INFLUENCE OF WATER ACTIVITY, TEMPERATURE, OIL CONTENT AND PROBIOTIC BACTERIA ON GROWTH AND OCHRATXOIN A PRODUCTION BY *ASPERGILLUS FRESENII* AND *ASPERGILLUS SULPHUREUS*

by

Yung-Chen Hsu

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STATEMENT OF COMMITTEE APPROVAL

Dr. W. T. Evert Ting, Chair

Department of Biological Sciences

Dr. Dawit Gizachew

Department of Chemistry and Physics

Dr. Scott Bates

Department of Biological Sciences

Approved by:

Dr. Robin W. Scribailo

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Ochratoxin A (OTA) is a ubiquitous mycotoxin produced by some species of *Aspergillus* and *Penicillium*. It has been detected in a variety of foods such as cereals, coffee, grapes, cocoa, wine, and spices. Consumption of OTA has been linked to kidney and liver diseases. The aims of this study were to determine the effects of (1) water activity and temperature (2) oil content and grinding and (3) probiotic bacteria on fungal growth and OTA production by *Aspergillus fresenii* and *A. sulphureus*. In the first study, the two fungi were grown on ground Niger seeds with 0.82, 0.86, 0.90, 0.94 or 0.98 a_w and incubated at 20, 30 or 37°C individually. The two species showed similar growth patterns on Niger seeds under all of the testing conditions. There was no fungal growth on ground Niger seeds with 0.82 a_w and the optimal growth condition for the two species on ground Niger seeds was 0.94 a_w at 30°C. However, the optimal conditions for OTA production by *A. fresenii* and *A. sulphureus* were different. The optimal conditions for *A. fresenii* to produce OTA on ground Niger seeds was 0.90-0.94 a_w at 37°C; whereas, *A. sulphureus* produced OTA optimally with 0.90-0.94 a_w at 30°C as well as 0.94-0.98 a_w at 20°C. Overall, *A. sulphureus* produced higher levels of OTA than did *A. fresenii*. The highest concentration of OTA (643 µg/kg) produced by *A. fresenii* was detected on seed samples with 0.90 a_w incubated at 37°C for 15 days, while the highest concentration of OTA (724 µg/kg) produced by *A. sulphureus* was detected on samples with 0.98 a_w incubated at 20°C for 10 days.

In the second study, growth and OTA production by the two fungi on ground Niger seeds with different oil content (10, 25 and 35%) and on whole Niger seeds at 30°C were compared. All seed samples were adjusted to 0.94 a_w in this study. The two fungi grew most rapidly on ground seeds with 35% oil content, producing high concentrations of OTA (229-453 µg/kg). On whole seeds, *A. sulphureus and A. fresenii* displayed slow growth until day 5 or 10, respectively, growing rapidly after that. The two species produced either non-detectable or below the limit of quantitation (<4 µg/kg) of OTA in ground seeds with 10 or 25% oil or in whole seeds during the 30-day incubation at 30°C.

In the third study, growth inhibition of *A. fresenii* and *A. sulphureus* by probiotic bacteria *Bacillus coagulans*, *B. coagulans* (strains unique IS2™ and GBI-306086), *Lactobacillus acidophilus* (strains LA-5 and LA-14), *L. plantarum* (strains 299V and LP115), and *L. rhamnosus*

was evaluated. Results of co-cultured method revealed that *L. plantarum* 299V had the highest levels of inhibition against the two fungal species; whereas, *L. plantarum* LP115, and *L. rhamnosus* showed only some inhibition effect against *A. sulphureus* and very little inhibition against *A. fresenii*. The two fungal species were not inhibited by *L. acidophilus* or *B. coagulans*.

Results from double-layer testing showed that the two *L. plantarum* strains and *L. rhamnosus* inhibited fungal growth completely when there were as few as 40-70 CFU probiotic bacterial colonies in the bottom layer of MRS agar; whereas, *L. acidophilus* inhibited fungal growth completely when the probiotic colonies were >125 CFU/plate. The three *B. coagulans* strains showed only partial growth inhibition against *A. fresenii* with 10^3 CFU/plate. *Bacillus coagulans* (unique IS2™ and GBI-306086) completely inhibited growth of *A. sulphureus* when there were as few as 40-70 CFU/plate; while *B. coagulans* completely inhibited the growth of *A. sulphureus* but only when there were $>10^3$ CFU plate. Even though the two fungal species were inhibited by some probiotic bacteria on MRS plates, the OTA production was not influenced.

CHAPTER 1. INTRODUCTION

Ochratoxins are toxic secondary metabolites of some *Aspergillus* and *Penicillium* fungi. The main forms of the toxin are ochratoxin A, B and C. Ochratoxin A (OTA) consists of para-chlorophenolic moiety containing a dihydroiso-coumarin group which is amide-linked to L-phenylalanine (Malir et al., 2016). Ochratoxin B (OTB) is a non-chlorinated form of OTA; while ochratoxin C (OTC) is an ethyl ester of OTA (Heussner and Bingle, 2015) (Fig. 1). In ochratoxin group, OTA is the most common form poisoning humans and animals, and is naturally abundant (Kőszegi and Poór, 2016).

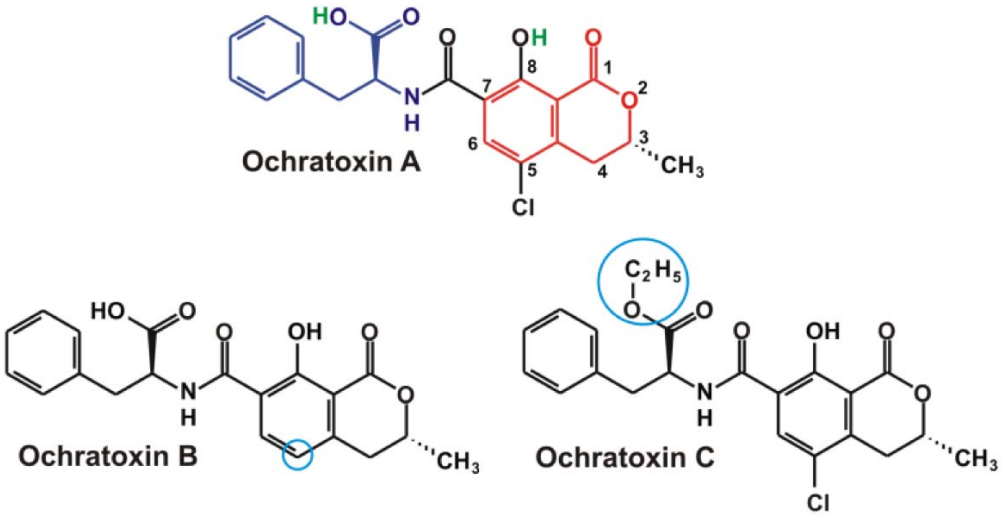

Figure 1. General structures of Ochratoxin A, B, C.
(Source: Zahra et al. (2016))

Ochratoxin A was first described as metabolite of *Aspergillus ochraceous* in 1965 (Van Der Merwe et al., 1965). This mycotoxin is produced by certain of *Aspergillus* species such as *A. ochraceous*, *A. carbonarius* and *A, niger* as well as by some species of *Penicillium*. It has been detected worldwide in various foods and feed such as cereals and cereals products. A European report estimated the contributions to the total human adult OTA exposure at 44% for cereals, 10% for wine, 9% for coffee, 7% for beer, 5% for cacao, 4% for dried fruits, 3% for meats and 3% for spices (European Commisson, 2002). Global occurrence data from 2006 to 2016, list the incidence

of OTA contamination in raw cereal grains at 29%, with the maximum concentration of OTA found in raw cereal grains at 1164 ng/g (Lee and Ryu, 2017).

Ochratoxin A contamination of food has become the global concern as it is a frequent and unpredictable problem that posting a difficult challenge for food safety. Moreover, OTA is a stable molecule which cannot be easily eliminated by most food processing methods such as baking, roasting, or brewing (Alshannaq and Yu, 2017). Consuming OTA contaminated foods or feeds cause a variety of adverse health effects; thus, it is a serious health threat for both humans and animals. These effects include kidney damage, interference of fetal development, mutagenicity, immunotoxicity, and neurotoxicity (Fink-Gremmels, 1999). Moreover, experimental and epidemiological evidence suggest that OTA exposure is associated with human cancer. In 1993, International Agency for Research on Cancer listed OTA as a possible carcinogen of group 2B (IARC, 1993).

Mycotoxin production is dependent on the fungal strain and can be influenced by a variety of factors including temperature, water activity, oxygen and CO_2 level, growth substrate or interactions with other microbes (Hesseltine, 1976; Jiménez and Mateo, 1997). Numerous studies have shown that OTA is usually formed in crops when storage conditions are favorable (Alshannaq and Yu, 2017; Magan and Aldred, 2005; Scudamore, 2005). Magan and Aldred (2005) reported that limiting moisture content for any potential OTA production in wheat grain is at approximately 17-18% (0.80-0.83 a_w).

1.1 Chemical Properties of OTA

Ochratoxin A is a weak organic acid and the molecular weight is 403.8 g/mol. Under the UV light, it shows green fluorescence under acid condition and blue fluorescence under alkaline condition (Pitt, 2013). Under acidic and neutral pH conditions, OTA is soluble in polar organic solvents, slightly soluble in H_2O and insoluble in saturated hydrocarbons; while under alkaline pH conditions, OTA is soluble in aqueous sodium bicarbonate and other alkaline solutions (Khoury and Atoui, 2010). The toxin is very resistant to heat, with previous study showing that OTA cannot be completely inactivated after dry heating to 250 °C for 10 min (Boudra et al., 1995).

1.2 OTA in Food and Feed

Ochratoxin A has been found in a wide variety of raw and processed foods, such as cereals, cocoa, coffee, dried fruit, grapes, nuts, spices, and wine (Palumbo et al., 2015), as the results of fungal growth on crops before and after harvest. Considering that mycotoxins can be transferred through the food chain, OTA may be found in animal products such as meat, milk and eggs when the animals are fed with moldy feed (Bennett and Klich, 2003; Gizachew et al., 2016). Krogh et al. (1976) found that pig kidneys contained the highest OTA concentration (25.7 ng/g) after bacon pig were administering 43 ng/g of OTA for one month.

According to a report from European Commission (2002), cereals are the major source of OTA contamination. The average OTA concentration in foods of plant origin, such as bean, beer, cereals, cocoa products, coffee, olives, pumpkin seeds, raisins, tea, and wine, ranged from 0.1 to 100 ng/g. Spices such as black pepper, chili powder, dried red pepper contain 1 to 100 ng/g of OTA. The average OTA concentration in feed of plant origin also ranged from 1 to 100 ng/g (Directorate General-Health and Consumer Protection, 2002; Ostry et al., 2013). Shotwell et al. (1969) found 110-150 ng/g OTA in corn obtained from commercial markets. Scott et al. (1972) detected the OTA in 18 out of 29 samples of heated grain, with the OTA concentrations ranging from 30-2700 ng/g.

In foods of animal origin such as chicken and pork, or dry-cured ham, the average concentration of OTA ranged from 0.1 to 1 ng/g. The same levels of OTA were also found in ingredients of animal origin in pet food (EFSA., 2005; Ostry et al., 2013).

