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Mechanistic Insight on Vibrio Gazogenes Mediated Intervention of Aflatoxin Biosynthesis in Aspergillus Flavus

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MECHANISTIC INSIGHT ON VIBRIO GAZOGENES MEDIATED INTERVENTION OF AFLATOXIN BIOSYNTHESIS IN ASPERGILLUS FLAVUS

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DEDICATION

I dedicate my research study to my loving parents, Dr. Md Shafiqul Aktar and Razina Yasmin who taught me not to give up no matter how difficult it could be. To my husband, Dr. Mohsin Sajjad for his constant support and love that helped me to complete this journey. To my beloved adorable son, Rohaan Labib Sajjad who shared my experience while staying inside me.

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ABSTRACT

Aflatoxin is a liver carcinogen, mycotoxin, and a secondary metabolite that is produced by some plant pathogens within the genus Aspergillus when they infect crops such as corn, peanuts, and cotton. Given the significant adverse health and economic impacts of aflatoxin and a predicted rise of crop contamination with mycotoxins caused by a changing climate, there is a pressing need for exploring new aflatoxin mitigation methods. Previous studies in our laboratory have shown that metabolites from *Vibrio gazogenes*, an estuarine non-pathogenic bacterium, can inhibit aflatoxin synthesis. However, a direct interaction study between *V. gazogenes* and aflatoxin producing strains has not been conducted thus far. Here we show that administration of *V. gazogenes* in the growth medium of *Aspergillus flavus*, an aflatoxin producing pathogen in corn, results in an almost complete inhibition (>99%) of aflatoxin biosynthesis at the level of gene expression. Light, electron and confocal microscopy suggested that *V. gazogenes* dependent aflatoxin inhibition was associated with internalization of bacterial cell materials but not intact bacteria, in endosome-like compartments. Administration of equal concentrations of heat-inactivated non-viable *V. gazogenes* cells to the growth medium resulted in a similar reduction in aflatoxin production, supporting that bacterial viability was not necessary for this inhibitory effect. We show that sclerotial development which depends on hyphal fusion decreased by more than 2-fold as well. Mycelia upon *V. gazogenes* administration showed early conidiation; however, conidia harvested from the treated colonies produced ~2-fold less aflatoxin than the earlier generation. We also show

that, *V. gazogenes* uptake results in an almost complete (>98%) block of hyphal fusion and an ~3-fold decrease of polar growth, processes that depend on endosomal functions, and transport. We have also found that *A. flavus* cannot uptake the same amount of *V. gazogenes* in the presence of endocytosis inhibitors which also affect the aflatoxin inhibition. In another study, we observed that, presence of *A. flavus* activates *V. gazogenes* lysing enzymes. Treating *A. flavus* with non-viable other gram-positive or gram-negative or prodigiosin producing bacterium did not have the same aflatoxin inhibitory effect. This indicates that the anti-aflatoxigenic activity is specific to *V. gazogenes* and prodigiosin is not the only aflatoxin inhibitor. Our study showed that, Intact *V. gazogenes* cells most effectively and efficiently decrease aflatoxin production (>99%) rather than different fractionated parts of *V. gazogenes* cells. Collectively, our study introduces a novel cellular perturbation tool through *V. gazogenes* that can allow us to identify the interconnected molecular and cellular mechanisms that co-regulate secondary metabolism and hyphal development in filamentous fungi, hence providing a novel starting point for the discovery of a safe pharmaceutical and green fungicidal product that can efficiently prevent aflatoxins which have profound impacts on agriculture, environmental sustainability, and human health. The study ultimately unlocks an opportunity for furthering fundamental science and developing practically applicable strategies for intervening in *Aspergillus* toxin accumulation in the environment.

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LIST OF ABBREVIATIONS

AFs.....Aflatoxins

AFB1.....Aflatoxin B1

AFB2.....Aflatoxin B2

AFG1..... Aflatoxin G1

AFG1..... Aflatoxin G2

AFM1.....Aflatoxin M1

AFM2.....Aflatoxin M2

DON.....Deoxynivalenol

IARC..... International Agency of Research on Cancer

ARMs.....Aflatoxin Response Metabolites

NOR.....Norsolorinic Acid

AVN.....Averantin

HAVN.....5¢-Hydroxy-Averantin

OAVN.....Oxoaverantin

AVNN..... Averufanin

AVF..... Averufin

VHA.....Versiconal Hemiacetal Acetate

VAL ..... Versiconal

VERB.....Versicolorin B

VERA.....Versicolorin A

DMST ..... De-methyl Sterigmatocystin

DHDMST.....Dihydro De-methyl Sterigmatocystin

ST..... Sterigmatocystin

DHST.....Dihydro Sterigmatocystin

OMST .....O-methyl Sterigmatocystin

DHOMST..... Dihydro-O-methyl Sterigmatocystin

ELISA ..... Enzyme-linked immunosorbent assay

HPLC .....High-Pressure Liquid Chromatography

RT-PCR.....Reverse Transcription Polymerase Chain Reaction

DNA.....Deoxyribonucleic Acid

RNA..... Ribonucleic Acid

OD..... Optical Density

YES..... Yeast Extract Sucrose

PDA.....Potato Dextrose Agar

GMM.....Glucose Minimal Medium

SMs .....Secondary Metabolites

EPS.....Exopolysaccharide

CPS ..... Cellular Polysaccharide

CFU..... Colony Forming Units

DMSO.....Dimethyl Sulfoxide

UV/Vis..... Ultraviolet-Visible

EtOH .....Ethanol

DMSO.....Dimethyl Sulfoxide

UV/Vis.....Ultraviolet–Visible

CHAPTER 1

INTRODUCTION

1.1 AFLATOXINS

Aflatoxins (AFs) are remarkably potent, most acutely toxic, an ever-increasing number of chemical carcinogenic mycotoxin. They were discovered because of their toxicity to turkeys, chicks, and ducklings in British hatcheries in 1960 (Bennett and Christensen, 1983). The poultry deaths were traced to a mold-contaminated Brazilian peanut meal and received publicity as a mysterious "Turkey-X disease." The mold from the toxic peanut meal was identified as *Aspergillus*; extraction of a purified toxic principle from cultures of *Aspergillus flavus* was reported by two groups in 1962 (Nesbitt et al., 1962; Van Der Zijden et al., 1962). A study on the nature of the toxin suggested its origin from the fungus *A. flavus*. Thus, the toxin was named "aflatoxin" by its origin from *A. flavus* (Brase S, 2013). This was the event that stimulated scientific interest and gave rise to modern mycotoxicology. Research on aflatoxins led to a "golden age" of mycotoxin research during which several new mycotoxins were discovered (Negash, 2018).

Aflatoxins (AFs) are a group of homologous estrogenic mycotoxin and pervasive, are low molecular weight molecules of structurally related toxic secondary metabolites mainly produced by *Aspergillus* genus including *A. flavus* and *A. parasiticus* and *A. nomius*, during favorable growth conditions of oxygen, moisture (3-18%), warm temperatures (24-35^{o}C) and substrate (sugar) (Gummadidala, 2018; Iqbal et al., 2014; Ji

and Xie, 2020; Zhuang et al., 2016). The aflatoxins, B1, B2, G1 and G2 (AFB1, AFB2, AFG1 and AFG2) are the major four toxins among at least 20 structurally related toxins which are named based on their respective blue and green fluorescence under long-wave ultraviolet (UV) light and their relative mobility by thin-layer chromatography on silica gel (Bennett and Christensen, 1983; Pitt, 2000; Yu, 2012) and aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2) are the hydroxylated metabolites of AFB1 and AFB2 produced through different metabolic processes and expressed in animals and animal products (Kumar et al., 2016). Aflatoxin is biosynthesized by many Aspergillus species such as *A. flavus, A. parasiticus, A. nominus, A. pseutotamarii, A. bombycis, A. toxicarius, A. parvisclerotigenus, A. minisclerotigenes, A. arachidicola,* and *A. pseudocaelatus*. But primarily aflatoxins are produced in copious amounts by *A. flavus* and *A. parasiticus*, which can cohabit and flourish on practically any crop or food including but not limiting to maize, oilseeds, spices, groundnuts, tree nuts, milk, and dried fruit (Bennett and Klich, 2003a; Strosnider et al., 2006). Aflatoxin B1 is predominant, the most toxic and most potent hepatocarcinogenic natural compound ever characterized, as well as heat resistant over a wide range of temperatures, including those reached during commercial processing conditions (Sirot et al., 2012). Aflatoxins (AFs) are polyketide-derived secondary metabolites and their structures are composed of bis-furan-containing dihydrofuran furan and tetrahydrofuran moieties (5 rings) fused with a substituted coumarin (an aromatic ring), a lactone ring and either a pentanone or a lactone ring (Brase S, 2013; Yu et al., 2005) **(**Figure 1.1**)**.

Aflatoxin is the only mycotoxin known to contaminate crops both pre- and post-harvest (potentially along the entire value chain), making it difficult to target prevention

and control interventions (Ayalew et al., 2016; Visser et al., 2020). *Aspergillus* can synthesize aflatoxin during postharvest handling of storage, transportation, and food processing (Wu and Khlangwiset, 2010). The Food and Agriculture Organization estimates that 25% of the world's crops are contaminated with aflatoxins, resulting in annual losses of around 1 billion metric tons of foods and food products (Alexandratos and Bruinsma, 2012). Recent reports from our group and others suggest that the risk of consuming foods with various mycotoxins is increasing steadily (Chen et al., 2018b; Gummadidala et al., 2019b; Herrera et al., 2019; Ortiz et al., 2018). Over 5 billion people in developing countries are at risk of chronic dietary exposure to aflatoxins, the hepatocarcinogenic mycotoxins. Losses due to aflatoxin alone range from $52.1 to $1.68 billion annually, and it is predicted that the impending climate change will increase mycotoxin contamination in crops, foods, and feed (Battilani et al., 2016; Mitchell et al., 2016b; Wu and Mitchell, 2016). Recent studies show that > 50% of ~ 40 million units of infant foods or breakfast cereals sold per week in the USA contain at least one (mycotoxin) (Zhang et al., 2018a). Despite years of efforts, there is still no precise method to prevent diseases and economic losses resulting from fungal infections and dietary exposures to aflatoxin and other mycotoxins (Herrman et al., 2020). In the USA alone, the number of samples tested positive for aflatoxin increased by 6% from 2012 to 2013. FDA limits for aflatoxin human consumption is 20 ppb, animal feeds is 300 ppb and for aflatoxin M1 in milk is 0.5 ppb (FDA, 2011). National estimates of dietary exposure to aflatoxins indicate differences between developed and developing countries. In developed countries, mean aflatoxin dietary exposures are generally less than 1 ng/kg body weight (BW) per day (a nanogram is one billionth [1×10^{-9}] of a gram), whereas estimates for some sub-Saharan African

countries exceed 100 ng/kg BW per day, although these latter estimates are often based on very few data (World Health and Joint, 2017).

Aflatoxins have both hepatotoxic and carcinogenic actions, depending on the level and duration of exposure. It contaminates crops, produce, food, nuts, cereal, milk, juices, homes, wood, etc., and can be ingested into intestines and enter systemic circulation (Figure 1.2) and cause acute illness and deaths (Alshannaq et al., 2018). The ingestion of aflatoxins in contaminated food or feed causes a disease called aflatoxicosis. Acute aflatoxicosis is produced when moderate to high levels of aflatoxins are consumed. Symptoms include acute liver damage, acute necrosis, cirrhosis, or in severe cases, acute liver failure, and death (Fung and Clark, 2004). Aflatoxins in the liver irreversibly bind to protein and DNA to form adducts such as aflatoxin B1-lysine in albumin (Skipper and Tannenbaum, 1990). Disruption of the proteins and DNA bases in hepatocytes causes liver toxicity (Azziz-Baumgartner et al., 2005). In humans, patients experience high fever, rapid progressive jaundice, edema of the limbs, pain, vomiting, alteration in digestion, absorption and/or metabolism of nutrients and swollen livers(Yu et al., 2005). Aflatoxin B1 has been categorized as class 1A human carcinogen by the International Agency of Research on Cancer (IARC) because it causes hepatocellular carcinoma. It is projected that 25,200-155,000 cases of liver cancer worldwide are attributed to aflatoxin exposure (Wu et al., 2011).

1.2 BIOSYNTHESIS OF AFLATOXINS

Aflatoxins, typical of other secondary metabolites, are produced during idiophase, after primary growth has slowed. Induction of aflatoxin biosynthesis appears to involve the

interplay of transcriptional regulatory elements and physiological factors that affect fungal metabolism (Payne and Brown, 1998). The hallmark discovery of a color mutant that accumulates the brick-red pigment, norsolorinic acid (NOR), in *A. parasiticus* marked a milestone in the understanding of the chemistry of aflatoxin biosynthesis (Bennett, 1979; Bennett et al., 1997; Bennett and Klich, 2003b). This discovery led to the identification of other key aflatoxin intermediates and established the early step metabolites in the aflatoxin pathway. It provided the opportunity to isolate the first aflatoxin pathway gene that encoding a reductase for the conversion from NOR to eventually aflatoxins (Ehrlich et al., 2008; Hsieh et al., 1973; Hsieh and Mateles, 1970; Yu, 2012).

The biosynthetic pathway of aflatoxins consists of 21 enzymatic steps for conversion from acetyl-CoA, and at least 30 genes encoding the enzymes and regulatory pathways have been cloned and characterized (Yu et al., 2004b). Genes (*aflR* and *aflJ*) coding for proteins shown to be involved in transcriptional activation of most of the structural genes are also part of the cluster (Bhatnagar et al., 2003). The aflatoxin gene cluster has been widely studied in *A. flavus* and *A. parasiticus*. The homology of the clustered genes between the two fungal species is 90%–99% (Fung and Clark, 2004). One of the main differences between the two species is their ability to produce B and G type aflatoxins (Caceres et al., 2020). In *A. flavus* and *A. parasiticus* the aflatoxin pathway genes are clustered within a 75-kb region of the fungal genome on chromosome III roughly 80 kb away from telomere (Chang et al., 2005; Trail et al., 1995; Wilson, 1989; Yu et al., 2004a) (Figure 1.3) and is regulated by the regulatory gene, *aflR* (Price et al., 2006; Yabe and Nakajima, 2004)

Aflatoxins are polyketide-derived secondary metabolites produced via the following conversion path (Bhatnagar et al., 2003; Yabe et al., 1988; Yu et al., 2004b):

acetate → polyketide → anthraquinones → xanthones → aflatoxins.

Initially, acetate and malonyl-CoA are converted to a hexanoyl unit by a specialized fatty acid synthase. *aflA (fas-2), aflB (fas-1),* and *aflC (pksA)* are involved in the conversion of acetate to NOR. The polyketide then undergoes multiple enzymatic conversions in the pathway. In the middle of the of the pathway, intermediate versicolorin B synthase catalyzes an important step that is responsible for the bisfuran ring required to bind aflatoxin to DNA (Payne and Brown, 1998). Versicolorin B is situated at the branching step of the pathway where the conversion of Versicolorin B to Sterigmatocystin (ST) leads to the production of aflatoxin B1 and the conversion of versicolorin B to dihydrode methyl sterigmatocystin leads to the production of aflatoxin B2 (Mitra et al., 2017).

1.3 ASPERGILLUS FLAVUS

The genus *Aspergillus* was first described by the Florentine priest and mycologist P. A. Micheli in 1729 and named based on the structural similarity of its conidiophore structure to the aspergillum, a liturgical implement used to sprinkle holy water (Bennett and Klich, 1992). This common genus has been classified based on morphology many times (Samson, 1992) and currently contains over 200 species. It is a common saprophytic aerobic fungus. It belongs to the family of Aspergillaceae in the phylum of Ascomycota and is mainly distributed in the warm temperate zone (Klich, 2007). *Aspergillus flavus* was described by Link in 1809 and has been known as an asexual species that only produces asexual spores, conidia, and the overwintering asexual fruiting bodies, sclerotia. Recently,

the sexual stage of *A. flavus* has been reported and classified as *Petromyces flavus* (Amaike and Keller, 2011; Horn et al., 2009). Most *A.flavus* strains are susceptible to antifungal therapy but the minimum inhibitory concentrations are at least two-fold higher than for other *Aspergillus* species (Frawley et al., 2020; Krishnan et al., 2009). Furthermore, recent discoveries revealed the presence of active multidrug-resistant genes in *A.flavus* strains increasing their potential for drug resistance and pathogenicity (Tobin et al., 1997). Aflatoxin B1 synthesized by *A.flavus* is extremely stable and cannot be detoxified by cooking or autoclaving and thus pollutes many food groups being ingested by humans and animals (Gummadidala, 2018).

Aspergillus flavus have worldwide distribution and can infest a wide range of agricultural products. It is not only a common pathogen of plants but also a human pathogen, and long-term exposure to *A. flavus* or more than 20 other species in humans and animals can induce asthma, exogenous alveoli, and allergic bronchial aspergillosis (Hedayati et al., 2007b). It has pathogenic ability causing aspergillosis in immuno-compromised humans effecting the skin, oral mucosa, and subcutaneous tissues. According to the centers for disease control and prevention (CDC) approximately 4.8 million cases of aspergillosis were diagnosed worldwide and *A.flavus* is the second most leading cause. *A.flavus* also infects corn, peanuts, and cotton by releasing aflatoxins. Aflatoxin B1, an *A.flavus* secondary metabolite, has been categorized as class 1A human carcinogen by the International Agency of Research on Cancer (IARC). During the growth, transportation, and storage of crops, *A. flavus* can induce various crop diseases like ear rot in corn, algarot in peanuts, and boll rot in cotton (Yu et al., 2005). Food safety issues caused by *A. flavus* are due to its representative secondary metabolites: aflatoxins (AFs), cyclopiazonic acid

(CPA), and aspertoxin, aflatrem and aspergillic acid (Li et al., 2019; Soni et al., 2020). The growth of *A. flavus* and synthesis of aflatoxins depends on substrate, pH, temperature, moisture, aeration, and competing micro-flora. It produces airborne conidia, which easily disperse by air movement or by insects. *A. flavus* is considered as an opportunistic pathogen that takes advantage of an opportunity not normally available, such as a host with a weakened immune system, and altered micro-biota for colonization (Tahira Aziz Mughal et al., 2020). It is not only a common pathogen of plants but also a human pathogen, and long-term exposure to *A. flavus* or more than 20 other species in humans and animals can induce asthma, exogenous alveoli, and allergic bronchial aspergillosis (Hedayati et al., 2007b). *A. flavus* colonization does not necessarily reduce yield but causes economic losses by contaminating seed with aflatoxin.

1.4 INHIBITORS TO STOP AFLATOXIN BIOSYNTHESIS

In recent decades, several feasible and cost-effective strategies have entered the market aiming to mitigate the effects of feed mycotoxin contamination in food and animal feeds. Technologies to reduce the incidence of mold and mycotoxin contaminations of silages can be employed in one of the three main phases (preharvest, harvest, ensiling) of silage production. Aflatoxin mitigation has been well documented in the recently released Synthesis of the Research on Aflatoxin in Health, Agriculture, and Trade (USAID, 2012). The pre-harvest solutions currently available are based on good Agricultural Practices, which typically include use of fungal resistant crops, good tillage and weeding practices, appropriate use of fertilizers, irrigation, and crop rotation (Ehrlich et al., 2008; Kimanya, 2008; Leslie et al., 2008; Waliyar et al., 2013) while post-harvest screening to remove contaminated seed appears to be a promising means to reduce or to eliminate aflatoxin

(Hell et al., 2008). There are several processing methods like sorting, washing, dehulling grain mechanically, roasting, cooking, wet and dry milling, canning, baking, frying, and extrusion cooking can reduce aflatoxin contamination up to 80% in crops and grains after harvesting (Negash, 2018). According to (Bovo et al., 2013) toxin absorbents, chemical, and biological methods are used directly for reducing aflatoxin in milk.

Various synthetic compounds are used extensively to control the fungal infestation, but there are concerns about the environmental health hazards of using these chemicals. Use of ethylene and carbon dioxide inhibit aflatoxin synthesis in the laboratory-grown *A. parasiticus* on peanuts (Gunterus et al., 2007) while some phenolic compounds such as acetosyringone, and syringaldehyde can inhibit aflatoxin biosynthesis in A. flavus efficiently (Molyneux et al., 2007) but they also contaminate soil and water. There are some studies on using plant extracts such as asafoetida, turmeric, and Azadirachta indica leaf extract to prevent aflatoxin production in groundnuts (Ghewande and Nagaraj, 1987).

Physical and chemical methods lead to unwanted side effects, and adsorption-associated technologies still have limitations, such as aflatoxin residues in foods, feeds, and hosts. One of the best ways to remove aflatoxin is detoxifying action for successful decontamination. Accordingly, to compensate for these drawbacks, microbe-mediated biodegradation methods have been suggested for effective control of aflatoxin contamination at a higher rate. Indeed, many studies have reported aflatoxin-degrading fungal and bacterial strains isolated from soil, feces, and crops. However, the use of living microorganisms as a food additive may raise safety issues and consumers may be reluctant to eat microbe-supplemented foods. The use of aflatoxin-degrading enzymes, produced by fungi and bacteria, may be able to overcome those drawbacks. To meet the emerging need,

using microorganisms and their metabolites as biocontrol agents against aflatoxin and other mycotoxins become a fruitful solution. Some examples include studies with *Bacillus spp*. such as *Bacillus subtilis, Lactobacillus spp., Pseudomonas spp., Ralstonia spp*., and *Burkholderia spp*. (Chalivendra et al., 2018; Guo et al., 2019; Palumbo et al., 2006a; Strosnider et al., 2006), *Lactobacillus spp*. (Ahlberg et al., 2017; Gonda et al., 2019; Guimarães et al., 2018), *Pseudomonas spp*. and *Ralstonia spp.* (Spraker et al., 2014; Spraker et al., 2016), *Trichoderma spp*. (Waliyar et al., 2015), *Streptomyces spp.* (Sakuda et al., 2016; Yoshinari et al., 2010), *Aspergillus oryzae* (Alshannaq et al., 2018). Recently we discovered that a non-pathogenic estuarine bacteria *Vibrio gazogenes* inhibit aflatoxin biosynthesis by *A. flavus* without inhibiting the fungal growth (Gummadidala, 2018).

