore refracrtility which leads to the drop in O.D. of the spore suspensions (Gould, 1970).

Clove oil was inhibitory to germination of C. botulinum 67B (Figure 13A). The control (medium with 0.95% ethanol) showed the highest drop in O.D.; the drop reached a value of 62% of the initial O.D. in two hrs of incubation, whereas concentrations of 10, 50, and 100 ppm of clove decreased to 68, 77, and 83% of their initial values, respectively, during the incubation time of two hrs.

Figure 13B presents the effect of thyme on the germination of spores of C. botulinum 67B. As the concentrations increased, the fall in O.D. reduced. At 100 ppm, there was no drop in O.D during two hrs of incubation. With 0, and 10 ppm, the rate of drop of O.D. was high during the first 30 min as compared with the later period of incubation.

The oil of black pepper was effective in inhibiting the germination of C. botulinum 67B (Figure 13C). The O.D.

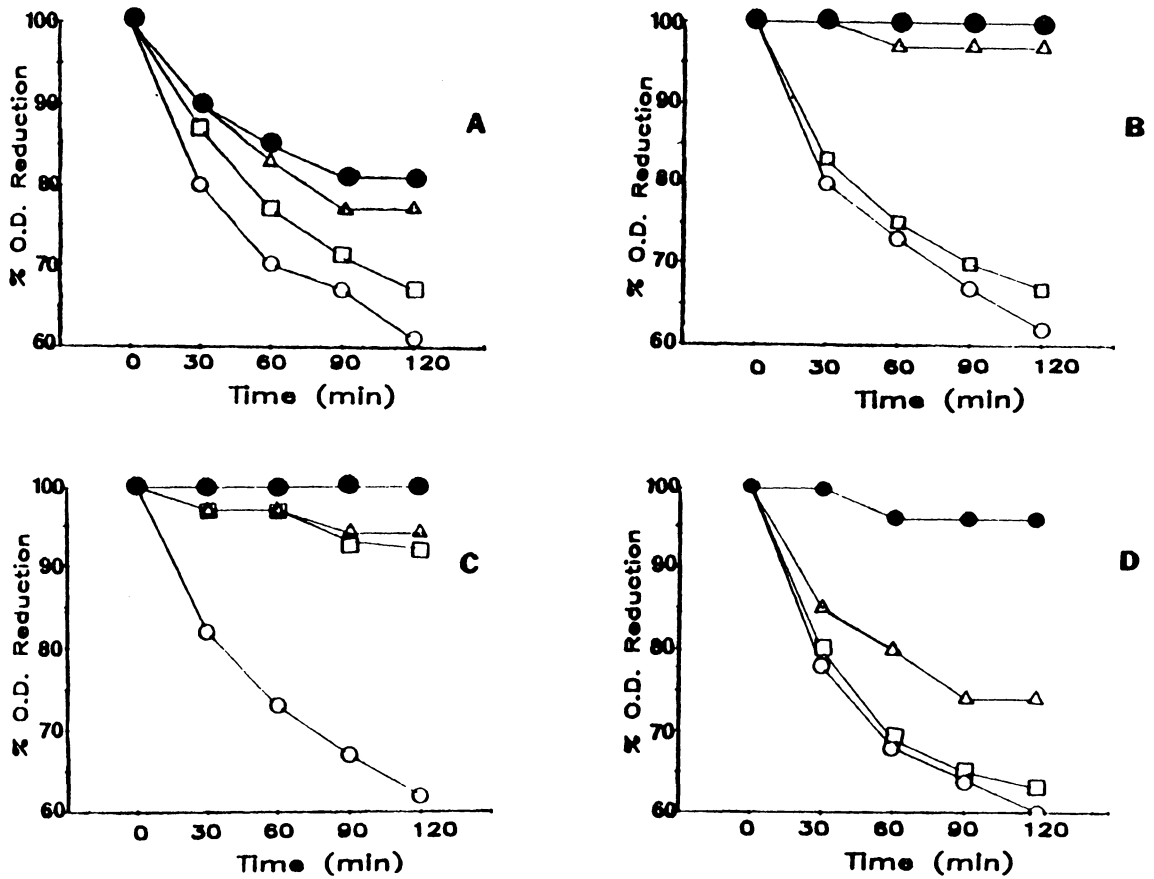


Figure 13. Effect of spice oils clove (A), thyme (B), black pepper (C), and pimenta (D) on germination of C. botulinum 67B spores suspended in GO medium. Symbols ○–○, □–□, △–△, and ●–●, refer to concentrations of 0 (control), 10 50, and 100 ppm, respectively.

reached 91% of the initial value after two hrs of incubation in the presence of 10 ppm black pepper. At 50 ppm, the O.D. dropped to 93% of the initial value, whereas 100 ppm showed no drop in O.D. during two hrs of incubation.

Figure 13D shows the effect of pimenta oil on germination of spores of C. botulinum 67B. The drop in O.D. reached 64, 75, and 97 percent of the initial values at concentrations of 10, 50 , and 100 ppm of pimenta, respectively. The plots showed that the rate of drop in O.D. was high in the first 30 min, except with 100 ppm, which showed no drop in O.D. during that time.

Origanum at 10 ppm showed little activity in inhibiting the germination of C. botulinum 67B (Figure 14A). The drop in O.D. at 10 ppm reached 64%, which was close to the drop in the control. At 50 ppm the O.D. dropped to 97% of the initial value during the first thirty min and maintained that value to the end of the two hrs of incubation. At 100 ppm there was no change in O.D., indicating a high level of effectiveness of the oil in inhibiting germination.

The fall in O.D. reached values of 72 and 94 percent at concentrations 10 and 50 ppm of garlic oil, respectively, compared to 63 percent in the control (Figure 14B). The rate of fall of O.D. was high at the first 30 min of incubation for the control and 10 ppm. At 100 there was no chnge in O.D. during incubation time of two hrs.

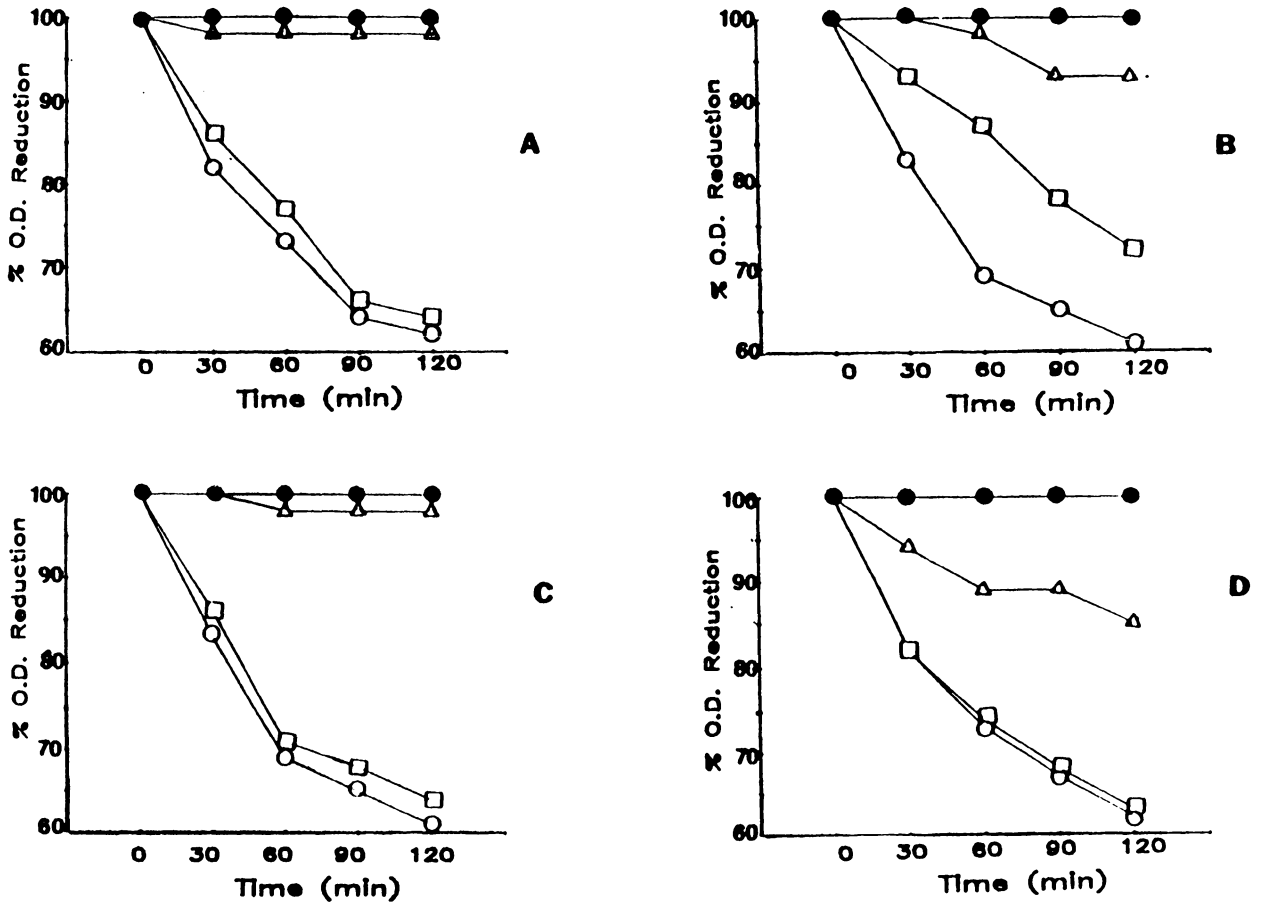


Figure 14. Effect of spice oils origanum (A), garlic (B), onion (C), and cinnamon (D) on germination of C. botulinum 67B spores suspended in GO medium. Symbols ○–○ , □–□ , △–△ , and ●–● , refer to concentrations of 0 (control), 10 50, and 100 ppm, respectively.

Figure 14C shows the effect of onion oil on the germination of C. botulinum 67B. At 50 ppm O.D. dropped to 98% of the initial value after one hr of incubation. At 100 ppm, no change in O.D. was observed during the two hrs of incubation. The fall in O.D. at 10 ppm and in the control reached 65 and 63%, of the initial values, respectively.

