# Strategies to detoxify the mycotoxin deoxynivalenol and improve food safety in the U.S.

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## ACADEMIC ABSTRACT

Mycotoxins are toxic secondary metabolites produced by fungi that are a threat to the health of humans and domestic animals. The most important mycotoxin in the U.S. is deoxynivalenol (DON), which causes symptoms such as vomiting, feed refusal, and weight loss in farm animals. The fungus *Fusarium graminearum* produces DON in staple crops such as wheat, barley, and corn. It is estimated that the economic losses associated with DON contamination alone exceed $650 million per year in the U.S. New strategies are needed to mitigate DON and improve food safety in the U.S. The overall goal of my research is to discover and employ microorganisms and enzymes to detoxify DON. The specific objectives are to: (1) discover and characterize microorganisms that detoxify DON, (2) use a cell free protein synthesis (CFPS) system to study enzymes that modify DON, (3) engineer yeast to detoxify DON with a metabolic engineering strategy, and (4) deliver a high school unit to teach high school students about mycotoxins in food. In Objective 1, two mixed cultures were identified from environmental samples that converted DON into the less toxic 3-keto-deoxynivalenol (3-keto-DON). In Objective 2, a CFPS system was used to express three known acetyltransferase genes to convert DON to 3-acetyl-DON (3-A-DON). In Objective 3, we identified a potential DON transporter from a library of randomly amplified fragments from the genomes of mixed cultures of microbes isolated from the environment. In Objective 4, we developed and delivered a unique high school unit to educate high school students about potential mycotoxins in food and feed products. The work presented here represents new and improved methods for mitigating mycotoxin contamination in the United States.

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## GENERAL AUDIENCE ABSTRACT

Some fungi produce dangerous toxins called mycotoxins that contaminate food and feed and cause adverse affects when consumed. The mycotoxin deoxynivalenol (DON) contaminates staple crops such as wheat, barley, and corn and when consumed by domesticated animals it can cause weight loss, feed refusal, vomiting, and even death. The goal of this research is to detoxify DON using miroorganisms such as bacteria or fungi as well as enzymes. The specific objectives are to: (1) discover and characterize microorganisms that detoxify DON, (2) utilize a cell free protein synthesis (CFPS) system to detoxify DON using known acetyltransferase genes, (3) engineer yeast to detoxify DON with a metabolic engineering strategy, and (4) deliver a high school unit to teach high school students about mycotoxins in food and strategies to mitigate them. For objective one, microorganisms were collected from plant and soil samples and incubated in solution containing 100 ppm DON. Two mixed cultures were discovered to convert DON to another metabolite, 3-keto-DON that is considered less toxic. In objective two, a cell free protein synthesis (CFPS) system was used to establish its functionality as a tool to screen for enzymes that will detoxify DON. Known acetyltransferase genes were expressed in the CFPS and DON was converted to the metabolite 3-acetyl-DON. The mixed cultures discovered in objective one were then utilized in objective three to determine what enzymes were responsible for the conversion of DON to 3-keto-DON. Objective four was established to shed light about the dangers of mycotoxins and how growers and scientists test for mycotoxins in food and feed.

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## ATTRIBUTIONS

CHAPTER 2

Nina Wilson and David Schmale planned, coordinated, and conducted most of experiments and coordinated the writing of the manuscript. Dash Gantulga and Cara Soyars conducted field and laboratory work to culture and identify DON-modifying microbes. Ken Knott conducted NMR assays and Niki McMaster conducted GC/MS assays. Susan McCormick provided materials for the work, and contributed to TLC methods. David Schmale and Ryan Senger provided oversight of the project. Nina Wilson and David Schmale wrote the manuscript, and all authors contributed edits to the manuscript.

CHAPTER 3

Nina M. Wilson and Jiayuan Sheng conducted cell free and western blotting experiments. Nicole McMaster performed GC/MS analysis. Xueyang Feng, Ryan S. Senger, David G. Schmale III planned and coordinated experiments.

CHAPTER 4

Nina M. Wilson and Celia Sanchez conducted experiments. Dash Gantulga generated the DON sensitive yeast. Nicole McMaster performed GC/MS analysis. Benjamin G. Freedman generated the DOP-PCR primers and method. David G. Schmale III and Ryan S. Senger planned and coordinated experiments.

CHAPTER 5

Cindy Bohland and her biology class piloted this activity. Nina Wilson, Shelbie Dashiell, and Niki McMaster conducted experiments. David G. Schmale III planned and coordinated experiments.

# CHAPTER I

# LITERATURE REVIEW

## FUSARIUM

### Overview

*Fusarium*, a genus of filamentous fungi in the phylum Ascomycota, causes disease in both plants (Nelson et al., 1994) and animals (Evans et al., 2004; Nucci and Anaissie, 2007). There are over 150 *Fusarium* species with some species cabable of producing secondary metabolites such as mycotoxins (Leslie and Summerell, 2006). Species most common in animal infections include *F. solani, F. oxysporum, F. verticillioides, and F. fujikuroi* (Nucci and Anaissie, 2007). *Fusarium* species cause nail, skin, bone, and intranasal infections (Nelson et al., 1994).

*Fusarium* infections in humans are a cause for concern, but the majority of infections occur in immunocompromised individuals (Nucci and Anaissie, 2007). *Fusarium* infections cause damage to agricultural crops, resulting in several billion dollars worth in losses for wheat and barley farmers (Windels, 2000). *Fusarium graminearum* is the main casual agent of Fusarum head blight (FHB) in the United States, however, *F. pseudograminearum, F. culmorum, and F. sporotrichoides* also cause FHB around the world.

The *Fusarium graminearum* genome has approximately 11,640 genes (Goswami and Kistler, 2004) with the most fluid DNA regions correlating with plant infection, which likely help the organism cause disease (Cuomo et al., 2007). There are four types of asexual spores called macroconidia (large), mesoconidia (medium), microconidia (small), and chlamydospores (Leslie and Summerell, 2006) that *Fusarium* species can. All asexual spores are haploid and germinate to form germ tubes, which then differentiate and grow into hyphae; hypha consists of one or more cells that can be divided by internal walls called setpa. Hyphae can form structures called conidiophores, which give rise to the asexual spores. *Fusarium* is the asexual stage (anamorph) and the sexual stage (telemorph) is called *Gibberella* (Desjardins, 2003). The sexual stage produces fruiting bodies called perithecia that produce ascospores (Desjardins, 2003). To produce perithecia and ascospores, a hyphal fragment from one mating type transfers a nucleus through a specialized structure called the trichogyne to the opposite mating type (Leslie and Summerell, 2006).

## Fusarium head blight

Fusarium head blight (FHB) causes significant reductions in yield and grain quality (Kang and Buchenauer, 2000; Parry et al., 1995). From 1998 to 2000, FHB of wheat and barley resulted in economic losses of $2.7 billion (Nganje et al., 2001). The causal agent, *Fusarium graminearum*, overwinters in decaying crop residue as a saprophyte (Parry et al., 1995) and during the spring and summer months, perithecia eject ascospores into the environment leading to infection of emerging wheat and barley heads (Goswami and Kistler, 2004).

*Fusarium graminearum* produces the mycotoxin deoxynivalenol (DON) to promote hyphal spread in wheat as DON is considered to be a virulence factor (Proctor et al., 1995, Desjardins et al., 1996). DON has demonstrated to contribute to *F. graminearum* aggressiveness (Mesterhazy, 2002) with higher aggressiveness associated with higher FHB severity, more *Fusarium* damaged kernels, and greater yield loss in wheat (Mesterhazy, 2002). Cultivar resistance determines the isolate's aggressiveness during infection (Mesterhazy, 2002). For example, resistant wheat cultivars were shown to accumulate low levels of mycotoxin, while the same isolate produced high levels of mycotoxins in susceptible cultivars (Mesterhazy, 2002).

# DEOXYNIALENOL

## Overview

The trichothecenes are a major class of mycotoxins containing over 150 toxic compounds (P.M.S, 1990). Trichothecenes are produced by a few different fungal species, with the most noteworthy being *Fusarium graminearum*, causal agent of Fusarium Head Blight in wheat and barley (Schmale and Munkvold, 2009). The fungus produces deoxynivalenol (DON), the most important mycotoxin in the United States (Tankaka et al., 1988, Salas et al., 1999, McMullen et al., 1997). DON contamination is a pressing problem in the U.S. DON causes feed refusal, skin disorder, diarrhea, reduced growth, and vomiting in animals (Pestka, 2010). The epoxide group on DON is responsible for its toxicity; it is required for the inhibition of protein synthesis (Ueno et al., 1973). Though a number of enzymes may modify DON and reduce its toxicity (Garvey et al., 2008, Kahtibi and Montanti et al., 2011, Kahtibi and Newmister et al., 2011, Poppenberger et al., 2003), targeting the epoxide group is generally regarded as the most reasonable (feasible) strategy (He et al., 2010, Karlovsky, 2011). Strains of Eubacterium (Binder, 2004) have been shown to de-

epoxidize DON, but much of this work was confined to anaerobic conditions. Recent work by Guan et al. suggests that DON can be de-epoxidized under aerobic conditions and a wide range of pH, though pure cultures of the microbes and the resulting enzymes have not yet been isolated (Guan et al. 2009).

**Occurrence and current management of DON**

DON is produced in the field and during post-harvest when conditions are favorable (Nelson et al., 1993). DON accumulates more rapidly at 25°C than at other temperatures tested; however, the maximum amount of DON accumulation was detected at 30 °C with water activity of 0.995 (Ramirez et al., 2006). The main sources of DON in human food and animal feed are contaminated wheat, barley and maize, as well as their processed products, e.g. breakfast cereals, bakery products, snack foods, beer, pet food and compound feeds made from small grains and maize (JECFA, 2001; Streit et al., 2013). Worldwide incidences of human food and animal feed contamination with DON are high (Binder et al., 2007; Pereira et al., 2014). Monitoring data of trichothecenes in Canadian cereal grain shipments from 2010 to 2012 showed that DON was the most frequently detected trichothecene, found in 76% of tested samples at concentrations up to 2.34 mg/kg (Tittlemier et al., 2013).

**Management of DON**

Briefly, the current practices to reduce or contain DON production in the field include cultivation practices, breeding for disease resistance, chemical control using fungicides, and biological control using antagonists (He et al., 2013). Although these approaches may reduce DON production by *Fusarium* spp. in the field, it is still possible that the plant pathogens can continually produce DON in grains after harvest and in storage, when environmental conditions become favorable for DON production (Ramirez et al., 2006).

Therefore, there is a need to decontaminate DON post-harvest by chemical, physical and biological means. Various physical (e.g. gravity and sieving separation, dehulling and washing procedures, and application of absorbents) and chemical techniques (e.g. treatments using ozone, sodium metabisulfite (Na2S2O5) and sodium hydroxide) have shown to decontaminate DON in grains. Biological transformation using microorganisms may be an additional approach to decontaminate DON (Karlovsky, 1999; Zhou et al., 2008).

## Microbial Transformation of DON

Aerobic and anaerobic microbial transformations have been studied. Microorganisms from ruminants, swine, poultry, fish, soil and agricultural commodities have shown DON transformation activities and is summarized and discussed in the Zhou et al., 2008 review.

Under anaerobic conditions, DON-to-DOM-1 reduction has been consistently observed in transformations by microorganisms from cows (King et al., 1984; Swanson et al., 1987, Fuchs et al., 2002), poultry (He et al., 1992; Young et al., 2007; Yu et al., 2010) and fish (Guan et al., 2009). In addition, hydrolysis was also noted in several transformations. For example, a rumen fluid transformed 3-A-DON to DON by deacetylation, and then to DOM-1 by deepoxidation reduction (King et al., 1984). Two bacterial isolates (LS100 and SS3) from chicken intestines transformed DON and eleven other trichothecenes into various products by deepoxidation and deacetylation (Young et al., 2007; Yu et al., 2010).

Under aerobic conditions, microorganisms from soils and plants transformed DON by hydroxylation, oxidation, isomerization or reduction. DON was oxidized to 3-keto-DON by Agrobacterium-Rhizobium sp. E3-39 (Shima et al., 1997), a cultural mixture (Völkl et al., 2004) and Devosia sp. 17-2-E-8 (Zhou and He, 2009; Zhou and He, 2010). Devosia sp. 17-2-E-8 reduced DON concentrations and also formed 3-epi-DON as the major product through epimerization (Zhou and He, 2009; Zhou and He, 2010). Recent studies showed that several strains of Nocardioides spp. and Devosia spp. formed 3-epi-DON as an intermediate product; however, other intermediate or terminal transformation products were not identified (Ikunaga et al., 2011; Sato et al., 2012). A bacterial culture from a mixture of 165 agricultural soils transformed DON to DOM-1 under both aerobic and anaerobic conditions (Islam et al., 2012). Marmoricola sp. MIM116 that was selected from wheat heads reduced DON concentration (Ito et al., 2012).

## DON Toxicity and Biosynthesis

DON's toxicity is associated with ribotoxic stress (Pestka and Smolinski, 2005; Pestka, 2010). DON binds to eukaryotic ribosomes and inhibits protein synthesis by interfering with peptidyl transferase during translation of proteins (Pestka, 2010). Cell signaling is disrupted

inducing mitogen-activated protein kinases (MAPKs), which are important transducers of downstream signaling events related to immune response and apoptosis (Iordanov et al. 1997; Laskin et al. 2002; Pan et al., 2013).

DON, nivalenol, and T-2 toxin are trichothecene mycotoxins that are sesquiterpenes derived from the mevalonic acid pathway (MVA) (Kimura et al., 2007). More specifically, DON is a type B trichothecene characterized by having a keto (carbonyl) function at C-8 (McCormick et al., 2011). The MVA pathway produces terpenes and is responsible for the production of secondary metabolites (Lange et al., 2000). Pyruvate produced from glycolysis is incorporated into the MVA pathway to produce isopentenyl diphosphate (IPP), modified to farnesyl diphosphate (Lange et al., 2000), which becomes the first substrate in DON biosynthesis (Foroud and Eudes, 2009). During the first step, farnesyl diphosphate is cyclized into trichodiene by trichodiene synthase (TRI5) (Foroud and Eudes, 2009). The next steps include a hydroxylation, , epoxidation, acetylation, and another hydroxylation. Hydroxylations are catalyzed by a P450 monooxygenase (TRI4) at C-2, C-3, C-11, C-12 and C-13 to produce isotrichodiol (Kimura et al., 2007). Two non-enzymatic isomerization steps produce the first trichothecene in the pathway called isotrichodermol (Foroud and Eudes, 2009). Trichothecene 3-O-acetylatransferase called TRI101 acetylates isotrichodermol at C-3 to produce isotrichodermin which is a form of protection against toxicity and then subsequently hydoxylated at C-15 by TRI11, a monooxygenase, to form 15-deacetylcalonectrin (Foroud and Eudes, 2009). This is followed by 15-O-acetyltransferase, TRI3, to produce calonectrin and afterward calonectrin is hyroxylated at C-7 and C-8 by TRI1, a monoxygenase, to produce 7,8 dihdroxycalonectrin (McCormick et al., 2011). The next product is 3,15-dideacetylcalonectrin is converted to 3-acetyldeoxynivalenol (3-A-DON) or 15-acetyldeoxynivalenol (15-A-DON) by the esterase TRI8 (McCormick et al., 2011). In the final step, the acetyltransferase TRI101 converts 3-A-DON or 15-A-DON to DON (McCormick et al., 2011).

DON has been determined to be a virulence factor in wheat, but not in barley (Jansen et al., 2005). Desjardins et al., obtained trichothecene-nonproducing mutants of G. zeae by disrupting Tri5, the gene encoding trichodiene synthase, which catalyzes the first step in the trichothecene biosynthetic pathway. Trichothecene-nonproducing mutants were less virulent than the trichothecene-producing parental strains as they demonstrated less disease (Desjardins et al., 1996).

**GENOMIC DNA LIBRARIES**

A DNA library is a mixed, heterogeneous collection of plasmids with a different fragment of DNA cloned into a specific position of the plasmid. The collection of fragments is designed to cover the entire genome of the organism(s) of interest. DNA libraries were initially constructed using a high-pressure nebulization to shear DNA followed by purification and an end-repair step to remove incompatible DNA ends (Sambrook, 2001). The DNA library was then phosphorylated and integrated into a linearized, dephosphorylated plasmid produced by using blunt-cutting enzymes (Sambrook, 2001). This method can be tedious and ineffective. A more efficient and effective way to generate DNA library fragments is to use degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) (Freedman et al. 2015). This form of PCR eliminates blunt-end cloning making for an easier transition into a plasmid. DOP-PCR uses degenerate PCR primers that bind randomly to genomic DNA and amplify different fragments during a PCR run. The *Taq* polymerase used with this procedure adds a poly "A" tail to PCR ends allowing for easy cloning into an entry vector and then a destination vector.

**CELL FREE PROTEIN SYNTHESIS**

DON-modifying microorganisms are mostly limited to oxidation or epimerization reactions (McCormick, 2015). Targeting the epoxide group is considered a feasible strategy to effectively eliminate DON toxicity (He et al., 2010). It is considered a good idea to utilize enzymes that will detoxify DON; however, there is no technology to screen for functional enzymes that detoxify DON (Abolmaali et al., 2008). Cell free protein synthesis (CFPS) systems are used to generate a protein of interest without living cells and only functional cellular lysate. In 1961, a CFPS was used to elucidate the genetic code and more recently CFPS technology has been used to generate protein for protein evolution and structural genomic studies (Nirenberg et al., 1961; Katzen et al., 2005). CFPS technology has made studying and understanding virus-like particles, membrane proteins, and protein evolution efficient and easier and most importantly has the ability to generate large quantities of protein (Carlson et al., 2012). The components consist of cell lysate containing ribosomes, aminoacyl-tRNA synthases, and other important factors for initiation and elongation of proteins (Carlson et al., 2012). NTPs, needed for an energy source, DNA or an mRNA template are

added to the cell lysate and incubated together until the energy source is depleted (Carlson et al., 2012).

## RESEARCH OBJECTIVES

The overall goal of this research is to discover and employ novel microorganisms and enzymes to detoxify DON in wheat and barley. The specific objectives are to: (1) discover and characterize microorganisms that detoxify DON, (2) use a cell free protein synthesis (CFPS) system to study enzymes that modify DON, (3) engineer yeast to detoxify DON with a metabolic engineering strategy, and (4) deliver a high school unit to teach high school students about mycotoxins in food.

**REFERENCES**

Abolmaali S, Mitterbauer R, Spadiut O, et al. Engineered bakers yeast as a sensitive bioassay indicator organism for the trichothecene toxin deoxynivalenol. *J Microbiol Methods*. 2008;72(3):306-312.

Binder EM BJ. Strain of Eubacterium that detoxifies trichothecenes. 2004:6/794-175.

Binder, E. M., Tan, L. M., Chin, L. J., Handl, J., and Richard, J. 2007. Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. Animal Feed Science and Technology 137: 265-82.

Carlson, Erik D., et al. "Cell-free protein synthesis: applications come of age." *Biotechnology advances* 30.5 (2012): 1185-1194.

Cuomo C.A., Guldener U., Xu J.R., Trail F., Turgeon B.G., Di Pietro A., Walton J.D., Ma L.J., Baker S.E., Rep M., Adam G., Antoniw J., Baldwin T., Calvo S., Chang Y.L., Decaprio D., Gale L.R., Gnerre S., Goswami R.S., Hammond-Kosack K., Harris L.J., Hilburn K., Kennell J.C., Kroken S., Magnuson J.K., Mannhaupt G., Mauceli E., Mewes H.W., Mitterbauer R., Muehlbauer G., Munsterkotter M., Nelson D., O'Donnell K., Ouellet T., Qi W., Quesneville H., Roncero M.I., Seong K.Y., Tetko I.V., Urban M., Waalwijk C., Ward T.J., Yao J., Birren B.W., Kistler H.C. (2007) The Fusarium graminearum genome reveals a link between localized polymorphism and pathogen specialization. Science 317:1400-2.

Desjardins A.E. (2003) Gibberella from A (venaceae) to Z (eae). Annu Rev Phytopathology 41:177-98.

Desjardins AE, Proctor RH, Bai G, et al. Reduced virulence of trichothecene-nonproducing mutants of Gibberella zeae in wheat field tests. Mol Plant-Microbe Interact. 1996; 9:775-781.

Evans J., Levesque D., de Lahunta A., Jensen H.E. (2004) Intracranial fusariosis: a novel cause of fungal meningoencephalitis in a dog. Vet Pathol 41:510-4.

Foroud N.A., Eudes F. (2009) Trichothecenes in cereal grains. Int J Mol Sci 10:147-73.

Freedman, B.G., Senger, R.S. (2015) Thermodynamic design of degenerate oligonucleotide primers for whole genome DNA amplification with reduced bias for use in

DNA libraries. Submitted to Genome Research.

Fuchs, E., Binder, E. M., Heidler, D., and Krska, R. 2002. Structural characterization of metabolites after the microbial degradation of type A trichothecenes by the bacterial strain BBSH 797. Food Additives & Contaminants 19: 379-386.

Garvey G.S., McCormick S.P., Rayment I. (2008) Structural and functional characterization of the TRI101 trichothecene 3-O-acetyltransferase from Fusarium sporotrichioides and Fusarium graminearum: kinetic insights to combating Fusarium head blight. J Biol Chem 283:1660-9.

Goswami R.S., Kistler H.C. (2004) Heading for disaster: Fusarium graminearum on cereal crops. Molecular Plant Pathology 5:515-525.

Guan S, He JW, Young JC, Zhu HH, Li XZ, Ji C, Zhou T (2009). Transformation of trichothecene mycotoxins by microorganisms from fish digesta. Aquaculture 290:290–295.

He, J., Boland, G. J., and Zhou, T. 2013. Chapter 70 Fusarium graminearum Schwabe (teleomorph Gibberella zeae (Schwein.) Petch.), Fusarium Head Blight Disease (Hypocreales: Nectriaceae) in Biological Control Programmes in Canada 2001- 2012. CAB International (CABI Publishing), Wallingford, UK.

He J, Zhou T, Young JC, Boland GJ, Scott PM. Chemical and biological transformations for detoxification of trichothecene mycotoxins in human and animal food chains: a review. Trends Food Sci Technol. 2010; 21:67-76.

Ikunaga, Y., Sato, I., Grond, S., Numajiri, N., Yoshida, S., Yamaya, H., Hiradate, S., Hasegawa, M., Toshima, H., Koitabashi, M., Ito, M., Karlovsky, P., and Tsushima, S. 2011. Nocardioides sp. Strain WSN05-2, isolated from a wheat field, degrades deoxynivalenol, producing the novel intermediate 3-epi- deoxynivalenol. Applied Microbiology and Biotechnology 89: 419–427.

Iordanov, M. S., Pribnow, D., Magun, J. L., Dinh, T. H., Pearson, J. A., Chen, S. L., and Magun, B. E. 1997. Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. Molecular Cell Biology 17: 3373–3381.

Islam, R., Zhou, T., Young, J. C., Goodwin, P. H., and Pauls, K. P. 2012. Aerobic and anaerobic de-epoxydation of mycotoxin deoxynivalenol by bacteria originating from agricultural soil. World

Journal of Microbiology & Biotechnology 28: 7-13.

Ito, M., Sato, I., Koitabashi, M., Yoshida, S., Imai, M., and Tsushima, S. 2012. A novel actinomycete derived from wheat heads degrades deoxynivalenol in the grain of wheat and barley affected by Fusarium head blight. Applied Microbiology and Biotechnology 96: 1059-1070.

Jansen C., von Wettstein D., Schafer W., Kogel K.H., Felk A., Maier F.J. (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted Fusarium graminearum. Proc Natl Acad Sci U S A 102:16892-7.

JECFA (Joint FAO/WHO Expert Committee on Food Additives). 2001a. Deoxynivalenol. Safety evaluation of certain mycotoxins in food. WHO Food Additives Series No. 47. World Health Organization (WHO), Geneva—International Programme on Chemical Safety (IPCS).