1.5 VIBRIO GAZOGENES

Vibrio gazogenes(Allen et al., 1983; Giovannoni and Margulis, 1981; Harwood, 1978; Ratcliffe et al., 1982) is a red-pigmented species that was described in 1978 by Harwood (Harwood, 1978). The organism was described as oxidase-positive and was named *Beneckea gazogenes*, but it was later classified in the genus *Vibrio* and the oxidase reaction (negative) was corrected. The original description was based on a single culture isolated from a saltwater marsh located near Woods Hole, Mass. (Giovannoni and Margulis, 1981). *Vibrio gazogenes* is a rod shaped, gram-negative facultative anaerobe marine gram-negative bacterium notoriously known for its synthesis of antifungal pigments. *V. gazogenes* is a non-pathogenic bacteria and fluorescent carboxylate nanoparticles have a positive effect on the growth of this bacterium (Jones, 2016).

Studies have shown that when *V.gazogenes* comes in contact with aflatoxin, the toxin induces *V.gazogenes* to synthesize antifungal and anti-aflatoxin compounds (Gummadidala et al., 2016). The utility of prodigiosin that are synthesized by *V. gazogenes* ATCC 43942, coupled with its ability to produce unique antibiotics and mycotoxin inhibitors under custom-designed environmental settings makes this strain "chemically gifted." In this context, its finished genomic sequence provides a necessary point of comparison with other *V. gazogenes* strains and bacterial species within the *Vibrio* genus for the elucidation of the molecular factors that govern its unique metabolic profile (Gummadidala et al., 2017). Understanding the mechanism by which *V. gazogenes* secrets aflatoxin inhibitory metabolic components which cause a decrease of aflatoxin and inhibits pathogenicity of *A.flavus* will help us further understand how to develop, design and target *A.flavus* pathogen and decrease mortality rates of fungal infected patients and plants.

1.6 BACTERIA-FUNGAL INTERACTION

Bacterial-fungal interactions are widespread in nature and there is a growing number of studies reporting distinct fungus-associated bacteria. However, little is known so far about how shifts in the fungus-associated bacteriome will affect the fungal host's lifestyle (Schulz-Bohm et al., 2017). Microbes ubiquitously occur in the environment and colonize almost every ecological niche. Because of this high abundance, different species coinhabit certain habitats and as a consequence interact with each other. Such encounters probably represent the driving force to produce secondary metabolites that regulate the coexistence and survival of different species (Scherlach et al., 2013). Hence, it is thought that microbial SMs play an important role in intraspecific and interspecific interactions, including cross-kingdom communication (Davies, 2013; Han et al., 2017; Schulz-Bohm et

al., 2017). SMs have profound impacts on the ability of these microbes to colonize and survive in complex biotic environments and that microbes respond to specific SM signals as part of a complex network of interactions (Spraker et al., 2018b). Moreover, fungal-bacterial associations are widely used for the preservation of the environment (e.g., mycorrhizae in reforestation), agriculture (e.g., food processing), and biotechnology (e.g., pharmaceutical research) (Scherlach et al., 2013). Beyond the most commonly observed microbial cell-cell interactions, there is a growing number of known endosymbioses where bacteria dwell within fungal hyphae (Bonfante and Anca, 2009; Frey-Klett et al., 2011; Kobayashi and Crouch, 2009; Lackner et al., 2009). Symbioses with endofungal bacteria are often overlooked, yet they may have a profound effect on the host's lifestyle. Bacterial endosymbionts of arbuscular mycorrhizal fungi, for example, might be implicated in the vitamin B12 provision for the fungus (Ghignone et al., 2012).

Epigenetics has been steadily gaining momentum in the last few decades in the world of transcriptional regulation. There is now growing evidence that microbial communication regulates epigenetic modifiers that in turn control mycotoxin biosynthesis. The SAGA complex, conserved across eukaryotes, induces transcription of genes by mediating histone acetylation of the corresponding promoters. Researchers showed that, *Pseudomonas piscium*, from the wheat head microbiome, secretes an antifungal agent, phenazine, against *F. graminearum*. Phenazine, upon entering the fungal cell, inhibits the histone acetyltransferase module of the SAGA complex which subsequently leads to an inhibition of fungal growth and pathogenicity in addition to a complete suppression of DON biosynthesis (Chen et al., 2018b). Another similar instance has been reported in *Aspergillus nidulans*—Streptomyces *rapamycinicus* association where the bacterium

induces histone modification mediated by the SAGA complex which results in the production of orsellinic acid and its derivatives by the fungus (Nützmann et al., 2011). It is indeed fascinating that microbes have evolved such well-tuned, intricately regulated mechanisms of interaction. Bacteria have been shown to contribute to the reduction of *Fusarium* mycotoxin accumulation in grains. Preventative application of *Pseudomonas fluorescens* strain before inoculation with *F. culmorum* resulted in a significant reduction in Fusarium head blight as well as DON levels in infected wheat grains(Khan and Doohan, 2009). Endophytes belonging to *Paenibacillus polymyxa*, isolated from wild teosinte, have been shown to produce fusaridins which contribute to the antifungal activity against *F. graminearum*. The co-existence of these bacteria with *F. graminearum* in grains during storage at room temperature resulted in a significant decrease in DON accumulation (Mousa et al., 2015). A recent review summarizes the different bacteria and fungi that can degrade mycotoxins including zearalenone and DON (Vanhoutte et al., 2016; Venkatesh and Keller, 2019). Another study showed that rice seedling blight fungus *Rhizopus microsporus* and its endosymbiont *Burkholderia rhizoxinica* form an unusual, highly specific alliance to produce the highly potent antimitotic phytotoxin rhizoxin (Moebius et al., 2014).

Yeast and several lactic acid bacteria can bind aflatoxins, decreasing aflatoxin bioavailability in feed or food. Because lactic acid bacteria prevent the growth of pathogenic bacteria by producing pathogen-inhibitory substances, and most are used as probiotics and generally regarded as safe, they are considered a desirable method for aflatoxin removal(Hernandez-Mendoza et al., 2009). Among lactic acid bacteria, physical adsorption by *Lactobacillus rhamnosus* GG has been extensively studied (Kim et al.,

2017). *Enterococcus faecium* strains isolated from the feces of healthy dogs have been shown to reduce the levels of aflatoxin B1 which were suggested as promising pet feed additives for aflatoxin decontamination. Some other examples are: *Saccharomyces cerevisiae* adsorb aflatoxin (Dogi et al., 2011; Luo et al., 2020; Pizzolitto et al., 2013), aflatoxin-binding bacterial strain, *Brevibacillus lacterosporus* also use for controlling aflatoxin contamination in feeds (Aftabi et al., 2015). Aflatoxin B1 biosynthesis and mycelial growth were inhibited and the inhibitory activity was occurred due to extracellular metabolites produced in cell-free supernatant fluids of the cultured broth of *Lactobacillus casei* (Chang and Kim, 2007).

It is expected that descriptions of bacteria mediated fungal growth and their aflatoxin inhibition will continue to accumulate as a result of growing interest in the field, as well as the greater appreciation by the scientific community of fungi as simple model systems. Also, the recognition that sophisticated cellular machinery previously associated with bacterial producing metabolic components in contact with fungal toxin has initiated an exciting area of research involving effector molecules and their fungal targets, which in turn could lead to new strategies for controlling fungal pathogens of plants. Those potential research could be extended to direct use of bacteria in the market or in the field to prolong the shelf life of commodities as a biological control agent. As an essential parameter, the safety of the antagonistic bacterium and its metabolites using a food model system currently needs to be evaluated. Bacteria-fungal interactions result in unique contributions to biogeochemical cycles and biotechnological processes. Thus, the interactions between bacteria and fungi are of central importance to numerous biological questions in agriculture, forestry, environmental science, food production, and medicine.

1.7 PREVIOUS WORK

Previous researchers from our lab, used *Vibrio gazogenes*, a non-pathogenic gram-negative marine bacterium, which was proven to synthesize antifungal and anti-aflatoxin metabolites. In their research study they used *Aspergillus parasiticus* and *Aspergillus flavus* – two saprophytic pathogenic fungi as aflatoxin-producing models. Their research study showed that, by treating *V. gazogenes* with aflatoxin, produced aflatoxin responsive metabolites (ARMs) that could significantly decrease aflatoxin synthesis by inhibiting the aflatoxin genes (*aflR, nor-1, ver-1*) and global secondary metabolism genes (*LaeA, VeA*). But the decrease in aflatoxin was only 40%. They treated the fungal cultures with the cells of *V. gazogenes* and the aflatoxin ELISAs revealed the significant decrease (>99%) in aflatoxin biosynthesis by the fungi. The aflatoxin inhibitory effect was very specific to *V. gazogenes* and not to other gram-positive or gram-negative bacteria. Fungal conidial growth and aflatoxin significantly decrease by 50% and 98% respectively after infecting corn kernels with *A. flavus* in the presence of the bacterium. Drosophila flies survival increased after treating with *V. gazogenes* before *A. flavus* infection. The research study also showed the uptake of the bacterium by the fungus into vesicles in scanning electron and transmission electron microscopies. RT-PCR assays revealed that live *V. gazogenes* cells significantly up-regulate aflatoxin genes (*aflR, nor-1, ver-1*) and global secondary metabolite genes (*laeA, veA*). The pathway through which *V. gazogenes* inhibits aflatoxin is complicating. A previous study from our lab had developed a novel tool (V. gazogenes) to inhibit the aflatoxin biosynthesis, which is acting at the cellular level rather than at the gene level.

1.8 CURRENT STUDIES FOCUSED ON THIS

To understand the toxigenicity of *A. flavus* in response to *V. gazogenes* cells, we focused on *Vibrio-Aspergillus* interaction experiments. Our first target to know, where *V. gazogenes* cells gone in the media when they meet *A. flavus*. We also want to understand if *V. gazogenes* internalized within hyphae during the inhibition of aflatoxin production. So, we determined whether bacterial cells were taken up by the growing mycelia. Given that we did not find any evidence of intact bacterium on the surface and inside of the fungal cells, we hypothesized that the viability of *V. gazogenes* was not essential for *V. gazogenes* -dependent aflatoxin reduction. To test this hypothesis, we sought to determine if heat-treated non-viable *V. gazogenes* could result in a similar reduction of aflatoxin production in *A. flavus* as the viable *V. gazogenes*. As a first step to determine the mechanisms underlying *Vibrio gazogenes* -dependent aflatoxin reduction, we determined whether the *V. gazogenes* treatment was able to restrict aflatoxin biosynthesis at the level of transcription activation of aflatoxin genes.

Due to our *Vibro-Aspergillus* interaction experiments, we found almost complete inhibition of aflatoxin when the bacteria internalized through endocytosis (vesicular uptake) by the fungus. So, we did some more relative experiments to observed sclerotia formation, hyphal polarization, and hyphal fusion etc. in *A. flavus*. Conidia and sclerotia contain aflatoxin and studies have positively correlated sclerotial development with aflatoxin production. we proceeded to test whether, sclerotia development is restricted, in a *V. gazogenes* -dependent manner. In consistence with our theory that *V. gazogenes* components upon internalization within hyphae, intervene in normal endosomal functions, we have also observed that hyphal elongation, a process that also relies on endocytosis and

normally functioning endosomes, was significantly inhibited. Since we observed *V. gazogenes* components in organelles that appeared to be endosomes, we proceeded to test whether hyphal fusion, a fundamental biological process of filamentous fungi, and which is coordinated by the endocytic machinery and endosomal transport, is impeded in a *V. gazogenes* -dependent manner.

To understand the effect of the uptake of *V. gazogenes* cells through endocytosis we treated *A. flavus* with endocytosis inhibitors (filipin and natamycin). Next, we investigated if *V. gazogenes* cells lysed in the presence of *A. flavus* because we did not observe any evidence of intact bacterium within the hyphal cells with transmission electron microscopy in our previous studies. To understand if the prodigiosin produce by *V. gazogenes* is the only aflatoxin inhibitor, we treated *A. flavus* with a gram-negative bacterium, *Serratia sp.*, which also secrete a host of virulence factors including prodigiosin. Since we observed prodigiosin is not the only functional component that is specific to *V. gazogenes,* we proceeded to test whether intact and fractionated *V. gazogenes* cells are impeded aflatoxin reduction in the same manner.

Our studies raise the idea of potentially using a food-grade *V.gazogenes* strain as a potential biocontrol agent to reduce *A. favus* aflatoxin contamination. Our research results, along with further studies, will eventually provide a novel product(s) that can be used as a natural anti-fungal and anti-aflatoxin agent. In particular, the increasing availability of sequenced genomes and modern molecular methods to facilitate high-throughput studies will undoubtedly improve our understanding of host/pathogen interactions in the context of both agricultural and human health sciences.

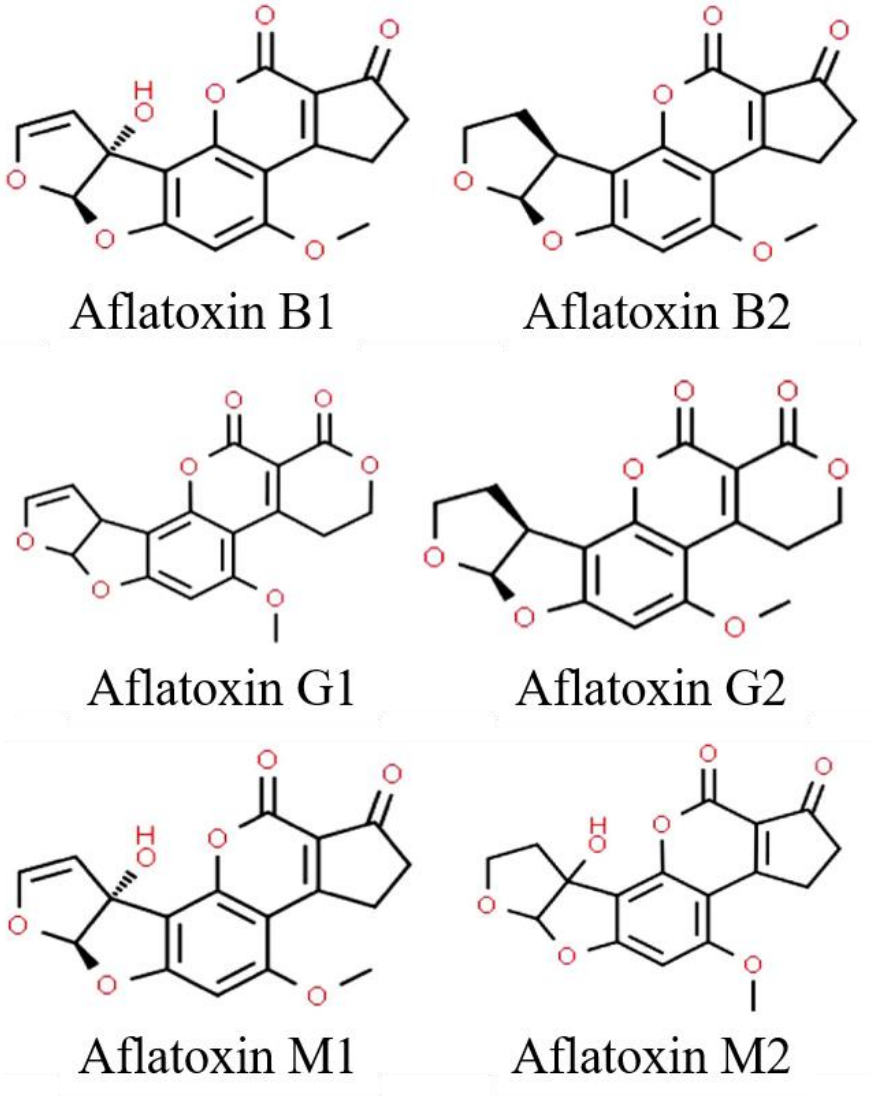


Figure 1.1: Chemical structures of the major types of aflatoxins (Peles et al., 2019)

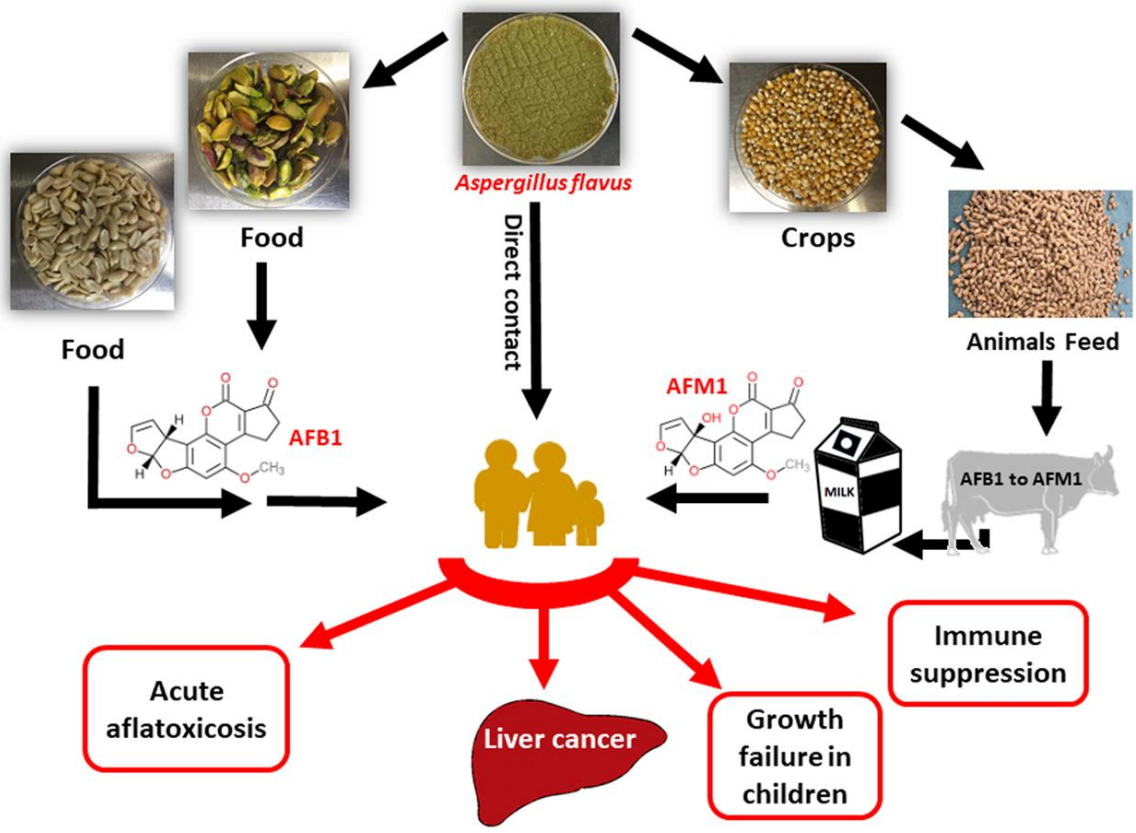


Figure 1.2: Schematic presentation summarizing the major AFB1 and AFM1 exposure routes and adverse health effect

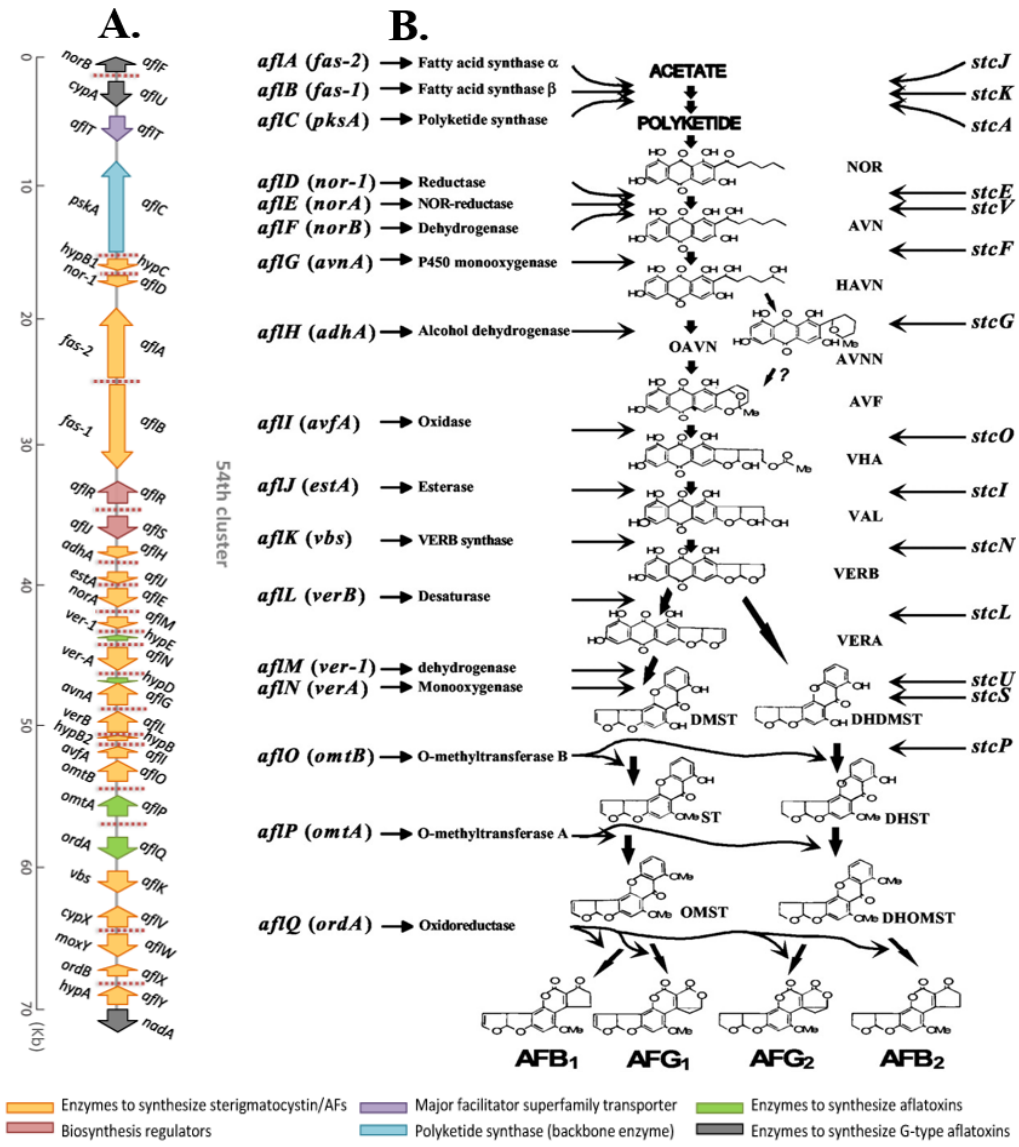


Figure 1.3: The gene cluster responsible for aflatoxin biosynthesis in *A. flavus* (A) Clustered genes (arrows indicate the direction of gene transcription) and (B) the aflatoxin biosynthetic pathway. The ST biosynthetic pathway genes in *A. flavus* are indicated at the right of panel B. Arrows in panel B connect the genes to the proteins they encode. Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5¢-hydroxy-averantin; *OAVN*, oxoaverantin; *AVNN*, averufanin; *AVF*, averufin; *VHA*, versiconal hemiacetal acetate; *VAL*, versiconal; *VERB*, versicolorin B; *VERA*, versicolorin A; *DMST*, de-methyl sterigmatocystin; DHDMST, dihydrode methyl sterigmatocystin; ST, sterigmatocystin; *DHST*, dihydro sterigmatocystin; *OMST*, O-methyl sterigmatocystin; *DHOMST*, dihydro-O-methyl sterigmatocystin (Yu et al., 2004b)

