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SAFETY STUDIES WITH PROTEOLYTIC *CLOSTRIDIUM BOTULINUM* IN HIGH-MOISTURE BAKERY PRODUCTS PACKAGED UNDER MODIFIED ATMOSPHERES

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Suggested short title:

C. botulinum in bakery products

FOREWORD

This thesis is written in a manuscript-based format. The first chapter consists of a general introduction and literature review of the theory and background information relevant to this topic. The following seven chapters and appendices A and B, each a complete manuscript, form the body of the thesis. The final chapter provides a general conclusion and summary. This thesis format has been approved by the Faculty of Graduate Studies and Research of McGill University and follows the conditions outlined in the "Guidelines for Thesis Preparation", section C. "Manuscript-based thesis", which are: "1). Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis (Reprints of published papers can be included in the appendices at the end of the thesis); 2) The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory; 3) The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: (a) a table of contents; (b)

an abstract in English and French; (c) an introduction which clearly states the rationale and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary; 4) As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis; 5) In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled: "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors and the co-authors of the co-authored papers."

While the work described in this thesis is the sole responsibility of the candidate, the research project was supervised by Dr. James P. Smith of the Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University as principal supervisor and by Dr. John W. Austin of the Bureau of Microbial Hazards of the Food Directorate of the Health Products and Food Branch of Health Canada as co-supervisor.

ABSTRACT

Initial challenge studies with spores of proteolytic *Clostridium botulinum* types A and B (~10^4 spores/g) showed that while air- and gas-packaged English-style crumpets (a_w 0.990) and pizza crust (a_w 0.960) were toxic after 42-days storage at ambient temperature (25°C), no neurotoxin was detected in bagels (a_w 0.944). Further challenge studies with similarly packaged crumpets inoculated with *C. botulinum* (~10^2 spores/g), pre- or post-baking, demonstrated that all crumpets were toxic within 4 to 6 days at 25°C and that toxigenesis preceded spoilage. Furthermore, reformulating crumpets to pH 8.3 and packaging in 100% CO_2 had little effect in delaying the growth of *C. botulinum* compared to crumpets formulated to pH 6.5 and packaged in 60% CO_2.

Subsequent studies were directed at determining the levels of additional barriers that could be used to ensure the safety of high-moisture MAP crumpets. While ethanol vapour proved to be an effective additional barrier in crumpets (100-g, [a_w 0.990, pH 6.5]) challenged with ~10^2 spores/g of *C. botulinum*, spoilage preceded toxigenesis due to absorption of ethanol from the package headspace by crumpets. Modelling studies in Trypticase Peptone Glucose Yeast (TPGY) broth confirmed the anti-botulinal nature of ethanol and showed that a level of ~4% (vol/vol) could be used for complete inhibition of this pathogen, depending on the a_w and pH of the growth medium. However, while ethanol vapour could be used to inhibit the growth of *C. botulinum* in high-moisture

crumpets, its anti-botulinal efficacy was influenced by the method of crumpet leavening (yeast *v* chemical).

Preliminary studies were also done to assess the potential of mastic oil, a novel inhibitor, against *C. botulinum.* While direct and indirect application of ethanolic extracts of mastic oil inhibited the growth of *C. botulinum in vivo*, they failed to do so in crumpets.

RÉSUMÉ

Les études initiales portant sur la souche protéolytique de *Clostridium botulinum* de type A et B (~10^4 spores/g) ont démontré que contrairement aux crumpets de style anglais (a_w 0.990) et aux croûtes de pizza (a_w 0.960) emballées avec de l'air ou sous atmosphère controllée, qui étaient toxiques après 42 jours de rangement à température ambiante, aucune toxine n'était détectée dans les bagels (a_w 0.944). Des études subséquentes effectuées avec des crumpets pré- ou post-cuisson inoculés avec *C.botulinum* (~10^2 spores/g) emballés sous conditions similaires ont démontré que tous les crumpets devenaient toxiques en 4 à 6 jours à 25°C et que la toxigénèse précédait la déterioration. Qui plus est, la reformulation des crumpets pour obtenir un pH de 8.3 de même que leur emballage sous atmosphère 100% CO_2 eurent peu d'effet pour ce qui est de retarder la croissance de *C. botulinum* si on la compare avec les crumpets reformulés afin d'obtenir un pH de 6.5 et emballés sous atmosphère contenant 60% CO_2.

Des études subséquentes furent entamées afin de déterminer les niveaux de barrières additionnelles nécessaires afin d'assurer la consommation sécuritaire des crumpets emballés sous atmosphère controllée. Alors que la vapeur d'éthanol s'avèra être une barrière additionnelle de grande efficacité pour les crumpets (100-g, [a_w 0.990, pH 6.5]) soumis à une inoculation aux environs de 10^2 spores/g de *C. botulinum*, la déterioration précéda la toxigénèse à cause de l'absorption de l'éthanol présent dans l'espace d'emballage résiduel

par les crumpets. Des études modelisées dans du bouillon de Levure de Glucose Tryptique Peptoné (TPGY) ont confirmé la nature anti-botulinale de l'éthanol et démontré qu'un niveau d'environ 4% (vol/vol) pourrait être utilisé pour obtenir une inhibition complète de ce pathogène, tout dépendamment de l'a_w et du pH du milieu de croissance. Cependant, alors que la vapeur d'éthanol pourrait être mise à profit pour inhiber la croissance de *C. botulinum* dans les crumpets à haut niveau d'humidité, son efficacité anti-botulinale se trouve influencée par la méthode utilisée pour faire lever les crumpets (levure *v* chimique).

Des études préliminaires furent aussi faites pour évaluer le potentiel de l'huile de mastic, un nouvel inhibiteur, envers *C. botulinum*. Alors que l'application directe et indirecte d'extraits éthanoliques d'huile de mastic résulta en l'inhibition de la croissance de *C. botulinum in vivo*, elle échoua à obtenir le même résultat avec les crumpets.

ACKNOWLEDGEMENTS

Although I was solely responsible for the work presented in this thesis, this project was greatly facilitated by the tremendous physical, emotional and financial support which was generously offered to me by many–for which I am sincerely grateful.

Drs. Jim Smith and John Austin, as co-supervisors, have given endless support and encouragement, and have provided constant direction and insight throughout this study. I thank them both for their kind patience, humour and friendship, in addition to providing a supportive atmosphere that greatly facilitated learning and allowed this project to develop and unfold.

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Finally, it cannot be overstated that this work would not have been possible without the unquestionable love of all those with whom I am fortunate to share my life. They have nurtured in me a desire to learn, believed in me, and unselfeshly have allowed me the freedom to pursue these studies. I return their love.

CONTRIBUTIONS OF AUTHORS OF MANUSCRIPTS AND PUBLICATIONS RESULTING FROM THIS WORK

1. Daifas, D. P., J. P. Smith, B. Blanchfield, B. Cadieux, G. Sanders, and J. W. Austin. 2003. Challenge studies with proteolytic *Clostridium botulinum* in yeast- and chemical-leavened crumpets packaged under modified atmospheres. J. Food Safety. (submitted for publication).

2. Daifas, D. P., J. P. Smith, B. Blanchfield, B. Cadieux, G. Sanders, and J. W. Austin. 2003. Effect of ethanol on the growth of *Clostridium botulinum*. J. Food Prot. 66(3) (In press).

3. Smith, J. P., D. P. Daifas, W. El-Khoury, A. A. El-Khoury, and J. Koukoutsis. 2003. Shelf life and safety concerns of minimally processed bakery products. Crit. Rev. Food Sci. Nutrit. (In press).

4. Smith, J. P., D. P. Daifas, and W. El-Khoury. 2003. Are minimally processed, high moisture bakery products packaged under modified atmospheres a botulism risk? International Food Information Services, Food Science Central, *FoodInfo Online Features* 6 January http://www.foodsciencecentral.com/library.html#ifis/11610.

5. Smith, J. P., D. P. Daifas, W. El-Khoury, and J. W. Austin. 2002. Microbial safety of bakery products. pp. 3-33. *In* J. Novak, G. M. Sapers, and V. K. Junega (eds.). Safety of minimally processed foods. Technomic Publishing Co. Inc., Lancaster, PA.

6. Smith, J. P., D. P. Daifas, W. El-Khoury, and J. Koutkousis. 2002. Food borne illnesses associated with bakery products. American Institute of Baking. Volume XXIV(4).

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8. Daifas, D. P., J. P. Smith, B. Blanchfield, and J. W. Austin. 1999. Effect of pH and CO_2 on growth of *Clostridium botulinum* in English-style crumpets packaged under modified atmosphere. J. Food Prot. 62:1157-1161.

9. Daifas, D. P., J. P. Smith, B. Blanchfield, and J. W. Austin. 1999. Challenge studies with *Clostridium botulinum* in bakery products packaged under modified atmospheres. Food Austral. 51:507-511.

10. Daifas, D. P., J. P. Smith, B. Blanchfield, and J. W. Austin. 1999. Growth and neurotoxin production by *Clostridium botulinum* in English-style crumpets packaged under modified atmospheres. J. Food Prot. 62:349-355.

The candidate, Daphne Phillips (Daifas), was primarily responsible for conducting all the research reported in these co-authored papers. However, Drs. Jim P. Smith and John W. Austin approved the research protocols and between them, edited all of the manuscripts. Burke Blanchfield assisted with preparation of spores of *C. botulinum* and sample inoculation, Brigitte Cadieux assisted in the preparation of growth curves while Greg Sanders processed micrographs of *C. botulinum*. All three of these co-authors also helped with the testing of samples for botulinum neurotoxin using the mouse bioassay. In addition,

Ilsemarie Tarte assisted with sample inoculation and the measuring of headspace ethanol. Finally, Jim Smith, Anis El-Khoury, John Koukoutsis, and Wassim El-Khoury contributed significantly to the review papers with sections relevant to their research areas (*B. cereus* and shelf-life extension of bakery products).

CLAIM OF ORIGINAL RESEARCH

This is the first comprehensive study that :

1. Demonstrates the potential of high-moisture, MAP bakery products to support the growth of and neurotoxin production by proteolytic *C. botulinum* and justifies the need for additional barriers to enhance the safety of such packaged bakery products.

2. Models the combined effect of ethanol concentration, a_w and pH on the probability of growth and neurotoxin production by proteolytic *C. botulinum* in broth studies and demonstrates that the inhibitory effect of ethanol is reversible.

3. Demonstrates the effectiveness of ethanol vapour generating sachets (Ethicap®) as potential additional barriers to the growth of and neurotoxin production by proteolytic *C. botulinum* in high-moisture crumpets and shows that the anti-botulinal efficacy of ethanol vapour is dependent on the method of crumpet leavening.

4. Demonstrates the strain-specific activity of mastic oil against proteolytic *C. botulinum* in media studies and the potential of mastic oil in combination with ethanol as vapour phase inhibitors against this pathogen.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CONTENTS OF APPENDICES

LIST OF TABLES IN APPENDICES

LIST OF FIGURES IN APPENDICES

ABBREVIATIONS USED

a_w	water activity
A	absorbance
ACMSF	Advisory Committee for the Microbiological Safety of Food
ANOVA	analysis of variance
AOAC	American Organization of Analytical Chemists
BoNT	botulinum neurotoxin
BSM	botulinum selective medium
CBI	*Clostridium botulinum* isolation
CDC	Centers for Disease Control
CFU	colony forming units
CMGA	Chios Mastic Grower's Association
D	decimal reduction time (minutes)
ELCA	enzyme-linked coagulation assay
ELISA	enzyme-linked immunosorbant assay
ERH	equilibrium relative humidity
F_0	total lethality
FSIS	Food Safety and Inspection Service
GMP	good manufacturing processes
GRAS	generally regarded as safe
HAACP	hazards analysis and critical control points
ICMSF	International Commission for the Microbiological Specification of Food
I.I.	inhibitory index
i.p.	intraperitoneal
IST	Iso-sensitest
LAB	lactic acid bacteria

MYPD	malt yeast peptone dextrose
MAP	modified atmosphere packaging (packaged)
MLD_{50}	minimum lethal dose-50%
MMT	modified McClung Toabe
NCYC	National Collection of Yeast Cultures
NG	no growth
NLV	Norwalk-like virus
NTHNN	non-toxic hemagglutinin
OD	optical density
ORP	oxidation-reduction potential
OGYEA	oxytetracycline glucose yeast extract agar
PA	putrifactive anaerobe
PCR	polymerase chain reaction
PHA	passive haemagglutination assay
ppm	parts per million
RCA	royal clostridial agar
RH	relative humidity
RIA	radio immunoassay
spp.	species
TEM	transmission electron microscopy
TPGY	trypticase peptone glucose yeast
TSC	sulfite cycloserine
USDA	United States Department of Agriculture
z	temperature sensitivity indicator

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The bakery industry, an important component of the North American economy, generates billions of dollars in revenue annually. In 1998, sales of bakery products in the United States exceeded 10 million metric tons and had a market value of ~$ 27 billion dollars (US)–a 14.5% increase over the previous four years (Kohn, 2000). In Canada, the bread and bakery industry shipped ~$2.3 billion dollars of products in 2000, an increase of 36.3% from 1988 levels and an amount that represented 4.2% of total food and beverage processing sector shipments (Agriculture and Agri-Food Canada, 2000). This sustained growth has been driven by consumer demands for convenient, premium baked goods that are fresh, nutritious, conveniently packaged and shelf-stable. At the same time, there has been an increase in in-store bakeries and a renewed interest in "organic", ethnic and artisan-type bakery products. Today, a diverse selection of bakery products can be found on supermarket shelves. Available products include unsweetened goods (breads, rolls, buns, crumpets, muffins and bagels), sweet goods (pancakes, doughnuts, waffles, cakes and cookies) as well as filled goods (fruit, cheese and meat pies, sausage rolls, pastries, sandwiches, cream cakes, pizza, and quiche). Most bakery products are marketed fresh and are stored at ambient temperature. However, other products, such as filled pies

and cakes, are stored under refrigerated conditions to achieve a longer shelf-life and to enhance their safety. Bakery products, like most processed foods, are subject to physical, chemical and microbiological spoilage. While physical spoilage (staling) and chemical spoilage (oxidative and/or hydrolytic rancidity) problems limit the shelf-life of low- and intermediate-moisture bakery products, microbiological spoilage, particularly mould growth, is the main concern in most high-moisture products. Losses due to mould spoilage vary between 1% and 5% of products depending on the season, product type, and method of processing (Mälkki and Rauha, 1978) and are therefore, of serious economic consequence to the bakery industry. Chemical preservatives such as sorbic acid, potassium sorbate and calcium propionate, are commonly used to prevent or retard mould spoilage in bakery products. However, with increasing consumer concerns about food preservatives, the bakery industry is now focusing its attention on modified-atmosphere packaging (MAP) for product shelf-life extension.

MAP has been defined as "the enclosure of a food product in a high gas barrier film in which the gaseous environment has been changed or modified to slow respiration rates, reduce microbiological growth, and retard enzymatic spoilage with the intent of extending shelf-life" (Young et al., 1988). Methods most commonly used to modify the gas atmosphere within a product include vacuum packaging, gas packaging and packaging with oxygen absorbents. Vacuum packaging is not widely used as a method of atmosphere modification due to its crushability effect on most bakery

products. However, it is used to prevent mould problems in flat breads (pita, naan etc.) and pizza crusts and is used to prevent rancidity problems in shortbread, a high fat product with a hard texture. Gas packaging, using mixtures of CO_2 and N_2, has been used to extend the mould-free shelf-life and keeping quality of a wide variety of bakery products stored at ambient temperature. Presently, more than 150 European bakery companies use this technology to extend the shelf-life of rolls, cakes, pizza, baguettes and sliced bread (Smith and Simpson, 1995; 1996). In Asia, oxygen absorbents and/or gas-generating sachets are widely used to prevent mould problems in intermediate- and high-moisture bakery products and this method of atmosphere modification is slowly gaining acceptance in the North American marketplace (Smith and Simpson, 1995; 1996).

Despite the increased use of MAP technology for shelf-life extension of high-moisture bakery products, there are safety concerns surrounding this technology. While physical and chemical hazards can compromise product safety, microbiological hazards, specifically foodborne pathogens, pose the greatest threat to consumer safety. A major microbiological hazard of concern in MAP high-moisture bakery products is the possible outgrowth of spores of strains of *Clostridium botulinum* types A and proteolytic B and subsequent neurotoxin production. Consumption of food in which *C. botulinum,* or closely related strains, has grown and produced neurotoxin often results in botulism, a rare but potentially fatal neuromuscular illness (Hatheway, 1993; Meng et al., 1997). The factors that contribute to the

concern over botulism resulting from high-moisture MAP bakery products are based on:

i. the ability of these spores, if present in flour or other ingredients, to survive the baking process;

ii. the anaerobic packaging conditions in MAP products which would be conducive to the growth of *C. botulinum*; and

iii. the inhibition of mould growth, the common indicator of spoilage in high-moisture bakery products.

In addition, several challenge studies have shown that this pathogen can grow to hazardous levels in gas-packaged food stored at ambient temperature while products remain organoleptically acceptable to the consumer at the time of toxigenesis (Hintlian and Hotchkiss, 1986; Farber, 1991). Recent product recalls in the U.S.A. and in Canada of MAP gluten-free breads containing potato flour and in Canada of naan breads would appear to exacerbate these safety concerns. However, since high-moisture MAP-bakery products have been on the marketplace for several years and have never been implicated in any botulism outbreaks, are these safety concerns justified? Do MAP high-moisture bakery products represent a serious botulism risk to consumers?

A risk has been defined as "the probability of a hazard occurring" (International Commission for the Microbiological Specification of Food [ICMSF], 1988) and Eklund (1993) has described several conditions that are critical in order for foods to be considered a botulism risk. These conditions include the food (or ingredients) being contaminated with *C. botulinum* spores or vegetative cells either from the environment or during further processing;

inadequate processing such that spores of *C. botulinum* are not inactivated (or the product becoming contaminated post-processing); the food supporting growth of and neurotoxin production by *C. botulinum;* and the food being acceptable and being consumed without further heating that would inactivate preformed neurotoxin.

Each of these conditions must be reviewed in detail in order to assess the botulism risk of MAP high-moisture bakery products stored at ambient temperature. If such products are indeed determined to pose a risk of botulism, then barriers to the growth of *C. botulinum* need to be incorporated into these products to prevent toxicity.

1.2 LITERATURE REVIEW

This review will focus on the potential hazards associated with bakery products, the microbiological causative agents of foodborne illness with specific reference to *C. botulinum* and the potential of high-moisture bakery products to support growth of and neurotoxin production by this pathogen as well as possible control measures.

1.2.1 Safety concerns in bakery products

Foodborne illness, which has been defined as "any illness associated with, or in which the causative agent is obtained by the ingestion of food" (Oblinger, 1988), can range from mild to fatal. Mead et al. (1999) estimated that, in the United States, there are approximately 76 million foodborne illnesses, including 325,000 hospitalizations and 5,000 deaths each year. While foods such as meat, fish, poultry, eggs and dairy products are the most common vehicles of foodborne illness worldwide, bakery products have also been implicated in foodborne disease outbreaks (Todd, 1996). In the United States, between 1988 and 1992, bakery products accounted for 29 outbreaks involving 820 cases out of a total of 2,423 reported foodborne disease outbreaks (Bean et al., 1996). In Canada, pizza, cheesecake, pies and tarts, bread, and muffins have

all been implicated in outbreaks of foodborne illness (Todd, 1996), while high-moisture English-style crumpets have posed safety concerns in Australia (Jenson et al., 1994).

The rest of the world is also subject to foodborne illnesses caused by bakery products. Todd (1996) reported that 35% to 47% of all foodborne disease outbreaks in Poland, Portugal, Bulgaria and Switzerland were caused by the consumption of bakery products, while several outbreaks in Brazil were traced to cream filled cakes (Potter et al., 1997). Ombui et al. (2001) identified maize flour, bread, scones and other wheat products, and lemon-pie pudding as major foods responsible for foodborne illness in Kenya between 1970 and 1993.

1.2.2 Potential hazards associated with bakery products

Several factors affecting the involvement of bakery products in foodborne illnesses are briefly discussed below:

1.2.2.1 Minimal processing

In order to achieve desirable textural and quality attributes, most bakery products only receive a minimal heat treatment. For example, although bread is baked at a high temperature, during baking the temperature in the center of the crumb rarely exceeds 100°C for more

than a few minutes. Furthermore, cream, cold custard, icing, spices, nuts, or fruit toppings or fillings, which may be incorporated in finished products may be prepared without any heating. According to Bryan et al. (1997), vegetative pathogenic microorganisms should be readily destroyed during baking because of their low thermal resistance (D-values) as shown in Table 1.1. However, spore-forming bacteria, which have much higher D-values (Table 1.1) will readily survive baking, and may grow to hazardous levels if packaging and storage conditions are conducive to their outgrowth (Bryan et al., 1997). While vegetative pathogens, such as *Salmonella* spp. and *Staphylococcus aureus*, as well as moulds and viruses, should be destroyed during baking, products may become contaminated post-baking from the air, equipment and handlers (Sugihara, 1977). Furthermore, cross-contamination may occur if bakery products are prepared or stored in the same area as raw foods such as eggs, meat or milk.

1.2.2.2 Hazardous products and/or ingredients

According to Farkas (1997), potentially hazardous foods have a pH of >4.5 and an a_w of >0.84. Many bakery products and ingredients have pH and a_w levels below this hazardous classification (Tables 1.2 and 1.3), and thereby, restrict microbial growth. Other products and

TABLE 1.1. *Thermal resistance of some specific bacteria*

Bacteria	Heat Resistance in Minutes		
	$D_{70°C}$	$D_{90°C}$	$D_{121°C}$
Vegetative			
Campylobacter jejuni	0.0001	—	—
Escherichia coli	0.001	—	—
Listeria monocytogenes	0.3	—	—
Salmonella spp.	0.001	—	—
Staphylococcus aureus	0.1	—	(<1)
Vibrio parahaemolyticus	0.001	—	—
Yersinia enterocolitica	0.01	—	—
Spore-forming [b]			
Bacillus cereus	—	10	—
Clostridium botulinum (Group I)	—	—	0.2
Clostridium botulinum (Group II)	—	1.5	—
Clostridium perfringens	—	—	0.15

Adapted from Report on Vacuum Packaging and Associated Processes (1992)

[a] Value in parentheses refers to the toxin

[b] Values refer to the spores

TABLE 1.2. *Water activity range of selected bakery products*

Product	a_w
Low-moisture content	
Cookies	0.20-0.30
Crackers	0.20-0.30
Intermediate-moisture content	
Chocolate coated doughnuts	0.82-0.83
Danish pastries	0.82-0.83
Cream filled cake	0.78-0.81
Soft cookies	0.50-0.78
High-moisture content	
Bread	0.96-0.98
Pita bread	0.90
Fruit pies	0.95-0.98
Carrot cake	0.94-0.96
Cheese cake	0.91-0.95
Pizza crust	0.94-0.95
Pizza	0.99

Adapted from Smith and Simpson (1995)

TABLE 1.3. *pH range of selected bakery products*

Product	pH range	Reference
High-acid		
Sourdough bread	4.2-4.6	Martinez-Anaya et al. (1990)
Apple pie	4.2	Smith and Simpson (1995)
Low-acid		
White bread	5.7	Rosenkvist and Hansen (1995)
Whole wheat bread	5.6	Rosenkvist and Hansen (1995)
Chocolate nut bread	6.2-6.6	Denny et al. (1969)
Date nut bread	6.1-6.7	Denny et al. (1969)
Non-acid		
Crumpets	6-8	Jenson et al. (1994)
Banana nut bread	7.2-7.9	Aramouni et al. (1994)
Carrot muffin	8.7	Smith and Simpson (1995)

ingredients; however, have levels conducive to the growth of microorganisms that are present in bakery ingredients. For example, the pH of custard, which is used in many filled baked products, is 5.8 to 6.6 and is ideal for the growth of *Salmonella* spp. (Bryan, 1976). Icing, which has a low a_w, is not usually conducive to microbial growth. However, the interface between the cake and icing may have a much higher a_w which enhances growth of microorganisms. Silliker and McHugh (1967) reported such an incident in which *S. aureus* grew at the interface of cake and icing. In addition, it is important to note that both the pH and the a_w of products may change during storage.

1.2.2.3 Storage conditions

Most bakery products, with the exception of cream-, custard- and meat-filled products, are held at ambient temperature for maximum storage quality; however, such non-refrigeration storage conditions may be conducive to microbial growth and may compromise safety. English-style crumpets, a high-moisture snack-food product typically stored at ambient temperature, have been implicated in several food poisoning outbreaks involving *B. cereus* (Jenson et al., 1994). Furthermore, since most products are "cook-and-hold products" and are not heated prior to consumption, there is no safety margin for destroying bacteria that may survive the baking process or may have

been introduced during handling or storage. For in-store bakeries, products are often displayed in bins or are loosely wrapped in paper. While this form of product display may be appealing to consumers, there is a potential for contamination of these products from self-serve bins if they are handled without the use of tongs or glassine paper.

Products such as cream-, meat- and cheese-filled cakes have an established history as vehicles of foodborne illness. While holding at refrigeration temperatures will delay microbial growth in these filled products, it may not be sufficient to prevent the growth of psychrotrophic pathogens such as *Listeria monocytogenes*. Furthermore, there is always a potential for temperature abuse at all stages of the processing, distribution and storage chain as well as in the home. If products are frozen, bacterial growth will be slowed, but once the product is thawed, growth may resume as has been shown in outbreaks involving *Salmonella* spp. (Schmidt and Ridley, 1985).

1.2.2.4 Modified atmosphere packaging

Modified-atmosphere packaging (MAP), using carbon dioxide (CO_2)-enriched gas atmospheres, vacuum packaging or oxygen absorbents, can extend the mould-free shelf-life and keeping-quality of a wide variety of high-moisture bakery products stored at ambient

temperature. Advantages and disadvantages of oxygen absorbent technology and gas packaging as methods of atmosphere modification for shelf-life extension of bakery products are outlined in Tables 1.4 and 1.5 respectively. Examples of gas-packaged products on the marketplace include bread, crumpets, sandwiches, pizza and muffins. Some flat breads (pita and naan bread) are packaged under vacuum while other products are packaged with oxygen absorbents (tortillas) or with combinations of gases and oxygen absorbents (gluten-free products). However, there are concerns about the safety of MAP technology since many pathogens can grow under a wide range of a_w, pH, temperature and packaging conditions as shown in Table 1.6. One pathogen of major concern in MAP products is *C. botulinum* which, if present in the raw ingredients, will readily survive the baking process. This concern would appear justified since this pathogen has been shown to grow to hazardous levels in MAP foods stored at ambient temperature while products were still organoleptically acceptable to the consumer (Hintlian and Hotchkiss, 1986; Farber, 1991; Korkeala et al., 1998; Lawlor et al., 2000). While MAP is widely used in Europe and is gaining acceptance in North America for extending the shelf-life of high-moisture, minimally-processed bakery products, there is a paucity of data on the safety of MAP bakery products stored at ambient temperature.

TABLE 1.4. *Advantages and disadvantages of oxygen absorbents*

Advantages	Disadvantages
❑ Inexpensive and simple to use	❑ Free airflow around sachet is needed
❑ Non-toxic and safe to use	❑ Cause package collapse
❑ Prevent aerobic microbiological spoilage	❑ May promote growth of *C. botulinum*
❑ Prevent rancidity/ off-flavours in fats and oils	❑ May cause flavour changes in: ▪ High moisture food ▪ High fat foods
❑ Maintain flavour by preventing oxidation of flavour compounds	❑ Consumer resistance to sachet use in packages. Possible mis-use of sachet
❑ Maintain quality without additives	
❑ Increase product shelf-life	
❑ Increase distribution radius	
❑ Reduce distribution losses	
❑ Replace chemical pesticides to prevent insect damage of foods	

Adapted from Smith and Simpson (1995)

TABLE 1.5. *Advantages and disadvantages of gas packaging*

Advantages	Disadvantages
❑ Increased shelf-life	❑ Initial high cost of films, equipment
❑ Increased market area	❑ Discoloration of meat pigments
❑ Reduction in production/ storage costs	❑ Leakage
❑ Improved presentation	❑ Fermentation and swelling
❑ Fresh appearance	❑ Potential growth of organisms of public health significance
❑ Clear view of the product	
❑ Easy separation of slices	

Adapted from Smith and Simpson (1995)

TABLE 1.6. *Minimum growth requirements of pathogenic bacteria* [a]

Bacteria	Temperature (°C)	pH	a_w	Gaseous conditions [b]
B. cereus	4	4.3	0.91	Facultative
C. jejuni	32	4.9	0.99	Microaerophilic
C. botulinum (Group I)	10	4.6	0.95	Anaerobic
C. botulinum (Group II)	3.3	5.0	0.97	Anaerobic
C. perfringens	15	5.0	0.95	Facultative
E. coli	7	4.4	0.95	Facultative
L. monocytogenes	0	4.3	0.92	Facultative
Salmonella spp.	6	4.0	0.94	Facultative
S. aureus	6 (10)	4.5 (5.2)	0.86 (0.90)	Facultative
V. parahaemolyticus	5	4.8	0.94	Facultative
Y. enterocolitica	-1	4.2	0.96	Facultative

Adapted from Smith et al. (2002b)

[a] Under optimal growth conditions

[b] Anaerobic, able to live in the absence of free oxygen; facultative, able to adapt to varying environments; microaerophilic, requiring very little free oxygen

[c] Values in parentheses refer to the production of enterotoxin

1.2.2.5 Recent market trends

Recent consumer trends have resulted in novel products such as preservative-free, low-fat and reduced-calorie baked-goods. However, modification of a product's formulation may also influence its a_w or pH to levels conducive to the growth of foodborne pathogens. Such novel products may be safe, but their safety must be assessed on an individual basis. This is even more critical if such products are packaged under modified atmospheres and stored at ambient temperature.

1.2.3 Causative agents of foodborne illness

Foodborne illnesses from bakery products can be caused by physical and chemical contamination of the final product. However, the majority of foodborne illnesses involving bakery products are caused by microorganisms, specifically moulds, viruses, and bacteria.

1.2.3.1 Moulds

Moulds, which often limit the shelf-life of high- and intermediate-moisture bakery products, can also be of public-health concern. Although mouldy bakery products will be rejected by consumers, some moulds, including *Alternaria, Aspergillus*, *Fusarium* and *Penicil-*

lium spp., may secrete potentially hazardous mycotoxins without visible signs of spoilage. Mycotoxins have been found in many foods, including cereals and grain products, nuts, fruits, vegetables and dairy products (Malloy and Marr, 1997). In a survey of flour, Weidenborner et al. (2000) found that *Aspergillus* species were the predominant isolates with 93.3% of these isolates being toxigenic.

The main mycotoxin producing moulds of concern in bakery products and their methods of control have been reviewed in detail in a recent American Institute of Baking technical publication (Bullerman, 2000) and therefore will not be discussed further in this review.

1.2.3.2 Viruses

Like moulds, viruses are ubiquitous in the environment and can cause viral gastroenteritis through the consumption of bakery products that are prepared or handled under unsanitary conditions (Hedberg and Osterholm, 1993). Norwalk-like viruses (NLVs) have been responsible for outbreaks in foodborne illness involving frosted bakery products, crumb cake, pie and rolls, hamburger buns and cookies, and custard slices (Kuritsky et al., 1984; Bean et al., 1996; Arness et al., 1999).

Another virus which can be transmitted from infected handlers to bakery products is Hepatitis A. Outbreaks of Hepatitis A have been

attributed to unbaked sherry trifle, breads, rolls and sandwiches and glazed pastries (Chaudhuri et al., 1975; Sockett et al., 1993). More recently, a community outbreak in 1994 in New York was traced to an infected bakery worker who had contaminated cooked doughnuts while applying a sugar glaze (Weltman et al., 1996). Methods to control viral gastroenteritis from bakery products include education of food handlers, good personal hygiene and proper sanitation practices.

1.2.3.3 Bacteria

While moulds and viruses have been implicated in outbreaks of foodborne illness involving bakery products, bacteria are by far the greatest source of concern. Foodborne illnesses caused by bacteria can be classified as either intoxication or infection. Foodborne bacterial intoxication is "any illness caused by the ingestion of food containing pre-formed bacterial toxin resulting from bacterial growth in food" (Oblinger, 1988). Foodborne infection, on the other hand, is caused by "the ingestion of food containing viable bacteria which then grow and establish themselves in the host resulting in illness" (Oblinger, 1988). Vegetative pathogenic bacteria, and spore-forming bacteria, which are of particular concern in high-moisture minimally-processed bakery products, will be reviewed. In addition, preventa-

tive measures to enhance the safety of such products will be briefly discussed.

1.2.4 Vegetatative pathogenic bacteria

Vegetative bacteria (i.e., non-spore forming bacteria) are most often implicated in outbreaks of foodborne illnesses. The vegetative bacteria of concern in high-moisture, minimally-processed bakery products are *Salmonella* spp., *S. aureus*, and *L. monocytogenes*.

1.2.4.1 *Salmonella* species

Numerous foodborne illnesses involving bakery products have been caused by *Salmonella* spp. (Table 1.7). The major sources of *Salmonella* in bakery products are unpasteurized eggs (Board, 1969; FSIS, 1998) which, although a potentially hazardous bakery ingredient, are invaluable for their foaming, emulsifying, and binding properties. In addition to unpasteurized eggs, a variety of other ingredients have been shown to be sources of *Salmonella* contamination of bakery products (Table 1.7).

Salmonella spp. are heat labile, and consequently, they should be inactivated during baking or cooking. However, for minimally-processed products such as cheesecake, or custard and meringue-

TABLE 1.7. *Characteristics of* Salmonella *species*

Sources of contamination	Raw eggs/dairy products, flour, cocoa and chocolate, coconut, peanut butter, fruit spices, and yeast flavourings
Symptoms of disease	Nausea, vomiting, abdominal cramps, diarrhea, fever, and headache
Causative agent	Infection
Onset of illness	6 to 48 hours
Duration of illness	2 to 7 days
Associated bakery products	Bread pudding, custard filled cakes and pastries, quiche, meringue pies, cheesecake, apple pie, tiramisu, and mousse
Preventative measures	Use of properly pasteurized eggs/dairy products, adequate processing temperature/time of products, proper refrigerated storage of raw ingredients/finished products (<4°C), good manufacturing practices (GMPs), good personal hygiene/sanitation, ongoing hygiene training, and hazard analysis critical control point (HACCP) application

Adapted from Smith et al. (2002a)

type pies, puddings or fillings, the mild heat-treatment necessary to produce an acceptable product may be inadequate for complete destruction of this pathogen (Hao et al., 1999). Other ingredients, such as cold custard, puddings, icings and toppings, may also support the growth of *Salmonella* spp. Although the a_w of some icings may provide a barrier to growth, the interface between the icing and the baked product may be conducive to growth (Silliker and McHugh, 1967). Infected eggs or other ingredients may also cross-contaminate equipment and result in multiple outbreaks. *Salmonella* spp. resist desiccation and can survive for long time periods in foods of very low a_w, such as spray-dried eggs, which may pose a safety concern when reconstituted (Dack, 1961).

Several bakery products have been implicated in foodborne outbreaks involving *Salmonella* spp. (Table 1.7). In addition, acidic products, such as fruit pies, have also been implicated (Table 1.7). Although the low pH (3.6) of the baked pie filling should have prevented the growth of this pathogen, cross-contamination of pies may have occurred during packaging. Another explanation could be that the egg glaze may have seeped through the high-fat pastry resulting in growth of *S. enteritidis* at the product's interface.

The most important control measures used to prevent *Salmonella* outbreaks in bakery products include the use of pasteurized

eggs, prevention of cross-contamination and strict temperature control of all hazardous raw ingredients and finished products.

1.2.4.2 *Staphylococcus aureus*

Staphylococcal food poisoning results from the consumption of food in which *S. aureus* (Table 1.8) has grown to sufficient numbers (~10^6 CFU/g) to produce enterotoxin. The illness is characterized by nausea, vomiting, abdominal pain and diarrhea, two to eight hours after eating food containing the enterotoxin (Tranter, 1990).

Since *S. aureus* is often isolated from cuts and nasal passages, as well as from skin and hair, food handlers are a major source of contamination of bakery products. *S. aureus* is also ubiquitous in air, water, raw milk, sewage, and on food contact surfaces. Several ingredients, such as inadequately pasteurized milk and cream, may also be sources of high numbers of *S. aureus* or its enterotoxin.

The main bakery products associated with foodborne outbreaks involving *S. aureus* have been baked goods containing fresh or synthetic cream (Table 1.8). Although imitation cream on its own does not contain sufficient nutrients to support the growth of this pathogen, growth can occur at the interface of the cream and the baked product (McKinley and Clarke, 1964). Due to good manufac-

TABLE 1.8. *Characteristics of* Staphylococcus aureus

Sources of contamination	Hair, hands, skin, throat, and nasal passages of humans, infected wounds, lesions, air, water, work surfaces, inadequately pasteurized milk/cream, and dried milk
Symptoms of disease	Nausea, vomiting, abdominal cramps, diarrhea, fever, and chills
Causative agent	Toxin in food
Onset of illness	2 to 8 hours
Duration of illness	1 to 2 days
Associated bakery products	Cream filled pastries (synthetic and fresh cream), apple muffins, cream puffs, long johns, flat bread, pizza, and coconut fillings
Preventative measures	Use of adequately pasteurized dairy products, strict personal hygiene/sanitation, good manufacturing practices (GMPs), strict temperature control of raw ingredients/finished products (<4°C), use of chemical preservatives (e.g., sorbates), and on-going hygiene training

Adapted from Smith et al. (2002a)

turing practices, these products are now seldom implicated in outbreaks of *S. aureus* food poisoning in North America (Todd et al., 1983). However, they continue to be a major source of illness in many temperate countries where refrigeration is inadequate (do Carmo and Bergdoll, 1990). Other bakery products which have also been implicated in outbreaks of staphylococcal food poisoning are oatmeal raisin cookies, apple muffins, cream puffs and long johns (Sumner et al., 1993). Pizza is also frequently involved, with contamination usually occurring as a result of poor manufacturing practices. Additionally, *S. aureus* can grow in high salt concentrations as found in many pizza ingredients.

Although *S. aureus* is destroyed by heating, its enterotoxin is heat resistant and is not inactivated by pasteurization (Bergdoll, 1989). Therefore, food poisoning outbreaks by *S. aureus* may still occur, even in the absence of viable cells, if pre-formed enterotoxin is present in the product as a result of temperature abuse of ingredients and/or fillings prior to baking.

There is an increasing variety of goods available from in-store bakeries which are held at ambient temperature. These products are often stored in self-serve bins and handled manually by both employees and customers. This combination of temperature and handling increases the potential for contamination by *S. aureus* and subsequent growth and/or enterotoxin production.

The number of staphylococcal food poisoning outbreaks attributed to the consumption of cream and custard filled pastries in the United States, has decreased dramatically in recent years. This is likely due to improved sanitation, temperature control, modification of product formulation and the use of preservatives. Good manufacturing practices (GMPs) have also effectively reduced the level of contamination by *S. aureus* of frozen cream-pies in North America. One survey of all plants manufacturing frozen pies in the United States reported that levels of *S. aureus* were consistently <10 CFU/g (Todd et al., 1983).

Other measures essential to the control of *S. aureus* inlcude the use of preservatives (Bryan, 1976), adherence to adequate temperature/time processes (Husseman and Tanner, 1947; Angelotti et al., 1961), and strict temperature control (<4°C) to restrict growth and enterotoxin production (Bryan, 1976).

1.2.4.3 *Listeria monocytogenes*

Listeria monocytogenes (Table 1.9) is widespread in nature–occurring in soil, vegetation, and water, as well as in many animal and plant products (Lovett and Twetd, 1988). Furthermore, it can grow over wide pH, a_w, and temperature ranges. Outbreaks of listeriosis have resulted from the consumption of soft cheese, cream and

TABLE 1.9. *Characteristics of* Listeria monocytogenes

Sources of contamination	Raw or inadequately pasteurized dairy products, bakery environment and/or post-processing contamination, and raw poultry and meat products
Symptoms of disease	Mild flu-like symptoms, septicemia, meningitis, encephalitis, and abortion in pregnant women
Causative agent	Infection
Onset of illness	2 to 35 days
Duration of illness	1 to 90 days
Associated bakery products	Pastries
Preventative measures	Use of properly pasteurized dairy products, strict temperature control of raw/finished products (<2°C), additional barriers (pH, water activity reduction, chemical preservatives), strict personal hygiene/sanitation, good manufacturing practices (GMPs), and ongoing hygiene training and HACCP

Adapted from Smith et al. (2002a)

butter (Linnan et al., 1988; Lyytikäinen et al., 2000) and therefore, *L. monocytogenes* is of potential concern in bakery products containing dairy ingredients. *L. monocytogenes* is also classified as a psychrotrophic pathogen (Table 1.9), which means that it can readily grow at the refrigerated storage conditions of cream- and cheese-filled pastries. Furthermore, since *L. monocytogenes* is ubiquitous in the bakery environment, post-processing contamination of finished products is possible. In a survey of 300 pastries from 100 bakeries in France, Ferron and Michard (1993) found that 14% of all pastries were contaminated with *L. monocytogenes*. One sample contained 7×10^5 CFU/g–a level that can cause listeriosis. The authors concluded that the risk of listeriosis from pastries was at least equivalent to that of meat and delicatessen products.

Control methods include adequate heating and baking of products to destroy this vegetative pathogen. Richter et al. (1991) demonstrated that heating flour inoculated with *L. monocytogenes* for five minutes at 80°C was sufficient to destroy the pathogen. *L. monocytogenes* is also unlikely to survive boiling of custards used in the preparation of certain filled products.

Strict hygiene and temperature control are essential in preventing contamination and growth of this pathogen. However, since temperature abuse is commonplace in the food industry, temperature alone is not an adequate barrier to ensure the safety of

contaminated products. *L. monocytogenes* can also grow in the presence of elevated levels of CO_2 used in gas-packaging. While the use of additional barriers is recommended to control the growth of *L. monocytogenes*, it can still grow at low pH and low a_w (Table 1.6). However, this method of control may not be commercially viable since reformulation to these levels of pH and a_w may result in sensory and textural changes of the finished product.

1.2.5 Spore-forming pathogenic bacteria

Spore-forming bacteria such as *Bacillus* and *Clostridium* spp., produce spores that readily survive the baking process. *B. cereus, B. subtilis* and *B. licheniformis* have been implicated in several outbreaks of foodborne illness involving bakery products (Todd, 1982; Kramer and Gilbert, 1989; Jenson et al., 1994; te Giffel et al., 1996).

1.2.5.1 *Bacillus cereus*

Bacillus cereus has been implicated in several outbreaks of foodborne illness involving bakery products in Australia (Jenson et al., 1994). There are two distinct forms of *B. cereus* toxin-mediated gastroenteritis. The emetic type is generally associated with cereal based foods while the diarrheal type is most frequently associated with proteinaceous foods (Table 1.10).

TABLE 1.10. *Characteristics of* Bacillus cereus

Sources of contamination	Flour, dairy products, spices, dried eggs, soy protein, yeast, improvers, dried fruits, cocoa, and rice	
	Type I (diarrheal type)	Type II (emetic type)
Symptoms of disease	Watery diarrhea, cramps, and occasionally nausea	Nausea, vomiting, malaise, and occasionally diarrhea
Causative agent	Toxin produced in the intestine	Toxin produced in food
Onset of illness	8 to 20 hours	1 to 5 hours
Duration of illness	1 day	1 day
Associated bakery products	Naan bread, English-style crumpets, pikelets, vanilla slices, meat-filled bakery products, pumpkin pie, and rice-filled pastries	
Preventative measures	Cool hot food rapidly (within 2 hours) to <15°C, strict temperature control of raw ingredients/finished products (<4°C), use of chemical preservatives (e.g., vinegar, propionates, calcium acetate), lactic acid sourdough cultures, bio-preservatives (nisin), and additional barriers (pH, salt)	

Adapted from Smith et al. (2002a)

Bacillus spores are ubiquitously found in soils, dust and water, and are commonly found in flour and flour-based products as well as in the bakery environment (Granum, 1997). Other sources of *Bacillus* spores in bakery ingredients include cream, dried milk, and whey concentrate, spices, dried eggs, soy protein, rice, yeast and improvers, dried fruits, and cocoa. The level of *B. cereus* required to produce toxin is approximately 10^5 spores/g of food (Lund, 1990). Foodborne illness caused by *Bacillus* species is under-reported as symptoms are generally mild and self-limiting (Table 1.10).

Although low numbers of spores may be present initially in flour, spores, which readily survive the baking process, can grow rapidly in products held under suitable conditions (Kaur, 1986; Rosenkvist and Hansen, 1995). Although the potential health hazard of *B. cereus* in bread is minimal, this pathogen is of greater concern in bakery products that receive a minimal surface heat-treatment (Lee, 1988; Cowden et al., 1995). Examples of such griddle-baked products include ethnic flat breads, crumpets, and waffles. Outbreaks of *B. cereus* gastroenteritis have been attributed to naan bread, crumpets and pikelets. Growth of *B. cereus* in these products is difficult to control since the heat treatment is insufficient to destroy spores and may actually act as a heat-shock, which enhances spore outgrowth at ambient storage temperature. *B. cereus* has also caused outbreaks of foodborne illness through the consumption of

bakery products containing dairy based custards or creams (Pinegar and Buxton, 1977).

Conventional methods of control include proper sanitation and testing of raw materials to reduce initial spore counts; however, these measures will not eliminate all spores or prevent germination and growth of *Bacillus* spp. in finished products. Growth of *Bacillus* spp. however, can be controlled by use of preservatives (propionates, sorbates, and acetates), sourdough, and strict temperature control (Kaur, 1986; Rosenkvist and Hansen, 1998). However, up to 14% of *B. cereus* strains may be psychrotrophic (i.e., capable of growing at refrigeration temperatures) (Granum, 1997). Thus, temperature alone may not be a practical control measure. Nisin, a commercially available bacteriocin, is approved for use in Australia to control the growth of *B. cereus* in high-moisture English-style crumpets.

1.2.5.2 *Clostridium perfringens*

Another spore-forming bacterium that is of potential concern, particularly in meat- and chicken-filled baked products (pies, sausage rolls, etc.), is *C. perfringens* (Table 1.11). Food poisoning due to *C. perfringens* (mainly toxin type A) usually occurs within six to 24 hours of ingestion of bakery products containing greater than 10^6 CFU/g. The illness follows ingestion of contaminated food and is

TABLE 1.11. *Characteristics of* Clostridium perfringens

Sources of contamination	Beef, turkey, chicken, cooked ground meat, gravies, and sauces
Symptoms of disease	Nausea, occasional vomiting, diarrhea, and intense abdominal pain
Causative agent	Toxin produced in the intestine
Onset of illness	6 to 24 hours
Duration of illness	1 to 2 days
Associated bakery products	Meat-filled bakery products
Preventative measures	Proper cooking of meat fillings, cooling baked products to <10°C within 2 to 3 hours, strict temperature control of stored products, reheating refrigerated products to reach an internal temperature of 75°C, strict personal hygiene/sanitation, good manufacturing practices (GMPs), ongoing hygiene training and HACCP

Adapted from Smith et al. (2002a)

caused by the production of an intracellular enterotoxin *in situ* by sporulating bacterial cells. Symptoms of the illness generally include severe abdominal pain and diarrhea that lasts about two days (McClane, 1997).

Since *C. perfringens* cannot grow below 15°C, rapid cooling of meat-filled products after baking and refrigerated storage of products are critical to prevent growth of this pathogen to hazardous levels. Furthermore, refrigerated products should be reheated to an internal temperature of 75°C prior to serving to destroy any vegetative cells remaining in the product (Labbe, 1989). Meat- and chicken-filled bakery products used to be a major cause of food poisoning by *C. perfringens.* However, by adhering to the above guidelines, very few incidents of *C. perfringens* involving these products have occurred in recent years. Good hygiene and personal sanitation are also essential to control the growth of this pathogen in meat-filled bakery products (McClane, 1997).

1.2.6 *Clostridium botulinum*

Foodborne botulism is a rare, potentially fatal neuromuscular illness that can result from the consumption of food in which *C. botulinum* has grown and produced neurotoxin (Hatheway, 1993; Meng et al., 1997). Infant botulism results when germinating spores of neurotoxigenic

clostridia colonize the infant intestine and produce neurotoxin *in situ* (CDC, 1998; Dodds, 1993c). Botulism has also resulted from similar colonization in people who are immuno-compromised (Griffin et al., 1997) and in addition, there is evidence that neurotoxigenic clostridial colonization may precipitate central nervous system (CNS) disorders (Siarakas et al., 1999; Sandler et al., 2000).

Belonging to the genus *Clostridium*, *C. botulinum* shares with it the following properties: i) the formation of heat- and chemical-resistant endospores; ii) the existence of a gram-positive cell wall structure in vegetative cells; iii) an anaerobic, fermentative metabolism, and iv) a low guanine:cytosine content (Johnson and Bradshaw, 2001). *C. botulinum* spp., and certain strains of *C. baratii*, and *C. butyricum*, as well as *C. argentinense*, have the ability to produce seven serotypes (A through G) of botulinum neurotoxin (BoNT) (Sugiyama, 1980). Types A, B, E, and F are the main causes of human botulism while types C and D are responsible for botulism in animals (Hatheway, 1993). Although these serotypes vary in their pathogenicity (Johnson and Bradshaw, 2001), all BoNTs, which are zinc-peptidases, are extremely specific for neurons (Schiavo et al., 2000) and are highly toxic ([~0.2 ng/kg in mice] Hatheway, 1993). Characteristics of Group I (A, B, and F) and Group II (B, E, and F) *C. botulinum* are summarized in Table 1.12. The neurotoxins are synthesized intracellularly during growth as single inactive proteins (~150 kDa) that are released during bacterial cell lysis and can

TABLE 1.12. *Characteristics by group of neurotoxigenic clostridia causing foodborne botulism*

	Group		*C. butyricum**
	I	II	
Neurotoxin type	A, B, F	B, E, F	E
Proteolysis	+	-	-
Optimal growth temperature (°C)	35-40	18-25	25
Minimal growth temperature (°C)	10	3	10-15
Minimum a_w for growth	0.95	0.97	NR
Minimum pH for growth	4.5	5.0	4.0-5.2
Spore heat resistance ($D_{-100°C}$) (minutes)	>15	<0.1	<1-5
Similar atoxic organism	*C. sporogenes* ($D_{112°C}$ 1.2)	(no spp. name assigned)	*C. butyricum*

* Strains producuing BoNT/E

Adapted From Hatheway (1993); Austin and Dodds (2001); Anniballi et al. (2002); Peck (2002)

NR not reported

be proteolytically cleaved to form an activte dichain molecule (Austin, 2001). Neurotoxin, *via* the blood stream, binds irreversibly at the neuro-muscular junctions of motor neurons, where it penetrates the plasma membrane, probably by receptor-mediated endocytosis. Within the nerve cell, the light chain of the BoNT acts specifically on proteins involved in neuroexocytosis to prevent the release of acetylcholine (Schiavo et al., 1992; Schiavo et al., 1993; Yamasaki et al., 1994). Foodborne botulism is characterized by bilateral descending flaccid paralysis and impaired motor and respiratory functions. Immediate treatment with anti-neurotoxin in addition to respiratory support has significantly decreased the fatality rate (Austin, 2001).

