To the Graduate Council:

I am submitting herewith a thesis written by Su-Main Chen entitled "Metabiotic Growth of Clostridium botulinum with Toxin Production in the Fresh Tomato." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

J. Orvin Mundt

J. Orvin Mundt, Major Professor

We have read this thesis
and recommend its acceptance:

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Graduate Studies and Research

METABIOTIC GROWTH OF *CLOSTRIDIUM BOTULINUM* WITH TOXIN PRODUCTION IN THE FRESH TOMATO

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Su-Main Chen

December 1981

DEDICATION

This manuscript is dedicated to my family, especially my parents, Shuang Chen and Hsia K'o Chen for their love and unfailing support. Also to my sister from whom I have always received encouragement.

ACKNOWLEDGMENTS

I would like to express my deep gratitude to my major professor, Dr. J. Orvin Mundt, for his encouragement, advice and invaluable aid. Special thanks go to Dr. F. Ann Draughon for providing C. botulinum Type B culture, and to Dr. Gary S. Sayler who served as thesis committee member.

ABSTRACT

The fungi Alternaria, Aureobasidium, Fusarium, Geotrichum, Mucor, Fusoma, Rhizoctonia, and Stemphilium were identified in molded tomatoes obtained in local markets. The pH of the infected tissue of tomatoes grown in Arizona was as high as 7.4, and of tomatoes grown in California as high as 8.3. The pH of the sound tissues of the center of some tomatoes and of the tissue across from the site of infection was raised as compared with the pH of normal tomato tissue. Bacillus megaterium, B. thuringensis, Enterobacter hafnia, Erwinia (6 species), Klebsiella pneumoniae and Staphylococcus were recovered from the juice cavities at the site of infection from 21 of 28 tomatoes examined, from the center of the tomato of 6 and across from the site of infection from 11 tomatoes. Mesocarps of sound, early mature tomatoes exposed by folding back incised skin were inoculated with Alternaria, Rhizoctonia, or Fusarium and with one loop heated cell suspension of either Clostridium sporogenes or C. botulinum. The tomatoes were incubated at 25°C and 30°C for three or more days. Visible deterioration was observed only at the site of infection. C. sporogenes was isolated from the juice cavities of 15 of 18 tomatoes adjacent the mesocarp, from tissue of 9 of 18 tomatoes 1 cm distant from the site of infection and from 4 tomatoes at the site across from the inoculation. The pH of the infected tissue ranged from 6.39 to 8.0. The pH of tissue 1 cm distant from the site of infection was between 4.36 and 4.9, and of tissue across from the site of infection between 4.3 and 4.46. C. botulinum was recovered from 19 of 27 juice cavities

adjacent the site of infection, from 6 of 26 tomatoes at a distance of 1 cm and from 1 of 21 tomatoes at the far side.

With an inoculum of 4×10^3 spores, the estimated numbers of cells of C. botulinum in infected tissue ranged from 2.4×10^6/g macerate of the fifth day when inoculated with Alternaria, to a maximum of 9×10^9 cells/g macerate when the tomato was inoculated with C. botulinum 4 days after inoculation with Fusarium. Toxic effects of the infected tissue were demonstrated by the injection of 0.09 ml of tissue supernatant diluted to 0.35 ml in gelatin-phosphate buffer into mice. Twelve of 16 mice receiving supernatants from tomatoes with associated growth of the molds Alternaria and Fusarium died, but only 1 of 8 mice died when Rhizoctonia was the associated mold. Three of 8 mice receiving heat-inactivated supernatant of Alternaria-C. botulinum died, and all injected with Fusarium-C. botulinum heated-inactivated supernatant survived. Mice given toxic supernatant and antitoxin survived.

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CHAPTER I

INTRODUCTION

Botulism has been recognized as a public health problem since 1793. It is caused by ingestion of a heat labile protein neurotoxin elaborated by the vegetative cells of Clostridium botulinum, a gram positive, anaerobic sporeforming rod. The bacterium was described by Van Ermengem in 1894 (Van Ermengem, 1979).

Outbreaks of botulism are rare compared to outbreaks caused by other food poisoning microorganisms, but it is still of great concern since the fatality rate is high, 23% in 1974 (Odlaug and Pflug, 1978). Botulism is primarily a hazard in home-processed low-acid canned foods. These foods were implicated in 67.3% of all outbreaks from 1899 through 1975. However, botulism is also a hazard in acid foods (pH 4.6). Home-canned acid foods were implicated in 34 (4.7%) of the 722 reported outbreaks of food-borne botulism from 1899 through 1975 (Odlaug and Pflug, 1978). Some type of tomato product was the vehicle for botulinal intoxication in 17 (50%) of the 34 home-canned acid food outbreaks. These outbreaks are of interest since C. botulinum reportedly will not grow and produce toxin at pH 4.7, although Raatjes and Smith (1979) observed C. botulinum to grow and produce toxin in homogeneous protein-rich substrates containing 3% or more soya or milk protein at pH 4.4.

