MICROBIOLOGY AND AFLATOXIN PRODUCTION IN BRAZIL NUTS

A Thesis
Submitted to the Faculty
of Graduate Studies

By

Katia Arrus

In Partial Fulfillment of the
Requirements for the Degree
of
Master of Science

Department of Food Science
University of Manitoba
Winnipeg, Manitoba

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

MASTER OF SCIENCE

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ABSTRACT

New regulations established by the European Community in 1998 have created concerns in exporting countries like Peru regarding the presence of aflatoxin in Brazil nuts. Tightened quality requirements in regards to total aflatoxin levels (maximum 4 ppb) and the presence of microbial contaminants including *Escherichia coli* and *Salmonella* have prompted research to help address current problems. The research conducted in the current study was divided into two parts.

In the first study, experiments were conducted to evaluate the effects of relative humidity (75, 80, 85, 97%) and temperature (10, 13, 15, 25, 30°C) on aflatoxin production in Brazil nuts. *Aspergillus flavus* Link isolated from Brazil nuts and previously screened for aflatoxin production was inoculated (10 µl; 10^5/ml) on the surfaces of shelled half-nuts (simulating damaged or trimmed nuts), shelled whole nuts and in-shell whole nuts. Maximum aflatoxin (total and B_1) was detected in nuts stored at 97% r.h. and at 25-30°C. Aflatoxin was not detected (detection limit of the ELISA kit was 1.75 ppb) in nuts maintained at either 10°C (97% r.h.) or at 30°C (75% r.h) in any of the nuts up to 60 d of storage. Shelled half-nuts contained the highest total (6817 ppb) and B_1 aflatoxin (4483 ppb) levels. In-shell nuts contained the least total and B_1 toxin with maximum levels of 93 and 49 ppb, respectively. Results of this study indicated that the limiting moisture concentration and water activity values affecting aflatoxin production (< 4 ppb) in whole shelled and in-shell nuts stored at 30°C are 4.5,

0.68 and 5.0, 0.75, respectively and that prompt and proper drying will help address the aflatoxin problem.

In the second study the exterior of tree-harvested Brazil nut pods from Peru were tested for yeast and molds including aflatoxigenic *A. flavus* and *Aspergillus parasiticus* Speare and various bacterial pathogens including *Escherichia coli* and *Salmonella*. *A. flavus* was detected in 3 of 14 pods evaluated. All isolates were aflatoxigenic (630 to 915 ppb aflatoxin). Yeast and molds including *Penicillium* spp., *Fusarium* spp., *Acremonium* Link ex Fr. and unidentified coelomycetes were also present on the exterior of all pods. *E. coli*, coliforms and salmonellae were not recovered from any of the pods. Whole, in-shell nuts obtained following opening of all pods yielded no *A. flavus* or *A. parasiticus*, however, yeasts and penicillia were isolated. Aflatoxin was not detected (detection limit 1.75 ppb) in any of the nuts. In addition, whole in-shell and shelled nuts from various unit process operations were all positive for *A. flavus*. *E. coli* and salmonellae were not recovered. Soaking of whole, in-shell nuts, performed prior to cracking or shelling, was shown to result in an increase in coliforms levels. It is recommended that the soaking operation be abandoned and other more hygienic pre-treatment methods adopted prior to cracking. The presence of *A. flavus* on tree-harvested pods has previously not been reported and additional studies should be performed in order to more clearly assess their occurrence.

FOREWORD

This thesis is composed of two papers prepared for journal publication with the addition of a literature review (Chapter 2), and consolidated introduction (Chapter 1), conclusion (Chapter 5) and references.

Chapter 3 is prepared in accordance with the format requested by the Journal of Stored Products Research. The manuscript contained in chapter 4 was prepared following the format requested by the International Journal of Food Microbiology, with minor changes to the format for the purposes of standardizing the presentation of this thesis.

1. INTRODUCTION

The Brazil nut or castaña tree (*Bertholletia excelsa* Humb. & Bonpl.) is indigenous to the Amazon region and grows mainly in Brazil, Bolivia and Peru (Province of Madre de Dios). It is well recognized that this sustainable resource has an important role in the preservation of the Amazon rainforest and represents one of the most important non-timber, forest products. Since 1999, the European Community has enforced stricter standards relative to microbiological and aflatoxin contamination (Chen et al., 2001; Agnes and Akbarsha, 2003). In this regard, the current standards call for rejection of nuts that contain total and B_1 aflatoxin levels above 4 and 2 ppb, respectively (EC, 1998).

Aflatoxins are secondary metabolites produced by certain strains of *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare. Similar to other tree nuts or groundnuts, Brazil nuts have tested positive for these fungi, (Holubova-Jechova, 1970; Castrillon and Purchio, 1988a; Freire et al., 2000).

Several environmental factors are known to influence aflatoxin production, however, temperature and relative humidity are considered to be the most critical. Studies performed on hazelnuts and pistachios suggested that the optimum temperature and relative humidity for aflatoxin production is 25-30°C and 97-99%, respectively (Diener and Davis, 1966, 1967; Schindler et al., 1967; Northolt et al., 1976; Simsek et al., 2002). Additional factors such as water activity, moisture, substrate composition

(Sakai et al., 1984) storage time, insect damage (Lynch and Wilson, 1991; Schatzki and Ong, 2001) and presence of a shell (Ayerst and Budd, 1960) also influence fungal growth and aflatoxin production.

In general, pods from the Brazil nut tree are harvested after they have fallen to the jungle floor. In many cases they are in direct contact with the soil for several days to weeks. Ostensibly, it is during this time that the pods become contaminated with *Aspergillus*. Bezerra (2001) for example, reported that contamination of the pods with *Aspergillus* reached 100% after a 50 d exposure to the soil. In addition, post harvest operations are also expected to have a major influence on further contamination of the nuts (Bayman et al., 2002). Once the pods have been collected they are opened using a machete, placed in jute bags and transported to a commercial shelling facility via truck or boat. In the shelling plant the in-shell nuts must be dried as soon as possible in order to minimize mold growth. In this respect they are spread onto warehouse floors and turned daily for 2-3 weeks. In preparation for shelling, the in-shell nuts are further dried in an oven to moisture content of about 4-6% and then soaked in water for 6-8 h. The combination of drying followed by soaking allows for easier shelling with fewer broken nuts, which command a lesser price. Nuts are cracked using a simple mechanical device and the shells are removed by hand. Shelled, whole nuts are sorted, trimmed of blemishes; oven dried, cooled and packed in plastic lined jute bags.

The objectives of this investigation were to:

1. To provide harvesters and processors information concerning proper storage of Brazil nuts in terms of temperature and relative humidity.

2. To investigate the origin and involvement of *A. flavus* and *A. parasiticus* during harvesting and processing of Brazil nuts.
3. To microbiologically evaluate several important unit operations involved in Brazil nut processing, and offer recommendations to producers in order to improve product quality.

2. LITERATURE REVIEW

2.1. Brazil Nut

2.1.1. Production

The Brazil nut or castaña tree (*Bertholletia excelsa* Humb. & Bonpl.) is indigenous to the Amazon region. It belongs to the *Lecythidaceae* family and is among the oldest and tallest trees in the Amazon; with some trees dating back more than 1100 years. Brazil nuts originate from wild trees scattered over an area encompassing more than one and a quarter million square miles and include the non-flooded areas of the Guianas, Amazonian Brazil, southeastern Colombia, southern Venezuela, eastern Peru and northern Bolivia. The trees grow in areas with an annual mean rainfall, temperature and relative humidity of 1400-2800 mm, 24 - 27°C and 79-86%, respectively (FAO, 1995). In Peru, Brazil nuts are produced mainly in the province of Madre de Dios (southeast), where they represent one of the most important non-timber forest products. Agreda (1999) calculated that approximately 20,000 people (22% of the population) in Madre de Dios depend directly or indirectly on the Brazil nut trade. Therefore, thousands of families depend on the economics of the Brazil nut trade for their income. However, the importance of the Brazil nut is not only based on economics but also on its ecological participation in the preservation of the Amazon rainforest. Since such a large population depends on the Brazil nut for income, their harvest is a potentially competitive economic alternative to deforestation, which in many areas of Amazonia is

occurring at alarming rates. Moreover, the Brazil nut is one of the last products of international trade that originates exclusively from wild rainforest trees.

The Brazil nut tree is one of the largest in the Amazon basin, ranging from 23 to 46 m tall with branches and fruits (pods) located only near the tops of the trees (Sun et al., 1987). The fruit of the tree resembles a woody-shelled capsule or pod and is generally round. It has a diameter of 10 –15 cm and a very tough outer shell. The pods weigh from 0.5 to 2.5 kg and contain 10 to 25 Brazil nuts arranged concentrically similar to orange segments (Fig. 2.1)

2.1.2. Composition

Brazil nuts are three sided with ivory colored meats. They possess a dark brown shell with a rough surface. They contain 15-17% protein by fresh weight (Woodrof, 1967; Beuchat, 1978; Franck and Betancourt, 1979) and approximately 50% protein by dry weight from defatted flour (Antunes and Markakis, 1977). The protein is rich in both sulfur-containing amino acids: methionine (18%) and cysteine (8%), and in glutamine, glutamic acid, and arginine (Sun et al., 1987). The nuts contain from 65 to 70% oil with significant amounts of linoleic (42-45% of crude oil) and oleic (30% of crude oil) acid (Woodrof, 1967; Beuchat, 1978; Franck and Betancourt, 1979). In addition, Brazil nuts provide a substantial source of selenium (40 ppm) (Antunes and Markakis, 1977), an important antioxidant associated with protection against tumor development (Ip, 1998; Finley et al., 2000).

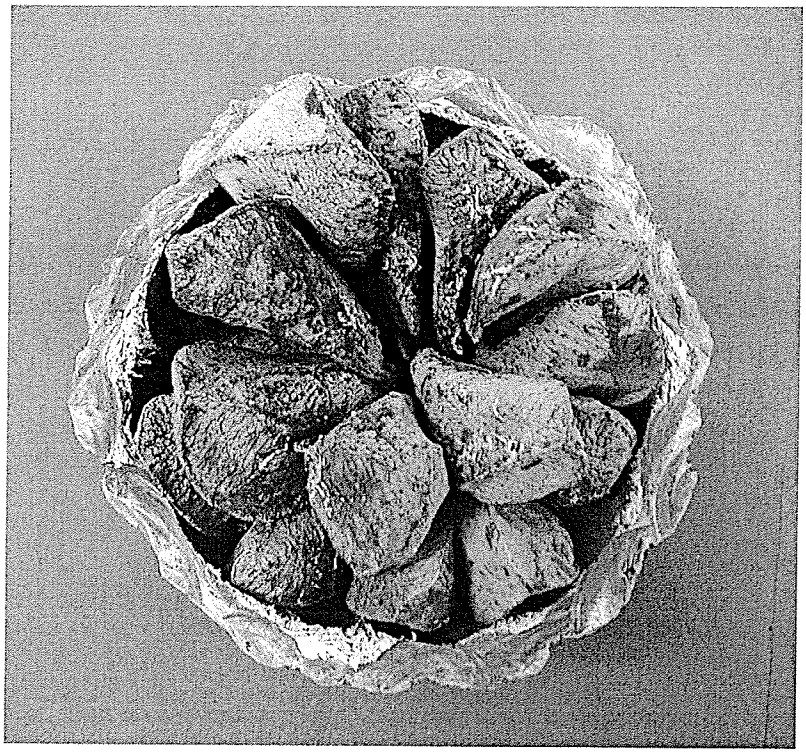


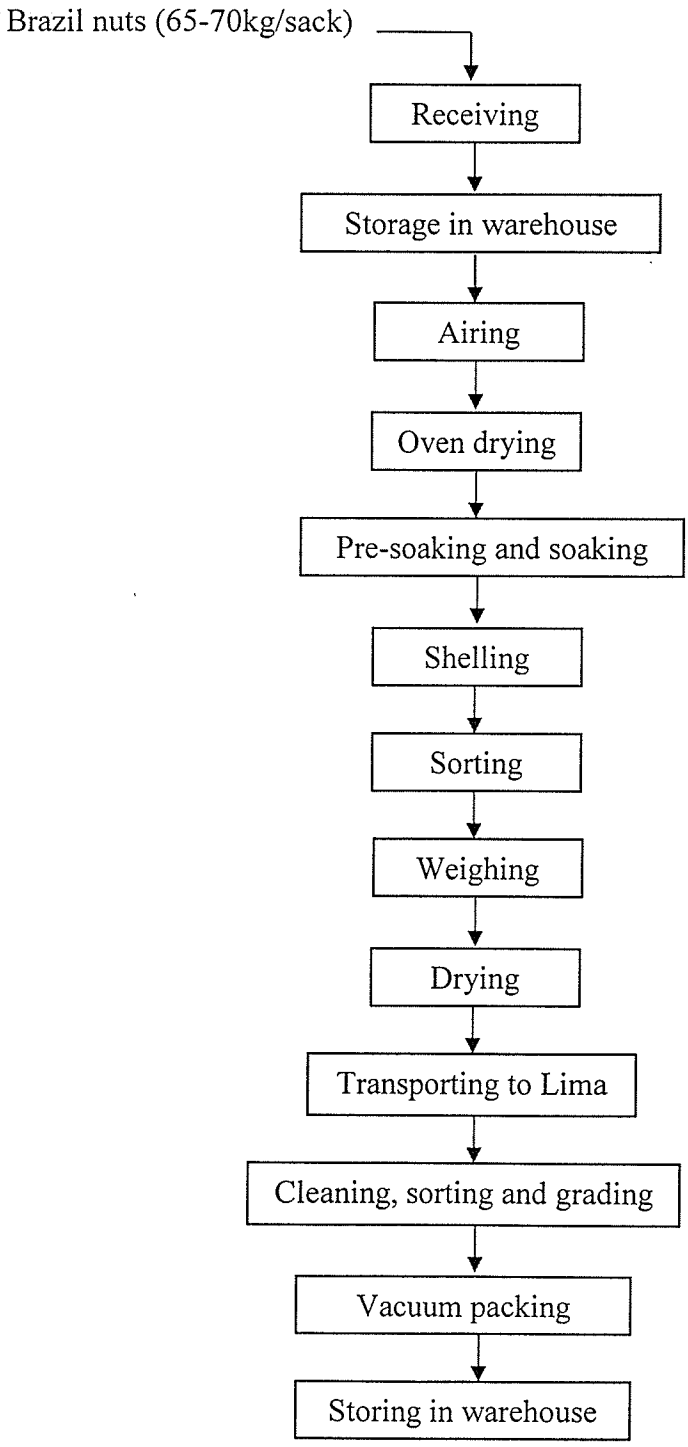
Figure 2.1. Pod containing Brazil nuts (overhead view)

2.1.3. Harvest and Post-harvest treatment

Mature trees may produce 200-400 pods yielding (high yield) 100-120 kg of in-shell nuts. However, production levels vary in that poor yields are common after a high yield year (FAO, 1995). Brazil nuts are collected mainly during the wet season. The pods begin to drop in late November and continue into early June. They are gathered following the rainy season when most of the pods have dropped to the ground. The exploitation of this natural resource is organized into concessions or 'castañales'. Concessions are areas of land that are leased from the government for harvesting pods. The 'castañales' range in size from several hundred to a few thousand hectares. The harvest is mainly a family business and consists of gathering the pods in a concession. The pods are normally opened with a machete in the rainforest and the nuts are emptied into jute sacks. This reduces the weight to be carried since in many instances the distance back to the main camp may be several kilometers and is achieved by walking. In Peru, there are two different areas where nuts are harvested: river and road concessions. In river concessions, the unshelled nuts are gathered in sacks and shipped to the city in canoes as soon as possible (up to 20 days). On the other hand, product from road concessions is sun dried for 2 days on tarps laid on the ground or on raised platforms called 'payoles'. They are then transported to a processing plant in 'barricas' or jute sacs (65-70 kg/sac). In the processing plant, the nuts are dried in a warehouse by spreading them on the floor. Here, they are turned and raked for at least 3 weeks in order to facilitate moisture reduction from 25-32% to 12-14%. The unshelled nuts are further dried with air (54°C) in a stationary oven (batch process) until they reach 4-6% moisture content (usually 30-36 h). Drying takes approximately 36 h. In Brazil and Bolivia, a brief burst of steam ('autoclave' process) is used to expand the shell and loosen the inner

skin (testa), producing a whiter kernel (FAO, 1995). In Peru, nuts are first presoaked in an enclosure using well water for 4 or 5 h in order to surface wash the nuts. The jute sacs or 'barricas' are then taken to another enclosure where they are soaked for 4 to 5 h in order to soften the shell before cracking. Following soaking the water is drained and the sacks containing the nuts are allowed to drain and dry for 3 to 4 h. The nuts are then transported to the cracking room. The individual nuts are cracked using a simple mechanical device that consists of a mechanical stage in which the nut is placed and cracked by means of hand-operated lever with action against a round metal bar. The shell is removed from the nut by hand. Shelled nuts are then placed in plastic containers and transported to a room where they are hand sorted. Defective nuts (discolored, containing eyes or brown blemishes and/or visible mold) are removed. The nuts are repacked in barricas (75 kg/each); oven dried (60°C for 12 h), and allowed to cool for at least 2-4 h. The final moisture content is around 3.5%. The nuts are then transported to Lima (Peru) by truck (3-4 days) where they are again cleaned, sorted and graded. Finally, shelled nuts are placed in thick plastic bags, vacuum packaged and placed in corrugated boxes for shipping (Vizcarra, personal communication, May 2002).

Figure 2.2. Process flow of shelled Brazil nuts

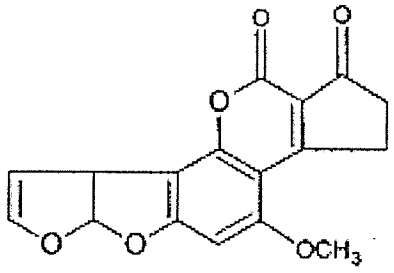


2.2. Aflatoxin

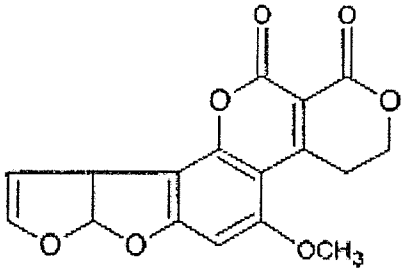
Aflatoxins are a group of secondary metabolites produced mainly by *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (Hocking, 2001). They were discovered in 1960, when the first outbreak of 'Turkey X disease' resulted in the death of hundreds of thousands of turkeys and ducklings after being fed Brazilian peanut meal contaminated with aflatoxins (Goldblatt, 1969). Since then, intensive research has been carried out to investigate factors that affect the production, biosynthesis and biological activity of the mycotoxin.