The effect of cinnamon oil on the germination of C. botulinum 67B is shown in Figure 14D. The rate of drop of O.D. at 10 ppm was essentially the same as the control for the entire incubation period. At 50 ppm cinnamon oil the O.D. reached 84% of the initial value after 2 hrs of incubation. 100 ppm of cinnamon showed no change in O.D. of the spores.

Overall, the results on the effect of spice oils on germination of C. botulinum 67B as determined by the optical density method indicated that the essential oils of garlic, onion, thyme, and origanum were the most potent inhibitors of germination. These oils prevented spore germination at concentrations of 100 ppm or higher. Clove and pimenta were the the least effective oils inhibiting germination. With the exception of black pepper, the drop of O.D. of the spore suspensions in the presence of 10 ppm of the spice oils was close to the drop of O.D. for the control (spore suspension in medium with 0.95% ethanol). The results for the inhibitory action of the oils as determined by the optical density method were comparable to those obtained for the

microculture method. For both methods, spice oils at 100 ppm or higher allowed only limited spore germination; while there was a high degree of germination in the presence of 10 ppm of the spice oils.

2. Duration of germination sensitivity to spice oils.

The influence of the time interval of the addition of cinnamon oil on inhibiting of the germination of C. botulinum 67B is shown in Figure 15. The drop in optical density was completely prevented when the cinnamon oils were added after 1, 5, 10, 15, and 30 min of exposure of the spores to the germinating medium (GO). This indicated the sensitivity of the spore germination of C. botulinum 67B to the inhibitory effect of the cinnamon oil even after 30 minutes of exposure of the spores to the germination medium. The components of oil of cinnamon may inhibit the trigger reaction and / or the sequence of the reactions following the trigger reaction. The components of cinnamon oil may either compete with the germinants in the medium for the receptor sites on the spores, or inhibit enzymes necessary for the metabolism of the germinants.

As with the oil of cinnamon, the oils of clove, thyme, origanum were tested for the duration of their inhibitory action on germination. All the oils inhibited the drop of O.D., even when added after 30 min of exposure of the spores to the germinating medium.

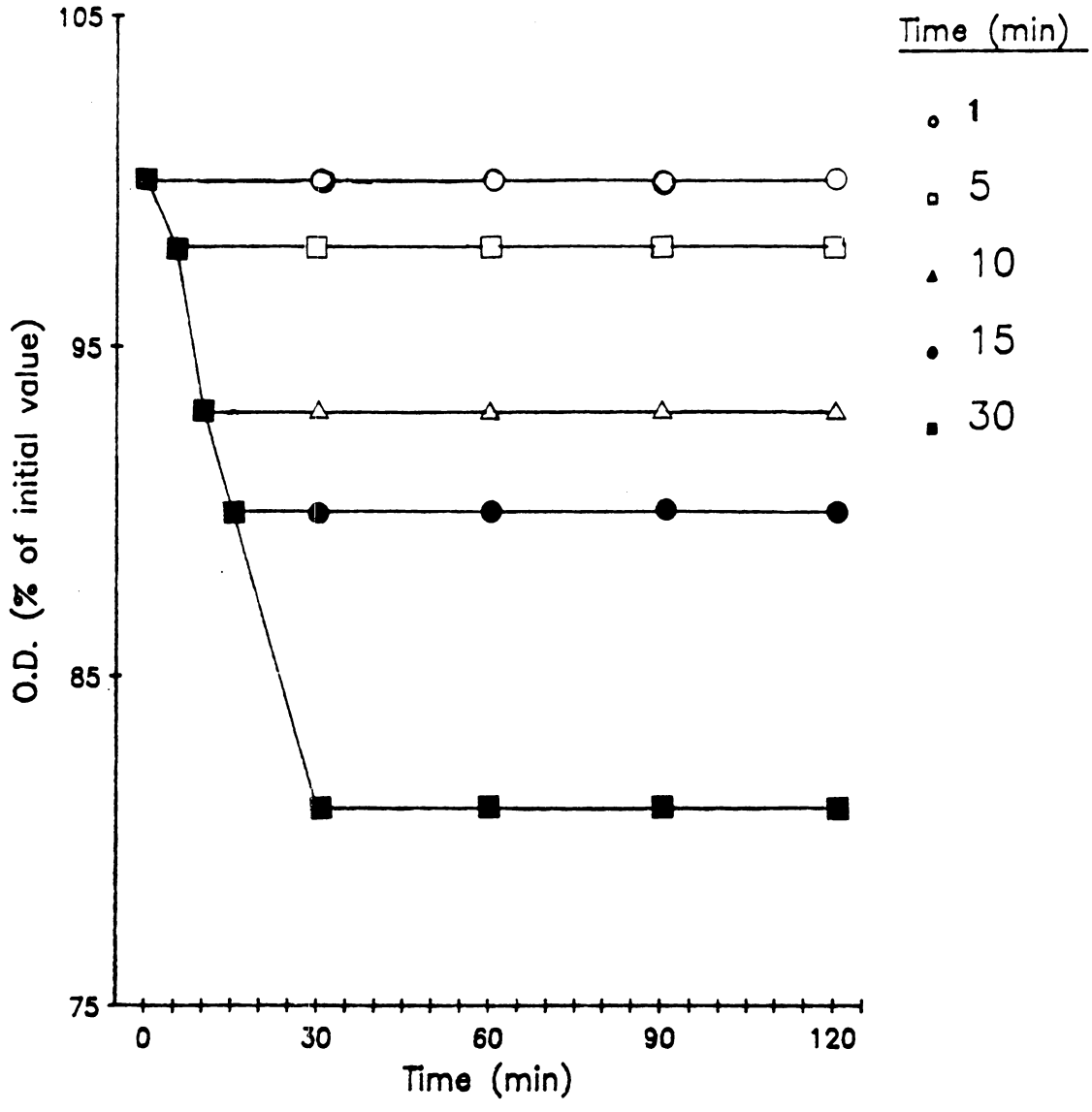


Figure 15. The influence of the time of addition of cinnamon oil (200 ppm) on inhibition of germination of C. botulinum 67B spores suspended in GO medium. The time (min) on the figure represent the interval between spore exposure to the medium and the addition of cinnamon oil.

3. Reversibility of inhibition of germination of C. botulinum 67B

The reversibility of C. botulinum 67B germination inhibition by the spice oils was studied. Spores were incubated in the presence of spice oils and then resuspended in a medium without the inhibitors. Inhibition of spore germination by the oils studied was in fact reversible (Figures 16-19). However, the drop in optical density of the resuspended spores in media without oils did not reach the O.D. of the control. This could be due to residual oil absorbed on the surface of the spores. The reversibility of the inhibition of germination indicated that the inhibition conferred no permanent damage to the spores. The spice oils at a concentration of 200 ppm or higher could be regarded as sporostatic but not sporocidal. These results are consistent with the results of Smoot (1981) and Cook (1982), in which they addressed the reversibility of the inhibition of germination by sorbic acid and BHA, respectively.

The mode of action of the spice oils, as inhibitors of germination, has not yet been reported. However, because of the high content of phenols and hydrophobic organic compounds in the oils, the mode of action of the spice oils may be similar to that of phenols or hydrophobic agents. It has been suggested that membrane fluidity is critical in triggering spore germination (Racine, 1981). Further, Blocher (1984) suggested that undissociated fatty acids,

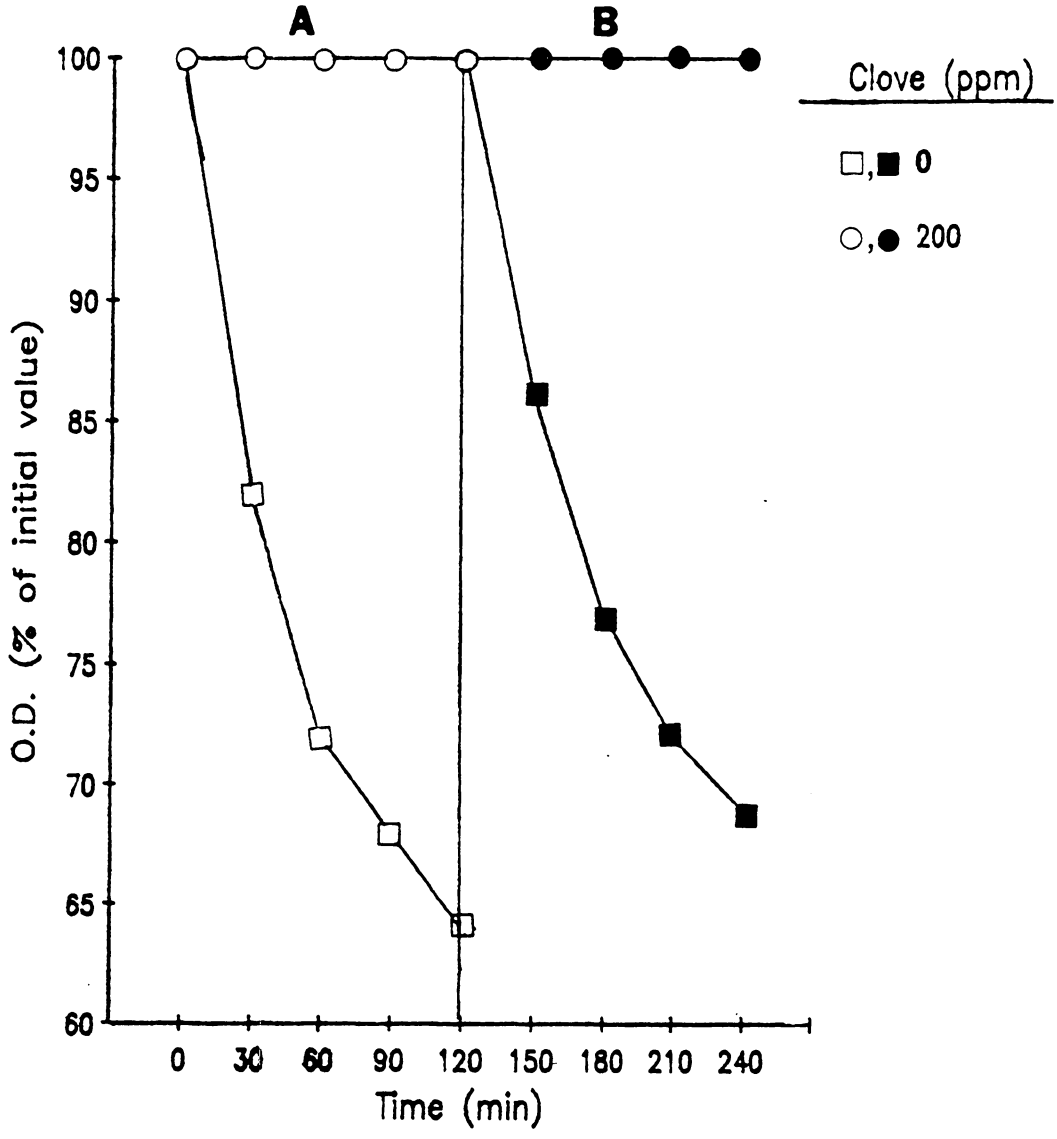


Figure 16. The reversibility of clove oil induced inhibition of C. botulinum 67B spore germination. (A) spores were suspended in GO medium with or without clove oil. (B) After 120 min., the spore suspension incubated with clove oil was separated by centrifugation and resuspended in GO medium with or without clove oil.