CHAPTER 2

Vibrio gazogenes DEPENDENT INHIBITION ON AFLATOXIN BIOSYNTHESIS IN *Aspergillus flavus*

2.1 INTRODUCTION

With changes in global climate, mycotoxin (aflatoxin) contamination of agricultural commodities from Aspergilli and other fungal pathogens continues to rise and poses a growing threat to human health (Bayman and Baker, 2006; Boonen et al., 2012; Buller, 1931; Carlile et al., 1994; CAST, 2003b; Chang and Kim, 2007; Cullen et al., 1994; Eaton and Groopman, 2013; Gow and Gadd, 2007; Ingold, 2012; Moss, 1986; Payne et al., 1993; Peñalva and Arst, 2002; Trail et al., 1995; Yin et al., 2008). While compounds that inhibit mycotoxin-synthesis (especially aflatoxin-synthesis) exist, infants and toddlers are still at risk of mycotoxin exposures (Gummadidala et al., 2019b; Zhang et al., 2018b), which can impair growth and development in children, weaken their immune system and make them vulnerable to infections and other health disorders including cancer.

Aflatoxin B1, a mycotoxin produced primarily by *Aspergillus flavus* is the most potent naturally occurring carcinogen (Coppock et al., 2018) and contamination levels are predicted to increase due to impending climate change (Battilani et al., 2016). Estimated U.S. losses range from $52.1 million to $1.68 billion annually for aflatoxin alone (Mitchell et al., 2016a). With liver carcinomas already being the third leading cause of cancer-related mortality worldwide, the global increase in the prevalence of hepatitis B virus (HBV) and

immunocompromised population has increased the risk of aflatoxin-induced liver cancer (Liu and Wu, 2010). NIH statistics indicate that 16,600 new cases of aflatoxin-induced liver cancer annually in the US (Kensler et al., 2011). The elimination of aflatoxin accumulation in food and feed, therefore, is of primary importance for reducing its global burden on public health and economy.

Despite years of efforts, there is still no precise method to reduce dietary exposures to aflatoxin and other mycotoxins. To address this need, the use of microflora and their metabolites as biocontrol agents against aflatoxin and other mycotoxins are becoming increasingly popular (Kagot et al., 2019; Khalid et al., 2018; Palumbo et al., 2006b; Spraker et al., 2018a). Bacteria and fungi can form a range of physical associations that depend on various modes of molecular communication for their development and functioning. These bacterial-fungal interactions often result in changes to the pathogenicity or the nutritional influence of one or both partners toward plants and animals (including humans). Here we present a structured research study of bacterial-fungal interactions, illustrated by examples sourced from many diverse scientific fields. We consider the general and specific properties of these interactions, providing a global perspective across this emerging multidisciplinary research area. We show that in many cases, parallels can be drawn between different scenarios in which bacterial-fungal interactions are important. Finally, we discuss how new avenues of investigation may enhance our ability to combat, manipulate, or exploit bacterial-fungal complexes for the economic and practical benefit of humanity as well as reshape our current understanding of bacterial and fungal ecology (Frey-Klett et al., 2011). Hence there is an urgent need to discover a novel drug that can successfully inhibit mycotoxin accumulation in food without the health concerns.

Researchers have tried different methods and the long-term goal is to understand how bacteria-fungal interactions influence to produce aflatoxin inhibitory molecules biosynthesis to discover novel molecules to eliminate aflatoxin accumulation in nature.

Our recent study (Gummadidala et al., 2016) has shown that the marine gram-negative bacterium, *Vibrio gazogenes*, which notably does not share the same ecological niches as aflatoxin producers, can produce metabolites that we termed 'aflatoxin response metabolites' (ARMs), which could result in partially (~2-3 fold) inhibit aflatoxin biosynthesis. However, a direct interaction study between *V. gazogenes* with an aflatoxin producer has not been conducted thus far. Hence in this study, we sought to further examine the aflatoxin inhibiting capacity of *V. gazogenes* by directly introducing the *Vibrio* cells in the *A. flavus* growth medium.

We present here the results from the *Vibro-Aspergillus* interaction experiments. Not only did the *Vibrio* cells result in almost complete inhibition of aflatoxin, but it also resulted in significant down-regulation of aflatoxin biosynthesis at the level of transcription activation of aflatoxin genes. Our data also revealed that, the dead and live *V. gazogenes* cells have similar aflatoxin inhibitory effects. We further conducted microscopic imaging and observed the uptake of *V. gazogenes* into endosomes like compartments in *A. flavus*. These data represent the possibility of the *Vibrio* cells perturbing the endosomal functions. The data also uncover the yet unstudied concept that *V. gazogenes* mechanism of aflatoxin inhibition occurs at the cellular level as well as at the gene level. Finally, our study has opened the door of understanding the bacteria-fungal and gene-level interactions which help us to inhibit the aflatoxin production and pathogenicity of plant and human pathogen *Aspergillus flavus*.

2.2 MATERIALS AND METHODS

2.2.1 Strains, media, and culture conditions

The fungus *Aspergillus flavus* wild type strain, CA14PyrG.1 (designated as CA14 hereafter), was used for this study. The strain was grown on 100 mm Petri dishes containing potato dextrose agar by inoculating $2x10^4$ spores in the center containing 10 mL of PDA and incubated in the dark at 29°C for 2 weeks. Fresh spores collected from these colonies were used for all the experiments in this study. In these experiments the fungus was grown in yeast extract sucrose (YES); a rich growth medium (containing 2% w/v yeast extract, 6% w/v sucrose, pH 5.8), by inoculation of $0.5x10^6$ spores per 50 mL of liquid medium and incubated in a dark orbital shaker at 29°C at 150 rpm.

The bacterium *Vibrio gazogenes* ATCC 43942 (Farmer et al., 1988), that was originally isolated from seawater, was grown in Difco Marine Broth (cat # 2216, BD Biosciences, Sparks, MD, USA) at 29°C in a shaking incubator (150 rpm) for 24 h by inoculating 10^5 colony forming units (cfu) /100 ml of growth medium.

2.2.2 Growth quantifications of *A. flavus* and *V. gazogenes*

All fungal growth in liquid media was quantified by using dry weight measurements. Briefly, the mycelia were filtered out of the growth media using a Miracloth (Millipore, Billerica, MA) and dried at 80°C for 6 hrs and the final weight was recorded. *A. flavus* growth on PDA media plate was estimated by measuring the mycelial colony diameter daily. After 9 days of incubation time, spores were manually collected from PDA plates using 1xPBS with 0.01% tween and resuspended in 50% glycerol. Spores were

counted using a haemocytometer. All *Vibrio* growth measurements were performed using absorbance readings of growth media at 600 nm. An absorbance of 1.2 on the UV/Vis spectrophotometer was considered as 10^6 cells/ml bacterial cell density and calculations were performed for 16 million bacterial cells appropriately.

2.2.3 Aflatoxin extraction and quantification

Since previous studies (Chanda et al., 2010) that have employed the fungal growth conditions used in the current study have shown that, aflatoxin biosynthesis and export into the growth medium occurs at peak levels at 40 h from the point of inoculation of fungal spores, we have performed all aflatoxin measurements in this study at 40h time-points. Aflatoxin was extracted from *A. flavus* cultured YES liquid media by using our standard chloroform-methanol extraction protocol (Kenne et al., 2018). According to this method, an equal volume of chloroform added to harvested media, and the organic layer was collected. Chloroform was evaporated from the organic layer and the residual aflatoxin was resuspended in 1 ml of 70% methanol. The resuspended aflatoxin extract was spun down at 15,000 rpm for 1 min at room temperature to remove extra debris from the media and mycelia. Aflatoxin was quantified in the extracts using ELISA and HPLC procedures as described previously (Gummadidala et al., 2019a). For analyzing aflatoxin using the Neogen Veratox Aflatoxin ELISA kit (cat # 8030) from Neogen (Lansing, MI, USA) and measured on a Stat Fax 4700 Microstrip Reader (Awareness Technologies, Palm City, FL, USA) as per kit's protocols. The ELISA has 2 ppb and 50 ppb as lower and upper limits of detection respectively. Thus, highly concentrated aflatoxin samples were diluted to fit within the detection range.

2.2.4 RNA extraction, purification, and cDNA synthesis

Total RNA was extracted from fungal cells harvested at 24h, 30h, and 40h post-inoculation in triplicates using a TRIzol-based (TRI Reagent®; cat # T9424, Sigma, Carlsbad, CA, USA) method as described previously (Gummadidala et al., 2016). The harvested mycelia were ground with a mortar and pestle in liquid nitrogen. The cold powdered mycelia were mixed with TRI Reagent® and chloroform and the mix was spun down at 10,000 rpm for 10 min at room temperature. The organic layer was mixed with equal volumes of isopropanol and incubated on ice for 15 min and later centrifuged at 10,000 rpm for 15 min at room temperature. The precipitated crude RNA was washed with 70% ethanol and resuspended in RNAse/DNAse free water (Sigma, Carlsbad, CA, USA). Within 24 h of extraction, RNA cleanup was performed using a Qiagen RNEasy Cleanup Kit (Qiagen, Valencia, CA, USA), as per kit's instructions and samples were stored at −80°C. Total RNA was then reverse transcribed to cDNA using iScript™ cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA) as per kit's instructions. All samples were checked for concentration and purity after each step using a NanoDrop 2000 Spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA). All cDNA samples were stored at −20°C until subsequent RTPCR quantification.

2.2.5 Transcript analysis by Quantitative Real-time PCR

Quantitative RT-PCR (qRT-PCR) was performed using SYBR Green I chemistry and CFX96 Real-Time PCR detection system (Bio-Rad). A pre-incubation at 95°C for 3 min, dye activation at 95°C for 10 s, primer annealing at 55°C for 30 s, elongation at 55°C for 50 s followed by a dissociation curve from 65°C to 95°C for 30 min (with 0.5°C

increments). The primers used for qRT-PCR are provided in Table 2.1. Gene-specific forward and reverse primers were designed using Primer3 online software (Ye et al., 2012). All RT-PCRs were performed in triplicate for each gene per sample. The 18s ribosomal DNA was used as a reference gene in the gene analysis. The gene expression values of *A. flavus* obtained from the threshold cycle values were normalized to the 18s rDNA of each sample. We choose 24, 30 and 40 h to study the transcripts of the genes (explained later in results). For the quantitative comparison of gene expressions, the values for each target gene at 30 h and 40 h were expressed as fold change relative to the 24 h time point of that specific treatment condition. Gene expression was normalized by ΔΔCT analysis (Livak and Schmittgen, 2001) to *A. flavus* β-tubulin gene (AFLA_068620) using the gene expression analysis software package (Bio-Rad CFX Maestro) of the Bio-Rad CFX96. A significant change in gene expression was considered if fold change was ≥ 2 and the p-value was < 0.01.

2.2.6 Statistical analysis

All statistical tests were performed using GraphPad Prism Software (GraphPad, CA, USA). All experiments were performed in triplicate. Microsoft Excel was used to generate graphs, perform statistical analysis, and calculate significance for dry weight measurements and aflatoxin analysis. Statistical analyses to determine for statistical significance of differences between control *versus* experimental groups were determined using one-way ANOVA (with sample size 3). Significance was set at $p < 0.05$. RT-PCR statistical analysis was performed using CFX Manager software with parameters set for significance at p-value <0.01 and gene expression fold change ≥ 2.

2.3 RESULTS

2.3.1 Introduction of the phenomena - *V. gazogenes* dependent aflatoxin reduction

I first sought to examine the toxigenicity of *A. flavus* (CA14) in response to 16 million *V. gazogenes* cells, when added to YES liquid media for 40 h as we found that at 40 h time-point *A. flavus* grow most vigorously and produce the highest amount of aflatoxin. We also try to understand if *V. gazogenes* internalized within hyphae during the inhibition of aflatoxin production. So, we determined whether bacterial cells were taken up by the growing mycelia. The uptake was clearly visible after 40 h time-point as the mycelial pellets showed similar orange-red color as *V. gazogenes* (Figure 2.1A). Harvested mycelial pellets that were grown in presence *V. gazogenes* showed a clear change of their original (Figure 2.1B). We also conducted a time-course measurement of the bacterial concentrations in the growth medium by recording the OD_{600} readings to determine if, during the mycelial growth, the bacterial cells were depleted in the growth media. As shown in Figure 2.1C, OD_{600} dropped steadily from 0h to 40h time-point, post-inoculation. By 40h time-point, OD_{600} readings were similar to that of the uninoculated growth medium, suggesting that *V. gazogenes* was taken up by the mycelia. Bright-field microscopy clearly showed bacterial components in discrete locations along the hyphae (spotted as orange dots using a regular camera in figure 2.1D-i). Hyphal micrographs observed with confocal laser scanning microscopy with a monochromatic camera suggested that the bacterial components were within the hyphae in endosome-like compartments (Figure 2.1D-ii). We also observed from our ELISA data (Figure 2.1E) that the administration of 16 M bacterial cells resulted in an almost complete inhibition (>98%) of aflatoxin production in liquid growth media while *V. gazogenes* was completely taken up by the fungal mycelia. To

further understand how aflatoxin production in *A. flavus* is influenced upon *V. gazogenes* administration, we have consistently used the same experimental set-up for the rest of this study with the administration of 16 M *V. gazogenes* cells.

2.3.2 *V. gazogenes* viability is not necessary for *V. gazogenes* - dependent aflatoxin reduction

Non-viable bacterial biomass had proven to be better and safer bio adsorbent for contaminants in the environment and is much preferred due to lack of nutrients and cultural conditions (Zeroual et al., 2006). Given that we did not find any evidence of intact bacterium on the surface and inside of the fungal cells, we hypothesized that the viability of *V. gazogenes* was not essential for *V. gazogenes* -dependent aflatoxin reduction. To test this hypothesis, we sought to determine if heat-treated non-viable *V. gazogenes* could result in a similar reduction of aflatoxin production in CA14 as the viable *V. gazogenes*. We boiled the bacteria at 100^{O}C for 10 min in a hot plate to make them unviable (bacterial cells were tested by inoculation into fresh marine broth media and observed for growth using spectrophotometric analysis over the next 40 h and confirmed the no growth of the *V. gazogenes* cells). 16 million dead or non-viable *V. gazogenes* cells were used to treat *A. flavus* and aflatoxin synthesis were observed. The scheme for this experiment is shown in figure 2.2A. The results of this experiment are shown in figure 2.2B, where we observed an almost complete inhibition of aflatoxin production (>95%), thereby confirming our hypothesis that *V. gazogenes* viability is not essential for *V. gazogenes* -dependent aflatoxin inhibition and non-viable and viable *V. gazogenes* cells almost equally inhibit aflatoxin biosynthesis.

2.3.3 *Vibrio gazogenes* dependent aflatoxin reduction occurred at the levels of aflatoxin gene expression

As a first step to determine the mechanisms underlying *Vibrio gazogenes* - dependent aflatoxin reduction, we determined whether the *V. gazogenes* treatment was able to restrict aflatoxin biosynthesis at the level of transcription activation of aflatoxin genes. Hence, we measured transcript levels of the aflatoxin pathway regulator gene (*aflR*) and three aflatoxin pathway genes, *pksA* (*aflC*), *nor-1* (*aflD*) and *ver-1* (*aflM*) at 24h and 40h time-points; the 24h corresponds to activation of aflatoxin biosynthesis and 40h correspond to the time-point when aflatoxin biosynthesis reached peak levels. The aflatoxin biosynthesis genes get activated by 24 h with transcript accumulation at 30 h and by 40 h the fungus is producing copious amounts of aflatoxin in YES liquid growth media (Roze et al., 2007) which make us to choose 24 h and 40 h time-point to study the transcripts levels of aflatoxin pathway genes. As shown in Figure 2.3A**,** while at 24h, we observed a significant downregulation of three of the four aflatoxin genes (with *aflR* showing greater than 2-fold reduction of gene expression), at 40h time-points we observed a significant reduction of transcript levels (>4-fold) of all four genes analyzed (Figure 2.3B).

2.4 DISCUSSION

I have brought forward through this study, a novel phenomenon – *V. gazogenes* dependent aflatoxin reduction in *A. flavus*, that can allow us to study the uptake mechanisms of *V. gazogenes* cells through fungal hyphae. Using confocal laser microscopies, we observed the uptake of the bacterium by the fungus into endosome like compartments. The first and essential step for a bacteria-fungal interaction is the physical

contact and attachment of the bacterial cell with the cell surface of fungi (Rossmann et al., 2020). In bacterial cells, these interactions often result in the membrane and cellular damage occurring from multiple factors that include the release of metal ions near the membrane, membrane rupture, and leakage (Wolfson et al., 2020). These interactions enable the internalization of the bacterial cells into the fungal cells (Siahmoshteh et al., 2016). Endocytosis is the most common mechanism of internalization of bacterial cells (Erktan et al., 2020; Kim et al., 2017), which can be clathrin-dependent (Cossart and Helenius, 2014; Veiga and Cossart, 2006) and receptor-mediated (Cossart and Helenius, 2014). Once in endosomes, the NPs can travel within cells using the available cellular machinery for endosomal transport (Bonazzi and Cossart, 2006). In a protoplast isolation and feeding experiment, the researcher showed that aflatoxin synthesis was happening in vesicles termed aflatoxisomes, which are endosomal like compartments (Chanda et al., 2009a; Chanda et al., 2009b). Previous researchers from our lab also showed *V. gazogenes* cells uptake by fungal endosomes using SEM and TEM microscopies (Gummadidala, 2018). In this research study, we have obtained clear evidence demonstrating that uptake of *V. gazogenes* into *Aspergillus* hyphae and this uptake resulted in significant aflatoxin reduction. Additionally, our observations from confocal microscopy support our theory that endocytosis is one key mechanism for bacterial uptake. Based on the results of this study, we propose a theoretical model (Figure 2.4) that represents a possible bacteria-fungal interaction model for *V. gazogenes*-dependent aflatoxin reduction. According to this model, the internalization of cellular components of *V. gazogenes* in hyphae results in the intervention of endosomal functions associated with aflatoxin biosynthesis.

Administration of equal concentrations of heat-inactivated non-viable V. gazogenes cells to the growth medium resulted in a similar reduction in aflatoxin production, supporting that bacterial viability was not necessary for this inhibitory effect. Previous researcher RT-PCR data revealed controversial gene expressions of aflatoxin pathway genes and global secondary metabolite regulatory genes in the presence of viable and non-viable *V. gazogenes*. Their data suggest that the viable and non-viable *V. gazogenes* aflatoxin inhibitory mechanisms are different (Gummadidala, 2018). In the future, it will need further investigation to demonstrate the effect of non-viable *V.* gazogenes mediated aflatoxin inhibition at the level of transcriptional activation of aflatoxin genes.

Since biosynthesis of aflatoxin occurs primarily in endosomal compartments (Chanda et al., 2009a; Lim and Keller, 2014b; Linz et al., 2012), we were curious to know if the *Vibrio*-dependent reduction of aflatoxin was at the level of cellular regulation only. Hence, we analyzed the expression levels of aflatoxin genes in the presence and absence of *V. gazogenes*, with the understanding that if inhibition of aflatoxin occurred only at the level of cellular regulation, there would be no significant difference of transcript levels of aflatoxin genes. However, to our surprise, we observed significant down-regulation (>90%) in the expression of all three aflatoxin genes (*nor-1, pksA,* and *ver-1*) and the aflatoxin pathway regulator *aflR* analyzed in this study, thereby demonstrating that aflatoxin inhibition occurred at the level of transcriptional activation.