2.2.1. Structure and Biosynthesis of Aflatoxins

There are four main aflatoxins: B_1, G_1, B_2, and G_2. The structures of aflatoxin B_1 and G_1 (Fig. 2.2.) were determined by Asao et al. (1965) and the absolute configurations of the aflatoxins were described by Brechbühler et al. (1967). The basic skeleton of the aflatoxin (AF) molecule consists of a condensed bisfuran/coumarin ring system. Aflatoxins B_2 and G_2 are dihydro derivatives of aflatoxin B_1 and G_1, respectively (Asao et al., 1965). During metabolism of AFB_1, several metabolites including aflatoxin M_1 (AFM_1) are produced (Wild and Turner, 2002). AFM_1 have been reported in milk and urine after animal or human ingestion of AFB_1 (Qian et al, 1994; Wang et al., 2001).



(1)



(2)

Figure 2.3. The structures of (1) aflatoxin B1 and (2) G1

Aflatoxins B_1 and B_2 fluoresce blue when exposed to long-wave ultraviolet light (360 nm) while aflatoxin G_1 and G_2 fluoresce green under the same conditions (Goldblatt, 1969). This fact has permitted the determination of these compounds using several physicochemical methodologies such as thin layer chromatography. Aflatoxins are soluble in several solvents, including methanol, hexane, acetone and chloroform (Goldblatt, 1969) and many are used for substrate extraction. These solvents are also combined with water to optimize extraction (Goldblatt, 1969).

Aflatoxins are acetate-malonate-derived compounds classified as polyketides. Bennett and Christensen (1983) describe polyketide formation as analogous to fatty acid biosynthesis but lacking the systematic dehydration and reduction steps. Polyketides are formed by condensation of an acetyl unit with three or more malonyl units, followed by decarboxylation. Individual components are produced by subsequent aromatization and modifications such as ring cleavage, oxidation, reduction, and methylation (Bennett and Christensen, 1983). Several precursors and biosynthetic pathways have been proposed, however, pathway details have not been clearly established. So far, six compounds have been recognized as intermediates in the biosynthesis of aflatoxins: B_1-norsolorinic acid, averantin, averufin, versiconal hemiacetal acetate, versiolorin A and sterigmatocystin (Zaika and Buchanan, 1987). The general steps in aflatoxin biosynthesis are: acetate $\rightarrow$ anthraquinones $\rightarrow$ sterigmatocystin $\rightarrow$ aflatoxin B_1 (Bennett and Lee, 1979). Zaika and Buchanan (1987) have reviewed compounds affecting the biosynthesis of aflatoxins.

2.2.2. Biological Activity

Aflatoxin metabolism has been extensively studied in both animals and humans (Moss, 2002; Wild and Turner, 2002). Aflatoxin B_1 (AFB_1) is metabolized in the liver after consumption (oral route) of contaminated food. AFB_1 metabolism produces highly reactive intermediate products such as AFB_1-8,9 exo-epoxide and AFB_1-8,9-endo-epoxide. The former product binds DNA to form 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB_1 (AFB_1-N7-Gua). The binding of these intermediaries to DNA results in the disruption of DNA transcription and abnormal cell proliferation with the potential to lead to mutagenesis (Wild and Turner, 2002). Other metabolites are also formed from AFB_1, including aflatoxin Q_1 (AFQ_1), AFM_1 and aflatoxin P_1 (AFP_1). These metabolites and other natural occurring aflatoxins (G_1, G_2, and B_2) are poorer substrates for epoxidation and consequently are less mutagenic, carcinogenic and toxic than AFB_1.

The toxicity assessment of aflatoxins is determined by acute oral lethal dose (LD_{50}). These values are based on an analysis of aflatoxin oral intake. LD_{50} values vary depending on age, sex and size of the animal. Therefore, susceptibility of animals to AFB_1 varies. For example, hamsters are 35 times more resistant to AFB_1 than rabbits (Moss, 2002). The LD_{50} for hamsters is 10.2 mg/kg, while for rabbits it is only 0.3 mg/kg. In addition, female rats are 3 times more resistant than males. This variability in aflatoxin toxicity is due to a wide difference among individuals in regards to metabolism, activation and detoxification since the species specific sensitivity to AFB_1 is strongly correlated with AFB_1 biotransformation. By extrapolation from both molecular biology studies and epidemiology of reported cases of acute aflatoxin poisoning (aflatoxicosis), a LD_{50} for humans was estimated to be about 5 mg/kg. (Moss, 2002).

2.2.3. Acute and Chronic Toxicity

An important outbreak involving aflatoxin contamination occurred in Northwest India in 1974, where 397 people suffered severe aflatoxicosis and 108 individuals died after consuming corn contaminated with 900 – 1100 ppb of the mycotoxin (Tandon et al., 1978). However, in developed countries, aflatoxin contamination rarely occurs at levels that cause notable aflatoxicosis in humans. Chronic toxicity, however, is of serious concern. Although, the effects involved in chronic toxicity have been extensively studied for many years, it is the carcinogenicity of AFB_1, which drives international concern regarding the occurrence of aflatoxins in food. Toxic effects include hepatic lesions and necrosis (Tuan et al., 2002), reduction of fertility (Ortatatli et al., 2002; Agnes and Akbarsha, 2003), acute gastrointestinal effects (Luzi et al., 2002), and cardiotoxicity (Abdel-haq et al., 2000). In 1993, the International Agency for Research on Cancer (IARC) designated aflatoxins as carcinogenic compounds. Aflatoxins are linked with hepatocellular carcinoma (HCC), the most common form of liver cancer, in populations with high incidence of Hepatitis B infection (Qian et al., 1994; Sylla et al., 1999; Li et al., 2001; Chen et al., 2001; Wang et al., 2001). Although, studies have not established a direct cause-effect relationship, a synergistic relationship between aflatoxins and the Hepatitis B virus must exist in order for HCC to occur. Reactive compounds formed during AFB_1 metabolism bind to DNA producing an AFB_1-N7-Gua adduct. This confers mutagenic properties to AFB_1 by suppressing gene p53 (Jackson and Groopman, 1999). Gene p53 produces a protein that prevents cell reproduction when DNA is damaged. Therefore, if gene p53 is suppressed, damaged cells will have less chance to be eliminated. On the other hand, the Hepatitis B virus is able to integrate

viral DNA in the human DNA, developing abnormal cells in the liver causing cirrhosis. Since abnormal cells can not be prevented from growing due to the suppression of gene p53, atypical cells will be replicated, generating HCC.

Even though the tolerable daily intake (TDI) of aflatoxins for humans is difficult to define, it has been established as 0.11-0.19 ng of AFB_1 / kg body weight (D'Mello, 2003). TDIs have been based on studies conducted in Southeast Asia and parts of Africa, where infection with Hepatitis B virus is an important carcinogenic factor (Sylla et al., 1999; Chen et al, 2001; Li et al., 2001; Wang et al., 2001).

2.2.4. Regulations

The acute and chronic toxicity and carcinogenic potential of aflatoxins has resulted in the imposition of regulations for maximum allowable levels in several countries. Regulations are established to reduce the amount of contaminated food and feed products that reach consumers and animals. In 1995, at least 77 nations had mycotoxin regulations, mainly for human food and animal feed (FAO, 1997). In 1998, the European Union agreed to a limit of 4 and 2 ppb for total and B_1 aflatoxin, respectively in a range of most commonly contaminated foods used for human consumption, including cereals, milk, nuts and dried fruits. This limit was imposed in order to harmonise standards and facilitate international trade (EC, 1998). The new regulations contain separate limits for AFB_1 and AFM_1 as well as the total aflatoxin content. The regulations impose limits for products intended for direct human consumption rather than for products intended for further processing (Table 2.1).

Table 2.1. Maximum allowable aflatoxin levels established by the European Commission[a]

Product	Aflatoxin: maximum permissible level (µg/kg)		
	B_1	Total	M_1
Groundnuts, nuts and dried fruit			
-Processed products for direct human consumption	2	4	-
-Subject to treatment before human consumption	8	15	-
-Ingredients	5	10	-
Cereals and processed products	2	4	-
Milk	-	-	0.05

[a]European Commission. 1998. Regulation No. 1525/98

2.2.5. Detection Methods for Aflatoxin

Various quantitative and qualitative methods have been proposed for aflatoxin detection in food commodities. Most methods require extraction of the toxins using a solvent or a combination of solvents and purification of the extract.

2.2.5.1. Enzyme Immunoassay

Immunoassay tests are based on the antigen-antibody reaction. The aflatoxin is extracted with an appropriate solvent. This extract is placed in contact with aflatoxin-specific antibodies. The antibodies bind to the aflatoxin present in the sample. An

aflatoxin-enzyme conjugate is then added, which reacts with any free antibodies not bound in the sample. Any unbound enzyme conjugate is then removed in a washing step. Following the addition of the enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine), the aflatoxin-enzyme conjugate is incubated, during which the bound enzyme conjugate converts the colourless chromogen into a blue product. The degree of colour change correlates with the aflatoxin concentration in the sample. The measurement is made photometrically at 450 nm. The detection limit of the analysis varies depending on the supplier, for example, Ridascreen® from Biopharm provides a detection limit of 1.75 ppb for total aflatoxin and 0.6225 ppb for aflatoxin B_1. The advantage of the ELISA test is that it is very specific, easy to perform and is not time consuming.

2.2.5.2. Thin Layer Chromatography

Thin layer chromatography (TLC) has been widely used as a rapid screening method for aflatoxins. It is based on the separation of aflatoxins using TLC plates coated with silica gel, coupled with the inherent fluorescence properties of aflatoxins under long-wave ultraviolet light. Several modifications have been evaluated using different solvents such as chloroform, methanol, acetone, benzene, ethanol, water or a combination of these solvents (Goldblatt, 1969). It is essential that aflatoxin standards be treated under the same conditions as the samples for the development of this method. TLC is commonly used due to its simplicity and practicality.

Using TLC, detection limits as low as 0.5 - 1.0 ppb have been reported in peanuts and peanut butter (Pons et al., 1966) and 1 - 4 ppb in cottonseed and cottonseed meal (Pons and Goldblatt, 1965).

2.2.5.3. Minicolumn Screening Method

Minicolumn screening is a qualitative method used for detection of >5 ppb total aflatoxins (B_1+B_2+G_1+G_2) (AOAC, 1996). Following purification with the appropriate solvents, the aflatoxin extract is passed through a 6 mm diameter minicolumn packed with layers of different adsorbents ($CaSO_4$, Florisil, silica gel, and neutral alumina). The columns, examined in a darkened room under UV light, will fluoresce at the top of the Florisil layer if aflatoxins are present. The minicolumn method is rapid and simple however, it is used only for aflatoxin detection since quantification is not possible using this method.

2.2.5.4. Immunoaffinity Column Method

This method is applicable for the determination of aflatoxin at >10ng total aflatoxin/g in corn, raw peanuts and peanut butter (AOAC, 1996). Subsequent to aflatoxin extraction and purification, the extract is applied to an affinity column containing monoclonal antibodies specific for aflatoxin B_1, B_2, G_1, G_2. Aflatoxins are then concentrated in the column and quantified by fluorescence measurement following a reaction with a bromine solution. Recoveries using this method are in the range 60-90%. This method has been used for several products. However, complex matrices may give false-positive results due to cross-reactions and interference.

2.2.5.5. High-performance Liquid Chromatography

Modern analysis of aflatoxin levels is commonly based on the use of high-performance liquid chromatography (HPLC). However, the negative aspect for the use

of HPLC is the high cost of instrumentation. Various chromatographic modes such as normal-phase, reverse-phase and ion exchange chromatography have been used in aflatoxin analysis. It is well suited for analysis of complex matrices such as extracts from cereals and feeds after proper purification. In addition, aflatoxins must be derivatized with trifluoroacetic acid to form a strongly fluorescent hemiacetal (Steyn et al., 2000). The mycotoxin is then separated by reverse-phase liquid chromatography and detected by fluorescence. This method can measure 0.1 ng of aflatoxin B_1, B_2, G_1, and G_2. The detection limit is 0.3 ng/g for each aflatoxin (AOAC, 1996).

2.2.6. Aflatoxin Occurrence in Foods

Aflatoxins are associated with a number of important food commodities such as nuts (peanuts, treenuts), cereals (sorghum, wheat, barley, corn), fruits (orange, grapefruit, apples, figs, dates) and spices (paprika). The highest incidence (1706 mg/kg) of total aflatoxin in foods was found in Brazilian ready to eat peanuts (Caldas et al., 2002). Contamination of dairy products with aflatoxins have also been reported since AFM_1 is excreted in milk when cattle are fed contaminated meal. In addition, conventional pasteurization and even ultra high temperature (UHT) pasteurization are not sufficient to completely deactivate AFM_1; for example aflatoxin M_1 has been detected (0.04 μg/L) in commercial milk (pasteurized or UHT) (Diaz et al., 1995). Therefore, products that are not completely detoxified via processing can be marketed and consumed containing residual toxins (carryover). The widespread consumption of food contaminated with aflatoxins is reflected by the presence of metabolites found during analysis of human blood and urine. In China, the occurrence of aflatoxin in humans has been estimated through evaluation of urinary markers (Qian et al., 1994)

while levels of serum AFB_1-albumin adducts have been used to reflect aflatoxin exposure in China (Wang et al., 2001) and Taiwan (Chen et al., 2001).

2.2.6.1. Aflatoxin Occurrence in Tree Nuts

Among tree nuts, pistachios, almonds, walnuts and pecans have been reported to contain aflatoxin (Wood, 1989; Stoloff et al., 1969). The aflatoxin level detected varies among different tree nuts. Schatzi (1996) found an overall aflatoxin level of 0.67 ng/g in almonds after evaluating 78% of 1993 U.S. production. Wood (1989) reported that 32% of pistachio nuts tested contained an average of 58 μg of aflatoxin /kg of nut. Aflatoxins have also been detected in hazelnuts and walnuts collected from markets in Egypt (Abdel-Hafez and Saber, 1993). Approximately 90% of the hazelnut samples (25-175 μg/kg) and 75% of the walnut samples (15-25 μg/kg) were positive for the mycotoxin.

Schatzki and Pan (1996) reported that 90% of the aflatoxin in pistachios was concentrated in just 4.6% of (low-quality) product, demonstrating that aflatoxin distribution is heterogeneous. In addition, sorting low-quality nuts removes a large part of aflatoxin at harvest. Schatzki (1995) reported that aflatoxin content in the crops that underwent sorting were 2-4-fold lower than in the crops that did not go through the sorting process. Mahoney and Rodriguez (1996) also indicated that injuries in nuts components, including hulls (mesocarps and epicarps), seed coats (testas) and kernels (seed), all contribute to variable aflatoxin content in pistachios. The authors inoculated shelled pistachio kernels with intact and damaged hull (hulls were sliced along the shell suture to simulate early-split pistachios) and they found 37 times more aflatoxin in damaged than in undamaged kernels after 10 d of incubation.

2.2.6.2. Aflatoxin Occurrence in Brazil Nuts

Several studies have demonstrated the occurrence of aflatoxins in Brazil nuts (Raymond, 1966; Castrillon and Purchio, 1988b; Freire et al., 2000; Steiner et al., 1992; Caldas et al., 2002). The highest aflatoxin B_1 concentration reported in Brazil nuts (imported from Brazil – 1988 crop) was 4.0 mg/kg (Steiner et al., 1992).

The distribution of aflatoxins in Brazil nuts is also heterogeneous (Frank et al., 1980; Steiner et al., 1992). Stoloff et al. (1969) found that only 5% of 100 individual nuts contained aflatoxins at levels ranging from 50 to 25,000 ug/kg. Similar findings were observed by Castrillon and Purchio (1988b) after analyzing samples from Manaus (Brazil). Castrillon and Purchio (1988b) reported that only 3% of 100 nuts were naturally contaminated with B_1 and G_1 aflatoxins in the range of 0.1 to 2.25 ppm and 0.075 to 1.5 ppm, respectively. However, Caldas et al. (2002) evaluated Brazil nuts from Brasilia (Brazil) and reported that 33.3% (3/9) of the samples were contaminated with aflatoxins, mainly AFB_1. In addition, Steiner et al. (1992) indicated that most of the aflatoxins were located in brown spotted nuts or kernels containing yellow fluorescence when screened under UV light at 360 nm. However, fluorescence is not emitted by the aflatoxin itself but by kojic acid, a compound produced by *A. flavus*. Moreover, Freire et al. (2000) reported that aflatoxins were detected only in nuts that appeared to be contaminated with fungi, shriveled and/or cracked (poor quality nuts). Concentration of AFB_1 and AFB_2 were 27.1 μg/ kg and 2.11 μg/ kg, respectively.

2.3. Aflatoxin Production

2.3.1. Aflatoxigenic Mold

The production of aflatoxin is limited mainly to strains of *A. flavus, A. parasiticus* and *A. nomius*. However, Atalla et al. (2003) showed that *Aspergillus oryzae*, *Aspergillus terreus*, *Aspergillus versicolor* were also able to produce aflatoxin when inoculated in wheat. Moreover, different fungal isolates possess different capabilities for toxin production. *A. parasiticus* isolates generally produce a greater amount of aflatoxins than *A. flavus*. Koehler et al. (1975) indicated that the average amount of aflatoxin B_1 produced by toxigenic *A. parasiticus* isolates was over twice that produced by aflatoxin producing strains of *A. flavus* in pecans. In addition, not all *A. flavus* and *A. parasiticus* isolates are able to produce aflatoxins. Diener and Davis (1966) isolated *A. flavus* from different food sources such as peanuts, corn, and feed and found that about 80% of 37 cultures produced aflatoxins. In contrast, Blaser and Schmidt-Lorenz (1981) evaluated food samples, such as nuts, almonds, peanuts, Brazil nuts and corn and indicated that only 27.5% of 218 pure cultures of *A. flavus* were aflatoxigenic. The difference between these results may be due to the use of different culture media for aflatoxin production. Hocking (1982) indicated that approximately 95% of Australian *A. flavus* and *A. parasiticus* isolates from peanuts were aflatoxigenic. Moreover, Doster and Michailides (1994) showed that all strains of *A. flavus* and *A. parasiticus* isolated from pistachios, with an abundance of small sclerotia, were aflatoxin producers, but only 43% of the isolates of strains with a few large sclerotia produced aflatoxin. Also, Koehler et al. (1975) determined that 93% of the *A. parasiticus* isolates, but only 54% of the *A. flavus* isolates were capable of producing toxins in pecans. The differences in toxin producing

ability by *Aspergillus* species seem to be due to their genetic background (Atalla et al., 2003) and growth conditions (Hocking, 1982)

There are also differences between *A. flavus* and *A. parasiticus* in regards to the types of aflatoxin produced. Goldblatt (1969) reported that 82% of *A. parasiticus* isolates produce both aflatoxins B_1 and G_1. However, among the *A. flavus* strains, the majority produce only aflatoxin B_1, while a lesser number yield both B_1 and G_1. Diener and Davis (1966) suggested that 90% of *A. flavus* isolates produce AFB_1 and only 10% produce AFB_1 and AFG_1.