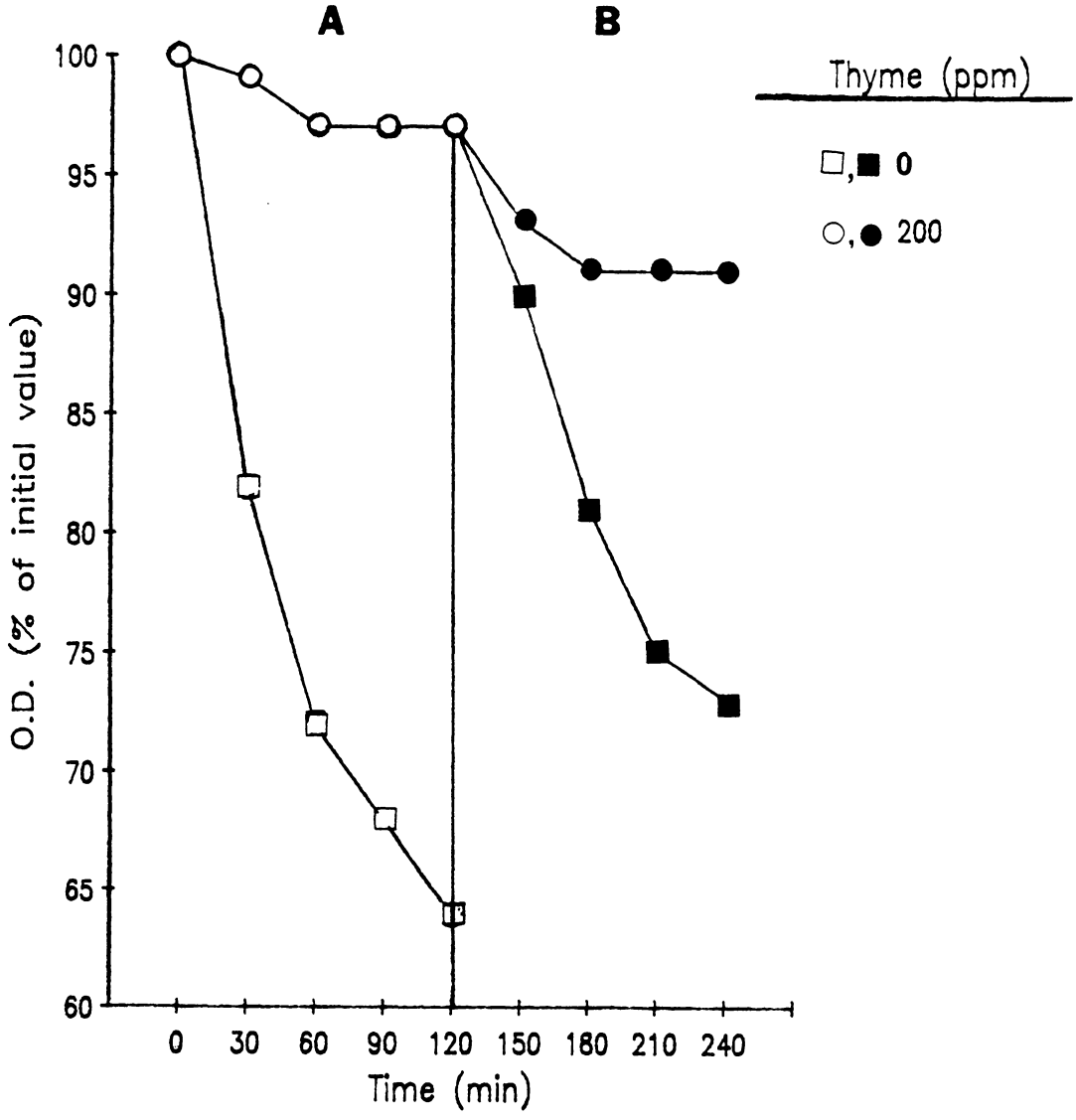


Figure 17. The reversibility of thyme oil induced inhibition of C. botulinum 67B spore germination. (A) spores were suspended in GO medium with or without thyme oil. (B) After 120 min., the spore suspension incubated with thyme oil was separated by centrifugation and resuspended in GO medium with or without thyme oil.

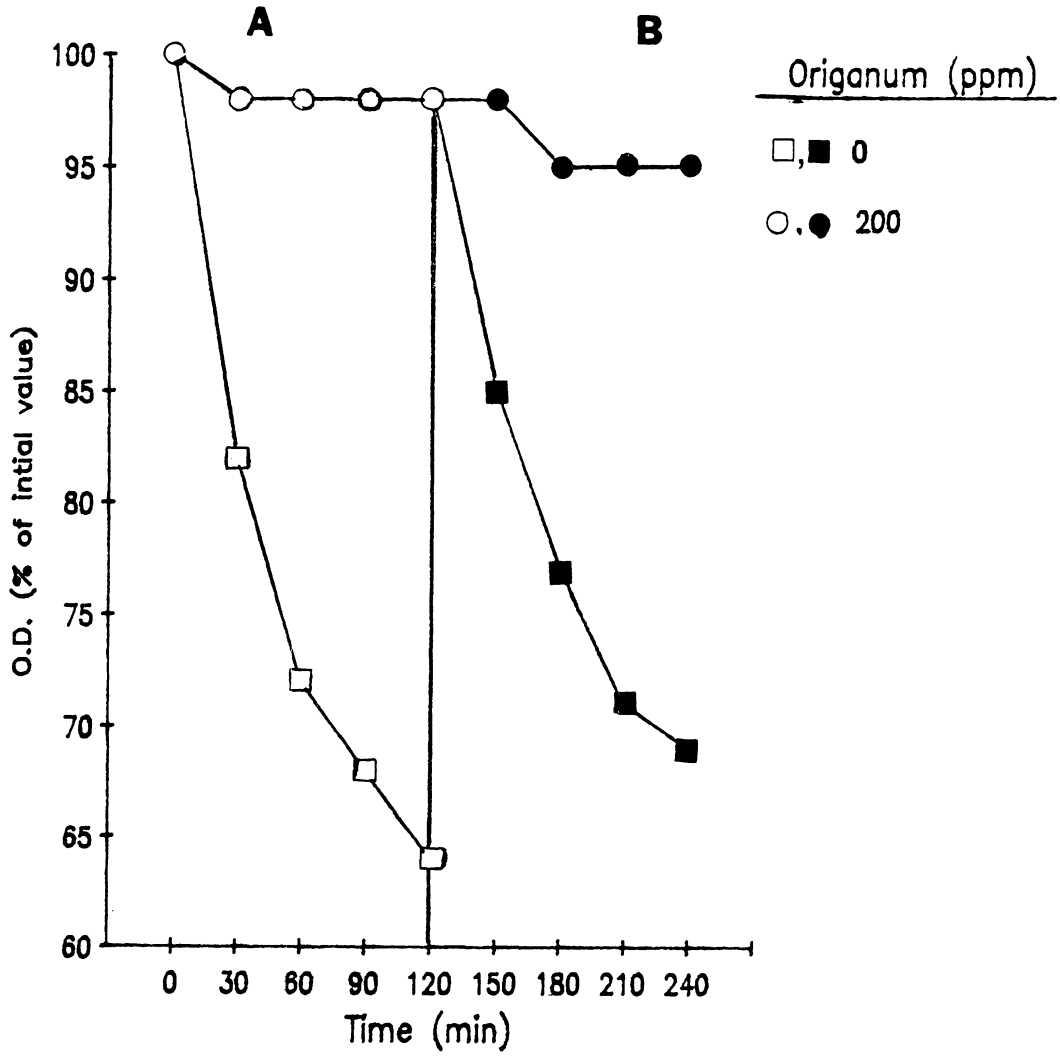


Figure 18. The reversibility of origanum oil induced inhibition of C. botulinum 67B spore germination. (A) spores were suspended in GO medium with or without origanum oil. (B) After 120 min., the spore suspension incubated with origanum oil was separated by centrifugation and resuspended in GO medium with or without origanum oil.