1.3 OTA Producers

Ochratoxin A is mainly produced by species of *Aspergillus* and *Penicillium* (Table 1). The OTA producing *Aspergillus* species can be divided into two main section: *Circumdati* (*A. ochraceus* group) and *Nigri* (*A. carbonarius* and *A. niger*) (Hayat et al., 2012). The *Circumdati* section of *Aspergillus* can grow at low water activity and at moderate temperature (Ostry et al., 2013). They are largely responsible for OTA production in coffee and long stored dried grains (Pitt, 2013). The *Nigri* section of *Aspergillus* grow well at high temperature and are responsible for OTA production in dried vine fruits and grapes or, grape products including grape juice and wines and (Somma et al., 2012) and as well as in coffee in some regions (Hayati et al., 2011).

Aspergillus spp. are considered the main source of OTA production in relatively warmer regions, while *Penicillium* spp. are the main OTA producers in relatively colder area (Wang et al., 2016). In 1969, Walbeek et al. isolated OTA from mycelium and culture filtrate of *Penicillium verrucosum* which was grown on yeast extract (Walbeek et al., 1969). *Penicillium verrucosum* usually contaminated cereal in the cool temperature zones, being the major OTA producers for stored cereals in Northern Europe and Canada (Ostry et al., 2013). In 2001, *P. nordicum* was confirmed as the second OTA-producing *Penicillium* species (Larsen et al., 2001). *Penicillium nordicum* generally contaminates cheese, meat, and meat products which are rich in protein and NaCl (Schmidt-Heydt et al., 2012).

Table 1. Ochratoxin A producing fungi.

Genera	Section	Species	Source
Aspergillus	*Circumdati*	*A. cretensis*	citrus, soil
		A. flocculosus	saltern
		A. ochraceus	cereal, coffee, beverage, grape, maize
		A. seudoelegans	soil
		A. roseoglobulosus	decaying leave of Rhizophora mangle
		A. sclerotiorum	fruit, soil
		A. steynii	green coffee bean, rice, soybean
		A. sulphureus	alkaline soil
		A. westerdijkiae	rice, beverage, green coffee bean, spics, corn, grapes
	Flavi	*A. alliaceus*	great barrier reef, kemiri nut, soil
	Nigri	*A. carbonarius*	grape, beer, coffee
		A. lacticoffeatus	coffee bean, soil
		A. niger	grape, beer, cereal, coffee,
		A. sclerotioniger	coffee bean, green coffee
Penicillium	*Viridicata* (Samson et al., 2004)	*P. nordicum*	cheese, fermented meats
		P. verrucosum	cereal, grape, wheat, rye, barely

Source: Wang et al. (2016)

1.4 OTA Toxicity

Previous studies showed that OTA may have nephrotoxic, immunotoxic, neurotoxic and teratogenic effects on several animals species, and can cause kidney and liver tumors in mice and rats (Duarte et al., 2011; Hagelberg et al., 1989; IARC, 1993; Kuiper-Goodman and Scott, 1989) . However, OTA toxicity varies depending on the animals species and sex, and the cellular type of the tested animals (O'Brien et al., 2001).

1.4.1 Nephrotoxicity

Epidemiological studies showed that OTA usually correlated to the porcine and poultry nephropathy (Elling et al., 1985) and is also associated with human nephropathy such as Balkan endemic nephropathy (BEN) (Malir et al., 2016). Balkan endemic nephropathy is a chronic kidney disease which could be associated with urinary tract tumors (Pfohl-Leszkowicz et al., 2002). The pathogenic characters of the disease are progressing atrophy and sclerosis of all structures in the kidney (Pavlović, 2013). This disease posts a high prevalence rate in the south-eastern Europe including Bosnia, Bulgaria, Croatia, Herzegovina, Romania and Serbia (Pavlović, 2013).

1.4.2 Neurotoxicity

During fetal development, nerve tissue seems to be very susceptible to the harmful effects of OTA (Paradells et al., 2015). Previous studies found that OTA can decrease the viability of neural cell and induce apoptosis in primary neurons (Sava et al., 2007; Zhang et al., 2009). In vitro studies, OTA was found to inhibit the proliferation and differentiation process of neural stem cells (Hong et al., 2000); while in vivo studies, OTA was shown to induce malformation in the central nervous system in the pregnant animals (Pfohl-Leszkowicz and Manderville, 2007). Moreover, Sava et al. (2006) demonstrated that the administration of 3.5 mg/kg OTA to mice resulted in oxidative stress in six brain regions.

1.4.3 Teratogenicity

Ochratoxin A is a potent teratogen in rats (Mayura et al., 1984), mice (Hsuuw et al., 2013), hamsters (Hood et al., 1976) and chick embryos (Gilani et al., 1978). Maternal OTA can move across the placenta and accumulate in fetal tissues which causes various anomalies (Khoury and

Atoui, 2010). Hayes et al. (1974) reported that pregnant mice treated with 5 mg/kg OTA during gestation period resulted in increased prenatal mortality, decreased fetal weight and caused various fetal malformation.

1.4.4 Carcinogenicity

In 1993, the International Agency for Research on Cancer (IARC) classified OTA as a possible human carcinogen (group 2) because of the sufficient evidences of OTA carcinogenicity shown in experimental animal studies (IARC, 1993). Early studies showed that hepatocellular neoplasm, renal tumors (renal tubular adenomas and renal cell carcinomas) were observed in male rats which had OTA administered in the diet (Pfohl-Leszkowicz and Manderville, 2007). In another study, metastases from renal cell carcinomas to liver, lung and lymph nodes was observed in male and female rats after gave 70 or 210 g/kg OTA (Boorman, 1989).

1.5 Main Factors Influence OTA Production

1.5.1 Temperature

Ochratoxin A is frequently produced at sub-optimal growth temperatures. Mitchell et al., (2004) showed that the optimal temperatures for *A. carbonarius* strains to grow on synthetic grape juice medium were 30-35°C, while the maximum OTA production occurred at 20°C. Alborch et al. (2011) found that *A. niger* grew most rapidly on maize kernels at 25-40°C while the maximum OTA production was at 15°C.

1.5.2 Water activity

Previous studies showed that high concentrations of OTA were often produced in high water activity conditions. Pardo et al. (2004) observed that the optimal water activity for OTA production by *A. ochraceus* in barely grain was 0.99. Moreover, Esteban et al. (2006) found that OTA accumulation of *A. niger* increased with increasing water activity ranging from 0.92-0.98 in Czapek Yeast Extract agar (CYA) and Yeast Extract Sucrose (YES) agar. Rodríguez et al. (2015) reported that *Penicillium verrucosum* produced OTA on dry cured sausage with 0.90 to 0.97 a_w during ripening process and the maximum concentration of OTA was found at 0.97 a_w.

1.5.3 Growth Media and Substrate

Previous studies have also shown substrate is an important factor influencing the mycotoxin production. Esteban et al. (2006) found that OTA produced by *A. niger* was significantly higher in YES medium than in CYA medium. Liu et al. (2016) reported that maltose, glucose, sucrose could induce aflatoxin production by *A. flavus* up to 26-fold. Fanelli and Fabbri (1989) observed that aflatoxin production by *A. parasiticus* was much higher in oily seeds than in starchy seeds. In addition, Mellon et al. (2000) found that aflatoxin production by *A. flavus* was decreased more than 800-fold when *A. flavus* was grown on the cottonseed lacking oil. As grains have a complex nutrient composition, mycotoxin production is not only related to the lipid content, but may also be associated with other grain nutrients. Stossel (1986) found that zinc could increase aflatoxin biosynthesis in soybean. However, there is a lack of studies examining how nutrients in different grains may influence OTA production.

1.6 Detection of OTA

The current detection methods can be roughly categorized into two type, instrumental analyses and immunoassays. Instrumental analyses include high-performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC-MS/MS). Advantages of the chromatographic methods include high sensitivity, low detection limits and minimum requirements for sample preparation. The limit of detection (LOD) of HPLC and LC-MS/MS can be as low as 0.05 μg/kg (Pena et al., 2005) and 0.01 ng/ml (Solfrizzo et al., 2011), respectively. In the recent years, LC-MS/MS is frequently used for analysis of multiple mycotoxins residue in sample when a QuEChERS (quick, easy, cheap, effective, rugged and safe) sample preparation method was introduced (Schenzel et al., 2010; Shanakhat et al., 2018). The QuEChERS method reduces the error associated with different physicochemical properties of mycotoxins. Even though these chromatographic methods are highly sensitive and versatility, the analytical instruments are rather expensive, and they require well-trained technicians.

Enzyme-linked immunosorbent assay (ELISA) is widely used for OTA analysis because of its simplicity, capacity for parallel analysis of multiple samples, cost efficiency and quick results (Shanakhat et al., 2018). ELISA has been the preferred method to detect and quantify a variety of mycotoxins in beverages, bodies fluids, food and feed (Meulenberg, 2012). However, the ELLISA

method is not as sensitive as HPLC for detection of mycotoxin. The LOD of ELISA is around 5~0.2 ng/ml (Urusov et al., 2015; Meulenberg, 2012). A comparative study showed that ELISA tended to underestimate the OTA content as compared to the HPLC method (Matrella et al., 2006). In recent year, many commercial ELISA kits for mycotoxin detection have been developed. Also, it has been found that using immunoaffinity chromatography (IAC) to purify samples prior to the ELISA analysis may improve the sensitivity of mycotoxin detection by ELISA. Meulenberg, (2012) validated a procedure using an OTA ELISA kit in conjunction with an IAC kit for analysis of OTA in wine. The LOD was 0.054 ng/ml and the working range was 0.25~9 ng/ml, which are generally better than ranges reported for HPLC.

1.7 Control of OTA

1.7.1 Current Regulation of OTA in food

Ochratoxin A is subject to legal regulations at both national and international levels (Malir et al., 2016). According to a Food and Agricultural Organization (FAO) report in 2003, 37 countries had legal regulation limits on OTA in food and feed (FAO, 2003). At the global level, the Joint FAO/ World Health Organization (WHO) Expert Committee on Food Additives (JECFA) established the maximum limit (5 µg/kg) of OTA in wheat, barley and rye. In addition, JECFA established a provisional tolerable weekly intake (PTWI) which is 100 ng/kg body weight (b.w.) of OTA (FAO/WHO, 1996). The regulation of the limits on OTA in foods was first set in place by the Commission Regulation (EC) No.472/200 in the European Union (EU) in 2001 and has been amended several times since then (Figure 2). The current maximum limits for OTA in foods set by European Commission Regulation (EC) No 1881/2006 is summarized in Table 2. For example, up to 5 and 3 µg/kg of OTA are allowed in unprocessed cereals and products derived unprocessed cereals respectively in EU. However, there is no advisory or regulation action limits on OTA in food or feed in the USA.

