NOVEL METHODS TO CONTROL THE GROWTH OF *BACILLUS CEREUS* IN ENGLISH-STYLE CRUMPETS

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Abstract

NOVEL METHODS TO CONTROL THE GROWTH OF *BACILLUS CEREUS* IN ENGLISH-STYLE CRUMPETS

Bacillus cereus has been responsible for several food poisoning outbreaks involving high moisture-high pH (a_w ~0.98, pH ~8.8) English-style crumpets. This spore-forming microorganism, which is commonly present in flour, dried milk powder, cocoa, and spices, can easily survive the baking process and grow to dangerous levels within 3-5 days at ambient storage temperature. Chemical preservatives e.g., propionic and sorbic acid, are commonly used to extend the shelf life and keeping quality of bakery products. However, these preservatives have been ineffective in high pH crumpets due to the complete dissociation of these acids at high pH levels. Therefore, two novel methods, involving sorbohydroxamic acid (SHA) and mastic essential oil (MO), were evaluated for their potential to inhibit the growth of this pathogen and other selected spoilage and foodborne pathogens in high moisture, high pH English-style crumpets.

Initial studies were done to evaluate the effect of pH (5 to 9), levels of sorbic acid and SHA (0 to 0.3% w/w), and MO (0 to 1% w/w), alone and in combination with each other, to control the growth of *B. cereus* in agar plate studies. While sorbic acid only controlled the growth of *B. cereus* at pH 5 and 5.5, SHA proved effective at all pHs and concentrations under investigation. MO also failed to inhibit the growth of *B. cereus* when added directly to agar plates. However, subsequent studies showed that MO was effective at a concentration of ~1% (w/w) in ethanol indicating its potential as a vapour phase inhibitor. However, the type of packaging material influenced the efficacy of this vapour phase inhibitor.

Based on these results, subsequent challenge studies focused on the control of *B. cereus* in high pH (~8.8) crumpets stored at ambient temperature. Products

were unacceptable when counts increased from 10^3 CFU/g to 10^6 CFU/g or sensory scores reached < 3 on a scale of 5. Only SHA (0.3% w/w) proved effective in high pH crumpets and all products had acceptable sensory scores, even after 14 days at ambient temperature.

These preliminary studies showed that SHA has the potential to control the growth of *B. cereus* in high pH crumpets without compromising product quality.

RÉSUMÉ

DE NOUVELLES MÉTHODES POUR LE CONTRÔLE DU *BACILLUS CEREUS* DANS LES CRUMPETS ANGLAIS

Le *Bacille cereus* a été inpliqué dans plusieurs manifestations d'intoxication alimentaire impliquant des crumpets anglais élevés en humidité et pH (aw ~0.98, pH ~8.8). Ce micro-organisme générateur de spores, qui est généralement présent dans la farine, le poudre de lait, le cacao, et les épices, peut facilement survivre le procédé de cuisson et atteindre des niveaux dangereux dans 3-5 jours à la température ambiante. Les préservatifs chimiques, l'acide propionique et sorbique, sont généralement employés pour étendre la durée de conservation et la qualité des produits de boulangerie. Cependant, ces préservatifs sont inefficaces dans les crumpets élevés en pH dus à la dissociation complète de ces acides aux niveaux élevés en pH. Alors, deux nouvelles méthodes, incluant l'acide sorbohydroxamique (SHA) et l'huile essentielle de mastic (MO), ont été évalué pour leur potentiel d'empêcher la croissance de cette bactérie et d'autres micro-organismes responsables pour la détérioration des aliments de haut humidité et haut pH comme les crumpets anglais.

Les études initiales ont été faites pour évaluer l'effet de pH (5 à 9), le niveau d'acide sorbique et SHA (0 à 0,3% w/w), et MO (0 à 1% w/w), seul et en combinaison, pour contrôler la croissance de *B. cereus* dans des études de agar dans des boites de Petri. Tandis que l'acide sorbique a contrôlé seulement la croissance du *B. cereus* au pH 5 et 5.5, SHA a prouvé efficace à tout les pHs et concentrations étudiées. Le MO également n'a pas empêché la croissance du *B. cereus* une fois ajouté directement aux boites d'agar. Cependant, les études ultérieures ont prouvé que le MO était efficace à une concentration de ~1% (w/w) en éthanol indiquant son potentiel comme inhibiteur en phase de vapeur.

Cependant, le type de matériel d'emballage a influencé l'efficacité de ce inhibiteur en phase de vapeur.

Basé sur ces résultats, des études ultérieures concentrées sur le contrôle du *B. cereus* dans les crumpets élevés en pH (~8.8) entreposé en température ambiante. Les produits étaient inacceptables quand les niveaux ont augmenté de 10^3 CFU/g à 10^6 CFU/g ou lorsque les scores sensoriels avaient atteint < 3 sur une échelle de 5. Seulement SHA (0,3% w/w) a démontré des effets pertinents dans les crumpets élevés de pH et tous produits avaient des scores sensoriels acceptables, même après 14 jours à température ambiante.

Ces études préliminaires ont prouvés que SHA offre des possibilités intéressantes de contrôler la croissance *B. cereus* dans les crumpets élevés en pH sans compromettre la qualité du produit.

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Chapter 1

Introduction and Literature Review

1.1. Introduction

Bread and other cereal based products have been part of a balanced diet since ancient times. Indeed, some of the earliest records on baking can be found in Egyptian tombs. Many of these tombs depict scenes of bread making and the remains of bread have been found buried with mummified corpses. Commercial bakeries were operating in 500 B.C., first in Egypt and other Mediterranean regions with leavened bread being reported in Egypt in 800 B.C. The production of bread and other bakery products has evolved from a primitive, cottage industry into a large-scale, modern, manufacturing industry generating billions of dollars in revenue and employing thousands of personnel. In 1998, sales of bakery products in the United States exceeded 10 million metric tons and had a market value of $27 billion dollars, 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 accounted for 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. The increased demand is being met by various new processing and packaging technologies, including modified atmosphere packaging, a technology which has increased the availability and extended both the shelf life and market area of a wide variety of bakery products. At the same time, there has been an increase in in-store bakeries and a renewed interest in "organic" (i.e., whole, natural grain and preservative-free), ethnic and artisan type bakery products.

Today, a wide variety of bakery products can be found on supermarket shelves. These include unsweetened goods, sweet goods and filled goods. Examples of products within each category are shown in Table 1. Most bakery products are marketed fresh and are stored at ambient temperature. However, other products, such as cream, fruit and meat filled pies and cakes, are stored under refrigerated or frozen storage conditions to achieve a longer shelf life and enhance the safety. Bakery products, like most foods, are subject to physical, chemical and microbiological spoilage. While physical and chemical spoilage problems limit the shelf life of low and intermediate moisture bakery products, microbiological spoilage is the main problem in high moisture products. Furthermore, high moisture bakery products have also been implicated in outbreaks of foodborne illness and therefore pose safety concerns.

Table 1. Categories of bakery products found on supermarket shelves

Categories of bakery products	Types within each category
Unsweetened goods	Bread: sliced, crusty, part-baked, ethnic
	Rolls: soft, crusty
	Crumpets
	English muffins
	Croissants
	Pizza base
	Raw pastry
Sweet goods	Large cakes: plain, fruited
	Pancakes
	Doughnuts
	Waffles
	Cookies
	Biscuits
	American muffins
	Buns
	Wafers
Filled goods	Tarts: fruit, jam
	Pies: meat, fruit
	Sausage rolls
	Pasties
	Cakes: cream, custard
	Pizza
	Quiche

Adapted from Blakistone (1998)

1.2. Safety concerns of bakery products

Each year, thousands of North American consumers suffer from some form of foodborne illness with symptoms ranging from mild to fatal. Foodborne illness has been defined as "any illness associated with, or in which the causative agent is obtained by, the ingestion of food" (Oblinger, 1988). Mead *et al.* (1999) estimated that in the United States there are approximately 76 million foodborne illnesses, 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 illnesses (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 illnesses (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 pose safety concerns in Australia (Jenson *et al.*, 1994).

The rest of the world is not immune to foodborne illnesses caused by bakery products. Todd (1996) reported that 35-47% of all foodborne illnesses 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). There are several reasons why bakery products are involved in foodborne illnesses. These are:

1.2.1. Minimal processing

In order to achieve desirable textural and quality attributes, most bakery products only receive a minimal heat treatment. For example, bread is baked at high temperature; however, during baking, the temperature in the center of the crumb rarely exceeds 100°C for a few minutes. Furthermore, some baked products include cream, cold custard, icing, spices, nuts, or fruit toppings or

fillings, which may be prepared without any heating. According to Bryan *et al.* (1996), vegetative pathogenic microorganisms should be readily destroyed during baking due to their low thermal resistance (D values) as shown in Table 2. However, spores of spore-forming bacteria will readily survive baking due to their high D values (Table 2) and may grow to levels of public health concern if packaging and storage conditions are conducive to their growth (Bryan *et al.*, 1997). While vegetative pathogens, such as *Salmonella* species and *Staphylococcus aureus*, as well as molds and viruses, should be destroyed during baking, products may be subject to post baking contamination from the air, equipment and food 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. Hazardous products/ingredients

According to Health Canada (Personal Communication), potentially hazardous foods have a pH of >4.5 and an a_w of >0.84. Many bakery products and their ingredients fall within this hazardous category. While many bakery products/ingredients have pH and a_w levels which restrict microbial growth, others have levels conducive to the growth of microorganisms present in bakery ingredients. For example, the pH of custard, used in many filled baked products, is 5.8-6.6 and is ideal for the growth of *Salmonella* species (Bryan, 1976). It is also important to note that both pH and a_w may change during storage. Icing, which has a low a_w, does not usually support microbial growth. However, the interface between the cake and icing may have a much higher a_w which enhances growth. Silliker and McHugh (1967) reported such an incident in which *S. aureus* grew at the interface of cake and icing.

Table 2. Heat resistance of specific bacteria

Bacteria	Heat Resistance in Minutes		
	$D_{70°C}$	$D_{90°C}$	$D_{121°C}$
Vegetative	0.0001	-	-
Campylobacter jejuni			
Escherichia coli	0.001	-	-
Listeria monocytogenes	0.3	-	-
Salmonella spp	0.001	-	-
Staphylococcus aureus [a]	0.1	-	(>1)
Vibrio parahaemolyticus	0.001	-	-
Yersinia enterocolitica	0.01	-	-
Spore Forming [b]			
Bacillus cereus	-	10	-
Clostridium botulinum (Group 1)	-	-	0.2
Clostridium botulinum (Group 2)	-	1.5	-
Clostridium perfringens	-	-	0.15

(Adapted from Smith *et al.*, 2002)

[a] Value in parentheses refers to the toxin

[b] Values refer to spores

1.2.3. 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 storage conditions may enhance microbial growth and may compromise safety. Furthermore, since most products are "cook and hold" and are not heated prior to consumption, there is no safety margin for destroying bacteria which may survive the baking process or may have been introduced during handling or storage. English-style crumpets, a high moisture snack food product stored at ambient temperature, have been implicated in several food poisoning outbreaks involving *B. cereus* (Jenson *et al.*, 1994). For in-store bakeries, products are often displayed in bins or are loosely wrapped in paper. While customers enjoy this form of product display, 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 *Listeria monocytogenes*. Furthermore, there is always a potential for temperature abuse at all stages of processing, distribution and storage chain and 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* species (Schmidt and Ridley, 1985).

1.2.4. Modified atmosphere packaging

Modified atmosphere packaging (MAP) using CO_2 enriched gas atmospheres, vacuum packaging and oxygen absorbents can extend the mold

free shelf life and keeping quality of a wide variety of high moisture bakery products stored at ambient temperature. 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 (tortilla) or with combinations of gases and oxygen absorbents e.g., 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 3. One pathogen of concern in MAP products is *Clostridium 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 food stored at ambient temperature yet products were still organoleptically acceptable to the consumer (Smith *et al.*, 2002). While MAP is widely used in Europe and is gaining acceptance in North America to extend 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.

1.2.5. 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 capable of supporting 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.

Table 3. Minimum growth requirements of pathogenic bacteria [a]

Bacteria	MINIMUM REQUIRED				
	Temperature		pH	A_w	Gaseous Conditions [b]
	°C	°F			
B. cereus	4	39	4.3	0.91	Facultative
C. jejuni	32	90	4.9	0.99	Microaerophilic
C. botulinum (Group 1)	10	50	4.6	0.95	Anaerobic
C. botulinum (Group 2)	3.3	37	5.0	0.97	Anaerobic
C. perfringens	15	59	5.0	0.95	Anaerobic
E. coli	7	45	4.4	0.95	Facultative
L. monocytogenes	0	32	4.3	0.92	Facultative
Salmonella spp.	6	43	4.0	0.94	Facultative
S. aureus [c]	6	43	4.5	0.86	Facultative
	(10)	(50)	(5.2)		
V. parahaemolyticus	5	41	4.8	0.94	Facultative
Y. enterocolitica	-1	30	4.2	0.96	Facultative

(Adapted from Smith *et al.*, 2002)

[a] Under optimal growth conditions

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

[c] Values in parentheses refer to the toxin

1.3. 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 molds, viruses, and bacteria.

Molds, which often limit the shelf life of high and intermediate moisture bakery products, can also be of public health concern. Although moldy bakery products will be rejected by consumers, some molds, including *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium* spp., may secrete mycotoxins of public health concern into bakery products without visible signs of spoilage.

Mycotoxins have been found in many foods, including cereals and grain products, nuts, fruits, vegetables and dairy products (Smith *et al.*, 2002). In a survey of flour, Weidenborner *et al.* (2000) found that *Aspergillus* species were the predominant isolates with 93.3% being toxigenic.

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

Viruses are also ubiquitous in the environment and can cause viral gastroenteritis in bakery products prepared or handled under unsanitary conditions (Smith *et al.*, 2002). Norwalk Like Viruses (NLVs) have been responsible for outbreaks of foodborne illness involving frosted bakery products, crumb cake, pie and rolls, hamburger buns and cookies, and custard slices (Smith *et al.*, 2002).

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 pastries covered with glaze or icing applied after baking (Smith *et al.*, 2002). More recently, a community outbreak in 1994 in New York was traced to an infected bakery worker who contaminated cooked doughnuts while applying a sugar glaze (Weltman *et al.*, 1996). Methods to control viral gastroenteritis in bakery products include education of food handlers, good personal hygiene and proper sanitation.

While molds 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 food intoxication or food 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). The majority of food poisoning outbreaks involving bakery products have been caused by two vegetative pathogenic bacteria, *Salmonella* species and *St. aureus*. Another vegetative pathogen of concern particularly in dairy filled baked goods is *L. monocytogenes*. Sources of these microorganisms in bakery products, the products involved in outbreaks, and the methods to control the growth of these pathogens in bakery ingredients and baked products are summarized in Tables 4, 5, and 6.

Spore forming bacteria have also been involved in food poisoning outbreaks. *Clostridium* species are spore-forming bacteria that readily survive the baking process and are of concern in minimally processed bakery products. *C. perfringens* (*welchii*) has been involved in several outbreaks in meat and chicken filled baked products such as pies, sausages, rolls, etc. However, very few incidents of *C. perfringens* involving these products have occurred in recent years due to strict temperature control of the raw ingredients and the baked products (Table 7). Good hygiene and personal sanitation are also essential to control the growth of this pathogen in meat filled bakery products.

Table 4. *Salmonella* spp. and bakery products

Salmonella spp.	
Sources of contamination:	Raw eggs/dairy products Flour Cocoa and chocolate Coconut Peanut butter Fruit, spices, and yeast flavorings
Symptoms of disease:	Nausea, vomiting Abdominal cramps Diarrhea, fever, headache
Causative agent:	Infection (>10^6CFU/g)
Onset of illness:	6 - 48 hours
Duration of illness:	2 - 14 days
Associated bakery products:	Bread pudding Custard filled cakes, pastries Quiche Meringue pies Cheese cake Apple pie Tiramisu Mousse
Preventive 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 On-going hygiene training Hazard analysis critical control point (HACCP) application

(Adapted from Smith *et al.*, 2002)

Table 5. *Staphylococcus aureus* and bakery products

Staphylococcus aureus	
Sources of contamination:	Hair, hands, skin, throats & nasal passages of humans Infected wounds, lesions Air, water, work surfaces Inadequately pasteurized milk/cream Dried milk
Symptoms of disease:	Nausea Vomiting Diarrhea Abdominal cramping Chills
Causative agent:	Toxin production in food when sufficient growth occurs (~10^6 CFU/g)
Onset of illness:	30 mins - 8 hours
Duration of illness:	1 - 2 days
Associated bakery products:	Cream filled pastries (synthetic and fresh cream) Apple muffins, cream puffs, long johns Flat bread Pizza Coconut fillings
Preventive measures:	Used 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 On-going hygiene training/HACCP

(Adapted from Smith *et al.*, 2002)

Table 6. *Listeria monocytogenes* and bakery products

Listeria monocytogenes	
Sources of contamination:	Raw/Inadequately pasteurized dairy products Bakery environment/post-processing contamination Raw poultry/meat products
Symptoms of disease:	Mild flu-like symptoms Septicemia Meningitis Encephalitis Abortion in pregnant women
Causative agent:	Infection
Onset of illness:	2 - 35 days
Duration of illness:	1 - 90 days
Associated bakery products:	Pastries
Preventive measures:	Use of adequately pasteurization 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) On-going hygiene training/HACCP

(Adapted from Smith *et al.*, 2002)

Table 7. *Clostridium perfringens* and bakery products

Clostridium perfringens (welchii)	
Sources of contamination:	Beef, turkey, chicken Cooked ground meat Gravies, sauces
Symptoms of disease:	Nausea Occasional vomiting Diarrhea Intense abdominal pain
Causative agent:	Toxin production in the intestine
Onset of illness:	6 - 24 hours
Duration of illness:	1 - 2 days
Associated bakery products:	Meat filled bakery products
Preventive measures:	Proper cooking of meat fillings Cooling baked products to <10°C within 2-3 hours Strict temperature control of stored products Reheating refrigerated products to reach an internal temperature of 70°C Strict personal hygiene/sanitation Good manufacturing practices (GMPs) On-going hygiene training/HACCP

(Adapted from Smith *et al.*, 2002)

A spore forming pathogen of concern in minimally processed bakery products, particularly those packaged under modified atmosphere is *C. botulinum*. Fortunately, there have been no reported outbreaks of botulism caused by bakery products in North America. However, in India, a suspected outbreak of foodborne botulism affecting 34 children, including three fatalities, was attributed to the growth and production of neurotoxin type E by *C. butyricum 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 (Smith *et al.*, 2002). In Italy, an outbreak of botulism resulted from the consumption of tiramisu made with contaminated mascarpone cheese (Smith *et al.*, 2002).

Although cases of foodborne botulism are rare, the severity of the intoxication means that the potential for growth and toxin production in a bakery product should be considered and additional barriers to growth and toxin production by this pathogen need to be incorporated into the product and/or packaging. Recently, Health Canada (Personal Communication) has recommended that bakery products, based on previous empirical data, be formulated to an a_w of ~0.94 and a pH of ~5.7. The levels of these barriers have proved effective against the growth of *C. botulinum* and should ensure the safety of most minimally processed MAP bakery products stored at ambient temperature (Table 8).

Table 8. *Clostridium botulinum* and bakery products

Clostridium botulinum (Type A and B spores)	
Sources of contamination:	Mozzarella, soft and processed cheeses Mascaponi cream cheese Vegetables e.g. pepper, onions, tomatoes, mushrooms Spices Garlic cloves Olives Peanuts, hazelnut puree Honey
Symptoms of illness:	Neurotoxicity Shortness of breath, blurred vision Nausea, vomiting, fatigue Paralysis of muscles Death
Causative agent:	Toxin in food (<10 CFU/g)
Onset of illness:	12 - 72 hours
Duration of illness:	1 -10 days
Associated bakery products:	Canned bread* Canned steam chocolate nut bread* English-style crumpets* Fruit breads* Crisp bread Tiramisu
Preventive measures:	Strict temperature control of raw ingredients/finished products (<4°C) Additional barriers (pH, water activity reduction) Ethanol vapor Strict personal hygiene/sanitation Good manufacturing practices (GMPs) On-going hygiene training/HACCP

*Laboratory studies only

(Adapted from Smith *et al.*, 2002)

1.4. *Bacillus cereus*

1.4.1. Sources of contamination

While foodborne illness caused by *Clostridium* species in bakery products is relatively rare, a spore forming pathogen of concern is *B. cereus* which has been implicated in several outbreaks of foodborne illnesses involving bakery products (Jenson *et al.*, 1994). There is also evidence that *B. subtilis* and *B. licheniformis,* well known as rope forming spoilage bacteria of bread, can also cause foodborne illness (Todd, 1982; Kramer and Gilbert, 1989; te Giffel *et al.,* 1996). *B. cereus* has two distinct forms of toxin-mediated gastroenteritis (Table 9). The emetic type is generally associated with cereal-based foods while the diarrheal type is most frequently associated with proteinaceous foods (Lund, 1990).

Bacillus species form spores that are ubiquitously found in soils, dust and water, and are commonly isolated from plants and animal products (Granum, 1997). *Bacillus* spp., attach to wheat that is milled into flour (Kirschner and Von Holy, 1989). Graves *et al.* (1967) examined flour from 11 U.S. mills and found that 18.2% of samples contained *B. cereus*, 9.1% contained *B. licheniformis* and 45.3% contained *B. subtilis*. The level of contamination of *B. cereus* in wheat flour was generally less than 10^3 spores/g (Kaur, 1986; Kim and Goepfert, 1971; Rizk and Ebeid, 1989). Therefore, *Bacillus* spores are commonly found in flour and flour-based products, as well as in the bakery environment. *Bacillus* spores are heat resistant, will survive baking, and under favorable conditions may grow to levels associated with toxin production. Survival of spores during baking depends on the type of product, the internal temperature reached during baking as well as the thermal resistance of the spore.

Bacillus spores have also been found in milk and can survive pasteurization. Hence, they are a source of concern in dairy products such as cream, dried milk (Slaghuis *et al.,* 1997; Larsen and Jorgensen, 1997), and whey concentrates (Pirttijarvi *et al.,* 1998). Although *B. licheniformis* was initially found

in higher numbers than *B. cereus* when milk products were held at room temperature, *B. cereus* rapidly dominated and reached levels associated with enterotoxin production (Crielly *et al.*, 1994). Harmon and Kautter (1991) reported a six- to seven-fold increase in *B. cereus* in reconstituted non-fat dried milk held at room temperature for 10.5 h. Cold adapted strains can even produce toxin at refrigerated temperatures (Foegeding and Berry, 1997), especially in aerated products such as whipped cream (Christiansson *et al.,* 1989) (Table 9).

Spices, such as ginger, mace, allspice, cinnamon, garlic, and pizza spice usually contain low levels of *Bacillus* spores; however, higher levels of spores associated with toxin production have also been found in these bakery ingredients (Powers *et al.,* 1976; Pafumi, 1986; Kneifel and Berger, 1994)(Table 9).

Other bakery ingredients which may be sources of *Bacillus* spp. include dried eggs (Shafi *et al.,* 1970), soy protein (Becker *et al.,* 1994), rice (Chung and Sun, 1986), yeast and improvers (Collins *et al.,* 1991; Bailey and von Holy, 1993; te Giffel *et al.,* 1996), dried fruits (Moreno *et al.,* 1985; Aidoo *et al.,* 1996), and cocoa (Gabis *et al.,* 1970; te Giffel *et al.*, 1996) (Table 9).

Table 9. *Bacillus cereus* and bakery products

Bacillus cereus		
Sources of contamination:	Flour, Dairy products Spices Dried eggs Soy protein Yeast and improvers Dried fruits and cocoa Rice	
Symptoms of illness:	Type I (Diarrhea type) Watery diarrhea Cramps Nausea	Type II (Emetic type) Diarrhea Cramps Nausea & vomiting
Causative agent:	Toxin produced in intestine	Toxin produced in food
Onset of illness:	6-15 hours	30-45 mins
Duration of illness	1 day	1 day
Associated bakery products:	Naan bread English-style crumpets Pikelets Vanilla slices Meat filled bakery products Pumpkin pie Rice filled pastries	
Preventive measures:	Cool hot food rapidly to <15°C (within 2 hours) Strict temperature control of raw ingredients/finished products (<4°C) Use of chemical preservatives e.g. vinegar, propionates, calcium acetate Lactic acid sourdough cultures Biopreservatives (nisin) Additional barriers (pH, salt)	

(Adapted from Smith *et al.*, 2002)

1.4.2. Associated outbreaks

The levels of *B. cereus* required to produce toxin is approximately 10^5 spores/g of food while higher spore levels (10^6-10^9 spores/g) are required for *B. licheniformis* and *B. subtilis* (Lund, 1990). Foodborne illness caused by *Bacillus* spp. is under-reported as symptoms are generally mild and self-limiting (Terranova and Blake, 1978).

Although low numbers of spores may be present initially in flour (Kaur, 1986), spores which survive baking can grow rapidly in products held under suitable conditions (Rosenkvist and Hansen, 1995). *B. cereus* does not survive baking while *B. subtilis,* which has a $D_{100°C}$ of 14 minutes, has been found in baked bread. (Leuschner *et al.*, 1998) Kaur (1986) reported that *B. cereus,* inoculated at ~10^4 spores/g, did not survive baking in 400 g loaves but survived baking in 800 g loaves. Although the actual number of *B. cereus* which survives baking is dependent on the loaf size and oven temperature/time combinations, the risk of foodborne illness from bread is minimal (Kaur, 1986; Collins *et al.*, 1991; Rosenkvist and Hansen, 1995; Thompson *et al.*, 1993). Kaur (1986) estimated that it would take three days at 27.5°C for *B. cereus* which survived the baking process to reach levels of 10^5 CFU/g in bread.

Some strains of *B. subtilis* and *B. licheniformis* cause "ropiness" resulting in the sensory rejection of product. However, this is not always the case and bread may have high numbers of *B. subtilis* yet still be organoleptically acceptable to the consumer. Illness attributed consumption of bread with high numbers of *B. subtilis* or *B. licheniformis* has been reported (Todd, 1982; Sockett, 1991; Thompson *et al.,* 1993). Therefore, control of *B. cereus*, *B. subtilis* and *B. licheniformis* is critical to the safety of bread products.

