Assessment of aflatoxin presence in the Rwandan feed and milk value chains and mitigation potential using high voltage atmospheric cold plasma

by

Kizito Nishimwe

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

Program of Study Committee:
Dirk E. Maier, Major Professor
   Kevin Keener
   Gary Munkvold
   Wilson Rumbeiha
   Charles Hurburgh
   Hugo Ramirez-Ramirez

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2020

Copyright © Kizito Nishimwe, 2020. All rights reserved.
# TABLE OF CONTENTS

Page

LIST OF FIGURES ........................................................................................................ iv

LIST OF TABLES ........................................................................................................ vi

NOMENCLATURE ....................................................................................................... vii

ACKNOWLEDGMENTS .............................................................................................. x

ABSTRACT ................................................................................................................ xi

CHAPTER 1. GENERAL INTRODUCTION ................................................................ 1
  Introduction .............................................................................................................. 1
  Thesis Organization ................................................................................................. 3
  References ................................................................................................................ 4

CHAPTER 2. RESEARCH OBJECTIVES ..................................................................... 6

CHAPTER 3. A REVIEW OF MYCOTOXIN DECONTAMINATION METHODS AND
  LEARNED LESSONS FROM AN ASSESSMENT OF THE RWANDAN FEED VALUE
  CHAIN ....................................................................................................................... 7
  Abstract ................................................................................................................... 7
  Introduction .............................................................................................................. 7
  Current intervention technologies to decontaminate mycotoxins in cereals .......... 15
  Learned lessons from an assessment of the Rwandan feed value chain .............. 26
  References .............................................................................................................. 29

CHAPTER 4. ASSESSMENT OF AFLATOXIN AND FUMONISIN CONTAMINATION
  AND ASSOCIATED RISK FACTORS IN FEED AND FEED INGREDIENTS IN
  RWANDA .................................................................................................................. 38
  Abstract ................................................................................................................... 38
  Introduction .............................................................................................................. 39
  Materials and Methods .......................................................................................... 43
  Results ..................................................................................................................... 50
  Discussion ................................................................................................................. 56
  Conclusions .............................................................................................................. 62
  References .............................................................................................................. 64

CHAPTER 5. PRELIMINARY SURVEY OF AFLATOXIN M1 CONTAMINATION IN
  RAW MILK IN DAIRY FARMS USING FEED INGREDIENTS FROM RWANDA ....... 70
  Abstract ................................................................................................................... 70
  Introduction .............................................................................................................. 71
  Materials and Methods .......................................................................................... 75
  Results ..................................................................................................................... 79
  Discussion ................................................................................................................. 82
Conclusions ................................................................. 85
References ............................................................. 86

CHAPTER 6. EFFICACY OF HIGH VOLTAGE ATMOSPHERIC COLD PLASMA TO DEGRADE AFLATOXIN B1, B2, G1, AND G2 ................................................................. 90
Abstract ................................................................. 90
Introduction .............................................................. 91
Materials and methods .................................................. 93
Results ........................................................................ 96
Discussion .................................................................. 103
Conclusions ............................................................. 105
References ............................................................. 106

CHAPTER 7. IN VITRO CYTOTOXICITY ASSESSMENT OF AFLATOXIN B1 DEGRADATION PRODUCTS AFTER HIGH VOLTAGE ATMOSPHERIC COLD PLASMA TREATMENT USING HEPG2 CELL LINE ................................................................. 109
Abstract ................................................................. 109
Introduction .............................................................. 110
Material and methods .................................................. 112
Results and Discussion .................................................. 117
Conclusions ............................................................. 124
References ............................................................. 125

CHAPTER 8. DEGRADATION OF AFLATOXIN IN SHELLED MAIZE INOCULATED WITH ASPERGILLUS FLAVUS USING HIGH VOLTAGE ATMOSPHERIC COLD PLASMA (HVACP) AND HVACP EFFECTS ON LIPID OXIDATION AND COLOR CHANGE IN MAIZE ................................................................. 129
Abstract ................................................................. 129
Introduction .............................................................. 129
Material and methods .................................................. 131
Results ........................................................................ 136
Discussion .................................................................. 140
Conclusions ............................................................. 142
References ............................................................. 143

CHAPTER 9. SUMMARY AND RECOMMENDATIONS ........................................... 146

APPENDIX 1 DAIRY FARMERS: CHECKLIST AND QUESTIONNAIRE ................. 151
APPENDIX 2 POULTRY FARMERS: CHECKLIST AND QUESTIONNAIRE ............ 155
APPENDIX 3 ANIMAL FEED VENDORS: CHECKLIST AND QUESTIONNAIRE .... 159
APPENDIX 4 ANIMAL FEED PROCESSORS: CHECKLIST AND QUESTIONNAIRE .... 165
APPENDIX 5 COMPARISON OF METHODS FOR AFLATOXIN DETECTION .......... 170
APPENDIX 6 POLICY BRIEF - BREAKING AFLATOXIN CONTAMINATION CYCLE. 171
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1</td>
<td>Aflatoxin chemical structures</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Chemical structure of fumonisin B1 and Sphingosine</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Molecule structure of ZEA</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Chemical structure of DON</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Chemical Structure of OTA</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Ammoniation of aflatoxin [adapted from Piva et al., 1995]</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Degradation pathways of AFB1 after ozone treatment</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Proposed AFB1 degradation pathways after UV treatment in aqueous solution</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Two proposed AFB1 degradation pathways after HAVP treatment</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Origin of purchased feed ingredients</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>Map of Rwandan provinces and districts</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Total aflatoxin contamination</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>Total fumonisin contamination</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Total aflatoxin means by sample type</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Total aflatoxin means among geographical districts</td>
<td>55</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>Percentage of milk samples contaminated with AFM1 by province</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>Schematic of the experimental setup of the HVACP system</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>OAS concentration (in parts per million by volume, ppmv)</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>Aflatoxin concentration residues after HVACP treatment</td>
<td>97</td>
</tr>
</tbody>
</table>
Figure 20. AFB1 chromatogram without HVACP treatment ................................................. 98
Figure 21. AFB1 chromatogram after 20 min HVACP treatment ........................................ 98
Figure 22. AFB2 chromatogram without treatment .......................................................... 99
Figure 23. AFB2 chromatogram after 20 min HVACP treatment ................................ .... 99
Figure 24. AFG1 chromatogram without treatment .......................................................... 100
Figure 25. AFG1 chromatogram after 20 min HVACP treatment ................................ .... 100
Figure 26. AFG2 chromatogram without treatment .......................................................... 101
Figure 27. AFG2 chromatogram after 20 min HVACP treatment ................................ .... 101

Chapter 7
Figure 28: Schematic of the experimental setup for HVACP treatment ............................. 114
Figure 29. Percentage of AFB1 remaining residues after HVACP treatment ...................... 118
Figure 30: Percentage of HepG2 cell viability exposed to AFB1 treated with HVACP .......... 120
Figure 31: Caspase-3 activity of hepG2 cells exposed to AFB1 treated with HVACP .......... 121
Figure 32: DNA fragmentation level in HepG2 cells exposed to AFB1 HVACP- treated ..... 122
Figure 33: Carbonyl content levels in HepG2 cells exposed to AFB1 treated with HVACP .... 123

Chapter 8
Figure 34. Schematic of the experimental setup of the HVACP system ............................... 132
Figure 35. OAS concentration (in parts per million by volume, ppmv) ............................... 136
Figure 36. AFB1 levels (µg/kg) in control samples, and maize samples HAVCP-treated ....... 137
LIST OF TABLES

Chapter 4
Table 1. Origin of animal feed among dairy and poultry farmers ........................................ 27
Table 2. Duration of complete feed and feed ingredients among feed value chain actors ....... 29
Table 3. Aflatoxin and fumonisin mean, standard deviation (SD) and median values in feed .... 50
Table 4. Socio-demographic characteristics of study participants......................................... 58

Chapter 5
Table 5. Analysis of AFM1 in milk samples across different provinces................................. 80
Table 6. Aflatoxin contamination in dairy feed........................................................................ 81

Chapter 6
Table 7. Aflatoxin and degradation products obtained using Q-TOF LC/MS ......................... 102

Chapter 8
Table 8. Reduction in *A. flavus* CFU/ g maize after HVACP treatment ............................ 138
Table 9. Measurement of color indicators before and after HVACP treatment . ................. 139
Table 10. PV and TBARS values measured before and after HVACP treatment ................. 140
# NOMENCLATURE

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>Aflatoxin B1</td>
</tr>
<tr>
<td>AFB2</td>
<td>Aflatoxin B2</td>
</tr>
<tr>
<td>AFG1</td>
<td>Aflatoxin G1</td>
</tr>
<tr>
<td>AFG2</td>
<td>Aflatoxin G2</td>
</tr>
<tr>
<td>AFM1</td>
<td>Aflatoxin M1</td>
</tr>
<tr>
<td>AFU</td>
<td>Arbitrary Fluorescence Unity</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ASF</td>
<td>Animal Source Foods</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>AU</td>
<td>Africa Union</td>
</tr>
<tr>
<td>CAADP</td>
<td>Comprehensive Africa Agriculture Development Program</td>
</tr>
<tr>
<td>CP</td>
<td>Cold Plasma</td>
</tr>
<tr>
<td>DBD</td>
<td>Dielectric Barrier Discharge</td>
</tr>
<tr>
<td>DBE</td>
<td>Double Bond Equivalent</td>
</tr>
<tr>
<td>DFF</td>
<td>DNA Fragmentation Factor</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNPH</td>
<td>2,4-dinitrophenylhydrazine</td>
</tr>
<tr>
<td>DP</td>
<td>Degradation of Product</td>
</tr>
<tr>
<td>DVO</td>
<td>District Veterinary Officers</td>
</tr>
<tr>
<td>EAC</td>
<td>East Africa Community</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EC</td>
<td>European Commission</td>
</tr>
<tr>
<td>EGTOP</td>
<td>Expert Group for Technical Advice on Organic Production</td>
</tr>
<tr>
<td>ELEM</td>
<td>Equine Leukoencephalomalacia</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ERs</td>
<td>Estrogen Receptors</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization of the United Nations</td>
</tr>
<tr>
<td>FB1</td>
<td>Fumonisin B1</td>
</tr>
<tr>
<td>FB2</td>
<td>Fumonisin B2</td>
</tr>
<tr>
<td>FB3</td>
<td>Fumonisin B3</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HSD</td>
<td>Honestly Significant Difference</td>
</tr>
<tr>
<td>HVACP</td>
<td>High Voltage Atmospheric Cold Plasma</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>LC-MS-MS</td>
<td>Liquid Chromatography with tandem Mass Spectrometry</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of Detection</td>
</tr>
<tr>
<td>MD</td>
<td>Median</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NISR</td>
<td>National Institute of Statistics of Rwanda</td>
</tr>
<tr>
<td>OAS</td>
<td>Optical Absorption Spectroscopy</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>OTA</td>
<td>Ochratoxin A</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PACA</td>
<td>Partnership for Aflatoxin Control in Africa</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PPE</td>
<td>Porcine Pulmonary Edema</td>
</tr>
<tr>
<td>QCM</td>
<td>Quality Control Materials</td>
</tr>
<tr>
<td>QTOF</td>
<td>Quadrupole and Time-of-Flight technology</td>
</tr>
<tr>
<td>RGS</td>
<td>Reactive Gas Species</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>UHT</td>
<td>Ultra High Temperature</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ZEA</td>
<td>Zearalenone</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

I would like first to thank my beloved family: lovely wife, Christine Mwubahamana; daughter, Raena; mother, Marthe Kamuyumbu; and sisters, Sophie Uwizera, Honorine Nyiramahoro, and Linda Iradukunda. Thank you for your support and encouragement throughout the PhD journey.

My major professor, Dr. Dirk Maier, I will always be grateful for being part of your research group. I would like to express my gratitude for your leadership and mentorship during the 3+ years passed at Iowa State University. Please, accept my heartfelt and warmest thanks.

I would like to thank my committee members, Dr. Kevin Keener, Dr. Gary Munkvold, Dr. Wilson Rumbeiha, Dr. Charles Hurburgh, and Dr. Hugo Ramirez-Ramirez for their guidance and support throughout the course of this research.

Sincere thanks to Dr. Erin Bowers, thank you for guidance, technical support and a strong listening ear. To Dr. Carl Bern, thank you for your time and inputs in this work.

To the current members of Dr. Maier’s research group: Sam Cook, Roger Guy, Michelle Friedmann, Hory Chikez, Mike Sserunjogi, and Ma Cristine Ignacio, thank you for your availability and assistance.

Lastly, this work could not have been completed without the support of different labs. I am very thankful to Veterinary Diagnostic Lab, Dr. Reddy’s lab, Dr. Boylston’s lab, Dr. Munkvold’s lab, Dr. Keener’s lab, and Dr. Anatharam’s lab. Many thanks to Isaac Agbemafle for helping me set up and optimize cell culture and bioassay experiments. Thank you for your technical advice and your guidance in completing my lab experiments.

I am thankful to the Borlaug Higher Education for Agricultural Research and Development (BHEARD) program for funding my PhD fellowship at Iowa State University (ISU), USA and my home university, the University of Rwanda, for providing the necessary support to complete my PhD.
ABSTRACT

Mycotoxins, particularly aflatoxins, are fungal metabolites, and under favorable growth conditions contaminate crops and animal feeds. They are associated with liver cancer, immunosuppression, and growth impairment. Human exposure to these mycotoxins is the result of ingestion of contaminated foods, or indirectly from consumption of animal source foods (e.g., dairy products and eggs) derived from animals previously exposed to aflatoxins in feeds. They also have an economic impact because contamination levels of aflatoxins and other mycotoxins in foods and feedstuffs can lead to rejection of these products in international markets.

However, there has been a lack of data on the presence of aflatoxins in the food and feed supply of Rwanda. The absence of published data on the presence of aflatoxins in Rwandan feeds raises the possibility of underestimating the present risk, and missing opportunities to reduce the risk of mycotoxin contamination of animal source food (ASF), particularly milk, via consumption of contaminated animal feeds by livestock. The overall objective of this study was to assess the prevalence of aflatoxin and fumonisin contamination and associated risk factors in feed and feed ingredients, and aflatoxin M1 contamination in raw milk in Rwanda, and to explore the potential of high voltage atmospheric cold plasma (HVACP), a low-cost technology in its nascent form, to mitigate aflatoxin contamination of maize, a major ingredient of feeds in Rwanda.

In total, 3328 feed and feed ingredient samples from the feed value chain in Rwanda were analyzed for aflatoxins and fumonisins using Enzyme-Linked Immunosorbent Assay (ELISA). Mean aflatoxin levels of 108.83 µg/kg (Median (MD): 43.65 µg/kg), 103.81µg/kg (MD: 48.4 µg/kg), 88.64 µg/kg (MD: 30.90 µg/kg) and 94.95 µg/kg (MD: 70.45 µg/kg) were determined for dairy farmers, poultry farmers, feed vendors and feed processors, respectively. Mean
Fumonisin levels were 1.52 mg/kg (MD: 0.71 mg/kg), 1.21 mg/kg (MD: 0.56 mg/kg), 1.48 mg/kg (MD: 0.76 mg/kg) and 1.03 mg/kg (MD: 0.47 mg/kg) for dairy farmers, poultry farmers, feed vendors and feed processors, respectively. Aflatoxin contamination was significantly affected by time of sampling and district from which feed samples originated (p<0.05). Fumonisins did not show any correlation trends. Ninety-two percent of survey participants were unaware of aflatoxins and fumonisins and their adverse effects.

In total, 170 raw milk samples were collected during one sampling period; the mean AFM1 concentration in these samples was 0.89 ± 1.64 µg/L (median: 0.33 µg/L) with a maximum of 14.5 µg/L. Ninety-one percent of milk samples exceeded 0.05 µg/L (the European legal limit) and 38% of samples exceeded 0.5 µg/L, the legal limit and maximum level established by the U.S. Food and Drug Administration (USFDA) and Codex Alimentarius, respectively.

HVACP treatment significantly degraded pure AFs (each AF individually) powder on a glass slide exposed directly at 85 kV and 180 W (60 Hz) at laboratory conditions (25°C, 80-90 % relative humidity) for 2, 5, 10, and 20 min using air. The electrode gap distance was maintained at 4.44 cm. After the treatment, AFs were extracted with methanol and the final concentration was 200 µM. AFB1 and AFG1 were more susceptible decreasing by 90% and 74% after 2 min, respectively comparing to the non-treated samples (0 min). After 10 and 20 min, AFG1 was not detected in samples. AFB2 and AFG2 were less susceptible decreasing by 38% and 79% after 20 min, respectively. Despite observed degradations, only one AFB1 DP (m/z: 331.08) was characterized presumably due to the limit of quantification of the technique used (< 10 mg/kg). AFB1 DP resulting in hydroxylation of the double bond in the position C8-C9 AFB1 was previously reported in published studies.
HepG2 cells, a human hepatoma cell line, were exposed to AFB1 treated at 85 kV with HVACP for 0, 2, 5, 10, and 20-minute periods for 72 hours (37°C, 5% CO₂). After HVACP treatment, AFB1 was extracted with DMSO (100 mL) and added to DMEM media (9.9 mL) for 100 µM AFB1 final concentration. AFB1 endpoint toxicities, i.e., cell viability (MTT assay using 1×10⁵ cells/mL), caspase-3 activity (2×10⁷ cells/mL), DNA fragmentation (1×10⁵ cells/mL), and protein carbonyl (80% confluent in 75-cm² cell culture flask), were assessed for each treatment time. After 10 and 20 min treatment, cell viability in HepG2 cells exposed to AFB1 DP did not show a significant difference compared to non-exposed HepG2 cells (negative control) (p>0.05) but significantly differed from HepG2 cells exposed to AFB1 (positive control) (p<0.05). Caspase-3 activity, DNA fragmentation values, and carbonyl contents in HepG2 cells exposed to AFB1 DP after 20 min treatment were similar to those in non-exposed HepG2 cells (p>0.05) but significantly differed from those in HepG2 cells exposed (p<0.05).

Maize kernels (25 g) inoculated with A. flavus spores (10⁶ spores/mL) were incubated for two weeks and treated with HVACP directly and indirectly at 85 kV and 180 W (60 Hz) at laboratory conditions (25°C, 80-90 % relative humidity) for 7, 15, and 30 min. HVACP significantly reduced AFB1 in all treatment times. After 7 and 15 min, AFB1 decreased by 81.3 - 96.3 % and 78.1 – 85.0%, respectively. The 30 min treatment showed AFB1 decreasing from below the LOQ (< 5 µg/kg) to 88.9% ± 10.3. HVACP treatment inhibited A. flavus growth significantly reducing it by 2.4 and 1.9 log₁₀ for direct and indirect exposure, respectively. A. flavus reduction was below the limit of detection for the 15 and 30 min treatments. HVACP treatment resulted in a slight change in yellow color but neither induced lipid oxidation nor changed flavor profile in treated maize samples.
As a result of this research, it was concluded that HVACP is a potential technology to mitigate AF in maize destined as an ingredient in the Rwandan feed supply chain without influencing organoleptic or nutrient characteristics. Future investigations are needed to quantify HVACP performance to degrade aflatoxins as a function of electric power, voltage, gap size between electrodes, exposure time, relative and absolute air humidity, plasma reactive species completely enveloping maize kernel surfaces, and maize moisture content without negatively affecting any organoleptic properties, other quality characteristics, and end use value of treated maize.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Aflatoxins and fumonisins are public health concerns worldwide, particularly in Africa, through the contamination of commodities and animal feeds but also because they have a negative economic impact. To mitigate aflatoxin contamination, the African Union, through the Comprehensive Africa Agriculture Development Programme (CAADP), a policy framework for agricultural transformation, has established aflatoxin mitigation as a high priority research area, and initiated the Partnership for Aflatoxin Control in Africa (PACA) (PACA, 2018). However, in many countries, particularly in Rwanda, data determining mycotoxin incidence and severity in food, feed, and animal source foods (ASF) are lacking. This dissertation research focused on the assessment of aflatoxin and fumonisin levels along with exploring one aflatoxin mitigation technology for treating contaminated maize, a major animal feed ingredient in Rwanda.

Aflatoxins and fumonisins cause several negative human health impacts. Mycotoxin contamination above threshold levels renders foods and feeds unsafe for human and animal consumption, and when detected is rejected by local and international markets (Bandyopadhyay et al., 2007). In addition to health concerns, aflatoxins and fumonisins have a negative impact on trade by reducing end use value of contaminated crops. For example, it was estimated that Africa lost $670 million annually in trading with Europe due the European Union mycotoxin threshold regulations. Europe has the stringent regulations for aflatoxin in grains and nuts; 0.02 µg/kg for aflatoxin B1 (AFB1) and 0.04 µg/kg for
total aflatoxins. While Africa contributed more than 90% of the supply to international markets for peanuts during the 1960s, the implementation of strict AF regulations by the EU and other countries have reduced the contribution to less than 5% (Xiong & Beghin, 2012).

Aflatoxins pose significant social and economic problems for Africa in general (Bankole et al., 2008) and more particularly for countries like Rwanda that have put in place programs of crop intensification, particularly cereals to ensure the achievement of food security and rural poverty alleviation (Cantore, 2011). Occurrence in milk is another concern. Milk production is estimated at 17.5 million Liters in 2017-18 (NISR, 2018). This is due to the Rwandan government effort to combat malnutrition through different programs, i.e., “Girinka” and “Inkongoro” (literally translated “May you have a cow” and “One cup per child”) intended for vulnerable families in order not only to increase incomes but also to reduce malnutrition.

While there has been lack of data on the presence of aflatoxins and fumonisins in the food and feed supply of Rwanda, it is noted that in the East Africa region where data are available, as in Kenya (Owaga et al., 2011), Tanzania (Kimanya et al., 2010) and Uganda (Lukwago et al., 2019), aflatoxins pose a severe concern to public health. In 2005, aflatoxicosis was observed in Kenya with 317 cases of acute hepatic failure in eastern Kenya, and 125 persons died after consuming maize highly contaminated with aflatoxins (Lewis et al., 2005). Gizachew et al. (2016) reported that all samples of animal feeds
collected in the Greater Addis Ababa milk shed of Ethiopia were contaminated with aflatoxins. The milk from that region was also contaminated with Aflatoxin M1 (AFM1).

The absence of published data on the presence of aflatoxins and fumonisins in Rwandan feeds raises the possibility of underestimating the present risk, and missing opportunities to reduce the risk of aflatoxin and fumonisin contamination of animal source food (ASF), particularly milk, due to contaminated animal feeds. It also places a constraint on the development of policies to mitigate aflatoxin and fumonisin contamination and limits the growth of commercial markets and trade because Rwandan feedstuffs are contaminated with aflatoxins and fumonisins. In addition, there is no single approved technology to decontaminate aflatoxin contaminated crops, and there is certainly a need to find an affordable, safe, and environmentally friendly technology which can help decontaminate aflatoxins.

The Food and Agriculture Organization (FAO) of the United Nations has set standards for aflatoxins in food and feed (codex stand 193 – 1995). For instance, the standard is 10 µg/kg for read-to-eat almonds, peanuts and pistachios, and 0.5 µg/kg in milks (FAO/WHO, 2015).

**Thesis Organization**

This dissertation comprises nine chapters. The first chapter describes the aflatoxin contamination context in the East African Region, and in Rwanda, particularly. The second chapter highlights research objectives. The third chapter is a review on current methods that
are used to degrade aflatoxins in food commodities, their potentials, and their limitations, as well. Chapter 4 discusses the aflatoxin and fumonisin contamination in feeds and feed ingredients, and associated risk factors. Findings were published in the Toxins Journal (K. Nishimwe et al., 2019). AFM1 contamination in raw milk in dairy farms using feed ingredients is discussed in chapter 5. Chapter 6 explores the high voltage atmospheric cold plasma technology to degrade aflatoxins, while chapter 7 comprises the in vitro cytotoxicity for AFB1. Investigations on the HVACP capacity to degrade aflatoxins in A. flavus inoculated maize kernels and lipid oxidation in treated maize kernels are included in chapter 8. Finally, chapter 9 summarizes all results for this dissertation and gives future research directions stemming from our findings.

References


CHAPTER 2. RESEARCH OBJECTIVES

The overall hypothesis of this dissertation research is that a large portion of animal feeds in Rwanda is contaminated with aflatoxins and fumonisins, that some amount carries into animal source foods such as milk, and that high voltage atmospheric cold plasma is a technology with potential to decontaminate aflatoxins in maize, a major feed ingredient in Rwanda.

Specifically, this research focused on:

1. Assessment of aflatoxin and fumonisin contamination and associated risk factors in feed and feed ingredients in Rwanda (Chapter 4).

2. Assessment of AFM1 contamination in raw milk from dairy farms using feed ingredients in Rwanda (Chapter 5).

3. Efficacy of High Voltage Atmospheric Cold Plasma to degrade aflatoxin B1, B2, G1, and G2 and characterization of degradation products (Chapter 6).

4. In vitro cytotoxicity assessment of aflatoxin B1 degradation products after high voltage atmospheric cold plasma (HVACP) treatment using HepG2 cell line (Chapter 7).

5. Degradation of aflatoxin in A. flavus inoculated maize using High Voltage Atmospheric Cold Plasma (HVACP) and HVACP effects on maize organoleptic properties (Chapter 8).
CHAPTER 3. A REVIEW OF MYCOTOXIN DECONTAMINATION METHODS AND LEARNED LESSONS FROM AN ASSESSMENT OF THE RWANDAN FEED VALUE CHAIN

Abstract

Since aflatoxin discovery in the 1960s, research efforts to minimize mycotoxin contamination have received much attention exploring different methods to decontaminate mycotoxins in foods and feeds. The present review discusses different methods applied to decontaminating mycotoxins and their limitations, and highlights the potential of cold plasma as a future direction to degrade mycotoxins in food and feed commodities and ingredients.

Introduction

Cereals constitute the principal staple food for many people across the globe and are primary animal feed resources. Cereals are high in nutritive values for energy (carbohydrate-based energy) and other nutrients such as proteins (Jespersen & Munck, 2009). Maize\(^1\), wheat, and rice are three major worldwide produced types of cereals, and their productions were estimated at 1134, 771, and 769 million tons, respectively (FAO, 2019a). However, more than one-third of global cereal production is lost or wasted (FAO, 2019b) due to pests, insects, and fungi infestations. For the latter, fungi cause cereal losses either by spoiling cereals and making them unsuitable for human and animal consumption or by producing toxins (secondary metabolites known as mycotoxins) under favorable growth conditions. Mycotoxins are very toxic to humans and animals.

\(^1\) Maize is also known as corn (generic term commonly used in the U.S.A)
Bennet defined mycotoxins as “natural products produced by fungi that evoke a toxic response when introduced in low concentrations to higher vertebrates and other animals by a natural route” (Bennett 1987). This definition excludes mushroom and yeast poisons from the mycotoxin category. The ingestion of mycotoxins generates diseases in humans and animals, known as mycotoxicosis (Gil-serena, Vázquez, & Patiño, 2019). Three dominant fungal genera produce mycotoxins: *Aspergillus*, *Fusarium*, and *Penicillium*. Aflatoxin is produced by *Aspergillus flavus* and *A. parasiticus*, fumonisins by *Fusarium verticilloides*, and *F. proliferatum*. Zearalenone (ZEA) and deoxynivalenol (DON) are produced by *F. graminearum* and *F. culmorum*, and *Penicillium expansum* produces patulin (Alshannaq & Yu, 2017).

Diseases caused by the fungal contamination of cereals were documented since the Middle Age. St. Antony’s fire or ergotism was a disease that resulted from the consumption of rye bread contaminated by *Claviceps purpurea*. The disease symptoms were the central nervous system disorders, gangrene of limbs, and finally death (Van Dongen and de Groot 1995). Aflatoxins were discovered in early 1960 following a mysterious turkey “X” disease in Great Brain. During the outbreak, many turkeys were dying of an unknown disease and investigations linked it to feeds manufactured at one feed mill in London. It was discovered that groundnut meal imported from Brazil was contaminated with fungal toxins, afterward named aflatoxins. More than 100,000 turkey pouls died during the outbreak (Richard, 2008). Other mycotoxins were since discovered. Over 300 mycotoxins are documented, but five are of health and economic importance. There are aflatoxins, fumonisins, zearalenone
(ZEA), deoxynivalenol (DON), and ochratoxin A (OTA) (Marin et al., 2013). Each mycotoxin category will be discussed separately in the following sections.

In the climate change context, with CO\(_2\) (350 ppm versus 650 -1200 ppm), temperature increases (2 -5°C), and drought stress will inevitably have a significant effect on mold growth and mycotoxin production and put at risk susceptible food commodities and feed (Baranyi et al., 2015; Medina et al., 2015; Moretti et al., 2019). In Europe, it was predicted that under the +2°C scenario, aflatoxin contamination in maize would be an issue for food safety (Battilani et al., 2016).