2.3.2. Aspergilli Isolated from Brazil Nuts

Several studies have demonstrated the presence of *Aspergillus* in Brazil nuts bought from retail, including *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus ochraceus* and *Aspergillus wentii* Wehmer. *A. flavus* was the dominant species, followed by *A. niger* and *A. parasiticus* (Holubova-Jechova, 1970; Castrillon and Purchio, 1988a; Freire et al., 2000). These studies evaluated nuts for the presence of *Aspergillus* spp.; however, no precise levels of contamination were determined.

The presence of *A. flavus* and *A. parasiticus* in Brazil nuts has been also evaluated. In most of these studies, nuts were surface sanitized, using 2% sodium hypochlorite (0.5% chlorine) or 95% ethanol, prior to mycological examination. Castrillon and Purchio (1988a) indicated that aspergilli were present in 29% of Brazil nuts. In addition, Bayman et al. (2002) reported a 61% and 41% incidence of *A. flavus* in non-sanitized and sanitized Brazil nuts, respectively. In addition, Freire et al. (2000)

reported a 27.5% incidence of *A. flavus* on surface sanitized shelled Brazil nuts. The incidence of *A. niger, A. tamari, A. fumigatus* and *A. nidulans* in Brazil nuts was found to be in the range of 12-14%, 2.4- 20%, 4-4.5% and 8%, respectively (Freire et al., 2000; Bayman et al., 2002).

Bayman et al. (2002) indicated that the presence of *Aspergillus* on the nuts could originate from airborne contamination in the field and during harvest and/or processing. Conidial penetration through the floral parts of the young kernel was also mentioned as a contamination source (Freire et al., 2000).

Spencer (1921) reported that Brazil nuts surface colonized by *Aspergillus* might give no indication of their internal condition until advanced stages of decay (kernel appears shrunken and mycelia penetrates center of the nut).

2.3.3. Factors Influencing Aflatoxin Production

Since the discovery of aflatoxin, the importance of substrate composition and environmental growth conditions has been examined as a means of effectively controlling aflatoxigenic mold growth and aflatoxin production. Sakai et al. (1984) evaluated the growth and aflatoxin production of *A. parasiticus* in various plant materials. They indicated that aflatoxin production was especially active in high lipid content substrates, such as nuts. However, environmental conditions such as temperature and relative humidity were considered to be the most critical factors for aflatoxin production. Several studies performed on nuts (peanuts, hazelnuts and pistachios) suggested that the optimum temperature and relative humidity for aflatoxin production is 25-30°C and 97-99% relative humidity (Diener and Davis, 1966; Diener and Davis, 1967; Schindler et al., 1967; Northolt et al., 1976; Simsek et al., 2002). Additional

factors such as water activity, moisture content, pH, storage time and insect damage were shown also to influence the production of aflatoxins. For example, aflatoxin production by *A. parasiticus* was optimal at 0.99 a_w in peptone-glucose-glycerine medium; however, no detectable quantities of aflatoxin were formed at 0.83 a_w incubated at 24°C for 5 d using the same medium (Northolt et al., 1976). Moisture content also plays an important role in aflatoxin production. Aflatoxins were formed by *A. flavus* in corn at moisture levels above 17.5% and at temperatures of 24°C (Trenk and Hartman, 1970). Moreover, maximal aflatoxin production was obtained at pH 6-6.5 (Davis et al., 1966). Furthermore, the presence of a shell is considered to be an important obstacle against aflatoxin production since it provides protection against fungal growth. Finally, it is important to realize that the interaction of these factors may provide different effects in the same substrate, enhancing or inhibiting aflatoxin production.

2.4. Microbiology of nuts

2.4.1. Pathogens in nuts

The presence of bacterial pathogens in nuts is of particular concern since most of them are eaten raw. Several studies have demonstrated that nuts are a source of microbial contamination. Weinzirl (1927) indicated that shelled nuts were an important source of *Escherichia coli* contamination in candies containing nuts. In addition, Ostrolenk and Hunter (1939) isolated *E. coli* from 8 of 10 varieties of nuts bought from retail markets. *E. coli* was present in 45% of black walnuts, 20% of cashew nuts, 16% of pignolias, 13% of almonds, 12% of pecans and 4-10% of Brazil nuts and English walnuts. Moreover, Meyer and Vaughn (1969) reported *E. coli* contamination in black

walnuts ranging from 0.4 to 4.9 MPN/g. Chipley and Heaton (1971) obtained shelled and unshelled pecan meat samples from commercial operations and found that 20% tested positive for *E. coli*. They also found that 10% tested positive for *Pseudomonas effusa*, *Clostridium* species, *Corynebacterium paurometabolum*, and *Proteous vulgaris*; also 20 and 30% tested positive for *Leuconostoc mesenteroides*, and *Enterobacter aerogenes*, respectively. Furthermore, Marcus and Amling (1973) collected pecans from nongrazed and grazed orchards and reported that 4% and 23%, respectively, were contaminated with *E. coli*. Finally, Freire and Offord (2002) detected the presence *Bacillus cereus*, *Salmonella* Typhimurium and *Staphylococcus aureus* in Brazil nut kernels purchased from retail suppliers in Brazil.

The presence of *E. coli* in food usually reflects improper sanitary practices since several studies (Ostrolenk and Hunter, 1939; Hyndman, 1963; Chipley and Heaton, 1971) have demonstrated that nutmeats with unbroken shells and aseptically shelled nuts contain no *E. coli*. However, contamination may take place prior to nuts reaching the processing plant. For example, as soon as nuts fall from the tree, they become contaminated with soil microorganisms (Hyndman, 1963; Kokal and Thorpe, 1969). Kokal and Thorpe (1969) indicated that there was a direct relationship between soil contact and *E. coli* incidence in nuts. No *E. coli* was detected in almonds collected from the tree, but 2% of the almonds became contaminated after they were knocked to the ground. Moreover, the incidence of contamination increased after windrow sweeping and subsequent prehulling stages, reaching 30 to 40%. Contamination via contact of the nut with the soil carried over into the processing plant. Further process operations may also enhance cross contamination. Beuchat (1973) indicated that nuts become

contaminated during shelling and grading where the shell containing microorganisms may contaminate the nutmeat. The tempering process (soaking nuts in water) was also listed as another hazard for contamination. Meyer and Vaughn (1969) reported that *E. coli* present on the walnut husk might contaminate the water used for soaking and remain viable after the tempering process. The water could also contaminate the walnut meats via separate sutures located in the shell. Beuchat and Heaton (1975) also support the assumption of the separate sutures as a route for microbial contamination. The authors suggest that soaking nuts with contaminated water allows the inshell pecan to absorb liquid through fibrovascular capillaries located in the suture separations at its apex, thus providing ports of entry for microorganisms. However, other studies indicated that cross contamination in nuts during soaking is not possible. Marcus and Amling (1973) reported that nutmeats from whole in-shell pecans soaked for 24 h in a lactose broth solution containing *E. coli* did not become contaminated. Nevertheless, the authors demonstrated that nuts soaked in water developed very small openings in the shell of the nut after 48 h of soaking, and none of the nuts completely closed when they were dried. This suggests a possible route for *E. coli* penetration to pecan kernels after soaking and drying.

3. AFLATOXIN PRODUCTION BY *Aspergillus flavus* IN BRAZIL NUTS

3.1. Abstract

Experiments were conducted to evaluate the effects of relative humidity (75, 80, 85, 97%) and temperature (10, 13, 15, 25, 30°C) on aflatoxin production in previously dried (3.5% m.c.) Brazil nuts. Initially *Aspergillus* spp. were isolated from the surfaces of whole in-shell Brazil nuts imported from Peru using Aspergillus flavus and parasiticus agar (AFPA). Isolates were subsequently screened for aflatoxin production using yeast extract sucrose medium and toxin was analyzed and confirmed using an ELISA technique and TLC, respectively. The surface of shelled half-nuts (simulating damaged or trimmed nuts), shelled whole nuts and in-shell whole nuts served as sites for inoculation (10 µl; 10^5/ml) using an aflatoxigenic isolate following a chlorine wash and water rinse. Maximum levels of aflatoxin (total and B_1) were detected in nuts stored at 97% r.h. and at temperatures of 25-30°C. Shelled half-nuts contained the highest total (6817 ppb) and B_1 aflatoxin (4483 ppb) levels. In-shell whole nuts contained the least total and B_1 toxin with maximum levels of 93 and 49 ppb, respectively. Aflatoxin was not detected (detection limit of the ELISA kit was 1.75 ppb) in nuts maintained at either 10°C (97% r.h.) or at 30°C (75% r.h) in any of the inoculation sites up to 60 d of storage. Maxima m.c. (%) and a_W values for nuts stored at these conditions were 4.50 and 0.78, and 9.14 and 0.92, respectively. Results of this study indicated that the limiting m.c. and a_W values affecting aflatoxin production (< 4 ppb) in whole shelled and in-shell nuts stored at 30°C are 4.5, 0.68 and 5.0, 0.75, respectively. Overall, increasing the relative humidity and temperature during storage resulted in an increase in aflatoxin and

were shown to be the most significant variables influencing toxin production in Brazil nuts.

3.2. Introduction

Brazil nut or castaña trees (*Bertholletia excelsa* Humb. and Bonpl.) are indigenous to the Amazon forests of South America and represent some of the oldest trees on earth, many of which date back more than 1100 years (FAO, 1995). Harvesting of Brazil nuts, a major non-timber forest product, not only helps to preserve the Amazon rainforest but also creates an economy on which thousands of low-income people depend.

The occurrence of aflatoxins, produced by *Aspergillus flavus* and *Aspergillus parasiticus*, in Brazil nuts has been confirmed in several studies (Castrillon and Purchio, 1988b; Steiner et al., 1992; Freire et al., 2000; Caldas et al., 2002). The highest concentration of aflatoxin B_1 (AFB_1) in Brazil nuts (Brazil crop of 1988), for example, was reported to be 4.0 mg/kg (Steiner et al., 1992). In many instances the presence of the mycotoxin was detected on the surface of shelled nuts exhibiting visible mold growth and or inside shriveled or cracked or brown spotted nuts (Freire et al., 2000). Shelled nuts exhibiting yellow fluorescence when screened under UV light were also shown to be positive for aflatoxin (Steiner et al., 1992).

Several environmental factors are known to influence aflatoxin production, however, temperature and relative humidity are considered to be the most critical. Studies performed on hazelnuts and pistachios suggested that optimum temperature and relative humidity for aflatoxin production is 25-30°C and 97-99%, respectively (Diener and Davis, 1966, 1967; Schindler et al., 1967; Northolt et al., 1976; Simsek et al., 2002).

Additional factors such as water activity, moisture, substrate composition (Sakai et al., 1984) storage time; insect damage (Lynch and Wilson, 1991; Schatzki and Ong, 2001) and presence of a shell (Ayerst and Budd, 1960) also influence fungal growth and aflatoxin production. It is important to also recognize, however, that the interaction of all these factors may provide for varying results in regards to fungal growth and mycotoxin production even on identical substrates.

The presence of aflatoxin is a serious concern for exporters of Brazil nuts especially since 1998 when the European Community decreased the maximum tolerable limit of total and B_1 aflatoxins to 4 and 2 ppb, respectively (EC, 1998). Moreover, since temperature and relative humidity are considered important factors for aflatoxin production, it was of interest to evaluate the effect of these parameters on aflatoxin production during storage of Brazil nuts. The objective of this study was to provide Peruvian Brazil nut harvesters and processors necessary information concerning proper storage of Brazil nuts in terms of temperature and relative humidity in order to limit aflatoxin production and thereby help maintain a viable, export trade. By maintaining this trade, forests that are managed for Brazil nut harvest can be legally protected against deforestation.

3.3. Materials and methods

3.3.1. Brazil nuts and isolation of *Aspergillus flavus*

Whole shelled (WS) and whole in-shell (WIS) Brazil nuts (February-March 2002 harvest) were imported from a Peruvian Brazil nut processor and 50 nuts of each type were randomly transferred onto the surface of deep petri dishes (100 x 20 mm) pre-

poured with Aspergillus flavus and parasiticus agar (AFPA; Oxoid Inc., Nepean, ON). Following incubation (30°C, 3 d) the dishes were examined for fungal growth. Yellow/orange (reverse of the colony) was used to indicate presumptive *A. flavus/parasiticus* (Hocking, 1982; Pitt et al., 1983). Presumptive cultures were subsequently purified and cultivated on both Czapeck and malt agar (Difco, Detroit, USA) at 28°C until extensive formation of conidia was observed. The isolates were then compared to known *A. flavus* and *A. parasiticus* strains (grown on the same media) supplied by the Canadian Grain Commission (Winnipeg, MB). *A. flavus* strains were identified tentatively and several were confirmed by the National Fungal Identification Laboratory, Agriculture and Agri-Food Canada (Ottawa, ON). No *A. parasiticus* strains were recovered. Cultures were maintained on AFPA slants at 5°C following growth at 25°C for 5 d.

3.3.2. Aflatoxin screening of fungal cultures

A. flavus isolates were screened for aflatoxin production using a procedure described by Davis et al. (1966). Flasks (500 ml) containing 100 ml sterile YES medium (2% yeast extract and 20% sucrose) were inoculated with a single conidium obtained from each isolate and incubated statically for 7-10 d in the dark at 21°C. Following incubation the flask contents were filtered (Whatman No.1), dried at 70°C for 24 h in an air drying oven and weighed (Davis et al., 1966). Individual filtrates (50 µl) were quantified for total aflatoxin using an enzyme immunoassay (Ridascreen®, R-Biopharm AG, Darmstadt, Germany). The remainder of filtrates was freeze-dried (Virtis Unitop 600L) dissolved (1 g) in chloroform (100 ml) vortexed and aflatoxin was confirmed by spotting extracts (10 µl) onto pre-coated TLC plates (20 x 20 cm) of silica gel (SIL G25-

HR, Machery-Nagel, Duren, Germany) and plates were developed in a solvent consisting of acetone:chloroform (9:1) at room temperature (AOAC, 1996). To detect fluorescence, plates were illuminated in the dark using a UV light (365 nm). A standard aflatoxin solution (R-Biopharm, AG) prepared in chloroform was loaded concurrently on the plate.

3.3.3. Aflatoxin production on Brazil nuts

A. flavus strain AF-3 was grown on malt extract agar slants at 25°C until extensive formation of conidia was observed (10 d). Conidia were harvested by washing the slants with peptone (0.1%) containing Tween 80 (0.1%; Andrews, 1996) and inocula were standardized to 10^7 conidia per ml using a Neubauer counting chamber (VWR Scientific, West Chester, Pa.) Spore concentrations were confirmed using serial dilution and direct plating (AFPA; 30°C, 3 d).

Three sample types or sites were selected for mold inoculation: half-shelled nuts or HS (simulating damaged or trimmed nuts), whole shelled nuts (WS) and whole in-shell nuts (WIS). In all cases the nuts used for inoculation were carefully examined for visible signs of damage and mold growth. WS and HS nuts exhibiting any discoloration or blemishes were discarded. HS nuts were prepared by slicing through their longitudinal axis using a utility knife. All nuts were surface disinfected by immersion in chlorine (0.4%, 2 min) rinsed in sterile water and dried overnight on paper towels in a laminar flow hood (Pitt et al., 1992).

For WS nuts 5 µl (10^5 conidia) inocula were pipetted at each of their ends following the creation of small-bore holes to help contain the inoculum. For HS nuts, each half was inoculated with 5 µl (10^5 conidia). WIS nuts were inoculated (5 µl; 10^5

conidia) at each end of the locule (Fig 3.1.). All samples were transferred aseptically to deep petri dishes (100 x 20 mm; 4 nuts per dish) and placed on perforated porcelain plates in grease-sealed glass desiccators, adjusted to specific relative humidity values via the use of saturated salt solutions: 97±1%, potassium sulfate; 84±1%, potassium chloride; 80±1%, ammonium sulfate and 75±1%, sodium chloride (Merck, 1996; Rockland, 1960). Temperature was maintained by placing the desiccators in thermostatically controlled incubators. Two experimental protocols were used in this study. In the first protocol WIS nuts were maintained at 30°C under 75, 80, 85 and 97% relative humidity. For each temperature-relative humidity combination, eight replicates consisting of four nuts per replicate were used. At 30 and 60 d, four replicates (16 nuts) were assessed for aflatoxin (total and or B_1). A similar procedure was used for WS and HS.

In the second protocol WIS nuts were maintained under 97% r.h. and at temperatures of 10, 13, 15, 25 and 30°C. For each relative humidity-temperature combination, eight replicates consisting of four nuts per replicate were used. At 30 and 60 d four replicates were assessed for total and B_1 aflatoxin. A similar procedure was used for WS and HS nuts. Relative humidity and temperature were monitored on a daily basis using a hygrometer (±5% accuracy) and thermometer (±1°C accuracy). The m.c. of the nuts was determined following oven drying (Beuchat, 1973) while available moisture was assessed using a water activity meter (A_W Sprint Th-500, Novasina) after 30 and 60 d of storage. Analyses were performed in duplicate.

3.3.4. Determination of aflatoxin

Total and aflatoxin B_1 were determined using enzyme immunoassay kits (Ridascreen®; Leszczynska et al., 2000). The detection limits for the total and B_1 test kits were 1.75 and 0.625 ppb, respectively. Preparation of Brazil nut samples including extraction and aflatoxin determination was performed according to the manufacturer's instructions. Aflatoxin B_1 standard (R-Biopharm) dissolved in methanol (concentration range: 0.5 to 40.5 ng/ml) was used to prepare calibration curves.

