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MANAGEMENT OF MICROBIAL DECAY OF FRESH AND PEELED CHESTNUTS IN MICHIGAN

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MANAGEMENT OF MICROBIAL DECAY OF FRESH AND PEELED CHESTNUTS IN MICHIGAN

By

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ABSTRACT

MANAGEMENT OF MICROBIAL DECAY OF FRESH AND PEELED CHESTNUTS IN MICHIGAN

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Edible chestnuts (*Castanea* spp.) represent a worldwide growing commodity. Worldwide including Michigan, postharvest losses due to microbial activity, including several filamentous fungi are problematic for the industry. To determine the organisms involved with shell mold and kernel decay, a survey for microorganisms associated with fresh chestnut was performed. Eleven species of molds were found to negatively impact fresh chestnuts; two species had never been found on chestnut prior to this study. Microbial populations were dependent on harvest methods and the farms from which the chestnuts were collected. A survey of peeling equipment to determine the source of contaminants on peeled chestnuts showed that the skin separator and the sorting belt were sources of contamination by two bacterial species and one species of yeast. To reduce microbial growth on fresh and peeled chestnut, several sanitizers were evaluated. Hydrogen peroxide and trifloxystrobin significantly reduced shell mold severity and kernel incidence on fresh chestnuts. X-ray irradiation, hydrogen peroxide and hot water immersion significantly reduced spoilage on peeled chestnuts. Information from this study adds levels of protection against postharvest chestnut mold, decay and spoilage when combined with other good manufacturing and agricultural practices.

To my parents, sister, brother and Yvonne, who can never know how much their love, encouragement and moral support has meant to me through the development of this thesis. With lots of love this is for you…

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CHAPTER 1

LITERATURE REVIEW

Introduction

Chestnut (*Castanea* spp.) is one of the most popular nut-bearing trees in the Mediterranean region, as well as many countries of Asia including China, Japan and Korea (Ridley, 1999). Chestnuts grown in Europe and Asia account for more than 70% of the worldwide production with chestnut production currently increasing in Australia, New Zealand and Chile (Fulbright and Mandujano, 2000; Grau and France, 1999; Klinac et al., 1999; Ridley, 1999; Mencarelli, 2001). In the United States chestnuts are rare; people are more likely to be familiar with the unrelated and poisonous horse chestnut (*Aesculus* spp.) (Fulbright and Mandujano, 2000) than with any of the edible, sweet chestnut species (*C. dentata* Borkh., *C. mollissima* Bl., *C crenata* Sieb and Zucc., *C. sativa* Mill., *C. seguinii* Dode, *C. pumila* Mill, *C. henryi* Rehd. and Wils.) (Anagnostakis et al., 1998; Fulbright and Mandujano, 2000; Miller, 2003). This is partially due to chestnut blight (*Cryphonectria parasitica* Murril and Barr), which virtually eliminated the once-widespread American chestnut (*C. dentata*) during the first half of the twentieth century (Merkel, 1905; Metcalf, 1912; Gravatt and Marshall, 1926). Despite advances in chestnut blight research, chestnut blight still remains one of the major limitations to growing American chestnuts (Fulbright and Mandujano, 2000; MacDonald and Double, 2006; Anagnostakis et al., 1998). Nevertheless, commercial orchards have been established in different locations in the United

States, and have been increasing over the years (Fulbright and Mandujano, 2000). In the past, especially in Europe and in China, chestnut consumption was predominant in rural areas and was considered as a food for the poor. Currently, due to their unique nutritional value, chestnuts are broadly consumed and used as a cooking ingredient with health-related benefits (De la Montaña Miguelez et al., 2004; Borges et al., 2008; Xu, 2005). Recently, the number of chestnut plantations has increased in the United States, including Michigan, and both fresh and peeled domestic chestnuts are becoming more common. Relatively recent domestic availability of chestnuts has triggered an increase in consumption. Vossen (2000) claimed that the United States has a great potential for chestnut production and a small rise in domestic consumption could be worth up to $800 million annually. Similar to patterns in Europe, chestnuts consumed in the United States, are most commonly prepared by roasting the fresh product (Harte et al., 2003) but other diverse culinary applications are also available (CGI: Chestnut Growers, Inc 2008; Kelley and Behe, 2002). In studies performed in Wisconsin and California to evaluate consumer preferences for chestnuts, between the years 2000 and 2004, most of those interviewed had never tasted a chestnut, but were interested in exploring them as a new food. Quality and nutritional value were listed as the most important attributes influencing purchase and consumption decisions (Gold et al., 2004; Vossen, 2000).

The quality of fresh chestnuts is limited due to microbial decay after harvest and during storage. Organisms that cause postharvest decay of fresh commodities are found worldwide and in most cases, it has been estimated that losses due to postharvest decay could be greater than 25 percent of all harvested

foods. Under poor storage conditions, and if adequate precautions are not taken, microbial growth is favored and losses caused by postharvest diseases may be greater than the economic value of the non-decayed portion of the crop (Narayanasamy, 2006). Chestnuts are not an exception. Destruction by various filamentous fungi, commonly called molds, is an important and predominant factor in worldwide economic and postharvest losses (Jerimini et al., 2006; McCarter et al., 1980; Narayanasamy, 2006).

Postharvest decay of chestnuts reduces product quality and leads to unmarketable chestnuts. Although a certain amount of affected product may be sold in less demanding markets, this practice results in important economic and potential market losses due to consumer rejection. A number of molds can cause postharvest decay and disease problems in chestnuts. Among these, *Penicillium* spp., *Aspergillus* spp., *Fusarium* spp., *Phomopsis castanea, Acrospeira mirabilis,* and *Sclerotinia pseudotuberosa* have been repeatedly identified in France, Italy, Australia, Chile, United States and other countries (Ellis and Ellis, 1985; Jerimini, et al., 2006; Montealegre, J. and Gonzalez, S. 1986; Paglietta and Bonous, 1979; Ridé, M. and Gudin, C. 1960; Vettraino et al., 2005 Washington et al. 1997; Wright, 1960). In 2007, more than 25 percent of the total Michigan crop was lost due to postharvest decay, equivalent to approximately 11,500 pounds of fresh chestnuts, which reflects a potential annual economic loss of approximately $30,000 or more for the young Michigan start-up chestnut growers' cooperative, Chestnut Growers, Inc. (CGI).

Furthermore, some fungi are capable of secreting substances that are potent, acute toxins or carcinogens to both animals and humans. These toxic

agents are called mycotoxins and their impact on domestic animals in terms of decreased growth rate, abnormal reproduction and early death has long been recognized (Adams and Moss, 2000; Bullerman and Bianchini, 2007; Cho et al., 2008; Mateo and Jiménez, 2007; Schollenberger et al., 2008; Tanaka et al., 2007; Varga, et al., 2007). The most important mycotoxins are aflatoxins, deoxynivalenol (DON), zearalenone, fumonisin B1, T-2 toxin, and ochratoxin A. These substances have been predominately isolated from grains such as wheat and corn, but can also been found in chestnuts as well as in other products including rice, grapes, beer, wine, vegetable oil, vegetables, peanuts, pecans and processed foods (Adams and Moss, 2000; Bullerman and Bianchini, 2007; Cho et al., 2008; Mateo et al., 2007; Lillard et al., 1970; Schollenberger et al., 2008; Tanaka et al., 2007; Varga, et al., 2007). In a nationwide survey in Canada from 1998 to 1999, penitrem A, chaetoglobosin A and C, emodin and ochratoxin A were found on imported chestnuts sold by grocery stores (Overy et al., 2003).

As an alternative to fresh chestnuts, peeled chestnuts can be sold as a value-added product to extend the chestnut market beyond seasonal sales, providing the opportunity to expand markets and their utilization. In Michigan, peeled chestnuts can be obtained by processing fresh chestnuts with a commercial peeling system obtained from Italy in 2001 (Boema; Neive, Italy) (Appendix B). After peeling, the chestnuts are vacuum-packed and stored frozen (Guyer et al., 2003). Other mechanical methods, including air-impingement de-shelling are also available but are not used in Michigan (Gao et al., 2008). Peeled frozen chestnuts, often develop a sticky, yellow ooze over the surface of the nut, within twelve days after thawing that affects quality and acceptability (B. Harte

and D.W. Fulbright, personal communication). Similar problems have not been previously reported in peeled chestnuts, but are common for processed fruits, vegetables, juices, ready-to-use salads and meats. In general, the growth of spoilage microbes, including several bacteria and yeasts, is usually accompanied by the accumulation of metabolites, such as ethanol, lactic acid, and ethyl acetate. These organoleptic changes due to microbial activity are associated with the enzymatic oxidation of various compounds leaking from the injured tissues. Spoilage is detectable by sensory and microbiological methods. When the microbial population attains a specific level, spoilage is dependent on the species in question and the ingredient assayed. In extreme cases, the refrigerated (4 °C) shelf life of several commercial products may not exceed five days (Brightwell et al., 2007; Guerzoni et al., 1996; James et al., 2005; Ng, 2007; Ragaert et al., 2006; Tournas, 2005).

All these issues regarding postharvest mold, decay, post-processing spoilage of peeled chestnuts and safety concerns, reflect the need for more effective microbial reduction strategies after harvest and processing. In response to these needs, one specific goal of this research was to determine the potential organisms that interact with both fresh and peeled chestnuts. Elucidating these organisms will help assess the efficacy of various physical, chemical, and biological treatments for reducing the microbiological populations ensuring the quality, safety and prolonged shelf-life of fresh and peeled chestnuts.

Chestnut fruit

Many true nuts, including chestnuts, are wrapped in a papery or spiny husk called the involucre, more commonly known as a bur (Appendix C). This structure is usually mistaken as the fruit, but it is actually a whorl of modified leaves around the flower or flowers. Inside the involucre, depending on the species or cultivar, one or several chestnuts can be found (usually three). In chestnuts, the ovule or ovules that develop into a seed or several seeds are called kernels, structures that are formed inside an ovary, which will swell and give rise to what is known as the shell, but botanically is a fruit called an achene (pericarp). This structure has an external brown wall with a woody and shinny appearance. Two large cotyledons, forming the kernel, surround the embryo, which contains the radicle, hypocotyls and epicotyls. Between the shell and the kernel, is a thin brown papery-like structure, commonly called a pellicle, which is botanically known as a seed coat, testa or episperm (Mencarelli, 2001; Miller G., 2003). Usually there is only one kernel per shell, but in certain cases there may be two or more (commonly called a double embryo). In different regions of Europe, including Italy and Spain, the term "marron" denotes a single kernel nut, while the term "chatâigne", "castaño" or "castaña" denotes double or even more kernels per shell. Therefore, from a botanical point of view, a chestnut is a fruit (achene) containing one or more seeds (kernel or edible part) with creamy, yellow-colored cotyledons that are covered by a membrane called the pellicle (episperm) (Mencarelli, 2001; Miller G., 2003).

When chestnuts start to ripen in late summer or autumn, the bur changes color from light green to yellow-brown and releases the chestnuts. Sometimes the bur opens on the tree, releasing the chestnuts, but the bur can also drop and open on the ground (Anagnostakis et al., 1998; Mandujano et al., 1998; Mencarelli, 2001; Miller, 2003; Willis et al., 2007).

Unlike other nuts like almonds, hazelnuts and walnuts, the chestnut kernel is starch-based (40 – 90 percent[1]), slightly hard, containing high fiber (14 - 19 percent), protein (6 - 10 percent) and low lipids (0.4 - 10 percent), which are 90 percent unsaturated fatty acids. Chestnuts are also rich in sugars mainly sucrose, glucose and fructose (40 -60 percent) and its water content is relatively high (>50 percent) (Anagnostakis and Devin, 1999; Fulbright, 2003). Furthermore, chestnuts are a source of vitamin A, calcium, iron, fiber with and antioxidants (Biomhoff et al., 2006; Gao et al., 2008). Biomhoff et al. (2006) and Anagnostakis and Devin (1999) reported that all these characteristics are highly beneficial for human health and are needed for proper nutrition and protection of animal cells, ranking chestnuts as a healthy food for consumers.

After harvest, respiration of most seeds is usually characterized by a low and constant rate without any peak, reflecting a non-climacteric respiration pattern. The same relative pattern can be found in chestnuts, but when compared to other seeds and to most other nuts, chestnuts have a higher respiration rate. Subsequently, due to their high respiration rate, water loss and starch conversion to sugars is high (Kader, 2002; Willis et al., 2007). Based on several respiration

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[1] Nutrients in chestnuts expressed as the percentage of dry weight

rate studies (Harte et al., 2003), chestnuts were comparable to certain berries (*i.e.*, blueberries) (Perkins-Veasie, 2004) and iceberg lettuce (Smyth and Cameron, 1998; Kader, 2002), and exhibited a lower respiration rate than cut broccoli (Talasila et al., 1994), peas, asparagus, sweet corn and mushrooms (Kader, 2002).

Therefore, the most common changes that occur in chestnuts after harvest are moisture loss, starch conversion to sugar, fungal decay and insect damage (Wells, 1980). Only starch conversion to sugar (commonly called curing) positively affects chestnut quality, enhancing the flavor, sweetness and acceptability of the product (Harte et al., 2003). The other three major factors have a negative affect on quality and storage potential of the final product (Harte et al., 2003; Jerimini et al., 2006; Montealegre and Gonzalez, 1986; Paglietta and Bonous, 1979; Wells, 1980). Because quality can only be maintained and not improved after harvest (Kader, 2002; Willis et al., 2007), efforts have focused on increasing the storability of chestnuts by reducing microbes and insects and maintaining the quality at harvest. Vossen (2000), reported that during three months storage, temperatures between -2 °C to 0.5 °C were recommended and Dooley et al. (1980) and Jian et al. (2002) concluded that controlled atmosphere (CA) plays an important role in

inhibiting fungal decay, increasing storage and reducing moisture loss. Currently, storage practices by growers and cooperatives have been unable to efficiently maintain chestnuts quality after harvest (Harte et al., 2003). This affirms the

urgent need to develop better postharvest chestnut handling and storage practices.

World chestnut industry

According to FAO in 2002, at least 25 countries produced chestnuts. With the exception of France, chestnut production has experienced continuous growth (FAO, 2002; Vossen, 2000) during the past 10 years. Chestnut production worldwide was estimated of over 500,000 tons (454,000 metric tons) during 2000-2001 and was distributed as follows: China, 23.7 percent; Korea, 23.3 percent; Italy, Turkey, and Japan, about 10-15 percent each; France, Spain, and Greece, about 5 percent each; and the United States, Australia, New Zealand, Chile, among others, less than 1 percent each (FAO 2002) (Appendix D). This production pattern is mainly due to the increase in cultivation areas and changes in consumer preferences towards healthier and more convenient foods (Gold et al., 2004; Kader, 2002; Vossen, 2000).

China is the largest low-cost producer and exporter of chestnuts with an estimated production of 117,000 tons (105,300 metric tons) and exports about a third of their chestnuts to Japan. Most chestnuts are consumed fresh or roasted with an unspecified amount used in Chinese cuisine to develop a broad variety of dishes.

Korea, the second largest producer yields almost the same amount of chestnuts as China per year (115,000 tons = 103,500 metric tons), approximately half of which are exported to Japan and some to the United States.

Japan is the largest chestnut importer and is among the largest consumer group, even though it is not the biggest producer. In Korea and Japan, local or imported chestnuts are primarily stored under refrigerated conditions of 4 – 7 °C and consumed fresh, boiled or as an ingredient in diverse dishes. Some are also stored dry or peeled for further use.

Within Europe, Italy is the largest chestnut producer and leads the world in production of delicacy-processed products such as *marrone glacé* (preserved chestnuts in sugared liquor). In Europe, the use of dry chestnuts and chestnut flour in cooking has recently declined, but the popularity of these products is increasing elsewhere, especially in the United States. These value-added products, such as processed, dried, peeled, and frozen chestnut have reached more than US $3.00 per pound, prompting moves to expand the chestnut industry (Vossen, 2000). Europe's second largest producer is France, with up to 25,000 tons (23,000 metric tons) per year. Irrespective of the decline in production over the past 10 years, due to other more valuable crops and urbanization, France is one of the biggest importers of chestnuts in Europe, mostly from Italy, Spain and Portugal.

Recently the United States, Australia, New Zealand, Chile and other countries in the Southern Hemisphere have begun to produce chestnuts and have established economically sustainable industries, mainly for export (FAO, 2002; Vossen, 2000; Fulbright and Mandujano, 2000). The United States, has at least of 2,500 acres of chestnut tree plantations. Of these, approximately 1,500 acres of young (less than 10 years) chestnut trees are distributed among

Michigan, Oregon, Washington and Ohio (Fulbright, 2008; Vossen, 2000). These commercial plantations are primarily from the cultivar 'Colossal' (European-Japanese hybrid = *C. sativa* x *C. crenata*). This cultivar has been broadly used, because it produces large nuts (up to 30 g), high yields (> 55 kg/tree), and is commercially sold at nurseries (Miller, 2003; Fulbright and Mandujano, 2000). Other cultivars such as 'Dunstan Hybrids' (a patented seedling of a third generation cross between American and Chinese chestnut hybrids = *C. dentata* x *C. mollissima*), 'Skookum', 'Layeroka', 'Myoka', 'Skioka', 'Eaton' which have apparent Chinese characteristics have also been planted (Miller, 2003; Fulbright and Mandujano, 2000; Vossen, 2000).

In recent years chestnut average production has been increasing (Vossen, 2000). As a point of reference, in 2003, approximately 3,000 pounds of chestnuts were harvested by the Chestnuts Grower Incorporation (CGI) in Michigan, while in 2007 a total of 45,000 pounds were harvested (Blackwell, 2006; Fulbright D. W., 2008). An important chestnut state is California where the oldest commercial plantations are found. Vossen (2000) indicated that most of the early chestnuts orchards established in California were brought by immigrants during the Gold Rush and are mostly seeds from European chestnuts.

Michigan's chestnut harvest starts in mid-September and proceeds through the first week of November but may be slightly later in northern or colder locations. Product is primarily sold through CGI a producer owned and controlled marketing cooperative with about 40 members. Most chestnuts are sold from Thanksgiving through Christmas, via sales to specialty ethnic markets, retail stores, food processors, restaurants, holiday festivals, farmers' markets and

individual consumers. Fresh and frozen peeled chestnuts compose up to 60 and 30 percent, respectively of outlet sales. Most of the remaining chestnuts are sold as flour, breading and new dehydrated products such as chips and slices, and puree (Blackwell, 2006; Fulbright D. W., 2008).

Since freshness and microbial quality are sales factors in chestnut appearance, domestic production has a definite advantage over imported chestnuts. Due to this advantage, storage conditions and postharvest management strategies are both important in preventing the chestnuts from molding and decaying. Regardless of quality and lack of freshness due to long-term storage and transport of imported chestnuts, the United States annually imports between 10 and 20 million pounds (4.5 to 9 million kg) of fresh European and Chinese chestnuts, at a retail price of approximately 40 million dollars. In order to the United States to expand its markets, replace imported chestnuts, and fulfill its all local needs, more than 5,000 acres (2,000 ha) of production would be required. Furthermore, if chestnuts are marketed efficiently and domestic consumption increases by only half a pound (0.22 kg) per capita, the United Sates would require over 50,000 acres (20,200 ha) of mature production to meet their demand. Following this growth, the industry would be worth more that $300 million annually, but may be worth more than $800 million annually, if the increase in consumption is even higher (Vossen, 2000).

Taken together, these suppositions indicate that the opportunity for expanding chestnut markets is a feasible venture. Fulbright and Mandujano (2000) indicated that establishing chestnut orchards in United States, including Michigan, has not been an easy task, but if they are managed appropriately and

planning is done efficiently, it may be economically successful. Nevertheless, regardless of the location, anyone considering investing in a chestnut orchard should evaluate individual production costs, and earning potential, and is advised to make comparisons with alternative investments.

Postharvest organisms in fresh and peeled chestnuts

Although Adams and Moss (2000) defined spoilage as *"The change in characteristics in a food, making it no longer acceptable"*, spoilage in foods can be considered subjective. Different factors, such as insects, dehydration and environment conditions, cause spoilage; but by far, one of the primary causes comes from microbial activity (Adams and Moss, 2000). Fungi have been identified as the major agents of postharvest fresh chestnut decay and rot (Jerimini et al., 2006; Jian et al., 2002; Montealegre and Gonzalez, 1986; Miller G., 2003; Paglietta and Bonous, 1979; Rutter et al., 1990; Vettraino et al., 2005; Vossen, 2000; Washington et al., 1997). A broad number of organisms have been identified that cause chestnut decay, colonizing the product after harvest.

Few of all these organisms, have been characterized as true plant pathogens, capable of initiating infection prior to harvest. One true plant pathogen *Sclerotinia pseudotuberosa* Rehm (syn. *Ciboria batschiana* Zopf., anamorph *Rhacodiella castaneae* Bainier), is responsible for chestnut black rot and one of the most important postharvest diseases of acorns (*Quercus* spp.) and chestnuts, especially in Europe (Vettraino et al., 2005; Washington et al., 1997). Infection usually occurs when chestnuts fall to the ground during harvest and rapidly become contaminated with ascospores secreted by fungal apothecia, previously

formed by dormant sclerotia lying on the ground (Agrios, 2005; Vettraino et al., 2005). However, studies by Delatour and Morelet (1979) and Vettraino et al., (2005) have also found infected chestnuts attached to the tree, and have identified the pathogen present in different plant parts, suggesting that the pathogen may occur symptomatically and asymptomatically as an endophyte. Another uncertain but possible real pathogen, which affects chestnuts is *Phomopsis castanea* Sacc., causing phomopsis chestnut rot. This organism has been predominantly reported in Australia and Chile and was recently identified as an endophyte in chestnuts. Although the disease cycle is still unclear, this fungus colonizes nuts via the peduncle and hilum (Appendix C). The disease can also be found in both chestnuts attached to the tree, and those in storage, but it appears that natural infection occurs in the field (Washington et al., 1998). Partial control of the disease can be achieved by postharvest management practices including low temperature storage and controlled atmosphere storage following an initial period in a controlled carbon monoxide atmosphere (Washington et al., 1998). Natural infection occurs in the field before harvest, since this disease can be found in chestnuts both before and immediately after harvest. Therefore, control measures are needed to restrict disease development in the field. Few report the use of fungicides for control of Phomopsis chestnut rot or related diseases in chestnuts. Montealegre (1984) indicated that captan, dodine or a combination of benomyl and mancozeb showed good control of *P. castanea* in vitro. Washington et al. (1998), reported that benomyl, imazalil, prochloraz and propiconazole were most effective in vitro against mycelial growth in Australia.

Opportunistic fungi involved in chestnut mold and rot after harvest include *Phomopsis endogena* Speg. (cif. *Phoma endogena* Speg.), *Phomopsis viterbenis Camici*, *Diplodia castanea* Prill and Del., *Alternaria* sp, *Aspergillus* sp., *Penicillium* spp., *Botrytis cinerea* Pers., *Gloesporium* sp., *Fusarium* spp., *Dothiorella* sp., *Cytodiplospora castanea* Oud., *Pestalotia* spp., *Diplodia* spp., *Acrospeira mirabilis* Berk. and Broome and *Rhizopus* spp (Breisch, 1993; McCarter et al., 1980; Montealegre and Gonzalez, 1986; Paglietta and Bonous, 1979; Pratella, 1994; Puttoo et al., 1988; Ridé and Gudin, 1960; Washington et al., 1997; Wright, 1960).