The toxicity of the neurotoxins that cause botulism necessitates a reliable method of detecting them. The mouse bioassay is typically used to detect BoNT, and this method relies on i.p. injection of mice along with neutralization with type-specific antisera to BoNT (Figure 1.1). Survival of mice protected with antisera in addition to symptoms of botulism in and/or death of unprotected mice indicates that neurotoxicity is attributable to *C. botulinum* (Austin and Blanchfield, 1996). Neutralization is necessary since false-positive results may occur due to the action of non-specific toxins (eg. toxins produced by *C. perfringens*). Although the bioassay is highly sensitive–detecting approximately five mouse 50% minimum lethal dose units (5 MLD_{50})–there are several disadvantages associated with the method (Table 1.13). Completing the test requires up

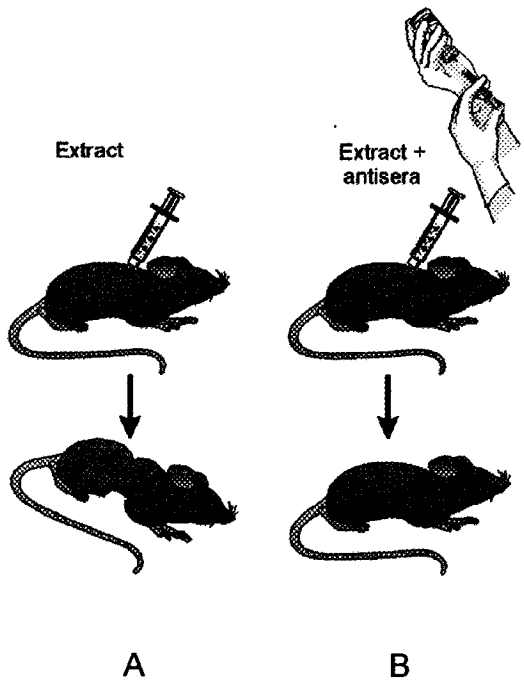


FIGURE 1.1. *Schematic showing the principle of detection of BoNT using the mouse bioassay: A, symptoms of botulism or death in duplicate mice injected with sample; and B, survival of duplicate mice with simultaneous injection of sample and antisera to BoNT*

TABLE 1.13. *Comparison of methods to detect botulinum neurotoxin*

Method	Principle	Sensitivity	Advantages	Disadvantages	Reference(s)
In vivo					
Mouse bioassay	Following intraperitoneal injection with BoNT, mice protected with antisera to BoNT survive while unprotected mice do not	5-10 MLD_{50}/mL	Commonly used, sensitive	Involves use of live animals; requires up to 72 hours for detection plus 2 to 3 days for typing; not specific (requires neutralization); expensive	Austin and Blanchfield (1996); AOAC (1995)
In vitro					
Passive haemagglutination assay (PHA)	RBCs coupled to anti-neurotoxin aggregate in presence of specific BoNT	0.8-2.0 MLD_{50}	Reaction visible to eye	Cross reactions between serotypes; inability to distinguish between serotypes	Johnson et al. (1966); Balding et al. (1973)
Radioimmunoassay (RIA)	Competitive binding to specific anti-neurotoxin by radiolabeled BoNT	400-3,000 MLD_{50}/mL to 80-100 MLD_{50}/mL	Specificity	Poor sensitivity, use of radioactive materials	Boroff and Chen (1973); Ashton et al. (1985)

TABLE 1.13. *Comparison of methods to detect botulinum neurotoxin* (cont'd)

Method	Principle	Sensitivity	Advantages	Disadvantages	Reference(s)
Gel diffusion assay	BoNT diffuses through gel to form complex (precipitin) with bound antisera	0.2-2.5 MLD_{50}/mL to 10-100 MLD_{50}/mL	Specificity	Poor sensitivity; time consuming, complex procedure	Vermilyea et al. (1968); Guilfoyle and Mestrandrea (1980)
Enzyme-linked immunosorbant assay (ELISA)	BoNT is captured (directly or indirectly) by anti-neurotoxin to solid plate and then detected with another labeled anti-neurotoxin	50-100 MLD_{50}/mL to <10 pg/mL	simple, rapid, high throughput capacity; amplification steps can increase sensitivity; polyclonal antibodies can increase specificity; ease of typing	expensive reagents; inability to distinguish between active and inactive BoNT; cross reactivity with other serotypes or NTHNN complex proteins; low sensitivity (possibly)	Notermans et al. (1978); Dezfulian and Bartlett (1984; 1991);Ferreira et al. (1987); Modi et al. (1988); Gibson et al. (1987;1988); Potter et al. (1993); Doellgast et al. (1994); Ekong et al. (1995); Szilagyi et al. (1999); Ferreira (2001)
Selective media/ immunoblot/ ELISA	BoNT produced by *C. botulinum* colonies isolated with selective agar are detected by ELISA	10-10,000 MLD_{50}/mL	rapid (2^+ days), high through put	low sensitivity	Dezfulian and Bartlett (1985); Goodnough et al. (1993); Rodriguez and Dezfulian (1997)

TABLE 1.13. *Comparison of methods to detect botulinum neurotoxin* (cont'd)

Method	Principle	Sensitivity	Advantages	Disadvantages	Reference(s)
Endopeptidase - ELISA/ fluorogenic assay	detects proteolytic cleavage of peptide substrates (solution or solid phase) by BoNT using ELISA	0.1-0.2 ng/mL to 0.6-4.5 ng/mL to 3.5-5 pg/mL	rapid, simple, sensitive, specific, can estimate neurotoxin activity; minimal cross reactivity	expensive; not commercially available	Hallis et al. (1996); Ekong et al. (1997); Witcome et al. (1999a, b); Anne et al. (2001)
PCR and DNA probes	DNA-antiBoNT antibodies react with BoNT and are amplified by PCR	5 pg	rapid, sensitivity, specificity	false negatives from sequence variability	Ferreira et al. (1992; 1994); Szabo et al. (1994); Wu et al. (2001)
Slot blot	Membrane immobilized BoNT is probed with anti-BoNT antibodies and detected with immuno-chemiluminesence	100 pg 2 to 16 MLD	rapid, simple, inexpensive, can quantitate neurotoxin, excellent specificity	low sensitivity	Cadieux (2001)

MLD_{50} (minimum lethal dose: the highest dilution that results in the death of 50% of mice)

NTHNN: Non-toxic hemagglutinin

to 72 hours, and a further 2 to 3 days may be required for neurotoxin typing. Another disadvantage of the bioassay is the use of live animals (Table 1.13). Therefore, several alternatives to the mouse bioassay have been suggested (Table 1.13). These alternative methods have relied mainly on immunological reactivity using enzyme-linked coagulation assays (ELISAs), or catalytic activity. While such assays are promising and may offer advantages over the mouse bioassay including simplicity, rapidity, the capacity for high throughput, as well as increased specificity, these methods need to be individually standardized against the bioassay before they are adopted (Johnson and Bradshaw, 2001).

Spores of *C. botulinum* are widely distributed in the environment although type-specific distribution varies with location (Dodds, 1993a). This pathogen is commonly isolated from soil and since spores are resistant to environmental stresses, they are also found, at various levels, in a wide number of food products (Dodds, 1993b). In addition, several foods including meats, fish, vegetables as well as prepared and processed foods have been implicated in outbreaks of foodborne botulism (largely attributed to *C. botulinum* types A, B, and E) throughout the world (Hauschild, 1993 [Table 1.14]). Although most outbreaks of botulism resulting from vacuum-packaged foods have involved the production of type E neurotoxin in vacuum-packaged fish (Korkeala, 1998), vacuum-packaged ready-to-eat foods have also been involved (Table 1.14). Food packaged under MAP has been identified as a risk factor for the growth

TABLE 1.14. *Foods implicated in selected examples of botulism outbreaks*

Outbreak	Food[a]	Cases/deaths	Contributing factors	Clostridium/BoNT
1984 Japan	Vacuum-packaged lotus root	36/11	Temperature abuse	proteolytic *C. botulinum* BoNT/A
1987 USA	MAP shredded cabbage	1/0	Temperature abuse	proteolytic *C. botulinum* BoNT/B
1989 UK	Hazelnut yogurt	27/1	Under processing of hazlenut puree	proteolytic *C. botulinum* BoNT/B
1993 Italy	Eggplant in oil	7/0	Temperature abuse	proteolytic *C. botulinum* BoNT/B
1994 USA	Vacuum-packaged soup	2/0	Temperature abuse	proteolytic *C. botulinum* BoNT/A
1994 USA	Skordalia (potato salad)	30/0	Unsafe storage of foil-wrapped potatoes	proteolytic *C. botulinum* BoNT/A
1994 China	Home-prepared fermented salted soybeans and gourd	6/3	Unsafe process and storage	*C. butyricum* BoNT/E
1996 Italy	Mascarpone cheese	8/1	Usafe process and storage	proteolytic *C. botulinum* BoNT/A
1996 India	Sevu (gram-flour crisp)	34/3	Unsafe process and storage	*C. butyricum* BoNT/E
1997 Canada	Smoked salmon	1/0	Unknown	non-proteolytic *C. botulinum* BoNT/E
1997 Iran	Cheese in oil	27/1	Unsafe process	proteolytic *C. botulinum* BoNT/A
1998 UK	Bottled mushrooms	2/1	Unsafe process and storage	proteolytic *C. botulinum* BoNT/B
1998 Germany	Vacuum-packaged smoked fish	2/0	Undetermined	non-proteolytic *C. botulinum* BoNT/E
1999 Japan	Vacuum-packaged curried hash	1/0	Temperature abuse	proteolytic *C. botulinum* BoNT/A

[a] Commercially prepared unless otherwise stated

Adapted from Aureli et al., (1996); Chaudhry et al., (1998); Korkeala (1998); IASR (1999); Peck (2002)

of and neurotoxin production by *C. botulinum* (Farber, 1991; Hintlian and Hotchkiss, 1986; Eklund, 1993). According to Eklund (1993), several conditions must be met for foods to be considered a botulism hazard. These conditions are:

i. the food (or ingredients) must be contaminated with *C. botulinum* spores or vegetative cells either from the environment or during further processing;

ii. the process treatment must be inadequate to inactivate *C. botulinum* spores or the product must become contaminated after processing;

iii. the food must support the growth of and neurotoxin production by *C. botulinum;* and

iv. the food must be acceptable and must be consumed without further cooking, or after insufficient heating, to inactivate pre-formed neurotoxin.

Each of these conditions must be reviewed in detail in order to assess the botulism risk of MAP high-moisture bakery products stored at ambient temperature. Microbiological challenge testing of foods is valuable for providing information on the kinds of micororganisms that will grow in a food product as well as identifying the conditions under which growth can occur and therefore can be useful in assessing the risk of botulism in bakery products. If such products are indeed determined to pose a risk of botulism, then barriers to the growth of *C. botulinum* need to be incorporated into these products to prevent toxicity.

1.2.7 Contamination of bakery ingredients and final baked products with proteolytic spores or vegetative cells of *C. botulinum*

Contamination of finished bakery products could potentially result from the presence of spores in the bakery ingredients or from post-processing environmental contamination.

1.2.7.1 Flour

Clostridium botulinum types A and B spores are ubiquitously found in soil and in agricultural and animal products (Austin and Dodds, 2001; Austin, 2001). Since wheat-flour is the major ingredient used in most bakery formulations, it could be a potential source of contamination of spores of *C. botulinum*.

Several extensive surveys have been conducted on the microbiology of flour (Holtman, 1935; Castell, 1944; James and Smith, 1948; Thatcher et al., 1953; Hesseltine and Graves, 1966; Capparelli and Mata, 1975; Rogers and Hesseltine, 1978; Seiler 1988; Rosenkvist and Hansen, 1995). All of these surveys have shown a consistent pattern of contamination of flour with a heterogeneous mixture of moulds, yeasts and bacteria. In an extensive survey of flours from fifty Canadian mills, Thatcher et al. (1953) reported that the microflora of flour consisted of moulds, mesophilic nonspore-forming and spore-forming bacteria as well as aerobic and anaerobic thermophilic spores (Table 1.15). The dominant mould species were *Asper-*

gillus and *Penicillium* spp. while *Flavobacterium, Bacillus, Achromobacter* and *Serratia* spp. were the predominant bacteria found in the finished flours (Thatcher et al. 1953). Capparelli and Mata (1975) examined the microflora of corn flour used to make tortillas. A similar contamination profile of wheat flour was observed with yeasts, coliforms, *B. cereus*, *Staphylococcus* spp. and facultative, non-pathogenic thermophilic clostridia being isolated from all corn-flour samples tested (Capparelli and Mata, 1975). Rogers and Hesseltine (1978) examined the microflora of both wheat and wheat flour from six areas of the United States. Bacterial counts were low and ranged from 5,000 to 10,000 CFU/g with aerobic thermophilic sporeforming and thermophilic flat-sour spore-forming bacteria being detected, albeit it in low numbers (<10 spores/g [Rogers and Hesseltine, 1978]). Seiler (1988) examined a large number of flours from UK mills over an eight year period. These results confirmed previous surveys that found flours to be contaminated with mostly mould spores, non-spore-forming bacteria (pseudomonads, *Erwinia* spp., *Escherichia coli* and *S. aureus*), with *Bacillus* spp. being the predominant spore-forming bacteria found. More recently, Rosenkvist and Hansen (1995) found *B. subtilis* (70%), *B. licheniformis* (24%), *B. pumilus* (2%), and *B. cereus* (2%) to be the predominant spp. in wheat flour used in the manufacture of whole wheat bread. Accordinging to Elliot (1980), the "occasional spores of *C. botulinum* would be expected to be present in flour". Furthermore, Bell and Kyriakides (2000) have also suggested that "the contamination of both wheat and flour with *C. botulinum*

TABLE 1.15. *Range of microbial counts in flours from fifty Canadian mills*

Microbial group	Counts (per g)
Mesophiles	
Total bacterial count	400-4,516,000
Bacterial spores	0-3,600
Moulds	0-18,500
Thermophiles	
Aerobic spores	0-68
" Flat-sour" spores	0-32
Anaerobic spores	0-23+

Adapted from Thatcher et al. (1953)

types A and B spores is inevitable". Hauschild et al. (1988) found that out of 40 samples of dry rice-, oat-, barley- and mixed cereal, one sample of rice cereal was contaminated with spores of *C. botulinum.* Spores of proteolytic *C. botulimun* type B have been found in barley, rye and other grain haylage as well as bakery waste fed to cattle that fatally contracted botulism (Divers et al., 1986; Notermans et al., 1981b, Keltch et al., 2000). Similarly, *C. botulinum* type C or D has been isolated from corn and bakery waste fed to cows that died from bovine botulism (Gray and Bulgin, 1982; Heider et al., 2001). While *C. botulinum* types C and D do not cause human botulism, the fact that they have been found in grain and bakery products does suggest the possibility of types A and B spores also being present. However, none of these surveys specifically reported the presence of spores of proteolytic *C. botulinum* in wheat or in flour. Indeed, while several extensive surveys have been done on the microbiology of flour, there is a paucity of data regarding both the incidence and levels of *C. botulinum* in grain-based bakery ingredients. While clostridial spores have been detected in flour, the species tend to be thermophilic in nature and more of a spoilage concern, particularly in canned products where they may be used as ingredients (Elliot, 1980). This does not mean that spores of proteolytic *C. botulinum* are absent in these ingredients since the methodology used in all of the previous microbiological surveys of flour and spices would not specifically detect *C. botulinum.* This can only be achieved by adding designated quantities of these ingredients directly to broth medium capable of

supporting the growth of *C. botulinum* (i.e., cooked meat medium [CMM] or trypticase peptone glucose yeast [TPGY] broth) and incubating at 35°C to 37°C for several days. Cultures would then have to be assayed for botulinum neurotoxin (BoNT) using the mouse bioassay. Only cultures that were toxic to mice, and subsequently neutralized with appropriate type-specific antisera to BoNT, would be considered positive for *C. botulinum*. However, according to Notermans et al. (1997), even though statistically valid sampling plans may be used in surveys, they are unlikely to provide a reliable indication of the true level of contamination since i) only a small proportion of food can be tested and ii) the microorganisms in foods are rarely uniformly distributed. In the absence of sound empirical data confirming the presence and levels of spores of proteolytic *C. botulinum* in flour, spices, and other ingredients, it is impossible to determine the probability of contamination by this pathogen or accurately assess the botulism risk of these bakery ingredients.

1.2.7.2 Spices, herbs, fruits, and vegetables

While flour is the main ingredient in most bakery products, other ingredients such as spices, herbs as well as fresh or dried fruits or vegetables could be potential sources of contamination by spores of proteolytic *C. botulinum* (Bell and Kyriakides, 2000). According to the Advisory Committee for the Microbiological Safety of Food (ACMSF, [2000]), "the addition of

spices to some par-baked breads stored at ambient temperature could potentially contaminate the products with spores of *C. botulinum*, as well as leading to localized areas of higher a_w and/or pH".

Extensive surveys have also been done to assess the microbiological quality of dried spices and herbs. According to Pivnick (1980) spices are not major contributors to foodborne disease. However spore-forming microorganisms capable of causing foodborne illness (i.e., *B. cereus* and *C. perfringens)* have been found in small numbers in several spices. Kim and Goepfert (1971) examined a large number of spices and other dry food ingredients and found *B. cereus* in most products at levels between 1,000 and 5,000 CFU/g. The incidence of contamination with this sporeforming pathogen was seasoning mixes (55%), spices (40%), dry potatoes (40%) and milk powder (37.5%). Kadis et al. (1971) examined several spices used in the production of gravy bases and found anaerobic, thermophilic spore-forming bacteria in all spices tested and concluded that the risk of foodborne illnesses from gravies prepared from commercial gravy bases was minimal. Julseth and Deibel (1974) examined the microbial profile of selected spices and herbs at import, and while thermophilic spore-formers were found, no pathogenic bacteria were found in the products tested. Powers et al. (1976) examined several commercial spices including bay leaves, red pepper, chili powder, cinnamon, garlic powder, mustard powder and oregano. *C. perfringens* was found in 15% of samples with counts ranging from 50 to 2,850 CFU/g. However, *B. cereus* was again the predomi-

nant spore-forming microorganism isolated, and was found in 53% of spices–with counts ranging from 50 to 8,500 CFU/g. Draughton et al. (1980) examined the microbial flora of spices used in the formulation of Mexican-style sauces. The most commonly isolated microorganisms were again *Bacillus* spp. (83% of samples) while *C. perfringens* was present in low numbers in a few samples. Rozanova et al. (cited by Notermans [1993]) isolated *C. botulinum* from fresh parsley and dill. However, although *C. botulinum* spores are frequently isolated from fresh produce, there are few specific reports of their presence in herbs and spices. As with surveys of flour, this paucity of information on the incidence of *C. botulinum* may be a result of the methodology used in surveys of spices.

Even if *C. botulinum* is present in spices in low numbers, there is some evidence that certain spices and their essential oils may have an anti-botulinal effect (De Wit et al., 1979; Huhtanen, 1980; Hall and Maurer, 1985; Ismaiel and Pierson, 1990a,b; Kim and Foegeding, 1993a). Hall and Maurer (1985) reported that extracts of mace (31-ppm), bay leaf (125-ppm) and nutmeg (125-ppm) inhibited neurotoxin production by proteolytic strains of *C. botulinum* in turkey frankfurter slurries. Ismaiel and Pierson (1990b) studied the effects of clove, thyme, black pepper, pimenta, origanum, garlic, onion and cinnamon oils on the growth and germination of *C. botulinum* types A, B and E. They found that while garlic best inhibited spore germination, 50% to 100% inhibition of growth of *C. botulinum* resulted from clove, thyme, cinnamon, pimento and origanum oils at levels of 100 ppm to 200

ppm (Ismaiel and Pierson, 1990b). Liu (2000) has also shown an anti-botulinal effect of spices in formulated value-added seafood products. However, the anti-botulinal effect of spices and/or their essential oils cannot be assumed to negate the potential hazard of outgrowth of contaminating spores of *C. botulinum.* Outbreaks of botulism have resulted from consumption of garlic bread prepared from a garlic-in-oil product (Solomon and Kautter, 1988; Morse et al., 1990) and fried onions (MacDonald et al., 1985; Solomon and Kautter, 1986) although both oils have been shown to have an anti-botulinal effect (De Wit et al., 1979; Ismaiel and Pierson, 1990).

Fruits and vegetables, particularly those in contact with soil or organic material, may be contaminated with *C. botulinum* spores (Notermans, 1993; Hauschild, 1989). Although good agricultural and horticultural practices may reduce the level of contamination, according to Notermans (1993), the "presence of *C. botulinum* on raw fruits and vegetables must always be considered". Growth of *C. botulinum* and neurotoxin production has been demonstrated in several fruits and vegetables including potatoes, spinach, carrots, mushrooms, pumpkin, squash, tomato, apricots, and figs particularly under non-acid conditions (Insalata et al., 1970; Hauschild et al., 1975; Ito et al., 1978; Dodds, 1989; Notermans, 1993; Austin et al., 1998). Furthermore, outbreaks of foodborne botulism have been caused by a variety of fruits and vegetables (potatoes, peppers, tomatoes, potatoes, mushrooms, onions, garlic, olives, peanuts, and hazelnut purée (Chou et al.1988; Angulo et al., 1998; Barker et al., 1977; O'Mahony et al., 1990; Casillas et al., 1978;

Solomon and Kautter, 1988; Notermans, 1993) all of which have been used in the production of sweet and savory bakery foods. Several cases of botulism have been linked to the consumption of garlic-buttered bread. The causative agent in this case was garlic-in-oil which had not been stored at refrigerated conditions and contained pre-formed botulinum neurotoxin (St. Louis et al., 1988; Morse et al., 1990).

1.2.7.3 Other ingredients

While the presence *of C. botulinum* spores in flour has never been specifically reported, they have been occasionally isolated from other potential bakery ingredients when appropriate methodology was used to detect this pathogen in foods. Dairy products, which are used in many bakery formulations, have an extremely low incidence of *C. botulinum* spores and are seldom implicated in outbreaks of foodborne botulism (Collins-Thompson and Wood, 1993). However, spores of proteolytic *C. botulinum* type B were recovered from powdered baby formula milk that was implicated in a case of infant botulism (Peck, 2002). Although soft cheeses have occasionally been responsible for outbreaks (Meyer and Eddie, [cited in Collin-Thompson and Wood, 1993]), a recent survey of dairy foods in Italy found no spores of *C. botulinum* in raw, pasteurized or clotted milk, butter, pasteurized cream or ricotta cheese. However, low levels (<10 spores/g) of this pathogen were found in mozzarella and soft- and processed-cheeses

commonly used as pizza ingredients. Furthermore, 32% of 1,017 samples of mascarpone cream-cheese were contaminated with *C. botulinum* spores (Franciosa et al., 1999). Botulinum neurotoxin type A was also detected in seven of 878 samples of mascarpone produced at a plant involved in an outbreak of foodborne botulism (Aureli et al., 1996; Franciosa et al., 1999).

Honey is also used in some bakery formulations such as breads, cakes, cookies, muffins and bagels. Schocken-Iturrino et al. (1999) found 7% of 85 honey samples contaminated with proteolytic *C. botulinum.* Honey has also been shown to contain low levels (<1 to 10 spores/kg) of *C. botulinum* spores (Hauschild et al., 1988; Dodds, 1993b) although higher levels (10^3 to 10^4 spores/kg) have been reported in honey associated with infant botulism (Dodds, 1993a). Therefore, while flour and spices appear to be relatively low botulism risk ingredients, other bakery ingredients may represent a higher botulism risk based on the incidence and levels of *C. botulinum* found in these ingredients. For this reason, it is best to assess the botulism risk of each bakery product independently based on the ingredients used in its formulation.

1.2.7.4 Final baked products

Although bakery products have contributed to foodborne illness (Bean et al., 1996; Todd, 1996; Potter et al., 1997; Jenson et al., 1994), and MAP high-moisture bakery products have been on the North American market-

place for over twenty years, there has never been a reported outbreak of botulism involving these products, or indeed any high-moisture bakery product (Todd, 1996). While this suggests that *C. botulinum* occurs rarely, if ever, in bakery products, the number of studies that have specifically examined bakery products for *C. botulinum* are very few. Early challenge studies with *C. botulinum* type A and B spores in bread failed to detect neurotoxin in any of the uninoculated control samples (Kadavy and Dack 1951; Wagenaar and Dack 1954; Wagenaar and Dack 1959). Again, these control studies do not mean that endogenous spores of *C. botulinum* were absent in these bakery products. Spores may have been present in extremely low numbers and either failed to grow or produce neurotoxin to levels detectable by the mouse bioassay.

While outbreaks of botulism caused by bakery products have not occurred in North America, in India, a suspected outbreak of foodborne botulism that affected 34 children and resulted in three fatalities was attributed to the growth and production of botulinum neurotoxin type E by *C. butryicum* in sevu, a crisp flat-bread prepared from gram- (pulse) flour. Growth and neurotoxin production was attributed to improper storage of the implicated crisp-bread (Chaudhry et al., 1998). In Italy, an outbreak of botulism resulted from the consumption of tiramisu, a composed (i.e., un-baked) bakery product made with contaminated mascarpone cheese (Aureli et al., 1996).

While sales of bakery products with extended shelf-life are increasing in today's global marketplace, epidemiology suggests that the botulism risk of such products may be low. However, despite the outstanding safety track record of the bakery industry world wide with respect to botulism, there is still concern that the safety of high-moisture, MAP bakery products "may be reliant on a low likelihood of spores being present, which would not be a satisfactory basis for safety" (ACMSF, 2000). Furthermore, according to Lioutas (1988) "an extremely low probability of contamination does not negate the hazard of botulism, it only reduces the risk".

1.2.8 Effect of heat treatment on spore survival

The second condition for foodborne botulism to occur is that the treatment process must be inadequate to inactivate any *C. botulinum* spores that are present, or the product must become contaminated with spores or vegetative cells after processing (Eklund, 1993).

Inadequate thermal processing and consequently, survival of spores of *C. botulinum* in canned products has resulted in outbreaks of botulism (Townes et al., 1996; Franciosa et al., 1997; Squarcione et al., 1999). In order to achieve desirable textural and quality attributes, most bakery products receive only a minimal heat treatment. Bread, for example, is baked at high temperature (~200°C); however, during baking, the temperature in the centre of the loaf rarely exceeds 100°C for more than a few minutes. Therefore, such heat treat-

ments will be inadequate to inactivate spores of many spore-forming bacteria due to their high thermal resistance (D-values [Bryan et al.,1997]). Spores of proteolytic *C. botulinum* are very heat resistant with some strains having a reported $D_{100°C}$ value of 25 minutes (Austin and Dodds, 2001; Austin, 2001). Since the internal temperature of bread during baking rarely reaches >100°C, these spores will readily survive baking and have the potential to germinate, outgrow and produce neurotoxin in products stored under favourable conditions (Soloski and Cryns, 1950; Denny et al., 1969; Marston and Wannan, 1976). Aramouni et al. (1994) monitored the heating profile of home-style canned quick-breads inoculated with *C. sporogenes* PA-3679 (10^4 spores/g), a non-toxigenic clostridium that is closely related to proeteolytic strains of *C. botulinum.* Temperatures at the centres of the quick-breads reached 106°C, 107°C and 108°C when baked in Mason jars at oven temperatures of 177°C, 191°C and 204°C respectively for 50 minutes. These internal temperatures are higher than those desirable to produce breads of acceptable quality. Nevertheless, *C. sporogenes* spores survived the baking process and subsequently grew when products were stored at 35°C. These studies show that spores of proteolytic *C. botulinum,* if present in the flour or any other raw ingredient, could readily survive baking temperatures and could grow to toxic levels under suitable conditions. This scenario has been illustrated by an outbreak of botulism resulting from the consumption of foil-wrapped potatoes that had been baked and then held at ambient temperature prior to being used in the preparation of salad (Angulo et al., 1998). Furthermore, if products are

contaminated with either *C. botulinum* spores or vegetative cells post-processing, *C. botulinum* could grow and produce neurotoxin in the packaged products when stored at ambient temperature.

1.2.9 Growth of and neurotoxin production by *C. botulinum* in modified atmosphere packaged bakery products

The third criterion for MAP bakery products to be implicated in foodborne botulism is that both the product and the surrounding packaging atmosphere must support the growth of and neurotoxin production by *C. botulinum* (Eklund, 1993). High-moisture bakery products typically have a_w between 0.95 and 0.99 (Smith and Simpson, 1995) which exceeds the growth-limiting a_w of 0.94 and 0.97 for proteolytic and non-proteolytic strains of *C. botulinum* respectively (Table 1.12 and Austin and Dodds, 2001). While some high-moisture bakery products such as fruit pies and sourdough breads may have low pH (<4.5), which restricts the growth of *C. botulinum*, most products have pH levels conducive to the growth of this important pathogen. However, it is also important to understand that both the pH and a_w of bakery products may fluctuate during storage. Such changes may result from moisture redistribution, and chemical or microbiological activity, and can therefore play a critical role in product safety. For example, icing which has a low a_w, is not usually a microbiological problem. However, the interface between cake and icing may have a much higher a_w due to moisture migration which encourages microbial growth. Silliker and McHugh (1967) reported such an incident in which *S. aureus* grew at the interface of

cake and icing. The microbial ecology of a product can also influence product pH. Although lactic acid bacteria may reduce the pH and enhance product safety (Lyver et al., 1998; 1999), yeasts and moulds such as *Penicillium, Mycoderma, Trichosporon*, and *Cladosporium* spp. may increase local pH to levels conducive to growth, although the overall pH may remain <4.6 (Odlaug and Pflug, 1979; Notermans, 1993). Botulinum neurotoxin was detected in low acid tomatoes (<pH 4.6) in which *B. licheniformis* had grown and increased product pH to levels favorable to the growth of *C. botulinum* (Montville, 1982). These studies show that food is a complex substrate and that the effect of possible physical, chemical, enzymatic and microbiological events need to be taken under consideration when assessing the potential of the product to support growth of *C. botulinum.*

1.2.9.1 Packaging conditions

Packaging conditions such as gas packaging that excludes O_2, vacuum packaging, or packaging in airtight conditions including jars or cans in which spoilage organisms may consume residual headspace O_2, also favour the growth of *C. botulinum* (Aramouni et al., 1994; Austin et al., 1998). Packaging atmospheres for MAP bakery products include CO_2. Microorganisms differ considerably in their sensitivity to CO_2 and this sensitivity is related to their requirements for O_2. The antimicrobial effect of CO_2 is influenced by the microbial load, the stage of growth, the concentration of CO_2, temperature

and package permeability (Smith et al., 1990). Carbon dioxide is most effective against aerobic bacteria and moulds, with concentrations of CO_2 as low as 5% to 10% being used to suppress growth of these microorganisms (Legan and Voysey, 1991; Ooraikul, 1991; Smith, 1992). However, gas-packaged bakery products are typically stored with higher amounts of CO_2 (Table 1.16). *C. botulinum* is not inhibited by elevated levels of CO_2 commonly used in gas-packaging of bakery products and spore germination may actually be stimulated by elevated levels of this gas. Although Coyne (1933) reported that CO_2 inhibited *C. sporogenes*, Parekh and Solberg (1970) reported no difference on the growth of *C. perfringens* in 100% CO_2 or 100% N_2. Doyle (1983) showed that growth and neurotoxin production by vegetative cells of *C. botulinum* were delayed with one atm pCO_2 relative to one atm pN_2; however, even at elevated pressures (8.8 atm pCO_2), neurotoxin production was not completely prevented. Furthermore, there is evidence that CO_2 enhances germination of spores and growth of *C. botulinum* depending on the concentration (Foegeding and Busta, 1983; Kim and Foegeding, 1993b).

Although most atmospheres used for gas-packaged bakery products are composed of mixtures of CO_2 and N_2 only, the exclusion of O_2 in the package atmosphere is not a prerequisite for growth of and neurotoxin production by *C. botulinum*. There is substantial evidence that *C. botulinum* can grow in air-packaged foods and in products containing high levels (20% to 100% [vol/vol]) of O_2 in the package headspace (Snyder, 1996; Clavero et

TABLE 1.16. *Typical gas mixtures used in gas-packaged bakery products*

	% Gas (vol/vol)		
	CO_2	N_2	O_2
Sliced bread	100	—	—
Rye bread	100	—	—
Buns	100	—	—
Brioche	100	—	—
Cakes	100	—	—
Maderia cake	65	35	—
Madeira cake	80	20	—
Tea cakes	50	50	—
Danish pastries	50	50	—
Crèpes	60	40	—
Croissants	100	—	—
Crumpets	100	—	—
Crumpets	60	40	—
Pita bread	99	1	—
Pita bread	73	27	—

Adapted from Goodburn and Halligan (1988); Ooraikul (1991)

al., 2000; Dufresne et al., 2000a, b). Whiting and Naftulin (1992) found that spores of proteolytic *C. botulinum* germinated in ≤1% oxygen and that vegetative cells could grow in 15% oxygen. Spore germination can occur at higher oxidation-reduction growth. However, once germination is initiated, growth may be rapid as the ORP is rapidly reduced due to spore metabolic activitymination can occur at higher oxidation-reduction potential (ORP) values than (Kim and Foegeding, 1993b). Furthermore, pockets of food may be sufficiently anaerobic, or have ORPs conducive to germination and growth and although the optimum ORP for growth is -350 mV, growth may be initiated at +30 to +250 mV (Kim and Foegeding, 1993b). Several researchers have recommended that atmospheres containing altered levels of CO_2 be considered cautiously (Hintlian and Hotchkiss, 1986; Farber, 1991; Austin et al., 1998). It is also important to realize that respiratory and fermentative activity by spoilage microorganisms can contribute to the CO_2 level within the packaged product (Smith and Simpson, 1995).

1.2.9.2 Substrate

The potential of bakery products to be a suitable substrate for the growth of proteolytic strains of *C. botulinum* has been well established in several inoculation studies. Although Edmonston et al. (1923) were unable to recover *C. botulinum* from two loaves of bread that had been inoculated before baking and then stored at room temperature for two days, Bever and

Halvorson (1947) demonstrated that neurotoxin could be produced by *C. botulinum* in a medium of sterile bread crumbs containing calcium or sodium propionate at levels of 0.2% to 1.4% when the pH of the medium was between 4.5 and 9.6. Concern was also expressed about the safety of canned bread intended for military rations (Soloski and Cryns, 1950). Subsequent studies confirmed that canned bread could support growth and neurotoxin production and determined that the conditions necessary for the safe production of this product would be a pH <5.4 and a moisture content of 35% (corresponding to a_w of ~0.95 [Ulrich and Halvorson, 1949; Soloski and Cryns, 1950; Kadavy and Dack, 1951]). However, increasing the pH of the product, even slightly, resulted in toxigenesis. Growth and neurotoxin production was observed in inoculated canned bread of pH 5.8 and a moisture content >36% and in canned steamed chocolate nut-bread at pH 6.8 and 36% moisture (Wagenaar and Dack, 1954;1960). Bread of pH 4.8 was determined to be safe as no growth occurred, and furthermore, the number of inoculated spores decreased during ambient storage in an equilibrium relative humidity (ERH) of 97% (Ingram and Handford, 1957). However, the safety of the bread depended on maintaining its pH throughout storage as spores were still viable after six months. Temperature differences within the canned bread that occurred during freezing, thawing or reheating, resulted in moisture migration and uneven moisture distribution in the bread (Weckel et al., 1964). The pH of thawed canned bread was unchanged but the pH (5.5 to 6.7) of the moisture that developed within the can was high

enough to support the growth of *C. botulinum*. These early studies demonstrate the need for the incorporation of multiple barriers into a food product to ensure its safety, particularly in products stored at ambient temperature. Canned military ration bread has been replaced by a bread product that is re- formulated to include preservatives and humectants and that is packaged with an oxygen absorbent (Powers and Berkowitz, 1990). Although bread is no longer canned for military rations, canned breads are commercially available in Japan and in South Africa (Lombard et al., 2000).

Home-style canned quick-breads, often containing fruits, nuts and vegetables, are baked sometimes in Mason jars and subsequently hermetically sealed before cooling to form a vacuum. Such breads are available by mail order and are promoted in magazines and on the Internet and claim to have a shelf-life at room temperature of six months or longer. Furthermore, although no longer recommended by suppliers of commercial canning jars, instructions for home preparation and storage of canned quick-bread and cake products are still available in print and on the Internet. Aramouni et al. (1994) demonstrated the survival and growth of *C. sporogenes* PA 3679 spores inoculated into home-style canned banana nut bread, and in addition, isolated endogenous spores of *C. sporogenes*. These authors concluded that further work was needed to determine the safe processing procedures for this type of product.

All of these challenge studies clearly demonstrate that many high-moisture bakery products, depending on their a_w and pH, are ideal

substrates for the growth of *C. botulinum*. Furthermore, if contaminated with spores of proteolytic *C. botulinum*, either pre- or post-baking, spores could outgrow and produce neurotoxin regardless of the packaging atmosphere surrounding the product.

1.2.10 Acceptability and consumption of products containing pre-formed botulinum neurotoxin

The final condition necessary for botulism to result from consumption of contaminated bakery products is that the food must be acceptable and must be consumed without further cooking, or after insufficient heating, to inactivate pre-formed neurotoxin.

1.2.10.1 Sensory and shelf-life studies

Although MAP can extend the shelf-life of bakery products by reduction of spoilage microorganisms, the loss of competitive microflora may enhance the growth of and neurotoxin production by *C. botulinum*. Consequently, toxigenesis may precede spoilage, which is a highly dangerous scenario. Toxigenesis has been shown to precede spoilage in fresh pasta (Glass and Doyle, 1991), in raw and vacuum-packaged baked potato (Sugiyama et al., 1981; Solomon et al., 1998), in N_2 packaged sandwiches (Kautter et al., 1981), and in cold- and hot-smoked fish packaged under various modified atmospheres (Dufresne et al., 2000b).

While MAP extends the shelf-life and keeping quality of high-moisture bakery products, secondary spoilage problems can still occur. Examples of these problems include discoloration, off-odors, staling and gas production by surviving spore-forming bacteria (e.g. *C. sporogenes* or *Bacillus* spp.) or by post-processing contaminants, such as heterofermentative strains of lactic acid bacteria or yeasts from the bakery environment. Such gas production can result in packages or cans having a blown appearance and being rejected by consumers (Powers et al., 1988; Aramouni et al., 1994; Smith and Simpson, 1995; 1996). Mould growth can also occur in MAP bakery products as a result of low levels of residual O_2 in the package headspace. Smith et al. (1986) showed that moulds were capable of growing in as low as 0.6% O_2, and even grew in the presence of elevated levels (60% to 80%) of headspace CO_2. All of these spoilage problems can occur alone or in conjunction with each other to limit the sensory quality, and hence shelf-life of MAP bakery products. While MAP can extend shelf-life by reducing spoilage microorganisms, the loss of competitive microflora may enhance the growth of other spore-forming bacteria in addition to *C. botulinum*. Background microflora such as *Bacillus* spp. and *Pænibacillus polymyxa* may also influence product safety through production of peptides with anti-botulinal activity as shown in recent challenge studies with value-added fish nuggets (Lyver et al., 1998; 1999) and vegetable purées (Girardin et al., 2002).

Nevertheless, there is a paucity of data on the sensory acceptability and safety of MAP bakery products if they are contaminated with *C. botulinum* spores. Such information is critical to the determination of whether spoilage precedes toxigenesis or *vice versa* and the margin of safety in such MAP products. These studies confirm earlier studies that bakery products are a suitable substrate for the growth of *C. botulinum*. Furthermore, they demonstrate that if such high-moisture products are contaminated with *C. botulinum*, they could, depending on their shelf-life, pose a serious health hazard since they could be toxic yet still be acceptable to the consumer. In challenge studies, spoilage preceding toxigenesis is generally considered to decrease the risk of toxic products being consumed. However, overt spoilage cannot always be regarded as a reliable barrier of food safety. Fortunately, there have been no reported outbreaks of botulism caused by bakery products in North America. However, it must be assumed that the crisp-bread (sevu) involved in the reported outbreak of botulism in India was sensorially acceptable at the time of toxigenesis (Chaudhry et al., 1998 and Table 1.14) and in addition, the tiramisu responsible for a botulism outbreak in Italy was consumed although it was described as being malodourous at the time of consumption (Aureli et al., 1996 and Table 1.14).

Clearly, more research is required on the safety, shelf-life and sensory characteristics of high-moisture bakery products with extended shelf-life. Sensory data from one set of challenge studies cannot be extrapolated to

other products due to differences in products' a_w, pH, packaging and storage conditions.

1.2.10.2 Effect of heating on pre-formed neurotoxin

Since most bakery goods are "cook and hold" products and are not heated prior to consumption, there is no additional safety margin for destroying neurotoxin that may have been formed in a product during storage. While some products may be toasted and par-baked products may be minimally baked to brown the crust, temperatures, which would only reach 70°C to 80°C at the centres of these products, may be insufficient to inactivate all pre-formed neurotoxin. Simpson (1993) showed that microwave heating for five and ten minutes at half- and full-power in a domestic microwave oven (800-W) destroyed pre-formed neurotoxin in a sous-vide spaghetti-meat-sauce product. The internal temperature of products heated at half- and full-power for 5 and 10 minutes ranged from 85°C to 95°C and from 95°C to 99°C respectively. While the levels of neurotoxin were not measured either prior to or following microwave heating, it was evident from the mouse bioassay that microwaving denatured the pre-formed neurotoxin in the sous-vide product. However, microwave heating or any other form of heating, cannot be regarded as an additional safety factor to reduce the botulinum risk of MAP minimally-processed bakery products. This can only be achieved through

good manufacturing practices as well as the use of additional barriers to control the growth of *C. botulinum* in order to ensure product safety.

1.2.11 Measures to control growth of *C. botulinum*

Although cases of foodborne botulism are rare, the severity of the intoxication means that the potential for growth of and neurotoxin production by this pathogen in bakery products should be seriously considered on a case-by-case basis and barriers to the growth of and neurotoxin production by this pathogen may need to be incorporated into the bakery product and/or its package to ensure product safety.

1.2.11.1 Water activity and pH

The a_w of bakery products is one of the most important factors influencing the growth and neurotoxin production by *C. botulinum*. Denny et al. (1969) demonstrated the importance of a_w on the growth of proteolytic *C. botulinum*. In seven varieties of inoculated canned, low-acid fruit- and vegetable-breads stored for up to six years, neurotoxin was produced at a_w >0.955 but not at $a_w \leq 0.955$. Of all breads with a_w between 0.955 and 0.977, 50% became toxic within two years. *C. botulinum* did not grow or produce neurotoxin in low acid, apple coffee cake or spice cake with an a_w of 0.93 when inoculated with 10^5 spores/g and incubated at 30°C (Powers et al., 1988). Reduction of a_w can be achieved through the addition of solutes such

as sugars, salts, polyalcohols or fractionated milk products. However, a limitation of this approach, particularly with high-moisture bakery products, is that the inhibitory level of required solutes may result in adverse changes to the sensory and textural properties of products. While the use of humectants (e.g., glycerol) may overcome this problem, it is a more costly approach.

Another barrier which can be used to enhance safety is pH. Reduction of pH can be achieved through the use of acidulants such as organic acids (e.g., citric, lactic, acetic acids) or cultures of lactic acid bacteria (e.g., sourdough cultures). Growth and neurotoxin production were inhibited in cooked shelf-stable noodles acidified to pH <4.6. However, neurotoxin production occurred in one sample of noodles in which the pH had increased as a result of microbial growth, demonstrating the importance of incorporating multiple barriers to the growth of *C. botulinum* (Ikawa, 1991).

Combinations of inhibitory factors often function interdependently in food. Studies in media (Baird-Parker and Freame, 1967; Montville, 1983) have demonstrated that decreasing either a_w or pH has the effect of increasing the minimum level of the other factor required for growth of *C. botulinum*. Dodds (1989) reported that neurotoxin production in vacuum-packaged cooked potato was significantly affected by storage temperature, a_w, pH as well as interactions between i) a_w and pH; and ii) a_w and storage time.

1.2.11.2 Selected additional barriers to the growth of *C. botulinum*

Leistner (1994) has described how combinations of restrictive factors (a_w, pH, temperature and competing microflora) can be used as multiple barriers that act in concert to provide a hurdle effect to microbiological growth by targeting distinct metabolic processes. Such hurdles may act individually, additively or synergistically to restrict growth.

Chemical or natural preservatives may have such action and as described by King (1981) should possess the following features: i) exhibit a broad antimicrobial spectrum; ii) be non-toxic to humans; iii) be effective at low concentrations; iv) have a minimal effect on pH of products; v) not affect the odour, colour, and/or flavour of products at the intended level of use; vi) be available in a dry form; vii) have good water solubility; viii) be non-corrosive; ix) be stable during storage; x) have no adverse effects on fermentation or loaf characteristics; and xi) be cost effective.

Sorbates and propionates have been traditionally used in bakery products as antimicrobial agents, largely to delay spoilage from moulds and *Bacillus* spp. (O'Leary and Kralovec, 1941; Seiler, 1968). The effectiveness of weak acid preservatives depends largely on the pH of the product and dissociation constant (pKa) of the acid since the undissociated molecule must penetrate the phospholipid bacterial cell wall. Factors which may decrease the effectiveness of preservatives in bakery products include pH >pKa of the preservative and decreased solubility at ambient temperature.

Propionic acid or its salts, at levels permitted in bread (0.2%), were ineffective in preventing neurotoxin production by *C. botulinum* in canned bread (pH 5.4 [Kadavy and Dack, 1951]) or in a medium of bread crumbs (Bever and Halvorson, 1947). However, propionates have been effective at higher concentrations (Miller et al., 1993). A level of 6% was needed before significant delay was observed. While higher levels of propionates may delay the growth of *C. botulinum,* such levels, even if they were legally permitted, would result in off-flavours and sensory rejection of baked products.

Sorbic acid can also inhibit growth and neurotoxin production at pH <6 (Ando, 1973; Smoot and Pierson, 1981). Lund et al. (1987) demonstrated that inhibition of *C. botulinum* by sorbic acid is mainly due to the undissociated acid and developed a model for the probability of growth of a single spore in media between pH 4.9 and 7.0. They concluded that 1,000 mg/kg sorbic acid, in combination with other inhibitory factors would likely provide effective control against the growth of *C. botulinum* in foods at pH 4.5 to 5.5. Briozzo et al. (1985) reported that 0.3% potassium sorbate effectively controlled *C. botulinum* neurotoxin production in a model cheese system when the a_w was reduced to 0.974 and the pH reduced to 5.7. However, since most bakery products, with the exception of sour-dough, have pH >5, chemical preservatives lose their antimicrobial effect due to their low dissociation constants.

1.2.11.3 Potential novel barriers

Consumers are becoming more aware and concerned about the use of food preservatives, and consequently, are demanding more preservative-free food. In a recent survey conducted by Agriculture and Agri-Food Canada, 75% of consumers were concerned about the levels of preservatives in food products while only 19% were concerned about irradiation. The response by the food industry to these concerns has been to use more natural preservatives such as cultured products and vinegar as well as MAP to extend the shelf-life of bakery products. Furthermore, the gases used in MAP (CO_2, N_2) are natural and do not need to be declared on a product's label (Smith and Simpson, 1996).

Ethanol, which is well known for its anti-mycotic effect, has been used in the shelf-life extension of MAP bakery products. Ethanol has been shown to increase the mould-free shelf-life of bread (Seiler, 1978; 1989; Seiler and Russell, 1991; Vora and Sidhu, 1987; Salminen et al., 1996), par-baked pizza (Seiler, 1989), pita bread (El-Khoury, 1999) and pasta (Giavedoni et al., 1994). Ethanol vapour has also been reported to inhibit fermentation in apple turnovers (Smith et al., 1987) as well as yeast and mould growth in pita bread (Black et al., 1993). Although Hall and Spencer (1964) observed a three- to five-day increase in the shelf-life of chickens that had been pre-dipped in ethanol, very little is known about the effect of ethanol on bacteria in food. In media, bacteria vary in their response to ethanol.

Yamamoto et al. (1984) found that low levels (0 to 2%) of ethanol inhibited germination of spores of *B. subtilis* and *B. pumilus* to vegetative cells. Cook and Pierson (1983), using a nutrient broth, inhibited *C. botulinum* type A with 10% ethanol; however, a minimum inhibitory concentration was not reported. Furthermore, it was not clear whether inhibition was reversible or not. Ethanol has been shown to be more effective in the vapour phase than in the liquid phase (Shapero et al., 1978). Therefore, a practical and safe method of generating ethanol vapour in MAP bakery products is through the use of ethanol vapour-generating sachets. These commercially available interactive sachets (Ethicap® or Antimold® 102; Freund Company Ltd., Japan) consist of food grade alcohol (55% by weight) absorbed on to silicon dioxide powder (35%) and contained in a sachet made of a copolymer of paper/ethyl vinyl acetate copolymer. Vanilla is also added to mask the smell of ethanol. Sachet sizes range from 0.6-G to 6-G containing 0.3 g to 3 g ethanol that can be evaporated and the choice of sachet depends on i) product weight; ii) product a_w; and iii) the desired product shelf-life. When food is packed with an Ethicap® sachet, moisture is absorbed from the food and ethanol vapour is released from encapsulation and permeates the package headspace. However, both the initial and final level of ethanol vapour in the package headspace is a function of sachet size and product a_w. Another type of ethanol vapour generator produced by Freund, is Negamold®. This dual functional sachet absorbs oxygen and generates ethanol, albeit it at ~50% of the ethanol vapour generated by Ethicap®

sachets. Ethanol vapour may warrant investigation as an agent to control growth of *C. botulinum* in high-moisture bakery proucts. However, the effectiveness of ethanol vapour may be limited by the water activity of high-moisture bakery products since ethanol is more effective at low a_w (Shapero et al., 1978).

Additional barriers to the growth of and neurotoxin production by *C. botulinum* in MAP bakery products, in addition to enhancing the safety and shelf-life of the food, should be ideally "natural barriers" since modified-atmosphere packaging appeals to consumers' and processors' concerns about "preservative-free" foods. Many such antimicrobial compounds can be found from microbial, plant and animal sources. Other novel barriers which may warrant investigation as anti-botulinal barriers are bio-preservatives (e.g., nisin) and antimicrobial essential oils, such as oil of mastic. These barriers have both proven effective in the control of *B. cereus,* a spore-forming pathogen of concern in high-moisture crumpets (Koukoutsis, 2002).

Biologically based preservation of food has been achieved using lactic acid bacteria (LAB) and/or their metabolic products, to improve or ensure the safety and quality of foods including fermented meats, dairy products, cereals and breads and are perceived as natural and healthy. LAB may inhibit spoilage and/or pathogenic microorganisms through several modes i) *in situ* production of lactic and acetic acids which can be controlled by manipulating the fermentable carbohydrate and temperature, ii) production

of diacetyl, hydrogen peroxide, and/or iii) production of bacteriocins. Sourdough cultures may have the potential to be used as an additional barrier to growth of *C. botulinum.* Sourdough bread is fermented by starter cultures which are characterised by lactic acid bacteria and yeasts which co-ferment to use otherwise unavailable carbohydrates and amino acids (Gobbetti and Corsetti, 1997). While there is abundant literature on the effects of sourdough on the flavour and improved shelf-life of bread, there is very little information available on the effect of sourdough culture on common foodborne pathogens and none to date on its effect on growth of and neurotoxin production by *C. botulinum.*

Sourdough has the potential to provide an additional barrier to growth of and neurotoxin production by *C. botulinum* in bakery products because i) such cultures are already used in the bakery industry; ii) fermentation can be controlled to result in pH that limits growth and neurotoxin production; iii) LAB used in starter cultures produce lactic acid, acetic acid, and H_2O_2 which may contribute an antibotulinal effect; and iv) inhibitory action may be achieved during fermentation and after baking. As well, adding sourdough has additional benefits of improving flavour, texture and colour (increased Maillard reaction) as well as reducing other microbial contamination.

Essential oils demonstrating antimicrobial activity generally contain a large number of compounds (Beuchat and Golden, 1989). Mastic "gum", a resinous exudate of *Pistacia lenticus* var. *chia*, is used as a food ingredient and flavoring agent, particularly in Mediterranean bakery products. Mastic

has antimicrobial activity which is largely associated with its high α-pinene content (Magiatis et al., 1999). Although this essential oil has shown inhibitory activity against many bacteria and fungi including *S. aureus, Lactobacillus plantarum, B. cereus, S. enteritidis* (Tassou and Nychas, 1995; Iuak et al., 1996; Hussain and Tabji, 1997), the anti-botulinal effect of mastic is not known.