Brazil nuts were macerated into a paste using a mortar and pestle. A portion (2 g) was subsequently mixed with methanol (70%, 10 ml) for 10 min on a shaker at room temperature. Following filtration (Whatman No.1) the filtrate (100 µl) was diluted with PBS buffer (900 µl); 50 µl was used per test. The absorbency of the samples (Multiskan MCC/340 MK II; 450 nm) was integrated into the software (provided by R-Biopharm AG) to obtain aflatoxin levels. Aflatoxin was confirmed by the Romer minicolum method following AOAC Official method 975.36 (AOAC, 1996). Efficiency of aflatoxin recovery was assessed using the Ridascreen® Total aflatoxin kit. Aflatoxin B_1 (1 mg) standard (R-Biopharm AG) was dissolved in methanol (70%) and diluted to 5, 50 and 100 ppb. Known quantities of the toxin were then added to macerated Brazil nut meal, extracted and quantified. Aflatoxin-free WS from Peru certified by an accredited laboratory (Inassa International Analytical Services S.A., Peru) were also evaluated for aflatoxin using the test kit.

3.3.5. Data Analysis

The data obtained in the study were analyzed using the analysis of variance (ANOVA) procedure of SAS (Cary, N.C.). Differences among means were compared using Tukey's test. Aflatoxin levels were transformed to log numbers before analysis.

3.4. Results

The efficiency for aflatoxin (B_1) recovery in Brazil nut meal following the addition of 5, 50, 100 ppb was 81.9, 85.1, 88.7%, respectively.

A. parasiticus was not recovered from any of the nuts examined. The majority of *Aspergillus* isolated on AFPA were identified as *A. flavus* and several of these isolates were subsequently screened for aflatoxin production (Table 3.1) Aflatoxin levels at 10 d ranged from 10 to 778 ppb and biomass in terms of mycelial growth did not appear to coincide with toxin production. Based on toxin production, isolate AF-3 was chosen for future challenge studies.

During the first two weeks of storage mold growth was not observed in any of the inoculated nuts. By the third week, however, mold was observed in nuts stored at 85 and 97% r.h. particularly at temperatures of 25 and 30°C. By four weeks growth was also observed on nuts maintained at 80 and 97 % r.h. and at temperatures of 30 and 15°C, respectively. However, mold was not observed at 97% (10°C) and only minimal growth occurred at 75% (30°C) even after 60 d. The most luxuriant growth was observed at 30°C on HS nuts. Overall, HS nuts, regardless of the relative humidity, appeared to be the most colonized.

Aflatoxin was not detected in any product stored at either 75% r.h. (30°C) or 10°C (97% r.h.) even after 60 d of storage. As shown in Fig 3.2. and 3.3. aflatoxin

production at 30°C was significantly ($p \leq 0.05$) affected by relative humidity (especially when it was increased from 80 to 85% r.h.) and inoculation site. Although maximum toxin levels were observed in HS nuts stored at 97% r.h. (3103 and 5047 ppb at 30 and 60 d, respectively) they were not significantly different from those levels in HS nuts maintained at 85% r.h. either at 30 or 60 d. In contrast, WIS nuts contained the least amount of toxin. For example, at 60 d and 97% r.h., the aflatoxin level in these nuts was approximately 1.6% of that in HS nuts.

Temperature was also shown to significantly affect mycotoxin production. As shown in Fig 3.4. and 3.5. when nuts were maintained at 97 % r.h., levels of aflatoxin generally increased with increasing temperature. Highest levels at 60 d, 6817 and 5046 ppb, were obtained at 25 and 30°C, respectively in HS nuts. In all cases the least amount of toxin occurred in WIS nuts; at 25 and 30°C levels of aflatoxin accumulation at 30 d in HS nuts were approximately 7 and 23 times greater than in WS nuts, respectively. It is interesting to note, however, that by 60 d total aflatoxin levels in HS nuts at 25 and 30°C were only 1.3 and 1.7 times greater, respectively than WIS nuts.

AFB_1 production in Brazil nuts at 30°C is shown in Fig 3.6. and 3.7. Similar to total aflatoxin production, synthesis of AFB_1 significantly increased with an increase in relative humidity especially from 80 to 85%. In this regard, the AFB_1 levels in HS nuts incubated at 85% r.h. increased by approximately 400 and 1870 ppb at 30 and 60 d, respectively. It is interesting to note that from 30 to 60 d, AFB_1 levels in nuts stored under 85% r.h. increased by approximately 1500 ppb, but only by 270 ppb at 97% r.h. Production of AFB_1 in nuts maintained at various temperatures under 97% r.h. is shown in Fig 3.8. and 3.9. Toxin levels were below the detection limit (1.75 ppb) at 30 and 60 d

for WS and WIS nuts maintained at 13-15°C. At 30 d the highest level of AFB_1 (1934 ppb) occurred in HS nuts kept at 30°C. By 60 d the level increased marginally to 2202 ppb. At 25°C, toxin levels increased by approximately 4483 ppb. AFB_1 levels on average comprised about 76-79 % of the total aflatoxin produced.

The moisture content and water activity of Brazil nuts maintained under various relative humidity environments is shown in Table 3.2. and 3.3. Initial moisture (3.5 %) and a_W levels (0.705) in all nuts (with the possible exception of HS nuts and WS nuts at 75 % RH) increased during storage; nuts stored at 97% r.h. exhibited the highest m.c. gain. Interestingly, by 60 d WIS nuts gained the highest amount of moisture regardless of storage relative humidity.

3.5. Discussion

A. parasiticus was not recovered from any of the nuts evaluated in this study. The majority of investigations dealing with Brazil nuts (Holubova-Jechova, 1970; Freire et al, 2000; Bayman et al., 2002) have reported similar findings with only one study (Castrillon and Purchio, 1988a) reporting the presence of *A. parasiticus.* In all these studies *Aspergillus* was the most dominant fungus recovered and as such the major aflatoxigenic species. Although all of the isolates recovered were aflatoxigenic, numerous authors have reported that not all *A. flavus* are capable of producing aflatoxin (Diener and Davis, 1966; Koehler et al., 1975; Blaser and Schmidt-Lorenz, 1981; Varma and Verma, 1987). During the first two weeks of storage, visible mold growth was not observed on any of the inoculated nuts and it is presumed that the low initial m.c. (3.5%) and or water activity (0.705) either prevented or inhibited growth. In this respect researchers have reported that the minimum a_W for growth of the mold is between 0.80

and 0.83 (Northolt et al., 1976; Pitt and Miscamble, 1995). Interestingly, in this study growth as evidenced by the presence of toxin (Diener and Davis, 1967; Yokoya et al., 1970), was detected on nuts having an a_W below this range.

When maintained at 75% r.h. and 30°C growth of *A. flavus* was not observed on any of the nuts even by 60 d. It was interesting to observe, however, that inoculated and especially control nuts grew *Aspergillus chevalieri* Mangin, a mainly tropical and subtropical species that proliferates under conditions of low moisture (Samson et al, 1995). It is assumed that the difference in the level of *A. chevalieri* growth between control and inoculated nuts may have been due to competition. Regardless of inoculation site, incubation at 30°C compared to 25°C resulted in more visible mold growth; however, total aflatoxin levels particularly by 60 d were normally higher in nuts stored at 25°C. Although growth of *A. flavus* is most favored between 29-35°C, (Holmquist et al., 1983; Schindler et al., 1967; Gqaleni et al., 1996) it is recognized that biomass is not closely associated with toxin production (Priyadarshini and Tulpule, 1978). Maximum mold growth was always observed on the HS nuts, regardless of temperature and r.h. Similar observations have been reported by other researchers when comparing damage in peanuts (Diener and Davis, 1967; Freire et al., 2000). HS nuts, which were used to simulate trimmed product or nuts damaged during shelling or handling and transportation, would be expected to have enhanced fungal colonization perhaps by providing an increase in surface area and nutrient availability.

Overall, aflatoxin production in Brazil nuts was significantly affected by r.h., temperature, and inoculation site and storage time. Of the variables investigated r.h. and temperature were the most significant relative to toxin production (high F ratio of 247 and 426 for r.h. and temperature, respectively; see Appendix 9 and 10). The importance

of these two factors was also evaluated in previous investigations (Snow et al., 1944; Diener and Davis, 1967; Schindler et al., 1967; Yokoya et al., 1970; Sanchis et al., 1988; Atalla et al., 2003). Maximum levels of aflatoxin were produced on HS nuts stored under 97% r.h. and at 25-30°C. Although aflatoxin production in challenge studies with Brazil nuts has not been reported, several researchers concur that maximum toxin production in nuts including hazelnuts and peanuts occurs with increasing r.h. to 99-100% (Diener and Davis, 1967; Chiou et al., 1984; Chiou and Tsao, 1997). According to Simsek et al. (2002), the maximum aflatoxin level in inoculated hazelnuts stored at 97% r.h. for 18 d was 905 ppb; with inoculated peanuts stored under similar environmental conditions for 21 d the level was 82300 (Diener and Davis, 1967) and 58700 ppb (Chiou et al., 1984). In addition, Chiou and Tsao (1997) detected 2880 ppb on peanuts inoculated with *A. parasiticus* and stored at 100% r.h. for 49 d.

Toxin was not detected in any product stored under 75% r.h. and total toxin levels at 80% r.h. on WS and WIS nuts at 60 d were below EC statutory levels of 4 ppb. In contrast, relative humidity levels for the safe storage of peanuts and hazelnuts were reported to be 85% (Diener and Davis, 1967; Simsek et al., 2002).

With regards to temperature, storage of nuts at 13°C yielded the lowest levels of aflatoxin which were also well below EC requirements. Considering that *A. flavus* is a mesophile, the results were not unexpected. In this study, storing Brazil nuts at 25-30°C yielded the highest level of toxin. In particular, total and AFB_1 toxin levels in WIS nuts gradually increased with increases in temperature. With WS and HS nuts these levels also increased but only up to 25°C thereafter decreasing.

The Brazil nuts used in this study were previously dried in a processing facility using a gas-fired oven to a m.c. of approximately 3.5%. It was imperative that the nuts

be dried prior to shipment in order to avoid deterioration during transit. During storage at 30°C, moisture and water activity levels in the WIS nuts were higher compared to WS nuts. In part this may be due to hygroscopic properties of the shell and or the ability of the shell to retain moisture, a phenomenon also observed by Chiou et al. (1984) with peanuts. Despite the higher m.c., toxin levels were lower for in-shell product indicating that the shell can only partially protect the nut from fungal invasion. For example, Frank et al. (1980) indicated that fungi including aflatoxin producers are able to penetrate the shells of Brazil nuts at r.h. greater than 75%. It should be noted that toxin production was detected in sound WIS nuts and therefore shell porosity (Spencer, 1921), cracks (Freire et al., 2000) and the presence of a locule (narrow open channel that extends through the entire length of the shell but does not appear to contact the nut) may influence nut stability.

Based on the results of this study it would appear that minimum moisture or a_W levels in order to control aflatoxin production (< 4ppb) in Brazil nuts is dependent on whether or not the product is in-shell and or the level of damage. In this respect it is recommended that at 30°C (typical ambient temperature in many nut gathering/storage and processing areas of Amazonia), WS and WIS nuts be maintained at moisture levels of 4.5% (a_w = 0.68) and 5.0% (a_W = 0.75), respectively. Damaged nuts or nuts which have been trimmed to remove blemishes etc. should be segregated and maintained at 3.5 % m.c. It should be pointed out that storage of these nuts was only maintained up to 60 d. According to Yokoya et al. (1970) WS Brazil nuts reach equilibrium after 8 months when they are stored under high relative humidity (88 to 97%) but only two months at 70%. It can be expected therefore that the m.c. levels in the nuts stored under 80-97 % r.h. would increase.

3.6. Conclusions

This study confirms earlier work demonstrating the importance of relative humidity and temperature on aflatoxin production in nuts. A relative humidity of 97% accompanied by temperatures in the range of 25-30°C was shown to promote aflatoxin production in infected Brazil nuts. Unfortunately, this worst-case scenario is representative of the harvesting season. Reduction of relative humidity and or temperature may not be an economic option. Air and or mechanical drying of the nuts could be used and would limit mold growth and toxin formation. In this respect whole shelled and in-shell nuts should be dried to m.c. of approximately 4.5 and 5.0%, respectively.

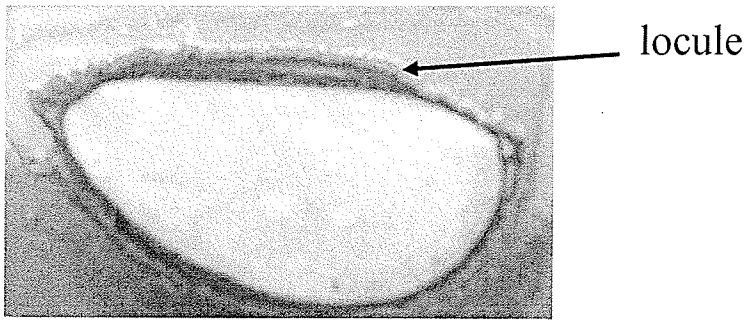
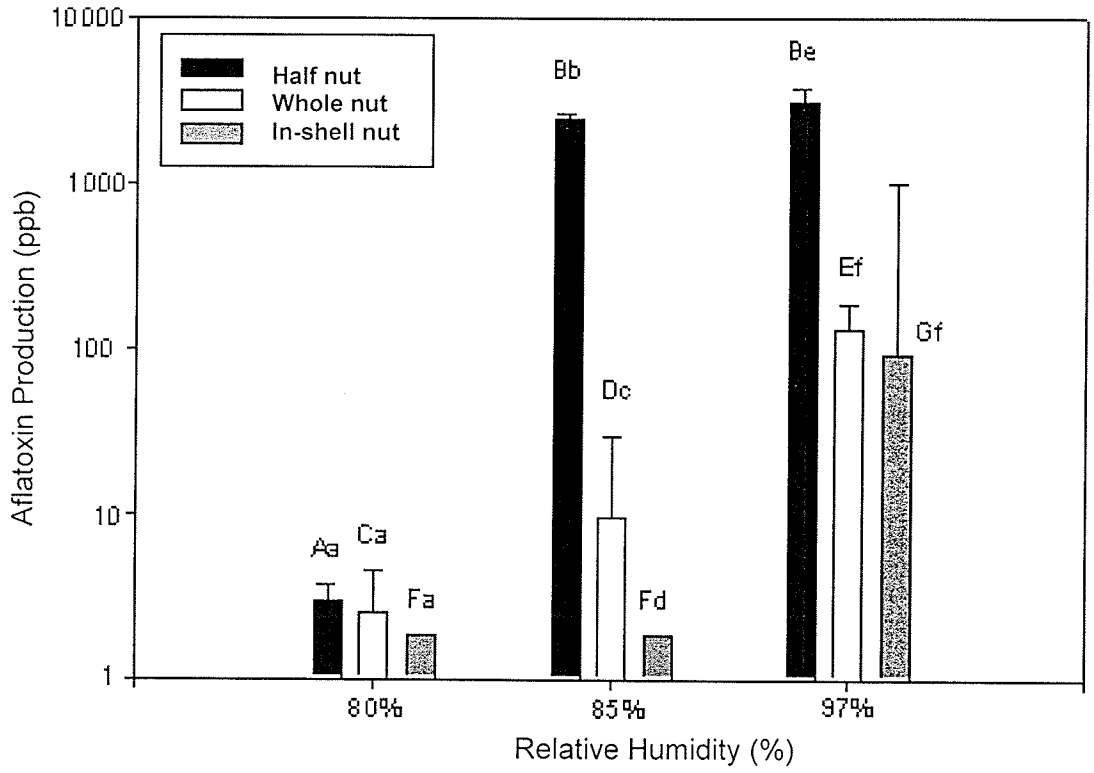


Figure 3.1. Transversal cut of Brazil nut

Figure 3.2. Total aflatoxin production in Brazil nuts after 30 d of storage at 30°C: influence of relative humidity and inoculation site [1,2,3]

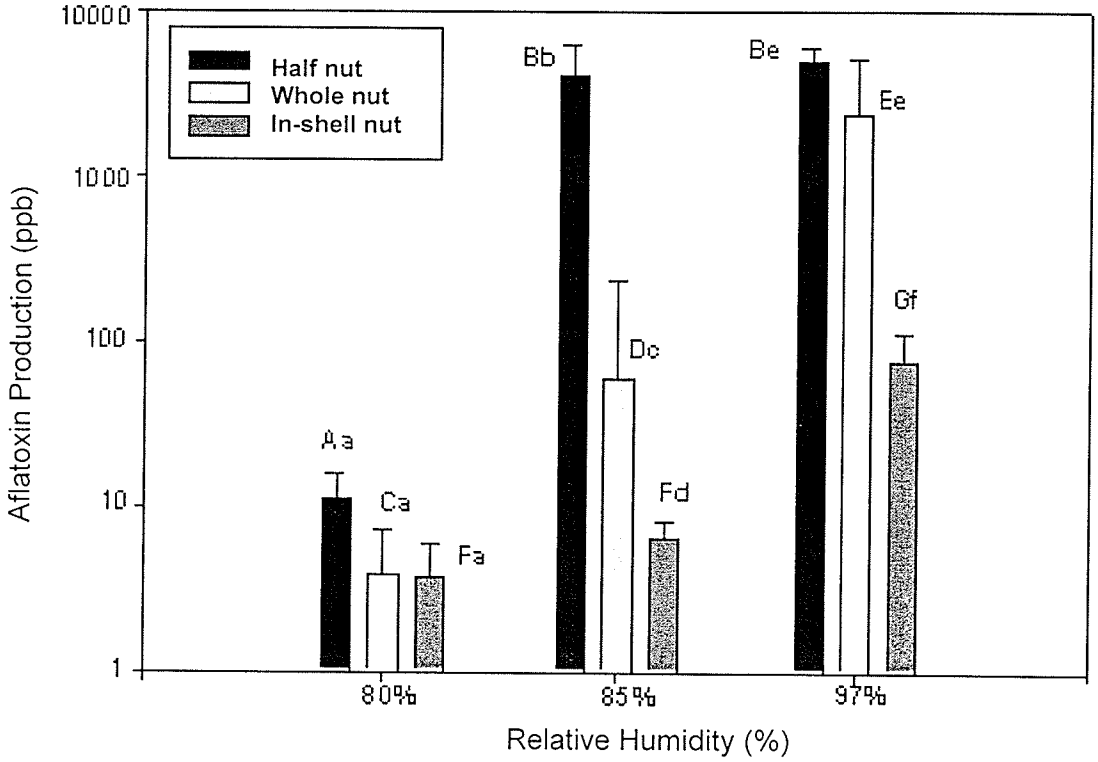


[1]Results are means of four trials analyzed in duplicate. Bars represent the standard deviation.

