Khalid Rehman Hakeem
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Aflatoxins in Food

A Recent Perspective

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Editors
Khalid Rehman Hakeem
Department of Biological Sciences
Faculty of Science
King Abdulaziz University
Jeddah, Saudi Arabia

Princess Dr Najla Bint Saud Al- Saud
Center for Excellence Research in Biotechnology
King Abdulaziz University
Jeddah, Saudi Arabia

Amir Ismail
Institute of Food Science and Nutrition
Bahauddin Zakariya University
Multan, Pakistan

Carlos A. F. Oliveira
Department of Food Engineering
School of Animal Science and Food Engineering
University of São Paulo
Pirassununga, SP, Brazil

ISBN 978-3-030-85761-5 ISBN 978-3-030-85762-2 (eBook)
https://doi.org/10.1007/978-3-030-85762-2

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

This book is dedicated to our Parents and the CoVid-19 frontline warriors.

Preface

Food is the basic need for life. Besides, providing nutrients necessary for the growth and development of the human body, food also serves as a carrier for the entry of several toxic compounds in the human body. Mycotoxins are the secondary metabolites of fungal species; more than 400 different types of mycotoxins are reported, but the most toxic among all the reported types of mycotoxins are aflatoxins. A wide variety of food commodities (particularly cereals, dry fruits, spices, milk, and milk products) are reported to have aflatoxin levels well beyond the maximum permissible limits, especially from the African and Asian countries. Aflatoxins are classified as group 1 category carcinogenic compound by the International Agency for Research on Cancer. Besides carcinogenicity, aflatoxins are also reported as teratogenic, mutagenic, hepatotoxic, neurotoxic, immunosuppressant, and growth retardant. More than 20 different types of aflatoxins are reported, but the most prevalent as well as the most toxic types are aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, aflatoxin M_1, and aflatoxin M_2. Based on the severe health implications of aflatoxins and their wide prevalence in the food commodities, countries across the world have established maximum permissible limits for aflatoxins. The maximum permissible limits for different types of aflatoxins vary from country to country, based on the economic condition of a country, its technological advancement level, and the level of the prevalence of aflatoxins in different food commodities. Researchers from different corners of the world are trying hard to explore safe and reliable methods for the degradation of aflatoxins in food commodities without disturbing the nutritional and sensory properties of food commodities. A number of chemical and microbiological methods are reported to have significant potential to remove/degrade aflatoxins present in the food commodities.

This book provides complete information on aflatoxins prevalence in food commodities, their history, types, chemical properties, factors affecting the production of aflatoxins, metabolic pathways involved, health implications of aflatoxins on different age groups, regulations adopted by different countries, detection and quantification of aflatoxins, decontamination strategies for the removal/degradation of aflatoxins, and impact of climate change on the prevalence of aflatoxins in different

food commodities. We believe that this book will initiate and introduce readers to state-of-the-art developments and trends in this field of study.

The book comprises 14 chapters, being written by experts in the field of aflatoxins research from different corners of the world. We hope that this volume would furnish the need for all researchers who are working or have interest in this particular field. Undoubtedly, this book will be helpful for the general use of research students, teachers, and those who have interest in aflatoxins/mycotoxins.

We are highly grateful to all our contributors for accepting our invitation, and for not only sharing their knowledge and research, but also for venerably integrating their expertise in dispersed information from diverse fields in composing the chapters and enduring editorial suggestions to finally produce this venture. We also thank Springer Nature team for their generous cooperation at every stage of the book production.

Lastly, thanks are also due to well-wishers, research students, and authors' family members for their moral support, blessings, and inspiration in the compilation of this book.

Jeddah, Saudi Arabia — Khalid Rehman Hakeem
Sao Paulo, Brazil — Carlos A. F. Oliveira
Multan, Pakistan — Amir Ismail

About the Book

Aflatoxins are the secondary metabolites of fungal species of especially Aspergillus origin that are highly toxic for humans and animals. Food commodities especially of African and Asian countries were reported to have alarmingly higher levels of aflatoxins, but due to the global trade of food and feed commodities, aflatoxins have now become a potential threat for the health of humans all across the world. Aflatoxins are of more than 20 different types but the most toxic as well as the most prevalent types in food and feed commodities are aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, aflatoxin M_1, and aflatoxin M_2. The last two types are present in milk and milk products only while the first four types of aflatoxins are collectively termed as total aflatoxins. The International Agency for Research on Cancer (IARC) has classified aflatoxins as group 1 category carcinogenic compound, and besides carcinogenicity aflatoxins are also reported as teratogenic, mutagenic, hepatotoxic, immunosuppressant, growth retardant, and neurotoxic.

Based on the severe health implications of aflatoxins, countries across the world have established maximum permissible limits for different types of aflatoxins that vary in different food commodities. European countries have especially highly stringent regulations for aflatoxins while the maximum permissible limits of developing countries are quite high based on their economic and technological advancement level. Regulatory bodies of all the countries are focusing on the adoption of strategies for the prevention of aflatoxins production in food commodities. Severe health implications of aflatoxins have compelled the researchers from different corners of the world to explore safe and reliable methods for the removal or degradation of aflatoxins without disturbing the nutritional quality, sensory properties, and safety of food commodities.

This book covers a wide range of topics, discussing aflatoxins biosynthesis, the aflatoxins toxicity impact on different age group of people, regulations adopted by different countries, removal/degradation of aflatoxins by adopting different methods, and the instrumental techniques adopted for the qualitative and quantitative analysis of aflatoxins. Furthermore, the impact of climate change on the production of aflatoxins is also discussed. In this book, we highlighted the working solutions as well as open problems and future challenges for aflatoxins research.

Contents

Contributors

Ismail Abiola Adebayo Microbiology and Immunology Department, Faculty of Biomedical Sciences, Kampala International University, Ishaka-Bushenyi, Uganda

Khurram Afzal Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Kamoldeen Abiodun Ajijolakewu Department of Microbiology, Faculty of Life Sciences, University of Ilorin, Ilorin, Kwara State, Nigeria

Saeed Akhtar Institute of Food Science & Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Kashif Akram Department of Food Science, Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Pakistan

Shinawar Waseem Ali Department of Food Sciences, Faculty of Agricultural Sciences, University of the Punjab, Lahore, Pakistan

Paula Alvito Food and Nutrition Department, National Institute of Health Dr. Ricardo Jorge (INSA), Lisbon, Portugal

Centre for Environmental and Marine Studies (CESAM), University of Aveiro, Aveiro, Portugal

Saber Amiri Department of Food Science and Technology, Faculty of Agriculture, Urmia University, Urmia, Iran

Mamoona Amir Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Adnan Amjad Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Ricardo Assunção Centre for Environmental and Marine Studies (CESAM), University of Aveiro, Aveiro, Portugal

Comprehensive Health Research Center (CHRC), Lisbon, Portugal

Laya Rezazad Bari Department of Horticultural Science, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran

Zahid Rafiq Bhat Laboratory of Epigenetics and Diseases, Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India

Carlos Humberto Corassin Department of Food Engineering, School of Animal Science and Food Engineering, University of São Paulo, Pirassununga, SP, Brazil

Renata Regina Pereira da Conceição Federal University of Minas Gerais, Institute of Biological Sciences, Avenida Antônio Carlos, Belo Horizonte, MG, Brazil

Marisa Matias de França Department of Food Engineering, School of Animal Science and Food Engineering, University of São Paulo, Pirassununga, SP, Brazil

Carlos Augusto Fernandes de Oliveira Department of Food Engineering, School of Animal Science and Food Engineering, University of São Paulo, Pirassununga, SP, Brazil

Caroline Corrêa de Souza Coelho Food and Nutrition Graduate Program—Federal University of State of Rio de Janeiro, UNIRIO, Rio de Janeiro, Brazil

Asim Faraz Department of Livestock and Poultry Production, Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, Pakistan

Ambreen Fazal Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Otniel Freitas-Silva Embrapa Food Agroindustry, Brazilian Agricultural Research Corporation, Rio de Janeiro, Brazil

Khalid Rehman Hakeem Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia

Princess Dr Najla Bint Saud Al- Saud Center for Excellence Research in Biotechnology, King Abdulaziz University, Jeddah, Saudi Arabia

Aneela Hameed Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Motahareh Hashemi Moosavi Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Majid Hussain Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Amir Ismail Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Tariq Ismail Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Zubair Ismail Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Alessandra V. Jager School of Pharmaceutical Sciences at Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

Sameem Javaid Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Muhammad Kashif Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Amin Mousavi Khaneghah Department of Food Science and Nutrition, Faculty of Food Engineering, University of Campinas, Campinas, SP, Brazil

Muhammad Latif Biochemical Lab, Center for Genetics and Inherited Disease, Taibah University, Madinah, Saudi Arabia

Suryyia Manzoor Institute of Chemical Sciences, Bahauddin Zakariya University, Multan, Pakistan

Parisa Mostashari Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Research Center of Nutrition and Organic Products (RCNOP), Shahrekord Branch, Islamic Azad University, Shahrekord, Iran

Khurram Muaz Institute of Food Science & Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Zarmina Mumtaz Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Iqra Naeem Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Yusuf Opeyemi Oyebamiji Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia

Muhammad Riaz Institute of Food Science & Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Regiane Ribeiro-Santos Federal Institute of Education, Science and Technology of Pernambuco—IFPE, Vitória de Santo Antão, PE, Brazil

Michael N. Routledge School of Medicine, University of Leeds, Leeeds, UK

School of Food and Biological Engineering, Jiangsu University, Zhenjiang, China

Muhammad Sajid Institute of Chemical Sciences, Bahauddin Zakariya University, Multan, Pakistan

Muhammad Usman Saleem Department of Biosciences, Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, Pakistan

Arif Shahzad Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

Candida P. Shirima Tanzania Bureau of Standards (TBS), Dar es Salaam, Tanzania

Samia Tahir Department of Food Sciences, Faculty of Agricultural Sciences, University of the Punjab, Lahore, Pakistan

Fernando G. Tonin School of Animal Science and Food Engineering, University of São Paulo, Pirassununga, SP, Brazil

Felipe Machado Trombete Department of Food Engineering, Federal University of São João del-Rei, Sete Lagoas, MG, Brazil

Bakhtawar Zawar Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

About the Editors

Khalid Rehman Hakeem, Ph.D. is Professor at King Abdulaziz University, Jeddah, Saudi Arabia. After completing his doctorate (Botany; specialization in Plant Eco-physiology and Molecular Biology) from Jamia Hamdard, New Delhi, India, in 2011, he worked as a lecturer at the University of Kashmir, Srinagar, for a short period. Later, he joined Universiti Putra Malaysia, Selangor, Malaysia, and worked there as Post Doctorate Fellow in 2012 and Fellow Researcher (Associate Prof.) from 2013 to 2016. Dr. Hakeem has more than 10 years of teaching and research experience in plant eco-physiology, biotechnology and molecular biology, medicinal plant research, plant–microbe–soil interactions as well as in environmental studies. He is the recipient of several fellowships at both national and international levels; also, he has served as the visiting scientist at Jinan University, Guangzhou, China. Currently, he is involved with a number of international research projects with different government organizations.So far, Dr. Hakeem has authored and edited more than 65 books with international publishers, including Springer Nature, Academic Press (Elsevier), and CRC Press. He also has to his credit more than 135 research publications in peer-reviewed international journals and 60 book chapters in edited volumes with international publishers.At present, Dr. Hakeem serves as an editorial board member and reviewer of several high-impact international scientific journals from Elsevier, Springer Nature, Taylor and Francis, Cambridge, and John Wiley Publishers. He is included in the advisory board of

Cambridge Scholars Publishing, UK. Prof. Khalid is recently elected as Fellow, Royal Society of Biology, UK. He is also a fellow of Plantae group of the American Society of Plant Biologists, member of the World Academy of Sciences, member of the International Society for Development and Sustainability, Japan, and member of Asian Federation of Biotechnology, Korea. Dr. Hakeem has been listed in Marquis Who's Who in the World since 2014–2019. Currently, Dr. Hakeem is engaged in studying the plant processes at eco-physiological as well as molecular levels.

Carlos Augusto Fernandes de Oliveira graduated in Veterinary Medicine in 1983 and received his Master's (1991) and Ph.D. (1994) in Public Health at the University of São Paulo (USP). Since 2009, he is Full Professor at the Department of Food Engineering, School of Animal Science and Food Engineering (FZEA) of USP. Professor Carlos Oliveira was the Head of Department of Food Engineering (2007–2011), and currently he is the Vice-Dean of FZEA/USP. He worked as a Visiting Researcher during 2016–2017 at the Toxinology Section of the Norwegian Veterinary Institute (NVI), Oslo, Norway. Professor Carlos Oliveira has participated in several international research projects with universities and scientific organizations in the United States, Germany, Denmark, Norway, France, and Portugal.The research interest of Professor Carlos Oliveira focuses on toxins produced by molds (mycotoxins), including their residues in animal products, the assessment of mycotoxin exposure in the human diet using biomarkers, animal toxicity trials, and decontamination approaches. Studies on detection, molecular identification, and prevention of pathogenic microorganisms in milk and dairy products are also developed, as well as potential applications of probiotics in dairy products.Professor Carlos Oliveira has published more than 200 articles in referred journals with 3200 citations and *h*-index of 33, as well as 32 chapters of book and 6 books. He currently serves as Co-Editor-In-Chief of the journal Food Science and Technology, also participating as an Editorial Board member of the journals Toxicology Reports (Elsevier), Quality Assurance and Safety of Crops and Foods (Wageningen/Codon), Dairy (MDPI) and Current Research in Nutrition and Food Science (Enviro Res. Publishers).

Amir Ismail is working as an Assistant Professor (since 2018) in the Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan—Pakistan. He started his career as a Research Fellow in a HEC-funded Research Project and later joined Bahauddin Zakariya University, Multan as Lecturer in the year 2014. Dr. Ismail completed his Ph.D. studies in 2017 and during his Ph.D. studies he went to the Department of Food Science University of Massachusetts Amherst, USA for research work related to microbial decontamination of aflatoxins in milk. He has a teaching experience of more than 8 years and has research collaborations with the leading scientists in the world in the field of food science and technology. He is also the Director Student Affairs and Food Science Undergraduate Program Coordinator in the Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan—Pakistan.Dr. Ismail has published more than 40 publications with a total impact factor of more than 100, he has published his findings as Principal Author in international journals like Food Control, Trends in Food Science and Technology, Toxin Reviews, Food Microbiology, Critical Reviews in Microbiology, Food Research International, Food Additives and Contaminants, and many others. He has also served as reviewer of articles published in International Journal of Dairy Technology, Food Additives and Contaminants, Applied Biochemistry and Biotechnology, Journal of Food Safety, Microbiology Open, Environmental Science and Pollution Research Journal, and so many others. Dr. Ismail is an Associate Editor of the International Journal of Food and Allied Sciences. He has won millions of research grants and has supervised more than 40 graduate students. His area of specialization is food safety, especially aflatoxins and has published a number of articles related to the prevalence of aflatoxins in Pakistani food stuff and the use of different techniques for the removal/degradation of aflatoxins in foodstuff. He has presented his research work in a number of national and international conferences and has represented his country in scientific events organized in Pakistan, the United Kingdom, and the United States. He is engaged in teaching courses related to food toxicology, food analysis, and food chemistry.

Chapter 1
Aflatoxins: An Introduction

Amir Ismail, Michael N. Routledge, Carlos Augusto Fernandes de Oliveira, Khalid Rehman Hakeem, and Candida P. Shirima

Abstract Food is the basic need for the survival of human life. Besides, being a source of nutrients, food may also get contaminated with several toxic compounds, the most prominent among which are aflatoxins. Developing countries, especially African and Asian countries, are reported to have alarmingly higher levels of aflatoxins in foodstuff like cereals, oil seeds, dry fruits, and spices. Aflatoxins are the highly toxic secondary metabolites of fungal species that may impart several health implications if ingested beyond their maximum limits. Based on the serious health implications posed by the aflatoxins, researchers around the world are striving hard to explore both preventive and control approaches to limit the exposure of aflatoxins by the human population.

Keywords Aflatoxins · Food · Metabolites · Contamination · Health effects

Food is the basic need for every form of life and is also among the basic human rights. The provision of a safe and nutritious food supply is essential to maintain a

A. Ismail (✉)
Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan
e-mail: amirismail@bzu.edu.pk

M. N. Routledge
School of Medicine, University of Leeds, Leeds, UK

School of Food and Biological Engineering, Jiangsu University, Zhenjiang, China

C. A. F. de Oliveira
Department of Food Engineering, School of Animal Science and Food Engineering, University of São Paulo, Pirassununga, SP, Brazil

K. R. Hakeem
Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia

Princess Dr Najla Bint Saud Al- Saud Center for Excellence Research in Biotechnology, King Abdulaziz University, Jeddah, Saudi Arabia

C. P. Shirima
Tanzania Bureau of Standards (TBS), Dar es Salaam, Tanzania

K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_1

healthy life. However, food commodities may get contaminated with different types of toxic compounds such as mycotoxins and a number of other toxic compounds. According to the estimates of the WHO, 0.6 billion people suffer from foodborne diseases, and around 0.42 million people die every year due to the consumption of unsafe food (WHO 2021).

Mycotoxins are the secondary metabolites of a limited number of fungal species that are capable of causing serious health implications, particularly in the vulnerable age groups (infants and elderly). The term mycotoxin originated from two words: "mykes" (Greek word) meaning fungus and "toxicum" (Latin word) meaning toxin or poison. Mycotoxins are suspected to contaminate around 25% of the world food. Food and feed commodities may get contaminated with mycotoxins either in the field or during storage (Oliveira et al. 2014). Mycotoxins are linked with a large number of health implications ranging from mild gastrointestinal disturbances to cancer of the liver and other body organs (IARC 2012). More than 450 different types of mycotoxins have been reported to date and are divided into different groups based on their structural similarities and toxicological properties. Aflatoxins are the most toxic and unfortunately most prevalent in the food and feed commodities among all the reported types of mycotoxins.

1.1 History of Aflatoxins

In 1960, more than 0.1 million young turkey birds died in the poultry farms located in the east and south of England because of an unidentified disease. The condition was referred to as the "X" epidemic, later renamed as the turkey "X" disease. The outbreak persisted over the year, and the symptoms of the disease were identical in all cases with the illness being generally short before death. The major symptoms observed in all affected turkeys were lack of appetite, drowsiness, lethargy, wings drooping, and sudden death. The neck and legs of turkeys were arched and stretched backwards when they died, liver lesions and hemorrhage were recorded, and the kidneys were often found congested and enlarged along with inflammation in the small intestine. Ultimately, after a cautious survey of early outbreaks, it was noticed that the disease existed only in London and was linked with "Brazilian peanut meal" manufactured at one of the feed mills of London. Studies conducted to understand the nature of peanut meal contaminants indicated that the diseases might have a fungal origin. An extensive analysis using thin-layer chromatography (TLC) of the alleged peanut meal was conducted, and it was soon discovered that the peanut meal was extremely toxic. Any known toxic compound by that time was found absent in the suspected peanut meal. Eventually, it was found that *Aspergillus flavus* was responsible for the production of toxin present in the feed (Austwick and Ayerst 1963). By the nature of this toxin's origin, it was named aflatoxin where "A" stands for *Aspergillus*, "fla" stands for flavus, and "toxin" for poison, meaning the toxic compound produced by *Aspergillus flavus*. During the examination of the peanut meal using TLC, four major aflatoxins were identified and isolated and were named as aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2). AFB_1 and AFB_2

fluoresce blue while AFG_1 and AFG_2 give green fluorescence under UV light. In the 1960s, a number of cases were reported where the consumption of aflatoxin-contaminated feed resulted in similar toxic effects in dairy animals, and the TLC analysis of the milk of such animals showed a compound that had a different relative to front (Rf) value but also gave blue fluorescence. The compound was later named "aflatoxin M_1" (AFM_1) due to its milk-based origin, and soon another hydroxylated derivate was discovered in milk and was termed as "aflatoxin M_2" (AFM_2). By the application of nuclear magnetic resonance and spectrophotometric analysis, the structural formulas of the aflatoxins were obtained. Structurally, aflatoxins were found closely related to pentacyclic structures of one or two six-membered lactone rings (Akhtar et al. 2020; Alleroft et al. 1961; Goldblatt 1969).

1.2 Production and Metabolism of Aflatoxins

Aflatoxin production has been reported in members of three sections of genus *Aspergillus*, namely, section Flavi (B and G types), section Nidulantes (AFB_1), and section Ochraceorosei (B types) (Pildain et al. 2008). Nevertheless, section Flavi species are the most prevalent and potent aflatoxigenic fungi with *A. flavus* and *A. parasiticus* being the most commonly encountered in agricultural commodities owing to their extensive prevalence in the agricultural environment and their adaptability to grow and produce aflatoxins under divergent environmental conditions (Norlia et al. 2019). Out of 33 species of section Flavi, 18 are known to be aflatoxigenic among which 16 are reported to produce all four major types of aflatoxins, i.e., AFB_1, AFG_1, AFB_2, and AFG_2, while the other two species are reported to produce either both AFB_1 and AFB_2 (*A. pseudotamarii*) or only AFB_1 (*A. togoensis*) (Benkerroum 2020). A detailed discussion on genes involved in aflatoxin production and the biosynthetic pathway is given in Chap. 2 (Aflatoxins Biosynthesis).

The production and relative composition of aflatoxins contaminating the agricultural crops are influenced by various abiotic and biotic environmental factors such as temperature, water activity (aw), substrate composition, storage time, carbon and nitrogen source, light, pH, the content of carbon dioxide (CO_2) and oxygen (O_2), loss of integrity of grains due to insects or thermal/mechanical damage, and the interaction between fungal species that grow in the same ecological zone (Medina et al. 2015; Paterson and Lima 2011; Vaamonde et al. 2006; Wu et al. 2011). Temperature is considered the main determinant of aflatoxin production by fungi. A warm and humid climate increases the susceptibility of aflatoxin contamination in agricultural commodities. The optimal temperature for aflatoxin production ranges between 24 and 30 °C with some alteration resulting from substrate and strain. In the case of substrates, for instance, shelled peanuts, cottonseeds, maize, and rice, the optimal temperature for aflatoxin production by *A. flavus* and *A. parasiticus* ranges between 20 and 30 °C, while a limited quantity of aflatoxins is produced at 10 °C or 40 °C (García and Heredia 2014). A detailed discussion of the factors influencing the production of aflatoxins is given in Chap. 2 (Aflatoxins Biosynthesis).

More than 20 different types of aflatoxins are currently known to occur naturally or as a consequence of the carryover effect in foods and feeds (Table 1.1). Thirteen

Table 1.1 Physical and chemical properties of aflatoxins

Aflatoxin type	Molecular formula	Molecular weight (g/mol)	Melting point (°C)	Physical description	Solubility
Aflatoxin B_1 (AFB_1)	$C_{17}H_{12}O_6$	312.06	268	Colorless to pale-yellow crystals or white powder exhibiting blue fluorescence	<1 mg/ml (at 72 °F) in methanol. In water, 16.14 mg/L at 25 °C
Aflatoxin G_1 (AFG_1)	$C_{17}H_{12}O_7$	328.27	237–299	Colorless to pale-yellow crystals exhibiting green fluorescence	In water, 477 mg/L at 25 °C
Aflatoxin B_2 (AFB_2)	$C_{17}H_{14}O_6$	314.29	287.5	Colorless to pale-yellow crystals exhibiting blue fluorescence	In water, 24.9 mg/L at 25 °C
Aflatoxin G_2 (AFG_2)	$C_{17}H_{14}O_7$	330.29	237–240	Fluffy and light crystalline solid exhibiting green-blue fluorescence	In water, 3.73 × 10 + 3 mg/L at 25 °C
Aflatoxin B_2a (AFB_2a)	$C_{17}H_{14}O_7$	330.29	NA	NA	NA
Aflatoxin G_2a (AFG_2a)	$C_{17}H_{14}O_8$	346.3	NA	NA	NA
Aflatoxin M_1 (AFM_1)	$C_{17}H_{12}O_7$	328.06	299	Solid exhibiting blue-violet fluorescence	NA
Aflatoxin M_2 (AFM_2)	$C_{17}H_{12}O_7$	330.29	293	Solid	NA
Aflatoxin M_2a (AFM_2a)	$C_{17}H_{14}O_8$	346.3	NA	NA	NA
Aflatoxin Q_1 (AFQ_1)	$C_{17}H_{12}O_7$	328.27	NA	NA	NA
Aflatoxin P_1 (AFP_1)	$C_6H_{10}O_6$	298.25	NA	NA	NA
Aflatoxin Q_2a (AFQ_2a)	$C_{17}H_{14}O_8$	346.29	NA	NA	NA
Aflatoxicol (AFL)	$C_{17}H_{14}O_6$	314.29	NA	NA	NA
Aflatoxicol H_1 ($AFLH_1$)	$C_{17}H_{14}O_7$	330.29	NA	NA	NA
Aflatoxicol M_1 ($AFLM_1$)	$C_{17}H_{14}O_7$	330.29	NA	NA	NA
Aflatoxin GM_1 ($AFGM_1$)	$C_{17}H_{12}O_8$	344.3	276	Solid	NA

(continued)

Table 1.1 (continued)

Aflatoxin type	Molecular formula	Molecular weight (g/mol)	Melting point (°C)	Physical description	Solubility
Aflatoxin GM_2 ($AFGM_2$)	$C_{17}H_{14}O_8$	346.3	270–272	Solid	NA
Parasiticol (AFB_3)	$C_{16}H_{14}O_6$	302.28	NA	NA	NA
Aflatoxin D_1 (AFD_1)	$C_{16}H_{14}O_5$	286.28	NA	NA	NA
Aspertoxin	$C_{19}H_{14}O_7$	354.3	NA	NA	NA

Modified from Benkerroum et al. (2020) and PubChem Database https://pubchem.ncbi.nlm.nih.gov/; NA = information is not available considered a member of difurocoumarolactone group despite the absence of six-membered lactone ring in its chemical structure (Benkerroum 2020; Cole and Kirksey 1971). Aspertoxin is a hydroxyl metabolite of O-methylsterigmatocystin (an intermediate metabolite of AFB_1 pathway) (Rodricks et al. 1968) and can be a parent compound in the biosynthetic pathway of AFM_1 and $AFGM_1$ (Yabe et al. 2012)

types of aflatoxins are produced by toxigenic fungus naturally, some of which can be biotransformed in animals, humans, or other microorganisms to produce metabolites that employ toxicity, though usually with a lesser potency than their parent compounds (Benkerroum 2020; Filazi and Sireli 2013). The epoxidation of aflatoxins (particularly AFB_1) in the liver by cytochrome P450 enzyme system results in the formation of AF-8,9-epoxide (AFBO) which has two isomeric forms: exo-8,9-epoxide and endo-8,9-epoxide. The AFBO, being electrophilic, reacts with DNA and/or proteins, forms adducts, and thus interfere in the functioning of these biological molecules (a detailed discussion on aflatoxin DNA and protein adducts is given in Chap. 5). The metabolism of aflatoxins by P450 family results in the number of hydroxylated products such as AFM_1, AFM_2, aflatoxin P_1 (AFP_1), aflatoxin Q_1 (AFQ_1), aflatoxin B_2a (AFB_2a), aflatoxin G_2a (AFG_2a), aflatoxin M_2a (AFM_2a), aflatoxicol (AFL), aflatoxicol H_1 ($AFLH_1$), and aflatoxicol M_1 ($AFLM_1$). Among these, AFP_1, AFQ_1, and AFB_2a are regarded as detoxification products of AFB_1 owing to their reduced DNA-binding ability (Guan et al. 2021; Shan 2019). AFL, which is formed by a NADPH reductase normally in the cytosol, maintains its DNA-binding potential and has been reported to be converted back into AFB_1 enzymatically, serving as a reservoir for AFB_1 scaling up its toxic effects (Lozano and Diaz 2006; Partanen et al. 2010). $AFLH_1$ has a chemical structure analogous to AFL that contains an additional hydroxyl group on the terminal cyclopentenone ring and has been reported to imply similar toxic effects (Salhab and Hsieh 1975). $AFLM_1$, another metabolite of AFL, has been isolated and identified in the microsomal liver preparations and has been reported to reconvert back into AFM_1 by carbon monoxide-insensitive dehydrogenase activity related to human liver microsomes, a process similar to the formation of AFB_1 from AFL (Salhab et al. 1977). AFD_1 is the degraded product of AFB_1 formed after ammonization and/or microbial treatment

(such as *Streptococcus thermophilus*, *Lactobacillus delbrueckii*, and *Pseudomonas putida*) and is regarded as less toxic than AFB_1 (Chen et al. 2015; Grove et al. 1984; Samuel et al. 2014). However, resetting AFD_1 in the acidified environment increases the risk of its conversion back into the AFB_1 (Grove et al. 1984).

1.3 Physical and Chemical Properties of Aflatoxins

Aflatoxins are colorless to pale-yellow crystals that exhibit fluorescence under ultraviolet (UV) light. Aflatoxins of the B group (AFB_1, AFB_2) fluoresce blue, while the G group (AFG_1, AFG_2) fluoresce green under UV light and thereby designated as B and G, respectively. Aflatoxins are slightly soluble in water (10–30 μg/ml), readily soluble in moderately polar organic solvents such as methanol, chloroform, and dimethyl sulfoxide while completely insoluble in non-polar solvents. Aflatoxins are unstable under UV light, in the presence of O_2, in extreme pH (<3, >10), and in the presence of oxidizing agents. Aflatoxins are highly sensitive to various chemical agents and can be degraded by the reaction to strong acids, alkalis, and ammonia (IARC 2012). The physical and chemical properties of aflatoxins are presented in Table 1.1.

The chemical structures of different types of aflatoxins and their metabolites are presented in Fig. 1.1. Structurally, aflatoxins are difuranocoumarin derivatives that consist of a coumarin center and are synthesized through a polyketide biosynthetic pathway in the fungus. On one side of the coumarin nucleus, there is a difurane moiety, while the other side consists of either a five-membered pentene ring (the group is named difurocoumarocyclopentenones) or a six-membered lactone ring (the group is named difurocoumarolactones). Aflatoxins of the B group and their derivatives such as AFB_1, AFB_2, AFB_2a, AFM_1, AFM_2, AFM_2a, AFP_1, AFQ_1, AFQ_2a, AFL, $AFLH_1$, and $AFLM_1$ are the members of the difurocoumarocyclopentenones group, while the aflatoxins of the G group and their derivatives such as AFG_1, AFG_2, AFG_2a, $AFGM_1$, $AFGM_2$, and $AFGM_2$ fall in the difurocoumarolactones group. Parasiticol (also known as AFB_3) is.

1.4 Toxicological Properties of Aflatoxins

Aflatoxicosis is the toxicity induced in humans or animals by the ingestion of food commodities contaminated with moderate to high quantities of aflatoxins. Aflatoxicosis may be acute (due to short exposure) or chronic in nature (due to long exposure). Acute aflatoxicosis may lead to gastrointestinal disturbance, necrosis, edema, increased risk of jaundice, fatigue, lethargy, and cirrhosis and extreme conditions (dose of 10–20 mg) may result in the failure of the liver and death. Chronic exposure to aflatoxins may result in cancer of the liver and other body organs, weakened immune system, teratogenic effects, mutagenic effects, and growth retardation

Fig. 1.1 Chemical structure of different types of aflatoxins and their metabolites. (Source: PubChem https://pubchem.ncbi.nlm.nih.gov/)

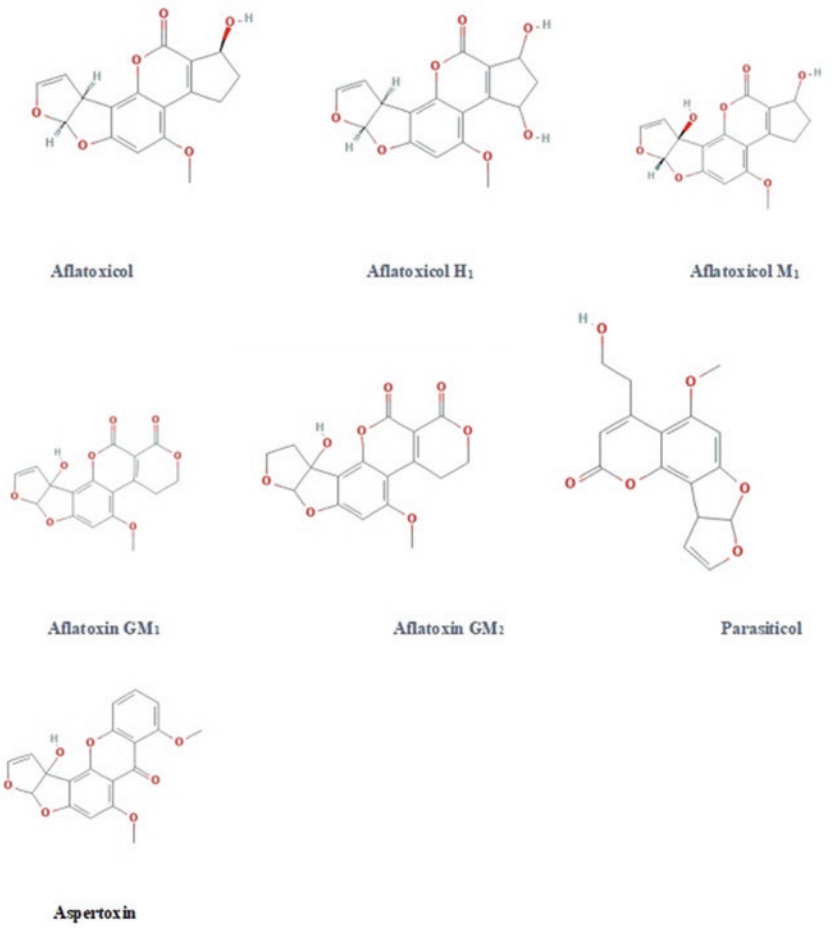

Fig. 1.1 (continued)

including wasting and stunting (Oliveira et al. 2014; Ismail et al. 2018). The impacts of aflatoxins on human health are influenced by the factors such as dose, duration of exposure, age, gender, animal type/species, overall health, and nutrition status and the exposure of other toxic compounds (Mishra and Das 2003).

The International Agency for Research on Cancer (IARC) has classified total aflatoxins (AFB_1 + AFB_2 + AFG_1 + AFG_2) and AFB_1 and AFM_1 alone as group 1 category carcinogenic compound (IARC 2012). The liver is the primary target organ of aflatoxins, and therefore hepatocellular carcinoma (HHC) is the major health implication of aflatoxins in humans. Globally, HCC is known as the seventh leading type of cancer in men and the ninth leading type of cancer in women. The primary target organ of hepatitis is also the liver, the chances of HCC multiply to many folds

in hepatitis patients with frequent exposure to aflatoxins, and both are common in developing countries like India, Pakistan, and Bangladesh; therefore these countries have the highest rate of HCC (Ismail et al. 2018; Wang et al. 2001).

A detailed discussion on the impact of different types of aflatoxins on different age groups including infants, children, adults, and elderly are given in Chap. 5 (Aflatoxin's Health Impacts on Infants and Children) and Chap. 6 (Aflatoxin's Health Impacts on Adults and Elderly).

1.5 Prevalence of Aflatoxins

Aflatoxins are produced by the fungal species of *Aspergillus* origin that are widespread. The environmental conditions and agronomic practices of African and South Asian countries are best suited for the production of aflatoxins by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins are produced in field conditions, but the maximum levels are reported to reach during post-harvest operations, especially during storage. The prevalence of aflatoxins in food commodities depends on a number of factors such as geographical location, a season of the year, type of food commodity, and post-harvest management practices, especially moisture content of food commodity/substrate and the relative humidity of the warehouse (Ismail et al. 2017). Food commodities especially reported to have alarmingly higher levels of aflatoxins are cereals, especially corn and rice; oil seed mainly groundnuts/peanuts; dry spices, especially red pepper; dry fruits, especially dates; and milk and milk products. The possible reasons behind the higher aflatoxin levels in the food commodities include suitable environmental conditions for the growth of fungus and production of aflatoxins, low literacy rate, lack of technological advancement, inadequate enforcement of rules and regulations, improper storage facilities, and poor economic conditions. Low levels of aflatoxins are reported in food commodities, especially from European countries; the possible reasons might include unfavorable environmental conditions for the growth of fungus and production of aflatoxins, technological advancement, and implementation of strict rules and regulations (Ismail et al. 2018; Oliveira et al. 2016).

A detailed discussion of the prevalence of aflatoxins in different food commodities is given in Chap. 3 (Worldwide Prevalence of Aflatoxins in Food and Feed).

Global warming due to climate change is currently an important issue that will probably cause shifts in the onset and length of growing and in the geographical range of certain crops (Thornton et al. 2014). The large impacts of global warming projected on crops worldwide will influence not only food security, by reducing yields and thus food availability, but also food and feed safety. From all the potential food safety hazards that could be affected by climate change, food contamination by mycotoxins is considered one of the most important factors, as detailed in Chap. 13 of this book (The Impact of Climate Change on Aflatoxin Contamination in Food Commodities).

1.6 Aflatoxin Regulations

Based on the toxicity of aflatoxins and their worldwide prevalence in food commodities, countries across the globe have established maximum limits for aflatoxins in different foodstuffs. A huge variation exists in the maximum limits of the same types of aflatoxins in the same food commodities, from country to country. Variation in the maximum permissible limits of aflatoxins is based on the economic condition of a country, type of food commodity, technological advancement of that country, rate of consumption of aflatoxin susceptible food, the overall health status of the population, and levels of aflatoxins contamination in different foodstuffs. Because aflatoxins are genotoxic, the most widely implemented criterion regarding the establishment of maximum permissible limits against highly toxic compounds such as aflatoxins, as proposed by the FAO/WHO, is built on the idea of "as low as reasonably acceptable" (ALARA). More than 100 countries have now established maximum limits against different types of aflatoxins in different food commodities ranging between 0.025 and 100 μg/kg (Ismail et al. 2018). The European Union (EU) has established the most stringent regulations against aflatoxins; the maximum permissible limit of total aflatoxins and AFB_1 in cereals and cereal-based products by the EU is 4 μg/kg and 2 μg/kg, respectively, while the EU maximum permissible limit for AFM_1 in milk is 0.05 μg/kg. Most of the countries now have maximum permissible limits for total aflatoxins, e.g., in Pakistan, it is 20 μg/kg, and no separate maximum permissible limit exists for AFB_1, while for AFM_1 in milk, it is 0.5 μg/kg (Akhtar et al. 2020; Medina et al. 2021).

A detailed discussion on the maximum limit of different types of aflatoxins in different foodstuffs is given in Chap. 7 (Regulations for Aflatoxins in Developing and Industrialized Economies).

1.7 Analytical Techniques

Since their identification, scientists all around the globe are working day and night to introduce more and more sensitive, reliable, repeatable, quick, and economically feasible methods for the detection and quantification of aflatoxins. As the toxic levels of aflatoxins are extremely low, the analytical approaches should be able to precisely detect their concentrations in foods at parts per billion. Moreover, the distribution of aflatoxins in food commodities is quite uneven, thus requiring suitable sampling techniques to avoid false results. The guidelines regarding sample collection for the quantification of aflatoxins are given in the EU Commission Regulations 401/2006. Samples all across the EU countries are collected according to the given protocol, and the method also compels that different performance indicators such as precision and recovery percentages are essentially performed. For the quantification of aflatoxins, samples are first extracted using suitable solvents such as methanol, chloroform, acetonitrile, and others. The extraction step is mostly

followed by the cleanup step involving the use of immune affinity columns (IAC). Then suitable techniques are adopted for the quantification of aflatoxins like HPLC, LC-MS, and others.

The most primitive type of method for aflatoxins analysis is TLC, which is still used in laboratories across the world because of its low cost when compared with other advanced techniques. In the TLC method, commercially available silica/alumina plates are now used as a stationary phase, and chloroform/methanol solutions (95:5 v/v) are mostly used as a mobile phase. The TLC method is a cheap method but has low limits of detection, requires technical expertise, and may be used only for qualitative purposes. The second most commonly employed method for the quantification of aflatoxins is enzyme-linked immunosorbent assay (ELISA). The ELISA method does not require high instrumental cost like high-performance liquid chromatography (HPLC) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) systems, and ELISA is also a convenient method. Some manufacturers are supplying ready-to-use ELISA kits for onsite testing purposes. However, the reliability, sensitivity, and repeatability of the ELISA method are a big hurdle for its acceptability, especially for research purposes. In most of the laboratories across the globe, aflatoxins are quantified by HPLC equipped with a fluorescence detector. Immunoaffinity columns are often required for cleaning purposes, before running the samples on HPLC to remove the interfering compounds. The C18 column is mostly used for aflatoxin separation, while mixtures of methanol, acetonitrile, and water are mostly recommended for use as a mobile phase for the quantification of total aflatoxins. The HPLC method is a convenient, reliable, repeatable, and acceptable method for the quantification of aflatoxins. The most reliable and most sensitive method for the quantification of aflatoxins is the LC-MS/MS method. In this technique, different types of aflatoxins are quantified simultaneously based on the liquid chromatography separation and mass-to-charge ratio (*m/z*) of aflatoxins. However, due to the huge amount of initial capital required for the purchase of LC-MS/MS, the use of this technique is not so common (Zhang and Banerjee, 2020; Yakubu and Vyas, 2020; Santini and Ritieni 2013). Besides these methods, gas chromatography (GC), GC-MS method, infrared spectroscopy method, radioimmunoassay technique, and different types of immunosensor assay techniques are also adopted for the quantitative/qualitative analysis of aflatoxins (Wacoo et al. 2014). Details on methods used for the analysis of aflatoxins are given in Chap. 8 of this book (Detection and Quantification of Aflatoxins).

1.8 Prevention and Control of Aflatoxins

Aflatoxins are reported in food commodities, especially of Asian and African origin. Despite the availability of suitable environmental conditions, the levels of these toxins may be managed/reduced by adopting two different approaches: (i) preventive approaches, i.e., by adopting good agricultural practices and proper management in the field, during harvesting and post-harvest operations including storage

and (ii) decontamination, i.e., by the degradation or removal of aflatoxins from food commodities (Benkerroum 2020). An overview of the methods described in the literature to tackle the aflatoxin contamination of foods is given in Chap. 9 (Removal and Detoxification of Aflatoxins).

1.8.1 Preventive Approaches

The first and best practice to limit the aflatoxin prevalence is the preventive approach. Aflatoxin production in different food commodities may be limited in a number of different ways, all of which are of equal significance and must be ensured especially in the areas where environmental conditions are favorable for the production of aflatoxins:

1. The first strategy to prevent the production of aflatoxins is the use of aflatoxin-resistant crop varieties. A number of different seed varieties are available that are resistant to the growth of aflatoxin-producing fungi and ultimately to the production of aflatoxins. Plant breeders from different parts of the world are doing research on the development of seed varieties having high yield potential but on the other hand having resistance against infestation by aflatoxin-producing fungi. The identification of different compounds in the plants that limit the production of aflatoxins will largely help in the control of aflatoxins.
2. The second approach that is especially getting fame in controlling the levels of aflatoxins in the crops of highest vulnerability such as maize is the use of biocontrol agents. The US Department of Agriculture (USDA) has introduced Aflasafe in African countries, which is a biocontrol agent in which non-aflatoxin-producing *Aspergillus flavus* is sprayed in the field to outcompete the growth of aflatoxin-producing strains of fungus. This biocontrol agent is usually sprayed 2–3 weeks earlier than the flowering stage.
3. The third strategy to prevent the production of aflatoxins is the adoption of good agricultural practices (GAPs). GAPs to limit aflatoxin production include crop rotation, maintaining proper plant density, proper nutrition, and irrigation. Weeds must be removed; insecticides and fungicides must be sprayed, and fertilizers must be applied when required and as recommended by agricultural experts. Harvesting must be done at the proper time as the moisture content of harvested crop is the single most important parameter that affects fungal growth.
4. The fourth step to limit aflatoxin production is the management of storeroom/warehouse conditions. Maintaining humidity and temperature; proper cleanliness; ventilation and arrangement of bags containing the food crops, insect, pest, and rodents' control; removal of infected grains; and fungicide spray as and when required are the essential prerequisites for limiting the growth of aflatoxin-producing fungal strains and ultimately for maintaining lower levels of aflatoxins (Benkerroum, 2020; García and Heredia 2014; Hell and Mutegi 2011; Ismail et al. 2018; Lizárraga-Paulín et al. 2013).

1.8.2 Decontamination

The prevalence of aflatoxins in food commodities well beyond the permissible limits has triggered researchers around the globe to find suitable methods for the decontamination of aflatoxins. A lot of research had been done in this area, especially during the last two decades. Aflatoxin decontamination in food commodities may be done by physical, chemical, and biological methods (Marshall et al. 2020; Yang 2020). A brief detail of these three aflatoxins decontamination approaches are given below.

1.8.2.1 Physical Methods

Physical methods for aflatoxin decontamination may be divided into two different approaches: (i) physical removal and (ii) degradation by physical means. In the first approach, aflatoxins are decontaminated/removed by the physical approaches, i.e., by separation techniques, solvent extraction methods, and the use of the mineral adsorbents. In the separation method, the damaged, discolored, dirty, and moldy grains are removed from the rest of the grains. Separation may be done by physical means involving human labor or by using the mechanical sorters (Oliveira et al. 2014a). In solvent extraction, suitable solvent mixtures, e.g., methanol and water, hexane and methanol, water and acetonitrile, and 80% isopropanol in water and 90% acetone in water are used for the removal of aflatoxins. The major limitation of the solvent extraction technique is that it cannot be used for food purpose but may find its applications for animal feed purpose (Ismail et al. 2018). A number of mineral adsorbents have shown a promising potential for the removal of aflatoxins. Recently, an activated carbon-based filter was used by Azam et al. (2020) for the removal of aflatoxins from roasted coffee samples. The activated carbon-based filter removed 80% of aflatoxins from the spiked samples (50 μg/kg). Adsorbent materials such as activated carbon bind aflatoxins on their surfaces and thereby reduce their bioavailability/absorption rate, and aflatoxins are removed from the body without being absorbed. Adsorbent materials are now frequently used in animal feeds, while their use for human food purposes is still not reported, but in very few cases. When proved to be safe, they may be used with human populations having severe exposure to aflatoxins through diet (Wang et al. 2008).

The second approach, i.e., aflatoxin degradation by physical means, includes degradation employing heating, ultraviolet radiations, microwave heating, extrusion, and irradiations. Aflatoxins though have shown heat resistance in most of the studies (Awasthi et al. 2012; Hassan and Kassaify 2014), but only very few studies have reported that the toxin can be degraded by the conventional heating techniques (Rastegar et al. 2017; Sani et al. 2014). Extrusion is reported to have the highest aflatoxin degradation potential among the reported physical approaches. Zheng et al. (2015) subjected peanut meal to extrusion processing (temperature of 150 °C and speed of 152 revolutions per minutes) and achieved 77% AFB_1 degradation,

while the initial AFB_1 level was 35.8 μg/kg. The details of physical approaches used by the researchers across the globe are reviewed by Marshall et al. (2020) and Pankaj et al. (2018).

The details of physical methods adopted for the decontamination/degradation of aflatoxins are given in Chap. 10 (Physical Decontamination and Degradation of Aflatoxins).

1.8.2.2 Chemical Methods

A number of different chemicals are used by the researchers from different corners of the world to evaluate their aflatoxin degradation potential and suitability for application in food products. Unfortunately, to date not a single chemical is permitted to be used in food products for the degradation of aflatoxins on a commercial scale. The chemicals that have shown a significant aflatoxin degradation potential include ammonia, hydrogen peroxide, sodium bisulfite, and ozone. Ozone gas has especially attained the attraction of researchers as it is already permitted for application in water treatment plants and for many other applications (especially germicidal) in the food industry. Ozone gas has the potential to degrade aflatoxins by attacking on the C8–C9 double bounds of the aflatoxin's furan ring structures (Ismail et al. 2018; Peng et al. 2018). Savi et al. (2015) has reported 81–95% degradation of aflatoxins in wheat samples by the application of 40–60 ppm O_3 for a time period of 30–180 min. A number of different plant extracts have also shown promising potentials to degrade aflatoxins. In a study conducted by Iram et al. (2015), aflatoxin degradation potential of eucalyptus (*Corymbia citriodora*) was investigated, and the authors reported 93–95% degradation of different types of aflatoxins (pH 8, temperature 30 °C, and incubation time were 72 h). The degradation rate of aqueous extracts increases with increase in incubation time and also varies with the type of plant used. Plant extracts have a number of advantages including environmental safety, cheaper rates, renewability, and biodegradability. However, the major limitation involved is the repeatability of results as identification of a plant needs expertise, and the bioactive compounds of a plant also vary too much.

Review articles about different chemical methods for the degradation of aflatoxins are given by Marshall et al. (2020), Pankaj et al. (2018), and Peng et al. (2018). The details of chemical methods adopted for the degradation of aflatoxins are given in Chap. 11 (Chemical Degradation of Aflatoxins).

1.8.2.3 Biological Methods

Based on the limitations of physical and chemical methods of aflatoxin decontamination, biological methods involving microbes (bacteria and fungi) serve as a better alternative. Microbial catabolic pathways are involved in the microbiological enzymatic degradation of aflatoxins to less toxic compounds, and sometimes a simple

adsorption process is involved (adhesion of aflatoxins with microbial cell walls). The major advantages of microbial applications against aflatoxins include their specificity in action, effectiveness, and their cheaper availability (Adebo et al. 2017). Lactic acid bacteria have "generally recognized as safe status (GRAS)" and are therefore especially explored by the researchers from different areas of the world to evaluate their aflatoxin degradation potential. Peltonen et al. (2001) evaluated aflatoxin degradation potential of 15 different strains of lactic acid bacteria and reported their aflatoxin degradation potential in the range of 6–58%. Among the yeast species, *Saccharomyces cerevisiae* also has GRAS and is already in use for the preparation of different food products. *Saccharomyces cerevisiae* is also reported to have a higher aflatoxin-binding potential. In a study conducted by Ismail et al. (2017a), AFM_1 decontamination potential of three different strains of lactic acid bacteria and a strain of *Saccharomyces cerevisiae* was evaluated. *Saccharomyces cerevisiae* was found to have the highest aflatoxin removal percentage (92% at the spiking level of 0.1 ng/ml). The highest aflatoxin removal percentage of *Saccharomyces cerevisiae* was associated with the large cell size of yeast cells as compared to lactic acid bacteria. Furthermore, the heat-killed cells of microbes are also reported to have a higher aflatoxin removal percentage as compared to live cells, and the removal percentage increases by increasing the concentration of microbial cells (Bovo et al. 2013; Ismail et al. 2017a).