For a botulism hazard to exist in an acid food, pH 4.6 or below, there are some contributing factors according to Odlaug and

Pflug: (1) contamination of the product with large numbers of C. botulinum spores, (2) contamination of the food with other microorganisms due to a process failure in delivery and/or postprocess contamination, (3) favorable composition of the food and storage conditions conducive to growth and toxin production of C. botulinum, and (4) metabiosis which is defined as the condition where the growth of one organism in foods makes conditions favorable for the growth of a second organism.

Studies of naturally molded tomatoes in our laboratory indicated that molds elevated the pH of fresh tomato tissue and also that bacteria frequently were present within the tomato. The observations lead to speculation that clostridia could be involved in a metabiotic relationship with molds in the fresh tomato; and if the bacterium were Clostridium botulinum, toxin might be produced. C. sporogenes, a non-toxigenic bacterium which is culturally identical with C. botulinum (Kinder et al., 1956), was used early in the studies to explore the hypothesis that clostridia would grow, then C. botulinum was used to demonstrate the production of toxin.

CHAPTER II

SURVEY OF THE LITERATURE

The effect of pH on growth and toxin production of Clostridium botulinum has been studied by many investigators. Dozier (1924; cited by Townsend et al., 1954), working with vegetative cells of 37 strains of C. botulinum in phosphate buffered double strength veal infusion-2% Difco peptone, found the minimum pH for growth to be 4.87. Later, Meyer and Gunnison (1929) did a study with the organisms isolated from canned pears in a botulism outbreak in 1927. They did not find toxin production when a Lactobacillus was present with C. botulinum. Townsend et al. (1954) inoculated tubes containing various foods at different pH levels with 2.0-2.5 X 10^6 spores of a mixture of C. botulinum Type A and Type B. The foods included green chili peppers, pimientos, eggplant with tomato sauce, pineapple-rice pudding, pork and beans, spaghetti sauce, spaghetti with tomato sauce and cheese, spaghetti with meat, prune pudding, strained pears and plums, vegetable juice, zucchini and banana puree. The tubes were incubated at 30°C until growth was evident, or for as long as one year if no growth was evident. The lowest pH at which toxin was formed was 4.8 in pineapple-rice pudding. The National Canners Association (1964) reported that spores of C. botulinum 62A would consistently grow and produce toxin at pH 5.0 or higher and growth would occur at pH 4.9 after a long incubation. Ohye et al. (1966) concluded that the pH 4.6 in foods where acidification was relied on to prevent growth was an ample

safety margin after all strains of C. botulinum which they studied failed to grow at pH 5.0. Roberts and Ingram (1973) indicated that pH 5.4 was inhibitory to C. botulinum Type A or B in a pork macerate medium. Hauschild (1975) indicated mushrooms acidified with acetic acid became toxic at pH 4.77. Huhtanen et al. (1976) using 1 X 10^3 spores of each of 10 strains of C. botulinum, four of Type A and six of Type B, inoculated tomato juice at different pH values. The minimum pH for the outgrowth of spores varied greatly for different strains. The lowest pH for the toxin production in tomato juice was pH 5.24. Ito et al. (1976) demonstrated toxin production in cucumber puree with a spore concentration of 10^6 per tube at pH 5.0, but not at pH 4.8. Townsend et al. (1954) showed that some products seemed to be less favorable for the germination of C. botulinum than others. Slocum et al. (1941) inoculated organisms isolated from canned tomatoes in a 1940 outbreak into 10 cans of commercially canned tomatoes and found no toxin after 32 days of incubation at room temperature.

The Canned Food Reference Manual (American Can Co., 1949) lists the pH of canned tomatoes as ranging from 4.0 to 4.5. Mundt et al. (1977) found that the pH of 366 home-canned tomatoes and 357 home-canned tomato juice ranged from 3.5 to 4.7. Fields et al. (1977) obtained one jar among 290 containers of home-canned tomatoes which had a pH of 4.8. The pH of none of 387 jars of tomatoes tested by Powers and Goodwin (1978) had a pH greater than 4.65. Anderson and Mendenhall (1978), studying 246 containers of home-canned tomatoes and tomato juice, found that the pH of all contents was less than 4.6 except for the contents of one container which was heavily molded.

Farrow (1963) observed that the pH of approximately 7% of nearly 15,000 tomatoes examined to be at 4.5; however, these are tomatoes produced for the canning industry, and would not be used by the housewife. The distribution of pH values in shown in Figure 1. The average pH of 4.3 almost coincided with an earlier study made by the National Canners Association listing the pH of tomatoes as ranging from 4.09 to 4.44. Wolf et al. (1979), in a study of 107 varieties of tomatoes grown under varying conditions in Minnesota, observed that the pH of 12 varieties exceeded 4.6 in the overripe stage of maturity. Burnham and Batson (1976) reported that the average pH of standard red cultivars of tomatoes introduced since 1970 is 4.26, or only 0.01 pH unit higher than the pH of cultivars grown before 1950. The average pH of 14 cultivars of tomatoes grown in Tennessee in 1976 ranged between 4.36 and 4.60 (Anon., 1977). The highest mean pH of 58 cultivars of tomatoes studied by Sapers et al. (1977) was 4.68 with a standard deviation of 0.09. They estimated that the frequency with which tomatoes would exceed pH 4.8 among four low-acid cultivars to be no greater than 0.0004.