Our study provides the first evidence, to the best of our knowledge, of an organism that demonstrates the ability to synthesize aflatoxin inhibitors, while not sharing ecological niches with aflatoxin producers at all. With the global changes in climate, *Vibrio gazogenes*

may be a chemically-gifted organism that has genetically evolved with the rising mycotoxin levels in the environment (Gummadidala et al., 2016; Kolpin et al., 2014; Rangel et al., 2015).

One important limitation of the theoretical model proposed here is that it is primarily founded on studies with one plant pathogen model. The endosomal localization of bacterial cells upon uptake is hypothesized because some of our preliminary unpublished data are in line with these findings. While we cannot rule out the possibility of localization of the bacterial cells in other subcellular organelles, especially cytoplasm, we also point out that pathogens within the genus *Aspergillus*, such as *A. parsiticus*, *A. nidulans*, *A. fumigatus*, and *A.oryzae*, have consistently been used as a filamentous fungal model and as a eukaryotic model to study fundamental cellular processes; most eukaryotic cellular processes are conserved in these models. We also bring to the readers' attention that the bacteria-fungal interaction model proposed is an attempted simplification of a possible complex response of a fungal cell to bacterial cells uptake and is focused on endosomal intervention only. However, given that the development of endosome to a toxisome involves a significant cross-talk of multiple organelles (including, but not limited to the endoplasmic reticulum, mitochondria, and peroxisomes) and vesicles from multiple protein sorting pathways, an endosomal intervention should affect the functions of other organelles as well and should be taken into account. As a first step to better utilize *V. gazogenes* as inhibitors of fungal toxins and virulence factors, it is essential to increase our understanding of (1) how *V. gazogenes* internalize into the endosomes, (2) how it affects the biochemical fate of endosomes, and (3) how the molecular processes critical to mycotoxin production and virulence influenced by the altered internalized *V. gazogenes*. The use of integrated

multi-omics (genomics, transcriptomics, proteomics, and metabolomics) approaches involving parallel experimental and computational aspects that will aim to identify the genes, proteins, and biochemical mechanisms associated with *A. flavus* response to *V. gazogenes* cells should provide a deeper understanding of the metabolic and regulatory networks of fungal cell systems that are the key player in bacteria -fungal interactions.

Table 2.1: List of PCR primers used in the study

Oligonucleotide primers used for the qRT-PCR	
Genes	Primers
pksA (aflC)	F 5'- CGCCACCTATTTTGCCGATG-3' R 5'-GTACTCAGACACAGACCGGC-3'
nor-1 (aflD)	F 5'- CAGCACCATCACCAACATGC -3' R 5'- CTGCACATGTCCTGGATCGA-3'
ver-1 (aflM)	F 5'- CGCCACCTATTTTGCCGATG-3' R 5'- GTACTCAGACACAGACCGGC-3'
aflR	F 5'- CTCAAGGTGCTGGCATGGTA-3' R 5'- CAGCTGCCACTGTTGGTTTC-3'

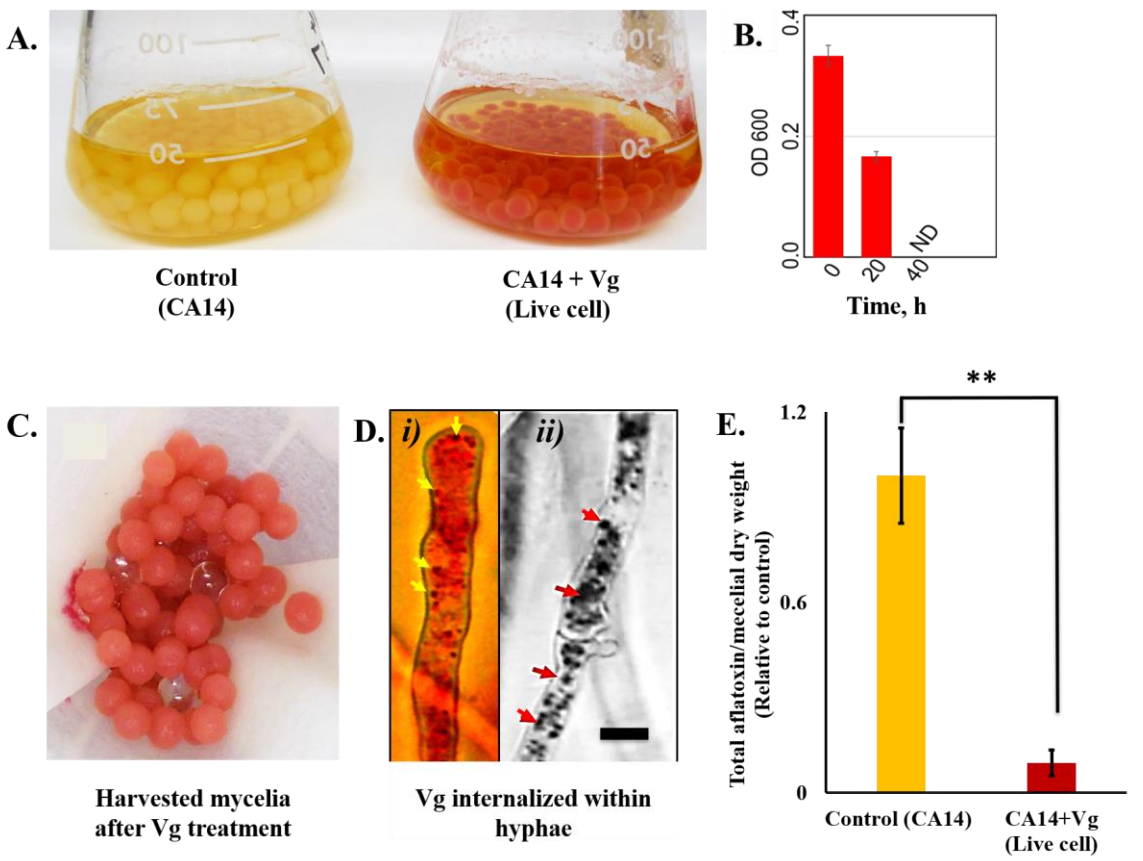


Figure 2.1: Fungal uptake and the effects of *V. gazogenes* treatment on aflatoxin levels in *A. flavus*. (A) Visual comparisons of mycelial pellets in growth medium without Vg (left flask) and with Vg (right flask) and, (B) Depletion of bacterial cells in the growth medium post spore inoculation. Bacterial density in the growth medium as quantified by measuring the optical density of the growth medium at 600nm (OD_{600}). (C) A photograph of harvested mycelial pellets that were grown in presence of Vg, (D) Brightfield images of CA14 hypha upon completion of Vg uptake after 40h growth; i) widefield micrograph captured with a digital microscope, yellow arrows indicating compartments with bacterial components ii) Confocal laser scanning micrograph captured with a monochromatic camera, red arrows indicating compartments carrying bacterial components, and (E) Total aflatoxin production per mycelial dry weight by *A. flavus* CA14 in liquid yeast extract sucrose (YES) growth media in presence and absence (denoted here as Control) of Vg. 16M (16x104 cells/mL medium) were used for the study. Aflatoxin was quantified by ELISA assay after 40 h time-point. The values in the y-axis show the aflatoxin levels relative to the corresponding controls. Error bars represent SEM, from triplicate experiments. Statistical significance of two-tailed *p*-values determined using an unpaired t-test for n=16. (*, $p<0.05$). Star indicates significance.

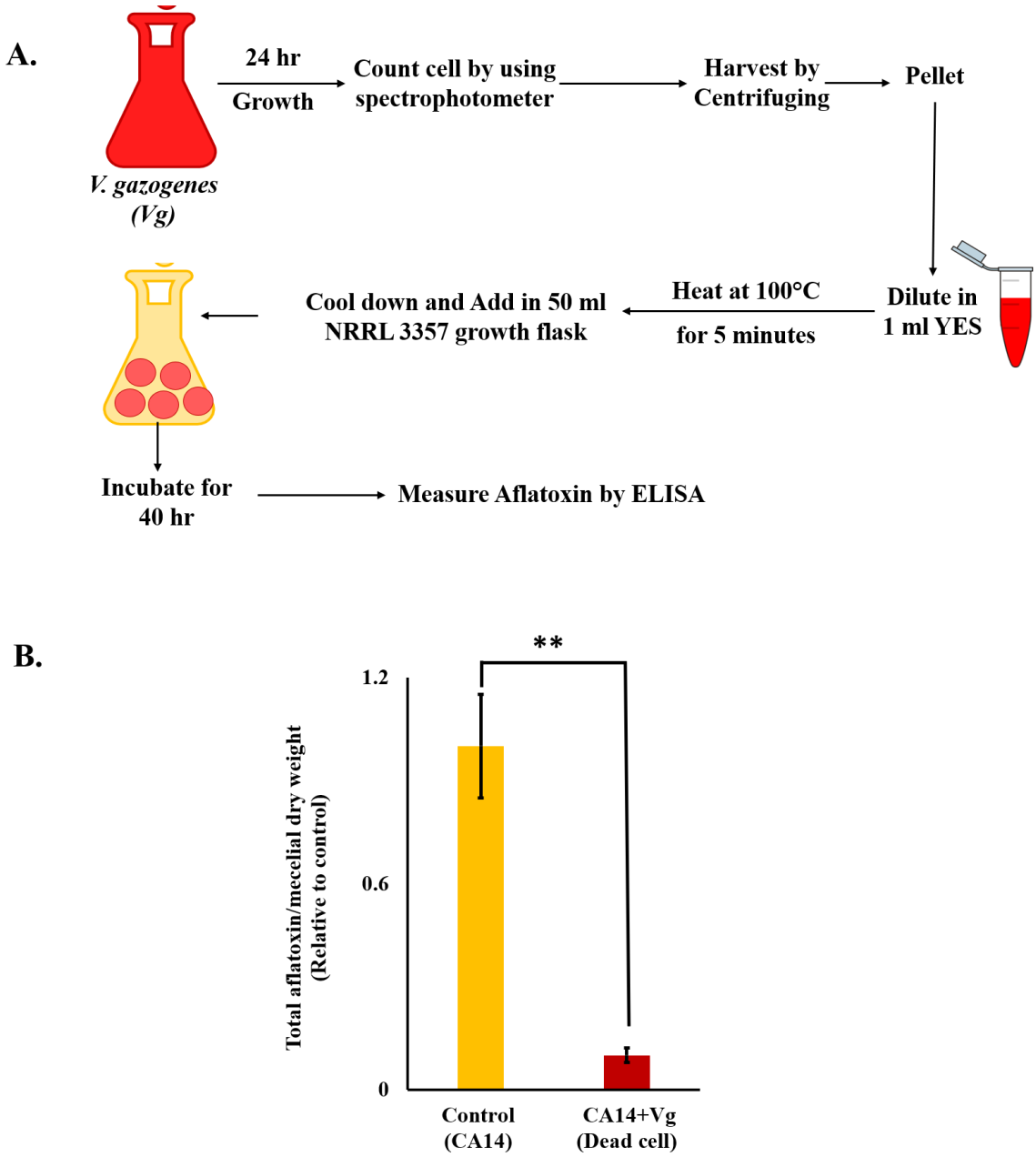


Figure 2.2: Effect of non-viable or dead *V. gazogenes* (denoted in figure as Vg) cells on aflatoxin production by *A. flavus*. (A) Systematic diagram of treatment procedure of *A. flavus* (CA14) by dead *V. gazogenes* cells (B) Total aflatoxin production per mycelial dry weight by *A. flavus* CA14 in yeast extract sucrose (YES) growth media in presence and absence (denoted here as Control) of Vg. 16M (16x104 cells/mL medium) were used for the study. The values in the y-axis show the aflatoxin levels relative to the corresponding controls. Error bars represent SEM, from triplicate experiments. Statistical significance of two-tailed p-values determined using an unpaired t-test for n=16. (*, $p<0.05$)

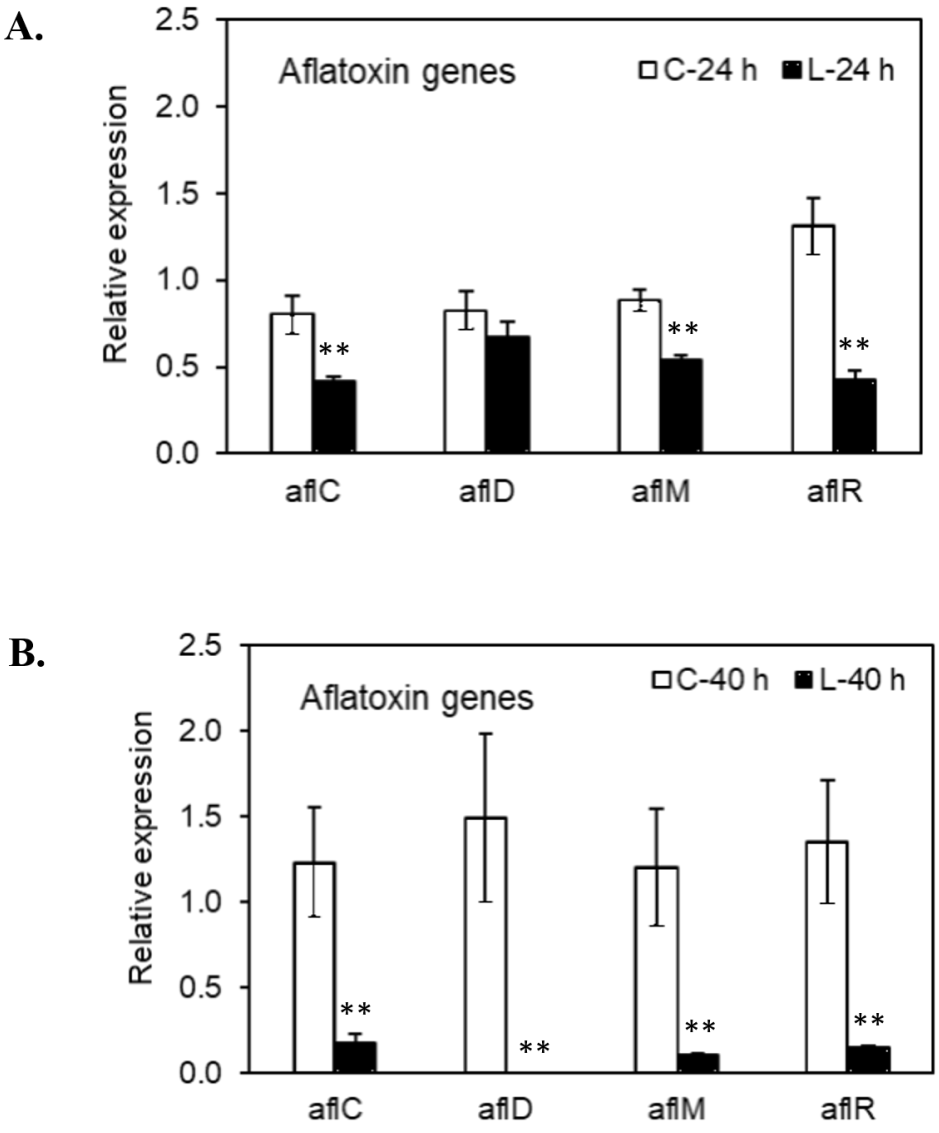


Figure 2.3: Influence of *V. gazogenes* on the expression of aflatoxin genes in *A. flavus*. (A) Comparison of expressions of genes *aflC*, *aflD*, *aflM*, and *aflR* in the control and *V. gazogenes* administered mycelia at 24 h time-point post-inoculation, and (B) comparison of the same genes at 40 h time-point post-inoculation. Error bars represent SEM, from triplicate experiments. Statistical significance of two-tailed *p*-values determined using an unpaired *t*-test for n=3. (**, $p<0.05$)

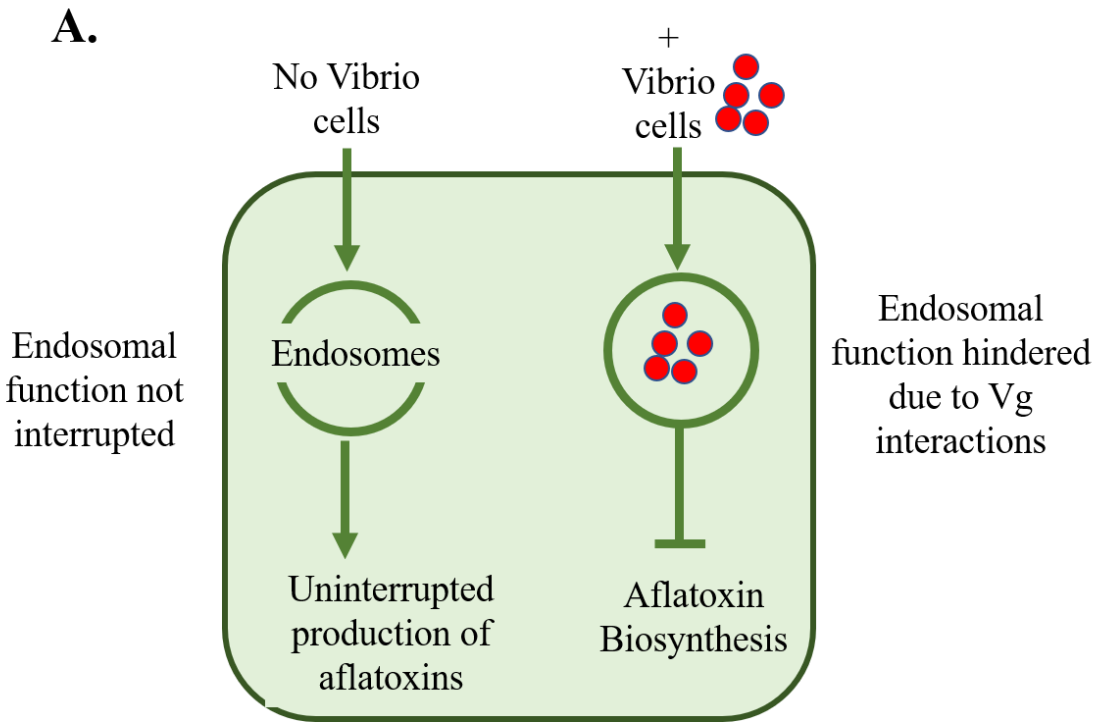


Figure 2.4: A possible bacteria-fungal interaction model for *V. gazogenes*- mediated growth-independent inhibition of secondary metabolism. (A) Proposed model. We hypothesize that *V. gazogenes* localize in endosomes after up taking by fungal cells. The endosomes, therefore, provide a platform for interactions between the bacteria and the endosomal contents: endosomal enzymes and the mycotoxin pathway intermediates. The interactions influence the molecular and biochemical mechanisms that drive the endosomal aflatoxin production. Based on our published studies (Gummadidala, 2018), the uptake of *V. gazogenes* into cells is determined by the transformations in the extracellular environment in contact with cells. Our unpublished confocal microscopy images of *Aspergillus flavus* cells exposed to *V. gazogenes* suggest that the bacteria internalize by early endosomes into the cells.

CHAPTER 3

INFLUENCE OF *Vibrio gazogenes* TREATMENT ON HYPHAL MORPHOGENESIS OF *Aspergillus Flavus*

3.1 INTRODUCTION

A. flavus is a well-known human pathogen that causes broad-spectrum diseases, which range from elevation of hypersensitivity to angio-invasive infections to causing invasive and non-invasive aspergillosis (Hedayati et al., 2007a; Morgan et al., 2005). Along with *Aspergillus fumigatus, A. flavus* is associated with allergic *bronchopulmonary aspergillosis (ABPA)* (Pihet et al., 2009; Sandhu et al., 1979; Schubert, 2009; Sehgal et al., 2018). *A. flavus* is used as model fungal pathogens for understanding the mechanisms of fungal pathogenicity since it causes invasive Aspergillosis which is the leading cause of infection-related mortality in the immunocompromised population. *A. flavus* is the primary producer of aflatoxin, which is classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (Gummadidala et al., 2016; Mitra et al., 2017). Aflatoxin exposure and hepatitis B and C virus infection are reported to be widespread and act synergistically to cause liver cancer (Tang et al., 2018). Given that aflatoxin is a common contaminant of weaning foods in parts of the world where childhood stunting is prevalent, it is essential to reduce aflatoxin exposure in foods consumed by children (Chen et al., 2018a). There is significant epidemiological evidence that human aflatoxin exposure is linked to the HIV/AIDS epidemic and the incidence of other

infectious diseases (Mak et al., 2018). Chronic low-level exposure to carcinogenic aflatoxins is a serious potential health threat in the US (Magnussen and Parsi, 2013) and the annual aflatoxin management alone costs $276 - $709 million (CAST, 2003a).

Consequently, innovative evidence-based technologies are urgently required to minimize aflatoxin exposure. Thus far, biological control has been developed as the most innovative potential technology of controlling aflatoxin contamination in crops, which uses the competitive exclusion of toxigenic strains by non-toxigenic ones. In our previous study we already introduced an effective non-pathogenic novel biocontrol agent (*Vibrio gazogenes*), which can inhibit aflatoxin production in *A. flavus* when the bacteria internalized through endocytosis (vesicular uptake) by the fungus. Due to bacteria-fungal interaction, bacterial active invasion by fungal cells can be happened along with endocytosis. The involvement of endocytosis in the apical growth of fungus hyphae, in the exchange of signaling molecules during the interaction of fungal cells with other microbes or plants, are studied (Moebius et al., 2014). Recent evidence has shown that hyphal growth not only depends on polarized secretion but also requires endocytosis, suggesting that the recycling of the membrane and sorting of vesicles is required for the fast elongation of hyphal tubes (Pruyne and Bretscher, 2000). Hyphal growth of filamentous fungi requires microtubule-based long-distance motility of early endosomes (Steinberg, 2014). Key players on the molecular level that direct tip growth and endocytosis in the fungal hyphae based on differential regulation of the actin cytoskeleton (Wendland and Walther, 2005).