The details of chemical methods adopted for the degradation of aflatoxins are given in Chap. 12 (Biological Decontamination and Degradation of Aflatoxins).

1.9 Concluding Remarks

Aflatoxins are the secondary metabolites of a few species of fungi and are group 1 category carcinogenic compound. Besides carcinogenicity, aflatoxins are also reported as teratogenic, hepatotoxic, growth retardant, and immune suppressant. More than 20 different types of aflatoxins are reported (13 are naturally produced), but aflatoxin B_1 is the most prevalent and most toxic type of aflatoxins. Aflatoxins are widely present in the food commodities, especially cereals (particularly maize), oil seeds (mainly peanuts), dry fruits, and spices (particularly red pepper). African and South Asian countries are especially reported to have high prevalence of aflatoxins in susceptible food crops mainly due to their hot and humid climate. Based on the health implications of aflatoxins, countries across the world have set maximum limits for different types of aflatoxins in different food commodities, while the EU countries have established the most stringent permissible limits against aflatoxins. A number of techniques are used for the detection of aflatoxins among which TLC is the most primitive technique (but still practiced), while LC/MS and HPLC are the most widely used confirmatory tools for the detection and quantification of aflatoxins.

National and international regulatory bodies along with all the stakeholders/food handlers must put their best efforts to curtail the level of aflatoxins in foodstuff.

Preventive approaches involving good agricultural practices at the farm level (resistant varieties and effective crop management) and good management practices for post-harvest operations (especially safe transport and controlled storage) must be ensured to reduce the contamination level of aflatoxins during field and after harvesting. To date, not a single method is available that is commercially implementable and acceptable for the degradation of aflatoxins in foodstuff (although for animal feed some acceptable methods exist and are in practice), and therefore further research is needed in this area. A number of in vitro studies have shown aflatoxin degradation potential of compounds that are safe, including ozone, plant extracts, and others, but further research is still needed involving in vivo studies to evaluate the impact of the said compounds on the toxicological, nutritional, and sensory properties of targeted food commodities.Conflict of InterestThe authors declare no conflict of interest.

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Chapter 2
Aflatoxin Biosynthesis

Khurram Muaz, Suryyia Manzoor, Saeed Akhtar, Muhammad Riaz, Mamoona Amir, Kashif Akram, and Amir Ismail

Abstract Aflatoxins are highly toxic contaminants synthesized by several toxigenic strains of *Aspergillus* as secondary metabolites. The biosynthesis of aflatoxins is a complicated process involving a chain of reactions which are catalyzed by various enzymes encoded by genes present on aflatoxin cluster. The genetic variations among different fungal strains can impact the final compound being produced. This chapter focuses on the biosynthetic pathway for aflatoxin production starting from acetate and finishing with the production of aflatoxin. The role of various genes and their encoded enzymes at every reaction has been described. Furthermore, the impact of different factors such as light, temperature, water activity, oxidative stress, carbon sources, nitrogen sources, and pH on aflatoxin biosynthesis has also been described. AFB_1 is the most common and toxic aflatoxin being consumed through various sources. Inside the human or animal body, AFB_1 is metabolized to different forms, making it either highly toxic or less toxic depending on the metabolism channel. The metabolism of AFB_1 has also been covered in this chapter.

Keywords Aflatoxins · Synthesized · Process · Reactions · Biosynthetic · Genes · Factors · Metabolism

2.1 Biosynthesis

Biosynthesis is an enzyme-catalyzed, multistep process involving the conversion of substrates into highly complex compounds within living organisms. During this process, the simpler compounds are modified either through conversion into other

K. Muaz · S. Akhtar (✉) · M. Riaz · M. Amir · A. Ismail
Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

S. Manzoor
Institute of Chemical Sciences, Bahauddin Zakariya University, Multan, Pakistan

K. Akram
Department of Food Science, Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Pakistan

K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_2

compounds or through joining together in order to form macromolecules. The biosynthesis of metabolites is carried out through certain metabolic pathways. Aflatoxins are synthesized by certain fungal species as secondary metabolites through a chain of reactions. The study regarding the biosynthesis of secondary metabolites generally involves the identification of reactions and their sequence through which the cells convert the primary metabolites into the final molecule. Additionally, the features regulating these processes are also identified. Even though a great variation may exist in chemical structures of metabolites, the biosynthesis of most secondary microbial metabolites may occur only through a certain number of biosynthetic pathways.

The biosynthetic pathway elucidation is a multistep procedure which may involve the identification of the primary metabolite from which the final molecule is being made and isolation of intermediate compounds formed along the pathway in order to hypothesize the sequence of reactions based on their chemical structures. The identification of enzymes involved at each point during the biosynthetic pathway and their isolation is also important in the understanding of this process. Furthermore, in order to completely understand the biosynthetic process, identification of regulatory factors and biosynthetic genes is also important.

2.2 Aflatoxin Biosynthesis

A complicated biosynthetic pathway consisting of at least 27 reactions catalyzed by different enzymes is involved in the production of aflatoxins (Roze et al. 2013; Yu 2012). The genes containing the codes for enzymes involved in the AF biosynthetic pathway are grouped in a cluster. The expression of these genes is controlled by two cluster-specific regulatory genes, namely, *aflR* and *aflS* (Chang 2003; Price et al. 2006). Additionally, the synthesis of AFs can also be influenced by environmental stimuli such as oxidative stress, nutrient sources, pH, and light which may initiate complex mechanisms through activation of various cell signaling pathways, hence modifying the expression of the genes playing a role in the production of toxin (Affeldt et al. 2014; Klich 2007; Montibus et al. 2015).

The DNA information in aspergilli is structured in eight chromosomes, and the genes resulting in AF synthesis are situated in the 54th cluster, 75-kb region of the fungal genome on chromosome III, 80 kb from the telomere of chromosome 3 (Georgianna and Payne 2009). This cluster consists of 30 genes (Fig. 2.1) and is mainly controlled by the regulatory genes *aflR* and *aflS* (Chang 2003; Price et al. 2006). The gene cluster involved in AF production has been extensively analyzed in *A. flavus* as well as in *A. parasiticus*. The studies have revealed the homology of the clustered genes among these species to be between 90 and 99% (Yu et al. 2000b). Due to the presence of these changes, one of the major differences existing among *A. flavus* and *A. parasiticus* is their ability to synthesize B- and G-type aflatoxins. The *A. flavus* majorly synthesizes aflatoxins B_1 and B_2, while *A. parasiticus* possesses the ability to produce both B and G types of aflatoxin. The functional genes

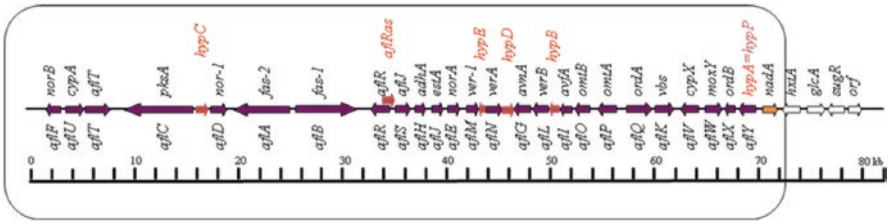

Fig. 2.1 Gene cluster for aflatoxin biosynthesis. This figure is adopted from the work of Yu (2012)

responsible for the synthesis of G-type aflatoxin include *aflF*, *aflU*, and *nadA* which, respectively, encode for an aryl alcohol dehydrogenase, a cytochrome P450 monooxygenase, and an oxidase (Ehrlich et al. 2004; Ehrlich et al. 2008). Furthermore, the elucidation of the aflatoxin-producing cluster has also been aided by studies on *A. nidulans* which has the capability to produce sterigmatocystin, an intermediate metabolite produced along the AF biosynthetic pathway. The *A. parasiticus* and *A. nidulans* exhibit the homology between 55 and 75%.

Birch (1967) first proposed the formation of aflatoxins through a polyketide pathway, and extensive studies have shown the involvement of at least 27 enzymatic reactions till now. Moreover, extensive studies have shown that along with other enzymes, cytochrome P450 also exhibits an important part in the production of aflatoxin. These enzymes are responsible for the attachment of functional groups (i.e., acetyl, methyl) during AF biosynthesis (Nelson 2011). Among the biosynthetic pathways for the production of mycotoxins known to date, the aflatoxin gene cluster has the highest number of cytochrome P450 (Roze et al. 2015). Thus owing to the number of oxidative changes involved, AF biosynthesis is among the longest and most complex processes (Minto and Townsend 1997).

2.3 Aflatoxin Biosynthetic Pathway

Aflatoxin biosynthetic pathway comprises of cascade of reactions catalyzed by various enzymes (Fig. 2.2).

2.3.1 Acetate to Norsolorinic Acid

Aflatoxins are the polyketide derivative furanocoumarin compounds. The biosynthesis of these substances require the hexanoate starter components (from acetyl-CoA and malonyl-CoA) which act as an initial substrate in a series of reactions ultimately leading to the formation of aflatoxins (Roze et al. 2013). The first step in the biosynthetic pathway for aflatoxins involves the formation of norsolorinic acid (NOR). The NOR is the first stable compound among the aflatoxin precursors

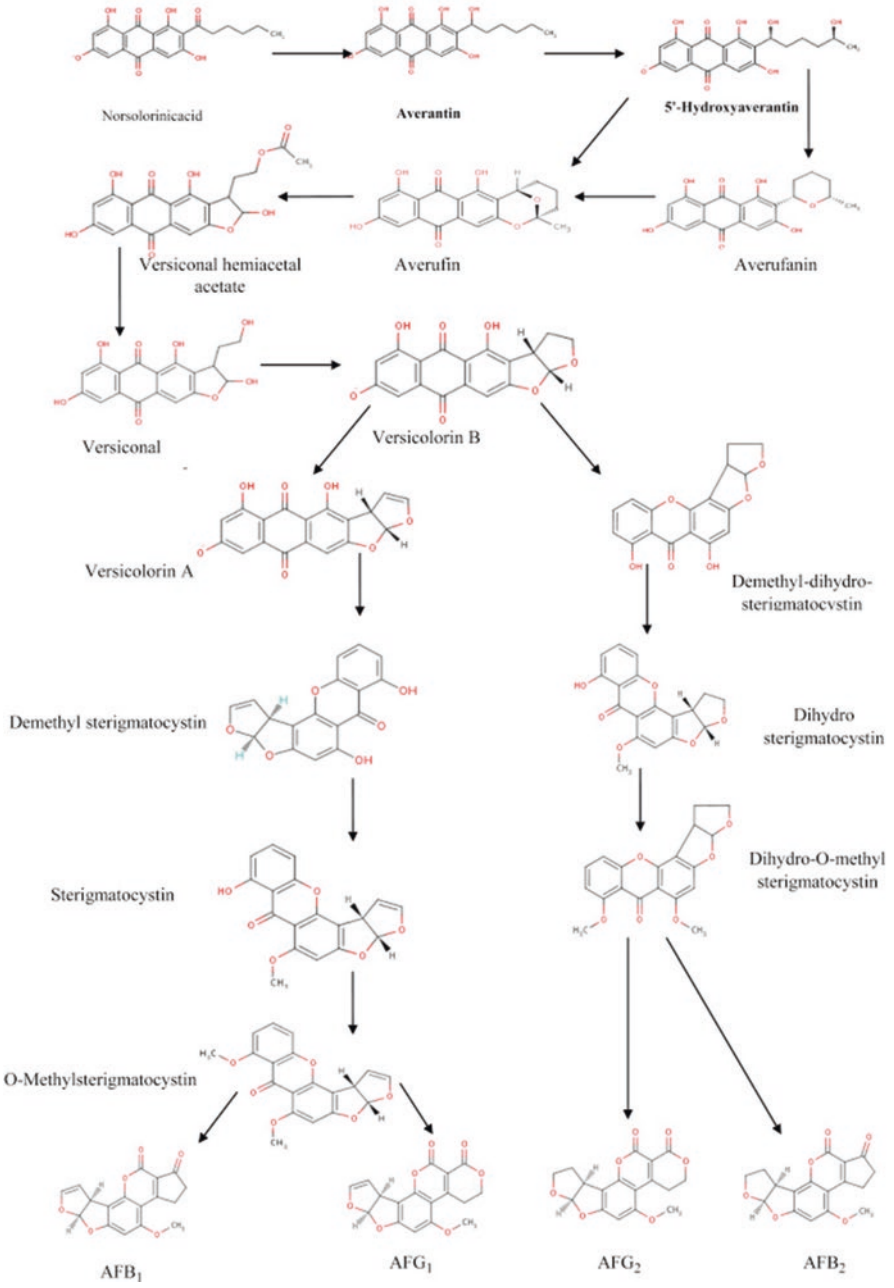

Fig. 2.2 Aflatoxin biosynthesis pathway. This figure is adopted from work of Gacem and El Hadj-Khelil (2016)

(Bennett et al. 1971). The formation of polyketide from a hexanoyl unit is assisted by a couple of fatty acid synthase enzymes and a polyketide synthase enzyme.

The enzymes catalyzing these changes are encoded majorly by four genes. The *afl*A and *afl*B were formerly known as *fas-2* and *fas-1*, respectively, because of their involvement in encoding fatty acid synthases. The *afl*A and *afl*B synthesize α and β protein subunits which are considered to play an important role in the formation of a polyketide structure from hexanoate units (Roze et al. 2013; Yu et al. 2004a). The gene *afl*C, also known as *pks*A, contains the coding regarding the synthesis of polyketide skeletons. The polyketide synthase is involved in the chain elongation that occurs in most of the secondary metabolites that are acetate derivatives. This enzyme has been found to further convert the polyketide structure into norsolorinic acid anthrone (NAA) (Roze et al. 2013). Furthermore, another gene, *hyp*C, is known to encode noranthrone oxidase, a 17-kDa enzyme that has been demonstrated to be involved in the catalytic transition of NAA to NOR (Ehrlich and Yu 2009). The *hyp*C is located in the region between *afl*C and *nor*-1. The *nor*-1 gene, due to its participation in aflatoxin biosynthesis, is also known as *afl*D.

2.3.2 Norsolorinic Acid to Averantin

The norsolorinic acid is further converted into averantin (AVN). The conversion of NOR into AVN is directed by the *afl*D (*nor-1*) gene. The cloning of the *afl*D gene was achieved through genetic complementation. The *afl*D gene encodes a ketoreductase that is required in order to convert the 1′-keto group of NOR to the 1′-hydroxyl group of AVN (Zhou and Linz 1999). Earlier it was predicted that the *aflE* (*norA*) and *aflF* (*norB*) genes present on the AF cluster were associated with this step. The short-chain aryl alcohol dehydrogenases, which may have the tendency to further assist the conversion of NOR to AVN, are encoded by these genes (Cary et al. 1996). However, they have also been shown to take part in other steps involved in the series of reactions catalyzed by enzymes during AFB_1 biosynthesis.

2.3.3 Averantin to 5'-Hydroxyaverantin

The earliest proof revealing the conversion of *Averantin* (AVN) to 5′-hydroxyaverantin (HAVN) was established through the experiments involving radioisotope incorporation (Bennett et al. 1980). The studies showed that in *A.* parasiticus, the transformation of AVN to averufin (AVF) is accomplished by two enzymatic reactions among which the first reaction involves the conversion of polyketide anthraquinone averantin into HAVN through hydroxylation. This reaction is catalyzed by P450 monooxygenase enzymes (Yabe et al. 1991). The gene *ord-1*, encoding the P450 monooxygenase, was cloned and disrupted by Yu et al. (1997). The studies of

ord-1 mutant in the presence of a substrate proved that HAVN is an intermediate compound formed during the transformation of AVN to AVF. The *ord-1* gene that exhibits a high degree of similarity in sequence to *A. nidulans stcf* (Brown et al. 1996) was renamed as *aflG* (*avnA*).

2.3.4 5′-Hydroxyaverantin to Averufin

Averufin (AVF) is among key intermediary compounds formed during the biosynthetic formation of aflatoxins through many studies (Keller et al. 2000). Furthermore, initially the involvement of several metabolites were reported during the transformation of AVN to AVF (Bhatnagar et al. 1992). However, the later studies negated the involvement of some of these metabolites as an intermediate in aflatoxin formation. One of such metabolites was averufanin (AVNN) which proved to be a shunt metabolite instead of being an intermediate in aflatoxin biosynthetic pathway (Sakuno et al. 2003; Yabe and Nakajima 2004).

An alcohol dehydrogenase-encoding gene cluster, *aflH* (*adhA*), was characterized by Chang et al. (2000) in *A. parasiticus*. The experiments showed that *adhA* deletion mutants resulted in predominant accumulation of HAVN. However, after prolonged growth periods, the mutant strains showed production of AVNN in minor quantities which was observed to be a shunt metabolite. Hence, the transformation of HAVN might be directly into the AVF or indirectly, as a result of the actions of additional cytosolic enzymes. Two cytosolic enzymes along with 5′-oxoaverantin (OAVN), a novel AF intermediate, were described during further studies by Sakuno et al. (2003). Among the series of intermediates formed during aflatoxin biosynthesis, OAVN was observed to be formed during the transformation of HAVN to AVF. The *aflH* (*adhA*) gene is responsible for encoding alcohol dehydrogenase enzyme that is involved in catalytic conversion of HAVN to OAVN. However, it was observed that the *adhA* deletion mutant was leaky, revealing the possible involvement of other genes or enzymes during transformation of OAVN to AVF. The study by Sakuno et al. (2005) showed the association of *aflK* (*vbs*) gene with the transformation of OAVN to AVF. Although initially the *aflK* gene was linked with the transformation of a versiconal compound into versicolorin B only, this was the first time described that the same enzyme can be involved in the catalysis of two reactions during AFB_1 biosynthesis. It was further hypothesized that this might have happened as a result of evolution in the gene cluster of AFB_1.

2.3.5 Averufin to Versiconal Hemiacetal Acetate

The oxidation of averufin (AVF) transforms it into versiconal hemiacetal acetate (VHA). VHA is known as a precursor to aflatoxin (Fitzell et al. 1977). The gene *aflI* (*avfA*) has been shown to take part in the biotransformation of AVF to VHA. Yu

et al. (2000b) revealed that when AVF-accumulating mutant, *A. parasiticus* SRRC 165, was complemented with *aflI* (*avfA*) gene from *A. flavus*, it restored the ability of strain transform AVF to VHA consequently producing aflatoxins, hence confirming the role of *aflI* (*avfA*) in this process. However, the precise role of *aflI* in the AVF oxidation has still not been completely clarified, though it has been commonly projected that the *aflI* encoded enzyme is involved in the catalysis of the ring closure step during the production of hydroxyversicolorone (HVN).

Furthermore, the gene *aflV*-encoded enzyme has been shown to be responsible for catalyzing the process of AVF transformation to HVN and the *aflW* gene product to be involved in conversion of HVN to VHA through a Baeyer-Villiger reaction. The gene *aflV* (*cypX*) is involved in encoding P450 microsomal monooxygenase, and *aflW* (*moxY*) has been found to encode a cytosolic monooxygenase (Wen et al. 2005).

2.3.6 Versiconal Hemiacetal Acetate to Versiconal

The subsequent phase after the formation of VHA includes its transformation into a versiconal (VAL). Various studies have revealed the involvement of an esterase in this conversion (Kusumoto and Hsieh 1996). The esterase enzyme was identified to have been encoded by *aflJ* (*estA*) (Yu et al. 2003). Furthermore, the esterase enzyme has also been isolated from *A. parasiticus* (Hsieh 1989; Kusumoto and Hsieh 1996). Additionally, the *aflJ* deletion mutants of *A. parasiticus* showed the accumulation of some metabolites such as versicolorin A (VERA) and VHA (Chang et al. 2004). In addition, trace amounts of versiconoc acetate (VOAc) along with other downstream metabolites in aflatoxin biosynthetic pathway including VAL and versicolorin B were also accumulated. The enzyme esterase is also known to exhibit involvement in the reversible transformation of VHA to VOAc. Furthermore, a study by Chang et al. (2004) confirmed the participation of esterase encoded by *aflJ* in transformation of VHA to VAL and VOAc to VOH during the biosynthesis of aflatoxins.

2.3.7 Versiconal to Versicolorin B

The conversion of VAL/VHOH to VERB was identified to have been catalyzed by a cyclase enzyme, named versicolorin B synthase (McGuire et al. 1996; Silva and Townsend 1997). This gene was cloned and named *vbs* (Silva et al. 1996). It was also observed during these studies that the recombinant proteins of the vbs gene exhibited the cyclase activity. The *vbs* gene was renamed as *aflK* due to its involvement in aflatoxin biosynthesis reaction cascade (Yu et al. 2004b). This enzyme is also involved in the conversion of OAVN into AVF as mentioned earlier. Furthermore, the closure of the bisfuran ring is also catalyzed by the *aflK*-encoded enzyme. The bisfuran ring is known to be responsible for the toxic character of aflatoxins as it binds with DNA after metabolization (Yu et al. 2004b).

2.3.8 Versicolorin B to Versicolorin a

The subsequent transformations of VERB are critical in determining the type of aflatoxin going to be synthesized. The VERB structure contains the tetrahydrofuran ring that is similar to the one present in AFB2/AFG2, hence forming the AFB2/AFG2 as a final product of aflatoxin biosynthetic pathway. Contrarily, the transformation of VERB to versicolorin A (VERA) leads to the formation of AFB1 or AFG1 eventually, as these toxins contain a dihydrobisfuran ring like VERA. The transformation of VERB to VERA involves the desaturation of the bisfuran ring (Yabe et al. 1993). It was identified that the *stcL*-disrupted *A. nidulans* did not synthesize sterigmatocystin (ST) compounds, consequently resulting in VERB accumulation (Kelkar et al. 1997). Furthermore, *aflL* (*verB*), a homologue of *stcL* in *A. parasiticus* and *A. flavus*, is considered to be involved in the conversion of VERB to VERA as it encodes a cytochrome P450 monooxygenase/desaturase (Kelkar et al. 1997).

2.3.9 Versicolorin a to Demethylsterigmatocystin and Versicolorin B to Dihydrodemethylsterigmatocystin

The biosynthetic pathway of aflatoxins involves the conversion of VERA to demethylsterigmatocystin (DMST) resulting in the formation of AFB_1 or AFG_1. Additionally, the transformation of VERB to dihydrodemethylsterigmatocystin (DHDMST) eventually leads to formation of AFB_2 or AFG_2. Henry and Townsend (2005) have described the changes occurring during this chain of reactions in detail.

Four genes have been observed to take part in the VERA to DMST conversion. The *ver-1* also known as *aflM* is a ketoreductase encoding gene that is similar to *nor-1*. The gene *aflM* was observed to be involved in the transformation of VERA to an intermediate compound which has still not been isolated. The homologous gene for *aflM* was also identified in *A. nidulans* as *stcU*. Furthermore, another gene, *aflN* (*verA*) responsible for coding a cytochrome P450-type monooxygenase, was shown to catalyze the transformation of VERA to another intermediate before converting into DMST. The *aflN* homologue in *A. nidulans* has also been identified as *stcS* (Yu et al. 2004a, b). Additionally, the disruption of *stcU* and *stcS* resulted in accumulation of VERA, hence confirming their requirement in the transformation of VERA to DMST ultimately (Keller et al. 1995). However, their exact function remains to be identified. The third gene involved in the transformation of VERA, *aflY* (*hypA*), is considered to encode a Baeyer-Villiger monooxygenase which appears to act as a mediator between two hypothetical structures during the transformation of VERA to DMST. The disruption of *aflY* in *A. parasiticus* resulted in accumulation of VERA which may suggest that it is involved as a part of the enzyme complex without permitting the development of intermediate compounds. The gene *aflX* (*ordB*) is further responsible for coding an oxidoreductase that is involved in the catalysis of oxidative decarboxylation and ring closure of the intermediate formed after *aflY*-catalyzed oxidation.

2.3.10 Demethylsterigmatocystin to Sterigmatocystin and Dihydrodemethylsterigmatocystin to Dihydrosterigmatocystin

The involvement of *O*-methyltransferases in aflatoxin biosynthesis was confirmed after studies on purified enzymes. It was revealed that *O*-methyltransferase I is involved in the catalysis of methyl transfer from S-adenosylmethionine (SAM) to the hydroxyls of DHDMST as well as DMST in order to produce DHST and ST, respectively. The *O*-methyltransferase I is a 43-kDa enzyme that has been isolated from *A. parasiticus*. The gene corresponding to this enzyme, *dmtA*, was isolated from *A. flavus*, *A. parasiticus*, and *A. sojae* and was later named *aflO* (*omtB*) (for O-methyltransferase B) (Yu et al. 2000b). The *stcP* gene in *A. nidulans* was identified to be homologous to the *aflO* gene. Furthermore, the disruption of *aflO* caused failure of DMST transformation to ST.

2.3.11 Sterigmatocystin to O-Methylsterigmatocystin and DHST to Dihydro-O-Methylsterigmatocystin

The *O*-methyltransferase catalyzes the transformation of ST to OMST and DHST to DHOMST. The gene containing the code for *O*-methyltransferase was first cloned using *A. parasiticus* through reverse genetics by producing antibodies against the *O*-methyltransferase isolated from *A. parasiticus* (Keller et al. 1993). The gene responsible for these transformations, *aflP* (*omtA*), was formerly named as *omt-1* and then *omtA* (Yu et al. 1993). The enzyme *O*-methyltransferase A is substrate specific; hence it cannot methylate DMST or DHDMST. In addition to *A.* parasiticus, the genomic sequence of *aflP* was cloned from *A. flavus* as well (Yu et al. 1995). Furthermore, the homologue for *aflP* was also identified in different other species of *Aspergillus*, either aflatoxigenic or non-aflatoxigenic (Klich et al. 1995). However, the orthologue of *aflP* is absent in *A. nidulans* which can explain the absence of aflatoxin as an end product and the presence of ST in this particular specie.

2.3.12 Formation of Aflatoxin B and Aflatoxin G

During the final conversions of OMST into AFB_1, various genes are involved including *aflQ*, *hypB*, *aflE*, and *hypE*. The roles of *aflQ* and *hypB* have been accurately defined in the final transformations; however partial roles have been reported for various other genes involved in AF biosynthesis. The gene responsible for encoding a P450 monooxygenase, *aflQ* (ordA), is present adjacent to *aflP* in the aflatoxin cluster. The expression of this gene ultimately leads to the transformation of OMST

into aflatoxin B_1 or G_1 and DHOMST into aflatoxin B_2 or G_2 (Ehrlich 2009). The *aflQ* transforms OMST into 11-hydroxy-O-methylsterigmatocystin (HOMST), a precursor of AFB_1 (Zeng et al. 2011). Nevertheless, it is still not clear whether two successive reactions involving monooxygenase are catalyzed by *aflQ* (*ordA*) gene product, OrdA, during the later steps of aflatoxin biosynthesis. The *hypB* gene encodes an oxidase reported to take part in conversion of HOMST into a seven-ring lactone (MW, 370 Da) and is expressed under conditions suitable for aflatoxin production (Ehrlich et al. 2010). This compound is converted into another unknown intermediary product through hydrolytic enzymes which do not belong to the aflatoxin cluster (Ehrlich 2009). The synthesis of G-group aflatoxins has been proposed to involve additional enzyme(s) (Yu et al. 1998). Further studies showed that cytochrome P450 monooxygenase encoded by *cypA* gene is involved in the formation of G-type aflatoxins (Ehrlich et al. 2004). Furthermore, the *nadA* gene that was earlier considered as a member of sugar utilization cluster (Yu et al. 2000a) was also shown to be a member of the adjoining aflatoxin cluster through microarray studies and participated in the formation of AFG_1/AFG_2 (Yu et al. 2011). The disruption of *nadA* gene showed that a recently observed AF intermediate, NADA (formed after OMST), is converted into G_1 type of aflatoxin through a cytosolic enzyme named NadA. Initially, the gene *aflE* (*norA*), homologous to *aflD* in the aflatoxin cluster, was believed to be involved in the conversion of NOR to AVN; however, further studies depicted the involvement of *aflE* in mainly final two transformations during formation of AFB_1. Although the role of *aflE* in AF biosynthetic pathway has been confirmed as its absence resulted in accumulation of deoxyaflatoxin, its exact position on AF gene cluster is still not known. Furthermore, the *hypB* was also predicted for its involvement in one of the steps exhibiting oxidation during the transformation of OMST to AFs. The involvement of *hypE* (*aflLa*) during the last steps of AFB_1 biosynthesis was also proposed because the disruption of *hypE* led to the production of an intermediary compound prior to the formation of deoxyAFB_1 synthesis. The *hypE* depicts homologies with several bacterial enzymes, and its participation along AF enzymatic pathway was suggested in combination with P450 monooxygenase (Ehrlich 2009). *A. flavus* is involved in the production of only B_1 and B_2 aflatoxins, while *A. parasiticus* is capable of producing aflatoxins B_1, B_2, G_1, and G_2. The presence of the intact *nadA* and *norB* genes has only been shown in *A. parasiticus* which is the G-group producer. The data proposes that *norB* is responsible for encoding another enzyme which is predominantly involved in the formation of AFG_1 and AFG_2 (Ehrlich et al. 2008).

2.3.13 Formation of Aflatoxin M

The aflatoxins M_1 and M_2 are the products of AFB_1 and AFB_2 biologically converted within mammals. These products were initially separated from bovine milk (Garrido et al. 2003). After aflatoxins enter a mammalian (humans or animals) body, the liver cytochrome P450 enzymes metabolize them, consequently converting them to a

reactive epoxide intermediate or hydroxylated aflatoxins M_1 and M_2. The epoxide intermediate is a more toxic compound exhibiting higher carcinogenicity, while aflatoxins M_1 and AFM_2 are less harmful metabolites of aflatoxins B_1 and AFB_2, respectively. Some recent studies involving feeding of aspertoxin (12c-hydroxy-OMST) (Yabe et al. 2012) revealed that *A. parasiticus* also exhibits production of some minor aflatoxins including M_1, M_2, GM_1, and GM_2. Furthermore, feeding of *O*-methylsterigmatocystin (OMST) to *A. parasiticus* resulted in production of AFM_1 and $AFGM_1$ along with AFB_1 and AFG_1, while feeding with DHOMST resulted in the production of aflatoxins M_2 and GM_2 in addition to B_2 and G_2. This revealed that *OrdA* is responsible for catalyzing the reaction involving 12c-hydroxylation resulting in transformation of OMST to aspertoxin and also the subsequent transformation from aspertoxin to AFM_1. In this scenario, the AFB_1 is not a precursor of AFM_1.

2.4 Factors Affecting Aflatoxin Biosynthesis

The aflatoxin biosynthesis is a complicated process based on a chain of reactions catalyzed by various enzymes coded by different genes. The synthesis of aflatoxins is affected by different nutritional and environmental factors which are discussed below.

2.4.1 Oxidative Stress

The aflatoxins are considered to be biosynthesized by *A. parasiticus* and *A. flavus* as a response by their cells against oxidative stress. A study showed that the aflatoxigenic strains of *A. parasiticus* require more oxygen as compared to non-aflatoxigenic strains during the phase of their active growth (Jayashree and Subramanyam 2000). It was further shown that aflatoxin synthesis in *A. parasiticus* is prompted by an increase in oxidative stress. The higher oxygen demand may consequently cause an increase in reactive oxygen species (ROS), hence increasing oxidative stress (Walsh et al. 2011). The relation of oxidative stress with aflatoxin synthesis was further confirmed as its alleviation resulted in a decrease in aflatoxin production (Huang et al. 2009). The microarray analysis revealed the downregulation of all the genes of *A. flavus* involved in AF biosynthesis after treatment with an antioxidant caffeic acid (Kim et al. 2008). Additionally, the toxigenic *A. parasiticus* possesses higher antioxidant activities by enzymes like superoxide dismutase in comparison to its nontoxigenic forms (Narasaiah et al. 2006). Catalases (CAT), superoxide dismutases (SOD), and glutathione peroxidase (GPX) play critical roles in the defense system of fungal strains against reactive oxygen species (ROS), hence facilitating the cellular defense to cope with oxidative stress. Among these, SOD provides frontline defense through conversion of the free radicals into H_2O_2 and O_2. Afterward,

peroxidases and catalases assist the transformation of H_2O_2 into H_2O and/or O_2 in to H_2O, respectively (Weydert and Cullen 2010). Aflatoxin production is considered to perform as a secondary defense mechanism after the primary mechanism that involves antioxidant enzymes (Hong et al. 2013). Furthermore, the AFB_1 biosynthesis is promoted in the availability of acetate units, and the beta-oxidation of fatty acids results in their degradation into acetate units among fungal strains (Maggio-Hall and Keller 2004), hence linking this pathway indirectly to AFB_1 production.

2.4.2 Carbon

The synthesis of secondary metabolites is well known for being dependent upon the availability of carbon and its source. Sugars are considered to be a favorable source of carbon aflatoxin biosynthesis as they produce the polyketide starter units (Davis and Diener 1968; Maggio-Hall et al. 2005). Various studies have shown the presence of simple sugars to be related to higher levels of aflatoxin synthesis in *A. parasiticus*, *A. flavus*, and *A. nidulans* (Bhatnagar et al. 2006; Calvo et al. 2002; Liu et al. 2016). In contrast, D-glucal (a glucose derivative), when used as the principle sugar source in the medium, was able to inhibit the aflatoxin production because D-glucal is not metabolized by fungi (Zhang et al. 2014). The carbon source utilization among *Aspergillus* is mediated by a sugar cluster which contains four genes grouped in a 7.5-kB cluster. This cluster of genes is positioned next to aflatoxin gene cluster in *A. flavus* and *A. parasiticus* (Bhatnagar et al. 2006; Yu et al. 2000a). Many genes present in the aflatoxin cluster have CreA-binding sites close to their promoter regions, which may be a basis of forming its relation with regulation of AF production (Georgianna and Payne 2009). CreA is a transcription factor that in combination with genes is involved in the process of carbon catabolic repression (CCR). The aspergilli use CCR as a strategic mechanism in order to preserve energy and regulate the carbon catabolism for using the most favorable carbon source (Deepika et al. 2016; Ruijter and Visser 1997). Furthermore, CreA also plays an important role in several other functions in addition to AFB_1 production in *A. flavus* (Fasoyin et al. 2018).

2.4.3 Nitrogen

Among *Aspergillus* species, the nitrogen sources are regulated by nitrogen metabolite repression mechanism. The nitrogen sources have been found to affect the synthesis of aflatoxin and ST in various ways (Calvo et al. 2002). In this perspective, AreA plays a critical role as it modulates the genes responsible for utilization of substitute nitrogen sources. Different nitrogen sources may affect the AF production differently as some substrates, such as asparagine, glutamate, and ammonium salts,

support the AF synthesis, while some substrates like sodium nitrate and tryptophan do not favor AF production (Yu 2012). It is because the nitrogen source media govern the under- and over-expression of the *areA* gene, consequently resulting in a higher or lower production levels of aflatoxin (Fasoyin et al. 2019).

2.4.4 pH

The lower pH levels are generally associated with high contents of aflatoxin production in *A. flavus*. A study showed a tenfold increase in AF production when the pH was reduced to 4.0, while an increase in pH caused a decrease in AF synthesis (Cotty 1988). For *A. parasiticus*, a decrease in pH level to <6.0 was found to stimulate the AFB synthesis, whereas the higher pH level favored the synthesis of G-type aflatoxin (Buchanan and Ayres 1975). The *aflM* expression is higher in acidic media as compared to that in neutral or alkaline media. Additionally, the fungal growth results in pH reduction, consequently enhancing AF production with time (Keller et al. 1997).

2.4.5 Light

The stimulus of light exerts a high impact on adaptation as well as survival of fungal strains. Light may have an impact on growth and morphological features of the fungus, consequently affecting the production of secondary metabolites as well (Rangel et al. 2015). The light induces a strong "velvet comlex" in aspergilli that is governed by a global regulator *veA* that controls a number of genetic elements including photoreceptors (Purschwitz et al. 2008). VeA regulates half of the gene clusters involved in secondary metabolite formation (28 out of the 56), including the AF gene cluster (Cary et al. 2015). The *veA* is an essential gene for the production of AFB_1 in *A. flavus* (Duran et al. 2009). The null mutants of *veA* and *IaeA* did not exhibit *aflR* expression (Amaike and Keller 2009). Deletion of *veA* in *A. parasiticus* resulted in the absence of versicolorin A, an aflatoxin intermediate, hence confirming its role in *aflR*/*aflS* expression (Calvo et al. 2004).

2.4.6 Temperature

Temperature is among the major influencing factors affecting aflatoxin synthesis. It is associated with promoting the expression of the structural biosynthesis genes (*aflD* and *aflO*). However, it does not induce expression of regulatory genes (*aflR* and *aflS*) (Gallo et al. 2016). A study by OBrian et al. (2007) showed that AF

biosynthetic genes were expressed more under the temperature of 28 °C as compared to higher temperature of 37 °C, but the aflatoxin pathway regulatory genes, *aflR* and *aflS*, did not show difference at these two temperatures. Furthermore, the genes involved in aflatoxin biosynthesis were found to be downregulated when exposed to elevated temperatures of 42 °C (Liu et al. 2017).

2.4.7 Water Activity

The water activity (a_w) may also affect aflatoxin biosynthesis. All the genes involved in AFB_1 biosynthesis and *laeA* were found to be inhibited under the a_w of 0.99 as compared to the a_w of 0.96, while highest expression was observed at a_w 0.92 (Liu et al. 2017).

2.5 Metabolism of Aflatoxin B_1

The metabolism of aflatoxin B_1 occurs through oxidative reactions catalyzed by the members of cytochrome P450 (CYP450) supergene family of isoenzymes. The CYP450 enzyme family is composed of hemoproteins and electron carriers that, during the cellular respiration, catalyze or enhance the oxidation-reduction reactions (Lamb et al. 2009). Earlier, it had been considered that CYP450 specifically originated from the liver, but later studies showed that they are distributed throughout the body (Ding and Kaminsky 2003). However, xenobiotics are mainly metabolized in the liver (Shimada et al. 1994). The isoforms of CYP450 involved in AFB_1 metabolism in the body include CYP1A1, CYP1A2, CYP3A4, CYP2Cs, CYP3A5, and CYP3A7 (Shimada et al. 1994). The glutathione S-transferase (GST) and AFB1-aldehyde reductase also catalyze the AFB_1 metabolism resulting in the formation of reactive metabolites, among which some can be used as the biomarkers for AF exposure (Bbosa et al. 2013). The metabolites of AFB_1 formed by actions of different CYP450 isoenzymes vary in their carcinogenic potential. The toxic impacts of AFB_1 are associated with activation as well as detoxification rate at the primary and secondary levels of metabolism (Neal et al. 1987). Furthermore, the fate of aflatoxin B_1 metabolism varies among and within humans and animals. In addition, the activation rate of aflatoxins varies among children and adults belonging to the same species as well, consequently affecting their resistance toward AFB_1 toxicity (Ramsdell and Eaton 1990). Additionally, AFB_1 metabolism varies among humans belonging to different regions of the world. The main pathways involved in AFB_1 metabolism include O-dealkylation, ketoreduction, epoxidation, and hydroxylation (Fig. 2.3). These reactions can result in formation of either highly toxic metabolite (AFBO and AFM1) or relatively nontoxic compounds (AFP_1, AFQ_1, or AFB_{2a}) (Wu et al. 2009).

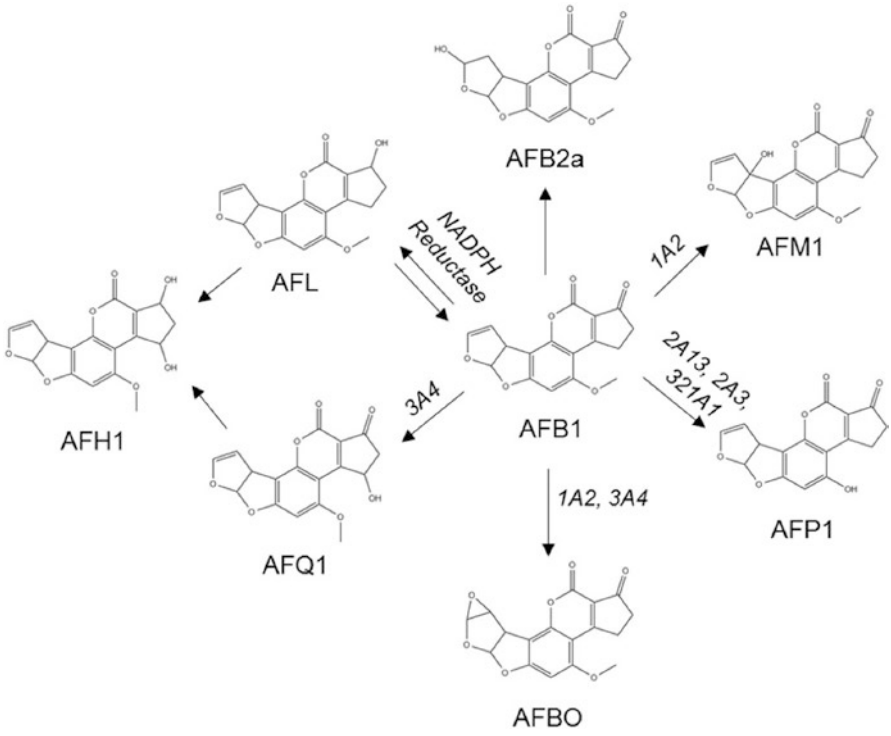

Fig. 2.3 AFB_1 metabolic pathway mediated by CYP450. This figure is adopted from the work of Rushing and Selim (2019)

2.5.1 Aflatoxin B1–8,9-Epoxide

The AFB_1 is metabolized to B1–8,9-epoxide (AFBO) through the help of enzyme system P450 in the liver. The AFBO has two isomers, *endo*-8,9-epoxide and *exo*-8,9-epoxide (Raney et al. 1992a). The isoenzymes, CYP3A4 and CYP1A2, are primarily responsible for this conversion. The CYP1A2 acts as a primary producer of AFBO when AFB_1 is in lower concentrations. In contrast, at higher AFB_1 concentrations, CYP3A4 majorly produces AFBO resulting in the formation of *exo* AFBO isomers only (Ueng et al. 1995). Also, the CYP1A2 produces more *exo* isomers as compared to CYP3A4 at lower AFB_1 levels (Gallagher et al. 1996). This intermediate exhibits extremely electrophilic character, allowing it to instantly react with amines of proteins as well as of nucleic acids. It reacts with DNA and attaches with N_7 position of guanine, consequently forming AFB_1-N_7-guanine adduct imparting AFB1-*exo*-8,9-epoxide highly carcinogenic character (Johnson and Guengerich 1997).

2.5.2 AFQ1

AFQ_1 is a relatively nontoxic metabolite of AFB_1 formed through hydroxylation mediated solely by CYP3A4 (Kamdem et al. 2006). It was first observed in monkey liver microsomal preparations exposed to AFB_1. Generally, AFQ_1 is produced in much higher amounts in comparison to AFM_1; however, rat microsomes were observed to produce lower amounts (Masri et al. 1974). It was shown that AFQ_1 occurred in humans frequently in amounts ranging from 1 to 11% of initial AFB_1 amounts (Yourtee et al. 1987). However, the potential of AFQ_1 to bind with DNA is significantly lower as compared to that of AFBO, hence making it a detoxification product of AFB_1 in comparison to AFM_1 which is toxic in nature (Raney et al. 1992b). Furthermore, another study showed the presence of AFQ_1 in levels higher than AFM_1 and AFB_1-N_7-guanine after monitoring the urinary and fecal samples. Moreover, the concentrations were found to be higher in fecal matter in comparison to urine, making it a potential biomarker source for evaluation of AFB_1 exposure (Mykkänen et al. 2005). Even though AFQ_1 is one of the most abundant metabolites of AFB_1, it is seldom used as a biomarker for AFB_1 exposure assessment.

2.5.3 AFP_1

AFP_1 is also a detoxification metabolite of AFB_1 produced by hydroxylation through P450 enzymes including CYP2A13, CYP2A3, and CYP321A1 (He et al. 2006; Niu et al. 2008). Studies have shown the presence of this metabolite in urine of individuals exposed to AFB_1 and those who had developed hepatocellular carcinoma (HCC) probably as a consequence of AFB_1 exposure (Ross et al. 1992).

2.5.4 Aflatoxicol

In contrast to other metabolites of AFB_1, aflatoxicol (AFL) is found in cytosolic fractions of liver preparations. The formation of AFL is mediated by NADPH reductase, typically in the cytosol (Partanen et al. 2010). Unlike AFP_1 and AFQ_1, AFL retains its DNA-binding capacity, consequently retaining its toxic nature. Therefore, it is not considered as a detoxification product of AFB_1. Furthermore, the AFL acts as a reservoir for AFB_1 as it has a tendency to be converted back to AFB_1 though enzyme actions, which further enhances the toxic effects (Partanen et al. 2010). The AFL is the only AFB_1 metabolite that can be transferred through the placenta of a human and can be formed from placenta as well. This metabolite has been observed in human urine as well as in breast milk of individuals exposed to AFB_1 (Kussak et al. 1998).

2.5.5 *AFH_1*

AFH_1 resembles AFL structurally with an additional hydroxyl group at the terminal cyclopentenone ring. Two enzyme systems are involved in the metabolic conversion of AFB_1 to AFH_1, namely, the microsomal hydroxylase and cytoplasmic reductase. However, it is not clear whether the AFH_1 is formed through hydroxylation of AFL or reduction of AFQ_1.

2.5.6 *AFB2a*

AFB2a was initially characterized as a product of AFB_1 formed as a result of acid catalysis. The mild acidic conditions promote the addition of waster across the 8,9-double bond to form the hemiacetal ring. This nonenzymatic transformation was observed in acidic media of molds that had been added with AFB_1 (Ciegler et al. 1966). It has been shown that AFB2a possesses lower toxicity as compared to AFB_1 due to lower DNA-binding capacity, hence making it a detoxification product of AFB_1. However, it has a unique tendency to bind with cellular proteins which can contribute to other cellular toxicities. In addition, this binding usually occurs with primary amines in alkaline conditions. The binding may take place on phosphoethanolamine head groups of phospholipids. It is one-of-a-kind structurally characterized aflatoxin-lipid adduct till now (Rushing and Selim 2017).

2.6 Conclusion

Aflatoxins are highly toxic compounds produced by various fungal strains belonging to *Aspergillus*. These toxins are produced as secondary metabolites through a complex biosynthetic pathway which has been under investigation for decades. The aflatoxin biosynthesis involves a number of reactions catalyzed by different enzymes which consequently produce intermediate compounds, ultimately forming aflatoxins. The aflatoxin formation is regulated by expression of genes at different steps in the chain of reactions. Studies on AF biosynthesis have helped reveal the type of enzymes and the genes responsible for encoding these enzymes taking part in the biosynthesis of aflatoxins. The production of aflatoxin is affected by several factors such as light, temperature, pH, water activity, and nutrient sources. The knowledge about the impact of these factors on gene expression can help propose effective strategies to prevent contamination of food as well as feed commodities with aflatoxins. Additionally, comprehensive understanding about AFB_1 metabolism can also help identify different biomarkers in order to assess the exposure of the population to aflatoxins. This can aid in determining an accurate estimate regarding the threat of aflatoxins being faced by populations of different regions around the world.Conflict of InterestThe authors declare no conflict of interest.

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Chapter 3
Worldwide Prevalence of Aflatoxins in Food and Feed

Yusuf Opeyemi Oyebamiji, Kamoldeen Abiodun Ajijolakewu, and Ismail Abiola Adebayo

Abstract Aflatoxins are poisonous toxins produced by *Aspergillus* spp. They are groups of highly toxic and carcinogenic secondary metabolic products, which contaminate food and feeds consumed by humans and animals. These adverse properties of aflatoxins cause economic loss and health-related problems such as chronic and acute effects and sometimes lead to death when severe. Aflatoxin contamination of crops is common and usually found in dietary staple foods such as maize, groundnut, rice, and milk due to fungal infection before and after harvest. This makes aflatoxins a real threat to food security, safety, as well as population growth. Identification, detection, and elimination of aflatoxins and the use of strategic management approaches have become necessities in order to guarantee food safety. This book chapter focuses on the occurrence of aflatoxin contamination in crops around the world.

Keywords Aflatoxins · Contaminate · Food · Feed · Threat · Population · Management

3.1 Introduction

Recently, the entire universe has encountered a major challenge of food security and protection as part of the major difficulties affecting the entire population of the world. Food safety and security are primarily defined by (i) adequate availability of

Y. O. Oyebamiji
Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia

K. A. Ajijolakewu
Department of Microbiology, Faculty of Life Sciences, University of Ilorin, Ilorin, Kwara State, Nigeria

I. A. Adebayo (✉)
Microbiology and Immunology Department, Faculty of Biomedical Sciences, Kampala International University, Ishaka-Bushenyi, Uganda
e-mail: ismail.abiola@kiu.ac.ug

K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_3

food; (ii) quality, nutritional, and cultural use of food for healthy life, and (iii) access to safe food (Nazhand et al. 2020). Factors connected to food scarcity, insecurity, and nutritional imbalance not only affect human health and well-being but also play key roles in the economic, political, and social outlook of a society. In respect to the foremost point, pre-harvest and post-harvest losses due to the contamination of mycotoxin are reported as one of the main factors causing insecurity of food since these substances occur along most food chains from farm to fork (Udomkun et al. 2017).