[2]Results followed by the same upper-case letter for similar inoculation sites are not significantly different ($p \leq 0.05$) according to Tukey's test.

[3]Results followed by the same lower-case letter for each relative humidity are not significantly different ($p \leq 0.05$) according to Tukey's test.

Figure 3.3. Total aflatoxin production in Brazil nuts after 60 d of storage at 30°C: influence of relative humidity and inoculation site [1,2,3]

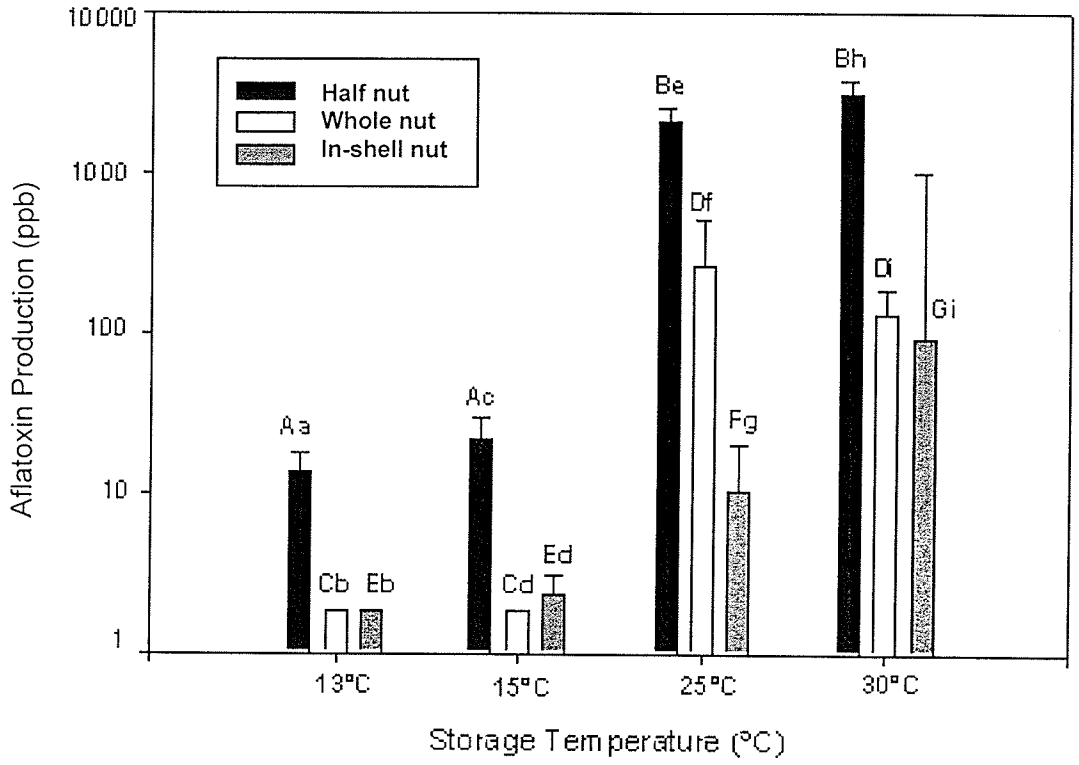


[1]Results are means of four trials analyzed in duplicate. Bars represent the standard deviation.

[2]Results followed by the same upper-case letter for similar inoculation sites are not significantly different ($p \leq 0.05$) according to Tukey's test.

[3]Results followed by the same lower-case letter for each relative humidity are not significantly different ($p \leq 0.05$) according to Tukey's test.

Figure 3.4. Total aflatoxin production in Brazil nuts after 30 d of storage at 97% r.h.: influence of temperature and inoculation site[1,2,3]

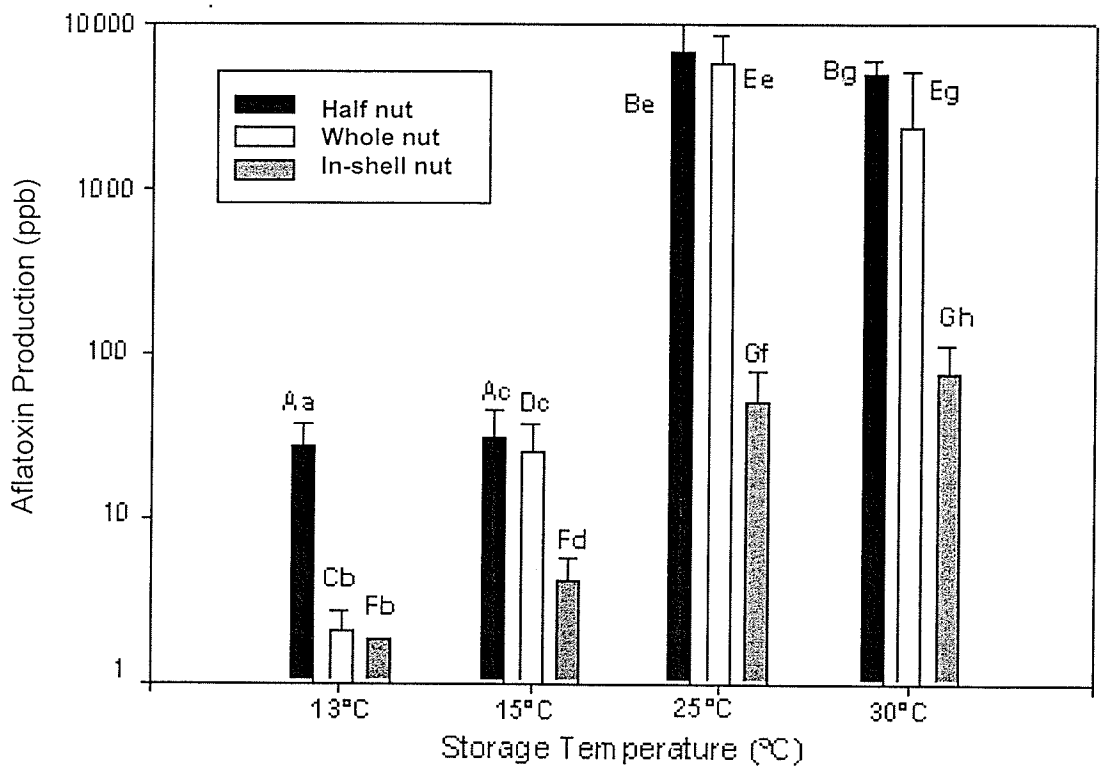


[1]Results are means of four trials analyzed in duplicate. Bars represent the standard deviation.

[2]Results followed by the same upper-case letter for similar inoculation sites are not significantly different ($p \leq 0.05$) according to Tukey's test.

[3]Results followed by the same lower-case letter for each temperature are not significantly different ($p \leq 0.05$) according to Tukey's test.

Figure 3.5. Total aflatoxin production in Brazil nuts after 60 d of storage at 97% r.h.: influence of temperature and inoculation site[1,2,3]

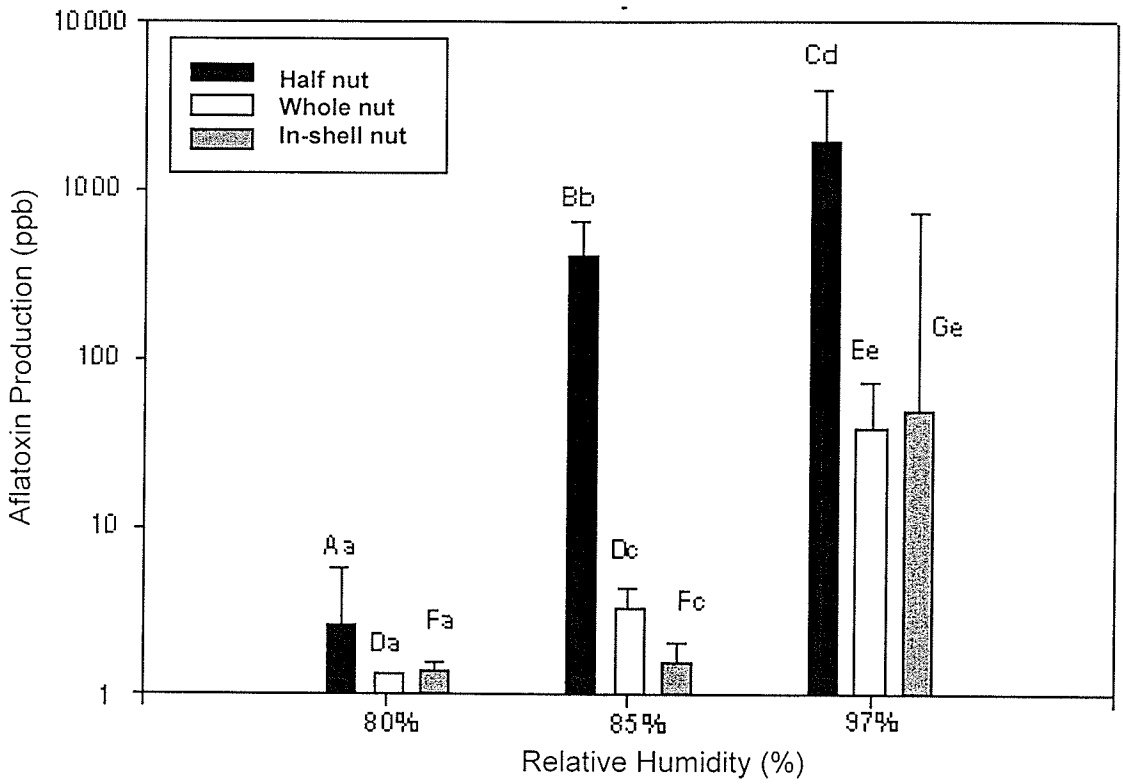


[1]Results are means of four trials analyzed in duplicate. Bars represent the standard deviation.

[2]Results followed by the same upper-case letter for similar inoculation sites are not significantly different ($p \leq 0.05$) according to Tukey's test.

[3]Results followed by the same lower-case letter for each temperature are not significantly different ($p \leq 0.05$) according to Tukey's test.

Figure 3.6. B_1 aflatoxin production in Brazil nuts after 30 d of storage at 30°C: influence of relative humidity and inoculation site [1,2,3]

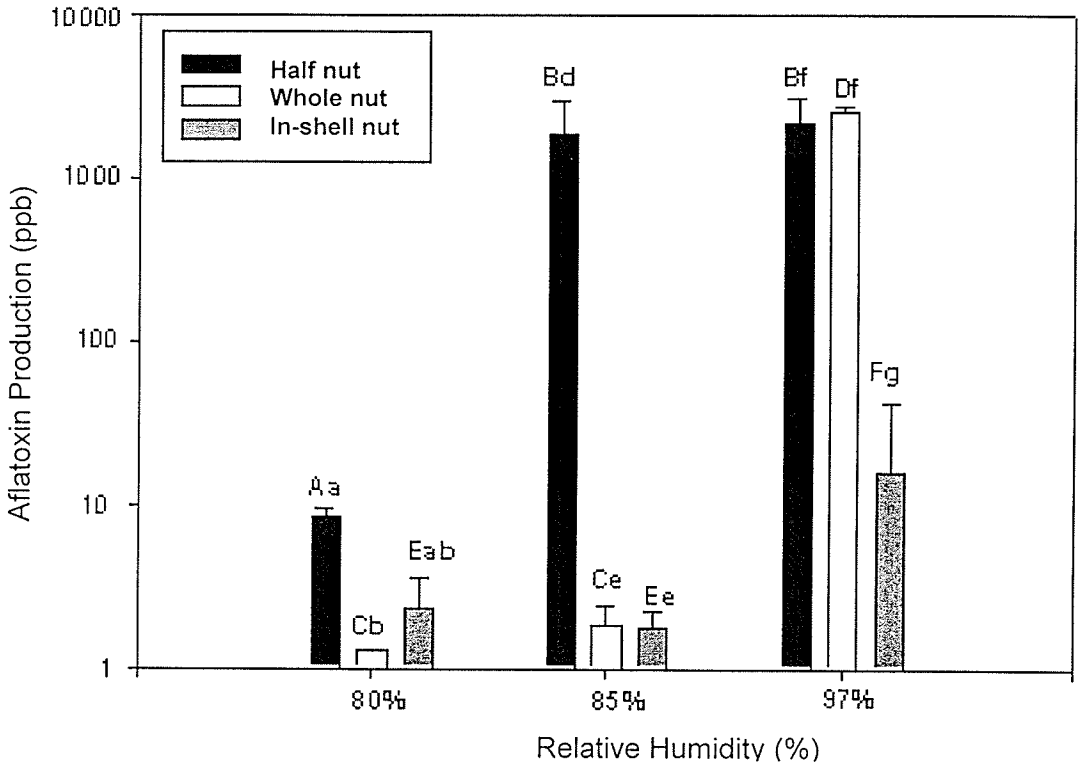


[1]Results are means of four trials analyzed in duplicate. Bars represent the standard deviation.

[2]Results followed by the same upper-case letter for similar inoculation sites are not significantly different ($p \leq 0.05$) according to Tukey's test.

[3]Results followed by the same lower-case letter for each relative humidity are not significantly different ($p \leq 0.05$) according to Tukey's test.

Figure 3.7. B_1 aflatoxin production in Brazil nuts after 60 d of storage at 30°C: influence of relative humidity and inoculation site [1,2,3]

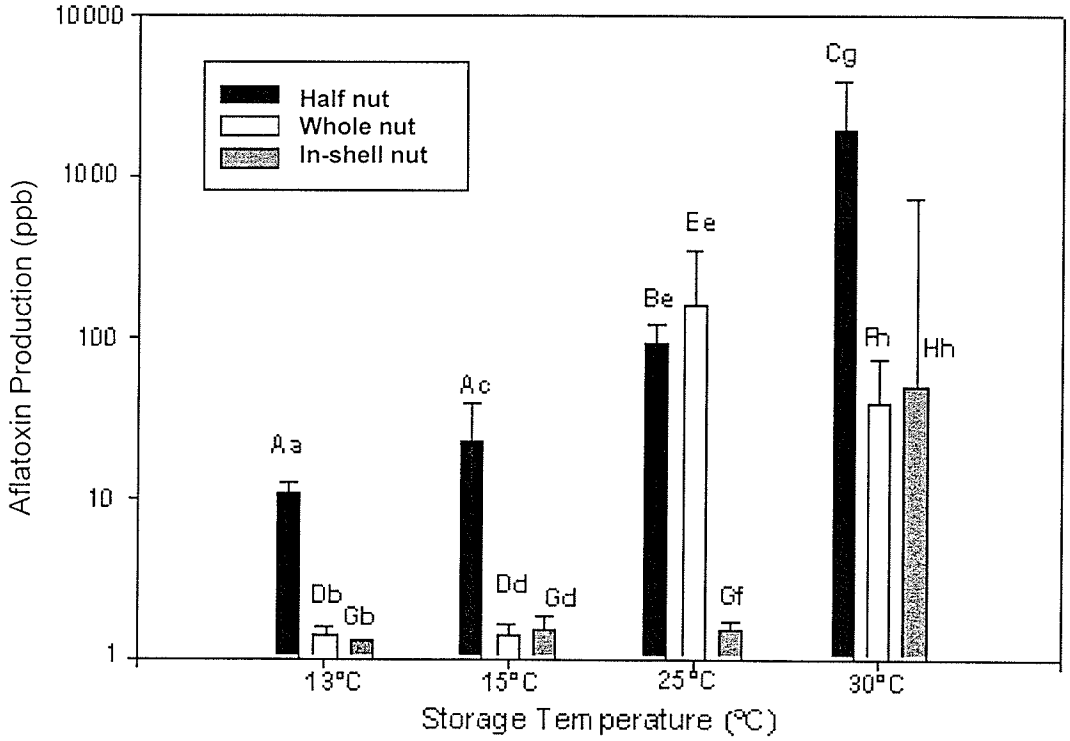


[1]Results are means of four trials analyzed in duplicate. Bars represent the standard deviation.

[2]Results followed by the same upper-case letter for similar inoculation sites are not significantly different ($p \leq 0.05$) according to Tukey's test.

[3]Results followed by the same lower-case letter for each relative humidity are not significantly different ($p \leq 0.05$) according to Tukey's test

Figure 3.8. B_1 aflatoxin production in Brazil nuts after 30 d of storage at 97% r.h.: influence of temperature and inoculation site[1,2,3]

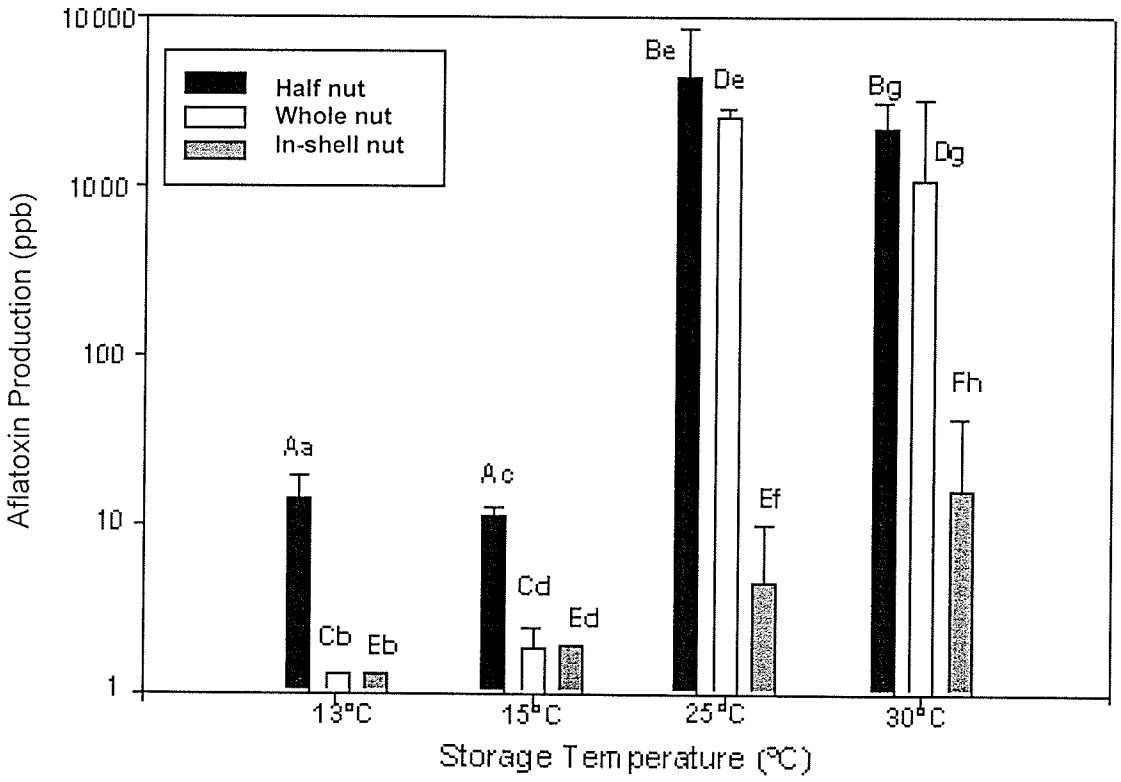


[1]Results are means of four trials analyzed in duplicate. Bars represent the standard deviation.