Kang Z., Buchenauer H. (2000) Ultrastructural and Cytochemical Studies on Cellulose, Xylan and Pectin Degradation in Wheat Spikes Infected by Fusarium culmorum. J. Phytopathology 148:263-275.

Katzen, Federico, Geoffrey Chang, and Wieslaw Kudlicki. "The past, present and future of cell-free protein synthesis." *Trends in biotechnology* 23.3 (2005): 150-156.

Karlovsky, P. 1999. Biological detoxification of fungal toxins and its use in plant breeding, feed, and food production. Natural Toxins 7: 1-23.

Karlovsky P. Biological detoxification of the mycotoxin deoxynivalenol and its use in genetically engineered crops and feed additives. Appl Microbiol Biotechnol. 2011; 91:491-504.

Khatibi PA, Montanti J, Nghiem NP, et al. Conversion of deoxynivalenol to 3-acetyldeoxynivalenol in barley-derived fuel ethanol co-products with yeast expressing trichothecene 3-O-acetyltransferases. Biotechnol Biofuels. 2011; 4:26.

Khatibi P.A., Newmister S.A., Rayment I., McCormick S.P., Alexander N.J., Schmale D.G., 3rd. (2011) Bioprospecting for trichothecene 3-o-acetyltransferases in the fungal genus fusarium yields functional enzymes with different abilities to modify the mycotoxin deoxynivalenol. Appl Environ Microbiol 77:1162-70.

Kimura M., Tokai T., Takahashi-Ando N., Ohsato S., Fujimura M. (2007) Molecular and genetic

studies of fusarium trichothecene biosynthesis: pathways, genes, and evolution. Biosci Biotechnol Biochem 71:2105-23.

King, R. R., McQueen, R. E., Levesque, D., and Greenhalgh, R. 1984b. Transformation of deoxynivalenol (vomitoxin) by rumen microorganisms. Journal of Agricultural and Food Chemistry 32: 1181-1183.

Lange B.M., Rujan T., Martin W., Croteau R. (2000) Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. Proc Natl Acad Sci U S A 97:13172-7.
Laskin, J.D., Heck, D. E., and Laskin, D. L. 2002. The ribotoxic stress response as a potential mechanism for MAP kinase activation in xenobiotic toxicity. Toxicology Science 69: 289–291.

Leslie J.F., Summerell B.A. (2006) The Fusarium Laboratory Manual Blackwell Publishing, Ames, Iowa.

McCormick SP. Microbial Detoxification of Mycotoxins. *J Chem Ecol*. 2013;39:907-918.

McCormick S.P., Stanley A.M., Stover N.A., Alexander N.J. (2011) Trichothecene: From simple to complex mycotoxins. Toxins 3:802-814.

McMullen M, Jones R, Gallenberg D. Scab of Wheat and Barley: A Re-emerging Disease of Devastating Impact. Plant Dis. 1997; 81:1340-1348.

Mesterhazy A. (2002) Role of deoxynivalenol in aggressiveness of Fusarium graminearum and F. culmorum and in resistance to Fusarium head blight. European Journal of Plant Pathology 108:675-684.

Nelson, P. E., Desjardins, A. E., and Plattner, R. D. 1993. Fumonisins, mycotoxins produced by Fusarium species: biology, chemistry and significance. Annual Review of Phytopathology 31: 233–252.

Nelson P.E., Dignani M.C., Anaissie E.J. (1994) Taxonomy, biology, and clinical aspects of Fusarium species. Clin Microbiol Rev 7:479-504.

Nganje W.E., Johnson D.D., Wilson W.W., Leistritz F.L., Bangsund D.A., Tiapo N.M. (2001) Economic Impacts of Fusarium Head Blight in Wheat and Barley: 1998-2000 North Dakota State University, Fargo, ND.

Nirenberg, Marshall W., and J. Heinrich Matthaei. "The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribonucleotides." *Proceedings of the National Academy of Sciences* 47.10 (1961): 1588-1602.

Nucci M., Anaissie E. (2007) Fusarium infections in immunocompromised patients. Clin Microbiol Rev 20:695-704.

Pan, X., Whitten, D. A., Wu, M., Chan, C., Wilkerson, C. G., and Pestka, J. J. 2013. Early phosphoproteomic changes in the mouse spleen during deoxynivalenol- induced ribotoxic stress. Toxicology Science 135:129-143.

Parry D., Jenkinson P., McLeod L. (1995) Fusarium ear blight (scab) in small grain cereals-a review. Plant Pathology 44:207-238.

Pereira, V. L., Fernandes, J. O., and Cunha, S. C. 2014. Mycotoxins in cereals and related foodstuffs: a review on occurrence and recent methods of analysis. Trends in Food Science & Technology 36: 96-136.

Pestka, J., and Smolinski, A. 2005. Deoxynivalenol: toxicology and potential effects on humans. Journal of Toxicology and Environmental Health Part B 8: 39-69.

Pestka JJ. Deoxynivalenol: Mechanisms of action, human exposure, and toxicological relevance. Arch Toxicol. 2010; 84:663-679.

P M S. TRICHOTHECENES IN GRAINS. Cereal Foods World. 1990; 35:661-666.

Poppenberger B., Berthiller F., Lucyshyn D., Sieberer T., Schuhmacher R., Krska R., Kuchler K., Glossl J., Luschnig C., Adam G. (2003) Detoxification of the Fusarium mycotoxin deoxynivalenol by a UDP-glucosyltransferase from Arabidopsis thaliana. J Biol Chem 278:47905-14.

Proctor R.H., Hohn T.M., McCormick S.P. (1995) Reduced virulence of Gibberella zeae caused by disruption of a trichothecene toxin biosynthetic gene. Mol Plant Microbe Interact 8:593-601.

Ramirez, M. L., Chulze, S., and Magan, N. 2006. Temperature and water activity effects on growth and temporal deoxynivalenol production by two Argentinean strains of Fusarium graminearum on irradiated wheat grain. International Journal of Food Microbiology 106: 291 - 296.

Salas B, Steffenson BJ, Casper HH, et al. Species Pathogenic to Barley and Their Associated Mycotoxins. Plant Dis. 1999; 83:667-674.

Sambrook, J. (2001). Cold Spring Harbor L: Molecular cloning: a laboratory manual/Joseph Sambrook, David W. Russell.

Sato, I., Ito, M., Ishizaka, M., Ikunaga, Y., Sato, Y., Yoshida, S., Koitabashi, M., Tsushima, S. 2012. Thirteen novel deoxynivalenol-degrading bacteria are classified within two genera with distinct degradation mechanisms. FEMS Microbiology Letters 327: 110–117.
Schmale DG, Munkvold G. Mycotoxins in crops: A threat to human and domestic animal health. Plant Heal Instr. 2009.

Shima, J., Takase, S., Takahashi, Y., Iwai, Y., Fujimoto, H., Yamazaki, M., and Ochi, K. 1997. Novel detoxification of the trichothecene mycotoxin deoxynivalenol by a soil bacterium isolated by enrichment culture. Applied and Environmental Microbiology 63: 3825-3830.

Streit, E., Naehrer, K., Rodrigues, I., and Schatzmayr, G. 2013. Mycotoxin occurrence in feed and feed raw materials worldwide: long-term analysis with special focus on Europe and Asia. Journal of the Science of Food and Agriculture 93: 2892–2899.

Swanson, S. P., Rood, J. H. D., Behrens, J. C., and Sanders, P. E. 1987b. Preparation and characterization of the deepoxy trichothecenes: deepoxy HT-2, deepoxy T-2 triol, deepoxy T-2 tetraol, deepoxy 15-monoacetoxyscirpenol, and deepoxy scirpentriol. Applied and Environmental Microbiology 53: 2821-2826.

Tanaka T, Hasegawa A, Yamamoto S, Lee US, Sugiura Y, Ueno Y. Worldwide contamination of cereals by the Fusarium mycotoxins nivalenol, deoxynivalenol, and zearalenone. 1. Survey of 19 countries. J Agric Food Chem. 1988; 36(5): 979-983.

Tittlemier, S. A, Gaba, D., and Chan, J. M. 2013. Monitoring of Fusarium trichothecenes in Canadian cereal grain shipments from 2010 to 2012. Journal of Agricultural and Food Chemistry 61: 7412−7418.

Ueno Y, Nakajima M, Sakai K, Ishii K, Sato N. Comparative toxicology of trichothec mycotoxins: inhibition of protein synthesis in animal cells. J Biochem. 1973; 74:285-296.

Völkl, A., Vogler, B., Schollenberger, M., and Karlovsky, P. 2004. Microbial detoxification of mycotoxin deoxynivalenol. Journal of Basic Microbiology 44: 147-156.

Windels C.E. (2000) Economic and social impacts of fusarium head blight: changing farms and rural communities in the northern great plains. Phytopathology 90:17-21.

Young, J. C., Zhou, T., Yu, H., Zhu, H., and Gong, J. 2007. Degradation of trichothecene mycotoxins by chicken intestinal microbes. Food and Chemical Toxicology 45: 136-143.

Yu, H., Zhou, T., Gong, J. H., Young, C., Su, X. J., Li, X-Z., Zhu, H. H., Tsao, R., and Yang, R. 2010. Isolation of deoxynivalenol-transforming bacteria from the chicken intestines using the approach of PCR-DGGE guided microbial selection. BMC Microbiology 10: 182-191.

Zhou, T., He, J., and Gong, J. 2008. Microbial transformation and its application in control of trichothecene mycotoxins in contaminated food and feed. World Mycotoxin Journal 1: 23-30.

Zhou, T., and He, J. 2009. Bacterial isolate, methods of isolating bacterial isolates and methods for detoxification of trichothecene mycotoxins. United States Provisional Patent Application No. 61/249,023. Filed October 6, 2009.

Zhou, T., and He, J. 2010. Bacterial isolate, methods of isolating bacterial isolates and methods for detoxification of trichothecene mycotoxins. Patent Cooperation Treaty Application No. 61/249,023. Filed October 6, 2010 (international).

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**CHAPTER II**

*mhArticle*

# Modification of the Mycotoxin Deoxynivalenol Using Microorganisms Isolated from Environmental Samples

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**Abstract:** The trichothecene mycotoxin deoxynivalenol (DON) is a common contaminant of wheat, barley, and maize. New strategies are needed to reduce or eliminate DON in feed and food products. Microorganisms from plant and soil samples collected in Blacksburg, VA, USA were screened by incubation in a mineral salt media containing 100 μg/mL DON and analysis by gas chromatography mass spectrometry (GC/MS). Two mixed cultures derived from soil samples consistently decreased DON levels in assays using DON as the sole carbon source. Nuclear magnetic resonance (NMR) analysis indicated that 3-keto-4-deoxynivalenol was the major byproduct of DON. 16s rRNA sequencing revealed these mixed cultures included mostly members of the genera *Acinetobacter*, *Leadbetterella*, and *Gemmata*. Incubation of one of these mixed cultures with wheat samples naturally contaminated with 7.1 μg/mL DON indicated nearly complete conversion of DON to the less toxic 3-epimer-DON (3-epi-DON). Our work extends previous studies that have demonstrated the potential for bioprospecting for microorganisms from the environment to remediate or modify mycotoxins for commercial applications, such as the reduction of mycotoxins in fuel ethanol co-products.

**Keywords:** mycotoxin, trichothecene, deoxynivalenol, bioprospecting, detoxification, *Fusarium*

## 1. Introduction

Mycotoxins are toxic secondary metabolites produced by fungi that are a threat to the health of humans and domestic animals [1]. This diverse class of compounds can contaminate commercial foods (e.g., wheat, maize, peanuts, cottonseed, and coffee) and animal feedstocks. Mycotoxins can be harmful even at small concentrations, creating significant food safety concerns [1,2]. The Food and Agriculture Organization estimated that approximately 1 billion metric tons of food is lost each year due to mycotoxin contamination [3]. Economic losses include yield loss from mycotoxin contamination [4], reduced value of crops [4], loss of animal productivity from health issues related to mycotoxin consumption [5], and even animal death [6,7].

The trichothecenes are a major class of mycotoxins containing over 150 toxic compounds and are toxic inhibitors of protein synthesis [8,9]. Trichothecenes are produced by several different fungi in the genus *Fusarium* [9,10]. One of the most economically important trichothecenes is deoxynivalenol (DON), which contaminates wheat, barley, and maize worldwide [11]. DON causes feed refusal, skin disorder, diarrhea, reduced growth, and vomiting in domestic animals [12]. Depending on the dose and exposure time of DON, there is also evidence that DON acts as an immunosuppressive [1]. It is among the most closely monitored mycotoxins in the US, and DON contaminations have resulted in estimated annual losses of up to $1.6 billion [13].

While there is structural variety, all trichothecenes share a core structure that includes the C-12,13 epoxide that is important to toxicity and protein inhibition [14,15]. DON is a type B trichothecene characterized by the presence of a keto group on the C-8 [16]. There are mechanisms the fungus *Fusarium* implements during the biosynthesis of DON to alter the structure making it less toxic, e.g. acetylating the C-3 position [16].

Microbial detoxification of mycotoxins has previously been reported [17,18]. Fuchs et al. (2002) were able to isolate an anaerobic eubacterium that converted DON to de-epoxy-DON [19]. A few years later, Völkl and colleagues (2004) reported that a mixed culture of organisms from soil samples converted DON to 3-keto-4-deoxynivalenol (3-keto-DON), but they were unable to identify the causal microorganisms responsible for the modification [20]. The product 3-keto-DON is

approximately 90% less toxic than DON, and represents a suitable detoxified product [21]. Shima et al. (1997) discovered a single organism in aerobic conditions from an environmental sample that converted DON into 3-keto-DON and He et al. (2015) isolated an aerobic organism, from the genus *Devosia*, converting DON to 3-epimer-DON (3-epi-DON) [21,22,23]. In 2010, Ikunaga et al. identified a bacterium from the genus *Nocardioides* that converts DON to 3-epi-DON [24]. Recently, He et al. (2016) discovered an aerobic culture of microorganisms converting DON to de-epoxy-DON [25]. The current study extends these prior investigations to a series of studies to isolate additional microorganisms from the environment that modify and remediate DON. While others have shown that soil bacteria can detoxify DON, the functional enzyme(s) responsible for conversion to 3-keto-DON remains elusive. Once the enzymatic mechanism(s) and genetic element(s) responsible are identified, yeast can be engineered to remediate DON during a fermentation process involving mycotoxin-contaminated feedstocks.

Based on previous work [21-25], we hypothesized that mixed cultures of microorganisms isolated from natural soil environments incubated with a mineral salt media using 100 μg /mL DON as the sole carbon source will detoxify DON. The specific objectives of this research were to: (1) identify microbes isolated from plant and soil samples taken in Blacksburg, VA that modify DON; (2) characterize DON metabolites using thin layer chromatography (TLC), gas chromatography mass spectrometry (GC/MS) and nuclear magnetic resonance (NMR); (3) identify bacterial components of mixed cultures with DON modification activity; and (4) determine if these microorganisms can modify DON in naturally contaminated wheat samples. Our work extends previous studies that have demonstrated the potential for bioprospecting for microbes that modify toxic secondary metabolites from grains and/or grain products, such as the reduction of mycotoxins in fuel ethanol co-products.

## 2. Results

### *2.1. Selection of Microbes in the Presence of High Concentrations of DON*

An initial screen of 11 plant and soil environmental samples incubated in mineral media containing 100 μg/mL DON as the sole carbon source identified 5 cultures in which no DON remained after 7 days. These 5 mixed culture samples that eliminated DON from the culture media (below the limit of quantification (<LOQ), which was 0.2 μg/mL) came from soil samples taken from a landscape plot, vineyard, and peach orchard and from plant samples taken in a small grain field and a vineyard. With further subculturing, 3 mixed culture samples had decreased DON levels in the culture media (Table S1) all of which were derived from the landscape plot. Only two samples from the landscape plot, mixed culture 1 and mixed culture 2 (Figure S1), consistently removed/modified DON in the culture media. Further assays with mixed cultures 1 and 2 (Table 1) suggested that the glycerol stocks were heterogeneous and likely contained mixtures of culturable and unculturable microbes; 4 sample replicates from mixed culture 1 and mixed culture 2 did not perform the same and had varying amounts of DON modification based on percentage of DON modified in each culture (Table 1). Mixed culture 3 replicates did not modify DON and thus were not studied further.

*2.2 Isolation of Individual DON Modifying Microbes*

There were 2 pure cultures, pure culture 1 and pure culture 2 from Table S1, of bacteria that were initially associated with decreased levels of DON within culture media. Pure culture 1 originated from the small grain field and pure culture 2 originated from the landscape plot. 16s rRNA sequencing revealed that pure culture 1 was from the genus *Achromobacter*, and the pure culture 2 was from the genus *Pseudomonas*. However, additional DON assays indicated that both pure culture 1 and pure culture 2 samples did not consistently modify DON (data not shown). The two pure cultures were inconsistent in their ability to eliminate DON from culture media, although preparation and culture conditions remained the same.

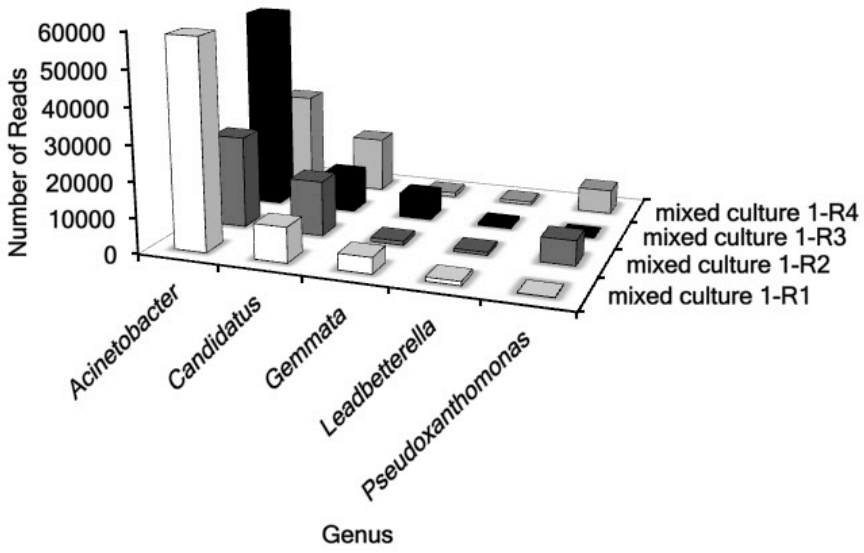
**Table 1.** DON modification by soil mixed cultures. Four sample replicates from mixed culture 1, mixed culture 2, and mixed culture 3 following incubation with 5 μg/mL DON.

| Culture Sample | Replicate | DON (μg/mL) Analytical Rep 1 | DON (μg/mL) Analytical Rep 2 | Mean DON (μg/mL) |
|---|---|---|---|---|
| mixed culture 1-R1 | 1 | 0.16 | 0.16 | 0.16 |
| mixed culture 1-R2 | 2 | 2.48 | 2.72 | 2.6 |
| mixed culture 1-R3 | 3 | 3.12 | 3.04 | 3.08 |
| mixed culture 1-R4 | 4 | 2.72 | 2.68 | 2.7 |
| mixed culture 2-R1 | 1 | <0.2 | <0.2 | <0.2 |
| mixed culture 2-R2 | 2 | 4.32 | 3.8 | 4.06 |
| mixed culture 2-R3 | 3 | <0.2 | <0.2 | <0.2 |
| mixed culture 2-R4 | 4 | <0.2 | <0.2 | <0.2 |
| mixed culture 3-R1 | 1 | 3.92 | 3.8 | 3.86 |
| mixed culture 3-R2 | 2 | 3.92 | 3.76 | 3.84 |
| mixed culture 3-R3 | 3 | 4.08 | 3.8 | 3.94 |
| mixed culture 3-R4 | 4 | 3.28 | 3.64 | 3.46 |
| Control-R1 | 1 | 4.46 | 4.48 | 4.47 |
| Control-R2 | 2 | 4.48 | 4.28 | 4.38 |
| Control-R3 | 3 | 3.84 | 4.04 | 3.94 |

*2.3. Identification of Mixed Cultures Using 16S Ribosomal Sequencing*

Sequencing of 16S of the mixed cultures mixed culture 1 and mixed culture 2 that consistently modified DON indicated that they contained mostly members of the genera *Acinetobacter*, *Leadbetterella*, and *Gemmata* (Figure 1). Mixed culture 1 consisted of members of the genera *Acinetobacter*, while mixed culture 2 was composed mostly of the genera *Leadbetterella*, and *Gemmata*. mixed culture 1- R1 was the only culture able to modify DON in culture media (Figure 1a). Mixed culture 1-R1 was composed mostly of the genera *Acinetobacter* and *Candidatus*. Mixed culture 2-R1, mixed culture 2-R3, and mixed culture 2-R4 modified DON (Figure 1b). Mixed culture 2-R2 was unable to modify DON in culture media and was the only culture that contained a large amount of *Burkholderia*.

(a)




(b)

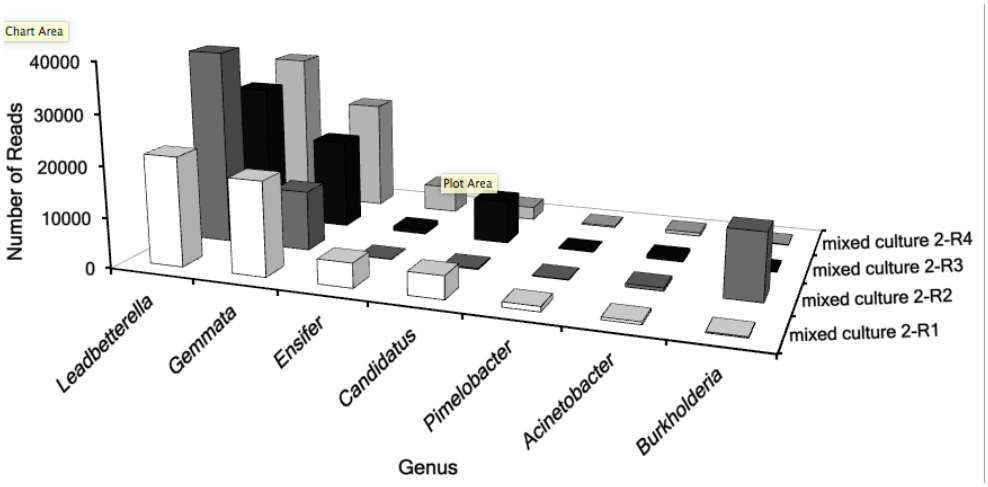



**Figure 1.** 16S rRNA sequencing data for within sample repetitions of (a) mixed culture 1 and (b) mixed culture 2. Each plot graph illustrates the major genera represented in each sample in accordance with number of reads resolved from sequencing. In (a) mixed culture 1-R1 was the only culture that was able to modify DON from the culture media. In (b) mixed culture 2-R1, mixed culture 2-R3, and mixed culture 2-R4 were able to modify DON from the culture media.

*2.4. Thin Layer Chromatography to Identify DON Derivatives*

TLC analysis of extracts of mixed culture 1 and mixed culture 2 showed a DON byproduct that was less polar than DON, 15-ADON, and 3ADON (Figure 2). Mixed culture 1 and mixed culture 2 byproducts showed similar properties to 3-keto-DON, which is nonpolar and migrated further up the TLC plate. Mixed culture 1 and mixed culture 2 byproducts were also dissimilar to the acetylated versions of DON, 15-A-DON and 3-A-DON. The product turned blue with NBP/TEPA, indicating that it contained an epoxide, and had a similar Rf (retention factor) as 3-keto DON.