Due to our *Vibro-Aspergillus* interaction experiments, we found almost complete inhibition of aflatoxin when the bacteria internalized through endocytosis (vesicular uptake) by the fungus. So, we did some more relative experiments to observed sclerotia

formation, hyphal polarization, and hyphal fusion etc. in *A. flavus*. Our experiments resulted in significant changes in hyphal development that could be related to the possibility of the *Vibrio* cells perturbing the endosomal functions. Also, we report here a set of intriguing alterations in hyphal development upon the *Vibrio* treatment that demonstrates the feasibility of using this experimental setup as a novel minimal cell perturbation approach for investigating how secondary metabolite synthesis and hyphal development are coordinated in filamentous fungi.

3.2 MATERIALS AND METHODS

3.2.1 Strains and culture conditions

The Strains used in this research are listed in Table 3.1 and stored as glycerol stocks at -80°C. *Vibrio gazogenes,* ATCC 43942, was used in this study and was grown in Difco Marine Broth 2216 (BD Biosciences, Sparks, MD, USA) at 28°C in a shaking incubator (150 rpm) for 24h before harvesting the cells for the interaction experiments. *Aspergillus flavus* wild type strain, NRRL 3357, was used in most of the interaction studies with *V.* gazogenes. We have used a *norA* mutant of *A. flavus* (MAEW4) that has a block in the aflatoxin pathway resulting in an accumulation of the orange-colored aflatoxin intermediate, norsolorinic acid when aflatoxin pathway is activated. YES (6% sucrose, 2% yeast extract, pH 5.8) medium was used for experiments with liquid culture, and potato dextrose agar (PDA) was used for experiments with solid culture. All auxotrophic strains were grown on glucose minimal medium (GMM) for spore production at 29°C. In some experiments using the *pyrG* auxotrophic strain, TJES 19.1, 0.56 g/L uracil, and 1.26 g/L uridine were supplemented and for the *argB* auxotrophic strain, TJES 20.1, 1 g/L arginine

was supplemented in the growth medium. In some cases, all three supplements were added as described below. Heterokaryon analysis was assessed on GMM+0.25% Triton X-100 medium.

3.2.2 Radial growth, spore production, and sclerotial formation

To estimate the radial growth of the strains, the diameter from leading hyphae on either side of the spore point inoculated plate of PDA and GMM was measured every day for 7 days and the experiment triplicated. Fungal growth in liquid media was quantified by using dry weight measurements. Briefly, the mycelia were filtered out of the growth media using a Miracloth (Millipore, Billerica, MA) and dried in an oven at 80°C for 6 h and the weight difference before and after drying was recorded. Spore production was assessed by creating a spore overlay. 10 mL of PDA cooled to solidify, and 10^6 cells/ml were center inoculated and grown at 30°C for one week. At the respective time point, spores were manually collected from PDA plates with the help of sterilized spreader by gently rubbing and homogenized in 3 ml 1xPBS with 0.01% tween and resuspended in 50% glycerol. Spore solutions were enumerated *via* hemocytometer and performed in triplicate.

The sclerotial formation was assessed on PDA media. 10^7 cells/ml were center inoculated on 100 mm PDA plates containing. These were incubated in the dark for 5 days, washed with 70% ethanol, sclerotia scrapped off, and weighed. The experiment was performed with four replicates.

3.2.3 Hyphal fusion assay and Heterokaryon formation

Hyphal fusion assay was assessed as previously reported (Zhao et al., 2017b). Briefly, the *pyrG* auxotroph TJES19.1, *argB* auxotroph TJES20.1, and mutant Δ*hamI* - TXZ9.16 were grown on GMM with supplements. To investigate if *V. gazogenes* treatment interfered in the hyphal fusion process, we mixed equal numbers of the *pyrG* auxotrophic strain (TJES 19.1) with *argB* auxotrophic strain (TJES 20.1) were spotted onto the GMM agar media containing arginine (1 g/L) and uracil (5 mM)/uridine (5 mM) with or without *V. gazogenes*. After incubation at 29°C for 5 days, the newly formed conidia were collected, and 10^5 conidia were spread onto the GMM+0.25% Triton X-100 medium with no added supplement (the 0.25% Triton X-100 restricts colony diameter to help with precise colony counts). Heterokaryotic colonies were counted after incubation for 3 days at 29°C. As a negative control we used a cross of the hyphal anastomosis mutant, Δ*hamI* - TXZ9.16, with TJES 20.1. This experiment was performed with triplicates and repeated twice.

3.2.4 Aflatoxin analysis

Aflatoxin was extracted from liquid cultures and solid cultures using our standard chloroform-methanol extraction protocol described previously (Kenne et al., 2018). Aflatoxin was quantified in the extracts using a Veratox for Aflatoxin ELISA kit (Neogen Food Safety, Lansing, MI, USA) and measured on a Stat Fax 4700 Microstrip Reader (Awareness Technologies, Palm City, FL, USA) and using HPLC procedures as described previously (Gummadidala et al., 2019b).

3.2.5 Statistical analysis

GraphPad Prism software (La Jolla, CA, United States) was used for statistical analysis. Statistically significant differences were determined by ANOVA and $P < 0.05$. The error bars in all figures indicate the standard error of the mean. All experiments were performed in triplicate.

3.3 RESULTS

3.3.1 *V. gazogenes* dependent aflatoxin inhibition associated with sclerotial formation

Conidia and sclerotia contain aflatoxin (Wicklow and Shotwell, 1983) and studies have positively correlated sclerotial development with aflatoxin production (Brown et al., 2009) although one process is not necessarily dependent on the other (Chang et al., 2016; Chang et al., 2017). Since we observed *V. gazogenes* have an impact on aflatoxin production and we found its components in organelles that appeared to be endosomes (Gummadidala, 2018), we proceeded to test whether, sclerotia development is restricted, in a *V. gazogenes* -dependent manner. For comparative quantification of sclerotia in the presence and absence of Vg, we have used a *norA* mutant of *A. flavus* (MAEW4) that has a block in the aflatoxin pathway resulting in an accumulation of the orange-colored aflatoxin intermediate, norsolorinic acid, when aflatoxin pathway is activated. We used this strain for this experiment because it would allow us to visualize the sclerotia side-by-side with norsolorinic acid. As shown in figure 3.1A, we observed significant depletion of sclerotia numbers in the areas where the fungi interacted with *V. gazogenes*; in the areas where red-colored *V. gazogenes* was present, norsolorinic acid depletion (absence of orange color) and a significant drop in sclerotia was clearly visible. After counting the

sclerotia, we observed a smaller number of sclerotia development in the treated area rather than the untreated area of the MAEW4 plate (Figure 3.1B).

3.3.2 *V. gazogenes* administration impeded hyphal elongation and resulted in the earlier development of conidia

In consistence with our theory that *V. gazogenes* components upon internalization within hyphae, intervene in normal endosomal functions, we have also observed that hyphal elongation, a process that also relies on endocytosis and normally functioning endosomes, was significantly inhibited (by ~3-fold) in a *V. gazogenes*-dependent manner (Gummadidala, 2018). Simultaneously, we observed an early asexual development (~2.5-fold higher conidia, as shown in figure 3.2A. To our surprise, these conidia, when grown back on PDA plates, produced significantly (~2 -fold) less aflatoxin that conidia collected from untreated NRRL 3357 colonies (Figure 3.2B).

3.3.3 *V. gazogenes* dependent aflatoxin inhibition associated with depletion of hyphal fusion

Since we observed *V. gazogenes* components in organelles that appeared to be endosomes, we proceeded to test whether hyphal fusion, a fundamental biological process of filamentous fungi, and which is coordinated by the endocytic machinery and endosomal transport (Fischer and Glass, 2019; Riquelme et al., 2018), is impeded in a *V. gazogenes* -dependent manner. We used a recently published hyphal fusion assay (Zhao et al., 2017a; Zhao et al., 2017b) that is based on the rationale that since heterokaryon formation requires hyphal fusion, any intervention of hyphal fusion would prevent heterokaryon formation. The scheme for this experiment is shown in figure 3.3A. Equal numbers of conidia

from *pyrG* auxotroph (TJES 19.1) and *argB* auxotroph (TJES 20.1) were mixed and grown on GMM medium supplemented with uracil/uridine and arginine. After 5 days conidia were spread onto GMM medium lacking supplementation where only conidia generated from heterokaryons could grow. As shown in figure 3.3 B and C, crosses between TJES 19.1 and TJES 20.1 in the presence of Vg, almost completely inhibited (by >95%) heterokaryon formation, an observation that was very similar to the negative control (crosses between the hyphal anastomosis mutant, *ΔhamI* and TJES 20.1).

3.4 DISCUSSION

We introduce through this study, a novel cellular perturbation tool (*Vibrio gazogenes*) that can allow us to study the mechanistic details of how fungal secondary metabolism and hyphal morphogenesis is coregulated at the level of endosomal uptake and transport; also from our previous studies, we found that this cellular perturbation does not lead to growth inhibition (Gummadidala, 2018). To know how aflatoxin synthesis and hyphal morphogenesis was disrupted in a *V. gazogenes* dependent manner we conducted this research. The internalization process of cellular components of *V. gazogenes* in hyphae results in the intervention of endosomal functions associated with hyphal morphogenesis and aflatoxin biosynthesis, was our main target to investigate in our study.

Since biosynthesis of aflatoxin occurs primarily in endosomal compartments (Chanda et al., 2009a; Lim and Keller, 2014a; Linz et al., 2012), we were curious to know if the *Vibrio*-dependent reduction of aflatoxin was associated with sclerotial formation. Hence, we have conducted an experiment to comparatively quantify sclerotial development in the presence and absence of the *Vibrio* treatment by using the *norA* mutant of *A. flavus*

(MAEW4). This strain produces bright orange-red color when aflatoxin pathway activated, which means the color indicates aflatoxin production visually. We observed that, in the presence of *V. gazogenes, A. flavus* (MAEW4) strain cannot produce orange-red color as well as aflatoxin, and the number of sclerotia was also depleted where we applied *V. gazogenes* in PDA plate with MAWE4 strain spreading.

An interesting observation connected with endosomal development, transport, and functions is the significant drop in hyphal elongation during polar growth upon *Vibrio* treatment. It is well established that the regular functioning of endocytic machinery is needed for hyphal elongation during polar growth (Riquelme et al., 2018; Upadhyay and Shaw, 2008). Hence, our observation in figure 3.2A (significantly less hyphal elongation) can support a theory that internalization of the *Vibrio* components interferes with the endocytic machinery that regulates polar growth. Another interesting observation was early conidiation that could be associated with the inhibition of hyphal fusion and reduced sclerotia development upon *Vibrio* administration. It is well known Aspergilli maintain a balance between sexual and asexual development through a cross-talk of light-dependent regulatory factors, the pathways regulating sexual and asexual reproduction and the secretion of oleic and linoleic-acid-derived oxylipins known as the psi factor (Chamberlain and Ingram, 1997; Krijgsheld et al., 2013; Park and Yu, 2012; Tsitsigiannis et al., 2004a; Tsitsigiannis and Keller, 2007; Tsitsigiannis et al., 2004b; Tsitsigiannis et al., 2005). These aspects will be investigated in more detail in our follow-up experiments.

Since we had observed the presence of the *Vibrio* components, albeit not intact cells, in endosome-like compartments, we were curious to know if these components were in any way, disrupting endosomal functions. Hence, we have conducted the hyphal fusion

assay in the presence and absence of the *Vibrio* treatment. Hyphal fusion occurs within and between fungal colonies to form heterokaryons that coexist in a common cytoplasm and it is currently understood that endosomes trafficking to and from the fusion site assist in the processes that are involved in the fusion (Daskalov et al., 2017; Fischer and Glass, 2019; Fleißner and Herzog, 2016; Fu et al., 2011; Sánchez-León et al., 2015). We have observed that the *Vibrio* treatment prevents the hyphal fusion between the uridine and arginine auxotroph mutants TJES19.1 and TJES20.1, respectively, resulting in a significant drop in the formation of heterokaryon containing conidia that could form colonies on GMM. Plates on which conidia obtained from *Vibrio* administered plates looked very similar to conidia obtained from the cross of *ΔhamI* (TXZ9.16) with TJES20.1 strain. Besides, we were also able to notice the effects of interruption of hyphal fusion on sclerotia numbers. Sclerotial development is a process that needs hyphal fusion (Erental et al., 2008), which could explain the significant drop in sclerotial counts upon *Vibrio* treatment. Previously published data show that cellular fusion in *A. flavus* is most clearly found in the inability to form heterokaryons or sclerotia. The consequence of heterokaryon formation and sclerotial loss is also found in decreased ability to synthesize aflatoxin in this strain (Zhao et al., 2017b). Another study showed that the deletion of the veA gene in *A. flavus* and *A. parasiticus* blocks the production of aflatoxin as well as sclerotial formation (Cary et al., 2007).

This study, therefore, unlocks a new door for research that can investigate how the *Vibrio* uptake into hyphae resulted in the down-regulation of aflatoxin and how this is linked with the alterations in hyphal morphogenesis that were observed in the current study. In the long run the research will lead to the discovery of novel cellular targets and

approaches to mitigate aflatoxin contamination to improve food and feed safety and human health. Our future studies will involve the use of integrated omics approaches to identify the molecular and cellular mechanisms coordinating secondary metabolic pathways and hyphal development that is targeted in the *Vibrio*-dependent aflatoxin inhibition.

Table 3.1: List of strains used in the study

Strain	**Genotype**	**Strain source**
NRRL 3357	*A. flavus* Wild type	(Payne et al., 1993)
MAEW4	norA	(Maggio-Hall et al., 2005)
TJES 19.1	Δku70, pyrG^{-}	(Zhao et al., 2017b)
TJES 20.1	Δku70, ΔargB:: A. fumigatus pyrG, pyrG^{-}	(Zhao et al., 2017b)
TXZ9.16	Δku70, ΔhamI:: A. flavus argB, pyrG^{-}	(Zhao et al., 2017b)

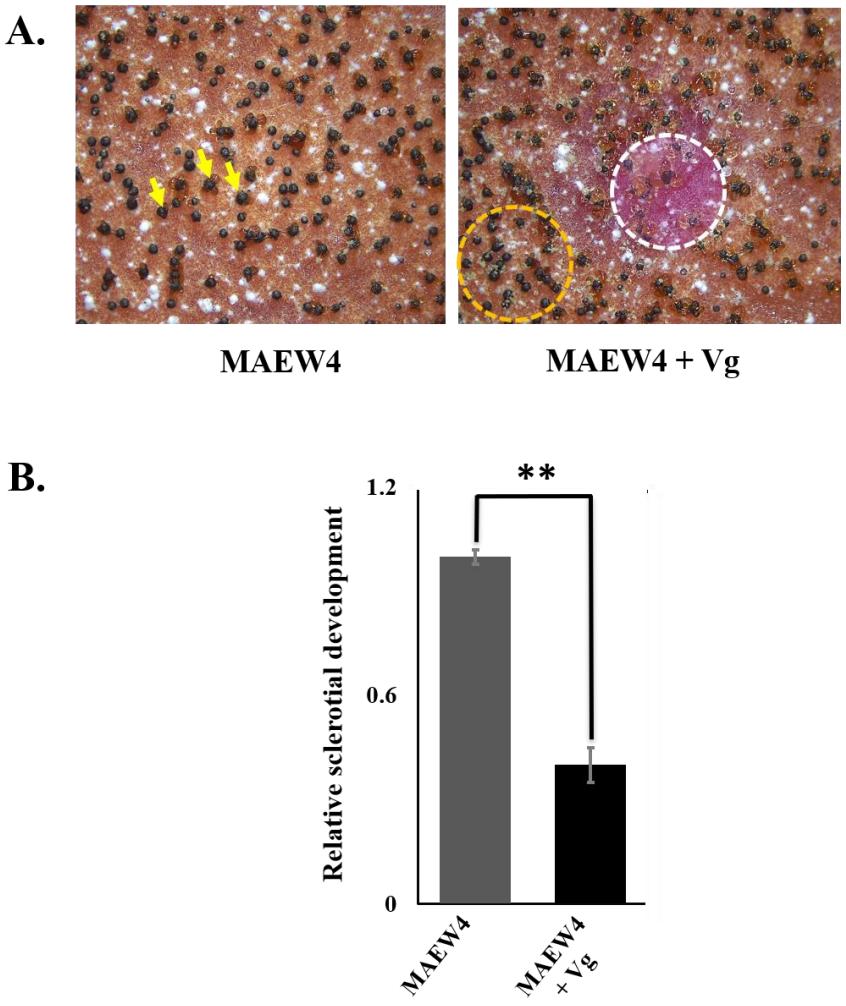


Figure 3.1: Influence of *V. gazogenes* (denoted in figure as Vg) on sclerotial development of *A. flavus*. (A) Visual comparisons of MAEW4 colonies in the presence and absence of Vg. White circle depicts an area with Vg treatment and the yellow circle is an area without Vg treatment. (B) corresponding sclerotia counts. Statistical significance of two-tailed *p*-values determined using an unpaired t-test for n=3. (**, $p<0.001$). Star indicates significance.

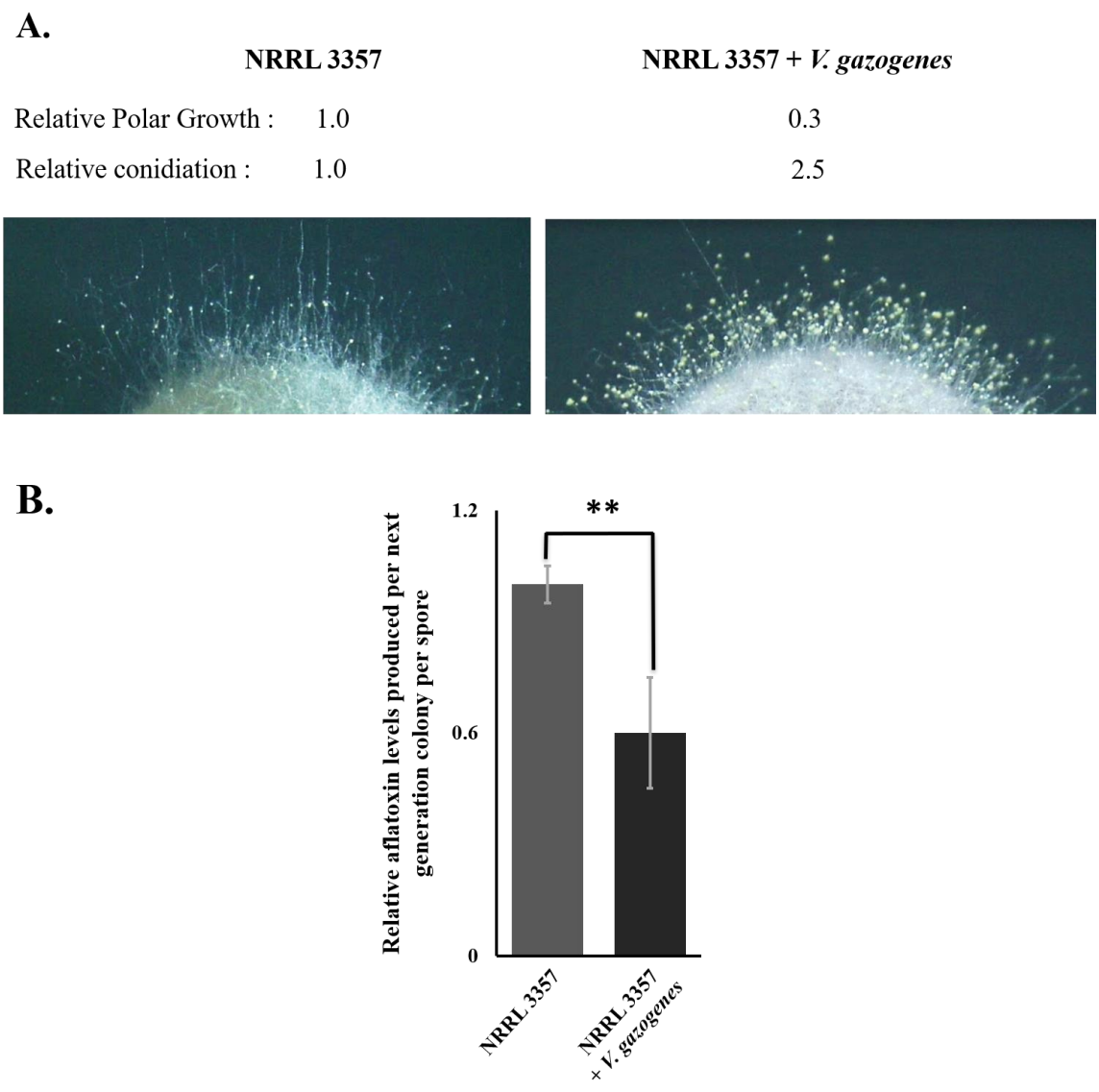


Figure 3.2 Influence of *V. gazogenes* uptake on polar growth, conidiation, and next-generation toxicity on *A. flavus*. (A) Dissection micrographs of sections of growing colonies of NRRL 3357, untreated and treated with *V. gazogenes*. Relative polar growth as measured by comparisons of tip growth measured by an average distance of the tip from the center of the colony; Relative conidiation was determined by conducting spore counts after 3days of growth. (B) Comparison of next generation toxicity was determined by determining total aflatoxin produced by mycelia growth from an equal number of spores harvested from untreated and *V. gazogenes* treated NRLL 3357 colonies. Error bars represent SEM, from triplicate experiments. Statistical significance of two-tailed *p*-values determined using an unpaired t-test for n=3. (**, $p<0.001$). Star indicates significance.