Meyer and Gunnison (1929) did experiments with the organisms isolated from canned pears in a 1927 outbreak. They found toxin when a yeast was present with the C. botulinum in the food. Tanner et al. (1940) detected botulinal toxin in acid fruits inoculated with C. botulinum spores where a Penicillium or Mycoderma had grown in the food and shifted the pH from 3.25 to 5.4 in raspberries and from 3.75 to 5.3 in cherries. Lagarde and Beerens (1970; cited by Odlaug and Pflug, 1978) observed that a contaminant such as Trichosporon was

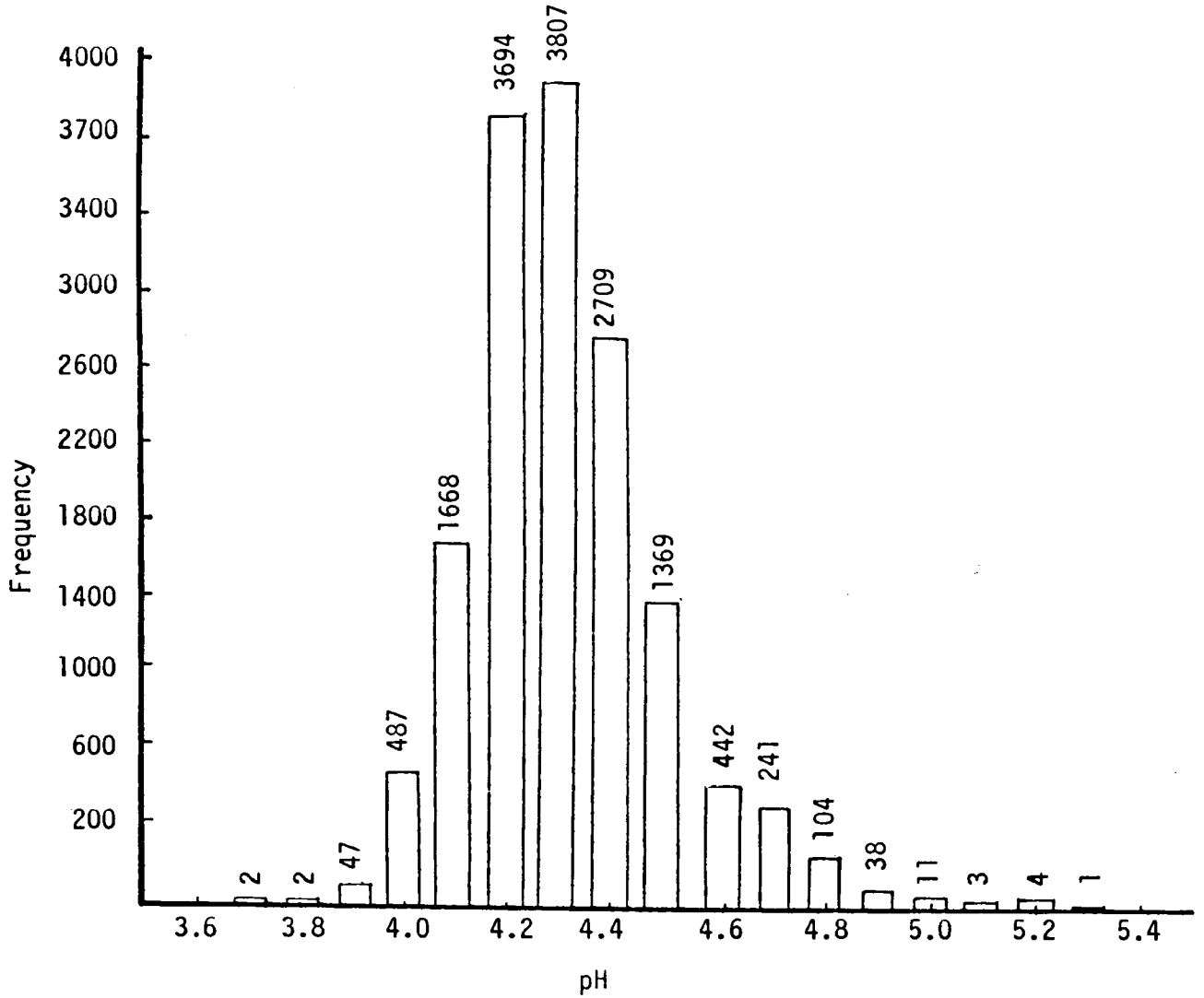

Figure 1. Histogram for pH values. (Adapted from Farrow, R. P., A Survey of pH Variation in Canning Tomatoes, Report 1-63, National Canners Association, Washington, D.C., 1963, from Powers, 1976).