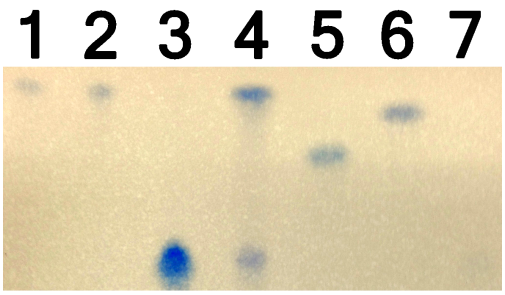



**Figure 2.** TLC analysis of DON byproducts and controls. Lane 1, mixed culture 1; Lane 2, mixed culture 2; Lane 3, DON; Lane 4, 3-keto-DON and DON mixture; Lane 5, 15-A-DON; lane 6, 3-A-DON, and lane 7, De-epoxy-DON.

*2.5. Nuclear Magnetic Resonance to Identify Structure of DON Derivatives*

NMR analysis showed that both the DON byproduct in both mixed culture 1 and mixed culture 2 was 3-keto-deoxynivalenol (3-keto-DON) (Table S2). The 3-keto-DON proton closely resembled that reported Shima et al. [21].

*2.6. DON Assays with Mixed Cultures with Naturally Contaminated Wheat Samples*

DON assays using wheat sample #13w-7 (41.0 μg/mL) did not show any DON reduction with mixed culture 1 or mixed culture 2. DON reduction was observed with wheat sample #13-v193 (7.1 μg/mL DON). In particular, two samples from mixed culture 1 (sample ID 2 and sample ID 3 in Table 2) showed nearly complete DON reduction compared to the control. Figure 3 shows a GC/MS chromatogram overlay of the DON control and the sample ID 2 from mixed culture 1, where significant DON reduction was observed. The DON peak had a retention time of 6.12 min, and the new peak at 6.33 min is postulated to be 3-epi-DON based on molecular weight and fragmentation. According to He et al. (2015), 3-epi-DON is significantly less toxic than DON. However, Ikunaga et al. [24] suggest that 3-epi-DON may still be just as toxic as DON since the epoxide ring is still present. DON and the postulated 3-epi-DON were detected in SIM mode with a target ion with a mass:charge ratio of 512.3 and had reference ions at 422.4 and 497.3.

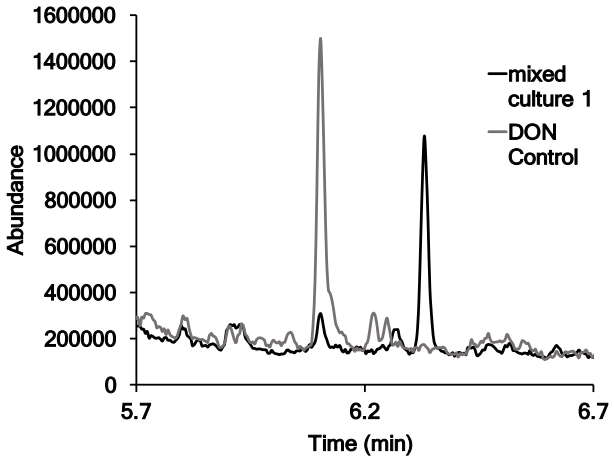



**Figure 3.** GC/MS chromatograph of DON derivatives in scan operating mode from sample ID 2 in Table 2 (mixed culture 1, wheat sample #13w-7, 7.1 μg/mL DON). DON (grey line; retention time of 6.12 min) was modified to 3-epi-DON (black line; retention time of 6.33 min).

**Table 2.** Grain culture extracts from naturally contaminated wheat sample #13v-193 (7.1 μg/mL DON) incubated with mixed culture 1 and mixed culture 2 were analyzed using GC/MS. Two separate assays were performed at different times with three replicates for mixed cultures 1 and 2 and the control. Sample ID 2 from mixed culture 1 in the first assay using wheat sample# 13v-193 showed significant DON reduction compared to the negative control (below the limit of quantification, which is 0.20 μg/mL).

| Sample ID | Culture ID | Assay | Replicate | Starting DON (μg/mL) in wheat | Final DON (μg/mL) in wheat |
|---|---|---|---|---|---|
| 1 | mixed culture 1 | 1 | 1 | 7.10 | 5.08 |
| 2 | mixed culture 1 | 1 | 2 | 7.10 | <0.20 |
| 3 | mixed culture 1 | 1 | 3 | 7.10 | 0.08 |
| 4 | mixed culture 1 | 2 | 1 | 7.10 | 7.04 |
| 5 | mixed culture 1 | 2 | 2 | 7.10 | 7.76 |
| 6 | mixed culture 1 | 2 | 3 | 7.10 | 5.88 |
| | | | | | **4.3 (mean)** |
| 7 | mixed culture 2 | 1 | 1 | 7.10 | 4.0 |
| 8 | mixed culture 2 | 1 | 2 | 7.10 | 6.4 |
| 9 | mixed culture 2 | 1 | 3 | 7.10 | 3.48 |
| 10 | mixed culture 2 | 2 | 1 | 7.10 | 7.56 |
| 11 | mixed culture 2 | 2 | 2 | 7.10 | 7.88 |
| 12 | mixed culture 2 | 2 | 3 | 7.10 | 7.28 |
| | | | | | **6.1 (mean)** |
| Control | control (no cultures) | | | | **7.10 (mean)** |

## 3. Discussion

New strategies are needed to reduce or eliminate DON in feed and food products. DON degrading activity restricted to anaerobic organisms limits the potential use of these microorganisms for industrial purposes as feed additives. Aerobic organisms pose their own problems, as many valuable organisms are unculturable in the lab. Even though culturing aerobic organisms from the environment can be tedious, Shima et al. [21] discovered strain E3-39 that can convert DON into 3-keto-DON under aerobic conditions and He et al. (2016) [25] discovered a microbial culture that converted DON to de-epoxy-DON. Here, we extend these prior studies by isolating mixed microbial cultures from the environment that modify DON, characterizing their DON derivatives, and characterizing the microorganisms present in cultures that modify DON.

Two mixed cultures were identified that consistently decreased DON in cultures in which DON was the sole carbon source in a minimal medium. From these mixed cultures, we were unable to isolate a pure culture that modified DON consistently in culture media. Several bacteria and

fungal colonies were initially selected and screened for DON modification, but only two bacteria eliminated DON from cultures containing DON as the sole carbon source. These two bacteria, an *Achromobacter* and *Pseudomonas* species, were not consistent in eliminating DON from cultures. This inconsistency could be due to the cultures being stored in glycerol stocks at -80°C, since the cold temperatures may have affected their ability to modify DON. Völkl et al. (2004) also were unable to identify a pure organism from the mixed culture (D107) that consistently modified DON [20]. Isolating pure cultures remains a challenge, as multiple microorganisms could be responsible for the metabolism or conversion of DON.

According to Shima el al. [19], 3-keto-DON is significantly less toxic than DON. Proton data from mixed culture 1 and mixed culture 2 were similar to 3-keto-DON reported by Shima et al. [21]. There is a discrepancy in the literature regarding proton data for 3-keto-DON reported by Völkl et al. [20]; the authors appear to have inadvertently switched some of the proton data for DON with the proton data for 3-keto-DON.

Results from 16s rRNA sequencing of mixed culture 1 and mixed culture 2 that consistently modified DON indicated that they contained mostly members of the genera *Acinetobacter*, *Leadbetterella*, and *Gemmata*. To our knowledge, these genera have not been reported previously to modify DON in culture. Strains of *Acinetobacter* have been associated with the modification of ochratoxin A [26]. He et al. reported *Pseudomonas* and *Achromobacter* genera in their microbial culture that converted DON to de-epoxy-DON; the two pure cultures we isolated that demonstrated activity in DON cultures could have lost functionality during storage in glycerol stocks at -80°C. Isolating DON modifying microbes is difficult, in part due to growth and function restrictions since some microbes may be inhibited by others [27]. Several microorganisms are likely responsible for the conversion of DON to 3-keto-DON, and with additional testing and analysis it may be possible to isolate the specific bacteria responsible for DON modification.

DON was nearly eliminated in two naturally contaminated samples of wheat (7.1 μg/mL DON) inoculated with mixed culture 1. GC/MS scans of the two samples showed the appearance of a peak with a similar mass:charge ratio as DON, but different retention times. This was postulated to be the DON metabolite, 3-epi-DON. A reduction of DON was not observed with the samples contaminated with a higher concentration of DON (41 μg/mL). The observed differences in the modification of DON in the assay with DON as the sole carbon source and the assay using naturally contaminated sources of wheat could be attributed to the naturally contaminated sources of wheat cultures containing additional carbon sources for the microbes to utilize. He et al. [28] were able to produce 3-epi-DON with their strain of *Devosia* with different carbon sources such as corn meal broth and a mixture of yeast and glucose. 3-epi-DON may have been produced from our naturally contaminated sources of wheat, since oxidation of 3-keto-DON may be further reduced to produce 3-epi-DON [29].

Our work extends previous studies that have demonstrated the potential to use mixed cultures of microbes to detoxify DON. Future work to assess how microbial assemblages change before, during, and after screening with DON will highlight what microorganisms are selected for under the pressure of DON. Additional work needs to be done to culture specific microorganisms that are unable to grow under the test conditions to greatly increase the probability of identifying an organism that can detoxify DON (e.g., the use of the iChip to identify the new antibiotic allowing scientists to screen for new microbes that are difficult to culture or unculturable with traditional

laboratory practices [30]. However, the transformation of DON to 3-keto-DON and to 3-epi-DON with our mixed cultures demonstrates the feasibility of our approach. Future work aims to elucidate enzymes responsible for modification of DON as Chang et al. did by modifying ochratoxin A using a carboxypeptidase enzyme from the species *Bacillus amyloliquefaciens* ASAG1 [31]. Engineering yeast that express DON-detoxifying enzymes and/or adding purified enzymes that can convert DON to less toxic byproducts will be of value in the fuel ethanol industry, where such strategies could reduce mycotoxins in fuel ethanol by-products destined for feed and food [27].

## 4. Materials and Methods

### *4.1. Field Collections*

Plant and soil samples were collected at Virginia Tech's Kentland Farm in Blacksburg VA, USA on September 13, 2013. Microbial samples were collected from fresh leaves or plant debris, and from soil samples collected with a soil corer (10x1 in. diameter galvanized steel soil sampler, Zoro). Eleven samples were collected from six different collection sites including a field of oat (*Avena sativa*), a field of corn (*Zea mays)*, a landscape plot, a vineyard (*Vitis vinifera*), a peach orchard (*Prunus persica*), and an apple orchard (*Malus domestica*).

### *4.2. Selection of Microbes in the Presence of High Concentrations of DON*

Plant samples were ground into a fine powder using a coffee grinder (Hamilton Beach, Model 80365, Southern Pines, NC). Soil samples were placed in one gallon zip lock bags and mixed thoroughly to displace soil clumps. Aliquots of 0.1 g of each sample were suspended in 1 mL of mineral salt medium (MM) [32] containing 100 μg/mL of DON as the sole carbon source. A negative control included 1 mL MM and 100 μg/mL DON without any environmental samples. Cultures were incubated on a shaker (New Brunswick Scientific Excella E-24 Incubator Shaker, Edison, NJ) for 7 days at 120 RPM and 28 °C. After 7 days, 10 μL of each culture was added to 1 mL of MM and 100 μg/mL of DON and incubated for another 7 days under the same conditions. This process of subculturing and incubation was repeated 4 additional times for a total of six weeks.

Resulting cultures were screened for the disappearance of DON using gas chromatography/ mass spectrometry (GC/MS) following standard protocols [33]. Each sample was diluted by adding 100 μl of culture to 1.9 mL of sterile water before GC/MS analysis; 250 μl of the dilution was added to 1.7 mL of acetonitrile and filtered through Whatman 1 qualitatiave paper. A 1 mL portion of the flow through was dried down in a glass tube using compressed air in a nitrogen evaporator set at 55°C. Dried samples were then derivatized at room temperature with a mixture of 99 μL of N-trimethylsilylimidazole (TMSI) and 1 μL of trimethylchlorosilane (TMCS) for 20 min. Then, 500 μL of isooctane containing 0.5 μg $g^{-1}$ of mirex (Sigma-Aldrich, St. Louis, MO, USA) was added to the glass tube and immediately vortexed followed by adding 500 μL of water to quench the reaction. From the top organic layer, 150 μL was transferred to chromatography vials for GC/MS analysis.

An Agilent 6890/5975 system was used for GC/MS analysis operating in selected ion monitoring (SIM) mode. An autosampler in splitless mode injected 1 μl of each sample onto an HP-5MS column (0.25 mm inner-diameter by 0.25 μm film thickness by 30 m length) to detect DON. The inlet temperature was set at 280°C with a column flow rate of 1.2 mL/min using helium. The

initial column temperature was held at 150 °C for 1 min, increased to 280 °C at a rate of 30 °C/min, and held constant for 3.5 min. A post run of 325 °C for 2.5 min was used to clean the column. DON was detected in SIM mode at a mass:charge ratio of 512.3 and had reference ions at 422.4 and 497.3. Mirex (hexachloropentadiene dimer) was used as an internal standard to check the quantitative precision of the instrument and was detected in SIM mode at a mass:charge ratio of 271.8 and had a reference ion of 275.8 [34]. A linear regression model was used to quantify DON with standards (Romer Labs, Austria and Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 0.05, 0.10, 0.25, 0.5, 1.0, 2.50, 5.0 μg $ml^{-1}$. Mycotoxin values were quantitated using a standard curve ranging from 0.05 to 1.0 μg $mL^{-1}$. Values determined to be greater than 1.0 μg $mL^{-1}$ were quantitated using a curve that included the 2.5 and 5.0 μg $mL^{-1}$ standards. The limit of quantification (LOQ) for the method was determined to be 0.2 μg $mL^{-1}$, based on standard protocols [33]. All cultures showing decreased levels of DON were transferred to 25% glycerol and stored at -80°C.

Each culture that demonstrated decreased levels of DON were then further analyzed 4X (quadruplicate) to show consistency of decreased DON in culture assays with 100 μg/mL DON. A 50 μL sample of the glycerol stocks mentioned above were cultured into four separate tubes of R2A (Reasoner's 2A; a medium for culturing slow-growing microorganisms; Sigma-Aldrich) liquid media for 2 days at 28°C. After incubation, 100 μL of each culture was then added to a new culture tube containing 1 mL MM and 100 μg/mL DON and allowed to incubate with shaking at 120 RPM at 28 °C for 7 days. Mycotoxin extraction and GC/MS analysis described above was used to determine the amount of DON in each sample. All samples were made into a 25% glycerol stock and stored at -80°C for future identification of microbes present.

### *4.3. Isolation of Individual DON Modifying Microbes*

Mixed cultures that resulted in decreased levels of DON were selected and 200 μL of each culture was plated on solid R2A media and incubated for 7 days at 28°C. After incubation, bacterial and fungal colonies of different morphologies were randomly selected and cultured in 1 mL of MM and 100 μg/mL DON for 7 days with shaking at 120 RPM at 28 °C. GC/MS preparation and analysis as described above was used to determine the concentration of DON in each sample after incubation. All pure cultures that demonstrated decreased levels of DON were made into a 25% glycerol stock and stored at -80°C. Individual microbes that demonstrated decreased levels of DON were sequenced using 16s primers (27F and 518R bacterial 16s ribosomal primers) at the Biocomplexity Institute at Virginia Tech.

### *4.4. Identification of Mixed Cultures Using 16s Ribosomal Sequencing*

To assist in the identification of the microorganisms present in mixed cultures, 100 μL of frozen stock from the mineral media cultures with 100 μg/mL DON was incubated in 2 mL of R2A liquid media for 2 days at 28°C. DNA from the mixed cultures was purified using a Thermo Scientific KingFisher mL nucleic acid purification machine and a Qiagen Puregene Yeast/Bac kit B. Samples of 40 ng/μl suspended in water were sent to MR DNA Laboratory in Shallowwater, Texas for 16S sequencing using the 27F primer. Illumina sequencing technology was used to generate an average of 20K reads.

*4.5. Thin Layer Chromatography to Identify DON Derivatives*

Thin Layer Chromatography (TLC) was used to detect new DON products in mixed culture extracts (GC/MS analysis of TMS derivatized samples run in SIM mode was used to measure the disappearance of DON). Samples were dried down using compressed air in the fume hood then 200 μL of acetonitrile was added and vortexed to ensure the DON derivatives were dissolved. Each sample was spotted 1 inch from the bottom of a 20x20 cm silica gel plate (60-F254, Millipore, Darmstadt, Germany). The plate was placed in a TLC tank (LxHxW 27.10 cm x 26.5 cm x 7.10 cm) using 48:92:10 hexane/ethyl acetate/methanol as the solvent. The solvent was allowed to run to approximately 3cm away from the top of the plate. The plate was dried in a fume hood, and then sprayed with NBP (nitrobenzylpyridine), heated for 30 min at 100°C, and then lightly sprayed with TEPA (tetraethylenepentamine). Products that contained an epoxide group were stained blue [35].

*4.6. Nuclear Magnetic Resonance to Identify Structure of DON derivatives*

In order to obtain sufficient product for nuclear Magnetic Resonance (NMR) analysis, samples were assayed in 10 mL of MM containing 100 μg/mL DON to produce enough byproduct, incubated for one week with shaking at 28°C, and were subsequently dried down using air. The residue was dissolved in 200 μL of acetonitrile and streaked on TLC plates as described above. Bands were visualized under UV light, marked with a pencil, and scraped off using a razor blade and placed in a glass vial. Deuterated chloroform was added to each vial and vortexed. $^{1}$H NMR spectra were recorded on a Bruker Avance II (500Mhz) equipped with a Prodigy cryoprobe. Chemical shifts were referenced to residual proton signal of theCDCl3 solvent. MNova 11 was used to analyze the $^{1}$H data.

*4.7. DON Assays with Mixed Cultures with Naturally Contaminated Wheat*

Two wheat (*Triticum aestivum*) samples naturally infected by *Fusarium graminearum* and containing different concentrations of DON were used: sample #13v-193 (7.1 μg/mL DON) and sample #13w-7 (41.0 μg/mL DON). GC/MS methods were used to determine the concentrations of DON present in the samples. Samples were ground to a fine powder using a Stein mill (Steinlite Corp).

To prepare for the assays, 50 μL of mixed culture was added to 2 mL of R2A liquid media and allowed to incubate for 2 days at 120 RPM and 28°C. A 1.0 g sub-sample of each wheat sample was added to a 125 mL flask topped with a foam stopper (21-26mm) and autoclaved on a dry cycle. Under sterile conditions, 4.5 mL of sterile water was added to each flask and 500 μL of each mixed culture was added to three flasks of each wheat sample, sample #13v-193 and sample #13w-7. Negative controls for each wheat sample were included without any mixed culture and only 5 mL of sterile water. All flasks were incubated for one week at 180 RPM and 28°C. To analyze DON concentration after incubation, each sample was dried down in an oven set at 55°C. Each 1.0 g sample was combined with 8 mL of an 84% (v/v) acetonitrile in DI water to extract DON, sonicated to release any clumps, then placed on a shaker at 200 RPM overnight at room temperature. The solvent was then cleaned by passing it through a column consisting of a 1:3 ratio of a 1.5 g mixture of C18 (40 um particle size) and aluminum oxide (active, neutral, 0.063 to 0.200 mm particle size range). An aliquot of 1 mL of the eluent was added to a glass test tube, dried and evaporated using a nitrogen evaporator set at 55 °C. Derivatization of samples was performed as described above and

GC/MS analysis was performed operating in scan mode analyzing from 5.7 to 8.8 minutes; all other parameters of the GC/MS method was kept the same as described above.

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**Author Contributions:** N.W. and D.S. planned, coordinated, and conducted most of experiments and coordinated the writing of the manuscript. D.G. and C.S. conducted field and laboratory work to culture and identify DON-modifying microbes. K.K. conducted NMR assays and N.M. conducted GC/MS assays. S.M. provided materials for the work, and contributed to TLC methods. D.S. and R.S. provided oversight of the project. N.W. and D.S. wrote the manuscript, and all authors contributed edits to the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Sobrova P, Adam V, Vasatkova A, Beklova M, Zeman L, Kizek R. Deoxynivalenol and its toxicity. *Interdiscip Toxicol*. 2010;3:94-99.
2. Placinta C., D'Mello JP., Macdonald AM. A review of worldwide contamination of cereal grains and animal feed with Fusarium mycotoxins. *Anim Feed Sci Technol*. 1999;78(1-2):21-37.
3. FAO. *The Global Forum for Food Security and Nutrition*. 2013.
4. Zain ME. Impact of mycotoxins on humans and animals. *J Saudi Chem Soc*. 2011;15(2):129-144.
5. Fink-Grernmels J. Mycotoxins: Their implications for human and animal health. *Vet Q*. 1999;21(4):115-120.
6. Pier AC. Major biological consequences of aflatoxicosis in animal production. *J Anim Sci*. 1992;70:3964-3967.
7. Pohland AE. Mycotoxins in review. *Food Addit Contam*. 1993;10(1):17-28.
8. P M S. TRICHOTHECENES IN GRAINS. *Cereal Foods World*. 1990;35:661-666.
9. Desjardins AE. *Fusarium Mycotoxins: Chemistry, Genetics and Biology*. APS Press; 2006. https://books.google.com/books?id=MYBFAQAAIAAJ.
10. Schmale DG, Munkvold G. Mycotoxins in crops: A threat to human and domestic animal health. *Plant Heal Instr*. 2009.
11. Tanaka T, Hasegawa A, Yamamoto S, Lee US, Sugiura Y, Ueno Y. Worldwide contamination of cereals by the Fusarium mycotoxins nivalenol, deoxynivalenol, and zearalenone. 1. Survey of 19 countries. *J Agric Food Chem*. 1988;36(5):979-983.
12. Pestka JJ. Deoxynivalenol: Mechanisms of action, human exposure, and toxicological relevance. *Arch Toxicol*. 2010;84:663-679.
13. Nganje WE, Kaitibie S, Wilson WW, Leistritz FL, Bangsund DA. *Economic Impacts of Fusarium Head Blight in Wheat and Barley: 1993-2001*. Department of Agribusiness and Applied Economics, Agricultural Experiment Station, North Dakota State University; 2004.