Peeled chestnuts, considered to be a fresh-processed (cut) product (IFPA, 2006) can be affected by the same microorganisms as the fresh product, but other opportunistic organisms may also play an important role in their spoilage. By removing the shell and pellicle, which are considered to be a weak but still natural physical barriers that protect the kernel, contamination with pathogens, opportunistic organisms and water loss can be facilitated, affecting their final quality and safety (Cantwell, 1995; Mencarelli, 2001). Therefore, consideration should be given to removing the shell and other plant parts, since natural products derived from plant parts may contribute to pathogen resistance and colonization by opportunistic organisms (Field et al., 2006). Such compounds, involved in protection from decay have not yet been isolated from chestnut kernels, however evidence suggests that undetermined substances in the shell and pellicle, may protect the kernel. These presumptive compounds may protect the chestnuts from decay, especially when the chestnuts are on the tree or stored fresh (Appendix E).

Even though Michigan peeled chestnuts have experienced microbial spoilage during cold storage (4 °C), there have never been reports that indicate similar spoilage problems in peeled chestnuts elsewhere. However, this problem is not unique because similar spoilage occurs with other vegetables and fruits, mainly after peeling, packaging or processing (Brightwell et al., 2007; Guerzoni et al., 1996; James et al., 2005; Ng, 2007; Ragaert et al., 2006; Tournas, 2005; Zagory, 1998). Zagory (1998) and Tournas (2005) indicated that after harvest, a wide range of economically important vegetables and specifically fresh-cut products, are often spoiled by a wide variety of microorganisms including many bacterial (*Curtobacterium*, *Rahnella*, *Erwinia*, *Pseudomonas* spp., among others) and several fungal species. Of those commodities evaluated, spoilage increases significantly when the product is injured or the skin, which acts as a physical barrier has been damaged or removed. Packaged sliced onions, shredded mixed lettuce and other commodities under ambient conditions and modified atmosphere, can become colonized by diverse spoilage microorganisms, including various yeasts as *Pichia fermentans*, *Cryptococcus laurentii*, and *Candida* spp., among others (Liu and Li, 2006; Ragaert et al., 2006).

Microbial populations typically increase from harvest, through processing, and cool storage, with the extent of growth impacting the shelf-life of the product. This can lead to a series of problems in both fresh and processed products, which includes several types of external molds, rots, internal decay and severe spoilage (Hammer, 1949; Tournas, 2005; Washington et al. 1997; McCarter et al., 1980; Zagory, 1998). These microbial changes, bring about undesirable

qualities and economical loss, thus confirming the importance of controlling microbial growth during production, harvest, processing, and during storage (Tournas, 2005; Zagory, 1998).

Food safety concerns

A critical factor in developing any industry is the ability to offer a safe high quality product. Chestnuts are not only subject to microbial decay, but without proper care, safety of the product may be subsequently compromised by accidental contamination with food-borne pathogens and an increase in endemic organisms or compounds. These organisms and compounds may cause immediate or long-term problems and diseases as well as intoxication of the consumers. Food has historically been associated with the transmission of diseases (CDC, 2008).

The World Health Organization (WHO, 1992) states: *"Even today, despite the increase of knowledge, foodborne disease is perhaps the most widespread health problem in the contemporary world and an important cause of reduced economic productivity"*. The Centers for Disease Control and Prevention (CDC) estimate that annually in the United States, at least 76 million cases of foodborne illness occur, more than 300,000 people are hospitalized and 5,000 die (CDC, 2008). In the United States, during 1998 to 2002, a total of 6,647 outbreaks of foodborne disease were reported, causing approximately 128,370 people to become ill (CDC, 2008). In 33 percent of the cases, the etiology was identified with bacterial pathogens causing the largest percentage of outbreaks (55 percent). Among bacterial pathogens, *Salmonella enterica* serotype Enteritidis and *Listeria monocytogenes* accounted for the largest number of outbreaks and

the majority of deaths. In addition, multistate outbreaks caused by contaminated fresh produce, such as spinach and lettuce, mainly caused by *Escherichia coli* O157:H7 have remained prominent. Viral pathogens, predominantly norovirus, caused 33 percent of outbreaks and increasing. Chemical agents, like mycotoxins, and parasites caused 10 and 1 percent of the outbreaks, respectively (CDC, 2006; CDC, 2008).

Thus far no foodborne disease outbreaks have been traced to chestnuts in the United States. Nevertheless, this cannot be discarded due to the fact that since 1998, more than 18 foodborne disease outbreaks in fruits and other nuts have been reported each year. Of those, in 2006, *Salmonella* serotype Tennessee and *Salmonella* serotype Thompson, together caused 107 cases of salmonellosis in Vermont and South Carolina, mainly from consumption of mixed nuts and peanuts (CDC, 2006). Furthermore, in 2004 an outbreak of salmonellosis has been linked to consumption of contaminated raw almonds, and hundreds of consumers across the United States may have been sickened. The recall was expanded to include millions of pounds of raw California almonds sold worldwide, significantly affecting the reputations and economy of this almond industry (CDC, 2006).

The number of outbreaks due to the presence mycotoxins is low, this natural toxins impact the safety of the final product, especially when long-term health effects are considered. Concerns over mycotoxins contamination has continued to climb in recent years. Recent recalls in several industries, including the grain and pet food industry in the United States have raised questions over ingredients sourcing and cross-contamination of the food chain. This affirms that

the economic impact of mycotoxins in feeds is in the hundreds of millions of dollars, including reduced production, monitoring, managing and control (CDC, 2008). Considerations must be taken in account, because diverse fungi colonize chestnuts and mycotoxins have been identified, in the fresh product during storage and retail sales (Overy et al. 2003).

New technologies such as modified atmosphere packaging (MAP) and other innovative safe postharvest treatments can reduce decay and spoilage organisms. Even though the food may look and smell appropriately, the product may not be safe to eat (Rajkowski and Baldwin, 2003). In diverse fresh-cut products such melons and other fruits that have been packaged under MAP, an increase in CO_2 and decrease in O_2 has been observed, which favor *Clostridium botulinum* growth and toxin formation, making these products unsafe for consumption (Enfors and Molin, 1978). When minimally processed and packaged foods are stored under refrigerated conditions (4 - 7° C) *Salmonella*, *Aeromonas*, *Yersinia*, *Vibrio cholerae, E. coli* O157:H7, and other foodborne pathogens can grow to undesirable levels (Novak et al., 2001). All of these food safety issues must be considered as possible safety concerns for fresh chestnuts, and could be especially devastating in the case of processed products, like vacuum-packaged, peeled, frozen chestnuts.

Due to these concerns, when any food product is being distributed, including fresh and peeled chestnuts, preventive measurements must be taken to assure that the product is safe to consume (CDC, 2006).

Sanitizers, postharvest and post processing treatments

With increased global trade and seasonality of agricultural products, fresh agricultural commodities are being transported over vast distances and stored for prolonged periods. Thus, effective cold-chain and product management practices are required to ensure that fresh products maintain their premium quality and safety. The fresh produce industry must develop several of the impact of postharvest treatments, sanitizers and practices that contribute to produce quality and safety (Kader, 2002).

Sapers (2003) stated that, "*Generally, it is more difficult to decontaminate products than it is to avoid contamination. Therefore, pre- and postharvest interventions that reduce risk of contamination will make the job of sanitizing the produce easier*". Due to this situation, the U.S. Food and Drug Administration (FDA) published a "Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables" in October 1998, which describes the important steps needed to remove field contaminant (dirt, twigs and more), chemical residues, and microorganisms responsible for quality loss, safety concerns and decay. This was a general, commonsense guide because, at that time, there was very little specific research to provide more concrete advice. In response to the continuing problems in particular sectors of the fresh produce industry, FDA has issued several warning letters and initiatives. In January 2004, FDA and CDC met with produce industry leaders to discuss numerous foodborne illness outbreaks associated with produce. Industry representatives agreed to take the lead on developing commodity-specific Good Agricultural Practices (GAPs) that would

provide additional guidelines tailored to individual commodities that had been implicated in recent foodborne illness outbreaks. In April 2006, in reaction to *E. coli* O157:H7 outbreaks on lettuce and spinach, the produce industry published its "Commodity Specific Food Safety Guidelines for the Lettuce and Leafy Greens Supply Chain." Nevertheless, until recently, little information was available concerning the efficacy of washing and sanitizing treatments (Sapers, 2003).

Historically, more is known about chemicals, such as fungicides, but consumer attitudes, safety concerns, regulations, and health and environmental issues regarding their use, inhibit their implementation. Nevertheless, these broadly used are efficient in reducing decay organisms (Gamage et al., 1997; Kader, 2002; Kicast, 1995; Narayanasamy, 2006; Sapers, 2003; Saroj et al., 2006; Smith and Pillai, 2004). Many less toxic have since been investigated including hydrogen peroxide, diverse acids, ozone, chlorine dioxide (gas and solution), hypochlorite, organic acids, natamycin, heat and more (Beuchat 1998; Block 1991; Brackett 1999; Crowe et al., 2005; Palou et al., 2002; Panagou et al., 2005; Perrera and Karunaratne 2001; Pierre et al., 2006, Sapers 2003; Suslow 2002; Wade et al., 2003). These compounds can be used to enhance safety, and shelf life of most type of fresh produce and including blueberries, citrus, sprouts, meat, leafy greens and many others. Several salts, oils and biocontrol agents that efficiently reduce decay also have been evaluated (Feng and Zheng, 2006, Kader, 2002; Mari et al., 2003; Mills et al., 2004; Porat et al., 2002; Sapers, 2003; Willis et al., 2007).

Incoming produce is typically delivered to a storage or distribution facility. After delivery, it is immersed in clean water containing an appropriate

concentration of a specific chemical sanitizer, or treated by other means (*e.g.* irradiation) with the final objective being the elimination of pathogens and the reduction of other organisms to an acceptable level (Kader, 2002; Sapers, 2003). The advantages, limitations, usage restrictions of a number of agents used to sanitize horticultural commodities and diverse foods are described in Table 1.

Table 1. Advantages, disadvantages and uses of some available sanitizing agents, salts, chemicals, bio-control agents, heat and irradiation for postharvest and postprocessing treatment of fresh fruits, vegetables and foods

Agent	Use Level	Advantages	Disadvantages	Use	Source
Chlorine (Sodium hypochlorite, liquid bleach, soda bleach liquor)	Specified for specific applications, usually between 50 to 200 ppm, but can be used up to 20,000 ppm for seeds	FDA approved Inexpensive General disinfection and broadly used Effective against a broad number of microbes Easy to monitor	Unstable in organic matter and variable pH Hazardous, possible carcinogenic, irritating Corrosive	Broadly used in food industry, ready-to-eat produce, horticultural commodities, water purification and more Reduces spoilage, postharvest pathogens and foodborne pathogens	(Crowe et al., 2005; Panagou et al., 2005; Spotts and Peters, 1980; Sapers, 2003)
Chlorine dioxide	1 to 5 ppm	FDA approved (for certain commodities- *e.g.* blueberries) Less corrosive compared with chlorine and ozone Not pH-dependent More potent that chlorine and effective against biofilms Residual	Corrosive Difficult to apply	Fresh horticultural commodities Not permitted for cut fruit or vegetables Reduces spoilage, postharvest pathogens and foodborne pathogens	(Mari et al., 1999; Roberts and Reymond, 1994; Sapers, 2003; Spotts and Peters, 1980; Wu and Kim, 2007; Zoffoli et al., 2005)

Table 1. (cont'd)

Agent	Use Level	Advantages	Disadvantages	Use	Source
Ozone	0.1 to 2.5 ppm	Generally recognized as safe (GRAS), but FDA review possible More potent than chlorine Not pH-dependent	May be expensive Special facilities to generate it on-site, including good ventilation Corrosive Not Residual Phytotoxic at high concentration Expensive and difficult to monitor OSHA concerns	Broadly used for ready-to-eat produce, horticultural commodities and water purification Reduces spoilage, postharvest pathogens and foodborne pathogens	(Gurol and Akata, 1996; Palou et al., 2002; Sapers, 2003; Sarig et al., 1996; Sharma et al., 2003; Suslow, 2002; Wade, Scouten et al., 2003)
Peroxyacetic acid	≤ 80 ppm	FDA approved Not pH-dependent More potent and effective against biofilms than chlorine Monitoring not difficult No on-site generation required	Hazardous and irritating Corrosive	Broadly used for ready-to-eat produce, horticultural commodities Reduces spoilage, postharvest pathogens and foodborne pathogens	(Mari et al., 1999; Mari et al., 1999; Pierre et al., 2006; Sapers, 2003

Table 1. (cont'd)

Agent	Use Level	Advantages	Disadvantages	Use	Source
Hydrogen peroxide	≤ 1000 ppm	FDA approved GRAS Produces no residue because it is broken down to water and oxygen	Phytotoxic to some commodities (*i.e.* berries and lettuce) Hazardous and irritating in high concentrations	Reduces spoilage, postharvest pathogens and foodborne pathogens	(Aharoi et al, 1994; Block, 1991;Sapers, 2003; Simmons, 1997; Ukuku and Sapers, 2001; Ukuku et al., 2001)
Organic acids (Lactic acid, acetic acid, caprylic acid, glycolic acid, capric acid, phosponic acid, propionate, sorbate, benzoate etc.)	Variable	Low mammalian toxicity Residual effect	May produce off-flavors in treated produce	Reduces spoilage, postharvest pathogens and foodborne pathogens	(Chakrabarti and Varma, 2000; Mills et al., 2004; Sapers, 2003)
Peroxidase-generated iodine, Copper (Copper sulfate pentahydrate, copper sulfate)	≤ 1 ppm	OSHA and EPA approved Low concentrations are highly effective	Toxic at high concentration May contaminate water source	Reduces spoilage, postharvest Algae control	(Sapers, 2003; Toxicology Group at NSF International, 2006)

Table 1. (cont'd)

Agent	Use Level	Advantages	Disadvantages	Use	Source
Organic and inorganic salt compounds (Bicarbonates, carbonates, calcium silicate, calcium chloride, calcium propionate, aluminum acetate, aluminum chloride, propyl-paraben, etc.)	Variable	Low mammalian toxicity Broad spectrum of modes of action Low cost	May produce off-flavors in treated produce	Reduces spoilage and postharvest pathogens Inhibit production of some mycotoxins (trichothecene and aflatoxin)	(DePasquale et al, 1990; El-Nebarawy et al., 1989; Karabulat, et al 2001; Mills et al., 2004; Ricker and Punja, 1991; Roinestad et al., 1993)
Chemicals (Mancozeb, thiabendazole, fludioxonil, trifloxystrobin)	Variable	Good control of spoilage microorganisms Residual effect	Increase of resistance in pathogens High mammalian toxicity Negative to environment Negative acceptability Lack of approval	Reduces spoilage and postharvest pathogens	(Caia et al, 1988; Griffiths, 1981; Mills et al., 2004)
Irradiation (Gamma irradiation, electron beam, X-ray)	≤ 10 kGy (spices and dry commodities), ≤ 1 kGy other foods (fruits and meats)	FAO/IAEA/WHO approved (products must be labeled properly using the Radura symbol) (*e.g.* meats) No toxic residues in commodities Alternative to chemicals and fumigants Ideal for processed foods (*i.e.* salsa, pico de gallo)	May damage produce tissue and leak nutrients by breaking down cell wall material May produce off-flavors in treated produce Viruses are highly resistant Expensive Special facilities to generate on-site Experienced personal Negative acceptability	Significant reduction of spoilage and pathogenic microorganisms Sprouting inhibition Insect and parasite disinfestations Delay of ripening Food can be sterilized (immunocompromised patients)	(Diehl, 1995; Gamage et al., 1997; Howard et al, 1995; Kilcast, 1995; Luh, 1997; Niemira, 2003; Saroj et al., 2006; Smith and Pillai, 2004)

Table 1. (cont'd)

Agent	Use Level	Advantages	Disadvantages	Use	Source
Bio-control agents (*Pseudomona syringae* Strain ESC-11, *Candida eleophila, Bacillus pumilis, B. amyloliquefaciens, Trichoderma harzianum, T pseudokoningii*)	Variable	No mammalian toxicity Value added commodity	New products still under experimentation Do not efficiently reduce foodborne diseases Application conditions are complicated and must be ideal for biological agent (intimate contact with pathogens) Irregular control	Reduces spoilage and postharvest pathogens	(Guizzardi et al, 1995; Mari and Guizzardi, 1998; Mari et al., 2003; Mari et al., 1996; Mari et al., 1996; Sugar and Spotts, 1999; Tronsmo and Raa, 1977)
Heat treatment (hot water dips or rinsing, vapor heat, hot dry air, far infrared radiation)	40° C – 90° C (15 – 30 min) (depends on commodity)	GRAS Can be used in any product that does not suffer from heat damage	May significantly damage produce susceptible to heat damage May be expensive Requires equipment to generate on-site	Reduction of spoilage and pathogenic microorganisms Insect and parasite disinfestations Reduce postharvest chilling injury Heat tolerance Inhibits anthocyanin synthesis	(Fallik, 2004; Hong et. al, 2007; Lamikanra and Watson, 2007; Lydakis and Aked, 2003; Mills et al., 2004; Siomos et al., 2005; Soto-Zamora and Yahia, 2005; Wolf and Bowen, 2004)

Sapers (2003) indicated that washing and sanitizing produce and equipment generally does not reduce the microbial populations greater than 2-logs (99 percent). Although these reductions may improve products shelf-life and quality, the possibility of human pathogen survival can not be excluded. Furthermore, diverse factors such as microbial adherence, formation of biofilms, and penetration of the microorganisms may compromise the efficacy of certain compounds. New washing technologies are being constantly evaluated and are needed to overcome these deficiencies. The new treatments must be superior in efficacy, but also must be safe and affordable to apply. Innovating technologies, such as food irradiation and heat treatments, which may be capable of reducing pathogens and other microorganisms (pasteurization treatments), greater than 5-logs might bring large improvements in the microbiological quality and safety of produce (Niemira, 2003).

Various chemical treatments have been evaluated for chestnuts in attempts to reduce mold and decay during fresh chestnut storage. Among the evaluated treatments, 95 percent alcohol, 1 percent copper sulfate, boric acid, 0.4 percent calcium chloride, formaldehyde, benzoic acid, sulfur dust and others were unsuccessful. Furthermore, these experiments indicated that certain treatments such as boric acid and formaldehyde, might also compromise the quality of the chestnuts by hardening the kernel (Hammer, 1949; Tan et al., 2007). Other materials exhibiting potential for decay control include 30 ppm iodine, 250 ppm carbendazim, 100-200 ppm sodium hypochlorite, 100 ppm peracetic acid (Morris, 2006; Panagou et al., 2005), 50 ppm natamycin, 1 percent

sorbic acid, 1 percent propionic acid, hot water dip at 90 °C for 10 minutes (Panagou et al, 2005) and immersion in certain fungicides like 2,6-dichloro-4-nitroaniline (Botran) (Wells, 1980). Water immersion at 15 °C for 15 minutes, 45-48 °C for 15 minutes and 52 °C for 5, 15, 30 or 60 min significantly reduced the insect damage and the presence of larvae of *Cydia splendana*, *Curculio elephas* and *Curculio sayi* during storage (Jerimini, et al., 2006; Sieber et al., 2007; Wells, 1980).

Even though various treatments may reduce microbial populations and insects in chestnuts water immersion at 52 °C warm bath for 60 minutes, may affect the final quality of the product, by decreasing soluble sugars and increasing starch during storage (Wells, 1980). Other options may be available to treat chestnuts after harvest, however there is still a need to find feasible safe alternative approaches to control decay of fresh chestnuts, during storage (Sapers, 2003).

Limited work has been done on strategies to control postprocessing spoilage of peeled chestnuts. However, many treatments used broadly for other food commodities may be useful in enhancing the microbial shelf-life of chestnuts (Sapers, 2003).

Objectives of this study

Several studies have identified the organisms that colonize chestnuts (Jerimini et al., 2006; Jian et al., 2002; Montealegre and Gonzalez, 1986; Miller G., 2003; Paglietta and Bonous, 1979; Rutter et al., 1990; Vettraino et al., 2005; Vossen, 2000; Washington et al., 1997). However, concerns regarding local

postharvest molds and decay in fresh chestnuts, as well as postprocessing microbial spoilage in peeled chestnuts still remain. Studying the organisms that colonize chestnuts and their interaction with each of the products should help us understand the behavior of these organisms before harvest, during storage and after processing. These studies will provide powerful tools to increase shelf life, leading to improved marketing of attractive, safe high quality chestnuts. Therefore, to help determine the impact of various microorganisms in the shelf life of fresh and peeled chestnuts, two objectives were set:

1. Determine the population of microorganisms and their impact on shell mold and kernel decay of fresh chestnuts in Michigan.

2. Determine the population of microorganisms and their impact on mechanically peeled chestnuts in Michigan.

Routine postharvest application of sanitizers, such as chlorine to chestnuts, does not appear to provide efficient control of the previously mentioned postharvest or postprocessing problems, leading to significant economic and quality losses. In reaction to this concern, two objectives were designed to identify more efficient microbial reduction strategies to minimize postharvest decay of chestnuts:

1. Evaluate the efficacy of postharvest treatments to reduce microbial decay of fresh chestnuts.

2. Evaluate the efficacy of postprocessing treatments to reduce microbial spoilage of peeled chestnuts.

With answers to these questions, I hope to help the young chestnut industry in Michigan provide high-quality chestnuts in future years.

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CHAPTER 2

SHELL MOLD AND KERNEL DECAY OF FRESH CHESTNUTS IN MICHIGAN

Abstract

Chestnut is a relatively new crop for Michigan and post-harvest loss due to decay has been problematic as production has increased each year. In 2007, more than 25 percent of the nuts were lost to postharvest decay, equivalent to approximately 5,300 kg of fresh product. To determine the organisms responsible for decay, a microbiological survey was performed in 2006 and 2007 to identify microorganisms involved in postharvest shell mold and internal kernel decay of chestnuts. Filamentous fungi including *P. expansum*, *P. griseofulvum*, *P. chysogenum, Coniophora puteana*, *Acrosperia mirabilis Acrospaeria mirabilis*, *Botryosphaeria ribis*, *Sclerotinia sclerotiorum*, *Botryotinia fuckeliana* (Anamorph *Botrytis cinerea*) and *Gibberella* sp. (Anamorph *Fusarium* sp.) were the predominant microorganisms that negatively impacted fresh chestnuts. Populations of microorganisms, including these fungi, varied between the farms, harvesting method and chestnut part. Overall, chestnuts harvested from the orchard floor were significantly ($p < 0.05$) more contaminated than chestnuts harvested directly from the tree, by more than 2-log CFU/g. In addition, a significant difference ($p < 0.05$) in the microbial population was seen between chestnuts submitted by different growers, with average count ranges of fungi, mesophilic aerobic bacteria (MAB) an yeast equal to 4.75-, 4.59- and 4.75-log CFU/g subsequently.