[2]Results followed by the same upper-case letter for similar inoculation sites are not significantly different ($p \leq 0.05$) according to Tukey's test.

[3]Results followed by the same lower-case letter for each temperature are not significantly different ($p \leq 0.05$) according to Tukey's test.

Figure 3.9. B_1 aflatoxin production in Brazil nuts after 60 d of storage at 97% r.h.: influence of temperature and inoculation site[1,2,3]



[1]Results are means of four trials analyzed in duplicate. Bars represent the standard deviation.

[2]Results followed by the same upper-case letter for similar inoculation sites are not significantly different ($p \leq 0.05$) according to Tukey's test.

[3]Results followed by the same lower-case letter for each temperature are not significantly different ($p \leq 0.05$) according to Tukey's test.

Table 3.1. Growth and aflatoxin production in YES medium[a] by *A. flavus* strains isolated from Brail nuts

Isolates	Aflatoxin[b] (ng/ml)	Mycelia[b] (g/100 ml YES)
AF-1	13.12 ± 5.23[c]	2.43
AF-2	627.60 ± 64.19	2.61
AF-3	778.09 ± 25.98	2.42
AF-4	12.58 ± 1.74	2.90
AF-5	12.88 ± 2.35	1.64
AF-6	10.02 ± 2.43	2.68
AF-7	612.32 ± 147.62	2.33

[a]Incubated at 21 ± 1°C for 10 d

[b]Mean of three values

[c]Mean value ± S.D.`

Table 3.2. Moisture content and water activity of Brazil nuts after 30 and 60 d of storage under various relative humidity at 30°C.

Relative humidity (%)	Half shelled nuts (HS)			Whole shelled nuts (WS)			Whole in-shell nuts (WIS)		
	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
75	3.50[a]	3.55	3.50	3.50	3.55	3.50	3.50	4.50	4.12
	(0.705)[b]	(0.695)	(0.682)	(0.700)	(0.695)	(0.680)	(0.711)	(0.780)	(0.720)
80	3.50	3.57	4.57	3.50	4.35	4.50	3.50	4.75	5.05
	(0.705)	(0.717)	(0.705)	(0.700)	(0.735)	(0.685)	(0.711)	(0.794)	(0.751)
85	3.50	6.43	6.60	3.50	5.34	5.35	3.50	6.73	6.90
	(0.705)	(0.895)	(0.823)	(0.700)	(0.775)	(0.815)	(0.711)	(0.880)	(0.886)
97	3.50	8.57	11.14	3.50	7.19	10.22	3.50	8.20	11.26
	(0.705)	(0.897)	(0.953)	(0.700)	(0.859)	(0.936)	(0.711)	(0.915)	(0.952)

[a]Moisture content (%). Mean of two values.

[b]Water activity. Mean of two values.

Table 3.3. Moisture content and water activity of Brazil nuts after 30 and 60 d of storage at various temperature under 97% r.h.

Temperature (°C)	Half shelled nuts (HS)			Whole shelled nuts (WS)			Whole in-shell nuts (WIS)		
	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
10	3.50[a]	5.40	9.14	3.50	6.32	6.45	3.50	4.15	5.50
	(0.705)[b]	(0.870)	(0.922)	(0.700)	(0.858)	(0.891)	(0.711)	(0.725)	(0.830)
13	3.50	5.71	9.43	3.50	6.38	7.26	3.50	4.52	7.86
	(0.705)	(0.890)	(0.933)	(0.700)	(0.864)	(0.900)	(0.711)	(0.745)	(0.913)
15	3.50	5.00	8.14	3.50	4.91	6.09	3.50	4.27	5.92
	(0.705)	(0.816)	(0.919)	(0.700)	(0.778)	(0.841)	(0.711)	(0.729)	(0.835)
25	3.50	8.57	11.00	3.50	7.38	8.57	3.50	7.88	8.96
	(0.705)	(0.904)	(0.917)	(0.700)	(0.898)	(0.904)	(0.711)	(0.894)	(0.933)

[a]Moisture content (%). Mean of two values.

[b]Water activity. Mean of two values.

4. MICROBIOLOGY OF BRAZIL NUTS

4.1. Abstract

Harvesting of Brazil nuts not only helps to preserve the Amazon rainforest but also provides income to individuals who would otherwise have no means of making a livelihood. Recently, many countries including the European Community have tightened the quality requirements for Brazil nuts particularly in regards to aflatoxin levels and microbiological contamination. The objectives of this research were to investigate the involvement of *Aspergillus flavus* and *Aspergillus parasiticus* during harvesting and nut processing, and to bacteriologically evaluate some of the unit operations involved in the processing. In this regard Brazil nut pods (5) were aseptically picked from trees located in each of three concessions of the Peruvian Amazon rainforest (Madre de Dios province). The exteriors of the pods were examined for yeast and molds including *A. flavus* and *A. parasiticus* and various bacterial pathogens including *Escherichia coli* and *Salmonella*. Brazil nuts obtained from various unit process operations were similarly evaluated. Scrapings from the exterior of Brazil nut pods (3/5) from only one concession yielded *A. flavus*. All isolates were aflatoxigenic (630 to 915 ppb aflatoxin). Yeast and molds including *Penicillium glabrum* (Wehmer) Westling, *Penicillium funiculosum* Thom, *Penicillium citrinum* Thom, *Penicillium sclerotiorum* van Beyma, *and Penicillium wortmanii* (Klocker) C. Benjamin were present on the exterior of all pods. *Fusarium spp.*, *Acremonium* Link ex Fr. and unidentified coelomycetes were also identified. *E. coli*, coliforms and salmonellae were not recovered from any of the pods.

Whole, in-shell nuts obtained following opening of all pods yielded no *A. flavus* or *A. parasiticus*, however, yeasts and penicillia were isolated. Aflatoxin was not detected (detection limit 1.75 ppb) in any of the nuts. Whole in-shell and shelled nuts from various unit process operations were all positive for *A. flavus*. *E. coli* and salmonellae were not recovered. Soaking of whole, in-shell nuts, performed prior to cracking or shelling, was shown to result in an increase in the levels of coliforms, however, levels of *A. flavus* decreased. The presence of *A. flavus* in tree-harvested pods has previously not been reported and additional studies will have to be performed in order to assess their occurrence.

4.2. Introduction

The Brazil nut or castaña tree (*Bertholletia excelsa* Humb. & Bonpl.) is indigenous to the Amazon region and grows mainly in Brazil, Bolivia and Peru (Madre de Dios). It is well recognized that this sustainable resource has an important role in the preservation of the Amazon rainforest. Brazil nuts represent one of the most important non-timber, forest products and approximately 20,000 people (22% of the population in Madre de Dios province) many of whom are impoverished, depend directly or indirectly on the Brazil nut trade (Agreda, 1999). Since 1999, the European Community has enforced stricter standards relative to microbiological and aflatoxin contamination (Chen et al., 2001; Agnes and Akbarsha, 2003). For example, the current standards call for rejection of nuts that contain total and B_1 aflatoxin at levels above 4 and 2 ppb, respectively (EC, 1998).

Aflatoxins are secondary metabolites produced by certain strains of *A. flavus* and *A. parasiticus*. These molds have been recovered from a variety of foods including ground and tree nuts. Although Brazil nuts have tested positive for these fungi, (Holubova-Jechova, 1970; Castrillon and Purchio, 1988a; Freire et al., 2000) the origin and involvement of these fungi during harvesting and processing are relatively unknown. Contamination of nuts including almonds, pecans, walnuts and Brazil nuts with bacterial pathogens including *Escherichia coli* has also been reported (Weinzirl, 1927; Ostrolenk and Hunter, 1939; Meyer and Vaughn, 1969; Chipley and Heaton, 1971; Marcus and Amling, 1973) and represents a major health concern since most are consumed without any further processing. Recently, Freire and Offord (2002) reported the presence of *Bacillus cereus*, *Salmonella* Typhimurium, *E. coli* and *Staphylococcus aureus* in shelled retail Brazil nuts.

In general pods from the Brazil nut tree are harvested after they have fallen to the jungle floor. In many cases they are in direct contact with the soil for several days to weeks. Ostensibly, it is during this time that the pods become contaminated with *Aspergillus*. Bezerra (2001) for example, reported that contamination of the pods with *Aspergillus* reached 100% after a 50 d exposure to the soil. In addition, post harvest operations are also expected to have a major influence on further contamination of the nuts (Bayman et al., 2002). Once the pods have been collected they are opened using a machete, placed in jute bags and transported to a commercial shelling facility via truck or boat. In the shelling plant the in-shell nuts must be dried as soon as possible in order

to minimize mold growth. In this respect they are spread onto warehouse floors and turned daily for 2-3 weeks. In preparation for shelling, the in-shell nuts are further dried in an oven to moisture content of about 4-6% and then soaked in water for 6 -8 h. The combination of drying followed by soaking allows easier shelling with fewer broken nuts, which command a lesser price. Nuts are cracked using a simple mechanical device and the shells are removed by hand. Shelled, whole nuts are sorted, trimmed of blemishes; oven dried (60°C for 12 h; Candela Peru standard), cooled and packed in plastic lined jute bags.

The objectives of the present study were to gain a better understanding regarding the origin and involvement of *A. flavus* and *A. parasiticus* during harvesting and nut processing, and to conduct a microbiological examination of various unit operations used in the processing of Brazil nuts.

4.3. Materials and methods

4.3.1. Brazil nut pods

Fourteen Brazil nut pods were aseptically harvested while still attached to trees located in three areas (concessions or castañales) of Amazonian Peru (Madre de Dios). The pods were placed into sterile bags and refrigerated until shipment (via air transport) to Winnipeg, and were received approximately 7 d following harvest.

4.3.2. Microbiological and aflatoxin analysis of the exterior of pods

The exterior of all pods were examined initially for cracks, holes and signs of insect damage and were subsequently scraped using a sterilized, hand-held metallic cheese grater. Scrapings (10 g) from each pod were homogenized (Stomacher LabBlender 400) in 90 ml Universal Broth (Difco Laboratories, Detroit, MI) for 30 sec and serially diluted as necessary using peptone water (0.1%w/v). The samples were analyzed for total colifoms and *Escherichia coli* using Petrifim™ (35°C, 48 h; 3 M Microbiology Products, St. Paul, MN). Yeasts and molds were enumerated using both potato dextrose agar (PDA, Difco) and dichloran rose bengal chloramphenicol agar (DRBC, Oxoid). *Aspergillus* was identified using: Aspergillus flavus and parasiticus agar (AFPA; Oxoid Ltd., Hampshire, UK). PDA and DRBC plates were incubated at 25°C for 5 d while AFPA plates were incubated at 30°C for 3 d. Mold isolates were identified according to standard taxonomic criteria (Samson et al., 1995). In addition, known cultures of *A. parasiticus* and *A. flavus* were provided by the Canadian Grain Commission (Winnipeg, MB) for comparison purposes. For *Salmonella*, the homogenized samples were incubated at 37°C for 24 h and portions (1.0 ml) were subsequently enriched in Selenite Cysteine (Difco) and Tetrathionate Broth (Difco) and incubated (24 h, 42°C). Samples from each primary enrichment broth were streaked on Brilliant Green and Salmonella-Shigella Agar (Difco) and incubated at 35°C for 24 h. Suspect colonies from each secondary enrichment agar were picked to Lysine Iron plus Triple Sugar Iron slants (Difco) and incubated (24 h, 35°C). Samples giving typical reactions were serogrouped with *Salmonella* O antisera (Difco).

Identified *A. flavus* isolates obtained from exterior scrapings of Brazil nut pods were screened for aflatoxin production according to Davis et al. (1966). Briefly, YES medium (100 ml) consisting of yeast extract (2%) and sucrose (20%) was inoculated with single spores of the *Aspergillus* isolates in 500-ml flasks and incubated for 7-10 d in the dark at 21°C. The filtrates (Whatman no.1) were analyzed for total aflatoxin using enzyme immunoassay (Ridascreen®; R-Biopharm AG, Darmstadt, Germany). The detection limit for the total aflatoxin kit was 1.75 ppb. Preparation of Brazil nut samples including extraction and aflatoxin determination was performed according to the manufacturer's instructions. Aflatoxin B_1 standard (R-Biopharm AG) dissolved in methanol (concentration range: 0.5 to 40.5 ng/ml) was used to prepare calibration curves.

4.3.3. Microbiological analysis of whole, in-shell and shelled nuts from tree-harvested pods

The Brazil nut pods were opened using a high-speed rotary cutting tool. The whole, in-shell nuts (WIS) were aseptically transferred to sterile bags (Whirl-Pak; Fisher Scientific Ltd., Toronto, Canada; 19 x 7.6 cm) and maintained under refrigeration until analyzed. Seven WIS nuts from each of the 14 pods were shaken in sterile peptone water (0.1 %, 100 ml) using a mechanical shaker for approximately 15 min. Prior to shaking, the entrance of the locules were sealed with molten wax. The peptone water from each set of WIS nuts was evaluated for yeast and mold using PDA and DRBC; AFPA was used to identify and enumerate potential *A. flavus* and *A. parasiticus* isolates. *Penicillia*

were confirmed by Agriculture and Agri-Food Canada, Biosystematics Research Institute (Ottawa, Canada).

In order to analyze whole shelled (WS) nuts, the WIS nuts which were previously shaken in peptone water were surface sanitized (4000 ppm chlorine, 2 min; Andrews, 1996) dried in a laminar flow cabinet (2 h) and cracked using a sanitized hand-held cracker. The WS nuts were subsequently cut in half along their longitudinal axis using a sanitized surgical blade. The HS nuts were again sanitized (4000 ppm chlorine, 2 min) and allow to air dry in a laminar flow hood. Halves from each WS nut were plated directly on PDA and AFPA. Leftover WIS nuts from each pod, which were not assessed for mold, were cracked, pooled and macerated using a mortar and pestle; samples (2 g) were subsequently evaluated for total aflatoxin (R-Biopharm AG).

4.3.4. Microbiological analysis of unit process operations

Twenty samples (500 g each) consisting of WS and WIS nuts from four unit process operations (airing, oven drying, soaking, shelling and drying) were collected from a commercial processing plant in Madre de Dios (Peru), dried to moisture contents of approximately 3.5 % and transported to our laboratory. The samples, part of the 2002-2003 harvests, were maintained under refrigeration and analyzed within 10 d of their arrival. Samples (50 g) were placed in sterile plastic bags containing Universal Broth (450 ml) and vigorously shaken (60 sec). Following serial dilution (0.1 % peptone), as required, the broths were analyzed for yeast, mold and aflatoxigenic mold by spread

plate using PDA, DRBC and AFPA as previously outlined. Samples were also assessed for *Salmonella*, total coliforms and *E. coli* as previously outlined.

The data obtained in this part of the study was subject to analysis of variance using the Statistical Analysis System (SAS Institute, Cary, N.C.). Differences among means were compared using Tuckey's test. Data were $\log_{10}$ transformed prior to analyses.

4.3.5. Microbial penetration

Commercially, WIS nuts are soaked for about 6 h in water in order to facilitate cracking, however, it is very likely that this process may contribute to high levels of cross-contamination. In order to evaluate the soaking step, five replicates consisting of 5 WIS nuts, previously inspected for visible cracks, were soaked in water containing an ampicillin resistant strain (02-8423; Health Canada) of *Salmonella* Typhimurium 02-8423 (10^7 cfu/ml) for 6 h at 30°C. Following soaking the nuts were allowed to drain and various surface sanitation protocols were evaluated: rapid immersion in 5% chlorine (liquid bleach); rapid immersion in 5% (v/v) chlorine followed by brushing and rapid immersion in 5% (v/v) chlorine; rapid immersion in 95% (v/v) ethanol which was burned off (Bayman et al., 2002); rapid immersion in 0.1% (w/v) mercuric chloride followed by three rinses in sterile distilled water (Marcus and Amling, 1973); immersion in 0.4% (v/v) chlorine for 2 min (Pitt et al., 1992) and incubation at 50°C for 15 min. After each treatment, the nuts were air dried for 60 min and evaluated for the presence of salmonellae as a means of assessing sanitation efficacy. In this regard, 5 nuts were

placed in sterile bags and weighted. An amount of peptone water (0.1% w/v) was added to obtain 10^{-1} dilution, shaken by hand for a total of 60 sec. and serially diluted as necessary using 0.1% (w/v) peptone water. Plating was performed using Xylose Lysine Deoxycholate Agar (Difco) containing ampicillin (100 ppm; Sigma Aldrich Co., MO). *Salmonella* survivors were assessed following incubation at 37° C for 24 h.

4.3.6. Dye penetration

Ninety WIS nuts without visible cracks were immersed in an aqueous solution containing methylene blue (0.4%) for 10 min (Meyer and Vaughn, 1969). Following air-drying the nuts were cracked and visually observed for staining.

4.4. Results

4.4.1. Mycological study of tree-harvested pods

The fungi isolated from the exterior of the Brazil nut pods and their incidence is shown in Table 4.1. Yeasts were present on all pods at levels of up to 10^7 cfu /g. Total mold counts ranged from 10^6 to 10^7 cfu/g. *Penicillium* appeared as the dominant genus (93%) followed by coelomycetes (79%), *Pestalotiopsis guepinii* (Desm.) Stey. (43%) and *Aspergillus flavus* (21%). *Acremonium* sp. was recovered on the surface of pods from only one concession. As shown in Table 4.2, three isolates were grown in YES medium for 10 d at 21°C and produced aflatoxin ranging from 630 to 915 ppb. *Salmonella*, *E. coli* or coliforms were not found on the outside of any of the pods (lowest dilution 10^{-1}).

