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## Effects of mastic resin and its essential oil on the growth of proteolytic *Clostridium botulinum*

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### Abstract

Studies were done to determine the effect of mastic resin and its essential oil, alone and in conjunction with ethanol, on the growth of proteolytic strains of *Clostridium botulinum* in media, and on neurotoxin production in challenge studies with English-style crumpets. Preliminary studies, using a spot-on-the-lawn method, indicated that high levels of mastic resin in ethanol (~ 8% w/w) were required for complete inhibition of all strains of *C. botulinum* tested, but mastic resin in ethanol had a greater anti-botulinal effect than ethanol alone. However, only low levels of mastic oil (~ 0.3% v/v) were required for inhibition of proteolytic strains of *C. botulinum*. Both studies showed a strain specific inhibition, with *C. botulinum* type A strains being more sensitive to mastic resin and its essential oil than type B strains. However, mastic resin in ethanol proved to be more effective when used as a vapor phase inhibitor applied to cotton pads and placed inside inoculated plates than when added directly to media. While both mastic resin and its essential oil inhibited the growth of proteolytic strains of *C. botulinum* *in vitro*, they failed to inhibit neurotoxin production in challenge studies with *C. botulinum* in English-style crumpets.

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**Keywords:** Mastic; Ethanol; *Clostridium botulinum*; Botulinum neurotoxin

### 1. Introduction

Modified atmosphere packaging (MAP), involving gas flushing and interactive oxygen absorbent sachets, is being increasingly used by the bakery industry worldwide to extend the shelf-life of high-

moisture bakery products. However, there are regulatory concerns about the safety of such products with extended shelf-life at ambient temperature, particularly with respect to the growth of *Clostridium botulinum*. While there have been no botulism outbreaks associated with commercially produced MAP bakery products, several challenge studies have shown that bakery products are a suitable substrate for the growth of *C. botulinum* (Daifas et al., 1999a). Daifas et al. (1999b) have shown also that MAP crumpets, a high-moisture minimally processed prod-

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uct, could support the growth of proteolytic strains of *C. botulinum* yet remain sensorially acceptable at the time of toxigenesis—a highly dangerous scenario. As a result of the potential of bakery products to support the growth of *C. botulinum*, it has been recommended that additional barriers, such as  $a_w$  and pH reduction, be incorporated into MAP bakery products to ensure their continued safety at ambient storage. However, reformulation of high-moisture products to lower  $a_w$  and pH levels is not always practical due to textural and sensory constraints. More recently, Daifas et al. (2000) investigated the potential of ethanol vapor as an additional barrier to enhance the safety of MAP crumpets. However, while this approach enhanced the safety of crumpets, their sensory shelf-life was compromised due to absorption of ethanol from the package headspace (Daifas et al., 2000). It is well known that two or more barriers, in conjunction with one another, can be far more effective than using each barrier separately. This “hurdle” approach is widely used by the food industry to extend both the shelf-life and safety of foods. A novel potential barrier that could be used, in conjunction with ethanol vapor, is mastic resin or its essential oil. Derived from the shrub *Pistacia lentiscus* var. *chia*, mastic resin, and its essential oil, have been used as flavoring for breads, as well as for other foods and beverages (Wyllie et al., 1990), and have also been shown to have antioxidant properties (Abdel-Rahman and Soud, 1975). The antimicrobial activity of mastic against several foodborne pathogens, including *Staphylococcus aureus*, *Salmonella enteritidis*, *Bacillus cereus*, *Escherichia coli* and *Helicobacter pylori*, as well as many yeasts and molds has also been reported (Alippi et al., 1996; Iuak et al., 1996; Tassou and Nychas, 1995; Huwez et al., 1998; Magiatis et al., 1999; Koukoutsis, 2002) with Gram-positive bacteria being generally more sensitive than Gram-negative (Hussain and Tabji, 1997; Ali-Shtayeh et al., 1998). However, the effect of mastic on *C. botulinum* has never been reported.

Therefore, the objective of this study was to determine the effect of mastic resin and mastic oil, alone and in conjunction with ethanol, on the growth of proteolytic strains of *C. botulinum* in media and on neurotoxin production in challenge studies with English-style crumpets.

## 2. Materials and methods

### 2.1. Preparation of inocula

Spores of proteolytic *C. botulinum* (A6, 17A, 62A, CK2A, MRB, 13983IIB, and 368B) were used in these studies. Spore crops of each strain were prepared separately, enumerated as described by Hauschild and Hilsheimer (1977), and stored at  $-80\text{ }^{\circ}\text{C}$ . Spores and overnight cultures of individual strains were used in the “spot-on-the-lawn” and microtiter assays respectively, while a composite spore inoculum was used in the agar plate study and in the challenge study with crumpets. All spores and spore mixtures were heat-shocked ( $75\text{ }^{\circ}\text{C}$  for 20 min) prior to sample inoculation. For cultures, each strain of *C. botulinum* was grown from frozen stock culture (Daifas et al., 2003) onto McClung Toabe agar (Difco, Becton-Dickinson; Sparks, MD, USA) and incubated anaerobically in an atmosphere of 10%  $\text{H}_2$ , 80%  $\text{N}_2$ , and 10%  $\text{CO}_2$  at  $37\text{ }^{\circ}\text{C}$ . Overnight broth cultures were prepared in Iso-sensitest (IST) broth (Difco) and incubated anaerobically to provide final inoculum levels of approximately  $10^8$  CFU/ml. Inoculum levels were verified using modified McClung Toabe (MMT) agar prepared from McClung Toabe agar (Difco) supplemented with 0.1% yeast extract (Difco) and egg-yolk as previously described (Daifas et al., 1999b).