Although the potential health hazard of bread is minimal, *B. cereus* is of greater concern in bakery products which receive a minimal surface heat

treatment. Examples of such griddle baked bakery products include ethnic flat breads, crumpets, and waffles. Outbreaks of *B. cereus* gastroenteritis have been attributed to Naan bread (Cowden *et al.*, 1995), crumpets (Lee, 1988), and pikelets (Murrell, 1978). Growth of *B. cereus* in these products is difficult to control since the heat treatment is insufficient to destroy spores and may actually heat shock spores which enhances their growth at ambient storage temperature. There is also concern for growth in dry, reconstituted rice cereals particularly when reconstituted with milk (Jaquette and Beuchat, 1998).

B. cereus has also caused illness in bakery products containing dairy based custards or creams. Pinegar and Buxton (1977) showed that custard used in the production of vanilla slices contained low numbers of spores (<500/g). However, immediately after cooking, levels of *B. cereus* increased to $>10^6$ CFU/g (Table 9).

B. cereus has also been isolated from prepared bakery products including meat filled bakery products (Khot and Sherikar, 1986), bread (Sadek *et al.,* 1985), pies and pastry (Pinegar and Buxton, 1977; Wyatt and Guy, 1981).

1.4.3. Control measures

Conventional methods of control include proper sanitation and testing of raw materials to reduce initial spore counts; however, these measures do not prevent germination and growth of *Bacillus* species in finished products. Growth of *Bacillus* spp., in baked products can be controlled with preservatives (Table 9). Propionic acid, calcium or potassium propionates, and calcium acetate can delay germination and growth of some *Bacillus* spp., particularly rope producers (Kaur, 1986; Kirschner and Von Holy, 1989; Rosenquist and Hansen, 1998). Thompson *et al.* (1998) reported that white bread containing vinegar was more effective at preventing rope production than calcium proprionate. Potassium sorbate, at a level of 2500 ppm, prevented growth of *B. cereus* in pumpkin pie (Wyatt and Guy,

1981), while 2000 ppm sorbic acid or 4000 ppm of potassium sorbate inhibited *B. subtilis* and *B. cereus* in the rice filling of Karelian pastry (Raevuori, 1976).

Lactic acid bacteria may also inhibit *Bacillus spp.* Corsetti *et al.* (1996) reported that 33% of 232 lactobacilli isolated from Italian sour dough inhibited strains of *B. subtilis*. Control of *B. subtilis* and *B. licheniformis* in bread was achieved by the addition of 10 to 15% sourdough fermented with *Lactobacillus plantarum* or *Lactobacillus sanfrancisco* L99. Bread had an increased acidity due to production of lactic and acetic acids and resulted in an increased shelf life without the addition of preservatives (Rosenquist and Hansen, 1998). Some strains of lactic acid bacteria also produce bacteriocins. Nisin is a commercially available bacteriocin produced by *Lactococcus lactis* and is the only bacteriocin shown to have an antimicrobial effect against *Bacillus* spp. Growth of *B. cereus* in high moisture English style crumpets was controlled in crumpets for up to 5 days at ambient storage temperature by the addition of 1 to 5 μg/g of nisin (Jenson *et al.*, 1994). However, nisin at levels up to 100 μg/g had no effect on the growth of either *B. subtilis* or *B. licheniformis* in wheat bread (Rosenquist and Hansen, 1998).

Spores of *Bacillus* are known for their heat resistance. Typical $D_{100°C}$ values for *B. cereus, B. subtilis* and *B. licheniformis* have been reported as 40, 14, and 56 minutes respectively (Wyatt and Guy, 1981; Leuschner *et al.,* 1998). Therefore, spores can readily survive the minimal heat processing conditions in baking. Thermal resistance may decrease by decreasing the pH of the product. The D value of *B. cereus* spores decreased from 3.7 to 3.1 minutes at 90°C when the pH of custard was decreased from 7.2 to 6.2 (Bassen *et al.,* 1989). A high pH (>9) may also provide some measure of control, however, very few bakery products are formulated to such pH levels (Leuschner *et al.,* 1998; El-Khoury 2001).

Cold storage may be a suitable control measure for some products, such as cream and custard type products. However, up to 14% of *B. cereus* strains may be psychrotrophic (Granum, 1997) and so temperature alone is not a practical control measure. Custards and fillings should be prepared in small batches, cooled rapidly and stored at 4°C.

Modified atmosphere packaging with CO_2 cannot control the growth of *Bacillus* spp., alone. Smith *et al.* (1983) reported that *B. subtilis* and *B. licheniformis* grew to 5×10^8 CFU/g after 3 days at 37°C or after 2 weeks at 20°C in crumpets packaged under modified atmospheres. However, combination treatments have proved effective to control this pathogen. El-Khoury (2001) controlled the growth of *B. cereus* in crumpets stored at 30°C for >28 days using a combination of 100% CO_2 and 600S Negamold®, an oxygen absorbent-ethanol vapor generator. However, products were organoleptically unacceptable due to the high levels of ethanol absorbed from the package headspace (El-Khoury, 2001).

Sutherland *et al.* (1996) examined the combined effects of ambient temperature, pH, salt and CO_2 on the growth of *B. cereus* in carbohydrate based foods. Quintavalla and Parolari (1993) also modelled the effects of pH, a_w, and temperature on the growth of *Bacillus* spp. isolated from bakery products. They reported a 12-15 day shelf life for products stored at 20°C and reformulated to pH 5.2 and a_w 0.93. Reformulation to pH 4.3 and a_w 0.92 extended product shelf life to 30 days at a similar storage temperature. However, the limitations of such models are that the data cannot always be extrapolated to food due to the complex nature of the food matrix.

Further studies conducted by El-Khoury (2001) on English-style crumpets, indicated that a_w alone was not a viable method to control the growth of *B. cereus*. Large amounts of salt and glycerol were required for complete inhibition of this pathogen and such levels would severely affect the organoleptic and textural qualities of crumpets. El-Khoury (2001) also showed that the growth of *B.*

cereus could be inhibited at pH 5 alone or in combination of potassium sorbate. However, several companies formulate English-style crumpets to pH levels >8 to enhance their color. However, most chemical preservatives are ineffective at high pH. El-Khoury (2001) showed that neither calcium propionate nor potassium sorbate were effective in controlling the growth of *B. cereus* at pH >5. While other methods (UV-light, bacteriocins, organic esters, and batter pasteurization) were effective in controlling the growth of *B. cereus* in media, they failed to inhibit its growth in crumpets, probably due to the complexity of the food system.

Therefore, novel methods are required to control the growth of this pathogen. Two such methods could be sorbohydroxamic acid, a chemical preservative active over a wide pH range, and mastic essential oil.

1.4.3.1. Chemical preservatives

Chemical preservatives are most commonly used by the bakery industry to prevent or retard microbiological spoilage. The Code of Federal Regulations (CFR) defines a chemical preservative as "any chemical that tends to prevent or retard deterioration when added to food" (CFR, 1992). The chemical preservatives most commonly used in bakery products include acetic acid, calcium and sodium propionate, sorbic acid and potassium sorbate. The minimum level of each preservative required to inhibit the growth of common spoilage microorganisms of concern in bakery products are shown in Table 10.

1.4.3.1.1. Acetic acid and acetates

Acetic acid and its salts (sodium acetate/di-acetate) can be used as preservatives in bakery products. The main reasons for using it as a preservative in such products are (i) low cost, (ii) availability, and (iii) low toxicity. Acetates have good antimicrobial activity, and can also be used as acidulants, flavoring

agents, and sequesterants. Acetic acid and its salts are most effective in baked goods with a pH <6 and have a greater activity against yeasts and bacteria than molds (Table 10). Sodium diacetate is commonly used since it is more effective against rope-forming bacteria.

1.4.3.1.2. Propionic acid and propionates

Propionic acid and its salts, propionates, are used to control mold spoilage and "ropiness" caused by *B. subtilis* (O'Leary and Kralovec, 1941). Although propionic acid has a higher antimicrobial activity, propionates (sodium and calcium) are more commonly used in bakery products due to their (i) greater solubility and (ii) odor-free characteristics. They are effective against molds, but have little activity against bacteria (with the exception of *B. subtilis*) and no activity against yeasts. This selective antimicrobial activity makes them ideal as mold inhibitors in yeast leavened products. A compound called "Soprodac" is also commercially available. It comprises of a 70% aqueous solution of equimolar amounts of sodium propionate and propionic acid and is commonly used as an antimycotic agent in pizza crust.

Table 10. Antimicrobial spectra of preservatives used in foods

Organic acids	Yeasts	Molds	*Enterobacteriaceae*	*Micrococcaceae*	*Bacillaceae*
	Minimum levels required for inhibition (%) (w/w)				
Acetic acid	0.5	0.1	0.05	0.05	0.1
Propionic acid	0.2	0.05	0.05	0.1	0.1
Sorbic acid	0.02	0.04	0.01	0.02	0.02

Adapted from Chichester and Tanner (1972)

1.4.3.1.3. Sorbic acid and sorbates

Sorbic acid and its sodium, calcium, and potassium salts are permitted preservatives in bakery products. Sorbates have been shown to be effective against yeasts, molds and various bacteria in baked goods. However, due to solubility and cost considerations, potassium sorbate is most commonly used as a preservative in bakery products.

Sorbic acid and potassium sorbate have a broad-spectrum of activity against yeasts and molds, but little activity against most bacteria, with the exception of *B. subtilis.* Sorbic acid and potassium sorbate are effective antimycotic agents at pH values up to 6. Hence, the antimicrobial activity of sorbates, as well as propionates, increases as the pH decreases. Sorbates, on a weight per weight basis, have been shown to be twice as effective as propionates to control mold growth in bakery products (Seiler, 1976). However, they have an adverse effect on yeast activity and reduce loaf volume as well as making the dough sticky and difficult to process (Legan and Voysey, 1991).

Sorbates can also be applied to bakery products in various ways to overcome their inhibitory effect on yeast fermentation during baking. These include encapsulation, spraying onto the product as an aerosol after baking, or incorporating it into the packaging material (Sofos, 1989). Sorbic acid can be encapsulated with fatty acids, such as palmitic acid, to produce sorboyl palmitate. This ester has been shown to be effective in controlling mold growth without interfering with the fermentation process. During baking, sorboyl palmitate is hydrolyzed and sorbic acid is released into the product to inhibit mold growth during storage (Sofos, 1989).

1.4.3.1.4. Sorbohydroxamic acid

Although sorbic acid is commonly used in the bakery industry to extend the mold free shelf life of bakery products, it is only effective in products with a pH of <6.5 (Figure 1). This limitation exists for all organic acids commonly used as preservatives in bakery products since they are all effective in the undissociated form which exists at lower pH levels. However, several bakery products have alkaline pH values, such as carrot cake, ginger bread cake, and some English style crumpets. Therefore, the choice of chemical preservatives to control mold spoilage in these products is limited. While parabens can be used for alkaline pH products, a major limitation to their use is cost and they can only be used with non-yeast leavened products (Dudman, 1963). Another possibility is the use of sorbohydroxamic acid (or sorbic hydroxamic acid), an ester of sorbic acid, which remains undissociated at higher pH values due to its high dissociation constant (pKa 8.8) (Dudman, 1963). Sorbohydroxamic acid has been shown to inhibit the growth of various mold species (i.e., *Aspergillus niger*, *Penicillium notatum*, and *Rhizopus* spp.) in grape juice at pH values ranging from 3.6-9.2, while sorbic acid was not effective at pH 5.7 and above (Table 11). Studies conducted by Troller and Olsen (1967) have shown that sorbohydroxamic acid was a much better mold inhibitor than sorbic acid in frostings (Table 12 and 13).

To date, the use of sorbohydroxamic acid as a food preservative has focused on the control of mold spoilage in bakery products. However, few studies have been done to determine its effect on bacteria of public health concern in bakery products, e.g., *B. cereus*. Furthermore, its ability to exert an antimicrobial effect over a wide pH range makes it promising as a potential preservative to control the growth of this pathogen, particularly in high pH bakery products.

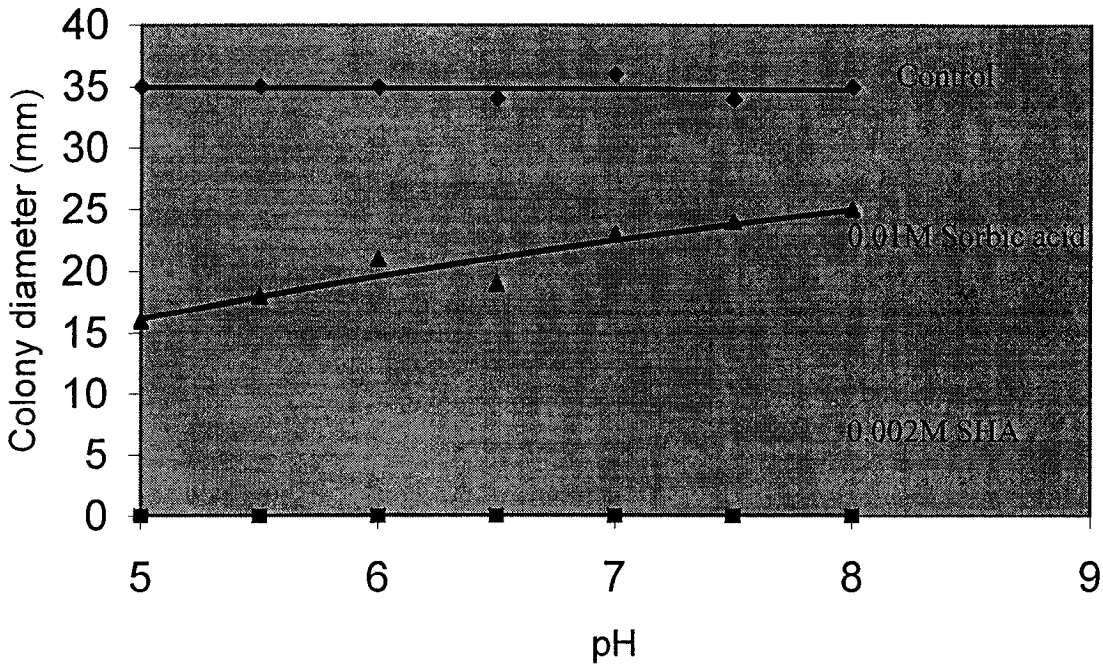


Figure 1. Comparison of the relative effectiveness of sorbohydroxamic acid and sorbic acid against *Aspergillus niger* JT-4 at various pH levels in agar plates stored at 25°C for 7 days

(Adapted from Troller and Olsen, 1967)

Table 11. Comparison of sorbohydroxamic acid (SHA) and potassium sorbate (KS) as antifungal agents in grape juice after 38 days at room temperature

Organism	pH	KS(%) (w/w)				SHA(%) (w/w)			
		0.1	0.05	0.025	0.012	0.1	0.05	0.025	0.012
Aspergillus	3.6	-	-	+	+	-	-	+	+
niger	5.7	+	+	+	+	-	-	+	+
	7.6	+	+	+	+	-	-	+	+
	9.2	+	+	+	+	-	-	+	+
Penicillium	3.6	-	-	-	+	-	-	+	+
notatum	5.7	+	+	+	+	-	-	-	+
	7.6	+	+	+	+	-	-	-	+
	9.2	+	+	+	+	-	-	-	-
Rhizopus spp.	3.6	-	-	-	-	-	+	+	+
	5.7	+	+	+	+	-	-	+	+
	7.6	+	+	+	+	-	-	+	+
	9.2	+	+	+	+	-	-	+	+

(Adapted from Dudman, 1963)

Table 12. Effect of sorbohydroxamic acid on the storage life of frosting

Storage time (days at 25°C)	Number of replicates with visible mold growth							
	Sorbohydroxamic acid (%) (w/w)						Sorbic acid (%) (w/w)	
	0	0.001	0.002	0.004	0.006	0.0075	0.005	0.01
0	0	0	0	0	0	0	0	0
13	5	0	0	0	0	0	0	0
55	5	2	0	0	0	0	2	1
104	5	2	0	0	0	0	3	1
160	5	5	1	0	0	0	5	3
216	5	5	5	1	0	0	5	5
223	5	5	5	2	1	1	5	5

Adapted from Troller and Olsen (1967)

Table 13. Effect of pH and concentration on the storage life of frostings preserved with sorbohydroxamic acid and sorbic acid

SHA (%) (w/w)	Sorbic (%) (w/w)		Samples with visible mold growth (%)						
			Storage time (Days)						
		pH	34	50	71	92	110	139	166
0	0	6.57	95	100	100	100	100	100	100
0	0	7.21	100	100	100	100	100	100	100
0.03	0	6.59	0	0	0	0	0	0	0
0.03	0	7.19	0	0	5	5	5	5	5
0.06	0	6.58	0	0	0	0	0	0	0
0.06	0	7.20	0	0	0	0	0	0	0
0.10	0	6.54	0	0	0	0	0	0	0
0.10	0	7.18	0	0	0	0	0	0	0
0	0.03	6.54	100	100	100	100	100	100	100
0	0.03	7.22	100	100	100	100	100	100	100
0	0.06	6.55	0	0	0	0	0	0	0
0	0.06	7.21	80	85	95	95	95	100	100
0	0.10	6.57	0	0	0	0	0	0	0
0	0.10	7.19	0	0	0	5	5	10	10

Adapted from Troller and Olsen (1967)

1.4.3.2. Natural preservatives

The use of chemical preservatives has resulted in increasing pressure by consumer groups for food manufacturers to either completely remove preservatives from food or to use more "natural" alternatives to extend a product's shelf life (Nychas, 1995). Many spices, herbs and their extracts possess antimicrobial activity, almost invariably due to their essential oil fraction (Deans and Ritchie, 1987). The essential oils of plants, such as oregano, thyme, sage, rosemary, clove, and coriander have all been shown to control food spoilage bacteria and molds (Salmeron *et al.*, 1990; Farag *et al.*, 1989; Aureli *et al.,* 1992; Stecchini *et al.,* 1993). The antibacterial and antimycotic activity of garlic and onion oil extracts have also been well documented (Mantis *et al.*, 1978; Sharma *et al.*, 1979; Saleem and Al-Delaimy, 1982; Conner and Beuchat, 1984 a, b).

The antimicrobial compounds of spices are commonly found in their essential oil fraction, which are also responsible for their characteristic aroma and flavor. According to Hargreaves *et al.* (1975), essential oils are defined as "a group of odorous compounds, soluble in alcohol and to a limited extent in water, consisting of a mixture of esters, aldehydes, ketones, and terpenes". Among the compounds having a wide spectra of antimicrobial activity are thymol from thyme and oregano, cinnamic aldehyde from cinnamon, and eugenol from cloves. Another oil which has been shown to have antimicrobial activity against *St. aureus* and *Pseudomonas fragi* is mastic essential oil (Tassou and Nychas., 1995). Lis-Balchin *et al.* (1996) examined the bioactivity of commercially available plant essential oil. They reported that it had strong activity against a cocktail of 25 bacteria species, 20 strains of *L. monocytogenes*, as well as *Aspergillus niger*, *Aspergillus ochraceus*, and *Fusarium culmorum*. However, while good results were obtained *in vitro*, such inhibition could not be extrapolated to food products. This was most likely due to the complexity of the food matrix and the lack of diffusion of the oil in the food. Essential oils are not commonly used in the bakery industry due to their strong aroma which may influence the sensorial properties of

products. However, the potential of mastic essential oil to control pathogenic bacteria, specifically *B. cereus* in bakery products warrants further investigation.

1.5. Mastic resin

1.5.1. Introduction

Mastic resin, and its use, can be traced back to ancient times. Ancient Greek scholars, e.g. Plinios, Theophrastus, Dioscouridis, and Galinos, wrote about mastic resin for its healing properties while its pharmaceutical properties were well known to Hippocrates and was first mentioned by Herodotus in the 5th century B.C. (Perikos, 1993). The Greek battlefield physician Dioscorides, wrote about the use of medicinal herbs, balms, treatments, and specifically mastic resin in his influential "De Materia Medica", a work which laid the foundation for modern pharmacology. Galen, the great second century physician, and successor to Hippocrates, reported the favorable therapeutic properties of mastic. Mastic was also used in Arabic medicine to treat upper abdominal pain and heartburn as well as gastric and intestinal ulcers (Perikos, 1993).

Mastic resin was probably the world's first "chewing gum", where it is reported to strengthen gums, deodorize the breath and act as an oral antiseptic. Mastic resin has also been shown to have powerful antimicrobial activity (Huwez and Thirwell, 1998; Al-Said *et al.*, 1986; Tassou and Nychas, 1995; Iauk *et al.*, 1996).

1.5.2. Sources of mastic resin

The genus *Pistacia* has eleven currently recognized species. However, only two are used as major sources of the gum resin. The first of these is mastic, while the second is known under a variety of names such as, Chios, Chio, or Chian turpentine, or Cyprus balsam (Mills and White, 1989).

Mastic resin is only produced on Chios, an island in the Aegean Sea, from a variety or cultivar of *Pistacia lentiscus* var. *Chia*. It is a slow growing evergreen shrub, which after 40-50 years reaches about 2-3 meters in height. However, when fully grown, it can be as high as 5 meters. Mastic resin is produced after five or six years, and after year 12 to 15, each tree produces 320 g to 1 kg of resin annually. Although the tree can live for ~200 years, the secretion of mastic resin declines after ~70 years (Browicz, 1987).

1.5.3. Cultivation and harvest of mastic resin

Mastic is the air-dried resinous exudate from *P. lentiscus* L. The mastic originates in special oleoresin reservoirs located in the inner bark of the trunk and branches. Mastic resin is harvested by two methods (1) traditional and (2) novel. In the traditional method of harvesting, mastic resin is obtained as a result of shallow incisions made into the bark with special tools, called "cedirini". These incisions are repeated several times from early July to the end of August and the exudate is usually collected manually, depending on the climatic conditions, at the end of August and in September. The resin exudes and either hardens in tears on the bark or drops to the ground where it is collected. There are different grades of resin tears depending on the degree of impurities from the soil and other foreign material. The mean yield of resin per tree is ~250-400 g over the five months harvesting season (Papanicolaou *et al.*, 1995 b). Several factors influence the yield of mastic resin including (i) length and number of incisions, (ii) frequency of incisions during the harvest season, (iii) climatic conditions, e.g. humidity and temperature.

The traditional method of resin harvesting has many disadvantages. Picking and cleaning the resin is labor intensive and thus costly. Also, a significant loss of volatile constituents occurs while the resin lies on the ground. It has been estimated that 70-80% of the initial level of essential oil from the resin is lost. Other undesirable reactions, such as oxidation and polymerization also,

seriously affect the final quality of mastic resin and more importantly mastic essential oil.

Several methods have been used to increase the production of mastic resin including a mixture of H_2SO_3 and a commercially available substance called "Ethrel" which are pasted directly to the bark of the mastic tree to stimulate resin secretion (Papanicolaou *et al.*, 1995 b).

In the novel method of harvesting, mastic resin is collected in a fluid form. The fluid resin is a yellow, clear sticky product of high viscosity under normal temperatures. Its essential oil content is approximately four times greater and is generally of superior quality than the essential oil obtained by the traditional method of harvesting (Melanitou *et al.*, 1995). However, a disadvantage of the novel method of harvesting is a loss of essential oil volatiles during storage and prior to further processing. For this reason, the traditional method of harvesting is still the preferred method of collecting the resin from the mastic trees (Melanitou *et al.*, 1995).

1.5.4. Chemical characteristics of mastic resin and mastic essential oil

Extensive research has been done on the chemical characteristics of both mastic resin and mastic essential oil both of which will now be briefly reviewed.

1.5.4.1. Mastic resin

Mastic resin consists of largely of triterpenoid molecules and a recently elucidated polymeric material. The polymeric fraction of mastic resin from *P. lentiscus* L. is 1,4-poly-myrcene (Van den Berg *et al.*, 1998). Moreover, the *cis* isomer is dominant 3:1 over the *trans* configuration. Analysis of the essential oil content of mastic teardrops (which composes only 2% by weight) showed that α-

pinene was the most abundant compound. In addition, β-myrcene and low amounts of limonene, camphene, and ß-pinene were identified (Van den Berg *et al.*, 1998). The chemical composition of mastic resin is: 1-3% essential oil, 4% a-mastichinic acid ($C_{23}H_{36}O_4$), 0.5% mastichonic acid ($C_{32}H_{48}O_4$), 18% b-mastichinic acid ($C_{23}H_{36}O_4$), 30% a-mastichorezene ($C_{35}H_{56}O_4$), and 20% b-mastichorezene ($C_{35}H_{56}O_4$) (Tassou and Nychas, 1995). Of these compounds, β-myrcene is the only compound with conjugated double bonds which are relatively prone to polymerization. Van den Berg *et al.* (1998) inferred that the tree produces relatively large amounts of β-myrcene that is polymerized once the resin exudes from the tree. Monoterpenes, such as β-myrcene, are relatively common components of the volatile fraction of tree balsams and are also secreted by butterflies (Van den Berg *et al.*, 1998). Polymers of isoprenes (natural rubber), polysesquiterpenoids and polyditerpenoids have been known for a long time. This new discovery is important since it is the first reported naturally occurring polymer of a monoterpene (Van den Berg *et al.*, 1998).

1.5.4.2. Mastic essential oil

The chemical composition of the essential oil produced from mastic resin has been studied by Papanicolaou *et al.* (1995 a). Its major components were found to be α-pinene, myrcene, β-pinene, camphene, limonene, linalool, and β-caryophylene, as shown in Table 14. Changes in the ratio of myrcene to α-pinene, which occur during storage, can be used as an indicator of the overall quality of the oil (Papanicolaou *et al.*, 1995 a). Generally, the higher the myrcene/α-pinene ratio, the better the quality of the oil.

Minor quantitative changes in other constituents of low concentration have also been observed during storage (Figure 2) (Papanicolaou *et al.*, 1995a). An increase in camphene, limonene, camphoraldehyde, linalool, γ-muurolene, anethol, and trans-carveol content of the essential oil has been observed (Figure 2). During storage, the viscosity of the oil increases and the profiles change

resulting in the appearance of four new peaks, which were tentatively identified as α-thujene, fenchene, sabinene, and myrtenal (Papanicolaou *et al.*, 1995 a). Myrcene, together with other major and minor constituents of the essential oil, also play a role in defining the odor and quality of the product. The identification of all these constituents are important, since changes in the oil levels commence immediately after harvesting and continue during storage of the mastic resin. These changes affect the characteristic odor and general organoleptic quality of mastic oil (Papanicolaou *et al.*, 1995 a). While most of these changes can be attributed to oxidation and polymerization, there is also a loss of volatile components due to evaporation.