1.3 RESEARCH OBJECTIVES

This work has been undertaken in view of the previously justified safety concerns about high-moisture MAP bakery products stored at ambient temperature, and in addition, due to the paucity of data concerning the safety of these products with respect to *C. botulinum*. The overall objectives of this work are to determine the effect of selected additional barriers: a_w, pH, level of inhibitors, ethanol, bio-preservatives, alone or in combination with each other on the growth of and neurotoxin production by proteolytic strains of *C. botulinum* in high-moisture bakery products packaged under selected modified atmospheres and stored at ambient temperature.

Specifically:

i. To determine the potential of selected modified-atmosphere packaged high-moisture bakery products to support growth of and neurotoxin production by proteolytic *Clostridium botulinum* and to determine the time-to-toxicity of selected high-moisture bakery products packaged under various modified atmospheres.

ii. To model the effects of a_w, and pH, in combination with selected additional barriers, on the growth of and neurotoxin production by *C. botulinum* in media and in a high-moisture bakery product.

iii. To investigate whether the nature of the effect of ethanol against *C. botulinum* is static or cidal.

iv. To determine the potential of mastic resin and oil as an additional barrier to the growth of and neurotoxin production by *C. botulinum*.

v. To compare a rapid immunoassay with the mouse bioassay for the detection of botulinum neurotoxin in microbiological challenge studies with bakery products.

PREFACE TO CHAPTER 2.0

A review of the literature has indicated that the potential exists for growth of *C. botulinum* in high-moisture bakery products packaged under modified atmospheres and stored at ambient temperature. Thus, a study was undertaken to determine if such bakery products could indeed support growth of and neurotoxin production by this pathogen.

2.0 CHALLENGE STUDIES WITH *CLOSTRIDIUM BOTULINUM* IN HIGH-MOISTURE BAKERY PRODUCTS PACKAGED UNDER MODIFIED ATMOSPHERES

2.1 INTRODUCTION

Modified-atmosphere packaging (MAP) is a relatively new technology for the shelf-life extension of food. Several methods can be used to modify the atmosphere within the package including vacuum packaging, gas packaging with mixtures of CO_2 and N_2, oxygen absorbents and ethanol vapour generators (Smith et al., 1986; Powers and Berkowitz, 1990; Smith and Simpson, 1995). Both vacuum and gas packaging have been used to extend the shelf-life of fresh and processed foods in Europe and Australia, while oxygen absorbent and ethanol vapour generator technologies have been most commonly used in Japan, especially for the shelf-life extension of bakery products (Smith et al., 1990). Studies in our laboratory have shown that gas packaging and oxygen absorbent technology can be used, in conjunction with reformulation, to delay both mould growth and staling for approximately six weeks at ambient temperature (Assouad, 1996). However, little is known about the public-health safety of such reformulated/packaged bakery products. MAP conditions may favour the growth of pathogenic bacteria, particularly *C. botulinum,* which may grow to hazardous levels before the

food is overtly spoiled (Hintlian and Hotchkiss, 1986; Farber, 1991; Austin et al., 1998).

Although MAP of bakery products is a recent packaging technology, early studies have shown that canned bakery products are a suitable substrate for growth of and neurotoxin production by *C. botulinum*. Bever and Halvorson (1947) demonstrated growth of *C. botulinum* in a medium of sterile canned bread crumbs, while others reported neurotoxin production by *C. botulinum* types A and proteolytic B inoculated into canned bread (Ulrich and Halvorson, 1949; Kadavy and Dack, 1951; Wagenaar and Dack, 1954, 1959; Denny et al., 1969). However, very little is known about the ability of commercially available, modified-atmosphere packaged bakery products to support the growth of *C. botulinum*.

Therefore, the objective of this study was to determine the potential of selected high-moisture bakery products, packaged under various modified atmospheres and stored at ambient temperature, to support growth of and neurotoxin production by proteolytic *C. botulinum*.

2.2 MATERIAL AND METHODS

2.2.1 Bakery products

Three high-moisture bakery products were used in this study: crumpets (a_w 0.990, pH 6.00), pizza crust (a_w 0.960, pH 5.62), and bagels (a_w 0.944, pH 5.63). Crumpets were prepared from all-purpose flour, sugar, instant yeast, reconstituted dry milk, salt, baking soda, and anti-staling amylase (Novamyl; Novo Nordisk; Danbury, CT) according to the method of Witty and Schneider Colchie (1979). Crumpets were baked by pouring 65 g of batter into greased, cylindrical moulds on an oiled griddle (Moffat Appliances; Montreal, QC) preheated to 204°C. Crumpets were baked for approximately 9 minutes until the surface was no longer wet and holes had formed on the top of the crumpets.

Pizza crusts were prepared from all-purpose flour, dry instant yeast, sugar, canola oil, salt, and amylase (Novamyl) as outlined by Hasan (1997). Pizza crusts were baked for 5 minutes at 177°C in a convection oven (Garland Convection Oven (t) TE-3,4-CH, Commercial Ranges Ltd; Mississauga, ON).

Bagels were formulated from hard-wheat white flour, malt, sugar, eggs, compressed yeast, canola oil, water, anti-staling amylase, honey and sesame seeds according to a commercial formulation. Bagels were

baked on metal sheets for 18 minutes (9 minutes per side) in a Garland convection oven at 204°C.

Following baking, all bakery products were cooled to room temperature, placed in polyethylene bags, and stored at -28°C for one week until needed.

2.2.2 Water activity

Water activities (a_w) of crumpets, pizza crusts and bagels were determined using a previously calibrated Decagon CX-2 water activity metre (Decagon Devices, Inc; Pullman, WA).

2.2.3 Preparation of spore inoculum/sample inoculation

A composite inoculum of *C. botulinum* spores was prepared from four type A strains (A6, 17A, 62A, and CK2A) and five proteolytic type B strains (MRB, IB1-B, 13983IIB, 368B, and 426B). Spore crops of each strain were prepared separately, enumerated as described by Hauschild and Hilsheimer (1977), and stored at -80°C. An equal number of spores of each strain were then combined to form a single suspension of approximately 3 x 10^7 spores/mL. The spore mixture was heat shocked at 75°C for 20 minutes prior to sample inoculation. Inoculum levels were verified prior to sample inoculation by plating appropriate dilutions, in duplicate, on McClung Toabe Agar (Difco, Becton-Dickinson; Sparks,

MD) containing 0.5% yeast extract (Difco) and egg-yolk and incubating anaerobically in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 at 35°C for three days. Bakery products were stab inoculated at six distinct locations with 180 µL (6 x 30 µL) of inoculum to provide an inoculum level of 5 x 10^4 spores/g. Control samples were inoculated in a similar manner with an equivalent volume of 0.1% peptone water.

2.2.4 Packaging

All inoculated and control crumpets, pizza, and bagels were packaged under the following atmospheres: i) air, ii) air with an AgelessFX®$_{200}$ oxygen absorbent (Mitsubishi Gas and Chemical Company; Japan) or iii) CO_2:N_2 (60:40). Samples (two crumpets, one pizza slice, or one bagel) (~85 to 90 g) were placed, in duplicate, in 210-mm x 210-mm high gas barrier Cryovac bags (O_2 transmission rate: 3 to 6 cc/m^2/day, 1 atm at 4.4°C, 0% RH; Cryovac; Mississauga, ON). For air-packaged samples, bags were sealed using an impulse heat-sealer. For samples packaged in air with an oxygen absorbent, an AgelessFX®$_{200}$ sachet was placed inside the bag prior to sealing with an impulse heat-sealer. Gas packaging was achieved using a Multivac chamber type heat-seal packaging machine (model SP-300H; Multivac, Inc; Kansas City, MO) to obtain the desired mix of CO_2 and N_2 (60:40). All packaged samples were stored at 25°C.

2.2.5 Headspace gas

Packaged bakery products were sampled for headspace gas composition by withdrawing gas samples using a 0.5-mL gas-tight pressure-Lok® syringe (Precision Sampling Co; Baton Rouge, LA) through a septum previously attached to the outside of each package. Headspace gas was analysed with a Varian gas chromatograph (model 3300, Varian Canada, Inc; Montreal, QC), fitted with a thermal conductivity detector and using Porapack Q and molecular sieve 5A (80-100 mesh) columns (Chromatographic Specialities; Brockville, ON) in series. Peaks were recorded and analysed with a Varian integrator (model 4270, Varian Canada, Inc; Montreal, QC).

2.2.6 Sensory analysis

Sensory analysis was carried out on packaged products by a three member untrained panel. Packages were evaluated subjectively for swelling and for the presence of visible mould growth. Products were also assessed for texture, colour and odour using a six-point hedonic scale (0 = no deterioration; 5 = extreme deterioration). To evaluate odour, packages were opened and the characteristic odour was described. For texture, colour and odour, a score of 2.5 was taken as the cut-off for acceptability and termination of shelf-life.

2.2.7 Enumeration of *C. botulinum*

Each sample was aseptically transferred to a stomacher bag. A 1:3 dilution was prepared by adding twice the sample weight of 0.1% peptone water and then stomaching for two minutes using a Colworth Stomacher (Seward Medical Stomacher, London, UK). Decimal dilutions were subsequently prepared from this initial dilution.

Growth of *C. botulinum* was determined as described previously (Austin et al., 1998; Lyver et al., 1998). *C. botulinum* was enumerated by spread plating 0.1 mL of appropriate decimal dilutions of the 1:3 homogenised sample, in duplicate, on *Clostridium botulinum* Isolation (CBI) Agar (Dezfulian et al., 1981). CBI plates were incubated in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 for 72 hours at 35°C. Lipase-positive colonies, characteristic of *C. botulinum*, were enumerated and identification was confirmed by neurotoxin neutralisation assays on trypticase peptone glucose yeast extract (TPGY) broth cultures of randomly selected colonies.

2.2.8 Neurotoxin assay

The remaining homogenised samples (1:3 dilution) were centrifuged at 23,500 x g for 20 minutes at 4°C. *C. botulinum* neurotoxin was detected as described previously (Austin et al., 1998). An aliquot of the sample supernatant was filtered through a 0.45-μm filter (Acrodisc,

Gelman Sciences; Ann Arbor, MI) and 0.5 mL of this filtrate was injected intra-peritoneally into each of two 20- to 28-g mice (Charles River; QC). Mice were observed for up to 72 hours for typical signs of botulism including ruffled fur, pinched waist, laboured breathing, limb paresis and general paralysis. Mice showing severe distress were euthanised by asphyxiation with CO_2. Neutralisation of neurotoxin was performed on randomly selected positive samples using antisera (Connaught Laboratories; North York, ON) to botulinum neurotoxins to confirm that toxicity was due to botulinum neurotoxin.

All raw ingredients used to formulate bakery products were screened for endogenous spores of *C. botulinum* as described by Austin and Blanchfield (1996). Detection of *C. botulinum* was confirmed by the mouse bioassay and neurotoxin neutralisation as described previously.

2.2.9 Changes in pH

The pH was determined using a previously calibrated Fisher Accumet® pH meter (Fisher Scientific; Ottawa, ON). Portions of sample supernatant were transferred to sterile glass tubes and pH measurement was made by immersing the electrode directly into the tubes.

2.2.10 Statistical analysis

Data were analysed using analysis of variance (ANOVA) for a randomised complete block design (Steel and Torrie, 1980) using Prism 2.01 (GraphPad Software, Inc.; San Diego, CA).

2.3 RESULTS AND DISCUSSION

In this screening study crumpets, pizza, and bagels were formulated with amylase to delay staling, inoculated with *C. botulinum* (5 x 10^4 spores/g), packaged in air and under MAP conditions and monitored for neurotoxin production after 42-days storage at ambient temperature (25°C). This storage period was chosen since previous studies had shown that bagels reformulated with amylase and packaged under MAP conditions could remain mould- and stale-free for up to 42 days at 25°C (Assouad, 1996).

2.3.1 Headspace gas

Changes in headspace gas composition are summarised in Table 2.1. For air-packaged crumpets, with and without an oxygen absorbent, headspace O_2 decreased to <1%, while CO_2 increased to 73% to 75%. For gas-packaged (CO_2:N_2 [60:40]) crumpets, CO_2 remained at ~60% while headspace O_2 was ~8% (Table 2.1), indicating leakage. Air-packaged crumpets, with and without oxygen absorbents, were visibly swollen, due to CO_2 production within the packages. Similar changes in headspace gas were observed for all packaged pizza (Table 2.1). All packages of pizza were visibly swollen. The changes in headspace gas can be attributed to the growth of spoilage microorganisms, specifically yeasts, moulds and bacteria. Smith et al. (1983), reported CO_2 production

TABLE 2.1. *Summary of changes[a] in bakery products inoculated with 5 x 10⁴ spores/g* C. botulinum *after 42-days storage (25°C)*

	Swelling	Mould	Sensory Scores[b]			pH	% Headspace Gas (v/v)			
			Colour	Texture	Odour		Initial		Final	
							O_2	CO_2	O_2	CO_2
Crumpets										
Air	+	+	2	5	5[f]	5.74	20	<1	<1	76
Air + oxygen absorbent	+	-	1	5	5[f]	5.84	20	<1	<1	73
CO_2 / N_2 (60:40)	-	+	1	5	5[f]	5.71	0	60	8	59
Pizza crust										
Air	+	+	0	4.5	4.5[f]	5.43	20	<1	<1	41
Air + oxygen absorbent	+	-	0	3	4.5[f]	5.59	20	<1	6	50
CO_2 / N_2 (60:40)	+	-	0	2.5	2.5[f]	5.44	0	60	<1	84
Bagels										
Air	-	+	0	2	3.5[s]	5.46	20	<1	<1	21
Air + oxygen absorbent	-	-	0	2.5	1.5[s]	5.60	20	<1	<1	<1
CO_2 / N_2 (60:40)	-	+	0	2.5	1.5[s]	5.61	0	60	<1	43

[a] Average of duplicate samples

[b] Scale of 0 to 5; 0 = no deterioration, 5 = extreme deterioration

[f] Fruity odour

[s] Stale odour

and swelling in modified-atmosphere-packaged crumpets due to the growth of heterofermentative lactic acid bacteria. Similar, but less dramatic changes in headspace gas were observed for all packaged bagels. However, none of the packaged bagels were visibly swollen after 42 days (Table 2.1).

2.3.2 Sensory evaluation

Changes in sensory analysis scores (colour, texture, and odour) for all packaged bakery products after 42 days are summarised in Table 2.1. All packaged crumpets were unacceptable after 42-days storage at ambient temperature. All crumpets, regardless of packaging conditions, were discoloured and had a soft, mushy texture, and a strong, sharp, "fruity" odour. Mould growth was observed on all crumpets with the exception of crumpets packaged in air with an oxygen absorbent. The presence of mould in CO_2 packaged crumpets can again be attributed to the leakage of headspace O_2 to levels conducive to mould growth.

Pizzas packaged in air with and without an oxygen absorbent, were sensorially unacceptable after 42 days at ambient temperature. Pizza had a crumbly texture and a strong "fruity" odour. Furthermore, mould growth was observed on all air-packaged pizza. However, pizza packaged in 60% CO_2 was marginally acceptable since both texture and odour scores approached the cut-off score for acceptability (>2.5). The

sensory deterioration of bagels at ambient temperature was less evident than the deterioration of either crumpets or pizza. Colour was unchanged after 42 days, and although odour scores were acceptable after 42 days, off-odours were described as "stale" and bagels had marginally acceptable texture scores. These results are in agreement with Assouad (1996) who extended the shelf life of bagels to approximately 42 days using a combination of amylase and MAP. Mould growth was again observed on air-packaged bagels and in one package of CO_2 packaged bagels that had elevated levels of headspace O_2 due to leakage.

2.3.3 Changes in pH

Changes in pH for all packaged bakery products are shown in Table 2.1. For all products, pH decreased slightly after 42 days. For crumpets, pH decreased from 6.00 to 5.82; for pizza from 5.62 to 5.48, while for bagels, pH decreased from 5.63 to 5.56. The slight decreases in pH may be attributed to lactic acid production by spoilage organisms or dissolution of CO_2 in the products. Since the minimum pH for growth of proteolytic *C. botulinum* is 4.6 (Dodds and Austin, 1997), the pH of all bakery products at the end of the 42-day storage period was conducive to the growth of this pathogen.

There was no significant difference between changes in headspace gas composition, pH and sensory evaluation scores for all inoculated and uninoculated bakery products ($P<0.005$).

2.3.4 Growth and neurotoxin production by *C. botulinum*

Growth and neurotoxin production results are summarised in Table 2.2. With the exception of bagels, counts of *C. botulinum* increased from an initial inoculum level of 5 x 10^4 spores/g to between 10^5 and 10^6 CFU/g after 42-days storage at ambient temperature (Table 2.2). For bagels, counts of *C. botulinum* after 42 days at ambient temperature were approximately 10^4 CFU/g, i.e., almost identical to the initial inoculum level (Table 2.2). Although growth was not observed in any bagels, spores remained viable. Ingesting spores does not generally result in botulism except in cases of infant botulism (Dodds, 1993c), and rarely, in cases of adult intestinal tract colonisation (Griffin et al., 1997).

Neurotoxin was detected in all inoculated crumpets and pizza, regardless of packaging atmosphere (Table 2.2). However, neurotoxin was not detected in any inoculated bagels after 42 days at ambient storage temperature (Table 2.2) or in any uninoculated crumpets, pizza or bagels. As well, no endogenous spores were found in any of the raw ingredients, although low levels of spores have been occasionally associated with some of the ingredients, specifically milk (Collins-

TABLE 2.2. *Toxin detection[a] in bakery products inoculated with 5 x 10^4 spores/g* C. botulinum *after 42-days storage (25°C)*

Packaging Treatment	Toxin[b]	*C. botulinum* (log CFU/g)
Crumpets		
Air	2/2	6.9
Air + oxygen absorbent	2/2	6.5
CO_2 / N_2 (60:40)	2/2	6.6
Pizza crust		
Air	2/2	5.0
Air + oxygen absorbent	2/2	5.9
CO_2 / N_2 (60:40)	2/2	5.6
Bagels		
Air	0/2	4.5
Air + oxygen absorbent	0/2	4.6
CO_2 / N_2 (60:40)	0/2	4.7

[a] Average of duplicate samples

[b] Number of samples testing positive out of number of samples analysed by mouse bioassay.

Thompson and Wood, 1993) and flour (Elliot, 1980)

The main inter-related factors that can influence the growth of and neurotoxin production by *C. botulinum* in foods are temperature, pH, a_w, redox potential, added preservatives and competing microflora. In this study, bakery products were stored at ambient temperature. Both product pH and storage temperature (25°C) were suitable for growth of and neurotoxin production by *C. botulinum* (Dodds and Austin, 1997). Crumpets (a_w 0.990) and pizza (a_w 0.960) supported growth and neurotoxin production while bagels with a lower a_w of 0.944, did not. This latter a_w approaches the limiting a_w of 0.94 for the growth of proteolytic *C. botulinum* (Dodds and Austin, 1997). The results observed in this study are in agreement with Denny et al. (1969), who found neurotoxin production in canned bread of a_w >0.950 but not in canned bread of a_w ≤0.950. Similarly, Wagenaar and Dack (1959) reported neurotoxin production in canned chocolate nut roll of 42% moisture and 27% sucrose but not in nut roll of identical % sucrose but a lower moisture content (29%). The a_w of these chocolate rolls were not reported. However, by using the equation of Money and Born (1951), developed for estimating a_w of confections, the a_w of the chocolate roll in which neurotoxin was detected would have been ~0.955, while the a_w of the non-toxic roll would have been ~0.930. Glass and Doyle (1991) also reported an a_w effect on neurotoxin production. Neurotoxin was detected in meat tortellini with a_w of 0.99 and 0.95 (stored at 30°C) after two and

six weeks respectively. However, neurotoxin was not detected in tortellini with an $a_w \leq 0.94$ stored at 30°C for 10 weeks.

It is evident from Figure 2.2 that counts of *C. botulinum* increased with increasing product a_w. However, this neglects all other factors such as the effect of different ingredients on available nutrients, which may influence growth and neurotoxin production. The pH of both pizza and bagels were similar (pH 5.2); however, the pH of crumpets was higher (pH 6.00). Interactions between a_w and pH have been reported (Dodds, 1989), and therefore, the observed increase in growth of *C. botulinum* with increasing product a_w must be interpreted cautiously.

All three atmospheres used in this study, including air, supported growth of and neurotoxin production by *C. botulinum*. It cannot be assumed that packaging in air or in MAP conditions that includes O_2 in the package headspace will protect against the growth of *C. botulinum* since headspace O_2 can become depleted due to the respiration of spoilage organisms. Most toxic crumpets had headspace O_2 of <1% at the end of 42-days storage. However, some toxic crumpets and pizza packaged under modified-atmospheres, had residual levels of headspace O_2 >5% at the end of storage–indicating possible leakage. Neurotoxin production has been previously reported in foods packaged with O_2 (Lambert et al., 1991a; Ikawa, 1991).

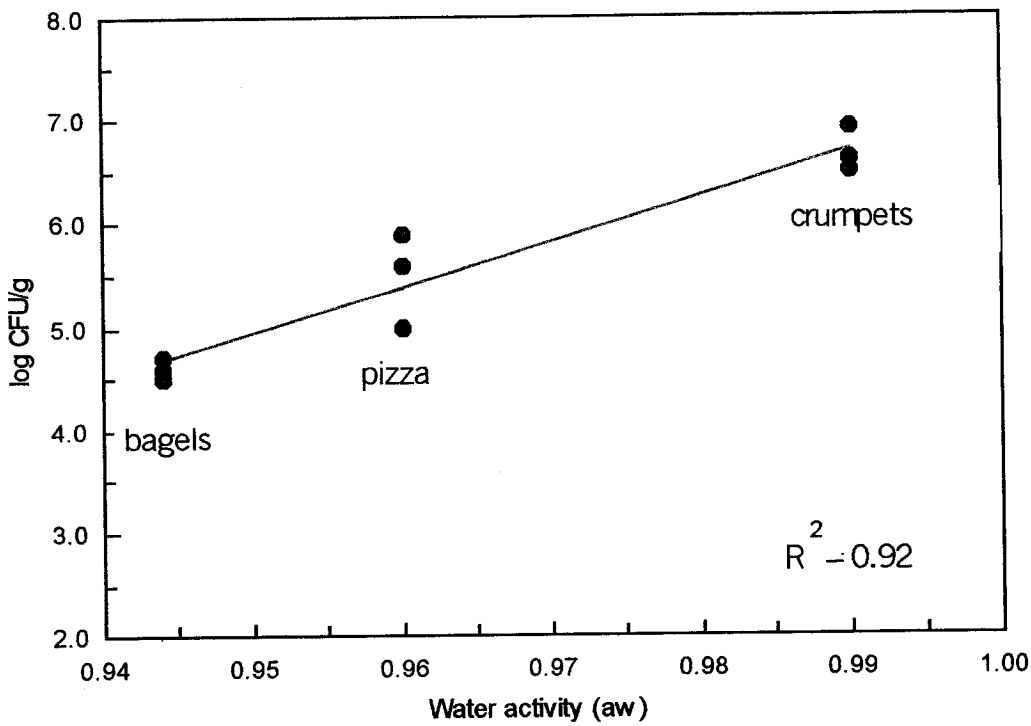


FIGURE 2.1. *Relationship between counts (log CFU/g) of* C. botulinum *in and water activity (a_w) of bakery products (pH 5.6-6.0) inoculated with 5 x 10^4 spores/g* C. botulinum *and stored for 42 days at 25°C*

The actual time to neurotoxin production in packaged crumpets and pizza could not be determined from this study. After 42-days storage, most bakery products were visibly spoiled as shown by sensory analysis scores >2.5 (Table 2.1) and, on the basis of their organoleptic quality, would be rejected by consumers.

2.4 CONCLUSION

In conclusion, these studies have shown that high-moisture, low-acid crumpets and pizza can support the growth of *C. botulinum*, regardless of the packaging atmosphere. Since no endogenous spores of *C. botulinum* were found in uninoculated crumpets, pizza, bagels, or in any of the raw ingredients, it may be assumed that the probability of contamination of these high-moisture bakery products with *C. botulinum* is low. However, because of the serious nature of botulism, and the increasing use of MAP for bakery products, it is recommended that barriers to the growth of *C. botulinum* be included when bakery products are packaged in high gas-barrier films under modified atmospheres. Preliminary studies have shown that reformulation of crumpets to lower pH (~4.5) using lactic acid bacteria could be used to delay neurotoxin production (results not shown). As well, reformulation studies are suggested to examine the effect of higher pH (>8) on growth of and neurotoxin production of *C. botulinum*. Further studies are suggested to determine the effect of additional barriers (ethanol vapour, novel anti-microbial agents etc.) on the safety of MAP crumpets stored at ambient temperature.

PREFACE TO CHAPTER 3.0

The potential of high-moisture bakery products, specifically pizza crusts and crumpets, to support growth of and neurotoxin production by *C. botulinum* was established in the previously described challenge study (Chapter 2.0). However, since it was not known from this study whether spoilage preceeded toxicity or *vice versa*, additional challenge studies were done to determine the safety, with respect to *C. botulinum*, of English-style crumpets packaged in air or under modified- atmospheres and stored at ambient temperature.

3.0 GROWTH OF AND NEUROTOXIN PRODUCTION BY *CLOSTRIDIUM BOTULINUM* IN ENGLISH-STYLE CRUMPETS PACKAGED UNDER MODIFIED ATMOSPHERES

3.1 INTRODUCTION

Bakery products are important staple foods in most countries and cultures. However, mould growth and staling are two problems limiting the shelf-life of both high- and intermediate-moisture bakery products (Brody, 1985; Parry, 1993; Smith and Simpson, 1995). Both problems can be overcome through reformulation and modified-atmosphere packaging (MAP), resulting in shelf-life extension of greater than six weeks at ambient temperature (Ooraikul, 1982; Smith et al., 1986; Smith et al., 1988; Powers and Berkowitz, 1990; Assoud, 1996). However, little is known about the public-health safety of such reformulated/packaged bakery products. The major safety concern with modified-atmosphere-packaged products is that the atmospheres may inhibit the microbial indicators of spoilage while enhancing the growth of pathogens, particularly *C. botulinum* (Hintlian and Hotchkiss, 1986; Farber, 1991).

Although MAP of bakery goods is a recent packaging technology, early studies have shown that bakery products are a suitable substrate for growth of and neurotoxin production by *C. botulinum*. Bever and Halvorson (1947) demonstrated growth of *C. botulinum* in a medium of sterile canned bread

crumbs, while others reported neurotoxin production by *C. botulinum* types A and proteolytic B inoculated into canned bread (Ulrich and Halvorson, 1949; Kadavy and Dack, 1951; Wagenaar and Dack, 1954, 1959; Denny et al., 1969). Furthermore, initial challenge studies have shown that crumpets (water activity [a_w] 0.990, pH 6.00) inoculated with a mixture of spores of proteolytic strains of *C. botulinum* (types A and B), packaged under MAP conditions and stored at ambient temperature were toxic after 42 days, while bagels (a_w 0.944, pH 5.63) similarly inoculated, packaged and stored, were not toxic (Daifas et al., 1999a). After 42-days storage at ambient temperature, toxic crumpets were visibly spoiled, and on the basis of their organoleptic quality, would be rejected by consumers. However, the time of toxigenesis was not determined and therefore, it was not known whether or not toxigenesis preceded spoilage.

Therefore, the objective of this study was to determine the actual time-to-toxicity in crumpets inoculated pre- and post-baking with proteolytic strains of *C. botulinum*, packaged under various atmospheres, and stored at ambient temperature.

3.2 MATERIALS AND METHODS

3.2.1 Crumpets

Crumpets used in this study were high-moisture English-style crumpets (a_w 0.990, pH 6.50) that were prepared from all-purpose flour, sugar, instant yeast, reconstituted dry milk, salt, baking soda, and anti-staling amylase (Novamyl; Novo Nordisk; Danbury, CT) according to the method of Witty and Schneider Colchie (1979). Crumpets were baked by pouring 65 g of batter into greased, cylindrical moulds on an oiled griddle (Moffat Appliances; Montreal, QC) preheated to 204°C. Baking time was approximately nine minutes, until the surface was no longer wet, and holes had formed on the top of the crumpets. Following baking, crumpets were cooled to room temperature, placed in polyethylene bags, and stored for one week at -28°C.

3.2.2 Preparation of spore inoculum/sample inoculation

A composite inoculum of *C. botulinum* spores was prepared from four type A strains (A6, 17A, 62A, and CK2A) and five proteolytic type B strains (MRB, IB1-B, 13983IIB, 368B and 426B). Spore crops of each strain were prepared separately, enumerated as described by Hauschild and Hilsheimer (1977), and stored frozen at -80°C. Equal numbers of spores

of each strain were then combined to form a single suspension of approximately 3 x 10^5 spores/mL. In the post-baking challenge study, the spore mixture was heat shocked at 75°C for 20 minutes prior to sample inoculation. Inoculum levels were verified prior to inoculation of samples by plating appropriate dilutions in duplicate on McClung Toabe Agar (Difco, Becton-Dickinson; Sparks, MD) containing 0.5% yeast extract (Difco) and egg-yolk and incubating anaerobically in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 at 35°C for three days. In the post-baking inoculation, crumpets were stab inoculated at six distinct locations with 180 µL (6 x 30 µL) spore suspension per 100-g sample to provide a final inoculum level of 5 x 10^2 spores/g. Pre-baking inoculation was achieved by replacing an appropriate volume of water in the formulation with an equivalent volume of spore suspension to give an inoculum level of 5 x 10^2 spores/g (baked weight basis). Control samples were inoculated in a similar manner with an identical volume of 0.1% peptone water.

3.2.3 Packaging

Both control and inoculated crumpets were packaged under the following atmospheres: i) air, ii) air with an AgelessFX®$_{200}$ oxygen absorbent (Mitsubishi Gas & Chemical Company; Japan) or iii) CO_2: N_2 (60:40). Samples (two crumpets weighing ~50 g each) were placed, in duplicate, in 210-mm x 210-mm high gas barrier Cryovac bags (O_2 transmission

rate: 3 to 6 cc/m²/day, 1 atm at 4.4°C, 0% RH.; Cryovac; Mississauga, ON). For air-packaged samples, bags were sealed using an impulse heat-sealer. For samples packaged in air with an oxygen absorbent, an Ageless FX®$_{200}$ sachet was placed inside the bag prior to sealing with an impulse heat-sealer. Gas packaging was achieved using a Multivac chamber type heat-seal packaging machine (model SP-300H) (Multivac, Inc; Kansas City, MO) to obtain the desired mix of CO_2 and N_2 (60:40). All packaged samples were stored at 25°C. Duplicate samples of crumpets were analysed at day 0 and daily until neurotoxin was detected or shelf-life was terminated.

3.2.4 Headspace gas

At day 0 and after each 24-hour storage interval, samples were analysed for changes in headspace gas composition. Gas samples were withdrawn using a 0.5-mL gas-tight pressure-Lok® syringe (Precision Sampling Co; Baton Rouge, LA) through a septum previously attached to the outside of each package. Headspace gas was analysed with a Varian gas chromatograph (model 3300, Varian Canada, Inc; Montreal, QC), fitted with a thermal conductivity detector and using Porapack Q and molecular sieve 5A (80-100 mesh) columns (Chromatographic Specialties; Brockville, ON) in series. Peaks were recorded and analysed with a Varian integrator (model 4270, Varian Canada, Inc.; Montreal, QC).

3.2.5 Sensory analysis

Sensory analysis was carried out on packaged products at day 0 and daily by a six-member untrained panel. Packages were evaluated subjectively for swelling and for the presence of visible mould growth. Products were also assessed for texture, colour and odour using a six-point hedonic scale (0 = no deterioration; 5 = extreme deterioration). To evaluate odour, packages were opened and the characteristic odour described. A sensory score of 2.5 was taken as the cut-off for acceptability and termination of shelf-life.

3.2.6 Sampling of crumpets

Each sample was aseptically transferred to a stomacher bag. A 1:3 dilution was prepared by adding twice the sample weight of 0.1% peptone water and then stomaching for two minutes using a Colworth Stomacher (Seward Medical Stomacher, London, UK). Decimal dilutions were subsequently prepared from this initial dilution.

3.2.7 Enumeration of *C. botulinum*

Growth of *C. botulinum* was determined as described previously (Austin et al., 1998; Lyver et al., 1998). *C. botulinum* was enumerated by spread plating 0.1 mL of appropriate decimal dilutions of the 1:3

homogenized sample, in duplicate, on *Clostridium botulinum* Isolation (CBI) Agar (Dezfulian et al., 1981). CBI plates were incubated in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 for 72 hours at 35°C. Lipase-positive colonies, characteristic of *C. botulinum,* were enumerated and identification was confirmed by neurotoxin neutralization assays on trypticase peptone glucose yeast extract (TPGY) broth cultures of randomly selected colonies.

3.2.8 Neurotoxin assay

The remaining homogenized samples (1:3 dilution) were centrifuged at 23,500 x g for 20 minutes at 4°C. Botulinum neurotoxin was detected as described previously (Austin et al., 1998). An aliquot of the sample supernatant was filtered through a 0.45-μm filter (Acrodisc, Gelman Sciences; Ann Arbor, MI) and 0.5 mL of this filtrate was injected intraperitoneally into each of two 20- to 28-g mice (Charles River; QC). Mice were observed for up to 72 hours for typical signs of botulism, including ruffled fur, pinched waist, laboured breathing, limb paresis and general paralysis. As required by Health Canada, mice showing severe distress were humanely asphyxiated with CO_2. Neutralization of neurotoxin was performed on the last positive samples of each treatment using antisera (Connaught Laboratories; North York, ON) to botulinum neurotoxins.

All raw ingredients used in the crumpet formulations were screened for endogenous spores of *C. botulinum* as described by Austin and Blanchfield (1996).

3.2.9 Changes in pH

The pH was determined using a previously calibrated Fisher Accumet® pH meter (Fisher Scientific; Ottawa, ON). Portions of sample supernatant were transferred to sterile glass tubes and pH measurement was made by immersing the electrode directly into the tubes.

3.2.10 Lethality of thermal process to spores of *C. botulinum*

The temperature profile of crumpets was determined by inserting a thermocouple (Type T, Model 600-1020; Barnant, Barrington, IL) into the centre of crumpets during baking and cooling. Total lethality (F_0) was determined using the Improved General Method as described by Ramaswamy and Abdelrahim (1991). F_0, the sum of the lethal rates (minutes at 121°C per minute at any measured temperature), was calculated as $\Sigma\left[10^{\left(\frac{T-121.C}{z}\right)}\right]\Delta t$, where the temperature (T) in Celsius degrees was measured each 0.5 minute (Δt), and the temperature sensitivity indicator (z) for *C. botulinum* was 10°C.

3.2.11 Predicted time to toxigenesis

The predicted time to toxicity was generated using the United States Department of Agriculture (USDA) Pathogen Modeling Program (version 5.1) (Whiting and Call, 1993; Whiting and Oriente, 1997) for proteolytic strains of *C. botulinum*. Actual values of a_w, pH, and inoculum used in this study were incorporated into the predictive model.

3.2.12 Statistical analysis

Data were analysed using analysis of variance (ANOVA) for a randomized complete block design (Steel and Torrie, 1980) using Prism 2.01 (GraphPad Software, Inc.; San Diego, CA).

3.3 RESULTS AND DISCUSSION

Initial screening studies had determined that crumpets (a_w 0.990, pH 6.00) packaged under similar atmospheres to this present study could support the growth of and neurotoxin production by *C. botulinum* (Daifas et al., 1999a). To determine the actual time until neurotoxin production and to determine if toxigenesis preceded spoilage, crumpets were inoculated with 5 x 10^2 spores/g of *C. botulinum* types A and B in two ways: i) post-baking in the first study and ii) pre-baking contamination by incorporating the spore inoculum directly into the batter prior to baking on the griddle in the second study.

3.3.1 Time to toxicity studies: post-baking contamination

3.3.1.1 Headspace gas composition

Changes in headspace gas composition for all post-baking inoculated crumpets are shown in Figure 3.1A-C. For both control and inoculated air-packaged crumpets, headspace O_2 decreased to <2% by day four, while CO_2 increased to ~40% by day seven (Figure 3.1A). All packages were slightly swollen due to CO_2 production. Smith et al. (1983) reported CO_2 production and swelling in modified atmosphere-packaged crumpets due to the growth of

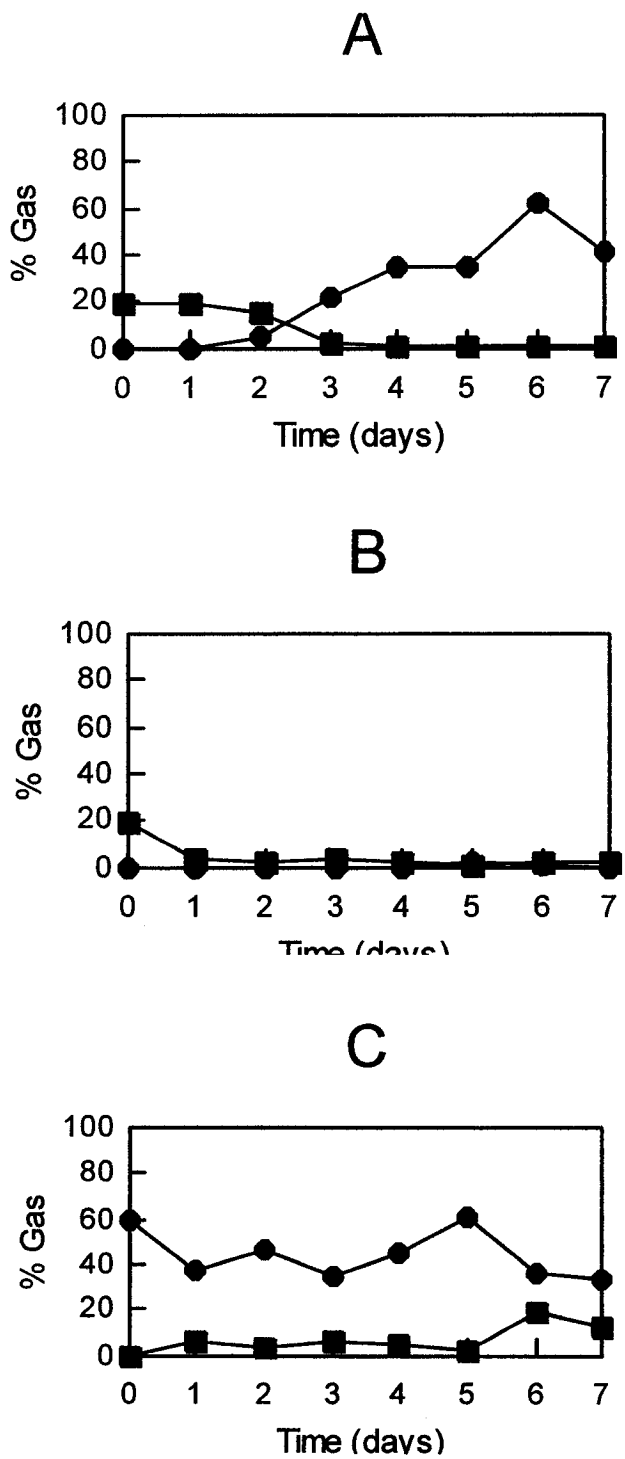


FIGURE 3.1. *Changes in headspace gas composition (* ■ O_2 ,● CO_2*) of crumpets inoculated post-baking with 5 x 10^2 spores/g* C. botulinum *and packaged A, in air, B, in air with an oxygen absorbent or C, in CO_2/N_2 (60:40) and stored at 25°C*

heterofermentative lactic acid bacteria. Similar patterns were observed for both control and inoculated crumpets packaged in air with an oxygen absorbent (Figure 3.1B). Headspace O_2 decreased to ~2% to 5% after day 1, while CO_2 increased gradually to ~2% by day 7 (Figure 3.1B). For all CO_2-packaged crumpets, headspace CO_2 initially decreased at day 1 (~40%), then increased to ~60% of headspace gas by day 5 and then decreased to ~35% by the end of storage (Figure 3.1C). Slight swelling was observed in some packages. The initial decrease in headspace CO_2 was attributed to absorption of CO_2 by the crumpets while the final decrease was attributed to package leaks. Headspace O_2 levels ranged from 3% to 10% by day 7 in several packages and may be due to leakage. However, bakery products can retain large quantities of air inside their porous structure and residual headspace O_2 is often observed in bakery products packaged under MAP conditions (Piergiovanni and Fava, 1997).

3.3.1.2 Sensory analysis

Sensory scores at the end of seven-days storage (Table 3.1) were almost identical for all control and inoculated crumpets. All crumpets had colour scores of 0 (on a scale of 0 to 5) and no visible mould was observed on any of the packaged crumpets. For control

TABLE 3.1. *Summary[a] of sensory analysis of post-baking inoculated crumpets after seven-days storage at 25°C*

Packaging Treatment	Swelling	Mould	Sensory scores[c]		
			Colour	Texture	Odour
Uninoculated crumpets					
Air	+	-	0	2	2[f]
Air + O_2 absorbent	-	-	0	1.5	2[f]
CO_2 / N_2 (60:40)	-	-	0	1.5	1
Inoculated crumpets[b]					
Air	+	-	0	2	2[f]
Air + O_2 absorbent	-	-	0	1.5	0.5
CO_2 / N_2 (60:40)	+	-	0	1.5	1.5[f]

[a] Average of duplicate samples.

[b] Inoculated with 5 x 10^2 spores/g *C. botulinum* types A and B.

[c] Scale of 0 to 5; 0 = no deterioration, 5 = extreme deterioration.

[f] Fruity odour.

crumpets, and for inoculated crumpets, texture deterioration scores were slightly higher (score = 2) for air-packaged than for modified-atmosphere-packaged crumpets (score = 1.5). Odour scores for all crumpets ranged from 0.5 to 2. Off-odours were described as "fruity" and all crumpets were rated as acceptable.

3.3.1.3 Changes in pH

The changes in pH of control and inoculated crumpets over the seven-day storage period were similar for all packaging conditions. By day 7, product pH was essentially unchanged from the initial pH of 6.50. The final pH values (day 7) for uninoculated control crumpets and for inoculated crumpets were 6.47 and 6.42, respectively. Since the minimum pH for growth of proteolytic *C. botulinum* is 4.6 (Dodds and Austin, 1997), the pH values of crumpets at the end of the seven-day storage period were conducive to the growth of this pathogen.

3.3.1.4 Enumeration of *C. botulinum*

Counts of *C. botulinum* (CFU/g) for all packaged crumpets are shown in Figure 3.2. Counts increased from 5×10^2 to 2.8×10^5, 1.0×10^5, and 2.6×10^5 after seven days in air-, air with an oxygen absorbent- and CO_2-packaged crumpets, respectively (Figure 3.2),

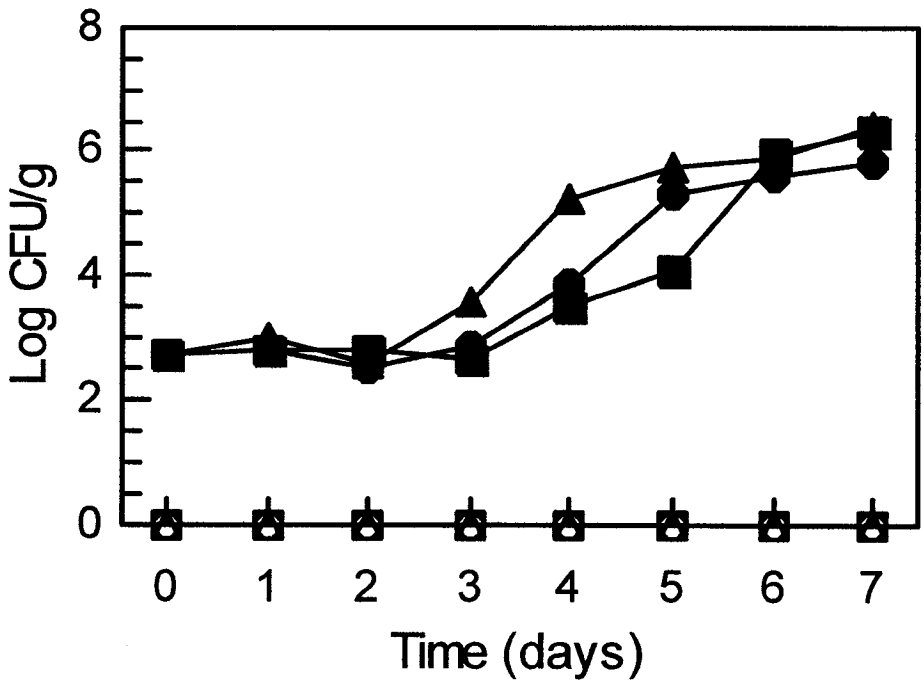


FIGURE 3.2. *Counts (log CFU/g) of* C. botulinum *in control crumpets (□,○,△) and crumpets inoculated post-baking with 5x10² spores/g* C. botulinum *(■, ●,▲) and packaged in air (□, ■), in air with an oxygen absorbent (○,●) or in CO_2 / N_2 (60:40 [△, ▲]) and stored at 25°C*

clearly indicating that all three atmospheres supported growth of *C. botulinum*. *C. botulinum* was not detected in any control crumpets.

3.3.1.5 Neurotoxin assay

Neurotoxin was detected after days 4, 5, and 6 in crumpets packaged in CO_2, in air with an oxygen absorbent and in air respectively (Table 3.2). Therefore, all three atmospheres, including air, supported growth of and toxigenesis by *C. botulinum*. Neurotoxin was not detected in any uninoculated control crumpets (Table 3.2). Neurotoxin production in foods packaged with O_2 has been reported previously. Lambert et al. (1991a) reported botulinum neurotoxin production after 19 days in packaged inoculated pork containing up to 20% headspace O_2. Similarly, Ikawa (1991) detected neurotoxin in inoculated cooked noodles packaged in O_2 permeable bags and stored aerobically at ambient temperature. Hintlian and Hotchkiss (1986) suggested that the inclusion of O_2 in the package headspace might delay the germination of *C. botulinum* spores. However, Whiting and Naftulin (1992) found that proteolytic spores of *C. botulinum* germinated in $\leq$1% O_2 while vegetative cells could grow in 15% O_2. If spores germinated in low O_2 tensions or in pockets of food with suitable conditions, then growth and neurotoxin production was possible. These authors concluded that a low food redox

TABLE 3.2. *Time of neurotoxin detection*[a] *in crumpets inoculated*[b] and *stored at 25°C*

Packaging treatment	Post-baking inoculation		Pre-baking inoculation	
	Day of neurotoxin detection	Corresponding log CFU/g	Day of neurotoxin detection	Corresponding log CFU/g
Inoculated crumpets				
Air	6	5.9	6	5.3
Air + O_2 absorbent	5	5.3	4	4.9
CO_2 / N_2 (60:40)	4.5	5.5	4	5.5
Uninoculated				
Air	ND	NA	ND	NA
Air + O_2 absorbent	ND	NA	ND	NA
CO_2 / N_2 (60:40)	ND	NA	ND	NA
Predicted[c]	3.4	not stated	3.4	not stated

[a] Average of duplicate samples.

[b] Inoculated with 5 x 10^2 spores/g *C. botulinum* types A and B

[c] Calculated values using USDA Pathogen Modeling Program (ver. 5.1) based on conditions of 25°C, pH 6.5, a_w 0.990 and 5 x 10^2 spores/g proteolytic *C. botulinum*

NA: not applicable

ND: not detected

potential was more significant, in terms of growth, than the level of O_2 surrounding the product. This study further confirms that O_2 surrounding a food cannot be regarded as a barrier to prevent the growth of *C. botulinum* since during storage microbial or other respiratory activity may lead to depletion of O_2 and generation of CO_2.

The actual time to toxigenesis (4 to 6 days) was within one to three days of the predicted time to toxigenesis (3.4 days) using the USDA Pathogen Modeling Program (Whiting and Call, 1993; Whiting and Oriente, 1997 [Table 3.2]). However, this model is based on microbial growth of pure cultures. Differences between actual versus predicted times may be due to strain differences, headspace gas composition, the food substrate, as well as the presence of competitive microorganisms, all of which may influence the growth of *C. botulinum* in food, but none of which were factored into the model.

This study confirms previous challenge studies in canned bread that high-starch foods stored at ambient temperature can support the growth of *C. botulinum* (Kadavy and Dack, 1951; Wagenaar and Dack, 1954; Denny et al., 1969). At the time of toxigenesis, all inoculated crumpets were organoleptically acceptable (Table 3.1). Furthermore, there was no significant difference between sensory analysis scores for control and inoculated crumpets (P <0.005). Therefore, if modified-atmosphere-packaged or air-packaged crumpets were contaminated with *C. botulinum,* they could pose a serious public health

threat since they would still be organoleptically acceptable to consumers. Toxigenesis has also been shown to precede spoilage in fresh pasta (Glass and Doyle, 1991), in raw, and baked vacuum-packaged potato (Sugiyama et al., 1981; Solomon et al., 1998) and gas-packaged (100% N_2) hamburgers and sausage sandwiches (Kautter et al., 1981).

3.3.2 Time to toxicity studies: pre-baking contamination

To determine the effect of baking on the safety of packaged crumpets, crumpet batter was inoculated with 5 x 10^2 spores/g (baked weight basis) prior to baking on the griddle. Almost identical results were observed for changes in headspace gas (Figure 3.3), sensory evaluation scores (Table 3.3) and changes in pH for all crumpets inoculated prior to baking as were observed in the post-baking study.