### 2.2. Mastic resin and oil

Commercially produced mastic resin (“gum”) and essential oil-of-mastic, obtained from the Chios Mastic Grower’s Association ([CMGA] Chios, Greece), were used throughout these studies. The sterility of both the resin and the oil was confirmed by aerobic and anaerobic plate counts at 25, 30 and  $37\text{ }^{\circ}\text{C}$ .

### 2.3. Media studies

The anti-botulinal activity of mastic resin and oil was examined by three methods: (i) a modified “spot-on-the-lawn” method to screen the resin, (ii) a microtiter plate assay to determine the effect of mastic oil against *C. botulinum*, and (iii) an agar plate study to investigate the anti-botulinal potential of mastic volatiles.

### 2.3.1. Spot-on-the-lawn

To determine the anti-botulinal activity of mastic resin, a modified “spot-on-the-lawn” assay was used (Lyver et al., 1998). Solutions of mastic resin (2, 4, 6, 8 and 10% w/w) in 95% food grade ethanol were prepared and then filter-sterilized using a 0.45- $\mu$ m filter (Acrodisc; Gelman Scientific, Ann Arbor, MI, USA). Plates of modified McClung Toabe agar (Difco) were spread with 0.1 ml of individual spore suspensions of *C. botulinum* ( $\sim 1 \times 10^6$  spores/ml) and allowed to dry at ambient temperature in an anaerobic chamber. Plates were then spotted with 5- $\mu$ l aliquots of the filter-sterilized mastic solutions (2–10%) and a control solution of 95% ethanol (0% mastic). All plates were incubated in an atmosphere of 10% H<sub>2</sub>, 10% CO<sub>2</sub>, and 80% N<sub>2</sub> at 37 °C for 3 days and observed for zones of inhibition of *C. botulinum*, that were scored qualitatively as complete, partial, or no inhibition.

### 2.3.2. Microtiter assay

To determine the inhibitory effect of oil-of-mastic alone on the growth of *C. botulinum*, a microtiter assay was used. Overnight cultures of individual strains of *C. botulinum*, prepared as described previously, were added to 10 ml of sloppy IST agar (Difco) supplemented with 0.15% Bacto-agar (Difco), pre-heated to 37 °C to provide final inocula of  $10^3$  cells/ml. The addition of agar was necessary to prevent separation of the mastic oil and the IST broth (Mann and Markham, 1998).

Mastic oil (0.3% v/v) was added to sloppy IST agar and 40- $\mu$ l portions of this mixture were dispensed into the first rows of microtiter plates. The mastic oil in the first row was subsequently serially diluted (1:2) in the plate using sloppy IST agar resulting in a final volume of 20  $\mu$ l IST agar per well. Spore inocula (180  $\mu$ l) were dispensed into appropriate wells to give final concentrations of mastic oil ranging from 0.3% to 0.008% (v/v). Positive and negative controls consisted of inoculated (containing 0% mastic oil) and uninoculated (containing 0.3% to 0.008% mastic oil) sloppy IST agar, respectively. Well contents were mixed seven times using a multi-channel pipeter. Plates were covered with sterile aluminum foil and incubated in an atmosphere of 10% H<sub>2</sub>, 10% CO<sub>2</sub>, and 80% N<sub>2</sub> at 37 °C for 48 h prior to reading absorbance at 450 nm using a microplate reader (model EL  $\times$  800 running

KC-Jr. software, Bio-tek Instruments; Summit, NJ, USA). The anti-botulinal activity of mastic was expressed as the inhibition index (I.I.) shown below:

$$\text{I.I.} = 1 - \frac{\Delta \text{ the experimental culture}}{\Delta \text{ the control culture}}$$

where  $\Delta$  is the change in optical density at 450 nm, and the experimental and control cultures were individual strains of *C. botulinum* grown in IST broth, with and without mastic oil, respectively. The I.I. ranged from 0 (no inhibition) to 1 (complete inhibition) as described by Chaibi et al. (1997).

### 2.4. Vapor-phase inhibition of *C. botulinum* in agar plates

The potential of mastic volatiles to inhibit the growth of *C. botulinum* on agar plates was investigated. Mastic resin solutions were prepared by dispersing powdered resin (1.9% w/w) in 95% ethanol (6.8% w/w) and distilled water (balance). This mixture was then diluted (1:10) in distilled water and both dispersions were filter sterilized using 0.45- $\mu$ m filters (Acrodisc, Gelman Scientific, Ann Arbor, MI, USA) to obtain clear solutions. Sterile cotton pads were placed inside the lids of Petri dishes and 1.5 ml of mastic solutions were dispensed onto pads to give 0.1% or 0.01% (w/w) mastic resin per agar plate (20 g). Control pads, containing equivalent amounts of ethanol without mastic, were prepared in a similar manner.