**Aflatoxins**

Aflatoxins are produced mainly by *Aspergillus flavus* and *A. parasiticus* and can contaminate crops, mostly cereals, especially maize, wheat, and rice (Alshannaq & Yu, 2017). *Aspergillus* spp are storage fungi, and maximal growth occurs at 33°C and high a\(_w\) (0.99) in vitro conditions (Holmquist et al.,1983). There are four different types of aflatoxins: aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) (Figure 1). They are characterized by their fluorescence under ultraviolet (UV) light and their retention time in thin-layer chromatography. AFB1 and AFB2 appear “blue,” while AFG1 and AFG2 appear “green.”(IARC, 2012). If dairy cows are fed with AFB1 contaminated feed, AFB1 is metabolized into AFM1 and excreted in milk. Up to 6% of AFB1 is estimated to be converted into AFM1(Iqbal & Asi, 2013).
It is estimated that 4.5 billion people frequently exposed to aflatoxins worldwide (Williams et al., 2004). The International Agency for Research on Cancer (IARC) has classified aflatoxins as carcinogenic to humans (Group 1) and are linked to liver cancer. Humans are exposed to aflatoxins directly by consuming contaminated commodities, or indirectly through contaminated milk if dairy cows are fed contaminated feed (IARC, 2012a). However, dietary exposure to aflatoxins is different in developed and developing countries; one ng/kg body weight (BW) in developing while the mean aflatoxin dietary exposures exceed 100 ng/kg BW in developing countries (WHO, 2018).

![Aflatoxin chemical structures](image)

Aflatoxin B1 \((C_{17}H_{12}O_6)\):
Molecular weight: 312.3

Aflatoxin B2 \((C_{17}H_{14}O_6)\):
Molecular weight: 314.3

Aflatoxin G1 \((C_{17}H_{14}O_7)\):
Molecular weight: 328.3

Aflatoxin G2 \((C_{17}H_{14}O_7)\):
Molecular weight: 330.3

Figure 1: Aflatoxin chemical structures [Adapted from IARC 2012]
Aflatoxins are globally regulated. In the USA, the Food and Drug Administration (FDA) has set 20 µg/kg in food commodities and dairy feed. For other animals, up to 300 µg/kg are allowed in feeds intended for finishing beef cattle (US FDA, 2019). For Codex Alimentarius and the East African Community (EAC), 10 µg/kg was adopted as the aflatoxin legal limit (EAC, 2019; FAO/WHO, 2015). Europe has the most stringent regulations on aflatoxin with 2 µg/kg and 4 µg/kg for AFB1 and total aflatoxins in cereals and groundnuts intended for human consumption (EC, 2006).

**Fumonisins**

Fumonisins are a category of mycotoxins mainly produced by *Fusarium verticillioides* and *F. proliferatum* (Cendoya et al., 2018). *Fusarium* spp. are known as field fungi and commonly occur in wheat and maize (Munkvold, 2017). There are more than 20 different types of fumonisins. Three types of fumonisins dominate, namely fumonisin B1, fumonisin B2, and fumonisin B3. Fumonisin B1 is the most predominant isomer (more than 60% of total fumonisin in contamination of maize) and the most toxic (Voss et al., 2011). In humans, fumonisin B1 toxicity is related to the chemical structure similarities with sphingosine (Figure 2), which is a part of the cell membrane lipids class surrounding nerve cell axons. The process disrupts cell growth and induces cell death (Smith 2018). The international agency for research on cancer (IARC) classifies fumonisin B1 as a possible carcinogen in humans (group 2B) because of inadequate evidence in humans for
carcinogenicity and sufficient evidence in experimental animals for the fumonisin B1 carcinogenicity (IARC, 2002).

Several epidemiological studies have correlated high esophageal cancer incidences associated with the consumption of high fumonisin-contaminated food (Van Der Westhuizen et al. 2003; Ahangarkani, Rouhi, and Azizi 2014; Wang et al. 2000). Another study has linked fumonisin exposure to the neural tube defect increase among Mexican-American women because of the consumption of large quantities of maize, primarily in the form of tortillas, highly contaminated with fumonisins (Missmer et al., 2006). In animals, fumonisins are known to cause equine Leukoencephalomalacia (ELEM) in horses (Voss, Smith, and Haschek 2007), and porcine pulmonary edema (PPE) in pigs (Smith et al. 1999).

![Chemical structure of fumonisin B1 and Sphingosine](image)

Figure 2. Chemical structure of fumonisin B1 and Sphingosine
Zearalenone

Zearalenone (ZEA) is a mycotoxin produced by *Fusarium* spp, namely *F. graminearum*, *F. culmorum*, *F. verticillioides*, *F. sporotrichioides*, *F. semitectum*, *F. equiseti*, and *F. oxysporum* (Figure 3). ZEA contamination in cereals occurs worldwide, mainly in temperate climates because of the *Fusarium* spp predominance in temperate climates. ZEA contamination occurs in the field at low concentration, but increase under storage conditions with high moisture content (greater than 30% - 40%) (Gupta et al., 2018).

ZEA is an endocrine disruptor. ZEA binds to estrogen receptors (ERs), which bind endogenous 17β-estradiol, followed by translocation to the nucleus. The ERs stimulation leads to protein synthesis and clinical signs of hyperestrogenism (Mostrom, 2011). In livestock species, ZEA interferes with the reproduction systems. Swine is the most commonly affected by ZEA effects and they present vulvovaginitis in gilts and sows, delayed estrus, prolapse of the vagina or rectum, mastitis, among other effects (Munkvold et al., 2019).

![Molecule structure of ZEA](image)

Figure 3. Molecule structure of ZEA
Deoxynivalenol

Deoxynivalenol (DON) or vomitoxin belongs to the group B trichothecene, a large group of mycotoxins produced by numerous Fusarium which share a common characteristic of the trichothecenes which is a tetracyclic 12,13-epoxytrichothec-9-ene ring that can be replaced at several positions, resulting in multiple derivative species including (Munkvold et al., 2019) (Figure 4).

The exposure to DON concentration in contaminated food causes vomiting (especially in pigs, the most sensitive species), abdominal discomfort, malaise, diarrhea, and anorexia (Pestka, 2007). Apart from the native (free) form of DON, there is another form known as the “masked” form in which DON is generated in conjugation other biological components. During digestion, the DON masked form could be reactivated in gastrointestinal tract and recover the native DON. The chemical structure of masked DON form also poses a challenge for analytical quantification since it could not be detected but remains toxics (Khaneghah et al., 2018).

Figure 4. Chemical structure of DON
Ochratoxin A

Ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus* and *Penicillium* genera (Figure 5). *Aspergillus ochraceus* is responsible for production of OTA in tropical regions, while *Penicillium verrucosum* produces OTA in temperate regions. OTA is nephrotoxic, genotoxic, carcinogenic, and immunosuppressive in animals (Patial et al., 2018). IARC classified OTA in Group 2B (possibly carcinogenic to humans) (IARC, 1993).

![Figure 5. Chemical Structure of OTA](image)

**Current intervention technologies to decontaminate mycotoxins in cereals**

Mycotoxin contamination in cereals is unavoidable. Since the aflatoxin discovery, research efforts to minimize mycotoxin contamination have received much attention. Temba et al. (2016), Pankaj et al. (2018) and Čolović et al. (2019) have extensively reviewed current technologies used to reduce mycotoxins in grains. Methods to decontaminate mycotoxins include physical, chemical, and biological technologies. Each category of methods will be discussed in the following sections.
• Chemical processing
  ✓ Ammoniation

Ammoniation treatment consists of treating feed material contaminated with mycotoxins in gaseous form (ammonia) or aqueous solution (ammonium hydroxide). The process has shown considerable success in reducing mycotoxin contamination and mycotoxin toxicity (Norred, 1979). The ammonia induces the degradation of AFB1 by hydrolyzing the AFB1 lactone ring to form two major non-toxic compounds, AFD1, and another compound with a loss of the cyclopentenone ring (Figure 6). However, the reaction can reverse if the ammoniation process is carried out under mild temperature, pressure, and gastric acid conditions (Piva et al., 1995). For the latter, this would affect the increase of AFM1 levels in dairy cows compared with the AFB1 quantity revealed analytically (Park, 1993; Piva et al., 1995).

![Figure 6. Ammoniation of aflatoxin (adapted from Piva et al., 1995)](image)

Additionally, the ammonia efficacy varies with the type of mycotoxins because of the chemical structure differences. The application of NH₄OH in feeds to reduce fumonisin toxicity did not show any toxicity decrease in laboratory animals after the
treatment (Čolović et al., 2019). For instance, the ammoniation process did not show any
effect on ZEA degradation in contaminated corn under conditions which do detoxify
aflatoxin-contaminated corn (Bennett et al., 1980). The ammoniation process increases
levels of total and non-protein nitrogen protein, ash, and soluble solids, and reduces level of
Currently, there is no any method approved by the FDA to detoxify aflatoxin
contamination. However, Texas, North Carolina, Georgia and Alabama have allowed the
use of ammoniation in maize and cottonseed under specific state policies. Ammoniated
maize is prohibited from interstate commerce and is subject to labeling and feeding
restrictions (Missouri Extension, 2019).

✓ Nixtamalization

Nixtamalization is the cooking process used for the preparation of maize for tortillas
in some countries like Mexico. Nixtamalization process consists of cooking maize grains in
a water-alkaline agent to soften the pericarp and endosperm in order to facilitate grinding.
(Santiago-Ramos et al., 2018). Guzmán-de-Peña et al. (2004) reported the AFB1
degradation during nixtamalization. Like ammoniation, nixtamalization degrades AFB1 by
opening AFB1 lactone ring and yields a water-soluble salt followed by decarboxylation.
However, the reaction reverts to AFB1 in acidic conditions (Price & Jorgensen, 1985),
which presents a challenge for nixtamalization as a mycotoxin decontamination strategy.
Feed additives

The European Commission (EC) defines mycotoxin-adsorbing agents as “substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action”. Feed additives include mycotoxin-adsorbing agents such as bentonites, montmorillonites, zeolites, and hydrated sodium calcium aluminosilicate (Boudergue et al. 2009). Mycotoxin-adsorbing agents can be mixed with feed diets, which bind mycotoxins in the gastrointestinal location, and reduce mycotoxin bioavailability. There are two different categories of mycotoxin-adsorbing agents: (a) adsorbing agents that bind mycotoxin and decrease their bioavailability, such as inorganic adsorbents (e.g., aluminosilicate clay) and organic adsorbent (e.g., yeast cell wall components of Saccharomyces cerevisiae). Despite the efficacy of mycotoxin-absorbing agents to reduce mycotoxin exposure, they present side effects due to the lack of selectivity, for example, binding essential nutrients (e.g., vitamins and mineral), and decreasing their bioavailability (Boudergue et al. 2009). Also, adsorbing agents can bind veterinary drugs when administered at the same time and subsequently reduce their efficacy (De Mil et al., 2015). The use of adsorbing agents has raised concerns about the practicality and ethics of use in human food as a food safety strategy to reduce mycotoxin contamination (Ahlberg et al., 2019).
• **Biological approach**

The biocontrol approach implies the competitive exclusion of an atoxigenic and toxigenic strains resulting in mycotoxin contamination reduction. The most well-known is the nontoxigenic *Aspergillus flavus*, which was developed at the industrial-scale to compete with toxigenic *A. flavus* in soil during crop growth. Two products using nontoxigenic *A. flavus* are available in commerce. Aflaguard® developed by Syngenta is a biocontrol approved in the USA to combat aflatoxin as preharvest strategy (US EPA, 2004) and Aflasafe®, developed by the International Institute of Tropical Agriculture (IITA), is largely adopted by numerous African countries as a forefront method to reduce aflatoxin contamination in the field (IITA, 2018). However, the efficacy of its practicability has raised concerns. For instance, Pitt (2019) critically discussed the practical use of biocontrol for reducing aflatoxin in maize in Africa. Furthermore, much attention was focused on the selection process, which prevents aflatoxin production and leaves behind other produced mycotoxins by *A. flavus* (e.g., cyclopiazonic acid). Also, the mentioned biocontrols do not affect other fungi producing mycotoxins (i.e., fumonisins), and their efficacy to reduce global mycotoxin contamination is questionable (Kagot et al., 2019).

• **Physical methods**

  ✓ **Grain sorting and cleaning**

  One of factors influencing the fungal growth and mycotoxin production in grains is defects of stored kernels caused by physical damages and/or insect activity. Molds take
advantage of cracked and broken kernels to invade stored grains and consequently produce mycotoxins (Mason, 2018). The remove of fine materials and broken kernels significantly reduce mycotoxin contamination in grains.

The sorting method consists of separating non-damaged (good) kernels from damaged (bad) kernels. Physically separating damaged, distorted and discolored kernels from “healthy” kernels showed to reduce aflatoxin levels significantly (Shi et al., 2014b). Also, Stasiewicz et al. (2017) used a single-kernel optical sorter to remove aflatoxin and fumonisin contaminated kernels from bulk samples and claimed to achieve 83% mean reduction in each toxin. Sorting and cleaning methods are low-cost methods that can be done at a small-scale farm. However, physical methods of cleaning and sorting are non-specific; mycotoxin contamination in individual kernels is very disparate, with a heterogeneous nature, and varies from one kernel to another (Campbell et al., 1986a). Aflatoxin contamination in individual maize kernels to another can range from 100 to 80,000 ng/g (Lee et al., 1980).

✓ Thermal processing

The thermal processing efficacy depends on different parameters: moisture content of the food matrix, toxin concentration, and thermal food processes used. Heat increase needed to achieve mycotoxin effects will have; however, negative effects on food product quality. In their study, Hale & Wilson (1979) showed that heating aflatoxin-contaminated maize at 160 - 180°C reduced total aflatoxins from 383 μg/kg to 60 μg/kg aflatoxin concentration,
but also reduced lysine and methionine, two essential amino acids, by 30 and 21%, respectively.

Investigations on microwave heating to reduce mycotoxins were shown to be slightly more effective (5-8%) than conventional heating to degrade AFB1 (Pankaj et al., 2018a). The degradation rate is proportional to microwave power and exposure time; the degradation increases with the increases of microwave power and exposure time (Farag et al., 1996).

✓ Nonthermal processing

➢ Ozone

Ozone is generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA) (US FDA, 2019). Ozone is a powerful oxidant gas and can inactivate fungi producing mycotoxins. Ozone has shown the efficacy to degrade aflatoxins with an electrophilic attack on the C8-C9 double bond of the furan ring, resulting in less toxic degradation products (Freitas-Silva & Venâncio, 2010). Luo et al. (2014) proposed degradation pathways after ozone treatment. They identified six degradation products with degradation of the furan ring (Figure 7). Despite the promising results, the application of ozone to reduce mycotoxins in commodities is limited by its cost-effectiveness at large scale. If maize is treated in a screw conveyor (θ=10.2 cm) at 200 -300 bu/h, the needed ozone would be 9788 - 14,666 g/h, much higher than what is currently available commercially (2000 g/h) (McDonough et al., 2011).
Figure 7. Degradation pathways of AFB1 after ozone treatment [adapted from (Luo et al., 2014)]

- **Radiation**

  Radiation includes ionizing (x-rays, ultraviolet [UV] rays, gamma-rays, electron beam) and non-ionizing radiations (microwave, infrared, radio waves, visible rays) (Pankaj et al., 2018a). The following discussion will focus on UV and gamma irradiation capacity to degrade mycotoxin since non-ionizing radiations (i.e., microwave) was discussed in the thermal processes.

- **Gamma irradiation**

  Radio-isotopes such as Cobalt-60 and Cesium-137 generate gamma rays with a wavelength of less than 0.01 nm. The mentioned radio-isotopes are commonly used in the food industry for food irradiation because energies emitted are too low to induce radioactivity in any exposed material. The irradiation process leaves radiolytic products such as hydrocarbons and 2-alkylcyclobutanones produced from the major fatty acids in
food, and some cholesterol oxides and furans (EFSA, 2011). The irradiation to control mycotoxins works at two levels: (i) action on mold viability, consequently reduce mycotoxin production and (ii) direct degradation or indirectly by free radicals generated by radiolysis (Calado et al., 2014). Calado et al. (2014) extensively reviewed the irradiation for mold and mycotoxin control. They stated that the degradation efficacy of irradiation technology to control mycotoxins depends on the irradiation dose, food matrix, and the combination with other treatment methods (e.g., heating). The negative consumers’ perception of the technology associated with nuclear radiation also limit its acceptability.

**Ultraviolet**

Ultraviolet (UV) rays have wavelengths between 10 and 400 nm. AFB1 absorbs UV radiation at 222, 265, and 362 nm, with the greatest absorption at 362 nm (Samarajeewa et al., 1990). At 362 nm irradiation, AFB1 becomes susceptible to degradation resulting in the formation of 3 degradation products when treated in aqueous solution (Figure 8) (Liu et al., 2010). However, the application of UV treatment for aflatoxin degradation is limited because UV rays do not penetrate deeply in food matrix and also leave residues with toxicity (Pankaj et al., 2018b). This would matter if the grain were internally contaminated with mycotoxins. Otherwise, UV will degrade mycotoxins are on the grain surface.
Cold plasma

Cold plasma (CP) is a technology in its nascent form with potential utilization in the food industry. Plasma refers to a cocktail of ionized gas, which contains ions (H\(^+\), H\(_3\)O\(^+\), O\(^+\), H\(^-\), O\(^-\), OH\(^-\), N\(_2\)\(^+\)), electrons, and high reactive species, including molecular species (N\(_2\), O\(_2\), O\(_3\), H\(_2\)O\(_2\)), and reactive radicals (O•, H•, OH•, NO•). In the plasma, the atmospheric generates reactive species are oxygen species (ROS) and nitrogen species (RNS) (Pankaj et al., 2018).

There are different modes to produce plasma, including corona and dielectric barrier discharges (DBD). Corona discharge implies low electrical discharges that take place at or near atmospheric pressure and involves use of thin wire-plate or sharp pin-plate (Chang et al., 1991), while DBD consists of two electric dielectric plates separated by a gap distance (few centimeters), and working gas between two plates. The application of enough high
voltage potential between two plates induces the gas breakdown and generates gas reactive species (GRS) (Bogaerts, 2002). Misra et al. (2019) have reviewed CP efficacy for fungal and mycotoxin control in foods. GRS in the plasma inactivate fungal cells by destroying the cell protein-membrane, inducing the DNA fragmentation, deforming of mycelial tip, and finally leading to cell apoptosis. Likewise, GRS degrade mycotoxins and change their chemical structures, resulting in less toxic products.

High voltage atmospheric cold plasma (HVACP) (a DBD form using high voltage at atmospheric pressure) caused the AFB1 to breakdown by 76% using a 5 min HVACP treatment in air having 40% relative humidity. The HVACP treatment generated six AFB1 degradation products. Two pathways were proposed: the degradation pathway involving two degradation products due to ozonolysis. The other AFB1 degradation pathway was due to other reactive species besides ozone (Figure 9). Despite the encouraging results on the AFB1 degradation using HVACP, there is need to understand the HVACP efficacy in degrading other aflatoxins (AFB2, AFG1, and AFG2). In addition, there is also a need to gain a more comprehensive understanding of AF degradation products safety after HVACP treatment in vitro, and vivo, as well.
Learned lessons from an assessment of the Rwandan feed value chain

A study was conducted in Rwanda to assess the prevalence of aflatoxins and fumonisins in animal feed and milk samples. Six rounds of sample collection were completed in all 30 districts of Rwanda between March and October 2017, yielding 3,328 feed samples. Samples were collected from four categories of participants: 10 feed processors (including feed mills selling complete feeds and concentrates, and maize mills selling maize bran as a feed ingredient), 68 feed vendors, 225 dairy farmers, and 309 poultry farmers. Key findings on the prevalence of aflatoxins and fumonisins in animal feed
and milk samples are presented and discussed in chapter 4 and chapter 5, respectively. In addition, the status of feed storage, the storage duration, and the origin of feeds were assessed among participants to understand practices favoring mycotoxin development and contamination, and findings are presented in this section.

More than 90% of dairy and poultry farmers declared purchasing feed instead of growing feed ingredients on-farm (s).

Table 1. The primary sources of feed supply are feed processors outlets (42%) and feed vendors (43%) (Figure 10). Findings also showed that farmers do not have control over the quality of their feed ingredients because the majority of farmers relies on feed quality and safety at points of sale. Farmers who grow maize and other grains sell them mainly for food purposes and buy-back feed ingredients from feed vendors and feed processor outlets.

<table>
<thead>
<tr>
<th>Origin of animal feed</th>
<th>Dairy farmers (%)</th>
<th>Poultry farmers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grown on farm</td>
<td>9.3</td>
<td>7.2</td>
</tr>
<tr>
<td>Purchased</td>
<td>90.7</td>
<td>92.8</td>
</tr>
</tbody>
</table>

Lack of mycotoxin control at the farm level in complete feed and feed ingredients means subsequent mycotoxin exposure to humans through ASF contamination (i.e., milk contamination). Mitigation strategies are needed to minimize ASF contamination.
Policy measures to mitigate mycotoxin contamination in animal feed need to focus on the upstream of the feed value chain to make sure that the quality and safety of feed ingredients at the farm level are adequate for both human and animal health. For instance, grain millers and feed processors need to be encouraged to adopt good post-harvest drying and storage practices to prevent molds associated with production of mycotoxins.

Unfortunately, few farmers in Rwanda can afford drying technologies. Thus, an alternative solution is needed to minimize aflatoxin contamination. For example, the inclusion of feed additives known as mycotoxin binders/sequesters in animal feed is an affordable solution to mitigate mycotoxins as discussed previously. In the same vein, the EAC Policy Brief (No 6) on mitigation of harmful effects of aflatoxin on animal productivity recommends research on efficacy and safety of binders in EAC partner states for regulatory framework guidance (EAC, 2018).

Complete feeds and feed ingredients are generally held in stock for three to four weeks at each step in the feed value chain, except for feed processors that prefer to ship
their products right away to their outlets across the country (Error! Reference source not found.). If prevention measures are not taken during storage, the production of mycotoxins is promoted and therefore increases the risk of mycotoxin accumulation in feed and feed ingredients. Findings on aflatoxin contamination in feed and feed ingredients are presented and discussed in chapter 5. Results showed high aflatoxin contamination presuming inadequate post-harvest prevention measures to mitigate mycotoxins along the feed value chain.

The assessment of the feed value chain in Rwandan revealed that farmers do not have control over the mycotoxin contamination of their feed ingredients. There is a need to adopt a holistic approach involving upstream stakeholders (i.e., feed millers, and feed vendors) and leveraging existing technologies (e.g., feed additives discussed above) to mitigate mycotoxin contamination in feed and feed ingredients.

Table 2. Duration of complete feed and feed ingredients among feed value chain actors

References


CHAPTER 4. ASSESSMENT OF AFLATOXIN AND FUMONISIN CONTAMINATION AND ASSOCIATED RISK FACTORS IN FEED AND FEED INGREDIENTS IN RWANDA

Kizito Nishimwe¹,², Erin Bowers³, Jean de Dieu Ayabagabo⁴, Richard Habimana⁴, Samuel Mutiga⁵ and Dirk E. Maier³

¹Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011, USA
²School of Agriculture and Food Science, University of Rwanda, PO Box 4285 Kigali, Rwanda
³Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, IA 50011, USA
⁴School of Animal Sciences and Veterinary Medicine, University of Rwanda, PO Box 4285 Kigali, Rwanda
⁵Biosciences Eastern and Central Africa and International Livestock Research Institute (BecA ILRI) Hub, ILRI Complex, Along Old Naivasha Road, PO Box 30709–GPO 00100 Nairobi, Kenya

Modified from a manuscript published in the Toxins Journal²

Abstract

Mycotoxins are fungal metabolites that contaminate crops, food, and animal feeds. Aflatoxins and fumonisins are among the mycotoxins that have been increasingly reported to affect health and productivity of livestock globally. Given that the health and productivity of livestock can directly influence human food safety and security, a study was conducted to assess the levels and factors for aflatoxin and fumonisin contamination in feed and feed ingredients in Rwanda. Aflatoxins and fumonisins were analyzed in 3328 feed and feed ingredient samples collected at six time points between March and October 2017 in all

30 districts of Rwanda. Of the 612 participants providing samples, there were 10 feed processors, 68 feed vendors, 225 dairy farmers and 309 poultry farmers. Enzyme-Linked Immunosorbent Assay (ELISA) was used for aflatoxin and fumonisin analyses. Mean aflatoxin levels of 108.83 µg/kg (Median (MD): 43.65 µg/kg), 103.81 µg/kg (MD: 48.4 µg/kg), 88.64 µg/kg (MD: 30.90 µg/kg) and 94.95 µg/kg (MD: 70.45 µg/kg) were determined for dairy farmers, poultry farmers, feed vendors and feed processors, respectively. Mean fumonisin levels were 1.52 mg/kg (MD: 0.71 mg/kg), 1.21 mg/kg (MD: 0.56 mg/kg), 1.48 mg/kg (MD: 0.76 mg/kg) and 1.03 mg/kg (MD: 0.47 mg/kg) for dairy farmers, poultry farmers, feed vendors and feed processors, respectively. Aflatoxin contamination was significantly affected by time of sampling and district from which feed samples originated (P<0.05). Fumonisins did not show any correlation trends. Ninety-two percent of survey participants were unaware of aflatoxins and fumonisins and their adverse effects. This study has provided the basic understanding of the extent of feed contamination across the country and has established a baseline for future interventions in Rwanda. Further studies are needed to explore strategies for mitigating mycotoxins in the feed value chain in Rwanda.

**Introduction**

Mycotoxins are secondary metabolites produced by some fungi under specific, favorable climate conditions. Aflatoxins and fumonisins are two classes of mycotoxins with widespread prevalence in cereal crops and feeds (Kimanya et al. 2008; Bankole et al., 2006;
Richard 2007; Binder et al. 2007; Biomin 2017). Aflatoxins are a group of structurally-similar compounds produced by Aspergillus fungal species, mainly *Aspergillus flavus* and *A. parasiticus* (Perrone & Gallo, 2017). Analyses for total aflatoxins typically include the sum of four principal aflatoxin moieties (B1, B2, G1 and G2). Fumonisins are predominantly produced by *Fusarium verticillioides* and *F. proliferatum* (Munkvold, 2017). Analyses for total fumonisins typically include the principal B1, B2, and B3 moieties. The optimum growth of *A. flavus* and *A. parasiticus* occur over a temperature range of 29° C- 37° C, with water activity (aw) of 0.99 (Holmquist, Walker, & Stahr, 1983). *F. proliferatum* and *F. verticillioides* produce fumonisins on maize grain at 25° C - 30° C with maximum produced at 0.956 and 0.968 aw (Marin et al., 1995).

Aflatoxins and fumonisins are a concern for public and animal health worldwide. Human health effects of fumonisins include disruption of sphingolipid metabolism and inhibition of folate transport, which can result in fetal neural tube defects (Marasas et al., 2004). Epidemiological studies suggest dietary fumonisin consumption may be associated with esophageal cancer (Norred and Voss 1994; Chu and Li 1994; Rheeder et al., 1992). Noteworthy detrimental health effects in animals resulting from fumonisin exposure include Equine Leukoencephalomalacia (ELEM) and Porcine Pulmonary Edema (PPE) (Wan Norhas et al., 2009). Aflatoxins are classified by the International Agency for Research on Cancer (IARC) in Group 1 as carcinogenic to humans and animals. They are associated with immunosuppression (Raisuddin et al., 1993; Shivachandra et al., 2003; Thaxton, Tung, & Hamilton, 1974) and childhood stunting (Gong et al., 2004; Gong et al.,
2002; Khlangwiset et al., 2011) and are lethal in high doses. An outbreak in Kenya in 2004 resulted in 317 acute poisonings and 125 human deaths attributable to aflatoxins (Azziz-Baumgartner et al., 2005; Lewis et al., 2005; Probst et al., 2007).

In Africa, mycotoxin contamination of commodities and animal feeds poses significant risk to the health and productivity of livestock consuming affected feed. Additionally, it poses a risk to humans that consume affected grain and animal source foods (i.e., milk) produced from animals fed mycotoxin-contaminated feed (Miller, 1995). Studies conducted in Eastern Africa revealed a high number of feed samples contaminated with aflatoxins in Kenya (Kang’Ethe & Lang’A, 2009; Kang’ethe et al., 2017; Senerwa et al., 2016), Uganda (Kaaya & Warren, 2005) and Tanzania (Nyangi et al., 2016). Livestock that consume aflatoxin-contaminated feeds experience reduced productivity and detrimental health effects. A dairy herd exposed to contaminated feeds (120 µg/kg of aflatoxins) for several months showed severe health problems and a decrease in milk production up to 28%. Up to 6% of dietary AFB1 transfers to milk in the form of AFM1 when lactating mammals (e.g., cows) are fed AFB1-contaminated feed (Britzi et al., 2013; Churchill, 2017). Aflatoxin M1 (AFM1) is a metabolite of aflatoxin B1 (AFB1) that is excreted in urine, bile, feces, and milk after dietary exposure of dairy cattle to contaminated feed (Becker-Algeri et al., 2016). In chicken, aflatoxins reduce body weight, feed conversion efficiency, average daily gain, feed conversion ratios and egg production (Atherstone et al., 2016). This results in additional potential for human dietary exposure. Aflatoxin and
Fumonisin feed contamination is a challenge throughout Africa and impacts livestock health and productivity, and food and feed safety (Atherstone et al., 2016).