In the 1960s, more than hundreds of deaths were reported in Turkey due to the consumption of groundnut meal which was infected by mold, which brought about the advent and discovery of aflatoxins. A toxin secreted by two fungi, which are *Aspergillus flavus* and *Aspergillus parasiticus*, in several cultivars of agricultural crops is known as aflatoxin (Khlangwiset et al. 2011). Aflatoxins are groups of highly toxic secondary metabolic products of some *Aspergillus* spp. such as *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius*, *Aspergillus fumigatus*, and *Aspergillus tamari*, though they are also produced by species such as *Emericella* spp. Aflatoxins are reported in feeds and foods during germination, cultivation, and storage stage. Both fungus (*Aspergillus flavus* and *Aspergillus parasiticus*) are predominant in crops, especially in groundnut, tree nuts, oil seeds, and maize, and in subtropical and tropical areas throughout the world (Khlangwiset et al. 2011). A study has shown that groundnuts are vulnerable and susceptible agricultural crops to aflatoxin contamination due to their relatively high moisture content, cultivation methods, and storage process which favored mold attack (Wu 2006). Furthermore, due to conducive social and environmental conditions, most countries which are developing and underdeveloped experience more cases of aflatoxin contamination in foods on a frequent basis (Ismail et al. 2015; Wu 2006).

More than 5 billion individuals are at risk of chronic exposure to aflatoxins, mostly in the developing countries. The tolerance level of the plant varieties to change in climate, rainfall pattern and drought, framing practices, and insect damage are factors that influence aflatoxin production by fungi. As earlier stated, the fungal contamination can occur during post-cultivation activities and crops such as groundnut and maize are highly vulnerable to *Aspergillus* attack due to the high level of consumption (Khlangwiset et al. 2011; Strosnider et al. 2006). Aflatoxins are of several types, but the popular ones are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB), aflatoxin G_1 (AFG_1), and aflatoxin G_2. AFG_2, are capable of poisoning the body through respiratory, mucous, or cutaneous pathways, which prompt excess activation of inflammatory response, while aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2) are found in milk and are the hydroxylated metabolites of AFB_1 and AFB_2 (Kumar et al. 2017). The level of toxicity of the aflatoxins increases in the order G_2, B_2, G_1, and B_1. Aflatoxins are known to be cancerous, mutagenic, and teratogenous in nature to humans and animals (Galvano et al. 1996). The International Agency for Research on Cancer (IARC) has categorized aflatoxins B_1 (AFB_1) as a group 1 carcinogen ("carcinogenic to humans") (Wu et al. 2009). The chain of transmission of aflatoxins from fungi to humans is shown in Fig. 3.1.

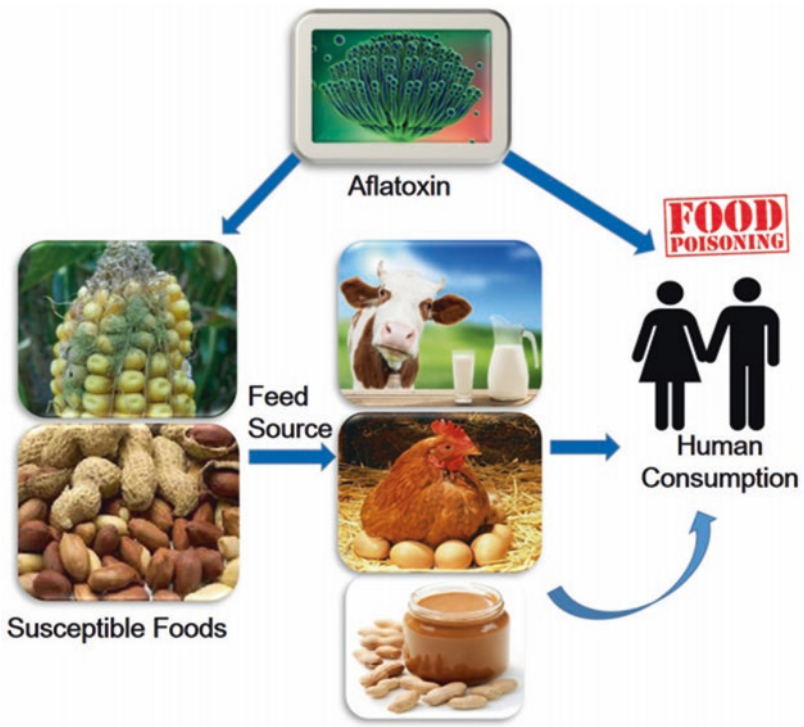

Fig. 3.1 Transmission chain of aflatoxin from fungi to humans. (Source: Kumar et al. 2017)

3.2 World Population Growth and Food Production Level

Figure 3.2 shows the increasing rate of population growth among the ten most populous countries around the world. These countries include China, India, The United States of America, Indonesia, Pakistan, Brazil, Nigeria, Bangladesh, Russia, and Mexico. China emerges as the most populous country in the world with 1,439,323,776 people followed by India and the United States of America with 1,380,004,385 and 331,002,651 people, respectively. As the world population increases, the demand, supply, and consumption rates of food produce such as cereals, legumes, oilseed, and vegetables increase which indicate that population growth is directly proportional to demand. Planting and cultivation of major cereal crops such as rice, maize, and wheat have also increased tremendously across the world. For example, the amount of cultivated rice, maize, and wheat has increased in multiple folds between 1968 and 2018 (Fig. 3.3). This increase is necessary in order to meet the frequent population growth and high consumption level of food worldwide. This continuous increase in the trend of all crops is essential in order to mitigate the threat to food security. Different factors such as *water use, pest infestation, efficiency of fertilizer use, production costs, government support, and change in farming systems have a*

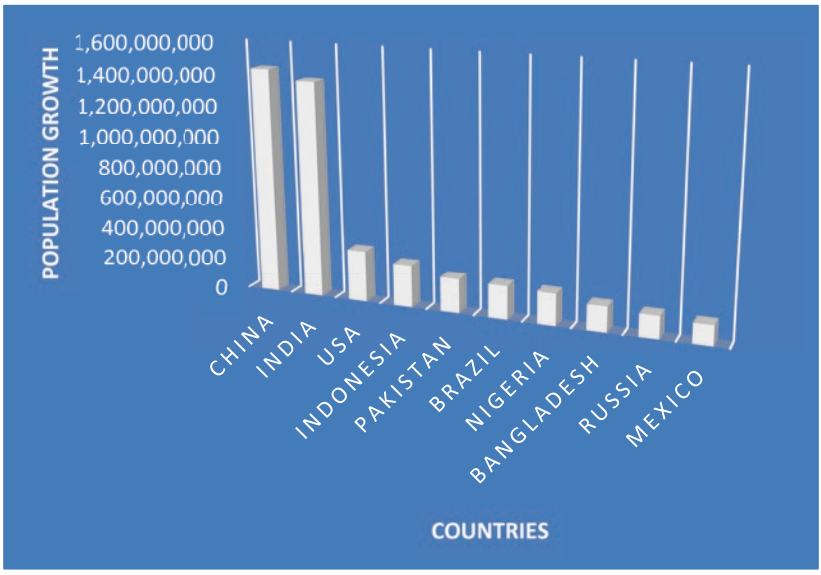

Fig. 3.2 Increasing population growth. (Source: Population by Country (2020) (Worldometers 2020))

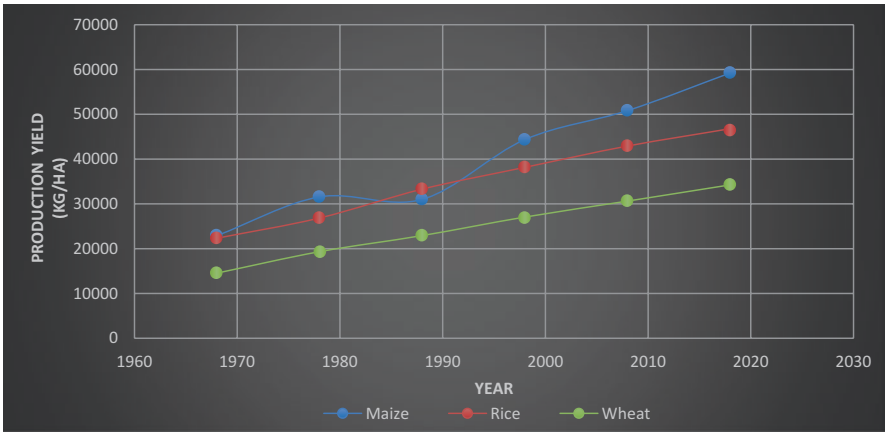

Fig. 3.3 World cereal production rate. (Source: FAOSTAT 2020)

major impact on crop production (Chang 1993). In 2016, the Food and Agriculture Organization (FAO) of the United Nations set the seventh sustainable developmental goal which is aimed to reduce hunger in different parts of the world by 2030. This may be difficult to achieve due to the advent of several threats which include environmental and abiotic stress such as drought and submergence and biotic stress such as pest, diseases, and fungi, especially aflatoxins that are having a massive effect on food production particularly cereals which are particularly vulnerable to the mycotoxins.

3.3 Occurrence of Aflatoxins in Feed and Food

The World Health Organization has declared aflatoxin as a threat to food security worldwide (Ali 2019). The presence of aflatoxin has been noticed in many food commodities and in animal feed. Certain crops and feeds which are predominately affected by aflatoxins include cereals (rice, corn, wheat, barley), oil seeds (groundnut, peanuts, almonds, pistachios, and other tree nuts), spices, fig, dried fruits, milk and dairy products, and other foods of animal origin (meat, offal, eggs).

3.3.1 Occurrence of Aflatoxins in Cereals

Cereals and their derivatives are the world's primary foodstuffs for human consumption. Grains of cereal crops such as barley, wheat, sorghum, and corn are prone to aflatoxin accretion, as a result of the presence of aflatoxigenic fungus. Aflatoxin complication which occurs in nature, especially in corn and rice, has become more severe due to frequent change in technological advancement in the agriculture sector. The problem of aflatoxin encountered in cereals is not confined to a particular climatic condition or geographical location of a place. Toxins occur in cereal crops at different stages such as on the field, in storage, on the seed, and in the entire plant (Filazi and Sireli 2013). It was reported that out of all the cereal crops tested for different types of aflatoxins, about 36.7% were infected. The degree of fungal growth and aflatoxin infection in cereals depends on multiple factors such as moisture content, temperature, types of soil, and mode of storage (Mahato et al. 2019). Table 3.1 highlights the occurrence of aflatoxin in cereal crops around the world.

Rice is the main cereal grain for half of the world's population followed by wheat, accounting for more than 19% of daily calories. Asia is considered the continent with the highest rice production and consumption. Rice is usually planted and harvested in a subtropical area under a hot and humid climatic condition which enhances the growth of fungus and the secretion of secondary metabolites. Rice may be infected with fungus-producing aflatoxins when the environment is conducive for their growth on the field, during cultivation, handling, and storage. Several studies have documented the occurrence of aflatoxins in rice which is highly predominant in many Asian countries (Ali 2019). Besides other mycotoxins, AFB_1 has been reported to have invaded rice in several countries such as China, the United Kingdom, the United States of America, India, Malaysia, Nepal, Iran, Pakistan, the Philippines, and Egypt (Mahato et al. 2019). The adverse consequences of this form of fungal attack include grain and/or husk discoloration, loss of viability, loss of quality, and contamination with toxins (Filazi and Sireli 2013). The high rate of invasion or occurrence of aflatoxins in rice and its derivatives has highlighted the significance of stringent control of this dietary staple food globally (Ali 2019).

Apart from rice, sorghum is a popular and staple food for most countries. Sorghum is planted in severe environmental conditions, whereby most other crops will not flourish or germinate well. Increasing and improving the

Table 3.1 The occurrence of aflatoxins in cereals

Crops	Year	Country	Total/positive samples	Aflatoxin	Range	Detection techniques	References
White rice	2016	Pakistan	34/14	AFB_1	Mean 7.74	HPLC	Iqbal et al. (2016)
Oat	2011	Malaysia	10/5	AFB_1	0.65–2.85	ELISA	Filazi and Sireli (2013)
Corn	2010	Brazil	214/82	AFB_1	0.2–129	–	Vargas et al. (2001)
Wheat	1999	Ethiopia	120/4.2	AFB_1	Mean 8.7	HPLC	Ayalew et al. (2006)
Rye	2018	Serbia	8/1	AFB_1	0.04	HPLC	Torović (2018)
Maize	2016	DRC	50/16	Total AFs	3.1–103.89	HPLC	Kamika et al. (2016)
Rice	2011	Canada	199/99	AFB_1	<0.002–7.1	HPLC	Filazi and Sireli (2013)
Rice	2018	Serbia	6/2	AFB_1	1.59–4.76	HPLC	Torović (2018)
Sorghum	2010	Tunisia	180	AFB_1	0.4–25.1	HPLC	Ghali et al. (2010); Mahato et al. (2019)
Sorghum	1990	India	150/4	Total AFs	0.02–0.06	TLC	Ramakrishna et al. (1990)
Cereal-based products	2013	Ghana	50/36	Total AFs	0.18–23.27	HPLC	Blankson et al. (2019)
Rice	2009	Iran	71/59	AFB_1	2.09	HPLC	Mazaheri (2009)
Rice	2015	China	370/235	Total AFs	Mean 0.65	HPLC	Lai et al. (2014)
Wheat	2008	Nigeria	–	AFB_1	17.01–20.53	TLC	Odoemelam and Osu (2009)
White corn	2019	Egypt	27/6	AFB_1	0.26–28.9	HPLC	El Sayed et al. (2019)
Maize	2016	DRC	50/16	AFB_1	1.5–51.23	HPLC	Kamika et al. (2016)
Sorghum	1999	Ethiopia	82/6.1	AFB_1	Mean 10.0	HPLC	Ayalew et al. (2006)
Breadfruit	2008	Nigeria	–	AFB_1	40.06–48.59	TLC	Odoemelam and Osu (2009)
Corn	2013	Serbia	10/8	AFB_1	3.88	HPLC	Torović (2018)
Maize	2014	India	150/28	AFB_1	48–58	HPLC/PCR	Mudili et al. (2014)
Rice		India	1511/581	AFB_1	5	HPTLC	Filazi and Sireli (2013)
Sorghum	2000	Brazil	140/18	AFB_1	Mean 7–33	–	Da Silva et al. (2000)
White yam	2011	Nigeria	100/21	AFB_1	<0.05–3.5	HPLC	Somorin et al. (2012)
Yellow corn	2019	Egypt	29/10	AFB_1	0.33–38.88	HPLC	El Sayed et al. (2019)

Rice flour	2016	Pakistan	30/11	AFB_1	Mean 3.51	HPLC	Iqbal et al. (2016)
Corn	2016	Serbia	600/30	Total AFs	1.3–6.9	ELISA	Kos et al. (2018)
White yam	2011	Nigeria	100/57	AFB_1	<0.02–3.2	HPLC	Somorin et al. (2012)
Corn	1990	India	102/45	Total AFs	0.02–74	TLC	Ramakrishna et al. (1990)
Wheat	20,020	Lebanon	25/3	AFB_1	1.05–2.20	HPLC	Joubrane et al. (2020)
Sorghum	2016	Ethiopia	30	Total AFs	11.44–344.26	HPLC	Weledesemayat et al. (2016)
Corn	2017	Vietnam	2370	AFB_1	1.0–34.8	ELISA	Ismail et al. (2018)
Maize	2017	Zimbabwe	388/80	AFB_1	0.56–26.6	HPLC	Murashiki et al. (2017)
Corn	2009	Italy	46/44	AFB_1	0.15–560	HPLC	Kos et al. (2018); Leggieri et al. (2015)
Maize	2006	China	73/71	Total AFs	Mean 0.99	–	Liu et al. (2006)
Rice	2019	Egypt	12/4	AFB_1	1.56–16.67	HPLC	El Sayed et al. (2019)
Brown rice	2016	Pakistan	28/15	AFB_1	Mean 8.91	HPLC	Iqbal et al. (2016)
Corn	2011	Italy	46/27	AFB_1	0.15–335	HPLC	Kos et al. (2018), Leggieri et al. (2015)
Rice	2009	India	1200/814	AFB_1	0.1–308	ELISA	Filazi and Sireli (2013)
Whole grain rice	2006	China	16/16	Total AFs	Mean 3.87	HPLC	Liu et al. (2006)
Corn	2015	Serbia	14/1	AFB_1	8.80	HPLC	Torović (2018)
Maize	2009	Mexico	41/36	AFG_1	1.59–57.1	HPLC	Espinosa et al. (2009)
Rice	2010	Nigeria	21/21	Total AFs	27.7–371.9	HPLC	Makun et al. (2011)
Corn	2012	Serbia	600/434	Total AFs	1.0–111.2	ELISA	Kos et al. (2018)
Wheat	2010	Tunisia	180	AFB_1	0.12–18	HPLC	Ghali et al. (2010); Mahato et al. (2019)
Wheat	2019	Egypt	11/4	AFB_1	1.59–8.13	HPLC	El Sayed et al. (2019)
Corn	2008	Nigeria	–	AFB_1	27.22–36.13	TLC	Odoemelam and Osu (2009)
Sorghum	2018	Tunisia	64/38	AFB_1	0.03–31.7	HPLC-FLD	Lahouar et al. (2018)
Barley	1999	Ethiopia	113/11.3	AFB_1	Mean 3.8	HPLC	Ayalew et al. (2006)
Rice	2009	Iran	71/59	AFB_1	1.89	HPLC	Mazaheri (2009)
Maize	2013	Pakistan	380/137	Total AFs	1.01–86.1	ELISA	Kos et al. (2014)

(continued)

Table 3.1 (continued)

Crops	Year	Country	Total/positive samples	Aflatoxin	Range	Detection techniques	References
Maize	2009	Mexico	41/–	AFB_1	5.03–465.3	HPLC	Espinosa et al. (2009)
Corn	2013	Serbia	600/148	Total AFs	1.2–65.2	ELISA	Kos et al. (2018)
Maize	1999	India	127/18	AFB_1	23–26.8	Indirect competitive ELISA	Janardhana et al. (1999)
Corn	2016	Serbia	32/18	AFB_1	4.20	HPLC	Torović (2018)
Corn	2019	Togo	55/21	AFB_1	Max. 256	HPLC-MS/MS	Hanvi et al. (2019)
Corn	2015	Serbia	600/220	Total AFs	1.1–76.2	ELISA	Kos et al. (2018)
Brown rice	2006	China	37/36	Total AFs	Mean 0.88	HPLC	Liu et al. (2006)
Millet	2008	Nigeria	–	AFB_1	34–40.30	TLC	Odoemelam and Osu (2009)
Sorghum	2019	Togo	12/3	AFB_1	6–16	HPLC-MS/MS	Hanvi et al. (2019)
Maize flour	2015	Turkey	100	AFB_1	0.041–1.12	HPLC	Kara et al. (2015); Mahato et al. (2019)
Corn	2006	Morocco	20/10	AFB_1	Mean 1930	HPLC	Zinedine et al. (2006)

DLLME-HPLC, Dispersive liquid-liquid micro-extraction coupled with high-performance liquid chromatography with fluorescence detection; IAC, immunoaffinity column; SPE, solid-phase extraction; nd, not detected; LOD, limit of detection; LOQ, limit of quantification; AfT, total aflatoxins (i.e., sum of AFB_1, AFB_2, AFG_1, and AFG_2); SK, super kernel

production level, easy accessibility, adequate storage, and process of this crop will significantly reduce the threat to food security and increase the nutritional level of the crop. Usually, sorghum is cultivated as fast as possible in order to allow the germination of other crops on the same field. In some cases, the cultivation of sorghum is done when there is a change in climatic conditions such as flooding, high rainfall, and hurricanes, thereby increasing the moisture content of the harvested crop which stimulates the growth of mycotoxin-producing fungus (Filazi and Sireli 2013).

3.3.2 Occurrence of Aflatoxins in Oil Seeds

Oil seed crops mainly include seed of flowers, soybeans, canola, safflower, rapeseed, peanuts, flaxseed, mustard seeds, and cotton seeds, used for different purposes such as cooking oil production, protein feed for animals, and commercial applications. Castor beans and sesame are other forms of known oil seeds. Following the removal of oil from the seeds, the remnants are a good source of protein, particularly for livestock meal, which includes press or oil cake (Filazi and Sireli 2013)**.** The crop and its derivatives are mostly eaten as snacks, and some of its ingredients are included in a normal human meal. Being aware of mycotoxin's existence has led to an increasing concern about their existence in edible materials. Oil seed crops are considered as a possible substrate for the secondary metabolites by a fungus, particularly the induction of aflatoxin by toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* (Kershaw 1982). Peanut's moisture content is one of the major factors which trigger the growth of fungus and production of aflatoxins. The presence of a suitable climatic condition enhances fungal growth, thereby promoting a high risk of liver cancer (Kamika and Takoy 2011).

Aspergillus parasiticus regularly attack young plants of peanuts in a systemic manner as a seedling from the seed, soil, and spread to the entire plant although the leaves and petioles are less affected compared to the stems and roots which experienced severe infection (Klich 2007). Aflatoxin infection are observed in tree nuts which include walnuts, pistachios, and almonds, although at a minimal rate as compared with cottonseed and corn, nevertheless the situation is worrisome to the producer due to (i) high unit value of the crop and (ii) the crop are mostly bought by the European markets which impose a significant limit as compared to other nations. The infection of peanuts by aflatoxins due to the attack of either *A. flavus* or *A. parasiticus* is a major concern in tropical semi-arid areas where plants are mainly rainfed, while the contamination of peanuts by aflatoxins does not in any way reduce the harvested yield. However, accumulation of a high amount of aflatoxins induced by the fungus in the nut could threaten the well-being of the mankind and animals' health (Filazi and Sireli 2013). Aflatoxin occurrence in oil seeds in different regions around the world is highlighted in Table 3.2.

Table 3.2 The occurrence of aflatoxins in oil seeds

Crops	Year	Country	Total/positive samples	Aflatoxin	Range	Detection techniques	References
Raw peanuts	2016	Zambia	92/51	Total AFs	0.015–46.60	HPLC	Bumbangi et al. (2016)
Peanuts	2006	Turkey	18/7	AFB_1	8–94	TLC	Gürses (2006)
Pistachio	2012	Iran	32/17	Total AFs	1–54	HPLC	Shadbad et al. (2012)
Musty peanuts	2010	China	5/5	Total AFs	1.2–1482.3	UHPLC–MS/MS	Huang et al. (2010)
Groundnut	2012	Ethiopia	120/93	Total AFs	15–11,900	ELISA	Gürses (2006)
Peanut butter	2007	Trinidad	32/0	AFB_1	ND	Charm II	Offiah and Adesiyun (2007)
Peanut butter	2008	Japan	21/10	AFB_1	Max. 2.59	LC/MS, HPLC, HPTLC	Kumagai et al. (2008)
Almond	2006	Turkey	13/3	AFB_1	1–13	TLC	Gürses (2006)
Cacao hazelnut cream	2005	Turkey	40/37	Total AFs	1–10	ELISA	Aycicek et al. (2005)
Nuts	2013	Iran	200/193	Total AFs	Mean 1.68	ELISA	Rezaei et al. (2014)
Cashew nuts	2013	Brazil	70/22	Total AFs	2–4	ELISA	Milhome et al. (2014)
Peanut products	1996	Brazil	80/41	Total AFs	43 to 1099	TLC	Freitas and Brigido (1998)
Peanuts	2003	Botswana	93/120	Total AFs	12.0–329	HPLC	Mphande et al. (2004)
Walnut	2012	Iran	26/20	Total AFs	1–54	HPLC	Shadbad et al. (2012)
Nuts	2013	Saudi Arabia	264/70	Total AFs	1–110	IAC/HPLC	El tawila et al. (2013)
Fresh peanuts	2010	China	35/14	Total AFs	Mean 2.5	UHPLC–MS/MS	Huang et al. (2010)
Nuts	2013	Iran	167/100	Total AFs	Mean 1.12	ELISA	Ali et al. (2013)

Peanuts	2001	Argentina	50/2	AFB_1	435–625	TLC	Fernandez Pinto et al. (2001)
Walnut	1993	Egypt	20/15	Total AFs	15–25	TLC	Abdel-Hafez and Saber (1993)
Peanut	2011	Malaysia	13/11	AFB_1	1.47–15.33	ELISA	Filazi and Sireli (2013)
Peanut	2012	Iran	6/2	AFB_1	<5	HPLC	Shadbad et al. (2012)
Walnut	2006	Turkey	24/6	AFB_1	3–28	TLC	Gürses (2006)
Dehulled hazelnut	2005	Turkey	51/20	Total AFs	1–10	ELISA	Aycicek et al. (2005)
Peanuts	2001	Argentina	50/2	AFG_1	83–625	TLC	Fernandez Pinto et al. (2001)
Pistachio	2010	Turkey	95/48	Total AFs	0.007–7.72	HPLC	Set and Erkmen (2010)
Peanuts		Taiwan	1827/597	Total AFs	0.2–513.4	HPLC	Chen et al. (2013)
Hazelnut	1993	Egypt	20/18	Total AFs	25–175	TLC	Abdel-Hafez and Saber (1993)
Peanut	2007	Trinidad	140/0	AFB_1	ND	Charm II	Offiah and Adesiyun (2007)
Hazelnut	2012	Iran	13/1	Total AFs	1–13	HPLC	Shadbad et al. (2012)
Peanut butters	2010	China	33/31	Total AFs	0.7–95.9	UHPLC–MS/MS	Huang et al. (2010)
Untreated nuts	2013	Iran	109/44	Total AFs	0–38.1	ELISA	Ostadrahimi et al. (2014)
Sunflower	2011	Malaysia	7/6	AFB_1	1.14–5.33	ELISA	Filazi and Sireli (2013)
Hazelnut	2006	Turkey	28/9	AFB_1	1–113	TLC	Gürses (2006)
Almond	2012	Iran	35/5	Total AFs	<5	HPLC	Shadbad et al. (2012)
Salt roasted nuts	2013	Iran	62/21	Total AFs	0–52.3	ELISA	Ostadrahimi et al. (2014)

(a) Total: $AFB_1 + AFB_2 + AFG_1 + AFG_2$
(b) ND: not detected

3.3.3 Occurrence of Aflatoxins in Spices

For many decades, spices have been utilized as fragrance, color, and preservatives for beverages, food, and flavor. In regard to the world commercial value, the most important spices include black pepper (*Piper nigrum* L.), chili (*Capsicum annuum* L.), nutmeg (*Myristica fragrans*), cumin (*Cuminum cyminum*), cinnamon (*Cinnamomum*), ginger (*Zingiber officinale*), turmeric (*Curcuma longa*), cloves (*Syzygium aromaticum*), and coriander (*Coriandrum sativum*) (Ozbey and Kabak 2012). Chili (*Capsicum annuum* L.) is among the spices which have gained popularity around the world, and it is mostly eaten as a food ingredient, especially in Southeast Asia and Latin America due to its taste, pungency, color, and flavor (Jalili and Jinap 2012). Due to its drying and processing method, climatic and environmental changes such as temperature, relative humidity, and insect and pest attack, spices tend to be highly infected by toxigenic and two mycotoxins which include aflatoxins (AFs) and ochratoxin A (OTA) (Ozbey and Kabak 2012)**.** It was reported that several cultivars of spices such as black pepper, cardamom, cinnamon, clove, cumin, coriander, and ginger are contaminated with aflatoxins in various regions (Mahato et al. 2019). Contamination of spices with *Fusarium* and *Alternaria alternata* was observed in fresh and sun-dried pepper (Iqbal et al. 2011). Fungal infection such as mold infection in spices can be witnessed at different stages which include the time of crop production in the field, after harvest and during storage as well as when conditions are suitable for the growth (Filazi and Sireli 2013). After harvest, sun-drying is a popular practice in certain countries around the world, which requires the pepper being spread out on the soil in a single layer. Hot pepper is contaminated with *A. flavus* and *A. niger* during storage. During storage, infection of spices with *A. flavus* consequently producing aflatoxins is regarded as one of the most severe problems threatening food security globally (Iqbal et al. 2011). Previous aflatoxin occurrence in spices across the world is being highlighted in Table 3.3.

3.3.4 Occurrence of Aflatoxin in Milk and Dairy Products

Milk is a highly nutritious food consisting of several nutrients which are crucial for the development and maintenance of human well-being. The health status of people in a given population is often reflected in the condition of their food-producing ecosystems. In addition, enforcing food legislation may be directly connected to the amount and quality of foods available. Therefore, consumers in developing nations, in particular the rural inhabitants, are faced with food security and animal safety problems as they rely on foods grown locally (Iqbal et al. 2015). When breastfeeding mammals such as sheep, goats, and cows consumed foods which are contaminated with aflatoxin B_1 (AFB_1) and B_2 (AFB_2), these metabolites are transformed to aflatoxins M_1 (AFM_1) and M_2 (AFM_2) (Filazi et al. 2010)**.** The quantity of AFM_1 in milk is directly proportional to the quantity of AFB_1 present in feed ingested by

Table 3.3 Occurrence of aflatoxins in spices

Crops	Year	Country	Total/positive samples	Aflatoxin	Range	Detection techniques	References
Red pepper	2010	Turkey	49/1	AFG_1	20	TLC	Demircioglu and Filazi (2010)
Red chili	2016	India	25/13	Total AFs	198.4	ELISA and TLC	Jeswal and Kumar (2016)
Red chili	2007	Turkey	100/100	AFB_1	<0.025–40.9	ELISA	Aydin et al. (2007)
Pepper	2011	Malaysia	4/4	AFB_1	0.65–2.1	ELISA	Filazi and Sireli (2013)
Turmeric	2016	India	25/9	Total AFs	65	ELISA and TLC	Jeswal and Kumar (2016)
Nutmeg	2001	Portugal	10/3	AFB_1	1–5	HPLC	Martins et al. (2001)
Paprika	2011	Spain	17	Total AFs	1.8–50.4	–	Santos et al. (2011)
Red chilies	2019	India	14/5	AFB_1	29.5–55.5	TLC	Nazir et al. (2019)
Ginger	2017	Nigeria	–	Total AFs	0.11–9.52	HPLC	Mahato et al. (2019)
Black chilies	2019	India	14/4	AFB_1	39.7–65.9	TLC	Nazir et al. (2019)
Paprika	2001	Portugal	12	AFB_1	1–20	HPLC	Martins et al. (2001)
Red chili	1996	Ethiopia	60/8	Total AFs	250–525	–	Fufa and Urga (1996)
Coriander	2016	India	25/12	Total AFs	116.5	ELISA and TLC	Jeswal and Kumar (2016)
Red chili	1995	Pakistan	176/116	AFB_1	–	TLC	Ahmad and Ahmed (1995)
Red pepper	2016	Iran	20/11	Total AFs	1.73–24.60	HPLC	Jalil (2016)
Black pepper	2006	Morocco	15/7	AFB_1	Mean 0.09	HPLC	Zinedine et al. (2006)
Smoked paprika	2011	Spain	4	Total AFs	22.3–83.7	–	Santos et al. (2011)
Cinnamon	2016	Iran	20/6	Total AFs	0.85–5.04	HPLC	Jalil (2016)
Ground red pepper	2004	Hungary	70/18	AFB_1	Max. 15.7	HPLC	Fazekas et al. (2005)
Red pepper	2010	Turkey	49/5	AFG_2	8–40	TLC	Demircioglu and Filazi (2010)
Cumin	2016	India	25/10	Total AFs	151	ELISA and TLC	Jeswal and Kumar (2016)

(continued)

Table 3.3 (continued)

Crops	Year	Country	Total/positive samples	Aflatoxin	Range	Detection techniques	References
Black pepper	2016	Iran	20/8	Total AFs	2.11–7.01	HPLC	Jalil (2016)
Dried ginger sliced	2017	India	10	Total AFs	3.64–7.52	HPLC	Bisht and Menon (2017)
Cayenne pepper	2007	Portugal	5/5	AFB_1	2–32	HPLC	Martins et al. (2001)
Paprika	2006	Morocco	14/14	AFB_1	Mean 2.88	HPLC	Zinedine et al. (2006)
Red pepper	2010	Turkey	49/11	AFB_2	3–60	TLC	Demircioglu and Filazi (2010)
Coriander	2019	India	14/6	AFB_1	33.4–67.9	TLC	Nazir et al. (2019)
Dried ginger powder	2017	India	10	Total AFs	2.99–5.25	HPLC	Bisht and Menon (2017)
Chillies	2017	USA	169/108	AFB_1	< 2	ELISA and TLC	Ismail et al. (2018)
Cumin	2006	Morocco	14/8	AFB_1	Mean 0.03	HPLC	Zinedine et al. (2006)
Ginger	2016	India	25/9	Total AFs	125	ELISA and TLC	Jeswal and Kumar (2016)
Black pepper	2016	Iran	40/5	Total AFs	1.44–3.21	HPLC	Barani et al. (2016)
Chili	2011	Spain	11	Total AFs	1.9–65.7	–	Santos et al. (2011)
Isot pepper	2004	Turkey	20/1	AFB + AFG	13.8	TLC	Erdogan (2004)
Turmeric	2016	Iran	20/7	Total AFs	1.48–5.68	HPLC	Jalil (2016)
Red-scaled pepper	2004	Turkey	44/8	AFB + AFG	1.1–97.5	TLC	Erdogan (2004)
Cumin	2011	Malaysia	3/2	AFB_1	1.89–4.64	ELISA	Filazi and Sireli (2013)
Red pepper	2016	Iran	36/36	Total AFs	4.26–30.2	HPLC	Barani et al. (2016)
Ginger	2006	Morocco	12/10	AFB_1	Mean 0.63	HPLC	Zinedine et al. (2006)
Powder pepper	2004	Turkey	26/3	AFB + AFG	1.8–16.4	TLC	Erdogan (2004)
Cumin	2019	India	14/4	AFB_1	24.9–63.9	TLC	Nazir et al. (2019)
Ginger	2016	India	25/2	Total AFs	25	ELISA and TLC	Jeswal and Kumar (2016)

AfT: $AFB_1 + AFB_2 + AFG_1 + AFG_2$

animals. AFM_1 infection of milk is posing a real threat to the human health, both in adults and infants, but it is more severe in infants due to high dependence on milk for their daily nutrition (Offiah and Adesiyun 2007).

Around 1–2% of AFB_1 present in animals' feed are converted to AFM_1 in milk although this varies from one animal to another, day to day, and depending on the type of milk consumed. When the consumption of AFB_1 has ceased, the concentration in milk reduces after 72 h to a level which cannot be detected (Filazi et al. 2010), but can be detected between 12 and 24 h of intake, and the bulk of AFB_1 and AFB_2 consumed by mammals are expelled from the body through feces and urine, but a few are bio-transformed in the liver and discharged simultaneously with milk in the form of AFM_1 and AFM_2, respectively (Filazi and Sireli 2013).

Occurrence of AFM_1 in milk and dairy products has been reported in many countries (Table 3.4). The existence of aflatoxin M_1 (AFM_1) in both dairy products and milk is a serious concern throughout the world, but it is more severe in developing nations (Iqbal et al. 2015), due to its carcinogenic, teratogenic, and mutagenic nature, which can lead to acute and chronic illness in humans and animals (Offiah and Adesiyun 2007). Contamination of milk with AFM_1 has been experienced in several countries**.** Different factors contribute to contamination of milk with AFM_1 such as change in climatic and environmental condition, change in feeding and agricultural practices, and the quality and safety control system of the food business operators in accordance with the different legislations. For instance, the Po valley (a province in Italy) which is the producer of almost all the milk consumed in the country, is regarded as the most susceptible region to AFM_1 due to its climatic conditions. AFM_1 can withstand heat deactivation process such as sterilization and pasteurization during food processing. So, cheese or any other products made from contaminated raw milk automatically contain AFM_1 (Serraino et al. 2019). To guard clients, mainly kids, from infected milk and dairy products, numerous nations have mounted regulation to adjust the levels of AFB_1 in feeds and AFM_1 in milk and cheese. The European Union limits for AFM_1 in milk and cheese are 50 ng/L and 250 ng/kg, respectively (Filazi et al. 2010).

3.4 Aflatoxin Safety Regulation

In order to control the level of aflatoxins intake in food, the European Union in 2007 set a safe limit for total aflatoxins and aflatoxins B_1 to be 4 μg/kg and 2 μg/kg accordingly for human consumption, where as in 2010 it was reviewed that the safe limit was set at 5 μg/kg and 10 μg/kg for aflatoxins B_1 and total aflatoxins, respectively. This has helped mitigate the level of aflatoxin in consumable agricultural commodities in Europe. Moreover, several other countries such as Taiwan, Canada, and the United States of America have set their safe limit at 10 μg/kg, 15 μg/kg, and 20 μg/kg, respectively (Ali 2019). The lowest safe limit for AFB_1 was at 1 μg/kg in Switzerland (Creppy 2002) and Bosnia and Herzegovina (Alessandra et al. 2011), while in Japan aflatoxins must be absent in crops for human consumption (Dadzie

Table 3.4 The Occurrence of aflatoxins in milk and dairy products

Crops	Year	Country	Total/positive samples	Aflatoxin	Range	Detection techniques	References
Yoghurt	2010	Iran	68/45	AFM_1	0.015–0.119	TLC	Fallah (2010)
Milk	2012	Pakistan	175/132	AFM_1	0.002–1.6	ELISA	Sadia et al. (2012)
Milk	2013	Brazil	129/111	AFM_1	<0.05	ELISA and HPLC	Picinin et al. (2013)
Yoghurt	2012	Iran	40/35	AFM_1	11.4–115.8	ELISA	Nilchian and Rahimi (2012)
Milk	2012	Pakistan	40/25	AFM_1	0.003–1.9	ELISA	Sadia et al. (2012)
Milk	2013	China	233/112	AFM_1	Mean 21.49	ELISA	Guo et al. (2013)
Yoghurt	2012	Iran	60/59	AFM_1	6.2–87	ELISA	Issazadeh et al. (2012)
Yoghurt	2013	China	178/8	AFM_1	Mean 27.10	ELISA	Guo et al. (2013)
UHT milk	2012	Saudi Arabia	96/79	AFM_1	0.01–0.19	ELISA	Abdallah et al. (2012)
Milk	2017	China	5650/267	AFM_1	0.05	ELISA	Ismail et al. (2018)
Milk	2012	Pakistan	17/15	AFM_1	0.002–0.794	ELISA	Sadia et al. (2012)
Feta cheese	2011	Egypt	15/10	AFM_1	7.14–122	ELISA	Ayoub et al. (2011)
Raw milk	2012	Palestine	40/34	AFM_1	3–80	ELISA	Zuheir and Omar (2012)
Cheese	2011	Lebanon	111/76	AFM_1	1.26–315	ELISA	Elkak et al. (2012)
White cheese	2009	Iran	116/93	AFM_1	52.1–744.5	ELISA	Fallah et al. (2009)
Raw milk	2012	India	45/45	AFM_1	0.1–3.8	HPLC	Siddappa et al. (2012)
UHT milk	2012	Turkey	40/8	AFM_1	<0.004–0.076	HPLC-FLD	Kabak and Ozbey (2012)
Sweets	2012	Pakistan	17/15	AFM_1	0.002–0.794	ELISA	Sadia et al. (2012)
White cheese	2010	Iran	72/59	AFM_1	0.030–1.200	TLC	Fallah (2010)
Cheese	2012	Iran	138/108	AFM_1	0.01–1.5	ELISA	Nilchian and Rahimi (2012)
Pasteurized milk	2012	India	7/3	AFM_1	1.8–3.8	HPLC	Siddappa et al. (2012)
Yoghurt	2020	Yemen	62/54	AFM_1	0.021–0.893	HPLC	Murshed (2020)
Milk	2009	Kuwait	309	AFM_1	BDL–80.8	ELISA	Dashti et al. (2009)
Fresh milk	2012	Nigeria	10/10	AFM_1	0.407–0.952	HPLC	Susan et al. (2012)
Cheese	2009	Kuwait	40/32	AFM_1	23.8–452	ELISA	Dashti et al. (2009)

Yoghurt	2010	Spain	72/2	AFM_1	13.22 ± 4.82	ELISA	Cano-Sancho et al. (2010)
Cheese	2020	Yemen	90/74	AFM_1	0.022–5.95	HPLC	Murshed (2020)
UHT milk	2009	Serbia	32/15	AFM_1	<0.01–0.05	TLC	Polovinski-Horvatovic et al. (2009)
Diary deserts	2011	Turkey	50/26	AFM_1	1.5–80	ELISA	Ertas et al. (2011)
Fresh milk	2013	Sudan	143/143	AFM_1	18–86	ELISA	Suliman and Abdalla (2013)
Milk	2010	Pakistan	84/81	AFM_1	0.69–100.04	HPLC	Muhammad et al. (2010)
Ice cream	2012	Iran	40/29	AFM_1	20.1–197.4	ELISA	Nilchian and Rahimi (2012)
Raw milk	2011	Egypt	48/37	AFM_1	3.41–137	ELISA	Ayoub et al. (2011)
Breast milk	2012	Iran	132/8	AFM_1	7.1–10.8	ELISA	Ghiasain and Maghsood (2012)
Pasteurized milk	2010	Iran	91/66	AFM_1	0.013–0.250	TLC	Fallah (2010)
Milk products	2012	Nigeria	10/10	AFM_1	0.248–2.510	HPLC	Susan et al. (2012)
UHT milk	2010	Spain	72/68	AFM_1	9.29 ± 2.61	ELISA	Cano-Sancho et al. (2010)
UHT milk	2012	India	45/29	AFM_1	0.06–2.1	HPLC	Siddappa et al. (2012)
Cream cheese	2009	Iran	94 /68	AFM_1	58.3–785.4	ELISA	Fallah et al. (2009)
Milk	2013	China	72/43	AFM_1	10–420	LC-MS/MS	Xiong et al. (2013)
Cow's milk	2014	Serbia	150/150	AFM_1	0.01–1.20	ELISA	Kos et al. (2014)
Milk	2009	Iran	196/196	AFM_1	Mean 77.92	ELISA	Sani et al. (2010)
Milk	2013	China	200/65	AFM_1	5.2–59.6	ELISA	Han et al. (2014)
Cow milk	2014	Croatia	194	AFM_1	3.65–162.3	ELISA	Bilandzic et al. (2014)
Ice cream	2010	Iran	36/25	AFM_1	0.015–0.132	TLC	Fallah (2009)
Milk	2016	Malaysia	53/19	AFM_1	3.5–100.5	ELISA	Nadira et al. (2016)
Butter	2010	Iran	31/8	AFM_1	0.013–0.026	TLC	Fallah (2009)
Cheese	2010	Spain	ND	AFM_1	<12.5	ELISA	Cano-Sancho et al. (2010)
Raw milk	2010	Sri Lanka	87/29	AFM_1	ND–0.085	–	Ismail et al. (2015)
Raw milk	2011	Turkey	50/43	AFM_1	1–30	ELISA	Ertas et al. (2011)

(continued)

Table 3.4 (continued)

Crops	Year	Country	Total/positive samples	Aflatoxin	Range	Detection techniques	References
Yogurt	2011	Egypt	22/17	AFM_1	9.70–89.3	ELISA	Ayoub et al. (2011)
Yoghurt	2011	Turkey	50/28	AFM_1	2.5–78	ELISA	Ertas et al. (2011)
Milk powder	2011	Egypt	19/5	AFM_1	2.15–16.5	ELISA	Ayoub et al. (2011)
Cheese	2011	Turkey	60/38	AFM_1	12–378	ELISA	Ertas et al. (2011)
Yoghurt	2011	Turkey	80/72	AFM_1	6–264	ELISA	Atasever et al. (2011)
Cow milk	2014	Croatia	143	AFM_1	2.69–44.9	ELISA	Bilandzic et al. (2014)
Milk	2006	Iran	72/72	AFM_1	230.2 ± 1.89	ELISA	Sefidgar et al. (2011)
Ice cream	2013	Iran	90/62	AFM_1	8.4–147.7	ELISA	Darsanaki et al. (2013)
Raw milk	2010	Thailand	240	AFM_1	0.014–0.197	HPLC	Ruangwises and Ruangwises (2010)
Lighvan cheese	2013	Iran	37/10	AFM_1	70.5–203	ELISA	Mohajeri et al. (2013)
Goat milk	2014	Croatia	32	AFM_1	2.78–40.8	ELISA	Bilandzic et al. (2014)
Yoghurt	2011	Turkey	80/70	AFM_1	10–475	ELISA	Atasever et al. (2011)
Milk products	2012	Nigeria	10/10	AFM_1	0.139–1.238	HPLC	Susan et al. (2012)
Powder milk	2020	Yemen	38/26	AFM_1	0.021–0.418	HPLC	Murshed (2020)
Raw milk	2011	Iran	122/122	AFM_1	4–112.4	ELISA	Kamkar et al. (2011)
Pasteurized milk	2011	Egypt	37/8	AFM_1	6.28–67.4	ELISA	Ayoub et al. (2011)
Liquid milk	2020	Yemen	60/40	AFM_1	0.021–2.89	HPLC	Murshed (2020)
Sheep milk	2014	Croatia	19	AFM_1	2.11–5.87	ELISA	Bilandzic et al. (2014)
Raw milk	2011	Iran	100/100	AFM_1	1.3–68	ELISA	Panahi et al. (2011)
Milk-based cereal weaning food	2007	Iran	80/72	AFM_1	3–35	ELISA	Oveisi et al. (2007)
Liquid milk	2007	Iran	128/128	AFM_1	31–113	ELISA	Oveisi et al. (2007)
Infant formula	2000	South Korea	26/18	AFM_1	0.032–0.132	HPLC	Filazi and Sireli (2013)
Infant formula	2007	Iran	120/116	AFM_1	1–14	ELISA	Oveisi et al. (2007)

ND: not determined

et al. 2019). Furthermore, in developed countries mentioned earlier, apart from the imposition of strict rules and regulation, other factors such as high rate of literacy and awareness among farmers and consumers, technological advancement both during processing and at storage stage has been associated with the low level of aflatoxins in such nations (Ismail et al. 2018).

However, in Africa and certain Asian countries, there are no strict safety regulations in place to curb the level of aflatoxins present in food commodities consumed by the population, which may be considered as one of the possible reasons for the presence of the high level of aflatoxins in food products. This has led to major health consequences among the people living in this part of the world. In addition, the presence of suitable environmental condition for aflatoxin development, technological hurdles, high rate of illiteracy among farmers and consumers and lack of awareness, poor storage condition and facilities, and overall high rate of poverty may also be considered as possible reasons for the high level of aflatoxins in Africa and certain Asia nations (Ismail et al. 2018).

3.5 Mycotoxins: Hidden Toxins

Mycotoxins are believed to be a part of the fungal chemical protection system that safeguards them from living creatures such as microorganisms, grazing animals, nematodes, insects, and humans. Mycotoxins can be found in food and several harvested crops and produce through many infection routes, at various phases of production, processing, transport, and storage. Mold and mycotoxin development are affected by numerous biotic and abiotic factors such as relative humidity, fungicides and fertilizers, temperature, insect infestation, kind of substrate and dietary factors, geographical place, genetic requirements, and interaction among the colonizing toxigenic fungal species (Rychlik et al. 2014). *Aspergillus*, *Fusarium*, *Alternaria*, and *Penicillium* are the most crucial fungal genera that produce mycotoxins which are found in foods and feeds. Mycotoxins which cannot be detected through traditional analytical methods due to modification of their form and structures inside the plants are referred to as masked mycotoxins (Berthiller et al. 2013). Nevertheless, the modified mycotoxins can be returned to their toxic nature during food processing and digestion through the process called hydrolysis. Parts of the altered toxins are found in various forms as complexes together with matrix compounds; for this reason, they can also be known as matrix-associated mycotoxins. Hidden fumonisin in its altered forms was returned to its toxic nature via hydrolysis and was eventually analyzed and determined via LC/MS/MS approach (Mahato et al. 2019). Several of the contemporary issues on the occurrence of masked mycotoxins are reported in different parts of the world including the United States of America, Africa, Europe, and some countries in Asia such as Japan and China. The highest prevalence rate of masked mycotoxins are reported in agricultural products, in particular cereal-based foods, which is threatening and detrimental to both humans and animals' health (Zhang et al. 2019). Therefore, the determination of masked mycotoxins is an essential part to ensure safety of feed, food, human lives, and animals.

3.6 Conclusion

Aflatoxins (AFs) are toxic secondary metabolites produced by *Aspergillus* species, which are found in susceptible agricultural products. Aflatoxins can cause substantial economic losses, and they have a detrimental effect on human and animal health. This book chapter summarizes the occurrence, effect, and implications of AF contamination in a wide range of agricultural crops around the world. Contamination of AFs can be found in both tropical and temperate regions of the world. Agricultural crops such as oil seeds, nuts, spices, dried fruit, beans, fruit, and cereals are the most important commodities affected by AFs. Maximum levels of aflatoxins were found in the food commodities of African and Asian countries. Due to the economic importance of AFs, regulations for major mycotoxins in agricultural commodities have been put in place in more than 100 nations, most of which are for aflatoxins, and maximum tolerated levels vary significantly across countries (Filazi and Sireli 2013). The inability to manage and at times even predict production of aflatoxin makes it a unique problem to food security. Although preventing aflatoxin contamination is the best control method, all forms of mycotoxin contamination cannot be avoided. Optimal conditions for post-harvest storage will reduce consumer exposure to most mycotoxins, but detoxification procedures may be needed in certain cases (Reddy et al. 2009).

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Chapter 4
Exposure Assessment of Aflatoxins in Humans

Iqra Naeem, Muhammad Usman Saleem, Sameem Javaid, Adnan Amjad, Majid Hussain, Tariq Ismail, Amir Ismail, Ambreen Fazal, and Zarmina Mumtaz

Abstract Aflatoxins pose serious health implications on all age groups, and therefore the exposure assessment of aflatoxins through all means is essential. Human exposure of aflatoxins is mainly through the ingestion route, but a limited amount of aflatoxins may also enter through the inhalation route. Aflatoxin exposure assessment may be performed through internal means, i.e., quantification of aflatoxins in blood/plasma/serum, in urine, or in breast milk, or through external means, i.e., estimation of the dietary intake of aflatoxins. In the chapter in hand, the exposure assessment of aflatoxins through external and internal means is discussed in detail.