Introduction

Chestnut (*Castanea* spp.) is considered to be the most popular nut-bearing tree among the Mediterranean countries of Europe and many countries in Asia with new production now starting in Australia, New Zealand and Chile (Fulbright and Mandujano, 2000; Grau and France, 1999; Klinac, Seelye, and Nguyen, 1999; Ridley, 1999; Mencarelli, 2001). The increased market for chestnuts is partially due to their nutritional value, low lipid content and antioxidant properties, (Anagnostakis and Devin, 1999; Biomhoff, Carisen, Andersen, and Jacobs, 2006; Gao, Lin, and Xiao, 2008). In the United States chestnuts are rare. People are more likely to be familiar with the unrelated poisonous horse chestnut (*Aesculus* spp.) (Fulbright and Mandujano, 2000) than with any of the edible, sweet chestnut species (*C. dentata* Borkh., *C. mollissima* Bl., *C crenata* Sieb and Zucc., *C. sativa* Mill., *C. seguinii* Dode, *C. pumila* Mill, *C. henryi* Rehd. and Wils.) (Anagnostakis, Gordon, and Hebard, 1998; Fulbright and Mandujano, 2000; Miller, 2003). This is partially due to chestnut blight (*Cryphonectria parasitica* Murril and Barr), which virtually eliminated the once-widespread American chestnut during the first half of the twentieth century (*C. dentate*) (Anagnostakis, 1992). Nevertheless, commercial orchards have been established in different locations in the United States, and have been increasing over the years (Fulbright and Mandujano, 2000; Vossen, 2000). The United States, has at least of 2,500 acres of chestnuts tree plantations. Of these, approximately 1,500 acres of young (less than 10 years) chestnut trees are distributed among Michigan, California, Oregon, Washington and Ohio (Fulbright, 2008; Vossen, 2000). Commercial

plantations are primarily composed of the cultivar 'Colossal' (European-Japanese hybrid = *C. sativa* x *C. crenata*). Other cultivars planted are composed predominately of Chinese chestnuts due to the naturally occurring chestnut blight resistance in this species (Miller, 2003; Fulbright and Mandujano, 2000; Vossen, 2000). Mature seedling Chinese chestnut trees (non-grafted trees) also have been planted in Midwest and eastern states with some orchards of seedling European chestnuts.

In the past ten years, domestic chestnut production has increased significantly. North American chestnuts are typically harvested from late-September to the first week of November. With most chestnuts sold from Thanksgiving through Christmas to specialty ethnic markets, retail stores, food processors, restaurants, holiday festivals, farmers' markets and individual consumers. In Michigan, fresh chestnuts and frozen, peeled chestnuts comprise up to 60 and 30 percent of outlet sales, respectively. The remainder is sold dehydrated as flour, breading and a new product called chestnut slices (Blackwell, 2006; Fulbright D. W., 2008).

Since freshness and microbial quality are factors in chestnut marketing, domestic production has a definite advantage over imported chestnuts. In Michigan, production is limited primarily due to the young nature of the industry and the microbial decay that occurs during storage. Postharvest decay of chestnuts causes severe losses, leading to completely unmarketable chestnuts. Several molds are known to cause severe postharvest decay and disease problems in chestnuts worldwide. Among these, *Penicillium* sp., *Aspergillus* sp., *Fusarium* sp., *Phomopsis castanea, Acrospeira mirabilis,* and *Sclerotinia*

pseudotuberosa (syn. *Ciboria batschiana, S. batschiana;* anamorphic from *Rhacodiella castanea,* syn. *Myrioconium castanea)* have been repeatedly identified in France, Italy, Australia, Chile and the United States (Ellis and Ellis, 1985; Jerimini, et al., 2006; Montealegre, and Gonzalez, 1986; Paglietta and Bonous, 1979; Ridé and Gudin 1960; Sieber et al., 2007; Vettraino et al., 2005; Washington et al. 1997; Wright, 1960.

Three studies were design to better understand the impact and possible effect of microorganisms involved in postharvest chestnut shell mold and internal kernel decay in Michigan. The first study was set up on one farm, with the purpose of evaluating the effect of different harvesting methods on microbial population. The second study qualitatively assessed the microbial quality (shell mold severity and incidence of kernel decay) from seven different farms. The third study identified microorganisms involved in shell mold and kernel decay on chestnuts collected from at least seven different farms.

Material and Methods

Collection and storage of chestnut samples

For the first study, were the objective was to evaluate the effect of different harvesting methods on microbial population, chestnuts of the cultivar 'Colossal' were collected from one farm in Livingston County, MI during the 2006 and 2007 growing season. Mature chestnuts were sampled using a stratified sampling method with two levels. The first level consisted of nine chestnut samples (~0.9 kg each) taken from the ground after less than 24 hours of contact. The second

level was composed of nine mature chestnut samples (~0.9 kg each) hand-harvested from different tree heights, in mature burs. Each sample was placed in a plastic bag and transported in a portable cooler to the storage facility. Before long-term storage, each chestnut sample was transferred to a mesh bag and randomly stored in a cooler at 4°C, located in Michigan State University (East Lansing, Michigan). Samples were separately placed on racks to prevent cross contamination. Microbial populations on chestnuts shells and kernels from two consecutive growing seasons (2006 and 2007) after harvest were assessed, and at 30, 60, 90 and 120 days of storage.

The second study consisted in evaluating differences between shell mold severity and kernel decay incidence (microbial quality), among farms in Michigan, three 0.77 kg samples of 'Colossal' chestnuts per farm were randomly collected before storage during the 2007-growing season. Each sample was randomly stored in mesh bags, as described above.

In addition, to engage the third study, a total of 200 individual 'Colossal' chestnuts from the 2006 and 2007 growing seasons, collected from at least seven different farms were randomly sampled twice during storage to identify the organisms that colonized the chestnuts. From this group, chestnuts showing shell mold or kernel decay symptoms were sorted for isolation and identification of the causal agents. Before identification, the observed symptoms were described, photographed and classified.

Microbial populations – First and third study

Shell

To determine the microorganisms found on shells, 25 g chestnut samples were placed in a sterile stomacher bag (Whirl-Pak, NASCO, International Inc.), diluted 1:5 in phosphate buffer solution (PBS) (pH = 7.4) and agitated for 1 min (2900/3500 rpm) in a pulsifier (Filtaflex Ltd., Almonte, Ont., Canada). After serial dilution, 100 µl aliquots were inoculated onto trypticase soy agar (Becton and Dikinson, Md, USA) containing 0.6% Bacto™ yeast extract (Becton and Dikinson) and 100 ppm of cycloheximide (Sigma-Aldrich, Mo, USA) for quantification of mesophilic aerobic bacteria (MAB) and onto potato dextrose agar (Becton and Dikinson) containing 20 ppm streptomycin (Sigma-Aldrich) and 50 ppm ampicillin (Sigma-Aldrich) for enumeration of yeasts and molds. The populations of MAB were determined after 48 h at 25° C (± 3° C). Mold and yeast plates, were counted after 72 h of incubation at 25° C (± 3° C) (APHA, 2001).

Kernel

Identically stored chestnuts from the same samples were flame sterilized after dipping in 99% ethanol. After manually removing the shell, each sample was weighed and placed in a sterile stomacher bag (Whirl-Pak), diluted 1:5 in PBS and homogenized for 1 min (normal speed) in a stomacher Model 400 (Seward Lab. System, England). The suspension was serially diluted, and quantitatively examined for MAB, yeast and molds as previously described (APHA, 2001).

Microbial mold severity and kernel decay incidence analysis – Second study

A sub-sample consisting of 3 randomly chosen stored chestnuts collected from each of seven farms was assessed for severity of shell mold and incidence of kernel decay after harvest and after 30 and 60 days of storage. Shell mold severity was calculated as the mean number of a 4-point visual qualitative measurement of decay, where 0 = 0 % decay, 1 = 1-25 % decay, 2 = 26-50 % decay, 3 = 51-75 % decay and 4 = 76-100 % decay. Incidence of decayed kernels was calculated as the mean number of chestnuts showing symptoms of decay.

Identification of microorganisms – Third study

All of the microorganisms isolated from symptomatically chestnuts, containing shell mold or kernel decay were picked and subsequently purified after isolation. MAB and molds were identified sequencing rDNA subunits (White, Bruns, Lee, and Taylor, 1990).

Genomic DNA was extracted from molds using the DNA QiAquick DNA extraction kit (Qiagen, Maryland, USA) as recommended by the manufacturer. A 0.5 to 1 mg sample of mycelia taken from five- to seven-day-old molds grown on potato dextrose agar, was immediately ground in a sterile mortar and pestle containing 500 µl of cetyltrimethylammonium bromide (CTAB) buffer pH 8.3 (Qiagen, Maryland, USA). After these the DNA was extracted followed the procedure proposed by Hamelin et al. (2000). The region including the two spacers (ITS4 and ITS6) was amplified. A total volume of 25 µl for each reaction

contained 60 ng of DNA, 23 µl of AFLP™ amplification core mix (Applied Biosystems, Foster City, Ca, USA), 2.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 10 pM of each primer. The primers used, ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990) and ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3') (Cooke et al., 2000) are complementary to the final portion of 18S rDNA adjacent to ITS3 and to the initial portion of 25S rDNA adjacent to ITS2, respectively (Cooke et al., 2000; White et al., 1990).

Bacterial genomic DNA was extracted using the protocol proposed by Jacobs et al. (2008). The region including the two spacers (UFLP and URPL) was amplified using the method of LiPuma et al. (1999). A total volume of 25 µl for each reaction contained 60 ng of DNA, 23 µl of AFLP™ amplification core mix (Applied Biosystems, Foster City, Ca, USA), 2.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 10 pM of each primer. The primers UFPL (5'-AGTTTGATCCTGGCTCAG-3') and URPL (5'-GGTTACCTTGTTACGACTT-3') were used for targeting the 16S rDNA region from the kingdom Procaryotae (bacteria) (LiPuma et al., 1999).

For molds and bacteria the extracted DNA region was amplified in a 2720 thermal cycler (Applied Biosystems). The program included a cycle at 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 71 °C for 1 min, and a final elongation at 71 °C for 10 min. The PCR product was placed in wells on an agarose gel (1.25 %), and electrophoresed for 3 hours at 70 volts. After electrophoresis, the amplified region was purified using a QIAquick PCR Purification kit (Qiagen, Maryland, USA). The PCR product was sequenced in

both directions using moderate throughput sequencing (Research Technology and Support Facility, MSU, East Lansing, MI, USA). Sequences were deposited in the Lasergene software (DNA Star Inc., Madison, WI., USA) and used as queries for similarity searches in the NCBI nucleotide database (NCBI, 2008). Species reported had a 98 to 100 % match in both directions.

Yeasts were inoculated on potato dextrose agar, incubated for 72 h at 25° C (± 3° C) and then sent to the DCPHA (MSU, East Lansing, Mi., USA) for further identification. Yeasts were identified using the API 20C yeast identification system together with microscopic morphology determinations (Michigan State University Diagnostic Center for Population and Animal Health (DCPHA)). This computer-assisted system for rapid identification of yeast provides results comparable to those obtained of conventional morphological methodologies (Land et al., 1979).

Statistical analysis

One-factor and repeated measurement design with analysis of variance (ANOVA) were done on all microbial count and microbial qualitative assessment data obtained from fresh chestnuts. Since all chestnuts were randomly assigned to the different treatment conditions, sphericity can be assumed making multivariate as well as degrees of freedom corrections unnecessary (Keselman, Algina, and Kowalchuk, 2001; Ott and Longnecker, 2001). Multiple analysis of variance (MANOVA) was also used to determine differences between the microbial populations in chestnuts from different farms. Significance between means was determined using the Tukey post-hoc multiple comparisons of means

test at the 95% family-wise confidence level (p=0.05). Calculations were performed using the statistical package "R: A language and environment for statistical computing" (R Development Core Team, 2007) (Ott and Longnecker, 2001).

Results

Shell mold symptoms

The harvested chestnuts were colonized by a broad range of microorganisms (Table 2), not all of which had the potential to cause shell mold. Fungi that infested and caused shell mold on fresh chestnuts were mainly composed of three species of *Penicillium* (*P. expansum*, *P. griseofulvum* and *P. chysogenum*) and *Coniophora puteana*. Dark-green to black spots with white mycelia were observed on chestnuts colonized by *Penicillium* species. These spots begin on the hilum (Figure 1-a and Appendix C). Once the mold grew, a mantle of white mycelia containing dark-green, blue, bluish-green or olive-green completely covered the shell (Figure 1-b). In extreme conditions, the fungi softened and discolored the shell tissue, subsequently affecting the quality of the fresh product (Figure 1-c). Symptoms from *Penicillium* were first observed after harvest, and rapidly developed during storage. *Penicillium* species belong to the phylum Deutoromycota, producing green, bluish-green or olive-green spores asexually. Various species cause blue mold rots and green mold rots. They are the most common and usually the most destructive of all postharvest diseases, affecting various fruits and vegetables, including commodities of importance like apples and citrus (Agrios, 2005). In addition to the losses caused, the fungus also

produces several mycotoxins, such as patulin and zearalenone (Adams & Moss, 2000).

Coniophora puteana, rarely infected the shell, but when present, the organism developed in pockets of white web-like mycelia around several chestnuts (Figure 1-d), which contained apparent high moisture content. After manually removing the mold, the appearance and quality of the chestnuts were not affected. This fungus belongs to the phylum Basidiomycota. It has never been reported affecting chestnuts or other commodities, but it can be found affecting indoor wood structures and stored wood, causing wet brown rot, significant decay and economical damage (Schmidt, 2007).

Table 2. Microorganisms isolated from stored chestnut shell and kernel during the 2006 and 2007 growing seasons in Michigan

Organism	Species	Shell[1]	Kernel[1]	Symptom Appearance[2]	Identification[3]	Comments and references
Fungi	*Penicilium* sp.	F	F	BH, DS	18S (99/99)-M	Genus has been reported to cause mold and decay in chestnuts (Breisch, 1993; Montealegre & Gonzalez, 1986; Paglietta & Bonous, 1979; Pratella, 1994; Ridé & Gudin, 1960; Rutter et al., 1990; Sieber et al., 2007) and other commodities of economical importance (Agrios, 2005).
	Penicilium expansum	F	F	BH, DS	18S (99/98)	
	P. griseofulvum	F	F	BH, DS	18S (99/98)	
	P. chysogenum	F	F	BH, DS	18S (99/98)	
	Coniophora puteana	O	NF	DS	18S (100/98)	Not reported in chestnuts before. Fungus of importance causing indoors wood-decay (Schmidt, 2007).
	Botryosphaeria ribis (Anamorph *Fusicoccum ribis*)	NF	O	DS	18S (100/98)	Not reported in chestnuts before. Fungus of importance, infecting many trees, including olives, pecans, pistachios, kiwi, apples and citrus. It causes cankers, dieback, death and black fruit rots (Sanchez-Hernandez et al., 2002; Slippers et al., 2005; Young et al., 2005).
	B. tsugae	NF	O	DS	18S (99/99)	
	Trichoderma viridae	NF	R	BH, DS	18S (100/99)	In this study it was found in chestnuts directly harvested from the tree and during storage. It has never been reported in chestnuts before and does not appear to cause any postharvest disease. Has been used as a biological control, belonging to the group or antibiotic producing microorganisms (Adeleye et al., 2004)

Table 1. (cont'd)

Organism	Species	Shell[1]	Kernel[1]	Symptom Appearance[2]	Identification[3]	Comments and references
Fungi	*Botryotinia fuckeliana* (Anamorph *Botrytis cinerea*)	NF	R	DS	18S (99/99)	Genus has been reported to cause mold and decay in chestnuts (Sieber et al., 2007) and other commodities of economical importance, including berries, apples and grapes during storage (Agrios, 2005).
	Phoma sp.	NF	R	DS	18S (100/99)	Genus has been reported to cause significant decay in chestnuts (Sieber et al., 2007) and other commodities of economical importance, including Papayas and kiwis (Nery-Silva et al., 2007; Young, Jae, & Jae, 2005).
	Sclerotinia sclerotiorum	NF	R	BH, DS	18S (99/99)	Has never been previously reported on chestnuts, but symptoms and pathogenesis may be similar to that of *Sclerotinia pseudotuberosa*, causal agent of chestnut black rot (Vettraino, Paolacci, & Vannini, 2005). Patoghen that significantly affects all annual vegetables, ornamentals and field crops (Agrios, 2005).
	Acrospaeria mirabilis	NF	F	BH, DS	M	Has been reported to cause significant decay in chestnuts, after harvest and during storage (Ellis & Ellis, 1985).

Table 1. (cont'd)

Organism	Species	Shell[1]	Kernel[1]	Symptom Appearance[2]	Identification[3]	Comments and references
Fungi	*Gibberella* sp. (Anamorph *Fusarium* sp.)	R	O	DS	18S (100/99)-M	Genus has been reported to cause mold and decay in chestnuts, specially during storage (Breisch, 1993; Montealegre & Gonzalez, 1986; Paglietta & Bonous, 1979; Pratella, 1994; Ridé & Gudin, 1960; Rutter et al., 1990; Sieber et al., 2007) and other commodities of economical importance, causing postharvest pink or yellow molds on vegetables, ornamentals, root crops, tubers, tomatoes, and bulbs (Agrios, 2005).
	G. moniliformis (*Anamorph F. verticillioides*)	R	O	DS	18S (100/99)	
	Giberella zeae (Anamorph *F. culmorum*)	R	O	DS	18S (100/99)	
	G. graminearum (Anamorph *F. graminearum*)	R	O	DS	M	
	Discula campestris	NF	R	DS	18S (99/99)	Has never been previously reported on chestnuts. Fungus of importance causing anthracnose in sugar maple, especially in seedlings (Stanosz, 1994).

Table 1. (cont'd)

Organism	Species	Shell[1]	Kernel[1]	Symptom Appearance[2]	Identification[3]	Comments and references
Yeast	*Candida* sp.	F	F	NS	API 20C	In this study, these microorganisms did not appear to cause postharvest decay in fresh chestnuts. Other micro flora survey studies indicated that after harvest, a wide range of economical important vegetables and other commodities, are often colonized but usually spoiled when they are processed, including chestnuts (Chapter 3 of this thesis), sliced onions and shredded lettuce. Sometimes they are also used as antagonist microorganisms, against certain postharvest diseases (Liu and Li, 2006; Ragaert et al., 2006; Sieber et al., 2007; Tournas, 2005; Zagory, 1998). The majority of these microorganisms, including *Rahnella* sp., *Candida* sp., and *Cryptococcus* sp. are widely distributed in nature (Brenner et. al, 1998; (Glushakova et al., 2004).
	C. guillermondii	O	O	NS	API 20C	
	Cryptococcus sp.	F	F	NS	API 20C	
	Cr. luteolus	O	O	NS	API 20C	
	Cr. laurentii	O	O	NS	API 20C	
Bacteria						
	Rahnella sp.	O	O	NS	16S (99/99)	
	Rahnella aquatilis	O	O	NS	16S (98/97)	
	Bacillus sp.	NF	R	NS	16S (99/99)	
	Methylobacterium sp.	O	O	NS	16S (99/99)	

[1] *F = Frequent (identified on ≥ than 80% of affected samples), O = Occasional (identified on ≤ than 25 % of affected samples), R = Rare (identified on ≤ than 5 % of affected samples), NF = Not found.*

[2] *BF = symptom appearance before storage, DS = symptom appearance during storage, NS = No symptoms.*

[3] 18S (% match-forward/reverse)-M = fungi identified using sequencing of 18S-rDNA subunit & microscopic morphology, 18S (% match-forward/reverse) = fungi identified using sequencing of 18S-rDNA subunit, M = microscopic morphology identification, 16S (% match-forward/reverse) = bacteria identified using sequencing of 16S-rDNA subunit, API 20C = yeast identified using API 20C yeast identification system & microscopic morphology.

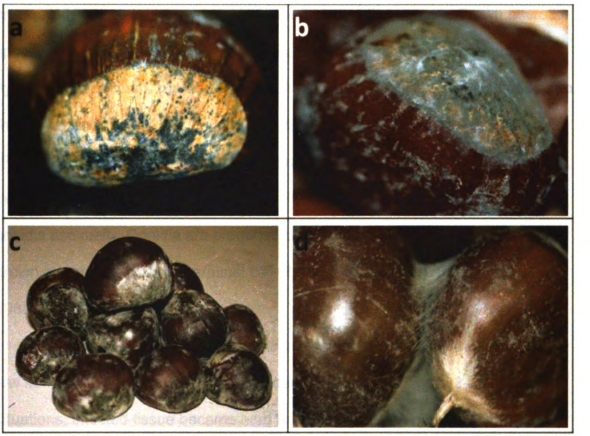

Figure 1. Chestnuts showing shell mold due to a-c) *Penicillium* spp. (*P. griseofulvum*, *P. expansum, P. chysogenum*) and d) *Coniophora puteana.* Image in this thesis is presented in color.

Kernel decay symptoms

Microorganisms infecting chestnut kernels primarily included *Penicillium* spp. (*P. griseofulvum*, *P. expansum*, *P. chysogenum*), *Acrospaeria mirabilis*, *Botryosphaeria ribis*, *Sclerotinia sclerotiorum*, *Botryotinia fuckeliana* (Anamorph *Botrytis cinerea*) and *Gibberella* sp. (Anamorph *Fusarium* sp.). *Penicillium* spp. (*P. griseofulvum*, *P. expansum*, *P. chysogenum*) produced white mycelia and dark-green, blue, bluish-green or olive-green spores (Figure 2-a). That sometimes penetrated deep into the kernel, resulting in extensive kernel rotting and complete kernel decay (Figure 2-b). *Acrospaeria mirabilis*, appeared as dark-brown spots (conidia), filling the space between the kernel cotyledons and kernel

cracks. Whitish, web-like mycelia developed around these spots. Brown necrotic spots were observed around the colonies, which sometimes enlarged, turned light brown, and finally dark-brown. The infection sometimes penetrated deep into the kernel, softening the tissue, and resulting in opaque brownish kernel decay. (Figure 2-c). This fungus causes considerable losses in chestnuts during storage, belonging to the phylum Deuteromycota. It lacks fruiting bodies, producing conidia asexually. Conidia are produced coiled, with 2 pale-cells and 1 dark-brown, verrucose, globose terminal cell (20-30 μ m diameter) (Ellis & Ellis, 1985).

Botryosphaeria ribis and *Sclerotinia sclerotiorum* were isolated from only a few kernels that presented an extreme dark-black decay (Figure 2-d). In some situations, infected tissue became hard and completely mummified. *Botryosphaeria ribis* and *Sclerotinia sclerotiorum*, belong to the Phylum Ascomycota. *Botryosphaeria ribis* produces conidia in pycnidia, while the fungus *Sclerotinia sclerotiorum* overwinters as sclerotia on or within infected tissues and in the ground. In spring or early summer sclerotia germinate and produce apothecia, which contain asci, producing ascospores (Agrios, 2005).

Botryotinia fuckeliana (Anamorph *Botrytis cinerea*) was isolated from grayish, completely decayed kernels. The mycelium appeared to rapidly invade the whole kernel, which became covered with a whitish-gray, cobweb-like mold. Infected kernels became soft, watery and acquired a dark gray to black coloration (Figure 2-e). Depending in its sexual stage, this fungus belongs to the phylum Ascomycota or Deuteromycota. It causes gray molds or rots of fruits and vegetables, both in the field and during storage. It produces abundandt gray

mycelium and long branches of conidiophores, which contain rounded apical cells bearing folorless or gray, one-celled, ovoid conidia (Agrios, 2005)

Gibberella sp. (Anamorph *Fusarium* sp.) caused white mold decay. Affected kernels appeared dry, whitish to light brown and extremely pale. A whitish web-like mycelium could be observed around decayed tissue, usually in the cracks, (Figure 2-f). Depending in its sexual stage, this fungus belongs to the phylum Ascomycota or Deuteromycota. The fungus usually produces colorless mycelia, at first, but with age it becomes cream-colored, pale yellow, pale pink, or purplish. It produces three kinds of asexual spores, microconidia, macroconidia and chlamydospores, but also produced sexual spores, throught perithecia, during its sexual phase (Agrios, 2005). In this study, this microorganism was usually mistaken for early infection by *Penicillium* spp., and usually both were present. In addition, the fungus also produces several mycotoxins, such as DON and zearalenone (Adams & Moss, 2000).