3.3.2.1 Enumeration of *C. botulinum*

Counts of *C. botulinum* again increased throughout storage. After seven-days storage at ambient temperature, counts (CFU/g) for crumpets packaged in air, in air with an oxygen absorbent and in 60% CO_2 had increased from an initial level of 5 x 10^2 spores/g to 5 x 10^6, 1 x 10^5, and 2 x 10^6 (CFU/g), respectively (Figure 3.4). It is obvious that the baking process was insufficient to destroy the

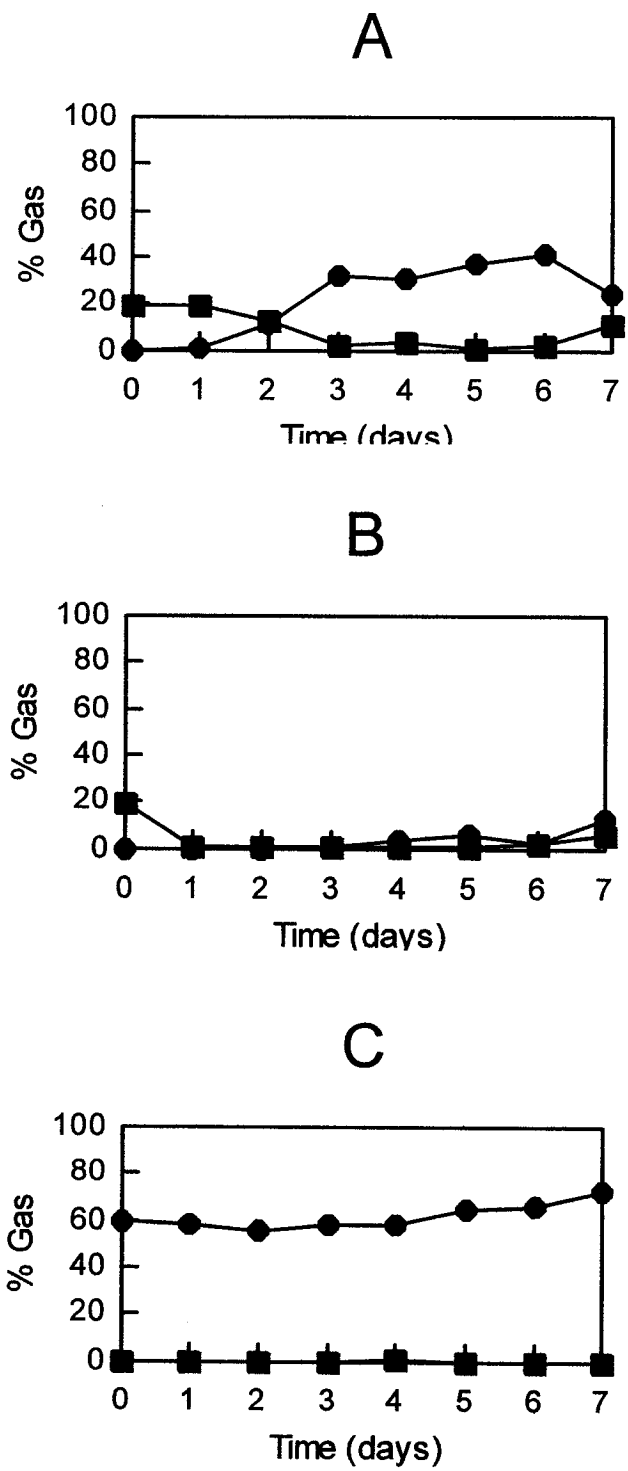


FIGURE 3.3. *Changes in headspace gas composition (■ O_2 ,● CO_2) of crumpets inoculated pre-baking with 5 x 10^2 spores/g* C. botulinum *and packaged A, in air, B, in air with an oxygen absorbent or C, in CO_2 / N_2 (60:40) and stored at 25°C*

TABLE 3.3 *Summary[a] of sensory analysis of pre-baking inoculated[b] crumpets after seven-days storage at 25°C*

Packaging Treatment	Swelling	Mould	Sensory scores[c]		
			Colour	Texture	Odour
Uninoculated crumpets					
Air	+	-	0	2	3[f]
Air + oxygen	+	-	0	1	1[f]
CO_2 / N_2 (60:40)	+	-	0	1	0.5
Inoculated crumpets[b]					
Air	+	-	0	1.5	2.5[f]
Air + oxygen	+	-	0	1	2
CO_2 / N_2 (60:40)	+	-	0	1	0

[a] Average of duplicate samples

[b] Inoculated with 5 x 10^2 spores/g *C. botulinum* types A and B

[c] Scale of 0 to 5; 0 = no deterioration, 5 = extreme deterioration

[f] Fruity odour

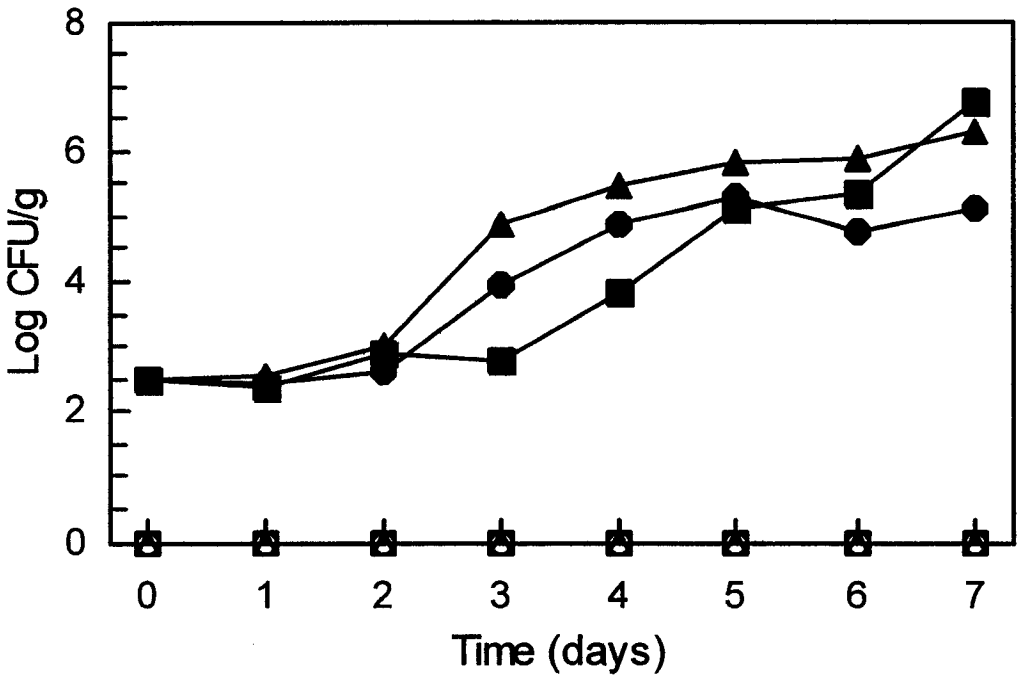


FIGURE 3.4. *Counts (log CFU/g) of* C. botulinum *in control crumpets (□,○,△) and crumpets inoculated pre-baking with 5 x 10^2 spores/g* C. botulinum *(■, ●,▲) and packaged in air (□, ■), in air with an oxygen absorbent (○,●) or in CO_2 / N_2 (60:40)(△, ▲) and stored at 25°C*

inoculated proteolytic spores. This is in agreement with Aramouni et al. (1994) who found that endogenous and inoculated spores of *C. sporogenes* PA 3679 survived baking in home-style quick-breads, thus demonstrating the potential for spores of *C. botulinum* to survive the baking process. Lund et al. (1988) reported neurotoxin production in inoculated vacuum-packaged potatoes after a double pasteurization process at approximately 90°C (total time of 27 minutes) and storage at ambient temperature. Similarly, spores of proteolytic strains of *C. botulinum* survived in vacuum-packaged potato heated in a water bath at 95°C for 40 minutes (Notermans et al., 1981a). To ensure spore destruction, retort temperatures (121°C) are necessary (Lynt et al., 1982) and heat processes should be equivalent to an F_0 of 3 minutes (Lund et al., 1988) even when pH is taken under consideration (Pflung et al., 1985). Since crumpets are cooked from the bottom only, they receive an uneven heat treatment. The maximum internal temperature reached during baking was 97°C (Figure 3.5A) and the total baking process (the area under the curve of Figure 3.5B) was equivalent to 0.03 minutes at 121.1°C (Figure 3.5B). This means that the heat treatment which resulted in crumpets of acceptable quality was only 1% of a target F_0 of 3 minutes and therefore, almost complete spore survival would be expected. As shown in Figure 3.4, counts of *C. botulinum* were >5 x 10^2 CFU/g by day 1, confirming that virtually all of the initial inoculum survived the baking process.

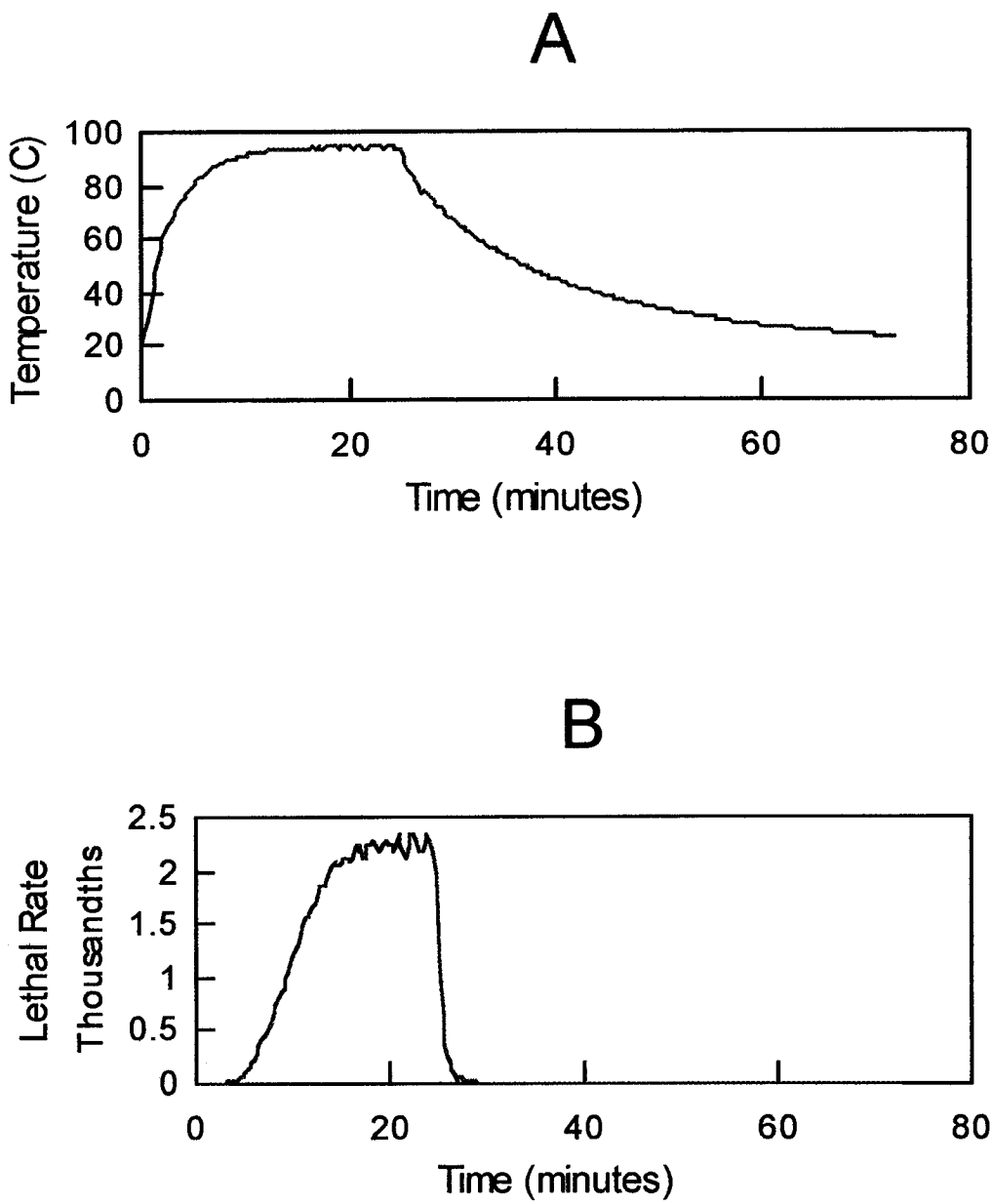


FIGURE 3.5. *A, Heat profile and B, total lethality (F_0) of crumpets*

3.3.2.2 Neurotoxin assay

As in the post-baking study, all crumpets inoculated pre-baking were toxic within one week, again, regardless of the packaging atmosphere (Table 3.2). Neurotoxin was detected first in modified atmosphere-packaged crumpets (day 4) and by day 6 in air-packaged crumpets. At the time of neurotoxin detection, counts were ~10^5 CFU/g (Table 3.2 and Figure 3.4). Time-to-neurotoxin production was within one to three days of the predicted time-to-toxicity (Table 3.2). It is evident from all sensory evaluation scores (Table 3.3) that toxigenesis again preceded spoilage, with crumpets packaged under modified-atmospheres being more acceptable than air-packaged crumpets.

3.4 CONCLUSION

In conclusion, these studies have shown that high-a_w high-pH crumpets can support the growth of *C. botulinum*, regardless of the initial gaseous atmosphere. However, no outbreaks of botulism have ever been attributed to either air- or modified-atmosphere-packaged high-moisture bakery products. The lack of reported botulism outbreaks may be attributed to several factors. Few bakery products have a water activity (a_w) high enough to support the growth of *C. botulinum.* This is supported by an initial screening challenge study that showed that high-moisture bagels (a_w 0.944) did not support growth of and neurotoxin production by *C. botulinum* (Daifas et al., 1999a). However, high-moisture pizza crust (a_w 0.960) and crumpets (a_w 0.990) supported growth of *C. botulinum* after six-weeks storage at ambient temperature, again regardless of the gaseous atmosphere (Daifas et al., 1999a). Another reason may be the presence of preservatives used to control mould growth and bacterial rope production in commercially-packaged bakery products. These preservatives, typically sorbates and propionates, may act either alone or in combination to inhibit the growth of *C. botulinum* (Sofos et al., 1979; Lund et al., 1987; Miller et al., 1993). In these studies, no preservatives were added. Perhaps the most important reason for the lack of reported botulism outbreaks due to bakery products is that low levels of spores are associated with ingredients, specifically milk (Collins-Thompson and Wood, 1993) and flour (Elliot, 1980). No endogen-

ous spores were found in these studies either in control crumpets or in any of the raw ingredients (results not shown). Therefore, the probability of spores of *C. botulinum* occurring in bakery products either through ingredients or as a result of contamination may be assumed to be low. However, these studies also have demonstrated that neurotoxin production preceded spoilage in crumpets inoculated with *C. botulinum*, especially in crumpets packaged under modified atmospheres. Growth of *C. botulinum* in crumpets to hazardous levels in the absence of organoleptic degradation is possible, and therefore, hurdles to the growth of this pathogen should be considered.

PREFACE TO CHAPTER 4.0

Crumpets inoculated with *C. botulinum* and packaged under various atmospheres, including 60% CO_2, became toxic within one week when stored at ambient temperature (Chapter 3.0). Therefore, subsequent challenge studies were conducted to determine the effect of product pH and higher levels of CO_2 on the time-to-neurotoxin detection.

4.0 EFFECT OF pH AND CO_2 ON GROWTH OF AND NEUROTOXIN PRODUCTION BY *CLOSTRIDIUM BOTULINUM* IN ENGLISH-STYLE CRUMPETS PACKAGED UNDER MODIFIED ATMOSPHERES

4.1 INTRODUCTION

Modified-atmosphere packaging (MAP), involving packaging in CO_2, alone or in combination with N_2, can be used to extend significantly the shelf-life of high- and intermediate-moisture bakery products (Seiler, 1978; Ooraikul, 1982; Brody, 1985; Smith et al., 1986). Carbon dioxide is used in MAP because it is both bacteriostatic and fungistatic. While most moulds and some yeasts can be inhibited by CO_2 (Skovholt and Bailey, 1933; Legan and Voysey, 1991), bacteria vary in their response to this gas (Hintlian and Hotchkiss, 1986; Farber, 1991). Carbon dioxide is effective in delaying the growth of gram-negative, aerobic spoilage bacteria commonly found in muscle foods (Daniels et al., 1985; Hintlian and Hotchkiss, 1986; Farber, 1991); however, several food pathogens are resistant to high levels of CO_2 while some are even stimulated by this gas (Dixon and Kell, 1989; Jager and Lück, 1997). Carbon dioxide has been shown to enhance spore germination of clostridia (Enfors and Molin, 1978; Foegeding and Busta, 1983) and, depending on its level, may enhance or inhibit growth of and neurotoxin production by *C. botulinum* (Doyle, 1983; Lambert et al., 1991a, b). Anaerobic spore-forming bacteria are present in flour (Thatcher et al., 1953), and

occasional spores of *C. botulinum* can be expected in both flour and milk (Elliot, 1980; Collins-Thompson and Wood, 1993). The incidence of naturally occurring spores of *C. botulinum* in bakery products is probably low; however, the health risk of botulism associated with growth of this pathogen is high. The safety of MAP products is therefore of concern because pathogenic bacteria, specifically *C. botulinum*, may flourish in an anaerobic environment without competition from normal aerobic spoilage microorganisms (Hintlian and Hotchkiss, 1986; Farber, 1991; Church and Parsons, 1995).

Recently, growth of and neurotoxin production by proteolytic strains of *C. botulinum* have been reported in English-style crumpets (a_w 0.990; pH 6.5) packaged initially in air, in air with an oxygen absorbent, or in 60% CO_2 (balance N_2) and stored at ambient temperature. Neurotoxin was first detected after four- to six-days storage for all packaging conditions (Daifas et al., 1999a, b). However, the effect of higher levels of CO_2 was not investigated. Furthermore, some commercially produced, high-moisture crumpets are formulated to pH >8 to enhance product colour, i.e., increased non-enzymatic browning, and very little is known about the growth of and neurotoxin production by *C. botulinum* in such alkaline bakery products. Thus, the objectives of this study were to determine the effects of product pH (6.5 and 8.3) and level of packaging CO_2 (60% and 100%) on the growth of and neurotoxin production by *C. botulinum* in English-style crumpets.

4.2 MATERIALS AND METHODS

4.2.1 Crumpets

English-style crumpets (a_w 0.990; pH 6.5 and 8.3) were prepared in a food laboratory (McGill University, Montreal, QC) from all-purpose flour, sugar, yeast, reconstituted dry milk, salt, $NaHCO_3$ and α-amylase (Novamyl; Novo Nordisk; Danbury, CN) as described previously (Daifas et al., 1999a). Crumpet pH, as measured using a calibrated pH meter (Fisher Scientific, Ottawa, ON), was set by varying the amount of $NaHCO_3$ in the formulation. The amount of $NaHCO_3$ (% wt/wt) required to formulate crumpets of pH 6.5 and 8.3 was determined from the regression equation $pH = 0.374x + 6.52$ (R^2 0.97) where x is the weight of $NaHCO_3$ (% wt/wt). Following baking, crumpets were cooled to room temperature, placed in polyethylene bags, and stored for one week at -28°C.

4.2.2 Preparation of spore inoculum and sample inoculation

A composite inoculum of *C. botulinum* spores was prepared from four type A strains (A6, 17A, 62A, and CK2A) and five proteolytic type B strains (MRB, IB1-B, 13983IIB, 368B, and 426B) obtained from the Bureau of Microbial Hazards (Health Canada, Ottawa, ON). Spore crops of each strain were prepared separately, enumerated as described by Hauschild

and Hilsheimer (1977), and stored frozen at -80°C. Equal numbers of spores of each strain were then combined to form a single suspension of approximately 3 x 10^5 spores/mL. The spore mixture was heat-shocked at 75°C for 20 minutes prior to sample inoculation. Inoculum levels were verified prior to inoculation of samples by plating appropriate dilutions in duplicate on McClung Toabe Agar (Difco, Becton Dickinson; Sparks, MD) containing 0.5% yeast extract (Difco) and egg-yolk (Austin and Blanchfield, 1996), and incubating anaerobically in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 at 35°C for three days (Austin et al., 1998). Crumpets were stab-inoculated by pipette ~0.5 cm beneath the surface at six distinct, equidistant locations on the top surface of the crumpet with 180 µL (6 x 30 µL) spore suspension per 100-g sample to provide a final inoculum level of 5 x 10^2 spores/g. This inoculum level was chosen based on the results of previous challenge studies in which crumpets, inoculated with 5 x 10^2 spores/g *C. botulinum*, became neurotoxin within one week (Daifas et al., 1999b). Control samples were inoculated in a similar manner with an identical volume of 0.1% (wt/wt) peptone water.

4.2.3 Packaging

All inoculated and control crumpets (pH 6.5 and 8.3) were packaged under the following atmospheres: i) 60% CO_2 (balance N_2) or ii) 100%

CO_2. Duplicate samples of crumpets (~50-g each, two per bag) were placed in 210-mm by 210-mm high gas barrier Cryovac bags (O_2 transmission rate: 3 to 6 cc/m^2/day, 1 atm at 4.4°C, 0% ERH; Cryovac, Mississauga, ON). Gas packaging was achieved using a Multivac chamber type heat-seal packaging machine (model SP-300H; Multivac, Inc., Kansas City, MO) to obtain the desired mix of CO_2 and N_2 (60:40 or 100:0). All packaged samples were stored at 25°C. Duplicate samples of uninoculated control crumpets were analysed at day 0 and after neurotoxin was detected in inoculated crumpets.

4.2.4 Headspace gas

At day 0 and after each 24-hour storage interval, samples were analysed for changes in headspace gas composition. Gas samples were withdrawn using a 0.5-mL gas-tight pressure-Lok syringe (Precision Sampling Co., Baton Rouge, LA) through a septum attached to the outside of each package. Headspace gas was analysed with a Varian gas chromatograph (model 3300; Varian Canada, Inc., Montreal, QC), fitted with a thermal conductivity detector and using Porapack Q and molecular sieve 5A (80 to 100 mesh) columns (Chromatographic Specialities, Brockville, ON) in series. Peaks were recorded and analysed with a Varian integrator (model 4270, Varian Canada, Inc.).

4.2.5 Sensory analysis

Each day, packaged crumpets were evaluated for package swelling, visible mould growth, texture, colour, and odour using a six-point hedonic scale (0 = no deterioration; 5 = extreme deterioration) as previously described (Daifas et al., 1999a, b). A sensory score of 2.5 was taken as the cut-off for acceptability and termination of shelf-life.

4.2.6 Neurotoxin assay

Each sample (two crumpets) was aseptically transferred to a stomacher bag. A 1:3 dilution was prepared by adding twice the sample weight of 0.1% (wt/wt) peptone water and then stomaching for two minutes using a Colworth Stomacher (Seward Medical Stomacher, London, UK). The homogenized samples were centrifuged at 23,500 x g for 20 minutes at 4°C. Botulinum neurotoxin was detected as described previously (Daifas et al., 1999b). An aliquot of the sample supernatant was filtered through a 0.45-μm filter (Acrodisc; Gelman Sciences, Ann Arbor, MI) and 0.5 mL of this filtrate was injected intraperitoneally into each of two 20- to 28-g mice (Charles River, QC). Mice were observed for up to 72 hours for typical signs of botulism, including ruffled fur, pinched waist, laboured breathing, limb paresis, and general paralysis. Mice showing extreme distress were humanely asphyxiated with CO_2 according to Health Canada Animal Care Committee guidelines

(personal communication). Neutralization of neurotoxin was performed on randomly selected positive samples, representative of all packaging conditions, using antisera (Connaught Laboratories, North York, ON) to botulinum neurotoxins. Survival of mice that were injected but not protected with antisera, were interpreted as confirmation that toxicity could be attributed to *C. botulinum* (Austin and Blanchfield, 1996).

4.2.7 Changes in sample pH

The pH of the samples was determined using a previously calibrated Fisher Accumet pH meter (Fisher Scientific, Ottawa, ON). Portions of filtered sample supernatant of the homogenized, centrifuged samples were transferred to glass tubes, and pH measurement was made by immersing the electrode directly into each tube.

4.2.8 Statistical analysis

Data were analysed using analysis of variance for a 2 x 2 factorial experimental design (Steel and Torrie, 1980) using Prism 2.01 (Graph-Pad Software, Inc.; San Diego, CA).

4.3 RESULTS AND DISCUSSION

4.3.1 Changes in CO_2 and pH

Changes in headspace gas composition for all crumpets are shown in Figure 4.1. For pH 8.3 crumpets packaged in 100% CO_2, headspace CO_2 decreased rapidly to ~78% after one day at ambient temperature, then increased to ~95% by day 2, and remained at this level throughout storage. A similar trend occurred with pH 6.5 crumpets packaged in 100% CO_2 (Figure 4.1). Headspace CO_2 also initially decreased for crumpets packaged in 60% CO_2; however, the initial decrease in headspace CO_2 was less marked in crumpets packaged in 60% CO_2 than was observed for crumpets packaged in 100% CO_2. For all crumpets (pH 6.5 and 8.3) packaged in 60% CO_2, headspace CO_2 decreased to ~50% to 55% after one day and then increased to, and remained at ~60% (Figure 4.1). The changes in headspace CO_2 can be attributed to the dissolution of CO_2 in the aqueous and lipid phases of the food and subsequent equilibration within the package atmosphere. For each level of packaging CO_2 (60% or 100%), headspace CO_2 was consistently higher for pH 6.5 crumpets than for pH 8.3 crumpets (Figure 4.1). This can be attributed to the decreased solubility of CO_2 at lower pH (Devlieghere et al., 1998) resulting in the higher levels of headspace CO_2 that

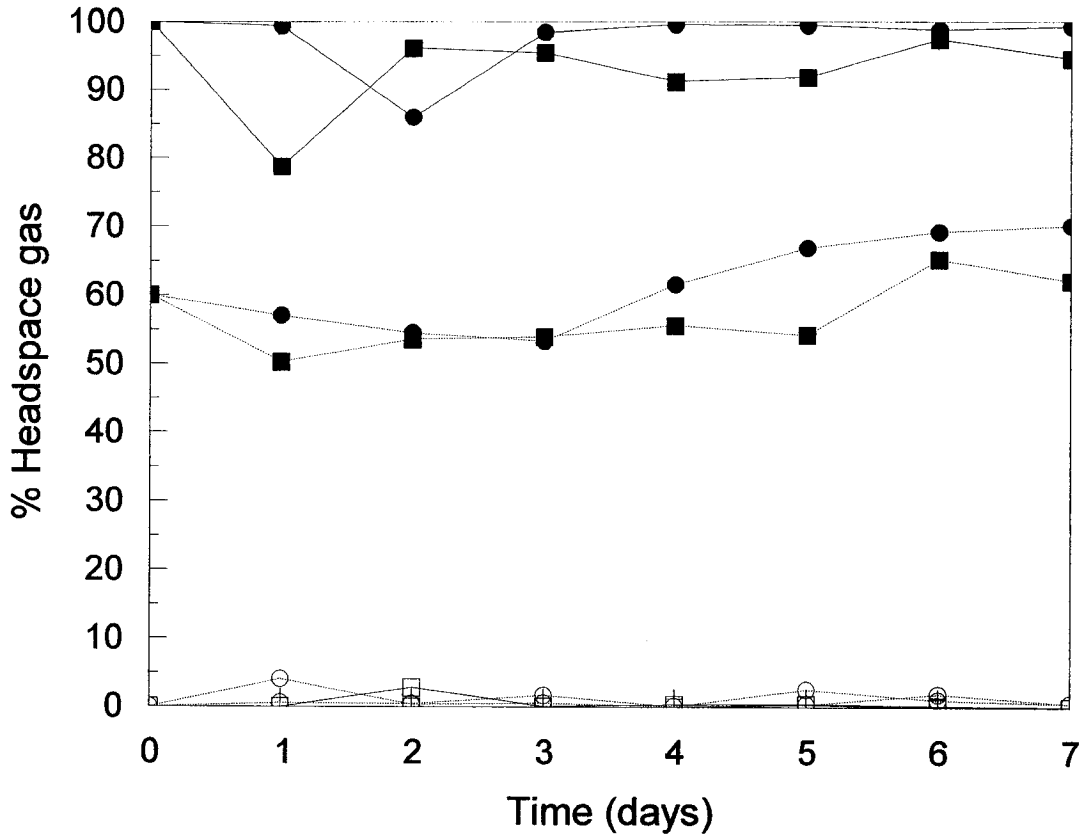


FIGURE 4.1. *Changes in headspace gas composition (O_2, open symbols; CO_2, closed symbols) of crumpets at pH 6.5 (●) and pH 8.3 (■) inoculated with 500 spores/g* C. botulinum, *packaged in 60% CO_2 (----) or in 100% CO_2 (—), and stored at 25°C*

6.5 crumpets. The low levels of headspace O_2 (<4%) observed in packaged crumpets (Figure 4.1) were attributed to residual O_2 trapped within the porous structure of the crumpets (Piergiovanni and Fava, 1997).

The effect of headspace CO_2 on pH changes is shown in Figure 4.2. For pH 6.5 crumpets packaged in 60% CO_2, pH remained constant throughout storage, while for crumpets of the same pH (6.5) packaged in 100% CO_2, pH decreased gradually from pH 6.5 to ~pH 5.5 by day 7 (Figure 4.2). For pH 8.3 crumpets, identical changes in pH were observed for crumpets irrespective of the level of packaging CO_2 (Figure 4.2). Alkaline crumpets (pH 8.3) were formulated to contain five times as much (wt/wt) bicarbonate as pH 6.5 crumpets. Whether packaged in either 60% or 100% CO_2, the pH of the alkaline crumpets (pH 8.3) decreased rapidly to approximately 7.3 within 24 hours and remained at this pH throughout storage (Figure 4.2). At pH 7.3, a buffering effect may have resulted because approximately equimolar amounts of dissolved CO_2 and bicarbonate would have been present (Daniels et al., 1985).

4.3.2 Neurotoxin detection

Neurotoxin was detected in inoculated crumpets regardless of product pH or level of packaging CO_2 (Table 4.1). However, the time at

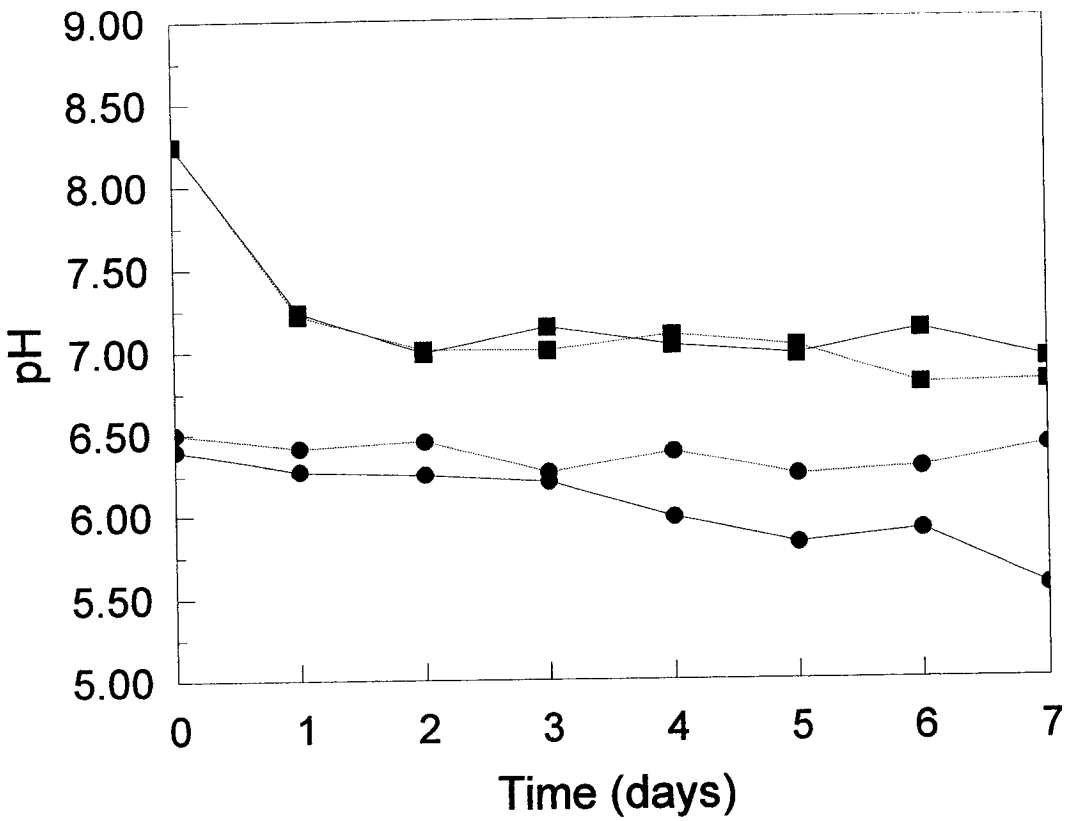


FIGURE 4.2. *Changes in pH of crumpets at pH 6.5 (●) and pH 8.3 (■) inoculated with 500 spores/g* C. botulinum, *packaged in 60% CO_2 (----) or in 100% CO_2 (—), and stored at 25°C*

TABLE 4.1. *Effect of pH and packaging atmosphere on sensory quality and toxicity of crumpets inoculated with 500 spores/g* C. botulinum *and stored at 25°C*

% gas (vol/vol)		pH		Sensory analysis scores[a, b]			Accepta-bility[b]	Time to toxicity[c]
CO_2	N_2	Initial	Final[b]	Colour	Texture	Odour		
60	40	6.5	5.9	0	1	1	+	4
60	40	8.3	7.1	1	0	0	+	4
100	0	6.5	5.9	0	1.25	1.5	+	5.5
100	0	8.3	6.9	0	2.5	2.5	±	7

[a] 0, no deterioration; 5 extreme deterioration; > 2.5, termination of shelf-life

[b] At the time of earliest neurotoxin detection

[c] Day of earliest neurotoxin detection

which neurotoxin was first detected was significantly influenced by an interaction between initial product pH and initial level of packaging CO_2 ($P < 0.001$). The time-of-earliest neurotoxin detection was not influenced by pH when crumpets were packaged in 60% CO_2 (Table 4.1). All crumpets (pH 6.5 and 8.3) initially packaged in 60% CO_2 and stored at 25°C, were toxic by day 4 (Table 4.1). However, product pH was a significant factor ($P < 0.015$) influencing time-of-earliest neurotoxin detection when crumpets were packaged in 100% CO_2. Spores of *C. botulinum* have been shown to germinate rapidly in 100% CO_2 (Foegeding and Busta, 1983; Kim and Foegeding, 1993b). However, packaging crumpets (pH 6.5 and 8.3) in 100% CO_2 resulted in a delay in the time of earliest neurotoxin detection of 1.5 days and 3 days, respectively (Tables 4.1 and 4.2). No neurotoxin was detected in any uninoculated crumpets after 10-days storage at ambient temperature (results not shown).

Whenever neurotoxin was detected in any crumpets, product pH at the time of detection was always ≤7.9. However, the upper pH limit for neurotoxin production by *C. botulinum* in MAP crumpets is probably somewhat higher than 7.9 since preliminary studies have demonstrated neurotoxin production at pH 8.7 in crumpets packaged in air with an oxygen absorbent and stored at ambient temperature (unpublished data). Several commercially produced bakery products have an alkaline pH (7 to 9) (Denny et al., 1969; Aramouni et al., 1994; Jenson et al., 1994; Smith and Simpson, 1995), and such high-pH bakery products have the

TABLE 4.2. *Neurotoxin detection in crumpets inoculated with 500 spores/g* C. botulinum *packaged in 60% or 100% CO_2 and stored at 25°C*

	% gas (vol/vol)		Neurotoxin analysis[a] (day)							
pH	CO_2	N_2	3	4	5	6	7	8	9	10
6.5	60	40	0/2	2/2	2/2	2/2	2/2	—[b]	—	—
6.5	100	0	0/2	0/2	1/2	2/2	1/2	2/2	0/2	2/2
8.3	60	40	0/2	2/2	2/2	2/2	2/2	—	—	—
8.3	100	0	0/2	0/2	0/2	0/2	2/2	0/2	—	2/2

[a] Number of samples testing positive/number of samples analysed

[b] No samples analysed for neurotoxin

potential to support growth of *C. botulinum* if packaged under MAP conditions. Foods are sometimes reformulated to pH <4.6, the minimum pH for growth and neurotoxin production by *C. botulinum* (Dodds and Austin, 1997), in order to ensure food safety. However, the upper pH limit (~pH 9) (Hobbs, 1976) for growth for *C. botulinum* is not generally considered to be as significant to food safety because the pH of most foods is generally <7. Furthermore, because the pH of food may decrease during storage as a result of microbiological and chemical changes, reformulation to exceed the upper limit (pH >9) may not be sufficient to ensure safety with respect to *C. botulinum*.

Although neurotoxin production by *C. botulinum* was not inhibited in crumpets packaged in CO_2, it was slightly delayed at a high CO_2 level (100%), particularly in crumpets with an initial pH of 8.3 (Table 4.1). This study is in agreement with previous studies that reported a delay in neurotoxin production by *C. botulinum* in elevated levels of CO_2. Doyle (1983) observed a one-day delay in neurotoxin production by *C. botulinum* in media under 100% CO_2 compared to media under 100% N_2. A delay in neurotoxin production has also been reported in MAP pork packaged in 45% to 75% CO_2 compared to pork packaged in 15% to 30% CO_2 (Lambert et al., 1991b).

When crumpets were packaged in 60% CO_2, neurotoxin was first detected after four-days storage at 25°C, regardless of pH (Table 4.2). Once neurotoxin was first detected in crumpets packaged in 60% CO_2, it

was consistently detected from day 4 to day 7 (Table 4.2). However, this trend was not observed for pH 6.5 or 8.3 crumpets packaged in 100% CO_2 (Table 4.2). This suggests that as well as delaying toxicity by 1.5 days to 3 days (Table 4.2), packaging in 100% CO_2 reduced the probability of detecting neurotoxin on subsequent sampling days after it was first detected. Several models, developed to predict i) the time-to-turbidity, or ii) the probability of neurotoxin formation in samples inoculated with *C. botulinum* under specified conditions, or ii) both, anticipate a lower probability of toxigenesis under less than optimal conditions (Whiting, 1995).

The mechanisms by which CO_2 delays neurotoxin production by *C. botulinum* is not entirely understood. However, it is generally accepted that the effect depends on the dissolution of CO_2 in the food (Daniels et al., 1985) and the movement of CO_2 or H_2CO_3 into the cell. One explanation for the delayed growth of and neurotoxin production by *C botulinum* in crumpets may be that at high levels, CO_2 may result in inhibition of decarboxylase reactions through mass action effects (Daniels et al., 1985).

4.3.3 Sensory analysis

Sensory scores were similar for all crumpets throughout storage irrespective of product pH or level of packaging CO_2 (results not shown).

For most crumpets, shelf-life was terminated after seven-days storage at ambient temperature. While colour scores remained optimal throughout storage, deterioration in both texture and odour scores was observed. Shelf-life was considered to be terminated when texture scores, odour scores, or both were >2.5. All products were organoleptically acceptable when they became toxic (Table 4.1), although pH 8.3 crumpets packaged in 100% CO_2 were marginally acceptable (score = 2.5) when neurotoxin was first detected (day 7 [Table 4.1]). Neurotoxin production by proteolytic *C. botulinum* prior to spoilage in foods at ambient temperature has been reported by several researchers (Kautter et al., 1981; Sugiyama et al., 1981; Austin et al., 1998; Solomon et al., 1998; Daifas et al., 1999a, b). Neurotoxin production by *C. botulinum* preceding spoilage of foods packaged under MAP conditions and stored at ambient temperature highlights the need for additional barriers to control the growth of this pathogen.

4.4 CONCLUSION

In conclusion, this study confirms that high-moisture high-pH bakery products, if contaminated with spores of *C. botulinum*, may become hazardous if packaged in CO_2. Although toxigenesis was delayed in crumpets (pH 6.5 and 8.3) packaged in 100% CO_2 and stored at ambient temperature, all products became toxic prior to spoilage. Further research is therefore required to determine the level of additional barriers to inhibit the growth of *C. botulinum* and ensure the safety of high-moisture low- and high-pH bakery products packaged under elevated levels of CO_2.

PREFACE TO CHAPTER 5.0

Previous studies had determined the need to incorporate barriers to inhibit the growth of and neurotoxin production by *C. botulinum* into high-moisuture bakery products, specifically English-style crumpets packaged under various atmospheres and stored at ambient temperature. The present study was designed to evaluate the potential of ethanol applied in the vapour state as a potential barrier to the growth of *C. botulinum* in packaged crumpets with the objective of ensuring the safety of these products at ambient temperature.

5.0 EFFECT OF ETHANOL VAPOUR ON GROWTH OF AND NEUROTOXIN PRODUCTION BY *CLOSTRIDIUM BOTULINUM* IN A HIGH-MOISTURE BAKERY PRODUCT

5.1 INTRODUCTION

Previous studies have demonstrated that high-moisture bakery products, when inoculated with spores of proteolytic *Clostridium botulinum* and packaged in high gas-barrier bags in air or under modified atmospheres, supported growth of and neurotoxin production by this pathogen at ambient temperature (Daifas et al., 1999a, b, c). The ability of such foods to become toxic prior to spoilage means that additional barriers need to be incorporated within the food product and/or packaging to ensure product safety.

Ethanol is well known for its antimycotic effect in foods, particularly bakery products. Ethanol has been shown to increase the mould-free shelf-life of bread (Seiler, 1978, 1989; Seiler and Russell, 1991; Vora and Sidhu, 1987; Salminen et al., 1996), par-baked pizza (Seiler, 1989), pita bread (El-Khoury, 1999) and pasta (Giavedoni et al., 1994). Ethanol vapour has also been reported to inhibit fermentation in apple turnovers (Smith et al., 1987) as well as yeast and mould growth in pita bread (Black et al., 1993). Although Hall and Spencer (1964) observed a three- to five-day increase in the shelf-life of chickens that had been pre-dipped in ethanol, very little is known about the effect of ethanol on bacteria in food. To the best of our

knowledge there have been no reports of the effect of ethanol, specifically ethanol vapour, on the growth of and neurotoxin production by *C. botulinum* in bakery products.

The objective of this study was to investigate the effect of ethanol vapour on the growth of and neurotoxin production by *C. botulinum* in English-style crumpets inoculated with *C. botulinum*, packaged in air in high barrier bags and stored at ambient temperature (25°C).

5.2 MATERIALS AND METHODS

5.2.1 Formulation of crumpets

English-style crumpets (a_w 0.990, pH 6.5) were prepared in a food lab (McGill University) from all-purpose flour, sugar, yeast, reconstituted dry milk, salt, $NaHCO_3$ and α-amylase (Novamyl; Novo Nordisk; Danbury, CT) and were baked in cylindrical moulds on a pre-heated griddle (204°C) as described previously (Daifas et al., 1999b). Baked crumpets measured approximately 8 cm × 1.6 cm and weighed approximately 50 g each.

5.2.2 Preparation of spore inoculum/sample inoculation

A composite inoculum of *C. botulinum* spores was prepared from three type A strains (17A, 62A, and CK2A) and three proteolytic type B strains (MRB, 13983IIB, and 368B) (Health Canada). Spore crops of each strain were prepared separately, enumerated as described by Hauschild and Hilsheimer (1977), and stored at -80°C. Equal numbers of spores of each strain were then combined to form a single suspension of approximately 3 x 10^5 spores/mL. The spore mixture was heat shocked at 75°C for 20 minutes prior to sample inoculation. Inoculum levels were verified prior to inoculation of samples by plating appropriate dilutions in

duplicate on McClung Toabe Agar (Difco, Becton Dickinson; Sparks, MD) containing 0.5% yeast extract (Difco) and egg-yolk (Austin and Blanchfield, 1996) and incubating anaerobically in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 at 35°C for three days (Austin et al., 1998). Crumpets were stab inoculated by pipette ~0.5 cm beneath the surface at six distinct, equidistant locations on the top surface of the crumpet with 180 μL (6 x 30 μL) of spore suspension per 100-g sample to provide a final inoculum level of 5×10^2 spores/g. Control samples were inoculated in a similar manner with an identical volume of 0.1% (wt/wt) peptone water.

5.2.3 Packaging

All inoculated and control crumpets were packaged by placing two crumpets (~50 g each) into 210-mm x 210-mm high gas barrier Cryovac bags (O_2 transmission rate: 3 to 6 cc/m²/day, 1 atm at 4°C, 0% RH; ethanol transmission rate: 0.21 g/m²/day, 1 atm at 25°C; Cryovac; Mississauga, ON). Crumpets were packaged in duplicate, with or without ethanol. Crumpets packaged with ethanol contained either i) 2-, 4- or 6-G sachets of Ethicap®, a commercially available ethanol vapour generator (Freund Industrial Company, Ltd.; Tokyo, Japan) or ii) sterile cotton wool pads saturated with 2, 4, or 6 g of filter-sterilized 95% ethanol. Ethicap® (Antimold 102) consists of a heat-sealed sachet containing food grade

ethanol adsorbed onto silicon dioxide releasing at least 55% ethanol on a weight basis. Trace amounts of vanilla, citrus or other flavours are included to mask undesirable flavours. The ethanol sachets and ethanol-soaked pads were added to the bags immediately prior to sealing with an impulse heat-sealer. All packaged samples were stored at 25°C. Duplicate samples of inoculated crumpets were analysed at days 0, 3, 5, 7, 8, 9, 10, 14, and 21. Duplicate samples of uninoculated control crumpets were analysed at days 0 to 7, 10, 12, and 14.

5.2.4 Headspace O_2 and CO_2 analysis

Changes in headspace O_2 and CO_2 were monitored using a previously calibrated Oxygen/Carbon Dioxide Analyser (Servomex, Food Package Analyzer Series 1400; Minneapolis, MN). Samples of headspace gas were withdrawn using a 0.5-mL gastight pressure-Lok® syringe (Precision Sampling Co; Baton Rouge, LA) through a septum attached to the outside of each package.

5.2.5 Oxidation reduction potential of crumpets

The oxidation reduction potential (ORP) of uninoculated crumpets was determined using a pH meter (Model 2220, Corning Glassworks; Corning, NY) fitted with a previously cleaned combination polished platinum oxidation reduction potential electrode containing a refillable

KCl/AgCl reference electrode (model 3131B; Hanna Instruments; Bedfordshire, UK). The electrode was activated by pre-treatment in a reducing solution of ferrous sulphate in dilute HCl (HI 7091L; Hanna Instruments) and calibrated against a standard solution of potassium hydrogen phthalate-quinhydrone measuring 200 to 275 mV (HI 7020; Hanna Instruments). The ORP of crumpets was measured by positioning the electrode ~1.3 cm below the surface of two crumpets packaged in a high gas barrier Cryovac bag (O_2 transmission rate: 3 to 6 cc/m^2/day, 1 atm at 4°C, 0% RH.; ethanol transmission rate: 0.21 g/m^2/day, 1 atm at 25°C; Cryovac; Mississauga, ON). The bag was subsequently sealed tightly around the probe to minimize ingress of oxygen.

5.2.6 Headspace ethanol

Changes in headspace ethanol were monitored using a Varian gas chromatograph (model 3300, Varian Canada, Inc; Montreal, QC), fitted with a flame ionization detector and using a Nukol column (30-M × 0.53-mm; Supelco, Canada, Ltd). Helium was the carrier gas with a flow rate of 30 mL/minute. The column temperature was set at 60°C and the injector port at 100°C. Headspace ethanol was anlaysed by withdrawing 0.5 mL of gas using a gastight pressure-Lok® syringe as described previously for headspace gas analysis and injecting into the chromatograph. Peaks were recorded and analysed with a Hewlett-Packard

integrator (model 3390A, Hewlett-Packard Canada, Ltd.; Kirkland, QC). The concentration of headspace ethanol (% vol/vol) was determined from a standard curve (R^2 0.998) generated by analysing, in triplicate, standard solutions of 95% food grade ethanol ranging in concentration from 0.25 to 10% (vol/vol).

5.2.7 Sensory analysis

On each sampling day, packages were evaluated for swelling and crumpets for visible mould growth. Texture, colour and odour of crumpets were evaluated by three untrained panellists using a six-point hedonic scale (0 = no deterioration; 5 = extreme deterioration) as previously described (Daifas et al., 1999a). A sensory score of 2.5 was taken as the cut-off for acceptability and termination of shelf-life.

5.2.8 Neurotoxin assay

Each sample (two crumpets) was aseptically transferred to a stomacher bag. A 1:3 dilution was prepared by adding twice the sample weight of 0.1% (wt/wt) peptone water and then stomaching for 2 minutes using a Colworth Stomacher (Seward Medical Stomacher; London, UK). The homogenized samples were centrifuged at 23,500 x g for 20 minutes at 4°C. Botulinum neurotoxin was detected as described previously (Daifas et al., 1999a, b, c). An aliquot of the sample supernatant was

filtered through a 0.45-μm filter (Acrodisc, Gelman Sciences; Ann Arbor, MI) and 0.5 mL of this filtrate was injected intra-peritoneally into each of two 20- to 28-g mice (Charles River; QC). Mice were observed for up to 72 hours for typical signs of botulism including ruffled fur, pinched waist, laboured breathing, limb paresis and general paralysis. Mice showing extreme distress were humanely asphyxiated with CO_2 according to Health Canada Animal Care Committee guidelines (Daifas et al., 1999c). Neutralization of neurotoxin was performed on randomly selected positive samples, representative of all packaging conditions, using antisera (Connaught Laboratories; North York, ON) to botulinum neurotoxins. Survival of mice that were injected with a positive sample supernatant while protected with antisera and death of mice that were similarly injected, but not protected with antisera, was interpreted as confirmation that toxicity could be attributed to *C. botulinum* (Austin and Blanchfield, 1996).

5.2.9 Changes in sample pH

The pH of the samples was determined using a previously calibrated (buffers pH 4.0 and 7.0) Fisher Accumet® pH meter (Fisher Scientific; Ottawa, ON). Portions of filtered sample supernatant of the homogenized, centrifuged samples were transferred to glass tubes and

pH measurement was made by immersing the electrode directly into each tube.

5.2.10 Ethanol content of crumpets

Changes in the ethanol content of crumpets were monitored using a Varian gas chromatograph (model 3300, Varian Canada, Inc; Montreal, QC). The column, carrier gas, carrier gas flow rate and column temperature were as described previously for headspace ethanol analysis; however, the injector port temperature was increased to 150°C. The ethanol content of crumpets was analysed by injecting 1 mL of supernatant of the centrifuged homogenized sample (1:3 dilution) into the chromatograph. Peaks were recorded and analysed with a Hewlett-Packard integrator (model 3390A; Hewlett-Packard Canada, Ltd.; Kirkland, QC). The ethanol content of crumpets (% wt/wt) was determined from a standard curve (R^2 0.996) generated by analysing, in triplicate, standard solutions of 95% food grade ethanol ranging in concentration from 0.25 to 10% (wt/wt).

5.2.11 Statistical analysis

Non-linear regression (Steel and Torrie, 1980) curves were fit using Prism 2.01 (GraphPad Software, Inc.; San Diego, CA).

5.3 RESULTS AND DISCUSSION

5.3.1 Changes in headspace oxygen, carbon dioxide and oxidation-reduction potential

Changes in headspace O_2 and CO_2 of crumpets inoculated with 500 spores/g *C. botulinum*, packaged in air with an ethanol vapour generator or with ethanol applied to a cotton pad and stored at 25°C, are shown in Figure 5.1A-B respectively. For control crumpets (inoculated and packaged in air without ethanol), headspace O_2 decreased to <1% while headspace CO_2 increased to ~45% by day 3. By day 7, headspace CO_2 increased to ~75%, a level it remained at for the duration of storage (Figure 5.1A-B).

Similar trends were observed for crumpets packaged in air with 2- to 6-G Ethicap® ethanol vapour generators or with 2 or 4 g of ethanol applied on pads. However, the rates at which headspace O_2 decreased and headspace CO_2 increased depended on the size of sachet or amount of ethanol applied to the pad (Figure 5.1A-B). The lowest levels of CO_2 were observed for crumpets packaged with greater amounts of ethanol. For crumpets packaged with 6 g ethanol per pad, headspace O_2 decreased only to ~10% while headspace CO_2 was <10% after 21-days storage at 25°C (Figure 5.1B). None of these packages was visibly swol-

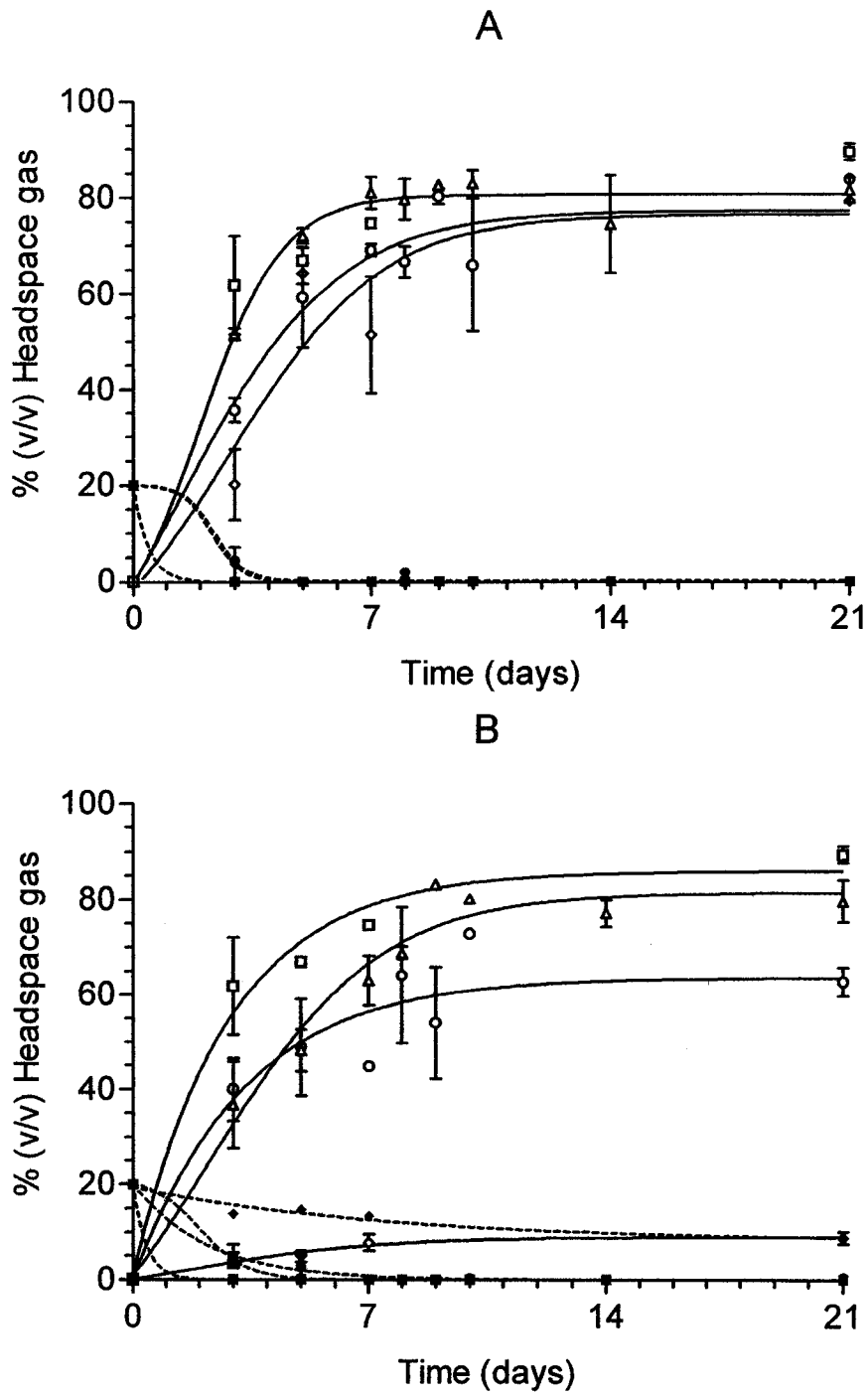


FIGURE 5.1. *Changes in headspace O_2 (---) and CO_2 (—) in crumpets inoculated with 500 spores/g* C. botulinum, *packaged in air without ethanol (*) or with A, 2-G (○), 4-G (□), or 6-G (△) Ethicap® or B, 2 g (●), 4 g (■), 6 g (▲) ethanol and stored at 25°C*

len; however, all crumpets were overtly spoiled before the end of storage.