Both, Wyllie *et al.* (1990) and Fleisher (1992) examined the oil volatiles of mastic oil obtained from the fruit of *P. lentiscus.* Analysis by high resolution GC and GC-MS indicated a complex composition consisting of 70 or more components (Wyllie *et al.,* 1990). Those compounds are listed in Table 15. The main components of the oil, comprising about 86% of the total extract were the monoterpenes myrcene (39%), α-pinene (28%), limonene (11%), β-pinene (5.4%), β-caryophyllene (2.4%) (Wyllie *et al.,* 1990). The aliphatic esters and ketones constituted the next most abundant group (7.3%), followed by phenolic derivatives (1.3%). The monoterpene hydrocarbons identified in the essential oil of *P. lentiscus*, such as myrcene, the ocimenes, limonene, δ-carene, and phellandrene, contribute flavor to many spices while sequiterpene, β-caryoplyllene, are considered as important contributors to the aroma of black pepper oil (Wyllie *et al.,* 1990). The phenolic derivatives, such as anethole, estragole, thymol, and carvacrol, are also common constituents of many herbs and spices and the sensory quality of these products. Thus, mastic essential oil is qualitatively similar in composition to several commonly used herbs, such as basil and oregano. The characteristic flavor of mastic oil makes it a useful addition to the range of herbs and spices commonly used in Mediterranean foods (Wyllie *et al.,* 1990).

Table 14. Comparative chemical composition of essential oil in mastic resin as determined by gas chromatography

Peak Number	Compound	Essential oil at harvest	Essential oil after 3 year storage
1	α-thujene	6.77	18.18
2	α-pinene	51.00	49.12
3	fenchene	NA	10.69
4	camphene	0.48	0.81
5	β-pinene	2.32	2.48
6	sabinene	NA	0.48
7	myrcene	30.99	14.75
8	limonene	0.55	1.11
9	ocimene epoxide	0.71	0.08
10	p-cymene	0.03	0.10
11	camphoraldehyde	0.92	1.10
12	perillen	0.09	0.12
13	dehydro p-cymene	0.05	0.12
14	linalool	0.20	0.33
15	β-caryophyllene	2.92	1.64
16	myrtenal	NA	1.41
17	α-humulene	0.23	0.23
18	neral	0.42	0.62
19	γ-muurolene	0.25	0.30
20	α-terpineol	0.37	1.23
21	myrtenol	0.28	0.22
22	anethol	0.28	0.36
23	trans-carveol	0.07	0.29
24	caryophyllene oxide	0.33	0.29
25	methyl-eugenol	0.03	0.02
26	cis-methyl-eugenol	0.03	0.02
27	trans-methyl-eugenol	0.65	0.29

NA – peak not available

Adapted from Melanitou *et al.* (1995)

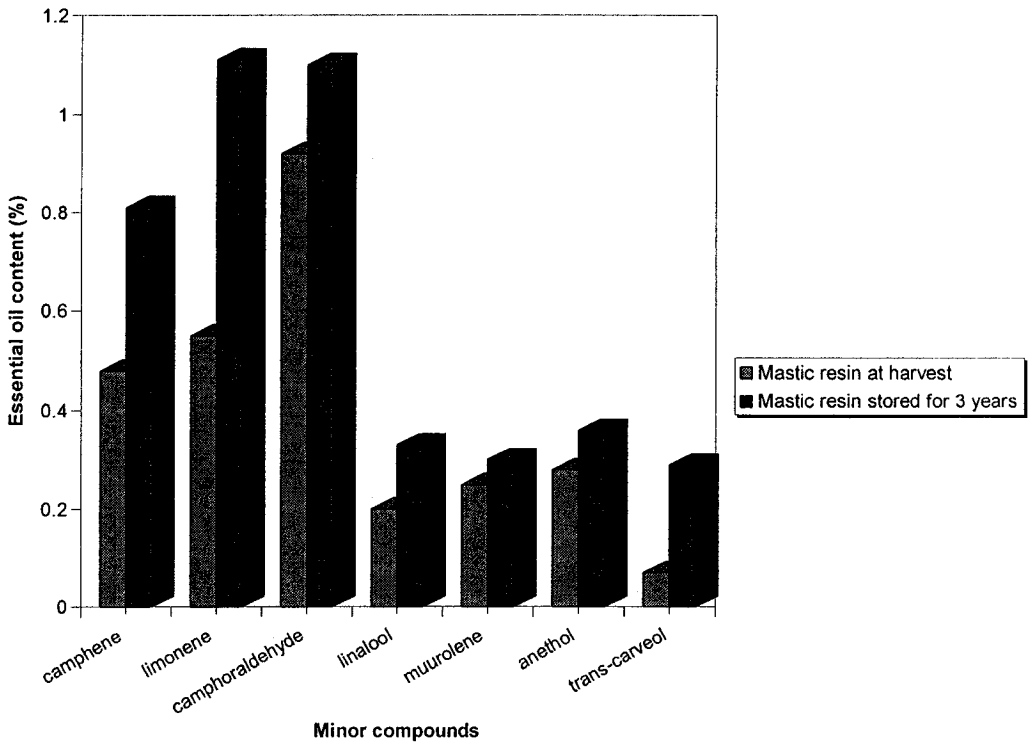


Figure 2. Quantitative changes in minor components of mastic essential oil during a 3 year storage trial at ambient conditions

Adapted from Melanitou *et al.* (1995)

Table 15. The main volatile constituents of mastic essential oil from the fruit of *Pistacia lentiscus*

Constituents	Area (%)
α-pinene	28
camphene	0.8
β-pinene	5.4
sabinene	tr*
myrcene	39
limonene	11
β-caryophyllene	2.1
undecan-2-one	6.9
α-terpineol	0.9
α-muurolene	0.16
δ-cadinene	0.6
anethole	0.6
carvacrol	0.6

* tr = trace amounts

Adapted from Wyllie *et al.* (1990)

1.5.4.3. Applications of mastic resin and mastic oil

Traditionally, mastic resin and mastic oil have been used for multiple purposes. When heated or burnt, it provides a pleasant scent and is used as incense. In ancient Greece, mastic resin was used as toothpaste to clean teeth and prevent bad breath (Perikos, 1993). Other uses of mastic resin include its addition to some liqueurs and to a white sticky jam known in Greek taverns as "ghliko tu kutaliu" (Perikos, 1993). It is also used to make varnish in lithography and used in the cosmetic industry. As a culinary aid, mastic resin and oil provide flavor to a range of traditional foods. For example, it is used in the preparation of "Tzoureki", a cake prepared for Easter in Palestine and Greece, in fondants, and in the Greek wine "Retsina". It is also used to flavor and stabilize ice cream in some Middle Eastern countries (Perikos, 1993).

Mastic resin and oil have been used by traditional healers for the relief of upper abdominal discomfort, gastralgia, dyspepsia and peptic ulcers. Recent research has shown that mastic resin/oil has antimicrobial activity against *Helicobacter pylori*, the microorganism responsible for many peptic ulcers (Huwez and Thirwell, 1998). Since, mastic is inexpensive and widely available in Third World countries and could be used as an inexpensive alternative to antibiotics in the treatment of peptic and gastric ulcers.

Mastic resin/oil is also a powerful antioxidant. Egyptian farmers have used mastic oil for many thousands of years to prevent rancidity in butter oil (Abdel-Rahman, 1975). Abdel-Rahman (1975) showed that mastic oil, was a better antioxidant compared to two commercial antioxidants, butylated hydroxyanisole (BHA) and Embanox 3 (EMB) which are composed of 20% BHA, 6% propyl gallate, and 4% citric acid in propylene glycol as solvent (Table 16). Mastic oil contains 44% terpenolic acids, thus 0.05% mastic oil contains approximately 0.02% terpenolic acids which is equivalent to the amount of BHA and EMB used. Moreover, it contains ~2% volatile oils which results in the desirable odor of preserved oils (Abdel-Rahman, 1975). At storage temperatures 25 and 35°C,

mastic improved the keeping quality of the oils. However, at 45°C, higher concentrations (>0.1%) of mastic oil were required to prevent rancidity and improve the shelf life of the oils during storage.

Table 16. Peroxide values of vegetable oil stored at different temperatures

Storage (days)	Peroxide values of sunflower seed oil				
	% BHA	% EMB	% mastic oil		
	0.02	0.02	0.02	0.05	0.10
			25°C		
0	8.4	8.4	8.4	8.4	8.4
20	8.2	8.5	10.1	8.6	8.4
50	10.1	10.3	16.1	10.2	9.5
			35°C		
0	8.4	8.4	8.4	8.4	8.4
20	9.1	9.1	10.5	9.1	8.8
50	10.0	12.7	19.2	12.9	9.5
			45°C		
0	8.4	8.4	8.4	8.4	8.4
20	9.8	9.9	13.8	11.6	9.3
50	16.7	16.8	24.6	18.8	14.7

Adapted from Abdel-Rahman (1975)

1.5.4.4. Antimicrobial activity of mastic essential oil

The excessive use of chemical preservatives and consumer concerns about their toxicity and carcinogenicity has resulted in increasing pressure on manufacturers to either completely remove chemical preservatives from their food products. There is considerable interest in the use of alternative food preservatives, to prevent either the growth of foodborne pathogens or to delay the onset of food spoilage bacteria. One naturally occurring compound which can fulfill both these objectives is mastic essential oil.

Tassou and Nychas (1995) examined the effect of various concentrations of mastic oil (0 to 1.5% by weight) on the growth of several Gram positive and Gram negative of food spoilage bacteria. In this study, *L. plantarum* and *S. aureus* were both completely inhibited by mastic essential oil, while the growth *P. fragi* and *S.enteritidis* were only partially inhibited (Tassou and Nychas, 1995). However, the essential oil in combination with EDTA (0.05% (w/v)), inhibited *P. fragi* but had no effect on *S. enteritidis*. The dissolution of essential oil was better with the addition of EDTA. The results are in agreement with previous investigations which showed that Gram positive bacteria were, in general, more sensitive to essential oils than Gram negative bacteria. Tassou and Nychas (1995) suggested that the antimicrobial activity of essential oils might be due to the impairment of enzyme systems involved in energy production and synthesis of structural components. However, no studies to date have examined the effect of mastic essential oil on *B. cereus*, a spore-forming microorganism of concern in high moisture bakery products.

1.6. Objectives of research

To date, few studies have been done to control the growth of *B. cereus* in minimally processed, high pH bakery products which have been implicated in several food poisoning outbreaks due to the growth this pathogen.

Therefore, the objectives of this research were:

1. To determine the potential of sorbohydroxamic acid (SHA) and mastic essential oil as novel antimicrobial agents against *Bacillus cereus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Salmonella enteriditis*, *Penicillium notatum*, and *Saccharomyces cerevisiae*, in model agar systems.
2. To determine the effect of a_w, pH, inoculum level, and concentration of SHA and mastic essential oil, alone, and in combination of each other, to control the growth of selected microorganisms in model agar systems.
3. To determine the potential of mastic essential oil as a vapor phase inhibitor.
4. To extrapolate the results *in vitro* to control the growth of *B. cereus* in high pH English-style crumpets.

Chapter 2

Use of Sorbohydroxamic Acid to Control the Growth of *Bacillus cereus In Vitro*

A limitation of most chemical preservatives is that they are totally dissociated at neutral pH. This study investigated the antimicrobial activity of sorbohydroxamic acid (SHA) over a wide pH range against selected microorganisms of concern in high moisture bakery products. This study examined the antimicrobial effect of MO against selected microorganisms of concern in high moisture bakery products. This study also examined the use of SHA and ME interactive sachets, alone and in combination, to inhibit the growth of *B. cereus* in English-style crumpets.

Chapter 2

Use of Sorbohydroxamic Acid to Control the Growth of *Bacillus cereus In Vitro*

2.1. Introduction

Bacillus cereus is a microorganism of public health concern in bakery products and has been implicated in several outbreaks of food poisoning due to the consumption of high moisture English style crumpets (Jenson *et al.*, 1994). While preservatives, such as sorbic acid, can control the growth of *B. cereus*, it is only effective in acid pH products (pH 5 to 6). However, several products are formulated to alkaline pH levels (8-9) to enhance their color. At such alkaline pH levels, most chemical preservatives are completely dissociated resulting in a decrease in their antimicrobial activity. Therefore, methods to control the growth of this pathogen are essential to ensure the safety of high pH, high moisture bakery products. El-Khoury (2001) showed that water activity (a_w) reduction through the addition of salt or the use of ethanol vapor generators could be used to achieve this objective. However, both approaches are not commercially viable since they required too much salt (~10% w/w) or ethanol vapor (~6 % v/v) to control *B. cereus* and resulted in products that were organoleptically unacceptable. Another approach is to use an antimicrobial agent that remains undissociated at high pH levels. One such preservative is sorbohydroxamic acid (SHA), an antimicrobial agent with a high pKa value (~9.0). However, little is known about the antimicrobial activity of SHA against *B. cereus* and other pathogenic and spoilage microorganisms of concern in high moisture bakery products.

2.1.1. Objectives

The objectives of this study were to determine the effect of pH and various concentrations of potassium sorbate and sorbohydroxamic acid, alone and in combination with each other, on the growth of *B. cereus* and other microorganisms of concern in high moisture bakery products in agar plate studies.

2.2. Materials and Methods

2.2.1. Microorganisms and inoculum preparation

Six microorganisms were used in this study – *B. cereus*, *B. subtilis* ATCC 6633, *S. enteriditis*, *L. monocytogenes*, *Saccharomyces cerevisiae*, and *Penicillium notatum*. These microorganisms were selected on the basis of their public health or their spoilage concern in high moisture bakery products. All microorganisms, with the exception of the yeast strain, were obtained from the culture collection of Dr. J.P. Smith (McGill University, Montreal, PQ, Canada). Cultures of *S. cerevisiae* were obtained from Ms. Barbara Butler, Department of Food Science and Agricultural Chemistry, McGill University. Both *B. cereus* and *B. subtilis* had been previously isolated from cereal based products (Lyver, 1997), while *S. enteriditis* and *L. monocytogenes* had been implicated in foodborne illnesses involving bakery products. *S. cerevisiae* and *P. notatum* were selected since they are common spoilage microorganisms associated with bakery products.

All bacteria were grown overnight in Tryptic Soy Broth (TSB) (Difco, Becton-Dickinson; Sparks, MD) at 30°C and then streaked onto Brain Heart Infusion (BHI) agar plates (Difco, Becton-Dickinson; Sparks, MD), incubated at 30°C for 24 h and checked for purity by examining their morphological characteristics on BHI plates and by Gram stains. Cultures were transferred every three weeks to ensure their viability.

A stock inoculum suspension of each strain was prepared by aseptically transferring a colony from BHI plates into 5 ml TSB supplemented with 0.6% yeast-extract (TSBYE) (Difco, Becton-Dickinson; Sparks, MD). All broths were incubated at 30°C for 24 h to give a stock suspension of ~10^9 CFU/ml. Appropriate dilutions of each stock suspension were made using 9 ml of sterile peptone water (0.1% w/v) to give a working suspension of 10^3 CFU/ml.

P. notatum was grown overnight on Potato Dextrose agar (PDA) (Difco, Becton-Dickinson; Sparks, MD) at 30°C and also transferred every three weeks to ensure viability. The mold inoculum was prepared by growing *P. notatum* on PDA at 30°C for 48 h. A stock mold suspension was prepared by washing spores from PDA plates with 9 ml of peptone water (0.1% w/v) and aseptically transferring them into a sterile test tube. Spores were enumerated using an Improved Neubauer Haemacytometer (Fisher Scientific, New Jersey, USA) and appropriate dilutions made, again using 9 ml of sterile peptone water (0.1% w/v), to give a working suspension of 10^3 spores/ml.

Stock and working yeast suspensions of *S. cerevisiae* were prepared in a similar manner to give a working suspension of 10^3 CFU/ml.

2.2.2. Preparation of test media plates

Two media (pH 5 to 9) were used throughout this study. Brain Heart Infusion (BHI) agar (Difco, Becton-Dickinson; Sparks, MD) was used as the test media for all bacteria, while Potato Dextrose agar (PDA, Difco, Becton-Dickinson; Sparks, MD) was used as the test media for yeast and mold growth. All media were prepared according to the manufacturer's instructions; however, distilled water was replaced with appropriate buffer solutions to achieve the desired pH. The pH of media was adjusted from 5 to 7, using citrate-phosphate buffer solution, and from pH 8 to 9, using TRIS buffer solution. All buffer solutions were prepared according to the method of Slowick and Kaplan (1955). Following pH adjustment, media were autoclaved (15

minutes at 121°C) and then cooled to 50°C in a water bath. Random samples were checked for pH using a previously calibrated pH meter (Model 220, Corning Glassworks, N.Y., USA) with an agal filled polymer body combined electrode with agal Ag/AgCl reference (Fisher Scientific, Model 13-620-104).

2.2.3. Chemical preservatives

Two chemical preservatives were used in this study, potassium sorbate (KS) (K & K Laboratories, Division of ICN Biomedicals Inc., Ohio, USA) and sorbohydroxamic acid (SHA) (Frinton Laboratories Inc., N.J., USA).

To monitor the activity of both KS and SHA, a UV absorption spectrum profile was performed both at pH 4.2 and 9.55 using the method described by Dudman (1963). Solutions of both KS and SHA (0.001% w/v) were made in distilled water and the pH adjusted to pH 4.20 using 6N HCl and to pH 9.55 using 6N KOH. The final pH of each solution was checked using a previously calibrated pH meter as described previously. The absorbance of each solution was monitored over a wavelength range of 220 nm to 320 nm using a spectrophotometer (Ultrospec 1000, Pharmacia Biotech., Cambridge, England).

2.2.4. Preparation of potassium sorbate (KS) and sorbohydroxamic acid (SHA)

A 10 % (w/v) stock solution of each preservative was prepared by dissolving 10 g of KS and SHA in 100 ml distilled water and stirring for ~10 min. The preservatives were then filter sterilized using a Seitz filtration unit (Nalge Company, Sybron International, N.Y., USA). Each preservative was stored in a dark bottle under refrigeration until use.

2.2.5. Effect of potassium sorbate (KS) and sorbohydroxamic acid (SHA) at various pH levels

Appropriate amounts of stock solution were added to each pH adjusted molten media (as described in section 2.2.2.) to give final concentrations ranging from 0 to 2000 ppm at 1000 ppm increments (Tables 17 and 18). The pH of the test media was again checked after the addition of preservatives and, where necessary, re-adjusted to pH 5, 6, 7, 8, and 9, using either 6N HCl (v/v) or 6N KOH (v/v) as shown in Table 19.

Table 17. Amount of KS stock solution (10% w/v) added to media to obtain final concentrations ranging from 0 to 2000ppm

Concentration (ppm)	Volume of KS (ml)	Volume of media (ml)
0	0	100
1000	1.0	99.0
2000	2.0	98.0

Table 18. Amount of SHA stock solution (10% w/v) added to media to obtain final concentrations ranging from 0 to 2000ppm

Concentration (ppm)	Volume of KS (ml)	Volume of media (ml)
0	0	100
1000	1.0	99.0
2000	2.0	98.0

Table 19. Levels of HCl or KOH used to adjust pH of media

Final pH	Volume of 6N HCl (ml)	Volume of 6N KOH (ml)
5	3.2	0
6	2.6	0
7	1.2	0
8	0.6	0
9	0	0.2

2.2.6. Microbial Analysis

All plates were inoculated, in duplicate, using a spread plate technique with 0.1 ml of each working suspension of test microorganism to give a final inoculum of 10^2 CFU/ml. Plates were incubated aerobically at 30°C and monitored daily up to 28 days for visible signs of growth. Growth was recorded on a scale of 0 to 5+, where 0 = no growth and 5+ = very heavy growth. The size of colonies (mm) was also measured at similar time intervals using a standard ruler.

Inoculum levels of each test suspension were confirmed by surface plating 0.1 ml of working suspension of each test microorganism onto appropriate agar plates. Plates were incubated aerobically at 30°C for 24 hrs and colonies were enumerated using a Darkfield Quebec Colony Counter (AO Scientific Instruments, Quebec, Canada). Counts were expressed as Colony Forming Units per ml (CFU/ml).

2.2.7. Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) for a randomized complete block design (Steel and Torrie, 1980). The software used for this study was StatGraphics Plus (version 3.1, 1997).

2.3. Results and Discussion

2.3.1. UV absorption spectra of KS and SHA

The UV absorption spectra of both KS and SHA (0.001% w/v), over a range of wavelengths (220 to 320 nm), are shown in Figures 3 and 4. The spectra of potassium sorbate had a single peak which increased to a maximum absorbance value at a wavelength of ~255 nm. The maximum absorbance values at pH 4.20 and pH 9.55 were 2.212 and 1.744, respectively. The spectrum of sorbohydroxamic acid at pH 4.20 was similar to that of KS, with a maximum absorbance value of 2.238 at a wavelength of ~255 nm. In alkaline solution, however, the spectrum of SHA showed a marked change, with a diminished maximum absorbance value of 1.719 at a wavelength of ~250 nm and the appearance of strong absorption in the ~270 to 320 nm regions. This change in the absorbance of SHA is due to the dissociation of SHA at pH ~8.8. Both absorption spectra of KS and SHA were in agreement with the spectra obtained by Dudman (1963) confirming the chemical activity of SHA at a wide pH range.

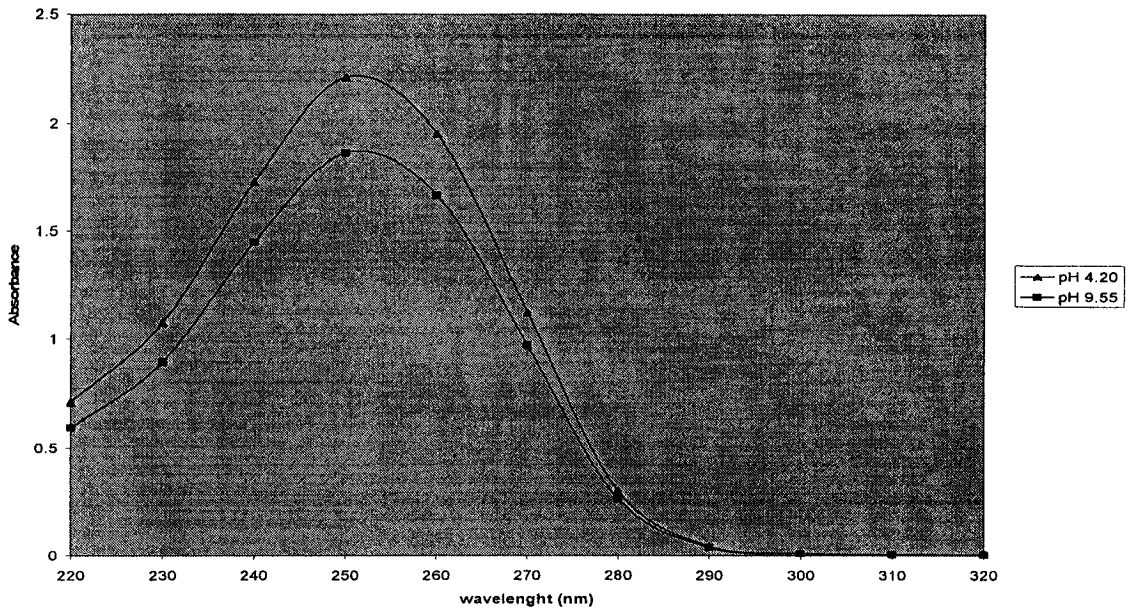


Figure 3. Ultraviolet absorption spectra of potassium sorbate in 0.001% (w/v) aqueous solution at pH 4.20 and 9.55

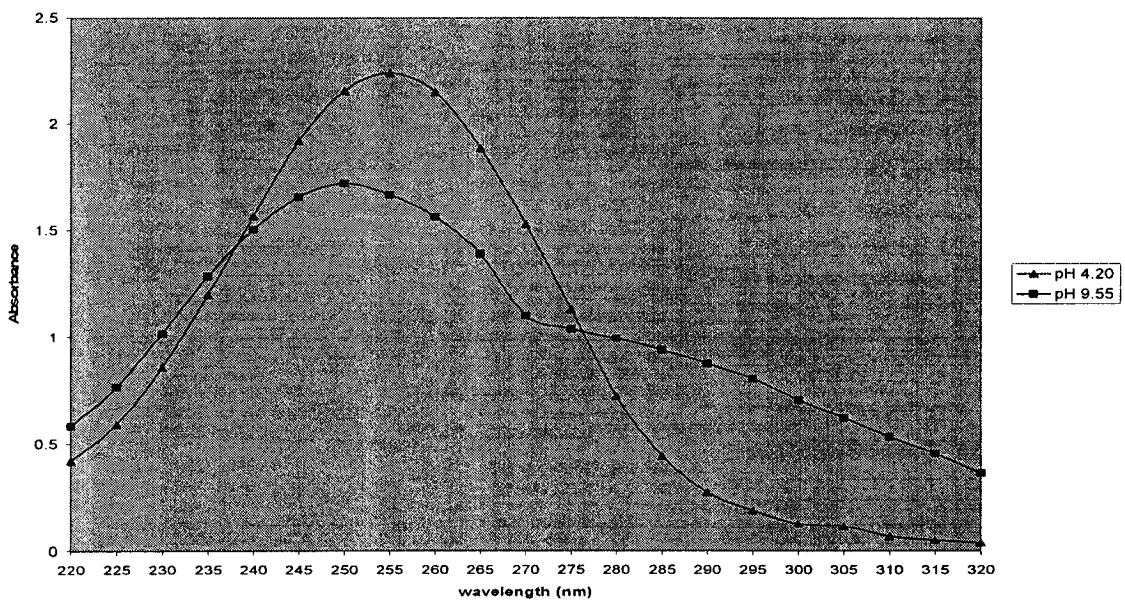


Figure 4. Ultraviolet absorption spectra of sorbohydroxamic acid in 0.001% (w/v) aqueous solution at pH 4.20 and 9.55

2.3.2. Antimicrobial effect of KS and SHA at various concentrations and pH levels

2.3.2.1. *Bacillus cereus*

The effect of both KS and SHA on the growth of *B. cereus* is shown in Table 20. Growth of *B. cereus* occurred in all control plates (0 ppm) after day 1 to 3 depending on the pH. Complete inhibition was obtained at all concentrations of KS ≥1000 ppm at pH 5 indicating an inhibitor effect on the growth of *B. cereus* (Table 20). At pH 7 and 9, ~1000 ppm of KS inhibited the growth of *B. cereus* for one day only. However, after day 1, growth of *B. cereus* was observed at both pH levels, regardless of the concentration of KS. This trend continued throughout 28 days storage at ambient storage (Table 20).