Keywords Aflatoxins · Exposure assessment · Ingestion · Inhalation · External · Internal

4.1 Introduction

Risk assessment is an eminent scientific tool for notifying risk management policy decisions developed by regulatory bodies, industries, advisory groups, and other entities to evaluate the food products for the safety of toxic substances and to facilitate their registration and approval before merchandising and making any regulatory decisions. In order to properly evaluate the health risk associated with a particular substance, the background knowledge of the frequency and magnitude of exposure

I. Naeem · S. Javaid · A. Amjad · M. Hussain · T. Ismail · A. Ismail (✉) · A. Fazal
Z. Mumtaz
Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan
e-mail: amirismail@bzu.edu.pk

M. U. Saleem
Department of Biosciences, Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, Pakistan

K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_4

and the population group potentially exposed is required. In other words, the risk assessment must include an accurate exposure assessment closely framed to the existing or required toxicological information (Ramasamy and Lee 2015).

Human exposure is an event that happens after contact of humans to the environmental contaminant or toxicant of specific concentration for a specific time interval (Alves et al. 2014). The individual's susceptibility to exposure is determined by certain characteristics, i.e., age, sex, immunity, nutritional status, potential to activate or detoxify the carcinogens, ability to repair DNA alterations, and socioeconomic status (WHO 1993). Human exposure assessment has a great significance in estimating the extent and likelihood of potential or actual exposure to the source of a hazardous substance, and it is an important part of epidemiological studies and any health risk assessment. Normally, it has been confined to estimating, at varying degree of certainty, the degree and connectivity among the source and levels of toxic substances and a human in the environment by understating various exposure pathways and the routes between them while considering the behavioral factors. As there is a need to establish a direct relationship between actual human exposure and adverse health effects, exposure assessment is a vital component of epidemiological research (Nieuwenhuijsen et al. 2006).

Estimating the outcomes of human exposure to aflatoxins entails the consideration of various facts. Firstly, not all of the ingested aflatoxin is biologically significant. In other words, a variable fraction of the consumed aflatoxin is detoxified from the body, and the effect of exposure on various biological systems is divergent as specified by the fraction that exercises through each biological pathway. Also, the total amount of toxin that enters the body through any competing pathway cannot be estimated by merely measuring the level of any particular biomarker. Secondly, the other dietary aspects such as modulation of aflatoxin toxicities by the dietary intake of several vitamins such as A, C, D, and E and antioxidants may impart a significant role in ascertaining the consequences of ingested aflatoxin. Thirdly, the biological exposure level is determined by infection with hepatitis B and C virus, and since this fact has been studied for its impact on cancer risk, it has not clearly been assessed for other known toxicological effects of aflatoxins. Therefore, the evaluation of severity and extent of biological exposure of aflatoxins to humans require more comprehensive data set from experimental analysis (Williams et al. 2004).

In the context of epidemiological studies, two main approaches are used to evaluate the human exposure to toxic substances, i.e. external or indirect exposure and internal or direct exposure. External exposure refers to the amount of a substance of concern in a source of contamination to human exposure. It implies the analysis of the incidence of mycotoxins in the food commodities and then combines this information with the data on the food consumption. While the internal exposure refers to the total amount of that substance or its metabolites within the body of a human, that specifies the integrated human burden of the exposure. It involves monitoring of biomarkers in human biological fluids or tissues (such as blood, urine, breast milk, hair, saliva, nail, feces, etc.) (Alves et al. 2014; Qian et al. 1994).

This chapter will focus on both external and internal exposure of aflatoxins in humans.

4.2 Human Biomonitoring of Aflatoxin Exposure

Human biomonitoring refers to the systemic collection of human biological fluids and tissues (such as blood, urine, breast milk, hair, or saliva) for the analysis of toxic substances and their metabolic and/or transformation products to promote exposure assessment (NRC 2006; Wilhelm 2014). It is a compendious tool used to evaluate the internal dose of a chemical substance or group of substances. Internal dose refers to the amount of substance stored in the individual or several body organs or in the entire body (Martins et al. 2020). Examining biological fluids or tissues for the presence of toxic substances or their metabolic products has been used frequently to form an association between toxin exposure and its biological effects (Groopman et al. 2005). This association is achievable due to the presence of biological markers that reveal cellular or molecular changes occurring in biological cells, fluids, or tissues (Groopman et al. 2005).

A biomarker or biological marker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Measurement of biomarker reflects an interaction between a biological system and a potential hazard that may be physical, chemical, or biological, and the measured response may be physiological, functional, biochemical (at the cellular level), or a molecular interaction (Strimbu and Tavel 2010). As biological markers are used to indicate the biological processes, detection of certain biomarkers may help identify, diagnose, and treat the individuals who are affected or at risk without showing any symptoms of disease (Jager et al. 2011). Biomonitoring contributes direct measurement of cumulative exposure at the sampling period, alternative to exposure measurements based on multiple estimates such as food, air, and soil that may have added to that exposure (Heffernan et al. 2014). Alternatively stated, the use of validated biomarkers of exposure may cover all possible sources of exposure with reduced uncertainties associated with occurrence and consumption data. Additionally, the information obtained can be used to set up population reference ranges and ascertain susceptible population and individuals with higher exposure (Heffernan et al. 2014).

The current state of the art in assessment of internal xenobiotic exposure by urine or blood analysis of target compounds, their metabolites, or reaction products is helpful to associate the exposures with health outcomes. Substances which are biologically persistent in nature can be characterized well with conventional analytical procedures. However, substances having short half-lives can be measured effectively only if the exposure is continual or continuous or if the exposure timings are known. Particularly, urinary excretion of mycotoxins primarily characterizes the recent exposure, while analysis of serum/plasma is more likely to present long-term exposure (Ediage et al. 2013). Preferably, a biomarker should be easily accessible and acquirable and must represent higher rate of sensitivity and specificity, show stability in its medium, and hence have minimum variability (Videira et al. 2020). Biomarkers can be conceptualized in respect of trait, rate, and state and are categorized into biomarkers of biologically effective dose, biological response, internal dose, and altered function/structure (Chahine et al. 2014).

The biological markers of mycotoxin exposure have been defined as the substances (e.g., the parent toxin and/or their metabolites) or the products generated as a result of their interaction with the target molecules (DNA or protein adduct or glucuronide conjugates) that are measurable in the biological fluids or tissues (such as serum/plasma, urine, breast milk) (Marin et al. 2018). These can be associated with the quantity of ingested mycotoxin and indicate the presence and magnitude of present and past exposure (Kensler et al. 2011; Marin et al. 2018).

The choice of biomarkers to be assessed in a particular matrix is crucial. Some of the mycotoxins such as aflatoxins absorb quickly after being ingested, and their level in the blood increases rapidly within a few hours of exposure. However, their removal from the body also occurs very rapidly except that they have bound to macromolecules to form adducts. The adducts of mycotoxins with the macromolecules in the blood have longer half-life, and these can provide important information on the toxic effects. One more preferred biological fluid used to assess the mycotoxin exposure is urine that might contain the parent compound or mycotoxin metabolic products. Therefore, it is important to understand the long- and short-term biomarkers of exposure along with their dose-response association and sensitivity in the biomarker's expression (Al-Jaal et al. 2019).

Biomarkers of aflatoxin exposure have played a significant role in understanding the aflatoxin-induced acute and chronic toxicities. These comprised of both biomarkers of exposure, serum AFB_1-albumin adduct (AFB_1-alb), and urinary aflatoxin M_1 (AFM_1) as internal dose indicators, and a biomarker of effect, concerning AFB_1-N^7-guanine adduct (AFB_1-N^7-gua) in urine as measure of biological effective dose (Kensler et al. 2011; Leong et al. 2012). These biomarkers of aflatoxin exposure are developed on the basis of the information that the reaction of parent compound AFB_1 with the superfamily of cytochrome p-450 enzyme system results in the formation of carcinogenic form of aflatoxin B_1-8,9 epoxide. The oxidation of AFB_1 by these enzyme systems also converts it into AFM_1, AFP_1, and AFQ_1 as well as a reduced form aflatoxicol. The reactive epoxide formed undergoes electrophilic attack to DNA and serum albumin and results in the formation of DNA and protein adducts, respectively, that have been validated as molecular dosimeters in both humans and experimental models (Gallagher et al. 1994; Shahbazi et al. 2017; Qian et al. 1994).

4.3 Aflatoxin Biomarkers in Blood

Blood is a non-homogeneous mixture of cells, lipids, proteins, and different metabolic products and can be conceptualized as comprising of serum, plasma, and cellular compartments (Chahine et al. 2014). Based on the type of biomarker, the analysis can be performed in the whole blood, plasma, serum, or particular cell type. From a scientific view point, blood is ideally a preferred matrix for a large number of toxic substances such as mycotoxins, pesticide residues, heavy metals, and drug residues as it is in direct contact with all the body tissues and is in equilibrium with

various body organs. However, being invasive in terms of phlebotomy, the application of blood in biomonitoring studies has several drawbacks. Firstly, the rate of participation of the study population is negatively affected. Secondly, the sample volume is often limited. Thirdly, sampling in infants, young children, and/or other susceptible population groups has ethical and practical downsides. Despite that, in comparison to urine, analysis of blood biomarkers gives more details about the mycotoxin concentration in the internal circulation and denotes a steady-state level (Fan et al. 2019) (Fig. 4.1).

In the systemic blood, aflatoxin and its metabolites are present both in free form and in the form of protein conjugates. The protein conjugation is permanent, and its presence in the systemic blood could be one of the main factors of reduced elimination of aflatoxin and its metabolic products in the urine (Nassar et al. 1982). Albumin is the only serum protein that binds well with the aflatoxin to form adducts (Sabbioni et al. 1990; Skipper et al. 1985), though binding of hemoglobin to AFB_1 has also been reported but with very low efficiency (Pereira and Chang 1981). It has been reported that around 1.4–2.3% of the ingested aflatoxin B_1 binds covalently with the serum albumin in humans (Gan et al. 1988). Non-covalent binding of aflatoxins with human serum albumin has also been reported with a binding constant of 10^4–10^5 dm^3/mol (Poór et al. 2017). AFB_1, AFG_1, AFB_2, and AFM_1 bind to human serum albumin with same affinity, whereas the complex of AFG_2 with human serum albumin is slightly unstable as compared to complexes of other types of aflatoxins with albumin (Poór et al. 2017). However, the aflatoxin-albumin adduct levels in serum are mainly the measure of AFB_1 ingested, owing to the fact that the existence of other types of aflatoxins in the food is rare (Williams et al. 2004).

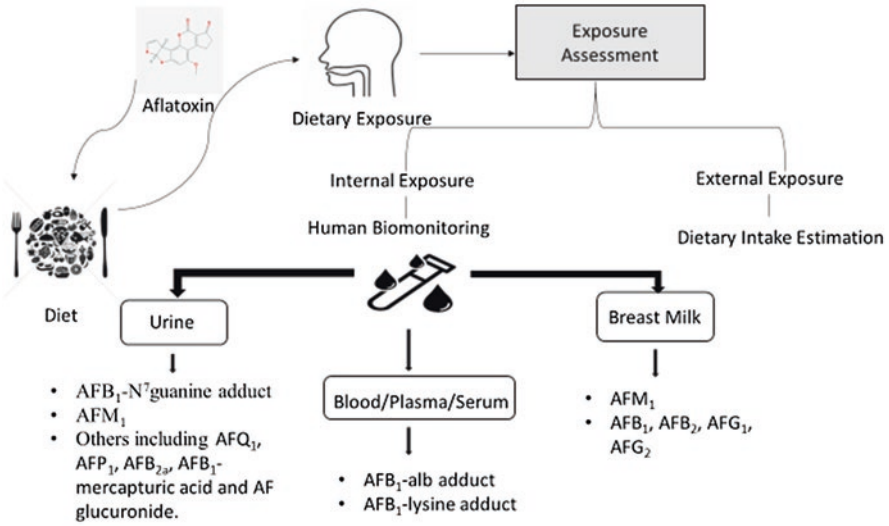

Fig. 4.1 Aflatoxins internal and external exposure assessment

In the last few decades, the application of serum AFB_1 albumin adducts as biomarkers of exposure has become standard in molecular epidemiology. This biomarker was first validated in experimental models (rats). Afterward, the mass spectrometric examination of rats' Pronase-digested serum albumin treated with AFB_1 resulted in the identification of chemical structure of AFB_1-lysine adduct (AFB_1-lys) (Sabbioni et al. 1990; Scholl and Groopman 2008). Aflatoxin epoxide in liver cells and aflatoxin dialdehyde in blood can bind covalently to the amino group of lysine in albumin to form aflatoxin albumin (AFB_1-lys) adducts. As aflatoxins form complexes with albumin, and albumin can readily be extracted from blood, it gives a fairly non-invasive estimate of the biologically effective dose of ingested aflatoxin. This method may be helpful in rapid screening of blood samples in case of acute exposure, and it also represents chronic exposure that cannot be estimated from other markers, for instance, the urinary aflatoxin- N^7-gua adduct (Leong et al. 2012). As the life span of human serum albumin is approximately 20 days, the chronic AFB_1 exposure results in the accumulation of 30-folds higher aflatoxin albumin adducts as compared to single exposure (Scholl and Groopman 2008).

The worldwide occurrence of aflatoxin biomarkers in the blood (either in serum or in plasma) has been investigated as reported in the literature and is summarized in Table 4.1. The findings obtained revealed that there is a huge and continuous exposure of humans to aflatoxins though the inconsistency has been observed owing to the variances among populations and countries and different analytic procedures. Aflatoxin albumin adducts (AFB_1-lys adduct) in the serum and plasma are the most frequently detected biomarker of aflatoxin exposure. Two reports (one from Ethiopia and one from Gambia) also reported the presence of AFM_1 in the serum samples analyzed, while the data regarding the presence of aflatoxicol in the blood was scarce. According to the studies included, the incidence rate of aflatoxin albumin adducts (AFB_1-lys adduct) in the analyzed samples from different countries was 9–100% with the average concentration of 0.63–58.2 pg/mg alb and maximum value of up to 999 pg/mg alb. Developing countries such as Kenya, Taiwan, Ethiopia, Egypt, Ghana, Malawi, Uganda, Nigeria, Mexico, and Malaysia revealed the worst scenario, while the levels of aflatoxins in the blood samples from the developed countries such the USA were low.

Several studies have also investigated the association between the serum AF-alb adduct level and dietary intake (Table 4.1). For this, food frequency questionnaire-based survey or 24-h dietary recall method was adopted. It was observed that the level of aflatoxins in the blood was significantly positively associated with the consumption of grains ($p = 0.046$), roots ($p = 0.000$), dark green leafy vegetables ($p = 0.000$), legumes ($p = 0.000$), yellow fruits $p = 0.006$), fruits and veg ($p = 0.000$), meat ($p = 0.000$), egg ($p = 0.000$), fish ($p = 0.000$), dairy foods ($p = 0.000$), sweets ($p = 0.000$) (Mahfuz et al. 2019), maize ($p = 0.0008$), lupine beans ($p = 0.007$) (Piekkola et al. 2012), chickpea ($p = 0.023$), millet ($p = 0.031$), groundnut ($p = 0.019$), soybean ($p = 0.018$), and lentil ($p = 0.003$) (Terefe 2020) while significantly negatively correlated with dried cassava ($p = 0.03$), and soya ($p = 0.04$) (Asiki et al. 2014).

Table 4.1 Worldwide occurrence of aflatoxin biomarkers in human blood

Country	Studied population	Biomarker	Blood type	Total/positive samples (%)	Mean/range (pg/mg alb)	Analytical method	References
Bangladesh	Children	AFB_1-lys	Plasma	208/20.8 (10.1)	1.34/NR	ID-MS/MS	Mahfuz et al. (2019)
				196/40.9 (20.9)	1.52/NR		
				173/30.9 (17.9)	3.43/NR		
				167/103 (61.7)	3.7/NR		
Brazil	Adult	AFB_1-lys	Serum	113/0	<LOD	HPLC-FD	Jager et al. (2016)
Egypt	Pregnant women	AF-alb	Serum	98/34 (34.7)	4.9d/NR	HPLC	Piekkola et al. (2012)
Ethiopia	Adults	AFB_1	Serum	100/37 (37)	36.9/NR a	LC-MS/MS	Terefe (2020)
		AFB_1-lys		100/27 (27)	72.3/NR a		
		AFM_1		100/38 (38)	69.9/NR a		
Gambia	Children	AFB_1	Serum	131/23.5 (18)	2.5/0.2–74	ELISA	Jonsyn-Ellis (2007)
		AFM_1		131/39 (30)	0.7/0.03–6.8		
		AFL		131/30 (23)	0.1/0.01–3.2		
Ghana	Adults	AFB_1-alb	Serum	507/NM	0.94/0.11–4.44b	HPLC	Tang et al. (2009)
Ghana	Pregnant women	AF-alb	Serum	755/755 (100)	10.9/0.44–268.73	HPLC-FD	Shuaib et al. (2010)
Guatemala	Elders	AF-alb	Serum	200/NM	5.11e/NR	ID-MS/MS	Alvarez et al. (2020)
Kenya	Adults	AFB_1-lys	Serum	600/468 (78)	NR/<LOD-211	LC-MS/MS	Yard et al. (2013)
Kenya	Adult women	AFB_1-lys	Serum	884/884 (100)	7.82d/NR	HPLC-FD	Leroy et al. (2015)
Kenya	Mother and children	AFB_1-lys	DBS	36/25 (69.4)	11.88/<LOD-999.42	HPLC-FD	Xue et al. (2016)
Kenya	Children	AFB_1-lys	Serum	327/NM	13.19e/NR	HPLC-FD	Wangia-Dixon et al. (2020)
Malawi	All age groups	AFB_1-lys	Serum	230/154 (67)	20.5/NR	ELISA	Seetha et al. (2018)
Malaysia	Adults	AFB_1-lys	Serum	170/164 (97)	7.67/0.2–23.16	HPLC-FD	Leong et al. (2012)
Mexico	Children	AFB_1-lys	Serum	31/13.95 (45)	5.6e/4.8–6.5	HPLC-FD	de León-Martínez et al. (2019a, b)
Mexico	Women	AFB_1-lys	Serum	34/28.22 (83)	3.48d/1.08–102.6	HPLC-FD	de León-Martínez et al. (2019a, b)

(continued)

Table 4.1 (continued)

Country	Studied population	Biomarker	Blood type	Total/positive samples (%)	Mean/range (pg/mg alb)	Analytical method	References
Nepal	Children	AFB_1-lys	plasma	85/85 (100)	3.62d/NR	UPLC-ID-MS/MS	Mitchell et al. (2017)
Nepal	Pregnant women	AFB_1-lys	cord blood	141/141 (100)	25.28d/NR	ID-MS/MS	Groopman et al. (2014)
Nigeria	Children	AFB_1-lys	Serum	58/58 (100)	2.6/0.2–59.2	LC-MS/MS	McMillan et al. (2018)
Taiwan	Elders	AF-alb	Serum	1282/1210 (94)	58.15/NRc	ELISA	Wu et al. (2009)
USA (East Texas 2007–2008)	All participant	AFB_1-lys	Serum	172/47 (27.33)	2.35e/NR	HPLC-FD	Xue et al. (2021)
USA (East Texas 2012–2013)				200/95 (47.5)	3.07/NR		
USA (East Texas 2013–2014)				398/398 (100)	4.34/NR		
USA (West Texas 2004–2005)				54/5 (9.26)	0.63/NR		
USA (West Texas 2006–2007)				160/52 (32.5)	2.72/NR		
USA (West Texas 2009–2010)				140/140 (100)	3.98/NR		
Uganda	Adults	AF-alb	Serum	100/100 (100)	9.7d/ 0–237.7	ELISA	Asiki et al. (2014)
	Children			96/96 (100)	11.5/NR		
Uganda	General population	AFB_1-lys	Serum	713/641 (90)	1.58e/0.4–168	HPLC-FD	Kang et al. (2015)
Uganda	General population	AFB_1-lys	Serum	985/706.25 (71.1)	1.33/<LOD-174	LC-MS/MS	Zitomer et al. (2020)

a, ng/ml; b, pmol/mg; c, fmol/mg; d, geometric mean; e, median; HPLC-FD, high-performance liquid chromatography coupled with fluorescence detector; ELISA, enzyme-linked immunosorbent assay; LC-MS/MS, liquid chromatography-mass spectrometry; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry; ID-MS/MS, isotope-dilution mass spectrometry; UPLC-ID-MS/MS, ultra-performance liquid chromatography coupled with isotope dilution tandem mass spectrometry

4.4 Aflatoxin Biomarkers in Urine

Urine is perhaps the most commonly used matrix to quantify the degree and extent of environmental exposure to toxic substances, particularly the substances having shorter half-lives. It is typically the preferred method of choice because the sample collection and analysis carry no related risk and samples are easily accessible in large volumes. Urinalysis involves two different types of sample collection, namely, 24-h and spot sample (Alves et al. 2014). Though collection of spot samples is easier, it has a drawback of differing chemical concentration and volume. This problem can be tackled by different methods, i.e., creatinine normalization, specific gravity, or osmolality. However, the most frequently used method is normalization of creatinine levels of the urine (μg mycotoxin L^{-1} urine/g creatinine L^{-1} urine) though specific gravity is perceived as less affected by gender, age, body size, and food intake (Sauvé et al. 2015).

In humans, the absorbed mycotoxins are mainly eliminated into the urine either as free mycotoxins or as metabolites or both (Fan et al. 2019). Different types of aflatoxins and their metabolites are present and have been detected in the human urine. This includes AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFQ_1, AFP_1, AFB_{2a}, aflatoxin glucuronide, AFB_1-mercapturic acid, AF-N^7-gua adduct, and aflatoxicol (Al-Jaal et al. 2019). However, the presence of AFM_1 and AF-N^7-gua adduct has been widely studied and correlated well with the dietary aflatoxin intake (Groopman et al. 1992; Zhu et al. 1987). It has been reported that 1.2–2.2% and 0.2% of the ingested AFB_1 are excreted as AFM_1 and AFB_1-N^7-gua adduct in the urine (Groopman and Kensler 1993; Zhu et al. 1987). Also, the presence of AFB_1 metabolites in the urine either AFB_1-N^7-gua adduct and AFM_1 was correlated with 4.0- and 3.3-fold increase in the incidence of hepatic cancer, respectively (Sun et al. 1999; Wang et al. 1996).

Both AFM_1 and AFB_1-N^7-gua adduct in the urine are biomarkers of acute aflatoxin exposure. Aflatoxin M_1 is the biotransformation product of AFB_1 metabolism in the liver that is excreted in the urine within the 24–48 h of exposure to AFB_1. While AFB_1-N^7-gua adduct is the major nucleic adduct of aflatoxin that was firstly excreted in the urine of aflatoxin exposed rats (Essigmann et al. 1977) and has spurred interest in using as possible biomarker for biological effective dose of aflatoxin B (Williams et al. 2004), N^7-guanine adducts are mainly formed by the adduct formation or DNA alkylation at nucleophilic sites in the chemical structure of DNA. In comparison to other DNA alkylation, N^7-guanine adducts seem to be effective biomarkers of internal dose owing to their higher abundance (Boysen et al. 2009). Experimental analysis revealed that around 30–40% of the AFB_1-N^7-guanine adduct generated is eliminated through urine within 24 h of exposure and more than 70% of this metabolite is eliminated within 48 h (Bennett et al. 1981; Groopman et al. 1992). Therefore, determination of urinary concentration of AFB_1-N^7-gua adduct has given useful information about the aflatoxin disposition following ingestion as a molecular dosimeter to better reflect the exposure in epidemiological studies (Groopman et al. 1985; Hsieh et al. 1988).

The worldwide occurrence of aflatoxin biomarkers in the urine is summarized in Table 4.2. The findings obtained revealed that there is a huge and continuous exposure of humans to aflatoxins through the lack of consistency that has been observed owing to the variances among populations and countries and different analytic procedures. Aflatoxin M_1 was the most frequently detected biomarker of aflatoxin in urine though the other forms such as AFB_2, AFG_1, and AFG_2 were also detected. Studies from Brazil, China, and the USA also reported the presence of AFB_1-N^7-gua adduct in the analyzed samples of urine with the values ranging from <LOD to 0.0065 ng/mg creatinine, while the data regarding the presence of AFP_1 and AFQ_1 in the urine was scarce. According to the studies included, the incidence rate of aflatoxins (AFM_1) in the analyzed urine samples from different countries was 0–100% with the average levels of 0.0003–2.68 ng/ml and maximum value of up to 70.8 ng/ml. Higher levels of urinary aflatoxins were reported from Ghana, Guinea, Malaysia, and Brazil, while lower levels were reported from the UK, the USA, Colombia, and Egypt. The higher inter- and intra-individual variability in urinary concentration of aflatoxins is due to their short half-lives, difference in the time of sample collection, and the changes in dietary aflatoxin exposure.

The probable daily intake (PDI) through urinary biomarker data is determined by multiplying the occurrence of aflatoxin with daily urine production of adults (supposed to be 1.5 L) and dividing it by the multiplication between body weight (kg) reported by the participants, urinary excretion ratio (ER) of aflatoxin (for AFM1 ER is 1.7% and 1.5% for male and female, respectively), and one thousand (Franco et al. 2019). Among the studies included in Table 4.2, only studies from Brazil, Cameron, and China described the exposure assessment based on estimated intake calculated through urinary biomarkers of aflatoxin exposure. The findings from these studies indicated that the PDI of AFM_1 (as calculated by urinary biomarker data) from Chinese population (0.41 μg/kg bw/day) was 9 times and > 400 times higher than from Cameron (0.042 μg/kg bw/day) and Brazil (0.001 μg/kg bw/day), respectively.

A few studies investigated the association of AFM_1 in urine and dietary intake. These studies reported that the level of aflatoxin in the urine was significantly positively correlated with the consumption of dairy products ($p < 0.01$) and eggs ($p = 0.03$) (Sulaiman et al. 2018). Until now, several studies have utilized urinary biomarkers of aflatoxin exposure as study endpoints; hitherto these analyses only evaluated a fraction of the overall spectrum of urinary metabolites of aflatoxins in both animals and humans. For instance, a demethylated metabolite AFP_1 and AFQ_1 and their glucuronides have also been found in the human urine. However, the practicality of these biomarkers has yet to be explored (Walton et al. 2001). Hence, development of a comprehensive methodology utilizing highly sensitive and specific analytical procedures to assess the predictive potential of these metabolic products as biomarkers of exposure or risk is imperative.

Table 4.2 Worldwide occurrence of aflatoxins biomarkers in human urine

Country	Aflatoxin biomarker	Studied population (n)	Total sample/+ive sample (%)	Mean/range (ng/ml)	Analytical method	References
Bangladesh	AFM_1	Adults	62/26.04 (42)	NR/0.002–0.20	HPLC-FD	Ali et al. (2017)
Bangladesh	AFM_1	Adults	93/92.8 (46)	0.08/0.03–0.35	ELISA	Ali et al. (2016)
Brazil	AFM_1	Adults	167/20.04 (12)	NR/0.0005–0.64a	LC-MS/MS	Franco et al. (2019)
	AFP_1		167/10.02 (6)	NR/0.01–0.08a		
	AFs		167/0 (0)	NR/0.0005–0.72a		
Brazil	AFB_1-N^7-guanine adduct	Adults	113/0 (0)	<LOD	HPLC-FD	Jager et al. (2016)
	AFM_1		113/73.45 (65)	NR/0.0004–0.002a		
Brazil	AFM_1	Adults	30/3 (10)	NR/2.75–70.4	ELISA	Kluczkovski et al. (2020)
Brazil	AFM_1	Adults	64/39.04 (61)	0.001/0.0002–0.01a	UPLC-MS/MS	Jager et al. (2014)
Cameroon	AFM_1	Children	220/30.8 (14)	0.86/NR	LC-MS/MS	Ediage et al. (2013)
Cameroon	AFM_1	Adults	145/14.5 (10)	0.05/<0.17–1.38	LC-ESI–MS/MS	Abia et al. (2013)
China	AFM_1	Adults	300/150 (50)	0.04/NR	HPLC	Mykkänen et al. (2005)
	AFQ_1			10.4/NR		
	AFB_1-N^7-gua			0.04/NR		
China	AFM_1	Adults	260/27.04 (10.4)	0.25/0.13–0.46b	UPHPLC-MS/MS	Fan et al. (2019)
China	AFB-N^7-gua	Adults	317/49.14 (15.5)	NR/0.3–1.81	HPLC	Qian et al. (1994)
	AFM_1		317/66.89 (21.1)	NR/0.17–5.2		
	AFP_1		317/52.94 (16.7)	NR/0.59–16		
Colombia	AFM_1	Children	96/40.03 (41.7)	0.02/<LOD–0.05	HPLC-FD	Sánchez and Diaz (2019)

(continued)

Table 4.2 (continued)

Country	Aflatoxin biomarker	Studied population (n)	Total sample/+ive sample (%)	Mean/range (ng/ml)	Analytical method	References
Egypt	AFB_1	Children	50/1 (2)	0.19/NR	HPLC	Polychronaki et al. (2008)
	AFB_2		50/5 (10)	0.001/0.001–0.002		
	AFG_1		50/2 (4)	0.08/0.06–0.08		
	AFG_2		50/12 (24)	0.002/0.001–0.008		
	AFM_1		50/4 (8)	0.01/0.005–0.006		
Ethiopia	AFB_1	Children	200/0 (0)	<LOD	LC-MS/MS	Ayelign et al. (2017)
	AFB_2		200/9 (4.5)	0.05/<0.05–0.06		
	AFG_1		200/5 (2.5)	0.06/0.05–0.07		
	AFG_2		200/6 (3)	0.07/0.06–0.07		
	AFM_1		200/14 (7)	0.064/0.063–0.07		
Ghana	AFM_1	Children	28/28 (100)	0.92/0.02–1.05a	HPLC	Kumi et al. (2015)
Ghana	AFM_1	Adults	91/82.99 (91.2)	1.80/0–11.56a	HPLC	Jolly et al. (2006)
Guinea	AFB_1	Children	50/8 (16)	2.68/0.179–18.0	HPLC	Polychronaki et al. (2008)
	AFB_2		50/29 (58)	0.06/0.001–0.04		
	AFG_1		50/1 (2)	0.709/NM		
	AFG_2		50/18 (36)	0.02/0.001–0.2		
	AFM_1		50/32 (64)	0.10/0.01–0.80		
Iran	AFM_1	Adults	70/14.7 (21)	0.005	ELISA	Mason et al. (2015)
Malaysia	AFM_1	Adults	160/98.08 (61.3)	0.02/0–0.02	ELISA	Redzwan et al. (2012)
Malaysia	AFM_1	Adults	444/197.49 (44.8)	0.65–5.34	ELISA	Sulaiman et al. (2018)
Nigeria	AFM_1	All age groups	120/17 (14.2)	0.3/0.05–1.5	LC-MS/MS	Ezekiel et al. (2014)
UK	AFM_1	Children	21/0 (0)	<LOD	LC-MS/MS	Gratz et al. (2019)
USA	AFB_1-N^7-gua	NR	20/16 (80)	0.0003/0–0.065 a	HPLC-MS/MS	Engner et al. (2006)

a, ng/mg creatinine; b, results of positive samples only; HPLC-FD, high-performance liquid chromatography coupled with fluorescence detector; ELISA, enzyme-linked immunosorbent assay; LC-MS/MS, liquid chromatography-mass spectrometry; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry; LC-ESI-MS/MS, liquid chromatography electrospray ionization-tandem mass spectrometry

4.5 Aflatoxin Biomarkers in Breast Milk

Human breast milk is regarded as one of the most admissible matrices for monitoring of environmental contaminants. Its application in biomonitoring is gaining popularity owing to the easy, non-invasive sample collection procedures and availability of huge chemical information. The great interest of analyzing the toxic substances in the breast milk is warranted not only by the consideration of maternal internal exposure but also the infant's external exposure during sensitive periods of growth and development. Though breastfeeding is regarded as the "gold standard" diet for infants, it has been questioned due to associated potential health hazards when mothers feed on contaminated diet (Fenton et al. 2005).

AFM_1 in breast milk is a carcinogenic metabolic product of AFB_1 and has been used as a biomarker of maternal-to-child transmission of this toxin (Zarba et al. 1992). In the liver, AFB_1 is monohydroxylated to AFM_1 that is transferred to the breastmilk via blood. When breastfeeding mothers feed on aflatoxin-contaminated diet, AFM_1 is eliminated in their milk at quite variable concentration (Altun et al. 2017). It has been estimated that around 0.3–6.2% of the ingested AFB_1 is excreted as AFM_1 in the milk. Ingestion of AFB_2 can also be monohydroxylated in the liver and excreted in milk as aflatoxin M_2, but very little information is available regarding the presence of this biomarker in breast milk (Diaz and Sánchez 2015).

The strong relationship between breast milk and blood compartments leads to rapid and high transference of lipophilic substances. However, the transfer of aflatoxin from blood to breast milk and overall incidence has never been explored sufficiently. Studies on experimental animals and human primates revealed that the ATP-binding cassette G2 efflux transporter protein (ABCG2) which is also known as breast cancer resistance protein (BCRP) is responsible for the transport of aflatoxin biomarkers (both AFM_1 and AFB_1) into the breast milk. This protein is highly expressed in the epithelial cells in several tissues such as the kidney, liver, placenta, intestine, mammary glands, and numerous blood-tissue barriers, and it facilitates the disposition and excretion of various toxins and drugs (Herwaarden et al. 2005; Zhang et al. 2020).

The worldwide prevalence of aflatoxin biomarkers, particularly AFM_1 in human breast milk, is summarized in Table 4.3. The findings of these studies indicated that the incidence rate of AFM_1 in the human breast milk samples from different parts of the world was 0–100% with the value in the range between 0.0001 and 19.0 ng/ml. The highest mean levels of AFM_1 were found in the breast milk of Egyptian mothers (7.0 ng/ml) followed by breast milk samples from United Arab Emirates (3.4 ng/ml) and Ecuador (0.22 ng/ml). Aflatoxin M_1 levels in the breast milk samples from Brazil and Malaysia were found to be under detection limit of the analytical procedure.

The analysis of dietary intake patterns of breastfeeding mothers revealed that the concentration of AFM_1 in the breast milk of most of the lactating mothers was significantly associated with the consumption of bread ($p = 0.04$, $p = 0.001$) (Azarikia et al. 2018; Jafari et al. 2017), cereals ($p \leq 0.05$) (Anthony et al. 2016; Omar 2012; Sadeghi et al. 2009), wheat flour ($p = 0.03$), wheat bulgur (for AFB_1) ($p = 0.003$), barley (for AFB_1) ($p = 0.01$), traditional dough ($p = 0.03$), egg (for AFB_1) ($p = 0.04$) (Azarikia et al. 2018), rice ($p = 0.01$, $p = 0.03$) (Bogalho et al. 2018; Gide et al. 2019), corn ($p = 0.01$) (Gide et al. 2019), wheatmeal ($p \leq 0.05$) (Adejumo et al.

Table 4.3 Worldwide occurrence of aflatoxin biomarkers in human breast milk

Country	Biomarker	Total sample/+ive sample (%)	Mean/range (ng/ml)	Analytical method	References
Brazil	AFM_1	86/0 (0)	<LOD	LC-MS/MS	Tonon et al. (2018)
Brazil	AFM_1	94/5 (5.3)	0.018/0.013–0.025	HPLC-FD	Ishikawa et al. (2016)
Cameroon	AFM_1	62/3.1 (5.0)	NI/(0.005–0.062)	HPLC-FD	Tchana et al. (2010)
Colombia	AFM_1	50/45 (90)	0.005/(0.0009–0.019)	HPLC-FD	Diaz and Sánchez (2015)
Cyprus	AFM_1	50/40 (80)	0.008/0.006–0.028	ELISA	Kunter et al. (2016)
Ecuador	AFM_1	78/10.92 (14)	0.22/0.02–0.46	HPLC-FD	Ortiz et al. (2018)
	AFB_1	78/7.02 (9.0)	0.15/0.01–0.29		
Egypt	AFM_1	150/98 (67.3)	7.1/0.2–19	ELISA	Tomerak et al. (2011)
Egypt	AFM_1	443/248 (56)	0.024/0.004–0.889	HPLC	Polychronaki et al. (2007)
	AFB_1	443/8.86 (2.0)	NI		
Egypt	AFM_1	10/2 (20)	2.75/0.5–5.0	HPLC	Alla et al. (2000)
Iran	AFM_1	84/84 (100)	0.004/0.003–0.009	ELISA	Kamali and Kamali (2020)
Iran	AFM_1	88/88 (100)	0.003/0.0001–0.014	ELISA	Azarikia et al. (2018)
	AFB_1	88/82 (93.2)	0.02/0.01–0.08		
Iran	AFM_1	250/39 (15.6)	0.005/0.01–0.039	ELISA	Jafari et al. (2017)
Iran	AFM_1	85/85 (100)	0.006/0.002–0.010	ELISA	Maleki et al. (2015)
Iran	AFM_1	160/156 (98)	0.008/0.0003–0.027	ELISA	Sadeghi et al. (2009)
Italy	AFM_1	82/4.1 (5.0)	0.055/<0.007–0.140	HPLC	Galvano et al. (2008)

Jordan	AFM_1	80/80 (100)	0.068/0.010–0.137	ELISA	Omar (2012)
Lebanon	AFM_1	111/104.1 (93.8)	0.004/0.0002–0.008	ELISA	Elaridi et al. (2017)
Malaysia	AFM_1	45/0 (0.0)	<LOD	HPLC-FD	Shuib et al. (2017)
Mexico	AFM_1	112/100.02 (89.3)	0.010/0.003–0.034	ELISA	Cantú-Cornelio et al. (2016)
Nigeria	AFM_1	225/225 (100)	0.004/0.002–0.007	HPLC	Ekeanyanwu et al. (2020)
Nigeria	AFM_1	50/48 (96)	0.06/0.05–0.09	HPLC	Gide et al. (2019)
Nigeria	AFM_1	100/82 (82)	0.057/NI	HPLC	Alegbe et al. (2017)
Nigeria	AFM_1	40/31 (77.5)	0.06/NI	HPLC	Anthony et al. (2016)
Nigeria	AFM_1	50/41 (82.0)	0.015/0.005–0.092	HPLC-FD	Adejumo et al. (2013)
Pakistan	AFM_1	125/93.75 (75)	NI/<0.001–0.044	ELISA	Khan et al. (2018)
Portugal	AFM_1	66/21.65 (32.8)	0.01/0.005–0.012	ELISA	Bogalho et al. (2018)
Serbia	AFM_1	10/6 (60)	0.01/0.006–0.022	ELISA	Kos et al. (2014)
Tanzania	AFM_1	143/143 (100.0)	NI/0.010–0.550	HPLC-FD	Magoha et al. (2014)
Turkey	AFM_1	100/53 (53)	0.007/0.005–0.008	ELISA	Muslu and Özdemir (2020)
Turkey	AFM_1	74/65.86 (89)	0.019/0.0096–0.08	ELISA	Altun et al. (2017)
Turkey	AFM_1	73/18 (24.6)	NI/0.001–0.006	ELISA	Atasever et al. (2014)
Turkey	AFM_1	75/75 (100)	NI/0.061–0.299	HPLC	Gürbay et al. (2010)
	AFB_1	75/75 (100)	NI/0.095–4.124		
UAE	AFM_1	140/128 (92)	3.4/NI	HPLC	Abdulrazzaq et al. (2003)

HPLC-FD, high-performance liquid chromatography coupled with fluorescence detector; ELISA, enzyme-linked immunosorbent assay

2013), traditional cream ($p = 0.01$), olives ($p \leq 0.001$), sour cucumber ($p \leq 0.01$) (Jafari et al. 2017), white cheese ($p < 0.05$) (Elaridi et al. 2017), egg ($p = 0.000$), cola drink ($p = 0.003$), sunflower seed oil ($p = 0.026$) (Cantú-Cornelio et al. 2016), chocolate ($p = 0.04$) (Bogalho et al. 2018), spices ($p \leq 0.05$), and vegetables ($p \leq 0.05$) (Muslu and Özdemir 2020).

Despite the efficacy of breast milk as biological fluid for monitoring of both internal and external exposure, its applications in human biomonitoring studies have several limitations. One of the main drawbacks of using breast milk as a non-invasively collected matrix for human biomonitoring is that only mothers and their infants can be included in the biomonitoring studies and only at specific periods of their lives, particularly during lactational periods. Also, the focus of most of the studies is determining the concentration of a toxic substance in the human breast milk without estimating the maternal tissue and/or serum level of toxin as well as levels of toxins in infant's serum. These hurdles in ascertaining the overall range of potential adverse effects from exposure to environmental contaminants on breastfeeding children are on account of poorly designed and/or performed studies, thus providing insufficient data which are not useful for evaluation of human health risk (Lakind et al. 2005).

4.6 Biomarkers of Aflatoxin Exposure in Hair

The analysis of scalp hair in human exposure assessment is receiving attention these days and has been emerged as specific and sensitive procedure complementary to urine and blood analysis because of its advantage of recurrent and non-invasively sample collection procedure, easier storage and transportation, and more stability toward environmental degradation as compared to urine, blood, and tissue samples (Bencko 1995; Smolders et al. 2009). The structure of hair consists of hair follicle, hair shaft, and adjacent dermal layer and is built up of 65–95% protein, 1–10% lipids, 0.1–5% pigments (melanin), and a fraction of polysaccharides, water, and trace elements (Robbins and Robbins 2012; Yu et al. 2017). Melanin has been anticipated as the chief-binding site for drugs and toxins (Karlsson and Lindquist 2016). Toxins are carried from the blood into the hair follicles by the capillaries, are perfused in the hair root, and become entrenched in the keratinized matrices (Sewram et al. 2001). Hair has been efficiently used to estimate both external and internal exposures to a wide array of toxic substances. The analysis of hair of varying length may reveal cumulative exposure over a period of several months, as hair grows around one centimeter/month and perhaps holds toxin permanently (Bermejo-Barrera et al. 1998; Wilkins et al. 1998).

Despite several advantages, the application of hair matrix in molecular epidemiology is facing several challenges, and these challenges are highlighted by the expert panel of the Agency for Toxic Substances and Disease Registry (ATSDR 2001). One of the major drawbacks of the hair matrix is difficulty in distinguishing the external contamination and internal exposure. Also, the lack of available data to predict adverse health effects through measurement of hair, lack of standard values for accurate interpretation, the absence of association blood, hair levels, and other

tissues are limiting the practicality of hair analysis. However, these limitations cannot mask the undeniable advantages of the hair matrix analysis for toxicological experiments and can be overcome by developing standardizing procedures for sample collection, sample preparation, and extraction of the substance to be analyzed and by better understanding of hair physiology (ATSDR 2001).

Human exposure to numerous environmental contaminants such as toxic metals, pharmaceutical drugs, and persistent organic pollutants has widely been studied using hair as biological matrix (Lehmann et al. 2018; Pereira et al. 2004; Sera et al. 2002; Sauvé et al. 2007; Tsatsakis et al. 2008; Wang et al. 2018). However, relatively little is presently known about the analysis of mycotoxins, particularly aflatoxins in human hair. The use of hair as a matrix for analysis of chronic mycotoxin exposure was first explored by Sewram et al. (2003). The authors investigated the level of fumonisins mycotoxins (FB1, FB2, and FB3) in the hair samples of South African population using high-performance liquid chromatography coupled with electrospray ionization-mass spectrometry (HPLC-ESI-MS) technique. The mean levels of FB_1, and FB_2 were recorded to be 27.7 μg/kg and 7.8 μg/kg, respectively. The method adopted resulted in the 81–101% recovery of the analyzed mycotoxin, thus illustrating the suitability of hair analysis in assessment of human exposure to mycotoxins. Later, Bordin et al. (2015) evaluated the dietary exposure of FB_1 by detection of FB_1 residues in hair samples of 56 individuals from Brazil. Fumonisin B_1 was found in 7.2% of the analyzed hair samples with an average concentration of 21.3 ng/g. In the same study, the FB_1 level in the hair samples of individuals was significantly associated with probable daily intake of FB_1 estimated from consumption of corn products.

To date, only a single study reported the AFB1 in human hair (Sabzevari et al. 2006). In this study, 50 mg of hair samples were obtained from the healthy volunteers, and AFB1 was analyzed using HPLC technique. There is a huge gap in consideration of aflatoxin biomarkers in hair, and this gap appears to be opportunistic for early proof-of-concept studies that could provide the basis for population-level surveillance studies.

4.7 Analytical Methods to Identify Aflatoxin Biomarkers in Biological Fluids

To carry out human biomonitoring studies, adequately sensitive and validated analytical procedures are required. Determination of mycotoxin biomarkers in the biological matrices may involve a direct, indirect, and non-targeted approach. Direct analysis employs standardized, properly optimized, and validated analytical procedures. This approach is pertaining to parent compounds only, as fewer modified substances are available as reference. To solve this issue, indirect approach can be used. In such a case, modified mycotoxins are converted into their free forms that can then be evaluated using routine procedures. Till now, both direct and indirect approaches based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been used for identification and quantification of unmodified and modified mycotoxins (Broekaert et al. 2015; Rychlik et al. 2014).

Various analytical procedures have been developed and adopted to quantify the metabolic products of aflatoxin metabolism in different biological matrices. Each procedure possesses exclusive specificity and sensitivity, providing the opportunity to select suitable procedures for particular applications. For instance, to measure a single AF metabolite, a method based on chromatography can separate the mixtures of aflatoxins into individual substances, as long as the interfering chemicals are not introduced by the extraction procedure. Antibody-based procedures are usually more sensitive than chromatographic methods; however they are not highly selective because of the danger of cross-reactivity of antibody with the aflatoxin metabolites. High-performance liquid chromatography (HPLC) procedure with immunoaffinity column cleanup was developed to measure and isolate the aflatoxin's metabolites in the biological fluid samples. Recent reports using isotope dilution mass spectrometry coupled with liquid chromatography separation have confirmed a surge in the sensitivity of overly 1000-fold over technologies used for the identification of aflatoxin biomarkers 15 years ago and are currently the "gold standards" for mycotoxin analysis (Kensler et al. 2011).

4.8 Exposure Assessment Through Dietary Intake

So far, the main approach to assess the human exposure to mycotoxins is the combination of consumption and mycotoxins' occurrence data, usually known as indirect or external approach. Human external exposure to aflatoxins can be estimated by calculating estimated daily intake (EDI), which is normally calculated as the product of aflatoxin concentration in the food and per day mass of the food intake and then divided by individual body weight (Akhtar et al. 2020; Ismail et al. 2020). This approach presents some constraints. The first arises from the assessment of levels of mycotoxins in the food commodities, as these are not homogenously distributed within the given food matrix. Additionally, several mycotoxins might be associated with the matrix substances or possibly be modified in the raw food by any chemical or biological process and are hence not detectable by the analytical methods. Further, there is complexity in obtaining the accurate information on food consumption. Lastly, the existence of these toxic substances in the food matrix does not certainly indicate that humans have been exposed to them. The bioavailability of these toxins can vary based on various factors, i.e., nature and composition of food, type of processing has gone through, and the inter- and intra-individual differences (Ediage et al. 2012; Ediage et al. 2013). Therefore, it is very difficult to achieve the accurate risk assessment by merely using these data. Hence, the monitoring of aflatoxins and their metabolites in human biological fluids and tissues is crucial for assessment of potentially hazardous exposure to this toxin.