14. Sato N, Ueno A (1977) Comparative toxicities of trichothecenes. In: Rodricks JV, Hesseltine CW, Mehlman MA (eds) Mycotoxins in human and animal health. Pathotox Publishers, Park Forest South, IL, pp 295–307
15. Ueno Y, Nakajima M, Sakai K, Ishii K, Sato N. Comparative toxicology of trichothec mycotoxins: inhibition of protein synthesis in animal cells. *J Biochem*. 1973;74:285-296.
16. Kimura M, Tokai T, Takahashi-Ando N, Ohsato S, Fujimura M. Molecular and genetic studies of fusarium trichothecene biosynthesis: pathways, genes, and evolution. *Biosci Biotechnol Biochem*. 2007;71:2105-2123.
17. McCormick SP. Microbial Detoxification of Mycotoxins. *J Chem Ecol*. 2013;39:907-918.
18. Zhou, T., J. He, and J. Gong. "Microbial transformation of trichothecene mycotoxins." *World Mycotoxin Journal* 1.1 (2008): 23-30.
19. Fuchs E, Binder EM, Heidler D, Krska R. Structural characterization of metabolites after the microbial degradation of type A trichothecenes by the bacterial strain BBSH 797. *Food Addit Contam*. 2002;19(4):379-386.
20. Völkl A, Vogler B, Schollenberger M, Karlovsky P. Microbial detoxification of mycotoxin deoxynivalenol. *J Basic Microbiol*. 2004;44(2):147-156.
21. Shima J, Takase S, Takahashi Y, et al. Novel detoxification of the trichothecene mycotoxin deoxynivalenol by a soil bacterium isolated by enrichment culture. *Appl Environ Microbiol*. 1997;63:3825-3830.
22. He, J. W., Bondy, G. S., Zhou, T., Caldwell, D., Boland, G. J., & Scott, P. M. (2015). Toxicology of 3-epi-deoxynivalenol, a deoxynivalenol-transformation product by Devosia mutans 17-2-E-8. *Food and Chemical Toxicology*, *84*, 250-259.
23. He, Jian Wei, et al. "An epimer of deoxynivalenol: purification and structure identification of 3-epi-deoxynivalenol." *Food Additives & Contaminants: Part A* 32.9 (2015): 1523-1530.
24. Ikunaga, Y., Sato, I., Grond, S., Numaziri, N., Yoshida, S., Yamaya, H., ... & Ito, M. (2011). Nocardioides sp. strain WSN05-2, isolated from a wheat field, degrades deoxynivalenol, producing the novel intermediate 3-epi-deoxynivalenol. *Applied microbiology and biotechnology*, *89*(2), 419-427.
25. He, Wei-Jie, et al. "Aerobic De-Epoxydation of Trichothecene Mycotoxins by a Soil Bacterial Consortium Isolated Using In Situ Soil Enrichment." *Toxins* 8.10 (2016): 277.
26. De Bellis, Palmira et al. Biodegradation of Ochratoxin A by Bacterial Strains Isolated from Vineyard Soils. Ed. Annie Pfohl-Leszkowicz. *Toxins* 7.12 (2015): 5079–5093. *PMC*. Web. 11 Dec. 2016.
27. Yu, H., Zhou, T., Gong, J. H., Young, C., Su, X. J., Li, X-Z., Zhu, H. H., Tsao, R., and Yang, R. 2010. Isolation of deoxynivalenol-transforming bacteria from the chicken intestines using the approach of PCR-DGGE guided microbial selection. BMC Microbiology 10: 182-191.
28. He, J. W., Hassan, Y. I., Perilla, N., Li, X. Z., Boland, G. J., & Zhou, T. (2016). Bacterial epimerization as a route for deoxynivalenol detoxification: the influence of growth and environmental conditions. *Frontiers in microbiology*, *7*.
29. Karlovsky, P. (2011). Biological detoxification of the mycotoxin deoxynivalenol and its use in genetically engineered crops and feed additives. *Applied microbiology and biotechnology*, *91*(3), 491-504.
30. Piddock, Laura JV. "Teixobactin, the first of a new class of antibiotics discovered by iChip technology?." Journal of Antimicrobial Chemotherapy 70.10 (2015): 2679-2680.
31. Chang, X., Wu, Z., Wu, S., Dai, Y., & Sun, C. (2015). Degradation of ochratoxin A by Bacillus amyloliquefaciens ASAG1. *Food Additives & Contaminants: Part A*, *32*(4), 564-571.
32. Sato I, Ito M, Ishizaka M, et al. Thirteen novel deoxynivalenol-degrading bacteria are classified within two genera with distinct degradation mechanisms. *FEMS Microbiol Lett*. 2012;327(2):110-117.

33. Khatibi PA, McMaster NJ, Musser R, Schmale DG. Survey of mycotoxins in corn distillers' dried grains with solubles from seventy-eight ethanol plants in Twelve states in the U.S. in 2011. *Toxins (Basel)*. 2014;6:1155-1168.
34. Mirocha, Chester J., et al. "Analysis of deoxynivalenol and its derivatives (batch and single kernel) using gas chromatography/mass spectrometry." *Journal of Agricultural and Food Chemistry* 46.4 (1998): 1414-1418.
35. Takitani S, Asabe Y, Kato T, Suzuki M, Ueno Y. Spectrodensitometric determination of trichothecene mycotoxins with 4-(p-nitrobenzyl)pyridine on silica gel thin-layer chromatograms. *J Chromatogr A*. 1979;172(1):335-342.

Supplementary Materials:

**Table S1.** Mixed and pure microbial samples, derived from either soil or plant material, that initially eliminated DON from cultures containing mineral media and 100 μg/mL of DON as the sole carbon source (GC/MS analysis indicated a value below the limit of quantification). Not all cultures were consistent at modifying DON in repeated assays. The percent of time each culture eliminated DON from the culture medium was based on how many times each culture eliminated DON from the culture over how many times each culture was assayed. Both mixed culture 1 and mixed culture 2 will modify DON in culture material 77% of the time they are assayed.

| **Sample** | **Collection Area** | **Mixed/ Pure Culture** | **Collection Method** | **% Time DON Eliminated from Culture** | **DON (μg/mL), Initial Screen** | |
|---|---|---|---|---|---|---|
| | | | | | **Rep 1** | **Rep 2** |
| Mixed culture 1 | Soil- Landscape Plot | Mixed | Soil Corer | 77 | <0.20 | <0.20 |
| Mixed culture 2 | Soil- Landscape Plot | Mixed | Soil Corer | 77 | <0.20 | <0.20 |
| Mixed culture 3 | Soil- Landscape Plot | Mixed | Soil Corer | 37 | <0.20 | <0.20 |
| Pure culture 1 | Plant- Small Grain Field | Pure - *Achromobacter* | Leaf material & debris | 33 | <0.20 | <0.20 |
| Pure culture 2 | Soil- Landscape Plot | Pure – *Pseudomonas* | Soil Corer | 50 | <0.20 | <0.20 |

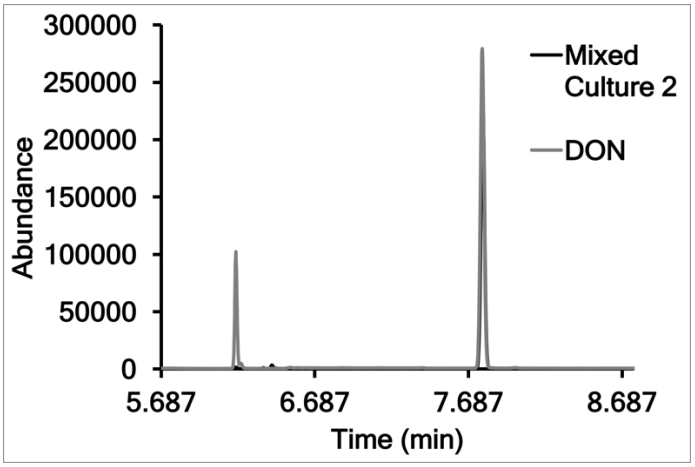


**Figure S1.** GC/MS chromatogram of mixed culture 2 (7.7 min; detected in SIM mode with a target ion with a mass:charge ratio of 438.2 and reference ions at 318.2 and 303.1) after incubation in mineral media with 100 μg/mL of DON as the sole carbon source. DON is represented by the peak at 6.1 min. The DON incubated with mixed culture 2 was below the limit of detection of 0.20 μg/mL.

**Table S2.** Proton data collected with a Bruker, Avance II, 500 MHz NMR for mixed culture 1 and DON. Proton data for 3-keto-DON was produced by Shima et al. [19]. Comparison of proton data for mixed culture 1 products with DON and 3-keto-DON confirm that mixed culture 1 contained 3-keto-DON. Proton data for mixed culture 2 was similar to mixed culture 1 (data not reported).

| Position | Mixed culture 1 | DON | 3-keto-DON |
|---|---|---|---|
| 1 | | | |
| 2 | 3.52 (s) | 3.65 (d, 4.48) | 3.52 (s) |
| 3 | | 4.56 (td, 4.55, 11) | |
| | 3.14 (d, 19.26), | 2.23 (dd, 14.63, 4.07), | 3.13 (d, 19), |
| 4 | 2.27 (br d, 19.30) | 2.11 (dd, 15, 11) | 2.28 (br d, 19) |
| 5 | | | |
| 6 | | | |
| 7 | 4.91 (br s) | 4,86 (br s) | 4.90 (br s) |
| 8 | | | |
| 9 | | | |
| 10 | 6.54 (br d, 5.92) | 6.63 (br d, 6.02) | 6.55 (br d, 6) |
| 11 | 4.58 (br d, 5.93) | 4. 83 (d, 5.89) | 4.57 (br d, 6) |
| 12 | | | |
| | 3.35 (d, 4.24), | 3.18 (d, 4.31), | 3.35 (d, 4), |
| 13 | 3.23 (d, 4.21) | 3.09 (d, 4) | 3.22 (d, 4) |
| 14 | 1.33 (3H, s) | 1.16 (3H, s) | 1.33 (3H, s) |
| | 3.90 (d, 11.72), | 3.92 (d, 12.25), | 3.90 (d, 12), |
| 15 | 3.73 (d, 12.07) | 3.76 (d, 12.25) | 3.74 (d, 12) |
| 16 | 1.90 (3H, b s) | 1.91 (3H, br s) | 1.90 (3H, br s) |

## CHAPTER III

# Modification of the Trichothecence Mycotoxin Deoxynivalenol Using a Cell Free Protein Synthesis System

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## Abstract

**Aim:** Engineer a cell-free protein synthesis (CFPS) system to express acetyltransferase enzymes (FsTRI101, FgTRI101, and FoTRI201) to modify the trichothecene mycotoxin deoxynivalenol (DON).

**Methods and Results:** When FsTRI101, FgTRI101, and FoTRI201 were expressed in the CFPSE system in the presence of 40 μg $mL^{-1}$ DON, the enzymes converted nearly all of DON (40 μg $mL^{-1}$) to 3-acetyl-deoxynivalenol (3-A-DON) within 30 minutes. Western blots confirmed the presence of all three proteins produced through the CFPS system. Two enzymes, FgTRI101 and FoTRI201, produced using the CFPS system were incubated at five temperatures (30°C, 40.3°C, 49.5°C, 60.5°C, and 69.5°C) and then exposed to 40 μg $mL^{-1}$ DON for 30 min. Results indicated that FgTRI101 and FoTRI201 were still able to convert DON to 3-A-DON at 30°C and 40.3°C, but not at the higher temperatures.

**Conclusions:** Three acetyltransferase genes expressed in the CFPS system converted DON to 3-A-DON. The acetyltransferases tested have limited activity beyond about 40°C.

**Significance and Impact of the Study:** This work highlights the potential application of a CFPS system to test for enzymes that will modify DON and other mycotoxins that threaten the health of humans and domestic animals. CFPS systems could be used to rapidly screen for functional enzymes (e.g., epoxide hydrolases, cyloisomerases, etc.) that can detoxify DON and be used to study other substrate/protein interactions.

Keywords: deoxynivalenol, cell free protein synthesis, 3-acetyl-deoxynivalenol, enzymes, detoxification

## Introduction

Deoxynivalenol (DON) is a toxic compound produced by the fungal species *Fusarium* causing harm to humans and animals when consumed (Da Rocha et al. 2014). DON inhibits protein synthesis by disrupting peptidyl transferase therefore interfering with initiation and elongation of polypeptides (Ueno et al. 1973; Shifrin and Anderson 1999; Pestka 2010). It is understood that the epoxide ring located on the 12, 13 carbons is responsible for DONs toxicity (Ueno et al 1973). Current DON mitigation technology primarily relies on traditional breeding strategies to introduce resistant or tolerant varieties of crops (Khatibi et al. 2012; Berger et al. 2014). Studies have reported discoveries of DON-modifying microorganisms, yet most of these microorganisms are limited to oxidation or epimerization reactions (McCormick 2013). Targeting the epoxide group is considered a feasible strategy to effectively eliminate DON toxicity (He et al. 2010; Karlovsky 2011). Abolmaali et al. (2008) generated DON sensitive yeast strains as a bioassay indicator to screen for DON degrading microbes; however, there is no technology to screen for functional enzymes that detoxify DON (Abolmaali et al. 2008).

Cell-free protein synthesis (CFPS) systems can be used to generate a protein of interest without the need for using living cells. CFPS was used in 1961 to elucidate the genetic code in *E. coli*, but more recently CFPS has been used to generate protein for protein evolution and structural genomic studies (Nirenber and Matthaei 1961; Madin et al. 2000; Katzen et al. 2005; Takai et al. 2010). CFPS technology has many potential applications such as studying and understanding virus-like particles, membrane proteins, and protein evolution and most simply to generate large quantities of protein (Carlson et al. 2012). The components of a CFPS system consist of cell lysate containing ribosomes and aminoacyl-tRNA synthases among other important factors for initiation and elongation of proteins (Carlson et al. 2012). Energy and nucleotide building-blocks in the form of NTPs as well as a DNA (or mRNA) template are added to the cell lysate and incubated until the energy source is utilized completely (Carlson et al. 2012).

In the current study, a CFPS system was engineered to express acetyltransferase enzymes (FsTRI101, FgTRI101, and FoTRI201) to modify DON. It was hypothesized that acetyltransferase genes, *TRI101* and *TRI201*, expressed in a CFPS system in the presence of DON would modify DON to 3-acetyl-deoxynivalenol (3-A-DON). The specific objectives of the study were to: (1) express acetyltransferase genes in a CFPS system in the presence of high

concentrations of DON, (2) express acetyltransferase genes in a CFPS system in the absence of DON; and (3) determine the activity of acetyltransferase enzymes produced in the CFPS system at increasing temperatures. This work highlights the potential application of a CFPS system to test for enzymes that will modify DON and other mycotoxins that threaten the health of humans and domestic animals.

## Materials and Methods

### Gene isolation and plasmid preparation

Acetyltransferase genes, *TRI101* and *TRI201*, were isolated from genomic DNA of the appropriate *Fusarium* species using primers as described in Khatibi et al. (2011) Khatibi et al. 2011). *TRI101* genes were obtained from *F. sporotrichioides* (FsTRI101) and *F. graminearum* (FgTRI101), and *TRI201* genes were obtained from *F. oxysporum* (FoTRI201) and *F. fujikuroi* (FfTRI201). In order to clone the TRI genes into a plasmid, XhoI-HF was used as the forward restriction cut site (except for FfTRI201, NcoI was used as the forward restriction cut site) and Bam-HI-HF as the reverse restriction cut site. PCR amplifications were carried out in 30 μl reactions using the high fidelity Q5 2X master mix (New England Biolabs, Ipswich, MA) with the following PCR parameters: one cycle at 95°C for 2 min; 30 cycles of 95°C for 40 s, 54°C for 30 s, and 72°C for 1 min; and finally one cycle at 72°C for 7 min.

Genes *TRI101* and *TRI201* were cloned into the rapid translation system plasmid pIVEX 2.4c (Roche Molecular Biochemicals, Pleasanton, CA) using the designated restriction enzymes and transformed into TOP10 chemically competent cells (Thermo Fisher Scientific) to generate high quality and quantity plasmid and purified using a GeneJET plasmid miniprep kit (Thermo Fisher Scientific). Plasmid purifications were stored at -20°C until use.

### Cell-free protein synthesis system

The CFPS lysate from BL21 *E. coli* cells and energy buffer were used as described in Swartz et al. (2004) and Jewett et al. (2004). Each reaction had a total volume of 101 μl consisting of 48 μl cell lysate, 40 μl energy buffer, 10 μl of plasmid (390 ng $\mu l^{-1}$ FsTRI101, 303 ng $\mu l^{-1}$ FgTRI101, and 288 ng $\mu l^{-1}$ FoTRI201), 4 μl of 1000X DON, and 1 μl of mRNA inhibitor creating a 40 μg $mL^{-1}$ reaction of DON. Each *TRI101* and *TRI201* was run in triplicate along with a positive green

fluorescent plasmid (GFP) control and a negative control containing no plasmid. All samples were then incubated for 3 hours at 500 rpm at 37°C. After incubation, 30 μl of each sample was dried down in a test tube to prepare for gas chromatography mass spectrometry (GC/MS).
For the CFPS and temperature gradient assays without DON, reactions were run with 4 μl of water to replace the volume of DON. A 32 μl reaction was used to perform the assay with DON added after expression of the acetyltransferase genes. A sample of 30 μl of the CFPS system of each *TRI101* and *TRI201* triplicate was taken and added to 40 μg $mL^{-1}$ of DON and placed in an incubator (New Brunswick Scientific Excella E-24 Incubator Shaker, Edison, NJ) at 30°C at 220 rpm for 30 min, 1 hr, and 2 hr. After the allotted duration of time, the samples were taken from the incubator and dried down to prepare for GC/MS analysis.

**Temperature gradient assay**

A 32 μl reaction was used to perform the temperature gradient assay. A 10 μl sample of each FgTRI101 and FoTRI201 experimental triplicate was pooled and placed in a thermocycler (Eppendorf Mastercycler ep Gradient S) set at 30°C, 40.3°C, 49.5°C, 60.5°C, and 69.5°C and incubated for 30 min. Then, 40 μg $mL^{-1}$ DON was added to each reaction and incubated for another 30 min. The samples were then immediately dried down in a test tube to prepare for GC/MS analysis.

**Gas chromatography mass spectrometry (GC/MS) analysis**

Each sample was dried down in a glass test tube using compressed air in an evaporator set at 55°C and then derivatized with a mixture of 99 μL of N-trimethylsilylimidazole (TMSI) and 1 μL of trimethylchlorosilane (TMCS) at room temperature for 20 min. Then, 300 μl of isooctane containing 0.5 μg $g^{-1}$ of mirex (Sigma-Aldrich, St. Louis, MO, USA; an internal standard to measure the precision of the GC/MS) was added to the glass tube and immediately vortexed. Then, 300 μl of deionized water was added to quench the reaction. A150 μl sample was taken from the top organic layer and transferred to chromatography vials for GC/MS analysis.

An Agilent 6890/5975 GC/MS (Santa Clara, CA) was used in selected ion monitoring (SIM) mode to detect DON and 3-A-DON. An HP-5MS column (0.25 mm inner-diameter by 0.25 μm film thickness by 30 m length) was loaded with 1 μl of each sample using an autosampler in splitless mode. The inlet temperature was set at 280°C and the column flow rate

was 1.2 mL/min using Helium as the carrier gas. The initial column temperature was held for 1 min at 150 °C, increased to 280 °C at a rate of 30 °C/min, and held constant for 3.5 min, followed by a post run of 325 °C for 2.5 min. The SIM mode detected DON and 3-A-DON with target ions at a mass:charge ratio of 512.3 and 392.2, respectively, with reference ions at 422.4 and 497.3 for DON and reference ions at 482.2 for 3-A-DON. The SIM mode detected mirex at a mass:charge ratio of 271.8 and had a reference ion of 275.8. DON and 3-A-DON were quantified using a linear regression model using standards (Romer Labs, Austria and Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 0.05, 0.10, 0.25, 0.5, 1.0, 2.50, 5.0 μg/ml$^{-1}$. A standard curve ranging from 0.05 to 1.0 μg mL$^{-1}$ was used to quantitate the values. If a value was greater than 1.0 μg mL$^{-1}$, a standard curve was generated to include the 2.5 and 5.0 μg mL$^{-1}$ standards. The method had a limit of detection of 0.05 μg mL$^{-1}$.

**Western blots**

For western analysis, 30 ul from the CFPS system incubated with and without DON was added to 12 μl 5X concentration loading buffer and 18 μl of water, boiled for 10 min, and then centrifuged for 1 min at maximum speed. A 12% SDS-PAGE gel was loaded with 15 μl of each reaction and run for 20 min at 140V in 1X SDS buffer in an electrophoresis chamber (BIO-RAD, Hercules, CA). The protein gel was then transferred to a 0.2 μm nitrocellulose membrane and run in the electrophoresis chamber with an ice block at 200 mA in 1X transfer buffer for 40 min. The membrane was then placed in a glass petri dish with Superblock blocking buffer in TBS (Thermo Fisher, Waltham, MA) and allowed to incubate for 2 hrs at 30°C with light shaking. The membrane was quickly rinsed with TBST. The primary antibody (3:10000 dilution), consisting of 9 mL of TBST, 1 mL of Superblock blocking buffer, and 3 μl of 6X anti-His mouse antibody (Thermo Fisher), was added on top of the membrane and placed in a 4°C overnight. The membrane was then washed 3 times for 15 min each with TBST. Then the secondary antibody (2.5:10000 dilution), consisting of 9 mL TBST buffer, 1mL blocking buffer, and 2.5 μl anti-mouse goat antibody with a horseradish peroxidase (HRP) conjugate (Thermo Fisher), was added on top of the membrane and incubated for 2 hrs at 30°C with light shaking. The membrane was washed 3 times in TBST for 15 min each. Clarity western ECL blotting substrate (BIO-RAD) was added to the top of the membrane for 1 min and visualized with a ChemiDoc MP Imaging System (BIO-RAD) on chemiluminescence setting.

## Results

### Cell free protein synthesis in the presence of DON

In the CFPS system, FsTRI101, FgTRI101, and FoTRI201 were expressed in the presence of 40 μg/ml DON. Based on GC/MS data, FsTRI101, FgTRI101, and FoTRI201 were able to convert DON to the less toxic metabolite 3-A-DON. The FfTRI201 enzyme did not convert DON to 3-A-DON and is not included in the data. Figure 1 illustrates the percent conversion of DON to 3–A-DON. FsTRI101 had the highest percent conversion with 97% of the DON being converted to 3-A-DON. FgTRI101 and FoTRI201 converted 95% of DON to 3-A-DON in the CFPS system in the presence of DON. Both the GFP plasmid control and negative control converted 1% of DON. In the CFPS system, FsTRI101 converted DON to 3-ADON (Figure 2) while the negative control, lacking any plasmid, did not convert any DON.

### Cell free protein synthesis in the absence of DON

The FsTRI101, FgTRI101, and FoTRI201 enzymes expressed in the absence of DON were all able to convert DON to the less toxic metabolite 3-A-DON in 30 min, 1 hr, and 2 hrs. Figure 3 is an illustration of percent DON converted to 3-A-DON after incubating FsTRI101, FgTRI101 and FoTRI201 with 40 μg $mL^{-1}$ DON for 30 min. FsTRI101, FgTRI101 and FoTRI201 all converted 94% of DON to 3-A-DON in 30 min. FfTRI201 did not convert DON to 3-A-DON and is not included in the data. Both the GFP plasmid control and negative control containing no plasmid converted 1.5% of DON.

### Temperature gradient assay

The FgTRI101 and FoTRI201 enzymes were all able to convert DON to the less toxic metabolite 3-A-DON at 30°C and 40.3°C, but not at the higher temperatures tested (49.5°C, 60.5°C, and 69.5°C) (Figure 4). FgTRI101 and FoTRI201 converted 90% of DON at 30°C and 86% of DON at 40.3°C. Conversion at higher temperatures was less than 1%. The negative control GFP plasmid consistently converted 1% or less of DON at all temperatures tested.

### Western blots

Western blots produced from FsTRI101, FgTRI101, and FoTRI201 enzymes expressed in the presence of 40 μg/ml DON and FsTRI101, FgTRI101, and FoTRI201 expressed without DON in the CFPS system (Figure 5). The molecular weight of FsTRI101, FgTRI101, and FoTRI201 is 50 kDa and the GFP is 25 kDa. Under both CFPS systems, with and without DON in the system, the western blots confirmed the expression of FsTRI101, FgTRI101, and FoTRI201.

## Discussion

New strategies are needed to mitigate mycotoxin contamination in feed and food products. A CFPS system was engineered to express acetyltransferases to modify the mycotoxin DON. The CFPS system offers several advantages over conventional cell-based protein expression methods as a time saving system since it produces proteins directly from a PCR fragment or an mRNA template without the need for molecular cloning, and bypasses time-consuming cell culturing (Endo and Sawasaki 2006). The CFPS system also achieves arguably the highest yields for numerous proteins, from hundreds of micrograms per milliliter to milligrams per milliliter in a batch reaction. As an open system, cell-free environment allows direct control over the transcription, translation in a non-physiological manner which is difficult to establish in vivo such as pH, redox potential and cytotoxicity additives (Ceres and Zlotnick 2002). In the current study, all acetyltransferase genes were expressed in the presence of 40 $\mu g\ mL^{-1}$ DON and without DON. FsTRI101, FgTRI101, and FoTRI201 converted DON to 3-A-DON in the CFPS system under both conditions. With these data, the CFPS system has potential to be used as a testing tool to screen for active, functional enzymes that will detoxify DON. This technology is ideal for screening microbial DNA fragments or modified enzymes.