SHA proved to be a slightly more effective inhibitor against *B. cereus* at all concentrations and pH levels under investigation (Table 20). The results with higher levels of SHA at pH 7 were interesting. While growth of *B. cereus* was inhibited by 2000 ppm of SHA at this pH level for 3 days, slight growth occurred at day 7 (Table 20). Possible reasons for these results include (i) adaptation of *B. cereus* to these levels of pH and inhibitor or (ii) utilization of the inhibitor as an energy source for growth. However, complete inhibition was obtained at either pH 5 or 9 with 2000 ppm of SHA (Table 20). Furthermore, there was a significant difference between KS and SHA preservatives at 2000 ppm levels over all pH levels above 5 ($P < 0.001$). Therefore, while KS was only effective at levels >1000 ppm at pH 5, SHA could be used to control *B. cereus*, particularly at the 2000 ppm level over a much wider pH range. Thus, SHA has the potential to inhibit the growth of this important pathogen in both acid and alkaline bakery products.

The effect of both inhibitors on the colony diameter *B. cereus,* which is also an indicator of the inhibitory effect of preservatives, is shown in Table 20. Potassium sorbate had no effect on colony diameter at pH 7 and 9 with the diameter of *B. cereus* reaching a maximum of 7-8 mm by day 14. Sorbohydroxamic acid, however, at levels of ≥1000 ppm, resulted in a significant reduction in colony

diameter reaching a maximum diameter of 3-4 mm by day 14. These results again confirm the greater inhibitory effect of SHA against *B. cereus* compared to KS.

The results obtained in this study are in agreement with those of El-Khoury (2001) and Chorin *et al.* (1997) who reported that KS inhibited the growth of *B. cereus* but only at pH <5.5. It is difficult to compare the results with SHA to other studies since no other work has been conducted on the antimicrobial activity of this preservative against *B. cereus*.

Table 20. Effect of pH and preservative levels of KS and SHA on the growth of *B. cereus* in BHI plates incubated aerobically at 30°C

Preservative level (ppm)	pH	Days to visible growth	Extent of growth [a]	Colony diameter (mm)
KS				
0	5	3	1+	3-4
	7	1	5+	7-8
	9	1	5+	7-8
1000	5	>28	0	0
	7	1	5+	7-8
	9	1	5+	5-6
2000	5	>28	0	0
	7	1	5+	7-8
	9	1	5+	5-6
SHA				
0	5	3	1+	3-4
	7	1	5+	7-8
	9	1	5+	7-8
1000	5	>28	0	0
	7	1	1+	4-5
	9	1	1+	3-4
2000	5	>28	0	0
	7	7	1+	3-4
	9	>28	0	0

[a] 0 = no growth
1+ = slight growth
2+ = growth
3+ = moderate growth
4+ = extreme growth
5+ = very extreme growth

2.3.2.2. *Bacillus subtilis*

The effect of both KS and SHA on the growth of *B. subtilis* is shown in Table 21. Similar results to those obtained with *B. cereus* were observed for *B. subtilis*. Growth of *B. subtilis* was evident at all concentrations of KS and all pH levels, with the exception of pH 5.0. At this pH level, a level of 1000 ppm of KS would be required to inhibit the growth of this spoilage microorganism of concern in high moisture bakery products (Table 21). Sofos (1989) reported that KS is mostly effective at lower pH levels against molds and yeasts and has little or no effect on bacterial cells with the exception of *B. subtilis.* Our results are in agreement with these observations and clearly demonstrate that the antimicrobial effect of KS decreases as the pH of the medium increases, i.e., as it moves further from its dissociation constant (pKa 4.76).

An almost identical trend in the antimicrobial spectra of SHA against *B. cereus* was observed for *B. subtilis*. However, visible growth of *B. subtilis* was observed at day 3 with 2000 ppm of SHA compared to day 7 for *B. cereus* (Table 21). Complete inhibition of growth was again observed at 2000 ppm of SHA at pH 5 and 9 (Table 21). Again, there was a significant difference between KS and SHA preservatives at the 2000 ppm level and at all pH levels >5 ($P < 0.001$).

The effect of KS and SHA on the colony diameter of *B. subtilis* is shown in Table 21. Again, a similar trend was observed to *B. cereus* i.e., the colony diameter of *B. subtilis* in plates containing SHA was ~25% of the diameter of colonies in plates with KS, regardless of pH and concentration.

Table 21. Effect of pH and preservative levels of KS and SHA on the growth of *B. subtilis* in BHI plates incubated aerobically at 30°C

Preservative level (ppm)	pH	Days to visible growth	Extent of growth [a]	Colony diameter (mm)
KS				
0	5	1	2+	4-5
	7	1	5+	7-8
	9	1	5+	7-8
1000	5	>28	0	0
	7	1	4+	7-8
	9	1	5+	7-8
2000	5	>28	0	0
	7	1	4+	7-8
	9	1	5+	7-8
SHA				
0	5	1	2+	4-5
	7	1	4+	7-8
	9	1	5+	7-8
1000	5	>28	0	0
	7	1	1+	4-5
	9	1	1+	3-4
2000	5	>28	0	0
	7	3	1+	3-4
	9	>28	0	0

[a] 0 = no growth
1+ = slight growth
2+ = growth
3+ = moderate growth
4+ = extreme growth
5+ = very extreme growth

2.3.2.3. *Salmonella enteriditis*

The effect of both KS and SHA on the growth of *S. enteriditis* is shown in Table 22. The antimicrobial effect of KS on the growth of *S. enteriditis* was similar to the results obtained for *B. cereus* and *B. subtilis* at levels >1000 ppm and at pH 5 and 7 (Table 22). SHA, however, had a greater inhibitory effect on the growth of *S. enteriditis* at levels >1000 ppm and over all pH ranges under investigation (Table 22). There was also a significant difference between KS and SHA at levels >1000 ppm at all pH levels >5 ($P < 0.001$).

The effect of KS and SHA on the diameter of *S. enteriditis* colonies is shown in Table 22. Similar trends were observed for *Bacillus* spp i.e., KS was less effective than SHA. All colonies were approximately four times the diameter on plates containing KS (with the exception of pH 5.0) compared to plates containing SHA.

2.3.2.4. *Listeria monocytogenes*

While *L. monocytogenes* is not a concern in unfilled bakery products, it is of concern in meat, cheese and cream filled bakery products. Furthermore, since this is psychrotrophic pathogen, refrigeration alone cannot always be relied upon to ensure the safety of these products and additional barriers may need to be considered to ensure their safety, particularly under mild to moderate temperature abuse conditions. The effect of both KS and SHA on the growth of *L. monocytogenes* and their effect on colony size are shown in Table 23. Both KS and SHA were not as effective against *L. monocytogenes* compared to *S. enteritidis*. Growth of *L. monocytogenes* was evident in all plates containing 1000 ppm of KS and SHA regardless of pH. However at pH 5, 2000 ppm of KS inhibited the growth of *L. monocytogenes* for 28 d at ambient storage temperature (Table 23). Furthermore, at this pH, KS proved to be a more effective inhibitor on a w/w basis against *L. monocytogenes* than SHA, reversing the trend observed for other

microorganisms under investigation in this study. Both inhibitors had a similar effect on the colony diameter of *L. monocytogenes* (Table 23). These results are in partial agreement with El-Shenawy and Marth (1988) who reported that KS inhibited or inactivated the growth of *L. monocytogenes* in broth depending on pH and concentration. Therefore, KS would appear to the preservative of choice to control the potential growth of this pathogen in cream or meat-filled bakery products.

Table 22. Effect of pH and preservative levels of KS and SHA on the growth of *S. enteriditis* in BHI plates incubated aerobically at 30°C

Preservative level (ppm)	pH	Days to visible growth	Extent of growth [a]	Colony diameter (mm)
KS				
0	5	1	1+	3-4
	7	1	5+	7-8
	9	1	5+	7-8
1000	5	>28	0	0
	7	1	2+	3-4
	9	1	5+	5-6
2000	5	>28	0	0
	7	1	1+	3-4
	9	1	5+	5-6
SHA				
0	5	1	1+	3-4
	7	1	5+	7-8
	9	1	5+	7-8
1000	5	14	1+	2-3
	7	3	1+	2-3
	9	3	1+	2-3
2000	5	>28	0	0
	7	>28	0	0
	9	>28	0	0

[a] 0 = no growth
1+ = slight growth
2+ = growth
3+ = moderate growth
4+ = extreme growth
5+ = very extreme growth

Table 23. Effect of pH and preservative levels of KS and SHA on the growth of *L. monocytogenes* in BHI plates incubated aerobically at 30°C

Preservative level (ppm)	pH	Days to visible growth	Extent of growth [a]	Colony diameter (mm)
KS				
0	5	1	1+	1-2
	7	1	5+	5-6
	9	1	5+	5-6
1000	5	3	1+	1-2
	7	1	3+	3-4
	9	1	5+	5-6
2000	5	>28	0	0
	7	1	2+	3-4
	9	1	5+	5-6
SHA				
0	5	1	1+	1-2
	7	1	5+	7-8
	9	1	2+	7-8
1000	5	3	1+	1-2
	7	1	1+	4-5
	9	3	1+	1-2
2000	5	7	1+	1-2
	7	3	1+	1-2
	9	3	1+	1-2

[a] 0 = no growth
1+ = slight growth
2+ = growth
3+ = moderate growth
4+ = extreme growth
5+ = very extreme growth

2.3.2.5. *Saccharomyces cerevisiae*

The inhibitory effect of sorbic acid and its salts against a wide variety of spoilage yeasts has been well documented (Sofos, 1989). As a result of this antimycotic activity, sorbic acid and its salts have been used to control yeast spoilage problems in a variety of products fruit juices, wines, cottage cheese, dried fruits and meat and fish products (Sofos, 1989). They have also proved effective in low pH and/or intermediate a_w products such as carbonated beverages, salad dressings, syrups, tomato products, candy, jellies and chocolate syrup (Sofos, 1989). This study has again confirmed the antimycotic effect of KS against *S. cerevisiae* and shown that its effectiveness is both pH and concentration dependent (Table 24). However, SHA proved to be a much more effective yeast inhibitor at all concentrations investigated. There was a significant difference between KS and SHA preservatives at levels ≥1000 ppm and all pH levels >5 ($P < 0.001$) with complete inhibition of yeast growth being obtained by 1000ppm of SHA (Table 24). Similar trends were observed on the effect of KS and SHA on yeast diameter (Table 24). Based on these results, similar levels of KS and SHA could be used to inhibit growth of *S. cerevisiae* in acid pH products. However, SHA could be used to control yeast spoilage problems more effectively than KS in neutral or alkaline pH bakery products.

Table 24. Effect of pH and preservative levels of KS and SHA on the growth of *S. cerevisiae* in BHI plates incubated aerobically at 30°C

Preservative level (ppm)	pH	Days to visible growth	Extent of growth [a]	Colony diameter (mm)
KS				
0	5	1	5+	7-8
	7	1	5+	7-8
	9	1	5+	7-8
1000	5	>28	0	0
	7	1	3+	5-6
	9	1	5+	5-6
2000	5	>28	0	0
	7	1	5+	7-8
	9	1	5+	5-6
SHA				
0	5	1	5+	7-8
	7	1	5+	7-8
	9	1	5+	7-8
1000	5	>28	0	0
	7	>28	0	0
	9	>28	0	0
2000	5	>28	0	0
	7	>28	0	0
	9	>28	0	0

[a] 0 = no growth
1+ = slight growth
2+ = growth
3+ = moderate growth
4+ = extreme growth
5+ = very extreme growth

2.3.2.6. *Penicillium notatum*

Extensive research has also been done on the use of sorbic acid and its salts to control mold spoilage in food products (Sofos, 1989). As a result, sorbic acid and its potassium salt have been used to extend the mold free shelf life of cheeses, butter, sausages, fruits cakes, grains, bread and smoked fish (Sofos, 1989). This study also confirmed the antimycotic effect of KS and SHA against *P. notatum*, a mold of spoilage concern in many intermediate and high moisture bakery products (Table 25). Furthermore SHA was again more effective than KS at all concentrations and pH levels under investigation in this study on both visible growth and colony mold diameter (Table 25) i.e., similar to the results obtained for *S. cerevisiae*. Again, there was a significant difference between KS and SHA preservatives at levels >1000 ppm over all pH levels >5 ($P < 0.001$). The results obtained in this study are also in agreement with the observations of El-Khoury (2001) who observed no visible growth of *P. notatum* in broth containing 1000 ppm KS at pH 5.0 but extensive growth at pH 6.0. Moreover, the results obtained for *P. notatum* are in agreement with the study of Troller and Olsen (1967) on the inhibitory effect of SHA on mold growth in frostings over a wide pH range.

Table 25. Effect of pH and preservative levels of KS and SHA on the growth of *P. notatum* in BHI plates incubated aerobically at 30°C

Preservative level (ppm)	pH	Days to visible growth	Extent of growth [a]	Colony diameter (mm)
KS				
0	5	1	5+	7-8
	7	1	5+	7-8
	9	1	5+	7-8
1000	5	>28	0	0
	7	1	3+	5-6
	9	1	5+	7-8
2000	5	>28	0	0
	7	1	3+	5-6
	9	1	5+	5-6
SHA				
0	5	1	5+	7-8
	7	1	5+	7-8
	9	1	5+	7-8
1000	5	>28	0	0
	7	>28	0	0
	9	>28	0	0
2000	5	>28	0	0
	7	>28	0	0
	9	>28	0	0

[a] 0 = no growth
1+ = slight growth
2+ = growth
3+ = moderate growth
4+ = extreme growth
5+ = very extreme growth

2.4. Conclusion

In conclusion, this study has confirmed that KS is effective against a range of microorganisms of both spoilage and public health concern in bakery products. However, a major limitation of its use is in products that are formulated at pH <6. Furthermore, it confirmed that KS is more inhibitory towards yeasts and molds than bacteria. This study also demonstrated the potential of SHA to control microorganisms of spoilage and public health concern in high moisture bakery products over a wider range of pH (5 to 9) than KS, particularly against molds and yeasts (Table 26). Based on these preliminary studies, SHA could also be used at a level of 2000 ppm to control the growth of *B. cereus,* a pathogen of concern in high-a_w, high-pH English style crumpets. Furthermore, since these products are chemically leavened, this level of SHA would not affect the product volume or texture. However, further studies are required to determine the effect of such high levels of SHA on the sensory quality of crumpets.

Table 26. Minimal inhibitory concentration (MIC) of SHA to control the growth of selected microorganisms in BHI plates adjusted to pH 9 incubated aerobically at 30°C

Microorganism	SHA level (ppm)
B. cereus	**~2000**
B. subtilis	**~2000**
S. enteriditis	**~2000**
L. monocytogenes	**NI[a]**
S. cerevisiae	**~1000**
P. notatum	**~1000**

[a] NI = no inhibition

Chapter 3

Use of Mastic Essential Oil to Control the Growth of Selected Microorganisms *In Vitro*

This study examined the antimicrobial effect of MO against selected microorganisms of concern in high moisture products.

Chapter 3

Use of Mastic Essential Oil to Control the Growth of Selected Microorganisms *In Vitro*

3.1. Introduction

Plant essential oils and extracts have been used for a wide variety of purposes for thousands of years (Jones, 1996). These purposes range from the use of rosewood and cedarwood in perfumery, to flavouring drinks with lime, fennel or juniper berry oil (Lawless, 1995), and the use of lemongrass oil for the preservation of stored food crops (Mishra and Dubey, 1994). The antimicrobial activity of plant essential oils and extracts has also formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Reynolds, 1996; Lis-Balchin and Deans, 1997). However, there is little quantitative data on the antimicrobial activity of most plant extracts (Davidson and Branen, 1993).

According to Hargreaves *et al.* (1975), essential oils are defined as "a group of odorous compounds, soluble in alcohol and to a limited extent in water, consisting of a mixture of esters, aldehydes, ketones, and terpenes". Among the compounds having a broad antimicrobial spectra are thymol from thyme and oregano, cinnamic aldehyde from cinnamon, and eugenol from cloves (Beuchat and Golden, 1989). Another plant oil which has been shown to have antimicrobial activity against several foodborne bacteria is mastic essential oil (Tassou and Nychas, 1995; Iauk *et al.*, 1996). Mastic essential oil has also been demonstrated to be effective against *Helicobacter pylori,* a bacterium responsible for causing peptic ulcers (Huwez and Thirwell, 1998). Thus, mastic essential oil has the

potential to control the growth of bacteria and warrants investigation as a novel method to inhibit the growth of *B. cereus* in high moisture bakery products.

Ethanol is well known as a powerful bactericidal agent at high levels (60-75%); however, at lower levels (2-20%), ethanol can also be used as an antimicrobial agent. Seiler (1989) evaluated the usefulness of food-grade ethyl alcohol to extend the shelf-life of bakery products. Results showed that ethanol was a powerful vapor phase inhibitor. Extensions in shelf-life of bakery products by ethanol were dependent on a_w of the product, permeability and integrity of the packaging film (Seiler, 1989).

A novel and innovative method of generating ethanol vapor is through the use of interactive sachets, such as Ethicap™. Originating in Japan, Ethicap™ sachets contain food-grade ethyl alcohol microencapsulated into silicon dioxide powder. When placed alongside food, ethanol is slowly released into the package headspace. Ethicap™ sachets are widely used to extend the shelf life of food in Asian markets and are commonly used to extent the mold-free and stale-free shelf life of bakery products (Takahashi *et al.*, 1984).

Recently El-Khoury (2001) investigated Ethicap™ to delay the growth of *B. cereus* in agar plate studies. While such an approach proved effective, it required high levels of ethanol in the package headspace (~ 8% v/v) to control the growth of this pathogen. It is well known that one or more barriers acting in synergy with each other is a far more effective approach to control microbial growth than using each barrier separately. This "hurdle" approach is widely used by the food industry to extend both the shelf life and keeping quality of foods. Therefore, the combined effect of mastic and ethanol may be a novel approach to control spoilage and pathogenic microorganisms of concern in high moisture bakery products.

3.1.1. Objectives

The objectives of this study were to determine the effect of mastic essential oil, alone or in combination with ethanol, on the growth of *B. cereus* and other selected microorganisms of concern in high moisture bakery products, in model agar systems.

3.2. Materials and Methods

3.2.1. Microbial and chemical analysis of mastic essential oil

The mastic essential oil used in this study was obtained from the Mastic Growers Association of Chios (Chios, Greece). Two of the major components of mastic essential oil, α-pinene and myrcene, were obtained from Sigma-Aldrich (Oakville, Ontario). Prior to its use, mastic oil was subjected to microbial and chemical analysis to ensure purity.

In the microbiological analysis, decimal dilutions of oil were prepared in 0.1% peptone water, mixed by vortex for 10 seconds and, immediately, aliquots (0.1 ml) were spread onto Brain Heart Infusion (BHI) agar in duplicate (Becton-Dickinson; Sparks, MD). For aerobic plate counts, plates were incubated aerobically at 30°C for 24–48 h. Anaerobic plate counts were done by placing plates in an anaerobic jar with a H_2 gas pack (BBL Microbiology Systems, Becton and Dickerson & Co., Cockeysville, Maryland, USA) and incubating at 30°C for 24-48 h.

The chemical composition of the commercial mastic essential oil was analyzed by Dr. Yaylayan (Department of Food Science and Agricultural Chemistry, McGill University) using a Varian CP-3800 gas chromatograph coupled to a Saturn 2000 mass selective detector. The column used was a fused silica DB-5MS (50m length x 0.2 mm i.d. x 33 um film thickness; J&W Scientific, ON). The GC column flow rate was maintained at constant flow of 1.5 ml/min.

Capillary direct MS interface temperature was set at 250°C and the ion trap temperature at 175°C. The ionization voltage of 70eV was used and EMV was set at 1750V. The GC oven initial temperature was set at -35°C for 10.30 min. and then increased to 50°C at a rate of 50°C/min.; after that the temperature was increased to 250°C at a rate of 8°C/min. and maintained at 250°C for 5 minutes. Liquid samples (0.2 μL) of mastic essential oil dissolved in ethanol were mixed with Silica gel (Merck, grade 60) prior to pyrolysis at 250°C for 20 seconds and concentration on SPT under the same conditions. The area of each peak was estimated using Varian software SatView (version 5.52). Peaks were identified from a library search of their retention times using the Sat View database (version 5.52).

3.2.2. Microorganisms and inoculum preparation

The microorganisms selected for this study were based on their potential to cause foodborne illness or spoilage in bakery products. The microorganisms used were - *B. cereus* FN 13, *B. subtilis* ATCC 6633, *S. enteriditis*, *L. monocytogenes*, *S. cerevisiae*, and *P. notatum*. The inoculum and working suspensions of each microorganism was prepared as described in section 2.2.1. All inoculum levels were checked by surface plating appropriate serial dilutions of each test microorganism onto agar plates using a spread plate technique. Plates were incubated aerobically at 30°C for 24-48 h, and colonies enumerated using a Darkfield Quebec Colony Counter (AO Scientific Instruments, Quebec, Canada).

3.2.3. Antimicrobial activity of mastic essential oil

The antimicrobial activity of mastic essential oil was tested by (i) direct methods of application (DMA) and (ii) indirect methods of application (IMA).

3.2.3.1. Direct Methods of Application

Two methods of DMA were investigated: (i) the agar diffusion test, (ii) the micro-dilution MIC resazurin assay.

3.2.3.1.1. Agar diffusion test

In the agar diffusion test, two media were used. Brain Heart Infusion (BHI) agar (Difco, Becton-Dickinson; Sparks, MD) was used for all bacteria, while Potato Dextrose agar (PDA) (Difco, Michigan, USA) was used for yeast and mold growth. The pH of the media was adjusted to ~ pH 8 using TRIS buffer solution. All media were prepared as described in the section 2.2.2 and plates inoculated, in duplicate, using a spread plate technique with 0.1 ml of each test microorganism to give a final inoculum of ~10^5 CFU/ml.

The solutions tested by this technique included mastic essential oil, mastic-ethanol (ME) solution (1% v/v) made by diluting 0.1 ml mastic oil with 9.9 ml food grade ethanol (95%), water-ethanol (WE) solution (1% v/v), and food grade ethanol (95% v/v). To test the antimicrobial activity of each of these solutions, wells were punctured in the agar using a sterile pipette tip. Then, 10 µl of each solution was dispensed into the wells which then diffused throughout the agar setting up a concentration gradient. Plates were incubated aerobically for 24 - 48 h at 30°C. Inhibition was evident as zones of no growth around the wells and was measured (mm) using a standard ruler.

3.2.3.1.2. Micro-dilution MIC resazurin assay

In the micro-dilution MIC resazurin assay, only bacteria were tested in this study by the procedure outlined by Mann and Markham (1998) since this method is not suitable for yeasts and molds. Serial dilutions of the selected bacteria were done in BHI broth to obtain working suspensions of 10^1 to 10^7 CFU/ml. Then, 1.7

ml of the various dilutions of each test microorganism was transferred aseptically into test tubes. To these tubes, 0.2 ml "sloppy" agar and 0.1 ml resazurin dye solution were added. Test tubes were then incubated at 37°C for 2 h and checked for color change. The lowest inoculum level of each bacteria that reduced the resazurin dye to a pink color was selected as the working inoculum level for the micro-dilution MIC resazurin assay. For most bacteria this was ~10^5 CFU/ml with the exception of *L. monocytogenes* which was ~10^6 CFU/ml. This inoculum was then subsequently diluted tenfold to obtain the desired level to be used in the assay.

In this assay, 96 well microtiter plates were used (Sero-Wel®, Bibby Sterilin Ltd., Stone, Staffs, UK). Serial, twofold dilutions of the mastic oil (MO) and ME solution were done in "sloppy" agar in the last row of the microtiter plate to give final concentrations of test solution ranging from 0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5, and 1%. Then, 170 µl of the final inoculum of each test microorganism was transferred aseptically to the first 7 rows containing 8 wells/per row (total of 56 wells). Twenty µl of each concentration of MO and ME solutions was then added to the first 7 rows, giving 8 replicates per test condition. Row 8 was left empty to separate test and control conditions. Row 9 was used as the positive control (170 µl of the final inoculum of each test bacteria + 20 µl "sloppy agar"). Rows 10 and 11 were used as the negative control and blank, respectively, by adding 190 µl "sloppy agar" to each well. All wells were mixed with a sterile pipette tip and incubated at 37°C for 3.5 h. Then, 10 µl resazurin dye (ICN Biochemicals Inc., Aurora, Ohio) solution was added to rows 1 to 10 while in row 11, 10 µl of distilled sterile water was added. After mixing, the microtiter plates were incubated for further 2 h at 37°C. Separate microtiter plates were used for each bacteria. The plates were then visually inspected for color change and the row where all wells remained blue was taken as the MIC. These wells, along with the adjacent rows were enumerated by plating serial dilutions, in duplicate, on Brain Heart Infusion (BHI) agar. Plates were incubated aerobically at 30°C for 24 – 48 h (*L. monocytogenes* for 48 h). All counts were done using a

Darkfield Quebec Colony Counter (AO Scientific Instruments, Quebec, Canada) and expressed as CFU/ml.

3.2.3.2. Indirect Methods of Application

In the indirect method of application (IMA), appropriate amounts of mastic-ethanol (ME) or water-ethanol (WE) solutions were applied to cotton pads. The solutions of mastic essential oil and ethanol were prepared by adding specific volumes of mastic essential oil to 95% food grade ethanol in sterile 250 ml Erlenmeyer flasks (Table 27). Flasks were then closed with rubber stoppers and mixed by inversion for ~1 min. Control solutions of distilled water and 95% food grade ethanol (WE) were also prepared in a similar manner (Table 28). All solutions were prepared fresh and used immediately.

Appropriate amounts (0 to 1 g in 0.2 g increments and 2 g) of each prepared ME and WE solutions were transferred aseptically to pre-weighed (1 g) circular cotton pads (5 cm x 5 cm) obtained from Jean Coutu (Monteal, Quebec).

In the IMA study, two media were again used. Brain Heart Infusion (BHI) agar (Difco, Becton-Dickinson; Sparks, MD) was used for all bacteria, while Potato Dextrose agar (PDA, Difco, Becton-Dickinson; Sparks, MD) was used for yeast and mold growth. Plates were prepared and inoculated with 0.1 ml of each test microorganism to give a final inoculum level of 10^2 CFU/ml as described previously (section 2.2.2). Plates (1 plate/ bag) were then inserted into 210 mm X 210 mm high gas barrier Cryovac bags (Cryovac Sealed Air Corporation, Mississauga, Ontario) and metallized bags (Vapor-Loc VF-52 Zipper, Mississauga, Ontario). Cotton pads were then inserted and taped to the inside of the bags that were then sealed using an impulse heat sealer (Model KM100-3M, Wolfertschwenden, Germany). Plates were incubated aerobically at 30°C and monitored daily for up to 28 days for visible growth. Growth was recorded on a scale of 0 to 5+, where 0 = no growth and 5+ = very heavy growth. When visible

growth was observed, the size of colonies was measured (mm) using a standard ruler.