As aflatoxins are known carcinogens in humans, exposure to these mycotoxins may result in growth impairment, immune suppression, and hepatic damage even at very low levels. Therefore, it is recommended that aflatoxin exposure should be "as low as reasonably achievable" (ALARA). The aflatoxin intake of even minor concentration such as 1.0 ng/kg bw/day is considered toxic to humans (EFSA 2020). The worldwide estimated daily intake of aflatoxins calculated from the consumption of aflatoxin contaminated food commodities is given in Table 4.4. From the

Table 4.4 Worldwide estimated daily intake of dietary aflatoxins

Country	Population (n)	Type of aflatoxin	Dietary exposure medium	EDI (ng/kg bw/day) mean/range	References
Brazil	Teenagers, adults, elders	AFB_1	Bakery products	0.2–5.2/NR	Bol et al. (2016)
Brazil	Adults	AFs	Peanuts and products	NR/6.6–6.8	Andrade et al. (2013)
Brazil	Adolescents	AFM_1	Milk	0.468/NR	Dos Santos et al. (2015)
	Adults			0.38/NR	
	Elderly			0.56/NR	
Cameroon	Children (108)	AFs	Maize-based dishes	43.8/NR	Nguegwouo et al. (2016)
	Adolescents (102)			31.9/NR	
	Adults (156)			27.4/NR	
China	Adults	AFB_1	Total diet	NR/0.2–2.8	Yau et al. (2016)
China	Children, teenagers, adults	AFB_1	Cereals and products, nuts, tea, vegetable oil	0.57/0.48–0.94	Zhang et al. (2020)
China	Children	AFB_1	Peanuts	NR/0.777–0.790	Ding et al. (2015)
	Adults			NR/0.34–0.35	
China	All age groups	AFM_1	Milk	0.009/NR	Guo et al. (2013)
Colombia	Adults	AFB_1	Arepa, bread, rice	1.14/0.004–2.80	Martinez-Miranda et al. (2019)
Egypt	Adults	AFB_1	Wheat	33.25/0–232.8	Hathout et al. (2020)
		AFs		32.57/29.32–232.80	
France	Adults	AFB_1	Total diet	NR/0.002–0.22	Sirot et al. (2013)
	Children	AFB_1	Total diet	NR/0.001–0.39	
France	Adults	AFs	Total diet	0.117/NR	Leblanc et al. (2005)
	Children	AFs	Total diet	0.323/NR	
Ghana	Infants	AFB_1	Processed cereals-based foods	146/11–852	Blankson and Mill-Robertson (2016)
	Children			108/9–657	
Ghana	Infants	AFs	Rice, cereal-based foods, pasta	342.43/NR	Kortei et al. (2019)
	Adults			95.97/NR	
Greece	Adults	AFM_1	Milk and yogurt	NR/0.350–0.499	Udovicki et al. (2019)
Iran	Adults	AFB_1	Dried fruits	0.065/NR	Heshmati et al. (2017)
Iran	Infants	AFM_1	Infant formula milk	0.074/NR	Hooshfar et al. (2020)
Iran	Adults	AFM_1	Milk	0.11/NR	Nejad et al. (2019)

(continued)

Table 4.4 (continued)

Country	Population (n)	Type of aflatoxin	Dietary exposure medium	EDI (ng/kg bw/day) mean/range	References
Iran	Adults	AFB_1	Rice, bread, puffed corn snack, and peanut	3.62/NR	Yazdanpanah et al. (2013)
Italy	All age groups	AFM_1	Milk	NR/0.03–0.34	Serraino et al. (2019)
India	Adults	AFM_1	Milk	1.33/NR	Sharma et al. (2020)
Jordan	Infants	AFM_1	Infant formula milk	1.56/NR	Awaisheh et al. (2019)
Kenya	Adults	AFs	Maize kernels	292/0.22–180,704	Kilonzo et al. (2014)
Kenya	Adults	AFM_1	Milk	0.8/NR	Ahlberg et al. (2018)
	Children			3.5/NR	
Kenya	NM	AFs	Maize kernel	292/1–180,704	Kilonzo et al. (2014)
			Maize meal	59/0–1144	
Lebanon	Adults	AFB_1	Total diet	NR/0.63–0.64	Raad et al. (2014)
Malaysia	Adults	AFB1	Nuts and products	0.36 and 8.89/NR	Leong et al. (2011)
Malaysia	Adults	AFB1	Total diet including peanuts	NR/24.37–34.0	Chin et al. (2012)
		AFs		NR/28.81–58.02	
Mexico	Children	AFM_1	Infant formula	NR/1.56–14	Quevedo-Garza et al. (2020)
Nigeria	Infants	AFs	Groundnut	1123.7/NR	Oyedele et al. (2017)
	Children			449.5/NR	
	Adults			187.3/NR	
	Children			763.3/NR	.
Nigeria	Infants and children	AFB_1	Complementary foods	NR/2.5–51,192	Ojuri et al. (2018)
		AFs	.	NR/25.7–54,892	.
North Central Nigeria	Adults	AFs	Rice, corn, millet, sorghum, garri, yam flour	65813.3/NR	Onyedum et al. (2020)
Pakistan	Adults	AFs	Rice	NR/19.1–26.6	Iqbal et al. (2012)
Pakistan	Adults	AFs	Black tea	0.337/NR	Ismail et al. (2020)
Pakistan	Adults	AFs	Spices	1.16/0.66–3.29	Akhtar et al. (2020)
Pakistan	Infants and adults	AFM_1	Milk	NR/0.22–5.45	Ismail et al. (2016)
Portugal	Children	AFB_1	Cereal-based food	0.003/NR	Assunção et al. (2018)

(continued)

Table 4.4 (continued)

Country	Population (n)	Type of aflatoxin	Dietary exposure medium	EDI (ng/kg bw/day) mean/range	References
.	.	AFM_1	.	0.058/NR	
Qatar	Adults	AFB_1	Cereals, dried fruits, nuts, grains, spices	18.74/NR	Al Jabir et al. (2019)
Saudi Arabia	Adults	AFB_1	Processed poultry meat, beef meat products	0.94/NR	Elzupir and Abdulkhair (2020)
		AFs		1.03/NR	
Serbia	Adults	AFM_1	Milk and yogurt	NR/1.238–2.674	Udovicki et al. (2019)
Spain	Adolescents	AFB_1	Coffee	3.0/NR	García-Moraleja et al. (2015)
		AFs		36/NR	
	Adults	AFB_1		1.0/NR	
		AFs		8.0/NR	
Spain	Children	AFB_1	Infants cereals	NR/0.12–29.06	Hernandez-Martinez and Navarro-Blasco (2010)
		AFs		NR/0.17–37.47	
Taiwan	All age groups	AFs	Peanut and products	0.03/NR	Wang et al. (2018)
Thailand	Children, teenagers, adults	AFB_1	Peanuts	0.66/NR	Kooprasertying et al. (2016)
		AFs		0.8/NR	
Tunisia	Adults	AFB_1	Millets	3.89/NR	Lasram et al. (2020)
Turkey	Adults	AFB_1	Surk cheese	0.203/NR	Sakin et al. (2018)
		AFM_1		0.057/NR	
Turkey	Adults	AFB_1	Hazelnuts	NR/0.014–0.016	Kabak (2013)
			Dried figs	NR/0.003–0.003	
		AFs	Hazelnuts	NR/0.018–0.023	
			Dried figs	NR/0.004–0.005	
Vietnam	Adults	AFB_1	Total diet	39.4/NR	Huong et al. (2016a, 2016b)
Vietnam	Adults	AFB_1	Rice	21.7/NR	Huong et al. (2016a, 2016b)
	Children			33.7/NR	
Zimbabwe	Adults	AFB_1	Maize	0.052/0.0076–0.355	Murashiki et al. (2017)

studies included in Table 4.4, it was observed that the human dietary exposure to aflatoxins ranged between 0 and 180,704 ng/kg bw/day with the average value of 0.0009–65,813 ng/kg bw/day. Higher dietary exposure to aflatoxin has been reported from developing countries such as Ghana, Nigeria, Kenya, Egypt, and Cameron as compared to developed countries such as Brazil, France, Turkey, and Zimbabwe. The data assembled revealed that the aflatoxin exposure from total diet studies is limited and the dietary exposure calculated by most of the authors is based on specific food types. The findings revealed that the dietary exposure of aflatoxin calculated from total diet studies was 0.2–2.8 ng/kg bw/day from China, 0.002–0.39 ng/kg bw/day from France, 24.37–58.02 ng/kg bw/day from Malaysia, and 39.4 ng/kg bw/day from Vietnam. The exposure measured from the consumption of cereals and products including maize was 0–180,704 ng/kg bw/day. The exposure measured from the consumption of nuts and nut products including peanuts was 0.03–1123 ng/kg bw/day. The exposure measured from the consumption of milk and milk products was 0.03–5.45 ng/kg bw/day. The exposure measured from the consumption of complementary food including infant milk formula was 0.12–852 ng/kg bw/day. There are differences in the demographics (such as age, body weight, etc.) and food consumption habits of people from different countries and regions. This difference affects the consumption of specific food types and its consumption frequency as well as the estimated exposure to carcinogens.

4.9 Conclusion

Exposure assessment can be performed using occurrence data in food and consumption data and by monitoring the biomarkers in the biological fluids. However, biomonitoring is the best approach to accurately measure the actual levels of aflatoxin exposure. AF-alb (AFB_1-lys) adduct, urinary AFM_1, urinary AF-N^7-gua, and AFM_1 in the breast milk have been the most widely studied biomarkers of aflatoxin exposure. Data on the presence of aflatoxin biomarkers in the human biological fluids showed higher dietary exposure to aflatoxins, particularly in developing countries. Though aflatoxins and their metabolites were reported very frequently in the biomonitoring assays worldwide, noticeable effort is imperative in the evaluation of aflatoxin biomarkers in high-risk areas in order to better estimate the actual exposure to dietary aflatoxins. Additionally, further investigations on toxicokinetics to better understand the aflatoxin metabolism in the humans and to investigate the potential of various products of aflatoxin metabolism to act as biomarkers of aflatoxin exposure and biological effective dose are required.

Based on our existing knowledge of relationships between external exposure, internal exposure, and the risk of adverse health effects, biomonitoring of toxic substances that are absorbed substantially and employ systemic toxicity may give different information. If only the association between the internal dose and external exposure is known, the biological parameter can be used as a benchmark of exposure, but it provides limited information on the health risk. While if a

quantitative association between internal exposure and adverse effects and the internal dose effects and internal dose-response relationships are known, biomonitoring studies provides information on direct health risk assessment and for effective prophylaxis for adverse health effects.Conflict of InterestThe authors declare no conflict of interest.

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Chapter 5
Health Effects of Aflatoxins in Fetus, Infants, and Children

Mamoona Amir, Arif Shahzad, Asim Faraz, Muhammad Sajid, Khurram Afzal, Iqra Naeem, Amir Ismail, and Zarmina Mumtaz

Abstract Aflatoxins are group 1 category carcinogenic compound that are reported in several food commodities well beyond the maximum permissible limits. Early days of life, i.e., intrauterine life, infancy period and early childhood days are the period during which the toxic compounds bring severe and permanent health implications. A number of researchers from all parts of the world have reported the prevalence of aflatoxins in mother food items, mother milk, infant foods, and children food items indicating the chances of serious health implications in these age groups. In this chapter, the impact of aflatoxins on human health during early days of life is summarized including aflatoxin exposure and birth defects, birthweight, preterm birth, possible mechanism of aflatoxin-induced birth defects and growth impairment, aflatoxin's impact on stunting, wasting and underweight, and aflatoxin's impact on immunosuppression and hepatic dysfunction.

Keywords Aflatoxins · Intrauterine life · Infancy · Early childhood · Health · Birth defects

5.1 Introduction

Intrauterine life, infancy, and early childhood are broadly considered as highly critical windows to exposures to most of the toxic substances such as heavy metals, endocrine-disrupting chemicals (EDCs), and mycotoxins. Also, continual exposure

M. Amir · A. Shahzad · K. Afzal · I. Naeem · A. Ismail · Z. Mumtaz
Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan

A. Faraz
Department of Livestock and Poultry Production, Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, Pakistan

M. Sajid (✉)
Institute of Chemical Sciences, Bahauddin Zakariya University, Multan, Pakistan
e-mail: dr.msajid@bzu.edu.pk

K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_5

to adulthood to a level of no significant concern might prompt irremediable health outcomes in the developing organism with everlasting sequelae. Although pathogenetic mechanisms are not fully understood, increasing evidence from both humans and experimental animals highlights the reduced capacity of the developing organism for rehabilitation after toxic insults to highly vulnerable processes (Frazzoli and Mantovani 2020).

Fetal exposure to toxic substances is in part due to their transfer from the maternal body via placenta (Gundacker and Hengstschläger 2012). Exposures that happen in utero can lead to latent and more subtle health consequences based primarily on the exposure dose, timing, particular toxic substance, administration route, and maternal or fetal genotype (Kilcoyne and Mitchell 2019). In the first week of gestation, exposure to toxic substances generates a tendency for greater outcomes which are either termination of pregnancy or continuation of development with the likelihood of effects that are not readily absorbable, i.e., impaired development of blastocyst cells. However, exposures from the second week of gestation to onward manifest as anomalies of varying extent and for various biological functions. The concentration of toxic substances in the maternal body can be increased from simultaneous exposures as well as exposure from mobilization of already stored toxic substances during pregnancy, thus exacerbating the fetal toxicity and developmental defects. The diffusion rate of toxic substances from the maternal body to the fetus increases from the fourth week of gestation especially in the late gestation (Behnke et al. 2013).

During infancy and early childhood, the exposure to toxic substances mainly occurs through the ingestion of breast milk and/or infant formula and weaning food (Coppa et al. 2019; Gummadidala et al. 2019). Though breast milk is a gold standard diet for neonates because of its dynamic and complete nutritional profile such as oligosaccharides, fats, peptides, growth factors, and immunoglobulins that ensure the optimal growth and development of the infant (Victora et al. 2016), it can be a source of environmental toxins that are harmful to the health of infants. It is reported that a single feeding can transfer 1–2% of the absorbed dose of a toxic substance from the maternal body to the infants. However, based on the substance, a greater amount can be transferred cumulatively over the entire breastfeeding period, perhaps contributing to the body burden of an infant. Around 0.3–6.2% of the ingested aflatoxin B_1 (AFB_1) has been reported to be secreted as AFM_1 in the breast milk (Ayar et al. 2007; Unusan 2006). Similarly, infant formula is a substitute of breast milk and has a composition that can satisfy the nutritional needs of infants during the first month of life up to the introduction of complementary food. Thenceforth, the infants from the sixth month on and young children (12–36 months or longer) are fed on follow-up formula and complementary food based on both animal and plant origin (de Mendonça et al. 2020). Aflatoxin contamination of breast milk (Khan et al. 2018; Sadeghi et al. 2009), infant formulas (Akhtar et al. 2017; Hooshfar et al. 2020; Omar, 2016; Quevedo-Garza et al. 2020), and complementary foods (Ayelign et al. 2018; Blankson and Mill-Robertson 2016) provides evidence of dietary exposure to aflatoxins during these critical life periods. Transition stage from mother feeding to baby foods is the major cause of aflatoxin-related growth

defects as baby foods have more aflatoxins as compared to mother milk (Gong et al. 2003).

Fetus, neonates, infants, and young children are particularly more susceptible to the exposure to toxic substances because of several reasons. After ingestion, toxic substances are absorbed through the surface of the gastrointestinal tract before their distribution in the various body compartments (Papadopoulou et al. 2019). In the first month of life, the gastrointestinal (GI) function is immature and is developing rapidly. The absorption of substances from the intestinal wall increases due to higher absorptive surface area of the small intestine. Also, as the GI motility is immature during early days of life, this leads to varying decreases in the absorbed dose or quicker absorption. While in early childhood, faster gastric emptying (above adult levels) possibly results in greater serum concentrations due to faster absorption, but not essentially a higher extent of absorption. During this period of life, most of the GI activities are mature, though the small intestine remains smaller as compared to adults. Moreover, the neonates have reduced plasma protein levels, qualitatively varying protein content, and increased levels of endogenous substrates, i.e., bilirubin and free fatty acids, that lead to reduced binding and rearrangement of xenobiotics from binding sites and ultimately increased systemic circulation of free forms of xenobiotics. Additionally, the higher body water percentage as compared to fat in early days of life, along with reduced protein-binding capacity, enhances the greater distribution of some xenobiotics, particularly polar substances. This can enhance the equilibrium concentration at target body organs and increase the toxicity potential of a toxic substance. Furthermore, for newborns, most of the metabolic processes are under-developed (except for glutathione conjugation and phase II sufation), leading to suppressed metabolic activities and prolonged removal of substances which are dependent on these metabolic pathways, while during infancy and childhood, the metabolic system is developing continuously and producing metabolites that differ from those of adults. Lastly, the renal and biliary elimination function is at lower levels during early life periods, leading to unproductive filtering and elimination of smaller fractions of protein-bound circulating substances (Hulin et al. 2014; Papadopoulou et al. 2019).

This chapter focuses on the potential adverse health outcomes of dietary aflatoxin exposure during early life particularly in fetus, infants, and children (Fig. 5.1).

5.2 Health Effects of Aflatoxins in Fetus, Infants, and Children

5.2.1 Aflatoxin Exposure and Birth Defects

Pregnancy is a critical period during which the fetal development is most likely to be at risk of harmful exposures. Birth defects are the primary cause of mortality in infants, contributing around more than 20% of all infant mortalities (Kenner and

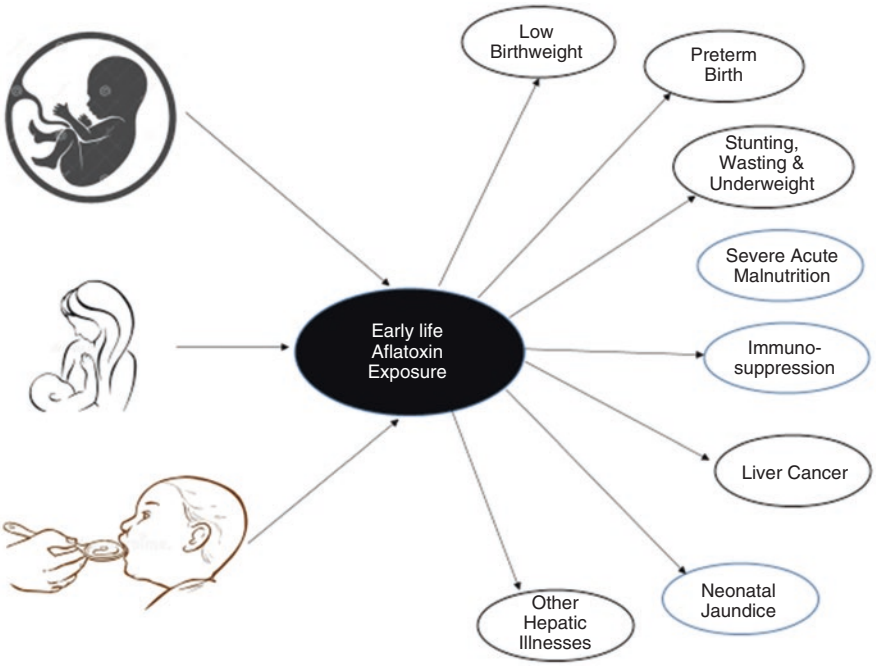

Fig. 5.1 Aflatoxins exposure during early life and health implications

Premji 2019). During pregnancy, many physiological changes take place in the maternal body that can affect the uptake, absorption, assimilation, metabolism, and elimination of the xenobiotics in both the mother and fetus. For instance, reduced intestinal motility and minimal gastric emptying during pregnancy can enhance the absorption of certain chemical substances (WHO 2006). Also, gestation and postpartum period have been related to significant alterations in the gut microbiota, fat redistribution, metabolic syndrome, increased epithelial permeability, reduction in plasma levels of albumin, and increased concentration of endotoxin in plasma (Kerr et al. 2015). All of these factors can affect the distribution and possible health outcomes of xenobiotics in the mother-child dyad.

The developing fetus is protected from exposure to toxic substances by several layers of defense. The first layer of defense is the maternal biotransformation system and metabolism. Besides terminating the toxicokinetics, the biotransformation accelerates the elimination of xenobiotics from the body, thus reducing the accessibility of the parent substance to the placenta. Placenta itself can act as a layer of defense (second layer of defense) (Blumenfeld et al. 2010). It is an important organ for embryonic and fetal growth and development during pregnancy and acts as a semipermeable barrier that segregates the maternal and fetal body and modulates the transfer of gases, nutrients, wastes, endogenous substances, and xenobiotics between maternal and fetal circulations. In addition, the fetal membrane, which

surrounds the fetus in utero, also plays an important role in protecting the fetal body from xenobiotics (Prouillac and Lecoeur 2010).

The transfer of toxic substances from the mother to fetus across the placenta is similar to the transport mechanisms across other biological membranes, thus increasing the exchange rate as the growth rate of the fetus increases (Porpora et al. 2013). Though these layers of defense acts as barriers for most of the toxic substances, these cannot act as an unpassable barrier against all the toxic substances (Partanen 2012). Some of the toxins such as aflatoxins have the ability to cross the placenta and impart adverse effects on the growth and development of a fetus (Partanen et al. 2009). It is reported that CYP3A7 in the human fetal liver can metabolize AFB_1 into aflatoxin B_1 epoxide (Li et al. 1997). Also, an in vitro study on human placental perfusions revealed that the human placenta has the capability to uptake AFB_1 and to metabolize it into a relatively less toxic form i.e., aflatoxicol (Partanen et al. 2009). Similarly, the presence of AFB_1-DNA adducts in 57.5% and 8.8% samples of human placenta and cord blood in concentration from 0.6 to 6.3 μmol/mol DNA (Hsieh and Hsieh 1993) also supports the evidence that AFB_1 and its metabolites are transported across the transplacental unit and are exposed to the developing fetus.

Studies in animal models showed that aflatoxin exposure, at varying doses and duration, resulted in reduced fetal weight (Butler 1971; El-Nahla et al. 2013; Schmidt and Panciera 1980; Wangikar et al. 2004, 2005;), reduced fetal growth (Schmidt and Panciera 1980), reduced birthweight (Kihara et al. 2000), reduction in crown to rump length (El-Nahla et al. 2013; Wangikar et al. 2004; Wangikar et al. 2005), and increase in fetal malformation and skeletal anomalies (Wangikar et al. 2004; Wangikar et al. 2005; El-Nahla et al. 2013). Several human studies reported the prevalence of aflatoxins in maternal and cord blood suggesting that human fetus is exposed to aflatoxins when mothers are feed to aflatoxin-contaminated diet (Castelino et al. 2013; Groopman et al. 2014; Lamplugh et al. 1988; Turner et al. 2007). In humans, the effect of aflatoxin exposure on fetal health has not been explored completely. Data only on the role of aflatoxins in intrauterine growth restriction (as measured by low birthweight, decreased birth size, and small-for-gestation age) and preterm birth is available.

5.2.1.1 Low Birthweight

Low birthweight is defined as a birthweight less than 2.5 kg and is assessed by the gestation period and the rate of fetal growth. An infant's low weight at birth is either because of preterm birth or due to small-for-gestational-age size (Kramer 2003). According to an estimate, more than 20 million newborns, representing around 15% of all child birth worldwide, are delivered with low birthweight, and about 96% of these cases are recorded in the developing countries. Babies born with low weight are at higher risk of morbidity and mortality, suppressed growth and cognitive development, and chronic illnesses later in life (WHO 2004). Various human studies

investigated the effect of maternal aflatoxin exposure on birthweight of newborns. Abdulrazzaq et al. (2002) measured the levels of aflatoxin in umbilical cord blood of mothers (n = 201) from the UAE who delivered consecutively to ascertain whether the fetuses had been exposed to aflatoxin significantly. The aflatoxin level in the cord blood was found to be significantly negatively correlated with the birthweight ($p<0.001$). These findings are consistent with the results of a prospective study by Shuaib et al. (2010a, b) who reported that pregnant women (n = 785) with the highest aflatoxin exposure (AFB_1-lysine level higher than 11.34 pg/mg albumin) were at higher risk of giving birth to low-birthweight babies as compared to those with lower aflatoxin exposure (AFB_1-lysine ≤ 2.67 pg/mg albumin) ($P_{trend} = 0.007$). Similarly, a prospective cohort study by Lauer et al. (2019) revealed that, apart from affecting the birthweight, in utero aflatoxin exposure imparted negative effects on z-scores and head circumference. In this study, increase in AFB_1-lysine levels in the mothers (median = 5.83 pg/mg) was significantly correlated with reduced birthweight (p = 0.04), weight-for-age Z values (p = 0.04), head circumference (p = 0.035), and head circumference for age z-score (p = 0.023) in the newborns. Being inconsistent with these report, no significant association between AFB_1-lysine levels in mothers (average = 1.4 pg/mg albumin) and birthweight of children was reported in Tanzania (Passarelli et al. 2019). The authors of the study declared that these inconsistent findings might be due to the fact that the studied participants were from urban areas having comparatively low exposure, and the mothers chosen were iron-depleted and anemic (Passarelli et al. 2019).

5.2.1.2 Small-for-Gestational Age

The term small-for-gestational age (SGA) represents a baby's birthweight of lower than 10th percentile for gestational age (Ng et al. 2019). Andrews-Trevino et al. (2019) investigated the relationship between maternal aflatoxin exposure and adverse birth outcomes including SGA in a birth cohort study from Nepal. Thirty-two percent (total = 1621) of the studied infants were SAG, and the maternal serum AFB_1-lysine level (average 1.37 pg/mg alb) was significantly correlated with SGA ($p < 0.05$). Fetal exposure to aflatoxin may also impart a direct effect on growth during early childhood. Turner et al. (2007) investigated the association of in utero aflatoxin exposure with impaired growth during the 1st year of life in Gambian infants. The authors reported that maternal AF-alb level (40.4 pg/mg alb) was found to be a strong predictor of height (p = 0.012) and weight gain (p = 0.012) of child who indicated the lower gain in children with higher exposure. Also, in the same study, it was found that reduction of AF-alb levels (from 110 pg/mg alb to 10 pg/mg alb) would lead to 2 cm height gain and 0.8 kg weight gain within the 1st year of life.

5.2.1.3 Preterm Birth

Preterm birth (PTB) or premature delivery occurs in earlier than 37 weeks of pregnancy (WHO 2004). It can be one of the most significant factors contributing to death and disease burden in neonates, mainly in the case of very early PTB that occurs earlier than 32 weeks of pregnancy. Each year, around 15 million newborns are premature, contributing more than 1 million deaths worldwide (WHO 2018). Studies have reported that PTB is linked with several health problems such as chronic lung disease, hearing and vision loss, neurodevelopmental disorders, and gastrointestinal disturbance (Harris et al. 2020). In utero aflatoxin exposure has been reported to cause maternal inflammation, which is the only pathological pathway having an obvious association with preterm labor (Humberg et al. 2020). Anemia in pregnant women can also be a diverse cause of preterm birth and has been significantly correlated with serum AFB_1-lysine concentration (Shuaib et al. 2010a). Passarelli et al. (2019) investigated the association of aflatoxin exposure and gestational age in the Tanzanian population and observed that an elevated natural log of AFB_1-lysine concentrations by one unit of pg/mg of albumin resulted in a significant decrease in the gestational age at birth (0.472 weeks; CI, −0.86 and −0.07). Andrews-Trevino et al. (2019) reported that 13% of the studied Nepalese infants from the AflaCohort were born preterm. However, in this study, no significant association was found between maternal serum AFB_1-lysine concentration and preterm birth.

These reports suggest that there is an association between in utero aflatoxin exposure and poor birth outcomes, particularly low birthweight. However, the available literature is not enough to conclude the relationship between aflatoxin exposure and other birth defects such as reduced birth size and preterm birth and highlights the need for further research.

5.2.1.4 Possible Mechanisms of Aflatoxin-Induced Birth Defects

The possible mechanism through which aflatoxins cause adverse birth outcomes is not completely understood yet. Nonetheless, it has been reported that aflatoxin causes modulation of cytokine expression (upregulation of pro-inflammatory cytokines and/or downregulation of anti-inflammatory cytokines), inflammatory and poor barrier response of the intestine that leads to systemic immune activation, toxicity of maternal organs resulting in systemic immune activation and placental and fetal developmental anomalies, and toxicity of fetal organs causing fetal inflammation and undermined fetal development (Smith et al. 2017). Additionally, in utero exposure to genotoxic substances may lead to epigenetic modifications in the fetus and newborn that may result in permanent adverse health outcomes (Goodrich et al. 2016). In a study by Hernandez-Vargas et al. (2015), maternal aflatoxin exposure during pregnancy was linked to DNA methylation in newborns on 71 CpG sites, with mean effect size of 1.7% alteration in methylation. It was observed that

aflatoxin exposure resulted in differential methylation of growth factor genes (such as *FGF12* and *IGF1R*) and immune-related genes (such as *CCL28*, *TLR2*, and $TGFB_1$).

5.2.2 Aflatoxin Exposure and Child Undernutrition

Child growth is globally considered as the prime indicator of a child's physical well-being as poor dietary practices, both in quality and quantity, and infectious diseases are the major factors that influence the growth and development in children. Growth impairment has been associated with an increased risk of cognitive impairment and infectious diseases in children and is regarded as a leading factor in child mortalities and economic damage in low- and middle-income countries. Each year, millions of death cases among children younger than 5 years are attributable to child growth impairment (Black et al. 2013). Undernutrition may be of three forms, i.e., underweight, wasting, and stunting (Mawa and Lawoko 2018). Stunting, a condition when child's height-for-age *z*-score (HAZ), is equal to or less than two standard deviations of the WHO child growth standard's median value (*z*-score ≤ -2) and is the most extensively used benchmark of chronic undernutrition. Children with weight-for-height *z*-score (WHZ) and weight-for-age *z*-score (WAZ) measurements equal to or below two standard deviation reference values of WHO are classified as wasted and underweight, respectively. Besides, children with WHZ or BMI-for-age *z*-score less than −3 SD of the median value or presence of bilateral edema or mid-upper arm circumference less than 115 mm are categorized in severe acute malnutrition, another form of child undernutrition (WHO 2017).

5.2.2.1 Stunting, Wasting, and Underweight

Dietary aflatoxin exposure has been reported to be implicated in the etiology of growth impairment, particularly child stunting, though the association between aflatoxin exposure and child underweight and wasting have also been investigated but with lesser consistency. Several studies have reported the association between dietary aflatoxin exposure and all three indicators of child undernutrition, and these studies have been periodically reviewed by various researchers (Chilaka and Mally 2020; Gong et al. 2012; IARC, 2015; Khlangwiset et al. 2011; Smith et al. 2012; Smith et al. 2015; Tesfamariam et al. 2020). These studies concluded that though aflatoxin exposure in early childhood had a significant effect on child growth and development and the weight of evidence supporting aflatoxin-induced growth suppression has been increasing over the last decades, establishing a causal link between aflatoxin exposure and child growth impairment is difficult (Gong et al. 2012; Khlangwiset et al. 2011) as some of the reports found no significant relationship between aflatoxin exposure and growth retardation during early childhood, implying that chronic dietary aflatoxin exposure at low doses might require more time to

show noticeable health effects, and their might be certain threshold dose of aflatoxin below which child continues to grow without any adverse effects (Chen et al. 2018; Mahfuz et al. 2020; Mitchell et al. 2017; Shirima et al. 2015).

Recently, a study by Rasheed et al. (2021) has estimated the health burden of aflatoxin-related stunting in children younger than 5 years from the four African countries including Benin, Togo, Tanzania, and Gambia by calculating the population attributable risk (PAR), and lifetime burden of disease for under 5-year-old children using both prevalence- and incidence-based approaches. The authors combined the prevalence data with a disability weight, estimating child stunting and co-occurrence of stunting and underweight to calculate years lived with disability (YLD), and estimated the years of life lost using mortality data. The disability-adjusted life years (DALYs) were estimated using probabilistic analysis and were compared with those provided by the Institute for Health Metrics and Evaluation's Global Burden of Disease (GBD) 2016's report. The study reported that the population attributable risk increased from 3% to 36% for aflatoxin attributable stunting and 14–50% for stunting-underweight co-occurrence. It was observed that aflatoxin attributable to child stunting resulted in 48,965.2 (95% uncertainty interval 45,868.75–52,207.53) DALYs per 10^5 individuals, while co-occurrence of child stunting and underweight resulted in 40,703.41 (95% uncertainty interval 38,041.57–43,517.89) DALYs per 10^5 individuals. The authors of the study declared that stunting DALYs could be saved up to 50% by minimizing the AF exposure up to undetectable concentration in the areas with high aflatoxin exposure.

5.2.2.2 Severe Acute Malnutrition

Severe acute malnutrition (SAM) is the most recurrent form of child undernutrition that kills children and mentally mutilates those who survive. Globally, around 20 million children younger than 5 years endure SAM, and around 1 million death cases are mainly attributable to SAM (Ahmed et al. 2014). SAM is mainly categorized into kwashiorkor (an edematous form with moderately acute wasting) and marasmus (a non-edematous form with severe emaciation) (Bhutta et al. 2017). Aflatoxins have been regarded as the underlying cause of SAM, particularly stunting (De Vries et al. 1989; Hendrickse et al. 1982). Since 1980s, several studies have investigated the association between kwashiorkor and aflatoxin exposure in children (Adhikari et al. 1994; Coulter et al. 1986; De Vries et al. 1989, 1990; Hatem et al. 2005; Hendrickse et al. 1982; Househam and Hundt 1991; McMillan et al. 2018; Onyemelukwe et al. 2012; Oyelami et al. 1997, 1998; Ramjee et al. 1992; Tchana et al. 2010). Children with kwashiorkor were observed to have more frequent serum concentration of aflatoxins and its metabolites. Recently, in a study of Nigerian children (aged between 6 and 48 months), who were facing SAM, aflatoxin exposure was correlated with different forms of SAM. The findings revealed that children with SAM had remarkably higher AFB_1-lysine levels in their serum (median 4.3 pg/mg albumin) against healthy children (0.8 pg/mg albumin) ($p < 0.05$). Also, serum AFB_1-lysine levels were higher in kwashiorkor children (median = 6.3 pg/mg

albumin) in comparison to marasmus children (0.9 pg/mg albumin) (Coulter et al. 1986; Hatem et al. 2005; McMillan et al. 2018). Additionally, aflatoxins were found more frequently in the body organs such as lungs (Oyelami et al. 1997) and liver (Apeagyei et al. 1986; Lamplugh and Hendrickse 1982) but not in the kidneys (Oyelami et al. 1998) of children who died with kwashiorkor. However, Onyemelukwe et al. (2012) and Ramjee et al. (1992) reported that aflatoxins were found intermittently and with relatively reduced concentration in the urine of children with kwashiorkor as compared to those of healthy children. These reports suggest that children suffering from kwashiorkor might have different aflatoxin metabolism as compared to healthy children and the children suffering from other types of SAM. Also, the urinary excretion of aflatoxins in kwashiorkor children might be inefficient in comparison to healthy groups (Coulter et al. 1986; Onyemelukwe et al. 2012). Aflatoxins have also been anticipated to act in a synergistic association with other etiological factors to expedite the kwashiorkor development (Onyemelukwe et al. 2012). This relationship is further strengthened by the spatial distribution of dietary aflatoxins and kwashiorkor, their compatibility in triggering metabolic syndromes, intestinal dysfunction, and immune system damage (Hendrickse et al. 1982; Wild and Gong 2009).

5.2.2.3 Possible Mechanisms of Aflatoxin-Induced Growth Impairment

The aflatoxin-related growth restriction is caused by several biological pathways that are not entirely understood yet. One of the proposed pathways by which aflatoxins may restrict the growth is environmental enteric dysfunction (EED) (Khlangwiset et al. 2011; Smith et al. 2012; Wild and Gong 2009). It is a complex enteric disorder arising from chronic exposure to intestinal pathogens and is pervasive among children living in areas with poor hygienic conditions. Continuous exposure to pathogens and their toxins results in changes in gut morphology such as blunting of the intestinal villa, epithelial damage, inflammation, and malabsorption of nutrients. The synergism between EED and malnutrition results in a cyclic association in which malnourished children are very likely to develop EED and consequently remain malnourished because of malabsorption of nutrients in the intestine and protein loss (Koyuncu et al. 2020). Human intestinal epithelial cells, like hepatocytes, express CYPs which convert AFB_1 into reactive epoxides (Zhang et al. 1998, 1999). Therefore, aflatoxin exposure results in compromised intestinal barrier function and ultimately chronic intestinal enteropathy by (i) disrupting the intestinal integrity, (ii) thinning the mucus layer, (iii) unbalancing immunological factors, and (iv) impaired functioning of microbial homeostasis.

Aflatoxin-induced injury of intestinal cells weakens the intestinal integrity leading to mucosal and systemic immune activation. In vitro studies on aflatoxin-induced modulation of intestinal epithelial cells showed that aflatoxin exposure (AFB_1 and AFM_1) on differential Caco-2 cells at varying concentration for different exposure time resulted in reduced cell viability (Huang et al. 2019; Nones et al. 2017; Zhang et al. 2015, 2018;) and induction in cell cycle arrest (Bao et al. 2019).

Additionally, aflatoxins have also been reported to cause modulation of intestinal tight junctions (TJ) by reducing the expression levels of TJ proteins and disturbing their structures such as zonula occludens-1, claudin-4, claudin-3, and p44/42 mitogen-activated protein kinase (MAPK) in differential Caco-2 cells (Gao et al. 2018). In animal models, aflatoxin exposure resulted in increased DNA fragmentation; downregulation of the caspase 3, caspase 9, Bax, CYP3A13, and p53 expressions, upregulation of TNF-α, Bcl-2, and their target protein expression (Jebali et al. 2018); increased serum lactulose/rhamnose ratio (Chen et al. 2016), serum diamine concentration in the jejunum (Liu et al. 2018); increase in surface area, crypt depth, and villus width in the duodenum and jejunum (Feng et al. 2017); decrease in villus absorptive area, villus height, and villus height to crypt depth ratio; increase in lymphoid follicular diameter; and increase in goblet cell counts in the jejunum (Jahanian et al. 2016; Jahanian et al. 2017). Moreover, aflatoxins (AFB_1 and AFM_1) have been reported to cause downregulation of pro-inflammatory cytokines (notably TNF-α, IL-1β, IL-6, and IFN-γ) production (Jebali et al. 2018; Taranu et al. 2019), reduction in transcript levels of secretory immunoglobulins A (sIgA) (Liu et al. 2018), and CD4+ cells (Kraieski et al. 2017) in the small intestine of animals.

Aflatoxin-related intestinal damage and resulting growth retardation may happen due to dysfunction of gut microbiota-related metabolism (Zhou et al. 2019). Children from Guatemala had aflatoxin exposure correlated with intestinal microbial dysbiosis and lower HAZ. Diversity analysis revealed significant differences in the beta-diversity of enteric microbiomes of shorter children (HAZ ≤ -2.45 SD) as compared to taller children (HAZ > -2.45 SD). Also, in the same study, it was reported that children with dietary aflatoxin exposure higher than 10 ng/kg bw/day were 24 times more likely to have dysbiotic intestinal microbiome as compared to children with lower dietary aflatoxin exposure (Voth-Gaeddert et al. 2019). Also, it is evidenced from the animal studies that aflatoxins cause reduction in the diversity of intestinal microflora, particularly *Bacteroidetes* and lactic acid bacteria (Wang et al. 2016) and increase in the diversity of gram-negative bacteria, notably *Escherichia coli*, *Klebsiella*, and *Salmonella* (Jahanian et al. 2016).

Malabsorption of micronutrients in the intestine is proposed to be a cause of aflatoxin-mediated enteric dysfunction and, ultimately, growth suppression (Smith et al. 2012). Studies have reported that aflatoxin exposure was significantly correlated with micronutrient deficiency, particularly vitamin A and zinc in both human adults and animals (Obuseh et al. 2011; Tang et al. 2009). However, insufficient evidence is available for the children group. Watson et al. (2016) measured the AF-alb adduct levels and levels of vitamin A, beta-carotene, and vitamin E in the blood of Guinean children (10–46 months) and found that the children having maximum exposure of aflatoxins (average AF-alb adduct = 57.1 pg/mg albumin) had 3.96 times and 1.98 times higher odds of vitamin A and zinc deficiency in comparison with the children having the lowest aflatoxin contact.

Lastly, aflatoxin-induced chronic liver injury might result in growth hormone resistance, and thus hepatotoxic effects of aflatoxins on growth hormone signaling is presented as putative mechanism responsible for aflatoxin-induced growth retardation (Khlangwiset et al. 2011; Smith et al. 2012; Wild and Gong 2009). Castelino

et al. (2015) measured the concentration of insulin-like growth factors (IGFs) and biomarker of aflatoxin exposure (AF-alb adducts) in Kenyan children aged (6–14 years). In the same study, the effects of AFB_1 on protein and gene expression of IGF axis in human hepatocyte line 16 cells (HHL-16) were also investigated in vitro. AF-alb concentrations in the serum (110.5 pg/mg alb) was significantly inversely correlated with both IGF1 (102.2 ng/ml) (β: −0.27, $p = 0.039$) and IGFBP3 (1902.3 ng/ml) (β: −0.39, $p = 0.046$) levels in the serum. Additionally, both IGF1 and IGFBP3 were positively correlated with child height (for IGF1 $\beta = 11.7$, $p < 0.001$; for IGFBP3 $\beta = 7.9$, $p = 0.001$) and weight (for IGF1 $\beta = 8.1$, $p < 0.001$ and for IGFBP3 $\beta = 5.5$, $p = 0.001$). Further, the path analysis showed that around 16% of the impact of aflatoxin albumin on the height of children can be assigned to reduced IGF1 levels. In vitro analysis showed that aflatoxin exposure revealed significant downregulation of both IGF1 and IGFBP3 gene expression and protein levels.

5.2.3 Aflatoxin and Immunosuppression

The immune system is the body's indispensable defensive mechanism that provides protection against invaders and xenobiotics such as mycotoxins. The responses of the immune system are divided into innate or natural responses and acquired or adaptive responses. The innate (natural) immune responses provides immediate defense against pathogens using natural killer cells (NKC) and inflammatory mediators (mast cells, basophils, and eosinophils) released by phagocytes (such as monocytes, neutrophils, and macrophages), while the adaptive (acquired) immune responses involve the proliferation of antigen-specific T and B cells and are further categorized into humoral and cellular immunity (Delves and Roitt 2000).

The development of the immune system in humans is a continuous process that starts in utero and continues during infancy, early childhood, adulthood, and to the decline of old age (Hertz-Picciotto et al. 2008; Winans et al. 2011). The early life stages are linked with increased susceptibility to infectious diseases, that is, because of an inefficient immune system. Fetal and early postnatal life is critical in terms of the development of the immune system in vertebrates. In the neonates, the cellular and humoral immune responses differ both quantitatively and qualitatively as compared to adults. This difference is anticipated in part due to lesser number and/or reduced functional capacity of leukocytes (i.e., natural killer cells, T and B lymphocytes, and myeloid lineage cells) which are an important part of early postnatal immune system (Holladay and Smialowicz 2000; Veru et al. 2014). Developmental immunotoxicity arises when a non-adult (fetus, newborn) encounters any xenobiotic exposure that alters the immune system prompting adverse health consequences (Dietert and Dietert 2008). The decreased immunity level due to mycotoxins may lead to vulnerability of individuals to infectious ailments, reduced efficiency of vaccines and other drugs, and several chronic diseases (Oswald et al. 2005).

Various in vitro and in vivo studies have reported that the immunosuppressive effects of aflatoxins are more likely to be associated with cell-mediated immunity as

compared to humoral immunity (Benkerroum 2020; Jolly et al. 2008; Mohsenzadeh et al. 2016). In humans, immunosuppressive impacts of aflatoxins have been comparatively less characterized, and there is a scarcity of data on the immunomodulatory effect of aflatoxins in children. In a prospective study of Gambian children ($n = 391$) aged 3–8 years, AFB_1 was significantly correlated with an increased risk of malarial infection ($p = 0.01$) and hepatitis B virus infection ($p = 0.04$) as measured by indirect immunofluorescent antibody test (IFAT) for antibodies to malaria parasites (*P. falciparum*) and hepatitis B surface antigen (HBSAg), respectively (Allen et al. 1992). In another cohort of Gambian children ($n = 472$) aged 6–9 years, the effect of dietary AF exposure (as measured by serum AF-alb adducts) was assessed on the CMI, sIgA levels, and antibody response to pneumococcal and rabies vaccine. Children with detectable levels of AF-alb adducts (23.3 pg/mg albumin) were found to have considerably lower sIgA levels (50.4 μg/mg protein) as compared to the children with non-detectable levels of aflatoxins (70.2 μg/mg protein) ($p < 0.0001$). For pneumococcal vaccine, antibody response to one of four serotypes (serotype 23) was weakly correlated with AF-alb adduct levels. But no response was recorded for antibodies to other serotypes (1, 5, 14) or rabies vaccine. Also, no correlation was observed between CMI responses to test antigens and AF-alb adduct levels (Turner et al. 2003).

Githanga et al. (2019) studied the immunomodulatory effects of dietary exposure to AFs in 433 children aged between 1 and 14 years in Kenya. Hepatitis B vaccine was presented for routine administration, and the effect of aflatoxin exposure (as measured by AFB_1-lysine adducts in serum) on immunogenicity of vaccines in childhood was assessed by estimating the hepatitis B surface antibodies (anti-HBs). It was observed that for a unit increase in the levels of serum AFB_1-lysine adducts (average 45.38 pg/mg), anti-HBs levels reduced by 0.91 mIU/ml. Also, aflatoxin exposure was negatively correlated with IL-2, IL-4, IL-6, IL-8, and IFN-gamma, while positively correlated with IL-10, TNF-alpha, and perhaps GM-CSF ($p > 0.05$). The authors concluded that, in spite of the inclusion of a larger population for routine immunization, a limited number of participants (less than one half) had developed immunity against hepatitis B and aflatoxin exposure was highly and weakly correlated with low anti-HBs, thus highlighting a potentially significant contribution of aflatoxins in reducing the vaccine effectiveness. The exact mechanism of immunomodulatory effects of aflatoxins have not been clearly understood yet; however studies have reported that inhibition of RNA, DNA, and protein synthesis by aflatoxins (particularly AFB_1) is responsible for aflatoxin-induced immunosuppression (Jolly et al. 2008).

5.2.4 Aflatoxin Exposure and Hepatic Dysfunction

The liver is the body's complex organ consisted of parenchymal cells (hepatocytes and cholangiocytes), perisinusoidal cells, and sinusoidal cells. It is essential for survival owing to its role in harmonization of metabolism in the body such as

glucose homeostasis and synthesis of various plasma proteins and steroid hormones. Besides, the liver is a remarkable organ because of its capacity to protect the individual from the injury caused by xenobiotics as it is the site where most of the toxic substances are metabolized, thus leading to either bio-activation and hepatic injury or detoxification and elimination. Though repair and regeneration capacity of the liver makes it a relatively robust organ, if the capability to repair and regenerate is inefficient, or if liver damage is severe, liver injury can lead to irreversible liver failure and death (Bischoff et al. 2018; Gu and Manautou 2012). Liver diseases such as hepatocellular carcinoma, cirrhosis, and non-alcoholic fatty liver disease encounter substantial disease burden. Globally, around 2 million deaths are mainly attributable to liver diseases: 1 million from cirrhosis and 1 million from HCC and viral hepatitis (Asrani et al. 2019). The prevalence of liver diseases is increasing in children, and the chronic liver diseases in the early childhood have been reported to progress in the early adulthood with long-term implications that continue throughout the life (Dhawan et al. 2017).

5.2.4.1 Liver Cancer

Chronic dietary aflatoxin exposure in early life might be a significant contributor of early inception of liver cancer (Polychronaki et al. 2008). There is scarcity of human data on early life aflatoxin exposure and the risk of cancer. However, in vitro and in vivo studies support the etiology. Rotimi et al. (2021) studied the effect of AFB_1 exposure using a perinatal rat exposure model (gestation through weaning) on epigenetic regulation in the infant by investigating the DNA methylation at *Tp53* gene, a tumor suppressor gene, and *H19* gene, environmentally responsive imprinted gene that has been associated with the risk of HCC. The DNA methylation, in both the liver and blood, was assessed following termination of exposure (3 weeks of age) and in early adulthood (3 months of age). The findings of the study revealed that DNA methylation on *Tp53* in aflatoxin-exposed rats was significantly elevated in the blood and reduced in the liver samples ($p < 0.05$) of both low dose (0.5 mg/kg) and high dose (5 mg/kg) aflatoxin-exposed rats. While the DNA methylation in the *H19* gene was elevated in both low- and high-aflatoxin-dose-treated rats and was lower in blood samples of rats (having 3 months of age) treated with higher aflatoxin dose ($p < 0.05$). The authors highlighted the need for further research to evaluate the role of genetic alterations caused by early life aflatoxin exposure in the development of HCC. HCC is likely to be more common in adults, while hepatoblastoma is the most frequent hepatic cancer of infants and young children (Wheatley 2018). The exact etiology of hepatoblastoma remains unknown. Although some studies have reported that aflatoxins play a certain role in the induction of cytotoxicity and DNA damage in hepatoblastoma cell line (HepG2) (Ju et al. 2016; Zheng et al. 2018; Zhu et al. 2015). Marchese et al. (2018) evaluated the effects of AFM_1 on cytokinomic and metabolomic profile, cell viability, and cell cycle of a hepatoblastoma cell line. AFM_1 induced both the blocking of the cell cycle in the G0/G1 phase and a decrease in the viability of HepG2 cell. Furthermore, the analysis of the

metabolomic profile indicated an increased concentration of IL-8, IL-6, and TNF-α and decreased concentration of IL-4 in HepG2 cells.

5.2.4.2 Neonatal Jaundice

Neonatal jaundice is a commonly occurring physiological process in newborns with 80% of the preterm babies and around half of the term babies showing clinical symptoms including yellowing of the skin and sclera caused by elevated serum concentration of bilirubin/reduced hepatic elimination of bilirubin and/or increased breakdown of red blood cells. Normally, hyperbilirubinemia resolves by natural transition within the 1st week of life with liver maturation; however, severe condition leads to re-hospitalization of newborns and has been reported to be associated with elevated concentration of unconjugated bilirubin and neurotoxicity resulting in long-term sequelae such as hearing loss, cerebral palsy, and kernicterus (Cohen et al. 2010; Maisels 2006; Scrafford et al. 2013). Aflatoxin exposure has been considered as a risk factor for neonatal jaundice (El-Shishtawy et al. 2006). Recently, a preliminary study was conducted to determine the serum AFB_1 concentration in full-term neonates (n = 24) with hemolytic jaundice secondary to glucose-6-phosphate dehydrogenase (G6PD) deficiency (Raafat et al. 2021). AFB_1 was present in 58% and 75% serum samples of neonates and their mothers, respectively. Also, it was found that serum AFB_1 concentration was significantly inversely correlated with G6PD activity ($r = -0.585$, $p = 0.03$) and birthweight ($r = -0.574$, $P = 0.032$), while positively associated with serum activity of alanine aminotransferase ($r = 0.536$, $P = 0.048$) (Raafat et al. 2021).

In Egypt, maternal aflatoxin exposure was highly significantly negatively correlated with the birthweight in neonates with jaundice ($p < 0.001$) (El-Shishtawy et al. 2006). Similarly, Abulu et al. (1998) investigated the presence of aflatoxins in cord blood from newborns with jaundice (n = 150) and without jaundice (n = 14) and found a significant reduction in the birthweight of neonates with jaundice who were found positive for the presence of aflatoxins in their maternal cord blood samples ($p < 0.05$). In this study, the authors found that neonates with jaundice had higher average AFB_1 levels (32.3 ng/ml and 35.6 ng/ml). The prevalence of aflatoxins was found to be higher in the wet season (81.8%) as compared to the dry season (50%). The authors of the study concluded that the exposure of aflatoxin in neonates was prenatal and that the prevalence of jaundice was raised in the wet and warm season. In a study from Nigeria, the blood samples of mothers (n = 80) and their jaundiced neonates (n = 327) and control group (60 non-jaundiced infants and 7 mothers) were analyzed for bilirubin levels, aflatoxin concentration, and naphthol levels (Sodeinde et al. 1995). Aflatoxins were found in the 27.45% of jaundiced infants, 17.2% of their mothers, 16.5% of non-jaundiced infants, and 14% of their mothers. Statistical analysis of the data revealed that either the presence of aflatoxins or the deficiency of G6PD enzyme is a risk factor in the occurrence of neonatal jaundice with odd ratios of 2.68 (CI, 1.18–6.10) and 2.97 (95% confidence intervals (CI), 1.31–6.74), respectively (Sodeinde et al. 1995).