able to raise the pH of pear syrup from 3.8 to 4.8 where C. botulinum produced toxin. Huhtanen et al. (1976) were able to demonstrate toxin production in tomato juice when a Penicillum or Cladosporium was present to raise the pH at the surface above 4.6. Odlaug and Pflug (1979) were able to demonstrate a pH gradient in tomato juice inoculated with mold when a heavy mold mat formed on the surface. The pH values near the mat were near neutrality, and the lower portions below the mat were more acid. Odlaug and Pflug showed that growth of C. botulinum was greatest at the surface and that the counts decreased with distance from the mycelial mat. Toxin was present throughout the tomato juice. Odlaug and Pflug suggested that growth of the mold at the surface of the tomato juice in a hermetic unit created a microenvironment within the mycelial mat or directly below the mycelial mat where the pH was greater than 4.6 and where C. botulinum spores germinated, reproduced, and produced toxin.

CHAPTER III

MATERIALS AND METHODS

Identification of Molds on Tomatoes

Tomatoes were obtained from the wholesale tomato markets. The tomatoes were grown in Arizona, California, Florida, and Michigan. Tomatoes in the early ripe stage were selected for all studies.

Molds of tomatoes were identified directly by morphological characteristics (Barnett and Hunter, 1972; Gilman, 1957).

Disinfection

Tomatoes to be opened for culture were surface-washed in a 1% aqueous dilution of nonylphenoxypoly (ethyleneoxy) iodine complex containing initially 1.75% ethanol-iodine complex, 14.25% iodine and 12.5% phosphoric acid. The same solution was used to disinfect knives and working surfaces. Tomatoes to be inoculated were dipped into a solution containing 1,000 micrograms per liter (1,000 parts per million) of available chlorine, prepared from commercial sodium hypochlorite.

Measuring the pH of Naturally Infected or Laboratory Infected Tomatoes

Each tomato was cut in half with a sterilized knife from the sound side to the infected side. Then the pH of the sound tissue at the opposite side of the infected tissue, 1 cm away from the infected tissue, and the infected tissue was measured by the insertion of the combination electride attached to an Orion Research Ionalyzer Model

399A pH meter. The pH of laboratory-infected tomatoes was measured at one, two, three, four, and five days of incubation.

Isolation and Identification of Bacteria in the Tomato

Tomatoes were cultured from four different locations, including sound tissue, 1 cm away from infected tissue, center of the tomato, and infected tissue, to phenol-red broth-0.5% glucose medium (PRBG). If growth occurred, the broth was streaked on nutrient agar. The plates were incubated at 30°C. The colonies were observed and picked into PRBG medium. All bacteria were identified with the aid of Bergey's Manual (Buchanan and Gibbons, 1974).

Mold Suspensions

Molds spores or suspensions were prepared on different media in order to get suitable growth. Rhizoctonia was grown on Sabouraud agar, Fusarium was grown on Mycophil agar, and Alternaria was grown on Malt Extract agar. These molds were used because they were the most frequently encountered field molds on tomatoes. Slants of the media in 16 X 150 mm tubes were inoculated with the molds and incubated at 30°C for 2 to 3 weeks. After incubation, the molds were harvested by adding 3 ml of 0.3% Tween 20 in distilled water solution to the test tube. The test tube was swirled, and the mycelial mat was brushed slightly with a sterile pointed spatula to dislodge the conidiospores or fragments of the mold.

Maintaining the Clostridia

Clostridium sporogenes ATCC# 7955 was subcultured in trypticase-peptone-glucose-yeast extract (TPGY: Speck, 1976), and fluid

thioglycollate (FT; Rohde, 1967) broths. One ml of 2% sodium azide was added to each 100 ml of medium to inhibit growth of nonclostridial microorganisms, including fungi.

Clostridium botulinum Type B# 1-24, obtained from Dr. Bruce Tompkin, was subcultured in cooked meat medium, TPGY, and FT broths. C. botulinum exhibited better growth in TPGY and FT broths than in cooked meat medium. All incubations were at 37°C for 4 to 7 days. Purity of the culture was assured by routinely preparing Gram stains.

Preparation of Spore Suspensions and Counting Spores

Since a sugar-free medium favored spore formation, C. botulinum was grown in the basal tryptone-peptone-yeast extract medium with sodium azide, but with sugar omitted. Tubes were incubated at 37°C for 4 to 7 days.

The broth culture was heated to 80°C for 10 minutes to destroy the vegetative cells. The numbers of spores in the inocula were counted by adding serial dilutions of the suspensions to Prickett tubes, and then immediately adding freshly melted and exhausted fluid thioglycollate agar. Spores were also enumerated directly by Gram staining 0.01 ml of broth spread over 1 cm area on a milk slide, and counting with a calibrated microscope. The number of spores per inoculum was estimated to be 4×10^3.