Table 27. ME solutions used for indirect methods of application

Solution (ratio)	Concentration (% v/v)	Mastic oil (ml)	Ethanol (95% v/v) (ml)
Mastic	100	10	0
5:1	83.3	8.3	1.7
4:1	80	8.0	2.0
3:1	75	7.5	2.5
2:1	66.7	6.7	3.3
1:1	50	5.0	5.0

Table 28. WE solutions used for indirect methods of application

Solution (ratio)	Concentration (% v/v)	Water (ml)	Ethanol (95% v/v) (ml)
Ethanol	100	0	10
5:1	83.3	8.3	1.7
4:1	80	8.0	2.0
3:1	75	7.5	2.5
2:1	66.7	6.7	3.3
1:1	50	5.0	5.0

3.2.4. Preparation of vapor generator sachets

Based on the results of the IMA study, it was shown that 1 g of a 1:1 ME solution gave the most consistent results. However, in this study the cotton pads were inserted directly into bags. This study was done to determine if incorporating the cotton pads into sachets would effect their antimicrobial activity. Three types of material were used to prepare sachets: (i) laminated paper (commercially available Ethicap™ sachets), (ii) perforated cellophane (typically used for baguettes), and (iii) bleached paper (tea bags).

The Ethicap™ sachets were prepared by cutting the top side of a 1G sachet (Freund Industrial Co. Ltd., Tokyo, Japan) and emptying its contents that were subsequently used as matrix material for the ME and WE solutions. Cellophane sachets were prepared by cutting two square 5 cm x 5 cm pieces of perforated cellophane film (Provigo, Baie d'Urfé) and heat-sealing three sides using an impulse heat sealer (Model KM100-3M, Wolfertschwenden, Germany). Finally, tea bag sachets were prepared by cutting the top side of 5 cm X 5 cm square tea bags (Tetley, Lipton, Canada) and emptying their contents. A 5 cm x 5 cm circular cotton pad (pre-weighed and adjusted to 1 g to ensure a constant amount of matrix material) was folded in half and inserted into pre-prepared empty sachets. Then, 1 g of the ME and WE solutions (1:1) were added to the cotton pads (Tables 27 and 28). Sachets were sealed using a impulse heat-sealer.

In addition, silica dioxide was also tested as a carrier matrix for the ME and WE solutions. The silica dioxide powder obtained from the Ethicap™ sachets was placed in an open Petri plate overnight to evaporate the adsorbed ethanol. The next day, the silica was microwaved for two 30 sec. intervals to drive off any residual ethanol and left to cool. Empty Ethicap™ sachets were then filled with 1g of "dry" silica, the appropriate amounts of ME and WE solutions were added and the sachets sealed as described previously.

Control and inoculated plates were packaged by placing plates (1 plate/bag) into high gas barrier Cryovac and metallized bags. ME and WE sachets were then inserted into the appropriate bags and taped to the inside to ensure the sachets did not make contact with the plates and to ensure a free flow of air surrounding each sachet. All bags were sealed using an impulse heat sealer (Model KM100-3M, Wolfertschwenden, Germany) and stored at 30°C. Plates were incubated aerobically at 30°C and monitored for visible growth on a daily basis for 28 days. Growth was recorded on a scale of 0 to 5+, where 0 =no growth and 5+ = very heavy growth. When visible growth was noticeable, the size of colonies was measured (mm) using a standard ruler.

3.2.5. Preparation of standard curves

Standard curves of ME and WE solutions were made from stock solutions of mastic essential oil and food grade ethanol (95% v/v). Standard solutions of ME (2, 4, 6, 8, 10, 15% (v/v)) were prepared from a stock solution of mastic essential oil and diluted with an appropriate volume of ethanol (Table 29). Similarly, WE standard solutions of 1, 3, 5, 7, 10, 15, 20, 25, 30, 40, 50% (v/v) were prepared from distilled water and ethanol (Table 30). Appropriate volumes of each standard solution were placed in 15 ml test tubes and overwrapped with parafilm, capped, and stored under refrigerated conditions until use to minimize volatilization of mastic volatiles and ethanol (Tables 29 and 30). Prior to measuring headspace volatiles, a septum was placed on the parafilm and the standard solutions were left at room temperature for ~ 24 h for the headspace to equilibrate. The result of the average of 3 injections of 0.5 ml of each standard solution using a Pressure-Lok® syringe (Baton Rouge, Louisiana, USA), were used to generate the standard curves of area versus mastic volatiles or ethanol concentration as shown in Figures 8 and 9.

3.2.6. Headspace analysis

Changes in headspace mastic essential oil volatiles and ethanol were monitored using a Varian gas chromatograph fitted with a flame ionization detector (FID), and a Nukol column (30 M x 0.53 mm) (Supelco, Canada Ltd.). Helium was used as the carrier gas at a flow rate of 30 ml/min. The column temperature at 60°C and the injector port at 100°C. Resultant peaks of mastic essential oil volatiles and ethanol were recorded on a Hewlett Packard integrator and the concentration of each was determined from the appropriate standard curves (Figures 8 and 9).

Differences in headspace composition over time were measured by GC/MS by Dr. Yaylayan (McGill University) as outlined in section 3.2.1. Headspace volatiles were manually withdrawn from pouches (5 x 1 mL) with a gas tight-precision syringe (Series A-2, Supelco, ON), 5 ml in total from each pouch and injected into the GC for concentration on a Sample Preconcentration Trap (SPT - Tenax GR) (adsorption at -35°C, desorption at 200°C) prior to separation on the GC column. Peaks were again identified from a library search of their retention times using the Sat View database (version 5.52).

Table 29. Mastic-Ethanol (ME) standard solutions used to prepare the headspace volatile standard curve

Concentration (% v/v)	Mastic oil (ml)	Ethanol (95% v/v) (ml)
0	0	10
2	0.2	9.8
4	0.4	9.6
6	0.6	9.4
8	0.8	9.2
10	1.0	9.0
15	1.5	8.5

Table 30. Water-Ethanol (WE) standard solutions used to prepare the headspace volatile standard curve

Concentration (% v/v)	Water (ml)	Ethanol (95% v/v) (ml)
0	0	10
1	0.1	9.9
3	0.3	9.7
5	0.5	9.5
7	0.7	9.3
10	1.0	9.0
15	1.5	8.5
20	2.0	8.0
25	2.5	7.5
30	3.0	7.0
40	4.0	6.0
50	5.0	5.0

3.2.7. Oxygen transmission rates (OTR) measurements

To measure the oxygen transmission rate (OTR) of the bags used in this study, a standard method (ASTMD 3985-81) was used, with 100% O_2 as the permeant gas. The OTR of the test film was measured by cutting a square of uniform size (5 cm x 5 cm) and placing it directly in the test cell. A 25 μm (1 mil) thick piece of Mylar (polyester) was used as a standard to measure any leakage and to determine any correction factor. All films were conditioned at the test temperature and a 100% relative humidity for 24 to 48 h prior to testing. For the OTR test, an Oxtran 2/20 Master (Mocon, Minnesota, USA) was used with an Oxtran 2/20 software package to monitor all the phases of testing, including entering test conditions (parameters), monitoring tests, and printing reports. Once the parameters were set, the computer controlled the components, gathered and logged data, printed all data in the form of tables and charts. Both cells of the Oxtran 2/20 are divided into chambers separated by the test film. Oxygen was passed through the upper chamber and humidified carrier gas (98% nitrogen and 25 hydrogen) passed through the bottom chamber to sweep the permeant gas to the sensor. A scanning automatic valve sent the sample gas, oxygen, to a specific coulometric sensor detector. The detector gave a current output directly proportional to the rate of oxygen arrival at the sensor. Therefore, the oxygen flux across the film was dynamically measured, the OTR expressed as $cc/m^2/day$ at 0% RH.

3.2.8. Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) for a randomized complete block design (Steel and Torrie, 1980). The software used for this study was StatGraphics Plus (version 3.1, 1997).

3.3. Results and Discussion

3.3.1. Results of microbial and chemical analyses of mastic essential oil

The microbial and chemical purity of the mastic essential oil (MO) used in this study was analyzed.

For the microbiological analysis, no growth occurred in any of the plates incubated under aerobic and anaerobic conditions, indicating that mastic essential oil was "sterile" and would not be a source of microbial contamination.

The chemical composition of mastic essential oil was analyzed and its chemical components identified (Table 31). Nine major peaks that correspond to MO appeared after ~32 to 34 minutes (Table 31). These major peaks of MO were identified according to library search as β-pinene, myrcene, ocimene, α-pinene, camphene, bicyclo (3.1.0) hex-ene, 4-methylene 1-(1-methyl ethyl), β-phellandrine, bicyclo (3.1.1) heptane, 6, 6-trimethyl-2-methylene-(1S), and limonene. Six of these peaks are in accordance with the chemical composition of the essential oil extracted from mastic resin and analyzed by Melanitou *et al.* (1995). The differences in chemical composition of MO are probably due to the more sensitive detection methods used in our department to analyse the oil. In addition, all the major peaks corresponded to the analysis performed by the supplier of the MO to determine its purity and quality.

Table 31. Mastic essential oil components identified using GC/MS

Peak number	Retention time (min)	Identified component
1	32.10	β-pinene
2	32.15	myrcene
3	32.35	ocimene
4	32.50	α-pinene
5	32.75	camphene
6	32.80	bicyclo (3-1-0) hex-2-ene, 4-methylene 1-(1-methylethyl)
7	33.15	β-phellandrine
8	33.35	bicyclo (3.1.1) heptane 6,6-trimethyl-2-methylene (1S)
9	34.40	limonene

3.3.2. Agar Diffusion test

The effect of mastic oil (MO), mastic-ethanol (ME), water-ethanol (WE) and food grade ethanol (95% v/v) solutions on the growth of selected microorganisms was investigated by the agar diffusion test.

MO had a negligible effect on the growth of most microorganisms with zones of inhibition ranging from 0-3 mm (Table 32). However, when the mastic essential oil was dissolved in ethanol (ME, 1% v/v), its antimicrobial effect was enhanced against most microorganisms tested with the exception of *L. monocytogenes* (Table 32). The bacteria tested were slightly more resistant to ME than yeasts and molds, as shown by the smaller zones of inhibition that ranged from 0-18 mm. Both *S. cerevisiae* and *P. notatum* were very sensitive to ME with zones of inhibition of 20 and 22 mm, respectively (Table 32). Thus, ME (1% v/v) has the potential to control the growth of most spoilage and pathogenic microorganisms of concern in bakery products.

The difference in the inhibitory activity between MO and ME is probably due to the greater diffusion of ME throughout the agar. Bennett *et al.* (1966) and Barry (1986) also found that the size of the zones of inhibition were dependent upon the rates of diffusion of essential oils throughout the test medium. Davidson and Parish (1989) showed that the agar diffusion test cannot be used to determine the antimicrobial activity of highly hydrophobic antimicrobial substances due to this diffusion problem. This lack of diffusivity can be overcome by the addition of surfactants or increasing the agar content of the medium. Another possibility is to dissolve the oil in ethanol thereby increasing its hydrophilic to hydrophobic ratio which facilitates its diffusion throughout the medium.

The WE solution (1% v/v) failed to inhibit the growth of the any of the microorganisms tested probably due to the dilution of ethanol in water (Table 32). However, ethanol (95% v/v) alone had a slight inhibitory effect against *S.*

enteritidis, *S. cerevisiae*, and *P. notatum* but not for any of the other microorganisms (Table 32). Therefore, while MO and ethanol alone had a slight inhibitory effect on most microorganisms tested, this antimicrobial activity was enhanced when the MO was dissolved in ethanol. The synergistic effect of ME against *B. cereus* and *S. cerevisiae* is demonstrated in Figures 5a, b. Furthermore, the inhibitory activity of the ME solution was significantly greater ($P < 0.001$) than all other solutions tested.

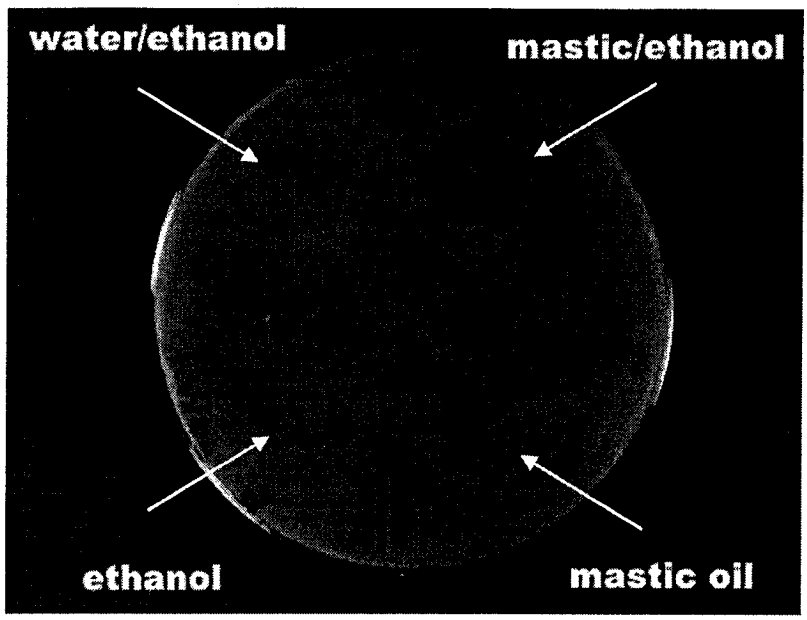
Table 32. Effect of MO, ME, WE and ethanol solutions on the growth of selected microorganisms by the agar diffusion test

Test Solution (10µl)	Zone of inhibition (mm) [a, b]					
	BC[c]	BS	LM	SE	SC	PN
MO	2	2	0	1	3	3
ME (1% v/v)	18	16	0	15	20	22
WE (1% v/v)	0	0	0	0	0	0
Ethanol (95% v/v)	0	0	0	1	2	2

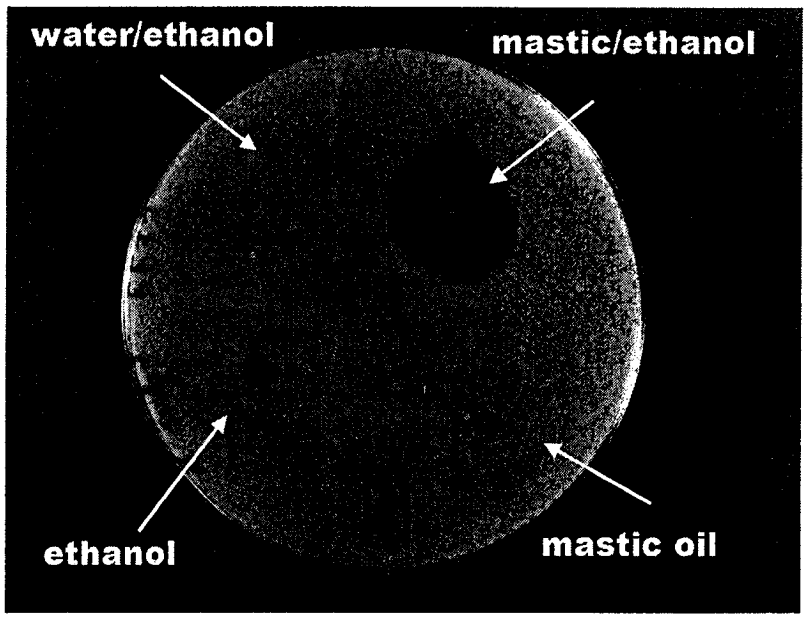
[a] average of duplicate samples

[b] inoculum (10^5 CFU/ml)

[c] BC = *B. cereus*
BS = *B. subtilis*
LM = *L. monocytogenes*
SE = *S. enteriditis*
SC = *S. cerevisiae*
PN = *P. notatum*



(a)



(b)

Figure 5. Agar diffusion test of 10 µl of various solutions (i) MO, (ii) ME (1% v/v), (iii) WE (1% v/v), and (iv) ethanol (95% v/v) on the growth of (a) *B. cereus* and (b) *S. cerevisiae* in BHI plates (10^5 CFU/ml)

3.3.3. Micro-dilution MIC resazurin assay

The antimicrobial effectiveness of a compound is often described in terms of its minimum inhibitory concentration (MIC). MIC has been defined as "the lowest concentration of the compound capable of inhibiting the growth of the challenging microorganism" (Mann and Markham, 1998).

A limitation of diffusion assays is that they cannot be used with accuracy to determine the MIC of hydrophobic substances such as essential oils, due to the partitioning of the oil throughout the agar (Southwell *et al.*, 1993). To overcome this problem, the assay can be modified by the addition of a chemically and microbially inert substance ("sloppy" agar) which acts as both an emulsifier and stabilizer to the added essential oil. This modification of the assay enables the MIC of essential oils to be determined more accurately.

The MIC of various concentrations of MO and ME solutions against selected bacteria (*B. cereus*, *B. subtilis*, *L. monocytogenes*, *S. enteriditis*) was determined using the assay developed by Mann and Markham (1998). The MIC of both MO and ME against the bacteria ranged from 0.125 to 0.5%. Growth of *Bacillus* spp. was inhibited by both MO and ME solutions (0.125% v/v) (Table 33, Figure 6). However, *L. monocytogenes* required an MIC of 0.5 % (v/v) (Table 33). This MIC for *L. monocytogenes* is in agreement with the observations of Del Campo *et al.* (2000) who studied the effect of rosemary extract, which has a similar chemical composition to MO, on the growth of this pathogen. However, Griffin and Markham (2000) reported that *B. cereus* and *B. subtilis* required an MIC of 0.3% (v/v) of tea tree oil compared to an MIC of 0.125% (v/v) used in this study. The results of this study are also in agreement with the observations of Mann and Markham (1998) who found that the micro-dilution resazurin assay was slightly more sensitive than the agar dilution assay for all microorganisms tested.

The counts of each test microorganism, at their respective MIC, are shown in Table 33. While the initial counts of each microorganism were at ~10^5 CFU/ml, counts at their MICs were ~10^3 CFU/ml (i.e., a 2 log reduction). These results are in agreement with Del Campo *et al.* (2000) who observed a 2-3 log reduction in microbial counts using rosemary extract. Based on these results, the sensitivity of the test microorganisms to MO and ME were BC and BS > SE > LM. Statistical analysis also showed that their was no significant difference between the MIC of the MO and ME solutions ($P < 0.05$).

The results of this present study disagree with the observations of Deans and Ritchie (1987) who reported no difference in the sensitivity of Gram positive and Gram negative bacteria to essential oils. However, our results are in partial agreement with the observations of Chao *et al.* (2000), Tassou and Nychas (1995), and Shelef *et al.* (1980) who reported that Gram positive bacteria were more sensitive to essential oils than Gram negative bacteria.

These results are also in partial agreement with the agar diffusion test which showed that *L. monocytogenes* was the most resistant microorganism to MO and ME solutions. Based on the MIC results, it is ~2-4 times more resistant to both MO and ME solutions than the other bacteria tested. However, based on the MIC, a concentration of ~0.5% (v/v) of both MO and ME solutions should inhibit the growth of this pathogen. Such an inhibition was not observed in the agar diffusion test even when higher levels (1% v/v) of both MO and ME solutions were dispensed into agar wells. The difference in the results between these two tests for *L. monocytogenes* can be attributed to a greater dispersion of the essential oil solutions throughout the medium in the micro-dilution MIC resazurin assay. Nevertheless, both tests confirm that *L. monocytogenes* is a difficult microorganism to control using MO and ME solutions by direct methods of application. Thus, indirect method of application of these solutions warrants investigation.

Table 33. Minimum inhibitory concentration (MIC) of MO and ME solutions by the micro-dilution resazurin assay

Solution	MIC [a] (% v/v)				Counts [c, d] (Log CFU/ml)			
	BC[b]	BS	LM	SE	BC[b]	BS	LM	SE
MO	0.125	0.125	0.5	0.25	3.1± 0.3	3.2± 0.2	2.8± 0.4	3.2± 0.2
ME	0.125	0.125	0.5	0.25	3.3± 0.2	3.2± 0.3	3.0± 0.3	3.1± 0.3

[a] Eight replicates of each bacteria

[b] BC = *B. cereus*
BS = *B. subtilis*
LM = *L. monocytogenes*
SE = *S. enteriditis*

[c] Inoculated with log 5.1 ± 0.3 CFU/ml

[d] Number of bacteria in broth containing MIC after 24h at 30°C ± SD calculated from 8 replicates

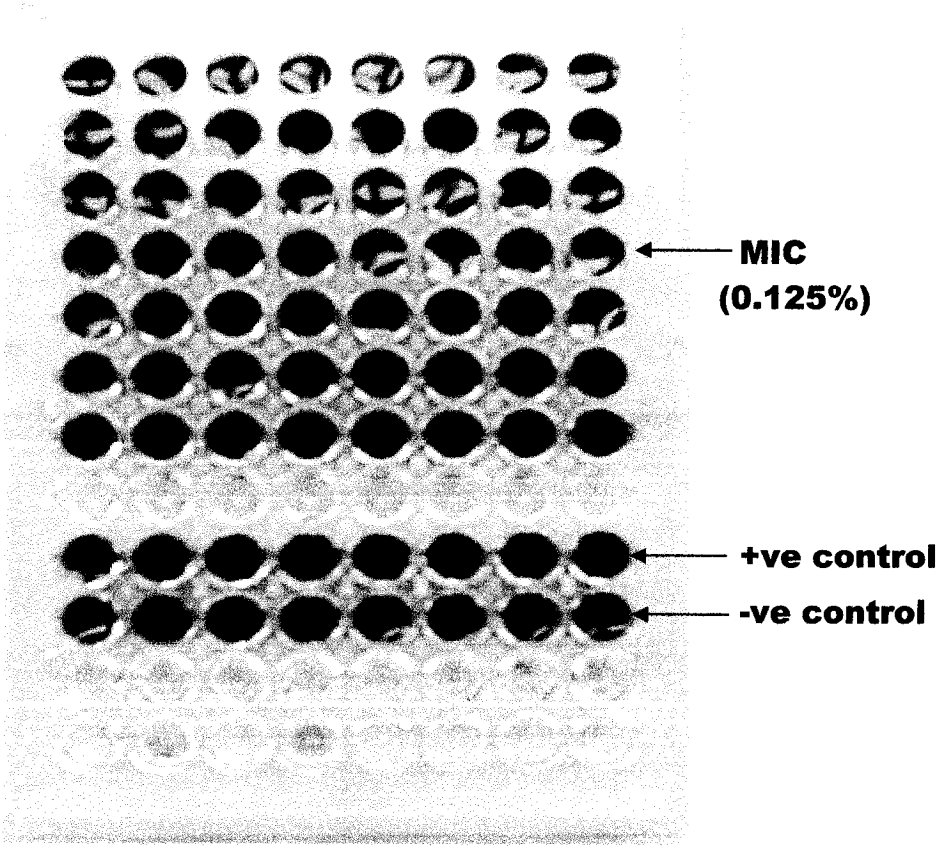


Figure 6. Micro-dilution MIC resazurin assay for MO against *B. cereus*

3.3.4. Indirect methods of application against *B. cereus*

The effect of various weights of several ratios of ME and WE solutions (1:1 to 5:1 ratio) applied to cotton pads and packaged in Cryovac and metallized bags, on the growth of *B. cereus* only is shown in Tables 34 and 35. This microorganism was selected to determine the optimum level of ME solution to control the growth of this pathogen which has been involved in several foodborne disease outbreaks involving high moisture bakery products.

Growth of *B. cereus* occurred at day 1 in all control plates and in all plates packaged in Cryovac bags with most levels of MO and ME (Tables 34 and 35). However, growth was delayed for 3 days in pads contained 2 g of MO (Tables 34 and 35). Similar results were obtained for the various ratios of ME solutions (3:1, 4:1, and 5:1) applied to cotton pads with growth of *B. cereus* occurring after 1-4 days (SD ± 1.4). However, growth was delayed for ~12 days (SD ± 10.5) in plates packaged alongside pads containing 1 and 2 g of ME solutions (2:1 and 1:1).

However, when WE solutions were applied to pads, growth of *B. cereus* occurred in all plates packaged in Cryovac bags after 1-2 days (SD ± 1.2) regardless of the weight or ratio used (Table 34).

The antimicrobial effect of the various ratios of ME and WE solutions applied to cotton pads on the growth of *B. cereus* in plates packaged in metallized bags is shown in Table 35. While growth of *B. cereus* occurred after 1 day for most ratios of MO and ME, it was delayed for >28 days when 2 g of the ME solutions was added to cotton pads regardless of the ratio used (Table 35). Furthermore, growth was delayed for >28 days using 1 and 2g of a 2:1 ratio of ME or 0.8 g of a 1:1 solution applied to pads.

All WE solutions again failed to inhibit the growth of *B. cereus* for more than 3 days in plates packaged in metallized bags regardless of the weights or ratios used. Statistical analysis showed that there was a significant difference ($P < 0.001$) between the antimicrobial effect of MO and ME and the type of

packaging material used. These studies confirm the synergy between MO and ethanol on the growth of *B. cereus*. Furthermore, they emphasize the importance of the packaging film for consistency of results. Based on these results, a 1:1 ME solution was selected for further investigation to control the growth of other microorganisms of spoilage and pathogenic concern in high moisture bakery products.