Aflatoxins also have negative economic impacts. In countries like the United States, there is financial impacts in the form of yield loss and product downgrading, discounting and rejection to the maize value chain due to aflatoxin contamination. Losses have been estimated to exceed $1 billion during years with warm summers and drought conditions based on models for estimating the potential market loss to the maize industry from aflatoxin contamination. In emerging economies, aflatoxins reduce farmer incomes due to low market value of contaminated products, production rejection and exclusion from high-value markets (Ladeira et al., 2017; Udomkun et al., 2017).

The livestock sector in Rwanda continues to grow. The percentage of livestock-owning households rearing cattle has increased from 34.4% in 2005/06 to 50.4% in 2013/14. The value of milk exports was $13,061,738 in 2016/17; a two-fold increase compared to 2011/12 according to the National Institute of Statistics of Rwanda (National Institute of Statistics of Rwanda (NISR), 2016.). The value of live poultry export exceeded $5 million in 2015/2016 (NISR, 2016). The government is contributing to growth in the livestock and poultry sectors through financial and technical assistance programs. Government efforts to increase dietary diversity and enhance human nutrition through consumption of animal source foods (ASF) (i.e., meat, milk, eggs) are evidenced in the countrywide Girinka (literally translated “One Cow per Poor Family”) Program (Minagri, 2018). There is a high demand for feeds to support the increasing production; however, the
feed sector faces challenges because of mycotoxins. A limited collection of 20 feed samples from feed vendors showed evidence of high aflatoxin contamination (Nishimwe et al. 2017). A study conducted in Kenya has shown that maize bran is the most contaminated of all ingredients used in feedstuffs (Nyangi et al., 2016). This is concerning because maize bran was reported to be the main basal feedstuff used in dairy cattle and poultry rations (Mbuza et al., 2017; Mutimura et al., 2013).

There is a scarcity of data regarding grain and feed contamination with aflatoxins and fumonisins in Rwanda that hampers mycotoxin management and mitigation efforts. The lack of data undermines government efforts to increase human health through increased consumption of ASF because they are likely also contaminated. Dietary diversity initiatives are ineffective when toxic contaminants, like aflatoxins, are present which are harmful to human health. This study was undertaken to assess the prevalence of aflatoxins and fumonisins in animal feeds and feed ingredients in Rwanda and to better understand risk factors contributing to mycotoxin contamination in animal feed.

**Materials and Methods**

**Study areas and identification of participants**

Six rounds of sample collection were carried out in all 30 districts of Rwanda (Figure 11) between March and October 2017. Four categories of participants were recruited for this study: feed processors (including finished feeds, and maize mills selling maize bran), feed vendors, dairy farmers, and poultry farmers. A comprehensive list of
eligible participants was obtained from the District Veterinary Officer (DVO) of each of the 30 districts. Recruitment targets for the study were 20 participants per district. When the number of potential participants per district was less than 20, all participants were included. For districts with more than 20 potential participants, a minimum of 20 participants were selected randomly. In the local Rwandan context, an individual with even one cow is considered a dairy farmer. There are a large number of farmers, particularly in rural areas, who rely on grazing and do not use animal feeds. To avoid such bias in the current study, we set two inclusion criteria prior to recruiting participants in the category of dairy farmers: they must have at least two cows and use feeds as the principal ration (or at least as a dietary supplement). Poultry farmers considered for participation in the study were broiler and layer farmers who sought to generate income from their products. A total of 612 participants were included consisting of 10 feed processors/grain millers, 68 feed vendors, 225 dairy farmers and 309 poultry farmers. Due to their relatively small number, all identified feed processors/grain millers were included.

**Questionnaire development and administration**

Structured questionnaires were designed to obtain socio-demographic information, participant knowledge/awareness of mycotoxins, and general feeding practices (i.e., ingredients used, sourcing and storage of ingredients or complete feeds). Questionnaires (Appendix 1, Appendix 2, Appendix 3, and Appendix 4) were specific to each of the four participant categories and were administered to participants by enumerators during the first
round of sample collection. In compliance with institutional ethics requirements (University of Rwanda and Iowa State University), participants gave their informed consent for inclusion before they participated in the study. Before signing the consent form, participants received an explanation of the objectives and anticipated outcomes of the project.

Figure 11. Map of Rwandan provinces and districts [Adapted from Kalisa et al. (2016)]

Sample collection

From each participant, a sample (approximately two kg) of feed or feed ingredients (complete feed, cotton cake, wheat bran, or maize bran) was collected. Samples were collected from the same participants for each of the six rounds of the study. If samples were
not available from some participants in a round, they were recorded as missing samples.

Samples were stored in a freezer (-20°C) as soon as possible after collection to halt fungal growth and additional aflatoxin accumulation.

**Sample preparation**

After removal from the freezer, complete feed and feed ingredient samples were allowed to equilibrate to room temperature before being ground in their entirety using a Romer Series II® sub-sampling mill (Romer Labs, Inc., Union, MO, USA). A minimum of 200 g ground sub-samples were kept and, again, stored in the freezer (-20°C) prior to analysis. To prevent cross-contamination, the mill was cleaned thoroughly between samples using a vacuum (according to manufacturer recommendation) and approximately the first 100 g of every ground sample exiting the mill was discarded. At least once per day, the mill was disassembled and an intensified dry cleaning was performed.

**Sample extraction**

A deviation from the Helica Total Aflatoxin Assay extraction protocol was made in consultation with the manufacturer to harmonize the extraction process for both mycotoxins. Our working procedure was as follows. A 20 g ground test portion was weighed from each sub-sample, mixed with 40 ml 90% methanol (ACS grade, Finar Ltd., Gujarat, India), and shaken in a sealed container for a minimum of two minutes. Particulate matter was allowed to settle; then five to 10 ml of extract was filtered through Whatman #1
filter paper. The resultant filtrate was collected and the pH was adjusted to 7.0 (+1.0), with 25% NaOH or 2M HCl. Approximately half of the collected filtrate was stored and refrigerated (5°C) in a labeled centrifuge tube to be used in the fumonisin ELISA assay. From the remaining collected filtrate, one ml was diluted in 1.5 ml 57% methanol in a centrifuge tube to bring the final concentration to 70% methanol, which is prescribed for the total aflatoxin ELISA assay. If samples contained aflatoxin or fumonisin levels exceeding the operating range of the assay, additional dilutions were made using 70% methanol. All 90% and 70% methanol extracts were stored in the refrigerator prior to laboratory analyses. Filtered extracts were not used after one week in the refrigerator due to loss of aflatoxin stability past this time.

**Sample analysis**

All collected feed and feed ingredient samples were analyzed using competitive Total Aflatoxin (AFB1 + AFB2 + AFG1 + AFG2) Enzyme-Linked Immunosorbent Assay (ELISA) (Catalog #941AFL01M-96) and Fumonisin (FB1 + FB2 + FB3) Assay (Catalog #951FUMO01C-96) (Helica Biosystems, Santa Ana, CA, USA) according to manufacturer specifications. For total aflatoxin kits, 200 μl of the aflatoxin-HRP conjugate was dispensed into each 96-well mixing plate and 100μl of either standard or sample was added to the appropriate mixing well containing conjugate. Wells were mixed by pipetting up and down at least 3 times. Content from each mixing well (100 μl) was transferred to a corresponding antibody coated microtiter well and incubated for 15 minutes. PBS-Tween wash buffer was
used to rinse the plate wells for five washes. A substrate reagent (100μl) was added to each antibody-coated microtiter well and incubated for 5 minutes. A stop solution (100μl) was added in the same sequence and at the same place as the substrate reagent was added. The optical density (OD) was read for each well of the plate using a plate reader with a 450-nm filter (Thermo Scientific Multiskan FC). For fumonisin kits, the assay procedure was the same as for the total aflatoxin assay procedure, with the following exceptions: 100 μl each of two conjugate solutions (A and B) were added to each well of the 96-well mixing plate, and incubation times were 10 minutes after transfer to the corresponding antibody-coated 96-well plate and 10 minutes after adding the substrate reagent. The OD readings generated by the standard solutions on each 96-well plate were used to generate a logit regression equation for that plate, which was subsequently used to calculate the total aflatoxin and fumonisin concentrations in sample extracts on the corresponding plate. The final concentration was adjusted according to the dilution factor. The operating range was 5 – 500 µg/kg and 1 – 6 mg/kg for total aflatoxins and fumonisins, respectively.

External validation

A subset of feed and ingredient samples that represented the varying types of matrices that were anticipated to be collected during our survey were obtained prior to round one sample collection. These samples were sent to Bioscience eastern and central Africa – International Livestock Research Institute (BecA-ILRI) in Nairobi, Kenya, for performance validation of ELISA assays relative to High Performance Liquid
Chromatography (HPLC) and fluorometry methods (VICAM Aflatest®, Watertown, MA, USA). Based on these results, an ELISA method was selected that performed best on the most prevalent matrices (Appendix 5).

**Internal validation**

For each ELISA 96-well plate, six ready-to-use standards were provided to establish a calibration curve ranging from 0.0-4.0 ng/ml for the total aflatoxin ELISA kit and 0.0-150.0 ng/ml for the fumonisin ELISA kit. The regression coefficient ($r^2$) was calculated for calibration curve linearity for each plate. The minimum acceptable level for the $r^2$ was set at 0.98. Aflatoxin and fumonisin quality control materials (QCM) (Biopure®, Romer Labs, Inc., Tulln, Austria) were included on select ELISA plates throughout the analysis period to monitor accuracy.

**Statistical Analysis**

A fixed effect model (SAS 9.4) was used to calculate the association between risk factors and the level of aflatoxins and fumonisins in feed samples using Analysis of Variance (ANOVA). Aflatoxin levels were log transformed due to the skewedness distribution. Gender, education, and age of the participants, origin of the sample (District), mycotoxin awareness, and round of the sample collection were fitted as fixed effect variables in the model. To calculate different descriptive statistic parameters, for samples lower than the limit of quantification of the test kit for total aflatoxins or fumonisins, the
data point was replaced by a value equal to half of the limit of quantification (2.5 µg/kg and 0.05 mg/kg for total aflatoxins and fumonisins, respectively) to avoid biasing the results. Samples exceeding the limit of detection (500 µg/kg and 6 mg/kg for total aflatoxins and fumonisins, respectively) were replaced by the values of 501 µg/kg and 6.1 mg/kg.

Results

Aflatoxin and fumonisin contamination among participants

A total of 3,328 feed and ingredient samples were collected in six rounds in all 30 districts of Rwanda from March to October 2017. Feed and feed ingredient samples in all four participant categories had high mean levels of total aflatoxin contamination (AFB1 + AFB2 + AFG1 + AFG2), and relatively low mean levels of total fumonisin contamination (FB1 + FB2 + FB3) (Table 3).

Table 3. Aflatoxin and fumonisin mean, standard deviation (SD) and median values in feed and feed ingredient samples among different participant categories

<table>
<thead>
<tr>
<th></th>
<th>Aflatoxins (µg/kg)</th>
<th>Fumonisins (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td><strong>Dairy farmers</strong></td>
<td>108.83</td>
<td>144.90</td>
</tr>
<tr>
<td><strong>Poultry farmers</strong></td>
<td>103.81</td>
<td>135.91</td>
</tr>
<tr>
<td><strong>Feed Vendors</strong></td>
<td>88.64</td>
<td>128.59</td>
</tr>
<tr>
<td><strong>Feed Processors</strong></td>
<td>94.95</td>
<td>103.19</td>
</tr>
</tbody>
</table>
Among different participants, mixed feed and maize had their lowest contamination during the June sampling period in dairy farmers (59.3 µg/kg and 86.6 µg/kg, respectively) and poultry farmers (74.6 µg/kg and 56.9 µg/kg, respectively) (Table S1). Statistical analysis did not show that participant category was a significant factor for aflatoxin and fumonisin contamination (p<0.05).

**Comparison of feed and ingredient sample contamination over sample collection period**

There was a significant difference in aflatoxin contamination among the six rounds of sample collection (p<0.0001). Samples collected in June (Round 3) had the lower aflatoxin contamination levels. Aflatoxin contamination level showed a decreasing trend from samples collected in March (Round 1) (mean: 140.78 µg/kg) to sample collected in June (Round 3) (mean: 70.01 µg/kg), then increased from June to October (Round 6) (Figure 12). Fumonisin level showed an increasing trend over the sample collection period. The lowest and highest levels of fumonisin contamination were 0.77 mg/kg and 2.33 mg/kg for Round 1 and Round 6, respectively (Figure 13). However, there is no significant difference among the six rounds of sample collection (p>0.05).

**Aflatoxin contamination level in feeds and feed ingredients samples**

Complete feeds were the most prevalent samples collected in the survey representing 56.3% of all samples.
Figure 12. Total aflatoxin contamination (µg/kg) mean, median, standard deviation, range, and outliers by round in feed samples (n = 3,328) collected between March and October 2017. Levels not connected by the same letter are significantly different.

Figure 13. Total fumonisin mean, median, standard deviation, range, and outliers by round in feed samples (n = 3,328) collected between March and October 2017. There is no statistical difference among different rounds of sample collection.
Complete feeds included feeds from commercial processors, which comprised 4.8% of total samples collected, and feeds mixed by vendors and/or farmers at their own facility or farm, which comprised 51.5% of total samples.

Feed ingredients comprised 43.7% of total samples collected with the most prevalent ingredient, maize bran, comprising 35.9% of total samples. Other ingredient samples collected were rice bran (2.6%), wheat bran (2.4%), brewery by-products (used only by dairy farmers) (2.3%), and whole maize (0.5%).

Aflatoxin contamination by sample type was significantly different (p-value: <0.05). The whole maize samples were most highly contaminated while brewery by-product samples were least contaminated (Figure 14).

**Aflatoxin contamination weighted average in feeds and feed ingredients**

The weighted mean illustrates the relative importance of each feed ingredient or complete feed according to their respective total samples (number of feed type samples/total number of samples X total aflatoxin contamination for feed type); in other words, an average which results from the multiplication of each feed ingredient or complete feed by a factor indicating its importance. The weighted average for mixed feed and maize bran were 54.5 µg/kg and 39.8 µg/kg, respectively. Other feed sample types had 4.5, 2.1, 2.4, 0.8 and 0.8 µg/kg for commercial feeds, rice bran, wheat bran, brewery by-products and whole maize, respectively.
Figure 14. Total aflatoxin means by sample type from the most frequent to least frequent feed and feed ingredient type. Levels not connected by the same letter are significantly different (p < 0.05).

*Aflatoxin contamination of feed ingredient types throughout sampling period*

The mean levels of aflatoxins in different feed ingredient types and complete feeds throughout the sampling periods were determined. The low levels for mixed feed, maize bran, rice bran and wheat bran were 72.4 μg/kg, 74.6 μg/kg, 40.7μg/kg and 9.9 μg/kg, respectively, occurred during the June sampling period. The brewery by-product ingredient had the overall lowest aflatoxin contamination for all sampling periods (Table 4A).
Aflatoxin contamination among geographical districts

Location was a significant factor for aflatoxin contamination level. Of the 30 districts in Rwanda, the Rubavu district had the lowest mean aflatoxin level (53.0 µg/kg) and the Gicumbi district had the highest (148.1 µg/kg) (Figure 15).

Maize bran and mixed feed were used across all districts. Rice bran was used in Gasabo, Huye, Nyamagabe, Kirehe, Rulindo and Gatsibo districts. Brewery by-product was used in Rubavu, Kicukiro and Musanze districts. Wheat bran was used in Gisagara, Nyaruguru, Gicumbi, Huye, Nyanza, Nyabihu, Rulindo and Musanze (Table 5A).
Socio-demographic characteristics of participants

Socio-demographic characteristics of study participants did not show any statistically significant differences to aflatoxin or fumonisin contamination of the samples collected from their locations (p-value: > 0.05). More than 20% of participants were female owners of dairy (8.1%) or poultry farms (12.5%). Additionally, about one fourth of participants (26%) had a university education. The majority of participants were in the age range of 26-40 years old (56.5%). Moreover, a high proportion of participants, 92.4%, were not aware of aflatoxins, fumonisins, and their adverse effects. Socio-demographic characteristics for feed processors/grain millers were not established, as they are cooperatives or commercial businesses (Table 4).

Discussion

The current study showed that feed ingredients and complete feeds collected from different participants (dairy farmers, poultry farmers, feed vendors and feed processors) that spanned the feed value chain had widespread contamination with aflatoxins and fumonisins. Co-occurrence of fumonisins and aflatoxins in feeds and feed ingredients has been reported in the East Africa region by several authors. In a report from Tanzania, fumonisin levels in co-contaminated samples ranged from 111 to 11,048 µg/kg (mean = 2157 µg/kg) and aflatoxins from 1 to 151 µg/kg (mean = 44 µg/kg) (Kimanya et al. 2008). Another study in Tanzania reported co-contamination in maize samples (Nyangi et al., 2016). Kang’Ethe et al. reported contamination of aflatoxins and fumonisins in feed
samples collected in two Kenyan counties (equivalent to provinces or states) with historical outbreaks of aflatoxin poisonings of people.

Up to 56% of feed samples were aflatoxin positive and 14.6% exceeded the 5 µg/kg limit set by the Food and Agricultural Organization/World Health Organization. The overall average mean was 3.84 µg/kg aflatoxins (range of 0.55 ug/kg to 7.13 ug/kg). Fumonisin B1 was detected in 81.8% of 22 feed samples analyzed for fumonisins (Kang’ethe et al., 2017). The relatively low aflatoxin contamination levels in these studies compared to our results can be explained, in part, by different interventions to mitigate mycotoxins in maize fields. For instance, the Africa Research in Sustainable Intensification for the Next Generation (Africa RISING/IITA) project intervened in the area of study in Tanzania (Nyangi et al., 2016) Farmers were trained on mitigating mycotoxin contamination along food and feed value chains In Kenya, different management and mitigation strategies were taken to reduce aflatoxin contamination following the 2005 aflatoxicosis outbreak.

Crop rotation, use of certified seeds, use of manure and fertilizers, sorting moldy grains, harvesting at physiological maturity, and proper storage were recommended by policymakers to mitigate aflatoxins and fumonisins at household level (Kang’ethe et al., 2017). However, more efforts are still need to eradicate completely aflatoxin in food and feed value chains.
Table 4. Socio-demographic characteristics of study participants.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Dairy farmers</th>
<th>Poultry farmers</th>
<th>Feed Vendors</th>
<th>F. Pr./G. M.(^a)</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#</td>
<td>%</td>
<td>#</td>
<td>%</td>
<td>#</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>176</td>
<td>29.2</td>
<td>234</td>
<td>38.9</td>
<td>50</td>
</tr>
<tr>
<td>Female</td>
<td>49</td>
<td>8.10</td>
<td>75</td>
<td>12.5</td>
<td>18</td>
</tr>
<tr>
<td><strong>Education</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>88</td>
<td>14.6</td>
<td>114</td>
<td>18.9</td>
<td>13</td>
</tr>
<tr>
<td>Secondary</td>
<td>74</td>
<td>12.3</td>
<td>98</td>
<td>16.3</td>
<td>36</td>
</tr>
<tr>
<td>University</td>
<td>53</td>
<td>8.80</td>
<td>87</td>
<td>14.5</td>
<td>19</td>
</tr>
<tr>
<td>None</td>
<td>9</td>
<td>1.50</td>
<td>9</td>
<td>1.50</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>0.20</td>
<td>1</td>
<td>0.20</td>
<td>0</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-25</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>2.59</td>
<td>13</td>
</tr>
<tr>
<td>26-40</td>
<td>108</td>
<td>48.0</td>
<td>193</td>
<td>62.5</td>
<td>39</td>
</tr>
<tr>
<td>&gt;40</td>
<td>117</td>
<td>52.0</td>
<td>108</td>
<td>35.0</td>
<td>16</td>
</tr>
<tr>
<td><strong>Awareness</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>8</td>
<td>1.30</td>
<td>27</td>
<td>4.50</td>
<td>9</td>
</tr>
<tr>
<td>No</td>
<td>213</td>
<td>35.3</td>
<td>278</td>
<td>46.0</td>
<td>59</td>
</tr>
</tbody>
</table>

\# Total number of participants in each category per each socio-demographic characteristic considered

\% Percentage of participants in each category per each socio-demographic characteristic considered

\(^a\)Feed Processors/Grain Millers were cooperatives or commercial companies. Several factors (i.e., gender, education and age) were not recorded.
Fumonisin levels in our study did not exceed European Union (EU) guidance values for feeds for poultry (< 4 months) set at 20 mg/kg, nor did fumonisin levels exceed the 50 mg/kg limit for adult ruminants (> 4 months) (Knutsen et al., 2018). Likewise, they did not exceed the United States Food and Drug Administration (FDA) guidance levels for maize and maize by-products intended for animal consumption (i.e., 30 mg/kg total fumonisins for breeding ruminants and breeding poultry) (USFDA, 2001). Aflatoxin contamination of feed vendor samples collected in the current study confirmed a previous study in which 21 feed samples collected in different open markets of Kigali had aflatoxin levels between 100.4 and 168.8 µg/kg (Nishimwe et al. 2017). More than 85% of feed ingredients and complete feed samples collected from dairy farms in the current study exceeded the 5 µg/kg of AFB1 limit established by the Rwanda Standards Board (RSB) for cattle feed supplements (RSB, 2017). This standard is the only published standard that addresses the mycotoxin issue in feeds in Rwanda. Aflatoxin contamination in feed ingredients and complete feeds affects animal health and productivity but also endangers public health through their transfer into ASF used as human food, such as the excretion of AFM1 in milk (Atherstone et al., 2016).

Aflatoxin contamination showed a significant difference over six sampling periods. Complete feeds and feed ingredients samples collected in June had the lowest overall mean of aflatoxin contamination. June corresponds to the maize harvest period, which is characterized by transition from the rainy to the dry season. At harvest, maize is expected to have the lowest level of aflatoxin contamination and, ideally, good post-harvest drying and storage practices would prevent molds and associated aflatoxin development. The practical reality is that most maize farmers in Rwanda do not have access to drying and storage technologies that would help them reduce maize to safe storage moisture content quickly and maintain it at safe storage moistures.
This lack of technology renders producers incapable of taking proactive measures to reduce the potential for mold growth and aflatoxin development. Subsequently, maize and maize-derived products have increased risk of aflatoxin accumulation the longer they are stored after harvest. Additionally, millers wet maize before hammer milling in order to remove the bran from the kernel more readily. The wetted maize bran is generally accumulated in a pile on the floor of the feed mill that can self-heat and induce further mold growth and aflatoxin development which continues once maize bran is bagged. Therefore, it is not surprising that the current study documented aflatoxin content in feed ingredients and complete feeds that contain maize bran or whole maize increasing the further away sample collection occurred from the June harvest period over the course of this study.

Mbuza et al. (2017) reported that maize bran is used as the main basal feedstuff in poultry and broiler farms in Rwanda, which was also observed in this study. Maize-based ingredients were either simple feed ingredient (maize bran) or mixed with other ingredients (mixed feed). The weighted averages were 39.8 \( \mu g/kg \) and 54.5 \( \mu g/kg \) for maize bran and mixed feeds, respectively. Consequently, the more contaminated maize-based ingredients are with aflatoxins, the higher the aflatoxin contamination in feeds. Feed ingredients and complete feeds also showed large standard deviations from the means and many outliers. The high standard deviations are explained by the heterogeneous nature of aflatoxin contamination (Campbell et al., 1986b). The fact that in this study most mean values were much greater than median values suggests the data were positively skewed, with many low values and fewer higher values.

The Rubavu district had the lowest level of aflatoxin contamination, which is likely due to the dominant feed ingredient, brewery by-products, from a major brewery located there. Brewery by-products had the lowest aflatoxin levels of any of the sample types collected in this
study, likely because of the proactive aflatoxin mitigation measures taken by the brewery (human beverage producer) to minimize aflatoxin contamination in raw ingredients (i.e., maize, barley).

More than 90% of participants in this study reported that they had never heard the words “mycotoxins or aflatoxins” nor their consequences. As a matter of fact, there is no equivalent word for “aflatoxins” in the local Kinyarwanda language. Therefore, an additional question was asked to assess participant’s knowledge of mold contamination in feeds and feed ingredients and their consequences. The vast majority (over 85%) reported to have seen moldy feed ingredients without knowing their consequences. A previous study, targeting maize flour vendors in Kigali’s open markets, reported that all participants (n=158) were unaware of aflatoxins (Nishimwe et al. 2017). Nyangi et al. reported that 62% of farmers in the Babati district of Tanzania were aware of mycotoxins and their consequences thanks to the intervention of Africa RISING/IITA and NGOs (Nyangi et al., 2016). Raising awareness of aflatoxins and their consequences has to be considered as a key element in any aflatoxin mitigation strategy. Implementation will not succeed if farmers do not first understand the danger of aflatoxins.

Absence of appropriate regulations contribute to the mycotoxin threat in Rwanda. All participants in this study were unaware of the one existing standard for aflatoxins in dairy feed supplements. In the African context, establishment of standards and enforcement of regulations is currently driven by trade and the desire to comply with export regulation (Matumba et al., 2017). The inability to enforce even the single, existing aflatoxin standard renders it useless and gives the false impression of controlling the situation (Magamba et al., 2017). Sirma et al. (2018) suggested that aflatoxin standards for food and feeds would be more effective if the context of local conditions were considered, such as typical crops of ingredients, capacity to enforce
regulations, differentiated aflatoxin contamination levels in food and feeds, regional standards and societal concerns (food and nutrition security).

Results of this study will inform current regional and continental efforts to leverage aflatoxin mitigation strategies. Recently, the East African Community (EAC), a regional intergovernmental organization, provided options for disposal and alternative uses of aflatoxin-contaminated commodities (EAC, 2018a). Moreover, ten years ago the African Union established already the Partnership for Aflatoxin Control in Africa (PACA), whose role is to provide leadership, and coordinate and increase effective aflatoxin control in Africa (PACA, 2018). This study will contribute to PACA’s resources and knowledge.

**Conclusions**

This study quantified aflatoxin and fumonisin contamination in the feed value chain of Rwanda over a 7-month period in 2017. The specific conclusions based on the results are:

1. Feed ingredients and complete feeds were contaminated with aflatoxins and fumonisins. Dairy farmers, poultry farmers, feed vendors and feed processors had mean aflatoxin levels of 108.83 µg/kg (Median (MD): 43.65 µg/kg), 103.81 µg/kg (MD: 48.4 µg/kg), 88.64 µg/kg (MD: 30.90 µg/kg) and 94.95 µg/kg (MD: 70.45 µg/kg), respectively. However, fumonisins did not exceed the EU and FDA guidance values for feeds for mature poultry set at 20 mg/kg, and 30 mg/kg for breeding ruminants and breeding poultry, respectively.

2. Considering the weighted average, mixed feed (54.5 µg/kg) and maize bran (39.8 µg/kg) were the two major contributors to aflatoxin contamination.

3. Two risk factors, district and sample period, showed a significant effect (p<0.05) on aflatoxin contamination of feed ingredients and complete feeds.
4. More than 90% of study participants were unaware of aflatoxins and fumonisins, and their consequences.

5. One standard for AFB1 in cattle feed supplements exists in published form in Rwanda and all study participants were unaware of the published standard.

Further studies are recommended to explore existing strategies for mitigating mycotoxins in the feed value chain in Rwanda. For instance, since there is no difference in aflatoxin contamination among participants, interventions will focus on early value-chain participants, since end-users are subject to what is available from suppliers. Standards and regulations adapted to the context are needed and an awareness campaign should be initiated to improve food and feed safety in Rwanda.

Acknowledgments

This study was made possible by the generous support of the American people through the United States Agency for International Development (USAID) and its Feed the Future Innovation Lab for Livestock Systems managed by the University of Florida and the International Livestock Research Institute, grant number No. AID-OAA-L-15-00003. This study is also supported by USAID, as part of the Feed the Future initiative, under the CGIAR Fund, award number BFS-G-11-00002, and the predecessor fund, the Food Security and Crisis Mitigation II grant, award number EEM-G-00-04-00013. The contents are the responsibility of the authors and do not necessarily reflect the views of USAID or the United States Government.
References


NISR. (2016). *NISR. National Institute of Statistics of Rwanda (NISR)*.