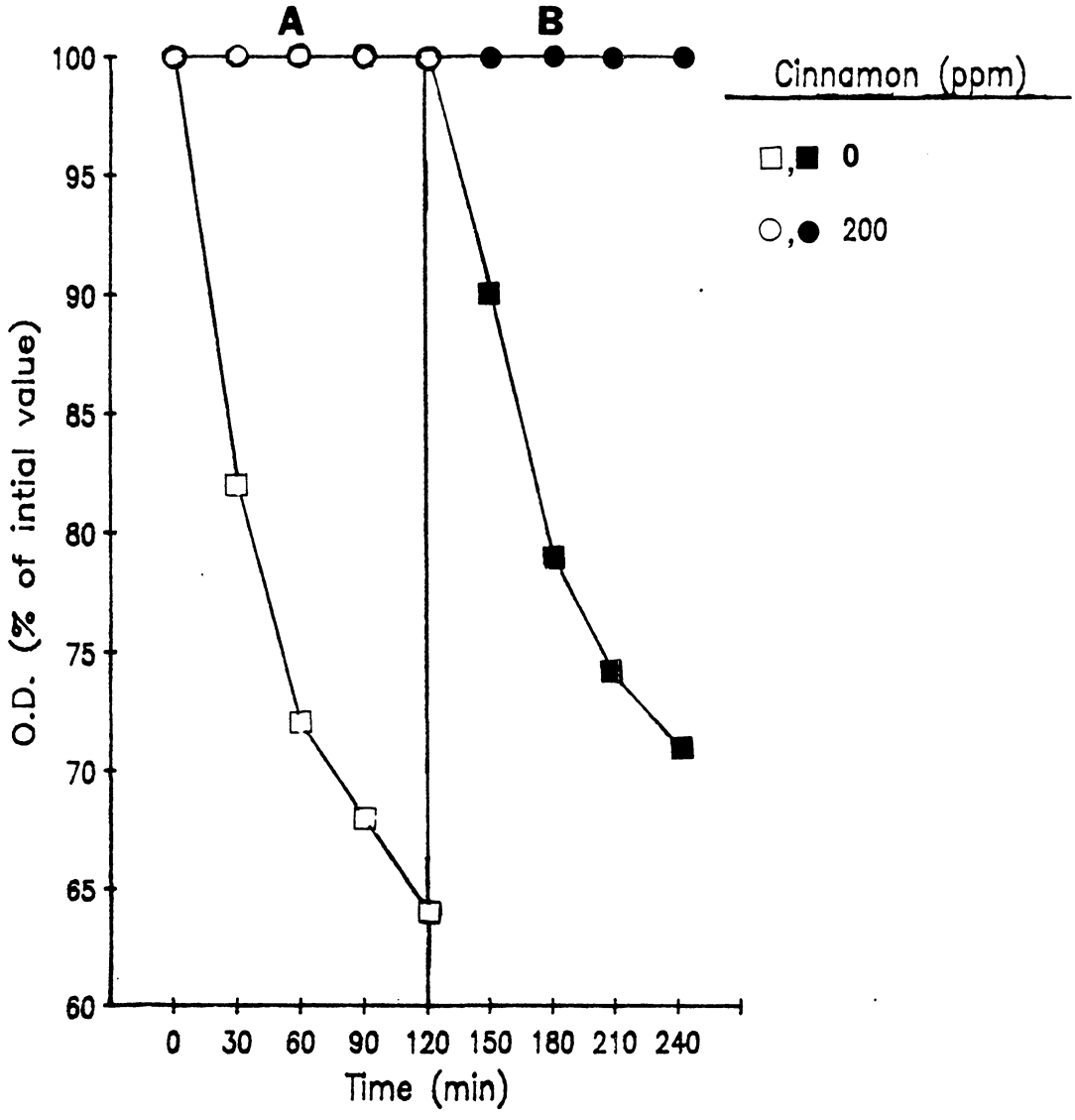


Figure 19. The reversibility of cinnamon oil induced inhibition of C. botulinum 67B spore germination. (A) spores were suspended in GO medium with or without cinnamon oil. (B) After 120 min., the spore suspension incubated with cinnamon oil was separated by centrifugation and resuspended in GO medium with or without cinnamon oil.

including sorbic acid , may inhibit spore germination by interacting with spore membranes and altering their fluidity. Spice oils, with their high content of hydrophobic compounds, may affect spore germination by altering the fluidity of the membranes, as with undissociated fatty acids.

4. Effect of spice oils on outgrowth and vegetative growth of C. botulinum 67B

To study the effect of spice oils on outgrowth and vegetative growth, spores of C. botulinum 67B were germinated in a medium which allowed germination but prevented outgrowth. The germinated spores were harvested, inoculated into a medium with spice oils, incubated and the growth determined (Figure 20). The reduction in growth ratio with increasing concentrations of the spice oils is obvious in all cases. Clove, cinnamon, and origanum were the most effective oils ; no growth occurred at concentrations of 150 and 200. Garlic and onion were the weakest oils, allowing slight growth even at 150 and 200 ppm. Black pepper was unique among all the oils; it allowed growth at concentrations of 50 ppm and higher, but the growth never reached an optical density of 0.35. Thus, a growth ratio of 0.05 was given to these concentrations to differentiate them from the concentrations which prevented growth completely. Ethanol (0.95%) in TYG medium showed a growth ratio of 0.81 which was the same as 10 ppm of thyme, garlic, and cinnamon.

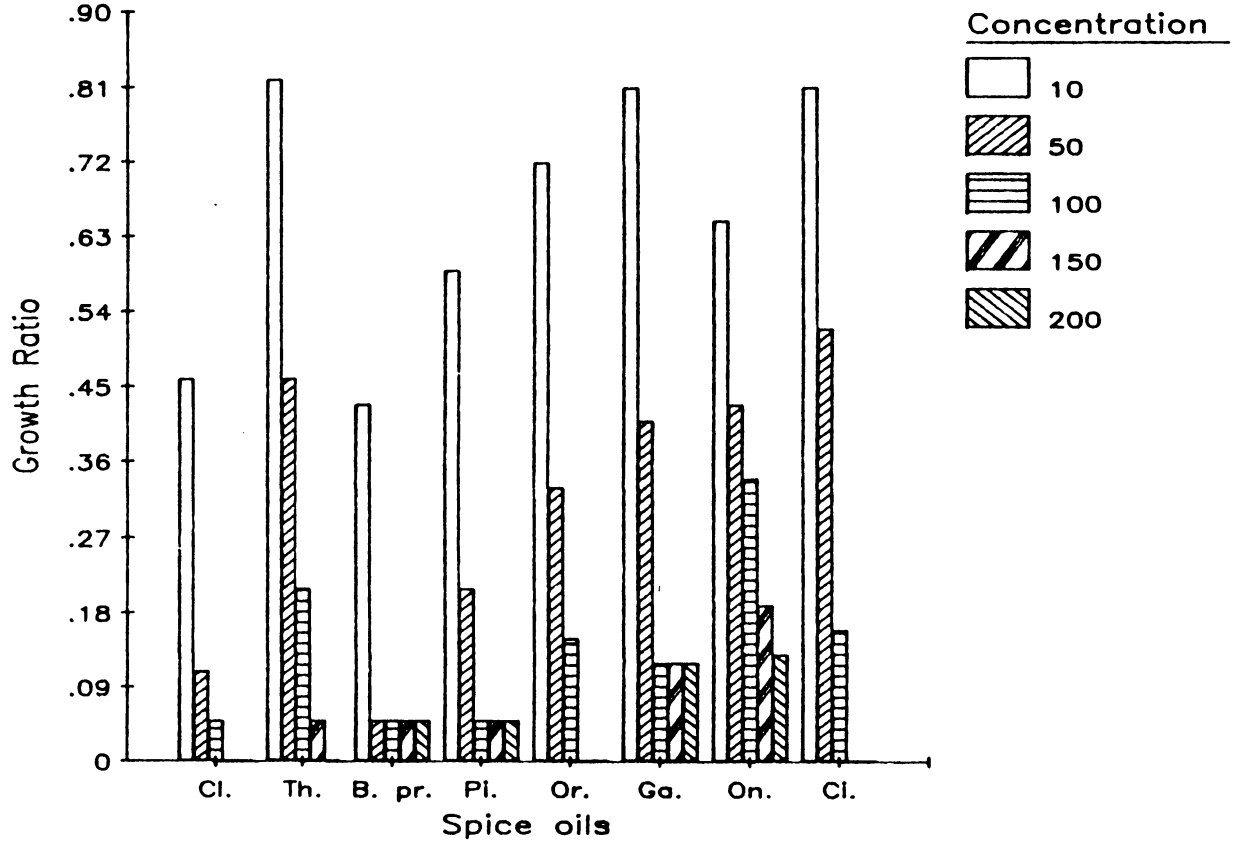


Figure 20. Effect of spice oils on growth (measured as growth ratios) of germinated spores of C. botulinum 67B in TYG medium. See Figure 2 for the abbreviations.

In another experiment the medium was inoculated with vegetative cells of C. botulinum 67B instead of germinated spores. Black pepper was very effective in preventing vegetative growth of the bacterium (Figure 21). Black pepper at concentrations of 100 ppm resulted in complete inhibition of growth during the one week incubation period. The spice oils of clove, origanum, and cinnamon, as in Figure 20, were effective in preventing the vegetative growth of the cells; these oils, at concentrations 150 and 200 ppm, prevented growth during one week of incubation. On the other hand, onion , garlic, pimenta, and thyme were the least effective oils on inhibiting vegetative growth; they allowed slight growth at the high concentrations (150 and/or 200 ppm). For the growth medium containing 0.95% ethanol, the ratio was 0.88. The growth ratios in the presence of 10 ppm of each spice oil was always less than 0.80.

A statistical analysis of the growth ratios represented in Figures 20 and 21 showed that there were no significant differences in the growth ratios of the two figures. Thus, regardless of whether the medium was inoculated with germinated spores or vegetative cells of C. botulinum, the time needed for growth to reach an O.D. of 0.35 was the same. This could be explained in two ways. First, there is no effect of the spice oils on outgrowth. Thus, the spice oils do not inhibit the polymerization processes such as