Table 34. Effect of MO, ME and WE solutions applied to cotton pads on the growth of *B. cereus*[a] in BHI plates packaged in high gas barrier Cryovac bags and incubated at 30°C

Solution	Days to visible growth [b]						
	Weight (g)						
	0	0.2	0.4	0.6	0.8	1	2
ME							
MO	1	1	1	1	1	1	3
5:1	1	1	1	1	1	1	3
4:1	1	1	1	1	1	1	4[c]
3:1	1	1	1	1	1	1	4[c]
2:1	1	1	1	1	3	12[d]	12[d]
1:1	1	1	1	1	12[d]	12[d]	12[d]
WE							
5:1	1	1	1	1	1	1	1
4:1	1	1	1	1	1	1	1
3:1	1	1	1	1	1	1	1
2:1	1	1	1	1	1	2[e]	2[e]
1:1	1	1	1	1	2[e]	2[e]	2[e]
Control	1	1	1	1	1	1	1

[a] Inoculum 10^2 CFU/ml
[b] Average of 4 samples
[c] Average of number of days to growth and SD ± 1.4
[d] Average of number of days to growth and SD ± 10.5
[e] Average of number of days to growth and SD ± 1.2

Table 35. Effect of MO, ME and WE solutions applied to cotton pads on the growth of *B. cereus*[a] in BHI plates packaged in metallized bags and incubated at 30°C

Solution	Days to visible growth [b]						
	Weight (g)						
	0	0.2	0.4	0.6	0.8	1	2
ME							
MO	1	1	1	1	1	1	3
5:1	1	1	1	1	1	1	>28
4:1	1	1	1	1	1	1	>28
3:1	1	1	1	1	1	1	>28
2:1	1	1	1	1	3	>28	>28
1:1	1	1	1	3	>28	>28	>28
WE							
5:1	1	1	1	1	1	1	1
4:1	1	1	1	1	1	1	1
3:1	1	1	1	1	1	1	1
2:1	1	1	1	1	1	3	3
1:1	1	1	1	1	3	3	3
Control	1	1	1	1	1	1	1

[a] inoculum 10^2 CFU/ml
[b] Average of 4 samples

3.3.5. Indirect methods of application against other selected microorganisms of concern in high moisture bakery products

Based on the initial study, this study was done to determine the antimicrobial effect of 1 g of a 1:1 solution of both ME and WE applied to cotton pads on the growth of microorganisms of concern in high moisture bakery products. The microorganisms tested were *B. cereus* FN 13, *B. subtilis, L. monocytogenes*, *S. enteritidis*, *S. cerevisiae*, and *P. notatum.*

The solutions were applied to pre-weighed cotton pads (1 g), inserted and taped in high gas barrier Cryovac and metallized bags containing plates inoculated with 10^2 CFU/ml of the microorganisms selected (Table 36). The time to visible growth was recorded and the size (mm) of the colonies at the time of growth was recorded.

For air packaged plates in Cryovac bags, growth of all test microorganisms occurred after 1 day. However, the ME solution (1:1) delayed the growth of all microorganisms for ~12-15 days (SD ± 2.8) again with the exception of *L. monocytogenes* which was visible after day 1 (Table 36). However, for plates packaged alongside pads containing 1 g of WE solution, growth of most microorganisms was observed after 2-3 days (SD ± 1.4) with the exception of *L. monocytogenes* (Table 36). The size of colonies at the time of visible growth, ranged from 1+ to 3+ (2-5 mm) for most bacteria and 3+ to 4+ (5-8 mm) for most yeast and mold colonies (Table 36).

For all air packaged plates in metallized bags, visible growth was again observed after 1 day (i.e., similar to Cryovac bags). However, the type of packaging material influenced the antimicrobial effect of the ME pads. Growth of all test microorganisms, with the exception of *L. monocytogenes,* was inhibited for >28 days when ME pads were inserted into metallized bags (Table 36).

Table 36. Effect of ME and WE solutions (1 g of 1:1 ratio) applied to cotton pads on the growth of selected microorganisms[a] on BHI plates packaged in Cryovac and metallized bags and incubated at 30°C

Packaging Treatment	Days to visible growth						Colony size at day of growth (mm)[e]					
	BC[d]	BS	LM	SE	SC	PN	BC	BS	LM	SE	SC	PN
Cryovac bags												
ME	12[b]	12[b]	1	12[b]	14	14	2+	2+	1+	2+	3+	3+
WE	2[c]	2[c]	1	2[c]	3[b]	3[b]	3+	3+	1+	3+	3+	3+
Air	1	1	1	1	1	1	3+	3+	2+	3+	4+	4+
Metallized bags												
ME	>28	>28	3	>28	>28	>28	0	0	1+	0	0	0
WE	3	3	1	3	5	5	3+	3+	1+	2+	3+	3+
Air	1	1	1	1	1	1	3+	3+	2+	3+	4+	4+

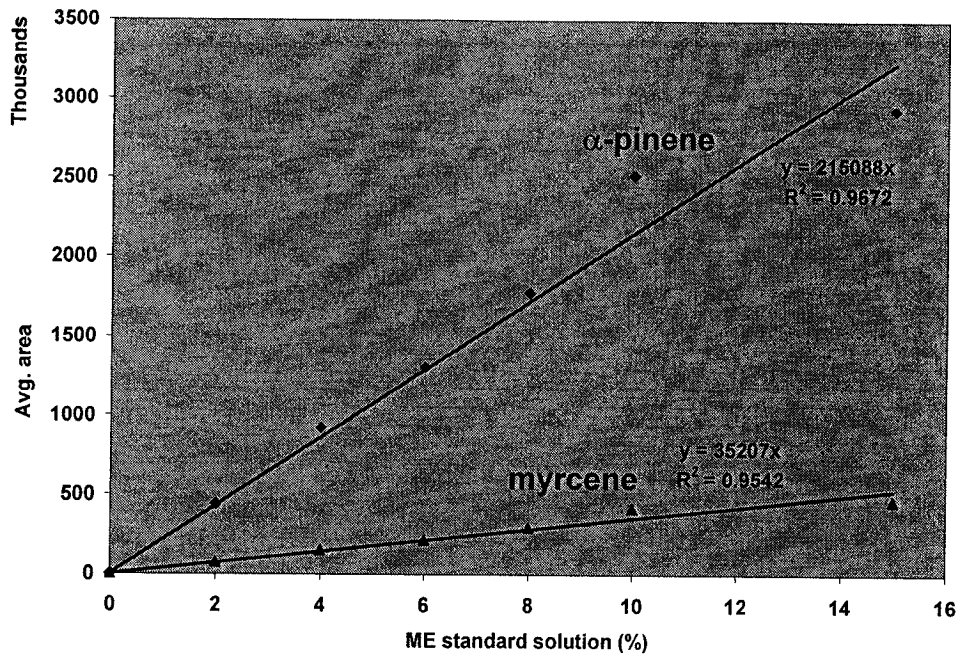
[a] Average of 2 samples, inoculum 10^2 CFU/ml
[b, c] Average of number of days to growth and SD ± 2.8, SD ± 1.4, respectively
[d] BC: *B. cereus*, BS: *B. subtilis*, LM: *L. monocytogenes*, SE: *S. enteriditis*, SC: *S. cerevisiae*, PN: *P. notatum*
[e] 0 = no growth, 1+ = 1-2 mm, 2+ = 3-4 mm, 3+ = 5-6 mm, 4+ = 7-8 mm, 5+ = >8 mm

The packaging material also had a slight effect on the antimicrobial activity of WE solutions applied to pads, as the growth of most microorganisms was delayed for ~3-5 days, again with the exception of *L. monocytogenes* (Table 36). The size of colonies on most plates, with the exception of plates packaged with ME pads, were similar to the results observed in plates packaged in Cryovac bags (Table 36).

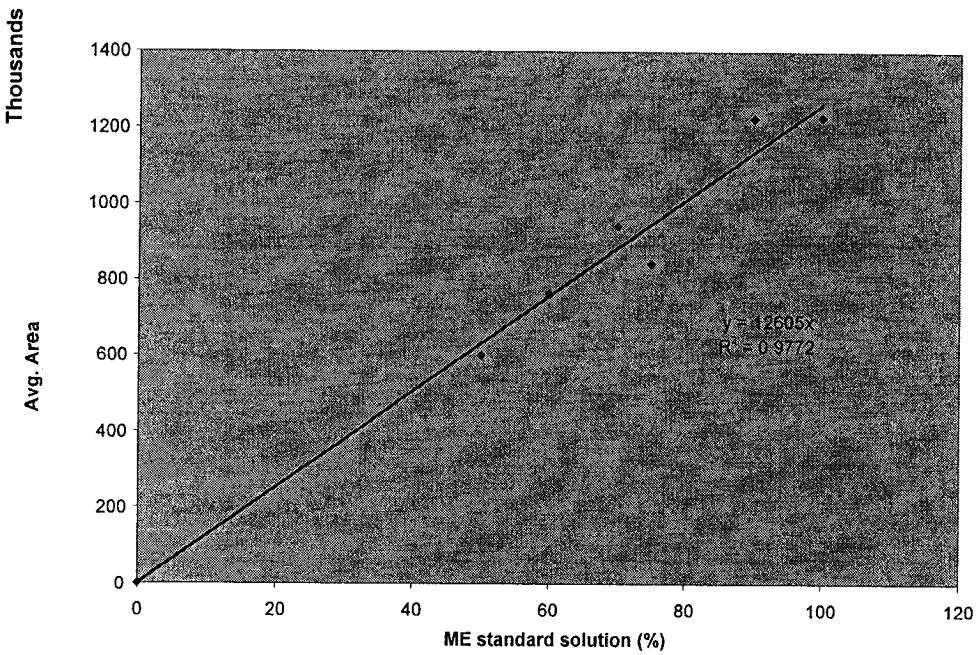
Headspace ethanol for plates packaged with pads containing ME and WE solutions was measured from standard curves shown in Figures 7 and 8, respectively. Ethanol gave one resultant peak with a retention time of 2.34 minutes. This is in agreement with studies conducted by El-Khoury (2001). Headspace mastic volatiles were measured from the ME standard curve (Figure 7) which resulted in two peaks with retention times of 3.25 and 6.45 minutes, respectively. These peaks were subsequently identified as α-pinene and myrcene i.e., major components of mastic essential oil. In all standard curves there was a highly significant R^2 value (~0.95 to 0.98) indicating the accuracy of fit of these curves to measure headspace ethanol and mastic volatiles.

Headspace ethanol, which was only measured at day 28 in all packages is shown in Table 37. No headspace ethanol was detected in any air packaged plates with the exception of plates inoculated with *S. cerevisiae*. In these bags, headspace ethanol ranged from ~0.3-0.5% (v/v) depending on the packaging material and can be attributed to yeast growth and metabolism. Similar results have been observed in yeast leavened crumpets packaged in air. For plates packaged with ME and WE pads in Cryovac bags, headspace ethanol ranged from ~1.2-1.8% (v/v) (Table 37). However, no significant difference ($P < 0.05$) was found between the levels of headspace ethanol generated by ME and WE pads.

For plates packaged under similar conditions in metallized bags, headspace ethanol levels were slightly higher reaching a maximum ~2.2% (v/v)



(a)



(b)

Figure 7. Standard curve of headspace (a) α-pinene and myrcene, and (b) ethanol of ME standard solutions

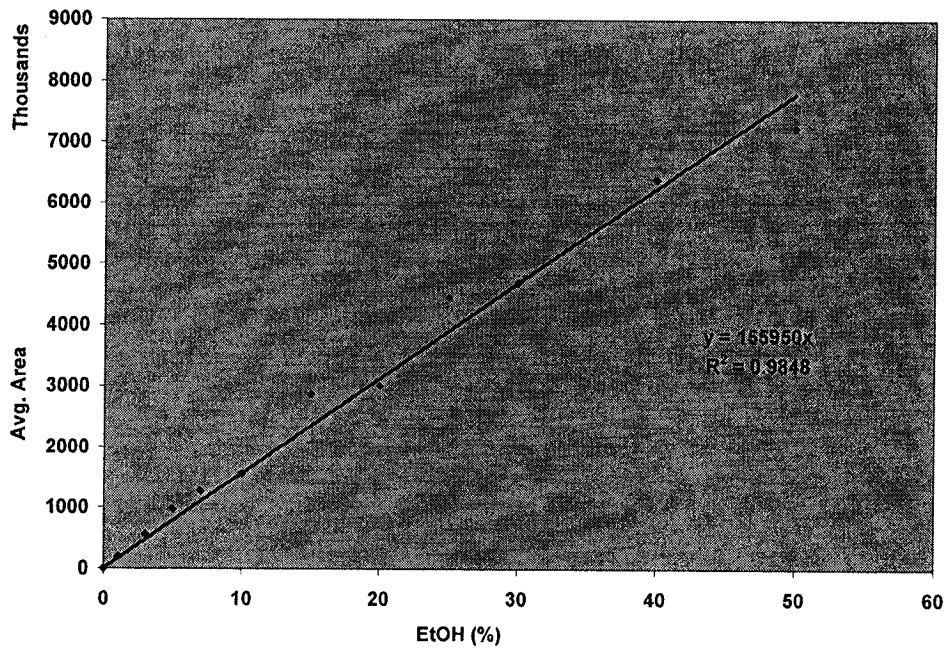


Figure 8. Standard curve of headspace ethanol of water-ethanol (WE) standard solutions

Table 37. Headspace ethanol, α-pinene and myrcene of ME and headspace ethanol of WE solutions (1 g of 1:1 ratio) applied to cotton pads on the growth of selected microorganisms[a] on BHI plates packaged in Cryovac and metallized bags and incubated at 30°C

Packaging Treatment	Headspace ethanol at day 28 (% v/v)						Headspace α-pinene at day 28 (% v/v)						Headspace myrcene at day 28 (% v/v)					
	BC[d]	BS	LM	SE	SC	PN	BC	BS	LM	SE	SC	PN	BC	BS	LM	SE	SC	PN
Cryovac bags																		
ME	1.2	1.3	1.3	1.5	1.5	1.4	0.9	1.0	1.0	1.2	1.2	1.1	0	0	0	0	0	0
WE	1.8	1.6	1.6	1.7	1.6	1.6	0	0	0	0	0	0	0	0	0	0	0	0
Air	0	0	0	0	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Metallized bags																		
ME	2.1	2.2	2.1	2.0	2.2	2.1	0	0	0	0	0	0	0.25	0.26	0.25	0.24	0.26	0.25
WE	2.4	2.4	2.3	2.2	2.6	2.5	0	0	0	0	0	0	0	0	0	0	0	0
Air	0	0	0	0	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Average of 2 samples, inoculum 10^2 CFU/ml
[b] BC: *B. cereus*, BS: *B. subtilis*, LM: *L. monocytogenes*, SE: *S. enteriditis*, SC: *S. cerevisiae*, PN: *P. notatum*

for the ME pads and ~2.4% (v/v) for the WE pads i.e., a difference of ~1% v/v between the Cryovac and metallized packages. Interestingly while α-pinene was detectable in the headspace of plates packaged in Cryovac bags, myrcene was not found. However, the opposite trend was observed in the headspace of plates packaged in metallized bags (Table 37). Furthermore, there was a significant difference ($P < 0.001$) between headspace ethanol and mastic volatiles in plates packaged in metallized bags compared to Cryovac bags.

These differences in levels of headspace volatiles were attributed to changes in the OTRs of the packaging materials with time (Figure 9). The OTR of the reference sample (Mylar) was ~60 cc/m2/day and this was within specification for the Mocon Oxtran. The initial OTRs of the Cryovac and metallized bags were ~12 and ~0 cc/m^2/day at 30°C, respectively. However, the final OTR of the Cryovac bags packaged with ME and WE pads was ~90 and ~18 cc/m^2/day at 30°C after 28 days, respectively. These results confirm that the ME had a greater plasticizing effect than WE pads on the Cryovac film as shown by a more pronounced decrease in its barrier properties and hence increase in OTR. However, OTRs of the metallized bags did not change over time indicating that the mastic volatiles or ethanol had no effect on the barrier characteristics of this packaging film.

In conclusion, the results of this study confirm that the type of packaging material influences the antimicrobial effect of ME pads. Furthermore they confirm that the antimicrobial effect of ME solutions is due to the MO dissolved in ethanol and not ethanol *per se*. These results also confirm our initial studies that the optimal inhibitory concentration of ME against most test microorganisms is 1 g of a 1:1 ME solution.

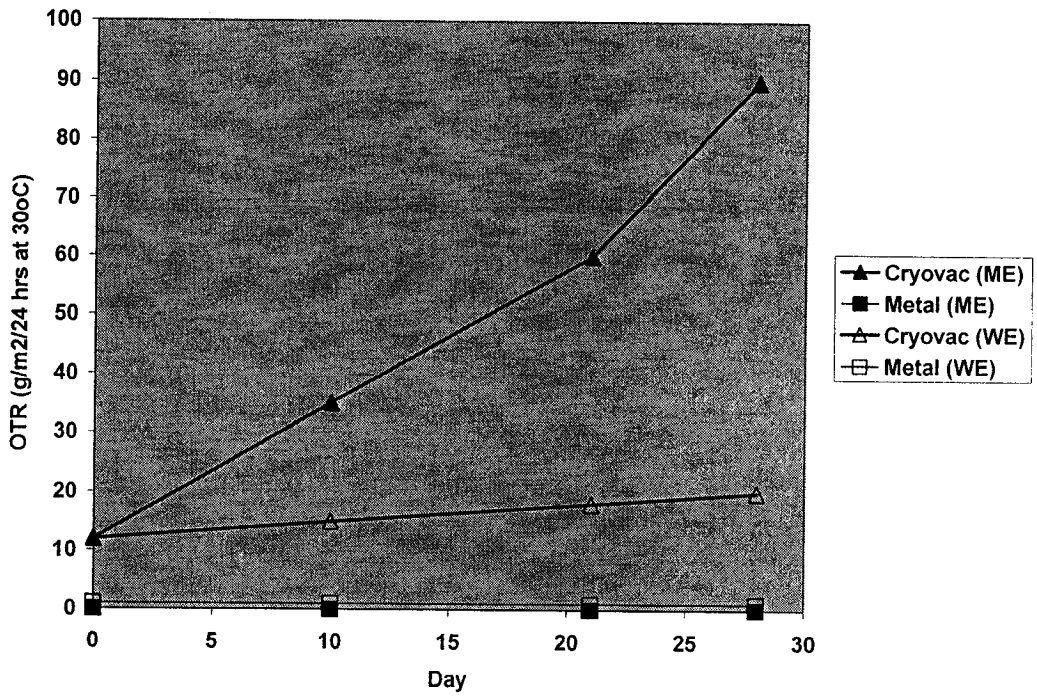


Figure 9. Oxygen transmission rates (OTR) of packaging materials used in vapor phase inhibition studies

3.3.6. Effect of interactive ME and WE sachets on the growth of selected microorganisms

In all previous studies, the ME and WE solutions were applied to cotton pads inserted and taped inside Cryovac and metallized bags. However, the contents of commercial interactive sachets do not come into direct contact with the food product. Thus, several types of sachet material were investigated to contain the ME and WE cotton pads. These included (i) laminated paper (commercially available Ethicap™ sachets), (ii) perforated cellophane (typically used for bagettes), and (iii) bleached paper (tea bags).

Growth of all test microorganisms occurred after 3-15 days or 3-28 days (Table 38) when 1 g of ME (1:1) was added to cotton pads and taped to the inside of Cryovac and metallized bags, respectively, confirming the results of previous studies. However, when pads were incorporated into various sachets, only the bleached paper sachets (tea bags) gave results similar to the free cotton pads taped to the inside of bags. In all other cases, pads inserted into laminated paper (Ethicap) and perforated cellophane sachets failed to inhibit the growth of test microorganisms.

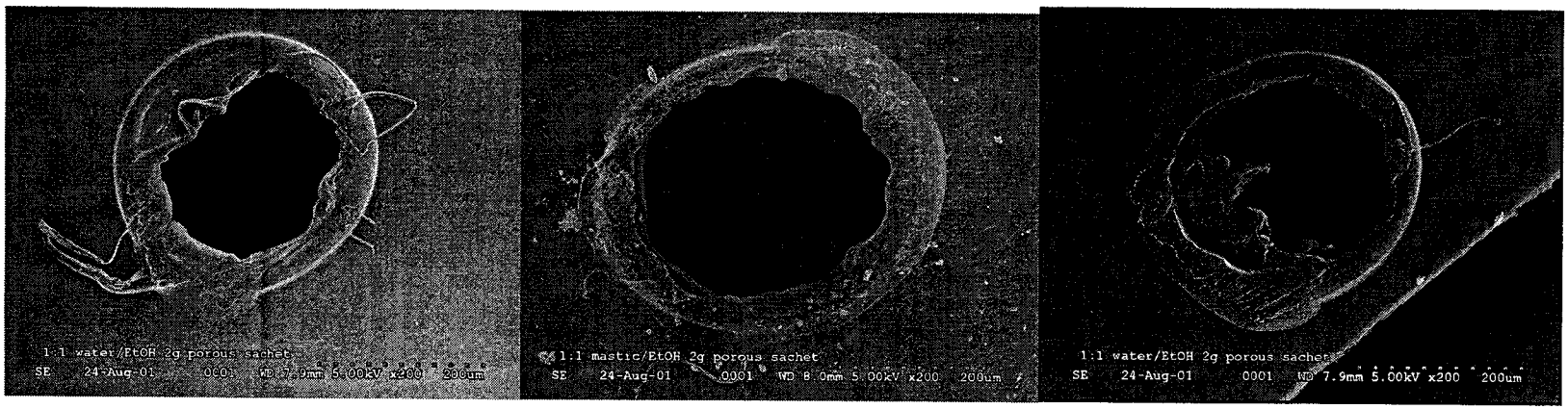
It was hypothesized that the mastic volatiles may have been trapped or clogged the perforations of the sachet materials containing a plastic component. However, electron microscopy scans of the various sachet materials (courtesy of Dr. John Austin, Health Canada) failed to confirm this hypothesis (Figure 10). Nevertheless, there was a significant difference ($P < 0.001$) in the antimicrobial effect of cotton pads alone or inserted into bleached paper sachets (tea bags) compared to the other sachet types. These results demonstrate the importance of the type of sachet material surrounding the cotton pad on the efficacy of the ME solution towards the test microorganisms.

Table 38. Effect of interactive ME and WE sachets on the growth of selected microorganisms[a] on BHI plates, packaged in Cryovac and metallized bags and incubated at 30°C

Packaging Treatment	Days to visible growth						Colony size at day of growth (mm)						Headspace ethanol at day 28 (% v/v)					
	BC	BS	LM	SE	SC	PN	BC	BS	LM	SE	SC	PN	BC	BS	LM	SE	SC	PN
Cryovac bags																		
Cotton	12[b]	12[b]	3[b]	12[b]	14	14	2+	2+	1+	2+	3+	3+	1.8	1.7	1.3	1.7	1.8	1.6
Cotton/ethicap	2[c]	2[c]	1	2[c]	3[b]	3[b]	2+	2+	1+	2+	3+	3+	1.3	1.2	1.4	1.1	1.4	1.2
Cotton/cellophane	2[c]	2[c]	1	2[c]	2[c]	2[c]	2+	2+	1+	2+	3+	3+	1.1	1.6	1.3	1.4	1.2	1.2
Cotton/tea bags	14	14	3[b]	14	14	14	2+	2+	1+	2+	3+	3+	1.6	1.7	1.3	1.5	1.5	1.6
Silica/ethicap	1	1	1	1	1	1	2+	2+	1+	2+	3+	3+	1.2	1.0	1.1	1.3	1.2	1.2
Metallized bags																		
Cotton	>28	>28	3	>28	>28	>28	0	0	1+	0	0	0	2.2	2.0	2.3	2.2	2.3	2.2
Cotton/ethicap	1	1	1	1	1	1	2+	2+	1+	2+	3+	3+	1.8	1.7	1.5	1.7	1.8	1.8
Cotton/cellophane	1	1	1	1	1	1	2+	2+	2+	2+	3+	3+	1.6	1.3	1.6	1.5	1.3	1.4
Cotton/tea bags	>28	>28	3	>28	>28	>28	0	0	1+	0	0	0	2.2	1.9	2.3	2.2	2.3	1.9
Silica/ethicap	1	1	1	1	1	1	3+	3+	2+	2+	3+	3+	1.9	2.0	2.1	2.2	1.8	2.0

[a] Average of 2 samples, inoculum 10^2 CFU/ml
[b, c] Average of number of days to growth and SD ± 2.8, SD ± 1.4, respectively
[d] BC: *B. cereus*, BS: *B. subtilis*, LM: *L. monocytogenes*, SE: *S. enteriditis*, SC: *S. cerevisiae*, PN: *P. notatum*
[e] 0 = no growth, 1+ = 1-2 mm, 2+ = 3-4 mm, 3+ = 5-6 mm, 4+ = 7-8 mm, 5+ = >8 mm

(a)



(b)

Figure 10. Electron scan microscopy of control, ME and WE sachets made from (a) porous cellophane and (b) bleached paper

Headspace ethanol was measured at day 28 in all packages. For plates packaged in Cryovac and metallized bags, headspace ethanol levels ranged from ~1.0 to 1.8% (v/v) and ~1.3 to 2.3% (v/v), respectively. Furthermore, there was a significant difference ($P < 0.001$) in headspace ethanol concentration between sachets packaged in Cryovac bags compared to metallized bags. This difference can again be attributed to changes in the OTR of the packaging materials with time (Figure 9).

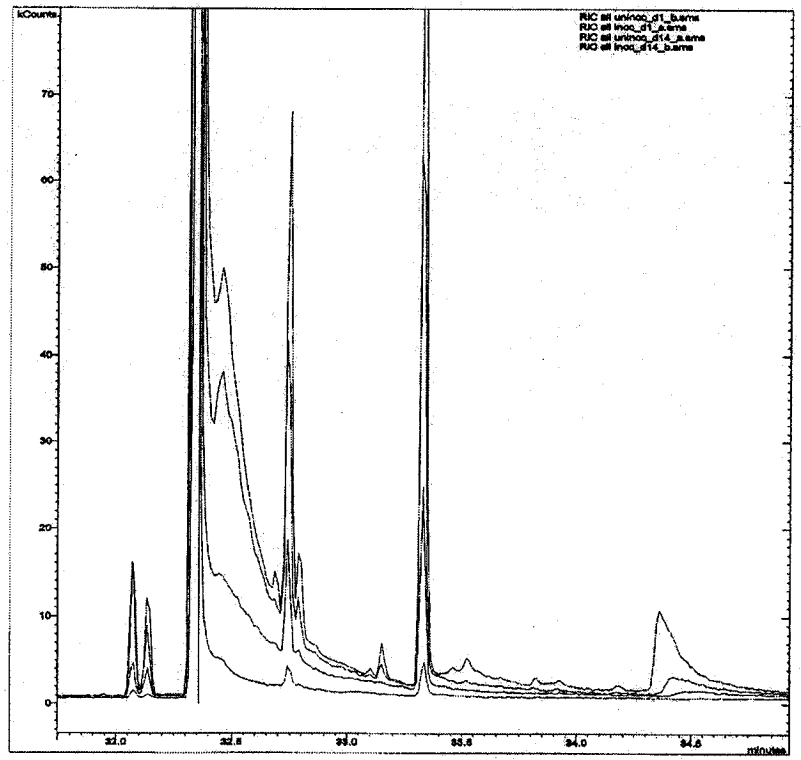
This study showed that the interactive sachets made cotton pads with 1 g of a ME solution (1:1) and inserted into bleached paper tea bags gave the most consistent results. Therefore, changes in headspace mastic volatiles of these sachets for both control and plates inoculated with *B. cereus* (10^2 CFU/ml) packaged in both Cryovac and metallized bags were monitored over a 28 days (Figure 11 and Table 39).