In the first experiment, FsTRI101, FgTRI101, and FoTRI201 were all able to convert DON to 3-A-DON while the protein was being expressed in the presence of 40 $\mu g\ mL^{-1}$ DON. Salehi et al. (2017) used the CFPS system to create a biosensor to detect endocrine disrupting chemicals. With this idea in mind, the CFPS system has the potential to rapidly screen for enzymes that will detoxify DON. In the second experiment, FsTRI101, FgTRI101, and FoTRI201 were all able to convert DON to 3-A-DON within 30 min. All acetyltransferase genes were expressed in the CFPS system first and then added to 40 $\mu g\ mL^{-1}$ DON. This system has the potential to be added to Dried Distillers Grains with Solubles (DDGS) material, a co-product of grain ethanol fermentation, to detoxify DON (Emiola et al. 2009). The addition of enzymes to DDGS is not a new concept. Research has shown that the addition of a non-starch degrading enzyme to DDGS material allows for better digestion in broiler chickens (Campasino et al. 2015).

FgTRI101 and FoTRI201 were both able to convert DON to the less toxic metabolite 3-A-DON at 30°C and 40.3°C, but not at higher temperatures. Previous studies reported thermal

melting temperatures ($T_m$) of 39.2 for FgTRI101 and $T_m$ of 38.2 for FoTRI201 (Khatibi et al. 2011). In the future, these enzymes could be engineered to be stable at higher temperatures, such as those encountered during fermentation (Shaw and Bott 1996).

The CFPS system can also be used to understand enzyme kinetics. In Khatibi et al. (2011), kinetic studies were performed in yeast with the *TRI101* and *TRI201* enzymes. Results from those kinetic assays showed that it took 15-24 hours to reach 90% conversion of DON when the enzymes were present in yeast. Our results with the CFPS system showed that it took 30 min or less to get near complete conversion of DON to 3-A-DON. The CFPS system could be used to get an improved understanding of the 'true' kinetic activity of enzymes.

Though CFPS is a powerful tool that has tremendous potential to understand substrate/protein interactions, there may be some limitations. First, we speculate that some DON is binding to ribosomes in the CFPS system and; thus, is unable to be quantified using GC/MS (Shifrin and Anderson 1999). Second, the expression of some enzymes may require certain co-factors or a certain pH for protein expression (Katzen et al. 2005; Sasaki et al. 2011; Welsh et al. 2011; Zawada et al. 2011). This quick and easy technology can go beyond screening for enzymes to detoxify DON or other mycotoxins, and, it could be used to shed light on a variety of substrate/protein interactions.

## Acknowledgements

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## Conflict of Interest

The authors declare no conflict of interest.

## References


Abolmaali, S., Mitterbauer, R., Spadiut, O., Peruci, M., Weindorfer, H., Lucyshyn, D., Ellersdorfer, G., Lemmens, M., Moll, W.D. and Adam, G. (2008) Engineered bakers yeast as a sensitive bioassay indicator organism for the trichothecene toxin deoxynivalenol. *Journal of Microbiological Methods* **72**, 306-312.

Berger, G., Green, A., Khatibi, P., Brooks, W., Rosso, L., Liu, S., Chao, S., Griffey, C. and Schmale III, D. (2014) Characterization of Fusarium head blight resistance and deoxynivalenol accumulation in hulled and hulless winter barley. *Plant Disease* **98**, 599-606.

Campasino, A., Williams, M., Latham, R., Bailey, C.A., Brown, B. and Lee, J.T. (2015) Effects of increasing dried distillers' grains with solubles and non-starch polysaccharide degrading enzyme inclusion on growth performance and energy digestibility in broilers. *The Journal of Applied Poultry Research* **24**, 135-144.

Carlson, E.D., Gan, R., Hodgman, C.E. and Jewett, M.C. (2012) Cell-free protein synthesis: applications come of age. *Biotechnology Advances* **30**, 1185-1194.

Ceres, P. and Zlotnick, A. (2002). Weak protein− protein interactions are sufficient to drive assembly of hepatitis B virus capsids. *Biochemistry* **41**, 11525-11531.

Da Rocha, M.E.B., Freire, F.D.C.O., Maia, F.E.F., Guedes, M.I.F. and Rondina, D. (2014) Mycotoxins and their effects on human and animal health. *Food Control* **3**6, 159-165.

Emiola, I.A., Opapeju, F.O., Slominski, B.A. and Nyachoti, C.M. (2009) Growth performance and nutrient digestibility in pigs fed wheat distillers dried grains with solubles-based diets supplemented with a multicarbohydrase enzyme. *Journal of Animal Science* **87**, 2315-2322.

Endo, Y. and Sawasaki, T. (2006) Cell-free expression systems for eukaryotic protein production. *Current Opinion in Biotechnology* **17**, 373-380.

He, J., Zhou, T., Young, J.C., Boland, G.J. and Scott, P.M. (2010) Chemical and biological transformations for detoxification of trichothecene mycotoxins in human and animal food chains: a review. *Trends in Food Science & Technology* **21**, 67-76.

Jewett, M.C. and Swartz, J.R. (2004). Mimicking the Escherichia coli cytoplasmic environment activates long-lived and efficient cell-free protein synthesis. *Biotechnology and Bioengineering* **86**, 19-26.

Karlovsky, P. (2011). Biological detoxification of the mycotoxin deoxynivalenol and its use in genetically engineered crops and feed additives. *Applied Microbiology and Biotechnology* **91**, 491-504.

Katzen, F., Chang, G. and Kudlicki, W. (2005) The past, present and future of cell-free protein synthesis. *Trends in Biotechnology* **23**, 150-156.

Khatibi, P.A., Berger, G., Liu, S., Brooks, W.S., Griffey, C.A. and Schmale III, D.G. (2012) Resistance to Fusarium head blight and deoxynivalenol accumulation in Virginia barley. *Plant Disease* **96**, 279-284.

Khatibi, P.A., Newmister, S.A., Rayment, I., McCormick, S.P., Alexander, N.J. and Schmale, D.G. (2011) Bioprospecting for trichothecene 3-O-acetyltransferases in the fungal genus Fusarium yields functional enzymes with different abilities to modify the mycotoxin deoxynivalenol. *Applied and Environmental Microbiology* **77**, 1162-1170.

Madin, K., Sawasaki, T., Ogasawara, T. and Endo, Y. (2000) A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. *Proceedings of the National Academy of Sciences* **97**, 559-564.

McCormick, S.P. (2013) Microbial detoxification of mycotoxins. *Journal of Chemical Ecology* **39**, 907-918.

Nirenberg, M.W. and Matthaei, J.H. (1961) The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribonucleotides. *Proceedings of the National Academy of Sciences* **47**, 1588-1602.

Pestka, J.J. (2010) Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. *Archives of Toxicology* **84**, 663-679.

Salehi, A.S., Shakalli Tang, M.J., Smith, M.T., Hunt, J.M., Law, R.A., Wood, D.W. and Bundy, B.C. (2017). A Cell-free Protein Synthesis Approach to Biosensing hTRB-Specific Endocrine Disruptors. *Analytical Chemistry* **89**, 3395-3401.

Sasaki, Y., Asayama, W., Niwa, T., Sawada, S.I., Ueda, T., Taguchi, H. and Akiyoshi, K., (2011) Amphiphilic Polysaccharide Nanogels as Artificial Chaperones in Cell-Free Protein Synthesis. *Macromolecular Bioscience* **11**, 814-820.

Shaw, A. and Bott, R. (1996) Engineering enzymes for stability. *Current Opinion in Structural Biology* **6**, 546-550.

Shifrin, V.I. and Anderson, P. (1999) Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. *Journal of Biological Chemistry* **274**, 13985-13992.

Shifrin, V.I. and Anderson, P. (1999) Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. *Journal of Biological Chemistry* **274**, 13985-13992.

Swartz, J.R., Jewett, M.C. and Woodrow, K.A. (2004) Cell-free protein synthesis with prokaryotic combined transcription-translation. *Recombinant Gene Expression: Reviews and Protocols* **267**, 169-182.

Takai, K., Sawasaki, T. and Endo, Y. (2010) Practical cell-free protein synthesis system using purified wheat embryos. *Nature Protocols* **5**, 227-238.

Ueno, Y., Nakajima, M., Sakai, K., Ishii, K., Sato, N. and SHIMADA, N. (1973) Comparative toxicology of trichothec mycotoxins: inhibition of protein synthesis in animal cells. *The Journal of Biochemistry* **74**, 285-296.

Welsh, J.P., Bonomo, J. and Swartz, J.R. (2011) Localization of BiP to translating ribosomes increases soluble accumulation of secreted eukaryotic proteins in an Escherichia coli cell-free system. *Biotechnology and Bioengineering* **108**, 1739-1748.

Zawada, J.F., Yin, G., Steiner, A.R., Yang, J., Naresh, A., Roy, S.M., Gold, D.S., Heinsohn, H.G. and Murray, C.J. (2011) Microscale to manufacturing scale-up of cell-free cytokine production—a new approach for shortening protein production development timelines. *Biotechnology and Bioengineering* **108**, 1570-1578.

Figure 1: GC/MS data collected after expressing FsTRI101, FgTRI101, and FoTRI201 in the cell free system in the presence of 40 μg $mL^{-1}$ DON. FsTRI101, FgTRI101, and FoTRI201 all converted DON to the less toxic metabolite, 3-A-DON. The GFP positive control and the negative control containing no plasmid were not able to convert DON to 3-A-DON.

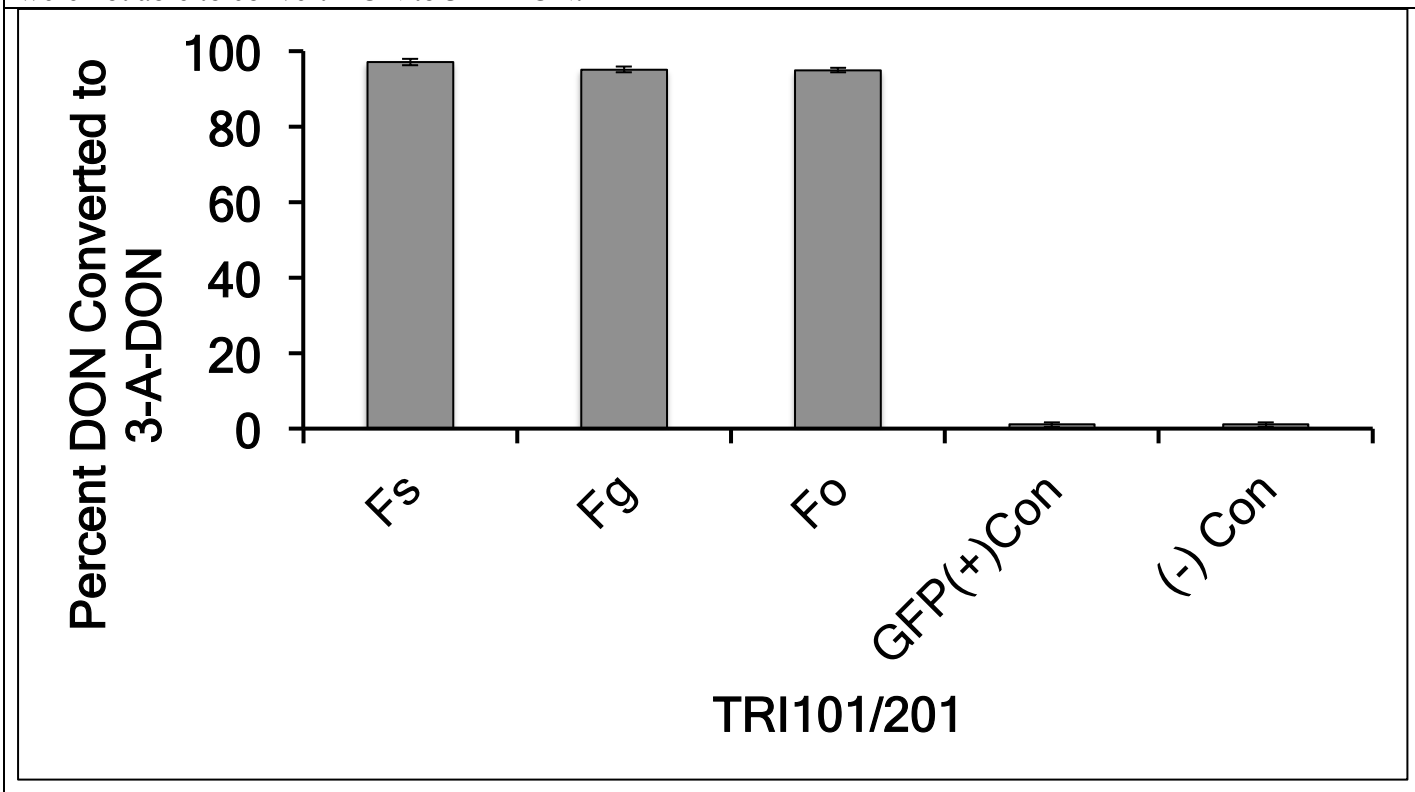


Figure 2: GC/MS chromatogram of FsTRI101 and the negative control containing no plasmid incubated in the presence of 40 μg $mL^{-1}$ DON. FsTRI101 converted DON to 3-A-DON (grey line; 6.4 min) and the negative control still contained mostly DON (black line; 6.0 min).

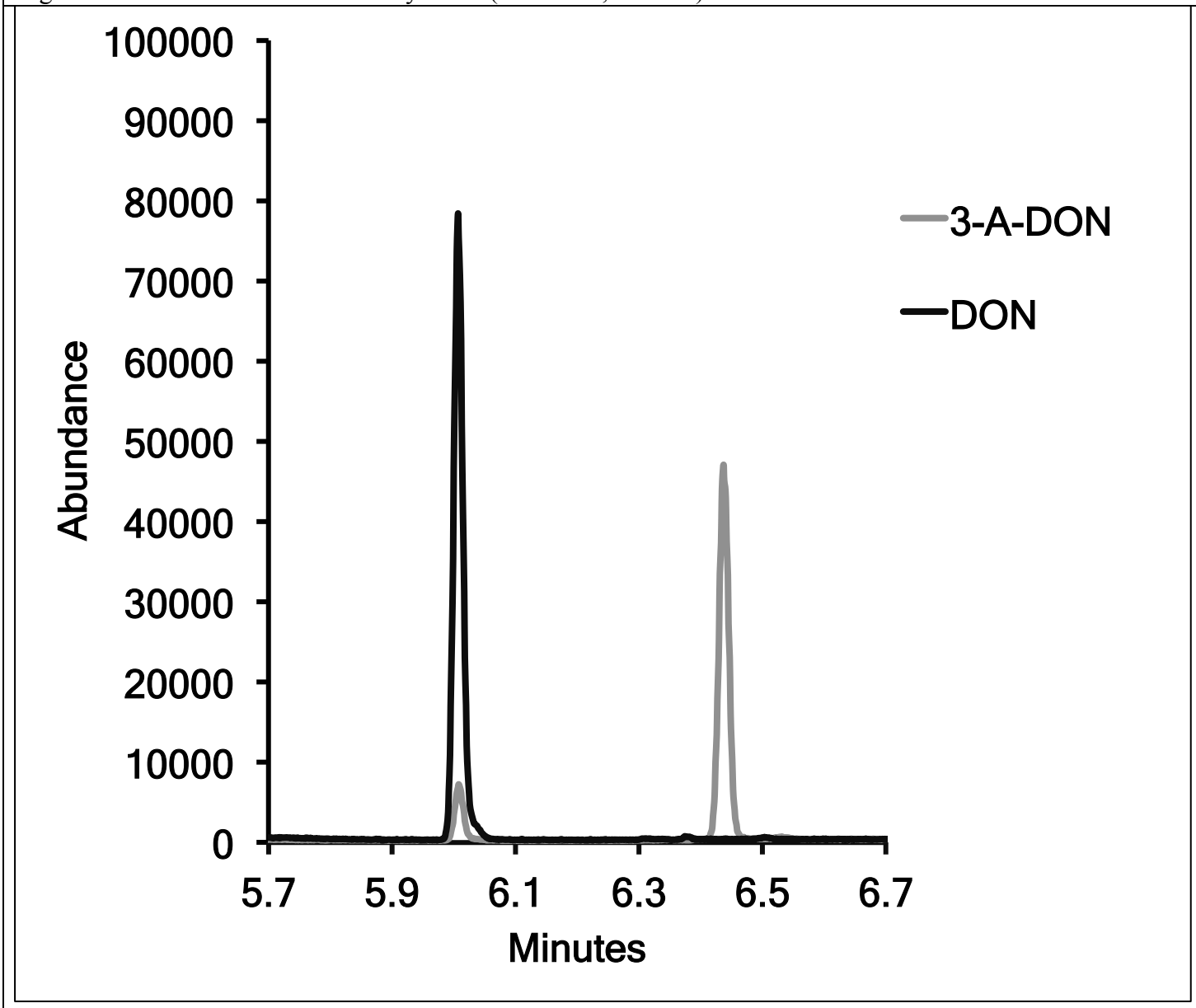


Figure 3: GC/MS data collected from FsTRI101, FgTRI101, and FoTRI201 after incubating with 40 μg $mL^{-1}$ DON for 30 min. FsTRI101, FgTRI101, and FoTRI201 all converted DON to the less toxic metabolite, 3-A-DON in 30 min. The GFP positive control and the negative control containing no plasmid were not able to convert DON to 3-A-DON.

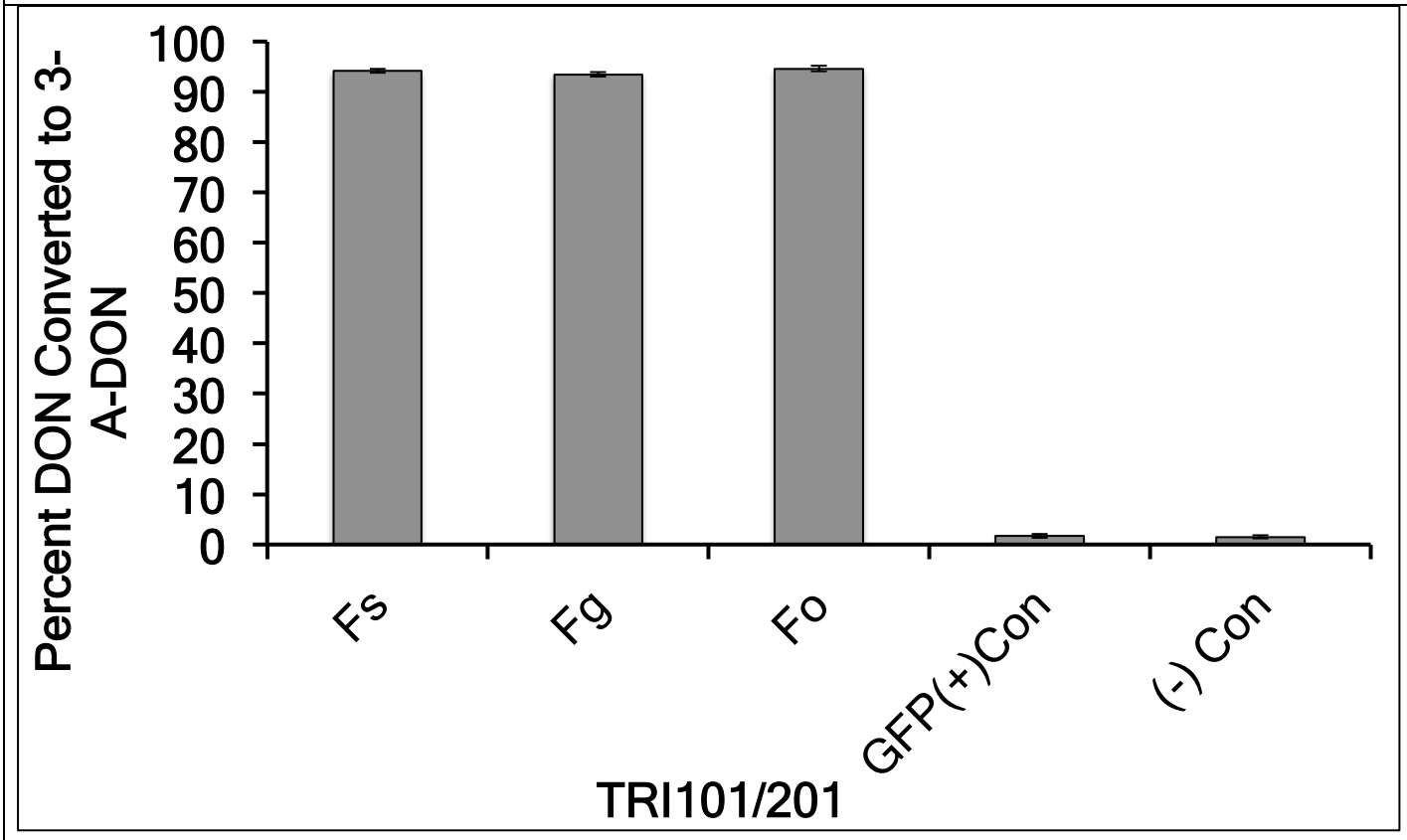


Figure 4: GC/MS data collected after incubating FgTRI101 and FoTRI201 at 30°C, 40.3°C, 49.5°C, 60.5°C, and 69.5°C with 40 μg $mL^{-1}$ DON for 30 min. FgTRI101 and FoTRI201 are still functional at 30°C and 40.3°C as DON is being converted to 3-A-DON. FgTRI101 and FoTRI201 lose functionality at 49.5°C, 60.5°C, and 69.5°C as there is no conversion of DON to 3-A-DON.