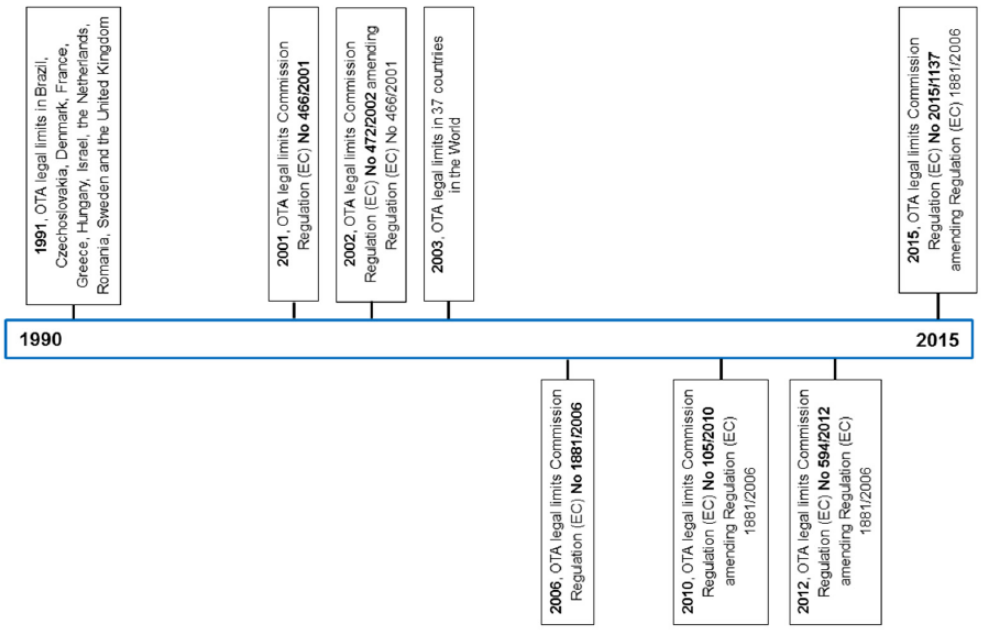

Figure 2. The milestone in evolution of legal regulation of OTA
(Source: Malir et al., (2016))

Table 2. Maximum levels of OTA in foodstuffs set by the European Union Commission Regulation (EC) No 1881/2006.

Food	Maximum Levels (µg/kg)
Unprocessed cereals	5.0
All products derived from unprocessed cereals, including processed cereals products and cereals product intended for direct human consumption	3.0
Dried vined fruit (currants, raisins and sultans)	10.0
Roasted coffee bean and ground roasted coffee	5.0
Soluble coffee (instant coffee)	10.0
Wine (including sparkling wine, excluding liqueur wine and wine with an alcoholic strength of not less than 15% vol) and fruit wine	2.0
Aromatized wine, aromatized wine-based drinks and aromatized wine-product cocktails	2.0
Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must	2.0
Processed cereal-based foods and baby foods for infants and young children	0.5
Dietary foods for special medical purposes intended specifically for infants	0.5
Spices including dried spices - White pepper, black pepper, nutmeg, ginger, turmeric - Whole or ground chilies, cayenne, paprika and chili powder - Mixtures of spices containing one of the above-mentioned spices	15.0
Liquorice root, ingredient for herbal infusion	20.0
Liquorice extract for use in food in particular beverages and confectionary	80.0
Wheat gluten not directly to the consumer	8.0

1.7.2 Prevention of fungal contamination

To control OTA in foods and feeds, preventing or eliminating fungal contamination at the pre-harvest phase is a first step. Plants damage caused by mechanical processes or insects can contribute to fungal contamination. However, agricultural practices such as crop rotation, timed plating and harvest, and the use of pesticides can reduce mycotoxin contamination in field crops (Park et al., 1999). Rousseau and Blateyron (2002) indicated that appropriate vineyard management could decrease 80% OTA occurrence in wine. However, in 2008, European Union established a strict legislation about the maximum residue levels of pesticides in the food and feed due to the increasing number of resistant fungal strains and the impact of fungicides on environment and human health.

Although the prevention of OTA contamination through pre-harvest management is the main goal of the agricultural and food industries, the contamination of *Aspergillus* or *Penicillium* species and subsequent OTA production is common under certain environmental conditions. In the post-harvest phase, storage and processing are the major stages where OTA contamination can be prevented. The strategies, which are focused on preventing fungal infection, include improvement of drying and storage conditions, use of irradiation, and fumigation with chemical or natural agents (Varga et al., 2010). Storage is the most critical post-harvest consideration in food handling. An accumulation of moisture and heat can enhance fungal invasion and lead to the occurrence of OTA contamination. In the processing stage, several physical and chemical methods have been developed to control and reduce fungal proliferation and subsequent mycotoxin production. Some chemical preservatives are able to prevent fungal growth and OTA formation in bread, including calcium propionate and potassium sorbate (Arroyo et al., 2005; Marín et al., 2002). Other antimicrobial food additives such as methyl para-hydroxybenzoic acid, propyl-paraben and sodium propionate have also been found to inhibit fungal growth and OTA production. (Barberis et al., 2009; Tong and Draughon, 1985).

In the last decade, researchers have shown an increasing interest on using bacteria, yeast and non-toxic fungi to inhibit the growth of OTA producing fungi. Lactic acid bacteria (LAB) and yeasts are potential biocontrol agents widely used in fermented foods and some of them are also a part of the intestinal flora in humans (Kapetanakou et al., 2012). Numerous studies have shown that particular LAB species can inhibit fungal growth (Fuchs et al., 2008; Dalié et al., 2010). This antagonistic effect owed to low-molecule-weight compounds produced by lactic acid bacteria such

as organic acid (acetic and lactic acid), hydrogen peroxide, proteinaceous compounds and reuterin. Pervious study also indicated that organic acids inhibits absorption of amino acids of fungi (Dalié et al., 2010). Moreover, reuterin, a compound resulted from glycerol fermentation by LAB under anaerobic condition, is known to suppress the activity of ribonuclease in *Fusarium* and *Aspergillus* species. (Langa et al., 2014).

1.7.3 Reduction of OTA

The ideal solution for reducing the risk of OTA is to prevent fungal infection in food sources. Unfortunately, the OTA contamination is difficult to completely avoid; therefore, decontamination or detoxification of OTA in food has been investigated. The OTA decontamination or detoxification can be classified into physical, chemical or (mico)biological approaches, aiming to reduce or eliminate OTA by degradation, modification and absorption.

The physical methods include heat treatment and irradiation. Urbano et al., (2001) found that the OTA in coffee beans was reduced up to 94% after roasting at 220°C for 15 mins. Using ϒ-irradiation to remove mycotoxins has been investigated by several researchers (Calado et al., 2014; Di Stefano et al., 2014). Kumar et al., (2012) reported that OTA in green coffee beans with 0.82 a_w was reduced by 90% after irradiation by 10kGy of ϒ-rays. However, the mycotoxin-degrading capability of ϒ-irradiation could depend on several factor, including type of mycotoxin, radiation dose, matrix composition, and water content of the product (Calado et al., 2018).

A wide variety of chemicals have also been found to destroy OTA. Ethyl acetate and dichlorometane were found to reduce up to 80% of OTA in coffee beans (Bortoli and Fabian, 2005). In cocoa, alkaline treatment could eliminate OTA levels more than 98% (Amezqueta et al., 2016). Moreover, ozone has been shown to remove OTA in grains, nuts or vegetables (McKenzie et al., 1997). However, chemical treatment is not allowed in the EU for products that are consumed by human (Varga et al., 2010).

Using microorganisms and their enzymes for mycotoxin detoxification have been widely studied. Numerous bacteria, protozoa, and fungi have been found to degrade or remove OTA in contaminated food (Varga et al., 2010). Khoury et al. (2017) showed that *Actinobacteria* strains were able to bind and remove OTA up to 52% in the ISP2 liquid medium. Latic acid bacteria have also been reported to remove up to 84% of OTA in the liquid media (Del Prete et al., 2016; Taroub et al., 2019). Since the degraded OTA products were not detected, the authors suggested that the

LAB remove OTA from the media by binding the OTA on their cell wall. Cell wall of LAB, which contains peptidoglycan matrices, neutral polysaccharides, teichoic acid etc., were able to adsorb mycotoxins (Bolognani et al., 1997; Zhang and Ohta, 1991). Fuchs et al., (2008) reported that *Lactobacillus acidophilus* was able to remove 97% of OTA in liquid media. Bielecki and Tramper (2000) investigated OTA removal from dough fermentation using LAB and found that *L. plantarum* removed 56% of OTA in the dough. Moreover, Haskard et al., (2001) showed that inactivated LAB exhibited higher OTA removal capacity, whereas, live LAB may release some of the mycotoxin adsorbed on cell wall over time.

1.8 Probiotic Bacteria

Probiotic bacteria have been added to a wide variety of food and feed to promote health of humans and animals respectively. Several studies have shown that some probiotic bacteria specially LAB were able to reduce the contamination of mycotoxin in media and wine. (Del Prete *et al*., 2016; Fuchs et al., 2008; Taroub *et al*., 2019). Since there is no safety concern of using probiotic bacteria in food or feed, they have been studied as potential biocontrol agents to reduce mycotoxin in foods (Kapetanakou et al., 2012; Meca et al., 2010; Taroub et al., 2019). However, there is a lack of studies examining the effect of probiotic bacteria on fungi growth and OTA production.

1.9 Niger Seeds

Niger (Guizotia abyssinica) seeds are used for extracting cooking oil and as bird feed. A previous study found heavy aflatoxin contamination in raw milk in the Greater Addis Ababa Milk shed, Ethiopia (Gizachew et al., 2016). Further investigation revealed that the cow was fed with aflatoxin contaminated Noug cake, which is a byproduct from Niger seeds after oil extraction. Apparently, Noug cake supported mold growth and aflatoxin production. Noug cake may also support production of other types of mycotoxins, such as OTA.

1.10 Objectives

Numerous studies have showed that mycotoxin productions are influenced by mold species, growth substrate, and incubation condition such as temperature, and water activity. Furthermore,

the lipid content in substrates has also been found to influence fungal growth and aflatoxin production (Fanelli and Fabbri 1989; Mellon et al. (2000). Nevertheless, the effects of water activity, temperature, and oil content on growth of *Aspergillus* species and OTA production on Niger seeds have not yet been studied.

The objectives of the study were to test (1) the effects of water activity and temperature on growth and OTA production by *A. fresenii* and *A. sulphureus*, on ground Niger seeds (2) the effects of oil content and grinding on growth and OTA production by the two *Aspergillus* species on Niger seeds, and (3) the effect of probiotic bacteria, including *Lactobacillus* and *Bacillus* species, on growth and OTA production by the two *Aspergillus* species on De Man-Rogosa-Sharpe (MRS) agar plates.

CHAPTER 2. EXPERIMENT METHOD

2.1 Effect of the Temperature and Water Activity on Fungal Growth and OTA Production

2.1.1 Fungal Cultures

Two OTA producing fungi, *Aspergillus fresenii* (NRRL 661) and *A. sulphureus* (NRRRL 4077) were obtained from the US Department of Agriculture (USDA) Culture Collection. *Aspergillus fresenii* was isolated from shelled Brazil nuts and *A. sulphureus* was isolated from the soil in India (ARS Culture Collection, n.d.). The two fungi were maintained individually on Czapek Yeast Extract Agar (CYA) slants. Stock cultures were maintained at 4°C. Working cultures were grown on CYA agar slants individually at 30°C for 5 days. Spore suspension of each strain was prepared in a sterile aqueous solution of 0.005% Tween 80. Each spore suspension was adjusted to 0.25 OD_{540mm} which contained approximately 10^6-10^7 conidia/ml.