The main components identified were ß-pinene, myrcene, ocimene, α-pinene, camphene, bicyclo(3-1-1)heptane 6, 6-trimethyl-2-methylene (BCHM), and limonene. These peaks make up about 84% of the volatile components of mastic oil identified by Wylie et al. (1990). Changes in headspace mastic volatiles of the ME interactive sachets placed inside Cryovac and metallized bags are shown in Table 39.

Headspace volatiles for ME sachets packaged in Cryovac bags changed dramatically during storage. All volatile components, with the exception of α-pinene, disappeared completely in both control and inoculated plates (Table 39). However, at the end of 28 days, ~6.5% and 2.8% of α–pinene remained in the package headspace.

Headspace ME volatiles also decreased in plates packaged for metallized bags, however a different trend was observed to Cryovac bags (Table 39 and Figure 11). While, a 100% reduction in headspace α-pinene was observed, all other components were still present, albeit at reduced levels (1-34% v/v) (Table

39). These results are in partial agreement with previous analysis of headspace mastic volatiles which showed that α-pinene was present in the headspace of plates packaged in both Cryovac but not packaged in metallized bags (Table 37). These differences can again be attributed to the significantly lower OTR of the metallized bags compared to Cryovac bags (Figure 9). Therefore, the more consistent antimicrobial activity of ME sachets in metallized bags may be due to the residual levels of these ME volatiles in the package headspace. Another possibility for their greater antimicrobial activity may be their absorbance into the media and not to their permeation through the packaging material as shown indirectly by the significant increase in the OTR of the Cryovac bags. Whatever the reason, it is evident that the type of packaging material influenced the antimicrobial activity of the ME interactive sachets. Based on these results, cotton pads can be inserted into bleached paper (tea bags) and over-wrapped with metallized bags to obtain optimal antimicrobial activity against the selected microorganisms used in this study.



R.t.	Compound
32.01	ß-pinene
32.13	myrcene
32.34	ocimene
32.45	α-pinene
32.74	camphene
33.33	BCHM
34.31	limonene

Figure 11. Composition of headspace mastic volatiles of ME solutions at day 1 and day 28 for uninoculated and inoculated BHI plates with 10^2 CFU/ml of *B. cereus* [from top curve to bottom: (i) uninoculated, day 1; (ii) inoculated, day 1; (iii) uninoculated, day 28; (iv) inoculated, day 28]

Table 39. Changes in headspace ME volatiles in BHI plates inoculated with *B. cereus*, packaged in Cryovac and metallized bags and incubated at 30°C[a] for 28 days

Packaging Treatment	Percent remaining (%)						
	ß-pinene	myrcene	ocimene	α-pinene	camphene	BCHM	limonene
Cryovac bags							
Control	0	0	100	6.53	0	0	0
Inoculated [b]	0	0	100	1.79	0	0	0
Metallized bags							
Control	4.25	5.15	13.53	0	19.34	21.98	23.97
Inoculated [b]	0.74	1.13	28.83	0	6.93	34.09	0

[a] average of duplicate samples
[b] 10^2 CFU/ml inoculum

3.4. Conclusion

In conclusion, this study has confirmed that low levels of headspace ethanol failed to inhibit the growth of *B. cereus* and other microorganisms of spoilage and public health concern in bakery products. However, this study demonstrated the potential combination of mastic essential oil and ethanol as vapor phase inhibitors to control the growth of microorganisms of spoilage and public health concern in high moisture high pH bakery products. Furthermore, this study demonstrated the importance of the using an appropriate sachet and packaging material to enclose the ME solution. Based on these preliminary studies, 1 g of a 1:1 ME solution dispensed on a cotton pad and inserted in bleached paper (tea bags) and over-wrapped in metallized bags could be used to control the growth of *B. cereus*, a pathogen of concern in high moisture, high pH English style crumpets.

Chapter 4

Novel methods to control the growth of *Bacillus cereus* in English-style Crumpets

This study examined the use of SHA and ME interactive sachets, alone and in combination with each other, to inhibit the growth of B. cereus in English-style crumpets.

Chapter 4

Novel Methods to Control the Growth of *Bacillus cereus* in English-style Crumpets

4.1. Introduction

Bacillus cereus has been responsible for several food poisoning outbreaks involving high moisture-high pH English-style crumpets (a_w ~0.98, pH ~9.0) (Jenson *et al.*, 1994). This spore-forming microorganism, which is commonly present in flour, dried milk powder, cocoa, and spices, can easily survive baking and grow to levels of public health concern within 3-5 days in bakery products stored at ambient temperature. Chemical preservatives e.g., propionic and sorbic acid, are commonly used to extend the shelf life and keeping quality of bakery products. However, these preservatives have been ineffective in controlling the growth of *B. cereus* in high pH crumpets due to the complete dissociation of these acids at high pH levels. Various methods were examined in previous chapters to control the growth of *B. cereus* in agar plate studies. Based on the results of these studies, two novel methods were evaluated for their potential to inhibit the growth of this pathogen of concern in a high-a_w, high-pH bakery products.

4.1.1. Objectives

The objectives of this study were to determine the effect of mastic-ethanol and water-ethanol vapor generators and sorbohydroxamic acid, alone and in combination with each other, on the growth of *B. cereus* in high-pH English-style crumpets.

4.2. Materials and Methods

4.2.1. Preparation of SHA stock solution

A stock solution (5% w/v) of SHA was prepared by dissolving 5 g of the preservative in 100 ml distilled water and stirring on a magnetic stirrer at medium speed for ~10 min. The solution was then filter sterilized using a Seitz filtration unit (Nalge Company, Sybron International, N.Y., USA) and the solution was then stored in a dark bottle under refrigeration (4°C) until use.

4.2.2. Crumpet formulation

High moisture English-style crumpets (a_w ~0.98-0.99, pH ~8.86) were prepared from all-purpose flour, sugar, instant yeast, reconstituted dry milk powder, salt, and baking soda, according to the method of Witty and Schneider Colchie (1979). Two formulations, with and without SHA, were prepared and the levels of ingredients used in each formulation are shown in Table 40. The final concentration of SHA used for this study was 3000 ppm and not 2000 ppm as used in the *in vitro* studies (Chapter 2), since higher preservative levels are usually required to give similar inhibitory activity *in vivo*. All dry ingredients were weighed on a Mettler Toledo Scale (PB-3001, Switzerland) in round stainless steel bowls and then transferred to a Hobart Mixer (A-200, Hobart Canada Inc., Don Mills, Ontario, Canada). The appropriate volume of milk and water were added and the contents mixed for 5 min until a homogeneous batter was formed.

Crumpets were formed by pouring crumpet batter (~65 g) into greased cylindrical molds on an oiled griddle (Moffat Appliances, Montreal, Quebec, Canada), pre-heated to 204°C and then baked for ~9 min 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, packaged in high moisture barrier bags (Ziploc®, S.C. Johnson and Son, Brantford, Ontario, Canada) and stored at

4°C until use. A flow process diagram outlining the various steps involved in the production of crumpets, with and without SHA, is shown in Figure 12.

Table 40. Ingredients used in crumpet formulations

Ingredient	Formulation 1[1] (g)	Formulation 2[2] (g)	Brand Name
Flour	600	600	Robin Hood Inc.
Instant dried milk powder	55	55	Carnation, Nestle
Sugar	1.6	1.6	Redpath
Salt	6	6	Windsor
Yeast (instant)	9	9	Fleischmanns (Quick-rise)
Baking soda	10	11.7	-
Water	890	800	-
SHA (5% w/v)	-	98	Frinton Laboratories

[1] without SHA [2] with SHA (3000 ppm)

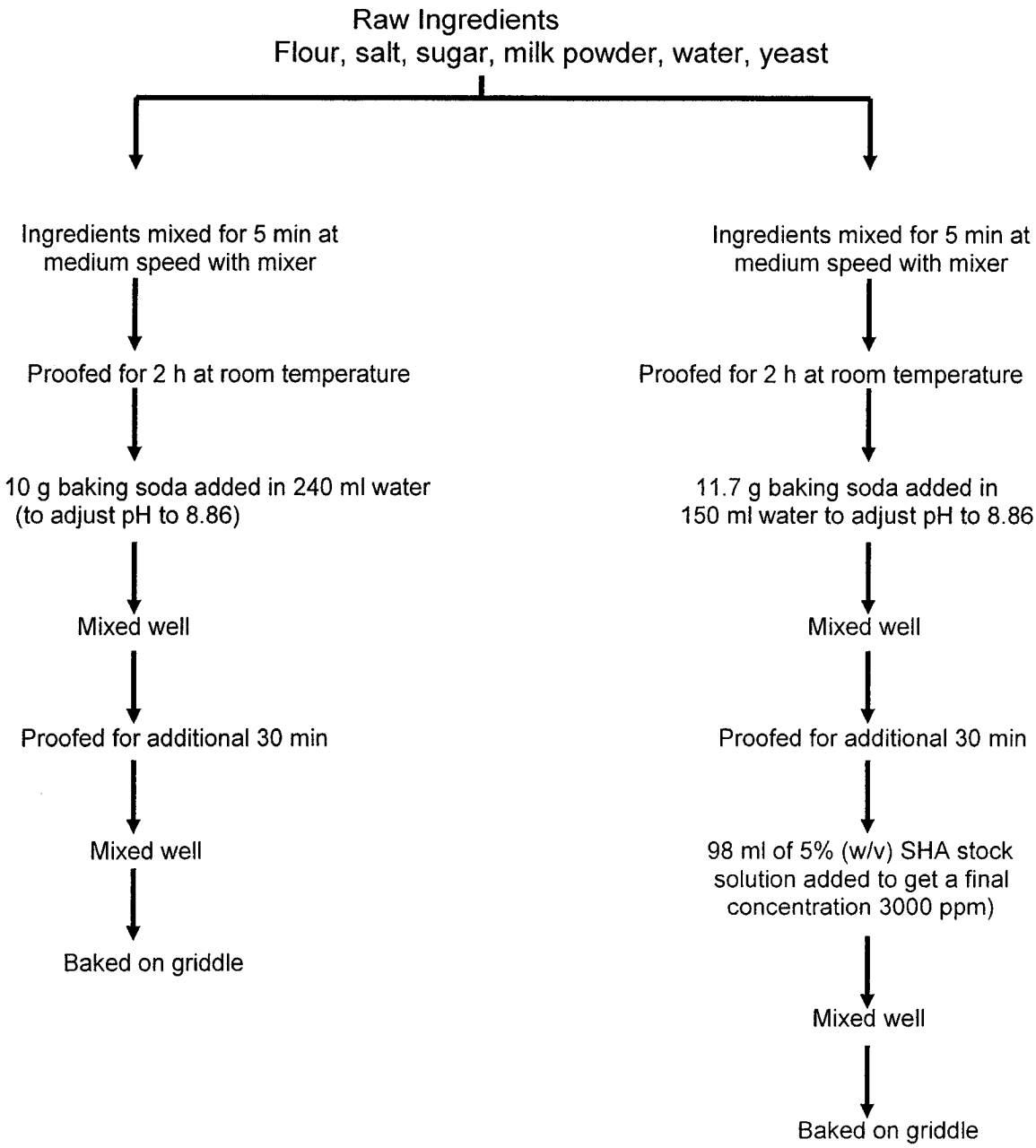


Figure 12. Flow-process diagram of preparation of crumpets used in this study

4.2.3. Preparation of vapor generator sachets

Two types of sachets were used in this study (i) Mastic-ethanol (ME) and (ii) water-ethanol (WE). A ME solution (1:1) was prepared by mixing equal volumes of mastic essential oil (Mastic Growers Association of Chios, Chios, Greece) and 95% (v/v) food grade ethanol in a sterile 250 ml Erlenmeyer flask. These solutions/ratios were chosen based on results obtained from previous *in vitro* studies (Chapter 3). The flask was closed with a rubber stopper and mixed by inversion for ~1 min. A control solution of 1:1 WE was prepared in a similar manner. Both flasks were overwrapped with aluminum foil and stored at refrigeration (4°C) until required.

Sachets were prepared from empty bleached paper tea bags (5 cm x 5 cm, Tetley Tea, Canada). Pre-weighed cotton pads (1 g) were inserted into the empty sachets and 1 g of the 1:1 ME and WE solutions were aseptically transferred onto individual pads. The sachets were then sealed using an impulse heat-sealer (Model KM100-3M, Wolfertschwenden, Germany).

4.2.4. Preparation of inoculum, crumpet inoculation and packaging

A strain of *B. cereus* FN 13 that had been used in all previous *in vitro* studies was again used in this challenge study. The inoculum and working suspension of *B. cereus* (10^3 CFU/ml) were prepared as described previously (section **2.2.1**) After the crumpets were cooled to room temperature, they were inoculated, in triplicate, with 0.1 ml of the working suspension to give a final inoculum of ~10^2 CFU/g. Control crumpets were inoculated in a similar manner with 0.1% peptone water.

All inoculated and control crumpets, with and without SHA, were packaged by placing 1 crumpet (~50 g each) into sterile metallized retort pouches (Vapor-

Loc, Mississauga, Ontario, Canada). ME and WE sachets (1 g) were then inserted into the appropriate metallized pouches and taped to the inside to ensure the sachets did not make contact with the crumpets and to ensure a free flow of air surrounding each sachet. The metallized pouches were then sealed using an impulse heat sealer (Model KM100-3M, Wolfertschwenden, Germany) and stored at 30°C. Triplicate samples of inoculated crumpets were analyzed after 0, 3, 7, 10, and 14 days, while control crumpets were analyzed at the beginning and end of the storage period (day 14).

4.2.5. Headspace mastic volatiles and ethanol analysis

Changes in headspace mastic essential oil volatiles and ethanol were monitored using a Varian gas chromatograph as described previously in section **3.2.6**.

The levels of headspace volatiles were determined from standard curves (R^2 = 0.95-0.99) generated by analyzing, in triplicate, standard solutions of mastic essential oil and food grade ethanol (see section **3.2.5**) (Tables 29 and 30, Figures 8 and 9).

4.2.6. Sensory analysis

Sensory analysis was carried out on packaged crumpets at day 0 and at each sampling day by a four-member untrained panel. Crumpets were ranked for color, odor, and texture using a five-point hedonic scale (0 = extreme deterioration, 5 = no deterioration). To evaluate these sensory variables, packages where opened and the characteristic odor and color described. Texture was evaluated by the "squeeze test" i.e., squeezing crumpets inside their packages. A sensory score of less than 3 was considered unacceptable for each parameter, implying that the sample would be rejected by the consumer and shelf life terminated.

4.2.7. Microbiological analysis

On the appropriate sampling day, crumpets were transferred aseptically into a stomacher bag. A 1:3 dilution was prepared by adding twice the sample weight with sterile 0.1% (w/v) peptone water and stomaching for 1 min using a Stomacher Lab Blender 400 (Seward Medical Stomacher, London, UK). Decimal dilutions were subsequently prepared from this initial dilution, again using 0.1% peptone water. *B. cereus* was enumerated by spread plating 0.1 ml of appropriate decimal dilutions in duplicate, onto polymyxin egg yolk mannitol bromothymol blue agar (PEMBA) (Oxoid LTD., Hampshire, England). Plates were incubated at 30°C for 24 h and colonies were enumerated using a Darkfield Quebec Colony Counter (AO Scientific Instruments, Quebec, Canada). Control plates were enumerated in a similar manner and all counts were expressed as CFU/g.

4.2.8. pH measurements

The pH of crumpets was measured using a previously calibrated pH meter (Model 220, Corning Glass Works, Corning, NY, USA) with a gel filled polymer body combination electrode with Ag/AgCl reference (Model 13-620-104, Fisher Scientific, Montreal, Quebec, Canada). The pH of crumpets was made, in duplicate, on the appropriate sampling day by placing the electrode directly into the 1:3 dilution of crumpet homogenate.

4.2.9. Statistical analysis

Data were analyzed using analysis of variance (ANOVA) for a randomized complete block design (Steel and Torrie, 1980). The software used for this study was StatGraphics Plus (version 3.1, 1997).

4.3. Results and Discussion

4.3.1. Changes in headspace ethanol

Changes in the headspace ethanol levels of control and inoculated crumpets, with and without SHA and packaged with Mastic-Ethanol (ME) or Water-Ethanol (WE) vapor generators in metallized pouches are shown in Figures 13 and 14, respectively.

In air packaged control crumpets formulated without SHA, headspace ethanol increased to ~0.5% (v/v) by day 14. This may be attributed to growth and fermentation of yeast that can survive the baking process (Daifas *et al.*, 2000). However, in air packaged crumpets formulated with SHA (3000 ppm), no headspace ethanol was detected over the 14 day shelf life study. These observations are in agreement with previous results (section 2.3.2.5), which showed that SHA had a powerful effect on yeast growth.

Changes in headspace ethanol for control crumpets formulated with and without SHA and packaged with ME and WE vapor generators are shown in Figure 13. Headspace ethanol levels reached a maximum of ~2.3% (v/v) by day 3 and then decreased to ~1.2 – 1.8% throughout storage due mainly to absorption of headspace ethanol by crumpets (Figure 13). Similar trends have been observed in crumpets and apple turnovers packaged with ethanol vapor generators (Smith *et al.*, 1988; El-Khoury, 2001).

A similar pattern in the levels of headspace ethanol was observed for crumpets inoculated with *B. cereus* compared to control crumpets (Figures 13 and 14). The levels of headspace ethanol in both control and inoculated crumpets packaged with ME vapor generators were also consistently lower than crumpets packaged with WE vapor generators regardless of their formulation. However, sachet types and crumpet formulation had no significant effect on the levels of

ethanol found in the package headspace ($P < 0.005$). Thus, differences in headspace ethanol can probably be attributed to variations in the standard curves used to determine the levels of headspace ethanol (Figures 13 and 14).

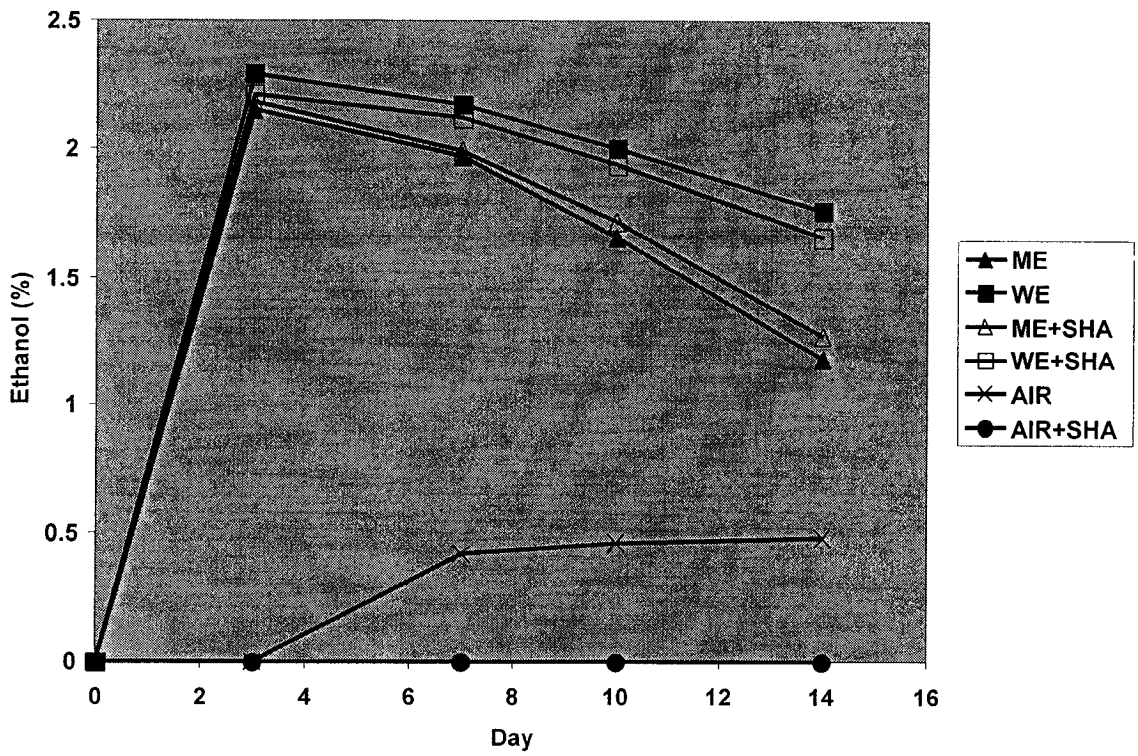


Figure 13. Changes in headspace ethanol of control crumpets formulated with or without SHA (3000ppm) and packaged alone, and in combination with ME or WE sachets (1 g), in metallized bags and incubated at 30°C

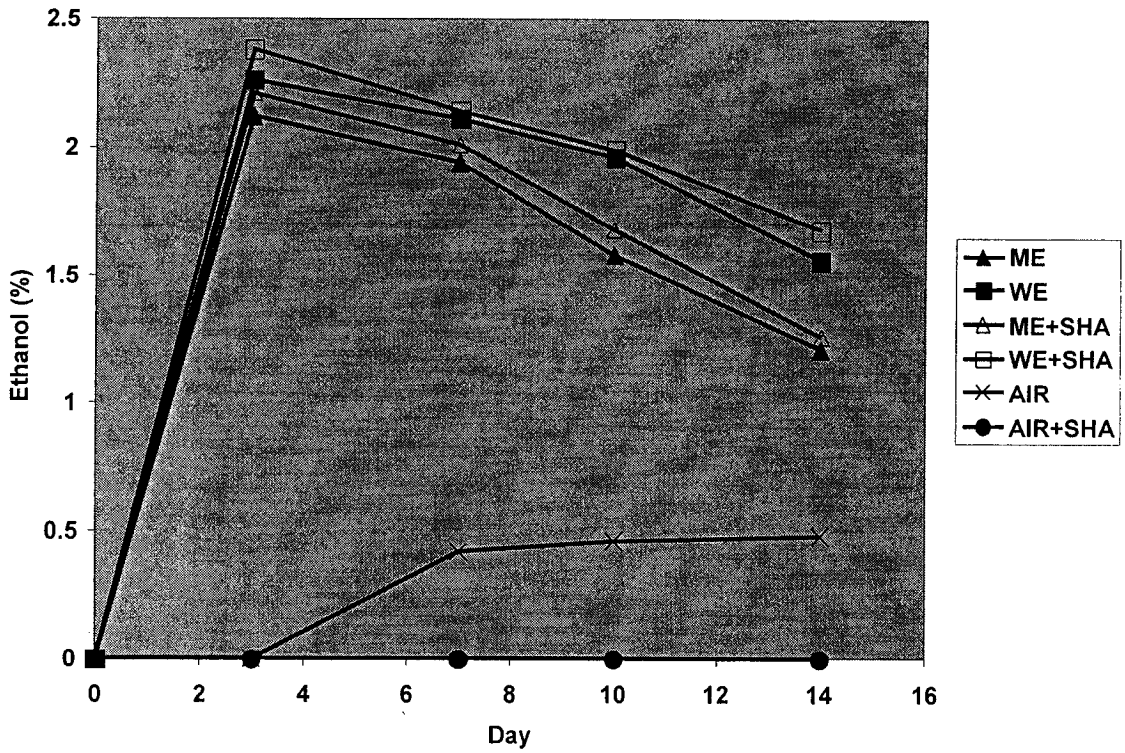


Figure 14. Changes in headspace ethanol of crumpets formulated with or without SHA (3000ppm), inoculated with *B. cereus* (10^2 CFU/g) and packaged alone, and in combination with ME or WE sachets (1 g), in metallized bags and incubated at 30°C

4.3.2. Changes in headspace mastic volatiles

Changes in the headspace mastic volatile levels of control and inoculated crumpets, formulated with and without SHA and packaged with ME or WE vapor generators in metallized pouches are shown in Figures 15 and 16, respectively.

For control crumpets packaged in metallized pouches with ME vapor generators (Figure 15), headspace mastic volatiles reached a maximum ~1.4 - 1.6% (v/v) by day 3 and then decreased to ~1.0 – 1.2% throughout storage due probably to absorption of headspace mastic volatiles by the crumpets.

Levels of headspace mastic volatiles for crumpets inoculated with *B. cereus* and packaged with ME vapor generators were similar to those of uninoculated crumpets (Figure 16). For crumpets (control and inoculated) packaged in other conditions (i.e., WE and air), headspace mastic volatile levels were 0%.

The levels of headspace mastic volatile of crumpets formulated with SHA and packaged with ME vapor generators were almost identical to crumpets formulated without SHA (Figures 15 and 16). Furthermore, no significant difference was found between headspace mastic volatiles in crumpets formulated with and without SHA ($P < 0.005$).