Almost identical trends in headspace gas changes were observed for uninoculated control crumpets (results not shown). This suggests that *C. botulinum* did not influence changes in headspace CO_2 which may instead be attributed to the inhibitory effect of ethanol vapour on endogenous microorganisms. Concentration dependent inhibition of microorganisms by ethanol has been reported. Seiler and Russell (1991) observed that the mould-free shelf-life of bread increased with increasing ethanol concentration. Similarly, Yamamoto et al. (1984) reported a concentration dependent logarithmic inhibition of bacteria by ethanol.

Although lower headspace CO_2 levels were associated with higher amounts of ethanol, headspace CO_2 in all crumpets (with the exception of those packaged with 6 g ethanol/pad) still increased to >50% after seven days with most packages having a blown appearance. Although no microbiological testing (with the exception of botulinum neurtotoxin) was conducted in the present study, previous studies had shown that the background microflora of crumpets included yeasts, moulds, *Bacillus* spp. and heterofermentative lactic acid bacteria (results not shown). This is consistent with Smith et al. (1987) who reported that these microorganisms are common contaminants of crumpets. Therefore, these microorganisms may have contributed to the increase in headspace CO_2 observed in this study.

The oxidation-reduction potential (ORP) of uninoculated control crumpets (packaged without ethanol) decreased rapidly to approximately -100 mV by day 3 (Figure 5.2), corresponding to a decrease in headspace O_2 to <0.1%. This rapid decrease in ORP may again be attributed to microbial respiration. When uninoculated crumpets were packaged with 6-G Ethicap®, the ORP decreased gradually to +50 mV by day 5 and remained at this level (Figure 5.2) even though headspace O_2 eventually decreased to <0.1%. A positive ORP of a food is largely attributed to the presence of dissolved O_2; however, positive conditions can also result from microbial oxidation of carbon sources in the absence of oxygen (Voet and Voet, 1990). The reported maximum ORP at which germination and growth of *C. botulinum* can be initiated ranges from +30 mV to +250 mV (Kim and Foegeding, 1993a). Therefore, although the minimum measured ORP of crumpets packaged with 6-G Ethicap® was +50 mV, it was within the range at which growth has been reported.

5.3.2 Headspace ethanol analysis

It was possible to measure headspace ethanol in packages of uninoculated crumpets only. Since no difference in changes in headspace O_2 and CO_2 was observed between uninoculated and inoculated crumpets (results not shown), changes in headspace ethanol of inoculated crumpets were assumed to be similar to those observed for

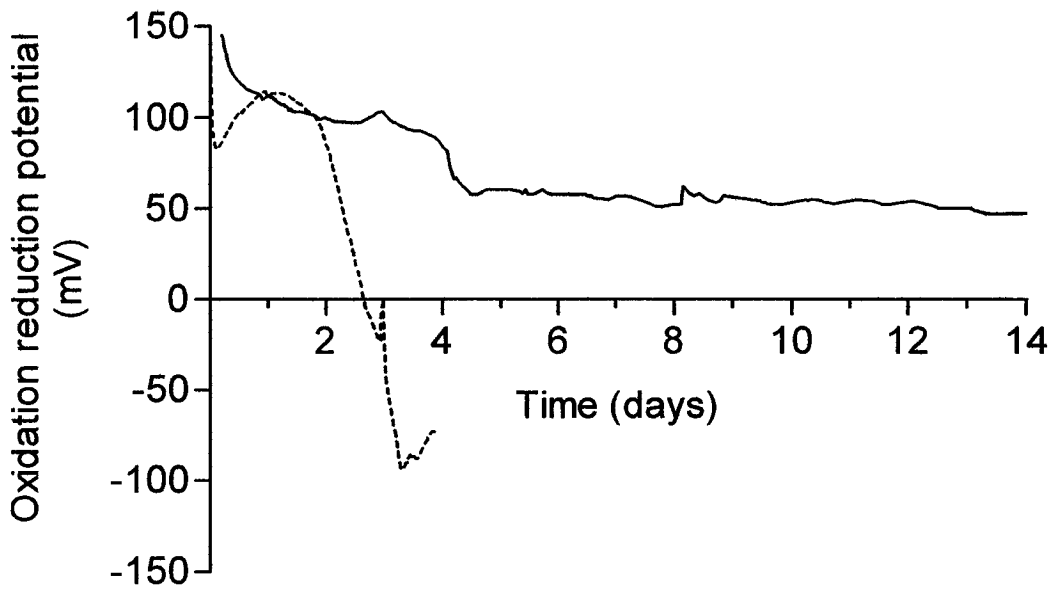


FIGURE 5.2. *Changes in oxidation-reduction potential of crumpets packaged without* (---) *and with* (—) *6-G Ethicap® at 25°C*

uninoculated crumpets. Headspace ethanol of packaged uninoculated crumpets increased with the size of Ethicap® or amount of ethanol that crumpets were packaged with. After one-days storage headspace ethanol ranged from 2.3% to 5.8% (vol/vol) for 2- to 6-G Ethicap® and from 2.8% to 9% (vol/vol) for 2 to 6 g ethanol/pad (Figure 5.3). Headspace ethanol for crumpets packaged with 6 g ethanol was initially high [9% (vol/vol)]; however, headspace ethanol decreased to ~6% (vol/vol) after four days and remained at this level throughout storage (Figure 5.3). These trends are consistent with Salminen et al. (1996) and Smith et al. (1987) who also reported significant correlation between headspace ethanol and the size of ethanol vapour generator packaged with bakery products.

Crumpets packaged without ethanol had an initial headspace ethanol content ~0.5% ([vol/vol] day 1) which increased gradually to ~3% (vol/vol) after 14-days storage at 25°C (Figure 5.3). This increase in headspace ethanol can be attributed to yeast fermentation. Crumpets are cooked in metal rings on a griddle just until the top surface is no longer wet and yeast may survive baking.

5.3.3 Sensory evaluation

The shelf-life of crumpets inoculated with 500 spores/g of *C. botulinum* and packaged with various sizes of ethanol vapour genera-

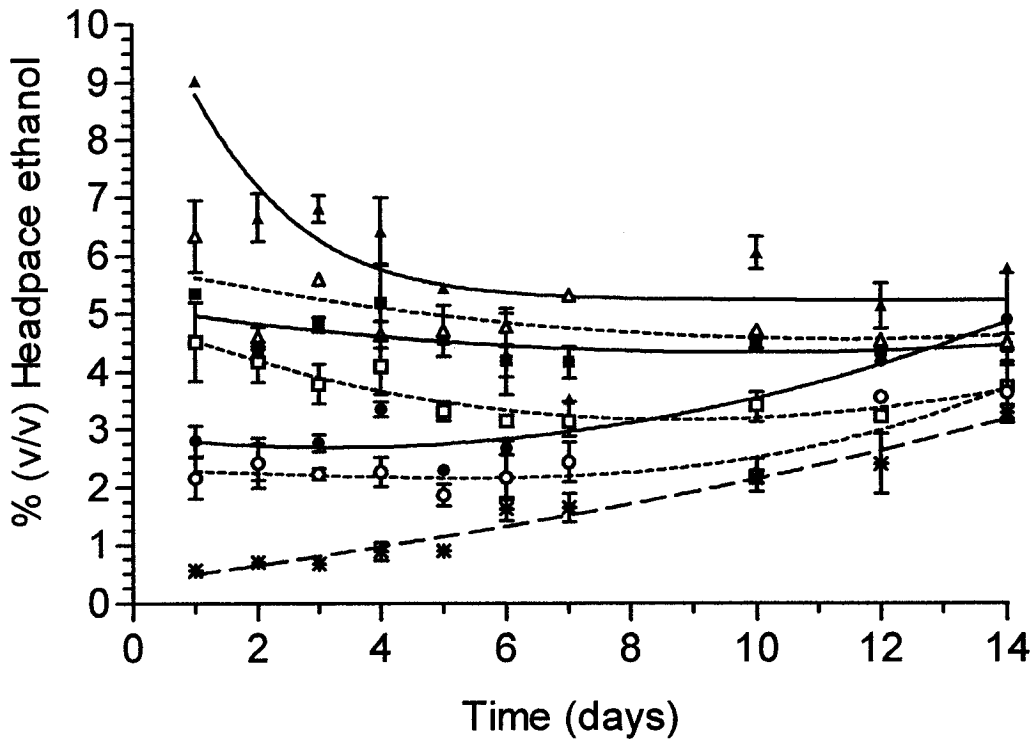


FIGURE 5.3. *Changes in headspace ethanol in crumpets inoculated with 500 spores/g* C. botulinum, *packaged in air without ethanol* (*), *with 2-G* (○), *4-G* (□), *6-G* (△) *Ethicap®* (---) *or with 2 g* (●), *4 g* (■), *6 g* (▲) *ethanol* (—) *and stored at 25°C*

tors or ethanol pads is shown in Table 5.1. Shelf-life of crumpets was terminated when odour and/or texture scores were ≥2.5. At these scores crumpet texture was characterized as soft and mushy while off-odours were described as "fruity" and/or "alcoholic". No visible mould was observed on any crumpets throughout storage.

The shelf-life of control crumpets was terminated after six-days storage at ambient temperature, i.e., one day after neurotoxin was detected in these control crumpets (packaged in air without ethanol vapour) (Table 5.1). Packaging crumpets with ethanol vapour resulted in, at most, a three-day extension of sensory shelf-life (Table 5.1). When crumpets were packaged with 2-G Ethicap® ethanol vapour generators, no shelf-life extension was observed, i.e., crumpets were overtly spoiled by day 6. However, spoilage preceded toxigenesis as neurotoxin was first detected in these crumpets at day 10. Neurotoxin was not detected during the 21-day storage period in any other crumpets (packaged with 4-G or 6-G Ethicap® or with 2, 4 or 6 g ethanol/pad). However, all crumpets were overtly spoiled by day 9 (Table 5.1).

This slight increase in shelf-life was significantly less than the 16-day extension reported for bread (200- to 300-g) stored at ambient temperature in air with 1% (wt/wt) ethanol (Vora and Sidhu, 1987) or in air with 2-G to 3-G Ethicap® (Salminen et al., 1996). In these two studies, the amount of ethanol used (≤1% [wt/wt]) did not affect changes in the sensory quality of the bread. Similarly, Seiler (1978) showed that cake

TABLE 5.1. *Neurotoxin detection and shelf-life in crumpets inoculated with 500 spores/g* C. botulinum, *packaged in air with ethanol and stored at 25°C*

Ethanol/100-g crumpets	Day neuro-toxin first detected[b]	Day shelf-life terminated[c]	% Absorbed ethanol[d,e] (wt/wt)	Estimated water activity[d, f]
Control				
None	5	6	1.2	0.971
Ethicap® ethanol vapour generator				
2-G [a]	10	6	2.3	0.964
4-G	>21[b,*]	6	2.7	0.960
6-G	>21	7	4.1	0.945
Ethanol (95% [wt/wt]) applied to pad				
2 g	>21	6	2.6	0.961
4 g	>21	7	3.2	0.955
6 g	>21	9	4.4	0.942

* No samples were analysed for neurotoxin after day 21

[a] Ethicap® releases 55% (wt/wt) ethanol, i.e., 2-G releases 1.1 g ethanol (Freund, 1987)

[b] Neurotoxin was detected by mouse bioassay

[c] Shelf-life terminated when average sensory evaluation score was >2.5 where 0 = no deterioration and 5 = extreme deterioration

[d] Ethanol content of crumpets not inoculated with *C. botulinum.*

[e] At time of first neurotoxin detection or at the end of storage.

[f] Calculated using Ross equation (1975).

and bread treated with 1% (wt/wt) of ethanol were sensorially acceptable but when treated with >2% (wt/wt), alcoholic flavours were very noticeable and products were rejected on the basis of flavour and/or odour. Alcoholic odours were often described in sensory analysis of crumpets used in this study irrespective of the amount of ethanol included in the package. Crumpets have a much higher water activity (a_w 0.99) than bread (a_w 0.94 [Salminen et al., 1996]) and therefore, would absorb more ethanol vapour from the package headspace than would bread–resulting in significant changes to the odour of crumpets. The higher ethanol content of crumpets may be acceptable as crumpets are usually toasted before consumption and heating products that have been packaged with ethanol vapour has been shown to reduce the ethanol content (Smith et al., 1987; Giavedoni et al., 1994).

5.3.4 Neurotoxin detection

Neurotoxin was detected after five days in all control crumpets inoculated with *C. botulinum*, packaged in air without an ethanol sachet or pad and stored at 25°C (Table 5.1). These results are consistent with previous studies in which neurotoxin was first detected after six days in crumpets packaged in air and stored at ambient temperature (Daifas et al., 1999b). However, neurotoxin production was delayed when inoculated crumpets were packaged in air with ethanol vapour (Table 5.1).

Neurotoxin was first detected after 10-days storage at ambient temperature in crumpets packaged with a 2-G Ethicap® ethanol vapour generator. Headspace ethanol for uninoculated crumpets packaged with 2-G Ethicap® was ~2.3% (vol/vol) from days one to ten (Figure 5.3). When crumpets were packaged with larger sizes of ethanol vapour generators (4- or 6-G Ethicap®) or with 2 to 6 g of ethanol applied to pads, neurotoxin was not detected during 21-days storage (Table 5.1). The corresponding headspace ethanol in uninoculated crumpets packaged with larger sizes of Ethicap® (4-G, 6-G) or 2 g to 6 g ethanol on pads was always ≥2.8% (Figure 5.3). No neurotoxin was detected in any uninoculated crumpets (results not shown).

These results agree with previous yeast and/or mould inhibition studies which showed that the effectiveness of ethanol vapour as an antimicrobial agent was dependent on growth conditions of the medium or food, particularly water activity (a_w). At lower a_w, smaller sizes of sachets and hence, lower levels of ethanol vapour, were required to inhibit microbial growth. As growth conditions became more optimal, the level of ethanol required for complete microbial inhibition increased (Smith et al., 1987). Salminen et al. (1996) reported that small ethanol emitters (0.6-G and 1-G Ethicap®) had no influence on the mould-free shelf-life of 200-g slices of rye bread. However, larger emitters (2-G and 3-G Ethicap®) extended the mould-free shelf-life for 8 days to 12 days

and 26 days to 27 days respectively at ambient storage temperature. Shapero et al. (1978) also reported that a combination of low a_w and 2% to 4% (wt/wt) could be used to inhibit the growth of *S. aureus* in model agar studies.

5.3.5 Changes in pH

The pH of all inoculated crumpets packaged with ethanol vapour decreased over storage as a function of the size of the ethanol vapour generator or amount of ethanol per pad. The initial pH of crumpets was 6.5. The final pH at the end of storage (day 21) of crumpets packaged without ethanol was 5.5. In comparison, the final pH of crumpets packaged with the highest level of ethanol (6 g) was essentially unchanged at 6.2. This trend may be attributed to the inhibitory effect of ethanol on the growth and metabolism of spoilage microorganisms. However, the pH of all crumpets remained, throughout storage, conducive to the growth of *C. botulinum*.

5.3.6 Ethanol content of crumpets

It was possible to measure the ethanol content of uninoculated crumpets only. The ethanol content of uninoculated crumpets also increased as the size of ethanol vapour generator or amount of ethanol per pad increased.

At the time at which neurotoxin was first detected (day 10) in crumpets packaged in air with 2-G Ethicap® the corresponding ethanol content of uninoculated crumpets (packaged with 2-G Ethicap®) was ~2.3% (wt/wt) (Table 5.1). However, uninoculated crumpets packaged in air with 2 g ethanol per pad contained, at day 10, ~2.6% (wt/wt) ethanol (Table 5.1). Complete inhibition of neurotoxin (>21 days) was observed in inoculated crumpets packaged with 2 g ethanol/pad. Assuming that headspace levels were similar for inoculated and uninoculated crumpets, this difference may be related to the slightly higher levels of headspace and/or absorbed ethanol in crumpets packaged with 2 g ethanol/pad than in crumpets packaged with 2-G Ethicap® (Figure 5.3 and Table 5.1). Therefore, since no neurotoxin was detected in any other crumpets packaged with higher amounts of ethanol, it would appear that 2% (wt/wt) of ethanol may be the approximate threshold level required to inhibit neurotoxin production by 500 spores/g *C. botulinum* in crumpets stored at 25°C. In previous studies in agar inoculated with 10^3 spores of proteolytic *C. botulinum* and exposed to ethanol vapour, 3% (wt/wt) was needed to completely inhibit growth (unpublished results). Inhibition of *C. botulinum* in crumpets at a lower level of ethanol (2% [wt/wt]) may be partly due to the effects of pH, a_w or the composition of the crumpets. However, the inhibitory effect of ethanol is principally attributed to its vapour pressure (Leirci et al., 1996). Although Seiler (1989) found no difference in the inhibitory effect of ethanol applied as liquid or as

vapour, Smith et al. (1987) reported that lower levels of ethanol were required in the vapour phase compared to direct addition to a medium or food for complete microbial inhibition.

The ethanol content of crumpets packaged without ethanol increased from 0.7% after baking to ~3% (wt/wt) at the end of storage and may be attributed to fermentation by yeast. Yeasts are generally less sensitive than moulds and bacteria to ethanol (Lücke and Jager, 1997). Some breads of acceptable quality may contain up to 1% to 2% ethanol (dry weight basis [Bonetto and Bortoli, 1996]); however, high levels of ethanol resulting from spoilage problems due to growth of *S. cerevisiae* in par-baked apple turnovers has been reported (Smith et al., 1987). In this study, the ethanol content of crumpets (including control crumpets) at the end of shelf-life (Table 5.1) exceeded the permitted level of 2% (wt/wt) in pizza crusts (Federal Register, 1974). The higher levels of ethanol detected in crumpets is due to their higher a_w (0.990). Smith et al. (1987) and Leirci et al. (1996) observed that the amount of ethanol generated and absorbed in a medium or product was greater at higher a_w than lower. As ethanol is absorbed by the crumpets, the a_w will decrease. However, because of the volatility of ethanol it is impossible, after packaging with ethanol vapour, to measure the a_w of crumpets. The a_w of the crumpets packaged with ethanol vapour was estimated using Raoult's Law and the Ross equation (Ross, 1975). After 10-days storage at ambient temperature, at the time neurotoxin was

detected in crumpets packaged with ethanol vapour, the a_w of crumpets packaged with 2, 4 and 6 g ethanol was estimated to be 0.961, 0.955 and 0.942 respectively (Table 5.1). This lowering of a_w from 0.99 may have contributed to the delay and/or inhibition of neurotoxin observed during storage as the minimum a_w for growth of and neurotoxin production by *C. botulinum* is ~0.95. However, inhibition of microorganisms by ethanol has been shown to be due to the presence of ethanol and not simply due to a reduction of a_w (Shapero et al., 1978; Smith et al., 1987).

In this study, neurotoxin production by *C. botulinum* in English-style crumpets was delayed at least five days using 2-G Ethicap® ethanol vapour generators capable of generating ~1.1 g ethanol. Complete inhibition of neurotoxin was observed for 21 days when crumpets were packaged with larger sizes (4-G or 6-G Ethicap®) or with 2 g to 6 g ethanol/pad, corresponding to ≥1.9 g ethanol. However, the shelf-life of all crumpets was terminated by day 10. Several studies have been done to determine the antimicrobial action of ethanol. Inhibition of DNA, RNA and protein biosynthesis has been reported in *Escherichia coli* exposed to ethanol while ethanol inhibited the glycolitic enzymes of yeast (Ingram and Buttke, 1984). However, although microoganisms vary in their response to ethanol, most studies agree that ethanol exerts its effect through disruption of the cell membrane structure and subsequent interference and/or inhibition of normal cell metabolic functions (Ingram,

1990). Whatever its mode of action, ethanol is a powerful antimicrobial agent and one that could be used to enhance the safety of food.

5.4 CONCLUSION

Based on these preliminary studies, ethanol vapour would appear to be an effective additional barrier to control the growth of and neurotoxin production by *C. botulinum* in high-moisture bakery products and increase the safety of these products at ambient temperature. While ethanol has GRAS status in the USA, it is only permitted at levels up to 2% by product weight in pizza whether the alcohol is added directly to the food or indirectly through a sachet. In these studies, the level of ethanol required to inhibit neurotoxin production by *C. botulinum* was >2%. However, while lower levels of ethanol would probably increase the sensory shelf-life of crumpets, product safety may be compromised.

PREFACE TO CHAPTER 6.0

Previous studies (Chapter 5.0) showed that while ethanol vapour had the potential to delay neurotoxin production by *C. botulinum* in high-moisture MAP crumpets, their sensory shelf-life was compromised. Therefore, in an attempt to determine if lower levels of ethanol could enhance safety of these crumpets, a subsequent study was conducted with media to investigate the effect of ethanol alone and in combination with other barriers on the growth of and neurotoxin production by *C. botulinum.*

6.0 EFFECT OF ETHANOL ON THE GROWTH OF *C. BOTULINUM*

6.1 INTRODUCTION

Modified-atmosphere-packaging (MAP), involving vacuum-packaging, gas flushing and oxygen absorbent technology, has been used to extend the shelf-life of high-moisture bakery products (Smith, 1992). However, concern has been expressed about the safety of these products, particularly with respect to *C. botulinum*, a spore-forming anaerobe that produces a potent neurotoxin. In minimally-processed foods such as bakery products, spores of proteolytic *C. botulinum*, if present in the raw ingredients, will readily survive baking. Since many high-moisture bakery products have a water activity (a_w) >0.95 and a pH >6 (Smith, 1992), these products have the potential to support growth of and neurotoxin production by this pathogen (Dodds and Austin, 1997). It is, therefore, important to ensure that the potential growth of *C. botulinum* in minimally-processed high-moisture MAP bakery products is prevented since the consumption of food in which this pathogen has grown and produced neurotoxin often results in fatal botulism.

To extend product shelf-life and increase safety, the food industry commonly uses a multi-barrier approach. This approach relies on several factors or hurdles which act in conjunction with each other, additively or synergistically, to inhibit or prevent microbial growth (Leistner, 2000). While appropriate combinations of low a_w and pH may be effective against the

growth *of C. botulinum* in bakery products, many products cannot be reformulated to such levels without loss of sensory acceptability. However, optimum combinations of inhibitory factors, at lower levels of each factor, can often result in enhanced safety of products.

One additional barrier that, in conjunction with reduced a_w and/or pH may prove effective in controlling the growth of *C. botulinum*, is ethanol. Recently, ethanol has been shown to inhibit growth of and neurotoxin production by proteolytic *C. botulinum* in English-style crumpets, a high-moisture bakery product that was toxic within one week in challenge studies with this pathogen at ambient temperature (Daifas et al., 1999b). Although ethanol delayed growth and neurotoxin production by *C. botulinum* in inoculated crumpets, the shelf-life of this product was limited due to absorption of ethanol from the package headspace (Daifas et al., 2000). Nevertheless, these studies demonstrated the anti-bolulinal effect of ethanol. However, little is known about the minimum levels of ethanol that could be used to enhance the safety of minimally processed bakery products without compromising product quality. Therefore, the objectives of this study were to model the effects of ethanol, alone and in combination with a_w and pH, on the growth of and neurotoxin production by *C. botulinum* in model broth studies.

6.2 MATERIALS AND METHODS

6.2.1 Growth curve

6.2.1.1 Cultures and growth conditions

A total of six proteolytic strains of Group I *C. botulinum* (17A, 62A, CK2A, MRB, 13983IIB, and 368B) were used throughout this study. Each strain of *C. botulinum* was grown from frozen stock culture onto McClung Toabe agar (Difco, Becton-Dickinson; Sparks, MD) and incubated anaerobically in an atmosphere of 10% H_2, 80% N_2, and 10% CO_2 at 37°C. Overnight broth cultures were prepared in trypticase peptone glucose yeast extract (TPGY) broth (Difco) and incubated anaerobically to provide final inoculum levels of approximately 10^8 CFU/mL. Inoculum levels were verified by plating appropriate dilutions in duplicate on McClung Toabe agar. Flasks of TPGY broth (500-mL), preheated to 37°C, containing 0, 1, 2, 3 or 4% (wt/wt) 95% ethanol were inoculated with 0.3 mL of the composite mixture of *C. botulinum* to provide a final inoculum level of 2×10^4 CFU/mL. All flasks were tightly covered with sterile aluminum foil and incubated in an atmosphere of 10% H_2, 80% N_2, and 10% CO_2 in an anaerobic chamber (Coy Laboratories, Detroit, MI) at 37°C. Growth was

monitored by plating (in the anaerobic chamber) appropriate decimal dilutions of inoculated TPGY broth onto TPGY agar and incubating anaerobically at approximately 90-minute intervals at 37°C for 28 hours. In addition to measuring viable cell counts, growth was simultaneously monitored by measuring optical density (OD) at 600 nm at 90-minute intervals using a Bausch and Lomb Spec 20 spectrophotometer (Fisher Scientific; Ottawa, ON) until the stationary phase was reached. At each sampling time, portions (4-mL) of culture from each flask were filter sterilized using a 0.22-μm filter (Acrodisc, Gelman Sciences, Ann Arbor, MI). Filtrates were held at 4°C until assayed for botulinal neurotoxin using the mouse bioassay.

6.2.1.2 Data treatment

Growth curve data (CFU/mL) were fitted to sigmoidal curves with a four parameter logistic equation using Prism 2.01 (GraphPad Software, Inc., San Diego, CA) and the first $\left(\frac{\partial CFU/mL\,(t)}{\partial t}\right)$ and second derivatives of the sigmoidal curves were determined using Mathcad 5.0 (MathSoft Inc., Cambridge, MA). The times at which the absolute growth rates were greatest were determined as the maxima of the curves of the first derivatives plotted versus time. The ends of the lag and exponential growth phases were analysed as the maxima and minima respectively of the second derivatives plotted versus time

according to the method of Buchanan and Cygnarowicz (1990). The predicted level of ethanol for complete inhibition of proteolytic strains of *C. botulinum* used in this study was obtained from extrapolation of linear regression analysis of the maximal absolute growth rates versus %(wt/wt) ethanol. The effect of ethanol concentration on the time-of-first neurotoxin detection was fit by regression analysis using Prism 2.02 (GraphPad).

6.2.2 Combined effect of ethanol, water activity (a_w) and pH

6.2.2.1 Preparation of spore inoculum and inoculation of TPGY broths

Equal numbers of spores of each strain were combined to form a single suspension of approximately 10^5 spores/mL. Inoculum levels were verified prior to inoculation of tubes of TPGY broth as described previously. The spore mixture was heat-shocked at 75°C for 20 minutes prior to inoculation. Flasks of TPGY broth were prepared at appropriate strengths to ensure that all broths would contain the same amount of medium after additions of ethanol. Flasks of TPGY broth were then adjusted with appropriate volumes of 0.1-N HCl or 0.1-N NaOH to provide a pH range of 6.2 to 8.2. The pH was checked using a previously calibrated Fisher Accumet® pH meter (Fisher

Scientific; Ottawa, ON). The pH-adjusted TPGY broths were subsequently adjusted with appropriate volumes of glycerol to provide a range of a_w from 0.953 to 0.997, taking into account that ethanol would have a depressant effect on the a_w of the broth (Ross, 1975). Water activity was determined using an Aqualab water activity meter (Decagon Devices, Inc.; Pullman, WA). After autoclaving and cooling to 25°C, portions of TPGY broth were aseptically dispensed into sterile tubes. Ethanol (0% to 8% [wt/wt]) was added to tubes of adjusted, sterile TPGY broth immediately prior to inoculating with 0.1 mL of spore suspension (10^5 spores/mL) to provide a final inoculum level of 10^3 spores/mL. Tubes were covered with approximately 3 mL of sterile Vaspar (45% paraffin, 55% white petroleum) and incubated at 25°C. The combinations of factors (ethanol, a_w, and pH) and the number of replicate tubes in each treatment condition were based on the primary model used previously by Whiting and Call (1993) and are shown in Table 6.2. Tubes were checked twice daily for turbidity and/or gas production. Tubes showing evidence of growth were assayed for neurotoxin using the mouse bioassay.

6.2.2.2 Data treatment

To determine the probability of growth as a function of time for a given condition (combination of %[wt/wt] ethanol, a_w, and pH), data

were fitted to a logistic equation using Prism 2.01 (GraphPad Software, Inc.; San Diego, CA). This cumulative probability distribution function has been used previously by Whiting and Call (1993), and Whiting and Oriente (1997) to model the time-to-turbidity for proteolytic and non-proteolytic *C. botulinum* respectively. As outlined by these authors, the parameters of this primary model describe the accumulating number of positive samples with increasing storage time and provide information about the time-to-turbidity, the rate at which samples show evidence of turbidity, as well as the proportion of samples which do not become turbid:

$$P_{(t)} = \frac{P_{\max}}{(1 + e^{k(\tau - t)})}$$

where:

$P_{(t)}$ is the cumulative probability of growth at time t, i.e., the probability that growth occurred between [0, t];

t is the time in days;

P_{max} is the maximum probability of growth after 365 days, i.e. the number of tubes showing evidence of growth (turbidity and/or gas production divided by the total number of tubes in that condition;

κ is the rate of increase in positive (i.e., turbid) tubes ($days^{-1}$);

τ is the time of the midpoint of the function (days), i.e., the time for 50% of the replicates that will eventually become toxic to show turbidity and/or gas production.

6.2.3 Transmission electron microscopy

Preparation for TEM essentially followed the method of Austin et al. (1990). Cells were fixed in 0.2-M cacodylate buffer pH 7.4 containing 2.5% (vol/vol) 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 (15 minutes each in 50%, 70% and 90% ethanol, followed by three 20-minute incubations in 100% ethanol). Samples were then infiltrated and embedded in Taab 812 resin (Marivac, Halifax, NS). 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) operating at 80 kV with the energy loss spectrometer in place.

6.2.4 Nature of the effect of ethanol

Portions (0.1-mL) from tubes of inoculated TPGY broths containing 0% to 8% ethanol (wt/wt) which did not show any evidence of growth (i.e.,

turbidity or gas production) or neurotoxin production after one year at 25°C were transferred into fresh TPGY broth to dilute the ethanol to less than 0.08% (wt/wt), and incubated anaerobically at 37°C and observed for evidence of growth. Supernatant from all subcultured tubes showing evidence of growth was subsequently assayed for neurotoxin.

6.2.5 Detection of neurotoxin

Botulinum neurotoxin was detected using the mouse bioassay. Vaspar (used in the second study only) was removed using a sterile Pasteur pipette prior to filtering vortexed cultures through a 0.22-μm filter (Gelman Scientific). A portion (0.5-mL) of filtrate was injected intraperitoneally into each of two 20- to 28-g mice (Charles River, QC). Mice were observed for up to 72 hours for typical signs of botulism including ruffled fur, pinched waist, laboured breathing, limb paresis and general paralysis. Mice showing severe distress were euthanised by asphyxiation with CO_2 according to Health Canada Animal Care Committee guidelines. Neutralization of neurotoxin was performed on randomly selected representative positive samples using antisera (Connaught Laboratories; North York, ON) to botulinum neurotoxins to confirm that toxicity was due to botulinum neurotoxin as described previously (Daifas et al., 1999b; 2000).

6.3 RESULTS AND DISCUSSION

6.3.1 Effect of ethanol on growth of *C. botulinum*

The growth profiles of the composite mixture of six proteolytic strains of *C. botulinum* grown in TPGY broth containing 0% to 4% (wt/wt) 95% ethanol as monitored by viable cell density (CFU/mL) and $OD_{600\text{-}nm}$ are shown in Figure 6.1A-B respectively. It is evident from Figure 6.1A-B that there was no linear relationship between the log of viable cell counts (CFU/mL) and $OD_{600\text{-}nm}$. It has been shown that a limitation of monitoring growth by OD is that cell numbers cannot be determined from OD since cell number is generally only proportional to OD over a very narrow range. Accordingly, estimation of microbial growth parameters is best achieved using viable counts (Begot et al., 1996, Dalgaard and Koutsoumanis, 2001). Therefore viable count growth curves (Figure 6.1A) were used to estimate growth parameters. Growth of *C. botulinum* was rapid in control flasks of TPGY broth (0% ethanol). However, growth, as measured by log viable cell counts, was inhibited as the concentration of ethanol increased.

Increasing levels of ethanol resulted in a concentration dependent extension of the lag phase when *C. botulinum* was grown in TPGY broth (Figure 6.2B). The end of lag phase growth is represented as the maxima of the curves as shown in Figure 6.2B. While the end of the lag phase

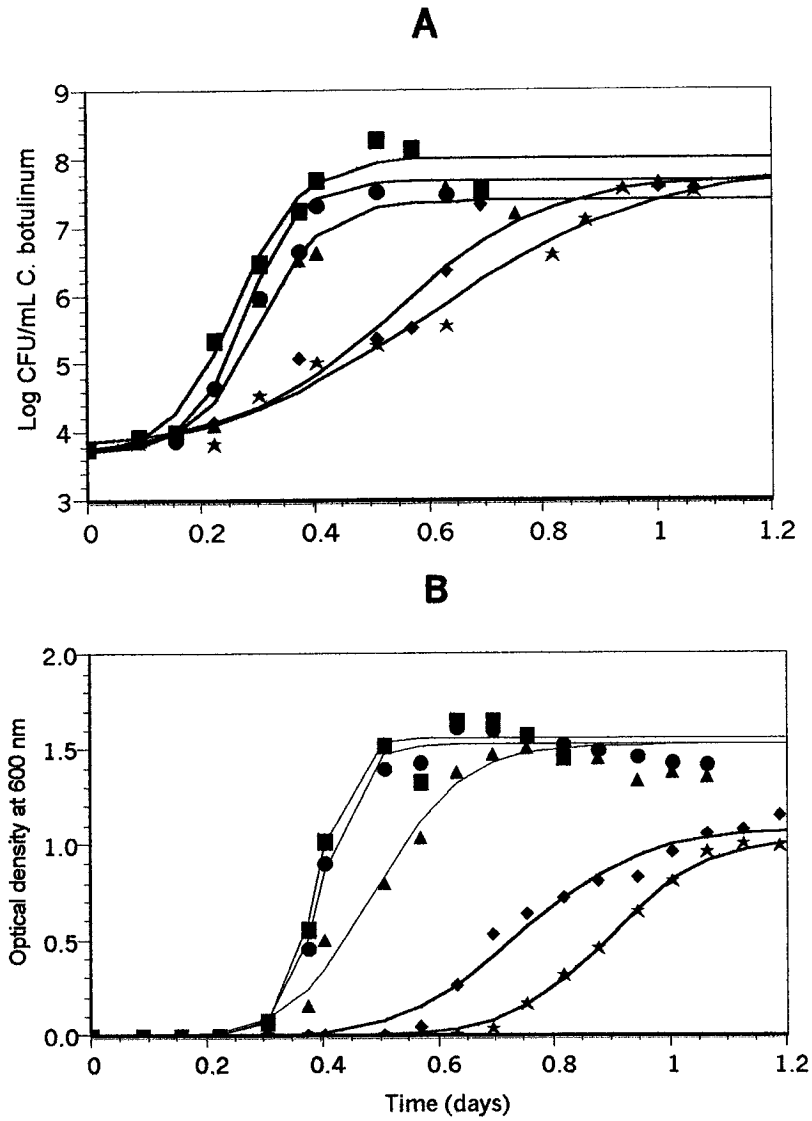
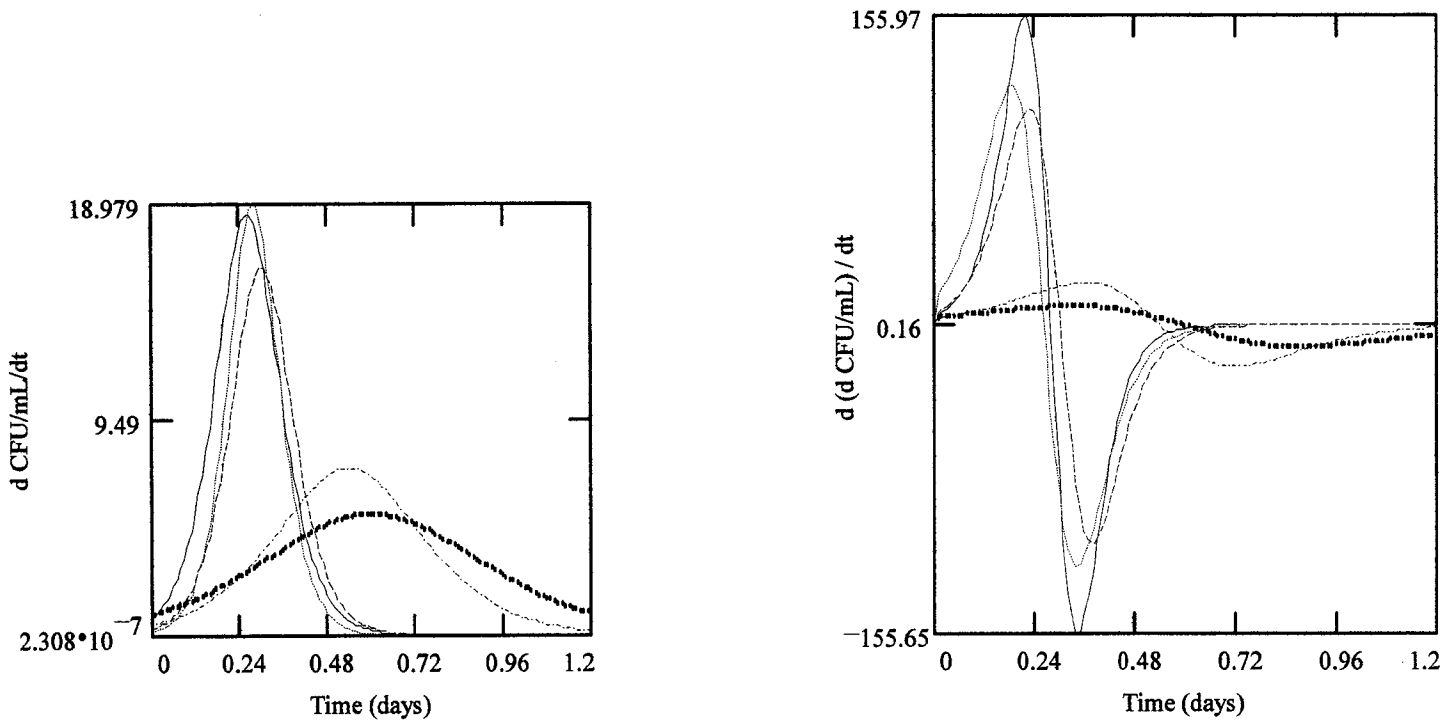


FIGURE 6.1. *Effect of %(wt/wt) ethanol (■ , 0; ●, 1; ▲, 2; ◆ , 3; ★, 4) on growth as measured by A, viable cell density (CFU/mL) and B, optical density at 600 nm of a composite mixture of proteolytic* Clostridium botulinum *in trypticase peptone glucose yeast broth at 37°C*



A B

FIGURE 6.2. *First (A) and second (B) derivative plots of growth curves (shown in Figure 6.1). Maximum absolute growth rates (maxima of curves in A) and the end of the lag phases (maxima of curves in B) for composite mixture of* Clostridium botulinum *grown in (—— , 0; ------, 1; — — 2; – — -, 3; ....., 4) % (wt/wt) ethanol*

occurred after 4.6 hours in control broths (0% ethanol), it was delayed to 5.2, 5.3, 9, or 9.5 hours respectively in broths containing 1, 2, 3 or 4% ethanol (Figure 6.2B).

Although ethanol (1% to 4%) delayed the lag phase, the growth of *C. botulinum* was not prevented. Growth, once initiated, was rapid; however, the rate of growth $\left(\frac{\partial CFU/mL\,(t)}{\partial t}\right)$ during the logarithmic phase was influenced by the concentration of ethanol in the medium. The times at which the maximum growth rates of *C. botulinum* occurred in TPGY containing ethanol (0% to 4%) are shown in Figure 6.2A. These times ranged from 6.4 hours for cultures grown without ethanol (0%) to 14.9 hours for cultures grown in 4% ethanol. Linear regression (R^2 0.879) and extrapolation of this data predicted that a level of 5.5% (wt/wt) ethanol would result in complete inhibition of growth of *C. botulinum* in TPGY broth at 37°C (Figure 6.3).

The maximum growth reached at the stationary phase was also decreased in the presence of ethanol. The maximum log viable cell count for *C. botulinum* grown in TPGY broth without ethanol was approximately 8.3 compared to 7.6 to 7.3 for cultures grown in broth containing 1% to 4% ethanol (Figure 6.1A) i.e., ethanol resulted in less than a one-log reduction in counts at the stationary phase regardless of the concentration of ethanol.

In summary, the effects of ethanol on the growth of the strains of *C. botulinum* used in this study were: i) an increased lag phase, ii) a decreased rate of exponential growth, and iii) a one-log reduction in counts at the station-

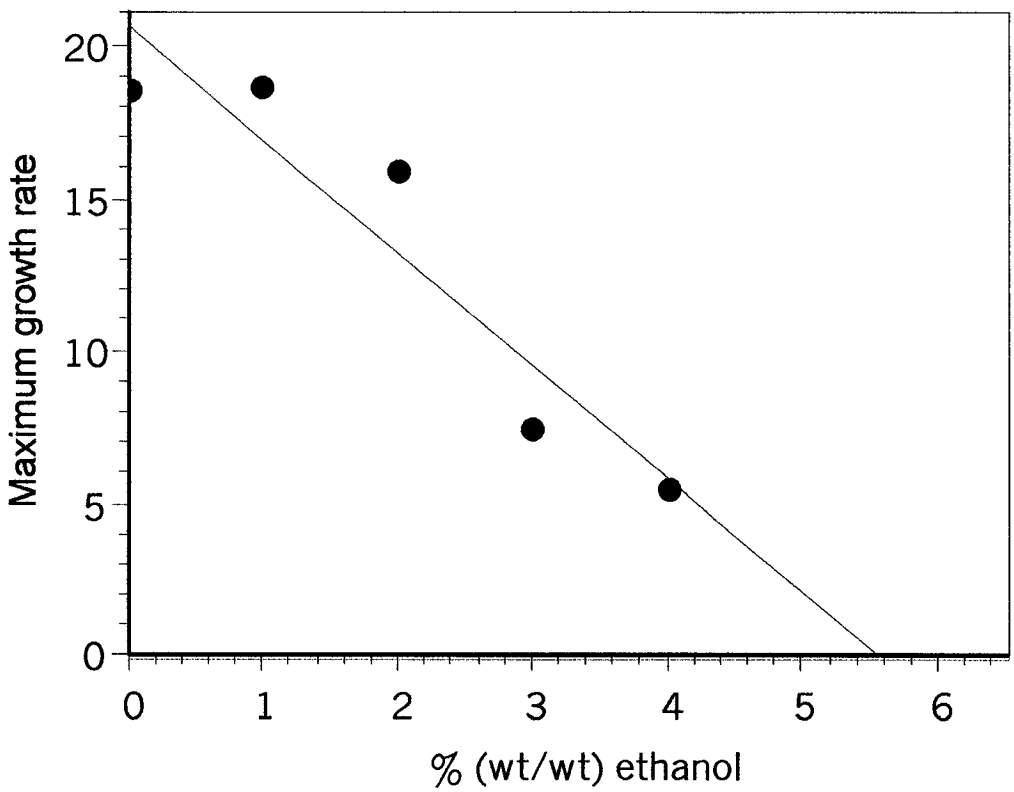


FIGURE 6.3. *Level of ethanol (%[wt/wt]) (intercept of x-axis) predicted to completely inhibit growth of mixture of proteolytic* Clostridium botulinum *in tripti-case peptone glucose yeast broth at 37°C*

ary phase. The effects of ethanol on growth of C. botulinum are consistent withthose of Yamamoto et al. (1984) who reported that ethanol exerted a concentration dependent inhibition of gram-positive food spoilage and pathogenic bacteria.

6.3.1.1 Neurotoxin production

Since TPGY broths were inoculated from an overnight culture of *C. botulinum*, sufficient neurotoxin was transferred with the inouclum to result in neurotoxin being detected in the freshly inoculated broths (time = 0). Therefore, it was necessary to dilute the cultures 1:100 before testing for neurotoxin using the bioassay to ensure that no neurotoxin was detected initially. The times at which neurotoxin was first detected in TPGY cultures (1:100) containing 0% to 4% ethanol are shown in Table 6.1. Ethanol resulted in a concentration dependent delay in the time at which neurotoxin was first detected (R^2 0.959). While neurotoxin was first detected after 9.8 hours in broths containing 0% and 1% ethanol, neurotoxin detection was delayed to 24.7 hours in broth containing 4% ethanol. For all concentrations of ethanol, when neurotoxin was first detected, log counts of *C. botulinum* were >7.0. While counts at the previous sampling times, at which neurotoxin was not detected, were approximately one-half log lower, these counts were, nevertheless, higher than expected. Previous studies found that the time-of-first neurotoxin detection in crumpets inoculated with 500 spores/g

TABLE 6.1. *Time of first neurotoxin detection in trypticase peptone glucose yeast broth[a] containing 0% to 4% (wt/wt) ethanol inoculated with ~10^4 cells/mL proteolytic* C. botulinum *and incubated at 37°C*

Ethanol % (wt/wt)	Time of first neurotoxin detection[b]				Time of last negative neurotoxin detection[c]			
	Time[d] (hours)	Neurotoxin	Growth of *C. botulinum*		Time (hours)	Neurotoxin	Growth of *C. botulinum*	
			log CFU/g	OD_{600nm}			log CFU/g	OD_{600nm}
0	9.8	+	7.8	1.12	8.8	-	7.4	0.51
1	9.8	+	7.4	0.90	8.8	-	6.6	0.45
2	12.3	+	7.2	0.82	9.8	-	6.5	0.48
3	18.0	+	7.0	0.65	15.4	-	6.4	0.55
4	24.7	+	7.5	0.95	24.1	-	7.4	0.82

[a] All TPGY cultures were diluted 1/100
[b] Time that neurotoxin was first detected using the mouse bioassay
[c] Corresponds to the time of the last sample before neurotoxin was detected
[d] The effect of % ethanol (x) on the time of first detection of neurotoxin (y) was fit to $y = 7.837e^{(0.2809x)}$ ($R^2 = 0.959$)

6.0 Effect of ethanol

C. botulinum corresponded to counts of approximately 10^5 CFU/g. The higher counts observed in this study may be due to the rapid exponential growth rates between sampling intervals. However, while growth of *C. botulinum* was inhibited by 1% to 4% (wt/wt) ethanol, neurotoxin was still detected in all cultures after 24-hours growth at 37°C, regardless of the concentration of ethanol.

6.3.2 Combined effect of ethanol, water activity (a_w) and pH

Based on extrapolation of the growth rate reduction by ethanol in the initial growth study, a level of 5.5% (wt/wt) ethanol was predicted to completely inhibit growth of the composite mixture of *C. botulinum* in TPGY broth at 37°C (optimal growth conditions). However, this level exceeds the maximum level of 2% (wt/wt) permitted for the shelf-life extension of certain bakery products, specifically, pizza crusts. Therefore, this second study was designed to determine the combined effect of ethanol, a_w and pH on the probability of growth of and neurotoxin production by a composite mixture of spores of the same six strains of proteolytic *C. botulinum* used in the initial growth study. Tubes of TPGY broth were observed for turbidity and gas production twice daily. For all broths containing 0% ethanol (conditions 1 to 10 [Table 6.2]), growth and neurotoxin production occurred within one to three days regardless of the levels of a_w or pH (P <0.005). Since growth was observed simultaneously in all replicate tubes of TPGY broths without ethanol, it was impossible to fit the parameters of the

logistic distribution ($P_{[t]}$) to the data at 0% ethanol. However, since 100% of tubes were positive within three days, the maximum probability of growth and neurotoxin production for conditions with 0% ethanol at ≥3 days was one ($P_{3\text{-days}} = 1$).

In broths containing 2% ethanol, growth and neurotoxin production occurred within 2 to 4 days (conditions 11 to 17 [Table 6.2]), again regardless of the levels of a_w or pH ($P < 0.005$). Since growth and neurotoxin production were observed simultaneously in all replicate tubes of TPGY broths at 2% ethanol, it was also impossible to fit the parameters of the logistic distribution to data at this level of ethanol. Nevertheless, the maximum probability of growth and neurotoxin production at ≥4 days was one ($P_{4\text{-days}} = 1$).