Plates of MMT agar were spread in triplicate with 0.1 ml of appropriate dilutions of a composite inoculum of *C. botulinum* to give a final inoculum of  $3 \times 10^1$ ,  $3 \times 10^2$  or  $3 \times 10^3$  spores/plate. The inoculated plates were immediately inverted over the lids and then placed (1 plate per bag) in high gas barrier bags (KM 542, 1.5 mil gauge, Oxygen Transmission Rate [OTR] 3–6 cm<sup>3</sup>/m<sup>2</sup>/atm/day, at 4.4 °C, 0% r.h., Cryovac Liquid Sealed Air, Mississauga, ON, Canada). An AgelessFX<sup>®</sup><sub>200</sub> oxygen absorbent (Mitsubishi Gas and Chemical, Tokyo, Japan) was taped to the inside of each bag to create an anaerobic environment and bags were then sealed with an impulse heat-sealer. Plates were incubated for three days at 37 °C and the effect of mastic on the inhibition of growth of *C. botulinum*, relative to the control, was determined as  $\log(N_0/N)$  where  $N_0$  and  $N$  were the counts (CFU/g) of

*C. botulinum* on plates without and with mastic resin, respectively.

### 2.5. Transmission electron microscopy (TEM)

Cells were prepared for TEM according to the method of Austin et al. (1990). Cells of *C. botulinum*, that had been grown anaerobically in trypticase peptone glucose yeast (TPGY, Difco) containing 0%, 0.15% or 0.3% mastic oil for 24 h at 37 °C, were fixed in 0.2-M cacodylate buffer, pH 7.4, containing 2.5% (v/v) glutaraldehyde and post-fixed in 0.2-M cacodylate buffer, pH 7.4, containing 1% osmium tetroxide. Cells were enrobed in 1% Noble agar and dehydrated through a graded series of ethanol solutions (15 min each in 50%, 70% and 90% ethanol, followed by three 20-min incubations in 100% ethanol). Samples were then infiltrated and embedded in Taab 812 resin (Marivac, Halifax, NS, Canada). Thin sections were cut on a Reichert-Jung Ultracut E ultramicrotome (C. Reichert Ag, Wien, Austria) and stained with uranyl acetate and lead citrate (Reynolds, 1963). Thin sections were examined in a Zeiss EM902 transmission electron microscope (Carl Zeiss, Thornwood, NY, USA) operating at 80 kV with the energy loss spectrometer in place.

### 2.6. Challenge studies with English-style crumpets

English-style crumpet batter was prepared, inoculated and baked as described by Daifas et al. (1999b). Appropriate amounts of mastic oil or powdered mastic resin were added to batches of crumpet batter (625 ml) to give baked crumpets with final concentrations of oil and resin ranging from 0.1% to 0.5% (v/w) and 1% to 3% (w/w), respectively. To determine if baking had any effect on mastic volatiles, similar amounts of mastic oil were also applied to the surfaces of another batch of crumpets post-baking. All crumpet batter was inoculated with a composite inoculum ( $3 \times 10^5$  spores/ml) of proteolytic *C. botulinum*, prior to baking on a griddle to give crumpets (50 g) with a final inoculum level of  $\sim 5 \times 10^2$  spores/g (Daifas et al., 1999a,b). Control crumpets (without mastic oil or resin) were inoculated in a similar manner with either *C. botulinum* or 0.1% (w/w) peptone water. Crumpets were packaged, in duplicate, in high gas barrier bags with an Agleless® FX<sub>200</sub> oxygen absorbent inside each bag. Bags were then sealed with an impulse heat-sealer and stored at 25 °C.

### 2.7. Detection of neurotoxin

Crumpets were analyzed for botulinum neurotoxin using the mouse bioassay at one-week intervals until neurotoxin was detected. On each sampling day, crumpets were mixed with 0.1% peptone water (1:3), stomached, centrifuged and filter-sterilized as described previously (Daifas et al., 1999b). Each filtrate (0.5 ml) was injected intraperitoneally into each of two 20- to 28-g mice (Charles River Laboratories, Quebec City, QC, Canada). Mice were observed for up to 72 h for typical signs of botulism including ruffled fur, pinched waist, labored breathing, limb paresis and general paralysis. Mice showing severe distress were euthanised by asphyxiation with CO<sub>2</sub> according to Health Canada Animal Care Committee guidelines. Neutralization of neurotoxin was performed on randomly selected representative positive samples using antisera to botulinum neurotoxins (Connaught Laboratories, North York, ON, Canada) to confirm that toxicity was due to *C. botulinum* as described previously (Daifas et al., 1999b).

## 3. Results and discussion

### 3.1. Preliminary screening

The effects of mastic resin solutions (0–10% [w/w]) in 95% ethanol against five strains of proteolytic *C. botulinum* are shown in Table 1. While the

Table 1  
Effect of mastic resin on *C. botulinum*

<i>C. botulinum</i>	% (w/w) Mastic <sup>a</sup>										
	0	2		4		6		8		10	
		I <sup>b</sup>	O <sup>c</sup>	I	O	I	O	I	O	I	O
A6	–	±	+	±	+	±	+	±	+	±	+
62A	–	±	+	±	+	±	+	±	+	±	+
17A	–	+	+	+	+	+	+	+	+	+	+
13983IIB	–	±	±	±	±	±	±	±	±	±	±
368B	–	±	±	±	±	±	±	±	±	±	±

–, no inhibition; ±, partial inhibition; +, complete inhibition.

<sup>a</sup> Mastic resin in 95% ethanol.