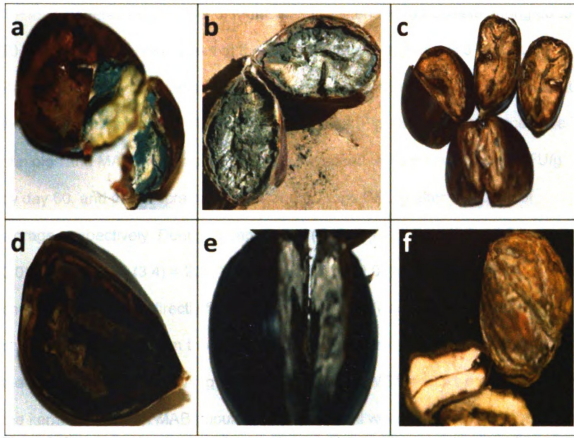

Figure 2. Chestnuts showing kernel decay due to a-b) *Penicillium* spp. (*P. griseofulvum, P. expansum, P. chysogenum*), c) *Acrospaeria mirabilis* d) *Botryosphaeria ribis* and *Sclerotinia sclerotiorum,* e) *Botryotinia fuckeliana* (Anamorph *Botrytis cinerea*), f) *Gibberella* sp. (Anamorph *Fusarium* sp.). Image in this thesis is presented in color.

Influence of chestnut harvesting method on microbial populations

Non-decaying chestnuts yielded low populations of MAB, molds an yeasts on the shells and in kernels when collected on day 0, from trees and the ground. The microbial population significantly increased during storage ($F_{(3,4)} = 176.61$, MSE = 5.81, $p < 0.01$) (Figures 3, 4 and 5). These populations ranged from 1.8 to 4.61 CFU/g log, depending on the portion of the chestnut examined (shell or kernel) and the harvesting method (ground or tree). With the shell yielding significantly higher ($F_{(1,4)} = 324.09$, MSE = 1.3 $p < 0.01$) microbial populations compared to the kernel.

In general, populations of molds and MAB tended to increase during 60 to 90 days of storage, regardless of the harvest procedure (Figures 3 and 4). Populations of microorganisms were generally highest in 90-day old chestnuts at the time of marketing (60 – 90 days after harvest) (Figures 3 and 4). In the case of molds and MAB, microbial populations increased up to 8 and 8.3 logs CFU/g by day 60, and then decreased to 6.1 and 6.7 logs CFU/g after 120 days of storage, respectively. During storage, mold ($F(3,4) = 177.59$, $MSE = 6.72$, $p < 0.01$) and MAB ($F(3,4) = 206.44$, $MSE = 5.27$ $p < 0.01$) populations from chestnuts harvested directly from the tree were significantly lower compared to chestnuts harvested from the ground. On day 0 and beyond, mold populations on the shell were significantly higher ($F(1,4) = 199.19$, $MSE = 12.41$ $p < 0.01$), than the kernel (Figure 3). MAB populations on the shell were significantly higher than the population in the kernel ($F(1,4) = 197.15$, $MSE = 5.31$ $p < 0.01$) (Figure 4).

Yeast populations did not reflect the same pattern in that the kernels of chestnuts harvested from the tree were significantly less ($F(1,4) = 2.81$, $MSE = 1.50$, *ns*), contaminated than those harvested from the ground (Figure 5). An irregular growth rate and significant population increase ($F(1,4) = 189.78$, $MSE = 17.29$, $p < 0.01$) was observed in yeasts colonizing the shell. With these populations on chestnuts collected from the orchard ground, significantly increasing ($F(1,4) = 189.78$, $MSE = 17.29$, $p < 0.01$) to 8.5 logs CFU/g during 120 of storage. Meanwhile, yeast populations from the shell of chestnuts collected directly from the tree, were more variable with a decline in population, from 4.1 logs CFU/g on day 30 down to 2.3 logs CFU/g 90 days later. Nevertheless, after 120 days a significant increase ($F(1,4) = 189.78$, $MSE = 17.29$, $p < 0.01$) up to

6.7 logs CFU/g was observed. During storage, yeast populations on the shell was significantly higher (F (1,4) = 256.65, MSE =8.71 $p < 0.01$) compared to those in the kernel. Populations of yeast on kernels collected from the tree or ground remained relatively constant during storage (Figure 5).

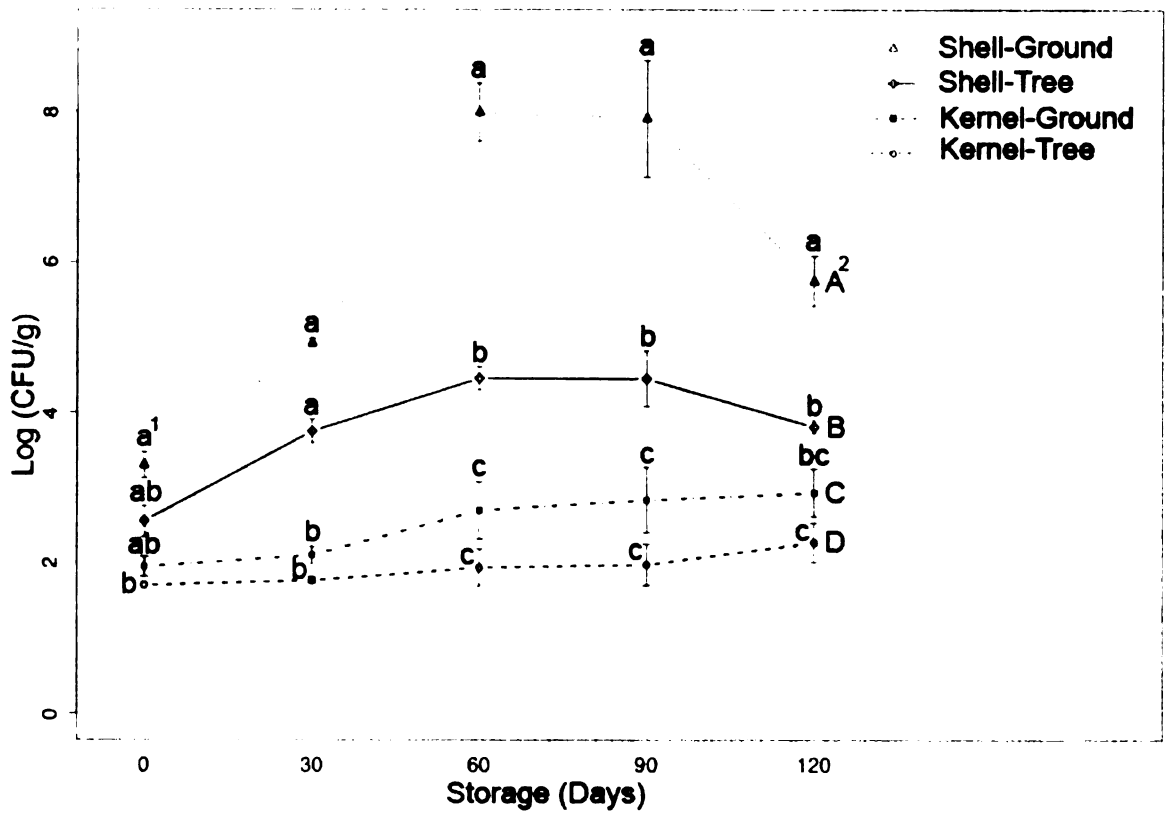

Figure 3. Mean mold counts (log cfu g^{-1}) on shells and in kernels of fresh chestnuts during 120 days of storage at 4° C. [1]Data points followed by the same lower case letter within the same day are not significantly different at $p = 0.05$ (ANOVA with post-hoc Tukey multiple comparison of means). [2]Overall data point followed by the same capitalized letter within chestnut part-harvesting method are not significantly different at $p = 0.05$ (Repeated measurement design – ANOVA with post-hoc Tukey multiple comparison of means). Error bars indicate standard deviation.

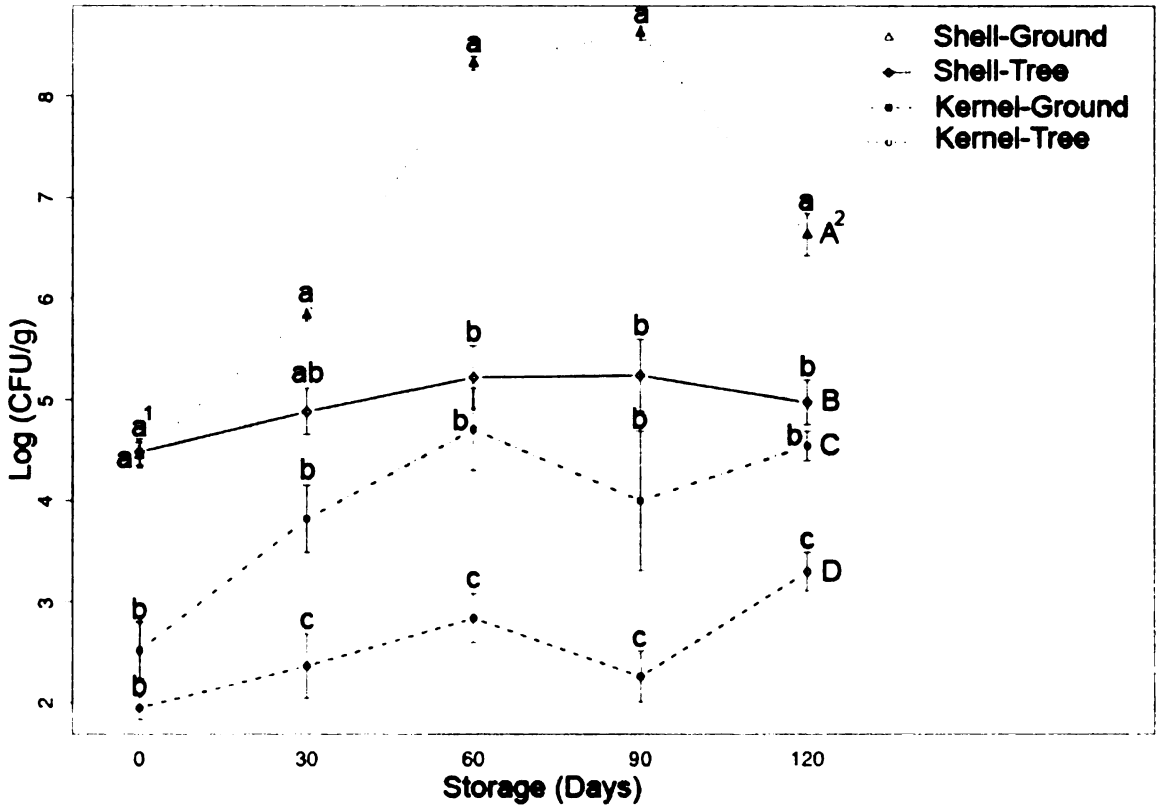

Figure 4. Mean MAB counts (log cfu g^{-1}) on shells and in kernels of fresh chestnuts during 120 days of storage at 4° C. [1]Data points followed by the same lower case letter within the same day are not significantly different at $p = 0.05$ (ANOVA with post-hoc Tukey multiple comparison of means). [2]Overall data point followed by the same capitalized letter within chestnut part-harvesting method are not significantly different at $p = 0.05$ (Repeated measurement design – ANOVA with post-hoc Tukey multiple comparison of means). Error bars indicate standard deviation.

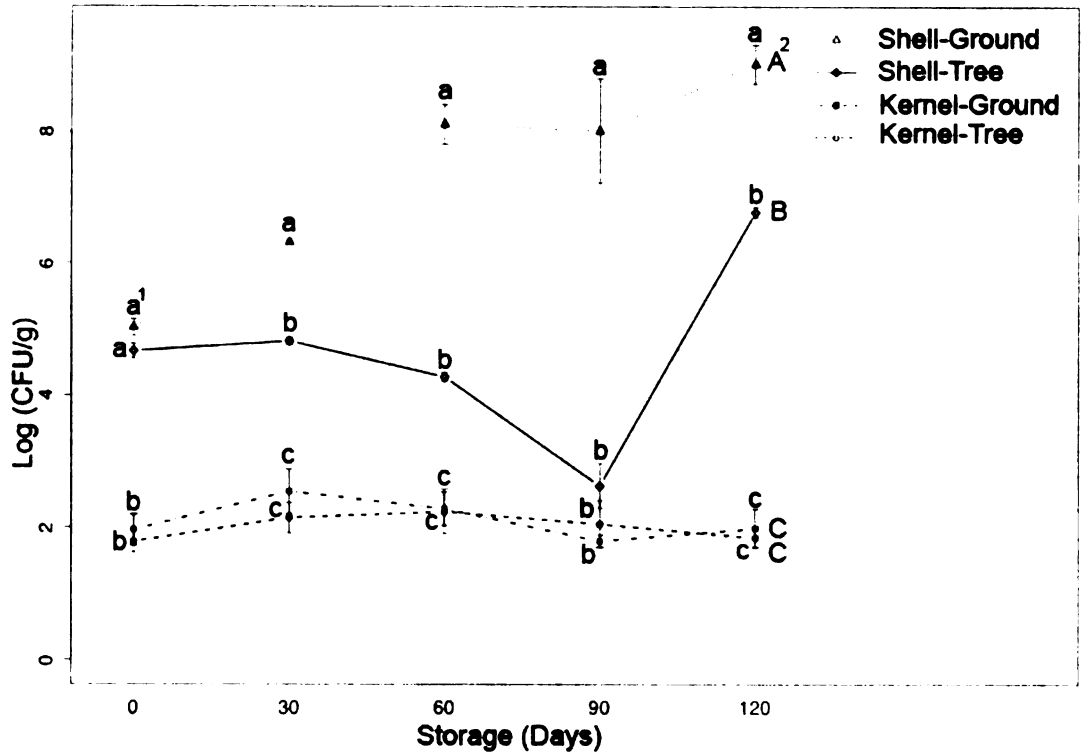

Figure 5. Mean yeast counts (log cfu g^{-1}) on shells and in kernels of fresh chestnuts during 120 days of storage at 4° C. [1]Data points followed by the same lower case letter within the same day are not significantly different at $p = 0.05$ (ANOVA with post-hoc Tukey multiple comparison of means). [2]Overall data point followed by the same capitalized letter within chestnut part-harvesting method are not significantly different at $p = 0.05$ (Repeated measurement design – ANOVA with post-hoc Tukey multiple comparison of means). Error bars indicate standard deviation.

Farm influence on microbial populations on shell and in the kernel of chestnuts

To determine the effect of different growing and harvesting conditions on postharvest microbial populations of chestnuts on various farms in Michigan, chestnuts were collected from seven different growers at the Chestnut Growers, Inc. on day of delivery. Populations on shell varied from farm to farm, with MAB, molds and yeasts ranging from 4.55 to 8.44, 3.94 to 7.74, 4.75 to 8.28 logs CFU/g respectively (Figure 6). A similar scenario was observed for the kernels

with MAB, molds and yeasts ranging from 2.34 to 7.63, 1.99 to 7.69, 2.68 to 7.24 logs CFU/g respectively (Figure 7). A significant difference was observed on molds ($F(6,1) = 11.43$, $p < 0.01$), MAB ($F(6,1) = 9.85$, $p < 0.01$) and yeasts ($F(6,1) = 27.82$, $p < 0.01$) on the shell (Figure 6). And in the kernel ($F(6,1) = 11.44$, $p < 0.01$), MAB ($F(6,1) = 4.39$, $p = 0.01$) and yeast ($F(15.04) = 19.64$, $p < 0.01$) (Figure 7). Based on multivariate analysis of variance (MANOVA - 'Wilks' test), all of the microbial populations (dependent variables) were significantly different from farm to farm, indicating a significant difference between microbial populations on shell (approx $F(18,3) = 8.92$, $p < 0.01$) (Figure 6) and kernel (approx $F(18,3) = 11.44$, $p < 0.01$) (Figure 7). Chestnuts from farm C yielded significantly higher microbial population compared to those from farm D. With the remaining farms being between farms C and D.

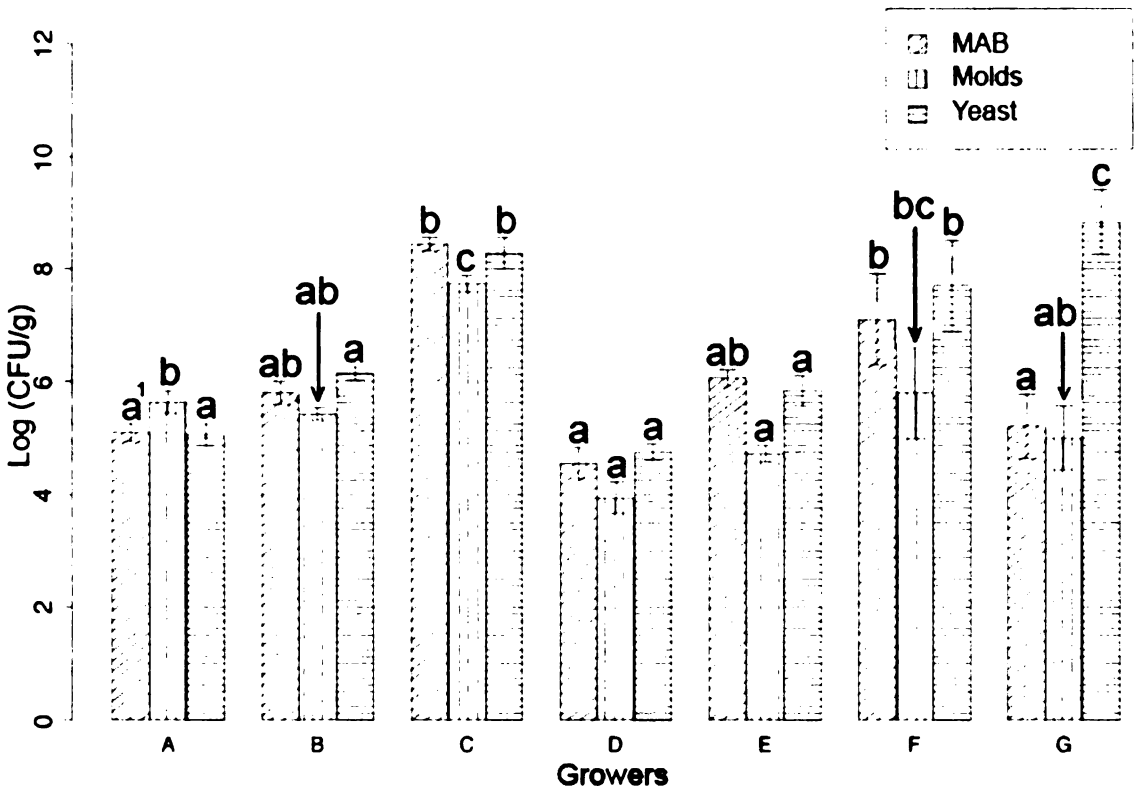

Figure 6. Total MAB, molds and yeast counts (log cfu g^{-1}) from the shells of fresh chestnuts after harvest from seven Michigan farms. [1]Values followed by the same letter within organisms are not significantly different at $p = 0.05$ (ANOVA) (Tukey multiple comparison of means). Error bars indicate standard deviation.

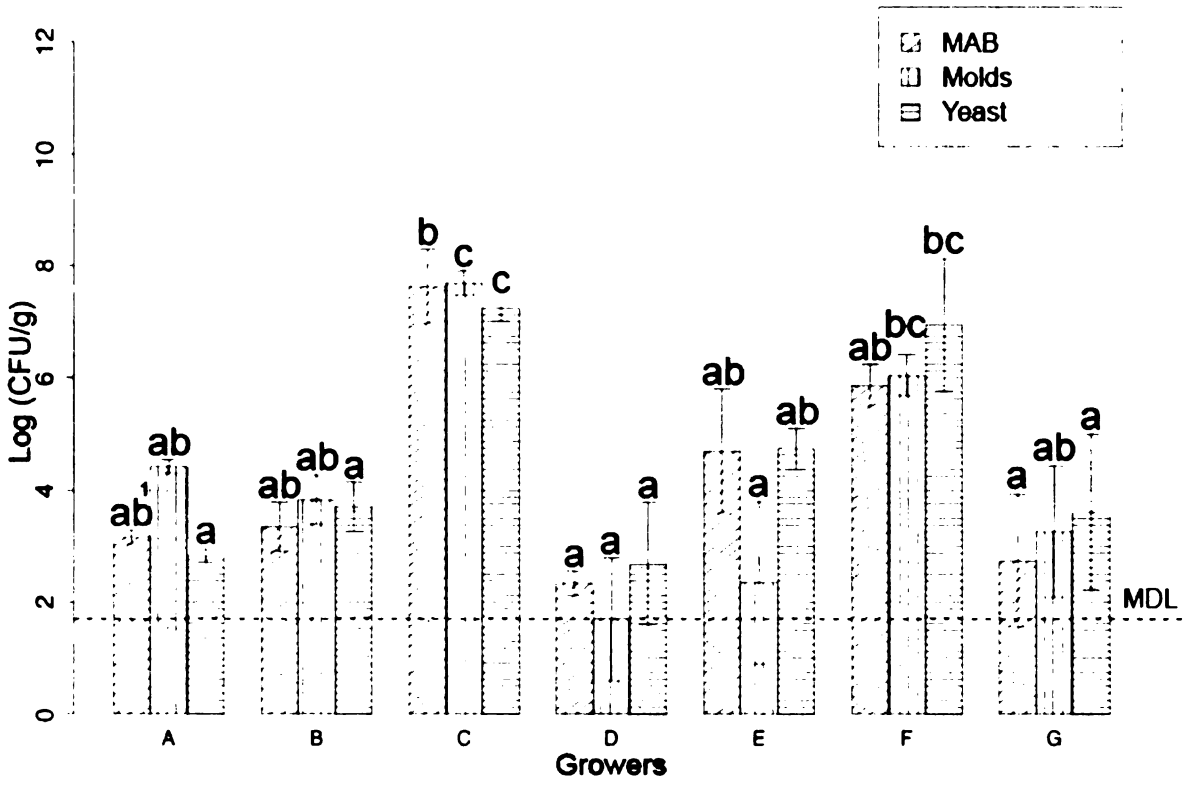

Figure 7. Total MAB, molds and yeast counts (log cfu g^{-1}) from the kernels of fresh chestnuts after harvest from seven Michigan farms. [1]Values followed by the same letter within organisms are not significantly different at $p = 0.05$ (ANOVA) (Tukey multiple comparison of means). Minimum detectable level (MDL) = Minimum possible count (0.5) x minimum dilution factor x inoculated aliquot (100 µl). Error bars indicate standard deviation.

Farm influence on microbial quality of chestnuts

To determine the effect of different growing and harvesting conditions on the severity of postharvest mold and kernel decay, chestnuts were collected from the seven different growers when received at Chestnut Growers, Inc. On receiving day (day 0) a significant difference in mold severity ($F(6,1) = 2.89$, MSE = 1.20, $p = 0.03$) and kernel decay incidence ($F(6,1) = 2.61$, MSE =0.11, $p = 0.04$), was observed between growers (Figure 8).