Contrary to this, Abdulrazzaq et al. (2004) observed no correlation between rates of jaundice or infection in neonates and AFM_1 levels in maternal or cord blood. Similarly, Ahmad et al. (1995) analyzed the cord blood samples of newborns (n = 37) who eventually developed jaundice and control/non-jaundiced babies (n = 40) for the presence of six major aflatoxin types and aflatoxicol from Nigeria. Also, in the second half of the study, serum concentration of aflatoxins in neonates with jaundice (n = 64), who were admitted from outside of the hospital, and non-jaundiced/control infants (n = 60) was also determined. Aflatoxin was found in the 37.8% and 22.5% samples of cord blood from jaundiced and non-jaundiced neonates, respectively, while more than 50% of jaundiced neonates were found positive for aflatoxins. The average cord blood and serum concentration of aflatoxin were high in the jaundiced group as compared to the non-jaundiced/control group with no statistically significant difference in frequency and concentration in both groups. Also, the study reported that there was no significant relationship between hyperbilirubinemia and serum concentrations of aflatoxin. These findings warranted further research to determine the effect of aflatoxin exposure on neonatal jaundice.

5.2.4.3 Other Hepatic Illnesses

Dietary aflatoxin exposure may also cause other hepatic illnesses such as hepatomegaly, i.e., an unusually large liver and cirrhosis (severe liver scarring seen at the last stages of chronic liver injury). Gong et al. (2012) reported that serum AF-alb adduct levels in children (aging between 6 and 17 years) of Kenyan suffering from hepatomegaly (176.6 pg/mg albumin) were significantly higher than normal children (79.9 pg/mg alb) (p = 0.03). Also, it was estimated that a unit log increase in AF-alb concentration in the blood might result in a significant (43%) increase in the prevalence of hepatomegaly after adjusting for confounding factors. There is scarcity of data on aflatoxin-induced liver cirrhosis in children, and only Amla et al. (1971) reported that aflatoxin exposure (through consumption of aflatoxin-contaminated peanut diet) in children (aged 1.5–5 years) was associated with varying proportions of hepatic lesions including cirrhosis, formation of fatty cysts, and fibrosis.

5.3 Conclusions

In utero, infant and early childhood days are sensitive stages for children's growth, and any exposure of toxic substances, particularly aflatoxins, during these stages of life through the maternal body, consumption of breast milk, and baby foods may lead to long-lasting health consequences. Aflatoxin exposure to fetus, infants, and young children has been associated with several adverse health outcomes such as birth defects, growth impairment, immune dysfunction, and hepatic dysfunction. Reduction and/or mitigation of aflatoxin exposure to fetus, infants, and children

demands setting awareness programs to pregnant and nursing mothers in order to promote dietary diversity that is associated with reduced dietary aflatoxin exposure while ensuring the nutrition and food safety of infants and young children. Nutrition-sensitive intervention programs targeting reduction of aflatoxin-induced adverse health outcomes, particularly child undernutrition, are required to reduce aflatoxin exposure to infants and young children. Randomized controlled trials should be conducted to further elucidate the casual relationship between aflatoxin exposure and adverse health outcomes in infants and young children. Moreover, future research focusing on elucidating the mechanism behind aflatoxin-induced impaired growth, immunosuppression, and hepatic dysfunction is imperative.Conflict of InterestThe authors declare no conflict of interest.

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Chapter 6
Aflatoxin's Health Impacts on Adults and Elderly

Saeed Akhtar, Muhammad Riaz, Muhammad Latif, Aneela Hameed, Bakhtawar Zawar, Muhammad Kashif, and Amir Ismail

Abstract Aflatoxins are the secondary metabolites of fungus that are reported in some food commodities well beyond the maximum allowable limits. The ingestion of aflatoxins may lead to severe health implications, and the magnitude of complications multiplies to many folds in people having weak immune system, e.g., infants, elderly, or diseased people. In this chapter, the impact of aflatoxins will be evaluated on the health status of adults and elderly people. The major health implications of aflatoxins on adults and elderly people include the impact of aflatoxins on hepatic functioning, the impact of aflatoxins on the urinary system, the impact of aflatoxins on the reproductive and immune system, and the impact of aflatoxins on the nervous system.

Keywords Aflatoxins · Adults · Elderly · Health · Hepatic · Urinary · Reproductive

6.1 Introduction

Aflatoxins are highly toxic polyketide compounds produced by a secondary metabolic pathway of various fungal species (more than 20) but predominantly by *Aspergillus flavus* and *Aspergillus parasiticus*. More than 20 different types of aflatoxins are reported, but the major types of aflatoxins are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), and aflatoxin M_2 (AFM_2). Aflatoxins may impact directly or indirectly on

S. Akhtar · M. Riaz (✉) · A. Hameed · B. Zawar · M. Kashif · A. Ismail
Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan
e-mail: riaz@bzu.edu.pk

M. Latif
Biochemical Lab, Center for Genetics and Inherited Disease, Taibah University, Madinah, Saudi Arabia

K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_6

both human and animal health, and their major health implications are carcinogenicity, teratogenicity, mutagenicity, growth retardation, and immunosuppression (Sarma 2010).

Since their discovery in the 1960s, aflatoxins emerged as a global challenge as they contaminate various food commodities including cereals (especially maize, rice, and wheat), dry fruits (especially peanuts, dates, apricots, figs, and others), spices (particularly red pepper and other dried spices), and milk and milk products. According to some estimates, 25% of the crops of the world are suspected to be contaminated with different types of aflatoxins but predominantly contaminated by AFB_1 which is also the most toxic type of aflatoxin among all reported types (Bhetariya et al. 2011). Around 4.5 billion people are estimated to be consuming aflatoxin-contaminated food commodities, and their health (especially of the vulnerable age group, i.e., infants and elderly) is at a huge risk due to the exposure to aflatoxins (Williams et al. 2004). The International Agency for Research on Cancer (IARC) has classified total aflatoxins (AFB_1 + AFB_2 + AFG_1 + AFG_2) and AFM_1 as group 1 category carcinogenic compound, but the highest carcinogenic potential is of AFB_1. Based on the serious health implications posed by aflatoxins on human health, countries across the world have now established maximum permissible limits for different types of aflatoxins. The Food and Drug Administration (FDA) and the US Department of Agriculture (USDA) have set 20 μg/kg allowable limit of total aflatoxins in various food items and 0.5 μg/L for AFM_1, while the EU maximum permissible limit for total aflatoxins in cereals is 4 μg/kg, and for AFM_1 it is 0.05 μg/L (Ismail et al. 2018).

The population of elderly people is increasing day by day. In 2004, about 461 million people were older than 65 years, and it is estimated that up to 2050, the strength of older people will be about 2 billion (Kinsella and Phillips 2005; UN 1999). Due to a weak immune system, caring for older people is more essential as compared to adult people. A scanty of data is available regarding aflatoxin's impact on elderly people. Like children, elderly people are also considered vulnerable groups against aflatoxins and their metabolites. Still, more research work is required to evaluate the impact of aflatoxins on elderly people. Fewer studies report that most of the body parts like the liver, urinary system, and reproductive system are influenced by aflatoxins.

The highly toxic nature of aflatoxins demands serious attention from health and regulatory agencies across the globe. The focus of the chapter in hand is on the health implications posed by aflatoxins on adults and elderly age groups (although very little data is available on the impact of aflatoxins on elderly people). The impact of aflatoxins on the hepatic functioning, urinary system, reproductive system, immune system, and nervous system will be reviewed in this chapter (Fig. 6.1).

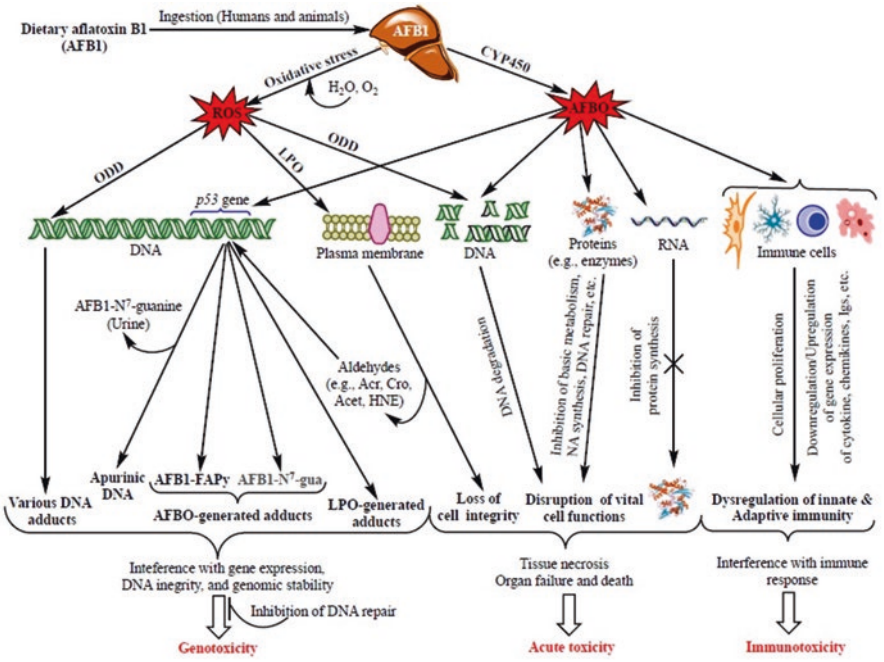

Fig. 6.1 Mechanism of aflatoxin-induced toxicity in humans. AFBO, aflatoxin B_1-exo-8,9 epoxide; ROS, reactive oxygen species; NA, nucleic acid; LPO, lipid peroxidation; HNE, 4-hydroxy-2-nonenal; Acr, acrolein; Acet, acetaldehyde; ODD, oxidative DNA damage; Cro, crotonaldehyde; Igs, immunoglobulins; uFA, unsaturated fatty acids; TNFα, tumor-necrotizing factor α; IL-1β, interleukin 1β; P-dG, cyclic propano-deoxyguanosine; IL-6, interleukin 6 (Benkerroum 2020)

6.2 Aflatoxin's Impact on Hepatic Functioning

Hepatocellular carcinoma (HCC), or liver cancer, is the third leading cause of cancer-related deaths worldwide, with around 0.83 million deaths in year 2020 only. Developing countries are reported to have the highest death rate due to cancer (WHO 2021). Since their discovery in the 1960s, it was known that aflatoxin's primary target organ in both humans and animals is the "liver." A number of studies from different corners of the world have reported the link between aflatoxins and HCC in humans. According to some estimates, 5–28% of cases of HCC worldwide are linked with the exposure to aflatoxins (Marchese et al. 2018). The countries having the highest prevalence of aflatoxins in their food commodities (African countries and Asian countries) are reported to have the highest prevalence rate of HCC (Smith et al. 2017). The primary target organ of aflatoxins is the liver, and the primary target organ of hepatitis virus infection (hepatitis B or C) is also the liver; therefore, the chances of HCC increase too many times in hepatitis patients having aflatoxin exposure through diet, and both of these contaminants are quite common in the developing countries (Wang and Tang 2005; Wogan et al. 2012; Wu 2013).

Aflatoxins after ingestion are absorbed by the duodenum and are converted into AFB_1-exo-8,9 epoxide (AFBO) mainly due to the activity of microsomal cytochrome P450 (CYP450) enzymes present inside the liver of humans and other animals. CYP450 enzymes (monooxygenases) catalyze the oxidation reaction of carbon number 8 and carbon number 9 double bond of the furan ring of aflatoxins that results in the formation of two metabolites AFBO and AFB_1-endo-8,9 epoxide. AFBO is a thousand-time more toxic compound in nature (highly toxic and unstable) as compared to AFB_1-endo-8,9 epoxide. AFBO reacts with cellular macromolecules including proteins, phospholipids, and nucleic acids that lead to disturbance of cellular structures, their signaling and metabolism, and other genetic disorders.

In a study conducted by Zhou et al. (2019) in Southern China, the HCC patients were recorded to have a higher concentration of aflatoxins in their serum cells and cancerous cells. The lipid profiling of blood serum was found altered in the HCC patients. Immunofluorescence staining and immunoblotting analysis revealed that S6K1 and CD36 expression increased and Aldo-keto reductase-7A (AKR7A) and ApoB expression decreased in HCC patients. The human HCC cell line (HepG2) also showed that exposure to high doses of aflatoxins results in decreased AKR7A expression, induced cell proliferation, and increased contents of TG, ApoA1, TC, and LDL. In another study conducted by, aflatoxin-induced liver cancer risk was estimated for the people living in Tanzania based on the aflatoxin exposure data (biomarker data), the prevalence of hepatitis B virus, and the total population of Tanzania. Disability-adjusted life years (DALYs) were computed to evaluate the burden of aflatoxin-related hepatic cancer. The estimated median value of aflatoxin exposure in the Tanzanian population was 105.5 pg/g bw/day. Based on aflatoxin exposure data, it was estimated that in year 2016, 1480 cases of hepatic cancer were added with an expected 1-year further life and the DALYs were 56,247. In a study conducted by Sharma et al. (2011), the risk of aflatoxin-associated liver cancer was estimated for people living in Bangladesh. The risk assessment was based on the presence of aflatoxins in the food commodities of Bangladesh, the average consumption of different food items, and the average weight of individuals. The estimated cases of per-year aflatoxin-associated liver cancer were 1311 or 44% of the total reported cases of liver cancer in Bangladesh. A study was conducted in Malaysia to analyze the impact of aflatoxins on adult human beings. Aflatoxin exposure was estimated by observing aflatoxin contents in food mixtures ($n = 236$) that were prepared as "ready for consumption." The study revealed that AFB_1 exposure through diet was 24.3–34 ng/kg body weight per day and peanut contents were found as the main contributor of aflatoxins. The estimated risk of liver cancer through this exposure was 0.61–0.85 cases of cancer per 100,000 population per year that contribute 12.4–17.3% of liver cancer cases (Chin et al. 2012). In another study that was conducted in Serbia, Greece, and Croatia to assess aflatoxin intake through maize products and to check aflatoxin's impact on the liver. Average aflatoxin exposure of adults by consuming maize in each country was among 0.44–5.59 ng/kg body weight/day. Per-year estimated cases of hepatocellular carcinoma were 105 individuals, whereas the cases for the occurrence of HBsAg+ were 0.020–0.026, 0.075–0.098, and 0.006–0.008 for Greece, Serbia, and Croatia,

respectively. Similarly, a study was conducted on Korean people having age greater than 20 years. The study used the monitoring data of AFB_1 in edibles with a high frequency of circulation in food groceries from May 2004 to June 2005. The monitoring data were compared with the dietary intake data of the adult Korean individuals (age < 20 years). The study estimated the average daily intake, excess cancer risk, and population risk of AFB_1. The results indicated that the daily intake of aflatoxins by adult Koreans through food intake was 0.064–0.361 pg/g body weight per day. Cancer risk due to the presence of aflatoxins in food for hepatitis B positive and hepatitis B negative population was 5.76×10^{-7}–3.25×10^{-6} and 1.47×10^{-5}–8.31×10^{-5}, respectively. The findings of the study indicated that the HCC cases in the Korean population were 0.6–3.3/million population with hepatitis negative and 15–83/million population having hepatitis positive. The risk of HCC among hepatitis-positive population was 25 times higher as compared to that population having hepatitis negative (Lee et al. 2009). The impact of aflatoxins is examined mostly through epidemiological studies, and therefore it is impossible to examine the risk of aflatoxin dose on the human population. A study was conducted by Liu et al. (2012) to quantify the load of cancer cases produced by aflatoxins in Asia and Africa. About 23% annually of all hepatocellular carcinoma cases were due to exposure to aflatoxins and for a total of around 172,000 cases/year. Similarly, in another study conducted by Liu and Wu (2010), it was analyzed that each year about 550,000–600,000 new hepatocellular carcinoma cases were reported due to exposure to aflatoxins. It was also examined that most of the reported cases were from sub-Saharan Africa, China, and Southeast Asia where people suffer from high HBV occurrence and aflatoxin exposure through food.

The number of HCC cases increases with age, and elderly people are most susceptible to it. HCC is less reported in individuals below the age of 40 years, while the maximum HCC cases in China are reported in the age range of 55–59 years, while in America and Europe, maximum HCC cases are reported in the age range of 63–65 years. In the countries/population having low-risk factors for HCC, the peak of HCC cases is reported above the age of 75 years. Men are reported to have higher incidence of HCC as compared to females (El-Serga 2012). In a study conducted by Yi et al. (2018), 0.504 million Korean people were examined during years 2002–2013. HCC happened in 2744 people. An increase in age resulted in an increase in the chances of HCC, and each 5-year increase in age was found to be associated with a 1.24-fold increase in the chances of HCC.

6.3 Aflatoxin's Impact on the Reproductive System

Infertility is a reproductive defect that is one of the major medical issues worldwide. A number of chemical agents including aflatoxins are reported to have the potential to cause infertility in both males and females. Apart from infertility, other negative effects of aflatoxins on males includes reduced fertility, altered epididymal function, spermatogenesis suppression (Kudayer et al. 2019; Murad et al. 2015), reduced

level and motility of sperm, deformed sperm morphology (Mohammadi et al. 2014), and multinucleated cell production in seminiferous epithelium (Faridha et al. 2006). Any abnormality in spermatogenesis (sperm production process) leads to decreased reproduction potential, reduced weight and size of the testes, degenerated seminiferous tubes, and sperm-level reduction (Fayjaloun et al. 2019; Komsky-Elbaz et al. 2018).

Steroidogenesis is the conversion of cholesterol into steroid hormones (testosterone and estrogens). Luteinizing hormone (LH) responds to synthesize testosterone in Leydig cells of the testes. The testosterone synthesis pathway involves the enzyme-mediated (P450) conversion of cholesterol to pregnenolone which further converts into testosterone in the endoplasmic reticulum (Faisal et al. 2008; Faridha et al. 2006). Aflatoxin ingestion interferes with the normal enzyme path and stimulates the liver to produce AFB_1-8,9-epoxide with the help of P450 enzyme. AFB_1-8,9-epoxide forms adducts with DNA and disturbs the normal working of enzymes involved in steroidogenesis which leads to infertility (Hasanzadeh and Amani 2013). Animal studies have reported the correlation between aflatoxin presence and infertility problems. The investigations carried out on mice to determine the aflatoxin effects revealed that the low dose of aflatoxins (20 mg/kg) caused deformations in sperm cells, formed coiled tails, swollen sperm heads, and nucleated spermatocytes, while the high dose (1500 μg/kg) caused swelling of sperm heads, decapitation, and reduction in sperm cell viability and motility (Faisal et al. 2008; Fapohunda et al. 2008; Mathuria and Verma 2008). In a study conducted by Hasanzadeh et al. (2013), the lower concentration of testosterone and LH in blood serum was examined when mice were fed for 48 days with 3.2 ppm dose of aflatoxin B_1. Some studies were carried out on rabbits which highlighted the marginal to severe anomalies imparted by aflatoxins. The dose-dependent (1000 ppb) effects of aflatoxin exposure were investigated, and the results disclosed degeneration of seminiferous tubules and spermatogonial cells, alterations in spermiogenesis, multinucleated cell formation, and reduced weight of the testes and sperm cells count (Ahmed et al. 2012). The other studies conducted on rabbits indicated the decreased number of spermatids, lower testes weight, reduced ejaculate volume, abnormal sperm output, and deformities in sperm morphology (Lakkawar et al. 2004; Salem et al. 2001).

A scanty of data is available regarding the effect of aflatoxins on the human reproductive system. Yet, fewer studies are in access specifying the unwanted effects of aflatoxins. In a study carried out by Murray et al. (2012), the semen of control and infertile patients were compared. Aflatoxin presence in the semen of infertile patients was confirmed in comparison with the control cases ($P < 0.05$). The aflatoxin level was in the range 700–1392 ng/ml, which became the cause of infertility and lower levels of spermatozoa. Mohammed et al. (2014) observed poor semen quality (volume, viscosity, motility, and sperm morphology) and declined fertility due to the presence of aflatoxins in the semen. Twenty-five percent semen samples of infertile men confirmed the prevalence of aflatoxin when compared with control ($P = 0.0007$). Hasanzadeh et al. (2013) examined the dose-dependent effects of aflatoxin B_1 on the level of sperm production. A significant difference ($P < 0.01$)

between the tested group and the control group was recorded. The study disclosed that as the level of aflatoxin exposure increases simultaneously, the level of sperms in the seminiferous tubes decreases. Another study by Ibeh et al. (1994) examined the effects of aflatoxins on the fertility of men. The findings of the conducted study highlighted the presence of aflatoxins up to 40% in the semen of infertile men ($P < 0.01$) when compared with the fertile men's semen. Upon semen analysis, abnormalities related to sperm count were found in the infertile individuals containing higher levels of aflatoxins.

The deleterious effects of aflatoxin ingestion on females include infertility, altered sexual maturation, abnormal growth of follicles, reduced hormone concentration, and defects in fetus growth (Kourousekos et al. 2015). In the infertile females, the level of luteinizing hormone (LH) increases due to increased secretion of gonadotrophin-releasing hormone. The increased LH levels decrease the levels of progesterone, which in turn effects the formation of corpus luteal (CL). The low levels of progesterone hormone and reduced size of CL are the common symptoms found in infertile females. Data supported the fact that aflatoxins interfere with the normal hormone balance and play a role in producing higher levels of LH which leads to infertility in females (Anwar et al. 2008; Balen and Michelmore 2002). El-Azab et al. (2010) conducted a study in Egypt to determine the presence of aflatoxins in the infertile females' blood. The study indicated that the presence of aflatoxins reduces the uterine and ovary size and implantation loss and increases the chances of intrauterine death in females. Polycystic ovary syndrome (PCOS) is a common problem among females characterized by the elevated levels of androgen (male hormone) which produces enlarged ovaries. Kandarakis et al. found the same symptoms of enlarged ovaries when they conducted a study on aflatoxin's health implications. In the study carried out by El-Azab et al. (2010), the same effects of aflatoxins in the infertile females were recorded. Due to the similarity between the structures of AFB_1 and steroid hormones, AFB_1 affects the receptors of steroid hormones which further interfere with estrogen and progesterone production and impart infertility in females. A number of studies have also reported the impact of aflatoxins on birth weight and height, jaundice, still birth, and other such issues due to the presence of aflatoxins in the maternal blood (Abdulrazzaq et al. 2004; Moslemi and Tavanbakhsh 2011; Sadeghi et al. 2009).

6.4 Aflatoxin's Impact on the Urinary System

Kidneys receive around 20–25% of the blood having toxic compounds such as aflatoxins, and therefore the functioning of the kidney may be altered or disturbed due to the exposure to the toxic compounds. Kidneys require a high amount of oxygen and nutrients for routine functioning, including filtration of one third of blood as well as reabsorption of salt and water up to 98–99%. Based on the direct contact of aflatoxins with contaminated blood, various of nephrons become visible to aflatoxins and its metabolites, and nephrotoxicity events may start before the aflatoxins are

excreted through urine. A number of studies have reported the direct link between aflatoxin exposure and renal tumors (Bbosa et al. 2013; Sharma et al. 2011). In a study conducted in Nigeria (an African country where the population is frequently exposed to higher levels of aflatoxins), the autopsy of the kidney of children showed the presence of aflatoxins. Animal studies and artificial human cell line studies have shown that the exposure of aflatoxins in the kidney affects the transport of phosphorus, sodium, and calcium in kidney cells and disturbance in the excretion of aflatoxin metabolites through the kidneys (Glahn et al. 1991, 1994; Oyelami et al. 1998). In a study conducted by Redzwan et al. (2014) in Malaysia, the impact of aflatoxins on kidney functioning was evaluated. Seventy-one people were included in this study, 52 of which were of age around 40 and the other 19 people were of age more than 40 years. A 5-ml fasting blood sample was collected from each participant. All the samples were found positive for aflatoxin B_1 lysine adducts, indicating the recent exposure of aflatoxins in 100% of the population. The kidney function parameters including serum creatinine level and glomerular filtration rate were found positively associated with the aflatoxin B_1 lysine adduct levels.

The impacts of AFB_1 and AFM_1 on the kidney were also studied by Li et al. (2018), and biochemical and pathological analyses were conducted to record its implications on the kidney. Two hundred ninety-three human kidney cells were used, and viability of these cells was determined through CCK-8 kit. At the same concentration (100 mg/L), AFB_1 had a greater inhibitory impact (26%) on cell viability as compared to AFM_1 (44%). Similarly, to analyze the impact of aflatoxins (AFB_1 and AFM_1) on kidney functions, three markers including creatinine, urea, and uric acid were measured by ELISA. After analysis, it was found that these three markers were sharply increased and eventually kidney malfunctioning was recorded. Moreover, in kidney pathological studies, after treating with aflatoxins (AFB_1, AFM_1), injury in the kidney cells was observed. After treatment with AFB_1 (0.5 mg/kg), some areas of the kidney had cytomorphosis, occasional severe inflammatory cell infiltration, edema, and hemorrhage, while in the case of AFM_1 (3.5 mg/kg), less severe renal injury was recorded indicating that AFM_1 has less toxic impacts on kidneys as compared to AFB_1. Taheri et al. (2017) evaluated the impact of various doses of aflatoxins (0.5, 0.7, and 1.4 mg/kg feed) on biochemical parameters of the kidney. It was observed that CAT and MDA levels were increased in the kidney at various concentrations of aflatoxins. All the analyzed concentrations of aflatoxins (0.5, 0.7, and 1.4 mg/kg feed) lead to oxidative damage to tissues of the kidney.

6.5 Aflatoxin's Impact on the Immune System

Aflatoxin-induced immunosuppression increases susceptibility to infectious diseases, decreases antibody production, and reduces cell-mediated immunity (Yin et al. 2016). Aflatoxin consumption-mediated disruptions in the immune system are evidenced by animal and human studies. Participants with the higher aflatoxin level experience decreased phagocyte efficiency; reduced number of macrophages, red,

and white blood cells; hypersensitivity reactions; affected lymphocytes; and suppressed T cell-dependent functions (Gao et al. 2017; Wang et al. 2018). The other impacts of aflatoxins include chronic infection reactivation, decreased response toward vaccination and inflammatory cytokine synthesis, decreased number of spleen cells, and adduct formation with DNA suppressing the cell proliferation essential for immune response (Jiang et al. 2008; Shirani et al. 2018; Yard et al. 2013).

In most studies, the toxic effects of AFB_1 are explained in reference to its highly unstable metabolite AFB_1-exo-8,9epoxide (AFBO). AFBO causes disruptions in metabolic, genetic, and cellular structures, induces toxicity in the immune system as well as affects the production of immune cells and immune response mediators, and disturbs the gene expression regulation by decreasing the cell proliferation (Mehrzad et al. 2014; Mohsenzadeh et al. 2016). Jiang et al. (2005) studied the effects of aflatoxin-albumin (AF-albumin) adducts on the functions of monocytes and leukocytes and indicated that high levels of AF-albumin cause impairment of T cells, which in turn weakens the immunity and increases the chances for infectious diseases. Aflatoxin inhibits monocyte activities (microbicidal and phagocytic) and also compromises the integrity of intestinal lining by destroying epithelial cells and decreasing goblet cell numbers which interfere with immune responses and elevate the invasion of toxins and pathogens (Meissonnier et al. 2008; Williams 2010; Yin et al. 2016).

The complementary system of innate immunity which plays an important role in the activation of phagocytosis was observed to be inhibited by aflatoxin exposure. The impact of aflatoxins in hosts with adaptive immunity is greater than those with innate immunity due to the fact that the host is already vulnerable to infections or may be the vaccination is weak (Benkerroum 2020). Jiang et al. (2008) conducted a study to examine the interaction of HIV (human immunodeficiency virus) and aflatoxins on suppression of the immune system. Immune parameters including high and low AFB_1 albumin adduct (AF-ALB) levels were compared. The high and low albumin levels were ≥0.91 pmol/mg albumin and <0.91 pmol/mg albumin, respectively. HIV viral burden and AF-ALB were analyzed in plasma. In addition, cytokine expression, leukocyte percentage, and immune phenotypes were determined by using flow cytometry. After analysis, it was reported that both HIV and aflatoxins suppress the immune system. Individuals, both HIV negative and positive, had lower expression of perforin on CD8+ T cells due to high AF-ALB. In HIV-positive individuals, CD4+ T regulatory cells ($P = 0.009$) and naive CD4+ T cells ($P = 0.029$) were significantly lower due to high AF-ALB as compared to those positive individuals having low level of AF-ALB. Moreover, percentage of B cells ($P = 0.03$) was also reduced in HIV-positive individuals due to high level of AF-ALB as compared to HIV positive with low level of AF-ALB. Besides immunosuppressive roles, aflatoxins are also reported to exert immune stimulatory impacts. In a study conducted by Valtchev et al. (2015), it was reported that on one side, the higher doses of aflatoxins suppress the immune system, but on the other hand, low doses of aflatoxins lead to immune stimulatory impacts in case of short-duration exposures. Mohammadi et al. (2014) also reported the upregulation of the transcription of membrane proteins TLR-2 and TLR-4 in human myeloid dendritic cells after the

exposure of AFB_1 doses in the range of 1–2 ppb (environment-related doses) for a period of 2–24 h.

Animals' studies also provided evidence for the immunosuppression effect caused by aflatoxin. In a rat study, a dose of 100 ppb AFB_1 was administrated for a period of 5 weeks, and it resulted in the decreased level of immunoglobulin and lymphocyte proliferation (Bahari et al. 2013). In another study the effects of lower aflatoxin dose (0.6 mg/kg) were examined in rats which altered macrophage functions and decreased the levels of lymphocytic IL-2 and immunoglobulin, and the same dose caused disruptions in the intestinal lining of broilers and thus altered the barrier function of the intestine and the phagocytic activities of dairy cows by depleting viability of neutrophils (Chen et al. 2016; Hinton et al. 2003).

6.6 Aflatoxin's Impact on the Nervous System

In neurotoxicity, aflatoxin degenerates the peripheral and central nervous system of humans and animals by changing biochemical parameters (Marchese et al. 2018). Epidemiological data concluded AFB_1 was a neurotoxic agent which disturbs the normal functioning of the brain and causes mild to severe nervous system disorders such as demyelinating disease, encephalopathy, neurocognitive deficits, and neuropathies. AFB_1 exposure also imparted coma, cerebral edema, and convulsion in humans while in some cases even death (Kawata et al. 2016). Aflatoxin via food gets absorbed in the blood stream through passive diffusion. In the liver, aflatoxin is bio-transformed into its epoxide form which, due to its lipophilic nature, easily finds a way to the blood circulation system. Blood supply toward the brain-containing aflatoxin epoxide disrupts the nerve cells by damaging the nerve myelin sheath (Kanbur et al. 2011).

Considering the effects of AFB_1 toward antioxidants, oxidative stress is regarded as the main mechanism of toxicity for metabolites of aflatoxins. AFB_1 poisoning triggers the formation of reactive oxygen species (ROS) which exert oxidative stress (Liddelow et al. 2017; Mehrzad et al. 2018). In aflatoxicosis, these ROS attack proteins, nucleic acids, and lipids, increasing the chances for lipid peroxidation and decreased antioxidant activity leading to cell function disruptions and cytolysis (Liao et al. 2014; Yilmaz et al. 2018). Aflatoxin-mediated increased lipid peroxidation and free radical's generation make the brain vulnerable to neuronal damage, and long-term AFB_1 exposure is potentially responsible for cytotoxicity by impairing membrane configuration with deteriorative effects on the blood-brain barrier and cause neurodegeneration (Chen et al. 2008).

Creatine kinase (CK) regulates brain cells' energy homeostasis by providing a large number of phosphocreatine (PCr). CK and PCr collectively (CK/PCr) make a system which transports energy from the buffer pool to the target cells. The Na + and K + -ATPase utilize ATPs produced by CK (Behrens et al. 2015). AFB_1 potentially interferes with the CK activity and promotes cerebral diseases (Uetsuka 2011). A study highlighted the effects of AFB_1 on inhibiting the homeostasis-regulating

enzymes (β-galactosidase and β-glucuronidase) and disturbing the energy balance. These alterations inhibit the activity of sodium-potassium pump and cause neuropathies (Bahey et al. 2015). Higher concentrations of AFB_1 assert oxidative effects in brain cells by elevating the level of superoxide dismutase and modifying the catalase activities which block the CK activity, and the brain cells are unable to receive enough ATPs. The whole scenario leads to the development of acute or chronic brain diseases depending on the level of aflatoxins. Brain CK release rapidly damages the brain cells in amounts larger than the other organs; hence, a diagnostic sign of brain damage is evidenced by several studies (Attia et al. 2012; Venkataraman et al. 2009). The elevated levels of CK correlated to AFB_1 exposure cause neurodegenerative disorders such as Huntington disease and Alzheimer's disease, and CK sensitivity to oxidation selectively damages the neurons (Nakajima and Masaoka 2014). CK activity also maintains neurotransmission and intracellular signals which got disturbed by decreased CK activity and increases the chances of neural cytotoxicity. As reported by Arun et al. (2012), suppressed CK activity is correlated with brain degeneration, and this decrease may influence severity in neural cytotoxicity by inhibiting CK isoenzyme (uMt-CK and BB-CK) expression in the brain.

Neurotransmitters transfer messages via neurons to the target cells. Studies have confirmed the role of AFB_1 to interfere with neurotransmitters and cause nervous system diseases (Bbosa et al. 2013). AFB_1 alters the tryptophan metabolism which in turn decreases the level of serotonin and dopamine. The dose-dependent relation between acetylcholine and AFB_1 causes toxicity in the brain cells (Tanaka et al. 2015). In the case of acetylcholine, AFB_1 exposure increases the level of acetyl cholinesterase (AChE), which is further involved in inflammatory and pathological process (Xie and Yang 2015). The increased concentration of AChE also affects the brain cognitive processes such as memory loss, disturbance in sleep cycle, restlessness, abnormal sensations, muscle tremor, and other neural disorders (Trebak et al. 2015). Studies have confirmed the effects of AFB_1 on mitochondrial DNA in the nerve cells. This DNA aberration disturbs the oxidative phosphorylation leading to the damage of proteins, lipids, and DNA, and this DNA damage leads to apoptosis (Wu and Khlangwiset 2010).

The chronic toxicity of AFB_1 fails the liver ammonia detoxification activity. The accumulated amount of ammonia is capable of crossing the blood-brain barrier, reaches to neuron cells, and increases the level of glutamate leading to a condition known as encephalopathy which further leads to insomnia, memory loss, coordination loss, and disorientation (Bbosa et al. 2013). AFB_1 has been recognized as an etiological agent in promoting Reye syndrome by causing neuronal degeneration and cerebral edema (Yaman et al. 2016). Sometimes, AFB_1 causes Reye syndrome along with the symptoms of encephalopathy. Glutathione being an antioxidant stabilizes the cell membranes by reducing the oxidative stress. AFB_1 decreases the glutathione levels and simultaneously increases the lipid oxidation reactions. These unfavorable reactions induce membrane impairment; destruction of lipids, proteins, and DNA; as well as pro-inflammatory and inflammatory responses (Obis et al. 2015). In some cases, AFB_1 increases the level of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and Toll-like receptors (TLRs), disrupts gene

expressions by interfering with the function of mRNA, and is also involved in cell apoptosis. Considering all consequences of AFB_1 on the human brain, it may be stated that most of the neural disorders happen when the energy pool (ATPs) declines or the reactions producing ATP are disturbed due to aflatoxin exposure (Zala et al. 2013).

6.7 Summary

Aflatoxins are the leading type of toxic compounds that are present beyond the maximum permissible limits, especially in the food commodities of African and Asian countries. In this chapter, the impact of aflatoxins on the health of adults and elderly people is reviewed, including the impacts on the functioning of the liver, kidney, nervous system, immune system, and reproductive system. The cytochrome enzymes present in the liver converts aflatoxins to different metabolic forms, the most toxic among which is AFBO that reacts with macromolecular components of cells, e.g., proteinaceous compounds and phospholipids, leading to metabolic and genetic disorders and altered cell structures. Elderly people being immunocompromised are greatly affected by the impacts of aflatoxins; therefore, the maximum cases of HCC are reported in elderly people. Aflatoxin's major target organ is the liver, and 5–28% cases of HCC are considered due to aflatoxins. In the case of aflatoxin-associated HCC, mutation occurs at codon 249, and normally transition occurs at the third base from G-T. Chances of HCC increase to many folds in case aflatoxin-contaminated food is being eaten by hepatitis patients due to the fact that the target organ of both (aflatoxins and hepatitis virus) is the liver. Chances of HCC are reported to increase with an increase in age, and therefore HCC cases are reported above 40 years of age (maximum above 55 years of age). Aflatoxin exposure by the kidneys may lead to kidney failure or kidney tumors. Aflatoxin exposure may lead to injured kidney cells, edema, and hemorrhagic condition of kidney cells, cytomorphosis, and inflammation in the cells of the kidney. Human exposure to aflatoxins also affects the function of different enzymes that participate in the production of male and female gametes leading to infertility in both genders. Aflatoxin exposure leads to anovulation and hormonal disturbance leading to low levels of progesterone and high levels of LH. Aflatoxin exposure leads to malfunctioning of B and T cells, and especially T cell activity is disturbed. Higher doses of aflatoxins lead to reduced production of IL and spleen cells, T cell impairment, and a weak immune system. The metabolism of AFB_1 inside the human body leads to the production of reactive oxygen species, the reactive metabolites leads to oxidation of fatty acids, and, ultimately, further radicals are generated. These free radicals are capable of crossing the blood-brain barrier (may even destroy it) and may cause different nervous system disorders. The levels of β-glucuronidase enzymes and β-galactosidase enzymes are increased in the brain, activity of the sodium-potassium pump is disturbed, tryptophan metabolism is disturbed, and acetylcholine levels are increased due to aflatoxin exposure, ultimately leading to serious disorders of the

nervous system. The presence of aflatoxins in food commodities and their serious implications on the health of adults and especially elderly people demands the adoption and implementation of strict regulatory measures. Educating farmers and other food handlers is essential to minimize the chances of fungal growth and, ultimately, the production of aflatoxins in food commodities.

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Chapter 7
Regulations for Aflatoxins in Developing and Industrialized Economies

Samia Tahir and Shinawar Waseem Ali

Abstract Among mycotoxins, aflatoxins are the most toxic as well as the most studied by the researchers across the world. Based on the prevalence of aflatoxins in food commodities and their health implications, countries across the world have adopted strict regulations and regular monitoring of aflatoxins. The regulations for aflatoxins vary based on the type of food commodity and type of aflatoxins. The regulations for aflatoxins in different food commodities are based on the economic condition of a country, its technological advancement level, the overall health and education status of the country, level of prevalence of aflatoxins in different food commodities, climatic conditions, and literacy rate.

Keywords Mycotoxins · Aflatoxins · Regulations · Economic · Health · Climatic condition

7.1 Mycotoxins

Mycotoxins are secondary metabolites produced by fungi, i.e., *Penicillium*, *Fusarium*, *Aspergillus*, etc. (Misihairabgwi et al. 2019). Mycotoxins are affecting the whole world, especially developing countries. About 300 different types of mycotoxins are known so far. More importance is given to ochratoxins, fumonisins, zearalenone, aflatoxins, and trichothecenes because of their toxicity (Guchi 2015). Mycotoxins are known to cause immunosuppression and cancer. Mycotoxin contamination can be pre-harvest or post-harvest due to poor storage practices (Afsah-Hejri et al. 2013).

S. Tahir · S. W. Ali (✉)
Department of Food Sciences, Faculty of Agricultural Sciences, University of the Punjab, Lahore, Pakistan
e-mail: shinawar.iags@pu.edu.pk

K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_7

7.2 Aflatoxins

Out of 300 known mycotoxins, aflatoxins are given more importance because of its occurrence and toxicity (Al-Zoreky and Saleh 2019). Aflatoxins are basically carcinogenic and toxic secondary metabolites of fungi. In 1960, aflatoxins were discovered in Europe. Aflatoxin was isolated from infected turkey after death of more than 1 lac turkey due to turkey X disease. Aflatoxin was transferred to them through consumption of peanut diet that was infected by *Aspergillus*. Aflatoxin shows acute toxicity in both animals and humans (Wogan 1966).

7.2.1 Aflatoxin Producers

Aflatoxins are produced specifically by *Aspergillus* species, which are widely distributed due to its universal adoption in a wide range of climates. This ultimately led to exploration of different secondary metabolites produced by these fungi.

7.2.2 Types of Aflatoxins

There are 20 different types of aflatoxins. Basically, aflatoxins are chemically classified into two groups. The first one is difurocoumarocyclopentenone series which includes AFB_1, AFB_2, AFB_{2A}, AFM_1, AFM_2, AFM_{2A}, and aflatoxicol, while the second one is difurocoumarolactone series which includes AFG_1, AFG_2, AFG_{2A}, $AFGM_1$, $AFGM_2$, $AFGM_{2A}$, and AFB_3. Out of all these well-known types, four that are of more importance are B1, B2, G1, and G2. *Aspergillus flavus* and *Aspergillus parasiticus* are involved in producing aflatoxin B1 and B2, while some *Aspergillus flavus* and *Aspergillus parasiticus* of group 2 produce aflatoxins G1 and G2. Aflatoxin B2 and G2 are dihydroxy by-products of aflatoxins B1 and G2, while aflatoxin M1 is chemically 4-hydroxy aflatoxin, and aflatoxin M2 is chemically 4-dihydroxy aflatoxin B2. They are distinguished based on their fluorescence under UV light, and aflatoxin B gives blue fluorescence, while aflatoxin G gives green fluorescence. AFB_1 and AFB_2 convert into less toxic metabolite, i.e., AFM_1 and AFM_2, respectively, in farming animals and humans by the activity of cytochrome P450 enzymes present in the liver. Aflatoxin Q1 is a metabolite of B1 in higher vertebrates.

7.2.3 Chemistry of Aflatoxins

Aflatoxins are produced as a secondary metabolite by molds on feed and food. More than 20 types of aflatoxin are known, out of which aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2 are the most common. Aflatoxins G2 and B2 are dihydro

by-products of original compounds. At room temperature they exist in pale yellow crystalline form. Aflatoxins are oxygenated hydrocarbons and are soluble in organic solvents like chloroform, methanol, etc. Usually dichloromethane is used for extraction because of its high solubility. Aflatoxins are sensitive to air, polar solvent, ultraviolet rays, light, and pH above 10 or below 3. Aflatoxins B1 and B2 have a melting point of 268–289 °C, while aflatoxins G1 and G2 have a melting point of 237–246 °C. Simple cooking process may not destroy aflatoxin, but it can be destroyed by autoclaving.

7.2.4 Aflatoxin in Food

Aflatoxin is usually common in developing and underdeveloped countries where cultivation and storage conditions are not up to the mark. Aflatoxin is mostly found on staple food like wheat, rice, maize, oat, cassava, groundnut, and oil seeds. Because of more susceptibility, ground nuts and maize are the main sources of aflatoxin transmission in humans and animals (Table 7.1).

7.2.5 Regulations Regarding Aflatoxin

In crops, mycotoxin contamination can occur before harvesting or after harvesting or during storage. Wheat, oats, milk, rice, cheese, corn, cotton seed, peanuts, cereals, and feed are reported to be contaminated with aflatoxins (Zheng et al. 2006).

More than 400 mycotoxins are presently reported in different research papers (Ali and Afzaal 2014). Among these 400, aflatoxins are more significant due to their carcinogenic, teratogenic, hepatotoxic, and mutagenic properties.

In Asia, cereals are the main source of carbohydrates and one of the main export commodities. Due to increase in cancer and hepatotoxicity, around 80 countries have implemented regulations regarding aflatoxins. These regulations are still not synchronized internationally because each country develops maximum limit according to the mean intake value of a particular food item.

Table 7.1 Commodities affected by aflatoxin

Aflatoxin	Commodity contaminated by aflatoxin
B1 and B2	Flour, sunflower, peanuts, rice, pea, sorghum, dairy products, maize, figs, cotton seed, apple and guava juice, meat, rape, and pistachio
G1 and G2	Figs, cereals, maize flour, peanuts, meat, corn, pea, sunflower seeds, dairy products, spices, and cotton seed

7.2.5.1 FAO Inquiry Regarding Regulations

The National Institute for Public Health and the Environment together with the Dutch embassy's agricultural services started a survey in as many countries as possible in 2002 in order to check which countries have regulation, which products are being regulated, law-enforcing authorities, and methods that are being used for mycotoxin analysis.

7.2.5.2 Survey's Results

Inquiry showed that approximately 99 countries with 87% of the world's populations had regulations for mycotoxins. These regulations mostly covered aflatoxins B1, B2, M1, and M2. Overall, a 30% increase was observed in 2003 as compared to 1995 with more products being regulated for mycotoxin contaminations. Figure 7.1 shows regulations of mycotoxin in the world.

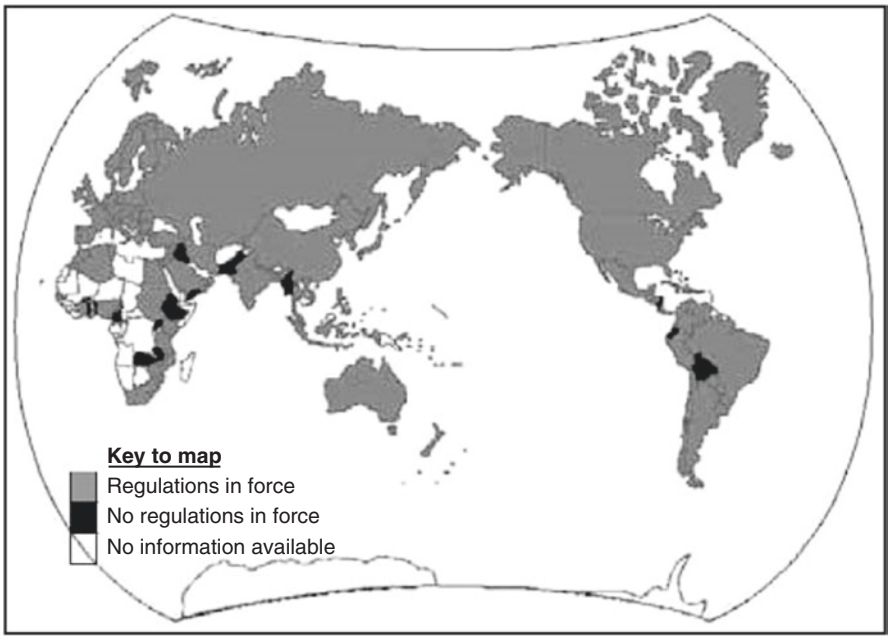

Fig. 7.1 Mycotoxin regulation (Source: FAO)

7.2.6 *Regulations in Specific Regions*

7.2.6.1 Africa

Approximately, 59% of the African population living in almost 15 countries had mycotoxin regulations. Most of the countries on this continent are still underdeveloped; that's why no specific regulation was observed, but cases of mycotoxins were reported in these countries that demanded regulations to be imposed (Figs. 7.2 and 7.3).

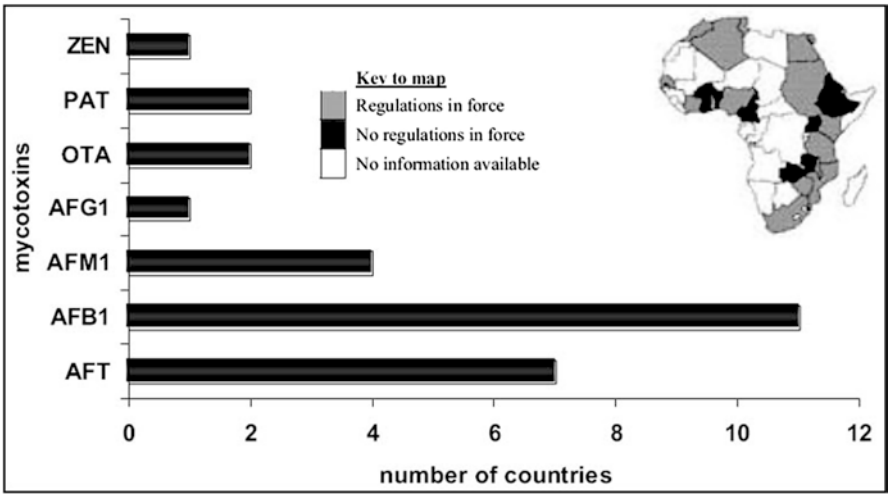

Fig. 7.2 Mycotoxin regulations in Africa (in food) (Source: FAO)

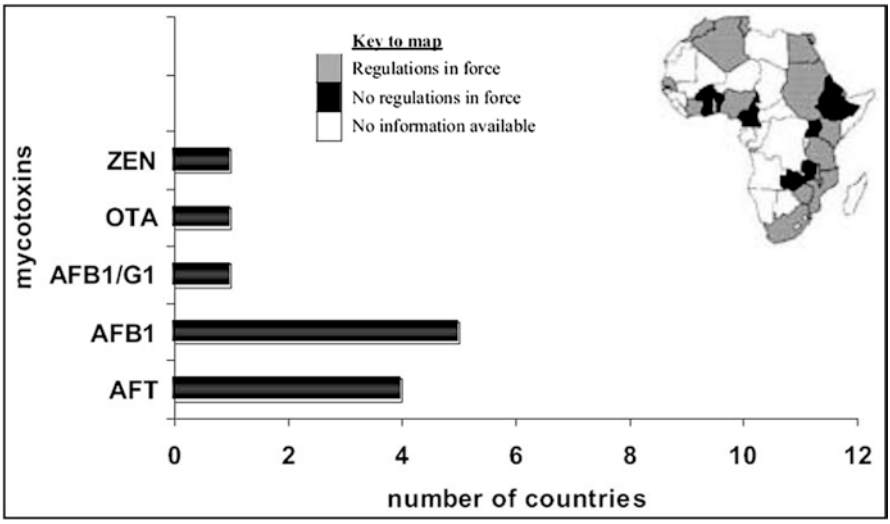

Fig. 7.3 Mycotoxin regulations in Africa (in feed) (Source: FAO)

7.2.6.2 Asia and Oceania

Asia and Oceania mostly include tropical and subtropical countries where fungi can result in mycotoxin contamination due to high temperature. Approximately, 88% of inhabitants of almost 23 countries in this region had mycotoxin regulations. In food all types of mycotoxins are being regulated, but in the case of feed, only B1 was regulated. Among these 23 countries, extensive and detailed regulations are observed in China and Iran (Figs. 7.4 and 7.5).