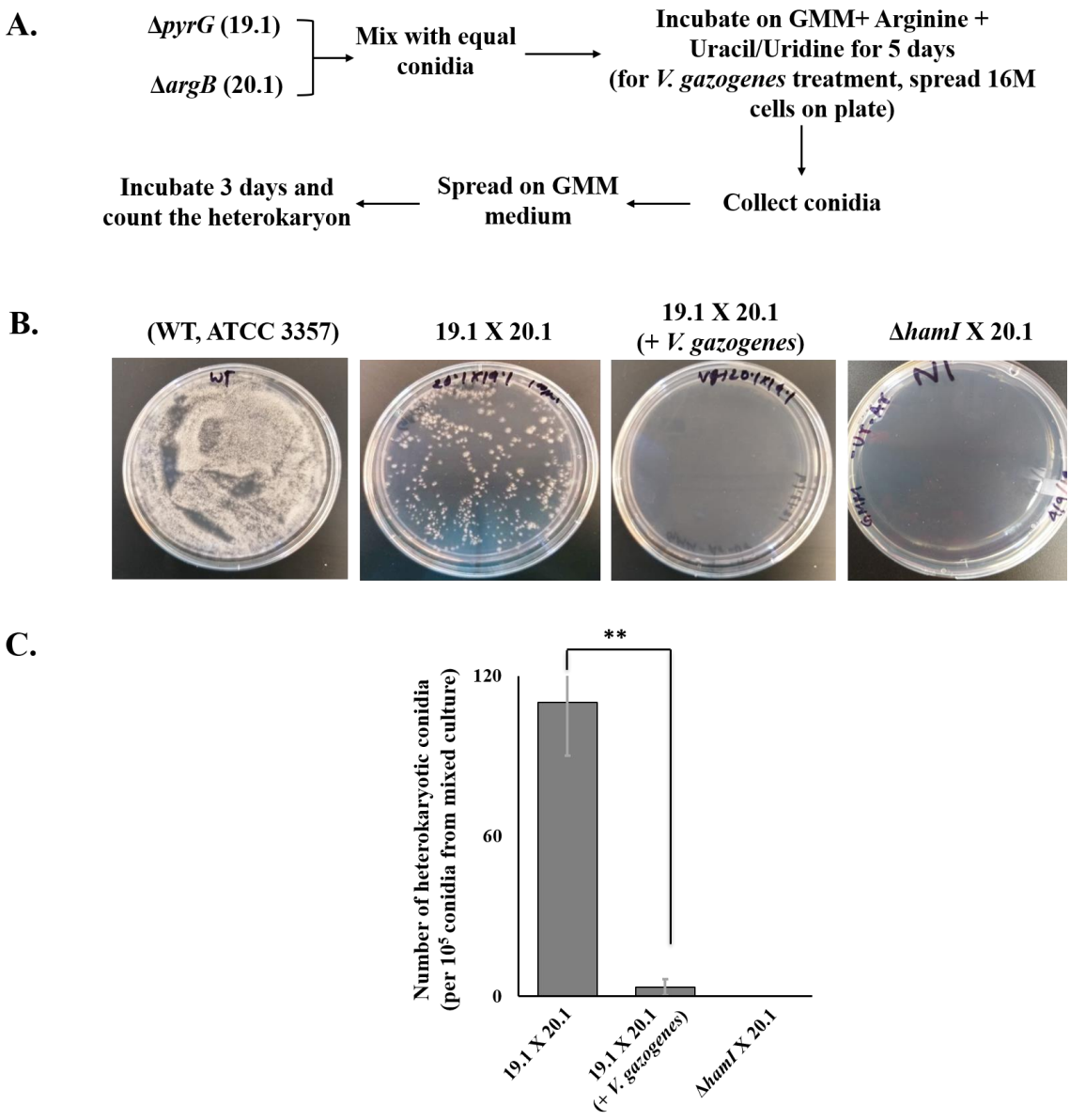


Figure 3.3: Influence of *V. gazogenes* uptake on hyphal fusion. (A). Outline of the method to measure hyphal fusion (based on the method described by (Zhao et al., 2017b) (B) Photographs of heterokaryon formation or lack of formation in crosses. Plates of mixed cultures were spread and incubated at 29°C for 3 days. (C) The number of heterokaryotic colonies formed for each cross. Error bars represent SEM, from triplicate experiments. Statistical significance of two-tailed *p*-values determined using an unpaired t-test for n=3. (**, $p<0.001$). Star indicates significance.

N.B.: ΔhamI (TXZ9.16) was uracil/uridine auxotrophic; 19.1 (TJES19.1), uracil/uridine auxotrophic; 20.1 (TJES20.1), arginine auxotrophic.

CHAPTER 4

UNDERSTANDING THE PROCESS OF FUNGAL UPTAKE OF *Vibrio gazogenes* CELLULAR MATERIALS LEADING TO AFLATOXIN DOWNREGULATION

4.1 INTRODUCTION

Filamentous fungal growth is characterized by an apical extension of the hyphal cell. This process requires the constant supply of membranes and proteins, such as cell wall-forming enzymes, to the growing hyphal tip. Endocytosis is common in filamentous fungi that support fungal morphology (Steinberg, 2014). Endocytosis is a complex process of absorption from the environment (and subsequent distribution within the cell) of soluble substances, macromolecules, microparticles, etc. utilizing vesicles developed by the cytoplasmic membrane (Olga et al., 2017). Bacterial secretomes and pathogens use endocytosis as a mode of entry to the cell interior (Dutta and Donaldson, 2012). The majority of reports on cellular uptake mechanisms suggest that endocytosis is the preferred route of cell entry of nutrients and components from their surrounding environments (Wattiaux et al., 2000). There are so many endocytosis inhibitors used in different researches, for examples: natamycin, dynazor, Pitstop 2, nocodazole, monodansylcadaverine, latrunculin A, nystatin, filipin, etc. used in *Rhizoctonia solani* (Olga et al., 2017), the polyene antimycotics nystatin, filipin and natamycin used in *Penicillium discolor* and *Aspergillus niger* (te Welscher et al., 2010; van Leeuwen et al., 2013; van Leeuwen et al., 2009).

Interactions between bacteria and fungi are widespread in nature and play pivotal roles in ecological and medicinal processes (Frey-Klett et al., 2011). Microbial secondary metabolites (SMs) have profound impacts on the ability of these microbes to colonize and survive in complex biotic environments. Microbiome also secretes various proteins into extracellular milieu which are known as microbial secretomes, under such interactions. Secretome proteins (e.g., receptors, transporters, adhesins, complex cell structures, secreted enzymes, toxins, and virulence factors) allow bacteria to interact with and adapt to their environment (Desvaux et al., 2009; Gagic et al., 2016; Zhou et al., 2010). Bacteria secrete the proteins by classical pathways like Sec and Tat pathways are known to be classically secreted proteins (Green and Mecsas, 2016), whereas many proteins that are found in the supernatant of many bacteria and lack signal peptides are known as non-classical secreted proteins (Wang et al., 2016). One school of thought suggests their secretion by some unknown secretion pathway (Zhao et al., 2018), while other supports that it is the cell lysis that makes these proteins come into the extracellular milieu as the majority of these proteins are cytoplasmic (Ebner et al., 2016; Oliveira et al., 2012; Zubair et al., 2020). Many species of bacteria are subject to lysis in the presence of some enzymes such as lysozyme mediated by other microbes in the same environment. *Penicillin* sp. causes the death of bacteria through enzyme-mediated lysis (Nelson et al., 2001). *Lactobacillus casei* showed higher anti-aflatoxigenic activity on *Aspergillus flavus* and individual antagonism assays, suggesting that the inhibitory activity was due to extracellular metabolites produced in cell-free supernatant fluids of the cultured broth of *L. casei* (Chang and Kim, 2007).

Prodigiosin, a red color secondary metabolite appears only in the later stages of bacterial growth produced by many strains of the bacterium *Serratia marcescens* (Bennett and Bentley, 2000; Harris et al., 2004), as well as other proteobacteria such as *Vibrio psychroerythrus*, *Vibrio gazogenes* and *Hahella chejuensis* (Williamson et al., 2006), *Pseudomonas magneslorubra*, *Serratia rubidaea*, *Alteromonas rubra*, *Rugamonas rubra* and Gram-positive actinomycetes, such as *Streptoverticillium rubrireticuli* and *Streptomyces longisporus ruber* (Elkenawy et al., 2017). The production of prodigiosin has been observed to be affected by several environmental factors, inorganic phosphate availability, media composition, temperature, and pH (Williamson et al., 2005). The number of differential and selective media has been used for the isolation and production of pigment from *Serratia sp.* (Darshan and Manonmani, 2015). *Vibrio gazogenes* is well-known for its ability to synthesize bactericidal and fungicidal pigments, magnesidin A (Imamura et al., 1994), prodigiosin and cyclo-prodigiosin (Allen et al., 1983). Previous studies have also shown that random mutations in this bacterium with 1-methyl-3-nitro-l-nitrosoguanidine expanded its metabolic output and activated the synthesis of additional bactericidal prodigiosin-related pigments, norprodigiosin and propyl prodigiosin (Alihosseini et al., 2010). Research study showed that aflatoxin exposure to *V. gazogenes* diminished prodigiosin released into the growth medium, but additionally resulted in the production of a new compound that demonstrated the ability to specifically-inhibit aflatoxin synthesis in the model aflatoxin producer, *A. parasiticus* (Gummadidala et al., 2016).

Microorganisms universally attach to and grow on a surface and produce extracellular polysaccharides, resulting in the formation of a biofilm (Donlan, 2001).

Biofilm formation, seems likely to provide a substantial survival advantage to aquatic organisms such as *Vibrio* species (Yildiz and Visick, 2009). Many studies on this rely on colony morphology as an indicator of biofilm formation. Flagella, pili, and polysaccharides are involved in the initial stages of biofilm formation by *Vibrio* sp. (Nakhamchik et al., 2008). The capsular polysaccharide (CPS) or exopolysaccharide (EPS) loci involved in biofilm formation have been identified from numerous *Vibrio* sp. (Yildiz and Schoolnik, 1999). The *Vibrio* sp. studied to date produce distinct exopolysaccharides, and some if not all have the potential to produce multiple types of polysaccharides (Enos-Berlage et al., 2005). In *Vibrio* species, regulation of exopolysaccharide production and biofilm formation is complex, and involves numerous transcriptional regulators, particularly two-component signal transduction and quorum sensing regulators (Fong and Yildiz, 2008; Yildiz et al., 2004). Quorum sensing is an important mechanism by which bacteria and fungi regulate developmental programs including biofilm formation and expression of virulence proteins through alteration of gene expression patterns based on population densities. Fungal secondary metabolites, including those produced by *Fusarium* spp., are involved in disrupting quorum signaling in bacteria (Martín-Rodríguez et al., 2014; Venkatesh and Keller, 2019). The exopolysaccharides are thought to play a significant role in the colonization of lactic acid bacteria to various ecosystems (Górska et al., 2010). Research investigating industrial applications is concentrated on the extracellular polysaccharides, which have simpler extraction and purification processes (Sutherland, 2002). Different methods can be used for extracting exopolysaccharide, among which ethylene precipitation usually use most (Comte et al., 2006; Ziadi et al., 2018).

These studies have prompted us to hypothesize that co-culturing *V. gazogenes* bacterium in the presence of endocytosis inhibitors (filipin and natamycin) with *A. flavus* fungus will have different inhibitory effects on the aflatoxin biosynthesis and the mycelial growth of *A. flavus*. In this study, we treated *V. gazogenes* cells with different media containing *A. flavus* only and a combination of *A. flavus* and *V. gazogenes*, to investigate the influence of *A. flavus* (CA14) in producing lysing enzymes by *V. gazogenes*. To understand the mechanism of action of *V. gazogenes* we further conducted one experiment where we treated *A. flavus* with heat mediated non-viable gram-positive and gram-negative and prodigiosin producing bacteria and observed that other bacteria don't have same anti-aflatoxigenic activity as *V. gazogenes*. To understand the mechanism of action of intact *V. gazogenes* cells we performed an experiment where we treated A. flavus with intact cells and different fractionated parts of *V. gazogenes* and surprisingly concluded that the intact *V. gazogenes* cells most effectively and efficiently decrease aflatoxin production (>99%) rather than other fractionated parts of *V. gazogenes* cells. Definitively our study establishes a novel cell biological approach for investigating how uptake mechanisms of cellular components of *V. gazogenes* leading to aflatoxin down-regulation.

4.2 MATERIALS AND METHODS

4.2.1 Strains, media, and growth conditions

The fungus *Aspergillus flavus* strain CA14PyrG.1 (designated as CA14 hereafter) (acquired from USDA Agricultural Research Service, New Orleans, LA, United States) and bacteria *Vibrio gazogenes* (ATCC 43942), *Serratia sp.* (ATCC 39006), *Escherichia coli* (ATCC 25922) *Staphylococcus aureus* (ATCC 25923), *Vibrio harveyi* (ATCC

14126), *Vibrio fischeri* or *Aliivibrio fischeri* (ATCC 7744) were used for this study. Yeast extract sucrose (YES) (2% yeast extract, 6% sucrose; pH 5.8) was used as the liquid growth medium and potato dextrose agar (PDA) was used as the solid growth media, for *A. flavus* for the entire study. Fungal cells were grown for 40 h (as required by experiments) by inoculating 10^7 spores per 50 mL of liquid growth medium and incubated at 29°C in a dark orbital shaker at 150 rpm. For growth of fungal mycelia on solid media, $2x10^4$ spores were center inoculated on 100 mm Petri dishes containing 10 mL of PDA and incubated in the dark at 29°C.

Difco Marine broth (cat # 2216, BD Biosciences, Sparks, MD) was used, as liquid growth media for *V. gazogenes* and the bacterial cells were grown for 24 h by inoculating 10^5 colony forming units (cfu) /100 ml of growth medium. The cultures were incubated at 29°C in a dark orbital shaker at 150 rpm. Nutrient broth (NB, cat # 234000, BD) was used as a liquid growth media for *Serratia sp.* Small inoculum was grown with 10^5 cfu/5 ml liquid growth media and incubated overnight at 26°C in a dark orbital shaker at 150 rpm. Tryptic soy broth (TSB, cat # 211825, BD Biosciences, Sparks, MD) was used as liquid growth media for *S. aureus*, and *E. coli*. Small inoculum was grown with 10^5 cfu/5 ml liquid growth media and incubated overnight at 37°C in a dark orbital shaker at 150 rpm. At the end of the incubation time, 100,000 cells were taken from the small inoculum and further used to inoculate 100 ml TSB and incubated for 24 h at 37°C in a dark orbital shaker at 150 rpm. Difco Photobacterium Broth (DPB, cat # 213050, BD) was used as liquid growth media for *Vibrio harveyi* and *Vibrio fischeri or Aliivibrio fischeri*. Bacterial cells were grown with 10^5 cfu/5 ml liquid growth media and incubated for 24 h at 26°C in a dark orbital shaker at 150 rpm.

4.2.2 *A. flavus* treatment with *V. gazogenes* and endocytosis inhibitors

To know the effect of endocytosis inhibitors in the uptake of *V. gazogenes* by fungal mycelia, we conducted an experiment by using two polyene antimycotics or endocytosis inhibitors named filipin (Sigma) and natamycin (Sigma) in their Minimal Inhibitory Concentration (MIC) (Van Leeuwen et al., 2010; van Leeuwen et al., 2009). Filipin was dissolved in 100% DMSO and natamycin in 85% DMSO (Brik, 1981). A 1-mmol l^{-1} stock solution of each endocytosis inhibitor was made and used for stepwise dilution. We grew *A. flavus* (CA14PyrG.1) in four separate flasks by inoculating 10^7 spores per 50 mL of YES medium and incubated at 29°C in a dark orbital shaker at 150 rpm for 40 h. In one flask we did not add anything, only CA14 growth started. In the second flask, we treated CA14 with *V. gazogenes*. In the third and fourth flasks, we treated CA14 with *V. gazogenes*. along with filipin and natamycin respectively in their 2X Minimal Inhibitory Concentration (MIC) recommended by (van Leeuwen et al., 2009). For filipin we used 3 µmol l^{-1} / 50 ml media and for natamycin we used 6 µmol l^{-1} / 50 ml media from our previously made 1-mmol l^{-1} stock solution.

4.2.3 Determination of color intensity score

Color intensity score was measured for fungal mycelial pellets in the absence and presence of *V. gazogenes* and endocytosis inhibitors (filipin and natamycin) after a 40 h time point from inoculation (Kaur et al., 2018). The cumulative score was determined by the color of *A. flavus* mycelial pellets which indicate the uptake of *V. gazogenes* cells by fungal cells in the presence of different endocytosis inhibitors (filipin and natamycin). The score for the color intensity was based on the following scale where; 0 = white natural *A.*

flavus (CA14PyrG.1) mycelial pellets with the absence of *V. gazogenes* and endocytosis inhibitors (no uptake of *V. gazogenes* cells); 10 = red CA14 PyrG.1 mycelial pellets treated with *V. gazogenes* but in absence of endocytosis inhibitors. (highest amount uptake of *V. gazogenes* cells by CA14PyrG.1). Hence, a higher color intensity score indicates more uptake of *V. gazogenes* cells by CA14PyrG.1.

4.2.4 lysis experiment of *V. gazogenes* in presence of *A. flavus*

For this experiment, we collected fresh 1 ml YES media (only YES-Y), 1 ml growth media from 24 h *A. flavus* (CA14) growth flask (YES+CA14 - YC) and 1 ml growth media from 24 h *A. flavus* with *V. gazogenes* growth flask (YES+CA14+Vg- YCV) respectively in three separate cuvettes. Then we added 50 µl of fresh 24 h growing *V. gazogenes* in each 1 ml media, respectively. We measured OD at 600 nm at 0 h, 6 h, and 12 h time points from each cuvette (Figure 4.1A).

4.2.5 *A. flavus* treatment with different non-viable bacteria along with non-viable *V. gazogenes*

The fungus *A. flavus* was treated with dead bacteria - *V. gazogenes, Serratia sp., E. coli, S. aureus, V. harveyi, V. fischeri,* or *A. fischeri*. The 24 h bacterial cultures were spun down at 4000 rpm for 15 min at room temperature and resuspended using YES media. With the help of absorbance measurements at 600 nm, 16 million cells from a 24 h bacterial culture (made unviable by boiling at 100^{O}C for 10 min in a hot plate) were sorted out and added to the 50 ml YES liquid media simultaneous with $5x10^{7}$ *A. flavus* spores and incubated at 29°C in a dark orbital shaker at 150 rpm for 40 h. Comparably 24 h bacterial cultures of *Serratia sp., E. coli, S. aureus, V. harveyi, V. fischeri, or A. fischeri.* were spun

down at 4000 rpm for 15 min at room temperature and resuspended using 100 x PBS. The same number of bacterial cells (unviable by boiling at 100^{O}C for 10 min in a hot plate) were sorted out and added to the 50 ml YES liquid media simultaneous with $5x10^{7}$ *A. flavus* spores and incubated at 29°C in a dark orbital shaker at 150 rpm for 40 h. The loss of viability in these cells was confirmed before experimentation by confirming their inability to grow in fresh growth medium. At the end of incubation, *A. flavus* mycelia were harvested by filtering the mycelia through a Miracloth (Millipore, Billerica, MA) and the cells subjected to appropriate processing for various growth measurements and aflatoxin analysis.

4.2.6 *A. flavus* treatment with intact *V. gazogenes* along with different fractionated part of the *V. gazogenes*

For conducting this experiment, we grew *V. gazogenes* in three separate batches for 24 hr, using Difco Marine broth liquid growth media by inoculating 10^{5} colony forming units (cfu) /100 ml of growth medium. Then, one batch of 24 h bacterial cultures were centrifuged down at 4000 xg for 15 min at room temperature and discarded the supernatant. We washed the bacterial cells with ethanol (EtOH) (two-fold volume of cells) at room temperature followed by centrifugation at 4000 ×g at 25ºC for 15 min. These steps resulted in the isolation of crude precipitate or washed bacterial cells (white color)(Bajpai et al., 2016; Paulo et al., 2012). We collected the supernatant which we called washate (red color) and reduced the volume of washate by evaporating the ethanol. Then we resuspended the washate in 1mL YES media before adding the cultures to the 50 ml fungal (*A. flavus*) growth medium. We also resuspended the crude precipitate or washed bacterial cells (white color) separately in 1mL YES media before adding the cultures to the 50 ml fungal (*A.*

flavus) growth medium. We prepared our second batch of bacterial cultures in the same manner. But after separating the washate and washed cells, we mixed them and resuspended the mixture (washate and washed cells) in 1mL YES media before adding the cultures to the 50 ml fungal growth medium. We directly resuspended our third batch 24 h bacterial cultures after centrifuging in 1mL YES media before adding the cultures to the 50 ml fungal (*A. flavus*) growth medium. We allowed all the fungal cultures to grow for 40 h with intact and different fractionated parts of *V. gazogenes*. At the end of incubation, *A. flavus* mycelia were harvested by filtering the mycelia through a Miracloth (Millipore, Billerica, MA) and the cells subjected to appropriate processing for various growth measurements and aflatoxin analysis.

4.2.7 Growth measurements of fungus and bacteria

All fungal growth in liquid media was quantified by using dry weight measurements. Briefly, the mycelia were filtered out of the growth media using a Miracloth (Millipore, Billerica, MA) and dried in an oven at 80°C for 6 h and the weight difference before and after drying was recorded. To estimate the growth of *A. flavus* on PDA media plates, the spread of mycelial colony was measured daily. After 9 days of incubation time, spores were manually collected from PDA plates using 1xPBS with 0.01% tween and resuspended in 50% glycerol. Spores were counted using a haemocytometer. All bacterial growth measurements were performed using absorbance readings of growth media at 600 nm. An absorbance of 1.2 on the UV/Vis spectrophotometer was considered as 10^6 cells/ml bacterial cell density and calculations were performed for 16 million bacterial cells appropriately.