CHAPTER 5. PRELIMINARY SURVEY OF AFLATOXIN M1 CONTAMINATION IN RAW MILK IN DAIRY FARMS USING FEED INGREDIENTS FROM RWANDA

Authors and Affiliations

Kizito Nishimwe1,2, Erin Bowers3, Jean de Dieu Ayabagabo4, Richard Habimana4, Samuel Mutiga5 and Dirk E. Maier3

1Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011, USA
2School of Agriculture and Food Science, University of Rwanda, PO Box 4285 Kigali, Rwanda
3Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, IA 50011, USA
4School of Animal Sciences and Veterinary Medicine, University of Rwanda, PO Box 4285 Kigali, Rwanda
5Biosciences Eastern and Central Africa and International Livestock Research Institute (BecA ILRI) Hub, ILRI Complex, Along Old Naivasha Road, PO Box 30709–GPO 00100 Nairobi, Kenya

Manuscript prepared for submission to World Mycotoxin Journal

Abstract

Milk is susceptible to aflatoxin M1 (AFM1) contamination when dairy cattle consume feed contaminated with aflatoxins. AFM1 is a public health concern and is classified (with other aflatoxins) as a group 1 human carcinogen by the International Agency for Research on Cancer (IARC). This study assessed the prevalence and amount of total aflatoxin contamination in dairy feed and ingredients and the corresponding AFM1 contamination in raw milk from samples collected at farms using local feed ingredients across Rwanda’s five provinces. In total, 170 raw milk samples were collected during one sampling period; the mean AFM1 concentration in these samples was 0.89 ± 1.64 µg/L (median: 0.33 µg/L) with a maximum of 14.5 µg/L. Ninety-one percent of milk samples exceeded 0.05 µg/L (the European legal limit) and 38% of samples exceeded 0.5 µg/L, the legal limit and maximum level established by the U.S. Food and Drug Administration (USFDA) and Codex Alimentarius, and East African Community (EAC),
respectively. Among the five types of dairy feed samples collected (maize bran, mixed feeds, rice bran, wheat bran and brewery by-products), maize bran was the most frequent feed ingredient used by dairy farmers (65.5%). Seventy-nine percent and 60% of dairy feed samples collected exceeded legal limits established for total aflatoxin contamination in cattle feed in Rwanda (5 μg/kg) and the USFDA limit in dairy feed (20 μg/kg), respectively. There is a need for intensive research along the milk value chain to determine risk factors for AFM1 contamination of milk, including seasonal influences, and the mitigation of that risk to consumers.

**Introduction**

Milk is an essential source of nutrients, including proteins, lipids, vitamins, and minerals (Haug et al., 2007). In Rwanda, milk production has increased from 628,266 tons in 2013 to 706,030 tons in 2014 according to the National Institute of Statistics of Rwanda (NISR) (NISR, 2015) and has generated $13,061,738 of export revenue in 2016/2017 (NISR, 2017). This increase is due to the Rwandan government's efforts to combat persistent malnutrition through different programs. The “Girinka” (which literally translates ‘one cow per poor family’) program, a government initiative, helps vulnerable families to have access to milk and dairy products by providing them with cows (Minagri, 2018). Currently, more than 80% of all milk produced in Rwanda is handled via informal value chains (milk producers sell directly to consumers or resellers) (Kamana et al., 2014), despite promotion of formal marketing via milk collection centers (MCCs) that have been established across the country, and predispose milk to consumption of non-compliant milk.
Milk is subject to the aflatoxin M1 (AFM1) contamination when lactating animals (including dairy cattle) consume feed contaminated with aflatoxin B1 (AFB1) (IARC, 2012). Aflatoxins are secondary metabolites produced by fungi, Aspergillus flavus and A. parasiticus, under favorable environmental conditions (10 – 43°C and 0.99 water activity, with an optimum at 33°C) (Holmquist et al., 1983). There are four different types of aflatoxins; AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2). AFB1 is metabolized in the liver into AFM1 and excreted in milk (IARC, 2012). The carryover of AFB1 from feed into AFM1 in milk (AFM1/AFB1 ratio) depends on different factors, such as animal species, feeding regimens, rate of ingestion, the health of the animal, hepatic biotransformation capacity, and production (Fink-Gremmels, 2008). In dairy ewes, the carryover was estimated at 0.112% 0.26 to 0.33% (Battacone et al., 2005) and in dairy cows, estimated at 5.8% and 2.5% in mid-lactation and late-lactation, respectively (Britzi et al., 2013).

Ruminants are relatively resistant to negative health and performance effects of dietary aflatoxin because rumen microbiota is capable of degrading aflatoxin (Upadhaya et al., 2010). In the rumen, a part of AFB1 is metabolized into aflatoxicol; another part is absorbed and metabolized in the liver into AFM1. AFM1 is either conjugated and excreted via bile, or enter the systemic circulation and excreted into milk, or urine (Fink-Gremmels, 2008). However, the consumption of highly contaminated feeds or prolonged exposure to moderately contaminated feed can lead to adverse effects (Alvarado et al., 2017; Zain, 2011). Holstein cows in mid-lactation fed 23 mg of aflatoxin B1 for seven days showed a decrease in feed intake and milk production (Applebaum et al., 1982). Ruminant health and productivity are impacted at levels of dietary aflatoxin exposure order of magnitude higher than dietary levels, which pose a risk for humans consuming milk and dairy products. For example, while the U.S. FDA has set a dietary
limit of 300 ppb for beef cattle, the limit for dairy cattle is 20 ppb, to account for the production of milk safe for consumers (US FDA, 2019). AFM1 is of worldwide public health concern; the International Agency for Research on Cancer (IARC) classified AFM1 in group 1—carcinogenic to humans (IARC, 2012b). AFM1 is heat resistant and cannot be eliminated by pasteurization or Ultra High Temperature (UHT) treatment of milk; therefore, AFM1 persists in final dairy products after treatment (Prandini et al., 2009). Regulations and recommended limits exist at country and regional levels to limit human exposure to AFM1. The European Commission (EC) has set a maximum level of 0.05 μg/kg AFM1 for raw milk (EC, 2006). The U.S. Food and Drug Administration (FDA) and the Codex Alimentarius Commission (a joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Food Standards Program) (FAO/WHO 2013) and the East African Community (EAC) (EAC, 2019) have set maximum levels for AFM1 in fluid milk at 0.5 μg/kg.

Rwanda is part of the EAC (consists of Burundi, Kenya, Rwanda, South Sudan, Uganda and Tanzania), which has a tropical climate suitable for the growth of Aspergillus fungi and subsequent production of aflatoxins. Key crops at risk for aflatoxin contamination include maize, cottonseed, and groundnuts (EAC, 2018). Feed contamination with aflatoxins is, therefore, a hazard as these ingredients and their fractionation and processing by-products are commonly used in animal feed resulting in AFM1 contamination in milk. In Singida, Tanzania, raw milk samples (37 samples) collected from dairy cows fed with sunflower seedcakes, or sunflower-based seedcake feeds had AFM1 levels ranging from 0.026 μg/L (the limit of detection) to 2.007 μg/kg, with 83.8% % and 16.1% exceeding 0.05 μg/L and 0.5 μg/L, respectively (Mohammed et al., 2016). A study conducted every month for one year in Kenya found that more than 50% of milk product (raw, pasteurized, UHT milk, yogurt and lala) samples (n=291) marketed in
Nairobi, Kenya exceeded 0.05 µg/kg AFM1, and three samples exceeded 0.5 µg/L. AFM1 levels significantly depended on household income (p<0.001), the time of year (lowest AFM1 levels in January), and products with UHT milk having lower levels (Lindahl et al., 2018). Another study conducted in two counties, Nandi and Makueni, Kenya, it was noted that 52% and 87% of milk samples (n=529) were contaminated with AFM1, respectively, and only 8% from Makueni exceeded 0.05 µg/Kg (Kang’ethe et al., 2017).

In Rwanda, dairy farmers rely on grasses (56%), parts of banana plants (21%), and residues of several crops (15%) (Klapwijk, 2011). Moreover, maize bran and rice bran are feed ingredients used by dairy farmers as supplements to increase production (Mazimpaka et al., 2017). Despite the lack of published data on AFM1 contamination of milk in Rwanda, a recent study on aflatoxin contamination in feed and feed ingredients showed that dairy feed and feed ingredients were contaminated with aflatoxins (Mean levels varied between 100.4 and 168.6 µg/kg, and maximum levels were 265 µg/kg) with 75% of animal feed samples exceeding 100 µg/kg AFB1 (Nishimwe et al. 2019), which presumably implies a risk of milk contamination with AFM1.

The absence of data on the prevalence of AFM1 contamination in Rwanda leads to an underestimation of the risk to consumers. Consequently, it constrains the development of adequate policy to mitigate the risk of AFM1 in milk and dairy products. This remains a missed opportunity to enhance consumer safety of milk and dairy products in Rwanda. This study aimed to assess the presence of aflatoxins in dairy feeds collected at farms across different provinces of Rwanda during one sampling period, and their influence on milk contamination with AFM1. A better understanding of the situation will lay a foundation for future intervention to thwart
aflatoxin health implications but also to minimize milk contamination, and therefore improve consumer safety.

Materials and Methods

Study design and participant selection

Briefly, in the local context, anyone with a cow is considered a dairy farmer. Consequently, dairy farmers in Rwanda would number in the millions. Two inclusion criteria were set for participation in the current study; dairy farmer participants must (1) have at least two cows and (2) use dairy feeds, as opposed to solely relying on grazing. These dairy farmers were of particular interest because they were more likely to be at AFM1 contamination risk, because of feed choice. Dairy farmers were identified in all five provinces (Eastern, Northern, Western, Southern and Kigali City) with the help of District Veterinary Officers (DVO). The DVOs are in charge of livestock at the district level; Rwanda has 30 districts in total. All identified dairy farmers consented to participation after they were informed of the aim of the study. This study received approval for compliance with institutional ethics requirements (University of Rwanda and Iowa State University). In July 2017, paired milk and feed samples (from the same farm at the same time) were collected for the assessment of aflatoxin M1 in milk (and aflatoxin and fumonisin contamination in feeds) from all 30 districts of Rwanda. This was part of a larger study measuring temporal and spatial contamination of Rwandan feeds and feed ingredients with aflatoxins and fumonisins, with particular attention to dairy and poultry feeds (Nishimwe et al., 2019).
Analysis of AFM1 in milk

A 50 mL raw milk sample was taken from the metallic or plastic bulk milk storage (~5 L) of each dairy farmer and transported in a cold box to the lab and kept in a freezer (-20°C) until further analysis. AFM1 in milk was analyzed using Afla M1 FL+ method (Vicam Company, Watertown, MA, USA) according to the manufacturer’s instruction. Briefly, milk samples were analyzed using immuno-affinity column fluorometry with a VICAM® fluorometer (Series-4EX, Source Scientific LLC, USA) and following the manufacturer procedure. Approximately 15 mL raw whole milk was centrifuged at 2000 X for 10 minutes. After centrifugation, the skim portion was removed without disturbing the top fat layer and 10 mL skim milk were collected for further analysis. The 10 mL collected skim milk was passed through an Afla M1 FL+ antibody-based affinity column (Vicam Company, Watertown, MA, USA) at a rate of about 1-2 drops/seconds until air comes through the column. Then, the Afla M1 FL+ column was washed twice with 10 mL methanol: water (10:90, v/v) solution at a rate of about 2-3 drops/second. AFM1 was eluted with 1 mL of methanol: water (80:20, v/v) at a rate of about an drop/second and collected in clean cuvette glass. One ml of diluted AflaTest® Developer was added to the eluate in the cuvette, mixed and was read using the calibrated fluorometer following the manufacturer’s instructions. AFM1 concentration was read after 60 seconds. The assay range was 0 – 1 µg/kg with a Limit of Detection (LOD) at 0.05 µg/kg. Any sample exceeding the assay range was re-tested using five mL (half of the required) skim milk and final AFM1 concentration was calculated by multiplying the AFM1 concentration as determined by the calibrated fluorometer by two. The VICAM® fluorometer method has been widely used for AFM1 analysis in milk (Ali, & Elseed, 2014; Hussain & Anwar, 2008; Mulunda & Mike, 2014). Previous validation tests have shown a good coefficient of variation (CV) (less
than 10%) for all AFM1-spiked raw milk samples ranging from 0 – 0.2 µg/kg and positive correlation (r =0.96) between liquid chromatography (LC) and VICAM fluorometer results (Liu & Powers, 2008).

**Analysis of aflatoxin in dairy feeds**

Dairy feed samples were analyzed using competitive Total Aflatoxin Enzyme-Linked Immunosorbent Assay (ELISA) (Catalog #941AFL01M-96, Helica Biosystems Inc, Santa Ana, CA, USA) as previously reported (Nishimwe et al., 2019). Briefly, for each identified dairy farmer, a two kg sample of dairy feed or feed ingredient was collected at the same time as the previously described milk sample. As soon as was possible, these samples were stored in a freezer (-20°C) until further analysis. Before analysis, feed ingredient samples were ground in their entirety using a Romer Series II® sub-sampling mill (Romer Labs, Inc., Union, MO, USA) at the finest grind setting. For each ground feed sample, a 200 g ground feed sub-sample was collected for analysis. A 20 g ground test portion was weighed from each sub-sample, mixed with 100 ml 70% methanol (ACS grade, Finar Ltd., Gujarat, India), and shaken in a sealed container for a minimum of two minutes. Particulate matter was allowed to settle; then, five to 10 ml of extract was filtered through Whatman #1 filter paper. The resultant filtrate was collected and the pH was adjusted to 7.0 (+1.0), with 25% NaOH or 2 M HCl. Aflatoxin-HRP conjugate (200 µL) was dispensed into each mixing well on an ELISA plate, and 100 µL of each standard or sample was added to the appropriate mixing well-containing conjugate. These were mixed three times by pipetting each mixing well, then 100 µL was transferred to a corresponding antibody-coated microtiter well and incubated at room temperature for 15 min. After the incubation, the contents were decanted and washed with PBS-Tween wash buffer five times. A
volume of 100 µL substrate reagent was added to each microwell and incubated at room
temperature in a dark place for five minutes. The stop solution (100 µL) was added in the same
sequence and at the same pace as the substrate reagent was added. Immediately, the optical
density (OD) was read for each well of the plate using a Thermo Scientific Multiskan FC plate
reader with a 450-nm filter (Thermo Fisher Scientific, Ratastie, Finland). A dose-response curve
was constructed using the OD values expressed as a percentage (%B/Bo) of the OD of the zero
standard against the aflatoxin content of standard (0.2, 0.5, 1.0, 2.0, and 4.0 ng/mL). The
calculated values for the aflatoxin standard calibration curve were semi-logarithmically
transformed using Excel (Microsoft, Inc. USA). The aflatoxin concentration corresponding to the
OD of each sample was read from the calibration curve. The linearity of the calibration curve
was assessed by calculating the regression coefficient (r2). The acceptable minimum for the r²
was set at 0.98. Aflatoxin quality control materials (QCM) (Biopure®, Romer Labs, Inc., Tulln,
Austria) were included on select ELISA plate to monitor accuracy and selected samples run in
duplicate.

**Statistical analysis**

AFM1 concentration in milk, corresponding aflatoxin concentrations in feeds, type of
dairy feed or feed ingredient, and provinces were evaluated and data analyzed using Analysis of
Variance (ANOVA) in JMP Pro 14 software (SAS Institute Inc., Cary, NC, USA). Any milk
sample lower than the LOD (0.05 µg/kg) was replaced by half the value of the LOD for avoiding
biased results. Aflatoxin concentrations were logarithmically transformed to reduce the natural
aflatoxin distribution skewness. A linear fixed model was used to calculate the interaction effects
between the provinces, aflatoxin levels in feeds, type of dairy feeds and AFM1 concentration in milk, with $\alpha=0.05$.

Results

AFM1 contamination in milk

In total, 170 milk samples were collected from dairy farms representing all five provinces in Rwanda. The overall mean of AFM1 level across all 170 samples was $0.89 \pm 1.64 \mu g/kg$ (mean ± standard deviation) with a median value of $0.33 \mu g/kg$. Ninety-one percent and 38% of all milk samples were contaminated AFM1 above the European (0.05 $\mu g/kg$) and U.S. FDA, Codex Alimentarius and EAC Standards (0.5 $\mu g/kg$) AFM1 maximum level limits, respectively (Error! Reference source not found.). AFM1 contamination in milk differed significantly by province ($p=0.02$) (Figure 16). The Eastern province, from which the largest number of milk samples were collected (n=60), was the origin of the sample containing the highest level recorded in the sampling (14.5 $\mu g/kg$). The Eastern province mean was also the highest of the five provinces ($1.23 \pm 2.29 \mu g/kg$), significantly higher than the Western province, which had the lowest mean AFM1 contamination ($0.33 \pm 0.46 \mu g/kg$).

Aflatoxin contamination in corresponding dairy feeds

Table 6 summarizes the total aflatoxin contamination (B1+B2+G1+G2) in 154 dairy feed samples collected simultaneously with the milk samples. During the time of sample collection, 16 dairy farmers were not able to provide feed samples
Table 5. Analysis of AFM1 in milk samples across different provinces

<table>
<thead>
<tr>
<th>Province</th>
<th>Number of Samples (% of total)</th>
<th>AFM1 (μg/kg)</th>
<th>Mean</th>
<th>Standard Deviation</th>
<th>Median</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eastern\textsuperscript{a}</td>
<td>60 (35.3)</td>
<td></td>
<td>1.23</td>
<td>2.29</td>
<td>0.46</td>
<td>14.5</td>
</tr>
<tr>
<td>Kigali City\textsuperscript{a,b}</td>
<td>34 (20)</td>
<td></td>
<td>1.01</td>
<td>1.44</td>
<td>0.41</td>
<td>5.4</td>
</tr>
<tr>
<td>Northern\textsuperscript{a,b}</td>
<td>20 (11.8)</td>
<td></td>
<td>0.65</td>
<td>1.2</td>
<td>0.26</td>
<td>5.5</td>
</tr>
<tr>
<td>Southern\textsuperscript{a,b}</td>
<td>36 (21.2)</td>
<td></td>
<td>0.65</td>
<td>0.93</td>
<td>0.37</td>
<td>4.95</td>
</tr>
<tr>
<td>Western\textsuperscript{b}</td>
<td>20 (11.8)</td>
<td></td>
<td>0.33</td>
<td>0.46</td>
<td>0.17</td>
<td>1.72</td>
</tr>
<tr>
<td>Total</td>
<td>170</td>
<td></td>
<td>0.89</td>
<td>1.64</td>
<td>0.33</td>
<td>14.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a, b}: Provinces sharing the same superscript letter do not differ significantly in mean AFM1 contamination.

Figure 16. Percentage of milk samples contaminated with AFM1 by province (and total) above 0.05 μg/L (the European regulatory limit) and 0.5 μg/L (both the U.S. FDA action level and Codex Alimentarius AFM1 maximum level).
Table 6. Aflatoxin contamination in dairy feed

<table>
<thead>
<tr>
<th>Dairy feed types</th>
<th>Province of sample collection</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Easter n</td>
<td>Kigali n</td>
</tr>
<tr>
<td>Brewery by-products</td>
<td>#Samples (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (µg/kg)</td>
<td></td>
</tr>
<tr>
<td>STD *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize bran</td>
<td>#Samples (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (µg/kg)</td>
<td></td>
</tr>
<tr>
<td>STD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixe Feeds</td>
<td>#Samples (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (µg/kg)</td>
<td></td>
</tr>
<tr>
<td>STD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice bran</td>
<td>#Samples (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (µg/kg)</td>
<td></td>
</tr>
<tr>
<td>STD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat bran</td>
<td>#Samples (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean (µg/kg)</td>
<td></td>
</tr>
<tr>
<td>STD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*STD: Standard Deviation
At the time of sample collection, maize bran was the principal dairy feed used by farmers representing 65% of the total samples. Dairy farmers also used mixed feeds, brewery by-products, wheat bran and rice bran comprising 21.5%, 6.5%, 3.9% and 2.6% of the total samples collected, respectively. However, feed ingredients did not show a significant different contribution to the AFM1 contamination (p: 0.07).

**Discussion**

This study reports AFM1 contamination in milk samples, and total aflatoxin contamination in dairy feeds collected from dairy farms (170 dairy farmers) in all five provinces of Rwanda. While the cattle herd in Rwanda is estimated at more than one million head and 50.4% of households are reported to be rearing cattle (NISR, 2016), the low number of dairy farmers identified during the sample collection was due to the inclusion criteria set for the study (farmers must use complementary feeds, as opposed to exclusive grazing, and have at least two cows). De facto, this study excludes dairy farmers with dairy cows distributed through different programs to combat the food insecurity (e.g., “Girinka” program) who rely on grazing, and the produced milk is primarily intended for household consumption. Many dairy farmers cannot afford to purchase dairy feeds and rely principally on grazing (including grasses and residues of crops), or other freely available feedstocks, like parts of banana plants (Klapwijk, 2011; Mutimura et al., 2013).

The period of sample collection (month of July) represents the high-risk period for AFM1 contamination. It is the hottest period of the year with a shortage of natural pasture availability. It is also the harvest period with more crop byproducts available (such maize bran and wheat bran), which are obtained after milling process and are used as feed ingredients, and they are used to compensate for the fresh grasses shortage. The current study revealed that dairy feeds were
contaminated with 94.49 µg/kg aflatoxin mean (median: 36.9 µg/kg), presuming milk contamination with AFM1. However, the situation would be reverted during the rainy seasons with fresh grasses availability. A study conducted in five counties in Kenya reported a trend for low AFM1 contamination during the rainy season compared to the dry season; they attributed this trend to the availability of natural pastures and the low use of feed ingredients during the rainy season due to fresh grasses availability (Senerwa et al., 2016).

The average AFM1 contamination in milk samples obtained in the current study was 0.89 ± 1.64 µg/kg (median: 0.33 µg/L) with a maximum of 14.5 µg/kg. As a human carcinogen (IARC, 2012), milk consumers are under risk of AFM1 adverse effects. Ninety-one percent of milk samples exceeded the European regulatory limit (0.05 µg/kg), and 38% exceeded 0.5 µg/kg, which represents both the U.S. FDA action level, Codex Alimentarius, and EAC maximum limit. AFM1 contamination in milk samples was high compared to the AFM1 contamination reported previously in Kenya. In one study, the AFM1 mean concentration was 0.105 ± 0.195 µg/kg. A total of 56% (n=349) of samples exceeded 0.05 µg/L, while 3% (n=19) exceeded 0.5 µg/L with a lower trend of aflatoxin levels in products available in mid-income are and in processed milk samples (Ahlberg et al. 2018). Another study also conducted in Kenya found that in total of 291 sampled milk products (raw, pasteurized, UHT milk, yoghurt and lala), more than 50% of the samples exceeded 0.05 µg/kg, but only three samples exceeded 0.5 µg/L (Lindahl et al., 2018).

Kagera et al. (2018) confirmed the same trend with 64% of samples exceeding the European legal limit of 0.05 µg/L. The difference with our study would be explained, in part, by the sample collection methods. In the mentioned studies, milk samples were marketed milk and milk products, while our study focused on raw milk collected at dairy farms. It can be hypothesized
that AFM1 contamination was diluted in marketed milk from different origins or reduced in milk products by lactic acid bacteria (Ahlberg et al., 2015).

The heterogeneous nature of aflatoxin contamination in dairy feeds (Lee, Lilliehoj, & Kwolek, 1980) would also explain the absence of a positive correlation between aflatoxin contamination in dairy feeds and AFM1 contamination in milk (i.e., dairy feeds with high aflatoxin contamination and low AFM1 contamination in milk from the same dairy farm). Since this study did not determine the aflatoxin quantity consumed by cows per day for each farm to estimate the AFB1 carry-over accurately or other parameters associated with AFM1 excretion, there is a need to understand the aflatoxin dynamic in the milk value chain to better characterize the potential exposure to AFM1 via milk in the Rwandan context.

Provinces differed significantly in AFM1 contamination in milk due to, in part, the availability of feed types within provinces. For instance, the Western province had the lowest number of milk samples (15%) exceeding 0.5 μg/kg AFM1, and brewery by-products with the lowest aflatoxin contamination level (mean: 1.06 μg/kg). Brewery by-products are provided by the largest brewer and soft beverage company, Bralirwa (from the French acronym “Bralisseries et Limonaderies du Rwanda”), located in the Western province, and using mainly barley and other cereals. Unlike other dairy feed types collected in this study with high aflatoxin contamination, brewery by-products are less aflatoxin-contaminated, most likely because of mitigation measures taken by the brewery to minimize aflatoxin contamination in raw ingredients (i.e., cereals). The estimation of the effectiveness of post-harvest interventions in preventing aflatoxin contamination along the feed and milk value will give a better understanding of aflatoxin mitigation strategies and lay a foundation for future intervention.
Before reaching the last consumer, milk from different dairy farmers will be collected at MCCs or through other intermediates. The milk mixture from different origins would concentrate or dilute AFM1 levels in milk depends on the initial contamination. The investigation of AFM1 contamination along the milk value chain will provide insight into the AFM1 contamination dynamics and the risk for the last consumer in the Rwandan context.

Conclusions

This study measured the AFM1 concentration and aflatoxins in 170 raw milk and in 154 dairy feed samples, respectively collected in five provinces of Rwanda. The specific conclusions based on the results are:

1. Milk samples were contaminated with AFM1 at a concentration of 0.89 ± 1.64 µg/L (median: 0.33 µg/L). Up to 91% of samples exceeded 0.05 µg/L, the European Union limit, and 38% of them exceeded 0.5 µg/L, the legal limit of USFDA, Codex Alimentarius, and EAC.

2. Among five different dairy concentrate ingredients sampled (brewery byproducts, maize bran, mixed feeds, rice bran, and wheat bran), maize bran was the most common one, representing 65.5% of all samples.

3. A year-round survey of the Rwanda milk value chain for AFM1 contamination is recommended to verify seasonal variations and better understand risk for consumers, and to lay the foundation for future interventions and mitigation strategies.

Acknowledgments

This study was made possible by the generous support of the American people through the United States Agency for International Development (USAID) and its Feed the Future
Innovation Lab for Livestock Systems managed by the University of Florida and the International Livestock Research Institute, grant number No. AID-0AA-L-15-00003. This study is also supported by USAID, as part of the Feed the Future initiative, under the CGIAR Fund, award number BFS-G-11-00002, and the predecessor fund, the Food Security and Crisis Mitigation II grant, award number EEM-G-00-04-00013. The contents are the responsibility of the authors and do not necessarily reflect the views of USAID or the United States Government

References


CHAPTER 6. Efficacy of High Voltage Atmospheric Cold Plasma to Degradate Aflatoxin B1, B2, G1, and G2

Authors and Affiliations

Kizito Nishimwe1,2, Hu Shi1, Kevin Keener4, Dirk E. Maier3
1Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011, USA
2School of Agriculture and Food Science, University of Rwanda, PO Box 4285 Kigali, Rwanda
3Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, IA 50011, USA
4School of Engineering, University of Guelph, Ontario, Canada

Manuscript to be submitted to Journal of Agricultural and Food Chemistry

Abstract

This study aimed to assess the efficacy of High Voltage Atmospheric Cold Plasma (HVACP) to degrade four different types of aflatoxin (AF), toxic metabolites produced by Aspergillus flavus and A. parasiticus, i.e., aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2), and to elucidate different potential degradation products (DP). AF (200 µM) on watch glass was treated with HVACP at 85 kV for 0, 2, 5, 10, and 20 min. AF residues were analyzed for each treatment time, and DP were elucidated in samples treated for the maximum treatment time of 20 min. Results revealed that AFB1 and AFG1 were more susceptible decreasing by 90% and 74% after 2 min, respectively, compared to the non-treated samples (0 min). AFG1 residues were under the limit of quantification (LOQ < 5 µg/kg) after 10 and 20 min. AFB2 and AFG2 were less susceptible decreasing by 38% and 79% after 20 min, respectively. The presence of a double bond at C8-C9 for AFB1 and AFG1 and its absence in AFB2 and AFG2 explains the susceptibility to HVACP treatment as it is readily degraded by the reactive species generated in the air stream. Despite observed degradations, only one AFB1 DP (m/z: 331.08) was characterized presumably due to the LOQ of the laboratory technique used.
AFB1 DP resulting in hydroxylation of the double bond in the position C8-C9. AFB1 was previously reported in published studies. HVACP proofed efficacious in degrading AF. However, future research on the understanding of resulting reactive species dynamics in plasma and their role to degrade different AF types is needed to optimize efficacy of the technology for scale-up.