<sup>b</sup> I (inner) concentric circle into which the mastic solution separated when applied to the agar.

<sup>c</sup> O (outer) concentric circle into which the mastic solution separated when applied to the agar.

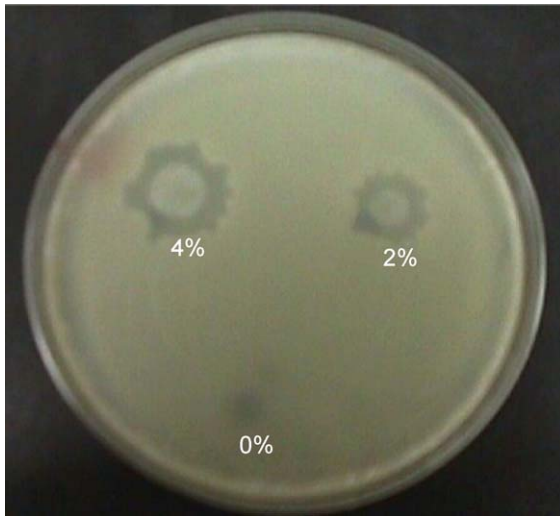


Fig. 1. Inhibitory effect of 0–4% (w/w) mastic in ethanol on *C. botulinum* 62A.

mastic solutions separated into two distinct, concentric phases in MMT agar plates—the outer of which had an iridescent, oil-like sheen (Fig. 1)—no such separation occurred with the control solution (0% mastic in ethanol). Inhibition of *C. botulinum* was consistently greater in the outer oil-like phase (Table 1) while partial inhibition was observed for most strains in the inner oil phase, with the exception of *C. botulinum* 17A, which was completely inhibited in both phases of the mastic resin solutions (Fig. 1). Most strains of *C. botulinum* type A were inhibited by  $\geq 2\%$  mastic resin, but higher ( $\geq 8\%$ ) concentrations of mastic resin were required for the complete inhibition of *C. botulinum* type B strains (Table 1). These results are comparable with the observations of De Wit et al. (1979) who reported that while garlic and onion oils partially inhibited *C. botulinum* type A strains, they had no effect on types B and E.

The major components of resinous mastic include 1% to 3% essential oil, 4%  $\alpha$ - and  $\beta$ -masticinic acid ( $C_{23}H_{36}O_4$ ), 0.5% masticolic acid ( $C_{23}H_{36}O_4$ ), 20%  $\alpha$ -masticonic acid ( $C_{23}H_{48}O_4$ ), 18%  $\beta$ -masticonic acid ( $C_{32}H_{48}O_4$ ), 30%  $\alpha$ -mastichorezene ( $C_{35}H_{56}O_4$ ) and 20%  $\beta$ -mastichorezene ( $C_{35}H_{56}O_4$ ) (Papageorgiou et al., 1997). The essential oil is a complex mixture of at least 70 compounds, primarily the monoterpenes myrcene (39%),  $\alpha$ -pinene

(28%), limonene (11%),  $\beta$ -pinene (5.4%) and  $\beta$ -caryophyllene (2.4%) with 7.3% aliphatic esters and ketones and 1.3% phenolic derivatives (Wyllie et al., 1990). Since no emulsifying agent was added to the filtered mastic-in-ethanol solutions, it is possible that phase separation occurred and that compounds with greater inhibitory activity were associated with the outer oil-like phase. However, since *C. botulinum* 17A was completely inhibited in both phases, it is clear that there are strain differences in the sensitivity of *C. botulinum* to mastic resin.

No inhibition was observed with 0% mastic (Table 1) indicating that mastic resin in ethanol had a greater inhibitory effect on proteolytic strains of *C. botulinum* than ethanol alone.

### 3.2. Effect of mastic oil on growth of *C. botulinum*

The effect of mastic oil alone on the growth of *C. botulinum* as shown by the inhibitory index (I.I.) in the microtiter assay is summarized in Table 2. The I.I., which reflects the relative change in optical density between cultures grown with and without mastic oil, ranges from 0 for no inhibition, to 1 for complete inhibition (Chaibi et al., 1997). While no inhibition was observed at  $<0.2\%$  mastic oil, strains of *C. botulinum* were partially inhibited by 0.2% mastic oil. However, the inhibitory indices at this level of oil were low, i.e.,  $<0.5$ . Greater inhibition of *C. botulinum* was only observed at levels of mastic oil of 0.3%. At this concentration of mastic oil, higher I.I.s were consistently observed for *C. botu-*

Table 2  
Inhibitory index<sup>a</sup> of mastic oil on strains of proteolytic *C. botulinum*

Strain of <i>C. botulinum</i>	% (v/v) mastic oil			
	0.30	0.20	0.10	0.01
62A	0.84	0.46	0	0
A6	0.83	0.25	0	0
CK2	0.78	0.33	0	0
17A	0.63	0.05	0	0
13983IIB	0.91	0.02	0	0
368B	0.63	0.10	0	0
MRB	0.50	0.24	0	0

<sup>a</sup> The inhibitory index is given as 1 minus the relative change in optical density (450 nm) between *C. botulinum* cultures grown with and without mastic.

*linum* type A strains than for type B strains, with the exception of *C. botulinum* 13983IIB, which had the highest I.I. of 0.91. These results are consistent with the “spot-on-the-lawn” assay and confirm a strain-specific inhibition by mastic resin and its essential oil.