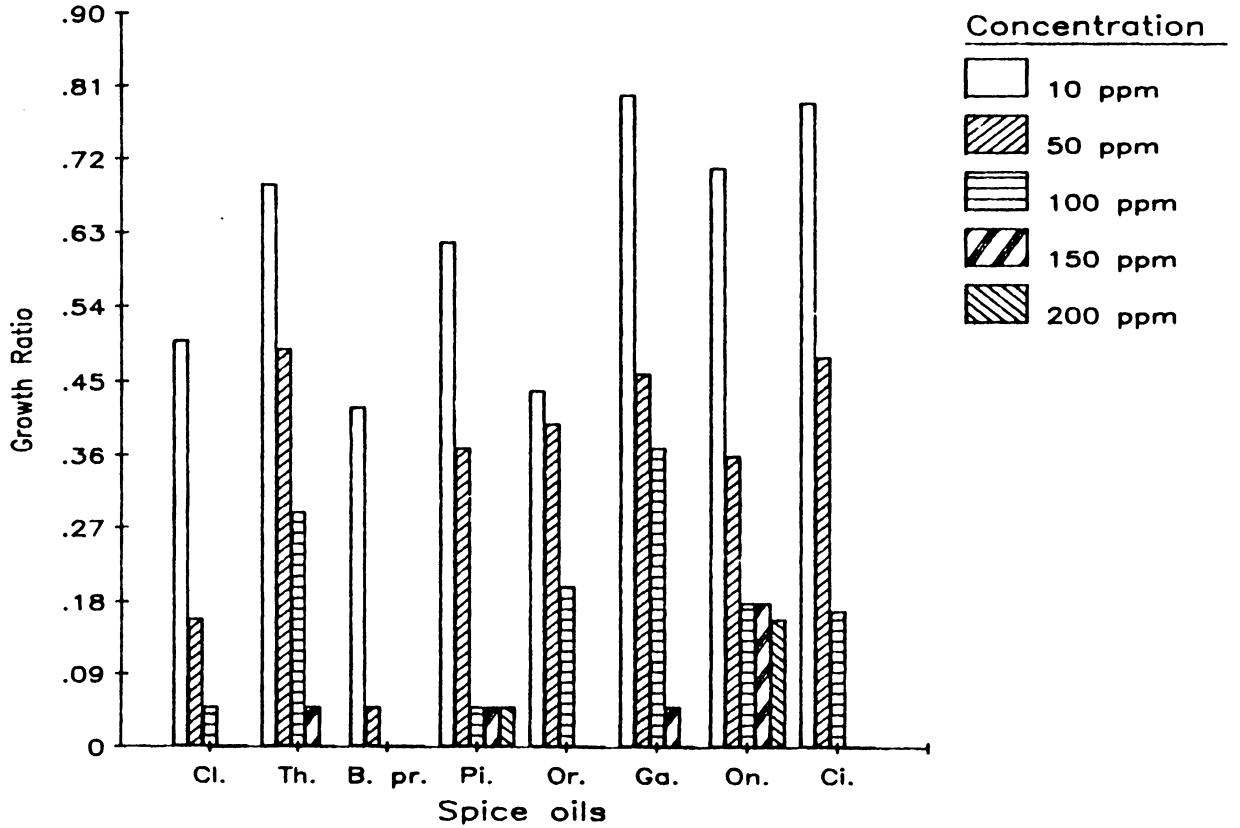


Figure 21. Effect of spice oils on growth (measured as growth ratios) of vegetative cells of C. botulinum 67B in TYG medium. See Figure 2 for the abbreviations.

synthesis of DNA, RNA, and proteins which occur during outgrowth. Second, because of the rapid outgrowth compared to the vegetative growth to O.D. of Ø.35, even if the oils caused a delay in outgrowth, the delay would not affect the long time needed for the vegetative growth to O.D. of Ø.35.

The mechanism of the effect of the oils on vegetative cell growth is not understood; however, due to the similarity in structure of the active components of the oils (Figure 1) with phenols, it has been suggested that the oils of these spices may affect vegetative growth by a mechanism similar to that of phenols (Davidson et al. 1983). Phenols affect the cell membranes of bacteria; thus, the oils could affect the permeability of the membranes, transport systems, electron transport, and energy production. The fact that both phenols and spice oils are more active on gram positive than gram negative bacteria (Davidsion, 1983; Dankert et al, 1979) supports the theory that their modes of action could be identical. The lipopolysaccharide layer in gram negative bacteria is responsible to prevent entrance of these substances into the cells, and render gram negative bacteria be more resistant towards their inhibitory actions (Sheu and Freese, 1973).

D. Effect of spice oils on toxin production

A summary of the effects of the spice oils on C. botulinum (mixed types A, B, and E) toxin production are presented in Table 7. All the spice oils at all the

Table 7. Effect of spice oils on toxin production by C. botulinum (mixed types 300 spores/ml) in prereduced TYG incubated at 32°C.

Spice oils	Conc. (ppm)	Growth and Toxin Production Days of Incubation 1	3	5	7
Control	-	5+[a]			
Control+Ethanol	-	5+			
Clove	50	0-	1+	5	
	100	0-	0-	2+	3
	200	0-	0-	0-	0+
Thyme	50	0-	5+		
	100	0-	4+	5	
	200	0-	0-	4+	5
B. pepper	50	0-	3+	5	
	100	0-	1+	5	
	200	0-	1+	5	
Pimenta	50	0-	3+	5	
	100	0-	1+	4	5
	200	0-	0-	0+	2
Origanum	50	0-	5+		
	100	0-	0-	5+	
	200	0-	0-	0-	0+
Garlic	50	5-	5+		
	100	0-	4+	4	5
	200	0-	1+	1	5
Onion	50	5-	5+		
	100	0-	5+		
	200	0-	1+	4	5
Cinnamon	50	0-	5+		
	100	0-	0-	2+	5
	200	0-	0-	0-	0-

[a] Number indicates the number of tubes out of five tubes that were positive for growth, -, no tubes contained toxin; +, tubes contained toxin.

concentrations used (50, 100, and 200 ppm) delayed toxin production in contrast to the control cultures. The time delay was dependent on the kind and concentration of the spice oil.

The longest delay in toxin production was caused by cinnamon at 200 ppm, which showed no toxin production after seven days of incubation. Clove and origanum at 200 ppm caused a delay of six days with respect to the control. On the other hand, garlic, onion, and black pepper showed slight delays in toxin production; at 200 ppm these oils delayed toxin production for only two days in contrast to the control.

Growth was not detected by visual observation during the entire 7 days of incubation of the inoculated media that contained 200 ppm of clove and origanum oil; however, the toxin was detected by mouse bioassay. This may be due in part to limited cell growth and subsequent cell lysis, and release of toxins to the medium. Costilow (1962) suggested that it is possible for nonviable spores of C. botulinum to produce toxin in an appropriate medium. However, his research involved the use of a much higher level of spores. Similar results were reported by Reddy et al. (1982).

F. Effect of origanum oil and sodium nitrite on the *C. botulinum* growth

1. In bacteriological medium

Origanum oil was used in combination with sodium nitrite as a possible partial replacement for sodium nitrite as an antibotulinal agent in meat products. Among all the spice oils, origanum oil was chosen because it was effective in inhibiting *C. botulinum* growth and toxin production. Moreover, Furia and Bellanca (1975) indicated that origanum oil can be used as a flavoring agent in cured meat products at concentrations as high as 500 ppm.

Origanum was effective in combination sodium nitrite in inhibiting *C. botulinum* growth (Table 8). At 10 ppm medium nitrite, all the replicates showed growth after two days of incubation; however, when origanum oil (100 and 200 ppm) was added with 10 ppm nitrite, the growth of *C. botulinum* was inhibited for the 21 days incubation period. Similarly , with 20 and 30 ppm sodium nitrite the addition of origanum oil (50, 100, and 200 ppm) prevented the growth of *C. botulinum*.

Heating sodium nitrite with the medium for five min enhanced the antibotulinal activity of nitrite (Table 9). For example, at 20 ppm of nitrite with no origanum oil, only one of the five tubes showed growth (after four days of incubation). At the same concentrations of sodium nitrite and origanum oil but without heating the sodium nitrite with

Table 8. Effect of origanum oil on growth of C. botulinum (A, B, and E) in TYG containing different concentrations of sodium nitrite added after autoclaving the medium.

Treatment #	Sodium Nitrite (ppm)	Origanum (ppm)	Replicate tube 1	2	3	4	5
1[a]	0	0	1[a]	1	1	1	1
2	0	50	2	3	3	3	4
3	0	100	4	5	11	13	13
4	0	200	18	19	20	-[b]	-
5	10	0	2	2	2	2	2
6	10	50	4	-	-	-	-
7	10	100	-	-	-	-	-
8	10	200	-	-	-	-	-
9	20	0	2	2	3	6	-
10	20	50	-	-	-	-	-
11	20	100	-	-	-	-	-
12	20	200	-	-	-	-	-
13	30	0	3	3	4	-	-
14	30	50	-	-	-	-	-
15	30	100	-	-	-	-	-
16	30	200	-	-	-	-	-
17	40	0	4	-	-	-	-
18	40	50	-	-	-	-	-
19	40	100	-	-	-	-	-
20	40	200	-	-	-	-	-
21	50	0	-	-	-	-	-
22	50	50	-	-	-	-	-
23	50	100	-	-	-	-	-
24	50	200	-	-	-	-	-

[a] Days to growth

[b] (-) refers to no growth after 21 days of incubation at 32°C

Table 9. Effect of origanum oil on growth of C. botulinum (A, B, and E) in TYG containing different concentrations of sodium nitrite added before autoclaving the medium.

Treatment #	Sodium Nitrite (ppm)	Origanum (ppm)	Replicate Tube 1	2	3	4	5
1[a]	0	0	1[a]	1	1	1	1
2	0	50	2	2	2	2	3
3	0	100	3	5	6	8	9
4	0	200	13	17	18	19	-
5	10	0	2	9	-	-	-
6	10	50	7	-	-	-	-
7	10	100	-	-	-	-	-
8	10	200	-	-	-	-	-
9	20	0	4	-	-	-	-
10	20	50	-	-	-	-	-
11	20	100	-	-	-	-	-
12	20	200	-	-	-	-	-
13	30	0	-	-	-	-	-
14	30	50	-	-	-	-	-
15	30	100	-	-	-	-	-
16	30	200	-	-	-	-	-
17	40	0	-	-	-	-	-
18	40	50	-	-	-	-	-
19	40	100	-	-	-	-	-
20	40	200	-	-	-	-	-
21	50	0	-	-	-	-	-
22	50	50	-	-	-	-	-
23	50	100	-	-	-	-	-
24	50	200	-	-	-	-	-

[a] Days to growth

[b] (-) refers to no growth after 21 days of incubation at 32°C

the medium (Table 8) four tubes out of five showed growth. The enhanced activity of the sodium nitrite with heating was most likely related to the formation of the Perigo-factor in the medium (discussed in the literature review).

Analysis of variance showed that the effect of the factors, sodium nitrite, origanum oil, and heat treatment were significant in preventing the growth of C. botulinum, ($P \leq 0.0001$, 0.0001, and 0.01, respectively). The interaction of sodium nitrite with origanum oil was also significant ($p \leq 0.01$), indicating that the two chemicals acted synergistically on the prevention of the growth of C. botulinum. This action may be explained by the mode of action of both the chemicals. Sodium nitrite is known to affect the outgrowth of germinated spores, whereas origanum oil affects the germination and growth. Origanum oil may enhance the antibotulinal activity of sodium nitrite by reducing the number of germinated spores on which sodium nitrite will act. At the same time, sodium nitrite could increase the inhibitory action of origanum by reducing the number of outgrown spores (cells).