**Introduction**

Aflatoxins (AF) are secondary metabolites produced by molds, *Aspergillus flavus* and *A. parasiticus*, when conditions are favorable: higher water activity (0.85 - 0.99) and temperatures (27°C - 33°C) (Holmquist et al., 1983). AF are very toxic to humans and animals and are classified by the International Agency for Research on Cancer (IARC) as Group 1 carcinogens (IARC, 2012a). They are also mutagenic, teratogenic (IPCS-WHO, 1998), immunosuppressive (Raisuddin et al., 1993; Shivachandra et al., 2003; Thaxton et al., 1974), and are linked with growth impairment (Gong et al., 2004; Khlangwiset et al., 2011; Watson et al., 2018). There are four different types of AF; aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2). They are all structurally different. AFB1 and AFG1 have a double bond at the C8-C9 position at the furan ring and are metabolized in the liver into an epoxide. The C8-C9 double bond does not exist in AFB2 and AFG2 (IARC, 2012a; JECFA, 2018). Currently, there is no official approved method to decontaminate AF in food matrices. However, cold plasma (CP) has shown to be a potential technology to degrade AF.

CP is a non-thermal emerging technology used in the food industry driven by consumer needs for food product safety and minimal effects on nutritional food quality attributes. CP refers to an ionized gas consisting of a mix of ions, electrons, neutral molecules, and a myriad of
reactive gas species (RGS) (Siddique et al., 2018). High Voltage Atmospheric Cold Plasma (HVACP) is a variant of CP and consists of a dielectric barrier discharge (DBD) system with unique characteristics of maintaining created reactive species (RS) inside a sealed container (Yepez & Keener, 2016). By using atmospheric air, reactive oxygen species (ROS) are generated such as ozone (O3), superoxide anions (‘O2−), singlet oxygen molecules [O2 (1Δg)], and hydroxyl radicals (•OH) (Misra et al., 2019). Different reactive nitrogen species (RNS), in addition to ROS, such as nitric oxide (NO), nitrite (NO2−) and nitrate (NO3−) are also generated in the HAVCP system. However, they are hard to produce because of the N2 triple bond, which requires significant energy to break (Lu et al., 2016). The generated RGS in the HVACP system can be further used to decontaminate food matrices.

Shi et al. (2017) demonstrated HVACP efficacy to degrade AFB1 by 76% using a 5 min treatment in air with 40% relative humidity. Based on the chemical structures of the degradation products (DP), the authors concluded that RGS degrade AFB1 at the C8-C9 double bond of the furan ring (Shi et al., 2017). Interestingly, the C8-C9 is linked to the structure-activity relationships in toxicity and carcinogenicity of AFB1 (Wogan & Newberne, 1971), and its degradation would reduce AFB1 toxicity. Since AF have different chemical structures, little is known about the HVACP efficacy to degrade other AF. It can be hypothesized that HVACP treatment of AF would result in different DP, which differ from AFB1 DP mentioned above. This study aimed to investigate HVACP capacity to degrade all AF (AFB1, AFB2, AFG1, and AFG2) and identify different DP.
Materials and methods

AF standards and Chemical Reagents

AFB1 was purchased from Cayman Chemical Inc. (Ann Arbor, MI, USA). AFB2 was purchased from ARC (St Louis, MO, USA). AFG1 and AFG2 were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Chloroform HPLC grade and methanol HPLC grade were purchased from Fisher Chemical (MA, USA).

High Voltage Atmospheric Cold Plasma (HVACP) System

The HVACP system (Phenix Technologies, Accident, MD, U.S.A.) has been previously used (Shi et al., 2017). It consists of two electrodes separated by dielectric barriers (Figure 17), and high voltage generated by a transformer from an electrical source. AF samples were treated at 85 kV and 180 W (60 Hz) at laboratory conditions (25°C, 80-90 %, relative humidity). The electrode gap distance was maintained at 4.44 cm.

![Figure 17. Schematic of the experimental setup of the HVACP system with treatment sample inside sealed bag placed between electrodes.](image)

AF sample preparation

For each AF standard, a stock solution was prepared by dissolving AF into chloroform for a final concentration of 0.1 mg/mL. From the stock solution, one mL of 200 µM AF was
prepared and pipetted onto a glass slide and set aside for 1 h to allow chloroform to evaporate entirely. The glass slide was placed inside a translucent polypropylene box (27.31 cm × 17.78 cm × 4.44 cm) (Grainger Inc., Lake Forest, IL, U.S.A.) and tightly sealed inside a high-barrier Cryovac B4170 film to prevent gas species leakage from occurring during the HVACP treatment. AF samples were HVACP treated for 2, 5, 10, and 20 min. For each treatment time, the experiment was conducted in duplicate. After the HVACP treatment, AF samples were stored overnight at room temperature in their sealed bags. The storage time allows GRS in the HVACP to recover to the atmospheric air status. Samples were further extracted with 5 mL chloroform into a vial and dried with nitrogen. The extract was then vortexed and dissolved into 5 mL methanol.

**Optical Absorption Spectroscopy**

Optical Absorption Spectroscopy (OAS) was used to characterize generated active species in the HVACP system. OAS was carried out using optical fiber (Ocean Optics USB2000+, Inc., Florida, USA) with 0.2 nm/pixel resolution. The spectral acquisition (200–800 nm of the electromagnetic spectrum) was carried out using a MATLAB® (The Mathworks, MA, USA) computer code developed in-house, that uses the instrument control toolbox. The spectrum was collected for every 10 secs for 4 hours (20 min for HVACP treatment and the system turned off the remaining time). The experiment was conducted in the dark to avoid any luminosity interference.
Analysis of AF residues in samples

The quantification of AF residues in each sample was carried out using the LC-MS/MS method (Varian, Inc., Walnut Creek, CA, USA) (Column: Polaris 5 C18-A 150x2 mm, Guard column: MetaGuard 2 mm Polaris 5u C18-A, Mobile Phase A: 5 Mm Ammonium acetate in 10% methanol aqueous solution with 1% acetic acid, Mobile Phase B: 5 Mm Ammonium acetate in methanol with 1% acetic acid, and Flow rate: 0.20 mL/min). Total AF were analysed, AFB1 (precursor ion: 313 m/z, product ion: 285 m/z), AFB2 (precursor: 315 m/z, product ion: 287 m/z), AFG1 (precursor ion: 329 m/z, product ion: 243 m/z), and AFG2 (precursor ion: 331 m/z, product ion: 245 m/z) with 0.8 µg/kg limit of detection and and 5 µg/kg limit of quantification.

Characterization of AF DP

The characterization of AF DP was conducted using AF HVACP-treated samples for the maximum time (20 min) and analysis LC-MS/MS instrument based upon quadrupole and time-of-flight technology (QTOF) (Agilent QTOF 6540, Agilent Technologies, Inc.Santa Clara, CA, USA) in the electrospray (ESI) positive mode over the range m/z 100 – 1,000. The flow rate was at 0.7 mL/min with mobile phase A, water (90%)/methanol (10%) and 0.1% formic acid (v/v) and B, methanol (90%)/water (10%) and 0.1% formic acid (v/v) with injection volume 1 µL. A linear gradient was set as follows: 1.5 min of 99% mobile phase A to 99% mobile phase B over 18 min with 2 min for column equilibration with initial conditions. The limit of detection (LOD) was 0.01mg/mL. Agilent Masshunter Software analyzed generated data.
**Statistical analysis**

Data were analyzed using JMP Pro 14 software (SAS Inc., Cary, NC, USA). Result values were expressed as mean ± SEM. Analysis of variance (ANOVA) was used to compare the differences between groups followed by Tukey's honestly significant difference (Tukey-HSD) test for pairwise comparisons within groups. The statistical significance was set at $P \leq 0.05$.

**Results**

Different GRS were measured in the plasma after 20 min of treatment. $O_3$, $NO_2$, $NO_3$, and $N_2O_4$ were the major GRS formed. $O_3$ was the most abundant reactive species and reached the saturation point after 300 s, followed by $NO_2$ (Figure 18).

![Figure 18. OAS concentration (in parts per million by volume, ppmv) measurement during HVACP treatment (maximum 20 min). Time is expressed in seconds (log10 scale). Different colors represent different generated reactive species in the HVACP system in air.](image-url)
AF residues after the HVACP treatment

Figure 19 shows the HVACP ability to degrade AF. HVACP significantly reduced AFB1 and AFG1. AFB1 and AFG1 decreased by 90% and 74% after 2 min, respectively. AFB2 and AFG2 were less susceptible to degradation. After 20 min, AFB2 and AFG2 decreased by 38.6% and 79.4%, respectively.

![Graph showing aflatoxin concentration (µM) residues after HVACP treatment for 2, 5, 10, 20 min (n=2 for each treatment). Values that do not have the same letter for each AF are significantly different (p < 0.05).]

Figure 19. Aflatoxin concentration (µM) residues after HVACP treatment for 2, 5, 10, 20 min (n=2 for each treatment). Values that do not have the same letter for each AF are significantly different (p < 0.05).
AF degradation products

- AFB1 DP characterization

For the non-treated AFB1, one identifiable peak corresponds to AFB1 (Figure 20). After 20 min of treatment, two peaks were identified (a) and (b) representing AFB1 DP (retention time: 6 min) and AFB1 (retention time: 6.6 min), respectively (Figure 21).

![Figure 20](image1.png)
Figure 20. AFB1 chromatogram without HVACP treatment with one identified peak (a) for 6.6 min retention time.

![Figure 21](image2.png)
Figure 21. AFB1 chromatogram after 20 min HVACP treatment with two identified peaks (a, b) for 6 and 6.6 min retention times, respectively.

- AFB2 DP characterization

Chromatograms before and after treatment are similar, and both have the same retention time (6.3 min) (Figure 22 and Figure 23).
Figure 22. AFB2 chromatogram without treatment with one identified peak (a) for 6.3 min retention time.

Figure 23. AFB2 chromatogram after 20 min HVACP treatment with one identified peak (a) for 6.3 min retention time.

- **AFG1 DP characterization**

  The non-treated AFG1 chromatogram showed one peak at 5.5 min retention time corresponding to AFG1 (Figure 23). After the 20 min treatment, there was no identifiable peak on the chromatogram (Figure 24).
Figure 24. AFG1 chromatogram without treatment with one identified peak (a) for 6.3 min retention time.

Figure 25. AFG1 chromatogram after 20 min HVACP treatment. There is no identifiable peak.

- **AFG2 DP characterization**
  
  Two chromatograms for Figure 26 and Figure 27 show the peak with retention time 5.7 – 5.9 min and corresponds to AFG2.
Figure 26. AFG2 chromatogram without treatment with one identified peak (a) for 5.7 min retention time.

Figure 27. AFG2 chromatogram after 20 min HVACP treatment with one identified peak (a) for 5.9 min retention time.

- **DP products summary**

  Table 7 summarizes the characteristics of identified peaks. For each peak, the retention time and corresponding mass were identified. Agilent Masshunter Software estimated double bond equivalent (DBE), mass difference and probability score for the proposed formula of the identified mass.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Identified peaks</th>
<th>retention time (mn)</th>
<th>m/z&lt;sup&gt;a&lt;/sup&gt;</th>
<th>calculated m/z&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Diff&lt;sup&gt;b&lt;/sup&gt; (ppm)</th>
<th>DBE&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Score</th>
<th>Proposed formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>0 a</td>
<td>6.6</td>
<td>313.0707</td>
<td>313.0707</td>
<td>-0.11</td>
<td>12</td>
<td>100</td>
<td>C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>20 a</td>
<td>6</td>
<td>331.08</td>
<td>331.0812</td>
<td>1.91</td>
<td>11</td>
<td>99.94</td>
<td>C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;14&lt;/sub&gt;O&lt;sub&gt;7&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>6.6</td>
<td>313.0697</td>
<td>313.0707</td>
<td>3.09</td>
<td>12</td>
<td>99.86</td>
<td>C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;14&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
<tr>
<td>AFB2</td>
<td>0 a</td>
<td>6.3</td>
<td>315.0848</td>
<td>315.0863</td>
<td>4.82</td>
<td>11</td>
<td>99.65</td>
<td>C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;14&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>20 a</td>
<td>6.3</td>
<td>315.0845</td>
<td>315.0863</td>
<td>5.78</td>
<td>11</td>
<td>99.5</td>
<td>C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;14&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
<tr>
<td>AFG1</td>
<td>0 a</td>
<td>5.5</td>
<td>329.0641</td>
<td>329.0656</td>
<td>4.51</td>
<td>12</td>
<td>99.69</td>
<td>C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;7&lt;/sub&gt;</td>
</tr>
<tr>
<td>AFG2</td>
<td>0 a</td>
<td>5.7</td>
<td>331.0801</td>
<td>331.0812</td>
<td>3.42</td>
<td>11</td>
<td>99.82</td>
<td>C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;14&lt;/sub&gt;O&lt;sub&gt;7&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>20 a</td>
<td>5.9</td>
<td>331.0802</td>
<td>331.0812</td>
<td>3.12</td>
<td>11</td>
<td>99.85</td>
<td>C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;14&lt;/sub&gt;O&lt;sub&gt;7&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>: The m/z of [M + H]+, <sup>b</sup>: mass difference, <sup>c</sup>DBE = double bond equivalents
Discussion

The plasma system is a mix of RS, ultraviolet (UV) radiation electrons, and neutral molecules. Since HVACP is a non-thermal plasma, the generated heat (< 45°C) is insignificant to cause AF degradation. UV has shown AF degradation capacity and the required intensity in the range of 1,820 – 13,000 µW/cm² (Liu et al. 2010). The UV radiation in the HVACP treatment is attributed to N₂ species transition and is equivalent to 50 µW/cm² (Shi et al., 2017a), which is negligible compared to the required UV radiation to degrade AF mentioned above. Thus, AF degradation is attributed only to GRS generated.

In this study, AFB1 and AFG1 were susceptible to HVACP treatment, whereas AFB2 and AFG2 were less susceptible to the treatment. Our results corroborate a previous study that reported the AFB1 and AFG1 susceptibility to cold atmospheric pressure plasma compared to AFB2 and AFG2 (Siciliano et al., 2016). After 2 min, AFB1 and AFG1 decreased by 90% and 74%, respectively, and after 10 min and 20 min, AFG1 residues were under LOD (< 5 µg/kg). The susceptibility difference between AFB1 and AFG1, on the one hand, and AFB2 and AFG2, on the other hand, is due to the chemical structure difference: the presence of a double bond at C8-C9 position for AFB1 and AFG1, and its absence for AFB2 and AFG2. Shi et al. (2017) demonstrated that HVACP treatment resulted in the modification of the AFB1 furofuran ring with C8-C9 degradation after the HVACP treatment. Likewise, results suggested that AFG1 underwent the same degradation pathway because they have a double bond at the C8-C9 position. Even though AFB2 and AFG2 showed resistance to treatment, there was a significant effect after 20 min with an increase of O₃, NO₂, NO₃, and N₂O₄. NₓOᵧ started increasing in the plasma after 200 seconds (~3.3 min) of treatment, while at the same time, O₃ reached the saturation point. O₃ would have little or no effect on AFB2 and AFG2 because at the maximum, O₃ did not induce any degradation whereas their degradation was correlated to the
NRS increase. Proctor et al. (2004) showed that O$_3$ was not able to degrade AFB2 and AFG2 because of the lack of the C8-C9 double bond, which is readily oxidized by AFB1 and AFG1. NRS, most likely, degrades AFB2 and AFG2 in the HVACP system.

The identification of AF DP was based on the comparison of non-treated AF and AF treated for 20 min (the maximum time of treatment) chromatograms. For each visible peak on the chromatogram, the most intense mass was extracted and analyzed with Agilent Masshunter Software. Apart from expected peaks of AF standards, only one peak was observed as AFB1 DP in an AFB1 sample treated for 20 min (Figure 21). The absence of visible peaks in samples with known degradation (for instance, after 20 min of treatment, AFG1 residues were under LOD) was due to the sensitivity of methods (LOD < 0.01 mg/mL). Therefore, if the degradation resulted in multiple DP with less than 10 ppm, they would not be visible on the chromatogram.

The only mass of identified AFB1 DP was analyzed with Agilent Masshunter Software to determine the proposed molecular formula. Since HVACP operated with atmospheric air, the degradation can only be formed by carbon, oxygen, hydrogen, and nitrogen. Numerous molecular formulas were proposed based on the generated DP mass. Mass differences, DBE and the score (the overall score ranges from 0 to 100%, with a score closer to 100% is better) were used to determine the most likely AFB1 DP chemical formula. For example, AFB1 has a known mass of ~312 MW. The software generated several possible chemical formulas; the two first ranked formulas are C$_{17}$H$_{12}$O$_6$ (Diff.: -0.11, DBE: 12, and score: 100) and C$_{18}$H$_8$N$_4$O$_2$ (Diff.: 4.17, DBE: 17, and score: 99.74). The combination of three mentioned characteristics of formulas ranked C$_{17}$H$_{12}$O$_6$ as the most likely AFB1 formula because of less Diff., and the highest score. The proposed formula corresponds to the known AFB1 formula. Using the same method, the AFB1 DP (331.08 m/z) has C$_{17}$H$_{14}$O$_7$ proposed formula. The DP would likely result in the hydroxylation of the double bond in the position C8-C9 AFB1. In their study, Shi et al.(2017)
also found AFB1 DP to be the significant DP after HVACP and five other different AFB1 DP. The AFB1 DP is also reported as a result of ozone treatment (Luo et al., 2013) and in the photodegradation pathway of AFB1 in water solution (Liu et al., 2010; Wang et al., 2011).

**Conclusions**

This study resulted in the following specific conclusions due to HVACP treatment of four aflatoxin types for 2, 5, 10, and 20 min at 85 kV:

1. AFB1 and AFG1 were more susceptible to the HVACP treatment versus AFB2 and AFG2. Degradation differences between AFB1 and AFG1 compared to AFB2 and AFG2 were the result of their chemical structure differences: AFB1 and AFG1 have a double bond at C8-C9 which is absent in AFB2 and AFG2.

2. AFB1 was significantly decreased for all treatment times by 72.4% - 90.1%. There were no significant differences between treatment times (p>0.05).

3. AFG1 was significantly reduced by HVACP treatment. The percentage decrease ranged from 74.3% to 90.4% after 2 and 5 min of treatment, respectively. AFB1 residues were under LOQ (<5 µg/Kg) after 10 and 20 min of treatment.

4. AFB2 was significantly decreased by 38.6% after 20 min of treatment (p<0.05). After 10 and 20 min of treatment, AFG2 was significantly reduced by 60.3% and 79.4% after 10 and 20 min, respectively.

5. Characterization of one AFB1 degradation product (m/z:331.08) revealed hydroxylation of the double bond in position C8-C9 which is consistent with previously published research.
6. Despite the observed degradation of AFB2, AFG1, and AFG2, this study was not able to reveal possible degradation products, presumably due to the limit of quantification of the technique used (< 0.01 mg/mL).

7. Additional research is needed to characterize reactive species generated in the HVACP air stream and understand their role in degrading the four primary aflatoxin types of concern for food and feed safety to optimize efficacy before scaling up the technology.

**Acknowledgments**

This project was funded in part by the government of Canada through the Business Platform for Nutrition Research (BPNR), hosted by the Global Alliance for Improved Nutrition (GAIN) through the Aflatoxin-control Challenge in Africa organized by the Partnership for Aflatoxin Control in Africa (PACA). This study was also supported by USAID, as part of the Feed the Future initiative, under the CGIAR Fund, award number BFS-G-11-00002, and the predecessor fund, the Food Security and Crisis Mitigation II grant, award number EEM-G-00-04-00013. The contents are the responsibility of the authors and do not necessarily reflect the views of USAID or the United States Government.

**References**


Abstract

Aflatoxin B1 (AFB1) is a secondary metabolite produced by *Aspergillus flavus* and *A. parasiticus*, and is a known carcinogenic in humans and animals. High voltage atmospheric cold plasma (HVACP) technology has already shown the capacity to decontaminate AFB1 in food and feed crops and ingredients. This study aimed to investigate the *in vitro* cytotoxicity of AFB1 degradation products (DP) after HVACP treatment. AFB1 (100 µM) was treated at 85 kV with HVACP for 2, 5, 10, and 20-minute periods. HepG2 cells, a human hepatoma cell line, were exposed to AFB1 and AFB1 degradation products (DP) for 72 hours and assessed for cell viability, caspase-3 activity, DNA fragmentation, and protein carbonyl for each treatment time. HVACP significantly reduced AFB1 for all treatment times (p < 0.05). After 2 minutes, AFB1 was degraded at 90%. After 5, 10, and 20 minutes of treatment, the AFB1 was degraded at 81%, 72%, and 88%, respectively. After 10 and 20 min treatment, cell viability in HepG2 cells exposed to AFB1 DP did not show a significant difference compared to non-exposed HepG2 cells (negative control) (p > 0.05) but significantly differed from HepG2 cells exposed to AFB1(positive control) (p < 0.05). Caspase-3 activity, DNA fragmentation values, and carbonyl contents in HepG2 cells exposed to AFB1 DP after 20 min treatment were similar to those in
non-exposed HeG2 cells (p>0.05) but significantly differed from those in HepG2 cells exposed (p<0.05). While this study showed *in vitro* AFB1 hepatototoxicity reduction after 20 min HVACP treatment, it did not explore all toxicity aspects related to AFB1 such as immunotoxicity, growth impairment, and mutagenicity. This should be explored in futures studies before HVACP treatment is scaled up.

**Introduction**

Aflatoxins are secondary metabolites produced by *Aspergillus* sp, mainly *Aspergillus flavus* and *A. parasiticus* under favorable conditions with an optimum of 33°C and 99% relative humidity (Milani, 2013). Aflatoxins are very toxic. The International Agency for Research on Cancer (IARC) classifies aflatoxins under Group 1 as carcinogenic to humans and animals: they have been known to cause liver cancer (IARC, 2012a). Aflatoxin B1 (AFB1) is the most toxic among the four different types of aflatoxins (JECFA, 2018); aflatoxins persist in food matrices once formed. Being heat-resistant, traditional thermal methods cannot degrade them (Arzandeh & Jinap, 2011; Hwang & Lee, 2006; Raters & Matissek, 2008; Sani, Azizi, Salehi, & Rahimi, 2014).

Numerous methods to degrade aflatoxins have been tested with limited success. For instance, physical methods consisting of cleaning and sorting are nonspecific, and results may vary from one kernel to another (Shi et al., 2014a). Although chemical approaches are efficacious, their residues and the preservation of nutritional characteristics of treated food and feeds are areas of concern (Piva et al., 1995). Cold Plasma (CP), a novel technology used in the food industry, offers an alternative for aflatoxin degradation in food matrices treatment (Misra et al., 2019).
High Voltage Atmospheric Cold Plasma (HACP), a dielectric barrier discharge (DBD), is an approach used to generate highly reactive species (RGS) from the air and are kept inside a sealed package (Yepez & Keener, 2016). At 40% relative humidity, HVACP has been shown to efficiently degrade AFB1 in maize from 62% to 82% after 1 and 10 minute treatments, respectively (Shi et al., 2017a). However, little is known about the safety of AFB1 degradation products (DP).

AFB1 toxicity is linked to the presence of a double bond at the C8-C9 position in an AFB1 chemical structure. In the liver, cytochrome P450 (primary CYP3A4 and CYP1A2) enzymes metabolize AFB1 into AFB1-8, 9-epoxide, which is either hydroxylated and conjugated to glutathione or gluconic acid and excreted, or binds to DNA with a nucleophilic attack on the N-7 guanine position. This DNA lesion leads to a genetic mutation with a G → T transversion on codon 249 of the p53 tumor suppressor gene causing a 249Arg → 249Ser of the p53 protein (Chu, 2003; Rushing & Selim, 2019). Moreover, AFB1-8, 9-epoxide triggers apoptosis, the cell self-destruction process, by (a) activating death receptors and inducing cascade reactions of caspases (Meik et al., 2001; Mughal et al., 2017; Zheng et al., 2017), and (b) through mitochondrial pathways caused by a cytotoxic agent, aberrant oncogene expression, p53, and Members of the Bcl-2 oncoprotein family (Saraste & Pulkki, 2000). The apoptosis ultimately leads to cell shrinkage, DNA fragmentation, and chromatin condensation (Peng et al., 2016).

Degradation of the C8-C9 double bond would most likely lead to AFB1 toxicity reduction. Shi et al. also showed that the generated RGS in the HVACP system attack the C8-C9 double bond as well as the modification of the lactone ring, cyclopentanone, and the methoxyl group. Based on the structure-bioactivity relationship of AFB1, they hypothesized a reduction of AFB1 toxicity (Shi et al., 2017). Therefore, this study aimed to gain a more comprehensive
understanding of AFB1 DP after HVACP treatment using HepG2 cells by exploring the AFB1 mechanisms of action: cell viability, apoptosis, and protein oxidative damage.

**Material and methods**

**Sample preparation**

One mL of 100 µM AFB1 (Cayman Chemical Inc., Ann Arbor, MI, USA) dissolved in a chloroform Fisher Chemical (MA, USA) was pipetted onto a glass slide and left for one hour, allowing the chloroform to evaporate and dry. A glass slide was placed in a translucent polypropylene box (27.31 cm × 17.78 cm × 4.44 cm) (Grainger Inc., Lake Forest, IL, U.S.A.) and tightly sealed inside a high-barrier Cryovac B4170 film to prevent gas species leakage generated during the HVACP treatment, and treated with the HVACP system for 2, 5, 10, and 20 minute periods. The experiment was run in duplicate for each treatment time. Samples were subsequently left overnight at room temperature to allow the generated reactive species to return to a normal air state.

Treated samples were extracted with 5 mL chloroform, placed into vials, and then dried with nitrogen. The extract was subsequently dissolved in 100 µL Dimethylsulfoxide (DMSO) (ATTC, Manassas, VA, USA), fully vortexed, and completed with 10 mL Dulbecco’s Modified Eagle Medium (DMEM) media (Gibco, NY, USA) for a final concentration of 1% DMSO, non-toxic concentration in DMEM media. The sample solution was vortexed thoroughly before usage.

**Analysis of AFB1 residues in samples**

The quantification of AFB1 residues (precursor ion: 313 m/z, product ion: 285 m/z), in each sample was carried out using the LC-MS/MS method (Varian, Inc., Walnut Creek, CA,
USA) (Column: Polaris 5 C18-A 150x2 mm, Guard column: MetaGuard 2 mm Polaris 5u C18-A, Mobile Phase A: 5 Mm Ammonium acetate in 10% methanol aqueous solution with 1% acetic acid, Mobile Phase B: 5 Mm Ammonium acetate in methanol with 1% acetic acid, and Flow rate: 0.20 mL/min).

Cell culture

HepG2 (HB-8065), the human hepatocellular carcinoma cell line, was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). HepG2 cells were grown in a T-75 cell culture flask (Corning®, Corning, NY, US) to form a monolayer culture in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS) (VWR International, West Chester, PA, USA) and 1% penicillin-streptomycin (Gibco, NY, USA). HepG2 cells were incubated in a humidified atmosphere with 5% CO2 at 37°C. The DMEM medium was replaced twice a week, and HepG2 cells were washed with Phosphate-Buffered Saline (PBS) (Gibco, NY, USA), trypsinized (Gibco, NY, USA), diluted, and passaged every week at a 1:3 ratio.

HVACP system

The HVACP system (Phenix Technologies, Accident, MD, U.S.A.) employed has been described (Shi et al., 2017b) as consisting of two electrodes separated by dielectric barriers (Figure 28), with high voltage generated by a transformer from an electrical source. Aflatoxin samples were treated at 85 kV voltage for a power of 180 W (80 Hz) under laboratory conditions (25°C, 80-90 %, relative humidity). The electrode gap distance was 4.44 cm.
Figure 28: Schematic of the experimental setup for HVACP treatment

MTT assays

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (EMD Millipore, Temecula, CA, USA) were used to assess cell viability according to manufacturer instructions. HepG2 cells were harvested and diluted with the culture medium at $1 \times 10^5$ cells/mL, and plated into a 48-well plate at 100 µL per well. Cells were left undisturbed overnight to allow recovery from handling. The used medium was then replaced with a fresh medium (100 µL per well) containing treated AFB1. Cells were incubated for 72 hours (37°C, 5% CO₂) and assessed for cell viability. After incubation, 10 µL MTT was added to each well and mixed by tapping gently on the side of the tray. The plate was further incubated at 37°C for four hours to allow MTT cleavage to occur. Subsequently, the MTT formazan produced in wells containing lice cells appeared as black fuzzy crystals on the well bottoms. After incubation, 100 µl isopropanol with 0.04 N HCl was added to each well and mixed thoroughly by repeated pipetting to change the red medium color to a yellow tone that would not interfere with MTT formazan measurement. The isopropanol dissolved the formazan to give a homogeneous blue solution suitable for absorbance measurement. The absorbance on an ELISA plate reader was measured at 570 nm wavelength within an hour (Biotek, Winnoski, VT, USA). Results were expressed as percentage cell viability of treated cells to negative control assay (viable, untreated cells) (% cell viability =
[(absorbance treated cells--absorbance blank)/(absorbance control cells--absorbance blank)]×100.