4.2.8 Aflatoxin measurements

Aflatoxin was extracted from *A. flavus* cultured YES liquid media by adding an equal volume of chloroform in a separating funnel and collecting the organic layer. Chloroform was evaporated from the organic layer and the residual aflatoxin was resuspended in 500 µl of 70% methanol. The resuspended aflatoxin extract was spun down at 15,000 rpm for 1 min at room temperature to remove extra debris from the media and mycelia. Quantification of aflatoxin for the aflatoxin uptake experiments was performed using a Neogen Veratox Aflatoxin ELISA kit (cat # 8030) from Neogen Food Safety (Lansing, MI, USA) and measured on a Stat Fax 4700 Microstrip Reader (Awareness Technologies, Palm City, FL, USA) as per kit's protocols. The ELISA has 2 ppb and 50 ppb as lower and upper limits of detection, respectively.

4.2.10 Stereo and bright field microscopy

For light imaging experiments *A. flavus* has grown a coverslip and treated with *V. gazogenes.* For visualization of growing colonies using dissection or stereo microscope (Leica EZ4), we grew colonies on 10 µl liquid YES media with or without *Vibrio* supplementation. For bright-field (Fischer microscope) imaging of hyphae growing in liquid medium, we used a growing colony generated from spores that were inoculated on 10 µL YES medium containing the *V. gazogenes* cells.

4.2.11 Confocal laser scanning microscopy

A. flavus hyphae was grown from spores by inoculating in 30 µL YES medium containing *Vibrio* cells on a 35 mm glass-bottom fluorescent dish for 6 h. The fungal

mycelia were fixed using 4% formaldehyde and washed using 1xPBS and 0.05% tritionX-100. The resulting mycelia were studied and imaged using Leica TCS SP5 confocal microscope (Leica Microsystems Inc., IL, USA) at 20x, 40x, and 100x magnification.

4.2.12 Statistical analysis

Statistical analyses for this study were conducted using the GraphPad Prism Software (GraphPad, CA, USA). Microsoft Excel was used to generate graphs, perform statistical analysis and calculate significance for dry weight measurements and aflatoxin analysis. The statistical significance of two-tailed p-values were determined using an unpaired *t*-test, using $n = 3$ and $p < 0.01$.

4.3 RESULTS

4.3.1 *V. gazogenes* cannot inhibit aflatoxin production in the same manner in *A. flavus* in the presence of endocytosis inhibitors (filipin and natamycin)

We sought to understand if *V. gazogenes* cells internalized and uptaken within hyphae in endosome-like compartments through endocytosis during the inhibition of aflatoxin production. To understand the effect of the uptake of *V. gazogenes* cells along with endocytosis inhibitors (filipin and natamycin) on *A. flavus* growth and aflatoxin synthesis, we treated *A. flavus* (in YES liquid media) with 16 million *V. gazogenes* cells for 40 hours with filipin and natamycin separately by using their Minimal Inhibitory Concentration (MIC) (Van Leeuwen et al., 2010). After harvesting the *A. flavus* mycelia at 40 h time point, we investigated if the mycelial color differed in the presence of endocytosis inhibitors. From our previous studies we found that, *V. gazogenes* bacterial components

were internalized within the hyphae in endosome-like compartments (Gummadidala, 2018). So, we initially checked the color of fungal mycelia by visualizing them using a stereo-microscope (Leica EZ4), which will help us to understand that the *V. gazogenes* cells uptake by fungal mycelial pellets was hampered with the presence of endocytosis inhibitors or not because if the uptake of bacterial cells is happening through endocytosis, then it will definitely reduce in presence of endocytosis inhibitors. Stereo microscopy clearly showed the color difference between different treatments (Figure 4.1A). The fungal mycelial pellets were dark red in color which were treated with only *V. gazogenes*, but the color eventually reduced in the presence of filipin and natamycin, respectively. We also found the color intensity score lower in presence of filipin which was 5.9 ± 0.15 and in presence of natamycin which was 3.2 ± 0.09, based on our color intensity scale: 0 (Whitish color *A. flavus* cell) -10 (dark orange-red color due to the presence of *Vibrio* cells) (Figure 4.1B).

The aflatoxin levels analyzed using the ELISA technique showed that there were nearly undetectable levels of aflatoxin in the YES liquid media in the presence of *V. gazogenes* (irrespective of cellular concentration) as compared to the untreated control. We observed that, in the presence of filipin and natamycin the total aflatoxin production (ng/g) also decreased at significant levels but not in the same manner which we treated with only *V. gazogenes* cells (Figure 4.1C). On the other hand, the dry weight analysis at the end of the incubation period revealed that the mycelial growth of *A. flavus* did not differ with 16 million *V. gazogenes* cells and the combination of *V. gazogenes* cells with filipin as compared to the untreated control (Figure 4.1D). But we observed that the mycelial growth

of *A. flavus* was significantly reduced while treated with natamycin along with 16 million *V. gazogenes* cells.

4.3.2 Presence of *A. flavus* activates production of *V. gazogenes* lysing enzyme

Next, we investigated if *V. gazogenes* cells lysed in the presence of *A. flavus* because we did not observe any evidence of intact bacterium within the hyphal cells with transmission electron microscopy in our previous studies (Gummadidala, 2018). To investigate the influence of *A. flavus* (CA14) in producing lysing enzymes by *V. gazogenes* followed some steps which is shown diagrammatically in figure 4.2A. We conducted a time-course measurement of the bacterial concentrations in the growth medium by recording the OD_{600} readings to determine if, during the mycelial growth, the bacterial cells were depleted or lysed in the growth media. When we added 50 µl of fresh 24 hr growing *V. gazogenes* cells in 1 ml YES (Y) media, OD_{600} readings did not change significantly after 6 h and 12 h time point. In the case of YES+ CA14 (YC) growth media, after adding 50 µl *V. gazogenes* cells, OD_{600} readings also did not change significantly at different time points. But, when we added 50 µl of fresh 24 hr growing *V. gazogenes* cells in 1 ml YES + CA14 + Vg (YCV) growth media, OD_{600} dropped steadily and significantly from 0h to 12h time-point (Figure 4.2B), suggesting that presence of *A. flavus* with *V. gazogenes* in YES media for 40 h activates the production of *V. gazogenes* lysing enzymes.

4.3.3 Prodigiosin is not the only aflatoxin inhibitor

To understand if the prodigiosin produce by *V. gazogenes* is the only aflatoxin inhibitor, we treated *A. flavus* with a gram-negative bacterium, *Serratia sp.*, which also secrete a host of virulence factors including prodigiosin. It also helped us to observe the

contribution of prodigiosin in aflatoxin inhibition. We also treated *A. flavus* with a gram-negative bacterium, *Escherichia coli,* and a gram-positive bacterium, *Staphylococcus aureus* to understand if the aflatoxin inhibitory effect was limited to *V. gazogenes*. Besides that, we treated *A. flavus* with two gram-negative, bioluminescent, marine bacterium *Vibrio harveyi* and *Vibrio fischeri* or *Aliivibrio fischeri* which were nonviable or dead to test the supposition that they were equally capable of decreasing aflatoxin levels in *A. flavus* cultural media like dead *V. gazogenes*.

Non-viable bacterial biomass had proven to be better and safer bio adsorbent for contaminants in the environment (Alshannaq et al., 2018; Farzaneh et al., 2016; Zeroual et al., 2006) which are much preferred due to lack of nutrients and cultural conditions. As far we know from our previous studies (Gummadidala, 2018) that, the unviable and viable *V. gazogenes* cells equally inhibit aflatoxin biosynthesis, we used 16 million dead bacterial cells to treat *A. flavus* and the fungus was harvested after 40 h. Dead bacteria cells were used to reduce the pathogenic interaction between the *A. flavus, Serratia sp., E. coli, S. aureus, V. harveyi,* and *V. fischeri or A. fischeri.* The growth of the *A. flavus* did not change in the presence of *Serratia sp., E. coli, S. aureus, V. harveyi*, and *A. fischeri* and the aflatoxin levels did not show any significant change except *Serratia sp.* (figure 4.3). As *Serratia sp*. also produces prodigiosin like *V. gazogenes,* it has some aflatoxin inhibitory effect on *A. flavus,* but it cannot inhibit aflatoxin production as the same level as *V. gazogenes* did which was almost ~95%. This data points out that the prodigiosin produce by *V. gazogenes* is not the only aflatoxin inhibitor and the aflatoxin inhibitory effect of *V. gazogenes* is exclusive to itself.

4.3.4 Intact *V. gazogenes* cell results in most efficient aflatoxin reduction

Since we observed prodigiosin is not the only functional component that is specific to *V. gazogenes*, we proceeded to test whether intact and fractionated *V. gazogenes* cells are impeded aflatoxin reduction in the same manner. We treated *A. flavus* with intact *V. gazogenes* cells for 40 h. Besides that, we washed *V. gazogenes* cells with ethanol (EtOH) precipitation method (Bajpai et al., 2016) and used that ethanol extract which we called washate (red color) and precipitate which we called washed cell (white color) to treat *A. flavus*. We used extracted washate and washed cells separately in two different *A. flavus* growth flasks. We also used extracted washate and washed cells together in another *A. flavus* growth flask. The scheme for this experiment is shown in Figure 4.3A. After 40 h growth of treated *A. flavus*, the mycelial growth and aflatoxin synthesis were observed. After 40 h the aflatoxin levels decreased with washate of *V. gazogenes* cells (Figure 4.3B) but not like intact *V. gazogenes* cells. The aflatoxin levels also decreased with washed cells and the combination of (washate + washed cells) of *V. gazogenes* cells but not like intact *V. gazogenes* cells. The fungal mycelial mass did not change in different treatments. This shows us that the intact *V. gazogenes* cells most effectively and efficiently decrease aflatoxin production (>99%) rather than different fractionated parts of *V. gazogenes* cells.

4.4 DISCUSSION

Understanding how fungal uptake of *V. gazogenes* cellular materials leading to aflatoxin downregulation is an area of agricultural and pharmaceutical promise and, in the case of fungal pathogens such as *A. flavus*, a process to better understand pathways required for aflatoxin synthesis and other virulence attributes. To date, research concerning the

internalization of the bacteria via endocytosis like mechanism (Guerra-Tschuschke et al., 1991). In this research, *V. gazogenes* allow us to study their secreted and synthesized metabolic components and fractionated cell components which are assumed to be the aflatoxin inhibitors. Based on the results of this study, we propose a theoretical model (Figure 4.5**)** that summarizes our current understanding of how aflatoxin synthesis and hyphal morphogenesis was disrupted in a *V. gazogenes* dependent manner. According to this model, the internalization of cellular components of *V. gazogenes* in hyphae results in the intervention of endosomal functions associated with hyphal morphogenesis and aflatoxin biosynthesis.

The result of our current study showed that, in the presence of endocytosis inhibitors (filipin and natamycin), *A. flavus* cannot uptake *V. gazogenes* in the same manner while they are absent. Researchers showed that, filipin disrupts the conidial plasma membrane of the filamentous fungus *Penicillium discolor*, whereas natamycin interferes with endocytosis in a dose and time-dependent manner (van Leeuwen et al., 2009). The findings reported in our study indicate that endocytosis inhibitors exposure induced decreased aflatoxin inhibition especially in the case of natamycin. Also, results showed that that the mycelial growth of *A. flavus* was significantly reduced while treated with natamycin along with 16 million *V. gazogenes* cells. Considering the existing data, we hypothesize that filipin and natamycin, two endocytosis inhibitors might be interfering with endocytosis which reduces the endosomal uptake of *V. gazogenes* cells by *A. flavus.*

A research study showed bacterial active invasion into living fungal cells which involves the secretion of chitinolytic enzymes by using rice seedling blight fungus *Rhizopus microsporus* and its endosymbiont *Burkholderia rhizoxinica*, where they form an

unusual, highly specific alliance to produce the highly potent antimitotic phytotoxin rhizoxin (Moebius et al., 2014). To investigate the bacterial secretome, proteins include secreted enzymes which usually help to invade into *A. flavus* cells, we experimented. We did not find any intact *V. gazogenes* cells inside the fungal hyphae in our previous studies, which driven us to examine how the uptaken of *V. gazogenes* cells happened. We assume that, *V. gazogenes* cells might be lysed in bacteria-fungal growth media or *V. gazogenes* cells might secrete lysing enzymes as secretome in bacterial-fungal growing media. The OD_{600} reading showed a steady and significant drop from 0h to 12h time-point while we use the media where we grew *A. flavus* and *V. gazogenes* together. This leads us to conclude that *V. gazogenes* cells secrete lysis enzymes as secretomes in the presence of 24 h growth media where *A. flavus* and *V. gazogenes* grow together.

It has been shown in our ELISA data that, *Serratia sp*. partially down-regulated aflatoxin production but not as *V. gazogenes* did. Previous studies revealed that, other fungal secondary metabolites trigger synthesis of metabolite fractions in *V. gazogenes* that demonstrate different HPLC traces compared to prodigiosin which has antimalarial, antifungal, immunosuppressant, and antibiotic properties (Hu et al., 2016; Williamson et al., 2007). Our previous researcher also showed that after treating *A. flavus* cultures (colorless or white) in YES liquid medium with live *V. gazogenes* (red color because of prodigiosin synthesis) for 72 h, optical density decreased over time and the fungal mycelia turned reddish pink (as attributed to the red pigment in the bacterial cells) and over a time period of 42 h the bacteria were completely depleted from the YES media and the media turned back to its original yellow (Gummadidala et al., 2016). In another study showed that, *Serratia marcescens* produce red pigment as secondary metabolites named

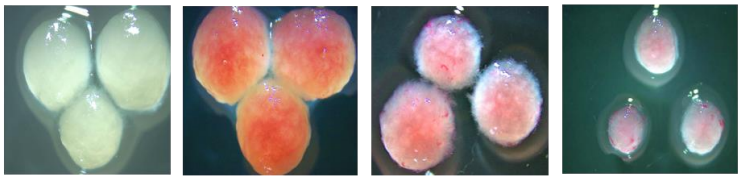
prodigiosin (de Assis Alcoforado Costa et al., 2019) which production can alter by using differential and selective media (Darshan and Manonmani, 2016). Researchers assume in their study report from there data that, mycotoxin triggered synthesis of mycotoxin inhibitors is a phenomenon that is conserved in the *Vibrio sp* (Alihosseini et al., 2010; Rameshkumar and Nair, 2009). We can hypothesize from our data that, prodigiosin might be a functional component which can downregulate aflatoxin production but it is not the only aflatoxin inhibitor and *V. gazogenes* might be synthesized some unique components such as metabolite consortium or aflatoxin responsive metabolites (ARMs) or secretomes which enable *V. gazogenes* to show the aflatoxin inhibitory effect specific to itself. Moreover, our research data also revealed that other non-viable gram-negative and gram-positive bacteria and some other *Vibrio sp.* have no aflatoxin inhibitory effect and mycelial growth effect on *A. flavus* which support our hypothesis again.

The present study reports the efficiency of intact *V. gazogenes* cells on aflatoxin reduction of *A. flavus*. In the bacterial-fungal biofilms, one form of association is the internalization of bacteria by fungi altering the fungal physiology (Frey-Klett et al., 2011). As far we know bacteria produce biofilm by which they can share nutrients and it acts as a shelter from harmful factors in the environment, such toxic materials and a host body's immune system, and exopolysaccharide (EPS) loci involved in biofilm formation have been identified from numerous *Vibrio sp*. (Yildiz and Visick, 2009). *V. gazogenes* cells produce significant biofilm outside the fungal cells when they grow together (Gummadidala, 2018). In our research, we targeted the intact cell of *V. gazogenes* for ethanol (EtOH) extraction which allows us to collect different metabolite fractions combine with exopolysaccharides (EPS) for further treatments. The findings reported in this study

indicate that different fractions of *V. gazogenes* cells (washate – red color, washed cells – white color and the combination of washate + washed cells) had separate aflatoxin inhibitory effects but nobody can down-regulate aflatoxin production as intact *V. gazogenes* cells.

In conclusion, our findings establish the foundation for a long-term study that will investigate the molecular, cellular, and biochemical mechanisms underlying the aflatoxin inhibitory effects of lysed and intact *V. gazogenes* cells, their secretomes and their secreted exopolysaccharide combined with metabolite fractions on *A. flavus*. Current studies in our laboratory reveal that fungal secondary metabolites trigger the synthesis of metabolic components like lysis enzymes in *V. gazogenes* that need to be brought under further investigation. Our future studies will shed light on revealing the individual components of *V. gazogenes* producing secretomes when in contact with *A. flavus*. Further testing and mechanistic understanding of ethanol consortium of *V. gazogenes* which related to the formation of biofilm should be continued. To fully understand the impact of intact *V. gazogenes* on aflatoxin down-regulation, the relative role of their individual fractionated components needs to be observed.

A.



	Control (CA14)	CA14 + Vg	CA14 + Vg + Filipin	CA14 + Vg + Natamycin
40 hr pellet				
Color intensity Score	0	10	5.9 ± 0.15	3.2 ± 0.09

B.

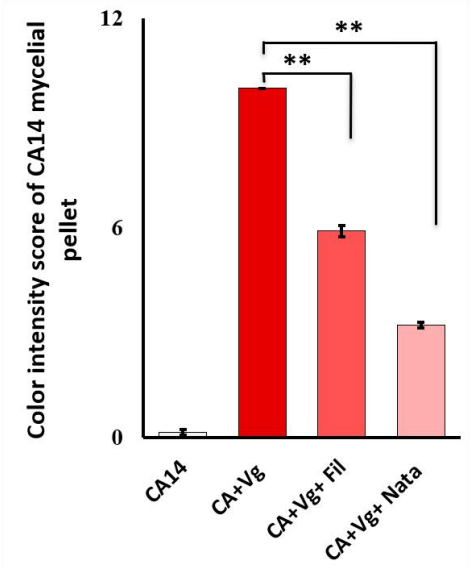


Figure 4.1: Influence of *V. gazogenes* (denoted as Vg in the figure) along with endocytosis inhibitors on aflatoxin production and growth of *A. flavus* (CA14). (A). Mycelial growth and color of mycelia in case of different treatments (Control- CA14 only, Ca14 with Vg, CA14 with Vg and filipin, and CA14 with Vg and natamycin) at 40 h time point. (B) Color intensity index of CA14 mycelial pellet in the presence of *V. gazogenes* endocytosis inhibitors (filipin and natamycin) based on clinical score. Error bars represent SEM, from triplicate experiments. Statistical significance of two-tailed *p*-values determined using an unpaired t-test for n =16. (**, $p<0.001$). Star indicates significance.

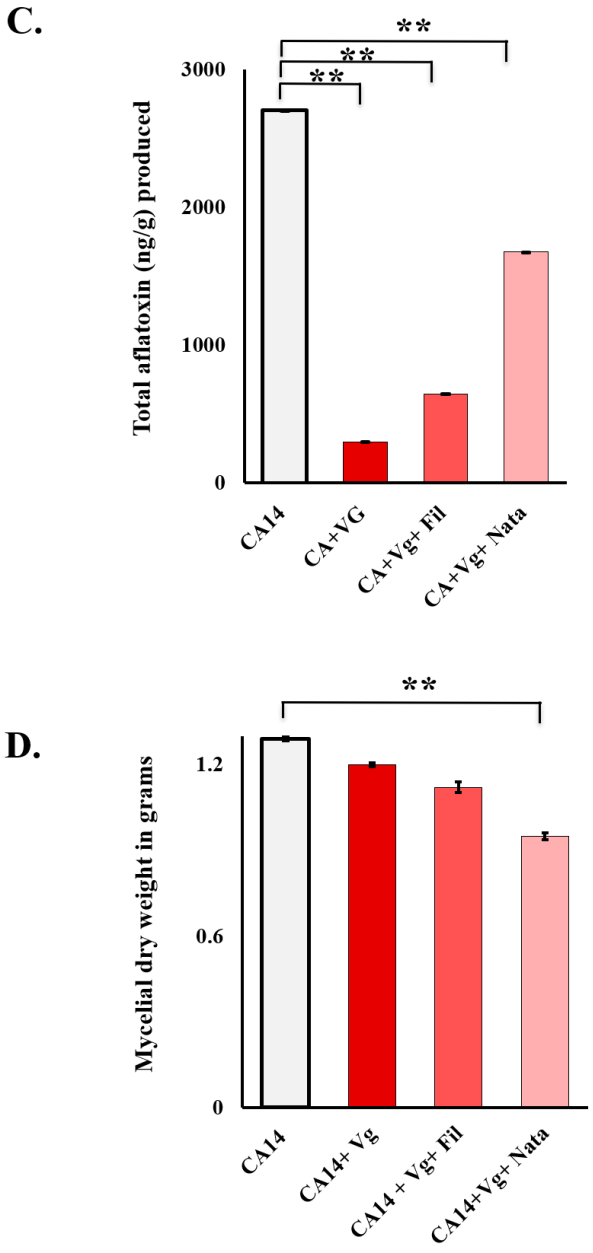


Figure 4.1(Continued): Influence of *V. gazogenes* and endocytosis inhibitors (filipin and natamycin) uptake on aflatoxin production and growth of *A. flavus* (CA14). (C) Total aflatoxin production (ng/g) per gram of mycelia at 40 hr time point, in the presence of *V. gazogenes* and endocytosis inhibitors (filipin and natamycin). (D) The percentage of *A. flavus* (CA14) mycelial dry weight in the presence of *V. gazogenes* and endocytosis inhibitors (filipin and natamycin) at 40 hr time point. Error bars represent SEM, from triplicate experiments. Statistical significance of two-tailed *p*-values determined using an unpaired t-test for n =16. (**, $p<0.001$). Star indicates significance.