2. In meat

The synergistic effect of the combination of origanum oil and sodium nitrite in a bacteriological medium led to a study on the combination of the same chemicals in comminuted vacuum-packaged pork inoculated with C. botulinum spores. Values for the chemical composition and total bacterial

content of the meat are shown in Table 10. These values are similar to those reported by Pierson and Reddy (1982).

Nitrite at 100 and 150 ppm reduced the number of toxic swollen samples during incubation at 27°C (Tables 11 and 12). However, the inhibitory effect of origanum oil was not clear. The maximum number of days for apprearance of swelling in packages inoculated with 300 spores per gram of meat was 27 days, compared to 51 days for packages inoculated with 3000 spores per gram of meat.

Duncan's multiple range test was performed on these data to determine the mean number of days for the appearance of swelling and toxin production at each combination of sodium nitrite and origanum oil. The results showed that for packages inoculated with 300 spores per gram of meat, there was no significant effect of origanum oil in the absence of nitrite on the mean number of days for the appearance of swelling and toxin formation (Table 13). This reflects the ineffectiveness of origanum oil on the growth and toxin production of C. botulinum in meat compared to bacteriological media.

The ineffectiveness of origanum oil in meat can possibly be attributed to the high solubility of the oils in the lipid portion of the meat thus reducing the concentration of origanum oil in the aqueous phase. Robach et al. (1977) and a recent NAS report (1982) suggested a similar explanation for the low antibacterial effect of BHA

Table 10. Proximate composition and total microbial count of pork.

Moisture (%)	72.3 ± 0.9
Fat (%)	5.4 ± 0.2
Protein (%)	20.2 ± 0.3
Total count (cfu/g)	3.9×10^4
Clostridium botulinum toxins	negative[a]

[a] Presence of *Clostridium botulinum* toxins was tested by mouse bioassay.

Table 11. Effect of origanum oil and nitrite in different combinations on swell formation and toxin production by C. botulinum (mixed types, A, B, and E) in packaged comminuted pork inoculated with 300 spores per gram and incubated at 27°C.

Nitrite (ppm)	Origanum (ppm)	# of toxic swollen and toxic samples at different incubation time (days)																
		2	3	4	5	6	7	8	9	10	12	13	15	16	18	21	24	27
0	0	1	4	1		1	1		1			1						
0	100	3	1		2		2						2					
0	200	3	2							1		2	2					
0	400	1	3		1				1	1		2						
50	0	2	3		2					1		1		1				
50	100	3		1	2					1		2						
50	200	2		1	1							1	2					
50	400	1	1	1	1						1		1	1			2	
100	0			1	1							1	3					2
100	100		1	1	1						1				1		1	2
100	200			1				1					1	3			2	1
100	400				2					1		2	2	1	1			
150	0		1	1						1		1	2					2
150	100				1					1	1	2				1		1
150	200	1	1								1	2						
150	400										1		2	1				1

Table 12. Effect of origanum oil and nitrite in different combinations on swell formation and toxin production by C. botulinum (mixed types, A, B, and E) in packaged comminuted pork inoculated with 3000 spores per gram and incubated at 27°C.

Nitrite (ppm)	Origanum (ppm)	# of toxic swollen and toxic samples at different incubation time (days)																		
		2	3	4	5	6	7	8	9	10	11	13	14	16	19	21	25	29	36	51
0	0	5	3	1																
0	100	3	1	1	1	2	1													
0	200	4	1			1					2		1							
0	400	5	1								1	1	2							
50	0	2	1								2	1		1	1					
50	100	1	2					2			1		1	1						
50	200	1	3		2	1					1	1			1					
50	400		1		1	1	1				2	2	1				1			
100	0	3		2		1				1	2	1								
100	100	1		2		2	1				2	1								
100	200	2	4		1									1						
100	400		3		1					1		1		1						2
150	0	1	1			1	1			2					1					
150	100	1	1			1				2						1	1		1	
150	200	1								2		1		1			1	1		
150	400						1			1		2			1			2		

Table 13. Effect of origanum oil with sodium nitrite on means of days for appearance of swelling in vacuum packaged pork inoculated with 300 per g of spores of C. botulinum (A, B, and E).

Concentrations in (ppm)		Means (days)[a, b]
Sodium nitrite	Origanum oil	for appearance of swelling
	0	5.3 a
0	100	6.3 a
	200	7.8 a
	400	12.1 a
	0	6.2 a
	100	11.6 a, b
50	200	16.4 a, b
	400	23.7 b
	0	24.1 a
	100	24.0 a
100	200	21.0 a
	400	17.0 a
	0	23.4 a
	100	28.1 a
150	200	34.3 a
	400	38.5 a

[a] For each nitrite concentration, means with different letters are significantly different ($p \leq 0.05$).

[b] Meat packages not swollen during the incubation time of 60 days were given the maximum incubation time (60 days) as the number of days for appearance of swelling to determine the means of days for C. botulinum growth and toxin production in the vacuum packaged meat within each treatment.

in crab meat and esters of organic acids in meat, respectively. Farbood et al. (1976) suggested that the absorption of the oils on the solid phase of meat was another reason for the low effect of spice oils in meat on several food poisoning bacteria including S. aureus, S. typhimurium, and E. coli.

The combination of the origanum oil with 100 and 150 ppm sodium nitrite, as with 0 ppm sodium nitrite, caused no significant effect on the mean number of days before swelling appeared. However, with 50 ppm of sodium nitrite, there were significant differences in the means with the addition of 0 and 400 ppm of origanum oil.

The effect of sodium nitrite on the mean number of days for the appearance of swelling was significant. The means ranged from 5.3-12.1, 6.2-23.7, 17-24, 23.4-38.5 days, at concentrations 0, 50, 100, 150 ppm, respectively. Comparing the effect of sodium nitrite in meat containing C. botulinum spores with bacteriological media inoculated with the same number and types of C. botulinum spores, sodium nitrite showed a higher antibotulinal activity in the media than in the meat. In the bacteriological medium (Table 8 and 9), 50 ppm of sodium nitrite prevented growth for over 21 days, whereas in meat at the same concentration of sodium nitrite (50 ppm), the mean number of days for growth appearance (swelling of the the meat packages) was 6.2 days (Table 12). Cassens et al. (1977) related the low inhibitory action of

sodium nitrite in meat compared to that in media to rapid reactions of nitrite with meat components such as myoglobin, sulfhydryl groups, nonheme protein, and lipid.

With the inoculation of 3000 spores per gram of meat in the packages, origanum oil had a significant effect on increasing the mean number of days for the appearance of swelling only when used in combination with 100 ppm of sodium nitrite (Table 14). At 100 ppm of sodium nitrite with 0 and 400 ppm of origanum, the mean number of days for the appearance of swelling were 6 and 26.3, respectively.

Overall, origanum oil at concentrations of 400 ppm with 50-100 ppm of sodium nitrite affected the growth and toxin production by C. botulinum in meat products; however, origanum oil did not show any significant effect when used with 0 and 150 ppm of sodium nitrite. This observation clearly indicated that origanum oil could be used in cured meat products to lower the concentration of sodium nitrite in cured meat products from 150 ppm to 50-100 ppm. At the same time factors other than antibotulinal effect of origanum oil such as its effect on color, taste, and structure of the cured meats need to be studied.

The probabilities of C. botulinum growth and toxin (p) production in meat packages after the incubation times of one and two weeks and for both inoculation sizes (300 and 3000 spores per gram of meat) were determined. The Log of

Table 14. Effect of origanum oil with sodium nitrite on means of days for appearance of swelling in vacuum packaged pork inoculated with 3000 per g of spores of C. botulinum (A, B, and E).

Concentrations in (ppm)		Means (days)[a, b]
Sodium nitrite	Origanum oil	for appearance of swelling
	0	8.3 a
0	100	9.7 a
	200	11.3 a
	400	6.5 a
	0	19.7 a
	100	13.0 a
50	200	7.0 a
	400	10.8 a
	0	6.0 a
	100	12.6 a, b
100	200	21.4 a, b
	400	26.3 b
	0	24.1 a
	100	27.4 a
150	200	28.8 a
	400	30.2 a

[a] For each nitrite concentration, means with different letters are significantly different ($p \leq 0.05$).

[b] Meat packages not swollen during the incubation time of 60 days were given the maximum incubation time (60 days) as the number of days for appearance of swelling to determine the means of days for C. botulinum growth and toxin production in the vacuum packaged meat within each treatment.

1/p values were plotted versus concentrations of sodium nitrite and origanum oil (Figures 22, 23, 24, and 25).

In packages inoculated with 300 spores per gram of meat and incubated for 1 week, the values of log 1/p at 0 and 50 ppm of sodium nitrite were much lower than at 100 and 150 ppm of sodium nitrite, indicating increased effect of sodium nitrite at concentrations of 100 ppm or above on growth of C. botulinum (Figure 22A).

On other hand, origanum oil at all the concentrations tested showed no significant effect on the range of log 1/p values (Figure 22B). The same effect of both sodium nitrite and origanum oil on p values can be seen after incubation time of the meat packages for two weeks (Figure 23A,23B). However, log 1/p values after two weeks of incubation were lower than those after one week of incubation. The increase in p values with increase in incubation time could be related to the depletion of sodium nitrite. The rate of depletion of sodium nitrite depends on many factors such as the incubation temperature of meat packages, pH, and the presence of isoascorbate or ascorbate (Christiansen, 1980).