2.1.2 Inoculation and Mycelial Growth Measurement

Niger seeds which were purchased from a local store were grinded and sterilized before use. The sterile ground seeds were adjusted to 0.82, 0.86, 0.90, 0.94 and 0.98 a_w by adding 1.2, 1.5, 2, 3.8 and 8 ml sterile deionized water respectively to 25 g seeds. Water activity of each seed sample was measured twice using a water activity meter (Aqualab, Pullman, Washington, USA). Before each measurement, the seeds were shaken gently to make sure that the water was distributed uniformly within the seeds. Ground seeds (2.5g) were placed in each petri dish and point inoculated with 10 µl of the spore suspension containing $10^{4\text{-}5}$ conidia at the center. Plates were incubated at 20, 30 or 37°C for 5, 10, 15 and 30 days. Duplicate plates were used for each test conditions. All plates were sealed with parafilm and incubated in close glass jars in the presence of a wet paper towel to maintain the water activity of the seeds. Fungal growth on each plate was examined after incubation for 5, 10, 15 and 30 days. Two diameters at the right angles of the fungal colony were measured (Fig. 3), and the mean of the two measurement was recorded as colony diameter.

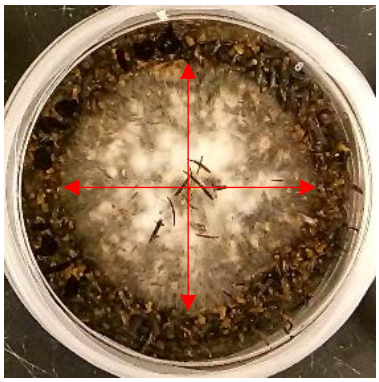

Figure 3. Measurement of fungal growth.

2.1.3 OTA Extraction and Purification

The same duplicate samples used for fungal growth measurements were removed from different incubation condition after 5, 10, 15 and 30 days and store at -20°C until OTA extraction. All ground seeds in each petri dish were transferred into a tube which contained 6 ml of 70% aqueous methanol. Each mixture was shaken at 150 rounds/min for 90 min and filtered through 0.22 µm sterile syringe filter (VWR® Syringe Filters). Each of the crude OTA extract was further purified using immunoaffinity column (VICAM, USA). One milliliter of the OTA extract was first diluted with 4 ml of PBS and loaded on the column. Then 5 ml of PBS and 5 ml of DI water were used to wash the column at a flow rate of 1 drop/sec. The OTA was eluted from the column with 1.5 ml of methanol at rate of 1 drop/sec.

2.1.4 Determination of OTA Production

Ochratoxin A concentrate in the seeds were determined and quantified by Thermo Scientific ultimate 300 High-Performance Liquid Chromatogram (HPLC) equipped with fluorescence detector. The chromatogram was recorded at 330 nm excitation and 460 nm emission wavelength using a reverse phase column C18, 4.6 mm × 150 mm, 3.5 mm, and isocratic mobile phase with a flow rate of 1 ml/min, consisting of the following gradients: acetonitrile, 57%; water, 41%; and acetic acid, 2% (HPLC-grade solvents from Fischer Scientific).

2.2 Effect of Oil Content and Grinding on Fungal Growth and OTA Production

2.2.1 Oil Extraction

The Niger seeds were pressed by an automated oil press machine (Vevor, CXZZC00, Shanghai, China). The seed oil was left at room temperature for one to two days to allow debris in the oil to settle down. The clear seed oil was removed and sterilized at 170°C, 90 min in a hot air oven (Fisher Scientific Isotemp oven 637G, USA). The pressed seeds were sterilized at 121°C for 15 min in an autoclave. The sterile pressed seeds and seed oil were stored at room temperature until use. Bligh/Dyer lipid extraction method was used to determine the oil content in the pressed seeds. First, 4 g of the pressed seeds were mixed with 10 ml chloroform and 20 ml of methanol and rotated for 20 mins on a shaker (Barnstead Thermolyne Labquake, USA). Second, additional 10 ml of chloroform was added to the mixture, rotated for 5 mins and centrifugated at 1500 rpm for 20 mins. The mixture was then filtered through 11 µm filter paper (Whatman filter No. 1) using a vacuum filter system. The filtrate was transferred into a graduated cylinder and allowed the mixture to separate into two phases. Third, the upper layer was discarded. The lower layer was transferred onto a pre-weighted aluminum plate and heated on a hot plate with low heat (40-50°C) in a hood until a thin layer of lipid was left on the plate. Forth, the aluminum plate was dried at 105°C for 15 mins in a hot air oven. The lipid in the aluminum plates were weighted. The lipid weight was used to calculate the oil content in the pressed seed.

Oil content (%) = (lipid weight / pressed seeds weight) x 100%

2.2.2 Preparation of Ground Seeds with Different Oil Content

Ground Niger seeds natural contain 35% oil and contain 10% oil after pressing. To adjust the oil content of the ground seeds, sterile seed oil was added to the pressed seeds. The pressed seeds and oil mixture were blended with a commercial blender (Waring Commercial, USA) for 5 mins to make sure that the oil was distributed uniformly within the seeds.

2.2.3 Inoculation and Mycelial Growth Measurement

Sterile ground seeds with different oil content and whole seeds were adjusted to 0.94 water activity as described previously. The 2.5 g of the ground seeds with different oil content or whole seeds were placed in each petri dish (52 mm in internal diameter). Plates were centrally point

inoculated with 10 µl of the spore suspension containing 10^{4-5} conidia and incubated at 30°C for 5, 10 and 15 days. All plates were sealed with parafilm and incubated in close glass jars in the presence of a wet paper towel to maintain the water activity of the pressed seed. Fungi growth on each plate was determined as described previously.

2.2.4 Determination of OTA Production

Ochratoxin A concentrate in the pressed seeds were determined and quantified by HPLC. The OTA extraction and OTA analysis were done as described previously.

2.3 Effect of Probiotic on Fungal Growth and OTA Production

2.3.1 Bacterial Culture

All probiotic bacteria were isolated from commercial probiotic dietary supplements (Table 3). The probiotics were maintained individually on De Man-Rogosa-Sharpe (MRS) agar slants. Stock cultures were stored at 4°C after cultivated at 37°C for 24 hr. Working cultures of *Lactobacillus* spp. were prepared by inoculating stock cultures into MRS broth tubes and cultivated at 37°C for 24 hr. Working cultures of *Bacillus* spp. were prepared by inoculating stock cultures into MRS broth tubes and cultivated at 45°C for 24 hr.

Table 3. The probiotic bacteria used in this study and their sources.

Probiotic bacteria	Sources
Lactobacillus rhamnosus GG	Culturelle
Lactobacillus plantarum LP115	The smarter Pearls
Lactobacillus plantarum 299V	Nature Made
Lactobacillus acidophilus LA-5	TruBiotics
Lactobacillus acidophilus LA-14	Meijer probiotic Pearls
Bacillus coagulans unique IS2™	Align PREbiotic +probiotic
Bacillus coagulans GBI-306086	Schiff Digestive Advantage
Bacillus coagulans	OLLY Purely Probiotic

2.3.2 Fungal Growth Inhibition by Probiotic Bacteria

2.3.2.1 Co-Cultured Method

The probiotic bacterial suspensions were prepared and diluted as described previously. MRS agar plates were spread with 100 µl of fungi spore suspension (10^6 to 10^7 conidia/ml) and placed in a biosafety cabinet to dry for 5 min. Then different dilutions of probiotic suspensions were spot inoculated on the plates. All plates were incubated at 37°C for 10 days. The plates were examined for the formation of inhibition zones surrounding the area with bacteria growth. Four widths at the right angles of the clear ring were measured (Fig. 4), and the mean of the four measurements was recorded as inhibition zones. When the mean width of the clear ring was >2mm, the inhibition effect against mold growth was consider positive. When the width of the clear ring was 1-2 mm the inhibition effect was consider marginal.

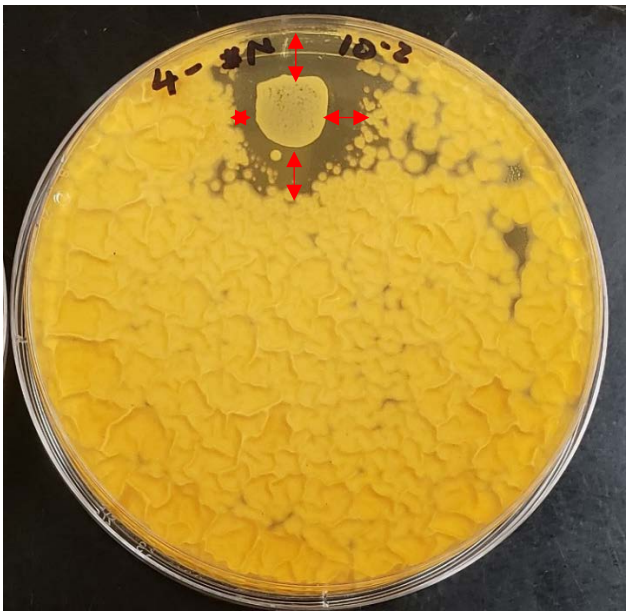

Figure 4. Measurement of clear ring surrounding growth of probiotic bacteria on MRS agar plate after incubation at 37°C for 10 days. This Figure illustrates a positive inhibition effect with a mean width of the clear ring > 2 mm.

2.3.2.2 Double Layer Method

Probiotic bacterial cells were harvested by centrifugation of a working probiotic culture at 15000 rpm for 15 min and the pellet was resuspended in phosphate-buffered saline (PBS) solution. Cell concentration was adjusted to 1.1 OD_{600nm} which equated to approximately 10^7-10^8 cells/ml. A serial 10-fold serial dilution of each probiotic suspension was prepared in PBS. Fifty µl of the diluted probiotic suspension was mixed with 10 ml of melted MRS agar and poured into a 60 mm petri dish. The MRS agar plates containing different dilutions of probiotic bacteria were placed in a sealed container containing a beaker of water to prevent the plates from drying. The MRS agar plates containing *Lactobacillus* bacteria or *Bacillus* bacteria were incubated at 37°C or 45°C respectively for 3 days. The plates were then overlaid with 5 ml of melted MRS agar (0.7%) containing 50 µl fungal spore suspension (10^6 to 10^7 conidia/ml). All the plates were incubated in sealed containers and incubated at 30°C. Fungal growth on each plate was examined after incubation for 10 days.

2.3.3 Influence of OTA Production by Probiotic Bacterial Cells

The same duplicate samples used for fungal growth inhibition in co-cultured method were removed after 10 days incubation. A 2.5 g agar plug which was covered by fungi mycelium in each petri dish was transferred into a tube containing 2 ml 70% aqueous methanol. The OTA extraction and determination were performed as described previously.