**Caspase-3 assays**

HepG2 cells were harvested and diluted with culture medium at 2×10^7 cells/mL and plated in a 6-well plate at 1.5 mL per well. Cells were left undisturbed overnight to negate the effects of handling. The used medium was removed and replaced with a fresh medium (1 mL per well) containing treated AFB1. Cells were incubated for 72 hours (37°C, 5%CO₂), then harvested with a cell scraper and quickly centrifuged at 700 rpm for seven minutes. After the cell pellet was washed with Gibco PBS (ThermoFisher Scientific, Waltham, MA, USA), it was re-suspended with a caspase assay buffer (50 mM Tris HCl ((pH 7.4)); 1 mM EDTA; 10 mM EGTA) containing TRITON® X-100 (ThermoFisher Scientific, Waltham, MA, USA) at a final concentration of 0.1%. Finally, it was vortexed vigorously for 15-30 seconds to lyse the cells, and incubated at 37°C for 20 minutes to allow for complete lysis. Lysates were subjected to centrifugation at 10,000 rpm for 15 minutes, and 190 µL supernatant incubated with 10 µL specific fluorescent substrate (final 50 µM), Ac-DEVD-AFC (Cayman, Ann Arbor, MI, USA) for one hour at 37°C. Caspase-3 activity was measured with excitation at 400 nm and emission at 505 nm using a fluorescence microplate reader (SpectraMax, M2). Caspase-3 activity was normalized to protein concentration and expressed as fluorescent units/mg protein.

**DNA fragmentation assays**

DNA fragmentation assays were performed using the Cell Death Detection ELISA (Roche Diagnostics, Mannheim, Germany) according to manufacturer instructions. HepG2 cells
were harvested and diluted with a culture medium at $1 \times 10^5$ cells/mL, and 500 µL ($5 \times 10^4$ cells/tube) were transferred into Eppendorf tubes. An equivalent of 500 µL culture medium with treated AF1 was added to tubes and closed loosely to further promote gas exchange. Tubes were incubated at 37°C, 5% CO$_2$ for 72 hours. After incubation, cell pellets were collected at 200× g for 5 minutes and resuspended in one mL of culture medium. Cell pellets were centrifuged at 1500× g for 5 minutes and resuspended with a 500 µL incubation buffer per tube provided in the kit for 30 minutes at room temperature. Lysates were centrifuged at 20,000× g for 10 minutes, and an equivalent of 400 µL supernatant was prediluted at 1:10 with incubation buffer ($1 \times 10^4$ cell equivalents/ml). The 100 µL diluted supernatant was incubated with a 100 µL anti-histone coated solution for 90 minutes. Additionally, an anti-DNA-POD conjugate solution (100 µL) was added to wells after washing away unbound components. A substrate solution (100 µL) was added to each well and incubated on a plate shaker at 250 rpm for 15 minutes until the color developed. Measurement absorbance was made at 405 nm for a substrate solution blank.

**Protein carbonyl assay**

HepG2 cells were grown into 75-cm$^2$ cell culture flask until 80% confluent. The used medium was removed, cells washed with Gibco PBS (ThermoFisher Scientific, Waltham, MA, USA) and replaced with a fresh medium (3 mL) containing treated AFB1. Cells were incubated for 72 hours (37°C, 5% CO$_2$), then harvested with a cell scraper and quickly centrifuged at 700 rpm for seven minutes. The oxidative damage to proteins was determined by measuring the protein carbonyl residues using DNPH (2,4-dinitrophenylhydrazine) (Cayman, Ann Arbor, MI, USA), which reacts with protein carbonyls according to the manufacturer instructions. This forms a Schiff base to produce the corresponding hydrazine, and can be analyzed
spectrophotometrically at wavelengths of 360-385 nm. In the same sample, the difference in absorbance between DNPH-reacted and nonreacted HCl was used to determine the carbonyl content, normalized to protein concentration. The results were expressed as carbonyl content (nmol/mg).

**Statistical analysis**

The data were analyzed using JMP Pro 14 software (SAS Inc., Cary, NC, USA). Result values were expressed as mean ± SEM. Analysis of variance (ANOVA) was used to compare the differences between groups followed by Tukey's honestly significant difference (Tukey-HSD) test for pairwise comparisons within groups. The statistical significance was set at P ≤ 0.05.

**Results and Discussion**

**AFB1 degradation after HVACP treatment**

HVACP significantly reduced AFB1 for all treatment times (p < 0.05). After 2 minutes, AFB1 was degraded at 90%. After 5, 10, and 20 minutes of treatment, the AFB1 was degraded at 81%, 72%, and 88%, respectively (Figure 29). These results confirmed the previous Shi et al. (2017b)’s work and HVACP efficacy to degrade AFB1, which showed 76% AFB1 degradation after 5 minutes HVACP treatment. However, AFB1 degradation did not show the same trend with HepG2 cells’ survival rate (Figure 30). After 2 minutes of HVACP treatment, the AFB1 degradation achieved a 90% reduction and the degradation was consistent over treatment times (p > 0.05), but the detoxification (or HepG2 cells’ survival rate) results were not achieved until much longer treatment time (between 10 and 20 minutes of HVACP treatment). This situation would be explained by the formation of intermediate AFB1 DPs, which are still toxic to HepG2.
cells. Structural elucidation of all AFB1 DPs for all treated times will give more insights on the AFB1 DPs chemical structure changes compared the AFB1 structure.

Figure 29. Percentage of AFB1 remaining residues after HVACP treatment for 0, 2, 5, 10, and 20-minute periods for 72 hours. Results are expressed in percentage (±SEM) of AFB1 remaining residues compared to non-treated AFB1. There is no significant difference in treatments with the same letters (p < 0.05).

**Cell viability**

The liver is the main target organ of AFB1 toxicity, and the HepG2 cell line is an excellent model for an *in vitro* liver toxicity study since it retains hepatic activities with various phase I and II enzymes (Knasmüller et al., 1998).

In the present study, 50% of the HepG2 cells (IC$_{50}$: inhibitory concentration) survived the 100 μM AFB1 exposure for 72 hours, which is in agreement with IC$_{50}$ previously reported (Liu et al., 2012). However, 10 μg/mL AFB1 (Reddy et al., 2006), and 200 μM AFB1 (Luo et al.,
were also reported as IC$_{50}$ in HepG2 cells. The growth status of cells could explain IC$_{50}$ differences, and AFB1 can cause different types of cell damages in various morphologies (Luo et al., 2014). HepG2 cells treated with 100 µM AFB1 DP after HVACP treatments of 2, 5, 10, and 20 minutes showed that 10 and 20 minute HVACP treatments did not significantly change HepG2’s survival rate compared to non-treated HepG2 cells (Figure 30). The HVACP system generates a mix of electrons, UV, and RS (nitrogen species and oxygen species) since it is a closed system using atmospheric air. This could indicate that only generated RS are capable of degrading AFB1 because UV and heat do not significantly cause AFB1 degradation. These results confirm the hypothesis of an AFB1 C8-C9 double bond breakdown during HVACP treatment associated with AFB1 toxicity (IARC, 2012b). It also renders degradation as non-toxic (Shi et al., 2017) which is related to AFB1 toxicity; therefore, it reduces AFB1 toxicity. Prior research corroborates the findings of the reactive nitrogen species (NRS) and reactive oxygen species (ROS). In one study Sakudo et al. (2017), generated species in nitrogen gas plasma degraded AFB1 treated for 1–30 minutes, and AFB1 DP exposed to HepG2 cells for 48 hours showed no significant differences between it and non-treated cells. Another study (Luo et al., 2014) showed that reactive oxygen species, ozone for instance, are capable of degrading AFB1 and rendering DP safe to HepG2 cells. The survival rate results revealed that the toxicity of AFB1 DP was significantly decreased or disappeared after 10 and 20 minutes of HVACP treatment.
Figure 30: Percentage of HepG2 cell viability exposed to 100 μM AFB1 treated with HVACP for 0, 2, 5, 10, and 20-minute periods for 72 hours. Results are expressed in percentage (±SEM) of cell viability compared to non-exposed HepG2 cells. There is no significant difference in treatments with the same letters (p < 0.05).

**Caspase-3 activity**

Caspase-3 activity was chosen for the study because it is a hallmark and plays an essential role in the apoptosis pathway (Chen et al., 2001; Porter & Jänicke, 1999), and AFB1 exerts its toxicity via the apoptosis pathway in cells. This study showed a significant gradual decrease in caspase-3 activity over time. The caspase-3 activity after 20 minutes of treatment did not significantly differ from non-treated HepG2 cells (Figure 31). The apoptotic reduction in HepG2 cells exposed to DP clearly indicates a decrease in their toxicity, and confirms HVACP efficacy to not only degrade AFB1, but also to degrade non-DP toxicity if exposed to HepG2 cells. Our present findings are in agreement with previous studies on AFB1 and the increase of
caspase-3 activity. The authors noted excessive apoptotic activity in the jejunum of one-day-old broilers exposed to 0.3 mg/kg AFB1 compared to controls initiated through mitochondrial and death receptor mediated pathways (Peng et al., 2014). In another study (Ribeiro et al., 2010), 40 Male Wistar rats that were 45 days-old and exposed to 9 nM AFB1, experienced apoptosis and cell death in primary hepatocytes that had been incubated for four hours. Since AFB1-8, 9-epoxide initiates apoptosis activity (Peng et al., 2016; Saraste & Pulkki, 2000), our findings support the hypothesis of C8-C9 double bond degradation during HVACP treatment by generating RS and making DP incapable of inducing apoptosis.

![Figure 31: Caspase-3 activity exposed to 100 μM AFB1 treated with HVACP for 0, 2, 5, 10, and 20 minute periods for 72 hours. Results are expressed in arbitrary fluorescence unity (AFU)/mg protein (±SEM). There is no significant difference in treatments with the same letters (p < 0.05)
DNA fragment

The HepG2 nucleosomes in cytoplasmic fractions exposed to AFB1 degradation products were significantly reduced after 20 minutes of HVACP treatment, and did not differ significantly from non-treated HepG2 cells (Error! Reference source not found.). The presence of nucleosomes is linked to DNA fragmentation which follows DNA damage during the apoptosis process. Upon activation, caspase-3 cleaves DNA fragmentation factor (DFF) 45 and dissociates from DFF 40 or the caspase-activated DNase (CAD). CAD then triggers the DNA fragmentation into nucleosomal units (Liu et al., 1997; Nagata, 2000; Zhang & Xu, 2000). The reduction of nucleosomes fraction after 20 minutes of HVACP treatment corroborates caspase-3 activity reduction in HepG2 cells exposed to AFB1 degradation products (Error! Reference source not found.), and confirms AFB1 DP toxicity reduction after HVACP treatment. As previously noted, the results are most likely explained by the reaction of RS and AFB1, and degradation of the C8-C9 double bond.

Figure 32: DNA fragmentation level in HepG2 cells exposed to 100 μM AFB1 treated with HVACP for 0, 2, 5, 10, and 20-minute periods for 72 hours. Results are expressed in measured absorbance (405 nm - 490 nm) (±SEM). There is no
significant difference in treatments with the same letters (p < 0.05).

**Protein carbonyl**

The results failed to demonstrate a significant difference between non-treated and treated HepG2 cells with DP after a 20-minute treatment. Carbonyl contents showed a decrease of HVACP over time (Figure 33). AFB1 exposure to cells enhances reactive oxygen species (ROS) formation and causes oxidative damage. ROS can then cause oxidative in proteins, lipids, and DNA (Marin and Taranu 2012). The presence of carbonyls in proteins is a marker of ROS mediated protein oxidation accompanied by the conversion of side chains of some amino acid residues (histidine, arginine, lysine, and proline) to carbonyl derivatives (Amici et al., 1989).

Previously, fish treated with AFB1 (100 µg/100 g BW) for six days showed a significant increase in protein carbonyl in the liver, kidney, and brain (Madhusudhanan et al., 2004). The reduced carbonyl content after 20 minutes of HVACP treatment indicates that DP did not induce the oxidative damage, which is explained by RS efficacy to degrade AFB1.
Figure 33: Carbonyl content levels in HepG2 cells exposed to 100 µM AFB1 treated with HVACP for 0, 2, 5, 10, and 20-minute periods for 72 hours. Results are expressed in percentage (±SEM) of cell viability compared to non-exposed HepG2 cells. There is no significant difference in treatments with the same letters (p < 0.05)

**Conclusions**

As a result of this study investigating *in vitro* cytotoxicity of HepG2 cells exposed to AFB1 DP after HVACP treatment, the following specific conclusions are drawn:

1. AFB1 degradation did not a significant difference over treatment time (p < 0.05). After 2 minutes, AFB1 was degraded at 90%. After 5, 10, and 20 minutes of treatment, the AFB1 was degraded at 81%, 72%, and 88%, respectively.

2. 100 µM AFB1 showed a statistically significant reduction (54%) of HepG2 cell viability after 72 hours of exposure.

3. Cell viability in HepG2 cells exposed to AFB1 DP for 10 and 20 min treatments did not statistically differ from non-exposed HepG2 cell viability (p>0.05).

4. Caspase-3 activity decreased overtime in HepG2 cells exposed to AFB1 DP; however after 20-min treatment it was not different from caspase-3 activity in non-exposed HepG2 cells (p>0.05).

5. A 20-min HVACP treatment of AFB1 did not induce significantly DNA fragmentation compared to non-exposed HepG2 cells (p>0.05).

6. Carbonyl content in HepG2 cells exposed to AFB1 DP after 20 min HVACP treatment was similar to the carbon content in non-exposed HepG2 cells (p>0.05).

7. Overall, the measured toxicity indicators (cell viability, caspase-3, DNA fragmentation, and protein carbonyl) did not show a statistically significant
difference compared to non-exposed HepG2 cells after 20 min HVACP treatment (p>0.05). This confirms the hypothesis of AFB1 C8-C9 double bond degradation during HVACP treatment and the loss of AFB1 DP toxicity.

8. This study did not explore all toxicity aspects related to AFB1 such as immunotoxicity, growth impairment, and mutagenicity. Therefore, futures studies should explore those aspects further.

Acknowledgments

This project was funded in part by the government of Canada through the Business Platform for Nutrition Research (BPNR), hosted by the Global Alliance for Improved Nutrition (GAIN) through the Aflatoxin-control Challenge in Africa organized by the Partnership for Aflatoxin Control in Africa (PACA). This study was also supported by USAID, as part of the Feed the Future initiative, under the CGIAR Fund, award number BFS-G-11-00002, and the predecessor fund, the Food Security and Crisis Mitigation II grant, award number EEM-G-00-04-00013. The contents are the responsibility of the authors and do not necessarily reflect the views of USAID or the United States Government.

References


CHAPTER 8. DEGRADATION OF AFLATOXIN IN SHELLED MAIZE INOCULATED WITH ASPERGILLUS FLAVUS USING HIGH VOLTAGE ATMOSPHERIC COLD PLASMA (HVACP) AND HVACP EFFECTS ON LIPID OXIDATION AND COLOR CHANGE IN MAIZE

Abstract

Aflatoxins (AF) are a significant concern for the global food and feed industry. High Voltage Atmospheric Cold Plasma (HVACP) has shown potential to degrade AF in maize. In this study, 25 g maize samples were inoculated with A. flavus spores ($10^6$ spores/mL), incubated for two weeks and then treated with HVACP for 7, 15, and 30 min. HVACP significantly reduced AFB1 in all treatment times. After 7 and 15 min, AFB1 decreased by 81.3-96.3% and 78.1-85.0%, respectively. The 30 min treatment showed AFB1 decreasing from below the LOQ (< 5 µg/kg) to 88.9% ± 10.3. HVACP treatment inhibited A. flavus growth significantly reducing it by 2.4 and 1.9 log_{10} for direct and indirect exposure, respectively. No A. flavus CFU formed for the 15 and 30 min treatments. HVACP treatment resulted in a slight change in yellow color but neither induced lipid oxidation nor changed flavor profile in treated maize samples. Future studies should focus on the optimization of HVACP treatment efficacy for AF reduction before the technology is scaled up.

Introduction

Aflatoxins (AF) are a category of mycotoxins produced mainly by Aspergillus flavus and A. parasiticus that contaminate crops under favorable conditions. They have attracted much attention because of their adverse effects; they are highly toxic to humans and animals (Ismaiel & Papenbrock, 2015). The International Agency for Research on Cancer (IARC) classifies AF in Group 1 as a human carcinogen (IARC, 2012a). AF contribute to 4.6-28.2% of overall hepatocellular carcinoma (HCC) each year, mainly in Africa and Asia due to the consumption of
aflatoxin-contaminated staple diets, mainly containing maize and groundnut (Liu & Wu, 2010). AF also pose an economic burden; 25% of the world’s food crop production is lost due to aflatoxin contamination (WHO, 2018). AF are common, and different methods have been tested to decontaminate AF in food matrices with limited results. For example, ozone is technically feasible but costly, and thermal technologies result in incomplete AF degradation and cause negative effects on food product quality (Pankaj et al., 2018a). There is a need for more research to explore other effective technologies that can reduce AF contamination, but also investigate whether their application to commercial scale is feasible and cost-effective for smallholder farmers.

Cold plasma (CP) technology offers potential applications for AF decontamination in food matrices (Misra et al., 2019). Plasma refers to quasi-neutral ionized gas, primarily photons, ions, electrons, and neutral molecules (Pankaj & Keener, 2017). The generated reactive species (GRS) in plasma cause AF breakdown resulting in less toxic degradation products (Misra et al., 2019). High Voltage Atmospheric Cold Plasma (HVACP) is a way to generate plasma using dielectric barrier discharge at atmospheric pressure in a sealed container. Shi et al. (2017) applied HVACP treatment to 25 g maize samples artificially coated with AFB1 and observed 82% AFB1 reduction after 10 min using 40% relative humidity air.

Since maize kernels are rich in easily degraded content (e.g., fat in maize germ degraded to fatty acids) (Sanjeev et al., 2014), lipid oxidation would be a concern because of GRS reaction with polyunsaturated fatty acids resulting in rancidity (Kamal-Eldin, 2006). Likewise, GRS would react with color pigments and cause color change. Rancidity and color change are essential characteristics that can influence consumer taste, and thus determine acceptability of the technology. Therefore, HAVCP scale-up requires understanding treatment effect on lipid oxidation and color change on natural AF contamination in maize. This study aimed to
investigate HVACP efficacy to (i) degrade AF naturally produced by *A. flavus* in maize kernels, and (ii) explore HVACP effect on lipid oxidation and color change of treated maize.

**Material and methods**

**Maize sample preparation**

Maize harvested during Fall 2018 at the Iowa State University (ISU) Agricultural and Agronomy Research and Demonstration Farm and allowed to dry naturally to 13.5% moisture content was tested for AF (< 5 µg/kg) and then inoculated with $10^6$ spores/mL *A. flavus* provided by Dr. Munkvold’s ISU seed pathology lab. Maize kernels (2.5 kg) were homogeneously spread with 18 mL spore in a rotary seed treater for 2 min to ensure uniform coverage on grain. Inoculated maize kernels were incubated at 30°C and 95% relative humidity (r.h.) for three weeks. Treated maize was then kept in a cold room (-18°C) until further analysis.

**HVACP system**

The HVACP system (Phenix Technologies, Accident, MD, U.S.A.) used was previously described by Shi et al. (2017). It consists of two electrodes separated by dielectric barriers (Figure 34). A transformer from an electrical source generates high voltage. Maize samples were treated at 85 kV and 180 W (60 Hz) at laboratory conditions (25°C, 80-90 % r.h.). The electrode gap distance was 4.44 cm.

**HVACP Treatment of inoculated maize samples**

A 20g inoculated maize sample was sub-sampled with a Humboldt Riffle (Elgin, IL, USA) and placed on a petri dish to form a monolayer for maximum HVACP exposure.
A Petri dish was placed inside a translucent polypropylene box (27.31 cm × 17.78 cm × 4.44 cm) (Grainger Inc., Lake Forest, IL, U.S.A.) and tightly sealed inside a high-barrier Cryovac B4170 film to prevent gas species leakage generated during the HVACP treatment. Samples were treated for 7, 15, and 30 min with direct (in the field of two electrodes) and indirect (out of the field of electrodes) exposure to the electrodes. For each treatment time, the experiment was conducted in triplicate. After HVACP treatment, maize samples were stored overnight at room temperature in their sealed box. The storage time allows GRS in the HVACP to recover into atmospheric air status.

**Aflatoxin analysis in treated maize samples**

Treated maize samples were ground with a Retsch ZM 200 (Retsch, Newtown PA, USA) to reduce sample particle size to a fineness similar to instant coffee with 95% passing through a 20-mesh screen. AF was extracted with HPLC grade methanol 80% (v/v) (Fisher Chemical, MA, USA), and analyzed with LC-MS/MS method (Varian, Inc., Walnut Creek, CA, USA) (Column: Polaris 5 C18-A 150x2 mm, Guard column: MetaGuard 2 mm Polaris 5u C18-A, Mobile Phase A: 5 Mm Ammonium acetate in 10% methanol aqueous solution with 1% acetic acid, Mobile Phase B: 5 Mm Ammonium acetate in methanol with 1% acetic acid, and Flow rate: 0.20
mL/min). Total AF, AFB1 (precursor ion: 313 m/z, product ion: 285 m/z), AFB2 (precursor: 315 m/z, product ion: 287 m/z), AFG1 (precursor ion: 329 m/z, product ion: 243 m/z), and AFG2 (precursor ion: 331 m/z, product ion: 245 m/z) were analyzed with 0.8 µg/kg limit of detection and 5 µg/kg limit of quantification.

**A. flavus CFU analysis in treated maize samples**

One gram of milled inoculated sample was diluted with 10 mL sterile distilled water (1/10). From the diluted suspension, 0.1 mL was spread on the *Aspergillus flavus* and *parasiticus* Agar (AFPA) media and incubated for four days. Three replications were made per dilution. The number of colonies was counted for each plate, the number of colonies on the three plates were averaged, and multiplied with a dilution factor of the sample used divided by the volume plated.

**Optical Absorption Spectroscopy**

Optical Absorption Spectroscopy (OAS) was used to characterize GRS in the HVACP system. OAS was carried out using optical fiber (Ocean Optics USB2000+, Inc., Florida, USA) with 0.2 nm/pixel resolution. The spectral acquisition (200-800 nm of the electromagnetic spectrum) was carried out using a MATLAB® (The Mathworks, MA, USA) computer code developed in-house that uses the instrument control toolbox. The spectrum was collected every 10 secs for 4 hours. After 30 min of treatment, the HVACP system was turned off and kept measuring reactive species for 4 hours. The experiment was conducted in a darkened lab to avoid any luminosity interference.

**HVACP treatment of non-inoculated maize samples**

Non-inoculated maize samples were treated under the same conditions mentioned above to compare against lipid oxidation and color change of treated maize kernels.
Lipid oxidation

Lipid extraction from maize

Lipids were extracted with n-hexane after a modified procedure as described by Song & Shurson (2013). Briefly, 50 g of ground maize was transferred to Erlenmeyer flask containing 100 mL n-hexane. The maize sample was then mixed with n-hexane at room temperature for 15 min by stirring. The mixture was filtered with Whatman #1 filter paper. The hexane solution containing lipids was collected into a 250mL, round-bottom flask. The step was repeated twice by adding 50mL n-hexane. The n-hexane solution was evaporated with a rotary evaporator for 30 min and left only maize oil. Maize oil extracts were stored at -20°C until further analysis.

Lipid peroxidation

The lipid peroxidation was measured using peroxide value (PV) and Thiobarbituric acid reactive substances (TBARS).

PV measurement

160 mg lipid extracts were mixed with 20 mL of a 3:2 (v:v) mixture of acetic acid – chloroform, the 1 mL of fresh saturated aqueous potassium iodide solution was added. The solution was left swirling for one min and then 20 mL of distilled water was added. Starch (0.5 mL) was added to the mixture as an indicator. A 0.01 N Na₂S₂O₃ solution was used to titrate until the blue-purple color disappeared, and the mixture became colorless. The PV of lipids was calculated using the equation:

\[ \text{PV (meq. of peroxide/kg oil)} = \frac{1000(S-B) \times N}{\text{weight of the sample}} \]

(S: sample titration, B: Blank titration), and N: Normality of Na₂S₂O₃)
**TBARS quantification**

0.2 g oil extract sample was added to 4.8 mL Trichloroacetic acid (TCA)/thiobarbituric acid (TBA) and mixed thoroughly. Mixtures were heated in a boiling water bath for 35 min and cooled to room temperature in an ice bath. Samples were then centrifuged at 1,000×g for 5 min. The absorbance was measured at 535 nm. The TBARS concentration was calculated versus the 1,1,3,3-tetraethoxypropane (1,1,3,3-TEP) solution standard curve equation, standardized to the weight of oil (nmol/g).

**Determination of color change**

The color change was determined with a MiniScan Ez colorimeter (HunterLab, Reston, Virginia, USA) at D65/10° and expressed as results of 3 parameters:

- **L***: lightness of color (L*=0 represents black and L*=100 represents white)
- **a***: color position between red and green (negative values indicate green and positive values indicate red)
- **b***: color position between yellow and blue (negative values indicate blue and positive values indicate yellow).

**Statistical analysis**

Data were analyzed using JMP Pro 14 software (SAS Inc., Cary, NC, USA). Result values were expressed as mean ± SEM. Wilcoxon test was used to assess HVACP efficacy to reduce AF contamination in maize while Kruskal-Wallis test was used to compare differences between exposure types (direct and indirect). The statistical significance was set at p ≤ 0.05.
Results

OAS measurement

Figure 35 shows different GRS measured in the plasma system using OAS during the HVACP treatment (30 min). Samples were left unopened for four hours (post-treatment). GRS measured were: Ozone ($O_3$), NO$_2$, NO$_3$, N$_2$O$_4$, and N$_2$O$_5$+H$_2$O$_2$. Ozone reached more than 3,000 ppm at 25 min of treatment then started decreasing. NO$_2$, NO$_3$, N$_2$O$_4$, and N$_2$O$_5$+H$_2$O$_2$ were also formed and persisted after the system was turned off.

![Diagram showing OAS concentration during HVACP treatment and post-treatment time.](image)

Figure 35. OAS concentration (in parts per million by volume, ppmv) measurement during HVACP treatment (maximum 30 min) and post-treatment time (3 hours and 30 min). Time is expressed in seconds (log$_{10}$ scale). Different colors represent different generated reactive species in the HVACP system in air.

AF levels before and after HVACP treatment

After incubation, maize kernels had 266.7 ± 24.03 µg/kg AFB1. Other AF (B2, G1, and G2) were also produced; however, their levels were below the LOQ (< 5 µg/kg), and were not
taken into consideration during HVACP treatment analysis. AFB1 levels at different treatment times (7, 15, and 30 min) showed a significant differences after HVACP treatment (p < 0.05) (Figure 36). After 7 min of HVACP treatment, AFB1 decreased at 96.3% ± 2.81 and 81.3% ± 17.8 for direct and indirect treatment. For 15 min HVACP treatment, AFB1 decreased by 85.0% ± 5.73 and 78.1% ± 20.9% for direct and indirect exposure, respectively. The 30 min HVACP treatment showed AFB1 decrease ranging from below the LOQ to 88.8% ± 10.3. Direct or indirect exposure to electrodes did not result in a significant difference (p > 0.05).

![Figure 36. AFB1 levels (µg/kg) in control (non-treated) samples, and maize samples HAVCP-treated for 7, 15, and 30 min. For each treatment time, samples were exposed to direct and indirect positions versus electrodes. Results are expressed in µg/kg ± SEM. Levels not connected by the same letter are significantly different (p < 0.05).](image-url)
A. *flavus* reduction before and after the HVACP

Table 8 shows *A. flavus* CFU/g maize reduction counted at $10^{-1}$ dilution. HVACP treatment significantly reduced *A. flavus* contamination ($p < 0.05$). After 7 min, *A. flavus* CFU/g maize were reduced by 2.4 and 1.9 log$_{10}$ for direct and indirect exposure, respectively. No *A. flavus* CFU/g maize was observed after 15 and 30 min.

Table 8: Reduction in *A. flavus* colony-forming units (CFU)/g maize after HVACP treatment for three times and direct and indirect exposure to the HVAC system electrodes.