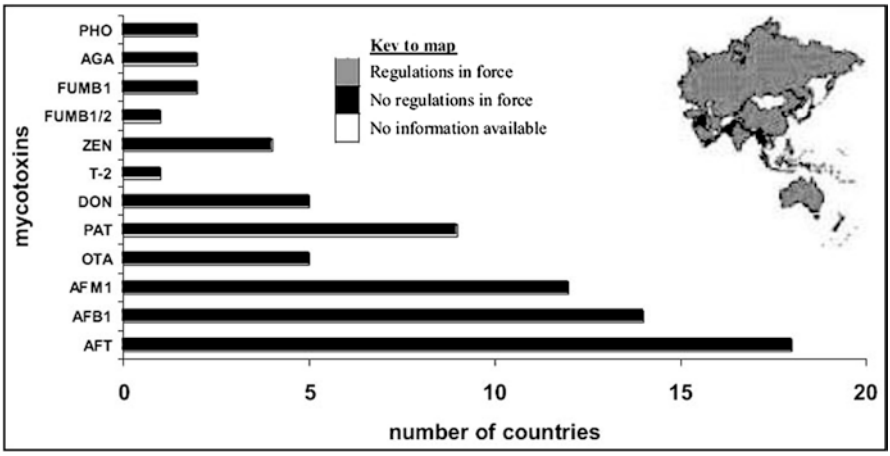

Fig. 7.4 Mycotoxin regulations in Asia (in food) (Source: FAO)

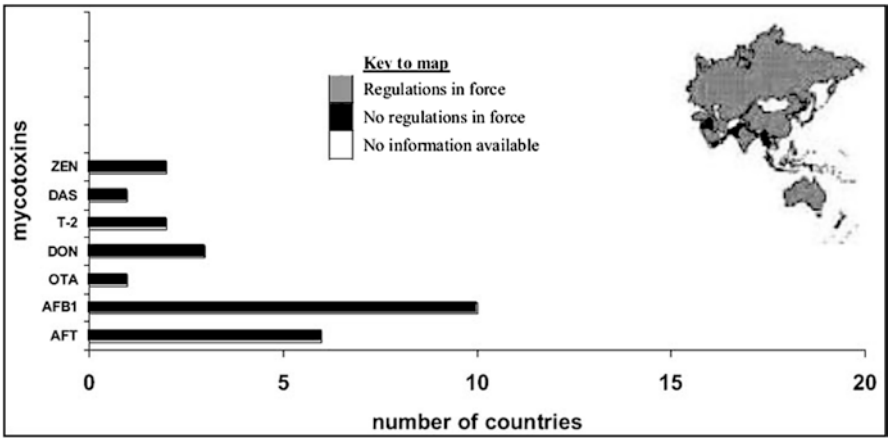

Fig. 7.5 Mycotoxin regulations in Asia (in feed) (Source: FAO)

7.2.6.3 Europe

In 2003, nearly 39 countries covering 99% of the total population of this region had extensive and detailed regulations regarding mycotoxins. Moreover, in Europe, regulations had been harmonized for various food stuffs, for example, aflatoxin M1 in milk, in the case of cereals ochratoxin A, and many more (Figs. 7.6 and 7.7).

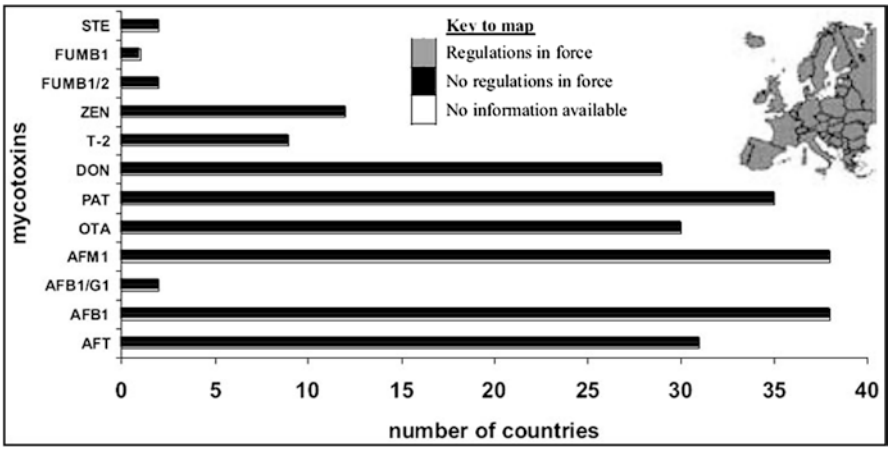

Fig. 7.6 Mycotoxin regulations in Europe (in food) (Source: FAO)

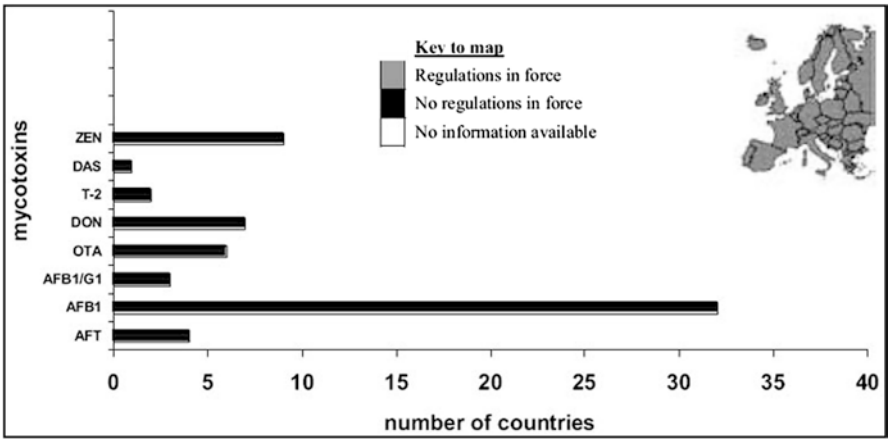

Fig. 7.7 Mycotoxin regulations in Europe (in feed) (Source: FAO)

7.2.6.4 Latin America

The major crops of Latin America include soybeans, sunflower, wheat, barley, maize, cotton, ground nuts, coffee, dairy products, and tree nuts. All these crops are known to be highly prone to fungal attack that in return results in toxin production; that's why regulations are necessary for this region. Nineteen countries had regulations for aflatoxin in Latin America according to a 2003 survey (Figs. 7.8 and 7.9).

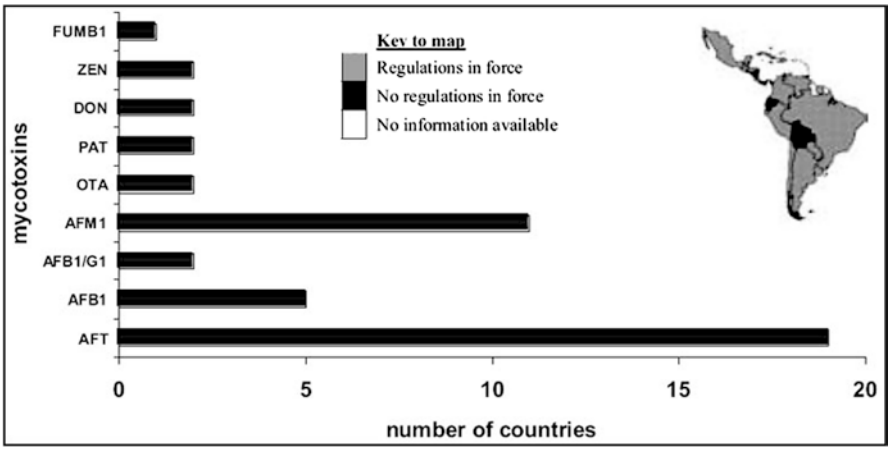

Fig. 7.8 Mycotoxin regulations in Latin America (in food) (Source: FAO)

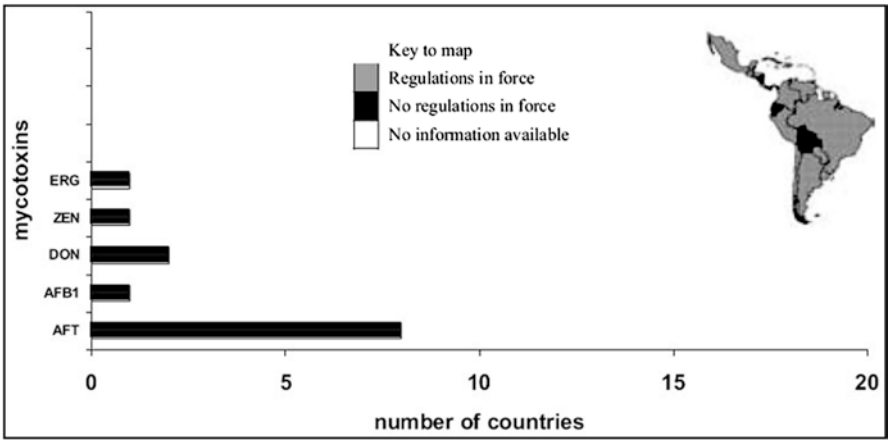

Fig. 7.9 Mycotoxin regulations in Latin America (in feed) (Source: FAO)

7.2.6.5 North America

The North American countries include the United States and Canada. Both countries are highly developed and not only have had regulations implemented related to mycotoxins but have also developed a highly advanced method of sampling for mycotoxin testing in food and feed (Figs. 7.10 and 7.11).

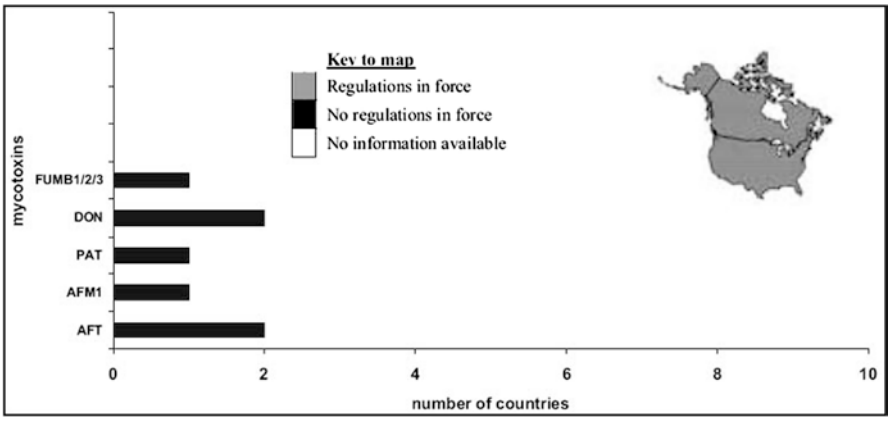

Fig. 7.10 Mycotoxin regulations in North America (in food) (Source: FAO)

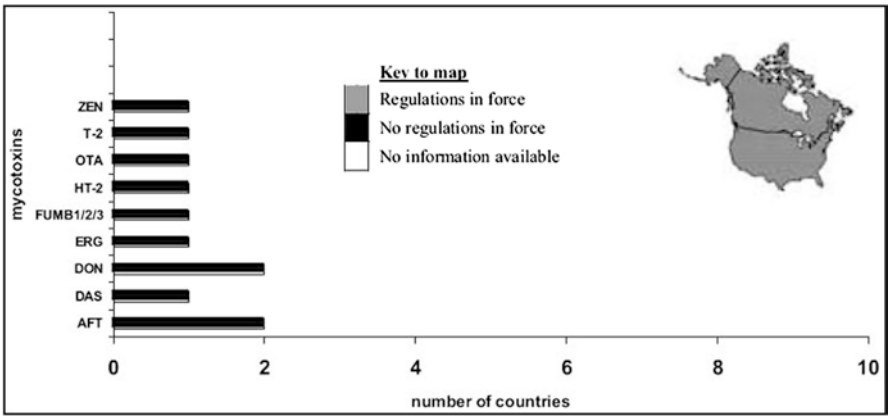

Fig. 7.11 Mycotoxin regulations in North America (in feed) (Source: FAO)

7.2.7 Worldwide Limitations of Aflatoxins

With the advancement in technology and awareness, a number of countries having mycotoxin regulations have been increased dramatically for the past years. Mostly in the case of food, more focus is on aflatoxin B_1, whereas in milk and milk products, aflatoxin M_1 is regulated. Aflatoxin B_1 has been regulated in almost every food; if a specific food is not present in the list, then for this type of food, regulations are the same as in same or closely related food category.

7.2.7.1 Aflatoxin B1 Regulations

According to a 2003 survey, approximately 29 countries set 2 μg per kg as the maximum limit for aflatoxin B1 in food and feed; most of these countries are in Europe, and most of these countries have already harmonized their regulations (Fig. 7.12).

7.2.7.2 Total Aflatoxin Regulation in Food

In most of the countries, aflatoxins are being regulated as a sum of all aflatoxins, that is, aflatoxin, B1, B2, G1, and G2 in general. The range of limit usually lies between 0 and 35 μg per kg with the median limit set at 10 μg per kg for most of the countries where 17 countries had set their limit to 20 μg per kg, most of these include Latin American countries with the United States being the first one to implement this limit for total aflatoxins (Figs. 7.13 and 7.14).

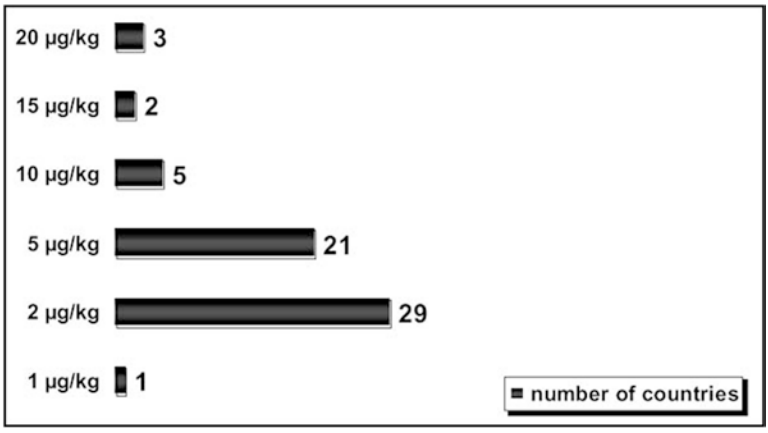

Fig. 7.12 Worldwide limits for aflatoxin B_1 in food (Source: FAO)

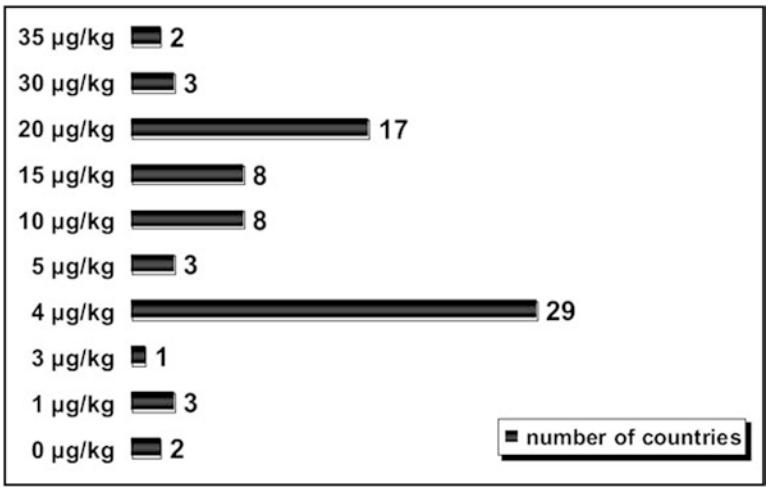

Fig. 7.13 Worldwide limits for total aflatoxin in food (Source: FAO)

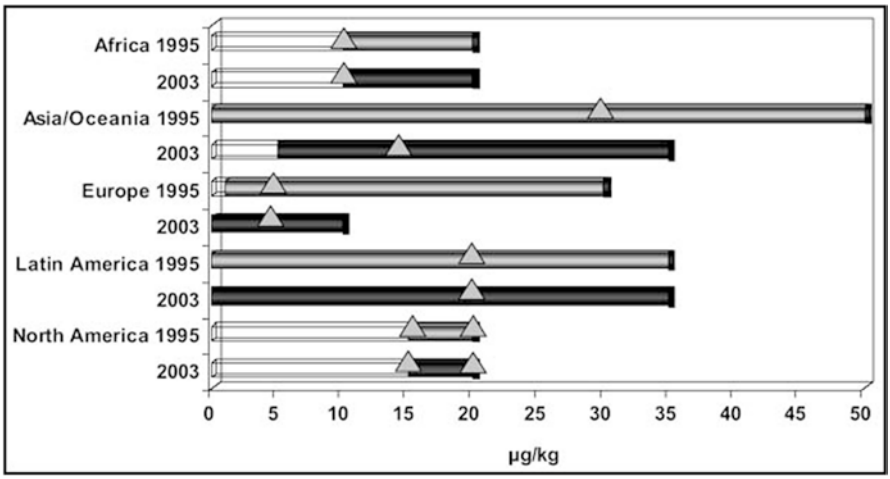

Fig. 7.14 Ranges and medians of limits for total aflatoxins in food per world region (Source: FAO)

7.2.7.3 Aflatoxin M1 Regulations

In 2003 only 60 countries had regulation regarding contamination of aflatoxin M1 in milk and its products with peak limit of 0.5 mg per kg (Fig. 7.15).

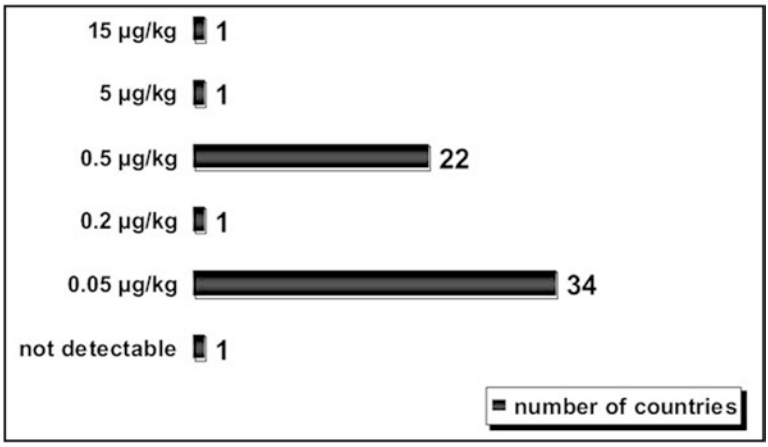

Fig. 7.15 Worldwide limitation of aflatoxin M1 (Source: FAO)

7.2.7.4 Aflatoxin B1 in Feed

Controlling aflatoxin B1 in feed will ultimately control the production of aflatoxin M1 in milk; that is why many countries have regulations for aflatoxin B1 in feed, especially in dairy feed. Almost 27 countries have set 5 µg per kg as the maximum limit of aflatoxin B1 in feed, most of these include European countries (Fig. 7.16).

7.2.7.5 Total Aflatoxin Regulations in Feed

In case of total aflatoxin, the limit was set at 20 µg per kg in most of the countries. The total aflatoxin limit is unusually high as compared to aflatoxin B1 due to its health effects. This limitation varies from countries to countries (Fig. 7.17).

7.2.8 Aflatoxin Regulation in Rice

7.2.8.1 Regulations in European Union

According to the EU, different maximum limits were assigned to different food products according to their intended use. EU countries have adopted the most stringent regulations for aflatoxins. In case of cereals, nuts, processed food commodities, dried fruits, and other food commodities, the maximum limit for aflatoxin B_1 is 2 ppb; for total aflatoxin, it should be less than 4 ppb; and for AFM_1 in milk, it should be less than 0.05 ppb (Ismail et al. 2018).

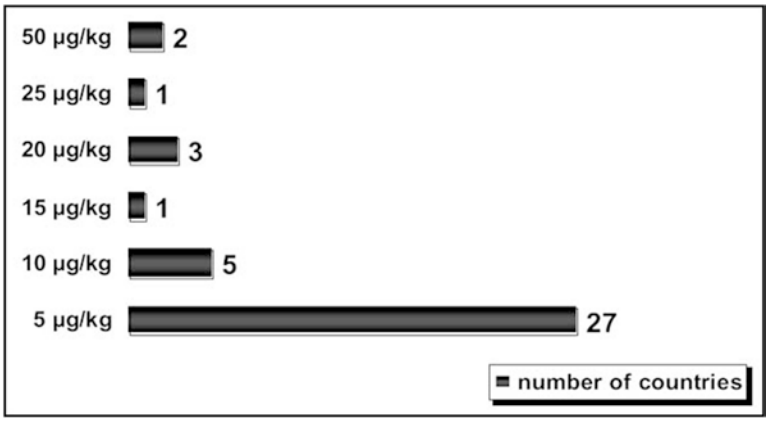

Fig. 7.16 Worldwide limitation of aflatoxin B1 in feed (Source: FAO)

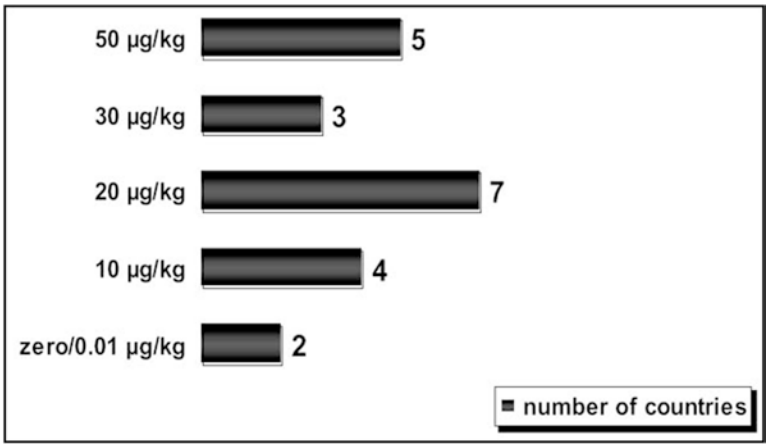

Fig. 7.17 Worldwide limitation of total aflatoxin in feed (Source: FAO)

7.2.8.2 Regulations in the United States

US regulations differ from the EU. In the United States, all food commodities excluding milk should have less than 20 ppb aflatoxin in total. There is no regulation for individual aflatoxin in the United States except for AFM_1 in milk, i.e., 0.5 ppb (Ismail et al. 2018).

7.2.8.3 Regulations in Asia

Regulations for the maximum limit of aflatoxin in different food commodities vary from country to country. In Japan, the maximum limit for total aflatoxin in all food commodities is 10 ppb. According to China, the maximum limit of total aflatoxin should be 10 ppb in paddy rice, brown rice, white rice, and all other products made from them. In Malaysia, for most of the food commodities, total aflatoxins should be less than 5 ppb. In case of Korea, the maximum limit for aflatoxin B_1 is 10 ppb, while total aflatoxins should be less than 15 ppb in grains, cereals, and its products. In Pakistan, the maximum limit for total aflatoxin is 20 ppb and for AFM_1 in milk is 0.5 ppb (Ismail et al. 2018).

7.2.9 Worldwide Limitations for Other Mycotoxins

7.2.9.1 Patulin

Patulin is considered the most regulated type of mycotoxin in the word with a maximum limit of 50 μg per kg in most of the countries, but in the case of the EU, the maximum limit was set at 10 mg per kg. Most of the regulations for patulin focus on fruit juices, especially apple juice (Fig. 7.18).

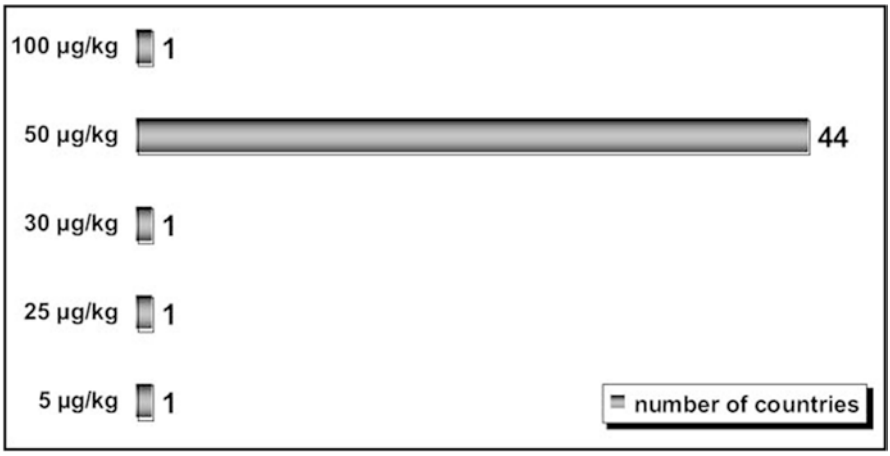

Fig. 7.18 Worldwide limitation of patulin (Source: FAO)

7.2.9.2 Ochratoxin A

In 2003, Ochratoxin A regulations were limited only to cereals with maximum limitation of 5 mg per kg in case of raw cereals and 3 mg per kg in case of processed cereals in Europe (Fig. 7.19).

7.2.9.3 Zearalenone

According to a 2003 survey, 16 countries had been successfully enforcing regulation regarding zearalenone which is estrogenic mycotoxin setting 1000 µg as maximum limit (Fig. 7.20).

7.2.9.4 Fumonisins

In 2003 fumonisins were the least regulated aflatoxin with only six countries implementing regulations with a maximum limit of 3000 µg per kg (Fig. 7.21).

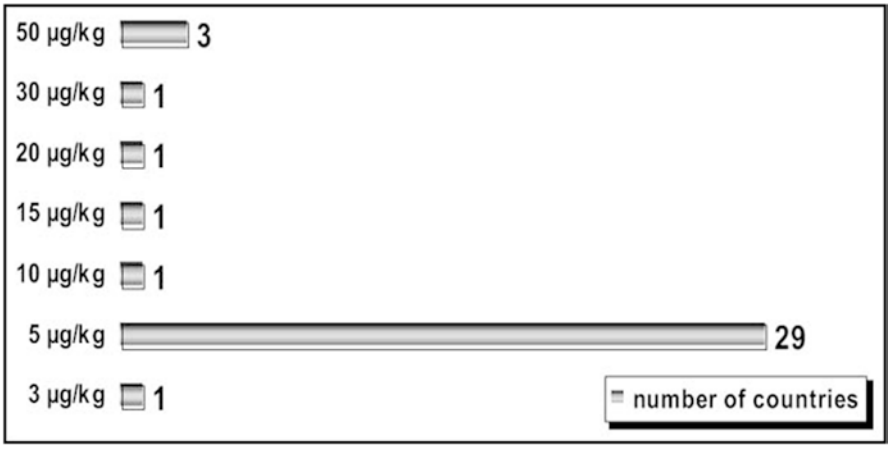

Fig. 7.19 Worldwide limitation of ochratoxin A (Source: FAO)

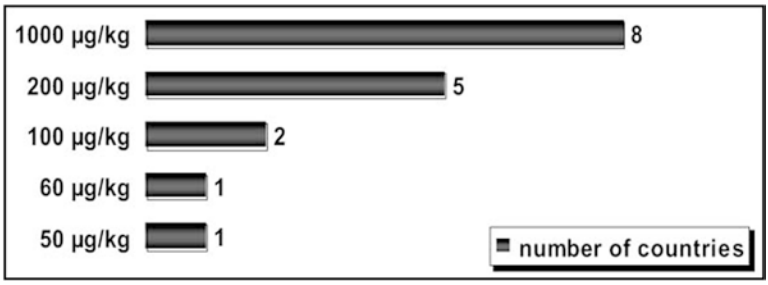

Fig. 7.20 Worldwide limitation of zearalenone (Source: FAO)

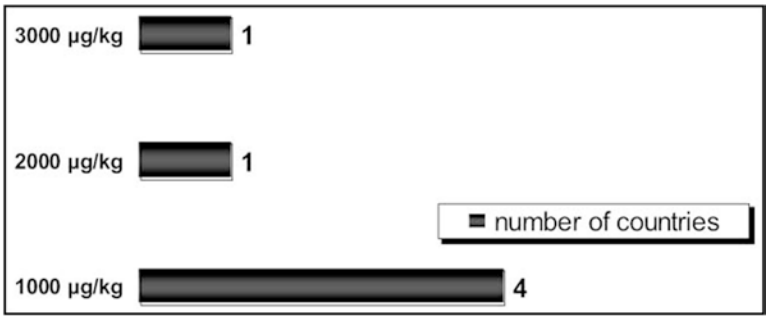

Fig. 7.21 Worldwide limitation of fumonisins (Source: FAO)

7.2.10 Harmonized Regulations in the World

7.2.10.1 ASEAN

Members of ASEAN countries include Cambodia, Brunei Darussalam, Indonesia, Malaysia, the Lao People's Democratic Republic, Myanmar, Singapore, the Philippines, Vietnam, and Thailand. These countries have similar and harmonized regulations.

7.2.10.2 MERCOSUR

Brazil, Uruguay, Argentina, and Paraguay are included in MERCOSUR countries. These have same regulations and detailed sampling method for aflatoxin in products like maize, milk, and peanuts.

7.2.10.3 European Union

The EU has regulated and harmonized their regulation related to aflatoxins since 1976. EU countries are following similar regulations for aflatoxin B1 and total aflatoxins in both food and feed.

7.2.10.4 Australia and New Zealand

Australia and New Zealand harmonized their regulation for aflatoxin in ergots, tree nuts, and peanuts. In addition, the harmonized regulations include unique limits for phomopsins in lupin seeds and products thereof and for agaric acid in food, containing mushrooms and alcoholic beverages.

7.2.10.5 Codex Alimentarius

The FAO and WHO join their hands to harmonized regulations and sampling methods for maximum countries to facilitate trade among them. In this aspect, the codex committee on food additives and contaminants has set maximum limits of aflatoxins in different commodities. Moreover, this committee also devises protocols that can help in the reduction of toxin production during different pre- and post-harvesting processes.

7.2.11 Worldwide Review of Aflatoxin Occurrences

Every year many cases of aflatoxins and aflatoxicosis are reported from around the world. According to the FAO (Food and Agriculture Organization), per year about 1000 million metric tons of agricultural commodities are contaminated with fungi due to poor agricultural practices, poor post-harvesting technique, improper handling, and environmental conditions like moisture content, temperature, and initial contamination in the environment.

7.2.11.1 Occurrence in Asia

An average contamination of about 0.5–0.6 ppb was found in all the sample of rice (29) collected from China from the area of Fusui, Huaian, and Huantai (Sun et al. 2011). Another research done by Wang et al. in 2007 shows an average contamination of 0.79 ppb. Conditions were much better in South Korea; only five samples were found to be contaminated with aflatoxin B1 out of 88 sample with a mean value of 4.8 ng/kg (Park et al. 2004). Similar results were found during another study done in 2014 (Ok et al. 2014).

According to a study, Indian rice was found to be heavily contaminated by aflatoxin as out of 1511 samples collected from 12 states, about 256 samples surpassed the maximum limits set by the Indian government that is 30 ppb. In 2009 another research shows a frequency of contamination to be 67.8% and the level of aflatoxin to be 0.5–308 ppb (Reddy et al. 2009). An average level of 1.75 ppb of aflatoxin B1 was found in nine samples out of 13 in Malaysia (Reddy et al. 2011).

Eighty-three samples were collected from different regions from Japan, and all of them were negative for all types of aflatoxin (Kumagai et al. 2008). In Vietnam with a mean level of 3.31 ppb for aflatoxin, 51 samples out of 100 surpassed the EU limit. In the Philippines where the mean aflatoxin contamination level was 1.53 ppb, 74 samples out of 78 showed positive results (Nguyen et al. 2007).

In the case of West Asia, 32 rice samples out of 100 samples collected from different places from Turkey surpasses the EU set value and according to Turkish regulations too (Aydin et al. 2011). In Iran, out of 71 samples, 59 samples were positive for aflatoxin B1 with average contamination level of 2.1 ppb (Mazaheri 2009).

7.2.11.2 Situation of Aflatoxin in Pakistan

In Pakistan, the average frequency of aflatoxin contamination was found 33 to 64%. The average level of aflatoxin was high in samples of paddy rice, brown rice, white rice, and parboiled rice with a range between 7.10 and 16.35 ppb (Iqbal et al. 2012). According to another research, nearly 70% of rice samples, from Pakistan, were found to be contaminated with aflatoxin (Ali and Afzaal 2014). The average level of aflatoxin B1 in sample collected from Sahiwal, Faisalabad, and Gujranwala was 16.77, 6.97, and 8.78 ppb, respectively, and total aflatoxin values were 8.94, 20.30, and 21.54 ppb (Majeed et al. 2013).

In cases of basmati rice, 18.3% were positive for aflatoxin, out of which 6% surpassed EU maximum limits for total aflatoxin. Condition of broken rice was found to be extremely worst with about 36.4% of positive samples, and all of them surpassed EU set limits (Firdous et al. 2014).

7.2.11.3 Situation of Aflatoxin in Europe

As most of the European countries import rice from Asian countries like Pakistan and India, all of the sample is collected from Austria, Sweden, Spain, and Switzerland. In samples of brown rice collected from Switzerland, the mean contamination value was 14.7 ppb for total aflatoxin (Ruadrew et al. 2013).

An average level of 1.97 ppb for total aflatoxin was found in samples collected from Spain. Out of 81 samples, 15 samples were found to be contaminated with aflatoxin in Austria (Reiter et al. 2010).

7.2.11.4 Situation of African Countries

Samples collected from Tunisia were free from aflatoxin (Ghali et al. 2010), whereas all samples collected from Nigeria and Ivory Coast were contaminated with aflatoxin with average value of 4.5 ppb for aflatoxin B1 and 82.5 ppb for total aflatoxin, respectively (Makun et al. 2011).

7.2.11.5 Situation of America and Latin America

The average aflatoxin value was 0.34–0.39 ppb in samples collected from Canada; out of 200 samples, 99 were found contaminated with aflatoxin (Bansal et al. 2011), whereas aflatoxin contamination was higher in Brazil as compared to Canada, where the mean contamination value was 13.13 ppb in Brazil, and 135 samples were found to be contaminated out of 235 (Almeida et al. 2012).

7.3 Conclusion

Aflatoxins are omnipresent as the climatic conditions of most of the countries support the growth of fungus responsible to produce aflatoxins and for the release of aflatoxins by fungi. The global trade of food commodities has also led to the spread of aflatoxins from the highest aflatoxin production regions to the regions where the overall climatic conditions and post-harvest management practices do not support the production and prevalence of aflatoxins. The criteria proposed by the Food and Agriculture Organization (FAO) are "as low as reasonably acceptable" (ALARA), but a huge variation exists in the regulations adopted for different types of aflatoxins in different food commodities by the countries across the world. European countries have the most stringent regulations for aflatoxins and have protected their people from the perils of aflatoxins not only by adopting the strict regulations but also by the regular monitoring of aflatoxins in food stuff, their technological advancement level, and investment in food safety and in the implementation of rules and regulations. The African countries have the highest prevalence level of aflatoxins in food commodities. The prevalence of aflatoxins in food commodities of African and Asian countries not only depends on their climatic conditions but also on the lenient regulations against aflatoxins due to their economic conditions, less implementation of rules and regulations, poor post-harvest management practices, low literacy rate, and lack of technological advancement.

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Chapter 8
Analytical Methods for Detection and Quantification of Aflatoxins

Alessandra V. Jager and Fernando G. Tonin

Abstract Aflatoxins are produced by filamentous fungi, primarily *Aspergillus flavus*, *A. parasiticus*, and *A. nomius*. These fungi occur naturally and might infest several food commodities throughout the food chain. The four major aflatoxins are Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1), and Aflatoxin G_2 (AFG_2). Aflatoxin M_1 (AFM_1) and Aflatoxin M_2 (AFM_2), the hydroxylated metabolites of AFB_1 and AFB_2, respectively, are excreted into the milk of mammals that have ingested contaminated feed. Aflatoxins are highly carcinogenic, teratogenic, and hepatotoxic to humans and animals; consequently, knowledge about their incidence and levels in food and feed is a matter of public health concern. Numerous countries have set specific regulations on the maximum permitted limits of these contaminants in foodstuffs. Therefore, identifying and quantifying aflatoxins by reliable analytical methods are paramount for compliance with these legal limits. This chapter presents the fundamentals and recent developments of sample extraction, cleanup procedures, and identification and quantification approaches for aflatoxins in food and feed.

Keywords Aflatoxins · Sample extraction · Immunoaffinity column · SPE · QuEChER · DLLME · Liquid chromatography · Thin-layer chromatography · Fluorescence · Mass spectrometry · ELISA · Method validation · Multiclass mycotoxin

A. V. Jager (✉)
School of Pharmaceutical Sciences at Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil
e-mail: alejager@usp.br

F. G. Tonin
School of Animal Science and Food Engineering, University of São Paulo, Pirassununga, SP, Brazil
e-mail: fgtonin@usp.br

K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_8

8.1 Introduction

Before the 1960s, episodes of farm livestock intoxication due to consumption of moldy feed had been described, but back then these episodes were not further investigated and remained unexplained (Richard 2008). However, in May 1960, after the acute poisoning of turkeys named Turkey "X" Disease in the United Kingdom, the correlation between an unknown toxin produced by fungus in the feed and its toxic effects was finally recognized (Sargeant et al. 1961; Lancaster et al. 1961). Shortly afterward, the unknown toxin was demonstrated to be carcinogenic to rats; it was also linked with liver cancer in humans (Le Breton et al. 1962). Chemical and physical characterization of the toxin, termed aflatoxin, was only accomplished some years later (Van der Zijden et al. 1962; Asao et al. 1963; Wogan 1966). Actually, aflatoxins belong to a larger group of toxic substances known as mycotoxins, which are produced by diverse fungi. Since the aflatoxins were characterized, scientists worldwide have scrutinized them, particularly in studies related to their synthesis and incidence in food and feed, adverse effects on human and animal health, and mitigation strategies (Rushing and Selim 2019).

Aflatoxins are produced by filamentous fungi, primarily *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* (Olsen et al. 2008). *Emericella astellata, E. venezuelensis, A. bombycis, A. ochraceoroseus, A. pseudotamari,* and *A. tamarii* also produce aflatoxins; however, these fungi are less common in crops. All the aforementioned fungi occur naturally and might infest several food commodities throughout the food chain: before and during harvesting; during storage, transportation, or processing; and even during consumption (Kaale et al. 2021). Groundnuts, cereals, oilseeds, and spices grown in tropical and subtropical regions under hot and humid climate conditions are the most susceptible to contamination (Williams et al. 2004).

Presently, around twenty aflatoxins have been characterized. The four major aflatoxins are Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1), and Aflatoxin G_2 (AFG_2). Aflatoxin M_1 (AFM_1) and Aflatoxin M_2 (AFM_2), the hydroxylated metabolites of AFB_1 and AFB_2, respectively, are also important: they are excreted into the milk of mammals that have ingested contaminated feed (Marchese et al. 2018). AFB_1 has been the most investigated and is the most prevalent in food and feed.

Aflatoxins are a group of difuranocoumarin derivatives (Fig. 8.1). AFB_1 and AFB_2 consist of a difuran ring fused to a coumarin nucleus with a pentenone ring; AFG_1 and AFG_2 contain a six-membered lactone ring instead of a pentenone ring (Dhanasekaran et al. 2011). Aflatoxins are insoluble in nonpolar solvents, very slightly soluble in water, and freely soluble in moderately polar solvents (e.g., methanol, acetone, chloroform, and dimethyl sulfoxide). AFB_1 and AFB_2 emit intense blue fluorescence under UV light, whereas AFG_1 and AFG_2 emit yellow-green fluorescence, hence the designations B and G, respectively (Antila et al. 2002). Ultraviolet (UV) light in the presence of oxygen, extreme pH values (<3 or >10), and oxidizing and chlorinating agents degrade aflatoxins (Budavari et al. 2001).

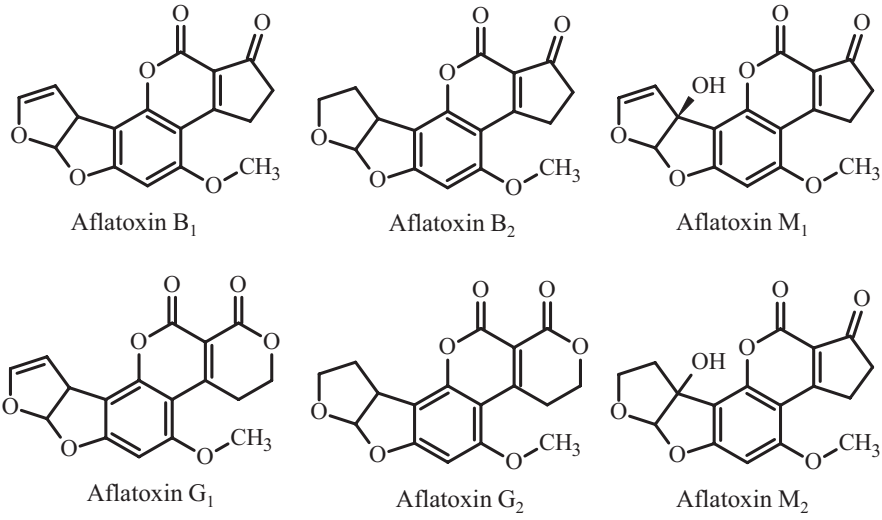

Fig. 8.1 Chemical structures of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2

Table 8.1 Physical and chemical properties of AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1

Aflatoxin	Molecular weight (g mol^{-1})	Molecular formula	log K_{ow}	Solubility (mg L^{-1})[a]	Fluorescence emission λ (nm)	Ultraviolet absorption λ_{max} (nm)[b]
AFB_1	312.27	$C_{16}H_{12}O_6$	1.23	918.3	425	223 265 362
AFB_2	314.29	$C_{17}H_{14}O_6$	1.45	585.4	425	265 363
AFG_1	328.27	$C_{17}H_{12}O_7$	0.50	3152	450	243 257 264 362
AFG_2	330.29	$C_{17}H_{14}O_7$	0.71	2009	450	226 265 363
AFM_1	328.27	$C_{17}H_{12}O_7$	−0.27	45,840	425	226 265 357

[a]Estimated in water at 25 °C
[b]In ethanol

Aflatoxins have high thermal stability, which prevents them from being degraded in heat treatments during food processing. Table 8.1 summarizes some physical and chemical properties of aflatoxins (Zhang and Banerjee 2020).

In 1987, over two decades after AFB_1 was discovered and extensively investigated, the International Agency for Research on Cancer (IARC) classified it as

Group 1 carcinogen (Antilla et al. 2002). Furthermore, AFB_1 is the most harmful and lethal among naturally occurring carcinogens. Another reason for IARC classifying AFM_1 as Group 1 is its association with immunosuppression, genotoxicity, mutagenicity, teratogenicity, and carcinogenicity (Womack et al. 2016).

As knowledge about the adverse effects of aflatoxins on human health evolved over the years, many countries set specific regulations on the maximum permitted limits of these contaminants in foodstuffs. As a general rule, the limits established for animal feed are usually higher than the limits set for food intended for direct human consumption.

The European Community legislation is undoubtedly the strictest and the most comprehensive regarding aflatoxins (European Commission 2006a). This legislation sets maximum levels not only for the sum of AFB_1, AFB_2 AFG_1, and AFG_2 (ΣAF), as listed in most countries, but also for AFB_1 individually. Maximum AFB_1 and ΣAF levels of 2.0 and 4.0 μg kg^{-1}, respectively, are allowed for groundnuts, nuts, dried fruits, and all cereals intended for direct human consumption. If further treatment is applied, AFB_1 and ΣAF levels in groundnuts must not exceed 8.0 and 15 μg kg^{-1}, respectively. Higher AFB_1 and ΣAF maximum levels, 5.0 and 10 μg kg^{-1}, respectively, are acceptable for nuts, dried fruits, and maize that are processed before human consumption or used as a food ingredient. The most restricted limit of 0.1 μg kg^{-1} AFB_1 is set for dietary food for particular medical purposes, cereal-based food, and baby food intended for infants and young children. Concerning animal feed, only AFB_1 is regulated (European Commission 2003). The maximum limit of 20 μg kg^{-1} AFB_1 is obligatory for all feed components and complete feedingstuff for cattle, sheep, goats, swine, and poultry, while 10 μg kg^{-1} AFB_1 is set for complete feedingstuff for calves and lambs. Feed for dairy animals has the lowest permitted level of 5 μg kg^{-1} AFB_1.

Legal limits adopted by the US Food and Drug Administration (US FDA) are more general and specify only levels for the sum of aflatoxins (ΣAF). A range between 100 and 300 μg kg^{-1} ΣAF is defined for grains intended for beef cattle, swine, and poultry depending on the maturity stage. Up to 20 μg kg^{-1} ΣAF is acceptable for animal feed other than corn and cottonseed meal, which includes grains intended for immature and dairy animals and human food (Jimenez Medina et al. 2021).

Asian countries do not have a harmonized regulation, and the maximum limits adopted there may vary considerably among countries (Anukul et al. 2013). Maximum ΣAF limits might be as high as 30 μg kg^{-1} for all food, as defined in India and Sri Lanka, or 35 μg kg^{-1} in Indonesia and Malaysia. Japan sets the ΣAF limit of 10 μg kg^{-1} for all food, and the AFB_1 limit of 10 and 5 μg kg^{-1} for rice and other grains, respectively. China regulates AFB_1 in corn and corn products and peanuts and peanut products at 20 μg kg^{-1}. Also, Chinese regulation lays down maximum AFB_1 limits of 10 μg kg^{-1} for rice and vegetable oils, except peanut and corn oils, and of 5 μg kg^{-1} for wheat, barley, beans, and other grains. For condiments, such as soy sauce, vinegar, and fermented paste that use grains as primary materials, the maximum AFB_1 value is 5 μg kg^{-1}. The lower AFB_1 limit of 0.5 μg kg^{-1} is set for food intended for particular dietary uses, like formula food and complementary

food for infants and young children. South Korea sets the AFB_1 limit of 10 μg kg^{-1} for grains, cereal products, dried fruits, streamed rice, and dried fermented soybeans. The lower AFB_1 limit of 0.1 μg kg^{-1} is set for baby food. Only Indonesia regulates ΣAF at 50 μg kg^{-1} for corn feed.

In 2011, the Brazilian National Health Surveillance Agency (ANVISA) reviewed regulations for mycotoxin, and maximum limits for aflatoxins that had previously been stated only for peanut, peanut butter, corn, and its derivatives were extended to various grains and other food items (ANVISA 2011). ΣAF concentrations in peanut, peanut butter, corn, and some spices must not surpass 20 μg kg^{-1}. The maximum tolerable ΣAF limit of 5 μg kg^{-1} is set for beans, chocolate, cocoa products, and grains except for corn. Cereal-based food and infant formulas for young children must have a maximum ΣAF limit of 1 μg kg^{-1}.

Regulations worldwide consider not only food (e.g., groundnuts) that may frequently be more contaminated with known aflatoxin-producing fungi, but also foodstuffs that are mainly applied as staple food, which are the primary source of nutrients and energy. There is also a relationship with the culture and eating habits of each country or region. For instance, there are special regulations for rice in Japan and China; corn, rice, and beans in Brazil; and dried fermented soybeans in South Korea because these are everyday food items in these countries.

AFM_1 essentially contaminates milk and dairy products. Given that children widely consume these products, several countries have specific regulatory limits for this toxin. The European Community has the lowest permitted AFM_1 level – 0.050 μg kg^{-1} – for raw milk, heat-treated milk, and milk for manufacture of milk-based products (European Commission 2006a). An even lower limit – 0.025 μg kg^{-1} – is fixed for infant formulas and dietary food for particular medical purposes intended for infants. The United States, Brazil, China, Indonesia, Philippines, Singapore, South Korea, and Taiwan permit 0.5 μg kg^{-1} AFM_1 for milk (ANVISA 2011; Jimenez Medina et al. 2021).

Finally, numerous developing countries do not have regulations for aflatoxins. Consequently, the population is vulnerable and is likely to consume inappropriate food (Ayelign and De Saeger 2020). Besides variance in consumer exposure and safety, all the divergences discussed in the previous paragraphs might severely impair the international trade of food commodities. In this scenario, it is important to identify and to quantify aflatoxins for compliance with these legal limits.

8.2 Fundamentals of Analytical Methods

Analytical chemistry is fundamental in countless areas. With respect to food safety, analytical methods are indispensable to confirm adulterations and to identify and to quantify xenobiotics that are harmful to human and animal health. The first and most essential decision regarding quantitative analysis is selecting the method. This choice is frequently complex and requires experience on the part of the analyst.