A. flavus was not recovered from any of the WIS and WS nuts; aflatoxin also was not detected in any of these nuts (detection limit was 1.75 ppb). All nuts were contaminated with yeast ($\leq 10^7$ cfu/g) and mold ($\leq 10^6$ cfu/g). With respect to molds, *Penicillium, Acremonium, Trichoderma* Pers. ex Fr. and *Verticillium* Nees ex Link were isolated (Table 4.3.).

Several molds were isolated from the WS nuts; however, none matched those found on their respective exteriors (shell or pod). A coelomycete (containing single celled spores, 28u x 10u, released in a light grey ooze) appeared as the dominant fungus (74%) followed by *Penicillium* (16.3%) and *Aspergillus wentii* (2%) (Table 4.4). A mycoparasite identified as *Nodulisporium* was detected with the coelomycete.

4.4.2. Microbiological analysis of unit process operations

Microbiological data concerning several of the unit operations involved in Brazil nut processing are presented in Table 4.5. WS nuts taken from all unit processes tested positive for *A. flavus* except following drying. However, *A. flavus* was detected in 5% of these nuts by direct plating using Aspergillus flavus and parasiticus agar. Neither *Salmonella* nor *E. coli* was detected in any of the samples (lowest dilution of 10^{-1}) despite a significant ($p \leq 0.05$) increase in colifoms following soaking. Shelled and dried nuts contained significantly ($p \leq 0.05$) lower numbers of coliforms and yeast and mold including *A. flavus*. It is interesting to note that oven drying of the in-shell nuts following airing did not result in any significant reductions for any of the microorganisms analyzed.

4.4.3. Microbial penetration

Penetration studies of *S.* Typhimurium into WIS nuts could not be performed since complete surface disinfection of salmonellae could not be achieved. Removal of the shell by any means would therefore result in cross-contamination to the inner nut. Several methods for surface sanitation were evaluated, however, the highest reduction ($\log_{10}$ 2.5) was achieved using chlorine (0.4%, 2 min). A brief dipping in mercuric chloride (0.1%) also resulted in a comparable reduction.

4.4.4. Dye penetration

Methylene blue was observed to penetrate the stem end or locule in 60% of the nuts examined. Dye was not observed on the nut itself (Fig. 4.1.).

4.5. Discussion

4.5.1. Mycological study of tree-harvested pod

Brazil nut pods picked from trees were placed in individual sterile bags and transported to the laboratory in corrugated cardboard boxes. Presumably during transport condensation developed since upon opening the bags several of pods appeared damp and molded. Nevertheless it is important to realize that these molds were previously present on the pods at the time of picking and that any condensation may have simply enhanced their numbers. It is therefore important to note that while *A. flavus* was detected on the exterior of some of these pods, their numbers (10^6 cfu/g) may be an exaggeration of their actual values. Since pods under go maturation during the warm (25-31°C) rainy season

(December to April), it would be reasonable to presume that accompanying changes in the natural microflora may also occur. Therefore, it would be of importance to determine whether environmental conditions prevailing during pod maturation have an effect on the microbial population and in particular on *Aspergillus.*

Although *A. flavus* was detected on the exterior of pods from only one concession (Palma Real) no evident differences in terms of environmental conditions, such as rainfall, were reported among the concessions that might account for its presence. Studies by Beuchat (1975) also indicated the presence of *A. flavus,* albeit low (2/146) on pecans that were hand picked from trees. The finding that aflatoxigenic molds are present on Brazil nut pods while still on the tree indicates that insects, birds and other visitors as well as aerial updrafts (Beuchat, 1975) may be included as vectors of spore transmission. Although many of the pods contained cracks and evidence of insect and or bird damage (bore holes) none of the WIS and WS nuts, including those from Palma Real, contained *A. flavus*; also no aflatoxin was detected. It is possible that damage to the pods was superficial and did not penetrate through the entire shell. Indeed when the pods were opened and the individual nuts removed, the interiors appeared entire and no insects were obvious. In general, the pods appeared quite robust having a tough, woody and fibrous shell, 5-6 mm in thickness. Similar to peanuts and other nuts, invasion of *A. flavus* into Brazil nut pods may not occur prior to harvest especially if they are intact and relatively undamaged (Lynch and Wilson, 1991; Schroeder and Ashworth, 1965). McDonald and Harkness (1964) also reported that preharvest development of aflatoxin in peanuts occurred only in kernels or broken pods. However,

once the pods fall to the ground, they can quickly become contaminated with *Aspergillus*. Bezerra (2001) reported that following 20 d of falling, pods became contaminated with *Aspergillus*; in-shell nuts were contaminated after 30 d. Structural damage caused by the fall increase the likelihood of internal contamination (Schroeder and Ashworth, 1965).

In addition to *A. flavus*, the exterior of the pods were contaminated with several fungi principally *Penicillium* and *Fusarium*. Many of these have been reported previously with WIS and WS Brazil nuts with the exception of *Pestalotiopsis guepinii* (Holubova-Jechova, 1970; Castrillon and Purchio, 1988a; Freire et al., 2000; Bayman et al., 2002). Overall, the number of fungal species recovered from the pods was relatively small; nevertheless, incidence rates were quite high. Since these trees grow well over 50 m, with branches only at the uppermost part, it is possible that only a select community of pollinators and or predators visits the developing pods. Also interesting was the finding that coliforms, *E. coli* and salmonellae were not detected (at 10^{-1} dilution) on the exterior of any of the pods despite likely encounters with insects and/or birds. Despite the apparent durability of the pods numerous rotten WIS nuts were retrieved. Similar to other nuts it is possible that microorganisms are capable of gaining entrance into the pod perhaps via the blossom end (Freire et al., 2000).

4.5.2. Microbiological analysis of unit process operations

WIS and WS nuts from various unit process operations were examined from a microbiological perspective. The nuts, obtained from a commercial processing facility in Peru, were dried and vacuum-packed prior to shipment.

Besides the substantial level of contamination that occurs once pods knock the ground, additional contamination occurs during harvest when Brazil nuts are collected from the ground and opened in the forest allowing the inoculation of in-shell nuts with field fungi. It was demonstrated that cracks in the shell generated during collection and handling creates another entrance for microbial contamination (Freire et al., 2000). Moreover, nuts are transported to the processing plant under conditions of high moisture content (25-32%), high relative humidity, and warm temperatures that favour fungal growth and aflatoxin production.

The soaking process, performed on WIS nuts prior to cracking as expected, contributed to a significant increase in coliforms, however, mold and in particular *A. flavus /A. parasiticus* levels were either not affected or decreased. The level of cross contamination that occurs during soaking not only depends on the quality of the water used but also on the cleanliness of the nuts (Chipley and Heaton, 1971). The temperature of the soaking water, partly influenced by the ambient temperature, would also have an effect on microbial numbers. Inclusion of sanitizers during processing of horticultural crops such as chlorine in wash water normally was shown to have little beneficial effects in reducing cross contamination because residual chlorine quickly dissipated due to high level of organics. Similar findings have been reported for tree nuts (Meyer and Vaughn,

1969). Although soaking is performed mainly in Peru, (autoclaving, for example, is performed in Brazil and Bolivia as a means to expand the shell from the kernel) it is interesting to note that the subsequent shelling and drying of the nuts resulted in a significant reduction in microflora. In particular aspergilli levels were 30 x 10^1 cfu/g. The terminal heat/drying process is not only lethal to many microorganisms but also reduces moisture such that any growth is inhibited. Although it was not possible to assess the degree to which soaking of WIS nuts contributed to an increase in internal contamination, a possible route for microbial penetration was observed by soaking nuts in methylene blue solution (0.4% aqueous). The dye penetrated at the stem end, extending along the narrow open channel known as locule. This observation suggests that the locule is a primary route by which microorganisms enter and contaminate the nut meat. However, shell porosity of in-shell Brazil nuts is also suggested as another potential entrance for mold contamination (Spencer, 1921).

4.6. Conclusions

Data presented in relation to tree-harvested pods is preliminary and should be substantiated by repetitive evaluation of pods from several production areas and at different harvest times during the year. Nevertheless, results from this study indicate that pods might be contaminated with *A. flavus/A. parasiticus* before they fall to the ground. Therefore, harvest and postharvest treatments, including drying, should be carried out promptly in order to delay mold growth and aflatoxin production. Intervention strategies to minimize fungal and bacterial contamination during processing must be evaluated. In

this respect, the drying operation is seen as a critical control point, which should be monitored closely; records and/or quality sheets should be maintained for verification purposes. Specifically time and temperature protocols should be evaluated and accomplished using Brazil nuts challenged with specific microorganisms including aflatoxigenic aspergilli. Since the WIS nuts are cracked and shelled by hand attention should also be paid to sanitation and hygiene. Water used for the soaking step should be safeguarded, monitored microbiologically and treated with a sanitizer. If possible, the soaking operation should be abandoned and a more hygienic pre-treatment method be adopted prior to cracking.

Table 4.1. Mycology (cfu/g) of exterior of tree harvested pods

	Location			
	Bajo Alegria	Palma Real	Alegria	Incidence (%)[f]
Aspergillus flavus	N.D.[c]	2.6×10^6 (3/5)	N.D.[e]	21
Penicillium spp.[a]	2.5×10^5 (3/4)[d]	8.7×10^5 (5/5)	2.6×10^6 (5/5)	93
Pestalotiopsis guepinii	2.0×10^4 (1/4)	3.0×10^6 (1/5)	6.8×10^5 (4/5)	43
Fusarium spp.[b]	1.2×10^4 (1/4)	1.5×10^4 (1/5)	N.D.[e]	14
Acremonium sp.	8.8×10^6 (3/4)	N.D.[e]	N.D.[e]	21
Unidentified Coelomycete	1.8×10^6 (4/4)	1.3×10^6 (5/5)	1.2×10^6 (2/5)	79
Total Mold	1.4×10^7 (5/5)	7.8×10^6 (5/5)	3.3×10^6 (5/5)	100
Yeast	1.7×10^7 (4/4)	5.2×10^7 (5/5)	6.8×10^7 (5/5)	100

[a]*Penicillium glabrum* (Wehmer) Westling, *Penicillium funiculosum* Thom, *Penicillium citrinum* Thom, *Penicillium sclerotiorum* van Beyma, *Penicillium wortmanni* (Klocker) C. Benjamin, other *Penicillium* sp. probably undescribed

[b]*Fusarium* spp. (Probably *Fusarium lateritium* Nees., *Fusarium proliferatum* (Matsushima) Nirenberg)

[c]Not detected in 4 pods analyzed using dilution method ($<30 \times 10^1$)

[d]Values in parenthesis indicate incidence per number of pods sampled

[e]Not detected in 5 pods analyzed using dilution method ($<30 \times 10^1$)

[f]Percentage of positive pods

Table 4.2. Growth and aflatoxin production in YES medium[a] by *A. flavus* isolated from the exterior of tree harvested pods

Isolates[b]	Aflatoxin[c] (ppb)	Mycelia[c] (g/100ml YES)
PR-1	630.3 ± 146.7	2.13
PR-2	915.5 ± 121.7	2.57
PR-4	814.0 ± 290.6	2.12

[a]Incubated at 21± 1°C for 10 d

[b]All isolates belong to pods taken from the Palma Real location

[c]Average of three replicates (dry weigth)

Table 4.3. Mycology of surface from in-shell nuts[a] (tree harvested pods)

	Location					
	Bajo Alegria[b]		Palma Real[c]		Alegria[c]	
	(cfu/g)	Range (cfu/g)	(cfu/g)	Range (cfu/g)	(cfu/g)	Range (cfu/g)
Yeast	2.4×10^6	$2.3 \times 10^5 - 7.6 \times 10^6$	1.4×10^7	$4.6 \times 10^4 - 6.3 \times 10^7$	1.2×10^6	$8.6 \times 10^1 - 4.7 \times 10^6$
Penicillium sp.[d]	5.7×10^6	$3.3 \times 10^4 - 2.2 \times 10^7$	2.9×10^6	$2.9 \times 10^4 - 1.4 \times 10^7$	1.6×10^5	$<30 \times 10^1 - 3.8 \times 10^5$
Other mold[e]	2.1×10^6	$<30 \times 10^1 - 6.3 \times 10^6$	1.2×10^6	$4.9 \times 10^4 - 3.7 \times 10^6$	2.6×10^6	$2.5 \times 10^3 - 6.4 \times 10^6$

[a]7 individual nuts were evaluated from each pod

[b]4 pods were tested

[c]5 pods were tested

[d]*Penicillium glabrum* (Wehmer) Westling, *Penicillium funiculosum* Thom, *Penicillium citrinum* Thom, *Penicillium sclerotiorum* van Beyma, *Penicillium wortmanni* (Klocker) C. Benjamin, other *Penicillium* sp. probably undescribed

[e]*Acremonium* Link ex Fr., *Trichoderma* Pers. Ex Fr., *Verticillium* Nees ex Link., unidentified Coelomycete

Table 4.4. Mycology of WS nuts from tree harvested pods

	Location			Total Incidence (%)[c]
	Palma Real[a]	Bajo Alegria[b]	Alegria[a]	
Aspergillus wentii	1/35[d]	1/28	N.D.[f]	2.0
Penicillium sp.	9/35	1/28	6/35	16.3
Unidentified Coelomycete	33/35	7/28	15/35	74.5
Unidentified mold	6/35	N.D.[e]	13/35	19.3

[a] Five pods evaluated

[b] Four pods evaluated

[c] Values indicate the percentage of positive shelled nuts

[d] Values indicate incidence per number of shelled nuts sampled (7 nuts from each pod)

[e] Not detected in 28 shelled nuts: evaluated using dilution method ($<30x10^1$)

[f] Not detected in 35 shelled nuts: evaluated using dilution method ($<30x10^1$)

Table 4.5. Influence of unit process operation on fungal and bacterial populations in WIS/WS Brazil nuts

Unit process operation	Mean[a] $\log_{10}$ cfu/g			
	Coliforms	Yeast	Mold	*A. flavus /A. parasiticus*[b]
Airing	1.39 ± 0.62^{cf}	4.63 ± 0.83^{ef}	4.99 ± 0.42^{e}	2.35 ± 0.43^{e}
Oven drying	1.71 ± 0.68^{f}	3.67 ± 0.43^{f}	4.65 ± 0.67^{e}	2.63 ± 0.42^{e}
Soaking	4.30 ± 0.95^{e}	5.58 ± 0.29^{e}	5.32 ± 0.83^{e}	1.71 ± 0.27^{f}
After shelling and drying	2.08 ± 0.91^{f}	1.81 ± 0.97^{g}	3.62 ± 0.23^{f}	N.D.[d]

[a]Average of 5 samples

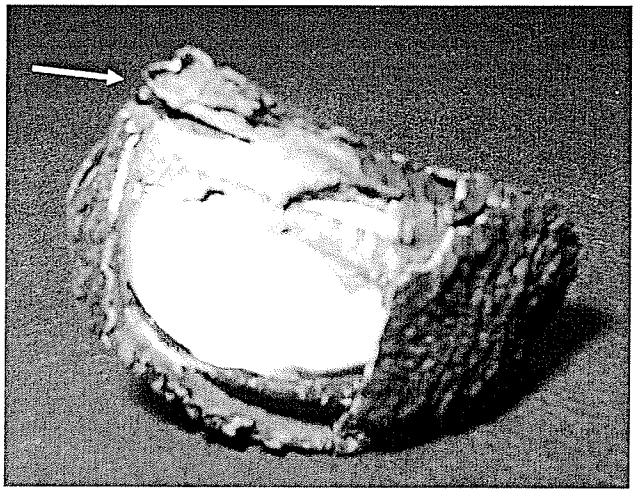
[b]Number of colonies that posses the characteristic for aspergilli (underneath orange) in A. flavus and parasiticus agar

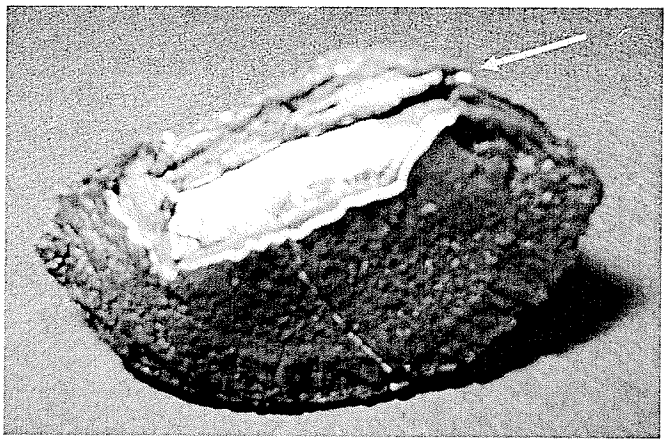
[c]Mean value±S.D.

[d]Not detected using dilution method ($<30x10^{1}$)

[e-g]Different letters within a column indicate significant differences among process ($p \leq 0.05$)

Figure 4.1. Dye penetration through the locule on Brazil nuts previously soaked in methylene blue solution (0.4%)





5. CONCLUSIONS

Data presented in relation to tree-harvested pods indicate that contamination of pods with aflatoxigenic *A. flavus* may occur at an early stage (before harvest). Therefore, any harvest or post harvest operations that would limit or reduce mold growth and mycotoxin production should be implemented. Of most importance, is the prompt drying of in-shell nuts during storage before cracking.

A high relative humidity of 97% accompanied by temperatures in the range of 25-30°C was shown to promote aflatoxin production during storage of infected Brazil nuts. Unfortunately, reduction of relative humidity and or temperature may not be an economic option. However, drying is a basic post harvest operation that will delay mold growth and aflatoxin formation. In this respect whole shelled and in-shell nuts should be dried to moisture concentrations of approximately 4.5 and 5.0%, respectively. Mechanical drying of sorted, shelled nuts should be considered a critical control point and therefore operational parameters must be monitored closely and recorded.

The soaking operation should be abandoned and a more hygienic pre-treatment method should be adopted prior to cracking. Also, the water used for soaking should be monitored microbiologically and treated with a sanitizer.

Since whole in-shell nuts are cracked and shelled by hand, attention should also be paid to sanitation and hygiene status of the personal as well as the facility to limit external contamination.