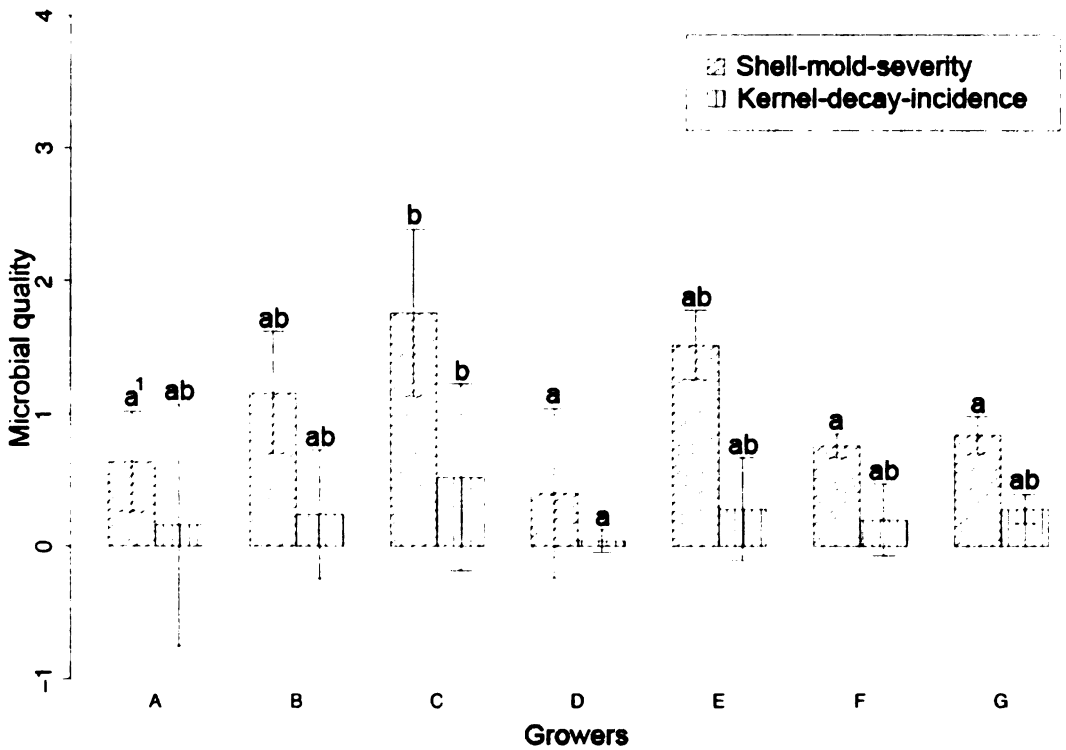

Figure 8. Shell mold severity and kernel decay incidence from seven Michigan farms at receiving day (Day 0). [1]Values followed by the same letter within quality index are not significantly different at $p = 0.05$ (Repeated measurement design - ANOVA) (Tukey multiple comparison of means). Error bars indicate standard deviation.

Overall, during 60 days of storage a significant difference in mold severity was observed on shells (F (6,2) = 5.03, MSE = 0.28, $p < 0.01$) but not in kernel decay incidence (F (6,1) = 2.78, MSE = 0.04, *ns*), between growers (Figure 9). Farms C and B had significantly higher shell mold severity compared to the other farms, with values of 2.8 and 1.5 logs CFU/g respectively.

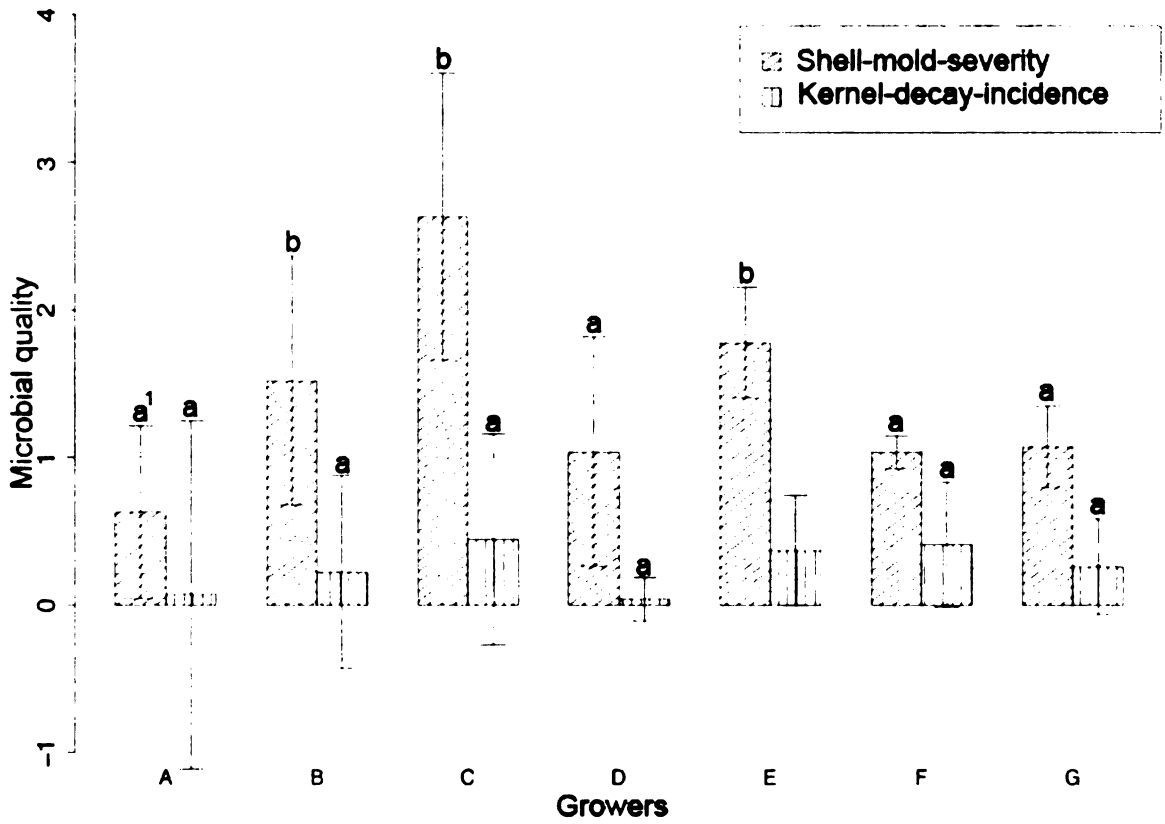

Figure 9. Shell mold severity and kernel decay incidence from seven Michigan farms, during 60 day storage at 4°C. [1]Values followed by the same letter within quality index are not significantly different at $p = 0.05$ (Repeated measurement design - ANOVA) (Tukey multiple comparison of means). Error bars indicate standard deviation.

Discussion

Postharvest shell mold and kernel decay affects a significant portion of chestnuts worldwide and about 20 percent of the chestnuts produced in Michigan. Results showed that fungi, bacteria and yeast significantly increased during storage. The extent of increase was dependent on the part of the chestnut (shell or kernel) and the collecting method (tree or ground). Highest populations were found on the shells of chestnuts collected from the ground. These results are not surprising as the shell is continually exposed to the environment while the

kernel is protected by the shell and pellicle. Molds were the most noticeable organisms associated with the shell and kernel decay of chestnuts during refrigerated storage (4° C). Similar problems, related to mold decay also have been reported in Asia, Europe, South America and North America (Jerimini et al., 2006; Jian et al., 2002; Montealegre and Gonzalez, 1986; Miller, 2003; Paglietta and Bonous, 1979; Rutter et al., 1990; Vettraino et al., 2005; Vossen, 2000; Washington, 1997). The fungal flora responsible for this infestation is diverse and appears to be strongly influenced by preharvest, harvest and storage conditions. In the traditional method of harvest, chestnuts that have fallen to the ground are collected by the workers or mechanical pickers (Anagnostakis, 2003). Attempts have been made to collect chestnuts in nets either on the ground or suspended above the ground, in Italy and France mainly to ease chestnut collection (Breisch, 1993; Bonous and Raccolta, 2002) and reduce molding, especially by *Sclerotinia pseudotuberosa* (Breisch, 1993). However, recent study indicated that suspended nets did not reduce chestnut mold after harvest or during storage (Sieber et al., 2007). Regardless, in this study, chestnuts harvested from the orchard floor were more heavily contaminated during storage, than those harvested directly from the tree.

Microbial populations are much higher on chestnut shells compared to the kernels. In general, only two fungal genera caused obvious mold symptoms with chestnuts shells after harvest and during storage. In order of importance, these were *Penicillium* spp. (*P. expansum*, *P. griseofulvum*, *P. chysogenum)* and *Coniophora puteana.* However, at least five different genera of filamentous fungi

were associated with infected chestnut kernels. These microorganisms which included *Penicillium* spp. (*P. griseofulvum*, *P. expansum*, *P. chysogenum*), *Acrospaeria mirabilis*, *Botryosphaeria ribis*, *Sclerotinia sclerotiorum*, *Botryotinia fuckeliana* (Anamorph *Botrytis cinerea*) and *Gibberella* sp. (Anamorph *Fusarium* sp.) were generally undetectable and caused the largest amount of loss. Other organisms might be associated with chestnut shell mold and kernel decay in Michigan, but during this two-season study, only these species were repeatedly associated with postharvest shell mold and kernel decay. Similar findings have been reported for chestnuts harvested in France, Italy, Australia, Chile, United States and other countries (Ellis and Ellis, 1985; Jerimini, et al., 2006; Montealegre and Gonzalez, 1986; Paglietta and Bonous, 1979; Ridé and Gudin, 1960; Vettraino et al., 2005 Washington, et al. 1997; Wright, 1960).

This was the first survey to associate *Coniophora puteana*, *Botryosphaeria ribis*, and *Sclerotinia sclerotiorum* with chestnut mold or kernel decay. Furthermore, *S. sclerotiorum,* which was found infrequently in our survey, has never been previously reported on chestnuts, but symptoms and pathogenesis may be similar to that of *Sclerotinia pseudotuberosa*, mold responsible for chestnut black rot, which is one of the most important postharvest diseases of acorns (*Quercus* spp.) in Europe (Vettraino, et al., 2005; Washington et al., 1997).

Findings concerning differences between mold severity in shell and microbial populations on chestnuts among farms, demonstrated that location, preharvest and harvesting conditions played an important role in quality and decay of chestnuts (Kader, 2002; Sieber, et al. 2007; Willis, et al., 2007).

Variation in microbial populations among farms, increases in populations during storage and variability of populations within the same field during storage, suggest multiple preharvest sources of contamination including soil, feces, irrigation water, water used to apply fungicides and insecticides, insects, inadequately composted manure, wild animals, and human handlers (Beuchat, 1996). Therefore, complementary studies on the efficacy of preharvest management, preharvest fungicide applications, and path of produce contamination, as well as harvesting methods and storage conditions must be considered. Furthermore, the interactions between climate and epidemiology of the fungal flora in the field also need to be better understood. Currently, all these factors must be taken into consideration and confirm the need to control the organisms prior to storage to avoid or reduce postharvest mold and decay during storage. These efforts must be in concert with a plan to efficiently identify and manage affected products during harvest and storage.

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CHAPTER 3

EFFICACY OF POSTHARVEST TREATMENTS FOR REDUCTION OF MOLD AND DECAY IN FRESH CHESTNUTS

Abstract

In Michigan where chestnut (*Castanea* spp.) cultivation is a pioneering industry, postharvest molds are responsible for significant economic losses. Molds contaminate chestnut before and at time of harvest and are exacerbated during transportation and storage. Consequently, various chemical sanitizers for fresh produce were evaluated for their effect on management of postharvest chestnut shell mold, kernel decay and weight loss during storage. Treatments were evaluated in chestnuts randomly collected after harvest from seven Michigan growers. Each treatment was repeated four times with 2.3 kg chestnuts per replication. Per replication a sub-group of 10 chestnuts was randomly picked and evaluated for microbiological quality (severity of shell mold and incidence of kernel decay) and weight loss. Overall, 2,700-ppm hydrogen peroxide + 200-pp, peracetic acid and 0.15-ppm trifloxystrobin significantly reduced both mold severity on the shell and the incidence of internal kernel decay compared to the other products. Other products and combinations of products may also play an important role in reducing the incidence of decayed kernels, even though they did not strongly reduce mold severity. Among these, were 0.92-ppm ozone solution, 7.5-mg chlorine dioxide/g of chestnuts, 100-ppm peracetic acid, 0.2-M sodium metabisulfite, and 1,350-ppm hydrogen peroxide + 100-ppm peracetic acid in

combination with 1,980-ppm caprylic acid, 1,333-ppm glycolic acid and 20-ppm capric acid. No product significantly reduced weight loss during storage. Based on these findings 2,700-ppm hydrogen peroxide + 200-ppm peracetic acid and 0.92-ppm ozone solution are best suited to protect against postharvest chestnut mold and kernel decay when combined with good agricultural practices and postharvest management.

Introduction

Postharvest decay of edible chestnuts (*Castanea* spp.) reduces nut quality and can lead to severe economic losses (Jerimini, et al., 2006; McCarter et al., 1980; Narayanasamy, 2006). A diverse range of filamentous fungi including *Penicillium* sp., *Aspergillus* sp., *Fusarium* sp., *Phomopsis castanea*, *Acrospeira mirabilis*, and *Sclerotinia pseudotuberosa* (syn. *Ciboria batschiana*, *S. batschiana*; anamorphic from *Rhacodiella castanea*, syn. *Myrioconium castanea*) have been most commonly responsible for postharvest decay of the shell and kernel in France, Italy, Australia, Chile, United States and elsewhere (Ellis and Ellis, 1985; Jerimini et al., 2006; Montealegre, J. and Gonzalez, S. 1986; Paglietta and Bonous, 1979; Ridé, M. and Gudin, C. 1960; Sieber, Jermini, and Conedera, 2007; Vettraino et al., 2005 Washington et al. 1997; Wright, 1960). In Michigan, postharvest decay has been identified as one of the major problems that negatively impacts fresh chestnuts, accounting for up to 25 percent of losses after harvest. Recently, several fungal species, have been isolated from fresh healthy Michigan chestnuts and chestnuts experiencing postharvest shell mold and kernel decay, including *Penicillium* spp. (*P. griseofulvum*, *P. expansum*, *P.*

chysogenum), *Acrospaeria mirabilis*, *Botryosphaeria ribis*, *Sclerotinia sclerotiorum*, *Botryotinia fuckeliana* (Anamorph *Botrytis cinerea*), *Gibberella* sp. (Anamorph *Fusarium* sp.), and *Coniphora puteana* (Chapter 2, this thesis).

Various chemical and non-chemical treatments have been previously evaluated for their impact on the quality of fresh chestnuts during storage. Among the treatments tested, 95 % alcohol, 1 % copper sulfate, 0.4% calcium chloride, boric acid, formaldehyde, benzoic acid, sulfur dust and others were unable to prevent storage molds (Hammer, 1949; Tan et al., 2007). Furthermore, certain treatments (boric acid and formaldehyde) compromised the chestnut quality by hardening the kernel (Hammer, 1949). Other chemical treatments potentially are able to minimize postharvest decay, including 30-ppm iodine, 250-ppm carbendazim, 100 to 200-ppm sodium hypochlorite, 100-ppm peracetic acid (Morris, 2006; Panagou, Vekiari, and Mallidis, 2005), 50-ppm natamycin, 1 % sorbic acid, 1 % propionic acid, hot water dip at 90° C for 10 min (Panagou, Vekiari, and Mallidis, 2005) and immersion in fungicides including 2,6-dichloro-4-nitroaniline (Botran) (Wells, 1980). Immersion in 15° C water for 15 min, 45-48 ° C water for 15 min and 52° C water for 5, 15, 30 or 60 min significantly reduced both the percentage of infected nuts during storage and the percentage of chestnuts exhibiting insect damage from the presence of larvae of *Cydia splendana*, *Curculio elephas* and *Curculio sayi*. (Jerimini, et al., 2006; Sieber, Jermini, and Conedera, 2007; Wells, 1980).

While various strategies can reduce both microorganisms and insects, some of these treatments, such as immersion in 52° C water for 60 min, will

decrease soluble sugars and increasing starch during storage, negatively affecting chestnut quality (Wells, 1980). Improved food processing and management practices can be used to enhance the safety, storage and shelf life of various commodities. These strategies primarily involve various sanitizers such as hydrogen peroxide, ozone, chlorine dioxide (gas and solution), hypochlorite, organic acids, natamycin, along with heat (Beuchat 1998; Block 1991; Brackett 1999; Crowe et al., 2005; Palou et al., 2002; Panagou et al., 2005; Perrera and Karunaratne 2001; Pierre et al., 2006, Sapers 2003; Suslow 2002; Wade et al., 2003). However several salts, oils and biocontrol agents have also been evaluated for reducing decay (Feng and Zheng, 2006, Kader, 2002; Mari et al., 2003; Mills et al., 2004; Porat et al., 2002; Sapers, 2003; Willis et al., 2007).

With the objective of understanding the effectiveness of postharvest treatment on microbial quality of Michigan fresh chestnuts, including the reduction of shell mold, kernel decay and weight loss during storage, sixteen different sanitizers were evaluated.

Material and Methods

Samples

A total of 454 kg Chestnuts (cv. 'Colossal') were obtained immediately after harvest, from seven commercial farms in Michigan and were used after 2 days of storage at 4 °C (MSU experimental station, Clarksville, MI, USA). All chestnuts were completely mixed, randomized and placed in pure water (~ 5,000 lt) where the majority of decayed, empty or damaged chestnuts were eliminated by their proclivity to float, as healthy chestnuts tend to sink.

Treatments and storage

Sixteen different treatments were tested are described in Table 3, with each treatment replicated four times using 2.3 kg of chestnuts.

Table 3. Postharvest treatments used in fresh chestnuts

Commercial name	Active ingredient	Concentration	Exposure time (min)
Scholar Fungicide (Syngenta Crop Protection, Inc. Greensboro, NC, USA)	50% Fludioxonil	0.03 ppm	3
Flint Fungicide (Bayer CropScience Int.)	50% Trifloxystrobin	0.15 ppm	3
CAF-06™ (BioSafe Systems, Glastonbury, CT, USA)	39.6% Caprylic acid, 26.67 emsorb6915, 14% glycolic acid, 13.3 light mineral oil and 0.4% capric acid	3,960 ppm caprylic acid, 2,667 glycolic acid, 40 ppm capric acid	3
Storox™ (BioSafe Systems, Glastonbury, CT, USA)	27% Hydrogen dioxide + 2% Peracetic acid	2700 ppm hydrogen dioxide + 200 ppm peracetic acid	3
Storox™ + CAF-06™ (BioSafe Systems, Glastonbury, CT, USA)	27% Hydrogen dioxide + 2% Peracetic acid + 39.6% Caprylic acid, 26.67 emsorb6915, 14% glycolic acid, 13.3 light mineral oil and 0.4% capric acid	1350 ppm hydrogen dioxide + 100 ppm 2 peracetic acid + 1,980 ppm caprylic acid, 1,333 glycolic acid, 20 ppm capric acid	3
Agri-Cide (Life Science Group, Inc. Monticello, IN, USA)	18.25 – 21.75% Copper sulfate pentahydrate	1 ppm	3
Chlorine dioxide gas (ICA TriNova, LLC Forest Park, GA)	ClO_2	7.5 mg of ClO_2/g chestnut	120
Chlorine dioxide solution (ICA TriNova, LLC Forest Park, GA)	$ClO_2 + H_2O$	10 ppm	3
Ozone solution (Aqua Air Technologies, Inc., Bloomfield, MI, USA)	$O_3 + H_2O$	0.92 ppm	3

Table 1. (cont'd)

Commercial name	Active ingredient	Concentration	Exposure time (min)
Sodium metabisulfite (Sigma-Aldrich™, St. Louis, MO, USA)	$Na_2S_2O_5$	0.2 M	3
Aluminum acetate (Sigma-Aldrich™, St. Louis, MO, USA)	$(CH_3CO_2)_2AlOH$	0.2 M	3
Bio-Save® 11 LP Biological fungicide (Jet harvest solutions, Longwood, FL, USA)	*Pseudomonas syringae* Strain ESC-11	1.65 g/L of water	3
Radiance™ (CH_2O, Olympia, WA, USA)	Shellac-based wax	Concentrated	45
Brilliance 3™ – CH_2O, Olympia, WA, USA	Caranauba-based wax	Concentrated	45
Peracetic acid (Lenntech™, Rotterdamseweg, Netherlands)	Peracetic acid	100 ppm	3
Chlorine solution (Champion packaging, Inc. Woodridge, IL, USA)	6% Sodium hypochlorite	1000 ppm of sodium hypochlorite	3

For each treatment a sub-group of 10 chestnuts was randomly picked and then tightly wrapped in a manually cut mesh bag (Figure 9-a). This sub-sample was then placed in the middle of remaining chestnuts containing 2.5 kg of chestnuts, thus creating a double-sac where a larger group of chestnuts from the same treatment surrounded the small sub-sample of 10 chestnuts, thereby protecting them from contact with untreated chestnuts and chestnuts receiving different treatments (Figure 9-b) (Ryser, 2007). All treatments solutions, except chlorine dioxide gas, ozone and waxes were thoroughly mixed, to achieve the desired concentration in 30 L of water. Experiment was performed once consisting in four samples per treatment, which were dipped at once in each solution for 3 min (exposure time) (Figure 9-c).

Chestnuts were exposed to 7.5 mg of ClO_2 gas /g of chestnuts inside a 20 L sealed bucket for 2 h (exposure time). Chlorine dioxide gas was generated in-situ using a 24 g commercial ClO_2 sachet (ICA TriNova, LLC Forest Park, GA) and circulated inside the sealed bucket using three 12 VDC/0.07-0.11A cooling fans (40x40x10 mm-case-fan) (Model MW-410H12C, AAVID, Bologna, Italy) that were attached to the bottom and opposite sides of the bucket (Figure 9-f). Ozone was generated using a commercial ozone generator (ozone solution, Aqua Air Technologies, Inc., Bloomfield, MI, USA) equipped with an oxygen concentrator (model CD 10/AD Corona Discharge ozone generator system, Clear Water Tech., Inc., San Luis Obispo, CA, USA). The gas was delivered through an inlet line directly into the water, which was constantly circulated through a 30 L container. Water containing up to 1-ppm ozone was produced within 20 min. The concentration of ozone in water used to treat chestnuts was constantly monitored by an electrochemical method (Protti, 2001) using a portable dissolved ozone meter (model OZ-21P, DKK-TOA Corporation, Takadanobaba, Shinjuku-ku, Tokyo, Japan).

Chestnuts were spray-waxed on clean Teletype paper (PM Company, Cincinnati, OH, USA) (Figure 9-e) using a manual pump spraying system model Jr. pump-up (JaniSan, Layton, UT, USA). After air-drying for 45 min, excess wax was manually removed with a clean polypropylene scrub brush (Fuller, Great Bend, Kansas, USA).

All treated samples were then stored submerged in one full 453.6 kg high-density polyethylene vented bin (MACX, Grand Rapids, MI, USA) of untreated

chestnuts which was held at 4 °C using a completely randomized design into four vertical strata (1 repetition per strata) (Figure 9-d) (MSU experimental station, Clarksville, MI, USA). Temperature (T) and relative humidity (R.H.) were continuously monitored in the 4 strata, using a data logger (Model: HOBO Micro station data logger - H21-002 with 4-Temp/RH sensor – S-THB-M002, MicroDAQ, Contoocook, NH, USA).