CHAPTER 3. RESULTS

3.1 Effect of Temperature and Water Activity on Fungal Growth and OTA Production

3.1.1 Fungal Growth on Ground Niger Seeds

Figure 5 shows the effect of a_w and temperature on growth rates and lag times of *Aspergillus fresenii* and *A. sulphureus on Niger seeds.* They displayed similar growth patterns on Niger seeds under all condition. Both were able to grow on Niger seeds with 0.86~0.98 a_w at 20, 30 and 37°C. Neither of the two fungi could grow on seeds with 0.82 a_w at the three incubation temperatures.

At 20°C, growth rates of the two fungi on Niger seeds were slow at all the water activities tested. The highest growth rates of the two fungi were detected from seeds with 0.94 a_w followed by 0.98, 0.90 and 0.86 a_w (Fig. 5a-5b). Only *A. sulphureus* reached confluent growth (52 mm in diameter) on the seeds with 0.94 a_w after incubation for 30 days at 20°C (Fig. 5b). On the seeds with 0.86 a_w, both fungi started the growth after a 10-day lag phase.

The highest growth rates of the two fungi were detected from seeds with 0.94 a_w at 30°C (Fig. 5c-5d) and confluent growth was found on seeds after 10 days of incubation. At 30°C, the reduction at water activity level from 0.94 to 0.86 resulted in a reduction of the growth rates. On the seeds with 0.90 a_w, confluent growth was reached after 15 days. The two fungi showed a 5-day lag time on the seeds with 0.86 a_w and confluent growth was found after 30 days of incubation. On the seeds with 0.98 a_w, two fungi grew during the first 15 days of incubation and the colonies diameter remined the same for the remaining incubation period.

When the incubation temperature increased to 37°C, both fungi started the growth after inoculation on the seeds with 0.86~0.98 a_w. On the seeds with 0.94 a_w, the growth rates of two fungi were slightly slower at 37°C as compared to those at 30°C. The confluent growth was found after 15 days of incubation. On the seeds with and 0.90 a_w, growth rates of the two fungi at 37°C were similar to those at 30°C and confluent growth was also reached after 15 days. On the seeds with 0.98 a_w, both fungi grew poorly at 37°C and the maximum mean diameters were <25 mm which were reached after 15 days of incubation.

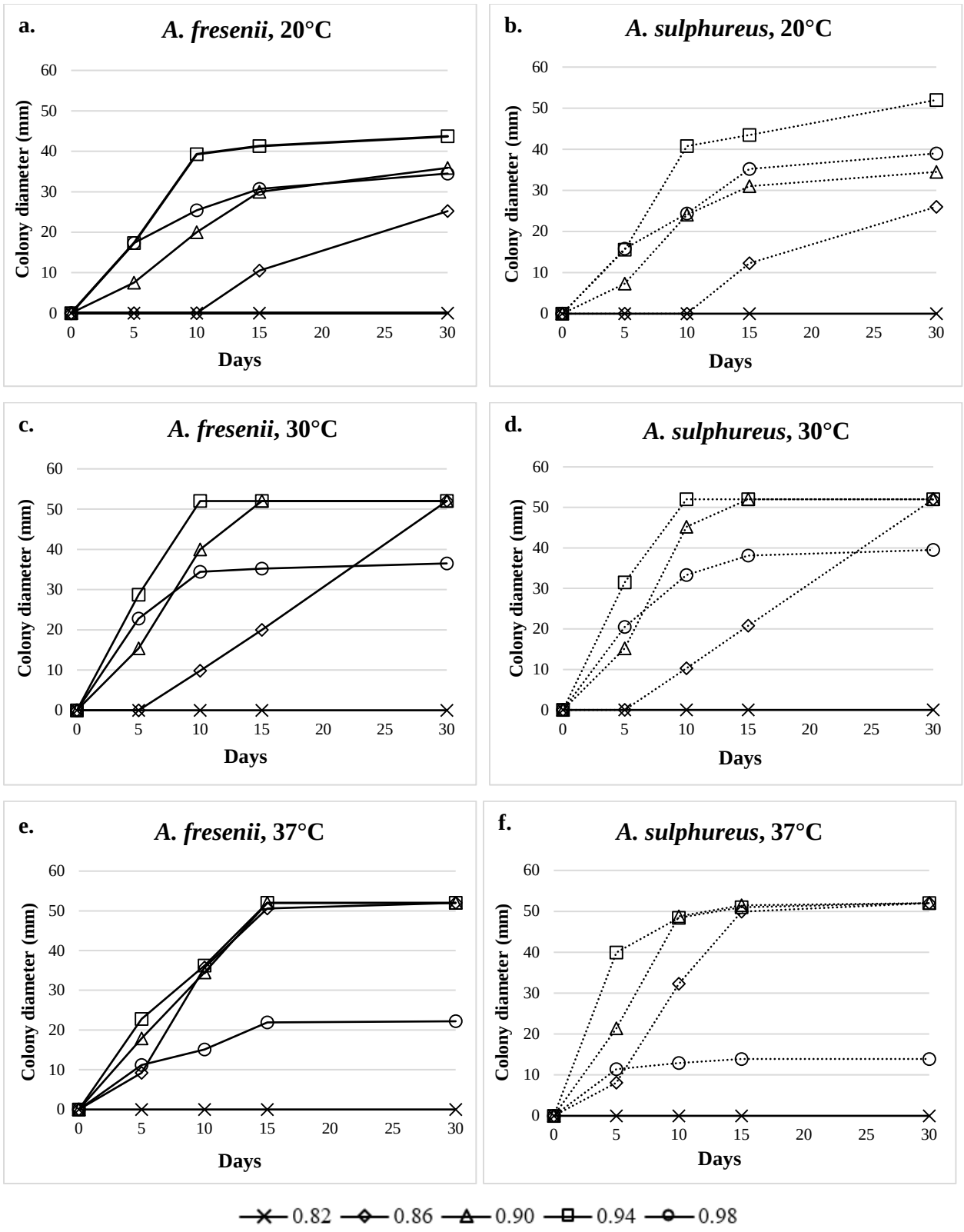

Figure 5. Mean colony diameters of *A. fresenii* (Fig. 5a, 5c, and 5e) and *A. sulphureus* (Fig. 5b, 5d, and 5f) on Niger seeds with 0.82, 0.86, 0.90, 0.94, or 0.98 a_w at 20, 30, and 37°C.

3.1.2 Ochratoxin A Production on Ground Niger seed

Table 4. summaries OTA concentrations detected from Niger seeds at each a_w, temperature and sampling incubation time. Even though two fungi displayed similar growth patterns on Niger seeds under all condition, the OTA production of two fungi showed a different trend. The OTA production of *A. fresenii* observed in the narrow range of condition and OTA production of *A. sulphureus* detected in the wide range of condition. The highest OTA (643 μg/kg) produced by *A. fresenii* was detected on the seeds with 0.90 a_w at 37°C within 15 days of incubation. The highest OTA (724 μg/kg) produced by *A. sulphureus* was detected on the seeds with 0.98 a_w at 20°C within 10 days of incubation.

At 20°C, OTA produced by *A. sulphureus* was appeared to increase with water activity of Niger seeds. The highest OTA (724 μg/kg) produced by *A. sulphureus* was detected on the seeds with 0.98 a_w (Fig. 6b) while *A. fresenii* produced low level of OTA at all testing water activity level (Fig. 6a).

When the incubation temperature increases to 30°C, the OTA production of *A. sulphureus* was decreased as the water activity increased from 0.90 to 0.98. The highest OTA concentration (650 μg/kg) was detected on the seeds with 0.90 a_w. However, the OTA production of *A. fresenii* was increased in the range of 0.90-0.94 a_w. The highest OTA concentration (332 μg/kg) was found in the seeds with 0.94 a_w.

At 37°C, the increasing of water activity level led to the lower OTA production. For *A. sulphureus*, the highest OTA production (286 μg/kg) showed on the seeds with 0.86 a_w. The OTA production for *A. fresenii* also decreased when the water activity increased from 0.90 to 0.98. The highest OTA concentration (643 μg/kg) was detected on the seeds with 0.90 a_w.

Table 4. Ochratoxin A produced by (a) *A. fresenii* and (b) *A. sulphureus* on ground Niger seeds at each water activity (a_w), temperature, and sampling incubation time.

a. *A. fresenii*

Day	OTA (µg/kg)											
	20°C				30°C				37°C			
	0.86	0.90	0.94	0.98	0.86	0.90	0.94	0.98	0.86	0.90	0.94	0.98
5	ND[1]	ND	ND	11	ND	ND	4	274	ND	4	15	11
10	ND	ND	ND	5	ND	ND	332	17	ND	57	31	12
15	ND	5	8	22	ND	37	65	ND	23	643	279	ND
30	8	ND	ND	ND	ND	ND	ND	ND	13	342	26	ND

b. *A. sulphureus*

Day	OTA (µg/kg)											
	20°C				30°C				37°C			
	0.86	0.90	0.94	0.98	0.86	0.90	0.94	0.98	0.86	0.90	0.94	0.98
5	ND	ND	ND	27	ND	11	24	27	ND	11	11	8
10	ND	9	41	724	ND	66	559	152	ND	8	18	7
15	ND	19	4	68	8	50	37	21	ND	30	31	14
30	63	142	680	4	50	650	5	ND	286	6	ND	ND

[1] ND: OTA was not detected.