Chaibi et al. (1997) reported inhibition of *C. botulinum* 62A by the essential oils of eucalyptus, chamomile, cedar, savage carrots, artemisia, grapefruit, vervain, and orange, while Ismaiel and Pierson (1990) reported inhibition of *C. botulinum* 67B with clove, thyme, cinnamon, pimento and origanum essential oils. However, in both these studies, low concentrations (0.01–0.04%) of these oils inhibited the strains of *C. botulinum* tested in this study similarly to 0.3% mastic oil. This can be attributed to differences in the profiles of the antimicrobial components of the other oils as compared to mastic oil.

### 3.3. Effect of mastic volatiles on *C. botulinum*

The inhibitory effect of mastic resin in ethanol applied directly to cotton pads on the growth of *C. botulinum* in MMT agar plates is shown in Fig. 2.

Low levels of ethanol had little to no inhibitory effect on the growth of *C. botulinum per se*. However, when mastic resin was added to ethanol, a 0.5–1 log reduction in the growth of *C. botulinum* was observed. When higher concentrations of mastic resin and ethanol were applied to pads, a 1.5- to 1.8-log reduction in counts of *C. botulinum* was observed. There also appeared to be an effect of inoculum size on the inhibitory effects of the mastic resin-ethanol solutions. This is indicated by the greater inhibition of growth obtained with an inoculum of 30 spores/plate compared to higher inoculum levels of 300 and 3000 spores/plate. Furthermore, since no inhibition of growth of *C. botulinum* was observed with control ethanol solutions, the observed inhibition of *C. botulinum* in the mastic resin-ethanol solutions can be attributed to the effect of mastic volatiles.

Volatile constituents of other oils (horseradish, wasabi, wormwood, garlic and ginger) have been shown to inhibit bacteria including *Bacillus subtilis* (Inouye et al., 1983; Yun et al., 1993; Delaquis et al., 1999). This study has also shown that lower levels of mastic resin in the vapor state were required to inhibit *C. botulinum* as compared with the amounts required when resin was directly added to media. One possible

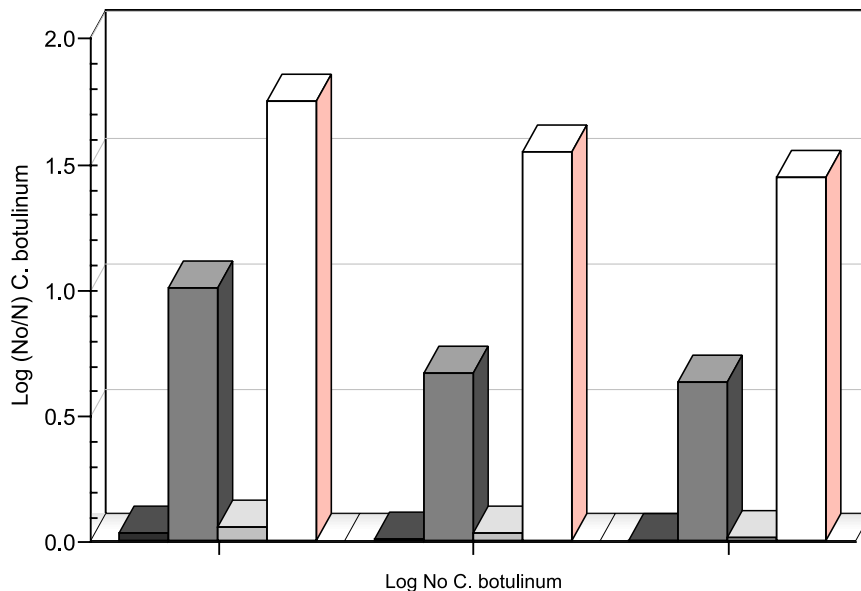


Fig. 2. Effect of volatiles of 0.01% mastic with 0.05% ethanol (■); 0.1% mastic with 0.5% ethanol (□); 0.05% ethanol (■); and 0.5% ethanol (■) [w/w] on a composite inoculum of proteolytic *C. botulinum* ( $3 \times 10^1$ ,  $3 \times 10^2$ , or  $3 \times 10^3$  spores/plate) grown on McClung Toabe agar at 37 °C.

reason for this observation may be a higher concentration of anti-botulinal mastic volatiles in the vapor phase. Another possible reason may be the conversion of volatile terpene alcohols, such as limonene and pinene, to more inhibitory, vapor-phase compounds (Megalla et al., 1990). A similar effect has been reported by Ahn et al. (1999). While these authors observed that vaporized isothiocyanates (ITCs) inhibited several microorganisms, including *B. subtilis* grown on agar, their antimicrobial activity was increased two- to ten-fold when acetic acid was combined with the ITCs. Whatever the reason, this initial study clearly indicates the potential of mastic resin as a vapor phase inhibitor of the growth of proteolytic strains of *C. botulinum*.

#### 3.4. Effect of mastic on cells of *C. botulinum*

The effect of mastic oil on vegetative cells of *C. botulinum* 62A is shown in Fig. 3. Micrographs showed that growth and cell division were evident in control cultures grown without mastic oil. Although spores were evident in control cultures (Fig. 3A), no spores were observed in TPGY cultures containing 0.15% or 0.30% mastic oil. Furthermore, structural changes were observed in all cells at these levels of mastic oil. In broths containing 0.15% mastic oil, areas of homogeneous, but granular-appearing inclusions, which lacked evidence of ribosomes, were noticeable (Fig. 3B). These effects were even more

pronounced in cells grown in TPGY broth containing 0.3% mastic oil in which cells had large, straight inclusions (Fig. 3C).