As with packages inoculated with 3000 spores per gram of meat, incubated for one week, log 1/p values for sodium nitrite at 150 ppm were higher than those at the other concentrations (Figure 24A). Origanum oil, on the other hand, had no apparent effect on log 1/p values specifically

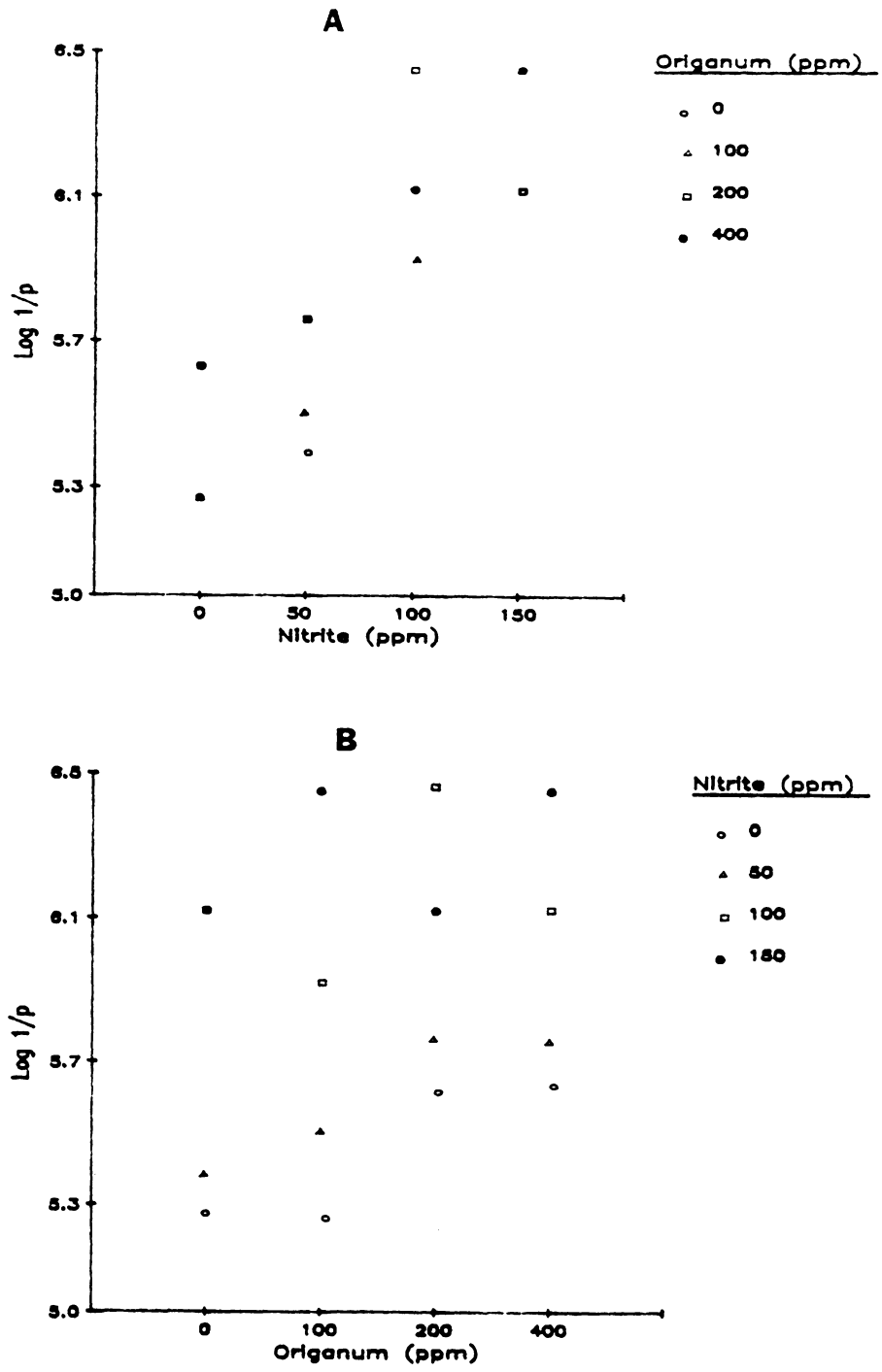


Figure 22. Effect of various levels of sodium nitrite (A) and origanum oil (B) on probability (p) of C. botulinum outgrowth and toxigenesis in vacuum packaged commminuted pork. The packages were inoculated with 300 spores per g, and were incubated at 27°C for 1 week.

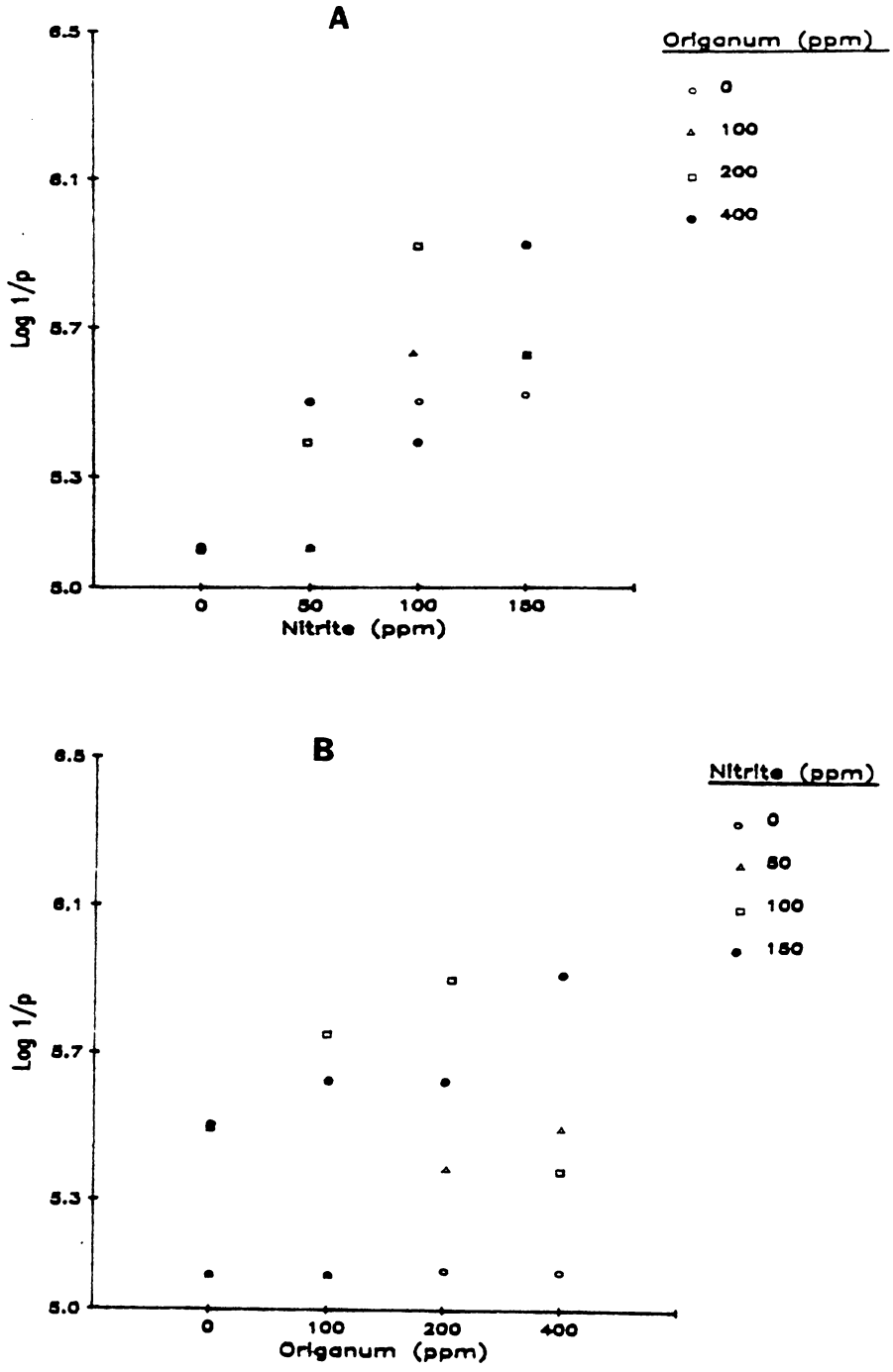


Figure 23. Effect of various levels of sodium nitrite (A) and origanum oil (B) on probability (p) of C. botulinum outgrowth and toxigenesis in vacuum packaged commminuted pork. The packages were inoculated with 300 spores per g, and were incubated at 27°C for 2 week.

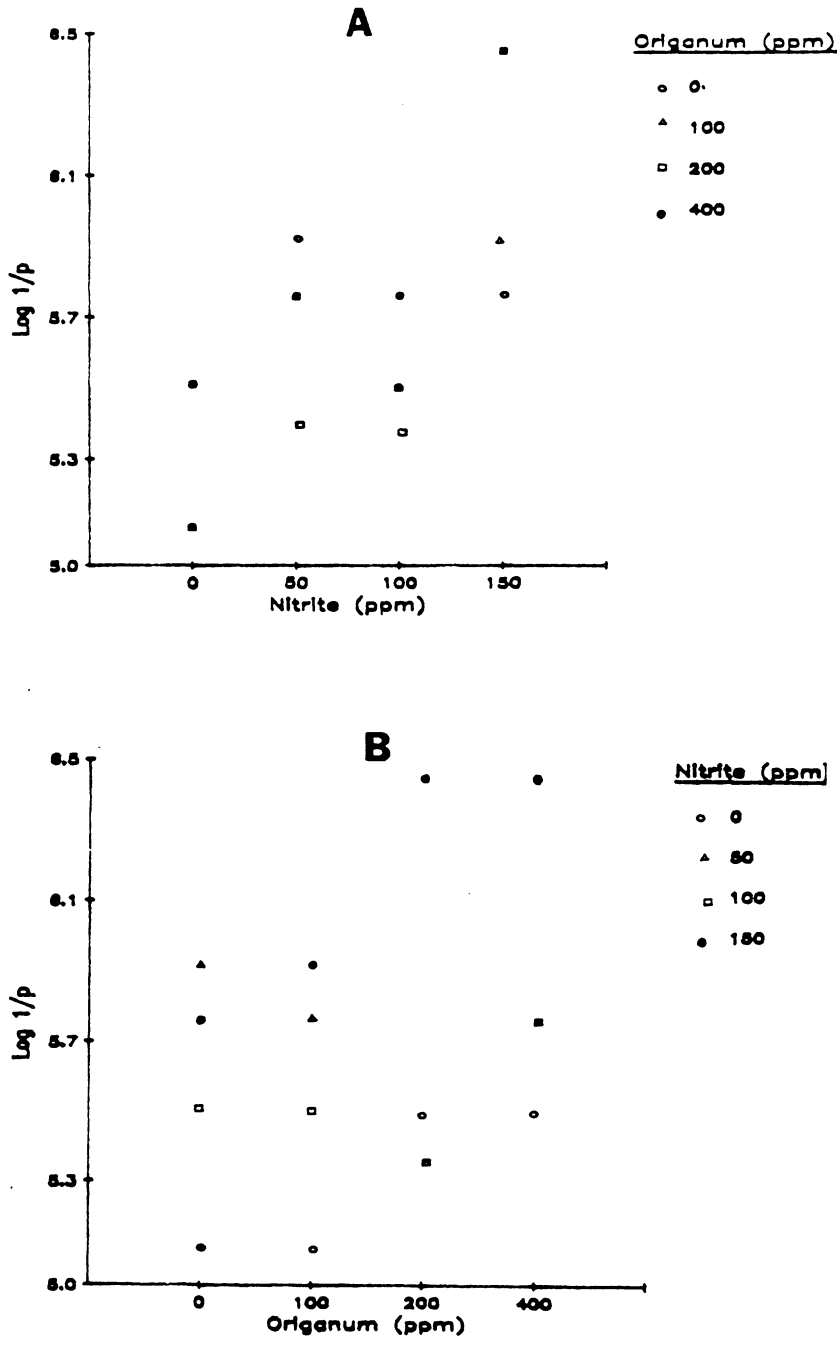


Figure 24. Effect of various levels of sodium nitrite (A) and origanum oil (B) on probability (p) of C. botulinum outgrowth and toxigenesis in vacuum packaged commminuted pork. The packages were inoculated with 3000 spores per g, and were incubated at 27°C for 1 week.