Inoculation of Tomatoes

Two cuts about 0.5 cm long were made to intersect at a 60 degree angle in tomato skin. The skin was folded back to expose the underlying mesocarp. This was inoculated with 1 loop of suspension of

the mold, and with 1 loop of heated suspension of clostridia immediately, or 4 days after inoculation with mold. The tomatoes were incubated in the dark at 25°C and 30°C in enamel pans which were covered with aluminum foil.

Observations on Inoculated Tomatoes

Tomatoes were surface-disinfected after incubation and cut with a sterile knife from the sound to the infected side. Tomato juice and tissue were removed with a sterile loop progressively from the sound area, 1 cm from the infected area and from the juice cavity adjacent the infected area to tubes of TPGY and FT broths. Smears were also made routinely for staining. The broths were not heated after inoculation with material from the tomatoes, since the clostridia do not sporulate below pH 5.7, as shown by failure to obtain outgrowths from heated media, lack of spores on the Gram stains, and unreported observations by Crossthwait (1979). The pH of each area was then measured by the insertion of the combination electrode into the desired area. Serial dilutions of infected tissue were cultured in FT agar in Prickett tubes.

Confirmation of Clostridia

The clostridia were recognized on stained smears from tomatoes and from broth by their distinctive morphology. Outgrowths in broth were also confirmed by subculture into reduced litmus milk.

Estimating Growth of C. botulinum in Infected Tomato

Numbers of C. botulinum in infected tissue were estimated by plating in TPGY and FT agars in Prickett tubes. Infected tissue was

macerated with a pointed knife in the juice cavity adjacent the infection. One ml of the macerate was withdrawn to a dilution blank made of 0.1% peptone-0.05% cysteine. Serial dilutions were prepared in the same fluid.

Confirmation of the Production of Type B Toxin by the Culture

The buffer used for dilution of toxin contained 0.2% gelatin and 0.4% dibasic sodium phosphate, and was adjusted to pH 6.8 with hydrochloric acid. C. botulinum was grown in TPGY for 7 days at 37°C. After growth, the supernatant fluid was obtained by centrifuging for 15 minutes at 2,800 rpm with a Sorvall CLC-2B centrifuge. The supernatant fluid was diluted with buffer in one to two dilution.

Eight three- to four-week old mice (C3H/HeJ) were obtained from Dr. McDonald at University of Tennessee Center for the Health Sciences. They were injected intraperitoneally with 0.35 ml aliquots of one to four dilutions of toxin. All mice died within 2 days.

Detection of Botulinal Toxin in Tomatoes

The infected tissues of tomatoes were diluted with an equal volume of buffer. The mixtures were centrifuged for 15 minutes at 2,800 rpm. The supernatant fluids were removed and stored at -15°C until used. At the time of use the fluids were thawed and diluted with equal amounts of gelatin-phosphate buffer. Mice were given 0.35 ml of fluid, representing 0.0875 ml tomato fluid, by intraperitoneal injection.

Confirmation of Toxin

Mice were given 0.35 ml of fluid, representing one to four dilutions of tomato material, by intraperitoneal injection. Injections given the mice included supernatant material prepared from uninoculated tomatoes, as controls; toxic supernatant fluids; toxic supernatant fluids which had been heated for 10 minutes in boiling water; and toxic fluids plus 0.1 ml Type B antitoxin. All mice were observed for 4 days after injection.

CHAPTER IV

RESULTS

Molds Isolated from, and the pH of Molded Arizona Tomatoes

Alternaria, *Fusarium*, *Geotrichum*, *Mucor* and *Stemphilium* were isolated from Arizona tomatoes (Table 1). All molds raised the pH of tissue at the site of infection, and the pH of one tomato which was severely bruised but without a mold lesion also had an elevated pH. The increase in pH extended to tissue in the center of the tomato in all instances, except one of the tomatoes molded with *Geotrichum*, and to tissue at the far side across from the mold lesion.

Molds Isolated from, and the pH of Molded California Tomatoes

In addition to the molds isolated from the Arizona tomatoes, *Fusoma*, *Aureobasidium* and *Rhizoctonia* were isolated from the California tomatoes (Table 2). In comparison with the pH of the sound tissue (4.4 to 4.6) the pH of the infected tissue in all instances except one was greater. Bacteria in association with *Geotrichum* may ferment the sugar to maintain a low pH in the tomato. The pH of center tissue was elevated, as compared with the pH of sound tissue, in all instances except the tomato infected with *Geotrichum* and *Aureobasidium*, and in all but three samples of sound tissue.