Huwez et al. (1998) reported that mastic oil induced ultrastructural changes in *Helicobacter pylori*, but these changes were not described. Although the antimicrobial action of essential oils has been established, their mechanism of action is unknown. Conner and Beuchat (1984a,b) suggested that essential oils might impair enzyme systems, such as those involved in energy production and structural component synthesis. Ultee et al. (1999) showed that carvacol, the major constituent of oregano oil, altered the permeability of the cell membranes of *B. cereus* to cations, resulting in impairment of essential cell processes and, finally, cell death. At non-lethal concentrations, *B. cereus* adapted to carvacol by changing its fatty acid composition (Ultee et al., 2000). While it is not clear how mastic oil inhibits the growth of *C. botulinum*, it is evident that changes occur in its cell structure particularly when grown in TGYB broth containing higher levels of mastic oil.

#### 3.5. Challenge study

The effect of mastic oil or resin on the growth of *C. botulinum* in crumpets challenged with this pathogen, packaged anaerobically and stored at ambient temperature, is summarized in Table 3. This storage temperature was selected since crumpets are stored at

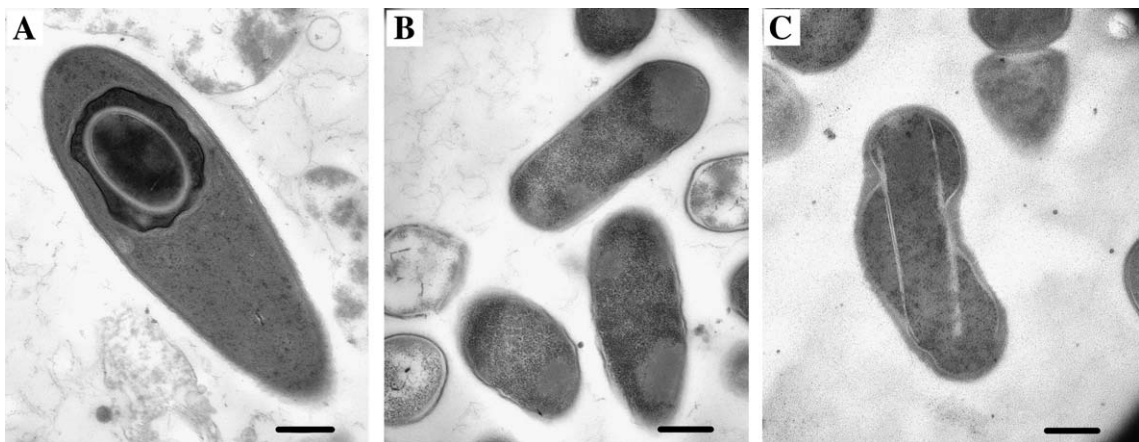


Fig. 3. Effect of (A) 0%, (B) 0.15% and (C) 0.30% (v/w) mastic oil on vegetative cells of *C. botulinum* 62A in trypticase peptone glucose yeast broth at 36 °C.

ambient temperature in retail stores. Botulinum neurotoxin was detected after 7 days in all inoculated crumpets, regardless of the level of mastic oil or resin used. While some mastic volatiles may have been lost during baking, the effect of heating on mastic resin or oil cannot be determined from this study. However, the results with mastic oil would indicate that baking may have had a negligible effect on its anti-botulinal efficacy since botulinum neurotoxin was detected at the same time regardless of whether mastic oil was added before or after baking.

No neurotoxin was detected in any control crumpets. While the spore inoculum was added to crumpet batter before baking, previous studies have established that such inoculation results in almost complete spore survival (Daifas et al., 1999b). Therefore, while mastic resin exhibited an anti-botulinal effect in media, it failed to prevent the growth of *C. botulinum* in crumpets, even when used at higher levels than *in vitro*. Similar results have been observed with other antimicrobial oils. While clove and oregano oils inhibited the growth of *Listeria monocytogenes* in media, they failed to inhibit this pathogen when added at similar levels to meat (Ting and Deibel, 1992; Aureli et al., 1992).

The reason for this lack of inhibition of *C. botulinum* by mastic resin and its essential oil in crumpets is not apparent. Food is a complex substrate and interactions between antimicrobials and food macromolecules, particularly proteins and fat, may reduce the activity of the antimicrobial (Shelef, 1983). Indeed, activity can be affected even by the composition of growth media (Blank et al., 1987).

Table 3

Numbers of samples testing positive for botulinum neurotoxin/number of samples tested in crumpets containing mastic oil or resin added before or after baking, inoculated with 500 spores/g *C. botulinum*, packaged with an oxygen absorbent and stored at 25 °C, after 7 days storage

Mastic addition	Mastic oil % (v/w)						Mastic resin % (w/w)			
	0	0.1	0.2	0.3	0.4	0.5	0	1	2	3
Before Baking	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
After Baking	2/2	2/2	2/2	2/2	2/2	2/2	NA <sup>a</sup>	NA	NA	NA

<sup>a</sup> NA, not applicable.