Figure 10. Treatment of fresh chestnuts before storage. a) sub-sample of 10 chestnuts, b) double-sac storage method, c) liquid-solution treatment application, d) Chlorine dioxide gas system used to treat chestnuts, e) wax application, f) treatment storage. Image in this thesis is presented in color.

Microbial mold severity, kernel incidence and weight loss analysis

Microbiological quality (severity of shell mold and incidence of kernel decay) and weight loss from chestnuts in the sub-sample of all 16 treatments was

evaluated after 60 days storage. Remaining chestnuts in treated samples were discarded. Shell mold severity was calculated as the mean number of a 4-point visual qualitative measurement of mold presence, where 0 = no mold, 1 = 1-25 % mold, 2 = 26-50 % mold, 3 = 51-75 % mold and 4 = 76-100 % mold. Incidence of decayed kernels was calculated as the mean number of chestnuts exhibiting decay. Weight loss was the difference from the in weight and after storage.

Statistical analysis

The mold severity, kernel decay and weight loss data collected after 690 days of storage were analyzed using one-factor analysis of variance (ANOVA. Significance between means was determined using the Tukey post-hoc multiple comparisons of means test at the 95% family-wise confidence level (p=0.05). Calculations were performed using the "R: A language and environment for statistical computing" statistical package (R Development Core Team, 2007) (Ott and Longnecker, 2001).

Results

A statistically significant difference was observed for shell mold severity ($F(15,1) = 0.89$, $p < 0.01$) and incidence of decay in kernels ($F(15,1) = 4.88$, $p < 0.01$) (Table 4), confirming that at least one treatment was statistically lower or higher in comparison with the other treatments. The most efficacious treatments for inhibiting shell mold included 3 minutes immersion time in 2,700-ppm hydrogen dioxide + 200-ppm peracetic acid, 0.15-ppm trifloxystrobin and 1,350-

ppm hydrogen dioxide + 100-ppm peracetic acid mixed with 1,980-ppm caprylic acid, 1,333-ppm glycolic acid and 20-ppm capric acid.

Table 4.Effect of fresh chestnut treatment application on shell mold[1], kernel decay[2] and weigh loss[3] stored for 60 days in control environment[4]

Treatment	Mold severity on shell[5]	Incidence of decay in kernel	Weight loss (g)
2700 ppm hydrogen dioxide + 200 ppm peracetic acid	0.17 ± 0.12 a	1.25 ± 0.95 a	0.38 ± 0.08 a
0.15 ppm Trifloxystrobin	0.20 ± 0.27 a	1.50 ± 1.33 a	0.40 ± 0.08 a
1350 ppm hydrogen dioxide + 100 ppm 2 peracetic acid + 1,980 ppm caprylic acid, 1,333 glycolic acid, 20 ppm capric acid	0.25 ± 0.19 a	3.50 ± 0.57 ab	0.22 ± 0.19 a
0.03 ppm Fludioxonil	0.27 ± 0.15 ab	1.25 ± 0.95 a	0.30 ± 0.13 a
3,960 ppm caprylic acid, 2,667 glycolic acid, 40 ppm capric acid	0.40 ± 0.21 b	2.75 ± 1.50 ab	0.27 ± 0.09 a
10 ppm ClO_2	0.40 ± 0.31 b	2.75 ± 0.95 ab	0.27 ± 0.17 a
0.2 M Sodium metabisulfite	0.48 ± 0.12 c	2.25 ± 1.50 a	0.25 ± 0.05 a
1000 ppm Sodium hypochlorite	0.58 ± 0.09 c	3.75 ± 0.96 ab	0.27 ± 0.12 a
0.2 M Aluminum acetate	0.63 ± 0.17 cd	3.25 ± 2.06 ab	0.35 ± 0.13 a
1 ppm Agri-Cide	0.85 ± 0.26 d	4.25 ± 1.50 ab	0.30 ± 0.14 a
2 L-concentrated Brilliance wax	0.98 ± 0.15 d	3.00 ± 1.41 ab	0.27 ± 0.15 a
0.92 ppm Ozone	1.12 ± 0.17 d	0.50 ± 0.58 a	0.35 ± 0.10 a
2 L-concentrated Radiance wax	1.12 ± 0.52 d	3.25 ± 1.50 ab	0.17 ± 0.15 a
7.5 mg ClO_2 gas/g chestnut	1.23 ± 0.26 d	1.25 ± 0.50 a	0.20 ± 0.16 a
16.5 g Bio-save 11 LP/L water	1.28 ± 0.15 d	4.50 ± 0.58 b	0.27 ± 0.05 a
100 ppm PAA	1.40 ± 0.36 d	2.00 ± 0.82 a	0.27 ± 0.19 a
Non-treated Control	1.68 ± 0.12 d	5.50 ± 1.73 b	0.2 ± 0.11 a

[1]Shell mold severity was calculated as the mean number of a 4-point visual qualitative measurement of mold presence, where 0 = no mold, 1 = 1-25 % mold, 2 = 26-50 % mold, 3 = 51-75 % mold and 4 = 76-100 % mold

[2]Incidence of decayed kernels was calculated as the mean number of chestnuts showing symptoms of decay (n=10).

[3]Weight loss was calculated as the mean of total weight before storage minus weight after storage.

[4]Treatments stored submerged in one full 453.6 kg high-density polyethylene vented bin of untreated chestnuts held at 4 °C using a completely randomized design into four vertical strata (1 repetition per strata) (mean T/R.H. = 7.2°C/96.5%).

[5]Numbers followed by the same letter within the column are not significantly different at $p = 0.05$ (Tukey multiple comparison of means).

Treatments that significantly reduced the number of decayed kernels in comparison with the non-treated control and other treatments were 0.92-ppm ozone, 7.5-mg ClO_2 gas/g chestnut, 0.03-ppm fludioxonil, 2,700-ppm hydrogen dioxide + 200-ppm peracetic acid, 0.15-ppm trifloxystrobin and 100-ppm peracetic acid. Of the evaluated treatments, none significantly reduced weight loss during storage ($F(15,1) = 0.02$, *ns*).

Discussion

Currently, the Michigan chestnut industry immerses harvested chestnuts in a 100-200 ppm chlorinated water bath to reduce contaminants and microbial population before storage. However, preliminary research indicated that the efficacy of this compound is not only limited, but may also have unpredictable side effects (Cena, 1998; Nguyen-rhe and Carlin, 1994). In recent years, important health and environmental concerns have arisen over the continued use of chlorine due to its reactivity with some food constituents to form toxic by-products (Cena, 1998; Graham, 1997). Thus, with the actual regulations for chemical use and possibility of future regulatory constraints on the use of chlorine as a sanitizer, alternative treatments are being sought to improve the microbiological quality and safety of agricultural commodities. Other laboratories including Beuchat (1998), Block (1991), Brackett (1999), Crowe et al. (2005), Palou et al. (2002), Panagou et al., (2005), Perrera and Karunaratne (2001), Pierre et al., (2006), Sapers (2003), Suslow (2002), and Wade et al., (2003), have evaluated other potentially safer sanitizers such as hydrogen peroxide, diverse acids, ozone, chlorine dioxide (gas and solution), hypochlorite, organic

acids and natamycin in addition to heat for enhancing the safety and shelf of a wide range of commodities. Several salts, oils and biocontrol agents that can reduce decay are also being used and evaluated (Feng and Zheng, 2006, Kader, 2002; Mari et al., 2003; Mills et al., 2004; Porat et al., 2002; Sapers, 2003; Willis et a., 2007).

The present work was conducted to evaluate the efficacy of 16 postharvest treatments, with the objective of improving microbiological quality of fresh chestnuts. Hydrogen dioxide significantly reduced both mold severity in shell and incidence of kernel decay. Hydrogen dioxide has been used at concentrations of 1 to 10%, to reduce microbial populations and extend the shelf life many fruits and vegetables (Crowe, Bushway, and Bushway, 2005; Sapers, 2003). This compound is approved by the Food and Drug Administration (FDA) and it is generally recognized as safe (GRAS), mainly because it produces no residues since it is reduced to water and oxygen (Sapers, 2003). Although treatments containing high concentrations of hydrogen dioxide may be beneficial in improving microbial quality and extending shelf life, use of this treatment may be limited for lettuce, blueberries and other commodities due to excessive surface oxidation, resulting in product damage (Aharoni, Copel, and Fallick, 1994; Block, 1991; Simmons, 1997; Ukuku and Sapers, 2001; Ukuku, Pilizota, and Sapers, 2001). Although hydrogen dioxide in combination with caprylic acid, glycolic acid and capric acid also significantly reduced mold shell severity, no reduction was seen in reducing kernel decay. Similar results have been obtained for blueberries and in green salads, where hydrogen dioxide in combination with citric acid suppressed the growth of microbial populations and extended the shelf life of

both commodities (Crowe, Bushway, and Bushway, 2005; Sapers, 2003). Furthermore, there is no evidence indicating that fresh chestnuts were negatively affected by any of the previously mentioned products.

This study demonstrated that certain sanitizers might also play an important role in reducing the incidence of decayed kernels, even though they were not as effective in reducing mold severity. Among these were 0.92-ppm ozone solution, 7.5-mg of chlorine dioxide gas/g of chestnut, 100-ppm peracetic acid, and 0.2-M sodium metabisulfite. Ozone, chlorine dioxide gas and peracetic acid are used in several sanitizing processes. They are all FDA approved and have played important roles in reducing spoilage organisms, postharvest pathogens and foodborne pathogens on ready-to-eat produce, and other diverse horticultural commodities.

In general, the previously mentioned sanitizers more potent than chlorine solutions, in FDA permitted concentrations (Gurol and Akata, 1996; Mari et al., 1999; Morris, 2006; Palou et al., 2002; Pierre et al., 2006; Panagou et al., 2005; Sapers, 2003; Roberts and Reymond, 1999; Sapers, 2003; Sarig et al., 1980; Sharma et al. 2003; Suslow, 2002; Wade et al., 2003; Wu and Kim, 2007; Zoffoli et al., 2005). Sodium metabisulfite, with relatively low mammalian toxicity and a broad-spectrum mode of action, was also effective in reducing kernel decay. It is widely used as a multi-functional antimicrobial agent and is a common preservative found in relishes, fresh and dried fruits and vegetables, tomato paste, mincemeat and potatoes (Mills et al., 2004; Porat et al., 2002).

Chemical treatments, including a broad range of fungicides such as mancozeb, thiabendasole, fludoxonil and trifloxystrobin can reduce decay in

different agricultural commodities (Gamage et al., 1997; Kader, 2002; Kicast, 1995; Narayanasamy, 2006; Sapers, 2003; Saroj, et al., 2006; Smith and Pillai, 2004). However, governmental regulations involving health and environmental issues impair their use. This study showed that trifloxystrobin significantly reduced shell mold severity and the incidence of kernel decay. Fludioxonil significantly reduced the incidence of kernel decay, compared with the control and other treatments, but was not among the best in reducing shell mold, when compared with other products.

Wax coatings are used to the prolong shelf life of produce by decreasing respiration rates, delaying fruit ripening, reducing shrinkage, reducing weight loss, improving product appearance and providing a carrier for postharvest treatment applications including the application of growth regulators. Edible coatings have been tried on various fruits such as mandarins (Bayindirly, Sumnu, and Kamadar, 1995), citrus fruits (Hagenmaier and Baker, 1993), pears (Sumnu and Bayindirly, 1994), bananas (Alzaemey, Fallana, and Thomson, 1989) and apples (Chai, Ott, and Cash, 1991). This study demonstrated that none of the postharvest treatments, including the two evaluated waxes (caranauba- and shellac- base waxes) significantly reduced weight loss of chestnuts during storage. However, future studies may confirm that the appearance of the chestnuts might be improved, a factor that was observed, but not measured in this study.

This study identified several compounds that may inhibit the growth of microorganisms responsible for postharvest mold and decay of chestnuts. Among these, was hydrogen dioxide, a product that could significantly control

postharvest mold and decay of chestnuts. Other compounds with the potential to inhibit internal decay include ozone, chlorine dioxide gas, peracetic acid and sodium metabisulfite. The prospect of commercially using these compounds as postharvest treatments needs to be further examined for potential advantages over conventional chestnut production practices. Efficient application and monitoring will need to be developed and examined for these new microbial reduction strategies to maximize product coverage and efficacy. Most compounds presently used in the food processing and agriculture industry are generally regarded as safe for human consumption at the appropriate concentration; hence hydrogen dioxide could be most easily adapted by chestnut producers to reduce postharvest mold and decay.

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CHAPTER 4

MICROBIAL CONTAMINATION IN PEELED CHESTNUTS AND THE EFFICACY OF POST-PROCESSING TREATMENTS FOR MICROBIAL SPOILAGE MANAGEMENT

Abstract

Peeled chestnuts represent a growing value-added commodity worldwide. Unlike most nuts, peeled chestnuts must be dehydrated or immediately frozen for later use. Concerns regarding the shelf life and spoilage of peeled chestnuts after thawing prompted a 2006-2007 survey in which chestnuts were quantitatively examined for various microbial contaminants after harvest as well as during and after peeling. Chestnuts (cv. 'Colossal') were randomly collected after harvest from at least seven Michigan farms, stored at 4 °C for 2 weeks, and peeled using a commercial brulage peeler. Microbial surveys indicated that average mesophilic aerobic bacteria (MAB), yeast and molds populations in chestnuts were 2.70, 2.74 and 2.51 at post harvest; 3.46, 3.27 and 2.40 during peeling; and 5.39, 3.09 and <1.70 log CFU/g after peeling, respectively. Overall, microbial populations in the water and environmental samples increased from < 1.7 to 5.46 log CFU/ml and <1.7 to 5.91 CFU/10 cm^2, respectively. During processing, the skin separator (brushes) and a sorting belt were identified as key points for contamination. Further studies indicated that two bacteria *Rahnella* sp., and *Curtobacterium* sp. and the yeast *Candida* sp. were the primary causes of spoilage. Trials were conducted to evaluate the efficacy of six sanitizer treatments, as well as X-ray irradiation and heat against these organisms on artificially inoculated chestnuts, during 18 days of storage at 4 °C. Overall, X-ray irradiation, 3 min immersion in

92 ppm hydrogen dioxide and 65 °C hot water were most effective in reducing MAB and yeast, compared to the other treatments.

Introduction

As an alternative to fresh chestnut sales, peeled chestnuts are processed and sold to diversify the Michigan chestnut industry. Demand for products such as peeled chestnuts, considered to be a fresh processed (cut) product, is increasing as a result of consumer desire for healthy, fresh, easy to use and appetizing foods (IFPA, 2006; Kader, 2002). In Michigan, fresh chestnuts are commercially peeled with a commercial peeling system made in Italy and purchased and imported in 2001 (Boema; Neive, Italy) (Appendix B). After peeling, the chestnuts are typically vacuum-packed and stored frozen (Guyer et al., 2003). Other mechanical methods of peeling, for example, air-impingement de-shelling technologies, are also available but are not used in Michigan (Gao et al., 2008).

Removing the shell and pellicle, which are considered to be a weak, but still natural physical barrier that protects the kernel, facilitates water loss and contamination of the kernel with pathogens and opportunistic organisms (Cantwell, 1995; Mencarelli, 2001), potentially affecting their final quality and safety (Field, Jordán, and Osbourn, 2006).

Within ten to fifteen days after thawing (~12 days), sticky, yellow ooze can develop on the surface of thawed peeled chestnuts, affecting their final quality and acceptability. Similar problems have never been reported in peeled chestnuts, but presence of slime or ooze is also common in processed fruits,

vegetables, juices, ready-to-use salads and meats (Brightwell et al., 2007; Guerzoni et al., 1996; James, Martin, and David, 2005; Ng, 2007; Ragaert et al., 2006; Tournas, 2005).

Growth of spoilage organisms is usually accompanied by the accumulation of metabolites, such as ethanol, lactic acid, and ethyl acetate, among others. The organoleptic changes due to such metabolic activity are associated with the enzymatic oxidation of various compounds leaking from the injured tissues. Spoilage is detectable by sensory and microbiological methods when the microbial population attains a certain microbial level. This population level is dependent on the microbial species in question and the ingredient characteristics. In extreme cases, shelf life of commercial products may not exceed five days under conditions of refrigeration temperature (4 °C) (Brightwell et al., 2007; Guerzoni et al., 1996; James et al., 2005; Ng, 2007; Ragaert et al., 2006; Tournas, 2005). Zagory (1998) and Tournas (2005) indicated that after harvest, a wide range of economical important vegetables and specifically fresh-cut products, are often spoiled by a wide variety of microorganisms including many bacterial (*Curtobacterium*, *Rahnella*, *Erwinia*, *Pseudomonas* spp., among others) and several fungal species. Spoilage significantly increases when the product is injured or the skin has been damaged or removed. Packaged sliced onions, shredded mixed lettuce and other commodities stored under air or modified atmosphere suffer from extreme colonization of diverse spoilage microorganisms, including bacteria, molds and several yeasts (*Pichia fermentans*, *Cryptococcus laurentii* and *Candida* spp., among others) (Liu and Li, 2006; Ragaert et al., 2006). All these usually lead to undesirable quality, economic loss and confirm

the importance of microbial growth during production, harvest, processing, and during storage (Tournas, 2005; Zagory, 1998).

Increasing attention has been focused on the microbial safety of processed fruits and vegetables, mainly on intervention methods to kill or remove human pathogens and spoilage microorganisms in fresh produce (Perish, et al., 2003; Sapers, 2003). In the majority of cases, sanitizing agents are added to processing water to reduce microbial populations and prevent cross-contamination of the product. Of these, chlorine has been used for several decades and is still the most widely used sanitizer in the food industry (Baur et al., 2004; IAFP, 2002). However, chlorine does not always reduce microbial populations, including foodborne pathogens and may be harmful due to the formation of toxic chlorine byproducts (Foley et al., 2004; Richardson, et al., 2000; Vitro et al., 2005). New technologies, *i.e.*, food irradiation, heat and others, which have been reported to achieve greater than a 5-log reduction of pathogens and other microorganisms (pasteurization treatments), have the potential to greatly improve the microbiological quality and safety of produce (Niemira, 2003).

The objectives of this study were to determine which spoilage organisms negatively impact peeled chestnuts before, during and after processing and how they were becoming established on the processed product. In addition, the efficiency of various postprocessing treatments to reduce microbiological populations and prolong the shelf life of peeled chestnuts was also assessed.

Material and Methods

Chestnut samples

During the 2006 and 2007 growing seasons, chestnut samples (cv. 'Colossal') from at least seven Michigan farms were assessed for microbial populations at two points before processing and five points during commercial processing (Boema; Neive, Italy), at the Michigan State University Rogers Reserve, Jackson. MI. Triplicate chestnut samples (250 g) were collected in Whirl-Pak bags (NASCO, International Inc.,120 mm x 60 mm) after transport to the facility (recollection buckets), pre-processing at the facility (receiver in chain batcher-A), passing through the burner on the elevator batcher-D, passing through the steamer-H, passing through the counter-rotating rollers and through the brushes-K, transport on the sorting belt-L, and before packaging at the end of the peeling line-M (Figure 10 and Figure 11). Samples were transported to the laboratory on ice and analyzed within 24 h of collection.

Environmental and water samples

Environmental samples were collected during processing (Figure 10 and Figure 11) on shoot before steamer-E, on shoot after steamer-H, on residue disposal shoot after brushes-J, from counter-rotating rollers in brushes-I, on shoot after brushes-K, on sorting belt-L and on shoot after sorting belt-M from 100 cm^2 area using sterile kim-wipes (Kimberly-Clark Professional, Rosewell, GA) in Whirl-Pak bags containing 10 ml of phosphate buffer solution (PBS) (pH = 7.4) (Figure 10 and Figure 11). 50 ml water samples were also collected from the tube

injecting water into the steamer-G, from tube leaking out of steamer after use-F, residue disposal shoot after brushes-J, after brushes-K and after the sorting belt-M in 50-ml sterile centrifuge tubes (Corning®, Life Science Group, Lowell, MA, USA) (Figure 10 and Figure 11). Samples were transported to the laboratory on ice and analyzed within 24 h of collection.

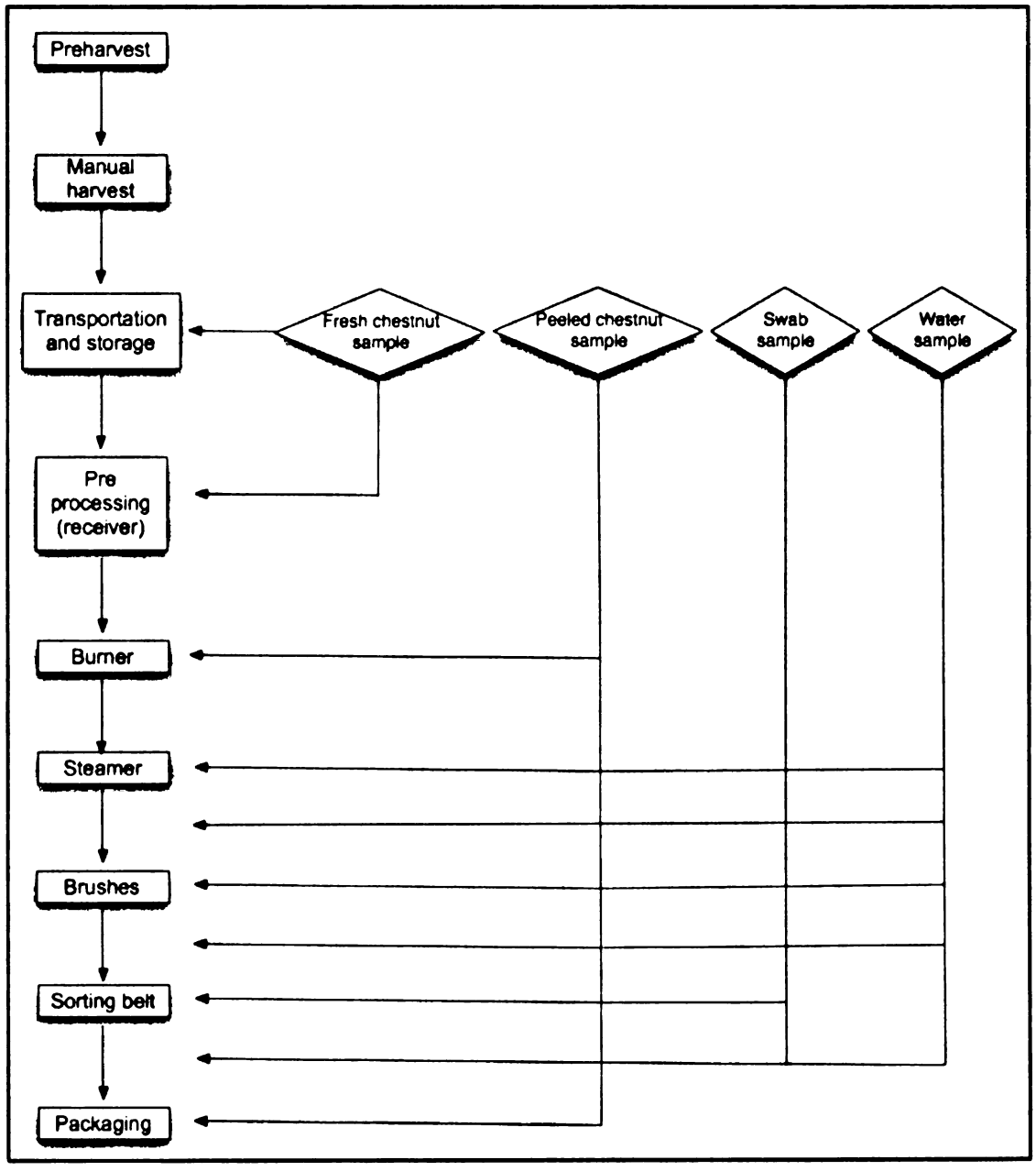

Figure 11. Sample collection points for fresh and peeled chestnuts. Swab and water samples were taken during chestnuts peeling.