<table>
<thead>
<tr>
<th>Exposure time (min)</th>
<th>Log$_{10}$ reduction in cfu/g, mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exposure</td>
</tr>
<tr>
<td></td>
<td>Direct</td>
</tr>
<tr>
<td>Control</td>
<td>4.92</td>
</tr>
<tr>
<td>7</td>
<td>2.52</td>
</tr>
<tr>
<td>15</td>
<td>N/O*</td>
</tr>
<tr>
<td>30</td>
<td>N/O</td>
</tr>
</tbody>
</table>

*N/O*: No CFU observed at $10^{-1}$ dilution

Color measurement before and after HVACP treatment

Color of maize kernels was measured using three indicators; L* (a greater value indicates a lighter color), a* (negative values indicate green and positive values indicate red), and b* (negative values indicate blue and positive values indicate yellow). L* and b* values did not show any significant difference in HVACP treated versus control samples ($p < 0.05$). However, b* showed a significant difference ($p > 0.05$) in HVACP treated versus control samples indicating a loss in yellow color of exposed maize kernels (Table 9).
Table 9. Measurement of color indicators (L*, a*, b*) before and after HVACP treatment for three times and direct and indirect exposure to the HVACP system electrodes.

<table>
<thead>
<tr>
<th>Exposure time (min)</th>
<th>Color indicator</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L*</td>
<td>a*</td>
<td>b*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Direct</td>
<td>Indirect</td>
<td>Direct</td>
<td>Indirect</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>88.6±0.4</td>
<td>4.0±0.06</td>
<td>26.8±0.4</td>
<td></td>
</tr>
<tr>
<td>7 min</td>
<td></td>
<td>87.4±0.4</td>
<td>88.3±0.1</td>
<td>4.4±0.1</td>
<td>4.8±0.2</td>
</tr>
<tr>
<td>15 min</td>
<td></td>
<td>89.0±0.4</td>
<td>88.5±0.1</td>
<td>4.3±0.02</td>
<td>4.3±0.2</td>
</tr>
<tr>
<td>30 min</td>
<td></td>
<td>87.6±0.1</td>
<td>87.8±1.1</td>
<td>4.5±0.2</td>
<td>4.2±0.3</td>
</tr>
</tbody>
</table>

Values with the same superscript letter in the same column are not significantly different.
PV and TBARS measurement before and after HVACP treatment

Table 10 presents PV values, which indicate the primary lipid oxidation, and TBARS values, which show the secondary lipid oxidation. PV values were not detected, and TBARS values did not show any significant difference before versus after HVACP treatment and different exposure time and type (direct and indirect) (P > 0.05).

Table 10. PV (in milliequivalents peroxide per kg oil) and TBARS (nmol per g oil) values measured before and after HVACP treatment for three times and direct and indirect exposure to the HVAC system electrodes.

<table>
<thead>
<tr>
<th>Exposure time (min)</th>
<th>PV (meq. Peroxide/kg oil)</th>
<th>TBARS (nmol/ g oil)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exposure</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Direct</td>
<td>Indirect</td>
</tr>
<tr>
<td>Control</td>
<td>ND*</td>
<td>8.9±2.9</td>
</tr>
<tr>
<td>7 min</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>15 min</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>30 min</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND: Not detected

Discussion

In the HVACP system, different reactive species (RS) were generated: reactive oxygen species (ROS), which contains O₃ or ozone, and reactive nitrogen species (RNS), which consists of NO₂, NO₃, N₂O₄, and N₂H₅+H₂O₂. Approximately 80 to 100 s after the HVACP system was turned on, O₃, N₂H₅+H₂O₂, and N₂O₄ were formed in the exposed air. In comparison to RNS, O₃ concentration increased immediately and reached a saturation point (i.e., formation and dissociation processes are equal) after ~400 s (6.6 min) then started decreasing gradually. O₃ formation is negatively affected by an increase in temperature (Whitehead, 2016), so its decrease can be associated with the temperature increase in the exposed air (45°C after 30 min of treatment). Increase RNS could also be another cause of O₃ depletion through their reaction with...
O₃. RNS persisted after turning off the HVACP system because of their stability and long half-life (Whitehead, 2016).

In this study, AFB₁ was significantly reduced for all treatment times, confirming HVACP efficacy to degrade AFB₁ produced by A. flavus in maize kernels. Exposure type (direct and indirect) and treatment times did not have a significant effect on AFB₁ reduction (p>0.05). Findings corroborate previous studies that showed the HVACP efficacy to degrade AF in maize kernels (Shi et al., 2017; Wielogorska et al., 2019). AFB₁ degradation in maize kernels is the result of ROS (O₃), and RNS (NO₂, NO₃, N₂O₄, and N₂H₅+H₂O₂), which attack and degrade AFB₁ in less toxic degradation products (Shi et al., 2017). Previous studies reported the efficacy of O₃ to degrade AF (Agriopoulou et al., 2016; Diao et al., 2013; Faroni et al., 2012; Luo et al., 2014). However, the use of O₃ for degrading AF has not been shown to be cost-effective because of the high concentration of ozone and long exposure time to ozone required (McDonough et al., 2011; Pankaj et al., 2018b). In addition to O₃ generated in HVACP, RNS have a synergistic effect for a better alternative to degrade AF in cereal grains because it requires short treatment time and uses free ambient air as working gas.

Findings showed that HVACP treatment significantly reduces A. flavus contamination in maize kernels and confirms the previous study on the HVACP potential to control A. flavus growth on different food matrices (Suhem et al., 2013). In exposed air, GRS destroy cell wall fungus, cause the leakage of intracellular components, and damage fungal DNA, which leads to fungal self-cell destruction or apoptosis (Misra et al., 2019). HVACP can be used to decontaminate maize before grain storage because it inhibits A. flavus growth and prevents AF contamination.
Organoleptic properties are essential factors for consumer acceptance of the products treated with HVACP technology. GRS are known to degrade polyunsaturated fatty acids (PUFA) contained in the maize germ because of lipid oxidation. Lipid oxidation (measured by peroxide values and TBARS) leads to rancidity and negatively impacts food quality (Gavahian et al., 2018). In this study, peroxide values were not detected and TBARS values did not change significantly before and after treatment. Maize kernels were hand-harvested and shelled, and carefully dried with little if any mechanical/physical damage done to the germs. PUFA in the germ were protected against GRS oxidation because of the protective pericarp layer covering the kernel, including germ.

HVACP treatment did not show a significant difference in the color change except the yellow color loss of exposed samples versus non-exposed samples (p<0.05). However, the yellow color change was minimal because it was not perceptible with a naked eye. The yellow color of the maize kernel is due to zeaxanthin; a carotenoid pigment present in pericarp and endosperm (Solovchenko et al., 2019), which was partially degraded by GRS.

**Conclusions**

As a result of this study investigating HVACP efficacy to degrade aflatoxin in shelled maize inoculated with *Aspergillus flavus*, and treatment effects on maize lipid oxidation and color change, the following specific conclusions are drawn:

1. HVACP effectively degraded AFB1 produced by *A. flavus* inoculated in maize kernels for all treatment times (7, 15, and 30 min) (p>0.05).

2. After 7 and 15 min, AFB1 decreased by 81.3-96.3 % and 78.1-85.0%, respectively. The 30 min treatment showed AFB1 decreasing from below the LOQ (< 5 µg/kg) to 88.9% ± 10.3.
3. HVACP treatment inhibited *A. flavus* growth significantly reducing it by 2.4 and 1.9 log\(_{10}\) for direct and indirect exposure, respectively. No *A. flavus* CFU formed for the 15 and 30 min treatments.

4. HVACP treatment resulted in a slight change in yellow color but neither induced lipid oxidation nor changed flavor profile in treated maize samples. Future studies should focus on the optimization of HVACP treatment efficacy for AF reduction before the technology is scaled up.

**Acknowledgments**

This project was funded in part by the government of Canada through the Business Platform for Nutrition Research (BPNR), hosted by the Global Alliance for Improved Nutrition (GAIN) through the Aflatoxin-control Challenge in Africa organized by the Partnership for Aflatoxin Control in Africa (PACA). This study was also supported by USAID, as part of the Feed the Future initiative, under the CGIAR Fund, award number BFS-G-11-00002, and the predecessor fund, the Food Security and Crisis Mitigation II grant, award number EEM-G-00-04-00013. The contents are the responsibility of the authors and do not necessarily reflect the views of USAID or the United States Government.

**References**


CHAPTER 9. SUMMARY AND RECOMMENDATIONS

Mycotoxins, especially aflatoxins, are present at high levels in human foods and animal feeds in tropical countries, particularly in countries of Sub-Saharan Africa (SSA). This study comprised two primary thrusts to address this concern.

The first focused on assessing animal feed and milk contamination by aflatoxins and fumonisins in Rwanda. To the best knowledge of the author, it is so far the most comprehensive study on the subject because samples were collected in all 30 districts in Rwanda during six near-monthly rounds from dairy farmers, poultry farmers, feed vendors and feed processors. It laid a foundation for recommendations and action steps for all stakeholders of Rwanda’s feed value chain.

The following findings and recommendations based on our scientific study results have been submitted to the Rwanda Ministry of Agriculture and Animal Production (Appendix 6):

- This study showed that in Rwanda maize bran as a feed ingredient and as an ingredient in mixed feed is the leading contributor to aflatoxin contamination in animal feeds. Even though other ingredients have also shown high levels of aflatoxin contamination, they are not as frequently included in animal feeds. Additionally, an increase in aflatoxin levels was observed in feed samples collected on dairy and poultry farms compared to samples collected from feed processors and feed vendors.

  - **RECOMMENDATIONS:** Feed processors and vendors should consider formulating feed to mitigate inclusion of high levels of aflatoxin in whole maize and maize bran in feed rations based on scientific animal studies with different species sensitivity and age,
and quantify the reduction in the carry-over of mycotoxins into animal source foods (i.e., milk and eggs) and the improvement in animal production.

- At the farm level, storage practices for feed ingredients and mixed feeds need to be improved in order to prevent an increase in aflatoxin levels. Farmers should be encouraged to dry and store feeds properly. They should transfer feed ingredients and mixed feeds after purchase from typical woven polypropylene bags into reusable hermetic plastic storage bags or barrels to preserve quality and prevent spoilage due to molds or insects.

- Little data has been available on mycotoxin contamination in foods and feeds in Rwanda. This lack of data has resulted in underestimating the mycotoxin risk and in lack of policies and standards despite the high potential impact on public and animal health.

- **RECOMMENDATION:** A year-round surveillance and early detection system in the Rwanda feed value chain is needed for mitigation of mycotoxin contamination in foods (especially dairy) and feeds (especially maize and maize bran).

- Most farmers and feed processors do not have control over the quality of their raw ingredients. Potential approaches for mitigating mycotoxin toxicity are the (1) addition of binding agents in feeds, and (2) diluting contaminated grain by blending (mixing) it with clean grain or other feeds, provided these practices are allowed in the East Africa Community (EAC).

---

3 As a result of this recommendation the newly established Food and Drug Administration in Rwanda has been tasked to explore the possibility to establish a year-round surveillance program of the Rwanda feed value chain.
o **RECOMMENDATIONS:** Initiate a scientific study to explore the potential usage of mycotoxin binders in dairy and poultry feeds, and quantify the reduction in the carry-over of mycotoxins into animal source foods (i.e., milk and eggs), and the improvement in animal production. Mycotoxin binders are defined as substances that bind mycotoxins and prevent them from being absorbed through the gut into the blood circulation from which they are excreted into animal products like milk. They are used as feed additives.

o Initiate a scientific study to explore the potential of diluting contaminated grain by blending (mixing) it with clean grain or other feeds and quantify the reduction of mycotoxins in the blended grain and the maximum level of mycotoxins in contaminated ingredients that can be reasonably mixed in provided these practices are allowed in the EAC.

➢ There is no single or simple solution to mitigating mycotoxins in feed ingredients and mixed feeds. Mycotoxin contamination of feeds is a challenge for the Rwanda feed value chain.

o **RECOMMENDATION:** Initiate collaboration among the different stakeholders of the Rwanda feed value chain from the private sector, academia and research as well as policymakers and farmers to pool their efforts and resources, and find the best approach for mycotoxin mitigation in Rwanda.

The second thrust of this study assessed potential strategies to mitigate aflatoxin presence in the Rwanda feed value chain, and specifically in whole maize and maize ingredients. Adding mycotoxin binders to feed was identified as having potential and will be pursued as part of a

---

4 As a result of this recommendation a study was funded by the USAID-funded Feed the Future Innovation Lab for Livestock Systems entitled: “Aflatoxin mitigation through education, intervention, and policy in Rwandan dairy value chain”. It commenced in January 2020.
newly funded research project in Rwanda. HVACP treatment was also identified as having potential because the technology may be cost-effective for treating 100 kg of maize at a time which is the typical amount held, handled and sold in bags from farmer to trader to miller in Rwanda and other SSA countries. However, before HVACP can be considered for scale-up and implementation, its efficacy to degrade aflatoxins (AFB1, AFB2, AFG1, AFG2) and the safety of its degradation products need further study. This research evaluated those aspects as well as investigated in vitro AFB1 cytotoxicity at different AFB1 toxicity endpoints, and HVACP capacity to decontaminate maize kernels contaminated with A. flavus without affecting lipid stability and other quality characteristics in maize kernels.

Findings and recommendations based on our scientific study results are:

- Results showed that AFB1 and AFG1 are susceptible to HVACP treatment and decreased by 90% and 74% after 2 min treatment, respectively. AFB2 and AFG2 decreased by 38% and 79% but only after 20 min treatment, respectively.
  - **RECOMMENDATION:** HVACP efficacy to degrade other mycotoxins such as fumonisin, vomitoxin, and ZEA that often occur in maize together with aflatoxin also needs to be assessed. The in vitro cytotoxicity test showed that AFB1 toxicity was significantly reduced after 20 min treatment in all tested assays (caspase-3, cell viability, DNA fragmentation, and protein carbonyl assays).
  - **RECOMMENDATION:** Future investigations should assess other AFB1 endpoint toxicities that were not addressed in this study, for instance, mutagenicity, immunotoxicity, and growth impairment before in vivo toxicity investigations.

- In A. flavus inoculated maize, HVACP reduced A. flavus by 2.4 log_{10} after 7 min of treatment, and no CFU was observed after 15 and 30 min treatment. AFB1 was reduced by
78 – 85% after 7 min treatment. HVACP treatment caused a slight decrease in yellow color in maize samples but did not increase peroxide values (PV) and thiobarbituric acid reactive substances (TBARS) values that are characteristic of lipid oxidation. Findings suggest the efficacy of HVACP treatment to degrade AFs within a short time (less than 7 min) without affecting maize quality characteristics significantly.

- **RECOMMENDATION**: Scale-up for HVACP treatment of 100 kg sacks of dry maize will need to assure AFB1 degradation as a function of electric power, voltage, gap size between electrodes, exposure time, relative and absolute air humidity, plasma reactive species completely enveloping maize kernel surfaces, and maize moisture content without negatively affecting any lipid oxidation and color change and other quality characteristics and end use value of treated maize.
APPENDIX 1 DAIRY FARMERS: CHECKLIST AND QUESTIONNAIRE

Dairy Farmers: Checklist and Questionnaire/ Aborozí b’inka zikamwa

Code:

I. Socio-demographic information about the farm owner / Iranga mimerere ya nyirubworozi
   a) Gender / Igitsina
      □ Male/ Gabo
      □ Female/ Gore
   b) Age group/ Icyiciro cy’imyaka
      □ 18-25 years/ imyaka
      □ 25-40 years/ imyaka
      □ Over 40 years/ imyaka
   c) Highest level of Education / Amashuri
      □ None / Ntiyize
      □ Primary/ Abanza
      □ Secondary / Ayisumbuye
      □ University / Kaminuza
      □ Others / Ayandi

II. Production/ Umusaruro
    a) Number of dairy cattle/ Umubare w’inka zikamwa:
    b) Milk production per cow/ Ingano y’amata akamwa ku nka imwe:
    c) Milk destination/ Aho amata ajya:
       □ MCC (Milk Collection Center)/ Ikusanyirizo ry’amata
       □ Local people (neighbors) / abaturanyi
       □ Home consumption / kunywererwa mu rugo
       □ Other / ahandi

III. Feeds and Feeding / Imigaburire
    a) What types of feeds do you provide your animals on farm? / Ni ubuhe bwoko bw’ibiryo ugaburira inka zawe ?
       □ Green grass (forage) / Ibyatsi
       □ Silage / silaje
       □ Hay / ibyatsi byumishijwe
If you mix your own feed or supplement purchased feed (or a diet of forages) with other ingredients, estimate the quantity: *Niba mujya mugura ibiryo bitandukanye mukivangira, nimugereranye ingano y’ibyo mugura buri cyumweru*

<table>
<thead>
<tr>
<th>Ingredients / ibiryo</th>
<th>Estimated Quantity (kg) / week Ingano (ibiro/ icyumweru)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize bran / buranda y’ibigori</td>
<td></td>
</tr>
<tr>
<td>Whole maize / ibigori</td>
<td></td>
</tr>
<tr>
<td>Rice bran / buranda y’umuceli</td>
<td></td>
</tr>
<tr>
<td>Sorghum / Amasaka</td>
<td></td>
</tr>
<tr>
<td>Sunflower cake / ibisigazwa by’ibihwagari</td>
<td></td>
</tr>
<tr>
<td>Cotton cake / ibisigazwa by’ipamba</td>
<td></td>
</tr>
<tr>
<td>Commercial feeds / ibiryo bivanze</td>
<td></td>
</tr>
<tr>
<td>Brewers / ibisigazwa byo mu nganda z’inzoga</td>
<td></td>
</tr>
<tr>
<td>Soybean / soya</td>
<td></td>
</tr>
<tr>
<td>Soybean cake / meal / ibisigazwa bya soya</td>
<td></td>
</tr>
<tr>
<td>Wheat / ingano</td>
<td></td>
</tr>
</tbody>
</table>

**b) Do you mix your own feed? / wivangira ibyo ugabura**
- □ Yes / Yego
- □ No/ Oya

**c) Do you feed your animals solely using ingredients grown on your farm? / Ugurira inka zawe ibyo wihiingira**
- □ Yes / Yego
- □ No / Oya
  - **If you purchase, do you:** Niba ugura , ujya :
    - □ purchase some or all ingredients and mix feed at home / Ugura Ibitandukanye ukivangira ?
    - □ purchase a complete feed that is already mixed? / Ugura ibivanze
  - **If you purchase feed and/or ingredients, where do you purchase them from? (you may select more than one option) / Niba ugura ibitavanze, ubigura he ? (Ushobora guhitamo ibisubizo birenze kimwe )
    - □ feed industry outlet / abacururiza inganda
☐ mixed feed vendors / abacuruza ibiryo bivangiye
☐ ingredient vendor / abacuruza ibiryo bitandukanye bitavanze
☐ directly from grain mill / Inganda z’amafu
☐ neighbor farmer / abandi borzoi muturanye
☐ other (please specify) / Ahandi

IV. Feed storage / Kubika ibiryo
a) Status of storage facility / Uko ububiko bumeze
☐ Pallet and dry / hashashemo imbaho humutse
☐ Pallet and humid / hashashemo imbaho hatose
☐ On floor / hasi nta gishashemo
☐ Concrete floor / hasi kuri sima cg amakaro
☐ Mud floor / hasi hatarimo sima cg amakaro (ibitaka)

b) Typical storage duration / Igihe ibiryo bibikwa nyuma yo kugurwa
☐ Less than one week / muni y’icyumweru
☐ One week to two weeks / hagati y’icyumweru cyimwe na bibiri
☐ Two weeks to three weeks / hagati y’hyumweru bibiri na bitatu
☐ Three weeks to four weeks / hagati y’hyumweru bitatu na bine
☐ More than one month / Birenza ukwezi

V. Mycotoxin Awareness / ubumenyi kuri afalatogisine
a. Have you ever seen moldy feeds? / Mwari mwabona ibiryo birimo uruhumbu?
☐ Yes / Yego
☐ No / Oya

b. Do you check ingredients for moldiness before buying them? / Ujya ureba niba ibiryo bitarimo uruhumbu mbere yo kubigura
☐ Yes / Yego
☐ No / Oya

c. If yes, what measures do you take when the ingredients are moldy? / Niba mubireba, iyo uruhumbu rurimo mubigenza mute?
☐ Buy / Ndabigura
☐ Reject / Ndabireka
☐ Buy for lower price / Mbigura kuri make
☐ Others…../ Ibindi

d. Do you check feeds for moldiness before feeding your dairy cattle? / Ujya ureba nib anta ruhumbu ruri mu biryo by’inka mbere yo Kubigabura?
☐ Yes / Yego
e. How do you handle moldy feeds? / Ibiryo birimo uruhumbu ubimaza iki?
   - Discard / ndabijugunya
   - Use / ndabikoresha
   - Dry before use / ndabanza nkabyanika mbere yo kubiha inka
   - 'Blending with non-moldy feeds / Mbivanga n’ibidaftite uruhumbu
   - Other (please specify) / Ibindi __________

f. What do you do to avoid moldiness in animal feeds? / ni iki mukora kugirango mwirinde uruhumbu mu biryo by’atungo?
   - Avoid long-term storage / kwirinda kubibika igihe kinini
   - Dry before storage / Kubyanka mbere yo kubibika
   - None? / Ntacyo nkora
   - Other / Ibindi

g. Have you heard of the word aflatoxin / fumonisin before? Mwigee mwumva afalatogisine cg fumonizine
   - Yes / Yego
   - No / Oya

1. If yes, are you aware of aflatoxin / fumonisin contamination in crops (cereal grains)? / Niba mwarigeze kubyumvaho, mwabyumvise mu binyampeke?
   - Yes / Yego
   - No / Oya

2. Are you aware of any harmful effects of aflatoxins / fumonisins on humans? / Mwaba muzi ingaruka zazo ku bantu?
   - Yes / Yego
   - No / Oya
   - If yes, list some harmful effects: / Niba muzi ingaruka mutubwiremo zimwe
     - Jaundice / uruhondo
     - Liver cancer / Kanseri y’umwijima
     - Stunting / kugwingira
     - Diarrhea / Impiswi
     - Immune depressing / Kugabanuka kw’ abasirikare b’umubiri

3. Are you aware of any harmful effects of aflatoxins / fumonisins on animals? / Mwaba muzi ko afalatogisine na fimonizine bishobora kugira ingaruka mbi ku matungo
   - Yes / Yego
   - No / Oya
APPENDIX 2 POULTRY FARMERS: CHECKLIST AND QUESTIONNAIRE

Poultry Farmers: Checklist and Questionnaire / Aaborozi b’inkoko

Code:

VI. Socio-demographic information about the farm owner / Irangamimerere y’umworzi

a) Gender / igitsina
   - Male / Gabo
   - Female / Gore

b) Age group / Icyiciro cy’imyaka
   - 18-25 years / Imyaka
   - 25-40 years / Imyaka
   - Over 40 years / Imyaka

c) Highest level of Education / Amashuri
   - None / Ntayo
   - Primary / Abanza
   - Secondary / Ayisumbuye
   - University / Kaminuza
   - Others / Ibindi

VII. Production / Umusaruro

a) Number of birds / Umubare w’inkoko

b) Eggs/Meat destination / Imikoreshereze y’umusaruro
   - Market / Isoko
   - Local people (neighbors) / Kugurisha ku baturanyi
   - Home consumption / Kubikoresha mu rugo
   - Other / Ibikoresho

VIII. Feeds and Feeding / Imigaburire

a) Feeding on farm / Ibiryo by’amatungo

<table>
<thead>
<tr>
<th>Ingredients / Ibiryo</th>
<th>Estimated Quantity (kg) / month Ingano (ibiroyi/ukwezi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize bran / buranda y’ibigori</td>
<td></td>
</tr>
<tr>
<td>Whole maize / Ibigori</td>
<td></td>
</tr>
<tr>
<td>Rice bran / Buranda y’umuceli</td>
<td></td>
</tr>
<tr>
<td>Sorghum / Amasaka</td>
<td></td>
</tr>
</tbody>
</table>
Sunflower cake / *Ibisigazwa by’ihwagari*

Cotton cake / *Ibisigazwa by’ipamba*

Commercial feeds / *Imvage z’inganda*

Brewers / *Ibisigazwa byo mu nganda z’inzoga*

Soybean / *Soya*

Soybean cake / meal / *Ibisigazwa bya soya*

Wheat / *Ingano*

### b) Origin of feeds / *Aho ibiryo biva*

<table>
<thead>
<tr>
<th>Origin of feeds / <em>Aho ibiryo biva</em></th>
<th>Percentage/ <em>Ijanisha (%)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Grown on own farm / <em>Ibyo yiezereza</em></td>
<td></td>
</tr>
<tr>
<td>Purchased from feed vendor / <em>Ibyo agura ku isoko</em></td>
<td></td>
</tr>
</tbody>
</table>

### c) If purchased from vendor / *Niba biva ku isoko*

- □ Already mixed (commercial feed) / *Bivangiwe ku isoko*
- □ Home-made (home-mixed) / *Bivangiwe mu rugo*

**IX. Feed storage / *Uko ibiryo bibikwa***

**a) Status of storage facility / *Imiterere y’ububiko***

- □ Pallet and dry / *hashashemo imbaho humutse*
- □ Pallet and humid / *hashashemo imbaho hatose*
- □ On floor / *Nta gishashemo*
- □ Concrete floor / *Harimo: sima, amakaro, amabuye, etc*
- □ Mud floor / *Nta gishashemo (ku butaka)*

**b) Typical storage duration (finished feeds OR for feed ingredients) / *igihe bimara bibitse***

- □ Less than one day / *Bitarenze umunsi umwe*
- □ One day to 3 days/ *Hagati y’umunsi umwe n’itatu*
- □ 3 days to one week/ *Hagatay’iminsi itatu n’icyumweru*
- □ More than one week / *Birenze ukwezi*

**X. Mycotoxin Awareness / *Ubumenyi ku ruhumbu***

**h. Have you ever seen moldy feeds? / *Mwigeze mubona uruhumbu mu biryo by’amatungo***

- □ Yes / *Yego*
□ No / Oya

i. Do you check ingredients for moldiness before buying them? / Mubanza kureba niba nta ruhumbu ruri mu biryo mbere yo kubigura?

□ Yes / Yego
□ No / Oya

j. If yes, what measures do you take when the ingredients are moldy? / Igihe mu rubonye, ni izihe ngamba mufata?

□ Buy / Kubigura
□ Reject / Kureka kubigura
□ Buy for lower price / Kubigura ku giciro gito
□ Others…… / ibindi……

k. Do you check feeds for moldiness before feeding your birds? / Mubanza kureba niba nta ruhumbu ruri mu biryo mbere yo kubigaburira inkoko

□ Yes / Yego
□ No / Oya

l. How do you handle moldy feeds? / Igihe murubonye, ni izihe ngamba mufata?

□ Discard / Kubijugunya
□ Use / Kubigabura
□ Dry before use / Kubyanika mbere yo kubigabura
□ ’Blending with non-moldy feeds / Kubivanga n’ibindi bitarimo uruhumbu
□ Other (please specify) / Ibindi __________

m. What do you do to avoid moldiness in animal feeds? / Ni iki mukora ngo mwirinde uruhumbu mu biryo

□ Avoid long-term storage / Kwirinda kubibika igihe ikrekire
□ Dry before storage / Kubyumisha / kubyanika mbere yo kubibika
□ None / Ntacyo
□ Other / ibindi

n. Have you heard of the word aflatoxin / fumonisin before? / Mwigeze mwumva ho ijambo afaratogiszine/ fimonizine?

□ Yes / Yego
□ No / Oya

1. If yes, are you aware of aflatoxin / fumonisin contamination in crops (cereal grains)? / Niba mwararyumviseho, muzi ko dushobora kuyisanga mu binyampeke?