with the packages containing 100 ppm of sodium nitrite or less (Figure 24B). After an incubation time of two weeks, the values of log 1/p for 150 ppm sodium nitrite were much higher than those at 0 ppm of sodium nitrite; at the same time there were no apparent differences in log 1/p values for sodium nitrite concentrations of 50 and 100 ppm (Figure25A). Again, origanum oil, at all the concentrations used, did not show any significant impact on log 1/p values (Figure 25B).

Overall sodium nitrite had a significant effect on the p values at all the concentrations used (0, 50, 100, and 150 ppm) $p \leq 0.0001$. Origanum oil also had a significant effect on p values ($p \leq 0.04$); according to the Duncan's multiple range test, the significant effect was related to the differences between p values at concentrations of 0 and 400 ppm. It is important to mention that factors other than origanum and sodium nitrite, such as pasteurization, sodium chloride, and sodium isoascorbate, also helped in lowering the p values. Moreover, the p values were obtained based on incubation of the meat packages at 27°C. The storage of cured meats at refrigerated temperatures lowers p values dramatically. Many publications have shown the effects of low temperatures on the growth of C. botulinum in meats. For instance, Pierson (1978) found that in bacon, inoculated with 1300 spores of C. botulinum per gram , and stored at

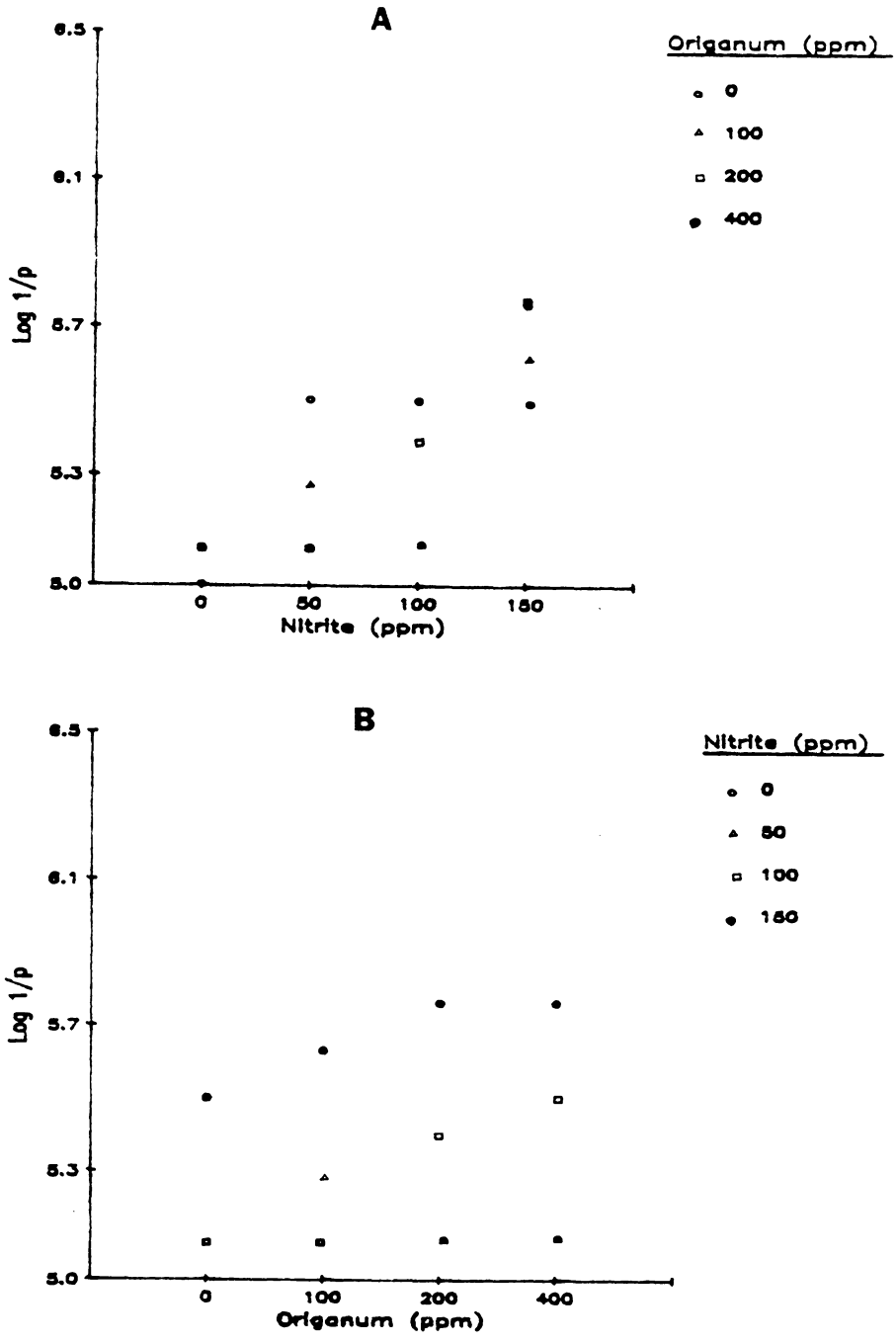


Figure 25. Effect of various levels of sodium nitrite (A) and origanum oil (B) on probability (p) of C. botulinum outgrowth and toxigenesis in vacuum packaged commminuted pork. The packages were inoculated with 3000 spores per g, and were incubated at 27°C for 2 week.

13°C, the growth and toxin production by C. botulinum were prevented during 180 days of incubation.

V. SUMMARY AND CONCLUSIONS

The inhibition of growth of six strains of C. botulinum by essential oils of eight spices was studied with a prereduced PY agar medium incubated for three days at 32°C. The oils of clove, origanum, pimenta, and cinnamon at concentration of 200 ppm completely inhibited growth (spore-to-cell transition) of C. botulinum strains. Strains of type A were more sensitive to the inhibitory action of the oils than strains of type B and E.

The effect of the oils on germination using the microculture method was different from the effect of the oils on growth. All the spice oils at a concentration of 100 ppm or above allowed a very low percentage of germination. Garlic oil was very effective in inhibiting germination. From the differences between the percent of germination and growth inhibition, it was obvious that the oils affected the rate of germination. In other words, with an increase in incubation time, the percentage of germination increased.

The results of the effect of spice oils on germination of C. botulinum 67B spores by the optical density method was identical to that obtained by the microculture method. Oils of clove and pimenta were the least inhibitory oils on germination; these oils allowed a drop in optical density of the spore suspensions even at a concentration of 100 ppm.

The duration of sensitivity of the spores to the inhibitory action of the oils indicated that the spores were

sensitive to the oils even after 30 min of exposure of the spores to the germination medium. This observation led to the proposal that the oil components were either competitive inhibitors of trigger reaction or inhibiting enzymes necessary for metabolism of the germinants. The inhibition of germination by the oils of clove, thyme, origanum, and cinnamon was reversible, indicating that the spores start germinating if the oils in the medium are either removed or depleted. This indicated that the components of the oils could be reversible inhibitor of some enzymes necessary for triggering the process of germination.

The oils of the spices had little or no effect on the outgrowth of C. botulinum 67B. Outgrowth involves the synthesis of protein, RNA, and DNA; no inhibition of these processes by spice oils or similar agents has ever been reported.

In contrast, vegetative growth was highly affected by the spice oils. Cinnamon, clove, and origanum oil at concentrations of 150 and 200 ppm caused a total inhibition of the vegetative growth of C. botulinum 67B.

All the oils caused a delay in toxin production by mixed types of C. botulinum. The ranking of the oils for their effect on toxin production was similar to that on growth. From this observation it can be concluded that the oils had a direct effect on growth, which indirectly affected toxin production.

The effect of the combination of sodium nitrite with origanum oil on C. botulinum (mixed types A, B and E) in microbiological media and meat was also studied. In microbiological medium origanum oil acted synergistically with sodium nitrite on the growth inhibition of C. botulinum. In meat (vacuum-packaged comminuted pork), origanum oil exhibited little activity in inhibiting C. botulinum, compared to the inhibition caused by the oil in the microbiological medium. Yet, the combination of 400 ppm of origanum oil with 50-100 ppm of sodium nitrite significantly delayed the growth and toxin production in meat packages. Additional studies are needed to evaluate the acceptability of cured meats containing origanum oil in combination with sodium nitrite.

When the equation of Hauschild (1982) was used to determine the probability of growth and toxin production by C. botulinum in meat packages (p), the results indicated that as the level of sodium nitrite was increased, p values decreased. Origanum oil, on the other hand, had no significant effect on reducing p values. In light of the low p values at the concentration of 150 ppm of sodium nitrite, it is recommended that sodium nitrite in cured meats be reduced to the level necessary for functions other than antibotulinal (coloring, flavoring, and antioxidant). It is important to mention that the p values were determined based on the incubation of the meat packages at 27°C. The

incubation of the meat packages at refrigeration temperatures significantly affects growth and thus p values.

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