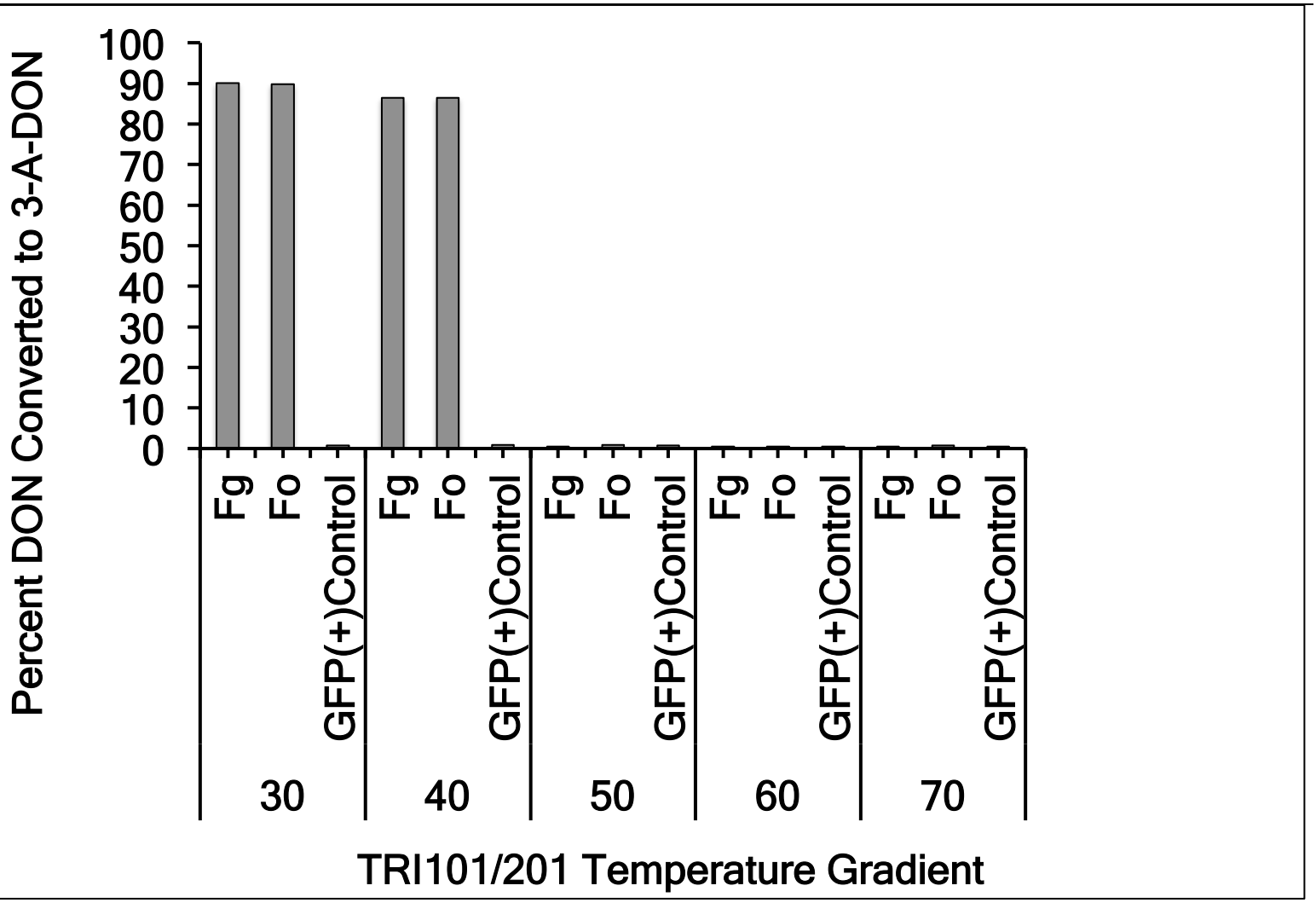
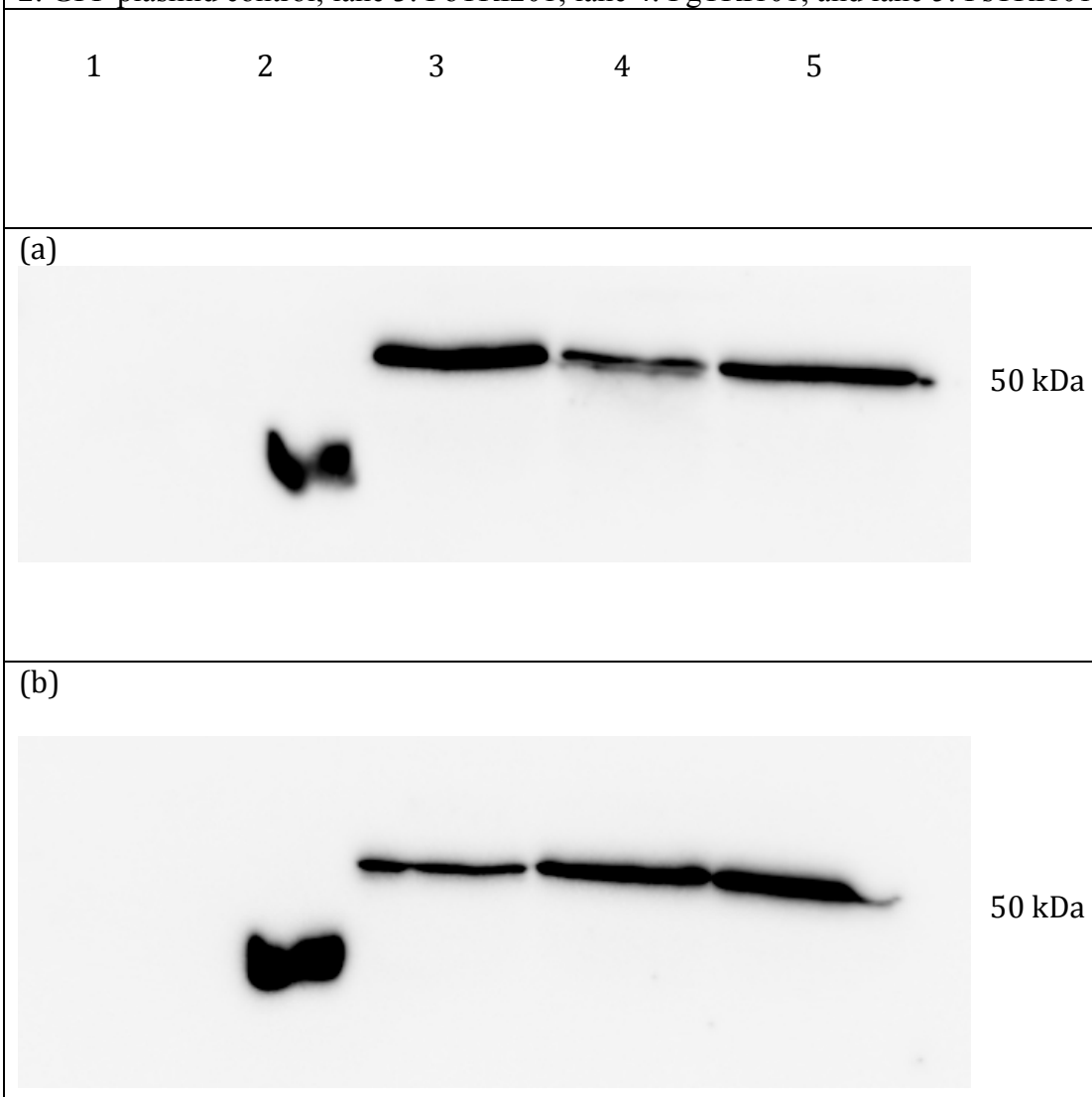
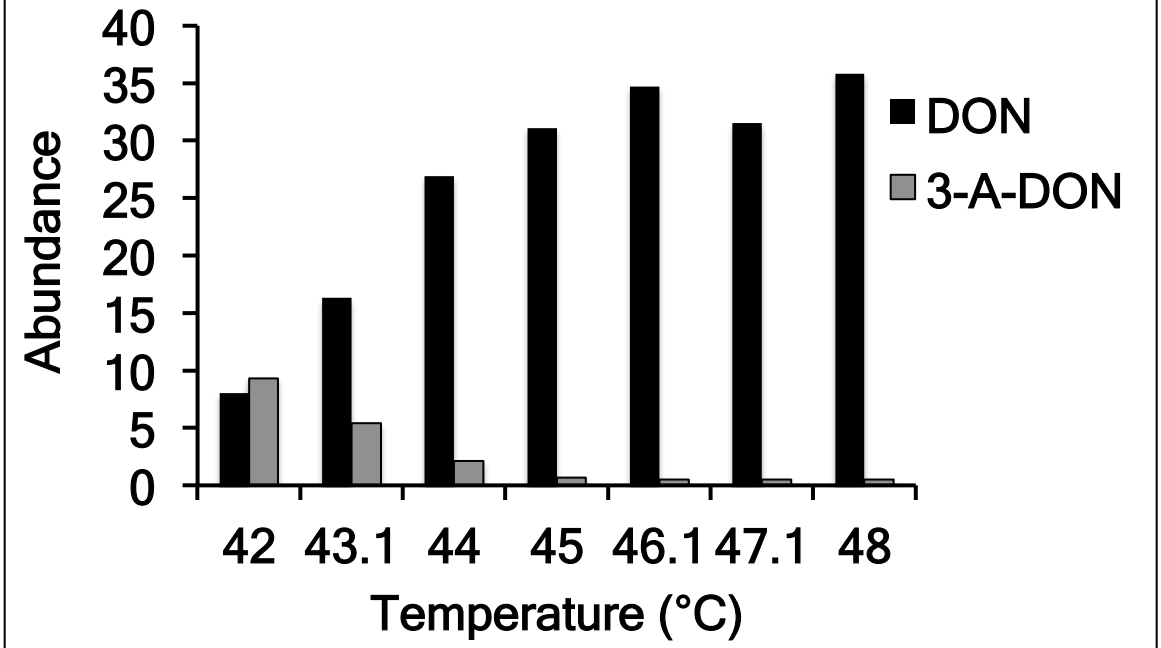


Figure 5: Western blots of (a) FsTRI101, FgTRI101, and FoTRI201 expressed in the presence of 40 μg $mL^{-1}$ DON and (b) FsTRI101, FgTRI101, and FoTRI201 expressed without DON in the CFPS system. Lane 1: negative control containing no plasmid; lane 2: GFP plasmid control; lane 3: FoTRI201; lane 4: FgTRI101; and lane 5: FsTRI101.



Supplementary Material:

Figure S1: FsTRI101 was tested between 42-48°C to observe at what temperature the enzyme loses function to convert DON to 3-A-DON.



# CHAPTER IV

## Screening Microbial Library Fragments Using Yeast to Identify Enzymes able to Detoxify the Mycotoxin Deoxynivalenol

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ABSTRACT

Deoxynivalenol (DON), a mycotoxin produced by *Fusarium* species, contaminates wheat, barley, and corn food and feed products causing numerous ill affects when consumed. Current technology to decrease DON contamination relies on timely and expensive fungicide applications and traditional breeding strategies to produce resistant or tolerant varieties of wheat and barley. New strategies to detoxify DON are needed. DON detoxifying enzymes can be sourced from microorganisms already known to detoxify DON and used to decontaminate wheat (*Triticum aestivum*) or barley (*Hordeum vulgare*) grain or by-products such as Distillers Dried Grains with Solubles (DDGS) produced from the fuel ethanol fermentation process. This work generated library fragments from microorganisms using degenerate oligonucleotide primed – polymerase chain reaction (DOP-PCR). The library fragments were cloned into DON sensitive yeast (*Saccharomyces cerevisiae*). The transformed yeast was then cultured in 100 μg/mL DON. Optical density ($OD_{600}$) measurements identified a transformed yeast culture/strain able to grow in the presence of DON. Sequencing revealed that the library fragment is a tellurite resistance gene. Additional assays with the tellurite resistance gene in yeast demonstrate activity closely associated to an efflux pump rather than a detoxification enzyme.

INTRODUCTION

A DNA library is a mixed, heterogeneous collection of plasmids with a different fragment of DNA cloned into a specific position of the plasmid. The collection of fragments is designed to cover the entire genome of the organism(s) of interest. DNA libraries were initially constructed using a high-pressure nebulization to shear DNA followed by purification and an end-repair step to remove incompatible DNA ends (Sambrook, 2001). The DNA library was then phosphorylated and integrated into a linearized, dephosphorylated plasmid produced by using blunt-cutting enzymes (Sambrook, 2001). This method can be tedious and ineffective. Despite advances to generate library fragments using more efficient ways such as mechanical or enzymatic shearing of DNA, there are still obstacles to produce large library fragments of good quality and quantity (Head et al., 2014). A more efficient and effective way to generate DNA library fragments is to use degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) (Freedman et al. 2015). This form of PCR eliminates blunt-end cloning making for an easier transition into a plasmid. DOP-PCR uses degenerate PCR primers that bind randomly to genomic DNA and amplify different fragments during a PCR run. The *Taq* polymerase used with this procedure adds a poly "A" tail to PCR ends allowing for easy cloning into an entry vector and then a destination vector.

Food and feed products can be contaminated with toxic metabolites produced by fungi called mycotoxins. Deoxynivalenol (DON) is a harmful mycotoxin mostly produced by the fungus *Fusarium graminearum* in the U.S. that contaminates staple crops such as wheat, barley, and maize (*Zea mays*) (Binder et al., 2007). It is known that DON causes deleterious effects when consumed by domestic animals including feed refusal, vomiting, reduced weight, and even death (Rotter, 1996). The mode of action for DON occurs via interfering with initiation and elongation of protein synthesis (Pestka, 2010; Ueno et al., 1973). Traditional breeding and fungicide application strategies have been the main means to mitigate DON in crops (Khatibi et al., 2012). However, new strategies are needed to mitigate DON and using enzymes is considered as a good strategy to detoxify DON. The epoxide ring present on C-12, 13 of DON is critical for toxicity (Karlovsky, 2011). A source of detoxifying/modifying enzymes lies within microorganisms able to detoxify or modify DON. Since the late 1990s, there have been numerous reports of microorganisms able to either modify or detoxify DON (McCormick, 2013). Microorganisms

able to oxidize DON to the less toxic metabolite 3-keto-4-deoxynivalenol (3-keto-DON) have been outlined in Shima et al. (1997) and Volkl et al. (2004) from an *Agrobacterium-Rhizobium* sp. E3-39 and a mixed culture D107, respectively. Ikunaga et al. (2011) reported a species in the genus *Nocardioides* that was able to epimerize DON to 3-epimer-deoxynivalenol (3-epi-DON). More recently, a mixed culture of bacteria discovered by He et al. (2016) was reported to convert DON to de-epoxy-DON (DED), a metabolite that is not toxic. This strategy of using enzymes from microorganisms to detoxify mycotoxins has been successful with another mycotoxin, ochratoxin. A carboxypeptidase enzyme was cloned from a *Bacillus* species in order to degrade ochratoxin (Chang et al., 2015).

In the current study, DNA microbial fragments were generated using DOP-PCR and cloned into DON sensitive yeast. Yeast strains that regained the function to grow in the presence of 100 μg/mL DON were selected for sequencing and additional assays. It was hypothesized that DNA microbial fragments expressed in a DON sensitive yeast system would identify enzymes able to detoxify or modify DON. This work will pave the way for identifying enzymes able to modify or detoxify DON. Resulting enzymes could be used to create transgenic crops, cloned into a yeast strain used for ethanol production, or purified and added to crops or distillers dried grains with solubles.

## MATERIALS AND METHODS

### DON Sensitive Yeast Strains

Two DON sensitive yeast strains were created as a tool to screen for microbial fragments containing functional DON detoxifying/modifying enzymes. DON sensitive yeast strains were cloned as described in Abolmaali et al. (2008). Wild-type *Saccharomyces cerevisiae* strains, RW2802 and JG436, have *PDR5* and *PDR10* or *PDR10* and *AYT1* knocked out, respectively. Sequences *PDR5* and *PDR10* code for transporter genes under stress conditions and *AYT1* encodes for an acetyltransferase gene.

DON sensitive yeast strains, RW2802 with *PDR5* and *PDR10* deleted (RW510) and JG436 with *PDR10* and *AYT1* deleted (JG10AY), were tested for sensitivity to 100 μg/mL DON. Each DON

sensitive yeast strain as well as the parental yeast strain RW2802 was cultured in 2 mL yeast peptone dextrose (YPD) media for 1 day at 28°C. A 10 μl sample of DON sensitive yeast along with parental yeast strain RW2802 was then assayed in triplicate in a 96 well plate in 200 μl of YPD media supplemented with 100 μg/mL DON, 100 μg/mL DED, 50 μg/mL DON and 50 μg/mL DED, and YPD media without any toxins. The yeast incubated for 24 hrs at 28°C with shaking. Then, optical density ($OD_{600}$) measurements were taken with a plate reader to measure growth.

Generating Microbial Library Fragments

Microbial library fragments were generated from one pure culture of bacteria (Pure Culture 1) and three mixed cultures (Mixed Culture 1, Mixed Culture 2, and Mixed Culture 3) from Wilson et al. (2017). A 50 μl sample from the glycerol stock was taken and cultured in 2 mL of Reasoner's 2A (R2A, VWR, Radnor, PA) broth. DNA was extracted from the four cultures using a DNeasy PowerSoil Kit (Mo Bio Laboratories, Carlsbad, CA). Freedman and Senger created primers for DOP-PCR in their lab to cover the genome(s) of microorganisms used in this study. The M set (M1, M2, M4 and M5) and Rand3 primers (Freedman and Senger, 2015) were used to generate library fragments from Pure Culture 1, Mixed Culture 1, Mixed Culture 2, and Mixed Culture 3 from Wilson et al. (2017). The DOP-PCR parameters involved 10 cycles of low stringency amplification with a 5 min denaturation at 95 °C followed by 10 cycles of: 94°C for 1 min, 30°C for 2 min, ramp to 68°C for 3 min, and amplification at 68°C for 8 min. Following this program, a high stringency program of 25 cycles was implemented. Each cycle consisted of: 94°C for 1 min, 55°C for 2 min, and 68°C for 8 min, with 5 s added for each cycle. The DOP-PCR products were then held at 68°C for 7 min. The low stringency cycles were designed to encourage random priming, and the high stringency cycles were designed to further amplify those randomly produced DNA fragments. A 50 μl DOP-PCR reaction was prepared with 2x GoTaq Green master mix (Promega, Madison, WI) so that resulting fragments had a poly A tail and were easy to clone into an entry vector. Fragments were visualized on a 0.8% agarose gel. All microbial library fragments from the four microbial cultures tested that produced a wide spread band on the gel were combined. Library fragments were generated with the M set primers (M1, M2, M4 and M5) and Rand3 primer (Figure 2) as described in Freedman and

Senger, 2015. Pure Culture 1 DOP-PCR reactions using M1, M2, and M3 primers were combined for cloning into the DON sensitive yeast strains. Mixed Culture 1 DOP-PCR reactions using all primers were combined for cloning into the DON sensitive yeast strains. Mixed Culture 2 DOP-PCR reactions using M1 and M2 primers were combined for cloning into the DON sensitive yeast strains. Mixed Culture 3 DOP-PCR reactions using M1, M2, M5, and Rand3 primers were combined for cloning into the DON sensitive yeast strains.

Cloning into DON Sensitive Yeast Plasmid

Vector pCR8/GW/TOPO TA (Life Technologies, Carlsbad, CA) was combined with 5 μl of the DOP-PCR DNA following manufacturer's directions and incubated for 30 min at room temperature before transforming into 50 μl of Clonetech® stellar competent cells (Clonetech Laboratories, Mountain View, CA) according to manufacturer's directions. Cells were pooled and grown up in 2 mL of lysogeny broth (LB) media with 50 μg/L spectinomycin.
Plasmid DNA in pCR8 was recombined with pYesDEST-52 by combining 150 ng of plasmid with 50 ng of the pooled pCR8 library DNA using the Invitrogen Gateway LR Clonase II kit (Thermo Fisher Scientific, Waltham, MA) and incubated overnight at 25°C. Aliquots of 5 μl transformation mix were used to transform into 50 μl vials of *E. coli* ccdB competent cells and grown in 2 mL of LB media with ampicillin (100 μg/L) selection. Plasmid DNA was purified using a GeneJet miniprep kit (Thermo Fisher Scientific, Waltham, MA). A 300-500 ng sample of purified plasmid DNA was then transformed into both DON sensitive yeast strains.

Library Enrichment Assays

Assays were prepared in a 96 well plate with 200 μl of synthetic dropout media without uracil (SD-U; Sigma) and 100 μg/mL DON and 10 μl of transformed yeast. Assays were incubated for 24 hrs at 28°C with shaking. After 24 hrs, $OD_{600}$ measurements were taken to determine growth of yeast cells able to grow in the presence of 100 μg/mL DON. Wells with noticeable growth compared to controls were plated on SD-U media for 2-3 days at 28°C until colonies were

clearly visible. For each plate, 12 yeast colonies were selected for sequencing with plasmid primers to determine the identity of microbial library fragments inserted into the plasmid.

## RESULTS

### DON Sensitive Yeast Strains

DON sensitive yeast strains, RW510 and JG10AY, were sensitive to 100 μg/mL DON as seen in Figure 1. Neither strain was able to grow in the presence of 100 μg/mL DON, while both were able to grow in the presence of 100 μg/mL DED, but were slightly sensitive to the mixture of DON and DED. The parent yeast strain RW2802 was able to grow in all conditions tested.

### Library Enrichment Assays

Library enrichment assays identified a transformed JG10AY yeast isolate able to grow in the presence of 100 μg/mL DON. Sequencing revealed the plasmid contained a gene similar to a tellurite resistance gene (Taylor, 1999). Further assays with this transformed yeast (TEL_JG10AY) indicated that DON was not being converted to a different metabolite. It is probable that TEL_JG10AY could be a transporter gene. Additional assays comparing TEL_JG10AY and a known DON efflux pump, *TRI12*, transformed into both DON sensitive yeast strains in the presence of an efflux pump inhibitor (ferulic acid; FA) indicated that TEL_JF10AY could be an efflux pump (Figure 3). TEL_JG10AY, TRI12_RW510, and TRI12_JG10AY all were inhibited by 5 mM FA and a combination of 5 mM FA and 100 μg/mL DON; however, the DON sensitive yeast strains were not inhibited by the presence of 5 mM FA. The parental yeast strains were able to grow in all test conditions.

## DISCUSSION

DON sensitive yeast strains, RW510 and JG10AY, were sensitive to 100 μg/mL DON. Both of these yeast strains provide a good tool to screen for microbial library fragments that can modify or detoxify DON. The DON sensitive yeast strains produced by Abolmaali et al. (2008) were

used to screen microbes that detoxified or modified DON. In the current study, continued research was undertaken to search for enzymes that are responsible for DON detoxification. Library fragments were generated using DOP-PCR with the M set primers (M1, M2, M4 and M5) and Rand3 primer. Using DOP-PCR is a useful method to generate library fragments that are easy to clone into an entry vector and other downstream applications. Fragments generated are large enough to house a gene that could be of interest. Primers that were unsuccessful at annealing to genomic DNA may need to be amended; however, using several primers increases the chances of producing library fragments that can be combined.

Library enrichment assays identified a transformed JG10AY yeast isolate, TEL_JG10AY, able to grow in the presence of 100 μg/mL DON. TEL_JG10AY did not detoxify or modify DON, so it is postulated to be a transporter/efflux pump. Comparing TEL_JG10AY with known efflux pump, *TRI12*, in the presence of an efflux inhibitor, FA, demonstrated that TEL_JG10AY behaves similarly *TRI12*. Additional testing needs to be done in order to confirm that FA is inhibiting the function of the efflux pumps, and not interfering with the pYesDEST-52 plasmid. Once FA is confirmed to inhibit efflux pumps, the microbial library can be enriched again in the presence of DON and FA to isolate the DON detoxifying enzymes.

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Nina M. Wilson and Celia Sanchez conducted experiments. Dash Gantulga generated the DON sensitive yeast. Nicole McMaster performed GC/MS analysis. Benjamin G. Freedman generated the DOP-PCR primers and method. David G. Schmale III and Ryan S. Senger planned and coordinated experiments.

REFERENCES

Abolmaali, S., Mitterbauer, R., Spadiut, O., Peruci, M., Weindorfer, H., Lucyshyn, D., Ellersdorfer, G., Lemmens, M., Moll, W.D. and Adam, G., 2008. Engineered bakers yeast as a sensitive bioassay indicator organism for the trichothecene toxin deoxynivalenol. *Journal of microbiological methods*, *72*(3), pp.306-312.

Binder, E.M., Tan, L.M., Chin, L.J., Handl, J. and Richard, J., 2007. Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. *Animal feed science and technology*, *137*(3), pp.265-282.

Chang, X., Wu, Z., Wu, S., Dai, Y. and Sun, C., 2015. Degradation of ochratoxin A by Bacillus amyloliquefaciens ASAG1. *Food Additives & Contaminants: Part A*, *32*(4), pp.564-571.

Freedman, B.G., Senger, R.S. (2015) Thermodynamic design of degenerate oligonucleotide primers for whole genome DNA amplification with reduced bias for use in DNA libraries. Submitted to Genome Research.

He, W.J., Yuan, Q.S., Zhang, Y.B., Guo, M.W., Gong, A.D., Zhang, J.B., Wu, A.B., Huang, T., Qu, B., Li, H.P. and Liao, Y.C., 2016. Aerobic De-Epoxydation of Trichothecene Mycotoxins by a Soil Bacterial Consortium Isolated Using In Situ Soil Enrichment. *Toxins*, *8*(10), p.277.

Head, S.R., Komori, H.K., LaMere, S.A., Whisenant, T., Van Nieuwerburgh, F., Salomon, D.R. and Ordoukhanian, P., 2014. Library construction for next-generation sequencing: overviews and challenges. *Biotechniques*, *56*(2), p.61.

Ikunaga, Y., Sato, I., Grond, S., Numaziri, N., Yoshida, S., Yamaya, H., Hiradate, S., Hasegawa, M., Toshima, H., Koitabashi, M. and Ito, M., 2011. Nocardioides sp. strain WSN05-2, isolated from a wheat field, degrades deoxynivalenol, producing the novel intermediate 3-epi-deoxynivalenol. *Applied microbiology and biotechnology*, *89*(2), pp.419-427.