Limit of quantification:4 µg/kg

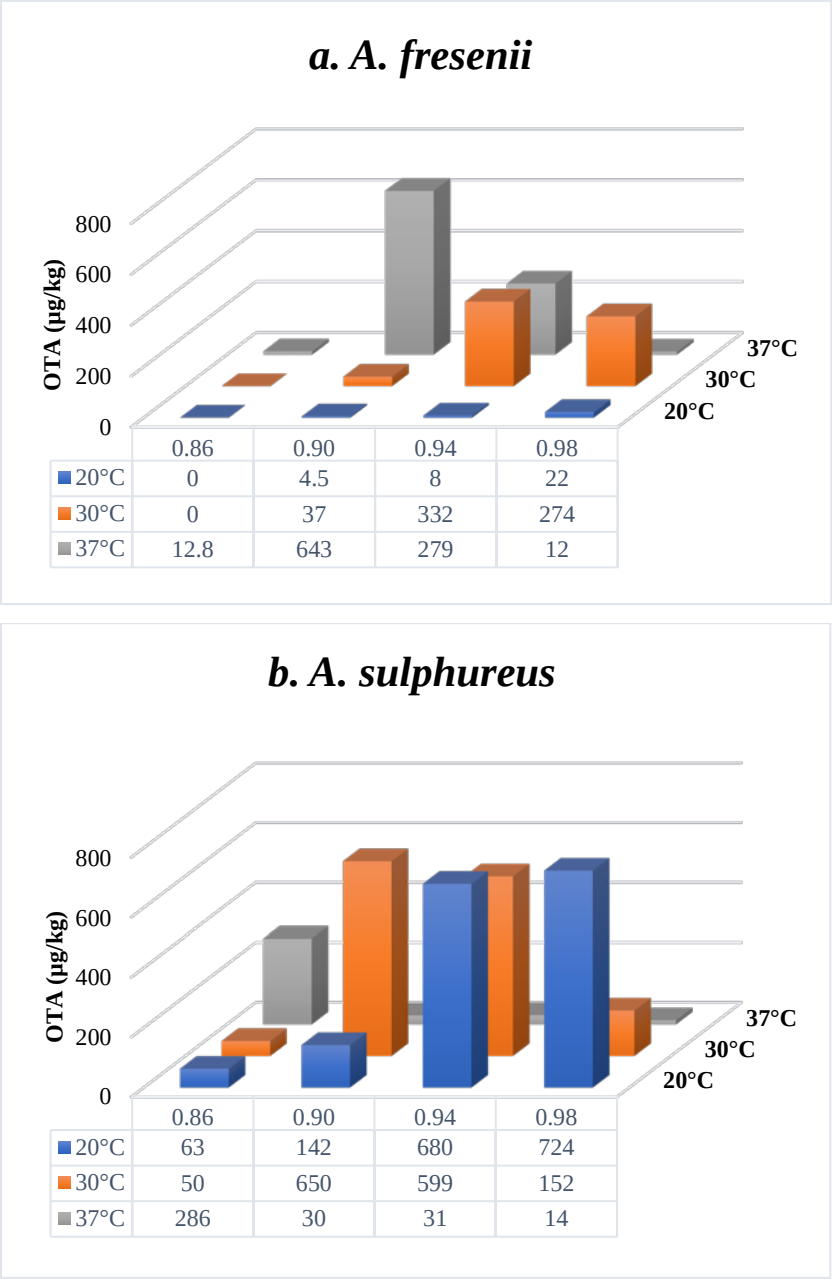

Figure 6. The maximum Ochratoxin A (OTA) production by (a) *A. fresenii* and (b) *A. sulphureus* on ground Niger seeds with 0.86, 0.90, 0.94 and 0.98 a_w during a 30-day incubation period at 20, 30 and 37°C.

3.2 Effect of Oil content and Grinding on Fungal Growth and OTA Production

3.2.1 Fungal Growth on Whole Niger seeds and Ground Niger Seeds with Different Oil Contents

Whole Niger seeds (35% oil content) and ground Niger seeds with 10, 25 or 35% oil contents were adjusted to 0.94 a_w. Growth of *A. fresenii* and *A. sulphureus* on these seed samples were compared at 30°C (Table 5). Both *A. fresenii* and *A. sulphureus* were able to grow on all the seed samples and they grew most rapidly on ground seeds with 35% oil (Fig. 7). Confluent growth was found after 10 days of incubation. Reduction of oil content in ground seeds resulted in reduction of the growth rates. On the ground seeds with 10% and 25% oil, the two fungi showed significant slower growth than on the ground seeds with 35% oil. The mean colonies diameters were 46-47 mm after 30 days of incubation. Both fungi showed slower growth on whole seeds than on ground seeds with the same oil content (35%) and the growth of *A. fresenii* was slower than the growth of *A. sulphureus*. The confluent growth of *A. fresenii* of *A. sulphureus* was found after incubation for 15 and 10 days respectively.

Table 5. Mean colony diameters of *A. fresenii* and *A. sulphureus* on ground Niger seeds with 10%, 25% or 35% oil content and whole Niger seeds at 30°C, 0.94 a_w.

Species	Day	Mean colony diameter ± SD (mm)			
		Oil content of ground Niger seeds			Whole seeds (35% Oil)
		10%	25%	35%	
A. fresenii	5	23.2 ± 1.2^{a1}	24.0 ± 1.5^{a}	27.8 ± 3.0^{a}	16.0 ± 1.0^{b}
	10	27.8 ± 2.3^{a}	33.6 ± 2.3^{b}	51.8 ± 0.3^{c}	36.4 ± 2.1^{b}
	15	34.7 ± 2.0^{a}	35.9 ± 2.5^{a}	52.0 ± 0.0^{b}	52.0 ± 0.0^{b}
	30	46.4 ± 2.1^{a}	47.4 ± 2.7^{a}	52.0 ± 0.0^{b}	52.0 ± 0.0^{b}
A. sulphureus	5	24.9 ± 2.0^{a}	24.5 ± 4.0^{a}	29.9 ± 2.3^{b}	21.7 ± 0.8^{a}
	10	31.2 ± 1.0^{a}	32.6 ± 2.5^{a}	52.0 ± 0.0^{b}	52.0 ± 0.0^{b}
	15	36.1 ± 1.6^{a}	37.5 ± 1.4^{a}	52.0 ± 0.0^{b}	52.0 ± 0.0^{b}
	30	46.9 ± 3.3^{a}	46.9 ± 3.7^{a}	52.0 ± 0.0^{a}	52.0 ± 0.0^{a}

[1]Mean colony diameters with different letters (a, b. and c) within a row are significant different ($P<0.05$).

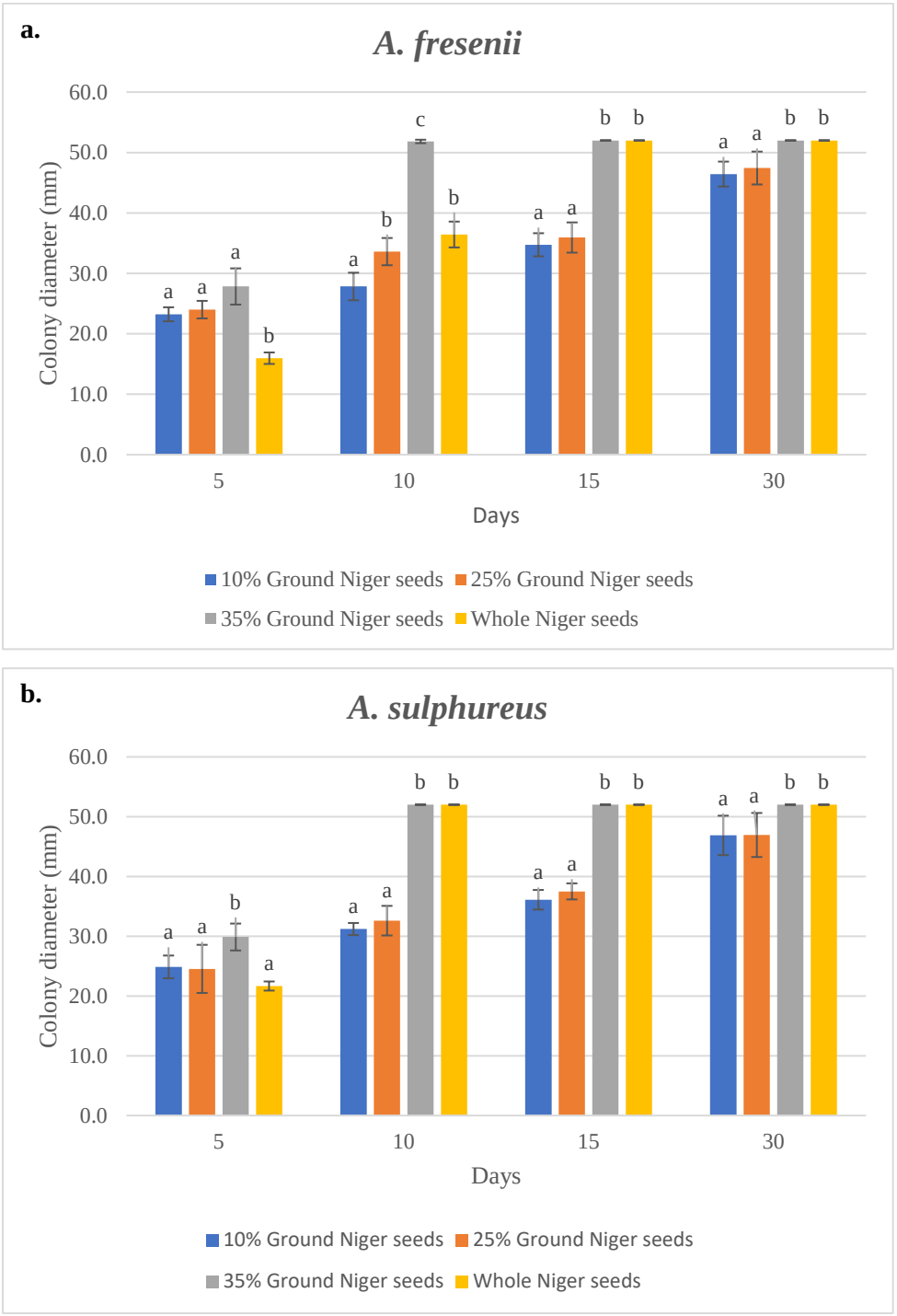

Figure 7. Mean colony diameters of (a) *A. fresenii* and (b) *A. sulphureus* on ground Niger seeds (0.94 a_w) with 10, 25 or 35% oil content and whole Niger seeds (0.94 a_w) at 30°C.

3.2.2 Production of OTA on Whole Niger Seeds and Ground Niger Seeds with Different Oil Contents

Table 6 summaries OTA concentrations detected from whole Niger seeds and ground Niger seeds with 10, 25 or 35% oil contents during a 30-day incubation at 30°C. In the ground Niger seeds with 35% oil contents, *A. fresenii* and *A. sulphureus* produced 229 and 453 µg/kg OTA respectively after incubation for 10 days. However, in seeds with 10 or 25% oil content, *A. fresenii* did not produced any OTA while *A. sulphureus* produced very low (<2 µg/kg) OTA during the 30-day incubation period although both fungi almost reached confluent growth on day 30 (Fig. 8). Similar result was found in the whole Niger seed samples. *A. fresenii* did not produced any while *A. sulphureus* produced up to 2.8 µg/kg of OTA during the 30-day incubation at 30 °C.

Table 6. Ochratoxin A (µg/kg) produced by *A. fresenii* and *A. sulphureus* in ground seeds with 10, 25% or 35% oil content and whole seeds at 30°C, 0.94 a_w.

Species	Day	OTA (µg/kg)			
		Oil content in ground seeds			Whole seeds (35% oil)
		10%	25%	35%	
A. fresenii	5	ND[1]	ND	ND	ND
	10	ND	ND	229	ND
	15	ND	ND	47	ND
	30	ND	ND	ND	ND
A. sulphureus	5	ND	0.6	ND	0.6
	10	1.8[2]	0.7	453	1.3
	15	1.7	1.0	24	2.8
	30	ND	ND	6.2	2.4

[1] ND: OTA was not detected.

[2]Limit of quantification is 4 µg/kg. OTA < 4 µg/kg was an estimated value.