6. RECOMMENDATIONS FOR FUTURE STUDIES

Additional studies should be performed in order to assess the occurrence of aflatoxigenic molds in tree-harvested pods. As such, pods from additional concessions located in Madre de Dios should be examined on a regular basis as a means of establishing a base line. Also, the presence and extent of *A. flavus* and *A. parasiticus* in tree picked pods during their maturation (December to March - April) should be evaluated. Tree-harvested pods positive for these aspergilli should be opened and in-shell nuts examined for mold and or aflatoxin. Sources for mold contamination should also be examined including the trees, pollinating insects and birds.

The final drying stage can be viewed as a critical control point in regards to eliminating pathogens such as *Escherichia coli* and salmonellae. Therefore, time-temperature relationships should be established and validated using shelled nuts spiked with various pathogens.

Contamination of non-aflatoxigenic *Aspergillus* strains in peanut crops have demonstrated a significant reduction in the growth of aflatoxigenic strains with a concomitant reduction in mycotoxin production. This method should be evaluated for storage of Brazil nuts.

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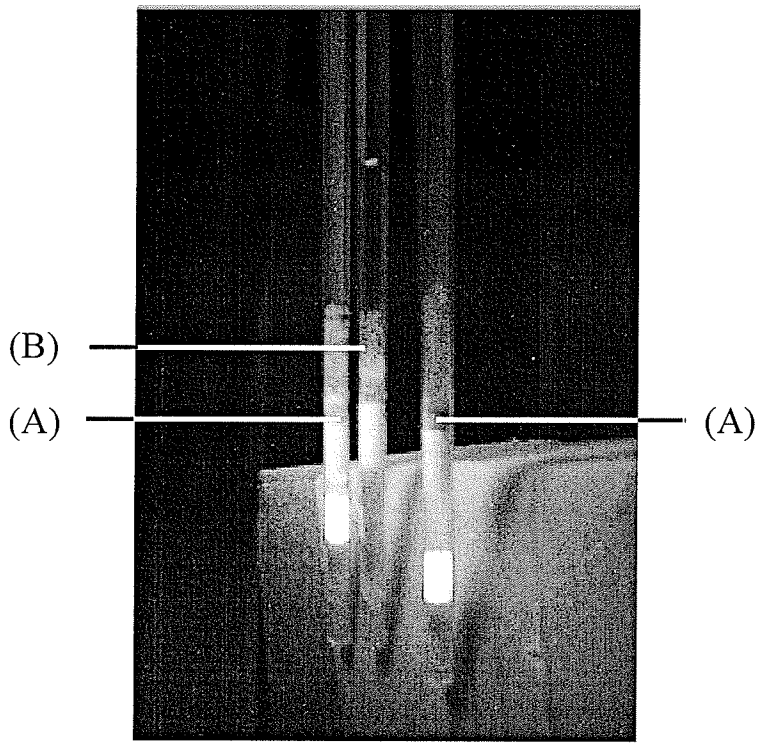
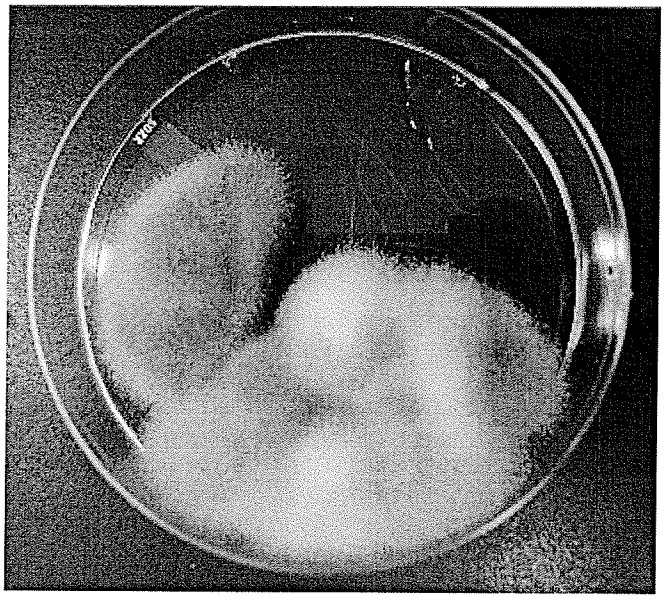
APPENDICES

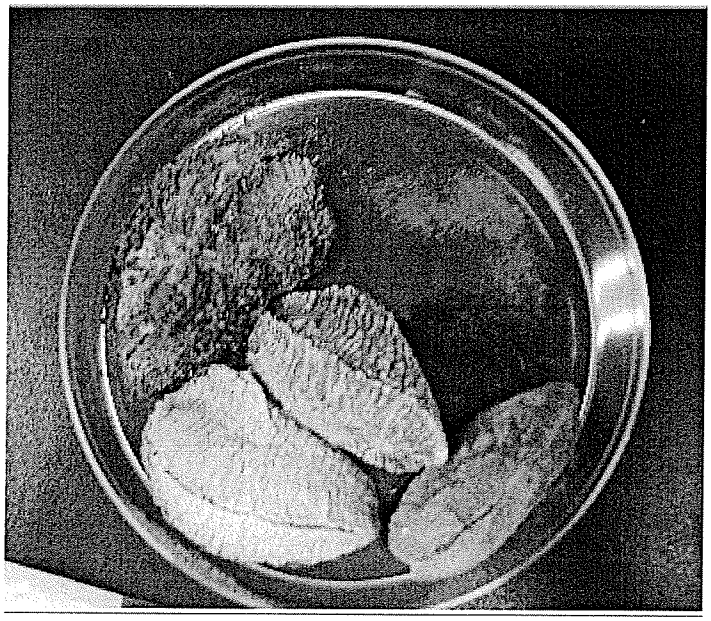
Appendix 1. Minicolumn method showing (A) positive and (B) negative results



Appendix 2. Inoculated shelled whole nuts after 60 d of storage at 25°C



Appendix 3. Inoculated in shell nuts after 60 d of storage at 25°C



Appendix 4. Total and B_1 aflatoxin production[a] (ppb) in Brazil nuts after 30 and 60 d storage under controlled conditions of relative humidity and temperature at different inoculation sites

Conditions	Half shelled nuts				Whole shelled nuts				In-shell nuts			
	30 d		60 d		30 d		60 d		30 d		60 d	
	Total aflatoxin	Aflatoxin B_1	Total aflatoxin	Aflatoxin B_1	Total aflatoxin	Aflatoxin B_1	Total aflatoxin	Aflatoxin B_1	Total aflatoxin	Aflatoxin B_1	Total aflatoxin	Aflatoxin B_1
75% 30°C	N.D.[b]	N.D.[c]	N.D.[b]	N.D.[c]	N.D.[b]	N.D.[c]	N.D.[b]	N.D.[c]	N.D.[b]	N.D.[c]	N.D.[b]	N.D.[c]
80% 30°C	2.00	2.19	10.70	7.59	1.90	N.D.[c]	3.45	1.23	N.D.[b]	N.D.[c]	2.98	1.54
85% 30°C	2519.3	438.1	4376.4	2036.8	12.20	2.40	134.2	81.5	N.D.[b]	N.D.[c]	5.52	1.0
97% 30°C	3154.8	2324.4	5118.2	2314.6	137.5	43.3	2971.1	1491.5	305.0	234.3	79.5	23.6
10°C 97%	N.D.[b]	N.D.[c]	N.D.[b]	0.96	N.D.[b]	N.D.[c]	N.D.[b]	N.D.[c]	N.D.[b]	N.D.[c]	N.D.[b]	N.D.[c]
13°C 97%	12.8	9.75	27.11	13.65	N.D.[b]	N.D.[c]	N.D.[b]	N.D.[c]	N.D.[b]	N.D.[c]	N.D.[b]	N.D.[c]
15°C 97%	21.5	24.9	31.5	10.4	N.D.[b]	N.D.[c]	26.2	1.06	N.D.[b]	N.D.[c]	3.38	0.88
25°C 97%	2152.5	93.4	8288.7	5184.7	311.6	198.4	6289.1	2551.9	10.9	N.D.[c]	53.7	4.55
30°C 97%	3154.8	2324.4	5118.2	2314.6	137.5	43.3	2971.1	1491.5	305.0	234.3	79.5	23.6

[a]Mean of four values

[b]N.D.: not detectable (below detection limit 1.75 ppb)

[c]N.D.: not detectable (below detection limit 0.625 ppb)

Appendix 5. Log transformed[a] total aflatoxin production[b] (ppb) in Brazil nuts after 30 and 60 d storage under controlled conditions of relative humidity and temperature at different inoculation sites.

Environmental conditions	Half shelled nuts		Whole shelled nuts		In-shell nuts	
	30 d	60 d	30 d	60 d	30 d	60 d
80% 30°C	0.467 ± 0.112	1.045 ± 0.161	0.399 ± 0.251	0.588 ± 0.272	0.273 ± 0	0.568 ± 0.205
85% 30°C	3.400 ± 0.035	3.608 ± 0.199	0.985 ± 0.481	1.775 ± 0.600	0.273 ± 0	0.804 ± 0.106
97% 30°C	3.492 ± 0.091	3.703 ± 0.086	2.121 ± 0.152	3.369 ± 0.352	1.971± 1.035	1.882 ± 0.162
13°C 97%	1.124 ± 0.131	1.432 ± 0.139	0.273 ± 0	0.324 ± 0.103	0.273 ± 0	0.273 ± 0
15°C 97%	1.337 ± 0.127	1.493 ± 0.160	0.273 ± 0	1.408 ± 0.168	0.373± 0.115	0.630 ± 0.120
25°C 97%	3.326 ± 0.091	3.834 ± 0.305	2.422 ± 0.286	3.776 ± 0.161	1.005± 0.291	1.710 ± 0.190
30°C 97%	3.492 ± 0.091	3.703 ± 0.086	2.121 ± 0.152	3.369 ± 0.352	1.971± 1.035	1.882 ± 0.162

[a]From appendix 4

[b]Mean of four log transformed values

Appendix 6. Log transformed[a] B_1 aflatoxin production[b] (ppb) in Brazil nuts after 30 and 60 d storage under controlled conditions of relative humidity and temperature at different inoculation sites

Environmental conditions	Half shelled nuts		Whole shelled nuts		In-shell nuts	
	30 d	60 d	30 d	60 d	30 d	60 d
80% 30°C	0.409± 0.341	0.931 ± 0.054	0.118 ± 0	0.118 ± 0	0.143 ± 0.049	0.369 ± 0.191
85% 30°C	2.603± 0.209	3.272 ± 0.210	0.522 ± 0.121	0.262 ± 0.116	0.202 ± 0.110	0.255 ± 0.096
97% 30°C	3.28± 0.311	3.343 ± 0.158	1.588 ± 0.273	3.034 ± 0.484	1.697 ± 1.162	1.200 ± 0.430
13°C 97%	1.026± 0.078	1.148 ± 0.139	0.145 ± 0.053	0.118 ± 0	0.118 ± 0	0.118 ± 0
15°C 97%	1.359± 0.233	1.054 ± 0.048	0.152 ± 0.067	0.262 ± 0.116	0.187 ± 0.081	0.273 ± 0.009
25°C 97%	1.962± 0.120	3.652 ± 0.282	2.204 ± 0.339	3.405 ± 0.050	0.173 ± 0.063	0.648 ± 0.348
30°C 97%	3.287± 0.311	3.343 ± 0.158	1.588 ± 0.273	3.034 ± 0.484	1.697 ± 1.162	1.200 ± 0.430

[a]From appendix 4

[b]Mean of four log transformed values

Appendix 7. Back transformed total aflatoxin production[a] (ppb) in Brazil nuts after 30 and 60 d storage under controlled conditions of relative humidity and temperature at different inoculation sites

	Half shelled nuts						Whole shelled nuts						In-shell nuts					
	30 d			60 d			30 d			60 d			30 d			60 d		
	AF[b]	S.D.[c]		AF	S.D.		AF	S.D.		AF	S.D.		AF	S.D.		AF	S.D.	
Conditions	(ppb)	Lower	Upper	(ppb)	Lower	Upper	(ppb)	Lower	Upper	(ppb)	Lower	Upper	(ppb)	Lower	Upper	(ppb)	Lower	Upper
80% 30°C	1.93	0.665	0.860	10.08	3.43	4.96	1.50	1.10	1.96	2.87	1.80	3.37	0.875	0	0	2.70	1.39	2.24
85% 30°C	2513	193.7	209.8	4050	1487	2349	8.66	6.47	19.61	58.57	44.6	177.4	0.875	0	0	5.37	1.38	1.76
97% 30°C	3103	584.7	720.3	5047	911.1	1112	131.3	39.1	55.5	2341	1301	2927	92.6	84.9	920.2	75.1	23.8	34.5
13°C 97%	12.30	3.47	4.70	26.06	7.40	10.19	0.875	0	0	1.11	0.445	0.564	0.875	0	0	0.875	0	0
15°C 97%	20.72	5.52	7.41	30.09	9.59	13.88	0.875	0	0	24.6	8.22	12.1	1.36	0.55	0.718	3.26	1.03	1.36
25°C 97%	2115	398.3	490.6	6817	3442	6950	263.2	127.4	246.1	5968	1851	2682	9.11	4.93	9.63	50.3	18.2	28.2
30°C 97%	3103	584.7	720.3	5047	911.1	1111	131.3	39.1	55.5	2341	1301	2927	92.6	84.9	920.2	75.1	23.8	34.5

[a]Back transformed log mean of four values

[b]Aflatoxin production

[c]Standard deviation (back transformed). Note that standard deviation values are not symmetric around the means (possess lower and upper limits) after transformation to the original scale.

Appendix 8. Back transformed B_1 aflatoxin production[a] (ppb) in Brazil nuts after 30 and 60 d storage under controlled conditions of relative humidity and temperature at different inoculation sites

	Half shelled nuts						Whole shelled nuts						In-shell nuts					
	30 d			60 d			30 d			60 d			30 d			60 d		
	AF[b]	S.D.[c]		AF	S.D.		AF	S.D.		AF	S.D.		AF	S.D.		AF	S.D.	
Conditions	(ppb)	Lower	Upper	(ppb)	Lower	Upper	(ppb)	Lower	Upper	(ppb)	Lower	Upper	(ppb)	Lower	Upper	(ppb)	Lower	Upper
80% 30°C	1.56	1.39	3.05	7.54	1.01	1.14	0.313	0	0	0.313	0	0	0.389	0.148	0.166	1.34	0.833	1.29
85% 30°C	400.2	153.3	248.0	1870	716.2	1161	2.33	0.810	1.08	0.827	0.427	0.557	0.594	0.357	0.460	0.799	0.356	0.444
97% 30°C	1934	988.9	2023	2202	673.0	969.1	37.8	18.1	33.9	1081	727.2	2215.4	48.8	46.3	672.9	14.9	9.96	26.8
13°C 97%	9.b62	1.74	2.08	13.1	3.85	5.30	0.396	0.161	0.182	0.313	0	0	0.313	0	0	0.313	0	0
15°C 97%	21.9	9.50	16.2	10.3	1.18	1.31	0.419	0.204	0.238	0.827	0.427	0.557	0.538	0.262	0.315	0.875	0.038	0.039
25°C 97%	90.6	22.1	29.15	4483	2143	4105	158.8	86.5	188.8	2539	278.1	312.3	0.488	0.202	0.234	3.44	2.45	5.46
30°C 97%	1934	988.9	2023	2202	673.0	969.1	37.8	18.1	33.9	1081	727.2	2215.4	48.8	46.3	672.9	14.9	9.96	26.8

[a]Back transformed log mean of four values

[b]Aflatoxin production

[c]Standard deviation (back transformed). Note that standard deviation values are not symmetric around the means (possess lower and upper limits) after transformation to the original scale.

Appendix 9. Analysis of Variance (ANOVA): influence of relative humidity and inoculation site on total and B_1 aflatoxin production in Brazil nuts after 30 and 60 d of storage at 30°C

Source	F	Type III SS	Mean Square	F Value	Pr > F
Inoculation site	2	33.96602231	16.98301115	144.12	<.0001
Relative humidity	2	58.44310301	29.22155150	**247.97**	<.0001
Storage time	1	3.485117853	3.48517853	29.58	<.0001
Relative humidity * Inoculation site	4	15.07412885	3.76853221	31.98	<.0001
Inoculation site * Storage time	2	0.84567909	0.42283955	3.59	0.0344
Relative humidity * Storage time	2	0.07486056	0.03743028	0.32	0.7992
Inoculation site * Relative humidity * Storage Time	4	1.62710734	0.40677683	3.45	0.0139

Appendix 10. Analysis of Variance (ANOVA): influence of temperature and inoculation site on total and B_1 aflatoxin production in Brazil nuts after 30 and 60 d of storage at 97% r.h.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Inoculation site	2	33.78099853	16.89049926	233.69	<.0001
Temperature	3	92.41273272	30.80424424	**426.20**	<.0001
Storage time	1	5.69306779	5.69306779	78.77	<.0001
Inoculation site * Temperature	6	7.93119912	1.32186652	18.29	<.0001
Inoculation site * Storage time	2	2.56575586	1.28287793	17.75	<.0001
Temperature * Storage Time	3	1.63453031	0.54484344	7.54	0.0002
Inoculation site * Temperature * Storage time	6	1.45915207	0.24319201	3.36	0.0056

Appendix 11. Sanitation protocols for Brazil nuts contaminated with *Salmonella* Typhimurium 02-8423

Sanitation method	Soaking water[a] (cfu/ml)	Attachment[b] (cfu/g)	After sanitation[b] (cfu/g)
Rapid immersion in 5% chlorine	2.8×10^7	4.7×10^6	2.6×10^5
Rapid immersion in 5% chlorine, brushed and reimmersed in 5% chlorine	1.2×10^8	2.2×10^6	3.9×10^4
Rapid immersion in 5% chlorine, brushed and reimmersed in 5% chlorine	7.0×10^4	4.3×10^3	9.0×10^2
Rapid immersion in 95% EtOH and flame off	5.2×10^7	4.2×10^5	1.3×10^4
Rapid immersion in 0.1% mercuric chlorine	3.3×10^7	5.2×10^6	5.5×10^4
0.4% chlorine and 2 min	3.3×10^7	5.2×10^6	4.6×10^4
Heat 50°C for 15 min	2.5×10^7	2.4×10^6	2.4×10^6

[a]Average of two values. Samples taken after 6 h of soaking

[b]Average of five sample: five nuts per sample