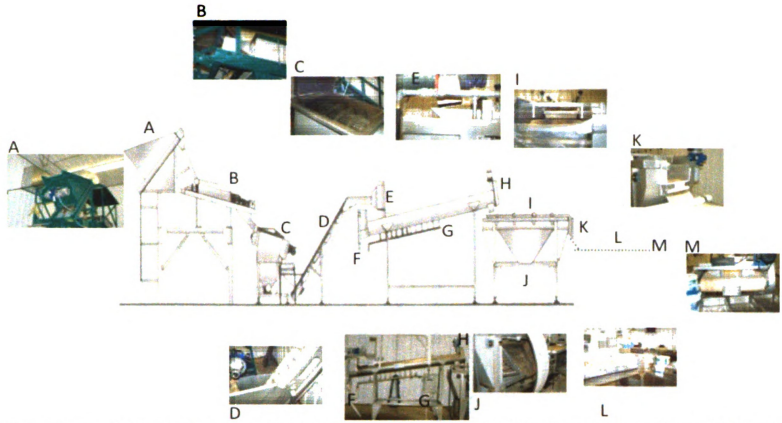

Figure 12. Schematic diagram of a brulage chestnut peeling line (Boema; Neive, Italy), describing chestnuts, environmental and water **sampling** points. A) Receiver-Chain batcher, B) Peeling ovens, C) Tangential cleaner, D) Elevator batcher, E) Shoot before steamer, F) **Tube leak**ing out of steamer after use, G) Injection water into steamer, H) Steamer, I) Rotating rollers in brushes, J) Residue disposal **shoot afte**r brushes, K) Brushes, L) Sorting belt, M) Shoot from end of peeling line-before packaging. Image in this thesis is presented **in color.**

Determination of microbial populations in fresh chestnut, peeled chestnut and environmental samples

Fresh chestnuts were surface sterilized by dipping each chestnut in 99% ethanol and flaming after which the shell was aseptically removed. Each sample was weighed (~ 25 g) into a sterile Whirl-Pak stomacher bag diluted 1:5 in phosphate buffer solution (PBS) (pH 7.4) and homogenized for 1 min (normal speed) in a stomacher Model 400 (Seward Lab. System, England). The same procedure, with exception of shell removal was repeated for peeled chestnuts. The suspension was serially diluted and 100 µl aliquots were inoculated onto trypticase soy agar (Becton and Dikinson Sparks, Md, USA) plus 0.6% yeast extract containing 100 ppm of cycloheximide (Sigma-Aldrich, St. Louis, Mo, USA) (TSA-YEC) for quantification of mesophilic aerobic bacteria (MAB) and onto potato dextrose agar containing 20 ppm streptomycin and 50 ppm ampicillin (PDA-SA) for enumeration of yeasts and molds.

Environmental swab samples were homogenized for 1 min (normal speed) in a stomacher Model 400 (Seward Lab. System, England) and then plated for MAB, yeasts and molds, along with 100 µl aliquots of the waters samples as just described. MAB plates were counted after 48 h of incubation at 25° C (± 3), whereas the mold and yeast CFU, were counted after 72 h of incubation at 25° C (± 3) (APHA, 2001).

Identification of microorganisms

Microorganisms isolated from 25 samples (25 g) of symptomatically thawed peeled chestnuts, containing sticky, yellow ooze on the surface were picked and subsequently purified after isolation. Spoilage symptoms were described, photographed and classified.

MAB were identified sequencing rDNA subunits (White, Bruns, Lee, and Taylor, 1990). Bacterial genomic DNA was extracted using the protocol proposed by Jacobs et al. (2008). The region including the two spacers (UFLP and URPL) was amplified using the method of LiPuma et al. (1999). A total volume of 25 µl for each reaction contained 60 ng of DNA, 23 µl of AFLP™ amplification core mix (Applied Biosystems, Foster City, Ca, USA), 2.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 10 pM of each primer. The primers UFPL (5'-AGTTTGATCCTGGCTCAG-3') and URPL (5'-GGTTACCTTGTTACGACTT-3') were used for targeting the 16S rDNA region from the kingdom Procaryotae (bacteria) (LiPuma et al., 1999).

The extracted DNA region was amplified in a 2720 thermal cycler (Applied Biosystems). The program included a cycle at 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 71 °C for 1 min, and a final elongation at 71 °C for 10 min. The PCR product was placed in wells on an agarose gel (1.25 %), and electrophoresed for 3 hours at 70 volts. After electrophoresis, the amplified region was purified using a QIAquick PCR Purification kit (Qiagen, Maryland, USA). The PCR product was sequenced in both directions using moderate throughput sequencing (Research Technology and Support Facility, MSU, East Lansing, MI,

USA). Sequences were deposited in the Lasergene software (DNA Star Inc., Madison, WI., USA) and used as queries for similarity searches in the NCBI nucleotide database (NCBI, 2008). Species reported had a 98 to 100 % match in both directions.

Yeasts were inoculated on potato dextrose agar, incubated for 72 h at 25° C (± 3° C) and then sent to the DCPHA (MSU, East Lansing, Mi., USA) for further identification. Yeasts were identified using the API 20C yeast identification system together with microscopic morphology determinations (Michigan State University Diagnostic Center for Population and Animal Health (DCPHA)). This computer-assisted system for rapid identification of yeast provides results comparable to those obtained of conventional morphological methodologies (Land et al., 1979).

Determining symptoms associated with microorganisms isolated from spoiled peeled chestnuts

Preparation of inocula

Based on visual observations, the identified cultures determined to be associated with spoilage in peeled chestnuts were used in further inoculation studies. Bacteria were sub-cultured on TSA-YEC and yeasts were sub-cultured onto PDA-SA and incubated for 48 h at 25 °C. A single colony of each strain was transferred to 5 ml of Lauria-Bertani (LB) soft media (Becton and Dikinson, Sparks, Md, USA), incubated for 24 h at 25 °C, and constantly aerated on a rotator shaker at 150 rpm (model Classic C10, New Brunswick Scientific Co. Inc., Edison, NJ, USA). Broth cultures were then sterilely transferred by pouring the

subculture into sterile 100 ml LB media. This process was exponentially repeated, each 24 hour until desired amount of inocula from each organism was acquired.

Sterilization and inoculation of peeled chestnuts

Whole chestnuts (cv. 'Colossal') were mechanically peeled, vacuum-sealed in low-density polyethylene bags (Kent Buthcers Supply, Grand Rapids, MI) stored at – 5 °C for five weeks until used and then were completely thawed for four hours at 25 °C before use.

The thawed chestnuts were sterilized by two successive 10 min immersions in 20% sodium hypochlorite (1 L per 0.5 kg of chestnuts) with constant agitation on a rotator shaker (190 rpm) (model Classic C10, New Brunswick Scientific Co. Inc., Edison, NJ, USA) and then subjected to three 15 min washing with sterile-deionized water (1 L per 0.5 kg of chestnuts) with agitation (190 rpm) to eliminate residual chlorine. Chestnuts were then placed on sterile blotting paper and air dried in a bio-safety hood with for 25 min.

To confirm chestnut spoilage associated with isolated microorganisms, individual organisms and equal organism cocktail, containing between 10^8 to 10^9 CFU/ml of each organism were used as inocula in the test. Sterilized chestnuts were placed in flasks contained mixed inocula (1 L per 1 kg of chestnuts) and agitated on a rotator shaker (200 rpm) for 15 minutes. Inoculated chestnuts were placed on sterile blotting paper and air dried in a bio-safety hood for 30 min.

Treatments of artificially inoculated peeled chestnuts, storage and microbial analysis

Whole chestnuts (cv. 'Colossal') were mechanically peeled, vacuum-sealed in low-density polyethylene bags (Kent Buthcers Supply, Grand Rapids, MI) stored at – 5 °C for five weeks until used and then were completely thawed for four hours at 25 °C before use. Eight treatments were implemented in an attempt to mimic industrial processes (Table 5). All liquid-solution treatments, except heat treatment (hot water), X-ray irradiation and ozone solutions, were thoroughly mixed, to achieve the desired concentration in 30 L of water. Inoculated ~500 g of chestnuts were immersed in each solution for 2 min. After treatment, three ~150g (~12 chestnuts) samples per treatment were collected.

For hot water treatments, peeled chestnuts were treated with heated water generated by a laboratory water bath (model Durabath™, Baxter Scientific, Melrose Park, IL). After the desired temperature was reached, inoculated chestnuts were immediately submerged in the water for 2 min. Temperature was constantly monitored using a 7 mm immersion thermometer (Fisherbrand Scientific by Ertco, UK). Five hundred grams of inoculated chestnuts were dipped at once in warm water for 2 min. After treatment, three ~150 g (~12 chestnuts) samples per treatment were collected

Ozone was generated using a commercial ozone generator (Aqua Air Technologies, Inc., Bloomfield, MI) equipped with an oxygen concentrator model CD 10/AD Corona Discharge ozone generator system (Clear Water Tech., Inc., San Luis Obispo, CA). Gas was delivered through an inlet line directly into the

water, which was constantly circulated through a 30 L water container. Water containing up to 0.7 ppm ozone was obtained within 25 min. After the desired concentration was achieved, ~500 g of inoculated chestnuts were immersed in the ozone solution for 2 min. The ozone concentration was constantly monitored using a portable dissolved ozone meter (model OZ-21P, DKK-TOA Corporation, Takadanobaba, Shinjuku-ku, Tokyo, Japan).

Inoculated peeled chestnuts were irradiated at target doses of 0.5, 1.0, 1.5, and 2.0 kG using a low-energy X-ray food irradiator (Rayfresh Foods Inc., Ann Arbor, MI). The sample consists of a single layer of chestnuts (~12 chestnuts, ~150 g) in a Whirl-Pak plastic bag. Incident dose was measured at six locations on the surface using radiochromic film dosimeters (GAF3001DS, GEX Corp., CO). To maximize dose uniformity on the surface a dual tube and double treatment technique was used. These means that target doses of low-energy X-ray, were uniformly distributed by two-tubes to the flat surface of each side of the chestnut sample by manually turning the sample once.

All populations of MAB and Y were determined on the day of treatment (day 0), and after 10 and 18 days of storage at 4° C.

Table 5. Post-processing treatments used in artificially inoculated peeled chestnuts

Commercial name	Active ingredient	Concentration
X-ray irradiation (Rayfresh Foods Inc., Ann Arbor, MI, USA)	low-energy X-ray irradiation	0.5, 1, 1.5, 2 kGy
Storox™ (BioSafe Systems, Glastonbury, CT, USA)	27% Hydrogen dioxide + 2% peracetic acid	2,700-ppm hydrogen dioxide + 200-ppm peracetic acid
Agri-Cide (Life Science Group, Inc. Monticello, IN, USA)	18.25 – 21.75% Copper sulfate pentahydrate	1 ppm
Chlorine dioxide solution (ICA TriNova, LLC Forest Park, GA)	$ClO_2 + H_2O$	10 ppm
Ozone solution (Aqua Air Technologies, Inc., Bloomfield, NI, USA)	$O_3 + H_2O$	0.70 ppm
Peracetic Acid (Lenntech, Rotterdamseweg, Netherlands)	Peracetic acid	80 ppm
Chlorine solution (Champion packaging, Inc. Woodridge, IL, USA)	6% Sodium hypochlorite	100 ppm
Sodium chloride (Sigma-Aldrich, St. Louis, MO, USA)	Sodium chloride	0.2 M
Hot water	Heat	65 °C

Statistical analysis

Microbial population in peeled chestnuts, environmental and water samples

One-factor analysis of variance (ANOVA) was done on all microbial populations obtained from peeled chestnuts. Multiple analysis of variance (MANOVA) was used to determine differences among the microbial populations from different sampling points. Significance among the means was determined by the Tukey post-hoc multiple comparisons of means test at the 95% family-wise confidence level (p=0.05). Calculations were performed by using the "R: A language and environment for statistical computing" statistical package (R Development Core Team, 2007) (Ott and Longnecker, 2001).

Microbial population in treated peeled chestnuts

Repeated measurement design with analysis of variance (ANOVA) was done to average microbial count data (MAB and yeast) obtained from treated peeled chestnuts. Since the assignment of samples to the different treatment conditions as well as the choice of subsamples at each point of measurement were completely at random, sphericity can be assumed making multivariate as well as degrees of freedom corrections unnecessary (Keselman, Algina, and Kowalchuk, 2001; Ott and Longnecker, 2001). This procedure will indicate if a treatment significantly reduce the microbial population during 18 days of storage. Significance among the means was determined using the Tukey post-hoc multiple comparisons of means test at the 95% family-wise confidence level (p=0.05. These calculations were performed using the same statistical package listed above.

Results

Microorganisms associated with spoiled peeled chestnuts

Two bacteria (*Rahnella* sp. and *Curtobacterium* sp.), and one yeast (*Candida* sp.) were consistently isolated from the 25 samples (~ 25 g) of vacuum-packaged, peeled chestnuts thawed and stored for 10 to 15 days at 4 °C. These three microbes were considered the primary cause of spoilage as they each produced yellow, sticky ooze when artificially inoculated on sterile chestnuts (Figure 12-a to 12-d). *Rahnella* sp. and *Candida* sp. were also found on fresh chestnuts in an earlier study (Chapter 2 of this thesis) and therefore the source of

contamination of peeled chestnuts may be fresh chestnuts that are naturally contaminated when brought from the field. Although some browning was also associated with the spoilage, this can occur without the development of ooze as the chestnuts oxidize in time.

In most cases, all three spoilage organisms were present in spoiled chestnut; however, each alone, was also capable of causing spoilage. No obvious visual difference in terms of symptoms produced if either one or more organisms causing spoilage were observed (Figure 12-b to 12-d). The chestnuts were visually spoiled when the MAB and yeast populations exceeded 8 log CFU/g.

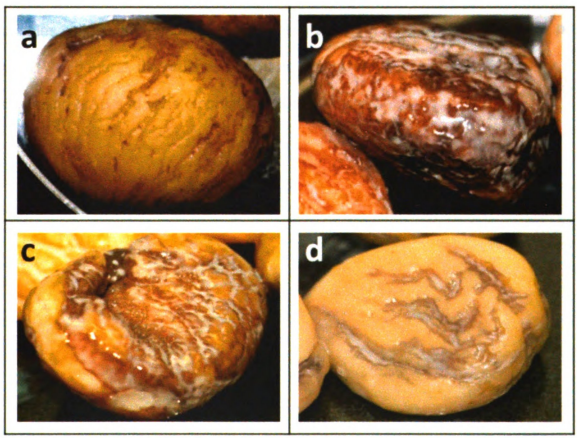

Figure 13. Peeled chestnuts, 10 days after inoculation and stored at 4 °C. a) Non-inoculated sterilized control, b) inoculated with *Curtobacterium* sp., c) inoculated with *Rahnella* sp., d) inoculated with *Candida* sp. Image in this thesis is presented in color.

Microbial population

Chestnut samples

Chestnut samples were collected at different locations before, during and after peeling, to determine how spoilage organisms were becoming established on the processed peeled product. Populations of MAB, molds and yeast before processing (buckets and receiver) varied from 2.50 to 2.90 logs CFU/g. After peeling, MAB ($F(6,2) = 362.19$, $p < 0.01$) and yeast ($F(6,2) = 8.66$, $p < 0.01$) increased significantly up to 5.39 and 3.09 logs CFU/g, respectively. In contrast, mold populations at the end of processing decreased to <1.7 logs CFU/g (Figure 13). A significant difference was seen between all microbial populations within samples collected before, during and after processing (approx $F(6,2) = 19.59$, $p < 0.01$). In general, the brushes played an important role in significantly increasing MAB and yeast populations in peeled chestnuts.

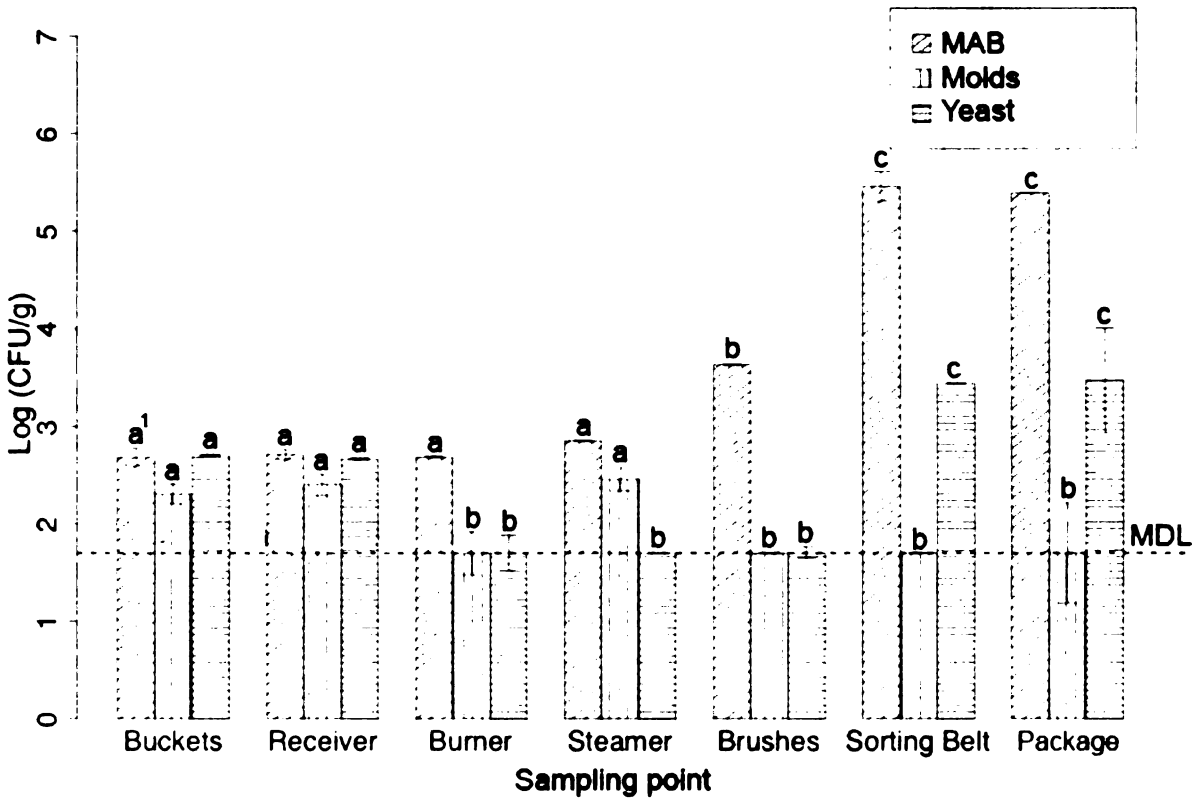

Figure 14. Total MAB, molds and yeast counts (log cfu g^{-1}) on peeled chestnuts before and during peeling process (Brulage peeler - Boema; Neive, Italy). [1]Values followed by the same letter within organisms are not significantly different at $p = 0.05$ (ANOVA) (Tukey multiple comparison of means). Minimum detectable level (MDL) = Minimum possible count (0.5) x minimum dilution factor x inoculated aliquot (100 µl). Error bars indicate standard deviation.

Environmental samples

Environmental samples were taken from points along the chestnut peeling line to help determine the point of chestnut contamination. Based on environmental swab samples, populations of MAB and yeast differed significantly along the peeling line. MAB, molds and yeasts were undetectable in the steamer and before brushes (< 2.7 logs CFU/cm^2). However, after the sorting belt and before packaging MAB ($F(5,2) = 243.06$, $p < 0.01$) and yeast ($F(5,2) = 15.05$, $p < 0.01$) populations increased significantly to 5.60 and 4.72 logs CFU/100 cm^2,

respectively. In contrast, mold populations after processing were <2.7 logs CFU/100 cm^2 (Figure 14). A significant difference was seen between all microbial populations within environmental samples collected during the peeling process (approx F (5,2 = 13.41, $p < 0.01$). These reaffirms that the brushes played an important role in significantly increasing MAB and yeast populations.

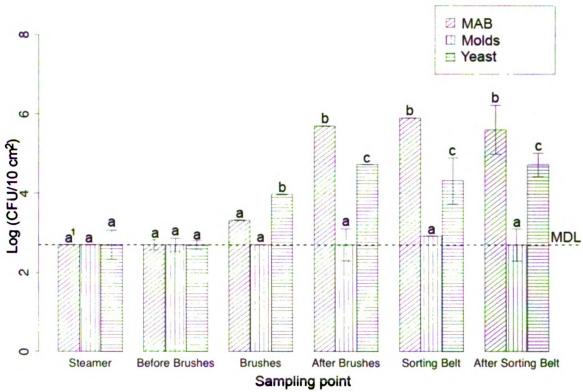

Figure 15. Total MAB, molds and yeast counts (log cfu cm^{-2}) on environmental samples during peeling process (Brulage peeler - Boema; Neive, Italy). [1]Values followed by the same letter within organisms are not significantly different at $p = 0.05$ (ANOVA) (Tukey multiple comparison of means). Minimum detectable level (MDL) = Minimum possible count (0.5) x minimum dilution factor x inoculated aliquot (100 µl). Error bars indicate standard deviation.

Water samples

Water samples were taken from points along the chestnut peeling line to help determine the point of contamination. MAB, molds and yeasts were undetectable in the steamer and after the steamer (< 1.7 logs CFU/ml). However, after the brushes, MAB ($F(4,2) = 1136.36$, $p < 0.01$) and yeast ($F(4,2) = 10.59$, $p < 0.01$) significantly increased to 3.73 and 4.98 logs CFU/ml, respectively. The mold population remained constant during the entire process (<1.7 logs CFU/ml) (Figure 15), but above population levels found in the steamer. A significant difference was seen between microbial populations within environmental samples collected during the peeling process (approx $F(4,1) = 428.37$, $p < 0.01$). In general, a significant increase in MAB and yeast was again observed in water collected from brushes.

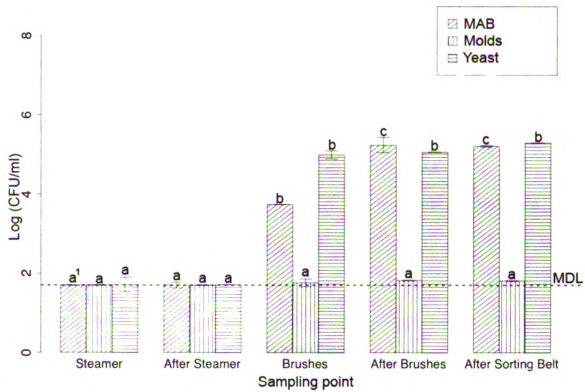

Figure 16. Total MAB, molds and yeast counts (log cfu g^{-1}) in water samples during peeling process (Brulage peeler - Boema; Neive, Italy). [1]Values followed by the same letter within organisms are not significantly different at $p = 0.05$ (ANOVA) (Tukey multiple comparison of means). Minimum detectable level = Minimum possible count (0.5) x minimum dilution factor x inoculated aliquot (100 µl). Error bars indicate standard deviation.