Karlovsky, P., 2011. Biological detoxification of the mycotoxin deoxynivalenol and its use in genetically engineered crops and feed additives. *Applied microbiology and biotechnology*, *91*(3), pp.491-504.

Khatibi, P.A., Berger, G., Liu, S., Brooks, W.S., Griffey, C.A. and Schmale III, D.G., 2012. Resistance to Fusarium head blight and deoxynivalenol accumulation in Virginia barley. *Plant Disease*, *96*(2), pp.279-284.

McCormick SP. Microbial Detoxification of Mycotoxins. *J Chem Ecol*. 2013;39:907-918.

Pestka, J.J., 2010. Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. *Archives of toxicology*, *84*(9), pp.663-679.

Rotter, B.A., 1996. Invited review: Toxicology of deoxynivalenol (vomitoxin). *Journal of Toxicology and Environmental Health Part A*, *48*(1), pp.1-34.

Sambrook, J., 2001. Molecular cloning: a laboratory manual/Joseph Sambrook, David W. Russell.

Shima, J., Takase, S., Takahashi, Y., Iwai, Y., Fujimoto, H., Yamazaki, M. and Ochi, K., 1997. Novel detoxification of the trichothecene mycotoxin deoxynivalenol by a soil bacterium isolated by enrichment culture. *Applied and Environmental Microbiology*, *63*(10), pp.3825-3830.

Taylor, D.E., 1999. Bacterial tellurite resistance. *Trends in microbiology*, *7*(3), pp.111-115.

Ueno, Y., Nakajima, M., Sakai, K., Ishii, K., Sato, N. and Shimada, N., 1973. Comparative toxicology of trichothec mycotoxins: inhibition of protein synthesis in animal cells. *The Journal of Biochemistry*, *74*(2), pp.285-296.

Völkl, A., Vogler, B., Schollenberger, M. and Karlovsky, P., 2004. Microbial detoxification of mycotoxin deoxynivalenol. *Journal of basic microbiology*, *44*(2), pp.147-156.

Wilson, N.M., McMaster, N., Gantulga, D., Soyars, C., McCormick, S.P., Knott, K., Senger, R.S. and Schmale, D.G., 2017. Modification of the Mycotoxin Deoxynivalenol Using Microorganisms Isolated from Environmental Samples. *Toxins*, *9*(4), p.141.

Figure 1: Assay with DON sensitive yeast in yeast peptone dextrose (YPD) media supplemented with 100 μg/mL DON, 100 μg/mL DED, 50 μg/mL DON and 50 μg/mL DED, and YPD media without any toxins. DON sensitive yeast strains, RW510 and JG10AY, were sensitive to 100 μg/mL DON.

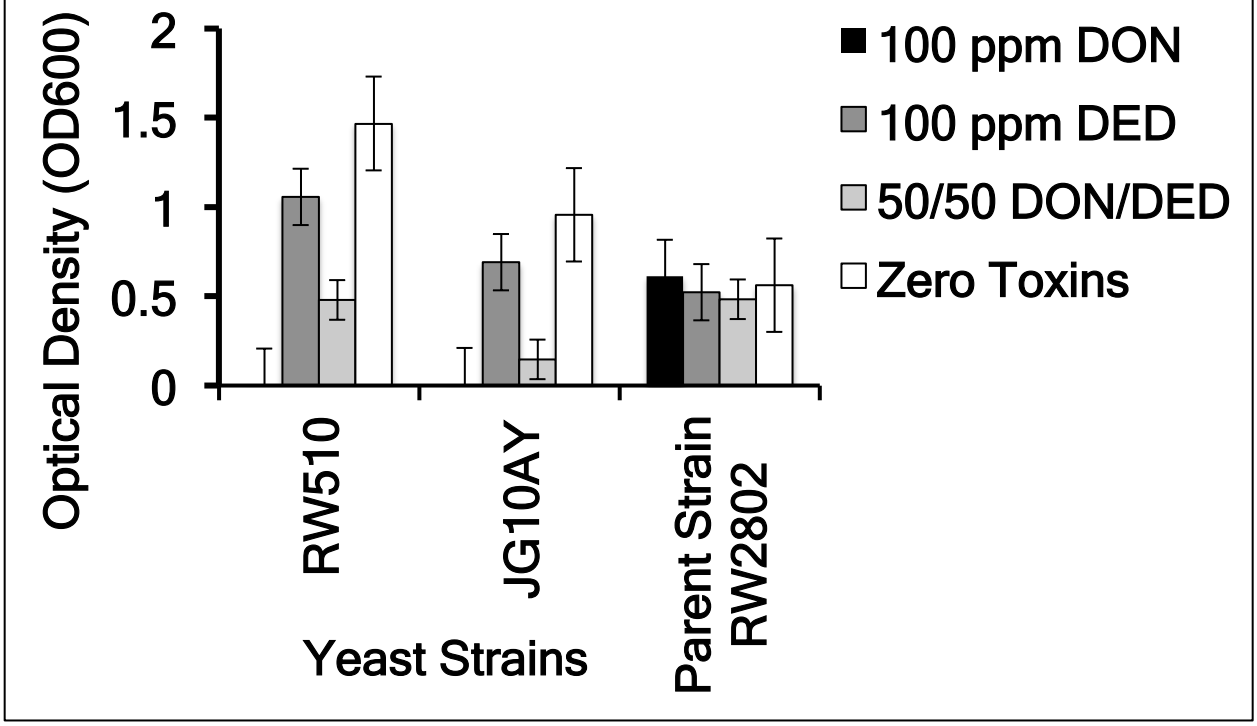


Figure 2: Gel electrophoresis image of the microbial library fragments. Red is Pure Culture 1, yellow is Mixed Culture 2, pink is Mixed Culture 3, and blue is Mixed Culture 1 with the M set primers and the Rand3 primer.

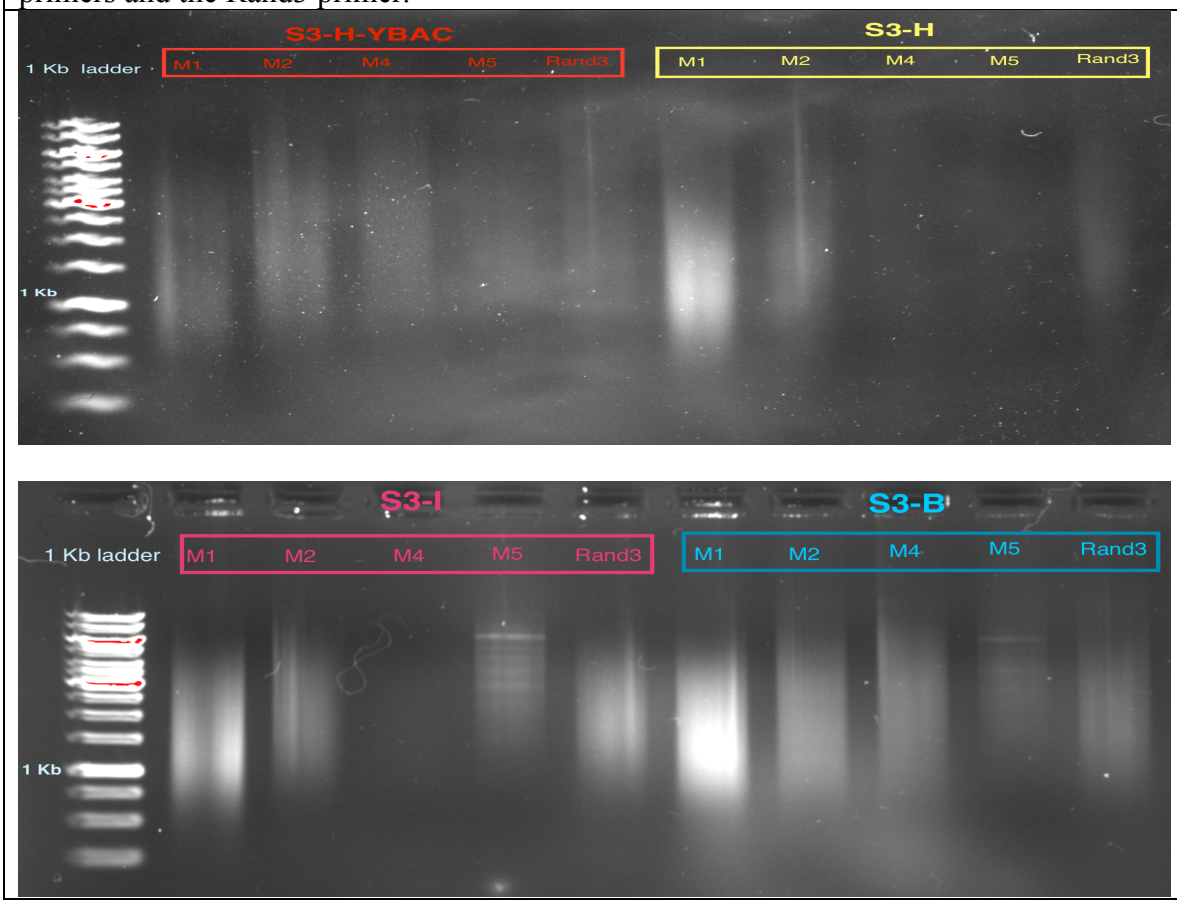
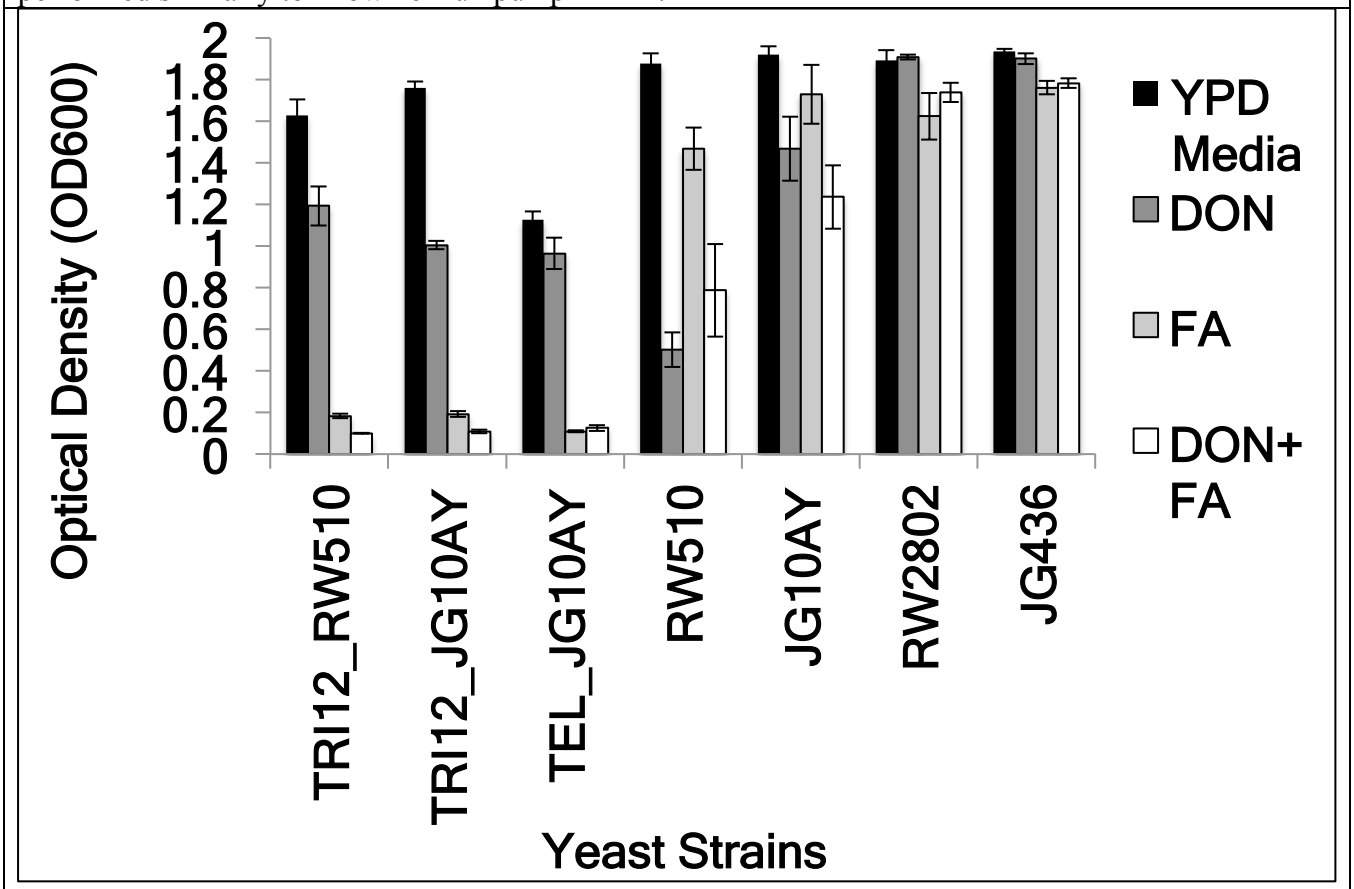


Figure 3: Transformed yeast strains tested in synthetic dropout media without uracil (SD-U) media, 100 μg/mL DON, 5 mM ferulic acid (FA), and a combination of 100 μg/mL DON and 5 mM FA. DON sensitive yeast strains and parent strains were tested in YPD media, 100 μg/mL DON, 5 mM FA, and a combination of 100 μg/mL DON and 5 mM FA. The putitive efflux pump, TEL_JG10AY, performed similarly to known efflux pump TRI12.



# CHAPTER V

**Title: Could Your Food be Contaminated with Toxins?**

Nina Wilson, Shelbie Dashiell, Nicole McMaster, Cynthia Bohland, and David Schmale

**Subtitle:** *Educating High School Students about Mycotoxins in Feed and Food Products*

Note: This manuscript was submitted to The Scientific Teacher January 2017.

ABSTRACT AND INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by fungi (Marin, S., 2013). They are important contaminants of livestock feed and human food products (J. Fink-Grernmels, 1999), and threaten the health of domestic animals and humans (Sobrova et al., 2010). Mycotoxins contaminate crops such as wheat, maize, peanuts, cottonseed, and coffee (Placinta et al., 1999). The Food and Agriculture Organization estimated that approximately 1 billion metric tons of food is lost each year due to mycotoxin contamination (FAO, 2013). Economic loss consists of yield loss from mycotoxin contamination, reduced value of crops, loss of animal productivity from health issues related to mycotoxin consumption, and lastly human health loss (Schmale, Munkvold, 2009).

The fungus *Fusarium graminearum* causes a disease known as Fusarium Head Blight (FHB) in wheat and barley. The fungus produces the mycotoxin deoxynivalenol (DON), which contaminates wheat, barley, and maize worldwide (Tanaka et al., 1988). DON contamination is a pressing problem in the United States (Salas et al., 1999). DON causes feed refusal, skin disorder, diarrhea, reduced growth, and vomiting in domestic animals, and humans may demonstrate symptoms of nausea, fever, headaches, and vomiting (Pestka, 2010). New approaches are needed to detect and monitor DON in feed and food products.

We developed a unit for advanced secondary school students that highlights the potential dangers of mycotoxins in feeds and foods. Students worked in small groups to detect the mycotoxin

(DON) from common grocery store products. A safe, easy-to-use enzyme-linked immunosorbent assay (ELISA) was used to determine if DON was present in these products. Students were asked to think about ways of mitigating these toxins in commercial scenarios, ranging from toxin removal strategies to policies to regulate them.

This activity, which aligns with the *Next Generation Science Standards* (see NGSS box), introduces students to dangers presented by mycotoxins in livestock feed and food products, while encouraging them to think critically about issues important to agriculture, food safety, and biotechnology. After completing this unit, students will be able to:

- Understand the potential for feed and food to be contaminated with mycotoxins
- Discuss different methods for detecting mycotoxins
- Consider principles of experimental design when sampling feed and food for mycotoxins, including potential pitfalls and limitations
- Conduct an experiment to detect the mycotoxin DON in common grocery store products and samples of wheat, barley, and corn naturally contaminated with DON using quick test kits
- Discuss ways to detect and control mycotoxins in commercial settings

**At the Intersection of Food and Biotechnology**

This unit will lead students though an interactive, interdisciplinary exercise to detect the mycotoxin DON in common food products. The activity allows students to think about the process of how food goes from farm to table. Students will consider how mycotoxins can affect the individual consumer, large groups of people, and even domestic animals. Students will learn

and discuss ways to test for mycotoxins and the regulations governments set for mycotoxins in food and feed.

## MATERIALS AND METHODS

### Part 1: Seeing Fungi Grow on Common Food Products (fungi, fungi, everywhere!)

The unit started with and introduction to fungi in common food products. Students looked at fungi that were cultured in humidity chambers from common grocery store items. This was a great way to get students thinking about fungi and mycotoxins. About a week berfore the lesson, small humidity chambers were built with plastic deep well petri dishes, 8.5 diameter Whatman filter paper, sterile water, and common food items from the grocery store. Unwashed wheat cereal, pretzels, organic and regular flour, organic and regular oats, a green bell pepper, and grapes were placed inside the humidity chambers and were incubated at room temperature for one week (**Figure 1**). The humidity chambers were sealed with parafilm to contain the fungi and other microbes that grew on the food products. The day of the lesson, students observed what kind of fungal contamination is present on the different products (the students kept the humidity chambers sealed, to prevent any potential harmful microorganisms from escaping from the chambers) (**Figure 1**).

### Part 2: Introduction and Lecture (20-30 min)

Following the viewing of the fungi in the sealed humidity chambers, we provided background information on the discovery of mycotoxins by telling the story about Turkey X disease and the discovery of aflatoxins (Spensley, P.C, 1963). Aflatoxins were discovered in the 1960s, on a

turkey farm in England. Turkeys were getting very sick and dying, which perplexed the growers raising the turkeys. Initially, people thought it was a disease spreading making the turkeys ill, so to prove it was a disease, the turkey farmers put healthy turkeys with the sick turkeys. However, none of the healthy turkeys got sick, so it wasn't a disease that was spreading from turkey to turkey. Eventually, the turkey farmers traced the sickness to the feed source; the turkey feed was contaminated with aflatoxins. The lesson then continued with information about the relative toxicity of aflatoxins, the fungi that produce them, and the foods and feeds where you might find aflatoxins (information provided in **Table 1**) (Catherine Matacic, 2016). We then introduced the mycotoxin deoxynivalenol (DON), the most important mycotoxin in the United States, and provided information on the crops where you might find DON, relative toxicity information for DON, and U.S. regulations for DON in feed and food products (information provided in Table 1) (Pestka, 2010). Methods to detect mycotoxins were presented next, such as gas chromatography mass spectroscopy (GC/MS) and enzyme linked immunosorbent assay (ELISA). Finally, we discussed the pros and cons of using each method (e.g., GC/MS analysis is expensive and accurate with low limits of detection, and ELISA is inexpensive and less accurate with higher limits of detection; **Table 2**).

**Part 3: Experiments to Detect DON in Feed and Food Products (75 min)**

After the short lecture, students were divided into small groups to conduct experiments to detect DON in different feed and food products. We had the students (ages 15-18) work in small groups of 3 or 4 students to quantify the mycotoxin DON from a variety of different materials including flour, oats, barley, corn as well as processed food items such as tortilla chips or wheat crackers (**Table 3**). All samples that were tested were ground using a coffee grinder and aliquoted as 1g

samples prior to the experiment, to allow more time for the students to conduct the experiment. We used naturally contaminated wheat and barley samples with low levels of DON as positive controls, and known samples without any DON as negative controls (these samples can be obtained from the Schmale lab at Virginia Tech). Safe, easy-to-use ELISA kits from Neogen (Agri-Screen for DON) with a detection limit of 1 ppm were used to determine if DON is present in these products.

We designed the experiment so that each group used six ELISA wells. Three wells were used for controls (the 1 ppm Neogen control and the positive and negative grain sample controls from the Schmale lab) and the students tested three unknown samples (two from **Table 3** and one from a product provided by the students or the teacher). Students followed the manufacturer's instructions for the kit, but used 1g of ground sample and 5 mL of water for extraction (instead of 5g of sample). Samples that tested positive for DON were supposed to turn pink, and samples that test negative were supposed to turn blue. Samples in **Table 3** were previously tested and determined to be within the United States governmental regulation of DON contamination for food and feed products and thus safe to use during the experiment. Images in **Figure 2** demonstrate some of the steps associated with running the experiment. **Table 4** presents materials needed and the associated cost for the lesson. All students should wear gloves and the appropriate personal protective equipment, as well as practice safe hygiene practices when working with samples and the ELISA kit.

**Part 4: Conclusion and Discussion (10 min*)***

Following the experiments, students had the opportunity to share their results and conclusions with the class. Each group reported on the colors observed for each of the samples tested. Not all

groups observed the same color change for the same samples, and one group even reported an unexpected color for their samples (green). This led to a discussion of the potential limitations of the design of the experiment, and possible sources of error. We then discussed real-world situations where a grower or a wheat mill operator might use this kit to determine if a crop is contaminated with DON, and what would happen if the crop was contaminated; the price for the contaminated grain would likely be reduced, and the grain might end up being used as feed or to produce fuel ethanol.

## RESULTS AND DISCUSSION

### Reflections and Assessment

A survey was distributed by the teacher (Cindy Bohland) after the lesson took place, to assess how effective the lesson was and what improvements could be made for future experiments. **Table 5** shows the responses to the teacher-administered survey. Overall, the students were able to appreciate the potential toxins in our food, clinical effects associated with mycotoxin consumption, importance of testing food and feed, and sources of error associated with mycotoxin testing. Things to improve and elaborate in detail were the differences between analytical methods of testing (GC/MS) and semi-quantitative/colorimetric assays such as ELISA. **Table 5** also demonstrates where improvements can be made in getting the message across to the students in a meaningful way that they can understand.

### Potential Modifications to the Unit

There are a number of potential modifications that could be made to improve the unit for additional offerings. First, it would have been helpful to have the 6 ELISA wells pre-labeled ahead of time, and have an associated image at the start of the lesson so the students knew what sample went in which vial. Second, it would have been helpful to have prepared aliquots of the Neogen reagents for each group prior to the lesson. This would have reduced any potential cross contamination among samples, and also would have reduced the total time required for the lesson. Third, the unit would have benefited from the use of filter pipette tips to reduce contamination in the pipettes and thus among the samples tested (some pipettes were observed to contain test material at the conclusion of the lesson). Fourth, it would be beneficial to take the students through the ELISA steps and explain what each reagent was doing in the process before starting the experiment. Fifth, this lesson could be taught over two days, with one day devoted to talking about mycotoxins, food safety, and plant pathology and the second day focusing on ELISA and the class experiments.

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**On the Web**

Information about mycotoxins:

*http://www.apsnet.org/edcenter/intropp/topics/Mycotoxins/Pages/default.aspx*

Information about fusarium head blight:

*http://www.apsnet.org/edcenter/intropp/lessons/fungi/ascomycetes/Pages/Fusarium.aspx*

GC/MS instructional video: *https://www.youtube.com/watch?v=wHbF2ZRSgy0*

Neogen's Agri-Screen for DON Kit: *http://foodsafety.neogen.com/en/agri-screen-don#documents*

REFERENCES

Marin, S., et al. "Mycotoxins: occurrence, toxicology, and exposure assessment." *Food and Chemical Toxicology* 60 (2013): 218-237.

Fink-Grernmels J. Mycotoxins: Their implications for human and animal health. *Vet Q*. 1999;21(4):115-120.

Sobrova P, Adam V, Vasatkova A, Beklova M, Zeman L, Kizek R. Deoxynivalenol and its toxicity. *Interdiscip Toxicol*. 2010;3:94-99.

Placinta C., D'Mello JP., Macdonald AM. A review of worldwide contamination of cereal grains and animal feed with Fusarium mycotoxins. *Anim Feed Sci Technol*. 1999;78(1-2):21-37. doi:10.1016/S0377-8401(98)00278-8.