Bacteria Isolated from Molded and Bruised Tomatoes

Bacteria were isolated from 21 of 28 tomatoes examined. They were isolated from 15 tomatoes at the site of mold infection, from

TABLE 1

MOLDS ISOLATED FROM ARIZONA TOMATOES AND THE pH OF TOMATO TISSUE

Mold(s)	Frequency	pH of Infected tissue	pH of Center tissue	pH of Sound tissue
Fusarium	3	4.6-7.7	6.0	4.5
Geotrichum	4	4.9-5.7	4.5-4.8	4.1-4.9
Mucors	3	6.4	4.7	4.8
Soft (no lesion)	2	4.5-6.0	4.7	4.6-4.8
Geotrichum and *Alternaria*	1	7.4	4.5	4.5
Alternaria	1	6.0	5.0	4.2
Alternaria and *Fusarium*	1	5.5	4.6	4.2
Geotrichum and *Fusarium*	1	4.8	4.4	4.3
Geotrichum and *Stemphilium*	1	7.1	6.6	4.6

TABLE 2

MOLDS ISOLATED FROM CALIFORNIA TOMATOES AND THE pH OF TOMATO TISSUE

Mold(s)	Frequency	pH of Infected tissue	pH of Center tissue	pH of Sound tissue
Alternaria	6	5.3-8.3	4.7-6.3	4.5-4.9
Fusarium	9	4.8-7.6	4.7-5.3	4.7-5.0
Stemphilium	2	5.2-6.1	4.7-5.7	4.6-4.9
Fusarium and *Fusoma*	1	7.4	5.5	4.9
Fusarium and *Rhizoctonia*	1	5.8	5.2	4.8
Fusarium and *Stemphilium*	1	7.3	4.9	4.8
Geotrichum and *Stemphilium*	1	7.8	4.9	4.9
Geotrichum and bacteria	1	4.5	4.6	4.4
Fusarium and *Alternaria*	7	5.2-8.3	4.9-5.3	4.7-5.0
Geotrichum and *Rhizoctonia*	7	5.7-7.7	4.8-5.3	4.6-4.9
Aureobasidium	1	5.5	4.8	4.8

6 tomatoes at the site of bruising, from the center of 6 tomatoes infected with mold, and from the far side of 9 infected tomatoes and of 2 bruised tomatoes (Table 3). Species of Erwinia were isolated most frequently. Two species of Bacillus were isolated once each, as was Staphylococcus, and Enterobacter hafnia and Klebsiella pneumoniae each were isolated from two tomatoes. The results are in agreement with those of Samish et al. (1961, 1963).

pH and Recovery of Clostridium sporogenes from Inoculated Michigan Tomatoes

The effect of the growth of mold and the recovery of C. sporogenes when tomatoes were inoculated simultaneously are shown in Table 4. Each of the three molds, Alternaria, Rhizoctonia and Fusarium, introduced to the external surface of the mesocarp, penetrated the mesocarp in 3 or more days, but deterioration was not visible beyond the inner surface of the mesocarp and the adjacent juice cavity. With each of the three molds, the pH of the mesocarp and the adjacent juice rose to 6.41 or higher in all instances. The pH of sound tissue 1 cm from the site of infection rose slightly in eight of the nine tomatoes, suggesting the establishment of a pH gradient.

C. sporogenes was recovered from the juice cavity adjacent the infected mesocarp of eight of nine tomatoes incubated at 25°C and seven of nine tomatoes incubated at 30°C. The isolations from sound tissue 1 cm distant from the site of infection were five and four, respectively, and from sound tissue at the far side of the tomato recoveries were three and two of nine tomatoes in each instance at

TABLE 3

BACTERIA ISOLATED FROM MOLDED OR BRUISED TOMATOES

Bacterium	Tomato	Frequency of isolation from Infected tissue	Center tissue	Sound tissue
Bacillus megaterium	Molded	1	0	0
Bacillus thuringensis	Bruised	1	0	0
Enterobacter hafnia	Molded	2	0	2
	Bruised	0	0	1
Erwinia carotovora	Molded	2	0	2
	Bruised	3	0	1
E. sipripedii	Molded	2	5	4
E. herbicola	Molded	4	0	0
E. quercina	Bruised	1	0	0
E. stewartii	Molded	2	0	0
E. salicis	Molded	0	0	1
Klebsiella pneumoniae	Molded	2	0	0
Staphylococcus	Bruised	1	0	0

TABLE 4

pH AND RECOVERY OF *CLOSTRIDIUM SPOROGENES* FROM MICHIGAN TOMATOES INCUBATED AT 25° and 30°C

Mold	Day cultured	Site cultured: Infected area pH	Infected area Recovery 25°C	Infected area Recovery 30°C	1 cm distant pH	1 cm distant Recovery 25°C	1 cm distant Recovery 30°C	Sound tissue pH	Sound tissue Recovery 25°C	Sound tissue Recovery 30°C
Alternaria	3	7.71	+	+	4.36	+	+	4.46	-	+
	4	7.11	+	-	4.47	-	-	4.34	+	-
	5	6.41	+	+	4.36	+	-	4.3	-	-
Rhizoctonia	3	6.95	+	+	4.52	+	-	4.3	+	-
	4	6.39	-	+	4.58	-	-	4.35	-	-
	5	6.65	+	+	4.51	+	+	4.35	+	-
Fusarium	3	8.0	+	+	4.8	-	-	4.45	-	-
	4	7.25	+	-	4.66	+	+	4.41	-	-
	5	7.37	+	+	4.91	-	+	4.43	-	+
Total			8/9	7/9		5/9	4/9		3/9	2/9

each temperature of incubation. The isolations suggest movement of the motile bacteria into the interior of the tomato.