□ Yes / Yego
□ No / Oya
2. Are you aware of any harmful effects of aflatoxins / fumonisins on humans? / Mwaba muzi ingaruka ishobora kugira ku bantu?
   □ Yes / Yego
   □ No / Oya
   □ If yes, list some harmful effects: / Niba muzizi muzitubwire
     □ Jaundice / Ighondo
     □ Liver cancer / Kanser i'umwijima
     □ Stunting / kugwingira
     □ Diarrhea / Impiswi
     □ Immuno-suppression / Kugabanuka k'ubudahangarwa bw'umubiri

3. Are you aware of any harmful effects of aflatoxins / fumonisins on animals? / Mwaba muzi ingaruka ishobora kugira ku matungo
   □ Yes / Yego
   □ No / Oya
APPENDIX 3 ANIMAL FEED VENDORS: CHECKLIST AND QUESTIONNAIRE

Animal Feed Vendors/ Abacuruza ibiryo by’amatungo: Checklist and Questionnaire

Code: [___] [___] [___] [___]

XI. Socio-demographic information about the responder / irangamimerere y’usubiza

a) Gender / Igitsina
   □ Male / Gabo
   □ Female / Gore

b) Are you the owner or the manager of the …..? / Murikorera cg hari uwo mukorera?
   □ Owner / Ndikorera
   □ Manager / Nkorera undi

c) Age group / Icyiciro cy’imyaka
   □ 18-25 years / Imyaka
   □ 25-40 years / imyaka
   □ Over 40 years / imyaka

d) Highest level of Education / Amashuri
   □ None / Ntayo
   □ Primary / Abanza
   □ Secondary / Ayisumbuye
   □ University / Kaminuza
   □ Other / Andi

e) Vendor profile / Icyiciro cy’umucuruzi
   □ Personal business / Iby’umuntu umwe
   □ Cooperative / iby’ ishyirahamwe (cooperative)

XII. Production details / Amakuru arambuye ku micururize

a) Equipment / Ibikoresho
   □ Hammer mill / Icyuma (imashini) gisya (Urusyo )
   □ Batch mixer / Icyuma (imashini) kivanga
   □ ....

b) Type of product / ubwoko bw’ibicuruzwa
   □ Ingredient only/if ingredient only, skip the next question / Ibiryo bitandukanye bitavanze
   □ In-store mixed feeds / Ibyo yivangyiye
   □ Mixed feeds from commercial industry/ Ibivanze byo mu nganda

c) Types and quantity of feeds sold (If outlet or vendor of industrial mixed feeds): / Ubwoko n’ingano by’ibiryo bicuruzwa
<table>
<thead>
<tr>
<th>Type/ Ubwoko</th>
<th>Estimated Quantity (kg) / month Ingano (Kg/ukwezi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy cattle/ Inka zikamwa</td>
<td></td>
</tr>
<tr>
<td>Poultry – Layers/ Inkoko z’amagi</td>
<td></td>
</tr>
<tr>
<td>Poultry – Broilers/ Inkoko z’inyama</td>
<td></td>
</tr>
<tr>
<td>Pigs / Ingurube</td>
<td></td>
</tr>
<tr>
<td>Fish / Amafi</td>
<td></td>
</tr>
</tbody>
</table>

**d) Types and quantity of ingredients sold or used for mixing:** / *Ubwoko n’ingano by’ibiryo bicuruzwa*  

<table>
<thead>
<tr>
<th>Ingredients /Ubwoko bw’ibiryo</th>
<th>Estimated Quantity (kg) / month Ingano (Kg/ukwezi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize bran / <em>buranda y’ibigori</em></td>
<td></td>
</tr>
<tr>
<td>Whole maize/ <em>Ibigori</em></td>
<td></td>
</tr>
<tr>
<td>Rice bran/ <em>Buranda y’umuceli</em></td>
<td></td>
</tr>
<tr>
<td>Sorghum / <em>Amasaka</em></td>
<td></td>
</tr>
<tr>
<td>Sunflower cake / <em>Ibisigazwa by’ihwagari</em></td>
<td></td>
</tr>
<tr>
<td>Cotton cake / <em>Ibisigazwa by’ipamba</em></td>
<td></td>
</tr>
<tr>
<td>Commercial feeds / <em>Imvage z’inganda</em></td>
<td></td>
</tr>
<tr>
<td>Brewers / <em>Ibisigazwa byo mu nganda z’inzoga</em></td>
<td></td>
</tr>
<tr>
<td>Soybean / <em>Soya</em></td>
<td></td>
</tr>
<tr>
<td>Soybean cake / meal / <em>Ibisigazwa bya soya</em></td>
<td></td>
</tr>
<tr>
<td>Wheat / <em>Ingano</em></td>
<td></td>
</tr>
</tbody>
</table>

**e) Source and quantity of ingredients/ mixed feeds:** / *Ingano n’aho ibiryo bicuruzwa bituruka*  

<table>
<thead>
<tr>
<th>Source/ Aho bituruka</th>
<th>Estimated Quantity(kg) / month Ingano (Kg/ ukwezi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local farmers/ <em>Abahinzi</em></td>
<td></td>
</tr>
<tr>
<td>Local farmer cooperatives / <em>Inshyirahamwe ry’abahinzi</em></td>
<td></td>
</tr>
<tr>
<td>Local middle sellers / <em>Abaranguza</em></td>
<td></td>
</tr>
<tr>
<td>Maize flour mills / <em>Inganda z’amafu</em></td>
<td></td>
</tr>
<tr>
<td>Breweries / <em>Inganda z’inzoga</em></td>
<td></td>
</tr>
<tr>
<td>Imported from neighboring countries (UG, TZ, DRC...) <em>Bivuye mu bihugu duturanye</em></td>
<td></td>
</tr>
<tr>
<td>Middle sellers importing from neighboring countries</td>
<td></td>
</tr>
</tbody>
</table>
Abaranguza babivana mu bihugu duturanye

Local animal feed industries / Inganda zo mu Rwanda z’ibiryo by’amatungo

f) Feed ingredients or mixed feed clients: / Abaguzi
   □ Individual farmers / Aborozi
   □ Farmer cooperatives / Ishyirahamwe ry’aborozi
   □ Other vendors / Abandi bacuruzi

XIII. Transport and Storage / Uburyo bitwarwa n’uko bibikwa
   a) Transport means by supplier / Uburyo bitwarwa n’ababigemura
      □ Small van / Utumodoka duto (Toyota Hilux)
      □ Lorry / Truck / Amakamyo
      □ Motorcycle / Moto
      □ Other (specify) / Ibindi
   b) Packaging by supplier / Uko biza bipakiwe
      □ Plastic sacks / Imifuka irimo amashashi
      □ Burlap sacks / Amagunira
         ○ if yes, size: / Ingano
      □ Other (specify) / Ibindi
   c) Transport duration by the supplier / Igihe bimara mu nzira
      □ Less than one day / Igihe kitarenze umunsi umwe
      □ One day to 3 days / Hagati y’umunsi umwe n’itatu
      □ 3 days to one week / hagati y’iminsi itatu n’icyumweru
      □ More than one week / Hejuru y’icyumweru
   d) Storage duration after receiving / Igihe bimara mu bubiko
      □ Less than one day / Igihe kitarenze umunsi umwe
      □ One day to 3 days / Hagati y’umunsi umwe n’itatu
      □ 3 days to one week / hagati y’iminsi itatu n’icyumweru
      □ More than one week / Hejuru y’icyumweru
   e) Do you have a warehouse? / ufite ububiko?
      □ Yes / Yego
      □ No / Oya
f) Warehouse status / Imitere y’ububiko

- Pallet and dry / hashashemo imbaho humutse
- Pallet and humid / hashashemo imbaho hatose
- Concrete floor / Harimo: sima, amakaro, amabuye, etc
- Mud floor / Nta gishashemo (ku butaka)

XIV. Ingredients/Feed quality check / Ibiryo no kugenzura ubuziranenge

a) Do you check ingredients and feed before buying? / Mujya mureba ubuziranenge bw’ibiryo mbere yo kubirangura?

- Yes / Yego
- No / Oya
  - If yes, what quality parameters do you check for (mark all that apply): Niba mubikora, ni ibihe bintu mugenzura

- Odor / Impumuro
- Cleanness / isuku
- Moisture content / Ubukonje/ ubutoheere
- Moldiness/ uruhumbu

b) Do you check the quality of ingredients and feed before selling? / mujya mureba ubuziranenge bw’ibiryo mbere yo kubigurisha ?

- Yes / Yego
- No / Oya
  - If yes, what quality parameter do you check for (mark all that apply): / niba mubikora ni iki mugenzura

- Odor / Impumuro
- Cleanness / isuku
- Moisture content / ubukonje
- Moldiness / uruhumbu

XV. Pricing / Ibiacro

a) At what prices do you sell the following ingredients? / Igiciro mugurishirizaho ibiryo

<table>
<thead>
<tr>
<th>Ingredients / Ibiryo</th>
<th>Price (RWF)/kg / igiciro (FRW/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize bran / buranda y’ibigori</td>
<td></td>
</tr>
<tr>
<td>Whole maize/ Ibigori</td>
<td></td>
</tr>
<tr>
<td>Rice bran/ Buranda y’umuceli</td>
<td></td>
</tr>
<tr>
<td>Sorghum / Amasaka</td>
<td></td>
</tr>
<tr>
<td>Sunflower cake / Ibisigazwa by’ihwagari</td>
<td></td>
</tr>
<tr>
<td>Cotton cake / Ibisigazwa by’ipamba</td>
<td></td>
</tr>
<tr>
<td>Commercial feeds / Imvage z’inganda</td>
<td></td>
</tr>
</tbody>
</table>
XVI. Staff capacity and experience / Ubushobozi n’uburambe by’abakozi
a) Which registered veterinarian do you collaborate with? / Ni uwuhe muntu wemewe n’urugaga rw’abize ubumenyi bw’amatungo mukorana?

☐ Yes / Yego  
☐ No / Oya  
  o If yes, what is the most recent time you had the training? / Niba mwarayagize, hashize igihe kingana iki?

b) Have you had a training in animal nutrition and feed management / mwigeze mugira amahugurwa ku biryo by’amatungo?

☐ Yes / Yego  
☐ No / Oya

XVII. Mycotoxin Awareness / Ubumenyi ku ruhumbu

a) Have you ever seen moldy grains or ingredients? / Mwigeze mubona uruhumbu mu biryo by’amatungo?

☐ Yes / Yego

☐ No / Oya

○ What do you do with moldy ingredients? / Igihe mu rubonye, ni izihe ngamba mufata?

☐ Buy / Kubirangura

☐ Reject / Kubireka

☐ Buy for lower price / Kubirangura ku giciro gito

☐ Othe / Ibindi

b) What do you do with moldy ingredients before selling? / Ni iki mukorera ibiryo byajemo uruhumbu mbere yo kubigurisha

☐ Discard / Kubimena

☐ Sell / Kubigurisha

☐ Sell for lower price / Kubigurisha ku giciro gito

☐ Dry before selling / Kubyumisha mbere yo kubigura

☐ Blend with non-moldy ingredients / Kubivanga n’ibindi bitari mo uruhumbu

☐ Other / Ibindi

c) What do you do to avoid moldiness in animal feeds?

☐ Store under dry conditions / kubibika ahantu humutse

☐ Prompt selling / Guhita bigurishwa
☐ Dry before storage/ Kubyanika mbere yo kubibika
☐ Nothing / Ntacyo
☐ Other / Ibindi
d) Are you aware of any health risks to humans or animals by moldy ingredients? /
   Mwaba muzi ko ibiryo birimo uruhumbu bigira ingaruka mbi ku buzima
   bwa’abantu n’amatungo ?
☐ Yes / Yego
☐ No / Oya
e) If yes, what should you do in case of moldiness in grains or ingredients? / Niba
   muzizi, ni iki cyakagombye gukorwa ku binyampeke birimo uruhumbu ?
☐ Consume / Kubirya
☐ Feed to animals / Kubiha amatungo
☐ Discard / Throw them away / Kubijugunya
☐ Other use: / Ibindi
f) Have you ever heard of mycotoxins before? / Mwigeze mwumva ijambo mycotoxine
☐ Yes ? Yego
☐ No / Oya
g) If yes, are you aware of aflatoxin / fumonisin contamination in crops (cereal grains)?
   Niba mwari mwarabyumvise, mwaba muzi ko dushobora gusanga afalatogisine mu
   binyampeke ?
☐ Yes/ yego
☐ No / Oya
h) Are you aware of the harmful effects of mycotoxins on humans? / Mwaba muzi ko
   afaaartogisine na fimonizine bigira ingaruka mbi ku buzima bw’abantu
☐ Yes / Yego
☐ No/ Oya
i) If yes, what harmful effects are you aware of: / Niba mubizi, mwatubwira zimwe
   muri izo ngaruka?
☐ Jaundice / Uruhondo
☐ Liver cancer / Kanseri y’umwijima
☐ Stunting / Kugwingira
☐ Diarrhea/ Impiswi
☐ Immuno-suppression / Kugabanuka k’ubudahangarwa bw’umubiri
j) Are you aware of the harmful effects of mycotoxins on animals? / Mwaba muzi se ko
   afalatogisine na fumonizinise bishobora kugira ingaruka mbi ku buzima
   bw’amatungo
☐ Yes / Yego
☐ No / Oya
APPENDIX 4 ANIMAL FEED PROCESSORS: CHECKLIST AND QUESTIONNAIRE

Animal Feed processors/ Abakora ibiryo by’amatungo: Checklist and Questionnaire

Code: 

2. Questionnaire (All question will be answered either at the time of the surveyor averaged over a normal year)

1. Production details / Amakuru arambuye ku bikoresho by’uruganda (amamashini)

| Hammer mill capacity (TPH)/ Ubushobozi bw’urusyo (Toni/isaha) |  |
| Ingredient mixer capacity (TPH) Ubushobozi bw’imashini ivanga (Toni/isaha) |  |
| Weighing system capacity (TPH) / Ubushobozi bw’imashini ipima ibiro (Toni/isaha) |  |
| Pelleting capacity (TPH) / Ubushobozi bw’imashini ikora pellets |  |
| Packaging capacity (TPH) / Ubushobozi bw’imashini ipakira |  |
| Installed capacity (TPH) / Ubushobozi bw’imashini zose zihari |  |
| Actual capacity (TPH) / Ubushobozi bw’imashini bukoreshwa (toni/isha) |  |
| Production (tones per day): Inago yibiryo bikorwa k’umunsi |  |

2. Major feed ingredients and their cost / Ibiryo by’ingenzi bikoreshwa mu gukora ibiryo by’amatungo n’uko birangurwa

<table>
<thead>
<tr>
<th>Ingredients/ibiryo</th>
<th>Price (RWF)/kg Igiciro/ kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize bran / Buranda y’ibigori</td>
<td></td>
</tr>
<tr>
<td>Whole maize/ Ibigori</td>
<td></td>
</tr>
<tr>
<td>Rice bran/ Buranda y’umuceli</td>
<td></td>
</tr>
<tr>
<td>Sorghum / Amasaka</td>
<td></td>
</tr>
<tr>
<td>Sunflower cake/ Ibisigazwa by’ibihwagari</td>
<td></td>
</tr>
<tr>
<td>Cotton cake/ ibisigazwa by’ipamba</td>
<td></td>
</tr>
<tr>
<td>Commercial feeds / Ibiryo byo mu nganda</td>
<td></td>
</tr>
<tr>
<td>Brewers/ Ibisigazwa byo mu nganda z’inzoga</td>
<td></td>
</tr>
<tr>
<td>Soybean / Soya</td>
<td></td>
</tr>
<tr>
<td>Soybean cake / meal/ Ibisigazwa bya</td>
<td></td>
</tr>
</tbody>
</table>
3. **Source of feed ingredients / Aho bituruka**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Imported (%) / Ibiva hanze</th>
<th>Domestic (%) / ibigurwa mu Rwanda</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize bran / Buranda y’ibigori</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole maize/ Ibigori</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice bran / Buranda y’umuceli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sorghum / Amasaka</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunflower cake/ Ibisigazwa by’ibihwagari</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotton cake/ ibisigazwa by’ipamba</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Commercial feeds / Ibiryo byo mu nganda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brewers/ Ibisigazwa byo mu nganda z’inzoga</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean / Soya</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean cake / meal/ Ibisigazwa bya soya</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat / Ingano</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4. **Annual commodities / Ibikoreshwa ku mwaka**

<table>
<thead>
<tr>
<th>Ingredients/ ibiryo</th>
<th>Quantity (MT/Y)/ ingano</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize bran / Buranda y’ibigori</td>
<td></td>
</tr>
<tr>
<td>Whole maize/ Ibigori</td>
<td></td>
</tr>
<tr>
<td>Rice bran / Buranda y’umuceli</td>
<td></td>
</tr>
<tr>
<td>Sorghum / Amasaka</td>
<td></td>
</tr>
<tr>
<td>Sunflower cake/ Ibisigazwa by’ibihwagari</td>
<td></td>
</tr>
<tr>
<td>Cotton cake/ ibisigazwa by’ipamba</td>
<td></td>
</tr>
<tr>
<td>Commercial feeds / Ibiryo byo mu nganda</td>
<td></td>
</tr>
<tr>
<td>Brewers/ Ibisigazwa byo mu nganda z’inzoga</td>
<td></td>
</tr>
<tr>
<td>Soybean / Soya</td>
<td></td>
</tr>
<tr>
<td>Soybean cake / meal/ Ibisigazwa bya soya</td>
<td></td>
</tr>
<tr>
<td>Wheat / Ingano</td>
<td></td>
</tr>
</tbody>
</table>
5. Warehousing capacity (MT): / Ubushobozi bw’ububiko (MT)

6. Warehouse condition/sanitation: // Imitere y’aho ibiryo bibikwa
- □ Pallet and dry / hashashemo imbaho humutse
- □ Pallet and humid / hashashemo imbaho hatose
- □ Concrete floor / Harimo: sima, amakaro, amabuye, etc
- □ Mud floor / Nta gishashemo (ku butaka)

7. Feed testing / Isuzuma ryibiryo by’amatungo
- □ Yes / Yego
- □ No/ Oya
  i. If yes, how: / niba mubikora, ni gute ?
    - □ In-house lab / Muri laboratwari y’uruganda
    - □ External lab (list name of lab): / Muri laboratwari yo hanze y’uruganda (izina)
    - □ Type of lab tests / ubwoko bw’isuzuma rikorwa
      - □ Feed composition / Ibigize ibiryo (intungamubiri ziri mu biryo
      - □ Moisture content / Ubukonje
      - □ Mycotoxins / Uruhummbu
        i. Aflatoxins/ afaratogisine
        ii. Fumonisins/ fimonizine
      - □ Other / Ibindi

8. Typical storage duration before processing: / igihe bibikwa mbere yo kubikoresha
- □ Less than one day/ Igihe kitarengeje umunsi
- □ One day to 3 days/ hagati y’umunsi umwe n’itatu
- □ 3 days to one week/ hagati y’iminsi itatu n’icyumweru
- □ More than one week / hejuru y’icyumweru

9. Annual feed production (in MT)/ Umusaruro ku mwaka :

10. Annual volume of feed produced per type / Ingano ya buri bwoko bw’ibiryo bikorwa ku mwaka

<table>
<thead>
<tr>
<th>Type of Mash</th>
<th>(MT)</th>
<th>(RWF/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chick Mash / Ibiryo by’imishwi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grower Mash / Ibiryo by’ibirwana</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layer Mash / Ibiryo by’izitera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broiler (Starter)/ imishwi y’inkoko z’inyama</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broiler (Grower)/ Ibirwana by’inkoko z’inyama</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dairy cow (supplement, concentrate, complete feeds….)/ Ibiryo by’inka zikamwa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigs (sow, finishing, piglet…)/ Ingurube</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

11. **Total value of annual sales in RWF: / amafaranga binjiza ku mwaka**

12. **Typical ways of selling feed products / Uburyo ibiryo bicuruzwa**
   - Directly to farmers (%):/ Aborozi :
   - Vendors (%):/ Abacuruzi
   - Outlets (%):/ depo y’uruganda

13. **Mycotoxin Awareness / Ubumenyi ku ruhumbu**
   a) Have you ever seen moldy feeds? / Mwigize mubona uruhumbu mu biryo by’amatungo
      - Yes / Yego
      - No / Oya
   b) Do you check ingredients for moldiness before buying them?/ Mubanza kureba niba nta ruhumbu ruri mu biryo mbere yo kubigura ?
      - Yes / Yego
      - No / Oya
   c) If yes, what measures do you take when the ingredients are moldy?/ Igihe mu rubonye, ni izihe ngamba mufata?
      - Buy / Kubigura
      - Reject / Kureka kubigura
      - Buy for lower price / Kubigura ku giciro gito
      - Others……/ ibindi ..... 
   d) Do you check feeds for moldiness before feeding your birds?/ Mubanza kureba niba nta ruhumbu ruri mu biryo mbere yo kubigaburira inkoko
      - Yes / Yego
      - No / Oya
   e) How do you handle moldy feeds? / Igihe murubonye, ni izihe ngamba mufata?
      - Discard/ Kubijugunya
      - Use / Kubigabura
      - Dry before use / Kubyanika mbere yo kubigabura
      - ‘Blending with non-moldy feeds/ Kubivanga n’ibindi bitarimo uruhumbu
      - Other (please specify) / Ibindi ____________
   f) What do you do to avoid moldiness in animal feeds?/ Ni iki mukora ngo mwirinde uruhumbu mu biryo
Avoid long-term storage / Kwirinda kubibika igihe ikrekire
Dry before storage / Kubyumisha/ kubyanika mbere yo kubibika
None / Ntacyo
Other / ibindi

Have you heard of the word aflatoxin / fumonis? / Mwigeze mwumva ho ijambo afaratogisine/ fimonizine ?
Yes / Yego
No / Oya

If yes, are you aware of aflatoxin / fumonis contamination in crops (cereal grains)? / Niba mwararyumviseho , muzi ko dushobora kuyisanga mu binyampeke ?
Yes / Yego
No / Oya

Are you aware of any harmful effects of aflatoxins / fumonisins on humans? / Mwaba muzi ingaruka ishobora kugira ku bantu ?
Yes / Yego
No / Oya
If yes, list some harmful effects: / Niba muzizi muzitubwire
Jaundice / Igihondo
Liver cancer / Kanseri y’umwijima
Stunting / kugwingira
Diarrhea / Impiswi
Immune depressing / Kugabanuka k’ubudahangarwa bw’umubiri

Are you aware of any harmful effects of aflatoxins / fumonisins on animals? / Mwaba muzi ingaruka ishobora kugira ku matungo
Yes / Yego
No / Oya
APPENDIX 5 COMPARISON OF METHODS FOR DETECTION OF AFLATOXIN AND FUMONISIN IN ANIMAL FEED FROM RWANDA

Comparison of methods for detection of aflatoxin and fumonisin in animal feed from Rwanda.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Fumonisin (ppb) detected by:</th>
<th>Aflatoxin (ppb) detected by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA</td>
<td>HPLC</td>
</tr>
<tr>
<td>B/3/Nyanz/3/A1 Mixture 4</td>
<td>&lt;100</td>
<td>378</td>
</tr>
<tr>
<td>B/6/HUYE/10/A1 Maize bran</td>
<td>100</td>
<td>933</td>
</tr>
<tr>
<td>B/6/HUYE/5/A1 Maize bran 4</td>
<td>640</td>
<td>191</td>
</tr>
<tr>
<td>B/6/KAMO/1/A1 mixture1</td>
<td>400</td>
<td>84</td>
</tr>
<tr>
<td>B/6/Nyanz/10/A1 Maize bran 1</td>
<td>561</td>
<td>340</td>
</tr>
<tr>
<td>B/6/Nyanz/3/A1 Maize-1</td>
<td>&gt;6000</td>
<td>0</td>
</tr>
<tr>
<td>B/6/Nyanz/4/A1 Mixture 2</td>
<td>1180</td>
<td>66</td>
</tr>
<tr>
<td>B/6/Nyanz/6/A1 mixture 3</td>
<td>560</td>
<td>134</td>
</tr>
<tr>
<td>B/6/Nyanz/8/A1 Maize bran 3</td>
<td>446</td>
<td>304</td>
</tr>
<tr>
<td>Cotton 1</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>Cotton 2</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>cotton 3</td>
<td>0</td>
<td>56</td>
</tr>
<tr>
<td>Cotton 4</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>Maize Grain 2</td>
<td>1758</td>
<td>1</td>
</tr>
<tr>
<td>Maize Grain 3</td>
<td>704</td>
<td>804</td>
</tr>
<tr>
<td>Maize Grain 4</td>
<td>858</td>
<td>576</td>
</tr>
<tr>
<td>Rice Bran 1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Rice Bran 2</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Rice Bran 3</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Rice Bran 4</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>
APPENDIX 6 POLICY BRIEF - BREAKING AFLATOXIN CONTAMINATION CYCLE IN FEEDS IN RWANDA: RESEARCH FINDINGS AND NEW WAY FORWARD

POLICY BRIEF:

BREAKING AFLATOXIN CONTAMINATION CYCLE IN FEEDS IN RWANDA: RESEARCH FINDINGS AND NEW WAY FORWARD

Kizito Nishimwe1,2, Erin Bowers3, Dirk E. Maier1,3

Introduction
Agriculture is the cornerstone of the Rwandan economy and contributes a third of the national Gross Domestic Product (GDP). The sector employs 80% of the population. The livestock and poultry sector contribute 3% to GDP with revenues reaching Rwf260 billion in 2017 from Rwf143 billion in 2012, according to the Rwanda National Institute of Statistics. Despite the strong performances of the livestock and poultry sector, feed quality and safety is challenged by mycotoxin contamination especially from maize and maize bran.

Mycotoxins are toxic compounds produced by fungi on field crops (e.g., maize, groundnuts) and in stored crops. They are a public health concern because they have a number of negative human and animal health impacts. Human exposure to these mycotoxins is the result of ingestion of contaminated foods, or indirectly from consumption of animal source foods (e.g., dairy products, eggs) derived from animals previously exposed to aflatoxins in feeds.

As part of the research funded by the Feed the Future Innovation Lab for Livestock Systems an 18-month project aimed at assessing aflatoxin and fumonisin contamination in feed ingredients and mixed feeds in Rwanda was conducted. Results were published in the Toxins Journal (Nishimwe et al., 2019).

Research Results to Date

More than 85% of 1180 samples of feed ingredients and mixed feeds (concentrated feeds and certain byproduct feeds) collected from dairy farmers exceeded the aflatoxin standard of 5 µg/kg for aflatoxin B1 established by the Rwanda Standards Board standard (RS: 100:2017) for feed ingredients and mixed feeds. While the survey covered feed samples from all 30 districts of Rwanda, it did not include samples of pasture, which is the main feed for cows in Rwanda, and...
which is not typically contaminated with aflatoxin. Fumonisin levels in the feed samples did not exceed the U.S. Food and Drug Administration (USFDA) guidance levels for maize and maize by-products intended for animal consumption (i.e., 30 ppm for breeding ruminants and breeding poultry, and 10 ppm for dairy cattle). Fumonisin levels in feed samples also did not exceed guidance values for feeds for poultry (< 4 months) and lambs set at 20 mg/kg, and 50 mg/kg set for adult ruminants (> 4 months) by the European Union (EU). U.S. and EU standards are commonly referenced international standards when country standards have not yet been approved.

A follow up study on a small subset of milk samples (a total of 170) from an unrepresentative fraction of dairy farms that fed ingredients or mixed supplements revealed average aflatoxin M1 concentrations that exceeded the action level of 0.5 µg/kg set by USFDA and RSB standard (RS EAS 67:2019) for aflatoxin M1 in milk and the maximum level set at 0.05 µg/kg by the EU. Although this suggests that feed aflatoxins can be transmitted to milk, it should be noted that the results do not reflect the milk from most cows in Rwanda, which are raised on pastures not typically contaminated with aflatoxin.

This research led to organization of a government-approved workshop with pertinent stakeholders that focused on strategies to prevent aflatoxin contamination of feed ingredients and mixed feeds. The recommendations from the workshop have been taken up by a government task force working on their implementation.

**Upcoming Research**

Under a new ENHANCE project, the potential use of binders in feed ingredients and mixed feeds to prevent absorption of AFB1 from diets and transfer to AFM1 in milk will be examined on-farm with a subset of dairy farmers that participated in the FOCUS project. The approach of using mycotoxin binders on-farm is a low-cost and practical mitigation strategy for reducing aflatoxin contamination in milk. Mycotoxin binders are substances that bind mycotoxins and hinder them from being absorbed through the gut into the blood stream, thereby preventing subsequent excretion into dairy milk.

**Recommendations**

- Mycotoxin contamination of feeds is a challenge for the Rwanda feed value chain, which starts in crop fields. There is no single or simple solution to mitigate mycotoxins in feed ingredients and mixed feeds, rather a number of complementary prevention and mitigation strategies can be deployed to safeguard the feed supply. Collaboration among the different stakeholders of the Rwanda feed value chain has been initiated and needs to continue among the private sector, academia, research institutions, policymakers and farmers (both crop and animal) in order to pool efforts and resources and find the best system approach for mycotoxin mitigation in Rwanda.
- Little data has been available on mycotoxin contamination in foods and feeds in Rwanda. This lack of data has resulted in underestimating the mycotoxin risk and in lack of policies, regulations and standards despite the high potential impact on public and animal health. A year-round surveillance and early detection system in the Rwanda feed value chain is key for
effective, long-term mitigation of mycotoxin contamination in foods (especially dairy) and feeds (especially maize and maize bran).

Reference:


Contact: Dr. Dirk E. Maier, dmaier@iastate.edu
Kizito Nishimwe, nishimwe@iastate.edu

1 Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011, USA
2 School of Agriculture and Food Science, University of Rwanda, PO Box 4285 Kigali, Rwanda
3 Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, IA 50011, USA

This work was funded in whole or part by the United States Agency for International Development (USAID) Bureau for Food Security under Agreement # AID-OAA-L-15-00003 as part of Feed the Future Innovation Lab for Livestock Systems. Any opinions, findings, conclusions, or recommendations expressed here are those of the authors alone.