Post-processing treatments

Sanitizers and hot water

To determine if the shelf life of chestnuts could be extended after thawing, several microbial reduction strategies were assessed using chestnuts inoculated with the three identified spoilage organisms. The mean plus or minus the standard deviation for populations of MAB and yeast were evaluated immediately after each treatment, and after 10 and 18 days of storage at 4° C (Figure 16).

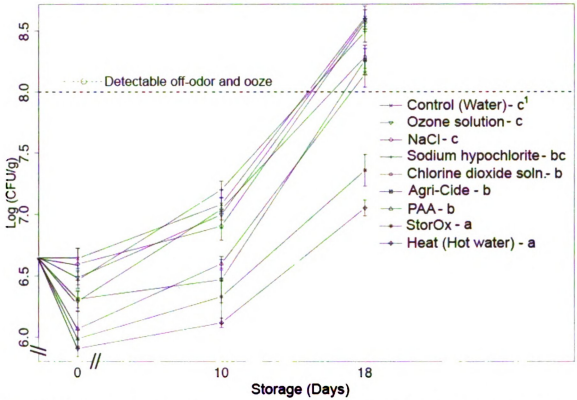

Figure 17. Mean MAB and yeast counts (log cfu g^{-1}) from peeled chestnuts treated with different sanitizers during 18 days of storage at 4° C. [1]Overall data point followed by the same letter within treatments are not significantly different at $p = 0.05$ (Repeated measurement design – ANOVA with post-hoc Tukey multiple comparison of means). Error bars indicate standard deviation.

Two minutes exposure time to 10 ppm Chlorine dioxide, 1 ppm copper sulfate pentahydrate and 80 ppm peracetic acid significantly reduced MAB and yeast population during 18 days of storage in comparison with the non-treated control ($F(8,2) = 43.89$, $p < 0.01$). However, on day 18 the microbial population had already surpassed the desired levels and spoilage could be observed. Only 2 min exposure time to 2,700-ppm hydrogen dioxide + 200-ppm peracetic acid and hot water treatment significantly reduced the mean MAB and yeast population during 18 days of storage in comparison with the non-treated control ($F(8,2) = 43.89$, $p < 0.01$). In both cases, the mean microbial population on day 18 was

significantly lower by 1.24 and 1.55 logs CFU/g respectively, in comparison with the non-treated control and no spoilage could be observed.

X-ray irradiation

To determine if spoilage could be managed and shelf life extended after thawing, various X-ray irradiation doses were tested on chestnuts inoculated with the three microbes associated with spoilage. The mean of MAB and yeast populations from the various doses of X-ray irradiation treatment were determined immediately after treatment, and 10 and 18 days of storage at 4° C (Figure 17).

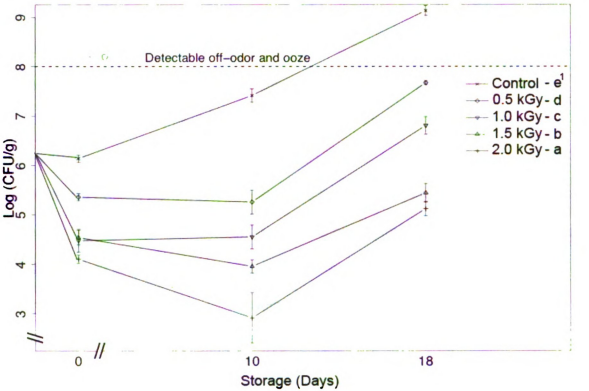

Figure 18. Mean MAB and yeast counts (log cfu g^{-1}) from peeled chestnuts treated with different X-ray irradiation doses during 18 days of storage at 4° C. [1]Overall data point followed by the same letter within dosis are not significantly different at $p = 0.05$ (Repeated measurement design – ANOVA with post-hoc Tukey multiple comparison of means). Error bars indicate standard deviation.

All X-ray irradiation doses significantly reduced the MAB and yeast populations during 18 days of storage in comparison with the non-treated control ($F(4,2) = 138.97$, $p < 0.01$). The mean microbial reductions when compared with the non-treated control on day 18 for 0.5, 1.0, 1.5 and 2.0 kGy were 1.47, 2.33, 3.69 and 4.02 logs CFU/g, respectively. After 18 days no spoilage could be observed in any of the treated chestnuts.

Discussion

Within 12 days after thawing, peeled chestnuts developed a sticky, yellow ooze over the surface that affected the final quality, acceptability and shelf life.

The three primary spoilage microorganisms identified included two bacteria *Rahnella* sp., *Curtobacterium* sp., and one yeast, *Candida* sp. There is no evidence indicating that filamentous fungi play an important role during spoilage.

Contamination of peeled chestnuts was strongly influenced by the peeling process. The traditional method of peeling chestnuts, (*e.g.* manual elimination of the shell and pellicle from fresh chestnuts) is laborious and requires heat and knives. In Michigan, peeled chestnuts are obtained by processing fresh chestnuts with a commercial-level peeling system (Boema; Neive, Italy) (Appendix B) after which the chestnuts are usually vacuum-packed and frozen (Guyer, et al., 2003). Other mechanical methods, such as air-impingement de-shelling, are also available but are not used in Michigan (Gao, et al., 2008). Michigan chestnut samples, and environmental and water microbial surveys indicated that average MAB and yeast populations significantly increased during peeling with the skin separator (brushes) and sorting belt identified as key points for contamination.

Similar results have been reported for other commodities and processing plants. Monitoring of several organisms, like *Listeria monocytogenes* during produce processing has indicated that cross-contamination from contaminated zones in the processing line, floor surface and gloves is critical and extremely difficult to control (Pappelbaum, et al., 2008). Others have reported that utensils, processing tables, and lines may also increase the levels of MAB, coliforms and pathogenic organisms like *Escherichia coli* 0157:H7 (Christison, Lidsay, and Von Holy, 2008). All of these studies have strongly indicated that the processing environment may play an important role in maintaining and enhancing pathogen populations. Because of these possibilities, and since MAB and yeast populations

significantly increased by more than 2 logs in chestnuts between harvest and after peeling, improved and more effective microbial reduction strategies after and during processing are needed to ensure the quality and prolong the shelf life of peeled chestnuts.

An alternative method to reducing microorganisms in the final product is to eliminate microbial presence by constantly disinfecting sections from the processing line, using different detergents, sterilizing agents and enzymes. These methods have been evaluated in dairy and meat processing lines (Manvi and Anand, 2001; Anon., 1970) and have also proven to be effective in removing microbial biofilms from dispensing equipment (Walker, Fourgialakis, Cerezo, and Livens, 2007). Neverhteless, further studies must be done to apply these methods to the chestnut peeler

Another alternative is to try to inactivate these spoilage microorganisms after processing and prior to storage. In most cases, sanitizing agents are added to processing water and consequently to the product, to reduce microbial populations and prevent cross-contamination of the processed commodity (Sapers, 2003). Hydrogen dioxide concentration of 1 to 10 percent have been used to reduce the microbial population and extend the shelf life of whole fruits, like blueberries, fresh-cut fruits and vegetables (Crowe, Bushway, and Bushway, 2005; Sapers, 2003). Hydrogen dioxide is Generally Recognized As Safe (GRAS), because it is reduced to water and oxygen after treatment (Sapers, 2003). Although treatments containing high concentrations of hydrogen dioxide may be useful in reducing microbial populations and extending shelf life, the use of these treatments in commodities like lettuce, blueberries and others, might be

limited because of possible plant surface oxidation resulting in product damage (Aharoni, Copel, and Fallick, 1994; Block, 1991; Simmons, 1997; Ukuku and Sapers, 2001; Ukuku et al., 2001). The present study indicated that hydrogen dioxide (1:100 dilution of Storox™, BioSafe Systems, Glastonbury, CT) significantly reduced the mean populations of MAB and yeast in peeled chestnuts, during 18 days of storage in comparison with the non-treated control.

Warm water (65 °C) was found to be among the best methods in significantly reducing spoilage as well as MAB and yeasts in peeled chestnuts, during 18 days of storage in comparison with the non-treated control. Heat treatments are often applied for a relatively short time (Fallik, 2004), as hot water dips or rinsing, vapor heat, hot dry air (Fallik, 2004) and other relatively new developed technologies like far infrared radiation (Tanaka et al. 2007). Historically, water has been the preferred medium for most applications since it is a more efficient heat transfer medium than air (Fallik 2004). Immersion of avocado fruit (cv. 'Hass') for 20–30 min at 40–42 ºC controls decay and enhances the storage quality (Fallik, 2004). Immersion of sweet chestnuts in 45 ºC water for 45 min enhanced storage quality of fresh product (Jerimini, *et al.*, 2006). A five log reduction of *Escherichia coli* O157:H7 was also observed after immersing apples in 80 and 90 °C water for 15 seconds (Fallik, 2004).

Studies have demonstrated that irradiation may also be capable of reducing pathogens and other microorganisms more than five logs in many foods. Irradiation is approved by the Food and Agriculture Organization (FAO)/International Atomic Energy Agency (IAEA)/World Health Organization (WHO), but irradiated products must be labeled properly using the Radura

symbol. Given the lack of evidence for toxic residues irradiation may become an attractive alternative to chemical sanitizers. However, irradiation remains expensive, requires special facilities and still lacks consumer acceptability (Diehl, 1995; Gamage, Faith, Luchansky, Buege, and Ingham, 1997; Howard, Miller, and Wagner, 1995; Kilcast, 1995; Luh, 1997; Niemira, 2003; Saroj, et al., 2006; Smith and Pillai, 2004). All evaluated X-Ray irradiation doses (0.5, 1, 1.5 and 2 kGy) significantly reduced the mean population of MAB and yeast during 18 days of storage and decreased spoilage in comparison with the non-treated control. Using the highest dose of radiation, population of MAB and yeast were 4 log CFU/g lower after 18 days of storage. Similar results have been reported for other commodities (Niemira, 2003). Nevertheless, it is important to consider that, both hot water treatments and irradiation may damage produce tissue and leak nutrients by breaking down cell wall material, producing off-flavors in the treated produce (Lydakis and Aked 2003; Niemira, 2003; Saroj, *et al.*, 2006; Soto-Zamora, 2005). There was no evidence indicating that the peeled chestnuts were negatively affected by any of the previously mentioned treatments, but further sensory evaluation is recommended to discard possible effects.

In conclusion, the present study showed that two bacteria *Rahnella* sp., and *Curtobacterium* sp. and the yeast *Candida* sp. were the primary causes of spoilage of mechanically peeled chestnuts. Overall, MAB and yeast in chestnuts, water and environmental samples significantly increased during peeling, with the skin separator (brushes) and sorting belt being key points for contamination. Nevertheless, several post-processing treatments were able to inhibit the growth of these microorganisms. Among these, 2,700-ppm hydrogen dioxide + 200-ppm

peracetic acid, immersion in hot water and X-ray irradiation were most effective. The prospect of adopting these methods as commercial post-processing treatments needs to be examined and may have several advantages for the peeled chestnut industry. Cost, efficient application, monitoring and implementation of each method must also be examined to maximize economic gains. All of the evaluated treatments are presently used in food processing and are generally regarded as safe at the appropriate concentration. These may be implemented as part of a sustainable integrated pest management strategy for peeled chestnut spoilage.

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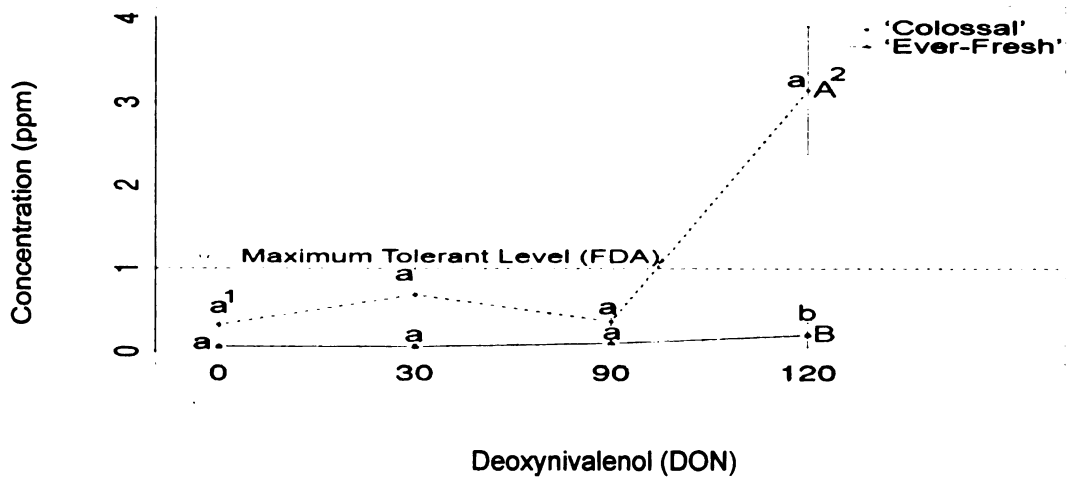
APPENDICES

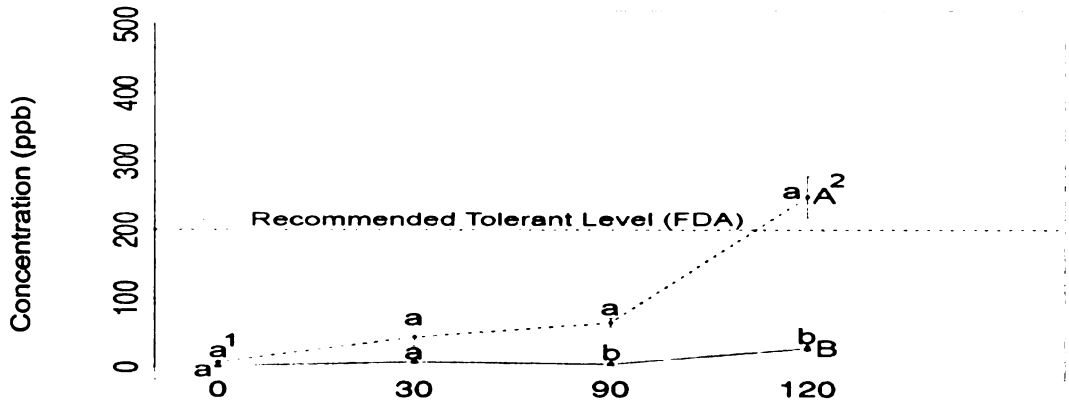
APPENDIX A

MYCOTOXINS IN FRESH MICHIGAN CHESTNUTS

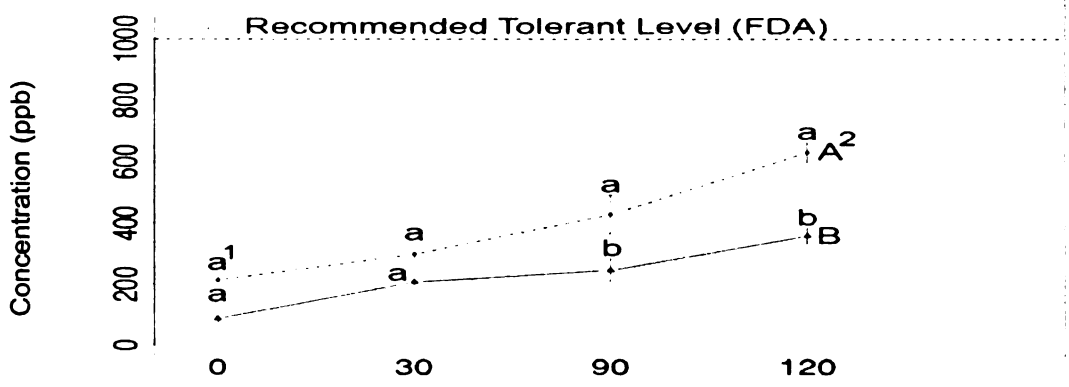
Figure 19. Presence of mycotoxins in two cultivars of fresh Michigan chestnuts. Five, 350 g of healthy kernel samples per cultivar were processed to extract mycotoxins and quantified by competitive direct ELISA test (Neogen Corporation, Acumedia Lansing, MI, USA) as specified by the manufacturer. [1]Data points followed by the same lower caseletter within the same day are not significantly different at $p = 0.05$ (T-test). [2]Overall data point followed by the same capitalized letter within chestnut part-harvesting method are not significantly different at $p = 0.05$ (Repeated measurement ANOVA). Error bars indicate standard deviation.



Deoxynivalenol (DON)



Ochratoxin

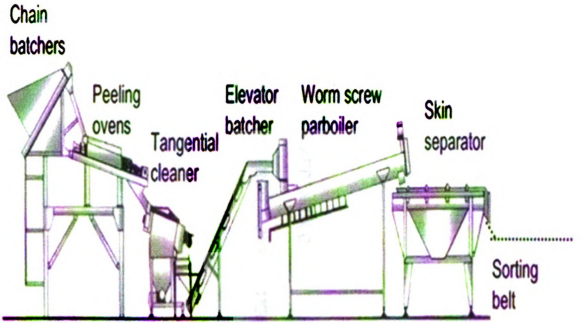


Zearalenone

Storage (Days)

APPENDIX B

COMMERCIAL-LEVEL PEELING SYSTEM (BOEMA; NEIVE, ITALY)



Source: Guyer, Fulbright, and Mandujano, 2003

Figure 20. Schematic diagram of a brulage chestnut peeling line (Boema; Neive, Italy)

APPENDIX C

CHESTNUT FRUIT MORPHOLOGY

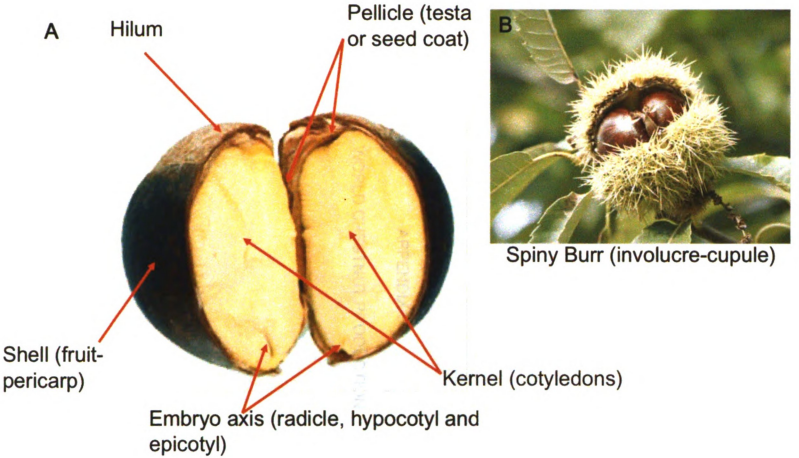
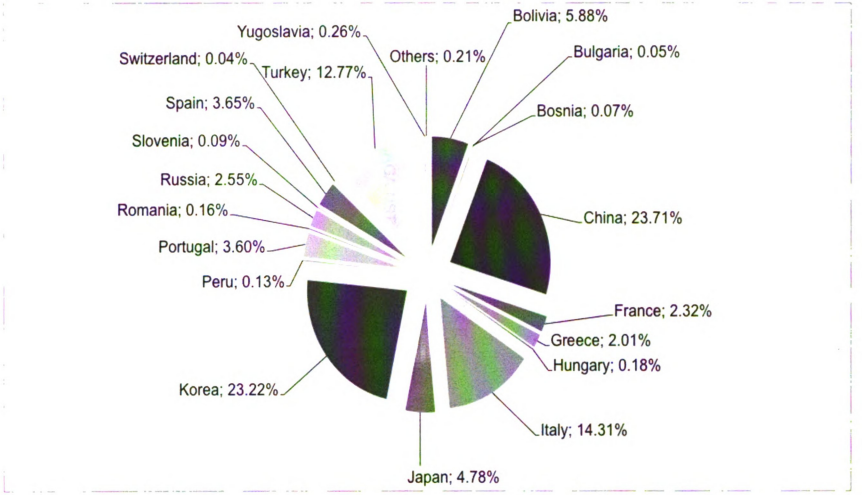


Figure 21. Chestnut fruit morphology. A. Chestnut fruit longitudinal cut, B. Spiny burr attached to chestnut tree with chestnuts. Image in this thesis is presented in color.

APPENDIX D

WORLD CHESTNUT PRODUCTION



Source: Food and Agriculture Organization (FAO), 2002

Figure 22. World chestnut (*Castanea* spp.) production in 2001

APPENDIX E

INHIBITION PROPERTIES OF PELLICLE, HILUM, SHELL AND KERNEL AGAINST *Cladosporium cucumerinum*

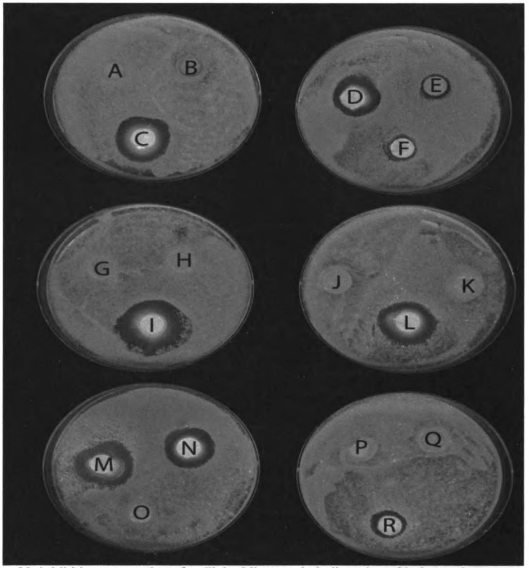


Figure 23. Inhibition properties of pellicle, hilum and shell against *Cladosporium cucumerinum*. To determine the inhibition properties of the different parts of the chestnuts (shell, hilum, pellicle and kernel), 70 percent acetone extract from these parts from two different cultivars were evaluated ('Everfresh' = *C. mollissima* and 'Colossal' = *C. sativa* x *C. crenata*). Paper disc agar diffusion method was employed, using filter paper discs of 10 mm diameter (Whatman, Shleicher and Schuell International Ltd.) impregnated with different concentration of the extracts. After the discs were put onto the agar, the organism was surface inoculated by spraying 10 ml of a sterile distilled-water solution containing between 220-300 spores/ml of the pathogen. The plates were sealed by the use of parafilm and stored inverted at room temperature (25° C ± 3°). A: 50 µl-shell-'Colossal', B: 100 µl-shell-'Colossal', C: 100 µl-pellicle-'Colossal', D: 50 µl-pellicle-'Colossal', E: 100 µl-hilum-'Colossal', F: 10 µl-pellicle-'Everfresh', G: 5 µl-hilum-'Everfresh', H: 100 µl-kernel-'Everfresh', I: 100 µl-pellicle-'Everfresh', J: Control (70 percent acetone), K: 5 µl-hilum-'Colossal', K: 100 µl-pellicle-'Everfresh', M: 50 µl-pellicle-'Everfresh', N: 50 µl-pellicle-'Colossal', O: Control (70 percent acetone), P: 50 µl-shell-'Colossal', Q: 100 µl-kernel-'Colossal', R: 10 µl-pellicle-'Everfresh'. Different levels of inhibition can be observed depending on the cultivar and different parts of the chestnuts. 'Everfresh' had higher levels of inhibition in comparison with 'Colossal'. The pellicle presented the highest zone of inhibition, followed by the shell and the hilum. The kernel did not inhibit the growth of the pathogen. Image in this thesis is presented in color.