All combinations of 4% ethanol, a_w, and pH (conditions 18 to 24 [Table 6.2]) also showed growth and neurotoxin production. However, the rate at which growth occurred in replicate tubes of broths containing 4% ethanol was influenced by the a_w and/or pH of the medium. Complete inhibition of growth was observed at 6% and 8% ethanol. Furthermore, no neurotoxin was detected for any combination of factors (ethanol, a_w, pH) at these levels of ethanol (conditions 25 to 30 [Table 6.2]) after one year (365 days) at 25°C. These results are consistent with the preliminary growth study which predicted inhibition of *C. botulinum* by 5.5% ethanol when overnight cultures were grown in TPGY at 37°C. Yamamoto et al. (1984) reported inhibition of gram-positive food spoilage and pathogenic bacteria with 9 to 11% ethanol while Cook and Pierson (1983)

reportedly inhibited *C. botulinum* type A with 10% ethanol. The inhibition of growth of *C. botulinum*

TABLE 6.2. *Effect of combinations of ethanol, water activity and pH of probability of growth and neurotoxin production* Clostridium botulinum *(10^3 spores/mL) in trypticase peptone glucose yeast broth at 25°C*

Condition	Levels of factors			Replicate tubes	Day of Growth[a]	Parameters of model[b]					
	% (w/w) Ethanol	Water activity	pH			P_{max}	s.e. P_{max}	τ	s.e. τ	κ	s. e. κ
1	0	0.956	7.7	10	3	NF	NF	NF	NF	NF	NF
2	0	0.960	7.2	10	3	NF	NF	NF	NF	NF	NF
3	0	0.966	6.2	10	3	NF	NF	NF	NF	NF	NF
4	0	0.976	8.2	10	3	NF	NF	NF	NF	NF	NF
5	0	0.980	6.7	10	2	NF	NF	NF	NF	NF	NF
6	0	0.997	6.2	5	1	NF	NF	NF	NF	NF	NF
7	0	0.997	6.7	5	1	NF	NF	NF	NF	NF	NF
8	0	0.997	7.2	5	1	NF	NF	NF	NF	NF	NF
9	0	0.997	7.7	8	1	NF	NF	NF	NF	NF	NF
10	0	0.997	8.2	5	3	NF	NF	NF	NF	NF	NF
11	2	0.954	7.7	20	3	NF	NF	NF	NF	NF	NF
12	2	0.959	7.2	20	4	NF	NF	NF	NF	NF	NF
13	2	0.965	6.2	15	3	NF	NF	NF	NF	NF	NF
14	2	0.970	8.2	15	3	NF	NF	NF	NF	NF	NF
15	2	0.972	7.2	15	3	NF	NF	NF	NF	NF	NF

Table continues

6.0 Effect of ethanol

TABLE 6.2. (cont'd). *Effect of combinations of ethanol, water activity and pH of probability of growth and neurotoxin production of 10^3 spores/mL* Clostridium botulinum *in trypticase peptone glucose yeast broth at 25°C*

Condition	Levels of factors			Replicate tubes	Day of Growth[a]	Parameters of model[b]					
	% (w/w) Ethanol	Water activity	pH			P_{max}	s.e. P_{max}	τ	s.e. τ	κ	s. e. κ
16	2	0.989	6.7	15	2	NF	NF	NF	NF	NF	NF
17	2	0.989	7.2	10	2	NF	NF	NF	NF	NF	NF
18	4	0.956	7.7	25	49 to >365[e]	0.18	0.01	210.2	0.4	0.92	0.04
19	4	0.959	7.2	25	60 to 143[e]	1	0.13	96.66	1.5	0.298	0.07
20[c]	4	0.962	8.2	25	14 to 104[e]	0.22	0.04	59.4	0	0.93	0.08
20[d]	4	0.962	8.2	25	192 to 263[e]	1	0.06	232.9	5.0	0.026	0
21	4	0.965	6.2	25	23 to 26[e]	1	0.1	24.49	0.2	1.33	0.2
22	4	0.967	7.2	25	6 to 9[e]	1	0.06	8.97	0.2	4.8	4.7
23	4	0.981	7.2	25	4	NF	NF	NF	NF	NF	NF
24	4	0.981	7.7	25	3	NF	NF	NF	NF	NF	NF
25	6	0.953	7.2	15	NG	0	NF	NF	NF	NF	NF
26	6	0.954	7.7	15	NG	0	NF	NF	NF	NF	NF
27	6	0.973	7.2	15	NG	0	NF	NF	NF	NF	NF
28	6	0.973	7.7	15	NG	0	NF	NF	NF	NF	NF

Table continues

6.0 Effect of ethanol

TABLE 6.2. (cont'd). *Effect of combinations of ethanol, water activity and pH of probability of growth and neurotoxin production of 10^3 spores/mL* Clostridium botulinum *in trypticase peptone glucose yeast broth at 25°C*

Condition	Levels of factors			Replicate tubes	Day of Growth[a]	Parameters of model[b]					
	% (w/w) Ethanol	Water activity	pH			P_{max}	s.e. P_{max}	τ	s.e. τ	κ	s. e. κ
29	8	0.964	7.2	5	NG	NF	NF	NF	NF	NF	NF
30	8	0.964	7.7	5	NG	NF	NF	NF	NF	NF	NF

a Growth was observed simultaneously in all replicates on this day.

b P_{max} is the maximum probability of growth after 365-days, i.e. the number of tubes showing evidence of growth (turbidity and/or gas production divided by the total number of tubes in that condition; κ is a rate constant (days-1); τ is the time of the midpoint of the function (days), i.e., the time for 50% of the replicates that will become toxic to show turbidity and/or gas production

c Data from days 1 to 190

d Data from days 191 to 365

e Indicates the range (days) when growth was first and last observed in replicate tubes

NG No growth during 365-days

NF Not fit

observed in this study was concentration dependent. For example, in broths of a_w ~0.954, pH 7.7, and 0, 2, 4 or 6% ethanol, growth was first observed on days 3, 3, 49, and not within 365 days respectively (Table 6.2). The addition of ethanol to a liquid medium results in the depression of its a_w. TPGY broths containing 6% and 8% (wt/wt) ethanol would, based on the Ross equation (1975), have estimated a_w of 0.971 and 0.964 respectively. However, since growth was observed within three days in TPGY broths containing 0% ethanol and adjusted to a_w <0.964 (Table 6.2), the inhibitory effect of ethanol on *C. botulinum* is attributed to simply more than a depression of a_w. Similar conclusions on the antimicrobial effect of ethanol have been reached by other authors (Shapero et al., 1978; Smith et al., 1987).

As described previously, the rate of growth of and neurotoxin production by *C. botulinum* in replicate tubes of TPGY broths containing 4% ethanol (conditions 18 to 24 [Table 6.2]) was influenced by the a_w and pH of the medium. These data were fitted to a mathematical model which describes the rate at which replicate tubes become toxic as a function of time. The fits of the model to each combination of 4% ethanol, a_w, and pH, and the standard error for each parameter are shown in Table 6.2. Such models, which estimate the probability of growth and neurotoxin production for a given set of conditions, are generally considered valid for evaluating the hazard of botulism associated with growth of *C. botulinum* in food. There is evidence that considerable growth is

required before measurable neurotoxin is produced and that most neurotoxin is produced during cell lysis (Siegel and Metzger, 1979; 1980). Although in the first study counts of *C. botulinum* were $>10^7$ CFU/mL when neurotoxin was first detected in TPGY containing 0% to 4% ethanol (Table 6.1), Daifas et al. (1999b) observed that the time at which neurotoxin was first detected in crumpets inoculated with 5×10^2 spores/g proteolytic *C. botulinum* and stored at 25°C corrsponded to counts of approximately 10^5 CFU/g. However, growth of this pathogen, once initiated, is rapid and is always associated with a neurotoxin of extreme potency. Therefore, any growth of *C. botulinum* is considered as unacceptable (Whiting and Call, 1993).

Selected conditions containing 4% ethanol which inhibited growth of *C. botulinum* are shown in Figure 6.4. Describing the effect of selected levels of barriers such as ethanol, a_w and pH on growth of *C. botulinum* is a practical approach to food safety, since the safety of bakery products with an extended shelf-life, therefore, can be predicted. Furthermore, the results of this study indicate that, while some combinations of barriers could be used to enhance the safety of a bakery product with a short shelf-life, other combinations would be more effective for products with a longer shelf-life. For example, for 4% ethanol, a_w 0.959 and pH 7.2 (condition 19 [Table 6.2]) the probability of neurotoxin at day 60 was 0.003 ($P_{60\text{-days}} = 0.003$). However, the probability of growth and neurotoxin production increased rapidly with time. At day 97, one-half (50%) of all

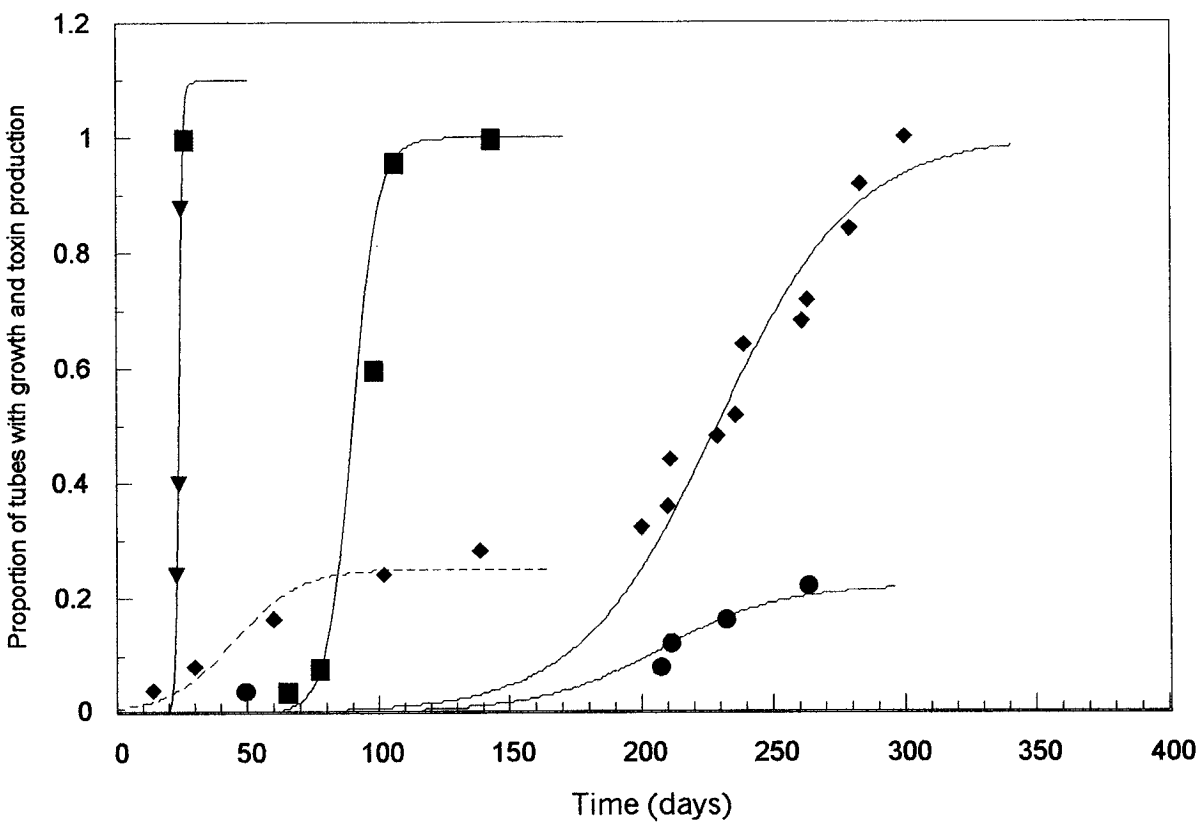


FIGURE 6.4. *Proportion of replicate tubes showing growth of and neurotoxin production by 10^3 spores/mL* Clostridium botulinum *for selected combinations of 4% (wt/wt) ethanol, a_w and pH (▼, 0.965, 6.2; ○, 0.962, 8.2; ■, 0.959, 7.2; ◆ 0.956, 7.7) in trypticase peptone glucose yeast broth at 25°C*

replicates were toxic ($P_{97\text{-days}}$ = 0.5) and by day 125, all replicates were toxic (P_{max} = 1 [Figure 6.4]). Therefore, this combination of barriers may be appropriate to enhance the safety of a bakery product with a shelf-life of <30 days. However, although safety may be enhanced by this combination, sensory quality may be adversely affected. Foods of intermediate a_w packaged with ethanol vapour have been shown to absorb less ethanol than similarly packaged foods of high a_w (Smith et al., 1987; Seiler and Russell, 1991). Therefore, this level of ethanol vapour may be more suitable for intermediate-moisture bakery products. Furthermore, some studies have shown that alcohol-like aromas may dissipate once packages are opened and/or products are heated (Seiler and Russell, 1991). Although many bakery products are consumed without heating, par-baked breads and rolls and crumpets are examples of products that are further heated prior to consumption and may be appropriate for this type of packaging.

The time-to-neurotoxin for the median number of positive replicates (τ) of broths containing 4% ethanol ranged from less than 3 days for broth of a_w 0.981 and pH 7.7 to 229 days for broth of a_w 0.962 and pH 8.2. With the exception of one combination of barriers (condition 18 [Table 6.2]), the P_{max} for all broths containing 4% ethanol was approximately one (P_{max} = 1). The most inhibitory combination of barriers (4% ethanol, a_w 0.956 and pH 7.7; condition 18 [Table 6.2]) had a P_{max} = 0.22 and τ = 207 days (Figure 6.4). Only data from one combination of barriers

(4% ethanol, a_w 0.962 and pH 8.2; condition 20 [Table 6.2]) did not fit the probability function used. The pattern of growth observed for these levels of barriers was different from all other growth conditions. At the onset of the study, several replicate tubes of this condition became positive and then no further growth was observed (Figure 6.4). Data from this condition (20, [Table 6.2]) fitted the model well for the first 200-days growth. Indeed, if this study had been terminated before 200 days, the P_{max} of neurotoxin production would have been assumed to be 0.25 (Figure 6.4). However, after 200 days, tubes again showed evidence of growth and this trend continued until all tubes were positive by 263 days. Although the pattern of overall growth observed for these levels of barriers did not fit the model well, there was a good fit for data from both separate periods of growth (Figure 6.4). While It is possible that this combination of barriers inhibited the germination or outgrowth of spores of *C. botulinum* initially, this inhibition was overcome in time, resulting in more tubes showing visible signs of growth and becoming toxic. McMeekin et al. (2000) proposed a point, the growth/no growth interface, at which interacting hurdles restrict growth, and emphasized the importance of considering the physiological mechanisms that occur in pathogens at this interface. It is possible that this combination of barriers (4% ethanol, a_w 0.962 and pH 8.2) was close to this growth/no growth interface.

6.3.3 Effect of ethanol on cells

These two studies have confirmed the inhibitory effect of ethanol on *C. botulinum*. In order to investigate the effect of ethanol on vegetative cells, overnight cultures of *C. botulinum* 62A were grown in TPGY broth containing 0, 2, 4, 5, or 6% ethanol at 37°C. No growth was visible in cultures grown in 5% or 6% ethanol, again supporting the predicted inhibitory level of 5.5% ethanol. Micrographs of these cultures grown in TPGY broth with 0, 2, or 4% ethanol are shown in Figure 6.5A-D. Growth and cell division are evident in cultures grown with 0% ethanol (Figure 6.5A). The effect of 2% and 4% ethanol on cells is shown in Figure 6.5B-D. Interference of growth, as demonstrated by cell elongation and septation at 2% ethanol (Figure 6.5B), is even more pronounced at 4% ethanol (Figure 6.5D). Reversible interference with cell division and elongation of cells of *E. coli* grown with ethanol has been reported (Fried and Novick, 1973), although these authors also isolated mutants in which ethanol stimulated cell division. Ethanol has been shown to act primarily on the cell membrane, either directly on the membrane and/or membrane associated enzymes or indirectly through impairment of biosynthesis of membrane components (Seiler and Russell, 1991). Empty, "ghost-like" cells, i.e., cells that were collapsed and devoid of intracellular contents were observed in cultures grown with either 2 or 4% ethanol, but not in cultures grown with 0% ethanol. This may be due to leakage of solutes

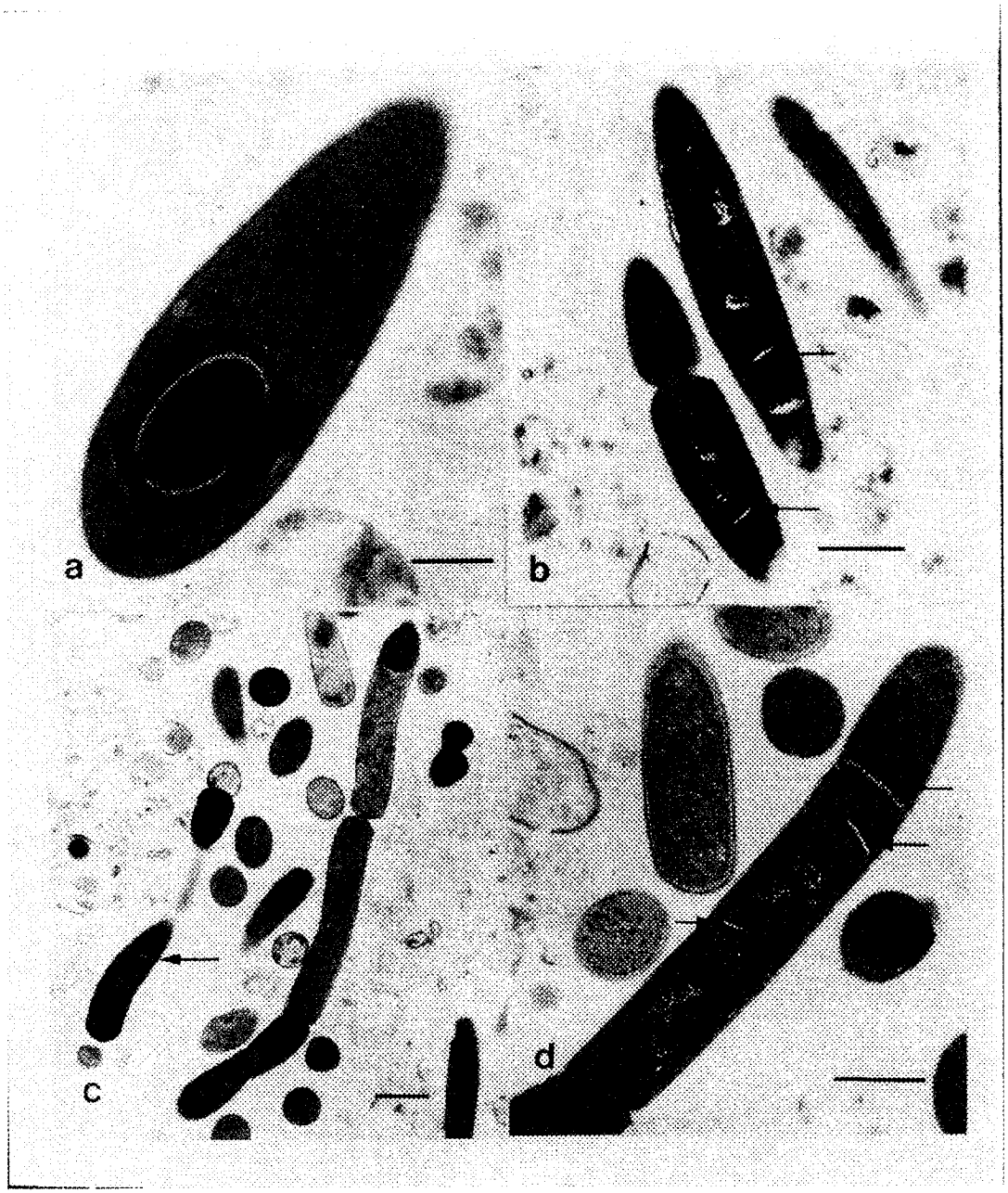


FIGURE 6.5. *Effect of A, 0% (bar represents 0.5 μm); B, 2% (bar represents 1 μm); and C-D, 4% (bars represent 2 and 1 μm respectively) (wt/wt) ethanol on vegetative cells of* Clostridium botulinum *in trypticase peptone glucose yeast broth at 37°C*

across the membrane or cell lysis following decreased peptidoglycan cross-linking in the growing cell wall (Ingram and Buttke, 1984).

6.3.3.1 Nature of the effect of ethanol

In the study on the combined effect of ethanol, a_w and pH, some tubes of TPGY broths showed no evidence of growth (turbidity and/or gas production) even after one year at 25°C. This included all broths containing 6 or 8% ethanol (conditions 25 to 30 [Table 6.2]) and 20 of 25 replicates containing 4% (condition 18 [Table 6.2]). To determine if ethanol had a cidal or a static effect at these levels, all tubes of TPGY broths showing neither turbidity nor gas production after one year were subcultured into fresh TPGY broth and incubated anaerobically at 37°C for 24 hours. All of these subcultured tubes showed turbidity and gas production within 24 hours and subsequently tested positive for neurotoxin by the mouse bioassay. Although it is not clear how inoculated spores were inhibited from growth during 365 days at 25°C, the inhibitory effect of ethanol was clearly reversible. Reversible inhibition of germination of spores of *Bacillus subtilis* var. *niger* and *B. pumilus* by low levels of ethanol has been reported by Trujillo and Laible (1970). Reversible inhibition was attributed to an enzymatic mechanism. Possible mechanisms involve ethanol directly inhibiting lytic enzymes, which degrade peptoglycan in the spore

cortex, or hydrophobically interacting with the spore-coat structure which, in turn effects germination enzymes. Chaibi et al. (1997) reported that ethanol at 3% or less, did not inhibit spore germination, outgrowth or cell multiplication of *C. botulinum* 62A. Cook and Pierson (1983) reported inhibition of germination of *C. botulinum* type A spores with 10% ethanol but it is not known if the inhibition was reversible or not.

6.4 CONCLUSION

In conclusion, these studies have confirmed that ethanol could be used to delay the growth of *C. botulinum*. Furthermore, modelling studies showed that the probability of growth and neurotoxin production is influenced by the concentration of ethanol, a_w and pH in the medium. However, model broth studies have certain limitations with respect to the safety of bakery products. These are: i) they are done using pure cultures of *C. botulinum* spores and do not consider the background microflora of products and ii) they do not consider the sensory aspect of a product to determine if toxigenesis preceded spoilage or *vice-versa*. While caution must be exercised when extrapolating results from model broth systems to food products, nevertheless, these studies have shown that ethanol has the potential to be a viable part of a multi-barrier food safety system for bakery products. However, a limitation of ethanol in the liquid form is that high levels (≥4% [wt/wt]) of ethanol would be required to be sprayed on the product surface to prevent growth of and neurotoxin production by *C. botulinum*. To overcome this limitation, the inclusion of ethanol vapour-generating sachets within the package might reduce the amount of ethanol required since ethanol has been shown to be most effective in the vapour state (Smith et al., 1987). Therefore, lower levels of ethanol applied in the vapour phase used in conjunction with a_w and/or pH reduction may give similar or greater inhibition

when compared to direct application of liquid ethanol onto the surface of bakery products.

PREFACE TO CHAPTER 7.0

Previous studies have shown that ethanol vapour can be used to control the growth of *C. botulinum* in yeast-leavened crumpets. However, while the microbiological safety of these crumpets was enhanced, their sensory shelf-life was limited due to the absorption of ethanol vapour from the package headspace. While this problem may be overcome by reformulation of crumpets using chemical leavening and packaging with ethanol vapour, little is known about the safety of such formulated packaged crumpets with respect to the growth of *C. botulinum.*

7.0 CHALLENGE STUDIES WITH PROTEOLYTIC *CLOSTRIDIUM BOTULINUM* IN YEAST- AND CHEMICALLY-LEAVENED CRUMPETS PACKAGED UNDER MODIFIED ATMOSPHERES

7.1 INTRODUCTION

Ethanol vapour-generating sachets (Ethicap®) are widely used in Asian markets to prevent mould spoilage and delay staling in high- and intermediate-moisture bakery products. Preliminary studies have shown that these interactive packaging sachets also have the potential to delay the growth of and neurotoxin production by *C. botulinum,* a pathogen of concern in high-moisture bakery products packaged under modified atmospheres. In challenge studies with yeast-leavened English-style crumpets, neurotoxin production by proteolytic strains of *C. botulinum* (500 spores/g) was completely inhibited for >21 days in crumpets (100-g) packaged in air with an Ethicap® sachet (4- or 6-G [Daifas et al., 2000]). However, a major limitation of using these larger sachets was that while the safety of high-moisture crumpets was enhanced, their sensory shelf-life was compromised. This was due to the high levels (3 to 4% [vol/vol]) of ethanol vapour in the package headspace, and its subsequent absorption by crumpets (Daifas et al., 2000). While such high levels of headspace ethanol were due mainly to its release from the interactive sachets, the metabolic activity of yeasts that survived the baking process also played a role. In air-packaged crumpets,

headspace ethanol levels of 1% to 3% (vol/vol) were detectable after 21 days at ambient storage temperature and were attributed to growth of and fermentation by endogenous yeasts. While a lower level of headspace ethanol and a longer sensory shelf-life of high-moisture crumpets may be achieved by product reformulation using chemical leavening and packaging with Ethicap®, little is known about the safety of such packaged crumpets. Therefore, the objective of this study was to determine the effect of leavening agent (yeast or chemical) on the anti-botulinal efficacy of ethanol vapour in challenge studies with *C. botulinum* in high-moisture crumpets.

7.2 MATERIALS AND METHODS

7.2.1 Preparation of crumpets

Two types of English-style crumpets were used in these studies– yeast- and chemical-leavened. Yeast-leavened crumpets were prepared from all-purpose flour, sugar, instant yeast, reconstituted dry milk, salt and baking soda as described by Daifas et al. (1999a). Chemical-leavened crumpets were prepared from a similar formulation; however, yeast and baking soda were omitted and replaced with baking powder. All dry ingredients in each formulation were calculated on a flour-weight basis. Crumpets were baked on an oiled griddle, preheated to 204°C, cooled to ambient temperature and subsequently stored at 4°C as described by Daifas et al. (1999a). Baked crumpets (pH 6.4, a_w 0.990) weighed approximately 50 g each.

Crumpets were designated into two groups: i) non-sterile crumpets for the first study and ii) sterile crumpets for the second study. For the second study, equal numbers of yeast- and chemical-leavened crumpets were sterilized in autoclavable bags at 121°C and 15 psi for 15 minutes. Following cooling to ambient temperature, all sterile crumpets were stored at 4°C for approximately 24 hours prior to inoculation and packaging. The sterility of randomly selected yeast- and chemical-leavened

crumpets was confirmed by aerobic and anaerobic plate counts at 25°C, 30°C and 37°C.

7.2.2 Preparation of spore inoculum and sample inoculation

In the first study, non-sterile crumpets were inoculated with *C. botulinum* only, while in the second study, sterile crumpets were inoculated with i) *C. botulinum* or ii) *C. botulinum* and *S. cerevisiae*.

7.2.2.1 *C. botulinum*

A composite inoculum of *C. botulinum* spores (17A, 62A, CK2A, MRB, 13983IIB, and 368B) was used in both studies. Spore crops of each strain were prepared separately, enumerated as described by Hauschild and Hilsheimer (1977), and stored at -80°C. Equal numbers of spores of each strain were then combined to form a single suspension of approximately 1.1 x 10^6 spores/mL. The spore mixture was heat-shocked at 75°C for 20 minutes prior to sample inoculation. Inoculum levels were verified prior to inoculation of samples by plating appropriate dilutions in duplicate on McClung Toabe agar (Difco, Becton-Dickinson, Sparks, MD) containing 0.5% yeast extract (Difco) and egg-yolk (Austin and Blanchfield, 1996) and incubating anaerobically in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 at 35°C for three days (Austin et al., 1998). In

both studies, crumpets were stab-inoculated by pipette approximately 0.5 cm beneath the surface at five distinct, equidistant locations on the top surface of the crumpet with a total of 45 μL of spore suspension per crumpet (50-g) to provide a final inoculum level of approximately 1 x 10^3 spores/g of *C. botulinum*.

In addition, uninoculated control crumpets were inoculated with an identical volume of 0.1% (wt/wt) peptone water.

7.2.2.2 *S. cerevisiae*

Cells of *S. cerevisiae* NCYC 1239 (National Collection of Yeast Cultures; Norwich, UK) were used in the second study only. The lyophilized yeast was rehydrated in malt yeast peptone dextrose (MYPD) broth (Difco), and incubated for 24 hours at 25°C on a platform shaker at 250 rpm (New Brunswick Scientific; New Brunswick, NJ). The yeast culture was subsequently stored in 50% glycerol (ICN Biomedicals, Aurora, OH) at -20°C. The yeast inoculum was prepared by transferring pure yeast culture into MYPD broth and incubating for 24 hours in a water-bath shaker (250 rpm) (New Brunswick Scientific) at 28°C. Cells from the stock solution were enumerated using an Improved Neubauer hemacytometer and diluted in 0.1% peptone water (Difco) to provide a final inoculum of 5.6 x 10^8 cells/mL. The inoculum was verified by plating appropriate decimal

dilutions on plates of oxytetracycline glucose yeast extract agar (OGYEA [Difco]) and incubating aerobically at 25°C for 48 hours. Only sterile yeast-leavened crumpets, previously inoculated with *C. botulinum* were surface inoculated with 45 µL of *S. cerevisiae* inoculum per crumpet (50-g) to provide a final inoculum of 5 x 10^5 cells *S. cerevisiae* per gram in addition to 1 x 10^3 spores/g *C. botulinum*. Sterile yeast- and chemical-leavened control crumpets were inoculated with an identical volume of 0.1% peptone water.

7.2.3 Packaging

In both studies, all inoculated and uninoculated control crumpets (50-g) were packaged in 210-mm x 210-mm high gas barrier Cryovac bags (O_2 transmission rate: 3 to 6 cc/m^2/day, 1 atm at 4°C, 0% RH.; ethanol transmission rate: 0.21 g/m^2/day, 1 atm at 25°C; Cryovac; Mississauga, ON). All crumpets (50-g [one per bag]) were packaged in triplicate, with either i) a 2-G sachet of Ethicap® (Freund Industrial Company, Ltd., Tokyo, Japan) releasing ~1.1 g ethanol or ii) 100% CO_2. Ethanol vapour generators were placed inside of bags that were then sealed with an impulse heat-sealer. Gas (CO_2) packaging was achieved using a Multivac chamber type heat-seal packaging machine (model SP-300H; Multivac Inc., Kansas, MO) to obtain the desired level of CO_2. All packaged samples were stored at 25°C. Triplicate samples of inoculated

crumpets were analysed at days 0, 5, 10, 15, 20, and 30 while triplicate samples of uninoculated control crumpets were analysed at day 0 and day 30 only.

7.2.4 Headspace O_2 and CO_2 analysis

In both studies, changes in headspace O_2 and CO_2 were monitored using a previously calibrated oxygen/carbon dioxide analyser (Servomex, Food Package Analyzer Series 1400; Minneapolis, MN). Samples of headspace gas were withdrawn using a 0.5-mL gastight pressure-Lok® syringe (Precision Sampling Co; Baton Rouge, LA) through a septum attached to the outside of each package.

7.2.5 Headspace ethanol

Changes in headspace ethanol were monitored using a Varian gas chromatograph (model 3300, Varian Canada, Inc; Montreal, QC), fitted with a flame ionization detector and using a Nukol column (30-M × 0.53-mm; Supelco, Canada, Ltd). Helium was the carrier gas with a flow rate of 30 mL/minute. The column temperature was set at 60°C and the injector port at 100°C. Headspace ethanol was anlaysed by withdrawing 0.5 mL of gas using a gastight pressure-Lok® syringe as described previously for headspace gas analysis and injecting into the chromatograph. Peaks were recorded and analysed with a Hewlett-Packard

integrator (model 3390A, Hewlett-Packard Canada, Ltd.; Kirkland, QC). The concentration of headspace ethanol (% vol/vol) was determined from a standard curve (R^2 0.992) generated by analysing, in triplicate, standard solutions of 95% food-grade ethanol ranging in concentration from 0.25% to 10% (vol/vol).

7.2.6 Enumeration of *C. botulinum*

On each sampling day, crumpets were aseptically transferred to a stomacher bag. A 1:3 dilution was prepared by adding twice the sample weight of 0.1% peptone water and then stomaching for two minutes using a Colworth Stomacher (Seward Medical Stomacher, London, UK). Decimal dilutions were subsequently prepared from this initial dilution. Growth of *C. botulinum* was monitored as described previously (Daifas et al., 1999b). *C. botulinum* was enumerated by spread plating 0.1 mL of appropriate decimal dilutions of the 1:3 homogenised sample, in duplicate, on *Clostridium botulinum* Isolation (CBI) agar (Dezfulian et al., 1981). CBI plates were incubated in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 for 72 hours at 35°C. Lipase-positive colonies, characteristic of *C. botulinum* were enumerated and identification was confirmed by neurotoxin neutralisation assays on trypticase peptone glucose yeast extract (TPGY) broth cultures of randomly selected colonies.

7.2.7 Enumeration of *S. cerevisiae*

Growth of *S. cerevisiae* was monitored by spread plating 0.1 mL of the appropriate decimal dilutions of the 1:3 sample homogenate, in duplicate, on OGYEA (Difco). Plates of OGYEA were incubated aerobically for 48 hours at 25°C. Large, round, white, shiny, elevated colonies, with a characteristic odour of baked bread, were presumptively enumerated as *S. cerevisiae* and randomly selected colonies were confirmed as yeast by wet cell mounts.

7.2.8 Neurotoxin assay

Botulinum neurotoxin was detected as described previously (Daifas et al., 1999a, b). An aliquot of the sample supernatant was filtered through a 0.45-μm filter (Acrodisc, Gelman Sciences; Ann Arbor, MI) and 0.5 mL of this filtrate was injected intraperitoneally into each of two 20- to 28-g mice (Charles River; QC). Mice were observed for up to 72 hours for typical signs of botulism including ruffled fur, pinched waist, laboured breathing, limb paresis and general paralysis. Mice showing extreme distress were humanely asphyxiated with CO_2 according to Health Canada Animal Care Committee guidelines (Daifas et al., 1999c). Neutralization of neurotoxin was performed on randomly selected positive samples, representative of all packaging conditions, using antisera (Connaught Laboratories; North York, ON) to botulinum neurotoxins.

Toxicity was attributed to *C. botulinum* since mice that were injected with a positive sample supernatant and antisera to botulinum neurotoxin survived while mice that were similarly injected, but not protected with antisera died (Austin and Blanchfield, 1996).

7.2.9 Changes in sample pH

The pH of crumpets was determined using a previously calibrated (buffers pH 4.0 and 7.0) Fisher Accumet® pH meter (Fisher Scientific; Ottawa, ON). Portions of filtered sample supernatant of the homogenized, centrifuged samples were transferred to glass tubes and pH measurement was made by immersing the electrode directly into each tube.

7.2.10 Statistical analysis

Data were analysed using analysis of variance for a 2 x 2 factorial experimental design (Steel and Torrie, 1980) using Prism 3.03 (Graph-Pad Software, Inc.; San Diego, CA).

7.3 RESULTS AND DISCUSSION

7.3.1 Challenge studies with non-sterile crumpets

In this first study, non-sterile crumpets (50-g) were inoculated with 10^3 spores/g *C. botulinum* only, packaged in air with an ethanol vapour generator (2-G Ethicap®) or in 100% CO_2 and stored at 25°C.

7.3.1.1 Changes in headspace carbon dioxide and oxygen

The levels of headspace CO_2 in all inoculated CO_2-packaged crumpets (yeast- or chemically-leavened) were consistently ≥95% (vol/vol) throughout 30-days storage while the levels of headspace O_2 in these crumpets were <2% for the same period (Figure 7.1A).

For all inoculated crumpets packaged in air with Ethicap® (2-G), headspace O_2 decreased to <1% while headspace CO_2 in these packaged yeast- and chemical-leavened crumpets increased to approximately 22% and 38% respectively after 15-days storage at 25°C (Figure 7.1B). While similar changes in headspace CO_2 were observed for both yeast- and chemical-leavened crumpets, levels of headspace CO_2 in chemical-leavened crumpets were consistently higher, throughout storage, compared to yeast-leavened crumpets.

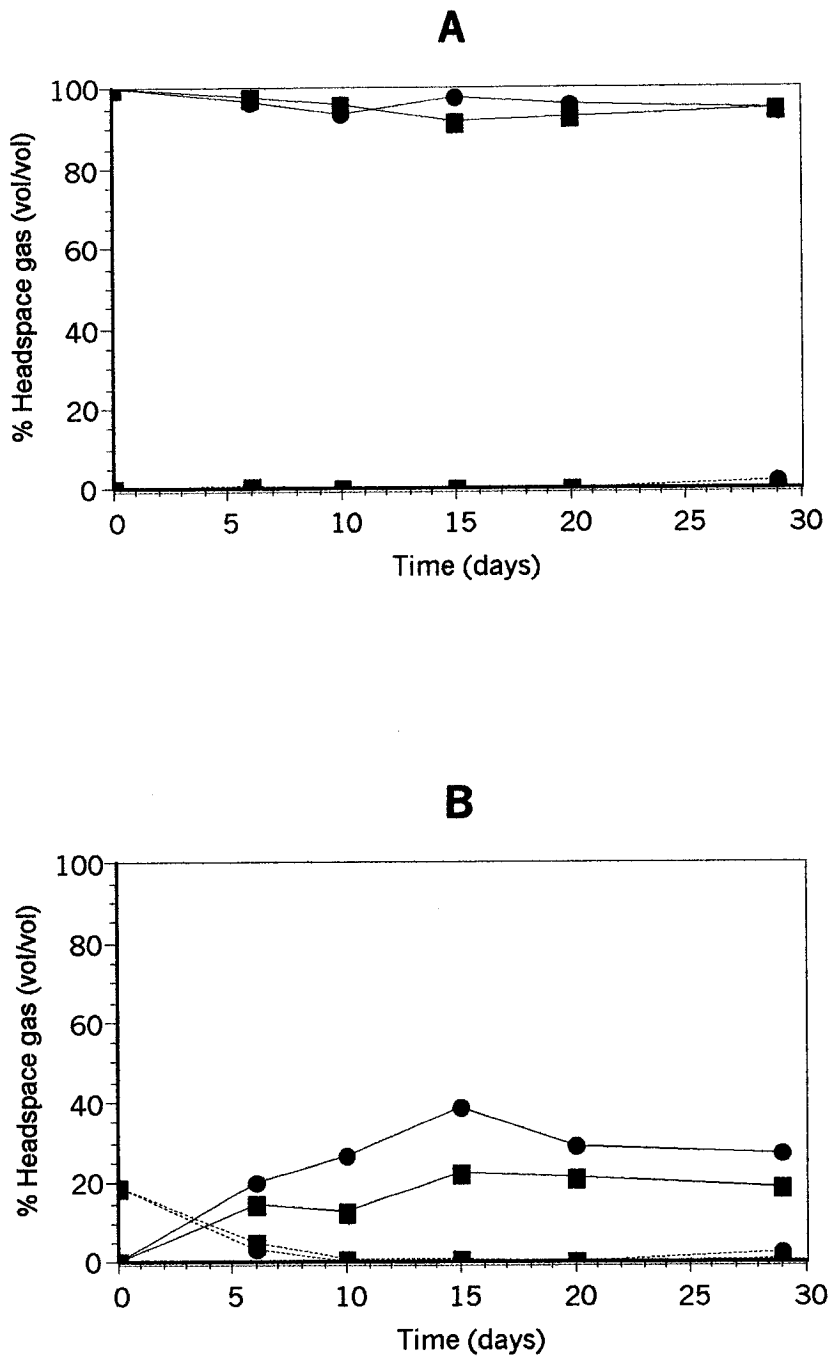


FIGURE 7.1. *Changes in headspace oxygen (----) and headspace carbon dioxide (—) in yeast-leavened (■), and chemically-leavened (●) crumpets inoculated with 10^3 spores/g* C. botulinum, *packaged in A , 100% CO_2; or B, air with 2-G Ethicap® and stored at 25°C*

These changes in headspace gas composition can be attributed to the metabolism of yeasts, lactic acid bacteria and *Bacillus* spp.–the predominant spoilage microflora of crumpets (Smith et al., 1987).

Similar headspace gas profiles were observed for uninoculated control crumpets (results not shown).

7.3.1.2 Headspace ethanol

The levels of headspace ethanol throughout storage in yeast- and chemically-leavened crumpets inoculated with *C. botulinum* and packaged in air with 2-G Ethicap®, or in 100% CO_2 are shown in Figure 7.2. Levels of headspace ethanol throughout storage were consistently higher in yeast-leavened crumpets packaged with Ethicap® and lower in chemically-leavened crumpets packaged with CO_2 (Figure 7.2). At the onset of storage (day 5), headspace ethanol in yeast-leavened crumpets packaged in air with Ethicap® was ~3% (vol/vol), a level that had increased to ~4.5% (vol/vol) after 30-days storage (Figure 7.2). The consistently higher headspace ethanol in yeast-leavened Ethicap®-packaged crumpets can be attributed to the fermentative activity of yeast. Headspace ethanol levels (~2.1% to ~2.8%) were similar in both yeast- leavened CO_2-packaged crumpets and chemically-leavened Ethicap®-packaged crumpets throughout

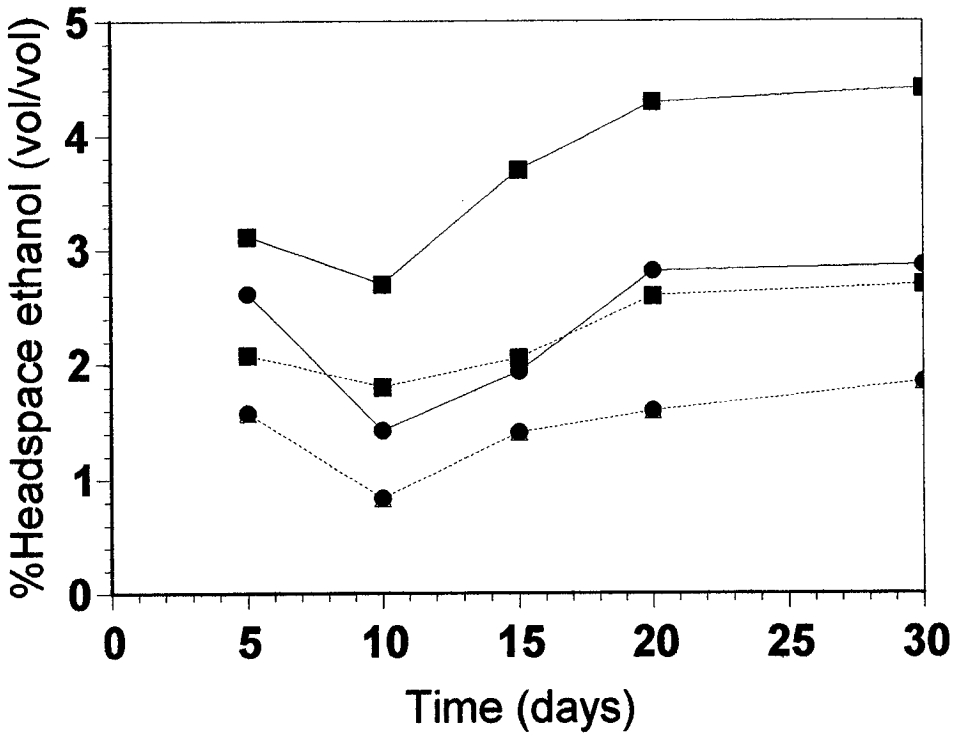


FIGURE 7.2. *Headspace ethanol in yeast-leavened (■), or chemically-leavened (●) crumpets inoculated with 10^3 spores/g* C. botulinum, *packaged in air with 2-G Ethicap® (—) or in 100% CO_2 (---) and stored at 25°C*

storage, while the lowest levels of headspace ethanol (~1.7% [vol/vol]) were observed in chemical-leavened CO_2-packaged crumpets (Figure 7.2).

Although organoleptic changes in crumpets were not monitored during the 30-day storage, at the termination of the study, all crumpets were overtly spoiled due to a strong alcoholic odour–regardless of the leavening agent or packaging atmosphere.

Headspace ethanol levels in uninoculated control crumpets were similar to those observed for similarly packaged inoculated crumpets, indicating that *C. botulinum* did not influence headspace ethanol levels (results not shown).

7.3.1.3 Changes in pH

The initial pH of crumpets of both formulations was 6.4. Minor changes in pH were observed throughout storage at ambient temperature. At the end of storage, the pH values of ethanol vapour- and CO_2-packaged crumpets were 6.4 ± 0.1 and 6.1 ± 0.1 respectively, regardless of the leavening agent (results not shown). The slightly lower pH of crumpets packaged in CO_2 may be due to dissolution of CO_2 in the aqueous phase of the crumpets.

7.3.1.4 Growth of yeast

In yeast-leavened crumpets inoculated with *C. botulinum*, total yeast counts were initially <1 CFU/g (Figure 7.3). However, by day 5, log yeast counts (CFU/g) had increased to >5, regardless of the packaging atmosphere (Figure 7.3). A similar, although more gradual trend was observed in chemical-leavened crumpets, packaged with either Ethicap® or CO_2, in which log yeast counts (CFU/g) at the end of storage were 5 and 2 respectively, i.e., lower than counts observed for yeast-leavened crumpets (Figure 7.3.). Similar results were observed in all control crumpets, again regardless of the packaging atmosphere (results not shown). The higher yeast counts observed for yeast-leavened crumpets can be attributed to *S. cerevisiae* that survived the baking process since crumpets are baked on a griddle and only receive a minimum heat treatment (Daifas et al., 1999b). However, while the yeast counts observed in the chemical-leavened crumpets were unexpected, they can be attributed to post-baking contamination. Such contamination of products in the bakery environment by *S. cerevisiae*, as well as by wild yeasts, is common (Legan and Voysey, 1991) and spoilage of crumpets due to growth of *S. cerevisiae* has been reported (Smith et al., 1983). It is also interesting to note that the high levels of ethanol vapour, particularly in yeast-leavened Ethicap®-packaged crumpets, did not inhibit yeast

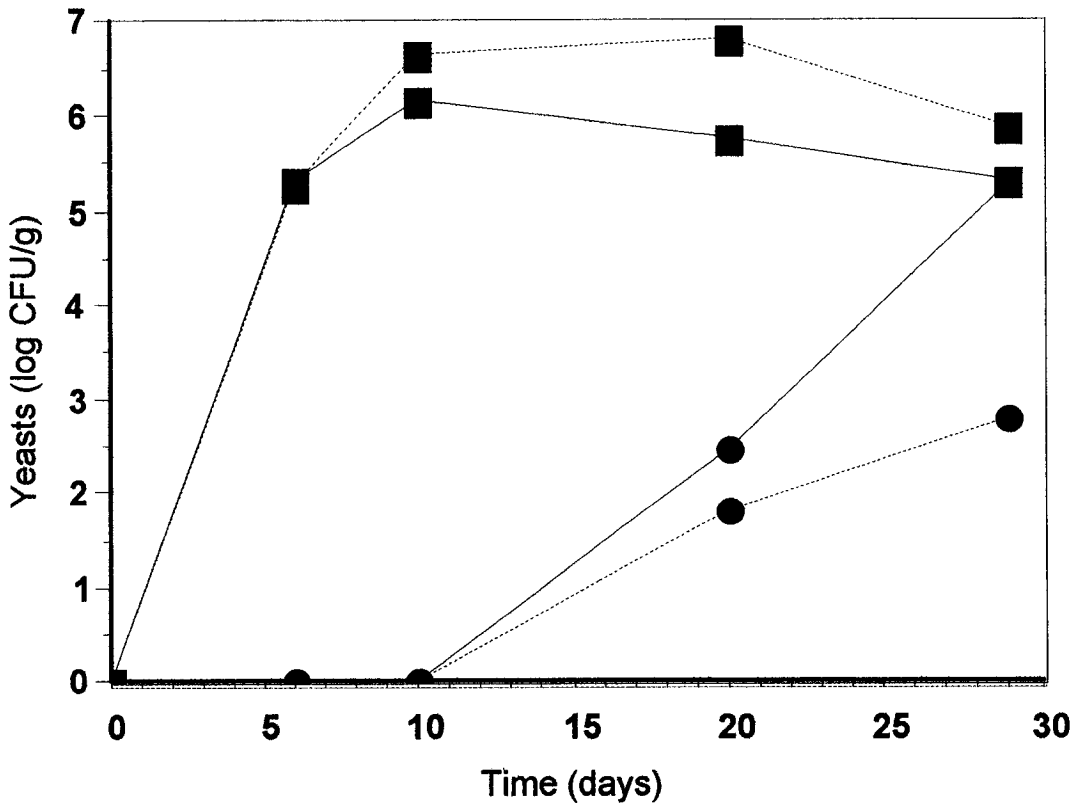


FIGURE 7.3. *Average growth of yeasts (log CFU/g) in yeast-leavened (■) and chemically-leavened (●) crumpets inoculated with 10^3 spores/g* C. botulinum, *packaged in air with 2-G Ethicap® (—) or in 100% CO_2 (----) and stored at 25°C*

growth (Figures 7.2 and 7.3). Sarais et al. (1996) reported that ethanol vapour failed to inhibit the growth of *S. cerevisiae* in cheese. However, ethanol vapour has been shown to control yeast growth in CO_2-packaged pita bread (Black et al.,1993) and in par-baked apple turnovers (Smith et al., 1987). Ingram (1986) reported that *S. cerevisiae* exhibited an ethanol tolerance due to ethanol-induced changes in its fatty acid and sterol composition. Similarly, Seiler and Russell (1991) reported that high (>8%) concentrations of ethanol may be required for complete inhibition of yeast growth while Bundgaard-Nielsen and Nielsen (1995) showed that the antimycotic effect of ethanol varies with yeast strain.

7.3.1.5 Growth of *C. botulinum*

Growth of *C. botulinum* was influenced by packaging atmosphere as well as by leavening agent. In all yeast- and chem- ical-leavened crumpets inoculated with 10^3 spores/g *C. botulinum* and packaged in CO_2, counts of *C. botulinum* increased during storage (Figure 7.4). After five-days storage at ambient temperature, the average log counts of *C. botulinum* in all CO_2-packaged crumpets were consistently >5. However, in chemical-leavened, CO_2-packaged crumpets, log counts of *C. botulinum* were consistently one log higher throughout storage compared to similarly packaged yeast-

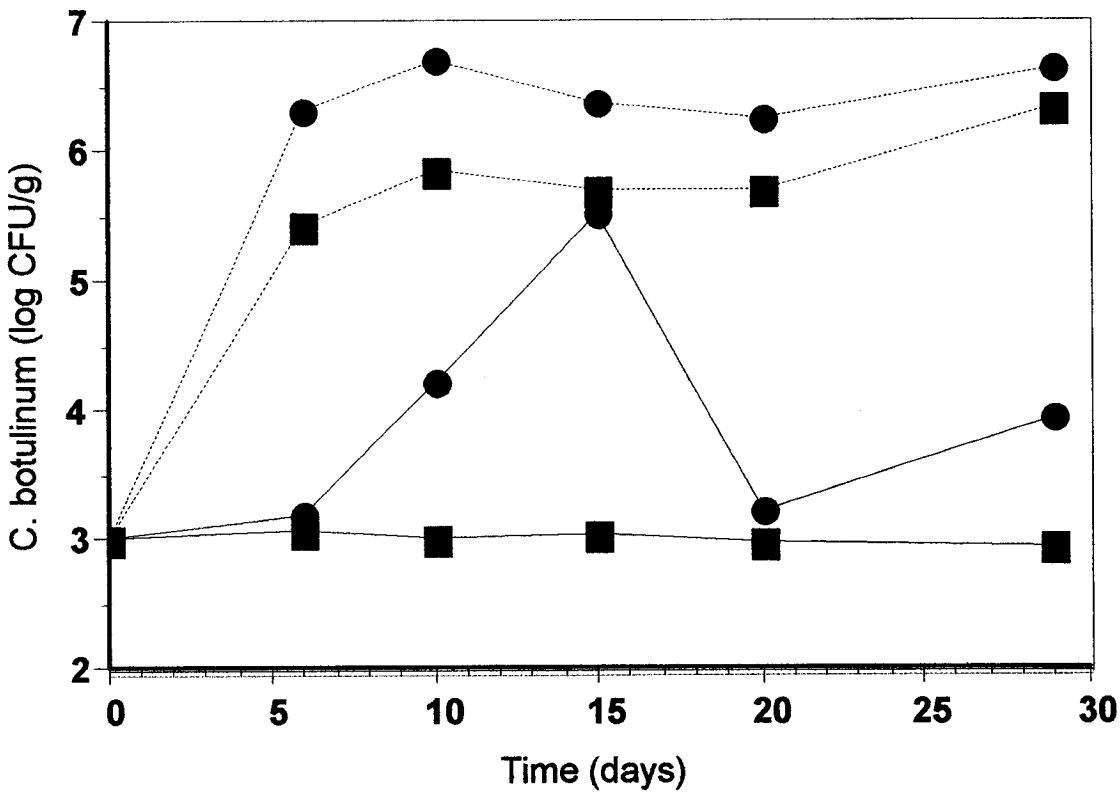


FIGURE 7.4. *Average growth of* C. botulinum *(log CFU/g) in yeast-leavened* (■) *and chemically-leavened* (●) *crumpets inoculated with* 10^3 *spores/g* C. botulinum, *packaged in air with 2-G Ethicap® (—) or in 100%* CO_2 *(----)and stored at 25°C*

leavened crumpets (Figure 7.4). Carbon dioxide and HCO_3^- are required for germination of *C. botulinum* (Kim and Foegeding, 1993b) and in addition, CO_2 may enhance or inhibit growth of and neurotoxin production by *C. botulinum* depending on its level in the package headspace (Doyle, 1983; Lambert et al., 1991a, b; Daifas et al., 1999c). In the present study, it is possible that the bicarbonate ion present in chemical-leavened crumpets contributed to the higher counts of *C. botulinum* in these crumpets, particularly those packaged in CO_2.