Although the lipid content of crumpets is low, flour and skim milk powder are rich sources of protein. Cornell et al. (1971) reported that the activity of butylated hydroxyanisole (BHA) was decreased when it bound reversibly to milk powder. Another possibility is that a potentiating agent, such as a chelator, may be needed to facilitate the entry of antimicrobials into the cell, thereby enhancing their activity (Kabara, 1991). Yet another possibility could be the loss of mastic volatiles through the high gas barrier bags. Although previous agar plate studies clearly showed that mastic volatiles had the potential to inhibit growth of *C. botulinum*, the interactive pads were placed directly inside the lids of Petri dishes which were then packaged in high gas barrier bags. While ethanol vapor per se has been shown to have a negligible effect on film permeability (Koukoutsis, 2002), little is known about the plasticizing effect of mastic volatiles on film permeability when the interactive mastic resin-ethanol sachets are placed directly inside bags and in close contact with the packaging film.

While higher levels of mastic may be necessary for the inhibition of *C. botulinum* in crumpets, such levels may lead to sensory rejection. At levels of  $\leq 0.3\%$  oil and 1% resin, crumpets had a pleasant, characteristic odor. However, formulation with higher levels of mastic may impart an objectionable odor. Although the sensory quality of crumpets was not quantitatively assessed in this study, it was obvious that crumpets formulated with mastic resin had a superior texture throughout storage than crumpets without mastic resin, indicating its potential as an anti-staling agent. The sensory quality of crumpets may also have been enhanced by the antimicrobial activity of mastic against spoilage microorganisms, specifically *Bacillus* spp., yeasts, molds, and lactic acid bacteria. Inhibition of spoilage microorganisms by mastic resin and its essential oil has been previously reported (Alippi et al., 1996; luak et al., 1996; Koukoutsis, 2002).

#### 4. Conclusion

This preliminary study has clearly shown the potential of mastic resin and its essential oil as a novel anti-botulinal agent in media. Since mastic is

used as a flavoring agent, it could have the additional advantage of being a “natural” preservative in bakery products. However, further studies are required to enhance the activity of both mastic resin and its essential oil against *C. botulinum* in bakery products. While the anti-botulinal activity of mastic appears to be greater in the vapor state in media studies, further studies are warranted to investigate this mode of application in packaged crumpets. Further studies are also required to determine the effect of baking and changes in packaging film permeability during storage on the antimicrobial efficacy of mastic oil volatiles.

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### References

- Abdel-Rahman, A.-H.Y., Soud, A.M.Y., 1975. Mastich as an antioxidant. *Journal of the American Oil Chemists' Society* 52, 423.
- Ahn, E.-S., Kim, J.-H., Shin, D.-H., 1999. Antimicrobial effects of allyl isothiocyanates on several microorganisms. *Korean Journal of Food Science and Technology* 31, 206–211.
- Alippi, A.M., Ringuet, J.A., Cerimele, E.L., Re, M.S., Henning, C.P., 1996. Antimicrobial activity of some essential oils against *Paenibacillus* larvae, the causal agent of American foulbrood disease. *Journal of Herbs, Spices, and Medicinal Plants* 4, 9–16.
- Ali-Shtayah, M.S., Yaghmour, R.M.-R., Faidi, Y.R., Salem, K., Al-Nuri, M.A., 1998. Antimicrobial activity of 20 plants used in folkloric medicine in the Palestinian area. *Journal of Ethnopharmacology* 60, 265–271.
- Aureli, P., Constantini, A., Zolea, S., 1992. Antimicrobial activity of some plant essential oils against *Listeria monocytogenes*. *Journal of Food Protection* 55, 344–348.
- Austin, J.W., Stewart, M., Murray, R.G., 1990. Structural and chemical characterization of the S layer of a *Pseudomonas*-like bacterium. *Journal of Bacteriology* 172, 808–817.
- Blank, G., Al-Khayat, M., Ismond, M.A.H., 1987. Germination and the resistance of *Bacillus subtilis* spores produced on clove and eugenol based media. *Food Microbiology* 4, 35–42.
- Chaibi, A., Ababouch, L.H., Belasri, K., Boucetta, S., Busta, F.F., 1997. Inhibition of germination and vegetative growth of *Bacillus cereus* T and *Clostridium botulinum* 62A spores by essential oils. *Food Microbiology* 14, 161–174.
- Conner, D.E., Beuchat, L.R., 1984a. Sensitivity of heat-stressed yeasts to essential oils of plants. *Applied and Environmental Microbiology* 47, 229–233.
- Conner, D.E., Beuchat, L.R., 1984b. Effects of essential oils from plants on growth of food spoilage yeasts. *Journal of Food Science* 49, 429–434.
- Cornell, D.G., Devilbiss, E.D., Pallansch, M.J., 1971. Binding of antioxidants by milk proteins. *Journal of Dairy Science* 54, 634–639.
- Daifas, D.P., Smith, J.P., Blanchfield, B., Austin, J.W., 1999a. Challenge studies with *Clostridium botulinum* in high moisture bakery products packaged under modified atmospheres. *Food Australia* 51, 507–511.
- Daifas, D.P., Smith, J.P., Blanchfield, B., Austin, J.W., 1999b. Growth and toxin production by *Clostridium botulinum* in English-style crumpets packaged under modified atmospheres. *Journal of Food Protection* 62, 349–355.
- Daifas, D.P., Smith, J.P., Tarte, I., Blanchfield, B., Austin, J.W., 2000. Effect of ethanol vapor on growth and toxin production by *Clostridium botulinum* in a high moisture bakery product. *Journal of Food Science* 20, 113–127.
- Daifas, D.P., Smith, J.P., Blanchfield, B., Cadieux, B., Sanders, G., Austin, J.W., 2003. Effect of ethanol on the growth of *Clostridium botulinum*. *Journal of Food Protection* 66, 610–617.
- Delaquis, P.J., Ward, S.M., Holley, R.A., Cliff, M.C., Mazza, G., 1999. Microbiological, chemical and sensory properties of pre-cooked roast beef preserved with horseradish essential oil. *Journal of Food Science* 64, 519–524.
- De Wit, J.C., Notermans, S., Gorin, N., Kampelmacher, E.H., 1979. Effect of garlic oil or onion oil on toxin production by *Clostridium botulinum* in meat slurry. *Journal of Food Protection* 42, 222–224.
- Hauschild, A.H.W., Hilsheimer, R., 1977. Enumeration of *Clostridium botulinum* spores in meat by a pour-plate procedure. *Canadian Journal of Microbiology* 23, 829–832.
- Hussain, H., Tabji, R.S., 1997. Antibacterial screening of some Libyan medicinal plants. *Fitoterapia* 68, 467–470.
- Huwez, F.U., Thirlwell, D., Cockayne, A., Ala'Aldeen, D.A.A., 1998. Mastic gum kills *Helicobacter pylori*. *New England Journal of Medicine* 339, 1946.
- Inouye, S., Goi, H., Miyauchi, K., Muraki, S., Ogihara, M., Iwanami, Y., 1983. Inhibitory effect of volatile constituents of plants on the proliferation of bacteria—antibacterial activity of plant volatiles. *Journal of Antibacterial and Antifungal Agents* 11, 609–615.
- Ismail, A.A., Pierson, M.D., 1990. Inhibition of germination, outgrowth, and vegetative growth of *Clostridium botulinum* 67B by spice oils. *Journal of Food Protection* 53, 755–758.
- Iuak, L., Ragusa, S., Rapisarda, A., Franco, S., Nicolosi, V.M., 1996. *In vitro* antimicrobial activity of *Pistacia lentiscus* L. extracts: preliminary report. *Journal of Chemotherapy* 8, 207–209.
- Kabara, J.J., 1991. Phenols and chelators. In: Russell, N.J., Gould, G.W. (Eds.), *Food Preservatives*. Blackie, Glasgow, pp. 200–214.