FAO. *The Global Forum for Food Security and Nutrition*.; 2013.

Schmale DG, Munkvold G. Mycotoxins in crops: A threat to human and domestic animal health. *Plant Heal Instr*. 2009.

Tanaka T, Hasegawa A, Yamamoto S, Lee US, Sugiura Y, Ueno Y. Worldwide contamination of cereals by the Fusarium mycotoxins nivalenol, deoxynivalenol, and zearalenone. 1. Survey of 19 countries. *J Agric Food Chem*. 1988;36(5):979-983.

Salas B, Steffenson BJ, Casper HH, et al. Species Pathogenic to Barley and Their Associated Mycotoxins. *Plant Dis*. 1999;83:667-674. doi:10.1094/PDIS.1999.83.7.667.

Pestka JJ. Deoxynivalenol: Mechanisms of action, human exposure, and toxicological relevance. *Arch Toxicol*. 2010;84:663-679.

Spensley, P. C. "Aflatoxin, the active principle in turkey'X'disease." *Endeavour* 22 (1963): 75.

Matacic, Catherine. "Fungal Toxins Are Poisoning Africa's Children, Says New Report." *Science | AAAS*. N.p., 31 Mar. 2016. Web. 13 Dec. 2016.

"Agri-Screen® for DON." *Agri-Screen® for DON*. Neogen, n.d. Web. 13 Dec. 2016.

**Figure 1.** Images of fungi growing on pretzels (top) and common grocery food products (bottom) in humidity chambers.

**Figure 2.** Images of students extracting potential mycotoxins from samples (top), pipetting samples into ELISA wells (middle), and observing color changes that indicate mycotoxins (pink samples contained DON, blue samples did not).

**Table 1.** Information about aflatoxins and deoxynivalenol (DON). Parts Per Billion (ppb) and Parts Per Million (ppm) are units for measuring and regulating mycotoxins.

| | **Aflatoxin** | **Deoxynivalenol (DON)** |
|---|---|---|
| **Toxicity** | Carcinogen, Liver Damage and Cancer, Weight Loss, Death | Vomiting, Feed Refusal, Weight Loss, Headaches, Death (Domestic Animals) |
| **Infected Crops** | Corn, Peanuts, Millet Seed, Cotton Seed | Corn, Wheat, Barley |
| **Areas Most Affected** | Africa | United States and Canada |
| **United States Regulations in Finished Foods and Feeds** | Food- 20 ppb<br>Feed- 300 ppb | Food- 1 ppm<br>Feed- 5 ppm |
| **Common fungi that produce the mycotoxin** | *Aspergillus flavus* | *Fusarium graminearum* |

**Table 2.** Pros and cons of using different detection methods.

| | Pros | Cons |
|---|---|---|
| **Gas Chromatography/Mass Spectrometry (GC/MS)** | • Highly Analytical<br>• Sensitive to Target Compound<br>• Detects Multiple Compounds | • Expensive<br>• Needs a Trained Technician to Operate<br>• Results Take Long to Acquire |
| **Enzyme Linked Immunosorbent Assay (ELISA)** | • Cheap<br>• Quick Results<br>• Easy to Use<br>• Detects Multiple Compounds | • Not Highly Analytical<br>• Easily Contaminated<br>• Detects Non-Target Compounds-False Positives |

**Table 3.** Unknown samples used in the classroom. Each group of students picked two of the below samples to test as unknowns.

| Sample Number | Sample Name | Source |
|---|---|---|
| 1 | White Rice | Grocery Store |
| 2 | Corn Meal | Grocery Store |
| 3 | White Flour | Grocery Store |
| 4 | Ground Corn Field Sample | Field Sample |
| 5 | Ground Sorghum Field Sample | Field Sample |
| 6 | Fritos | Grocery Store |
| 7 | Tortilla Chips | Grocery Store |
| 8 | Bran Cereal | Grocery Store |
| 9 | Grape Nuts Cereal | Grocery Store |
| 10 | Wheat Thins | Grocery Store |
| 11 | Animal Feed- Corn Glucose | Virginia Tech's Cattle Farm |
| 12 | Dog Food | Grocery Store |
| 13 | Wheat | Schmale Lab |
| 14 | Barley | Schmale Lab |
| 15 | Organic Flour | Grocery Store |

**Table 4.** Materials needed for the experiment. Costs will vary depending on size of classroom and materials already in possession.

| ***Provided with 1 Agri-Screen for DON NEOGEN kit*** | | Cost | Vendor |
|---|---|---|---|
| **Materials:** | **#:** | | |
| antibody coated microwells | 24 | | |
| red marked mixing wells | 24 | | |
| bottle of 1ppm DON Control | 1 | | |
| bottle of DON-HRP conjugate | 1 | | |
| bottle of K-Blue substrate | 1 | | |
| bottle of Red Stop solution | 1 | | |
| | | $167.00 | Neogen |
| ***Needed Per Student Group*** | | Cost | Vendor |
| **Materials:** | **#:** | | |
| grade 1 Filter Paper | 5 | $12.00 (100 circles) | Capitol Scientific |
| Funnel (65 mm) | 5 | $53.00 (pack of 12) | Thermo Fisher |
| 50 mL conical tube | 5 | $214.00 (case of 300) | Thermo Fisher |
| 25 mL graduated cylinder | 1 | $112.00 (case of 18) | Thermo Fisher |
| 15 mL conical tube | 5 | $248.00 (case of 500) | Thermo Fisher |
| 100-1000 uL pipette tips | 1 | $78.00 (pack of 560) | Fisher Scientific |
| 20-200 ul pipette tips | 1 | $90.00 (pack of 960) | Fisher Scientific |
| 100-1000 ul pipette | 1 | | |
| 20-200uL pipette | 1 | | |

**Table 5.** Post-unit survey results conducted by the biology teacher. Each student rated how much they gained from the lesson, from none, a little, to a lot, based on the statements in the table.

| Statement | None | A Little | A Lot |
|---|---|---|---|
| 1. Awareness of potential toxins in your food | 0 | 1 | 22 |
| 2. Methods to detect mycotoxins in a lab setting | 0 | 9 | 14 |
| 3. Regulations on food toxin limits | 0 | 11 | 12 |
| 4. Potential for error when sampling | 0 | 5 | 18 |
| 5. Importance of testing for mycotoxins when processing from raw materials into finished products (for example, ear of corn into Fritos corn chips) | 0 | 5 | 18 |
| 6. The importance of positive and negative in experimental design | 0 | 12 | 11 |
| 7. Analytical techniques (ELISA and GC-MS testing) used for detecting mycotoxins | 3 | 14 | 6 |
| 8. Understanding of limits for mycotoxins present in different detection methods | 4 | 11 | 8 |
| 9. Clinical effects of mycotoxins in humans and animals | 1 | 5 | 17 |
| **Total** | **8** | **73** | **126** |

**Connecting to the *Next Generation Science Standards* (NGSS Lead States 2013):**

**Standards**

**HS-ESS3 Earth and Human Activity**

| | **Performance expectations** | |
|---|---|---|
| | The chart below makes one set of connections between the instruction outlined in this article and the *NGSS*. Other valid connections are likely; however, space restrictions prevent us from listing all possibilities. The materials/lessons/activities outlined in this article are just one step toward reaching the performance expectations listed below.<br><br>**HS-ESS3-2.** Evaluate competing design solutions for developing, managing, and utilizing energy and mineral resources based on cost-benefit ratios.<br><br>**HS-ESS3-4.** Evaluate or refine a technological solution that reduces impacts of human activities on natural systems. | |
| **Dimension** | **Name and *NGSS* code/citation** | **Specific connection to classroom activity** |
| Science and Engineering Practices | **Engaging in Argument from Evidence**<br>• Evaluate competing design solutions to a real-world problem based on scientific ideas and principles, empirical evidence, and logical arguments regarding relevant factors (e.g. economic, societal, environmental, ethical considerations). (HS-ESS3-2)<br><br>**Constructing Explanations and Designing Solutions**<br>• Design or refine a solution to a complex real-world problem, based on scientific knowledge, student-generated sources of evidence, prioritized criteria, and tradeoff considerations. (HS-ESS3-4) | Students are able see from the ELISA tests that not all samples from the same source are uniform and that several trials of testing are needed and that other methods, such as the analytical GC/MS, may be better for testing for mycotoxins.<br><br>The data the students gathered from the ELISA test is used in a discussion of the potential sources of error when using the test and how reliable the test is and what people should do to get an accurate sampling representation. |
| Disciplinary Core Ideas | **Developing Possible Solutions**<br>• When evaluating solutions, it is important to take into account a range of constraints, including cost, safety, | Students discuss the pros and cons of using certain technologies to quantify mycotoxins and how certain groups of people may prefer one over the other. |

| | | |
|---|---|---|
| | reliability, and aesthetics, and to consider social, cultural, and environmental impacts. (secondary to HS-ESS3-2),(secondary HS-ESS3-4) | |
| Crosscutting Concept | **Science Addresses Questions About the Natural and Material World**<br>• Science and technology may raise ethical issues for which science, by itself, does not provide answers and solutions. (HSESS3-2)<br><br>---<br>**Influence of Science, Engineering, and Technology on Society and the Natural World**<br>• Analysis of costs and benefits is a critical aspect of decisions about technology. (HS-ESS3-2) | Mycotoxin contamination is presented and illustrates how these toxins have a significant impact on feed and food products, yet there are still sampling issues and different regulations on mycotoxin concentrations for different domestic animals and humans.<br><br>Students are made aware of the pros and cons of certain mycotoxin testing technologies |

# Sample Sign Up Sheet

| Sample Number | Sample Name | Group # | Group # |
|---|---|---|---|
| 1 | White Rice | | |
| 2 | Corn Meal | | |
| 3 | White Flour | | |
| 4 | Ground Corn Field Sample | | |
| 5 | Ground Sorghum Field Sample | | |
| 6 | Fritos | | |
| 7 | Tortilla Chips | | |
| 8 | Bran Cereal | | |
| 9 | Grape Nuts Cereal | | |
| 10 | Wheat Thins | | |
| 11 | Animal Feed- Corn Glucose | | |
| 12 | Dog Food | | |
| 13 | Wheat | | |
| 14 | Barley | | |
| 15 | Organic Flour | | |

**Objective:**

**Hypothesis:**

**Experimental Design:**

**Table 1.** Samples for Testing with ELISA Kit

| Sample Number | Sample Name |
|---|---|
| ***NEOGEN Control*** | **1ppm DON** |
| ***Control 1*** | **Trilogy Wheat** |
| ***Control 2*** | **Trilogy Barley** |
| 1 | White Rice |
| 2 | Corn Meal |
| 3 | White Flour |
| 4 | Ground Corn Field Sample |
| 5 | Ground Sorghum Field Sample |
| 6 | Fritos |
| 7 | Tortilla Chips |
| 8 | Bran Cereal |
| 9 | Grape Nuts Cereal |
| 10 | Wheat Thins |
| 11 | Animal Feed- Corn Glucose |
| 12 | Dog Food |
| 13 | Wheat |
| 14 | Barley |
| 15 | Organic Flour |

1. Each group must test the ***control samples*** seen above in Table 1. Identify the positive and negative control.
   Trilogy Wheat: _____
   Trilogy Barley: _____

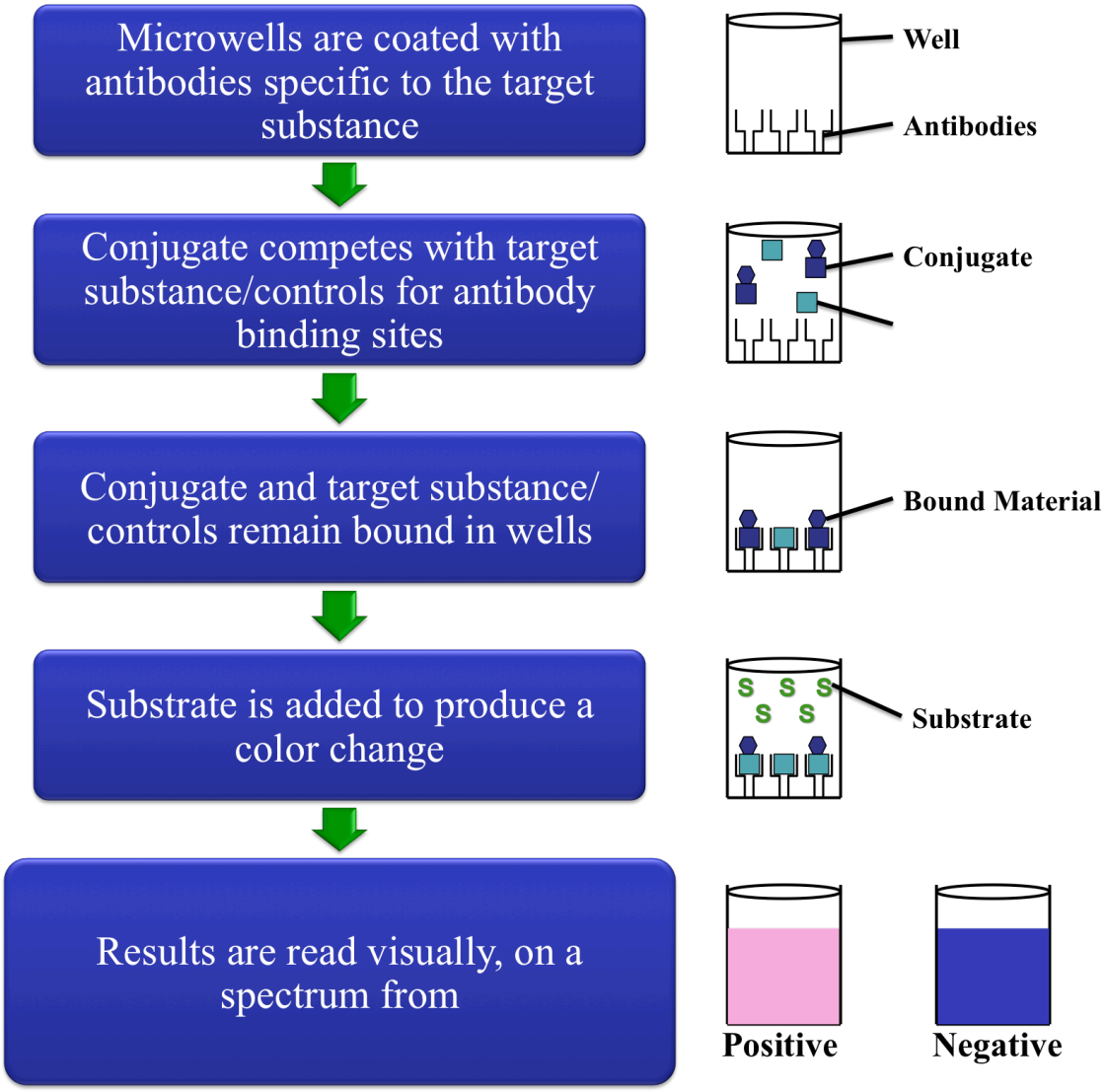
2. Each group must sign up for 2 other unknown samples on a sheet in the front of the classroom, each sample can only be tested twice (signups on a first come first serve basis). List the unknown samples that your group is testing in Table 2.

**Table 2.** Unknown Samples Being Tested

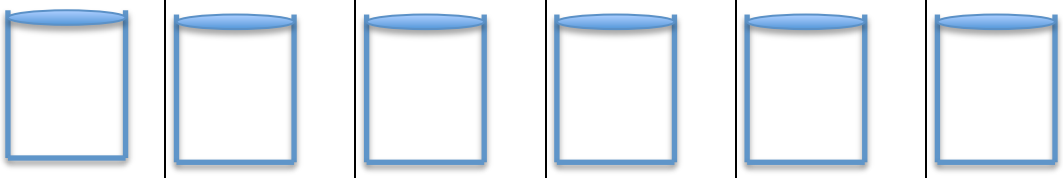
| Sample Number | Sample Name |
|---|---|
| | |
| | |

3. Weigh out 1g of each ground sample and transfer into **labeled** 15mL vials.
4. Measure 5mL of water in a graduated cylinder and add to the sample vials and shake vigorously for 3 minutes making sure it is well mixed.
5. Place vials in rack and let them settle and separate for 10-15 minutes or until there is enough supernatant. While samples are settling, set up extraction funnels and filter paper.
6. When needed separation is present in the vials, using the 200µL-1mL pipette, extract the supernatant and filter it in the extraction setup.
7. Label red mixing wells and clear wells with following conventions
    i. "C": NEOGEN Control
    ii. "+": positive control
    iii. "-": negative control
    iv. "unknown #": unknown sample number
    v. "unknown #": unknown sample number
    vi. "unknown #": unknown sample number
8. After extractions have filtered, red-mixing wells will be used.
9. Swirl the blue labeled conjugate reagent bottle, set pipette to 100µL and add reagent to each well. Discard the pipette tip.
10. Using a new pipette tip, add 100µL of the NEOGEN control from the yellow labeled bottle to the well labeled "C". Pipette this up and down to make sure conjugate and sample are well mixed. Discard tip.
11. Using a new pipette tip, add 100µL of the first extraction sample into it's corresponding labeled well and pipette this up and down to mix. Repeat this for all 5 extraction samples. *Make sure to change the pipette tip between each sample.*
12. Using a new tip for each, transfer 100µL from each red marked well into the corresponding antibody coated (clear) well.
13. Mix by sliding the wells back and forth on a flat surface for 10-20 seconds without splashing reagents. Wait 5 minutes.
14. Initial reaction is complete. Shake out the contents of the antibody-coated wells.
15. Fill each well with distilled water and shake out 5 times. After shaking out the last time, remove all droplets by turning wells upside down and tapping on paper towel.
16. Swirl the green labeled substrate reagent bottle and add 100µL of reagent to each well. Discard the tip.
17. Mix by sliding the wells back and forth on a flat surface for 10-20 seconds without splashing reagents. Wait 5 minutes.
18. Swirl the red labeled Red Stop reagent and add 100µL of the reagent to each well. Mix by sliding on flat surface and observe color change.

*Adaptations made from *NEOGEN Agri-Screen for DON kit manual*
The science behind the ELISA assays



**Results:**

| **Sample Name** | | | | | | |
|---|---|---|---|---|---|---|
| **Observation** |  | | | | | |
| **Interpretation** | | | | | | |

1. Compare your controls to another group. Do they differ? If so, what are some reasons? Could this affect how you interpret your results of unknown samples?

2. Were the results what you expected? Did they match your hypothesis? Why is making a hypothesis in the beginning of an experiment design important?

3. Possible sources of error in experiment:

4. Ways to improve this experiment in the future:

# CHAPTER VI

## Future Directions

Research opportunities that would continue to reduce DON contamination should be geared toward: (1) identifying individual isolates of microorganisms responsible for the conversion of DON to 3-keto-DON from mixed cultures, (2), testing the isolated microbes for stability and robustness of DON modification, (3) testing isolated microbes on naturally contaminated wheat and barley, (4) testing gene(s) responsible for DON conversion with naturally contaminated wheat and barley samples in small scale fermentation assays.

Since the 1980s, biological transformation of DON has been studied and only a few pure isolates of microorganisms have been identified (Shima et al., 1997; Fuchs et al., 2002; Young et al., 2007; Yu et al., 2010; Ikunaga et al., 2011; Sato et al., 2012; Ito et al., 2012). Isolating DON-transforming microorganisms has proven difficult due to restrictions associated with mechanism and growth such as: (1) the initial source material being diverse and microbial populations being very large (Fuchs et al., 2002; Yu et al., 2010); (2) growth or function of DON-transforming microorganisms may be inhibited by microorganisms (Yu et al., 2010); (3) common laboratory media or culture conditions may not be favorable for the growth or function of DON-transforming isolates (Fuchs et al., 2002; Yu et al., 2010); 4) the conversion of DON may be an induced by the presence of mycotoxins and the activity could be unstable without any mycotoxins (Ikunaga et al., 2011; Sato et al., 2012; Ito et al., 2012); or 5) multiple microorganisms could be responsible for the metabolism or conversion of DON (Sato et al., 2012). Further experiments to isolate microorganisms from mixed cultures should take into consideration the above factors.

Upon identifying single isolates of microorganisms, additional experiments should be performed to test for the stability and robustness of the microorganisms to modify DON. It is critical that individual microbes consistently modify DON for use in industrial applications such as a biological control agent. After testing robustness of the microorganisms, further experiments would need to be conducted to determine if pure isolates can modify DON in naturally contaminated wheat and barley samples. Using naturally contaminated samples will provide a better understanding if the pure isolates will be beneficial for industrial purposes. Once confirmed that isolated microbes can modify DON with naturally contaminated samples, these

microorganisms could be incorporated into a grower's fungicide regime to eliminate the toxicity of DON, which would increase the value of the crop.

Isolated microorganisms should be tested for function on crops to detoxify or modify DON; however, if microorganisms prove unstable or not functional, detoxification enzymes could be purified and produced for to spray alongside traditional fungicides. Further research with enzymes and genes that detoxify DON is needed. Characterization and purification of detoxification enzymes and genes can elucidate enzyme properties and kinetics. Library fragments from microorganisms can be used as a source for enzymes that detoxify DON. Additional experiments need to be conducted to test microbial library fragments in DON and the efflux pump inhibitor via culturing and sub-culturing methods. The cell free protein synthesis system can be utilized to test fragments capable of modifying or detoxifying DON.

Detoxification genes can be cloned and expressed in crops, such as wheat, barley, and corn to develop resistant varieties or which detoxify DON as well as cloned and expressed in commercial ethanol yeast strains to detoxify or modify DON in dried distillers grain (DDG) material. Such methods would prevent DON from being incorporated into human and animal foods and feeds. Therefore, it is essential that genes identified genes and enzymes be tested with naturally contaminated wheat or barley samples in small scale fermentation assays. Detoxification enzymes have tremendous potential to eliminate DON in crops and make food and feed safe to consume.

**REFERENCES**

Fuchs, E., Binder, E. M., Heidler, D., and Krska, R. 2002. Structural characterization of metabolites after the microbial degradation of type A trichothecenes by the bacterial strain BBSH 797. Food Additives & Contaminants 19: 379-386.

Ikunaga, Y., Sato, I., Grond, S., Numajiri, N., Yoshida, S., Yamaya, H., Hiradate, S., Hasegawa, M., Toshima, H., Koitabashi, M., Ito, M., Karlovsky, P., and Tsushima, S. 2011. Nocardioides sp. Strain WSN05-2, isolated from a wheat field, degrades deoxynivalenol, producing the novel intermediate 3-epi- deoxynivalenol. Applied Microbiology and Biotechnology 89: 419–427.

Ito, M., Sato, I., Koitabashi, M., Yoshida, S., Imai, M., and Tsushima, S. 2012. A novel actinomycete derived from wheat heads degrades deoxynivalenol in the grain of wheat and barley affected by Fusarium head blight. Applied Microbiology and Biotechnology 96: 1059-1070.

Sato, I., Ito, M., Ishizaka, M., Ikunaga, Y., Sato, Y., Yoshida, S., Koitabashi, M., Tsushima, S. 2012. Thirteen novel deoxynivalenol-degrading bacteria are classified within two genera with distinct degradation mechanisms. FEMS Microbiology Letters 327: 110–117.

Shima, J., Takase, S., Takahashi, Y., Iwai, Y., Fujimoto, H., Yamazaki, M., and Ochi, K. 1997. Novel detoxification of the trichothecene mycotoxin deoxynivalenol by a soil bacterium isolated by enrichment culture. Applied and Environmental Microbiology 63: 3825-3830.

Yu, H., Zhou, T., Gong, J. H., Young, C., Su, X. J., Li, X-Z., Zhu, H. H., Tsao, R., and Yang, R. 2010. Isolation of deoxynivalenol-transforming bacteria from the chicken intestines using the approach of PCR-DGGE guided microbial selection. BMC Microbiology 10: 182-191.