For yeast-leavened crumpets packaged in air with ethanol-generating sachets (2-G Ethicap®), counts of *C. botulinum* increased less than one log from the initial inoculum level of 10^3 spores/g (Figure 7.4). Again, as observed for CO_2-packaged crumpets, counts of *C. botulinum* were higher in chemical-leavened Ethicap®-packaged crumpets than in yeast-leavened crumpets packaged under similar conditions. In the chemical-leavened crumpets, log counts of *C. botulinum* were generally <4.5 although higher numbers (~10^5 CFU/g) were enumerated on day 15 (Figure 7.4).

The growth patterns of *C. botulinum* in both yeast- and chemical-leavened crumpets may have been influenced by levels of headspace CO_2 and ethanol in these packaged crumpets. Higher counts of *C. botulinum* were associated with higher levels of headspace CO_2. Indeed, the lowest increase in counts of

C. botulinum (<1 log) were observed in yeast-leavened Ethicap®-packaged crumpets in which levels of headspace CO_2 were ~20% (vol/vol [Figure 7.1]). However, while CO_2 has been shown to have a stimulatory effect on the growth of *C. botulinum*, it is unlikely that low levels of CO_2 in these yeast-leavened Ethicap®-packaged crumpets failed to be stimulatory, since growth at similar levels of CO_2 has been reported (Lambert, 1991a).

A more plausible explanation for the differences in the growth patterns of *C. botulinum*, particularly between yeast- and chemically-leavened crumpets packaged with an ethanol vapour sachet, is that growth was influenced by the level of headspace ethanol in these packaged crumpets. In the present study, growth of *C. botulinum* was associated with headspace ethanol levels ≤2.6% (vol/vol) (Figures 7.2 and 7.4) In yeast-leavened Ethicap®-packaged crumpets, headspace ethanol was >2.6% throughout storage and increased to ~4.5% (vol/vol) by day 30. By this time (day 30), headspace ethanol levels were <2.8% for yeast-leavened CO_2-packaged crumpets as well as for chemical-leavened Ethicap®-packaged crumpets (Figure 7.2). Daifas et al. (2003) have shown in model broth studies that 4% liquid ethanol, in combination with the a_w and pH of the growth medium, had an inhibitory effect on the growth of and neurotoxin production by *C. botulinum.* This present study confirms that levels of between 2.6% to 4% ethanol in the package headspace would be required to

inhibit the growth of this pathogen in high-moisture English-style crumpets. It is also apparent from these results that the method of leavening will influence the headspace ethanol and hence, the antibotulinal effect of ethanol vapour. Indeed, if Ethicap® sachets are to be used with chemically-leavened crumpets, then larger sachets would be required to enhance the safety of these crumpets.

7.3.1.6 Neurotoxin detection

The times at which neurotoxin was detected in yeast- and chemical-leavened crumpets packaged in air with 2-G Ethicap® or in 100% CO_2 and stored at ambient temperature are shown in Table 7.1. Neurotoxin was detected earlier in all CO_2-packaged crumpets than in crumpets packaged with ethanol vapour-generating sachets (Table 7.1). Neurotoxin was detected in all CO_2-packaged crumpets after five-days storage, regardless of the method of leavening. However, neurotoxin was first detected in chemical-leavened crumpets packaged with Ethicap® at day 10 (Table 7.1). More significantly, neurotoxin was not detected in any of the yeast-leavened Ethicap®-packaged crumpets during 30-days storage at ambient temperature (Table 7.1). Furthermore, no neurotoxin was detected in any control crumpets packaged under these atmospheres (results not shown).

TABLE 7.1. *Toxin detection in crumpets inoculated with 10^3 spores/g* C. botulinum, *packaged in air with ethanol vapour or CO_2 and stored at 25°C*

Leavening agent	Packaging atmosphere [a]	Neurotoxin analysis [b] (day)				
		5	10	15	20	30
Yeast	Ethanol	0/3	0/3	0/3	0/3	0/3
	CO_2	3/3	2/3	—[c]	—	—
Chemical	Ethanol	0/3	1/3	3/3	3/3	—
	CO_2	2/3	3/3	—	—	—

[a] Crumpets packaged in air with a 2-G Ethicap® sachet or in 100% CO_2 as indicated

[b] Number of samples testing positive/number of samples analysed by mouse bio-assay

[c] Not tested for neurotoxin since neurotoxin production previously established

These trends can again be related to changes in the counts of *C. botulinum*, as well as to the level of ethanol vapour within the package headspace. Counts of *C. botulinum* were consistently higher in CO_2-packaged crumpets, regardless of the method of leavening, than in crumpets packaged with ethanol vapour. In addition, counts of *C. botulinum* were also higher in chemical-leavened Ethicap®-packaged crumpets than in yeast-leavened crumpets packaged under similar conditions (Figure 7.4). The lower counts of *C. botulinum,* and hence longer time-to-neurotoxin, can also be related to changes in headspace ethanol (Figure 7.2) during storage. Ethicap® (2-G) releases up to 1.1 g ethanol per 50-g crumpet. Levels of headspace ethanol were consistently higher in yeast- than in chemically-leavened crumpets packaged with Ethicap®. While neurotoxin was first detected at day 10 in chemical-leavened crumpets, growth of and neurotoxin production by *C. botulinum* was inhibited for >30 days. The higher levels of ethanol consistently observed in these crumpets, and subsequent inhibition of neurotoxin, may be attributed to ethanol producing microorganisms such as *S. cerevisiae* and other indigenous microflora of crumpets.

This present study confirms previous challenge studies with proteolytic *C. botulinum* that showed that packaging yeast-leavened crumpets with 2% (wt/wt) ethanol inhibited the growth of and neurotoxin production by *C. botulinum* (Daifas et al., 2000). One possible

reason for the anti-botulinal effect of ethanol vapour may be an inhibition of spore germination. Ethanol-induced inhibition of L-alanine-initiated germination of *B. subtilis* spores has been reported (Curran and Knaysi, 1961; Trujillo and Laible, 1970). Whatever the reason, this study confirms the antibotulinal effect of ethanol vapour and demonstrates the importance of the method of leavening on the anti-botulinal efficacy of ethanol vapour. Bakery products typically contain ethanol, particularly if prepared with yeast, fruit, or alcohol. Ethanol contents of >1% have been reported for bourbon cake, apple cake, herb onion bread, and raisin bread (Logan and Distefano, 1998; Chiamoto and Maitani, 1984). While this study has shown that ethanol vapour can delay the growth of and neurotoxin production by *C. botulinum*, the ethanol content of a product can vary with its formulation.

7.3.2 Challenge studies in sterile crumpets

Based on the results of the initial study (section 7.3.1), there appeared to be an interactive effect between the method of leavening (yeast or chemical) of crumpets and the anti-botulinal effect of ethanol vapour. This was attributed to the survival of yeast (*S. cerevisiae*) in yeast-leavened crumpets and the subsequent growth of and ethanol production by these endogenous yeasts. This ethanol, in addition to the

ethanol vapour generated by Ethicap® sachets, may have resulted in headspace ethanol inhibitory to the growth of *C. botulinum.* However, other spoilage microorganisms such as LAB or *Bacillus* spp. in crumpets may have played a role by producing ethanol and/or other antimicrobial metabolites (Wentz et al., 1967; Lyver et al., 1999; Okereke and Montville, 1991; Giardin et al., 2002). Therefore, to determine the role of yeasts on the anti-botulinal efficacy of ethanol vapour, a subsequent study was conducted using sterile yeast- and chemical-leavened crumpets to control the effects of background microorganisms on growth of *C. botulinum*. Sterile yeast-leavened crumpets were inoculated with a co-inoculum of *C. botulinum* (10^3 spores/g) and *S. cerevisiae* (10^5 cells/g). Co-inoculation with *S. cerevisiae* was done to simulate survival of baker's yeast in yeast-leavened crumpets at a level that represented the average yeast counts in CO_2- and Ethicap®-packaged yeast-leavened non-sterile crumpets in the first study (Figure 7.3). Sterile chemical-leavened crumpets were inoculated with *C. botulinum* (10^3 spores/g) only. All inoculated sterile crumpets were packaged in air with 2-G Ethicap® or in 100% CO_2, stored (25°C) and monitored for changes in headspace gas, growth (CFU/g) of *S. cerevisiae* and *C. botulinum* and detection of botulinum neurotoxin.

7.3.2.1 Changes in headspace carbon dioxide and oxygen

The headspace gas composition of sterile yeast- and chemical-leavened crumpets packaged in 100% CO_2 remained constant throughout storage (CO_2 >96% [vol/vol] and O_2 <2% [vol/vol] [results not shown]) and were consistent with those observed in similarly packaged non-sterile crumpets in the first study (Figure 7.1A).

However, changes in headspace O_2 and CO_2 of sterile yeast- and chemical-leavened crumpets packaged in air with an Ethicap® sachet (2-G), were more noticeable, particularly for sterile crumpets co-inoculated with *C. botulinum* and *S. cerevisiae* (Figure 7.5A-B). In these co-inoculated yeast-leavened crumpets, headspace O_2 decreased steadily throughout storage, reaching levels of <2% (vol/vol) by day 15 while headspace CO_2 steadily increased to levels of ~14% over the same period (Figure 7.5A-B). These changes in the headspace gas composition of co-inoculated yeast-leavened Ethicap®-packaged crumpets can be attributed to facultative growth of and metabolism by *S. cerevisiae* only (Walker, 1998) since all uninoculated crumpets were sterile at the onset and end of storage (results not shown).

Furthermore, the headspace gas changes in these sterile co-inoculated crumpets were similar to those observed for non-sterile yeast-leavened crumpets inoculated with *C. botulinum* only and

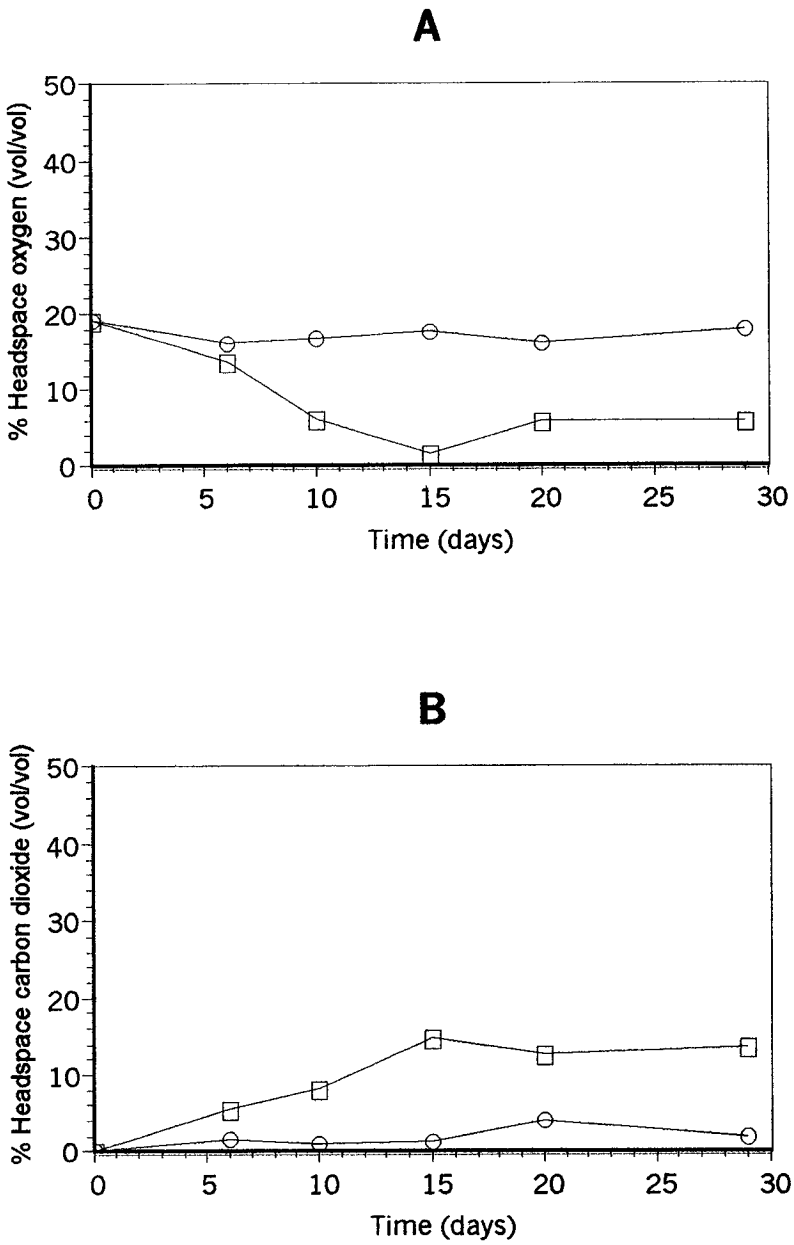


FIGURE 7.5. *Changes in A, headspace O_2 and B, headspace CO_2 in sterile yeast-leavened crumpets inoculated with 10^3 spores/g* C. botulinum *and 5 x 10^5 cells/g* S. cerevisiae *(□) and chemically-leavened crumpets inoculated with 10^3 spores/g* C. botulinum *(○), packaged in air with 2-G Ethicap® and stored at 25°C*

packaged in air with Ethicap® (Figure 7.1), and therefore confirm an important role played by yeasts in headspace O_2 depletion and CO_2 production–conditions conducive to the growth of *C. botulinum*.

In sterile chemical-leavened crumpets inoculated with *C. botulinum* only and packaged with an Ethicap® sachet (2-G), headspace O_2 and CO_2 remained >16% and <3% respectively, throughout storage (Figure 7.1).

Headspace O_2 and CO_2 levels in all 100% CO_2-packaged sterile uninoculated control crumpets were consistently <2% and >98% respectively throughout storage. Over the same period, headspace gas levels of uninoculated control crumpets packaged in air with Ethicap® were consistently >19% O_2 and <1% CO_2–further confirming the sterility of crumpets.

7.3.2.2 Growth of *S. cerevisiae*

Yeast counts (log CFU/g) for sterile yeast-leavened crumpets co-inoculated with *C. botulinum* and *S. cerevisiae* and packaged in air with Ethicap® or in 100% CO_2 are shown in Figure 7.6. Counts of *S. cerevisiae* increased gradually during storage and reached a level of ~$10^{6.5}$ CFU/g by day 20 (Figure 7.6). Yeast counts in these sterile crumpets were similar to similarly-packaged non-sterile crumpets inoculated with *C. botulinum* only (Figure 7.3). Although similar

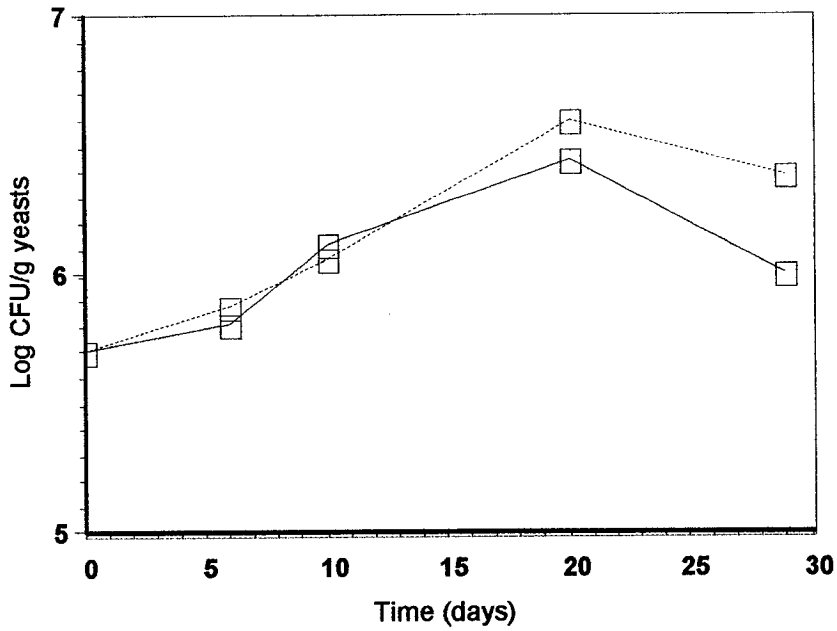


FIGURE 7.6. *Growth (average log CFU/g)* S. cerevisiae *in yeast-leavened crumpets inoculated with* 10^3 *spores/g* C. botulinum *and 5 x* 10^5 *cells/g* S. cerevisiae *(□), packaged in air with 2-G Ethicap® (—) or in 100%* CO_2 *(----) and stored at 25°C*

growth trends were again observed between Ethicap®- and CO_2-packaged crumpets, slightly higher counts were observed in all gas-packaged crumpets (Figure 7.6). Again, this trend is consistent with growth profiles of yeasts in non-sterile crumpets (Figure 7.3). Furthermore, these results, as indicated by the first study, confirm that *S. cerevisiae* can grow to similar levels in crumpets regardless of the packaging atmosphere and may deplete headspace O_2 to levels conducive to the growth of *C. botulinum* (Figure 7.5).

No yeasts were observed in any sterile chemical-leavened crumpets inoculated with *C. botulinum* only nor in uninoculated sterile control crumpets (results not shown).

7.3.2.3 Growth of *C. botulinum*

Changes in the counts (log CFU/g) of *C. botulinum* in sterile crumpets packaged in air with Ethicap® or in 100% CO_2 are shown in Figure 7.7A-B. Again, as observed for non-sterile crumpets inoculated with *C. botulinum*, clearly different growth patterns were observed between Ethicap®- and CO_2-packaged crumpets, with counts being consistently one to three logs higher in crumpets packaged in 100% CO_2 (Figure 7.7A-B).

In Ethicap®-packaged crumpets, counts of *C. botulinum* increased less than one log cycle from the initial inoculum level of

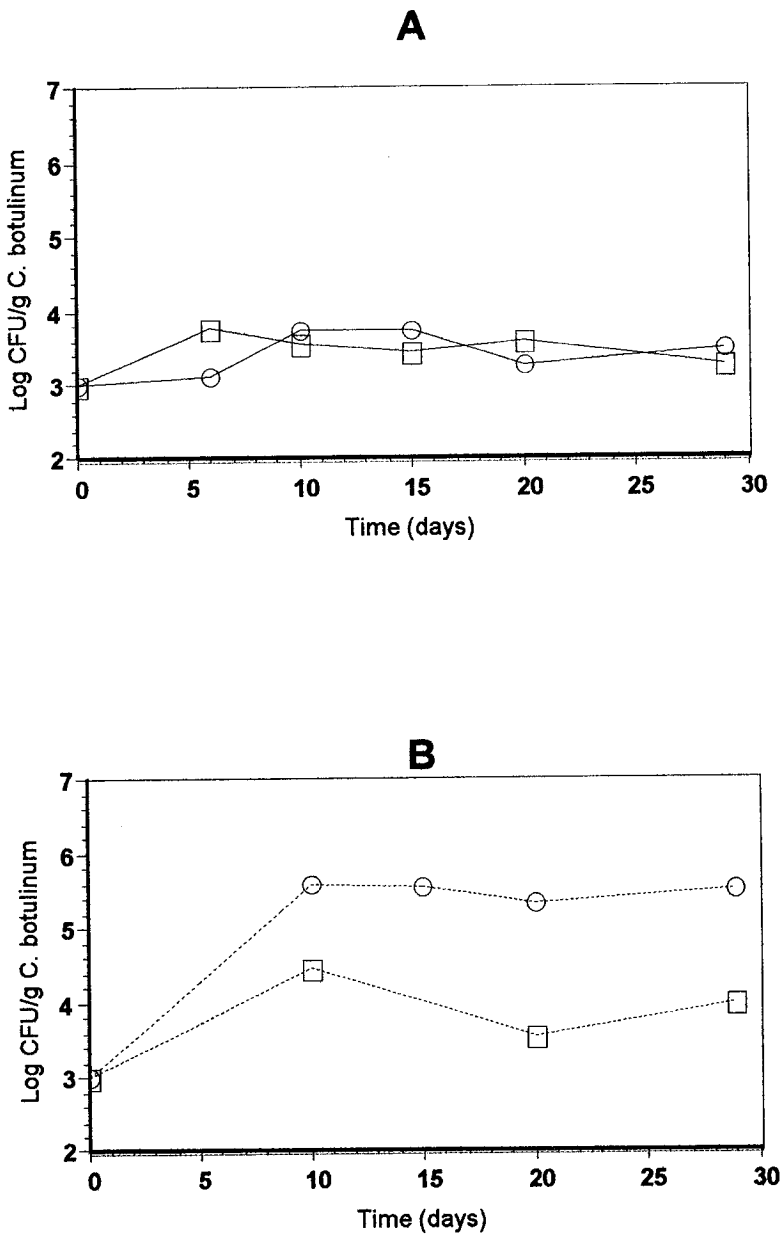


FIGURE 7.7. *Average growth of* C. botulinum *(log CFU/g)* in *sterile yeast-leavened crumpets inoculated with* 10^3 *spores/g* C. botulinum *and* 5×10^5 *cells/g* S. cerevisiae *(□) and sterile chemically-leavened crumpets inoculated with* 10^3 *spores/g* C. botulinum (○) *packaged in A, air with 2-G Ethicap® (—); or B, in 100%* CO_2 *(----) and stored at 25°C*

~10^3 spores/g throughout storage (Figure 7.7A) even though headspace O_2 and CO_2 levels were conducive to its growth after 15-days storage (Figure 7.5). This trend in the counts of *C. botulinum* was similar to that observed in the first study with non-sterile yeast-leavened Ethicap®-packaged crumpets (Figure 7.4). In the second study, headspace O_2 levels in sterile chemical-leavened Ethicap®-packaged crumpets remained high (>16%) throughout storage. However, although counts (log CFU/g) were low (<4.0), growth of *C. botulinum* was not completely prevented. Several studies have reported the growth of *C. botulinum* in elevated levels of headspace O_2 (Whiting and Naftulin, 1992; Clavero et al., 2000; Dufresne et al., 2000a, b).

In sterile CO_2-packaged crumpets, counts of *C. botulinum* were approximately two logs higher in chemical-leavened crumpets inoculated with *C. botulinum* compared with yeast-leavened crumpets co-inoculated with *C. botulinum* and *S. cerevisiae* (Figure 7.7B). Although headspace ethanol was not measured in the second study, the differences in counts between inoculated and co-inoculated crumpets can be assumed to be due to the inhibitory effect of yeast produced by *S. cerevisiae* in these co-inoculated sterile crumpets.

It is interesting to note that although similar trends were evident in the growth of *C. botulinum* and detection of its neurotoxin between non-sterile and sterile crumpets, counts were higher and neurotoxin

was slightly delayed in the first study using non-sterile crumpets compared to the second study with sterile crumpets. This difference was surprising; however, it may be due to several interrelated factors including: i) the activity of background microflora in non-sterile crumpets; and ii) the differences in headspace gas composition, particularly CO_2, which has been shown to enhance the growth of and neurotoxin production by *C. botulinum.*

No *C. botulinum* was detected in any sterile uninoculated crumpets after 30-days storage at 25°C.

7.3.2.4 Neurotoxin detection

The times at which neurotoxin was detected in sterile crumpets packaged in air with Ethicap® or in 100% CO_2 are summarised in Table 7.2. Packaging crumpets with CO_2 resulted in a highly significantly ($P<0.001$) increased frequency of detecting neurotoxin in crumpets. In addition, more chemical-leavened crumpets inoculated with *C. botulinum* were toxic than yeast-leavened crumpets co-inoculated with *C. botulinum* and *S. cerevisiae* ($P<0.001$). Neurotoxin was detected earlier i) in crumpets inoculated with *C. botulinum* only compared to those co-inoculated with *C. botulinum* and *S. cerevisiae*; and ii) in CO_2-packaged crumpets compared with those packaged with Ethicap® (Table 7.2). Indeed, in sterile yeast-leavened

Ethicap®-packaged crumpets co-inoculated with *C. botulinum* and *S. cerevisiae*,

TABLE 7.2 *Neurotoxin detection in sterile crumpets inoculated with* C. botulinum *or co-inoculated with* C. botulinum *and* S. Cerevisiae, *packaged in air with ethanol vapour or* CO_2 *and stored at 25°C*

Leavening method	Inoculum [a]	Packaging atmosphere [b]	Neurotoxin analysis [c] (day)				
			5	10	15	20	30
Yeast	*C. botulinum / S. cerevisiae*	Ethanol	0/3	0/3	0/3	0/3	0/3
	C. botulinum / S. cerevisiae	CO_2	0/3	3/3	3/3	3/3	3/3
Chemical	*C. botulinum*	Ethanol	0/3	0/3	1/3	1/3	1/3
	C. botulinum	CO_2	3/3	3/3	3/3	3/3	3/3

[a] Crumpets inoculated with 10^3 spores/g *C. botulinum* or with 10^3 spores/g *C. botulinum* and 10^5 cells/g *S. cerevisiae*

[b] Crumpets packaged in air with a 2-G Ethicap® sachet or in 100% CO_2 as indicated

[c] Number of samples testing positive/number of samples analysed by mouse bio-assay

neurotoxin was not detected during 30-days storage while sterile chemical-leavened crumpets inoculated with *C. botulinum* only and packaged in 100% CO_2 were toxic after five-days storage. While headspace ethanol was not measured in the second study, it can be assumed that the ethanol vapour generated by Ethicap® delayed neurotoxin production in chemical-leavened crumpets and completely inhibited neurotoxin production in all co-inoculated yeast-leavened crumpets due, again, to additional ethanol production by yeast in the co-inoculum.

In both studies, the time at which neurotoxin was detected generally corresponded to log counts of *C. botulinum* ≥4.4 (Figure 7.8). This is in agreement with previous studies of Daifas et al. (1999b) which showed that the time-to-neurotoxin in MAP crumpets coincided with log counts of *C. botulinum* of ~5.

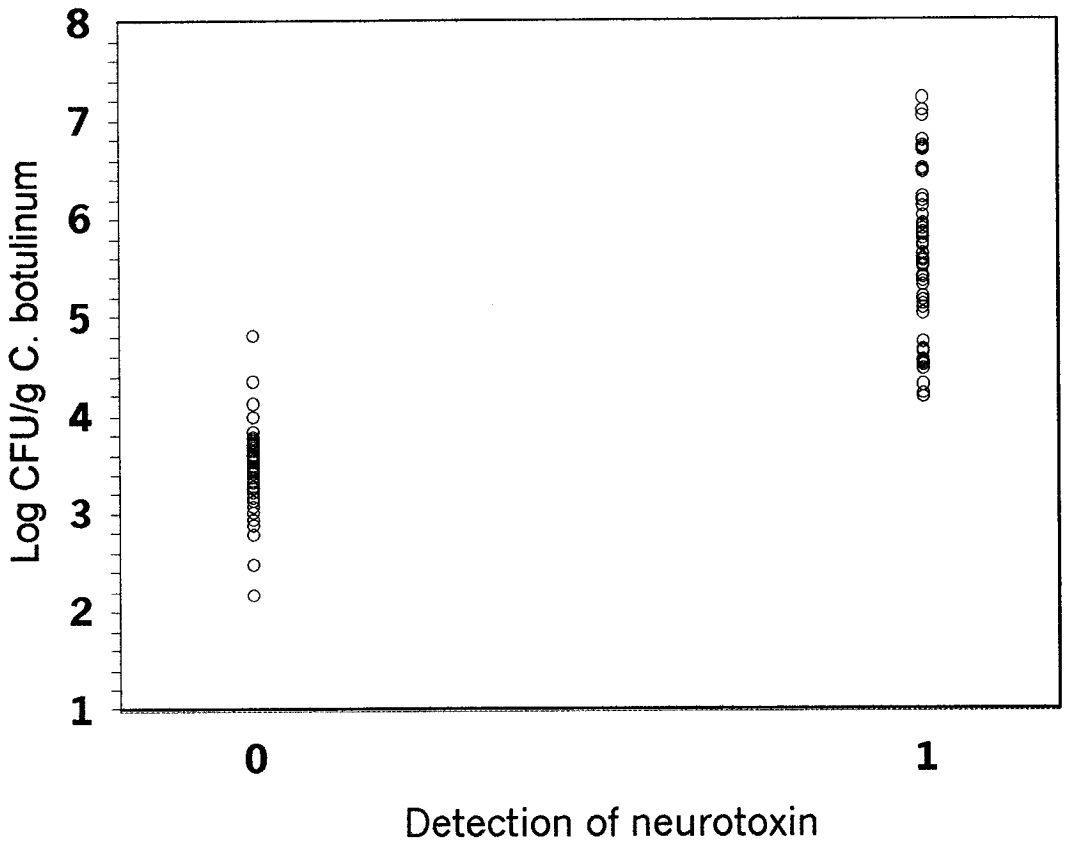


FIGURE 7.8. *Log count (CFU/g)* C. botulinum *in non-toxic (0) and toxic (1) non-sterile and sterile yeast- or chemically-leavened crumpets inoculated with* 10^3 *spores/g* C. botulinum *or* 10^3 *spores/g C. botulinum and 5 x* 10^3 *cells/g* S. cerevisiae, *packaged in air with 2G Ethicap® or in 100%* CO^2 *and stored at 25°C*

7.4 CONCLUSION

The results of both studies confirm the role of yeast, particularly in Ethicap®-packaged crumpets, in delaying neurotoxin production. Furthermore, both studies confirm that ethanol vapour is not as an effective anti-botulinal agent in chemical-leavened crumpets compared to those leavened by yeast. In the first study, the complete inhibition of neurotoxin production in yeast-leavened Ethicap®-packaged crumpets was assumed to be due to the additive effect of ethanol generated by i) the interactive sachets and ii) endogenous yeasts and other microorganisms. This second study with sterile crumpets confirms the significant role ($P<0.001$) played by *S. cerevisiae* in enhancing the anti-botulinal efficacy of ethanol vapour generating sachets. Based on these studies, the method of crumpet leavening appears to have a profound effect on the growth of and neurotoxin production by *C. botulinum* in CO_2- and Ethicap®-packaged crumpets.

In conclusion, Ethicap® sachets have the potential to provide an effective additional barrier to the growth of *C. botulinum* and to enhance the safety of high-moisture bakery products at ambient temperature. However, product safety must be assessed on a case-by-case basis, as formulation changes such as the method of leavening may influence the level of ethanol required to ensure product safety. While smaller Ethicap® sachets (2-G) could be used to enhance the safety of yeast-leavened crumpets, larger sizes (≥4-G) would be required to ensure the safety of chemical-leavened crumpets. However, regardless of the size of sachet used, the

sensory shelf-life of such packaged crumpets may be compromised due to absorption of ethanol from the package headspace.

PREFACE TO CHAPTER 8.0

While ethanol vapour can be used to control the growth of *C. botulinum* in high-moisture bakery products, it is only effective at high levels in the package headspace. An alternative approach to reduce the levels of ethanol in the package headspace would be to combine ethanol vapour with a natural antimicrobial, such as oil-of-mastic. Since little, if anything, is known about the antibotulinal effect of mastic resin or oil, studies were designed to investigate its potential of these agents to inhibit the growth of and neurotoxin production by *C. botulinum* in media and in crumpets.

8.0 STUDIES ON THE ANTI-BOTULINAL ACTIVITY OF MASTIC

8.1 INTRODUCTION

High-moisture minimally-processed bakery products such as crumpets, have been shown to have the potential to support growth of *C. botulinum* when packaged under modified atmospheres and stored at ambient temperature (Daifas et al., 1999b). Since reformulation of these high-moisture products is not always practical due to sensory constraints, it has been recommended that additional barriers to the growth of *C. botulinum* be included in the product and/or package to reduce the hazardous, albeit unlikely, event that products become toxic.

One novel approach of inhibiting microbial growth in food is the use of certain plant extracts and essential oils. Many plant essential oils contain antimicrobial substances including flavanoids, triterpenoids, sesqiterpenoids and phenols (Rojas et al., 1992; Nychas, 1995). Most pathogens including *C. botulinum*, *Bacillus* spp., *S. aureus, Salmonella* spp., and *E. coli*, in addition to yeasts and moulds, are sensitive to compounds found in many essential oils (Beuchat and Golden, 1989). De Wit et al. (1979) reported that *C. botulinum* type A was inhibited by garlic and onion while clove, pimenta and black pepper oil were active against *C. botulinum* 67B (Ismaiel and Pierson, 1990a) and *C. sporogenes* was shown to be sensitive to citral (the primary component of lemon grass oil) and oregano (Paster et al., 1990; Deans et al., 1992). While Hall and Maurer (1986) reported that mace, bay leaf, and nutmeg extracts inhibited botulinum neurotoxin production, Chaibi

et al. (1997) reported that cedar, eucalyptus, camomile, savage carrots, vervain, grapefruit and orange oils inhibited germination of *C. botulinum* 62A.

The shrub *Pistacia lentiscus* var. *chia*, produces an essential oil with a pleasant characteristic balsamic odour. Both the resin and its essential oil are used as flavouring for breads, as well as for other foods and beverages (Wyllie et al., 1990), and have been shown also to have antioxidant properties (Abdel-Rahman and Soad, 1975). Although present in all tissue (leaves, twigs and stems) the major source of the oil is mastic, the resinous exudate of the plant. The antimicrobial activity of mastic against several foodborne pathogens including *S. aureus, S. enteriditis, B. cereus, E. coli, Helicobactor pylori* as well as many yeasts and moulds has been reported (Alippi et al., 1996; Iuak et al., 1996; Tassou and Nychas, 1996; 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 not been reported.

The objective of this study was to determine the effect of resinous and essential oil-of-mastic on the growth of and neurotoxin production by *C. botulinum* i) in media and ii) in challenge studies with English-style crumpets.

8.2 MATERIALS AND METHODS

8.2.1 Preparation of spore inoculum

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°C. Spores of individual strains were used in the "spot on the lawn" and microtiter assays, while a composite inoculum was used in the agar plate study and in the challenge study with crumpets. For the agar plate and challenge studies, equal numbers of spores of each strain were combined to form a single suspension of approximately 3 x 10^6 spores/mL which was further diluted in 0.1% peptone water as required for individual studies. All spore mixtures were heat-shocked (75°C for 20 minutes) prior to sample inoculation and inoculum levels were verified using modified McClung Toabe (MMT) agar prepared from McClung Toabe agar (Difco, Becton-Dickinson; Sparks, MD) supplemented with 0.1% yeast extract (Difco) and egg-yolk as previously described (Daifas et al., 1999b).

8.2.2 Mastic

Mastic resin ("gum" [Chios Mastic Grower's Association {CMGA}; Chios, Greece]) and its oil (CMGA) were used throughout these studies. The sterility of both the resin and oil was confirmed by aerobic and anaerobic plate counts at 25, 30 and 37°C.

8.2.3 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 for anti-botulinal activity, ii) a microtiter plate assay to determine the inhibitory effect of mastic oil against *C. botulinum*, and iii) an agar plate study to investigate the anti-botulinal potential of mastic volatiles.

8.2.3.1 Spot on the lawn

Preliminary screening of the anti-botulinal activity of resinous mastic against outgrowth of spores of *C. botulinum* was done using a modified "spot on the lawn" assay. Mastic resin (CMGA) was dissolved in 95% ethanol to make a 10% (wt/wt) solution, which was further diluted in 95% ethanol to provide a dilution series of 2, 4, 6, 8 and 10% (wt/wt) mastic. Solutions were filter-sterilized using a 0.45-μm filter (Ascrodisc; Gelman Scientific, Ann Arbor, MI). Plates of

MMT agar were spread with spore suspensions (0.1 mL) of individual strains of *C. botulinum* (~1 x 10^6 spores/g) prepared as previously described. Inoculated plates of MMT agar were spotted with 5 μL each of the filter-sterilized mastic solutions (2% to 10%) or a control of 95% ethanol (0% mastic). Plates were incubated in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 at 37°C for three days. Following incubation, plates were observed and inhibition of *C. botulinum* was scored qualitatively as complete, partial, or no inhibition.

8.2.3.2 Microtiter assay

The antimicrobial activity of the essential oil-of-mastic (CMGA) was determined using a microtiter assay. Inocula used in this assay were prepared by adding an appropriate volume of spores of individual strains of *C. botulinum*, prepared as described previously, to 10 mL of Iso-sensitest (IST [Difco]) broth supplemented with 0.15% Bacto-agar (Difco) preheated to 37°C to provide final inocula of 10^3 spores/mL.

Forty (40) μL of 0.3% (vol/vol) essential oil of mastic (CMGA) in IST broth containing 0.15% agar to prevent separation (Mann and Markham, 1998) was dispensed into the first rows of microtiter plates and 1:2 row-wise serial dilutions were subsequently prepared leaving 20 μL per well. Spore inocula (180-μL) were dispensed in appropriate

wells providing a range of 0.3% to 0.008% (vol/vol) oil of mastic. Positive and negative controls consisted of inoculated (containing 0% mastic) and uninoculated (containing 0.3% to 0.008% mastic) IST broth respectively. Well contents were mixed seven times using a multi-channel pipetter. Plates were covered with sterile aluminium foil and were incubated in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 at 37°C for 48 hours prior to reading absorbance at 450 nm using a microplate reader (model ELx800 running KC-Jr. software, Bio-tek Instruments; Summit, NJ). The anti-botulinal activity of mastic was expressed as the inhibition index (I.I.)

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

where Δ 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 respectively. The I.I. ranges from 0 for no inhibition to 1 for complete inhibition (Chaibi et al., 1997).

8.2.3.3 Vapour-phase inhibition agar plate study

The potential of mastic volatiles to inhibit growth of *C. botulinum* was investigated using an agar plate study. Powdered mastic resin was dispersed in 95% ethanol and was subsequently diluted with

distilled water to give a dispersion containing 1.9% mastic and 6.8% ethanol (wt/wt). This dispersion was further diluted (1/10) with distilled water and both mixtures were filter-sterilized using a 0.45-μm filter (Ascrodisc). Sterile cotton pads were placed in the lids of petri dishes and 1.5 mL of mastic solutions were dispensed onto the pads to provide 0.1% or 0.01% mastic (wt/wt [based on 20-g agar]). Plates of MMT agar previously spread, in triplicate, with a composite inoculum of *C. botulinum* (5×10^1, 5×10^2 or 5×10^3 spores/mL), prepared as previously described, were immediately inverted over the lids prior to sealing each plate in a high gas barrier bag (O_2 transmission rate: 3-6 cc/m^2/day, 1 atm at 4.4°C, 0% RH; Cryovac; Mississauga, ON) containing an AgelessFX®$_{200}$ oxygen absorbent (Mitsubishi Gas & Chemical Company; Japan) with an impulse heat-sealer. Plates were incubated for three days at 37°C. The effect of mastic on *C. botulinum* was determined as log (N_0/N) where N_0 and N are the counts (CFU/g) of *C. botulinum* grown without and with mastic respectively. Controls consisted of solutions of equivalent amounts of ethanol without mastic.

8.2.4 Transmission electron microscopy

Preparation for TEM essentially followed 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% oil of mastic for 24 hours at 37°C, were fixed in 0.2-M cacodylate buffer pH 7.4 containing 2.5% (vol/vol) 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 (15 minutes each in 50%, 70% and 90% ethanol, followed by three 20-minute incubations in 100% ethanol). Samples were then infiltrated and embedded in Taab 812 resin (Marivac, Halifax, NS). 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) operating at 80 kV with the energy loss spectrometer in place.

8.2.5 Challenge study

8.2.5.1 Sample preparation, inoculation and packaging

Crumpet batter was prepared as described by Daifas et al. (1999a, b). Essential oil-of-mastic (40, 80, 120, 160, or 200 μL [CMGA]) or powdered mastic resin (0.4 g, 0.8 g or 1.2 g [CMGA]) was added to 625 mL crumpet batter. Seven hundred twenty (720) μL of a composite inoculum (3 x 10^5 spores/mL) of proteolytic *C. botulinum*, prepared and verified as described previously, was added prior to

baking on a griddle as described previously (Daifas et al., 1999b). This resulted in baked crumpets (50 g) containing 5 x 10^2 spores/g *C. botulinum* and 0.1% to 0.5% (vol/wt) oil-of-mastic or 1% to 3% (wt/wt) mastic resin. In addition to adding mastic to crumpets prior to baking, mastic oil (0.1% to 0.5% [wt/wt]) was dispensed evenly on the surface of inoculated crumpets by pipette post-baking. All crumpets were packaged, in duplicate, in high gas barrier bags (Cryovac) with an Agleless® FX_{200} oxygen absorbent (Mitsubishi), sealed with an impulse heat-sealer and were stored at 25°C. Controls consisted of i) crumpets inoculated with *C. botulinum* packaged without (0%) mastic and ii) crumpets inoculated only with 0.1% (wt/wt) peptone water and similarly packaged and stored.

8.2.5.2 Detection of neurotoxin

Crumpets were analysed for neurotoxin using the mouse bioassay at one week intervals until neurotoxin was detected. On each sampling day, crumpets were diluted 1:3 in 0.1% peptone water and 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, QC). Mice were observed for up to 72 hours for typical signs of botulism including ruffled fur, pinched waist, laboured breathing, limb paresis and general paralysis. Mice showing severe distress were euthanised by

asphyxiation with CO_2 according to Health Canada Animal Care Committee guidelines. Neutralization of neurotoxin was performed on randomly selected representative positive samples using antisera (Connaught Laboratories; North York, ON) to botulinum neurotoxins to confirm that toxicity was due to botulinum neurotoxin as described previously (Daifas et al., 1999b).

8.3 RESULTS AND DISCUSSION

8.3.1 Preliminary screening

The effect of mastic resin (0% to 10% [wt/wt]) in 95% ethanol on five strains of proteolytic *C. botulinum* is shown in Table 8.1. When applied to MMT agar, the mastic solutions separated into two distinct, concentric phases–the outer of which had an iridescent, oil-like sheen (Figure 8.1). However, no such separation occurred when the control (0% mastic in ethanol) was applied to the agar. Furthermore, in all cases, no inhibition was observed with 0% mastic (Table 8.1). For mastic solutions, inhibition of *C. botulinum* was greater at the outer oil-like phase (Table 8.1). While partial inhibition was observed in the inner phase for all concentrations of mastic, for *C. botulinum* A6 and 62A, complete inhibition of growth also occurred at the outer phase (Figure 8.1). *C. botulinum* 17A was completely inhibited by ≥2% mastic. Although type B strains of *C. botulinum* were partially inhibited by mastic at ≤6%, complete inhibition of both type B strains was associated with the outer, oil-like phase at higher (≥8%) concentrations of mastic (Table 8.1). Generally, strains of *C. botulinum* type A were more inhibited by mastic than type B strains were (Table 8.1). This is consistent with De Wit et al. (1979) who reported that while garlic and onion oils partially inhibited *C. botulinum* type A, types B and E were not inhibited.

TABLE 8.1. *Effect of resinous mastic on* C. botulinum

C. botulinum	% (wt/wt) Mastic [a]									
	0	2		4		6		8		10	
		I[b]	O[c]	I	O	I	O	I	O	I	O
A6		±	+	±	+	±	+	±	+	±	+
62A		±	+	±	+	±	+	±	+	±	+
17A		+	+	+	+	+	+	+	+	+	+
13983IIB		±	±	±	±	±	±	±	+	±	+
368B		±	±	±	±	±	±	±	+	±	+

[a] Mastic resin in 95% ethanol

[b, c] I (inner) and O (outer) concentric circles into which the mastic solution separated when applied to the agar.

- No inhibition; ±, partial inhibition; +, complete inhibition

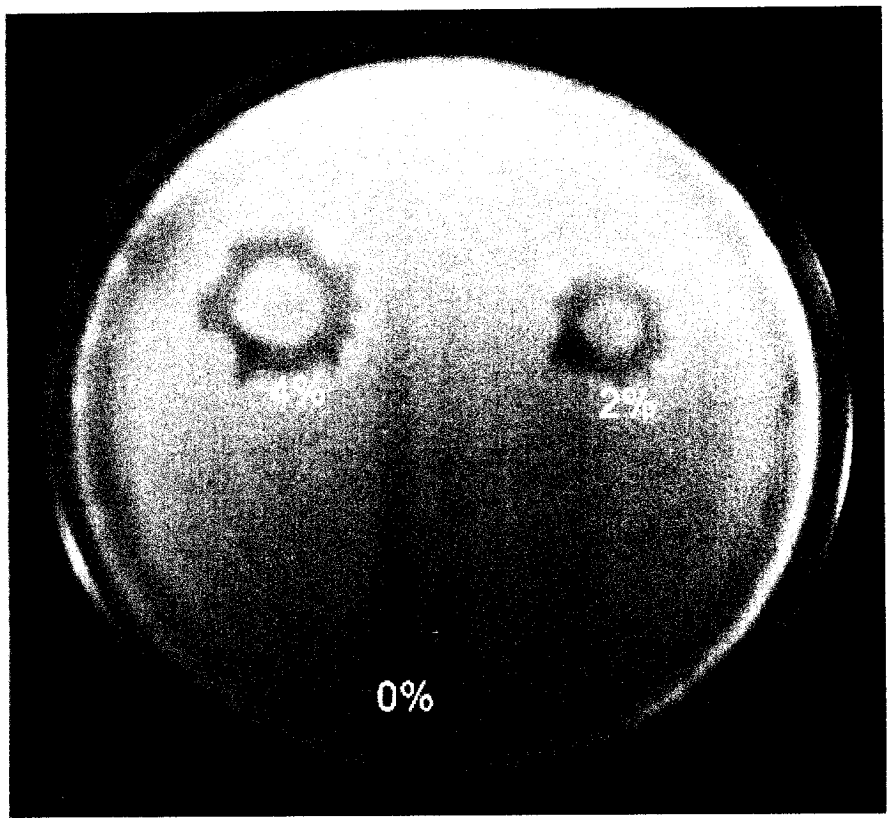


FIGURE 8.1. *Inhibitory effect of resinous mastic (0 to 3% [wt/wt]) in ethanol on* C. botulinum *62A*

The major components of resinous mastic include 1% to 3% essential oil, 4% α- and β-mastichinic acid ($C_{23}H_{36}O_4$), 0.5% masticholic acid ($C_{23}H_{36}O_4$), 20% α-mastichonic acid ($C_{23}H_{48}O_4$), 18% β-mastichonic acid ($C_{32}H_{48}O_4$), 30% α-mastichorezene ($C_{35}H_{56}O_4$) and 20% β-mastichorezene ($C_{35}H_{56}O_4$) (Papageorgiou et al., 1991). The essential oil is a complex mixture of at least 70 compounds–primarily several monoterpenes: myrcene (39%), α-pinene (28%), limonene (11%), β-pinene (5.4%) and β-caryophyllene (2.4%) as well as 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 for both phases, it is clear that there are strain differences in sensitivity to mastic.

Nevertheless, since greater inhibition was observed with the oil-like phase than with ethanol alone (0% mastic control), this study has indicated, qualitatively, that mastic resin in ethanol had a greater inhibitory effect on proteolytic strains of *C. botulinum* than ethanol alone.

8.3.2 Effect of mastic oil on growth of *C. botulinum*

The activity of mastic oil against growth of *C. botulinum* in IST broth in the microtiter assay is shown in Table 8.2. Oil-of-mastic, at levels of

TABLE 8.2. *Inhibitory index*[a] *of oil of mastic on strains of proteolytic* C. botulinum

Strain of *C. botulinum*	% (vol/vol) Oil of mastic			
	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

[a] 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

≥0.3% (wt/wt) had an inhibitory effect on *C. botulinum*. The inhibitory indices for the strains tested, as shown in Table 8.2, ranged from 0.50 to 0.91. The inhibitory index, which reflects the change in optical density between spores with and without mastic oil, can range from 0 for no inhibition to 1 for complete inhibition (Chaibi et al., 1997). Although some strains of *C. botulinum* were inhibited by mastic at 0.2% (wt/wt), the inhibitory indices at this level were low, i.e., <0.5. Furthermore, no inhibition was observed at <0.2% mastic (Table 8.2). Chaibi et al. (1997) reported inhibition of *C. botulinum* 62A by essential oils (eucalyptus, camomile cedar, savage carrots, artemisia, grapefruit, vervain, and orange), while Ismaiel and Pierson (1990a) inhibited *C. botulinum* 67B with clove, thyme, cinnamon, pimento and origanum oils. However, lower concentrations (0.01% to 0.04%) of these oils were required to achieve similar inhibition to that observed in this study with 0.3% mastic. In this study, greater I.I.s were observed for type A than for type B strains of *C. botulinum*, with the exception of *C. botulinum* 13983IIB. This is consistent with the results of the "spot on the lawn" assay in which *C. botulinum* type A also showed greater sensitivity to mastic. However, in the microtiter assay, the largest I.I. (0.91) was observed for *C. botulinum* 13983IIB, again confirming a strain-specific inhibition.