- Koukoutsis, J., 2002. Novel methods to control growth of *Bacillus cereus* in English-style crumpets. MSc Thesis, McGill University, Montreal, QC.
- Lyver, A., Smith, J.P., Nattress, F.M., Austin, J.W., Blanchfield, B., 1998. Challenge studies with *Clostridium botulinum* type E in a value-added surimi product stored under a modified atmosphere. *Journal of Food Safety* 18, 1–23.
- Magiatis, P., Mellious, E., Skaltsounis, A.-L., Chinou, I.B., Mitaku, S., 1999. Chemical composition and antimicrobial activity of the essential oils of *Pistacia lentiscus* var. *chia*. *Planta Medica* 65, 749–752.
- Mann, C.M., Markham, J.L., 1998. A new method for determining the minimum inhibitory concentration of essential oils. *Journal of Applied Microbiology* 84, 538–544.
- Megalla, S.E., Tel-Keltawi, N.E.M., Ross, S.A., 1990. A study of antimicrobial action of some essential oil constituents. *Herba Polonica* 26, 181–186.
- Papageorgiou, V.P., Bakola-Christianopoulou, N.M.B., Apazidou, K.K., Psarros, E.E., 1997. Gas chromatographic-mass spectroscopic analysis of the acidic triterpenic fraction of mastic gum. *Journal of Chromatography* 769, 263–273.
- Reynolds, E.S., 1963. The use of lead citrate as high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* 17, 208–212.
- Shelef, L.A., 1983. Anti-microbial effects of spices. *Journal of Food Safety* 6, 29–44.
- Tassou, C.C., Nychas, G.J.E., 1995. Antimicrobial activity of the essential oil of mastic gum (*Pistacia lentiscus* var. *chia*) on gram positive and gram negative bacteria in broth and in model food system. *International Journal of Biodeterioration and Biodegradation* 36, 411–420.
- Ting, E.W.T., Deibel, K.E., 1992. Sensitivity of *Listeria monocytogenes* to spices at two temperatures. *Journal of Food Safety* 12, 129–137.
- Ultee, A., Kets, E.P., Smid, E.J., 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Applied and Environmental Microbiology* 65, 4606–4610.
- Ultee, A., Kets, E.P., Alberda, M., Hoekstra, F.A., Smid, E.J., 2000. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. *Archives of Microbiology* 174, 233–238.
- Wyllie, S.G., Brophy, J.J., Sarafis, V., Hobbs, M., 1990. Volatile components of the fruit of *Pistacia lentiscus*. *Journal of Food Science* 55, 1325–1326.
- Yun, K.W., Kil, B.S., Han, D.M., 1993. Phytotoxic and antimicrobial activity of volatile constituents of *Artemisia princeps* var. *orientalis*. *Journal of Chemistry and Ecology* 19, 2757–2766.