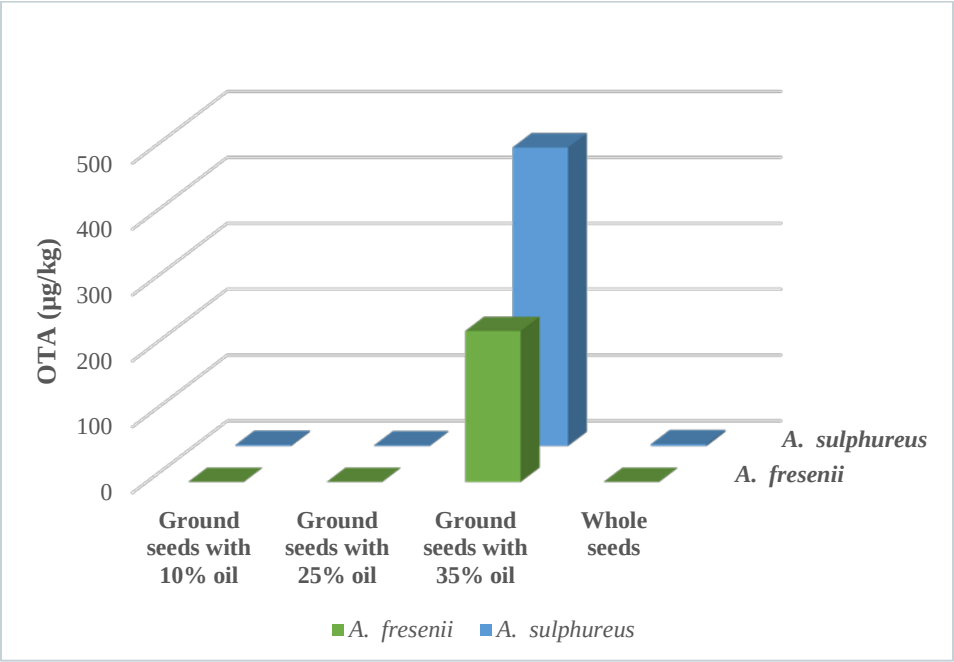

Figure 8. The maximum Ochratoxin A (OTA) production by *A. fresenii* and *A. sulphureus* on ground Niger seeds with 10, 25, 35% oil and whole seeds during a 30-day incubation period at 0.94 a_w, 30 °C.

3.3 Effect of Probiotic Bacteria on Fungal Growth and OTA Production

3.3.1 Fungal Growth Inhibition by Probiotic Bacteria

3.3.3.1 Co-Cultured Method

The growth inhibition effects of eight probiotic bacteria, *L. rhamnosus* GG, *L. plantarum* LP115, *L. plantarum* 299V, *L. acidophilus* LA-5, *L. acidophilus* LA-14, *B. coagulans* unique IS2^{TM}, *B. coagulans* GBI-306086, *B. coagulans* and *E. faecalis* (negative control) against *A. fresenii* and *A. sulphureus* was evaluated after co-culturing a probiotic bacterium and a fungus on the surface of MRS agar plate for 10 days at 30 or 37°C.

Table 7 summaries the growth inhibition effects of *L. rhamnosus* GG, *L. plantarum* LP1115 and *L. plantarum* 299V. *Lactobacillus rhamnosus* and *L. plantarum* LP115 both showed negative growth inhibition effect against *A. fresenii*. However, both of them showed positive growth inhibition effect (> 2 mm in width of the clear ring) against *A. sulphureus* at 37°C when 10^5 CFU/µl of probiotic bacteria were inoculated on MRS agar plate. In addition, at 37°C, *L. rhamnosus* and *L. plantarum* LP115 showed marginal growth inhibition effect (1-2 mm in width of the clear ring) against *A. sulphureus* when the probiotic concentrations were $\geq 10^4$ and 10^3-10^4 CFU/µl, respectively. *Lactobacillus plantarum* 299V showed marginal growth inhibition against *A. fresenii* when 10^5 CFU/µl of probiotic bacteria were inoculated on MRS agar plate. Moreover, at 30°C, *L. plantarum* 299V showed positive growth inhibition against *A. sulphureus* when probiotic concentration was 10^5 CFU/µl and showed marginal growth inhibition when probiotic concentration was 10^4 CFU/µl. At 37°C, *L. plantarum* 299V showed positive growth inhibition against *A. sulphureus* in all the testing probiotic concentration.

Table 7. Mean width (mm) of clear ring surrounding *L. rhamnosus* GG, *L. plantarum* LP115 and *L. plantarum* 299V on MRS agar at 30 and 37°C for 10 days

Probiotic isolates	Probiotic inoculated (CFU/µl)	Mean width (mm) of clear ring ± SD			
		A. fresenii		*A. sulphureus*	
		30°C	37°C	30°C	37°C
L. rhamnosus GG	10^5	-[1]	0.8 ± 0.4^b	-	3.6 ± 2.1^b (Positive)
	10^4	-	-	-	1.2 ± 0.3^b (Marginal)
	10^3	-	-	-	-
	10^2	-	-	-	-
L. plantarum LP115	10^5	0.7 ± 0.1^a	0.8 ± 0.1^a	0.8 ± 0.1^a	2.8 ± 0.3^b (Positive)
	10^4	0.1 ± 0.1^a	0.3 ± 0.3^a	0.2 ± 0.3^a	1.1 ± 0.2^b (Marginal)
	10^3	-	0.3 ± 0.3^a	-	1.0 ± 0.3^b (Marginal)
	10^2	-	-	-	-
L. plantarum 299V	10^5	1.8 ± 0.1^a (Marginal)	1.2 ± 0.4^b (Marginal)	3.7 ± 0.6^c (Positive)	4.0 ± 0.3^c (Positive)
	10^4	0.8 ± 0.4^a	0.7 ± 0.2^a	1.2 ± 0.1^a (Marginal)	2.9 ± 0.1^b (Positive)
	10^3	-	0.7 ± 0.3^b	-	2.6 ± 0.3^c (Positive)
	10^2	-	-	-	2.3 ± 0.4^b (Positive)

[1]: no growth inhibition

[2]Mean width of clear ring with different letters (a, b. and c) within a row are significant different ($P<0.05$).

3.3.1.2 Double Layer Method

Growth inhibition of *A. fresenii* and *A. sulphureus* by probiotic bacteria was also evaluated using a double layer method at 30°C (Fig. 9). All five *Lactobacillus* probiotic bacteria tested showed growth inhibition effect against the two fungi (Table 8). *Lactobacillus rhamnosus* and *L. plantarum* inhibited fungal growth completely when there were 40-70 CFU probiotic bacterial colonies in the bottom layer MRS agar; whereas, *L. acidophilus* inhibited the fungal growth completely when the probiotic colonies were >125 CFU/plate.

The three *B. coagulans* strains showed partial fungal growth inhibition against *A. fresenii* when there were $1x10^2$-$3x10^3$ CFU of probiotic colonies in the bottom layer MRS agar. However, the three *B. coagulans* strains showed a stronger inhibition effect against *A. sulphureus*. *Bacillus coagulans* unique IS2™ and GBI-306086 could completely inhibit growth of *A. sulphureus* when there were 40-70 CFU/plate; while *B. coagulans* showed partial inhibition against *A. sulphureus* when there were around 40-$3x10^2$ CFU in the bottom layer MRS agar.

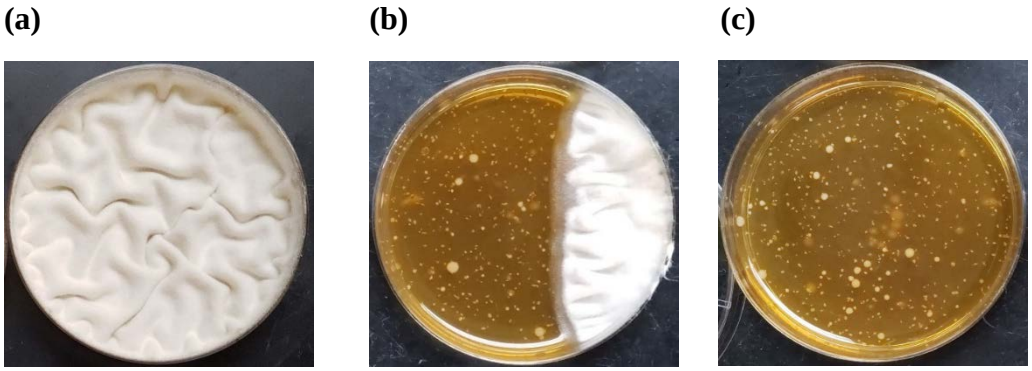

Figure 9. Appearance of (a) confluent fungal growth, (b) partial fungal growth, and (c) no fungal growth on the surface of double layer MRS agar plates with probiotic bacteria in the bottom layer of the plate.

Table 8. Growth of *A. fresenii* and *A. sulphureus* on the surface of double layer MRS agar plates with probiotic bacteria[1] in the bottom layer of the plate at 30°C for 10 days.

Probiotic Bacteria[1]	*A. fresenii*				*A. sulphureus*			
	Probiotic bacteria (CFU/plate) in the bottom layer of MRS							
	1-3x10^3	1-3x10^2	40 -70	<25	1-3x10^3	1-3x10^2	40 -70	<25
Lactobacillus rhamnosus GG	-[2]	-	-	+	-	-	-	+
Lactobacillus plantarum LP115	-	-	-	+	-	-	-	+
Lactobacillus plantarum 299V	-	-	-	+	-	-	-	+
Lactobacillus acidophilus LA-5	-	-	+	+	-	-	+	+
Lactobacillus acidophilus LA-14	-	-	+	+	-	-	+	+
Bacillus coagulans unique IS2™	+/-	+/-	+	+	-	-	-	+
Bacillus coagulans GBI-306086	+/-	+/-	+	+	-	-	-	+
Bacillus coagulans	+/-	+/-	+	+	-	+/-	+/-	+
Enterococcus faecalis (- control)	+	+	+	+	+	+	+	+

[1]Prior to the fungal growth inhibition study, *Lactobacillus spp.* and *E. faecalis* were grown in MRS agar plate for 3 days at 37°C while *B. coagulans* strains were grown in MRS agar plate for 3 days at 45°C.

[2]-: no fungal growth; +/-: fungal growth on part of the plate; +: confluent fungal growth

3.3.2 Influence of Probiotic Bacteria on OTA Production

3.3.2.1 Co-Cultured Method

Effect of probiotic bacteria on OTA production by *A. fresenii* and *A. sulphureus* on MRS agar was evaluated using the co-cultured method. As shown in Table 9, only *A. sulphureus* produced high levels of OTA on MRS agar at 37°C and the OTA concentration of positive control was 733.6 µg/kg. The OTA production of *A. sulphureus* on MRS agar plate was enhanced around 2.6% by *L. plantarum* LP115 (752.7 µg/kg) and reduced 9.7% by *L. rhamnosus* GG (662.2 µg/kg) and 3.2% by *L. plantarum* 299V (710.0 µg/kg). The variation of OTA production was all <10% which was considered as no influence.

Table 9. Ochratoxin A (µg/kg) produced by *A. fresenii* and *A. sulphureus* on MRS agar plates when co-cultured with probiotic bacteria at 30 and 37°C for 10 days.

Probiotic	OTA (µg/kg)			
	A. fresenii		*A. sulphureus*	
	30°C	37°C	30°C	37°C
Control	3.5	5.8	733.6	2.0
Lactobacillus rhamnosus GG	0.2	0.9	662.2	1.0
Lactobacillus plantarum LP115	2.9	0.8	752.7	0.8
Lactobacillus plantarum 299V	2.2	2.6	710.0	0.9
Bacillus coagulans unique IS2™	-[1]	1.4	-	0.9
Bacillus coagulans GBI-306086	-	2.1	-	2.7
Bacillus coagulans	-	5.6	-	1.4
Enterococcus faecalis (negative control)	1.0	1.2	732.8	2.0

[1] *Bacillus coagulans* did not grow at 30 °C

CHAPTER 4. DISCUSSION

4.1 Effect of Temperature and Water Activity on Fungal Growth and OTA Production

The results showed that growth and OTA production by *A. fresenii* and *A. sulphureus* on ground Niger seeds were influenced by water activity and temperature. The growth patterns of the two fungi on the seed samples were similar under all of the testing conditions. Optimum water activity level and temperature for fungi growth were at 0.94 a_w and 30°C. Minimum water activity for both fungi to grow was on the seeds with 0.86 a_w. On the seeds with a low water activity (0.86 a_w), growth rates of the two fungi increased as the incubation temperature increased; whereas, on the seeds with a high water activity (0.98 a_w), the growth rates of the two fungi decreased when the incubation temperature increased from 20 to 37°C.