8.3.3 Effect of mastic volatiles on *C. botulinum*

When solutions containing either 0.01% (wt/wt) or 0.1% (wt/wt) mastic were applied to cotton pads in the inverted lids of plates of MMT agar inoculated with 5 x 10^1, 5 x 10^2, or 5 x 10^3 spores/g *C. botulinum*, growth was partially inhibited (Figure 8.2). For 0.01% mastic, a ≤1 log reduction of *C. botulinum* (CFU/g) was observed, regardless of the inoculum level. However, the 0.1% mastic solution resulted in greater inhibition. At this higher concentration of mastic, a 1.5 to 1.8 log inhibition was observed for all inoculum levels (Figure 8.2). Furthermore, since there was no direct contact between the agar plates and the mastic/ethanol solutions, the observed inhibition of *C. botulinum* can be attributed to the mastic volatiles alone or from the 0.01% and 0.1% ethanol/mastic aqueous solutions. Volatile constituents of other oils (horseradish, wasabi, wormwood, garlic and ginger) have been shown to inhibit bacteria including *B. subtilis* (Inouye et al., 1983; Yun et al., 1993; Delequis et al., 1999). However, when *C. botulinum* was grown in IST broth containing mastic oil, anti-botulinal activity was evident only at concentrations ≥0.2%. The lower levels of mastic oil required to inhibit *C. botulinum* in this study may be due to several factors: i) mastic may have a greater anti-botulinal effect in the vapour state; ii) the anti-botulinal activity of the inhibitory compounds may be closely related to their solubility and

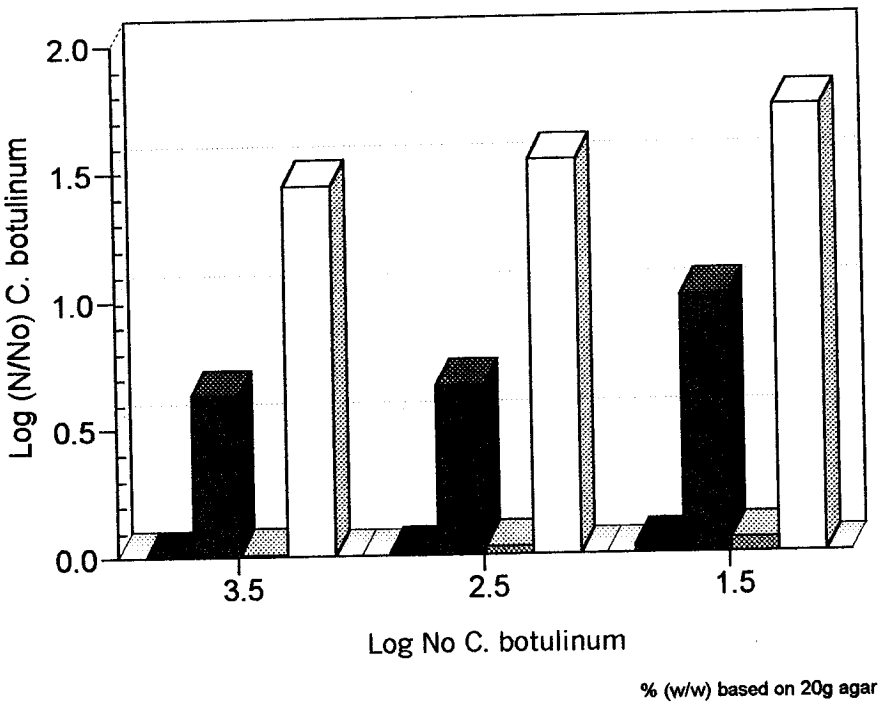
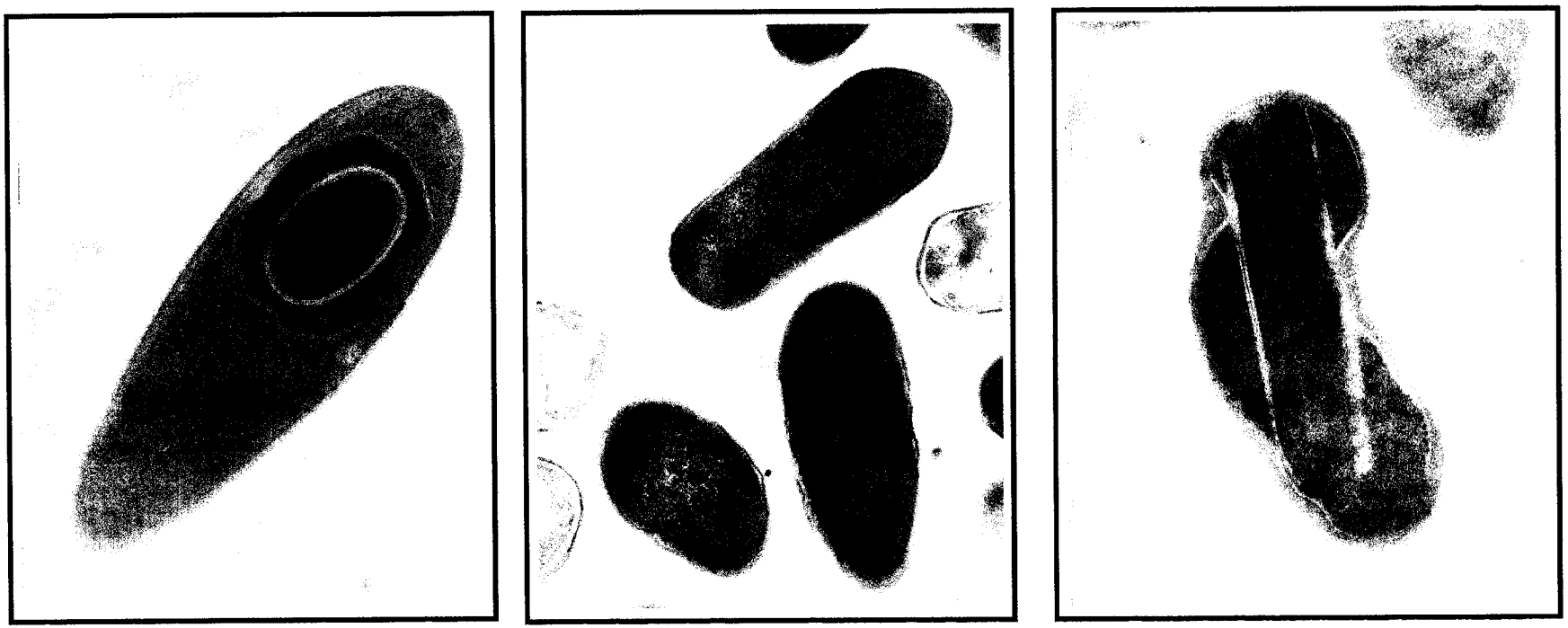


FIGURE 8.2. *Effect of volatiles of 0.01% mastic with 0.05% ethanol (■); 0.1% mastic with 0.5% ethanol (□); 0.05% ethanol (■); and 0.5% ethanol (▨) [wt/wt]) on a composite inoculum of proteolytic* C. botulinum *(10^1, 10^2, or 10^3 spores/g) grown on McClung Toabe agar at 37°C*

therefore, be dependent upon the solvent; and iii) volatile terpene alcohols that may be formed from the oxygenation of terpenes such as limonene and pinene, and which may possess greater activity than the parent compound (Megalla et al., 1990) may be present in the vapour. Furthermore, although ethanol in the vapour phase has been shown to be an effective antimicrobial (Smith et al., 1987, Daifas et al., 2000), the absence of inhibition observed for controls (0% mastic and ≤0.5% ethanol) indicates that ethanol at this level was sub-inhibitory. However, it is possible that the low, sub-inhibitory levels of ethanol enhanced the activity of the added mastic. Such synergistic effects of volatile inhibitors have been reported by Ahn et al. (1999). While these authors observed that vapourized 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.

8.3.4 Effect of mastic on cells of *C. botulinum*

In order to investigate the effect of mastic on vegetative cells, 18-hour cultures of *C. botulinum* 62A were grown in TPGY broth containing 0%, 0.15%, or 0.30% mastic oil at 36°C. Micrographs of these cultures grown in TPGY broth with 0%, 0.15%, or 0.30% oil-of-mastic are shown in Figure 8.3A-C. Growth and cell division were evident in con-



A B C

FIGURE 8.3. *Effect of A, 0%; B, 0.15%; and C, 0.30% (vol/wt) mastic oil on vegetative cells of* Clostridium botulinum *62A in trypticase peptone glucose yeast broth at 36°C*

trol cultures grown without (0%) mastic. Although spores were evident in control cultures (Figure 8.3A), no spores were observed in TPGY cultures containing 0.15% or 0.30% mastic. Furthermore, when cells of *C. botulinum* were grown with these levels of mastic, structural changes in the cells were observed. At 0.15% mastic areas of homogeneous but granular-appearing inclusions, which lacked evidence of ribosomes, were noticeable (Figure 8.3B). Effects were more pronounced in cells grown with 0.30% mastic. In these cells, large straight inclusions in the cells were evident (Figure 8.3C). Huwez et al. (1998) have also reported mastic induced ultrastructural changes in *H. pylori*, but these changes were not described. It is not clear how mastic inhibited *C. botulinum*. Although the antimicrobial action of essential oils has been established, the mechanisms of action are not known. 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, interacts with the membranes of *B. cereus* by changing its permeability for cations leading to impairment of essential cell processes and finally to cell death. At non-lethal concentrations, *B. cereus* adapted to carvacol by changing its fatty acid composition (Ultee et al., 2000). Nevertheless, it is clear that structural changes occurred in cells of *C. botulinum* grown with mastic, particularly at a level of 0.30%.

8.3.5 Challenge study

Crumpets, inoculated with 500 spores/g of *C. botulinum* containing 0% to 3% mastic resin added pre-baking or 0% to 0.5% mastic oil, added pre- or post-baking, were stored anaerobically at 25°C.

8.3.5.1 Neurotoxin detection

Crumpets were first tested for neurotoxin after seven-days storage at ambient temperature. As shown in Table 8.3, neurotoxin was detected at this time in all crumpets regardless of the level of mastic. Clearly, packaging crumpets with mastic resin (1% to 3%) or mastic oil (0.1% or 0.5%), either pre- or post-baking, did not prevent toxigenesis. In this study, the spore inoculum was added to batter before baking. However, previous studies have established that such inoculation results in almost complete spore survival (Daifas et al., 1999b). Furthermore, neurotoxin was also detected at the same time (day 7) in inoculated crumpets containing no mastic (0%), packaged and stored under similar conditions (Table 8.3). Therefore, while mastic at lower levels had an anti-botulinal effect in media studies, it failed to prevent neurotoxin production even when used at higher levels in crumpets. Similar results have been observed with other antimicrobial oils. Clove and oregano oils, which were bactericidal against *L. monocytogenes* in media failed to inhibit this organism in meat when added at similar levels (Ting and Deibel, 1992; Aureli et al., 1992).

TABLE 8.3. *Detection of botulinum neurotoxin in crumpets containing mastic oil or resin added pre- or post-baking, inoculated with 500 spores/g* C. botulinum, *packaged with an oxygen absorbent and stored at 25°C, after seven-days storage*

Mastic addition	Mastic oil % (vol/wt)						Mastic resin % (wt/wt)			
Pre-baking	0	0.1	0.2	0.3	0.4	0.5	0	1	2	3
Neurotoxin[a]	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
Post-baking	0	0.1	0.2	0.3	0.4	0.5	NA	NA	NA	NA
Neurotoxin	2/2	2/2	2/2	2/2	2/2	2/2	NA	NA	NA	NA

[a] Number of samples testing positive for botulinum neurotoxin using the mouse bioassay out of the number of samples tested

NA: Not applicable, crumpets were not packaged post-baking with mastic resin

The reason for the lack of inhibition of *C. botulinum* by mastic 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 media (Blank et al., 1987). Although the lipid content of crumpets is expected to be low, the flour and skim milk powder would provide rich sources of protein. Cornell et al. (1971) reported that the activity of butylated hydroxyanisole was decreased when the antimicrobial bound reversibly to dry milk. It is possible that higher levels of mastic may be necessary for inhibition of *C. botulinum* in crumpets, although higher levels may lead to sensory rejection. Even though the sensory quality of crumpets was not quantitatively assessed in this study, it was obvious that crumpets packaged with mastic retained a firmer texture throughout storage than crumpets packaged without mastic. Therefore, mastic may have had an anti-staling effect on crumpets. In addition, the sensory quality of crumpets may have been enhanced by the antimicrobial activity of mastic against spoilage microorganisms, such as *Bacillus* spp., yeasts, moulds, and lactic acid bacteria. Inhibition of such spoilage microorganisms has been reported (Alippi et al., 1996; Iuak et al., 1996; Koukoutsis, 2002). At levels of ≤0.3% oil and 1% resin, crumpets had a pleasant, characteristic odour. However, packaging with higher levels of mastic imparted a very strong odour, which may be considered objectionable.

It is also possible that heating diminished the activity of mastic. The effect of heating on the activity of mastic cannot be determined from this study since neurotoxin was detected at the same time in crumpets regardless of whether the mastic was added pre- or post-baking.

Another approach to enhancing the anti-botulinal activity of mastic in crumpets would be the addition of a potentiating agent. Chelators have been shown to increase antimicrobial activity by increasing cell entry (Kabara, 1991). Since the anti-botulinal activity of ethanol and ethanol vapour has been established, ethanol may be such an agent (Daifas et al., 2000; 2003). Furthermore, since lower levels of ethanol may, at reduced pH and a_w, be effective in delaying growth of *C. botulinum* (Daifas et al., 2002) and since the anti-botulinal activity of mastic may be greater in the vapour state, it may be worthwhile to investigate the activity of mastic oil or vapour in combination with ethanol vapour to enhance crumpet safety.

8.4 CONCLUSION

Studies using media clearly showed that mastic had a direct and an indirect inhibitory effect on strains of proteolytic *C. botulinum* and therefore mastic resin and oil have potential as an anti-botulinal agent. Since mastic is used as a flavouring agent in bakery products, it would have the additional advantage of being a "natural" alternative to preservatives typically used with bakery products. However, since packaging crumpets with mastic, either pre- or post- baking, failed to prevent neurotoxin production by *C. botulinum* in this study, further studies are required to determine the reason for the lack of inhibitory effect in food and the levels of additional agents, such as ethanol, that may enhance its anti-botulinal activity in the food matrix.

9.0 GENERAL CONCLUSION AND SUMMARY

Modified-atmosphere packaging using vacuum packaging, gas packaging with a mixture of gases or oxygen absorbent technology has been available for at least thirty years to extend the shelf-life of various food products. Most recently, this technology has been used extensively, particularly in Europe, to extend the mould-free shelf-life of many high-moisture bakery products. Despite the safety concerns about the growth of *C. botulinum* that are justifiably associated with this technology, few botulism outbreaks have been attributed to MAP products worldwide since the inception of this technology.

The safety record of the bakery industry, which generates sales in excess of $50 billion dollars in North America alone, is very good. Minimally processed bakery products have been implicated in several outbreaks of foodborne disease; however, they have been mainly caused by *Salmonella* spp., *S. aureus*, *B. cereus* and NLVs. While there are regulatory concerns about the safety, with respect to *C. botulinum*, of high-moisture MAP bakery products, there have been no reports of botulism from such products. These regulatory concerns, and specific recommendations for additional microbial barriers, appear to be based on early challenge studies with bread products hermetically sealed in cans. However, there are no known challenge studies to date that have assessed the botulism hazard in high-moisture bakery products stored at ambient temperature; hence, the reason for undertaking this research.

Preliminary challenge studies with proteolytic *C. botulinum* (500 spores/g) demonstrated that selected high-moisture bakery products (crumpets and pizza crusts) could support the growth of *C. botulinum*, regardless of the packaging atmosphere. Furthermore, growth of *C. botulinum* to hazardous levels occurred in crumpets within seven days at ambient storage temperature prior to sensory rejection–a highly dangerous scenario. These studies confirm that such packaged high-moisture bakery products have the potential to support the growth of and neurotoxin production by *C. botulinum*; and therefore, these products may pose a safety risk if contaminated with this pathogen. Furthermore, they confirm regulatory concerns with respect to the safety of such products and justify the need to incorporate additional barriers into such packaged products to ensure their safety at ambient storage temperatures.

To achieve this objective, subsequent studies focused on the use of traditional barriers to the growth of *C. botulinum* including a_w and pH as well as novel barriers, specifically ethanol vapour and mastic oil. Early challenge studies with proteolytic *C. botulinum* (500 spores/g) demonstrated the importance of a_w as a barrier to its growth to ensure the safety of MAP high-moisture bakery products stored at ambient temperature. While crumpets (a_w 0.990) and pizza crusts (a_w 0.960) became toxic, bagels (a_w 0.944) did not. However, not all bakery products can be reformulated to such a low a_w due to undesirable sensory and textural changes in the final baked product. Another practical and cost effective approach that could be used to enhance the safety of bakery products would be pH modification. Preliminary challenge studies indicated that although

reformulation of preservative-free MAP crumpets using lactic acid to pH 4.6 inhibited growth of and neurotoxin production by *C. botulinum*, product taste was compromised. However, since some commercially produced high-moisture crumpets are formulated to alkaline pH to enhance browning, subsequent challenge studies were done using crumpets reformulated to pH 8.3. While reformulation of crumpets to this pH level delayed the growth of *C. botulinum*, it did not, even in the presence of elevated (100%) levels of CO_2, prevent toxigenesis. A further concern was that all high-moisture high-pH crumpets were organoleptically acceptable at the time of toxigenesis. Therefore, such high-pH bakery products, if contaminated with spores of *C. botulinum*, could also become hazardous when packaged in atmospheres containing CO_2.

Subsequent studies focused on novel barriers (ethanol vapour, oil-of-mastic) to enhance the safety of crumpets. While the anti-mycotic effect of ethanol and ethanol vapour is well established, there was a paucity of data on its anti-botulinal effect. Extensive challenge studies have shown that ethanol, in the vapour phase, has the potential to enhance the safety of English-style crumpets stored at ambient temperature. However, a limitation of its use is that a high level of ethanol (~3% wt/wt) is required in the package headspace to prevent the growth of and neurotoxin production by proteolytic *C. botulinum*. Modelling studies in broth confirmed that a level of at least 4% liquid ethanol was required, depending on the a_w and pH of the growth medium, for complete inhibition of *C. botulinum*. However, while such high levels of ethanol promoted

product safety, the sensory quality of crumpets was compromised due to absorption of ethanol from the package headspace.

To address these sensory concerns, challenge studies were conducted to determine the effect of the method of crumpet leavening (yeast or chemical) on the anti-botulinal efficacy of ethanol vapour and the potential of mastic extracts and oil as additional barriers to the growth of *C. botulinum*. While chemical-leavening decreased the level of ethanol in the package headspace, growth of *C. botulinum* was not prevented. Survival of *S. cerevisiae* in yeast-leavened crumpets was shown to contribute significantly to the anti-botulinal effect of ethanol. This study confirms that a level of ~3% (wt/wt) ethanol vapour would be required to ensure the safety of high-moisture MAP crumpets. Furthermore, it also demonstrates the importance of the method of leavening and the need to asses product safety on a case-by-case basis.

Studies were also done to determine the anti-botulinal effect of mastic oil and ethanolic extracts of resinous mastic as a potential additional barrier to be used in conjunction with lower levels of ethanol. While mastic showed promise as a novel anti-botulinal agent *in vitro*, it failed to control the growth of and neurotoxin production by *C. botulinum* in challenge studies with crumpets. Further research is required to determine the reason(s) for the lack of inhibitory activity of mastic oil in the food matrix.

While these challenge studies would appear to confirm regulatory concerns about the safety of high-moisture MAP bakery products, the results of these studies must be interpreted with caution. All of these studies were done

using high inoculum levels of *C. botulinum* (10^2 to 10^4 spores/g)—levels that have not to date been verified in bakery ingredients or in final baked products. Based on this limitation, it is difficult to extrapolate these "worst-case-scenario" challenge studies to all high-moisture MAP bakery products.

A basic assumption in assessing the botulism risk of high-moisture, MAP bakery products is that they may be contaminated with spores of proteolytic *C. botulinum.* Although numerous surveys of flour and other bakery ingredients have shown a consistent pattern of microbial contamination with thermophilic and rope-forming *Bacillus* spores, few, if any surveys have specifically examined ingredients or products for spores of *C. botulinum*. While high levels of endogenous spores are indicative of the quality and spoilage potential of these bakery ingredients, there is no scientific evidence to verify that high levels of these spores are reliable indicators of the incidence and levels of contamination of spores of proteolytic *C. botulinum* in flour or other bakery ingredients such as herbs and spices. In the absence of such information, it is impossible to estimate the botulism risk of high-moisture MAP bakery products. While such surveys may provide useful empirical data on the levels of contamination of *C. botulinum* spores in bakery ingredients, they would be costly and impractical and not provide any information about a product's ability to support the growth of this important pathogen. Furthermore, it cannot be assumed, based on a limited number of challenge and modelling studies, that all high-moisture MAP bakery products, if contaminated with spores or vegetative cells of proteolytic *C. botulinum,* would support their growth. The composition, a_w, pH, and ORP of

the product substrate in addition to its complex background micro-flora may influence the ability of a bakery product packaged under modified atmospheres to support the growth of *C. botulinum*. Based on the fact that no endogenous spores of *C. botulinum* have been detected in these and other challenge studies with bakery products, a lack of epidemiological evidence, and the excellent safety record of the bakery industry, high-moisture MAP bakery products would appear to be extremely low-risk products with respect to botulism. However, in view of the severity of the disease and regulatory concerns about the safety of MAP bakery products with extended shelf-life, on-going research is required at the industrial, governmental and academic levels on shelf-life and safety studies of these products. Further studies are also required to examine the potential of novel barriers, such as oil-of-mastic or other antimicrobial oils and bio-preservative producing strains of LAB, on the growth of this important patho-gen. Only when such sound empirical data is available will it be possible to accurately determine the botulism risk and safe shelf-life of high-moisture MAP bakery products and to formulate realistic guidelines concerning the levels of additional barriers, if any, required to ensure their continued safety at ambient storage temperature.

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APPENDIX A. COMPARISON OF TWO SELECTIVE MEDIA FOR ENUMERATION OF *C. BOTULINUM* IN CHALLENGE STUDIES

I. INTRODUCTION

The growth of *Clostridium botulinum* in food products is hazardous since ingestion of food in which this pathogen has grown and produced potent botulinum neurotoxin (BoNT) can result in fatal botulism. Recently, minimally processed bakery products inoculated with spores of proteolytic *C. botulinum* (types A and proteolytic B) and packaged in air or under modified atmospheres in high gas barrier bags, were shown to support growth and neurotoxin production within four to six days at ambient storage temperature (Daifas et al., 1999a, b).

In foods such as bakery products that have the potential to support growth of and neurotoxin production by *C. botulinum*, selective agars may be useful for isolating this pathogen from food samples. Selective and differential media commonly used for the isolation of *C. botulinum* include *Clostridium botulinum* isolation (CBI) agar (Dezfulian et al., 1981) and botulinum selective medium (BSM) (Mills et al., 1985). While both media contain identical antibiotics for the selection of *C. botulinum*, BSM also contains thymidine phosphorylase which reportedly results in an enhanced antibiotic effect and decreased "background" micro-flora with clinical samples (Mills et al., 1985). However, few studies have evaluated these media for either isolation of

C. botulinum from foods or for monitoring its growth by plate counts in challenge studies.

Therefore, the objectives of this study were to compare BSM and CBI agar for the recovery and enumeration of *C. botulinum* from crumpets challenged with proteolytic strains of *C. botulinum*.

II. MATERIALS AND METHODS

1. Preparation of media

Three media were used in this study, *Clostridium botulinum* isolation (CBI) agar, botulinum selective medium (BSM) and modified McClung Toabe (MMT) agar. CBI agar (Dezfulian et al., 1981) was prepared using McClung Toabe agar (Difco; Becton-Dickinson; Sparks, MD), 0.5% yeast extract (Difco), 5% egg yolk and 2.5% cycloserine, 0.4% sulfamethoxazole, and 0.4% trimethoprim. BSM (Mills et al., 1985) was prepared from Brain Heart Infusion (Difco), Bacto-agar (Difco), 1.5% egg-yolk (Austin and Blanchfield, 1996), 2.5% cycloserine, 0.4% sulfamethoxazole, 0.4% trimethoprim and 100 IU/L of thymidine phosphorylase (Sigma-Aldrich Co.;St. Louis, MO). MMT agar was prepared as previously described for CBI agar, but without the addition of antibiotics (Austin and Blanchfield, 1996).

2. Sample preparation and inoculation

Crumpets, a high-moisture bakery product (a_w 0.99, pH 6.5), were prepared, and then were inoculated with 90 μL of a composite inoculum (5.6×10^5 spores/mL) to provide a final inoculum level of 500 spores/g of

C. botulinum, and packaged in high gas barrier bags in air or under modified atmospheres as described previously by Daifas et al. (1999b). Crumpets were sampled after three- and five-days storage at ambient temperature. On sampling days, appropriate decimal dilutions of homogenates of inoculated and uninoculated crumpets were prepared using 0.1% peptone water. Aliquots (0.1-mL) of appropriate decimal dilutions were then spread plated, in duplicate, on BSM and CBI agar plates until dry. In addition, on day one, decimal dilutions of the spore suspension were similarly plated on BSM, MMT, and CBI agar. Following spreading, plates were incubated anaerobically in an atmosphere of 10% H_2, 10% CO_2, and 80% N_2 at 35°C and were checked for growth at 18, 24, 36, 48 and 72 hours. Lipase-positive colonies on plates were identified and enumerated presumptively, as *C. botulinum*. Representative presumptive colonies of *C. botulinum* were sub-cultured and grown anaerobically in cooked meat medium (Austin and Blanchfield, 1996). All supernatants were tested by the mouse bioassay for confirmation of botulinum neurotoxin.

3. Detection of neurotoxin

A modification of the AOAC mouse bioassay (Austin and Blanchfield, 1996) was used. Culture supernatants (0.5-mL) were injected intraperitoneally into each of two mice (20- to 28-g; Charles River, QC). Mice were

observed for up to 72 hours for typical signs of botulism including ruffled fur, pinched waist, laboured breathing, limb paresis and general paralysis. Mice showing severe distress were euthanised by asphyxiation with CO_2 according to Health Canada Animal Care Committee guidelines. Neutralization of BoNT was performed on randomly selected representative positive samples using antisera (Connaught Laboratories; North York, ON) to BoNTs to confirm that toxicity was due to BoNT as described previously by Austin et al. (1998).

III. RESULTS AND DISCUSSION

1. Recovery of *C. botulinum* from spore inoculum

When decimal dilutions of the initial inoculum (5.6 x 10^5 spores/mL) were plated on BSM, MMT or CBI agar, there was no significant difference ($P<0.05$) in the numbers of lipase-positive colonies recovered from each medium (Table A-1). Equivalent recovery of *C. botulinum* using these three media has been reported previously (Dezfulian et al., 1981; Mills et al., 1985; Glasby and Hatheway, 1985). Furthermore, colony morphology of *C. botulinum* was similar on all media. However, *C. botulinum* colonies were larger and more elevated and had a more prominent (i.e., larger) lipase zone on MMT and CBI agar compared to plates of BSM.

2. Recovery of *C. botulinum* from crumpets

Equivalent numbers of *C. botulinum* colonies were recovered from inoculated crumpets (500 spores/g) on both BSM and CBI agar (Table A-2). On BSM, colonies of *C. botulinum* were visible after 18 hours with lipase activity evident at 24 hours, while growth and lipase activity were only visible on CBI agar after 24 and 36 hours respectively. Again, *C. botulinum* colonies were larger and more elevated on CBI agar

TABLE A-1. *Recovery*[a] *of* Clostridium botulinum *(5.6 x 10^5/mL) from modified McClung Toabe (MMT) agar,* Clostridium botulinum *isolation (CBI) agar and botulinum selective medium (BSM)*

MMT agar (log CFU/mL)	CBI agar (log CFU/mL)	BSM (log CFU/mL)
3.86	3.89	3.90

[a] Average of 12 replicate plates

Standard deviation (%): MMT 31%; CBI 25%; BSM 29%

TABLE A-2. *Comparison of* Clostridium botulinum *isolation (CBI) agar and botulinum selective medium (BSM) for enumeration of* C. botulinum *from crumpets inoculated with 500 spores/g* C. botulinum *types A and proteolytic B*

Storage Atmosphere[1]	Incubation time (days)	CBI (log CFU/g)	BSM (log CFU/g)
A	3	2.5	2.7
A	3	2.8	2.7
B	3	2.9	2.8
B	3	2.9	2.8
C	3	2.8	3.0
C	3	4.5	3.0
A	5	6.2	6.3
A	5	6.4	6.3
B	5	6.3	6.3
B	5	5.4	5.4
C	5	6.3	6.3
C	5	6.5	6.4

[a] Atmospheres: A, air; B, oxygen absorbent; C, 60:40 CO_2:N_2 (all crumpets stored in high barrier bags at 25°C)

compared to plates of BSM. Isolated lipase-positive colonies from BSM and CBI agar were grown on MMT agar, transferred to cooked meat medium (CMM) and confirmed as *C. botulinum* using the mouse bioassay as described by Austin and Blanchfield (1996).

No endogenous *C. botulinum* were recovered from uninoculated crumpets. Occasionally, lipase-positive colonies, uncharacteristic of the strains of *C. botulinum* used in this study, were isolated from both inoculated and uninoculated crumpets plated on BSM or CBI agar. BSM has been shown to suppress background microflora in clinical samples due to the incorporation of thymidine phosphorylase which enhances the antimicrobial action of trimethoprim-sulfamethoxazole (Mills et al., 1985). However, no difference in the suppression of background crumpet microflora was observed in this study (results not shown). Furthermore, these uncharacteristic, smaller lipase-positive colonies had "weaker" lipase activity (i.e., smaller and less opalescent lipase zones). In addition to being lipase-positive and non-toxic, all isolates were gram-positive, lecithinase-negative, spore-forming, motile rods. All isolates were strict anaerobes, growing preferentially at 37°C compared to 25°C. Dezfulian et al. (1981) reported that five out of 35 other species (i.e., not *C. botulinum*) of clostridia tested grew on CBI agar. Of these clostridia, only *C. sporogenes*, which phenotypically resembles *C. botulinum*, is lipase-positive. Although it is likely that the observed lipase-positive colonies were *C. sporogenes*, the isolates were distinguishable on the

basis of morphology and lipase activity patterns (as previously described) from the strains of *C. botulinum* used in this study. However, since *C. sporogenes* and *C. botulinum* are generally indistinguishble from each other on selective media, culture supernatants of isolates grown in CMM were tested for neurotoxin using the mouse bioassay. All supernatants of the cultures of the uncharacteristic lipase-positive isolates were negative for neurotoxin. *C. sporogenes* has been associated with flour and milk (ingredients used in preparation of crumpets) and, in addition, has been isolated from crumpets and canned quick-breads (Smith, 1982; Aramouni et al., 1994). However, the non-toxic lipase-positive colonies isolated from the crumpets in this study were <1% of the total lipase-positive colonies enumerated.

In summary, selective and differential media (CBI agar and BSM) enabled isolation of presumptive *C. botulinum* from inoculated crumpets. While these media were developed for the isolation of C. *botulinum* from clinical samples, there are few reports of their use with food. Cantoni et al. (1998) reported that CBI agar resulted in better recovery of *C. botulinum* type A from cheese than either royal clostridial agar (RCA), or sulfite cycloserine (TSC) agar. However, in the present study, *Clostridium botulinum* isolation (CBI) agar and BSM were equivalent in their recovery of *C. botulinum* and exhibited similar background microflora from inoculated crumpets. Furthermore, although growth of *C. botulinum*

was visible earlier with BSM, enumeration was easier with CBI agar since colonies of *C. botulinum* were larger and more elevated with CBI agar.

IV. CONCLUSION

Based on these results, CBI would be the preferred medium of choice for the isolation of presumptive colonies of *C. botulinum* in challenge studies with high-moisture bakery products.

Clostridium botulinum isolation (CBI) agar has been used in challenge studies to presumptively enumerate *C. botulinum*. Lyver et al. (1998) used this medium to enumerate non-proteolytic *C. botulinum* in inoculated value added surimi shrimp nuggets. Daifas et al. (1999a, b) also sucessfully used this medium to monitor growth of proteolytic strains of *C. botulinum* in challenge studies with high-moisture bakery products. However, it cannot be assumed, even in challenge studies, that all lipase-positive, lecithinase-negative colonies are proteolytic *C. botulinum*. Confirmation of *C. botulinum* can be achieved by using an assay method capable of detecting botulinum neurotoxin. While this is usually achieved using the mouse bioassay, rapid immuno-chemical methods such as the ELISA-ELCA® are being developed. However, prior to being used as an alternative to the mouse bioassay, such methods must be compared to the standard bioassay.

APPENDIX B. COMPARISON OF TWO ASSAY METHODS FOR DETECTION OF BOTULINUM NEUROTOXIN IN CHALLENGE STUDIES WITH BAKERY PRODUCTS

I. INTRODUCTION

Growth of and neurotoxin production by *C. botulinum* in foods packaged under modified atmospheres is of major food safety concern. The botulinum neurotoxins (BoNTs) are zinc endopeptidases and clostridia producing BoNTs can be divided on the basis of the antigenic specificity of their neurotoxins, into seven types A through G and four sub-types, A-b, B-a, A-f, and B-f. Alternatively, *C. botulinum* can be classified on the basis of physiologic and nucleic acid relatedness into four distinct groups–I through IV (Austin, 2001). Foodborne botulism is associated with proteolytic strains of Group I and non-proteolytic strains of Group II.

Growth of Group I strains of *C. botulinum* producing BoNT/A or B are of concern in bakery products since, if spores are present in the raw ingredients, they will readily survive baking (Daifas et al., 1999a, b). In bakery products such as crumpets that have the potential to support growth and neurotoxin production by *C. botulinum*, detection of the neurotoxin is necessary in order to ensure product safety. Selective media may be useful for isolating *C. botulinum* from foods or for monitoring its growth in challenge studies. However, since the neurotoxin produced by this pathogen is potent,

it is generally necessary to detect the neurotoxin itself. The standard method for detecting BoNTs is the AOAC-approved mouse bioassay (AOAC, 1995) which has a sensitivity of approximately 10 pg/mL neurotoxin. However, this method has several disadvantages. It is time consuming, taking from 3 to 6 days, and requires many mice. Furthermore, the method is not entirely specific for BoNT and positive samples must be confirmed by neutralization with antisera to BoNTs. There is also increasing public pressure about the use of *in vivo* tests, particularly those requiring the sacrificing of animals.

One alternative approach to the mouse bioassay for the detection of BoNT is the ELISA-ELCA® (Elcatech, Inc.; Salem, NC). This method couples an enzyme-linked immunosorbant assay, which relies on the antigenic specificity of Groups I and II neurotoxins, to an enzyme-linked coagulation based amplification step. This amplified ELISA has reportedly achieved equivalent sensitivity to the mouse bioassay for BoNT/E in challenge studies with *C. botulinum* type E with fish (Roman et al., 1994; Reddy et al., 1997). However, the use of the ELISA-ELCA® for the detection of BoNT/A and B in food has not been reported.

Therefore, the objectives of this study were to evaluate the ELISA-ELCA® as an alternative method to the mouse bioassay for the detection of BoNT/A and B in crumpets inoculated with spores of proteolytic *C. botulinum*.

II. MATERIALS AND METHODS

1. Sample preparation

Fifty-eight filter sterilized ([0.45-μm]; Acrodisc, Gelman Sciences; Ann Arbor, MI) extracts were used in this study. Forty-two extracts were from crumpets that had been inoculated with 500 spores/g of a composite inoculum of *C. botulinum* (A6, 17A, 62A, CK2A, MRB, 1B1-B, and 13983IIB), while the remaining 16 crumpet extracts were from uninoculated control crumpets. All extracts were stored at 4°C for a maximum of ten days prior to being tested as a group. Extracts were analysed using i) the mouse bioassay, and ii) the ELISA-ELCA® amplified immunosorbant assay using both antibodies specific for BoNT/A and B.

2. Detection of neurotoxin using the ELISA-ELCA®

BoNT was assayed for using an enzyme-linked immunosorbant assay coupled to an enzyme-linked coagulation assay (ELISA-ELCA®) according to the manufacturer's instructions (Elcatech, Inc.; Winston-Salem, NC). The assay methodology and reactions, which have been described in detail (Doellgast, 1987; Doellgast et al., 1993, 1994), are based on the solid- phase capture of the BoNT and the subsequent amplification of its detection using enzymes of the blood clotting cascade. At the end of the

amplification step, botulinal neurotoxin was detected as absorbance at 550 nm ($A_{550\text{-}nm}$) with simultaneous subtraction of $A_{450\text{-}nm}$ using a microplate reader (model ELx800 running KC-Jr. software, Bio-tek; Summit, NJ). Results were positive when $A_{550\text{-}450\text{-}nm}$ was twice the $A_{550\text{-}450\text{-}nm}$ of the background.

Positive controls of pure BoNT (Elcatech) in gelatine phosphate buffer and negative controls of gelatine phosphate buffer only, in triplicate, were included with every plate.

a. Optimization of assay

The assay was optimized for both sensitivity and reproducibility. Sensitivity was optimized by varying the amplification (enzyme-linked coagulation assay [ELCA]) and detection incubation times as per manufacturer's recommendation. As shown in Table B-1, the first ELCA step (thrombin formation) was varied from 25 to 45 minutes, in increments of 5 minutes, while the second ELCA step (fibrin formation) was arrested after either 30 or 60 minutes. Following fibrin formation, the $A_{550\text{-}450\text{-}nm}$ was read at 10-minute increments (up to 60 minutes) to determine the optimum timing for the detection step (hydrolysis of phenolphthalein monophosphate). The precision of the method was estimated by assaying plates of triplicate dilution series of BoNT/A and B ranging from 2,000 to 0 pg/mL prepared as

described above. Results were averaged and the standard deviation and coefficient of variation calculated.

3. Detection of neurotoxin using mouse bioassay

A modification of the AOAC mouse bioassay (Austin and Blanchfield, 1996) was used. Filter-sterilized crumpet extracts (0.5-mL) were injected intraperitoneally into each of two mice (20- to 28-g; Charles River, QC). Mice were observed for up to 72 hours for typical signs of botulism including ruffled fur, pinched waist, laboured breathing, limb paresis and general paralysis. Mice showing severe distress were euthanised by asphyxiation with CO_2 according to Health Canada Animal Care Committee guidelines. Neutralization of BoNT was performed on randomly selected representative positive samples using antisera (Connaught Laboratories; North York, ON) to BoNTs to confirm that toxicity was due to BoNT as described previously by Austin et al. (1998).

4. Statistical analyses

The presumptive results of the ELISA-ELCA® were compared to the modified mouse bioassay method using the NcNemar statistic $\chi^2 = \frac{(|a-b|-1)^2}{a+b}$ (Steel and Torrie, 1980) where a and b are the number of positive and negative results of the two assays as indicated:

		Mouse bioassay	
		+	-
ELISA-ELCA®	+	c	b
	-	a	d

The methods were significantly different when $\chi^2 > 3.84$ ($P < 0.05$). Agreement between methods was calculated as $(\frac{c+d}{a+b+c+d})$ (McClure, 1990) where a, b, c, d are the number of positive and negative results of both methods as previously indicated. Specificity was calculated as the proportion of extracts not containing BoNT which tested negative for neurotoxin. All uninoculated crumpet extracts were assigned a negative sample status since decimal dilutions of homogenized crumpets plated on *Clostridium botulinum* isolation (CBI) agar (Dezfulian et al., 1981) had not previously resulted in the recovery of any lipase-positive colonies characteristic of *C. botulinum* (Daifas et al., 1999b). The false-positive rate was determined as [1 - specificity] (McClure, 1990).

III. RESULTS AND DISCUSSION

1. Effect of ELCA incubation time on sensitivity

The optimized amplification (ELCA) incubation times were determined to be 40 minutes for the first ELCA step (thrombin formation), 60 minutes for the second ELCA step (fibrin formation) and 20 minutes for hydrolysis of phenolphthalein monophosphate ([40 + 60 + 20] [Table B-1]). Varying incubation times by as little as five minutes influenced the sensitivity of the assay. It was critical to adhere to the optimized times and protocol temperature since this assay depends on immunochemical and enzymatic reactions characterized by affinity and avidity. Using these optimized conditions, standard curves of purified neurotoxins A and B were prepared (Figure B-1). The resulting sensitivity, under optimum conditions, was < 12.3 pg/mL for BoNT/A and 12.3 pg/mL for BoNT/B (Table B-1). However, Doellgast et al. (1993), using the ELISA-ELCA®, reported a greater sensitivity–detecting 5 to 10 pg/mL of neurotoxin. Nevertheless, the sensitivity observed in this study was similar to that of the mouse bioassay which can detect approximately 10 pg/mL or <5 MLD_{50}/mL of purified BoNT (Kautter et al., 1984; Fernández and Ciccarelli, 1999).

Between and within plate reproducibility was determined by following the recommended timed steps supplied with the ELISA-ELCA® kit. Coefficients of variation ranged from 10% to 26%, with the highest

TABLE B-1. *Effect of varying each enzyme-linked coagulation assay (ELCA) step on the limit of detection for botulinum neurotoxin types A and B*

Time (minutes) of ELCA incubation steps *			Limit of detection (pg/mL) BoNT type	
I	II	III	A	B
25	30	30	37	111
25	30	60	111	333
35	60	10	>37	20.8
40	60	10	20.8	31.3
45	60	10	>37	>37
35	60	20	13.9	18.5
40	60	20	<12.3	12.3
45	60	20	>37	>37
35	60	30	<12.3	37
40	60	30	>37	>37
45	60	30	>37	>37

*ELCA incubation steps: I, thrombin formation; II, fibrin formation; and III, hydrolysis of monophosphase-phenolphthalein

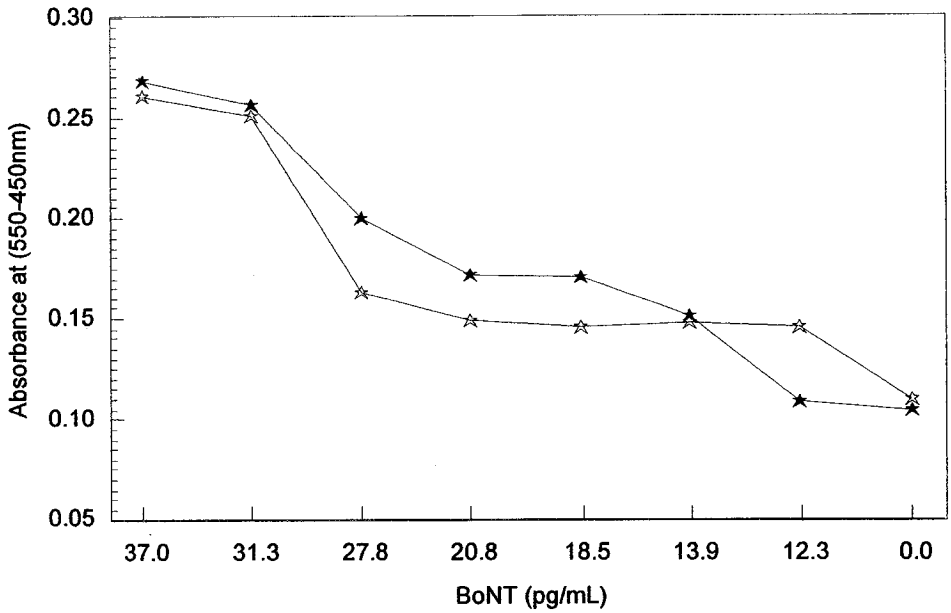

FIGURE B-1. *Standard curve using enzyme-linked immunosorbant assay-enzyme linked coagulation assay (ELISA-ELCA®) specific for type A (★) and type B (✡) botulinum neurotoxin*

values occurring near the limits of detection (results not shown). Similar results were observed for between plate reproducibility (results not shown).

2. Inoculated samples

No significant (α = 0.05) difference was observed between the mouse bioassay and the amplified immunosorbant assay (using anti-BoNT/A or B antibody) for the detection of botulinum neurotoxin in inoculated crumpets. Agreement between both methods was 95% when antibody specific for BoNT/A was used, and was 88% when antibody specific for BoNT/B was used (Table B- 2). The number of extracts that tested positive by either method generally increased with storage time of crumpets (Figure B-2). In the original challenge studies, counts of presumptive *C. botulinum* (CFU/g), using CBI agar, increased over seven days (Daifas et al., 1999b). It is probable that the amount of BoNT in the extracts increased as the counts increased and therefore the number of extracts testing positive also increased. On each day, the number of extracts testing positive by the immunoassay was as great as, or greater than, by the mouse bioassay.

For both serotypes, BoNT was detected with the ELISA-ELCA® as early as, or earlier than, with the mouse bioassay. Neurotoxin was first detected at day 3 by the immunoassay specific for BoNT/A and at day 4

TABLE B-2. *Comparison of mouse bioassay and ELISA-ELCA®* methods for detection of botulinum neurotoxin in crumpets inoculated with 500 spores/g* C. botulinum *types A and proteolytic B and stored at 25°C*

ELISA-ELCA result	Mouse bioassay result		Methods equivalent (α=0.05)	Agreement	ELISA-ELCA result	Mouse bioassay		Methods equivalent (α=0.05)	Agreement
	Inoculated crumpets					Uninoculated crumpets			
BoNT/A	+	-			BoNT/A	+	-		
+	20	2	yes	0.95	+	0	0	yes	1
-	0	20			-	0	16		
BoNT/B	+	-			BoNT/B	+	-		
+	17	2	yes	0.88	+	0	12	no	0.25
-	3	20			-	0	4		

* Enzyme-linked immunosorbant assay-enzyme-linked coagulation assay (Elcatec)

** Agreement is proportion of results which agree

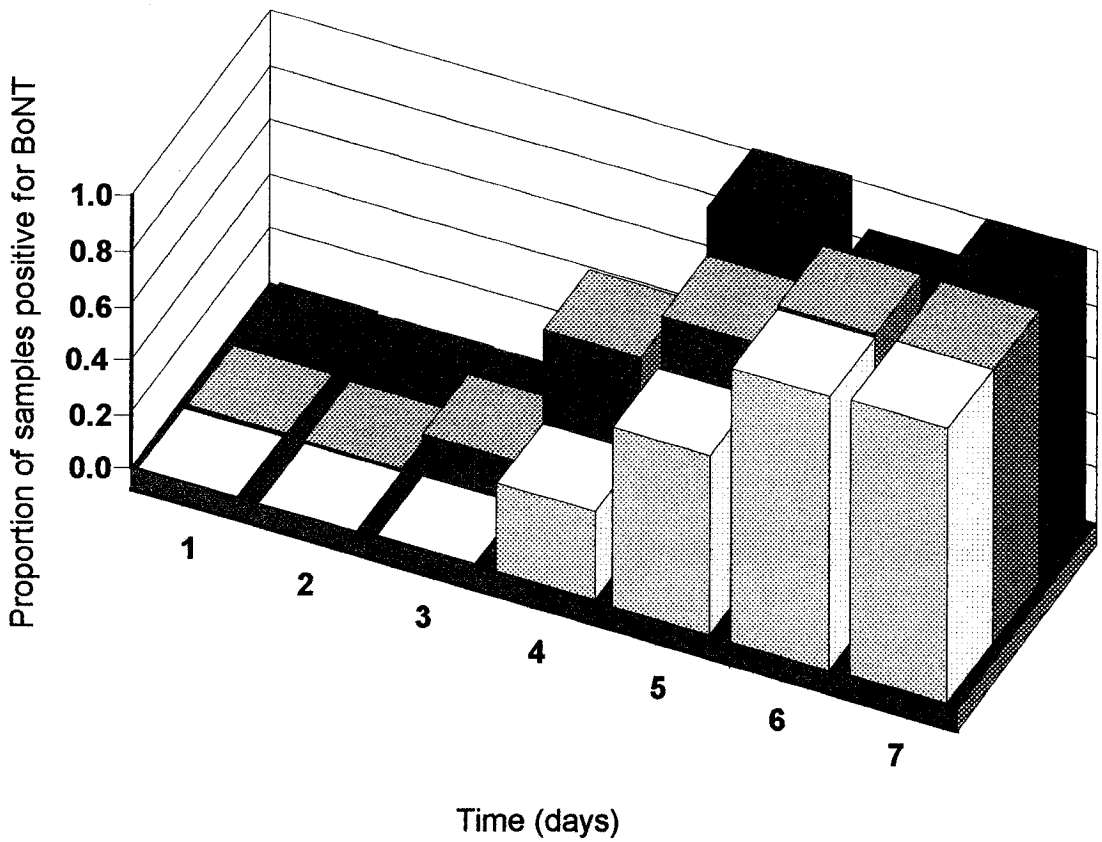

FIGURE B-2. *Detection of BoNT by the mouse bioassay, (▨) the immunoassay specific for BoNT/A (▩), or BoNT/B (■)*

by both the immunoassay specific for BoNT/B and the mouse bioassay (Figure B-2). This may be attributed to greater sensitivity of the immunosorbant assay for BoNT/A or may be attributed to a decreased specificity of the immunosorbant assay, i.e., a false-positive result. Since extracts from crumpets that had been inoculated with *C. botulinum* were used, the amount of BoNT in the extracts was not known.

One advantage of ELISA assays is that since neurotoxin type specific antibodies are used, the neurotoxin serotype can be easily determined. This offers an advantage over the bioassay when "cocktail spore inoculums" of multiple serotype strains are used in challenge testing. Neurotoxin typing, using the mouse bioassay, requires as many as 30 mice and additional time (up to three more days) (AOAC, 1995). In this study, BoNT/A and B were detected, indicating that strains of both proteolytic types of *C. botulinum* had grown and produced neurotoxin in inoculated crumpets.

However, one disadvantage of ELISA techniques is that strain specific variation in reactivity has been reported. Neurotoxins produced by different strains of *C. botulinum* within the same neurotoxin serotype can have different primary structures (Cordoba et al., 1995) and therefore can have anti-genic variation. ELISAs which use a single monoclonal antibody as a solid-phase capture system may be prone to false-negative results due to antigentic variation within serotypes (Hallis et al., 1996). Although Notermans et al. (1984) observed cross-reactivity between proteolytic and non-proteolytic strains of

C. botulinum, Doellgast et al. (1993) reported a non-proteolytic B strain having weak reactivity with the ELISA-ELCA®.

3. Uninoculated samples

When the mouse bioassay was compared to the amplified ELISA using antibody specific for BoNT/A, there was no significant difference (α = 0.05) between methods. Indeed, the methods agreed 100% of the time, with neither method detecting botulinum neurotoxin in uninoculated samples (Table B -3).

However, when the bioassay was compared to the immunoassay using anti-BoNT/B antibody, agreement between methods was only 25%, and thus, in this instance, the methods were significantly different (α = 0.05) (Table B-2). The false positive rate of the amplified ELISA using antibody for BoNT/B was 0.75. It could be assumed that uninoculated control crumpets contained no endogenous spores since no lipase-positive colonies, characteristic of *C. botulinum*, were recovered from these crumpets (Daifas et al., 1999b). Furthermore, in all studies to date, no uninoculated control crumpets that were stored under conditions conducive to growth have ever tested positive for botulinum neurotoxin using the mouse bioassay (Daifas et al., 1999a, b).

All positive controls of pure BoNT tested positive while all negative controls of gelatine phosphate buffer tested negative. Since all samples (from uninoculated crumpets) that tested positive by the ELISA-ELCA® were negative by the mouse bioassay, an interfering substance capable of cross-reacting with

BoNT/B antibody may have caused the observed false-positive results. The casein blocking agent and washing protocol supplied with the test kit are designed to eliminate all non-specific proteases. However, extracts were from uninoculated crumpets that had been stored for six to seven days at ambient temperature and were close to the end of their shelf-life. Microbiological analysis of these crumpets showed high counts of aerobic organisms, lactic acid bacteria, yeasts and *Bacillus* spp. (results not shown). It is possible that other neurotoxins or proteins originating either from these spoilage microorganisms, or from chemical reactions, may have reacted non-specifically, thereby resulting in false-positive results. Widely found in plants including wheat (*Triticum vulgaris*), and in some bacteria (Pusztai and Bardocz, 1996), lectins are heat stable and bacterial resistant proteins that bind specifically with sugar residues of polysaccharides, glycoproteins, or glycolipids. Notermans and Nagel (1989) hypothesised that proteins such as lectins, could cross-link with anti-BoNT antibodies. Since lectins are structurally similar to BoNT (Lacy et al., 1998), and are competitive antagonists of BoNT (Bakry et al., 1991) they could presumably cross-link the carbohydrate residues on the antiBoNT and BoNT antibody. The false positive rate (0.75) calculated in this study was for BoNT/B extracts from uninoculated crumpets only. It was not possible to calculate a false positive rate for inoculated crumpets since they were inoculated with *C. botulinum*, and thus, absence of growth and neurotoxin production could not be predicted. However, although the false positive rate could not be calculated for inoculated crumpets, there was no evidence of inconsistent or unusual

positive results. Inoculated crumpets were sampled over time. At days 1 and 2, all extracts were negative for both methods. On day 3, only one of six extracts was positive using the immunosorbant assay specific for BoNT/A while all extracts were negative using either the mouse bioassay or the amplified ELISA specific for BoNT/B (Figure B-2). These results suggest that prior to day 4, *C. botulinum* had not produced sufficient neurotoxin for detection. Nevertheless, these results are incongruent with the false-positive results observed for uninoculated crumpets. It is possible, however, that the activity or avidity of an interfering substance could have increased as the indirect result of different microbiological activity in the control and inoculated crumpets.

IV. CONCLUSION

In conclusion, this study has shown that the ELISA-ELCA®, using antibody specific for either type A or B botulinum neurotoxin, was statistically equivalent to the mouse bioassay. However, the false-positive rate (0.75) observed using the ELISA-ELCA® specific for BoNT/B makes the immunoassay, at this time, unacceptable for challenge testing or screening of bakery products for *C. botulinum*, especially with proteolytic *C. botulinum* type B. Further studies are required to determine the factors responsible for the false-positive results observed in this study from uninoculated control crumpets in order to optimize the reproducibility and accuracy of the ELISA-ELCA®.

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I James P Smith give my permission to Daphne Phillips (Daifas) and to McGill University to reproduce the following published papers, of which I am a co-author as part of the thesis entitled "Safety studies with proteolytic *Clostridium botulinum* in high-moisture bakery products packaged under modified atmospheres" submitted to the Faculty of Graduate Studies and Research of McGill University in partial fulfilment of the requirements for the degree of Doctorate of Philosophy.

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6. Daifas, D. P., **J. P. Smith**, I. Tarte, B. Blanchfield, and J. W. Austin. 2000. Effect of ethanol vapor on growth and toxin production by *Clostridium botulinum* in a high moisture bakery product. J. Food Safety. 20:113-127.
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