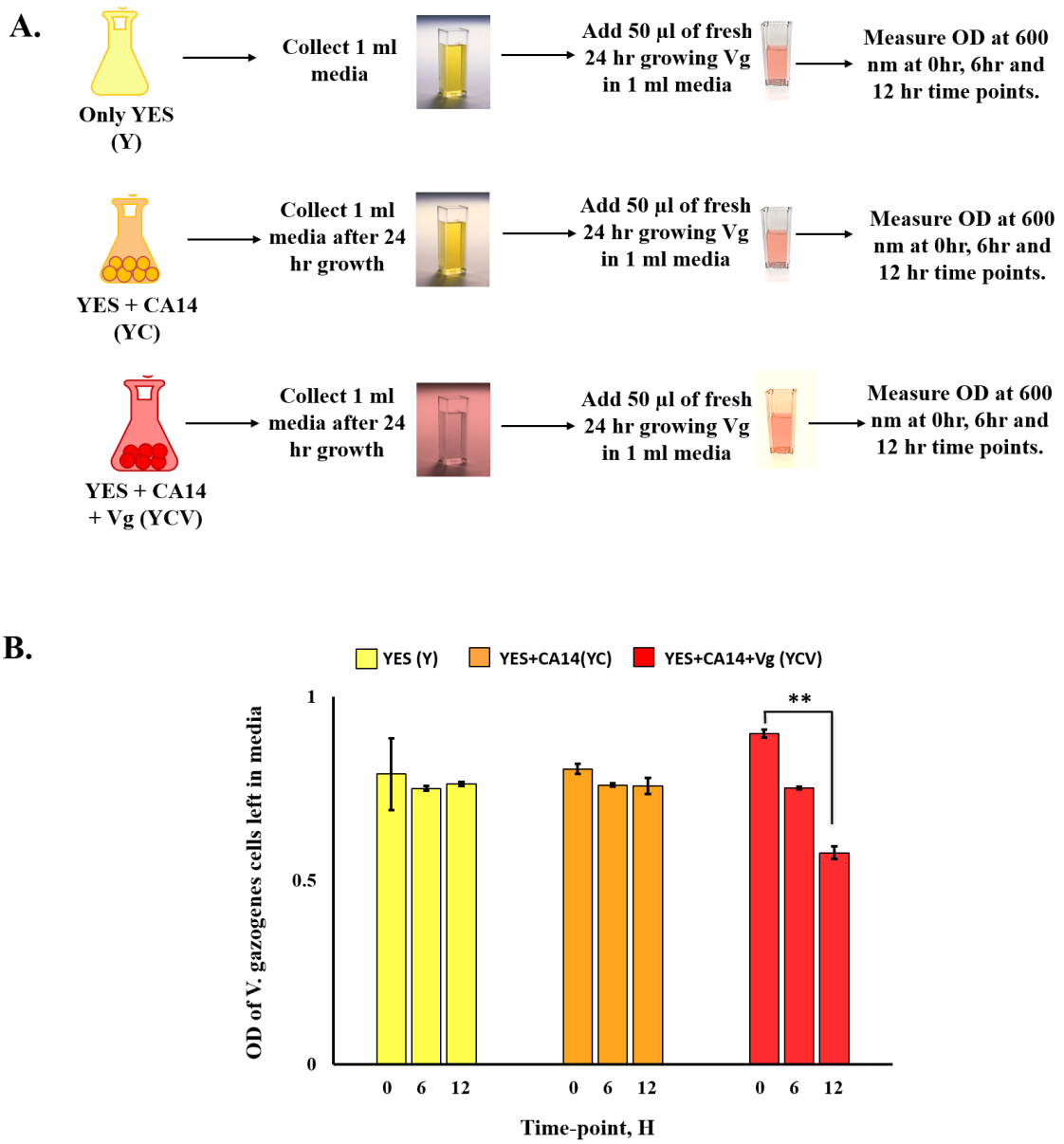


Figure 4.2: Influence of *A. flavus* in producing lysing enzymes by *V. gazogenes*. (A). Systematic diagram of treatment procedure of *V. gazogenes* cells by different media (with and without *A. flavus* (B) Lysis of bacterial cells in different growth medium post spore inoculation. Bacterial density in the growth medium as quantified by measuring the optical density of the growth medium at 600nm (OD_{600}). Error bars represent SEM, from triplicate experiments. Statistical significance of two-tailed *p*-values determined using an unpaired t-test for n =16. (**, $p<0.001$). Star indicates significance.

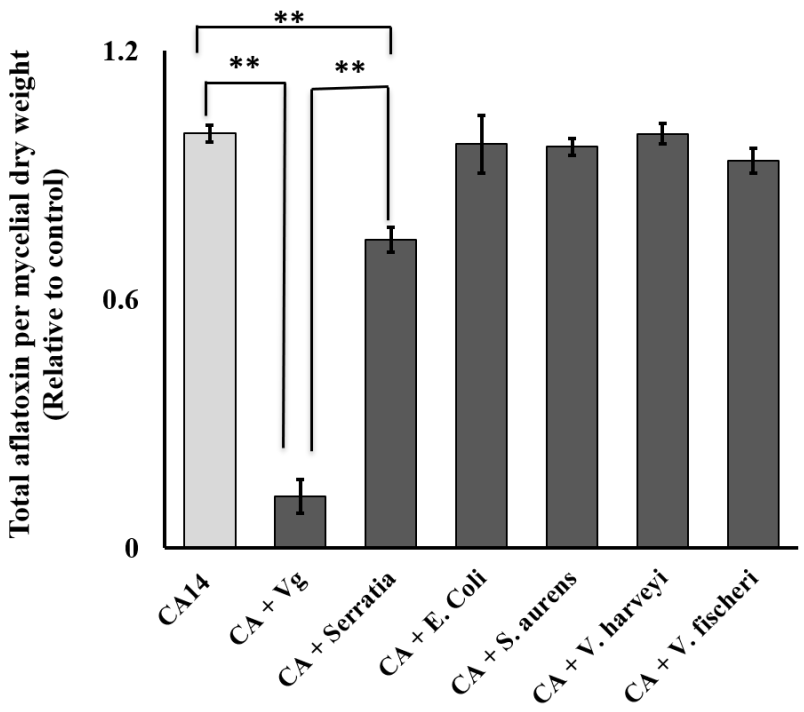


Figure 4.3: Influence of different bacteria along with *V. gazogenes* (non-viable) on aflatoxin production and growth of *A. flavus*. Total aflatoxin production (ng/g) per gram of mycelia at 40 hr time point was measured through ELISA. Error bars represent SEM, from triplicate experiments. Statistical significance of two-tailed p-values determined using an unpaired t-test for n =16. (**, $p<0.001$). Star indicates significance.

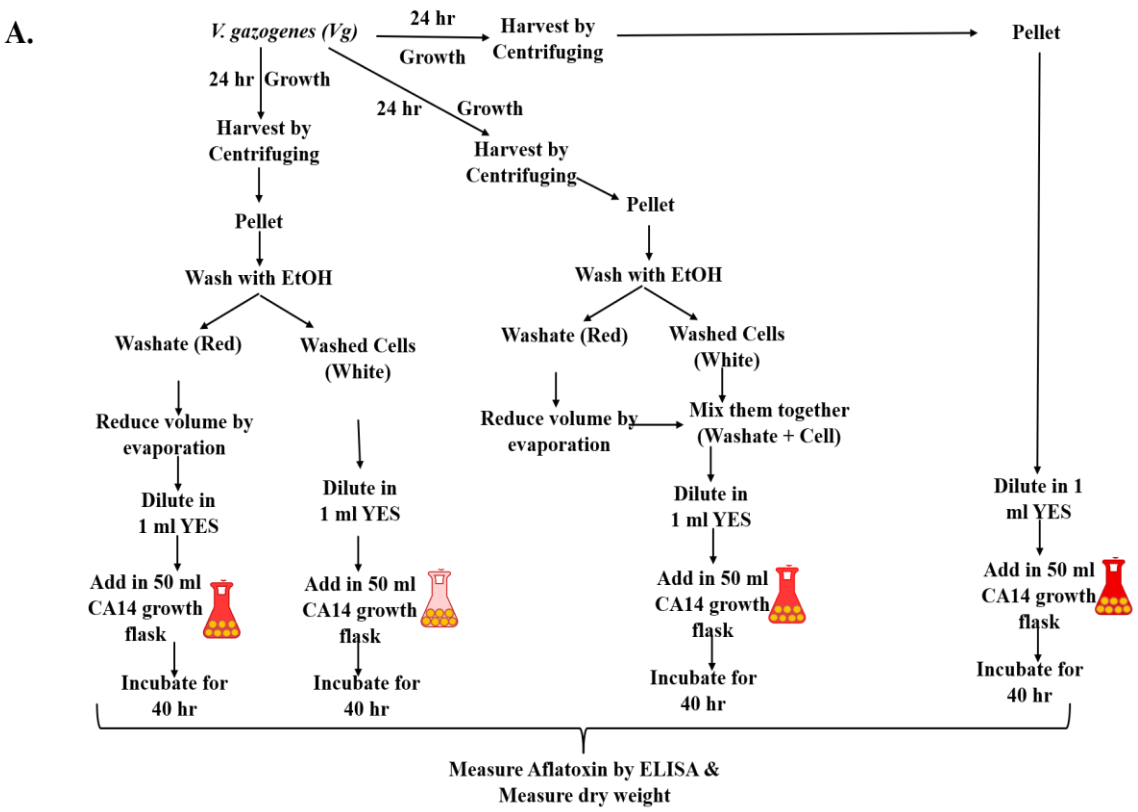


Figure 4.4: Influence of intact and different fractionated parts of *Vibrio gazogenes* cells on aflatoxin reduction of *A. flavus.* (A). Systematic diagram of treatment procedure of intact *V. gazogenes* cells by ethanol (EtOH) precipitation method to treat *A. flavus*

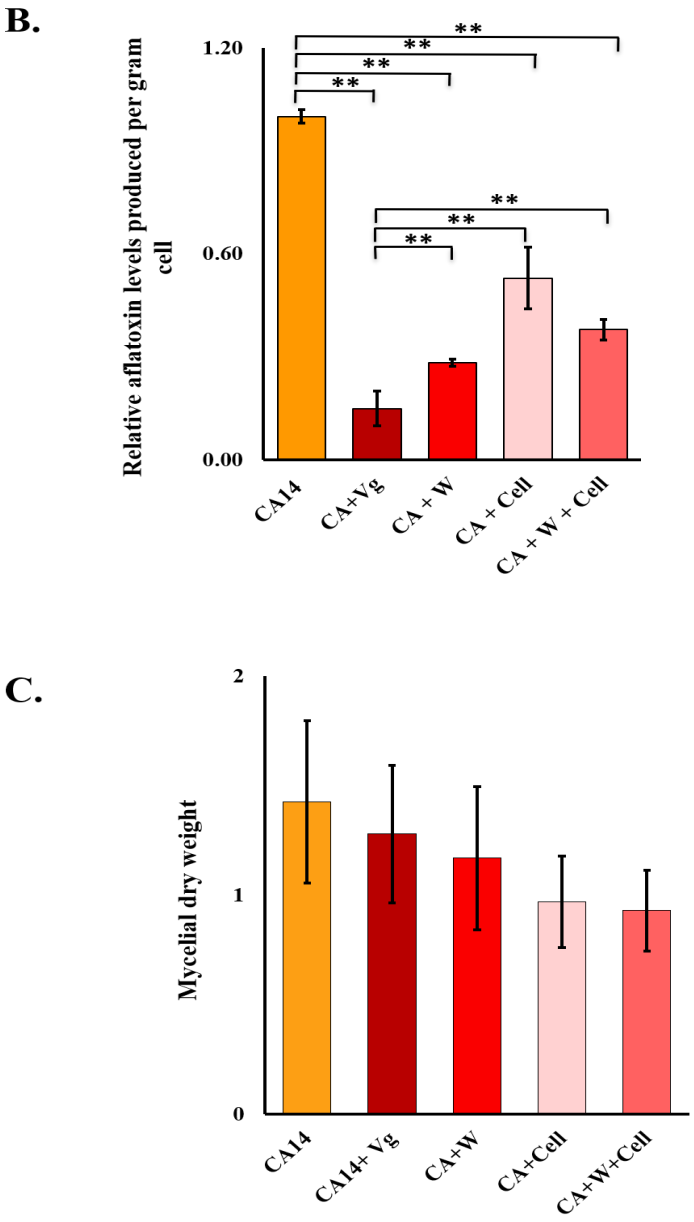


Figure 4.4 (Continued): Influence of intact and different fractionated parts of *Vibrio gazogenes* cells on aflatoxin reduction of *A. flavus.* (B) Relative aflatoxin production (ng/g) per gram of mycelia at 40 hr time point, in the presence of intact and different fractionated parts of *Vibrio gazogenes* cells. (C) The dry weight of mycelia was plotted on the y-axis and bars represent the measurements in grams. Statistical significance of two-tailed p-values were determined using an unpaired t-test for n=3. Error bars represent SEM. (**, $p<0.001$). Star represents the difference as compared to the control was statistically significant.

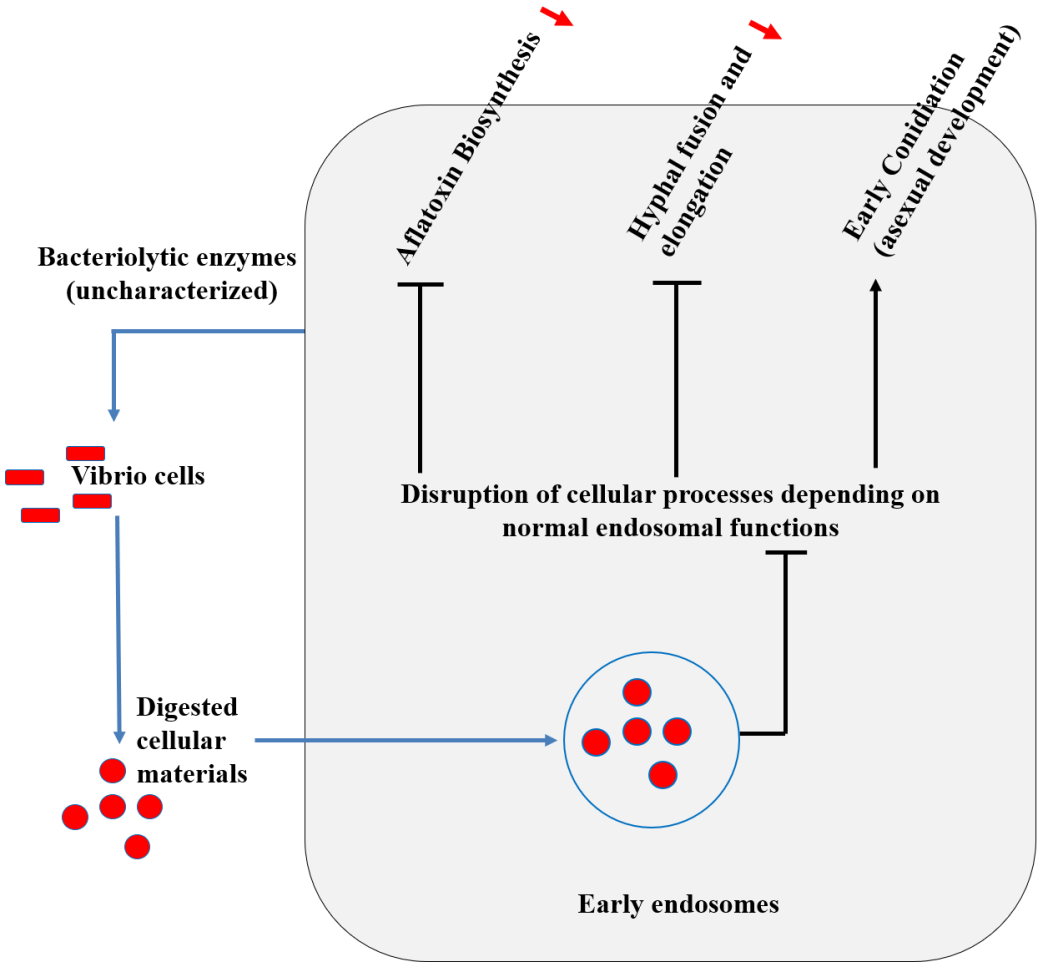


Figure 4.5: Theoretical model of disruption effects of *V. gazogenes* on aflatoxin synthesis and hyphal morphogenesis. According to this model, the internalization of cellular components of *V. gazogenes* in hyphae results in the intervention of endosomal functions associated with hyphal morphogenesis and aflatoxin biosynthesis

CHAPTER 5

CONCLUSIONS, FUTURE DIRECTIONS AND SIGNIFICANCE TO PUBLIC HEALTH

5.1 SUMMARY OF CONCLUSIONS

In summary, *Vibrio gazogenes* cells have a significant impact on aflatoxin reduction in *A. flavus* when interactions occur between them. Their cellular components and metabolites almost completely inhibited aflatoxin production upon endosomal uptake. The inhibition occurred at the levels of aflatoxin gene expression. Light, electron and confocal microscopy collectively suggested that *V. gazogenes* dependent aflatoxin inhibition was associated with internalization of bacterial cell materials but not intact bacteria, in endosome-like compartments. We have investigated the effects of different endocytosis inhibitors on endosomal uptake of *V. gazogenes* cells by *A. flavus*. The result showed that *A. flavus* cannot uptake the same amount of *V. gazogenes* which also affect the aflatoxin inhibition. In another study, we observed that presence of *V. gazogenes* in *A. flavus* growth medium leads to a production of bacteriolytic components (to be characterized in our future studies) that appeared to lyse the *V. gazogenes* cells; we hypothesize based on these observations, that this lysate through endosomal uptake inhibited hyphal morphogenesis, leading to reduced hyphal elongation, hyphal fusion, and sclerotial formation. We have developed a novel assay for hyphal fusion and tested the fusion process in the absence and presence of *V. gazogenes*. We also show that, *V.*

gazogenes uptake results in an almost complete (>98%) block of hyphal fusion and an ~3-fold decrease of polar growth, processes that depend on endosomal functions, and transport. The inhibition of sexual structures (sclerotia) was correlated with higher asexual spore formation. However, those spores generated colonies were ~2-fold less aflatoxigenic than the earlier generation. Treating *A. flavus* with non-viable other gram-positive or gram-negative or prodigiosin producing bacterium did not have the same aflatoxin inhibitory effect suggesting that *V. gazogenes* dependent aflatoxin inhibition was specific to *V. gazogenes* and prodigiosin is not the only aflatoxin inhibitor. An intriguing finding in this study was that only intact *V. gazogenes* cells when added to the growth medium resulted in the most effective decrease aflatoxin production (>99%) rather than different fractionated parts of *V. gazogenes* cells, suggesting that interactions between intact *V. gazogenes* and the fungus is needed for the best results. In conclusion, our study has opened a new research direction that leads to the development of a novel "green aflatoxin inhibitor" and enables us to understand the bacteria-fungal interactions related to the prevention of fungal infections and toxin contamination in the environment.

5.2 FUTURE DIRECTIONS

Our focus will be solely on the molecular and cellular mechanisms coordinating secondary metabolic pathways and hyphal development in our follow-up experiment. In the future, identifying the genes, proteins, and biochemical mechanisms associated with *A. flavus* response to *V. gazogenes* cells need to be done. Mining fungal secretomes are important for a range of applications, including identification of novel enzymes, understanding fungal interactions with the environment, investigating pathogenic mechanisms, epitope mapping, and identification of new vaccine candidates. Thus, we plan

to characterize the secreted metabolic components and secretomes of *A. flavus* in the presence and absence of *V. gazogenes* cells. To uncover the precise roles of the individual fractionated components of *V. gazogenes* on aflatoxin inhibition, we also plan a further investigation. Overall, our future studies are aimed at understanding the complex mechanisms underlying *V. gazogenes* and *A. flavus* interactions that will be leveraged to improve plant and human health.

5.3 BROADER SIGNIFICANCE TO PUBLIC HEALTH

The COVID-19 pandemic, also known as the coronavirus pandemic, is an ongoing pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). More than 7.18 million cases of COVID-19 have been reported in more than 188 countries, resulting in more than 408,000 deaths and it is still going on (WHO, 2020). Dealing with the unforeseen challenges caused by the COVID-19 pandemic has taken a significant toll on people across the world. Some reports suggest that patients recovering from COVID-19 infections have an increased likelihood of developing invasive Aspergillosis and other *Aspergillus* infections when exposed to *Aspergillus* spores or hyphae (Marion et al., 2020; Verweij et al. 2020; Prattes et al., 2020). Conversely, aflatoxin and other mycotoxin exposures that are appearing to increase in our foods with the changing climate, are consistently threatening our immune system, which in turn can make us vulnerable to COVID-19 and other microbial infections and other health disorders.

Besides that, one-fourth of the total crops produced in the world is wasted every year due to fungal spoilage. With the ongoing pandemic that has severely impaired the food supply chains worldwide, such wastages have increased significantly, and it predicted

that this spoilage would increase with the impending climate change. With the associated energy and water losses, this enormous food wastage is a critical emerging challenge to the Food/Water/Energy (FWE) nexus because the global population is expected to reach ~9.6 billion by 2050 and as a consequence, the global food demand will see a 70%-100% increase (FAO, 2018). My thesis directly addresses these ongoing public health challenges.

The knowledge from this research can be used in novel agricultural and pharmaceutical practices to protect and improve the health of plants, humans, and their communities in the future. It will lead to the development of “green antifungals” that can prevent mold infestations in crops and improve the overall environmental health. Simultaneously the research has the ability to generate a biopharmaceutical drug from our novel tool to reduce the morbidity and mortality rate in immunocompromised patients due to invasive aspergillosis disease. In summary, it will help to mitigate the toxic molds and mycotoxins that threaten food safety and human health, and thereby improve public health.

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