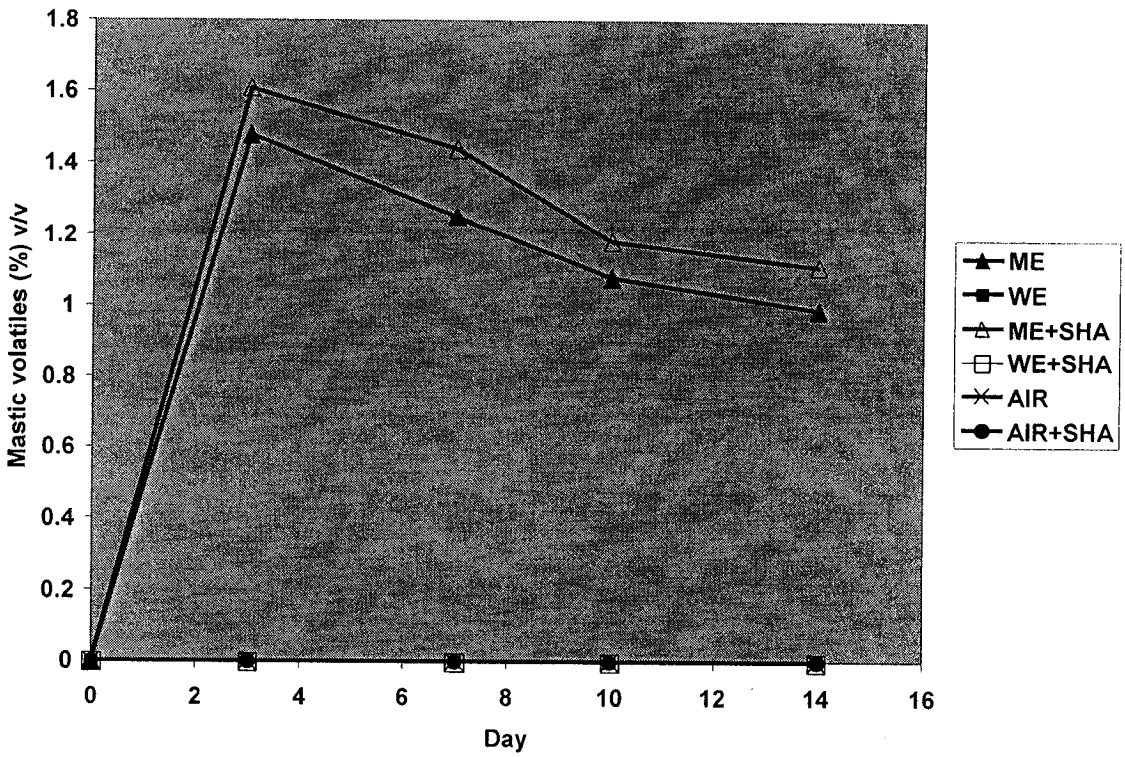


Figure 15. Changes in headspace mastic volatiles of control crumpets formulated with or without SHA (3000ppm) and packaged alone, and in combination with ME or WE sachets (1 g), in metallized bags and incubated at 30°C

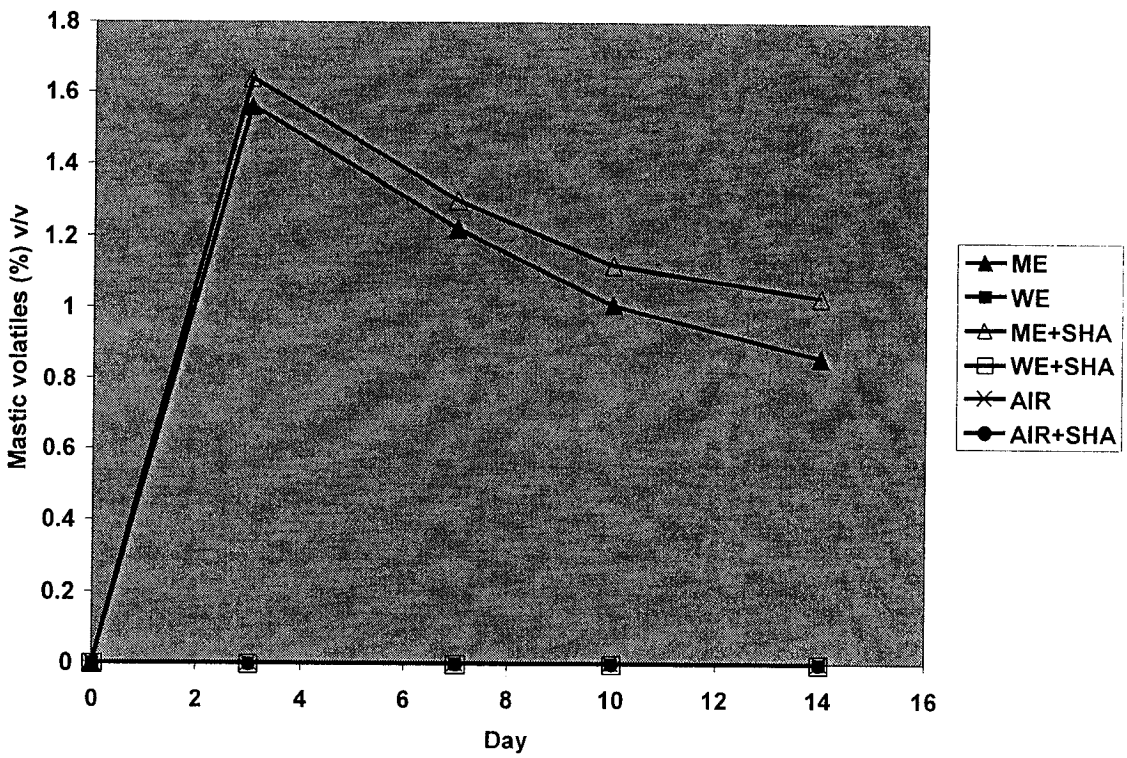


Figure 16. Changes in headspace mastic volatiles of crumpets formulated with or without SHA (3000ppm), inoculated with *B. cereus* (10^2 CFU/g) and packaged alone, and in combination with ME or WE sachets (1 g), in metallized bags and incubated at 30°C

4.3.3. Sensory analysis

Changes in the sensory scores of control and inoculated crumpets, formulated with and without SHA, and packaged under various conditions in metallized bags are shown in Table 41. All sensory parameters (color, odor and texture) were evaluated by a four-member untrained panel using a five-point hedonic scale (0 to 5) where (0 = extreme deterioration, 5 = no deterioration). A sensory score of <3 was regarded as the cut-off point of consumer acceptability for each sensory parameter.

All crumpets (control and inoculated) formulated without SHA and packaged with ME and WE sachets, developed an unacceptable gray color by day 14 and had sensory scores <3 (Table 41). However, crumpets formulated with SHA, alone and in combination with ME and WE sachets, had acceptable color scores, i.e., scores ≥3, by day 14 (Table 41). The higher color scores of crumpets formulated with SHA may be due to either a golden yellow color being imparted during baking due to the Maillard reaction, to the inhibition of microbial spoilage, or a combination of both.

The odor scores of crumpets formulated with SHA were all acceptable at the end of storage (day 14) regardless of the packaging conditions. However, crumpets packaged without SHA developed a fruity odor and were unacceptable by day 14 (Table 41).

Crumpets packaged with ME vapor generator sachets had a distinct mastic odor described by panelists as a "refreshing pine odor". While two members judged this odor as acceptable and gave scores of 4, the other two found it unacceptable and gave it a score of <3 (Table 41).

The texture scores for all crumpets are shown in Table 41. Control and inoculated crumpets formulated with SHA and packaged in air had the highest scores at day 14 having a firm dry texture at the end of the storage (Table 41). However, all other crumpets formulated without SHA had a sticky wet texture and

texture score of <3 by day 14, regardless of the packaging conditions. Furthermore, there was no significant difference between the sensory scores of the control and inoculated crumpets regardless of the packaging conditions ($P < 0.005$).

Table 41. Summary [a] of sensory analysis at day 14

Packaging Condition	Sensory scores [b]		
	Color	Odor	Texture
Control crumpets			
ME	<3 [c]	<3 [d, e]	<3 [f]
WE	<3 [c]	<3 [d]	<3 [f]
ME + SHA	4	3 [e]	<3
WE + SHA	4	4	<3
Air	<3 [c]	<3 [d]	<3 [f]
Air + SHA	5	5	5
Inoculated crumpets [g]			
ME	<3 [c]	<3 [d, e]	<3 [f]
WE	<3 [c]	<3 [d]	<3 [f]
ME + SHA	4	3 [e]	<3
WE + SHA	4	4	<3
Air	<3 [c]	<3 [d]	<3 [f]
Air + SHA	5	5	5

[a] Average of triplicate crumpets
[b] Scale of 0 to 5; 0 = extreme deterioration, 5 = no deterioration
[c] Gray color
[d] Fruity odor
[e] Mastic odor
[f] Wet texture (gummy)
[g] Inoculated with *B. cereus* (10^2 CFU/g)

4.3.4. Changes in the microbiological counts

Changes in the microbiological counts of control and inoculated crumpets, formulated with and without SHA, and packaged in air or with ME and WE sachets are shown in Figures 17 and 18.

No *B. cereus* was found in any of the control crumpets (Figure 17). However, all control crumpets had an initial *Bacillus* count of ~10^1 CFU/g. This is in agreement with previous studies by Stadhouders and Driessen (1992) and Eyles *et al.* (1989) who reported similar levels of *Bacillus* spp., in bakery ingredients, such as milk powder and flour. These species were tentatively identified as *B. subtilis* based on their morphological characteristics on PEMBA (i.e., flat, oval green colonies, ~5-8 mm in diameter). Furthermore, these colonies were lipase positive and lecithinase negative on PEMBA thereby differentiating them from colonies of *B. cereus* on this medium. In control crumpets, formulated without SHA, *Bacillus* spp. counts increased to ~10^4 CFU/g after 14 days (results not shown). However, for SHA formulated crumpets packaged alone or in combination with the ME sachets, counts remained at a level of 10^1 CFU/g throughout storage (results not shown).

In crumpets inoculated with *B. cereus* (10^2 CFU/g), counts ranged from 10^2 to 10^8 CFU/g after 14 days at ambient storage (Figure 18). For crumpets formulated with SHA, and packaged alone and in combination with ME and WE sachets, counts of *B. cereus* remained at an initial inoculum level of ~10^2 CFU/g after 14 days. However, in crumpets formulated without SHA, counts increased to ~10^6-10^8 CFU/g, regardless of the packaging conditions. There was a significant difference in the microbiological counts between crumpets packaged with and without SHA ($P < 0.05$). These results were contrary to those observed in agar plate studies. In these studies, both SHA and ME generators, alone and in combination with each other, were effective in controlling the growth of *B. cereus*. However, in crumpets only SHA proved effective to control the growth of *Bacillus* spp. of both spoilage and public health concern in high moisture, high pH

crumpets. The difference in results between *in vitro* and *in vivo* studies may be due to the lack of dispersion of the ME volatiles in the food matrix.

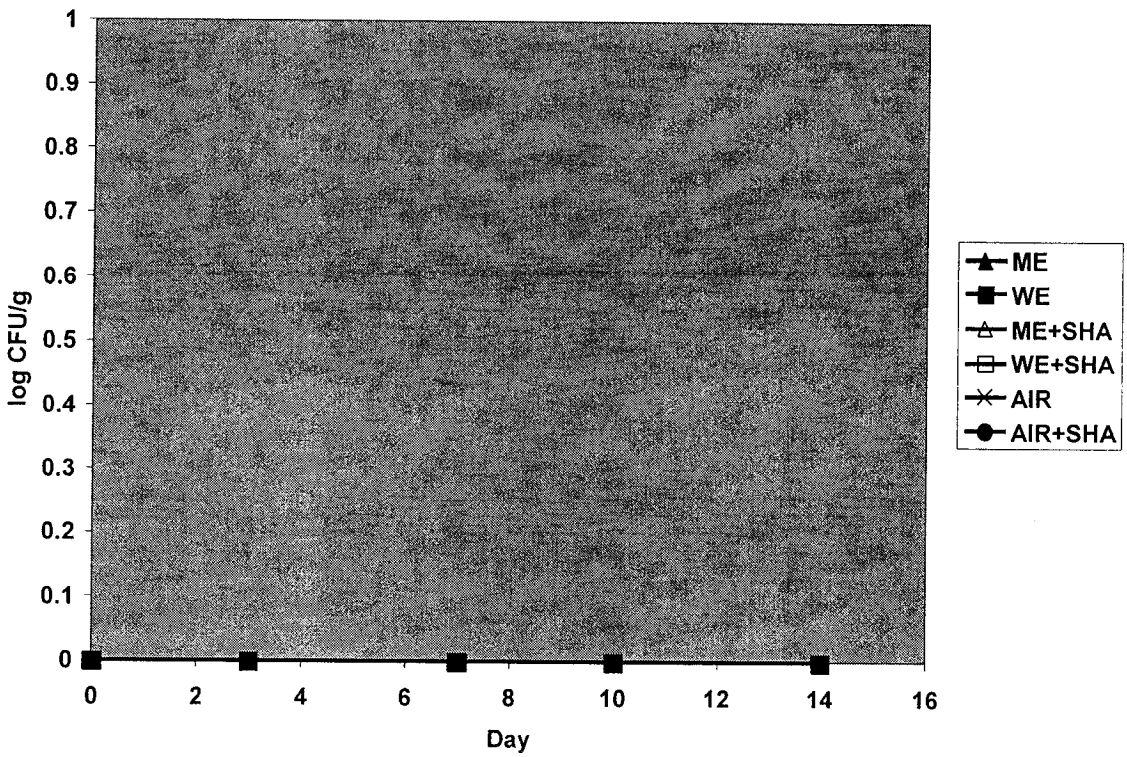


Figure 17. Effect of SHA, ME and WE vapor generator sachets, alone and in combination on the growth of *B. cereus* in control crumpets packaged in metallized bags and incubated at 30°C

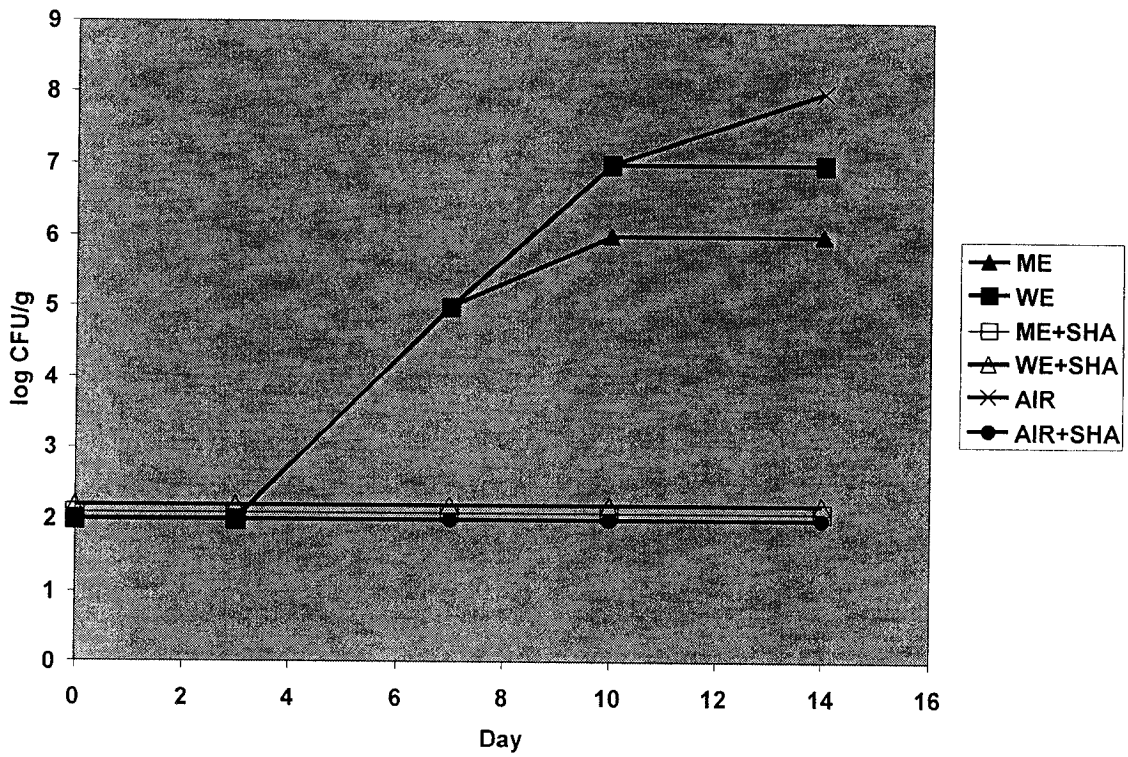


Figure 18. Effect of SHA, ME and WE vapor generator sachets, alone and in combination on the growth of *B. cereus* in crumpets inoculated with 10^2 CFU/g, in metallized bags and incubated at 30°C

4.3.5. Changes in pH

Changes in pH of control and inoculated crumpets, formulated with and without SHA, and packaged with various sachet types in metallized bags are shown in Figures 19 and 20.

The most dramatic decrease in pH was observed for all crumpets (control and inoculated) formulated without SHA. In these crumpets, the pH decreased from its initial value of ~pH 8.86 to ~pH 7.5 by day 14 at a storage temperature of 30°C. However, for all SHA formulated crumpets packaged alone or with vapor generator sachets, and decreased slightly from ~pH 8.86 to ~pH 8.3-8.5 at the end of storage. Furthermore, there was a significant difference between the pH changes of crumpets formulated with and without SHA ($P < 0.005$). The difference in pH change between crumpets formulated with and without SHA may be attributable to the inhibition of background *Bacillus* spp. by SHA as shown in both *in vitro* and *in vivo* studies. Lyver (1997) also found that pH changes in cooked breaded surimi nuggets were attributed to the growth of *Bacillus* species during storage.

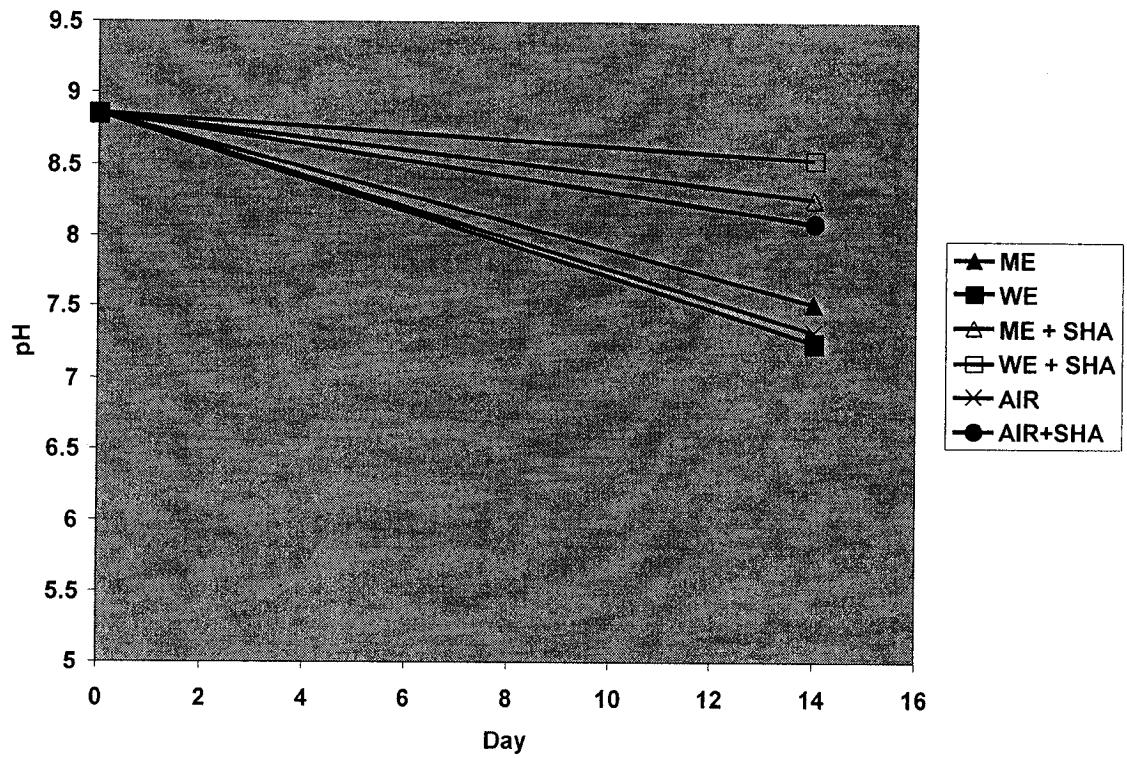


Figure 19. Changes in pH of control crumpets formulated with or without SHA (3000ppm) and packaged alone, and in combination with ME or WE sachets (1 g), in metallized bags and incubated at 30°C

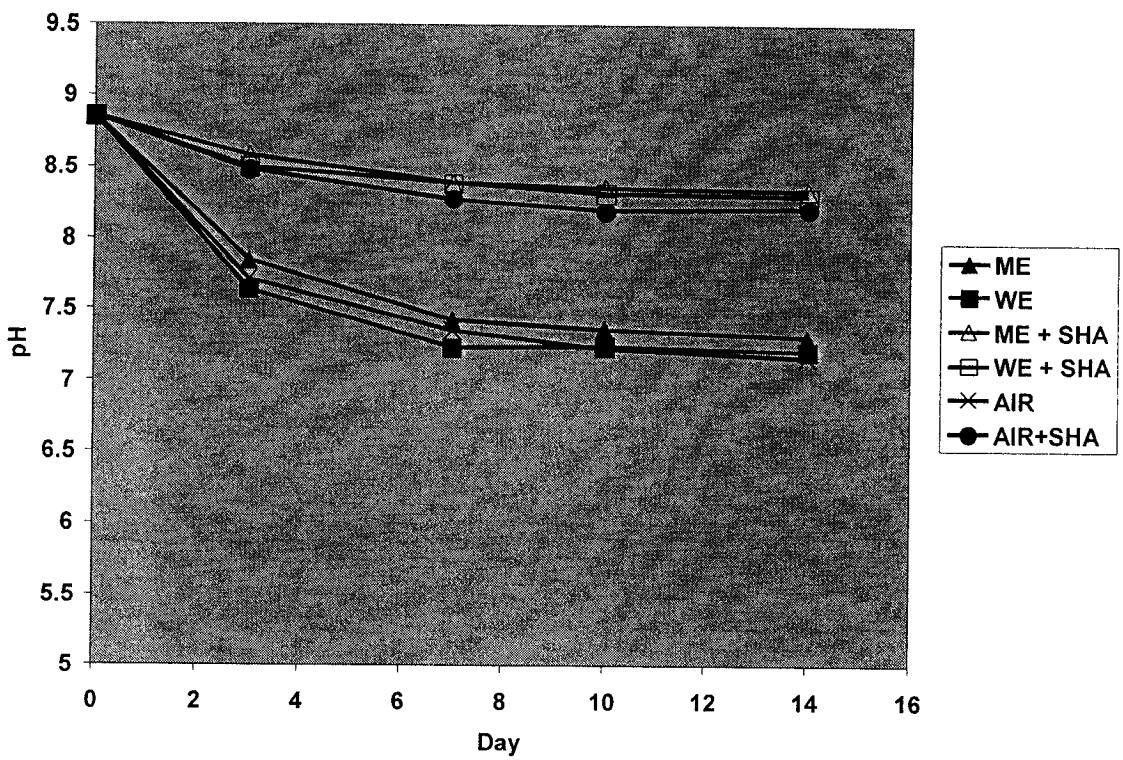


Figure 20. Changes in pH of crumpets formulated with or without SHA (3000ppm), inoculated with *B. cereus* (10^2 CFU/g) and packaged alone, and in combination with ME or WE sachets (1 g), in metallized bags and incubated at 30°C

4.4. Conclusion

In conclusion, sorbohydroxamic acid (3000 ppm), alone and in combination with ME and WE, was effective in inhibiting the growth of *B. cereus* for 14 days in high-a_w, high-pH English style crumpets. Furthermore, all SHA formulated crumpets packaged in air or with 1 g vapor generator sachets, had acceptable sensory scores, even after 14 days at ambient temperature. These studies agree with agar plate studies which showed that SHA at a concentration of 2000 ppm controlled the growth of *B. cereus*. However, while ME sachets (1 g) also proved effective in controlling the growth of this pathogen in agar plates, they failed to do so in English style crumpets. This lack of inhibition in the food matrix may be due to different absorption patterns of the antimicrobial ME volatiles from the package headspace into crumpets compared to agar. Whatever the reason, these preliminary studies have shown that SHA has the potential to control the growth of *B. cereus* in high pH crumpets without compromising product quality.

Chapter 5

General Conclusion

Bacillus species form spores that are ubiquitously found in soils, dust and water, and are commonly isolated from plants and animal products (Granum, 1997). *Bacillus* spp., attach to wheat which is milled into flour (Kirschner and Von Holy, 1989). The level of contamination of *B. cereus* in wheat flour is generally less than 10^3 spores/g (Kaur, 1986; Kim and Goepfert, 1971; Rizk and Ebeid, 1989). Therefore, *Bacillus* spores are commonly found in flour and flour-based products, as well as in the bakery environment. *Bacillus* spores are heat resistant, will survive baking, and under favorable conditions may grow to levels associated with toxin production. Survival of spores during baking depends on the type of product, the internal temperature reached during baking as well as the thermal resistance of the spore. English style crumpets are high moisture bakery products which have been implicated in several food poisoning outbreaks involving *B. cereus*. In the baking process of crumpets, the bottom is fully baked while the remainder of the crumpet receives a partial baking treatment. Consequently *B. cereus* spores survive the thermal process and subsequently germinate and proliferate to levels of 10^5 CFU/g within 3-5 days at ambient temperature.

Preliminary studies indicated that KS was effective against a range of microorganisms of both spoilage and public health concern in bakery products. However, a major limitation is that it can only be used in products formulated to pH <6. This study also demonstrated the potential of SHA to control microorganisms of spoilage and public health concern in high moisture bakery products over a wider pH range (5 to 9) than KS, particularly against molds and yeasts. Based on these preliminary studies, SHA could also be used at a level of

2000 ppm to control the growth of *B. cereus,* a pathogen of concern in high-a_w, high-pH English style crumpets.

Our present studies have shown that low levels of ethanol vapour failed to control the growth of *B. cereus* and other microorganisms of spoilage and public health concern in bakery products. However, this study demonstrated the potential of mastic essential oil in combination with ethanol as a vapour phase inhibitor to control the growth of these microorganisms. While, MO had no effect on the growth of *B. cereus* but ME solutions did inhibit the growth of this pathogen of concern in bakery products for >28 days. Furthermore, these studies have shown that the efficacy of ME is dependent on both the type of sachet material and packaging material enclosing the sachet. Based on these preliminary agar plate studies, 1 g of a 1:1 ME solution dispensed on a cotton pad and inserted in bleached paper (tea bags) and over-wrapped in metallized bags could be used to control the growth of *B. cereus*, a pathogen of concern in high moisture, high pH English style crumpets.

Sorbohydroxamic acid (3000 ppm), alone and in combination with ME and WE, was effective in inhibiting the growth of *B. cereus* for 14 days in high-a_w, high-pH English style crumpets. Furthermore, all SHA formulated crumpets packaged in air or with 1 g vapor generator sachets, had acceptable sensory scores, even after 14 days at ambient temperature. These studies agree with agar plate studies which showed that SHA at a concentration of 2000 ppm controlled the growth of *B. cereus* in agar plate studies. However, while ME sachets (1 g) also proved effective in controlling the growth of this pathogen in agar plates, they failed to do so in English style crumpets. This lack of inhibition in the food matrix may be due to different absorption patterns of antimicrobial volatiles from the package headspace into crumpets compared to agar. Whatever the reason, these preliminary studies showed that SHA has the potential to control the growth of *B. cereus* in high pH crumpets without compromising product quality.

In conclusion, the growth of *B. cereus* was controlled high moisture, high pH English style crumpets by using SHA (3000 ppm). However, toxicological tests are required on the safety of these levels for human consumption. Studies are also required to improve the dispersion of mastic essential oil in food matrix if it is to be used commercially to extend the safe shelf life and keeping quality of high moisture bakery products.

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