pH of Inoculated Tomatoes and Recovery of C. botulinum

C. botulinum was recovered from the juice cavities adjacent the infected sites of 30 of 50 tomatoes when the fluids were cultured in FT broth and of 25 of 50 tomatoes cultured in TPGY (Table 5). It was recovered from 11 of 49 tomatoes cultured 1 cm distant from the site of infection when the tissue was cultured in FT broth, and from 6 of 49 tomatoes cultured in TPGY, and twice from sound tissue at the opposite side of the tomato. The superiority of FT broth is indicated by 43 recoveries in this medium, as compared with 31 in TPGY broth. The results indicate that C. botulinum had penetrated the mesocarp to the juice cavity at the site of infection, and then migrated into the internal tissues of the tomato.

Increase in Numbers of C. botulinum in Molded Tomato

The numbers of spores with which tomatoes were inoculated and the numbers of C. botulinum per gram of infected tissue are shown in Table 6. The clostridia failed to grow in 11 inoculated tomatoes. They reproduced by a minimum of 2 log units in 12 tomatoes. They increased less rapidly when inoculated with Alternaria, a probable consequence of the slower growth of this mold as compared with growth of Rhizoctonia and Fusarium. Seven of the 12 tomatoes were inoculated with C. botulinum 4 days after inoculation with mold. Five of the 12 failures of C. botulinum to grow were among tomatoes inoculated with the bacteria 4 days after inoculation with mold.

TABLE 5

pH AND RECOVERY OF *CLOSTRIDIUM BOTULINUM* FROM INOCULATED TOMATOES

Mold	Infected tissue			Tissue 1 cm distant			Tissue, far side		
		Recovery in			Recovery in			Recovery in	
	pH	FT*	TPGY*	pH	FT	TPGY	pH	FT	TPGY
Alternaria	6.0-8.6	10/17**	9/17	4.3-5.7	3/17	3/17	4.3-5.7	1/10	0/10
Rhizoctonia	5.3-7.6	9/16	8/16	4.2-4.9	5/16	1/16	4.3-4.5	0/10	0/10
Fusarium	7.0-8.5	11/17	8/17	4.7-6.8	3/16	2/16	4.2-4.7	1/9	0/9
Totals		30/50	25/50		11/49	6/49		2/29	0/29

*FT, fluid thioglocollate broth; TPGY, tryptone-peptone-glucose-yeast extract broth.

**Number recovered per number inoculated.

TABLE 6

NUMBERS OF C. BOTULINUM INOCULATED AND RECOVERED FROM TOMATOES

Mold	Inoculum, number of spores	Days incubated	Numbers per ml infected tissue
Alternaria	4×10^5	6	1×10^7
	1×10^5	6	2×10^7
	4×10^3	5	2.4×10^6
Rhizoctonia	1×10^3	3	1×10^7
	4×10^3	4	2.5×10^8
	"	5	1.4×10^8
	"	6	7.0×10^7
Fusarium	4×10^3	3	3×10^6
	6×10^3	3	5×10^9
	4×10^3	5	8×10^7
	"	6	9×10^9
	"	6	2.4×10^8

Toxin Production

The number of lethality of infected tissue for mice are shown in Table 7. Juice and macerated tissue from the infected sites were mixed and diluted with an equal volume of buffer and centrifuged. The supernatant fluid was again diluted at the time of IP injection of 0.35 ml fluid containing 0.0875 ml toxic fluid. Eight mice were injected with fluid prepared from the 3 molded tissues. Seven of the Alternaria, 1 of the Rhizoctonia, and 5 of the Fusarium groups died. Control mice injected IP with 0.35 ml tomato serum were unaffected.

Five of 8 mice given heated fluid from the Alternaria tomato and all of the 8 mice given fluid from the Fusarium tomato were unaffected. The reason for the death of 3 mice given heated fluid is not known.

Six mice given Fusarium fluid and antitoxin survived with no visible symptoms of injury during more than 4 days of observation.

TABLE 7

LETHALITY OF INFECTED TISSUE FOR MICE

Mold	Numbers of mice injected with*					
	Fresh fluid	Died	Heated fluid	Died	Fresh fluid - antitoxin	Died
Alternaria	8	7	8	3	--*	
Rhizoctonia	8	1	--		--	
Fusarium	8	5	8	0	6	0

*0.35 ml tomato tissue in equal volume buffer.