The temperature and water activity requirement for fungi growth not only vary among different fungal species and strains but also are influenced by growth substrates. Pardo et al. (2004) reported that at 30°C, the minimum and the optimal water activity levels for three *A. ochraceus* isolates to grow on irradiated barley grains were 0.85 and 0.99 a_w respectively. Alborch et al. (2011) showed that the optimal water activity level range for *A. niger* and *A. carbonarius* to grow on maize kernels was 0.96-0.98 a_w. However, in this study, the optimum water activity level for *A. fresenii* and *A. sulphureus* to grow on Niger seeds were lower than the previous study. The result may due to the growth substrate using in this study. Previous study suggested that nutrient source can influence the optimum condition for fungal growth (Wearing and Burgess, 1979).

In this study, high OTA was usually observed when the fungi reached the confluent growth on the seed plates. However, not all seeds plates with confluent growth had OTA. In addition, the high OTA only presented in a short period of time and then decreased. Previous study showed that in the absence of sufficient nutrition, fungi could utilize OTA as a carbon source to maintain their metabolic rates (Valero et al., 2006). Abrunhosa et al. (2002) found that *A. carbonarius* was able to degrade OTA into ochratoxin α using carboxypeptidase enzyme. Varga et al. (2000) also detected this phenomenon with *A. niger* which degraded OTA into ochratoxin α within 7 days in liquid or solid media. Since the OTA analysis in this study only performed after 5, 10, 15 and 30 days of incubation. The OTA production between these sampling days may not be detected. In

additional, whether the two fungi degraded OTA to other compounds were not analyzed in this study.

Even though the two fungi displayed similar growth patterns on ground Niger seeds under all testing condition, the optimal conditions for OTA production by the two fungi were slightly different. *Aspergillus sulphureus* produced higher levels of OTA under a wide range of growth conditions. In contrast, *A. fresenii* produced less OTA under a narrower range of conditions (Fig. 5). Moreover, the range of water activity levels supporting OTA production by the two fungi was narrower than those supporting growth. Similar result also found in previous studies. Pardo et al. (2004) showed that the water activity range for three *A. ochraceus* isolates to grow and produce OTA on irradiated barley grains were 0.85-0.99 a_w and 0.90-0.99 a_w, respectively. Another study reported that *A. niger* and *A. carbonarius* were able to grow on maize kernels with 0.92, 0.96 and 0.98 a_w; whereas, both fungi only produced high OTA on maize with 0.98 a_w (Alborch et al., 2011).

4.2 Fungal growth and OTA production on whole Niger seeds and ground Niger seeds with different oil contents

Previous study showed that Noug cake which is a byproduct of Niger seeds after oil extraction supported fungal growth and aflatoxin production (Gizachew et al., 2016). The result of this study showed that both fungi were able to grow on the ground Niger seed with 10%, 25%, 35% oil and whole seeds. However, growth of the two fungi on the ground Niger seeds with 10% and 25% oil was significantly slower than the growth on ground Niger seeds with 35% oil. High levels of OTA production by the two fungi were only observed on ground seed with 35% oil. The OTA production of both fungi on ground seeds with 10% and 25% oil and whole seeds were either not detectable or lower than the quantitation limit (4 µg/kg). According to the results from study one, high OTA usually was observed when fungi nearly reached or reached the confluent growth on the seed plates. However, even the growth of both fungi on ground seeds with 10% and 25% oil nearly reached the confluent plates after 30 days incubation, the OTA production of both fungi was very low.

In this study, the reduction of oil content in Niger seeds resulted in the slower fungi growth and low OTA production. This result suggests that the oil in the seeds was important for fungal growth and mycotoxin production. Previous study mentioned that seed composition, notably lipid composition may affect the susceptibly of plants to fungi contamination and mycotoxin production (Scarpari et al., 2014). Reddy et al. (1992) studied the influence of different seed crops (groundnut,

paddy, sorghum, cowpea and green gram) with different lipid content on growth and aflatoxin production by *A. parasiticus*. They found that groundnut which had the highest lipid content (430 mg/g) also supported the highest aflatoxin B1 production (43000 µg/kg). Additionally, they found that slower fungal growth and lower aflatoxin B1 production in defatted power seeds materials. Another study also highlighted the important role of fatty acid on mycotoxin production. Dall'Asta et al., (2012) found that high linoleic acid content in maize resulted in high fumonisin contamination.

On the whole Niger seeds, the growth of *A. sulphureus and A. fresenii* were slightly slower than the growth on ground seeds with 35% oil during the first 5 and 10 days respectively. In addition, the two fungi did not produce any detectable OTA or produced low levels (≤ 2.8 µg/kg) OTA in the whole Niger seeds. The results may be due to the seeds shell of Niger seeds which retarded the growth of fungi and hindered the OTA production. Previous studies have found that seed coat integrity is an important factor in resistance of fungi contamination. Stossel (1986) reported that seeds coat integrity of soybeans affected the colonization of *A. flavus*. The raw damaged soybean supported vigorous mycelial growth, while, intact raw soybean supported less mycelial growth. He suggested that seed coat integrity is a barrier which increase the difficulty for fungi to access the substrate. Another study also showed the similar influence of whole seed on fungal growth and mycotoxin production. Reddy et al. (1992) found that *A. parasiticus* grew faster and consistently produced more aflatoxin B1 on powdered seed materials than on whole seeds. In addition, they reported that aflatoxin production of *A. parasiticus* on powered seed materials was higher than on whole seeds and defatted powered seed materials.

4.3 Fungal growth inhibition by probiotic bacteria

The results of this study showed that the growth of *A. fresenii* and *A. sulphureus* could be influenced by probiotic bacteria. In the co-cultured method, *L. plantarum* 299V showed best growth inhibition effect against the two fungi. Neither *L. acidophiles* nor *B. coagulans* showed any growth inhibition effect against the two fungi when evaluated by the co-cultured method. Since the fungal growth inhibition was not clearly shown by the co-culture method, a double layer method was also used to verify the fungal growth inhibition by the probiotic bacteria.

In double-layer method, fungi were inoculated on the MRS plates which had probiotic bacteria growing in the bottom layer MRS agar for three days. High concentration of antifungal metabolites

produced by probiotic bacteria had accumulated in the bottom layer of MRS. In contrast, in the co-cultured method, fungi and probiotic bacteria were inoculated simultaneously on the MRS agar plate. The concentrations of antifungal metabolites produced by probiotic bacteria were not very high in the MRS agar plates. Therefore, more obvious fungal growth inhibition was observed when the double-layer method was used.

Similar result also found in the study of growth and aflatoxin production by *A. parasiticus* in the presence of *Lactococcus lactis*. Wiseman and Marth (1981) reported that the growth of *A. parasiticus* was inhibited when the fungal spores were inoculated to a 3-day *L. lactis* culture. Similar growth inhibition was also found when both organisms were inoculated simultaneously. El-Gendya and Marth (1981) also showed that the growth inhibition of *A. parasiticus* was observed when the fungi were added to a 3-day culture of *Lactobacillus casei* in APT broth. In addition, there was stimulation of fungal growth when fungi and bacteria were inoculated to the medium at the same time. However, there were no stimulation of fungal growth in this studied.

In this study, *A. fresenii* did not produce much of OTA on MRS plate at either 30 or 37°C. Therefore, the influence of the probiotic bacteria on the OTA production by *A. fresenii* could not be determined. *Aspergillus sulphureus* produced high level of OTA (662 -734 μg/kg) on MRS agar plate at 30°C only but not at 37°C. There was no obvious increase or decrease of OTA production *Aspergillus sulphureus* when co-cultured with the eight probiotic bacteria.

The influence of probiotic bacteria on mycotoxin production varies among different probiotic bacteria species and strains. Previous studies showed that some of the probiotic bacteria had anti-mycotoxigenic effect. Gerbaldo et al. (2012) found that the aflatoxin production by *A. flavus* on MEA was reduced 95.7-99.8% by *L. rhamnosus* L60 and 27.5-100% by *L. fermentum* L23. Dallagnol et al. (2019) found that the OTA production by *A. niger* on CYA with 0.971 a_w was reduced 90% by *L. plantarum* CRL 778. However, some of the probiotic were able to stimulate the mycotoxin production. Wiseman and Marth, (1981) showed that when *A. parasiticus* and *Lactococcus lactis* were inoculated simultaneously, the aflatoxin production of *A. parasiticus* was enhanced at the first 3 days.

CHAPTER 5. CONCLUSION

The goals of this study were to test the influence of (1) water activity, temperature (2) oil content, grinding and (3) probiotic bacteria on fungal growth and OTA production. The following conclusions can be drawn from this study.

1) Fungal growth and OTA concentration on ground Niger seeds were influenced by both water activity and temperature and the optimal condition for OTA production did not necessarily correlate with the conditions for maximum fungal growth.
2) *Aspergillus fresenii* and *A. sulphureus* were able to grow and produce OTA on ground Niger seeds with 0.86, 0.90, 0.94 or 0.98 a_w at 20, 30 or 37°C.
3) The optimal condition for both *A. fresenii* and *A. sulphureus* to growth on the ground Niger seeds was observed at 0.94 a_w, 30°C.
4) The optimal condition for *A. fresenii* to produce OTA on ground Niger seeds was 0.90-0.94 a_w at 37°C and the optimal conditions *A. sulphureus* to produce OTA were on ground Niger seeds with 0.90-0.94 a_w at 30°C and seeds with 0.94-0.98 a_w at 37°C.
5) Maintaining water activity of Niger seeds at $\leq$ 0.82 a_w can prevent growth and OTA production of *A. fresenii* and *A. sulphureus*.
6) The oil content of Niger seeds played an important role on fungal growth and OTA production by *A. fresenii* and *A. sulphureus*.
7) The reduction of oil content in Niger seeds could reduce the growth and OTA production by *A. fresenii* and *A. sulphureus*.
8) The intact shell of Niger seeds not only retarded growth but also hindered the OTA production by *A. fresenii* and *A. sulphureus.*
9) The eight probiotic bacteria tested in this study showed various growth inhibition effects against *A. fresenii* and *A. sulphureus*. Such effects were more obvious when there was sufficient amount of anti-fungal metabolites accumulated in the MRS agar plate.
10) Among the eight probiotic strains evaluated in this study, *L. plantarum* 299V could be used as an agent inhibiting growth of *A. sulphureus*.
11) Neither increase nor decrease of OTA production by the two fungi which co-cultured with the eight probiotic bacteria on the MRS agar.

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