**Not done; no toxic material available.

CHAPTER V

DISCUSSION

This appears to be the first report of the growth of clostridia and the production of toxin by C. botulinum in a metabiotic relationship with mold in the fresh tomato. Growth of the bacterium in laboratory studies with canned tomato juice (Huhtanen et al., 1976; Odlaug and Pflug, 1979) followed the rise in pH produced by the associated mold growth. Growth of the bacterium in the fresh tomato appears to be dependent upon the same factor.

Each tomato used in this study must be considered as an individual. Even among a number of tomatoes inoculated with the same mold, the pH values differed. About 30% of the molded tomatoes inoculated with molds and C. botulinum do not support growth of the bacterium. It is not known whether the heat-shocked spores died before the action of the mold on the tomato could prepare the tomato to be receptive to clostridial growth, or whether other, subtle differences of the tomatoes are responsible.

The quantity of toxic material available for mouse toxicity studies was very limited. In contrast with test tube studies, where the amount of tomato involved can be large, this study was limited by the amount of tomato tissue that could be affected by the mold during the period of incubation. The time of incubation was limited by the ripening process and deterioration of the tomato upon aging. This in turn was limited by the type of tomatoes available. Shipper tomatoes have a thicker mesocarp than do garden tomatoes. The mesocarp

is very firm and growth of mold during the first several days after inoculation is slow. The extent of mold growth is moderate.

A further factor which may limit the amount of toxic material is the inability to reproduce mold lesions in the laboratory comparable to those produced in the field in locally grown tomatoes. Field lesions produced by Alternaria and Fusarium tended to be penetrating, while lesions in the laboratory are restricted to the surface. It is not known whether the difference is attributable to the tomato or to the laboratory conditions. In this study, it was necessary to use tomatoes which were in the early ripe stage to avoid the use of tomatoes undergoing autolytic tissue decomposition late in the incubation period. Tomatoes were incubated in pans covered loosely with aluminum foil and without humidification. When water was added to the pan of incubating tomatoes mucors overgrew the tomatoes. Finally, the numbers of spores of C. botulinum were as many in tomatoes inoculated with Rhizoctonia as in tomatoes inoculated with Alternaria and Fusarium, but less toxin was present in the macerate. No known reason accounts for the lack of toxin but, since the toxin is a protein, it is possible that Rhizoctonia digested it.

With the demonstration of growth of C. botulinum and toxin production in fresh tomatoes in a metabiotic relationship with mold, a reasonable hypothesis may be presented to account for botulinal intoxication following the consumption of home-canned tomatoes and tomato juice.

C. botulinum is normally present in approximately one-third of the soil samples examined (Meyer and Dubovsky, 1922a, b; Perry,

1946; Smith, 1970) and on the surfaces of fruits and vegetables. Hauschild et al. (1975) found 15 spores of C. botulinum per 100 grams of unwashed mushrooms and 41 spores per gram of washed mushrooms. In rare instances, C. botulinum may be present on the surface of a tomato at the site of a developing mold lesion, and as the pH is elevated, the spore will initiate growth. Lesions associated with metabiotic growth in this study appear to be restricted to the mesocarp with little if any discoloration of fluid in the juice cavity. If such tomatoes used for canning are closely trimmed, bacteria and toxin will be in the retained portion of the tomato.

Woolford and Schantz (1978) reported that foods with low pH, such as tomato soup at pH 4.1, provide more protection to toxin during heating than do foods at higher pH. They obtained a 50% inactivation of the toxin in tomato soup after 7 minutes of heating at 68°C, whereas in cream of mushroom soup at pH 6.2 and in beef pie blend at pH 5.9, more than 99% of the toxin was inactivated. After 30 minutes of heating tomato soup at 68°C, the initial toxin level was reduced by 90%, but in the quantities they used at the start, 6,000 mouse LD_{50}/ml remained. Powers (1976) stated that in some instances the center temperatures in jars of home-canned tomatoes would not get above 71°C. Woolford and Schantz showed that after heating for 30 minutes at 71°C, roughly 3% of the toxin had not been inactivated.

CHAPTER VI

CONCLUSIONS

The pH of infected tissue either in naturally contaminated or in laboratory infected moldy tomatoes was greater than 4.6 in a very high percentage of the samples. Increases in pH were noted in tissues 1 cm distant from the infected tissue and at the far side in some tomatoes.

Species of Bacillus, Enterobacter, Erwinia, Klebsiella, and Staphylococcus were observed in preparations for microscopic examination and in culture, in an apparent associated growth with mold.

Clostridium sporogenes and C. botulinum were recovered from the juice cavities adjacent the inoculated mesocarp, at a site 1 cm distant from the inoculation, and from the sound tissue of molded tomatoes. Quantities when 4×10^3 spores of C. botulinum were placed on the exterior surface of exposed mesocarp, as many as 9×10^9 cells/g of infected tissue were enumerated after 6 days incubation.

Toxin lethal for mice was produced during growth of C. botulinum in association with Alternaria and Fusarium, and to a lesser extent in association with Rhizoctonia. Mice were protected if supernatant fluid from infected tissue was heated, or of antitoxin was administered with the fluid.

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