Moreover, the required accuracy must be considered. Unfortunately, high reliability almost always demands considerable time and resources. The second decision concerns defining the number of samples, which also involves considering time and resources. Instrumental analysis might be an option if the number of samples is large, but reasonable time is spent on preliminary operations, such as assembling and calibrating instruments and preparing standard solutions. If the number of samples is small, an analytical method that dismisses the need for preliminary steps may be the best choice. Finally, the complexity of the sample and the number of components to be quantified also defines which method will be selected. Various analytical methods are available to determine aflatoxins. Because many countries have regulatory limits, analytical methods established by collaborative studies involving several laboratories or adopted by international organizations should be preferred, thus allowing analytical results to be compared. After validation according to international guidelines, new methods developed for analysis of aflatoxins are likely to become widely accepted in studies on the incidence of these toxins in food and human exposure to them (Berthiller et al. 2017).

The next decision regards sampling, which might be the most significant source of error. Sampling involves collecting a portion of the analyzed material; the composition of this portion must closely represent the entire material being sampled. The analyst must ensure that the laboratory sample is representative and must protect it from contamination and changes in composition before analysis. Contamination with aflatoxins originates from fungal metabolism and might not occur evenly throughout the sample, which poses an additional challenge (Wesolek and Roudot 2016). The European Commission regulation 401/2006 includes guidelines and plans for sampling mycotoxins, so that reliable qualitative and quantitative results can be achieved (European Commission, 2006b). Detailed discussion about sampling is beyond the scope of this chapter, but one must be aware of standard procedures before conducting a study (Galaverna and Dall'Asta 2012).

Processing the sample is another step in an analysis. Aflatoxins are determined in many solid samples, mainly grains, and a grinding step is mandatory before the analytical sample is removed. First, a solid sample is ground or milled, to reduce particle size. Then, it is mixed, to ensure homogeneity. After that, it is stored for some time before analysis. Zhang and Banerjee (2020) described dry, wet, and cryogenic grinding for analysis of aflatoxins in diverse food matrixes. The dry grinding protocol is extensively used to obtain homogeneous particle size for miscellaneous commodities like corn, wheat, peanuts, groundnuts, dried fruits, and spices (Spanjer et al. 2008). Although only dry grinding devices are available in most labs, and even though samples with bulky sizes must be handled or samples might contain high sugar or fat content, this type of grinding might cause obstruction or melting due to heat generated by the blades. An alternative is to turn to wet grinding, which provides samples with smaller particle sizes and uniform distribution of aflatoxins, but it is laborious and time-consuming (Spanjer et al. 2006). Wet grinding consists of mixing a sample with water or other solvents before the blending process, to form a slurry that provides test portions with better particle distribution and reproducibility than the test portions from samples processed by dry grinding. Lastly, cryogenic

milling allows heat-sensitive samples, typically fatty food, to be ground in teeny particle sizes (Liao et al. 2013). Cryogenic grinding requires that samples be frozen overnight and demands special milling devices and liquid nitrogen or dry ice as freezing agent. Compared to dry and wet milling, cryogenic grinding preserves the physical composition of samples. Many labs prefer using dry milling on a routine basis and only apply wet or cryogenic grinding to food matrixes that are unsuitable for dry milling.

Most analytical methods employ solutions of samples prepared in a suitable solvent. Ideally, the solvent should completely dissolve the analyte as fast as possible. The dissolution and extraction conditions must be sufficiently mild to prevent the analyte from being lost (Fifield and Kealey 2000). Many researchers have focused on the procedures and optimized conditions for efficiently dissolving and extracting aflatoxins from food and feed given that this is a decisive step for analysis of residual amounts of toxins. The choice of and the actual need for an extraction and purifica technique is closely related to the selected analytical method. If the analytical method is highly selective, purification may not be as extensive. On the other hand, if the analytical method cannot determine low concentrations like those found in samples, pretreatment must also pre-concentrate the analyte that will be later measured.

Once analytes are in solution, the next step eliminates any substances present in the sample that may interfere in the measurement, which frequently results in enrichment of the analyte. Interferent is any compound other than the analyte that affects the measurement. Few of the physical properties that are used to measure and to quantify an analyte are unique to a single chemical substance. In contrast, the measured properties are characteristic of a group of elements or substances. Initially, only absorption and fluorescence emission were employed to measure aflatoxins. Unfortunately, other substances from food matrixes also exhibit the same behavior. No fast and straightforward rules for eliminating interferents exist, and solving this problem may be the most critical aspect of analysis after sampling. Therefore, eliminating interferents to quantify aflatoxins has also been investigated. A crucial challenge is the diverse composition of the sample, which may contain fat, proteins, and carbohydrates as major components, comprising assorted substances that might interfere in the measurement. An ideal analytical method should determine many analytes in several matrixes while maintaining the same performance.

After interferents are eliminated, there is usually an intermediate step that is fundamentally represented by chromatographic separation techniques. Analysis of aflatoxins cannot be discussed without mentioning the first of all separation techniques, thin-layer chromatography (TLC). The years following the discovery of aflatoxins were also accompanied by the development of high-performance liquid chromatography (HPLC) and, lately, ultra-high-performance liquid chromatography (UHPLC). Chromatographic separation is not mandatory when it comes to identifying and quantifying aflatoxins, but it is undoubtedly available in most laboratories that determine these contaminants in food and feed.

Given that the analytical results depend on measuring a physical or chemical property of an analyte, this property must vary in a known and reproducible way as

a function of the concentration of the analyte. Early methods employed the natural ultraviolet absorbance or fluorescence emission of aflatoxins as a powerful identification and quantification tool. Indeed, these properties are still widely used and recommended by official protocols. In turn, combining HPLC or UHPLC with mass spectrometry (MS) provides analytical chemists with a remarkable technique to characterize and to quantify organic compounds.

Analytical results are incomplete without estimating reliability. Thorough method validation provides an analytical method with reliability and consistency (FDA 2019). Analytical validation is a quality assurance procedure that describes the conditions under which laboratory analysis are planned, performed, monitored, reported, and archived. Later, we will dedicate a topic to analytical validation parameters for identifying and quantifying aflatoxins in food and feed.

Knowledge about the presence of aflatoxins and their levels in food is a matter of public health concern. Therefore, one should not forget that many developing countries might lack resources for laboratory analysis and specialized personnel, maintenance, and supplies for high-tech instrumentation. On the other hand, resources for applying noninstrumental methods that can provide valuable data may be available. An important research area in analytical chemistry involves developing simple and inexpensive devices that can be used on-site and thus avoid that humans and animals consume contaminated and inappropriate food.

Developing analytical methods to determine aflatoxins goes hand in hand with knowledge about the presence of aflatoxins in food and their toxic effects. The greater the awareness and regulatory restrictions, the more selective and sensitive the analytical methods must be to meet these requirements. The general rule for analyzing almost any substance in food also applies to aflatoxins: extraction in a suitable solvent, elimination of interferents by a proper cleanup procedure, preconcentration when necessary, and identification or quantification on the basis of a physical or chemical property.

8.3 Sample Extraction and Cleanup for Determination of Aflatoxins

After a solid sample is properly sampled and powdered or ground, an extraction step is practically unavoidable (Reiter et al. 2009). Frequently, over 80% of the time required for an analysis is spent on sample preparation and cleanup. Several methods to extract aflatoxins from food and feed, like liquid-liquid extraction (LLE), solid phase extraction (SPE), immunoaffinity columns (IACs), dispersive liquid-liquid microextraction (DLLME), and QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe), have been established and extensively revised (Turner et al. 2009; Espinosa-Calderón et al. 2011; Zhang and Banerjee 2020; Miklós et al. 2020).

8.3.1 Liquid–Liquid Extraction

LLE is a classic and the oldest extraction technique. It is based on the solubility properties of the analytes in the aqueous or organic phase, or even in a system with two or more solvents. In the past, aflatoxins were primarily extracted by LLE because it was the only procedure available (Pons and Goldblatt 1965; Park and Melnick 1966). The first step entails extraction with methanol, acetone, chloroform, or a mixture of different polar organic solvents. Next, the extract is concentrated by evaporation. If necessary, a second extraction removes fats from the remaining residue by means of a polar and a nonpolar organic solvent such as hexane, cyclohexane, or petroleum ether. Some extractions include sodium chloride, which dissolves into the aqueous phase and increases its ionic force, facilitating extraction of aflatoxins into the organic phase, a process known as salting-out. A disadvantage of LLE is possible absorption of aflatoxins onto glassware, not to mention that LLE is a tedious, time-consuming procedure that requires large volumes of toxic solvents, which is not environmentally friendly.

Even though LLE is hardly sufficient to produce a clean extract to proceed with detection or quantification, some authors quantified aflatoxins in cereals, fish, spices, and beverages by using this extraction. Otta et al. (2000) extracted AFB_1, AFB_2, AFG_1, and AFG_2 from corn and wheat with a mixture of acetonitrile/water (9:1, v/v) and from fish with a mixture of methanol/water (7:3, v/v) on a vibrating shaker. The extracts were filtered, evaporated to dryness, and dissolved in dichloromethane/acetone (9:1, v/v), and the analysis was continued without any additional cleanup. Sheijooni-Fumani et al. (2011) quantified AFB_1 in rice, chickpea, and lentil by employing a two-step LLE. Well-milled solid samples were extracted with methanol/water (8:10, v/v); after vigorous shaking for 30 min, the supernatant was extracted again with chloroform. Then, after centrifugation for 5 min, the settled extraction phase was dried; the residue was dissolved in methanol; and the analyte was quantified. García-Moraleja et al. (2015) extracted aflatoxins from coffee beverages by LLE. The samples were freeze-dried, and the residues were extracted with ethyl acetate/formic acid (95:5, v/v) in three 5-min cycles. The supernatants were evaporated to dryness and reconstituted in methanol/water (1:1, v/v) before analysis. Sailaja et al. (2018) reported that a single extraction with chloroform at room temperature was adequate to extract aflatoxins from red chili. The chloroform extracts were filtered, washed with distilled water, and dried with anhydrous sodium sulfate. After being concentrated almost to dryness, the residues were dissolved in chloroform. Kokkonen et al. (2005) extracted AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1 from cheese with acetonitrile acidified with 0.1% formic acid and defatted with hexane. The mixture was filtered, and an aliquot of the acetonitrile phase was evaporated to dryness and dissolved in methanol for later quantification.

Although LLE is a low-cost, widely applicable method, LLE extracts are not entirely free of interferents, and additional cleanup is frequently required (Kamimura et al. 1985). Given that further cleanup becomes inevitable, some methods include a purification step on a minicolumn filled with silica, florisil, or alumina, to purify

the extract (Waltking 1970; Maia and Siqueira 2002). This has led to the well-recognized and commonly used SPE for analysis of aflatoxins

8.3.2 Solid-Phase Extraction

Samples can be purified by SPE, a process that has been available since the mid-1980s and which was developed as an alternative or supplement to LLE (Otles and Kartal 2016). SPE aims to reduce interference from components of the sample matrix and to improve detection sensitivity. The principle of purification is similar to that of solid-liquid chromatography. Sample separation, purification, and enrichment are mainly achieved by selective adsorption and desorption of components in the sample by a solid phase, placed inside a column or a disposable cartridge, which are widely commercially available. Commonly, two modes of SPE are employed: retention and elution of analytes or removal of interferents. The most usual method, which involves more steps, entails eluting the sample extract through the solid phase (adsorbent), to retain the analyte, followed by elution with a solvent of appropriate strength, to wash away impurities. Then, the analyte is eluted with a small volume of solvent, to achieve separation, purification, and concentration. Interfering impurities can also be selectively adsorbed while the analyte is allowed to elute, with cleanup comprising a single step. In addition, the SPE technique is a valuable tool for many purposes because it is versatile and might be optionally used in combination with other extraction techniques. Given that samples of food and feed designed for analysis of aflatoxins are solid, a single LLE usually precedes the SPE step, but purification and enrichment obtained in the latter step are much superior. Adsorbents that are used to purify extracts to determine aflatoxins are mostly prepared with C_{18}, florisil, silica gel, or alumina, and countless other adsorbents for SPE are commercially available (Zhang and Banerjee 2020).

Successful applications of SPE to clean up diverse samples aiming at determining aflatoxins have been reported. Several parameters such as type and amount of sorbent, elution solvent, and previous extraction or dilution with proper solvents are constantly being reevaluated for each type of sample. Romero-Gonzales et al. (2009) analyzed AFB_1, AFB_2, AFG_1, and AFG_2 in beer by just eluting 10 mL of degassed samples through a C_{18} commercial cartridge. After washing the column with water, aflatoxins were eluted with acetonitrile/methanol (60:40, v/v). Minicolumns prepared in-house and filled with aluminum oxide efficiently purified extracts of various grains including cornmeal, cottonseed, peanuts, almonds, and pistachios in one step. Ground samples were extracted with methanol/water (80:20, v/v), filtered, and diluted (1:1, v/v) with acetonitrile. The previous mixture was eluted through the columns. The purified extract was collected and directly injected into a LC system. Compared with other available commercial cartridges, these minicolumns allowed substantial savings (Sobolev and Dorner 2002). The same authors also showed that florisil adsorbed aflatoxins selectively in polar solvents (Sobolev 2007). Raw almonds, Brazil nuts, walnuts, hazelnuts, brown and white

rice, cornmeal, and dry-roasted pistachio nuts were first extracted in methanol/water (80:20, v/v). After a sequential wash with methanol/water (60:40, v/v), methanol, and chloroform/methanol (90:10, v/v), to remove impurities, aflatoxins were released from the column with acetone/water/formic acid (96:3.7,0.3, v/v). Sep-Pak Silica and Oasis HLB Waters™ were used to clean up extracts of cooked rice and medicinal herbs, respectively (Park and Kim 2006; Ventura et al. 2004).

8.3.3 Immunoaffinity Columns

IACs might be considered a subtype of SPE columns. IACs contain antibodies immobilized on inert support beads that exclusively retain aflatoxins (Ertekin et al. 2019). Due to their high specificity, IACs produce cleaner extracts with lower level of interfering matrix components than the less selective solid phases of SPE sorbents. IACs have been extensively applied because they are well established for cleaning up and concentrating sample extracts for analysis of aflatoxins (Scott and Trucksess 1997; Patey et al. 1991).

IACs are undoubtedly handy when a less selective detector is used, which is the case of ultraviolet or fluorescence detectors, and they have emerged as one of the most important sample preparation techniques. The processes involved in IACs are essentially the same as those described for SPE: the sample extract is eluted on the IAC, and the antibody retains aflatoxins. A sequential wash with water or buffer removes impurities, and aflatoxins are released by elution with an appropriate solvent, usually methanol, which breaks the aflatoxin-antibody bond. Several commercial IACs are available for AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1, but, unlike regular SPE cartridges, IACs are filled with phosphate-buffered saline (PBS) solution and kept refrigerated, to maintain the functionality of the antibody.

Another critical point in IAC development is the high content of solvents, such as acetonitrile, methanol, or acetone, in sample extracts: antibodies are not tolerant to high concentrations of organic solvents. Before being eluted through the column, extracts must be diluted with water or buffer. The problem is that this dilution increases the volume that has to be eluted and may yield insoluble compounds that interfere in the binding of aflatoxins to the antibodies (Uchigashima et al. 2009).

Despite the higher selectivity of IACs, special attention must be given to them when they are applied to food matrixes that have not been previously evaluated. Castegnaro et al. (2006) highlighted that some drawbacks of IACs must not be overlooked. Complex matrixes might contain unknown substances that obstruct the binding site of antibodies, thereby reducing the absorption of toxins and yielding inaccurate results. Furthermore, interaction with other substances might alter the structure of toxins, so antibodies will not recognize them.

IACs are manufactured for single extraction, and instructions clearly recommend that they be discarded after use. Unfortunately, many laboratories cannot afford their high cost, mainly when they are imported and purchased with foreign currency. This led numerous researchers to assess the reuse of different commercial

columns, but results regarding aflatoxins and the composition of samples are not consistent among manufacturers. Liao et al. (2020) evaluated regenerating columns for raw malt extracts. They found that washing the IAC with PBS and stocking it at 4 °C overnight maintained IAC performance for nine additional extractions of AFB_2, AFG_1, and AFG_2, but only two additional extractions of AFB_1. Iha et al. (2017) also attempted to reuse IAC by immediately washing it with PBS and storing it at 8 °C overnight for re-equilibration, for use on the following day. A naturally contaminated peanut sample was extracted six times with the same column, and the results obtained for aflatoxins were statistically equivalent, except for AFG_2. If only AFB_1 was quantified, the same selectivity was maintained for ten consecutive extractions. Liu et al. (2012) used reconditioned IACs twice to extract aflatoxins from medicinal herbs. Nevertheless, they highlighted that if the column was dirty with pigments or ingredients that did not elute with water, it should not be reused due to poor analyte recovery.

Divergences among assessments clearly demonstrate that caution must be taken when reusing IACs from different manufacturers and applying them to analyze food or feed extracts with different compositions from the previously assessed compositions.

8.3.4 Dispersive Liquid–Liquid Microextraction

To reduce solvent consumption in traditional LLE substantially, DLLME has been used to extract aflatoxins from milk, dairy products, fruit juices, oils, wheat, and eggs. DLLME is a miniaturized extraction technique that offers advantages such as simplicity, rapid operation, high throughput, and low cost. In a usual DLLME protocol, an appropriate mixture of extraction solvent and organic dispersant is rapidly injected into the aqueous sample with a syringe (Rezaee et al. 2006). This generates a cloudy solution consisting of microdroplets of the extraction solvent, which is dispersed entirely into the aqueous phase. After centrifugation, the fine particles of the extraction solvent are settled on the bottom and are removed for further analysis. Thus, the extraction solvent must have higher density and low solubility in water. Because the contact area between the organic solvent and the water sample is large, extraction is fast and efficient. The extent of enrichment of DLLME enhances with dispersion of the extraction solvent. The finer the droplet of the extraction solvent, the higher the enrichment performance. Some parameters affecting the extraction efficiency must be optimized, including the volume of extraction solvent and disperser solvent, extraction, and centrifugation time.

Most analytical methods that use this technique require previous extraction of the sample with a polar solvent given that the basic principle of DLLME is to extract substances from an aqueous or immiscible phase. Table 8.2 contains some examples showing that previous extraction with an aqueous solvent is always performed before DLLME.

Table 8.2 Dispersive liquid-liquid microextraction-based methods to extract aflatoxins from food and feed

Matrix	Toxin	Previous extraction	DLLME	Analytical method	Reference
Edible oils	AFB_1 AFB_2 AFG_1 AFG_2	(1) Sample + methanol/water (6:4, v/v) (2) Centrifugation at 4000 rpm/10 min 3) Dilution with water 4) IAC Afla Clean™	(1) 5.0 mL IAC extract in PBS + 500 μL ACN (disperser) + 120 μL $CHCl_3$ in (extractant) (2) Centrifugation 4000 rpm/3 min	HPLC – fluorescence detection LOD: 1.1 × 10^{-4} – 5.3 × 10^{-3} ng mL^{-1}	Afzali et al. (2012)
Dairy products	AFB_1 AFM_1	QuEChERS: (1) Sample + (water, ACN, NaCl, sodium citrate) (2) Centrifugation at 5000 rpm/45 min 3) Supernatant + ($MgSO_4$, PSA, C_{18}) 4) Centrifugation at 2700 rpm/5 min	(1) 3.0 mL previous extract (disperser) + 500 μL $CHCl_3$ (extractant) injected in 7.0 mL of deionized water (2) Centrifugation: 2700 rpm/10 min	HPLC – fluorescence detection LOD: 0.1 μg kg^{-1} AFB_1 0.1 μg kg^{-1} AFM_1	Karaseva et al. (2014)
Plant-based milk	AFB_1 AFB_2 AFG_1 AFG_2	(1) 5 mL sample + 6 mL ACN + NaCl (2) Centrifugation at 6000 rpm/5 min	(1) Previous extract (disperser) + 1500 μL $CHCl_3$ (extractant) injected in 5.0 mL of deionized water (2) Centrifugation: 6000 rpm/5 min	HPLC – fluorescence detection LOQ: 0.5 μg kg^{-1}	Hamed et al. (2019)
Egg	AFB_1	(1) Sample + ACN/water (80:20, v/v) + diatomaceous earth (2) Ultrasound/2 min (3) Filtration (4) Aqueous extract defatted with hexane	(1) 1.2 mL previous extract (disperser) + 240 μL $CHCl_3$ (extractant) injected in 3.0 mL of deionized water (2) Centrifugation: 4000 rpm/3 min	HPLC – fluorescence detection LOD: 0.12 μg kg^{-1}	Amirkhizi et al. (2018)
Fish	AFB_1 AFB_2 AFG_1 AFG_2	(1) Sample + 5 mL (60:40 ACN/PBS) (2) Ultrasound/7 min (3) Filtration	(1) 400 μL $CHCl_3$ (extractant) injected in 5.0 mL previous extract + 25 mg NaCl (disperser) (2) Centrifugation: 2500 rpm/10 min	LC – MS/MS LOD: 0.07–0.036 μg kg^{-1}	Jayasinghe et al. (2020)

(continued)

Table 8.2 (continued)

Matrix	Toxin	Previous extraction	DLLME	Analytical method	Reference
Peanuts	AFB_1 AFB_2 AFG_1 AFG_2	(1) Sample + methanol/water (80:20, v/v) (2) Ultrasound/30 min (3) Centrifugation at 2795 × *g*/5 min	(1) 1.0 mL previous extract (disperser) + 200 μL $CHCl_3$ (extractant) injected in 5.0 mL deionized water (2)Centrifugation: 2795 × *g*/5 min	HPLC – fluorescence detection LOD: 0.03–0.1 μg kg^{-1}	Chen et al. (2017)
Vegetable oils	AFB_1 AFB_2 AFG_1 AFG_2	(1) Sample + ACN (84% in water) (2) Shaken/30 min (3) Centrifugation at 4000 rpm/3 min	(1) 0.8 mL oil extract + 200 μL TFA (disperser) + 400 μL CH_2Cl_2 (extractant) injected in 3.2 mL deionized water (2) Centrifugation: 4000 rpm/5 min	HPLC – fluorescence detection LOD: 0.005–0.03 μg kg^{-1}	Wang et al. (2019)
Yogurt	AFM_1 AFB_1 AFB_2 AFG_1 AFG_2	(1) Sample centrifuged at 6000 rpm/5min (2) Sample + NaCl + ACN (3) Centrifugation at 6000 rpm/5 min	(1) 5.0 mL previous extract (disperser) + 1500 μL $CHCl_3$ (extractant) injected in 5.0 mL of deionized water (2) Centrifugation at 6000 rpm/5min	HPLC – fluorescence detection LOD: 0.0015–0.0055 μg kg^{-1}	Hamed et al. (2017)

LOD detection limit, *ACN* acetonitrile

8.3.5 QuEChERS

Although the previously described extraction procedures selectively isolate aflatoxins from a complex matrix and use much less solvent than LLE, modern analytical chemistry is continuously developing rapid, easy-to-perform, and low-cost analytical procedures (Perestrelo et al. 2019). QuEChERS is a simple dispersive solid-phase extraction (d-SPE) procedure that can be applied to numerous samples. QuEChERS consists of extracting the homogenized sample with an appropriate solvent, normally acetonitrile, by vortex-shaking for a few minutes. A liquid-liquid partition is created by adding excess salts or buffers to the mixture. After centrifugation, water might be removed with anhydrous $MgSO_4$, and the extract is further cleaned in an additional step in which the extract is mixed with a sorbent, such as primary-secondary amine (PSA), silica gel, C_{18}, or graphitized carbon black (Juan et al. 2017). QuEChERS was initially developed to simplify screening of pesticide residues in large numbers of agricultural samples, but now it has been applied for analysis of many other food contaminants, and aflatoxins are surely among them (Anastassiades et al. 2003; Michlig et al. 2016; Choochuay et al. 2018).

With a classic procedure QuEChERS, AFB_1 was extracted from wheat, rice, oat, rye, maize, and barley (Zhao et al. 2017). First, aqueous acetonitrile solution (95:5, v/v) was added to milled samples; then, the mixture was vortexed for 1 min and subjected to ultrasound for 3 min before NaCl and $MgSO_4$ were added. The extract was vortexed for 1 min and centrifuged for 5 min. Next, the supernatant was transferred to another tube containing PSA. After vortexing and centrifugation, the extract was ready for analysis.

Likewise, all other extraction procedures, solvent type, extraction time, and need for adsorbents or not must be optimized before the extraction procedure is effectively established. Also, analysts must always bear in mind that the extraction procedure is unquestionably committed to the detection system that will be used subsequently.

Lastly, even though research has focused on reducing extraction steps, most methods for analysis of aflatoxins still combine two or three approaches to achieve the best results.

8.4 Detection and Quantification of Aflatoxins

8.4.1 Thin-Layer Chromatography

TLC with fluorescence detection was the only chromatographic technique available in the 1960s. Although it was the method of choice of AOAC International (Association of Official Analytical Collaboration International) for an extended period, it is no longer widely used (Trucksess 2000). Despite being a low-cost procedure, the separation efficiency of TLC is low, and identification of aflatoxins might be susceptible to interferences from the fluorescence of other components with similar migration patterns in the sample. Eventually, when combined with a selective cleanup method, such as IACs, TLC is a robust and straightforward screening method (Stroka et al. 2000). Aflatoxins can be quantified if TLC is coupled to densitometry, becoming an alternative for labs that cannot afford more expensive chromatographic methods (Stroka and Anklam 2000; Marutoiu et al. 2004). In two-dimensional thin-layer chromatography (2D-TLC), the plate is rotated 180° after the first separation, and aflatoxins and other components of the sample are eluted once more with a second solvent (Durakovic et al. 2012). High-performance thin-layer chromatography (HPTLC) and bidirectional HPTLC are derived from the previous TCL and have been successfully applied for quantification of aflatoxins in food and feed (Tomlins et al. 1989; Ramesh et al. 2013).

8.4.2 High-Performance Liquid Chromatography Coupled to Fluorescence Detector

The most widely used strategy to determine aflatoxins in food and feed is separation by HPLC followed by fluorescence detection. However, although AFB_1 and AFG_1 exhibit natural fluorescence, it is less intense than the fluorescence of AFB_2 and AFG_2. Moreover, water, acetonitrile, and methanol, used as mobile phases in reversed-phase liquid chromatography, also quench the fluorescence of AFB_1 and AFG_1. Pre- or post-column derivatization methods are recurrently used to circumvent this issue, to increase the signal during analysis. Pre-column derivatization with trifluoroacetic acid (TFA) hydrolyzes AFB_1 and AFG_2, to form the more fluorescent hemiacetals AFB_{2a} and AFG_{2a}. Even though the time required for complete reaction is short, derivatization represents an additional step. Nevertheless, it is worth performing because derivatization enhances detection (Saito et al. 2020; Wang et al. 2020). Detection limits ranging from 0.1–0.2 μg kg^{-1} or tenfold lower are easily achieved and entirely meet the requirements of analytical methods for surveillance. Post-column derivatization is accomplished by adding pyridinium bromide-perbromide (PBPB) to the mobile phase after separation. Two atoms of the bromide are added at the 8,9-double bond of the dihydrofuran moiety of aflatoxin, enhancing the fluorescence signal (Stroka et al. 2001). Such bromination is more cost-effective, but bromine can also be produced by an electrochemical cell (Kobra™ Cell) in the post-column step by adding KBr to the mobile phase (Kok 1994; Omotayo et al. 2019). Extraction by IAC and quantification by reversed-phase high-performance liquid chromatography-fluorescence detector (HPLC-FD) without or with pre- or post-column derivatization are among the official methods adopted by AOAC International (AOAC International 2002). Although the mass spectrometry detector has continuously replaced the fluorescence detector, the latter is still widely employed for various purposes because it is spread among analytical chemistry laboratories, as shown in Table 8.3.

8.4.3 Liquid Chromatography Coupled to Mass Spectrometry Detector

Since the mid-1990s, when atmospheric pressure ionization (API) interfaces were developed and overcame the low sensitivity and ionization efficiency of thermospray, particle beam, and fast atom bombardment interfaces, liquid chromatography-mass spectrometry has emerged as the most powerful technique for identification and quantification of contaminants in food (Miklós et al. 2020).

Commercially available atmospheric pressure ion sources include ESI (electrospray), APCI (atmospheric pressure chemical ionization), and APPI (atmospheric

Table 8.3 Overview of HPLC-fluorescence detection-based methods for determination of aflatoxins

Matrix	Toxin	Pretreatment	Analytical method	LOD μg kg^{-1}	Reference
Milk (pasteurized and UHT)	AFM_1	(1) Water bath at 37 °C (2) Centrifugation (3) AflaM1 IAC	Column: Nucleosil C_{18} (250 × 4.6 mm, 5 μm) Mobile phase: water/acetonitrile: methanol (6:2:3, v/v/v), isocratic Detection: direct fluorescence detection	0.01	Mannani et al. (2021)
Milk, yogurt, milk powder, and ice cream	AFM_1 AFM_2	(1) LLE, methanol/water (7:3, v/v) + NaCl (2) Dilution with water (3) IAC	Column: agilent XDB C_{18} (250 × 4.6 mm, 5 μm) Mobile phase: water/methanol: acetonitrile (6:2:2, v/v), isocratic Detection: post-column derivatization, Kobra® cell	0.125 0.151	Lee and Lee (2015)
Surk cheese	AFB_1 AFB_2 AFG_1 AFG_2 AFM_1	(1) LLE, Celite + chloroform + NaCl (2) Dilution with PBS (3) IAC	Column: Inertsil ODS-3 (150 × 4.6 mm, 5 μm) Mobile phase: water/acetonitrile/acetic acid (49.5:49.5:1, v/v/v), isocratic Detection: post-column derivatization, electrochemical cell	0.033–0.061	Sakin et al. (2018)
Cereal flour (wheat, maize and rice)	AFB_1 AFB_2 AFG_1 AFG_2	(1) LLE, methanol/water (8:2, v/v) (2) Dilution with PBS (3) AflaTest IAC	Column: Inertsil ODS-3 (250 × 4.6 mm, 5 μm) Mobile phase: water/acetonitrile: methanol (6:2:3, v/v/v), isocratic Detection: post-column derivatization, Kobra® cell	0.014–0.028	Kara et al. (2015)

(continued)

Table 8.3 (continued)

Matrix	Toxin	Pretreatment	Analytical method	LOD µg kg^{-1}	Reference
Coffee (beans and powder)	AFB_1 AFB_2 AFG_1 AFG_2	(1) LLE, methanol/water (8:2, v/v) (2) Dilution with PBS (3) Easi-Extract IAC	Column: Novapack C_{18} (150 × 3.9 mm, 4 µm) Mobile phase: water/methanol: acetonitrile (64:18:18, v/v), isocratic Detection: pre-column derivatization with TFA	0.09–0.17	Al-Ghouti et al. (2020)
Dried figs	AFB_1 AFB_2 AFG_1 AFG_2	(1) LLE, methanol/water (3:2, v/v) + NaCl (2) Dilution with PBS (3) Aflatest IAC	Column: C_{18} (250 × 4.6 mm, 5 µm) Mobile phase: water/acetonitrile: methanol (6:2:3, v/v/v), isocratic Detection: post-column derivatization, Kobra® cell	0.13–0.46	Bakirci (2020)
Spices (turmeric, red pepper, black pepper, cinnamon)	AFB_1 AFB_2 AFG_1 AFG_2	(1) LLE, methanol/water (8:2, v/v) + NaCl (2) Dilution with water (3) Aflatest IAC	Column: Zorbax Eclipsed XDB (250 × 4.6 mm, 5 µm) Mobile phase: water/acetonitrile: methanol (5:2:3, v/v/v), isocratic Detection: post-column derivatization, electrochemical cell Libios-K01	0.1–0.3	Zareshahrabadi et al. (2020)

(continued)

Table 8.3 (continued)

Matrix	Toxin	Pretreatment	Analytical method	LOD μg kg^{-1}	Reference
Feed ingredients (corn, wheat bran, soybean, dried distillers grains with solubles)	AFB_1	(1) LLE, methanol/water (8:2, v/v) (2) Dilution with PBS (3) AokinImmunoClean IAC	Column: C_{18} (150 × 4.6 mm, 5 μm) Mobile phase: water/methanol (50:50, v/v), isocratic Detection: post-column derivatization, electrochemical cell AURA	0.03	Li et al. (2014)
Feed samples (mustard cake, cotton seed cake, soybean cake, groundnut cake, wheat bran, crushed wheat/maize)	AFB_1 AFB_2 AFG_1 AFG_2	(1) LLE, methanol/water (7:3, v/v) + NaCl (2) Dilution with water (3) AflaPure IAC	Column: Spherisorb C_{18} (250 × 4.6 mm, 5 μm) Mobile phase: water/methanol: acetonitrile (6:2:2, v/v), isocratic Detection: pre-column derivatization with TFA	0.06–0.92	Patyal et al. (2021)

pressure photoionization). Any of them can analyze aflatoxins; however, the ESI source is undoubtedly the most frequently found in laboratories and the most employed (Capriotti et al. 2010). After exiting the API source, the molecules enter the vacuum chamber and reach the mass analyzer through an ion transporting and focusing region. Single-stage or multistage (MS/MS) mass analyzers are commercially available. Except for in-source CID, there is no collision-induced dissociation (CID) in a single-stage mass analyzer, and molecular ions cannot be fragmented. Thus single-stage mass analyzers do not meet the European Union recommendations for analysis of residues in food because a precursor ion and two product ions are required to confirm a contaminant (European Commission 2002). This can be achieved by multiple reaction monitoring (MRM) of selected ions, a parameter that is experimentally optimized for each compound.

Aflatoxins can be analyzed on a MS/MS mass analyzer like triple quadrupole (QqQ), quadrupole time-of-flight (Q-TOF), quadrupole-linear ion trap (Q-TRAP), and Orbitrap. Although triple-quadrupole is the most regularly used for determining mycotoxins, not all analytical standards are commercially available. As a result,

interest in high-resolution mass spectrometry (HRMS), such as Orbitrap and time-of-flight (TOF) mass analyzers, has increased. HRMS has essential advantages: it records full scan spectra by measuring the accurate mass of analytes, screens untargeted compounds, and reviews analysis data, allowing analytes that had not been considered at the time of spectral acquisition to be investigated (Tittlemier et al. 2021).

One of the main advantages of LC-MS/MS systems is their remarkable sensitivity and selectivity, dismissing the need for extensive cleanup of sample extracts. Nevertheless, for some substances in very complex samples, cleanup cannot be entirely neglected when high sensitivity is desired. When it comes to the practical use of LC-MS/MS methods, co-eluting matrix components might significantly suppress or enhance the signals of the analytes. In other words, analyte ionization is prevented by competition of charges from the other components of the sample. This is the well-recognized "matrix effect" in mass spectrometry detection, and it harms the performance of LC-MS/MS methods, mainly in terms of the accuracy of quantification (Truffeli et al. 2011a, b). For instance, if a solvent calibration curve is prepared with the analytical standards for the quantification, differences in the signals of the analytical standards and sample extracts will directly impact the result. There is no universal approach, but understanding why signals are suppressed or enhanced might support the analyst's decision.

The most straightforward approach to avoid the matrix effect is to dilute the sample extract in an appropriate solvent (Stahnke et al. 2012). However, diluting the extract will also dilute aflatoxin, and the mass spectrometer might not be sensitive enough to overcome the loss in concentration. If calibration curves are prepared in blank extracts, a similar suppression or enhancement effect is predicted both for standards and sample extracts. This approach is named the matrix-matched calibration curve. Nevertheless, the availability of samples free from the contaminant should be verified before deciding on this method. The standard addition method is laborious and time-consuming, especially when more than one aflatoxin must be quantified. Another valuable approach is the stable isotope (SI) dilution assay. Deuterated and ^{13}C-aflatoxins are commercially available and considered the ideal Internal Standards. SI solutions can be added to sample extracts and calibration curves or incorporated in the sample before extraction and cleanup (Varga et al. 2012). This procedure provides enormous flexibility under the conditions of sample extraction and significantly improves the precision of the method, but SIs are expensive, which should be borne in mind before choosing the methodology.

Due to its outstanding performance, it is not surprising that LC-MS/MS has been widely and successfully employed to determine aflatoxins in food and feed. Table 8.4 depicts several applications of LC-MS/MS in diverse samples together with the extraction and cleanup procedures.

Table 8.4 Overview of LC-MS/MS-based methods for determination of aflatoxins

Matrix	Toxin	Pretreatment	Analytical method	LOD ($\mu g\ kg^{-1}$)	Reference
White cheese	AFM_1	(1) Extraction with Celite, CH_3Cl, and NaCl (2) Extract defatted with hexane (3) IAC	LC (Q-TRAP) Column: Optima ODS-H (150 × 2.0 mm, 5 µm) Mobile phase: (A) 10 mmol L^{-1} ammonium acetate, (B) 10 mmol L^{-1} ammonium acetate in methanol, gradient elution	0.0625	Kamel et al. (2017)
Milk and Jujube	AFM_1 AFB_1	Milk: (1) Dilution with deionized water, (2) Filtration (3) C_{18} Micro-SPE Jujube: filtration (1) Extraction with methanol/water (7:3, v/v) and sonication/20 min (2) Centrifugation at 4000 rpm/3 min (3) C_{18} Micro-SPE	LC (Q-TOF) Column: SB-C_{18} (50 × 4.6 mm, 1.8 µm) Mobile phase: (A) 0.1 % aqueous formic acid, (B) 0.1% formic acid in ACN, gradient elution	AFM_1: 0.049 AFB_1: 0.023	Du et al. (2018)
Milk, powder milk, and yogurt	AFM_1	(1) Extraction with acetonitrile, formic acid, and NaCl (2) Centrifugation at 13,000 rpm/5min	Online SPE-LC (QqQ) Online cartridge: BioBasic C18 Column: PFP (100 × 2.1 mm, 2.6 µm) Mobile phase: (A) 0.1% aqueous formic acid, (B) 0.1% formic acid in methanol, gradient elution	0.0005–0.0007	Campone et al. (2016)
Whole milk, milk-based infant formula, and animal feed	AFB_1 AFB_2 AFG_1 AFG_2 AFM_1	(1) Extraction with acetonitrile/water (50:50, v/v) (2) Centrifugation at 4500/15 min (3) Filtration	LC (Q-TRAP) Column: Kinetex XB-C_{18} (100 × 2.1mm, 2.6 µm) Mobile phase: (A) 0.1% aqueous formic acid, (B) 0.1% formic acid in methanol, gradient elution	0.005–0.0038	Zhang et al. (2018)

(continued)

Table 8.4 (continued)

Matrix	Toxin	Pretreatment	Analytical method	LOD (μg kg^{-1})	Reference
White rice and sorghum	AFB_1 AFB_2 AFG_1 AFG_2	(1) Extraction with acetonitrile/water (50:50, v/v) containing 0.1% formic acid (2) Centrifugation at 4500/5 min (3) Filtration (4) SPE with ISOLUTE Myco	LC (QqQ) Column: XB bridge C_{18} (100 × 2.1, 1.7 μm) Mobile phase: (A) 0.1% aqueous formic acid, (B) 0.1% formic acid in acetonitrile, gradient elution	0.28–0.90	Ok et al. (2016)
Baby food and feeds	AFB_1 AFB_2 AFG_1 AFG_2	(1) Extraction with acetonitrile/water (78:22, v/v) (2) Centrifugation at 5000/8 min (3) Evaporation of acetonitrile by rotoevaporation (4) Dilution with water (5) IAC	LC (QqQ) Column: BEH C_{18} (100 × 2.1, 1.7 μm) Mobile phase: 0.1 % formic acid methanol/ water (75:25, v/v), isocratic	0.003–0.008	Alfaris et al. (2020)
Ground maize	AFB_1 AFB_2 AFG_1 AFG_2	QuEChERS: (1) Extraction with methanol/water (60:40, v/v) (2) Addition of $MgSO_4$ and NaCl (3) Centrifugation at 4000/5 min (4) Filtration	LC (QqQ) Column: Zorbax Eclipse C_{18} (50 mm × 3.0, 1.8 μm) Mobile phase: (A) 5 mmol L^{-1} ammonium acetate, (B) 5 mmol L^{-1} ammonium acetate in methanol, gradient elution	0.11–0.36	Ouakhssase et al. (2019)
Corn powder, edible oil, peanut butter, and soy sauce samples	AFB_1 AFB_2 AFG_1 AFG_2	(1) Extraction with acetonitrile/water (50:50, v/v) (2) Sonication/20 min (3) Addition NaCl (4) Centrifugation at 8000/5 min (5) Filtration	Online TFC-SPE-LC (QqQ) TFC: Cyclone (50 × 0.50 mm, polymer type) Column: Phenomenex C_{18} (100 × 2.1 mm, 1.7 μm) Mobile phase: (A) 0.1% aqueous formic acid, (B) 0.1% formic acid in methanol, gradient elution	0.20–2.0	Fan et al. (2015)

(continued)

Table 8.4 (continued)

Matrix	Toxin	Pretreatment	Analytical method	LOD (μg kg^{-1})	Reference
Dark tea	AFB_1 AFB_2 AFG_1 AFG_2	(1) Extraction with acetonitrile/water (86:14, v/v) + NaCl (4) Pressure filtration (5) MFC (PriboFast MFC260) + IAC	LC (Q-TOF) Column: C_{18} (100 × 2.1 mm, 1.8 μm) Mobile phase: (A) water, (B) methanol, gradient elution	0.024–0.21	Ye et al. (2019)
Fish feed	AFB_1 AFB_2 AFG_1 AFG_2	(1) Extraction with acetonitrile/water, 0.1 mol L^{-1} KH_2PO_4 (60:40, v/v) (2) Sonication/7 min (3) Matrix Imprinted Polymer	LC (Q-TRAP) Column: Zorbax C_{18} (100 × 4.6 mm, 3.5 μm) Mobile phase: (A) 0.1% aqueous formic acid, (B) 0.1% formic acid in methanol, gradient elution	0.42–1.15	Jayasinghe et al. (2020)

TFC turbulent flow column, *MFC* multifunctional column

8.4.4 Screening and Rapid Methods

Enzyme-linked immunosorbent assay (ELISA) is the most common and extensively used screening method for determination of aflatoxins in agricultural raw materials. ELISA is easy to perform, is not as expensive as chromatography, does not use organic solvents, and is essential to provide on-site results about aflatoxins in a short period. Therefore, it is a standard and well-accepted method for monitoring aflatoxins. ELISA kits are commercially available and also provide quantitative results. Several samples can be analyzed without any extensive cleaning step. However, ELISA is highly matrix-dependent, so analysis of more complex samples requires that the manufacturer be consulted or the method be individually validated for the specific sample (Miklós et al. 2020).

To quantify AFM_1 in raw milk, Maggira et al. (2021) validated a commercial ELISA kit among three commercially available kits against an HPLC-FL method. The authors found that the ELISA kit was a faster and equally reliable alternative method to HPLC in routine analysis.

A calibration curve implanted enzyme-linked immunosorbent assay (C-ELISA) was developed to determine aflatoxin B_1 in wheat, corn, soybean, and peanuts (Wu et al. 2020). The new development implanted an optimized standard curve data into a matched analysis software, programmed by the researchers, to make data processing more convenient and faster. The new method proved rapid and sensitive and provided equivalent results to HPLC for all the AFB_1 concentrations in real samples.

AFB_1 detection by electrochemical immunoassays is sensitive and fast. Kong et al. (2018) used 2-aminoethanethiol to increase the speed and sensitivity of a conventional electrochemical immunoassay by assembling the thiol on the surface of a

gold electrode, to form self-assembled monolayers. Then, non-competitive immunoassays occurred on the surface, to give an electrochemical immunoassay sensor. The detection limit achieved by the new development was 0.1 ng mL^{-1} AFB_1.

Among new developments, Fan et al. (2020) established a time-resolved fluorescence immunoassay based on fluorescent microspheres containing a Eu^{3+} chelate named AFM_1-POCT. They used a portable fluorimeter, and the reaction took 5 min. The results were equivalent to the results of UHPLC-MS determination in the range of 0.0121–2.0 μg kg^{-1}, so the method met the detection limits of 0.05–0.5 μg kg^{-1} required by the regulatory organization and enables on-site sampling.

8.5 Analytical Method Validation

Analytical method validation essentially involves evaluating whether a new or a modified literature method applies to the routine of a certain laboratory. The main objective is to demonstrate that the analytical method is appropriate, ensuring that it is accurate, reproducible, and applicable to the substance intended for identification and quantification. The method is considered validated when it is evaluated according to a series of at least some preestablished parameters. The evaluated performance parameters will vary depending on the intended use of the method, its type (quantitative or qualitative), and the degree to which it has been previously validated (FDA 2019). For example, new quantitative methods should include at least the following performance characteristics: selectivity, detection limit, quantification limit, linearity, or other calibration models, range, accuracy, precision, measurement uncertainty, ruggedness, confirmation of identity, and spike recovery (FDA 2019).

Vast literature is available to assist the validation process for those who intend to proceed with an in-house validation. The most comprehensive and maybe widely accepted guidance is the European Commission Council Directive concerning the performance of analytical methods and the interpretation of results (European Commission 2002). National guidelines might also be compulsory and might be accessed by region or country, if necessary. Raposo and Ibell-Bianco (2020) presented a valuable discussion about general analytical method validation, which was extremely useful because the authors presented discrepancies and controversies among different guidelines for obtaining and interpreting the most required parameters in analytical method validation.

Considering how aflatoxins are formed in food and feed, validation should be best evaluated with Certified Reference Materials (CRM), if available. Matrix reference materials with naturally occurring mycotoxins are preferred over fortified ones because the incurred mycotoxins are incorporated deeper within the matrix (Tittlemier et al. 2021). Using a spiked matrix with standard solutions to determine recovery might yield unrealistic values (Dzuman et al. 2014). Finally, proficiency tests or interlaboratory studies are crucial. Numerous collaborative studies have been carried out by AOAC International (Bao et al. 2012; Stroka et al. 2001).

Thus, assuming the importance of this topic associated with strong regulations of some segments, laboratories must attend to their analytical methods and their adequate validation whenever appropriate.

8.6 Multiclass-Mycotoxin Methods of Analysis

Unfortunately, aflatoxin-producing fungi are not the only fungi that infest crops, and even fungi related to the synthesis aflatoxins might produce other mycotoxins. Besides aflatoxins, the most investigated mycotoxins in food and feed are Fumonisin B_1 (FB_1), Fumonisin B_2 (FB_2), Ochratoxin (OTA), Deoxynivalenol (DON), Nivalenol (NIV), Zearalenone, (ZEA), T-2, and HT-2, among others.

Current mycotoxin regulations and acceptable levels in food and feed apply to many different individual mycotoxins in a single food, which emphasizes the importance of establishing methods that can simultaneously determine multiple mycotoxins in a single analysis (European Commission 2006a). To achieve these goals, methods for determining a group of mycotoxins became common (Lago et al. 2021). Due to the various structures and physical and chemical properties of different mycotoxins, extraction and analysis parameters must be adapted to the intended matrixes, to meet the minimum requirements of recovery, sensitivity, and selectivity. Routine determination of multiple mycotoxins is only possible, thanks to significant advances in extraction and purification techniques and notable increase in the sensitivity of triple quadruple mass spectrometers frequently coupled to liquid chromatography, as already mentioned. Diverse multi-mycotoxin analytical methods are described in the specialized literature (González-Jartín et al. 2021)

8.7 Multi-Residue Methods of Analysis

Besides the multiple mycotoxins that are likely to contaminate food and feed, other harmful substances exist. Other toxic substances such as pesticides, veterinary drugs, hormones, and plant alkaloids might also be incorporated along the food production chain. Multi-residue methods for several analytes differing in polarity, structural formulas, and physicochemical properties are becoming regularly accessible (Steiner et al. 2021a, b). Because the surveillance of aflatoxins in food and feed is mandatory and well-recognized, these contaminants are recurrently included in the list of multiclass methods. When a method for multi-residue determination is employed, almost all efforts are directed to increasing the number of identified or quantified analytes and reducing the time required for analysis. Analyzing more analytes in a shorter time means saving resources and making faster decisions, especially decisions related to accepting a raw material batch or not or releasing a final product to consumers.

With single methods, aflatoxins can be quantitatively extracted after proper cleanup and determined with a suitable detector. In contrast, a compromise among all the components is needed when developing multiclass methods, especially for complex samples, in which the applicability of analyte-specific extraction is not practicable (Steiner et al. 2020). Multi-residue analytical methods also expand the possibility of screening before a more specific method is applied. The numerous investigations and efforts in this research area brought impressive advances in approaches for sample preparation, identification, and quantitation (Dzuman et al. 2015). Diverse applications are easily found in the specialized literature (Steiner et al. 2021a, b).

8.8 Conclusions

Since aflatoxins were discovered, they have attracted a lot of attention, effort, and resources. The search for new analytical methods to determine mycotoxins is undoubtedly a fertile field of research. Protocols for sample preparation have been continuously improved and optimized for analysis of aflatoxins in various food and feed matrixes, having progressed from laborious liquid-liquid extractions to solid-phase extraction and modern immunoaffinity columns, culminating in the simple dilute and shoot and QuEChER approaches. Faster cleanup has allowed significant advances in high throughput analysis. Different analytical protocols for determining aflatoxins in food commodities have advanced and gradually improved. Despite drawbacks such as low sensitivity and poor accuracy, TLC was the most used chromatographic technique for quantification of aflatoxins until the 1980s. Thereafter, it was steadily replaced with HPLC coupled to ultraviolet and fluorescence detectors. IAC cleanup with liquid chromatography separation and fluorescence detection is widely used as a gold analytical method in laboratories worldwide because it has been extensively validated in collaborative studies and recognized by regulatory boards. Mass spectrometry detectors impressively enhanced the selectivity and sensitivity of methods for the determination of aflatoxins and multiple mycotoxins. Liquid chromatography coupled to mass spectrometry is the most potent tool for monitoring and controlling the levels of aflatoxins in food and feed. Due to its vast applicability, LC-MS/MS instruments have become the mainstream device in almost all research and routine laboratories. However, its inclusion in several laboratories is hampered by the high costs for acquiring, maintaining, and training personnel for the instrument. Finally, screening and fast methods, represented mainly by ELISA, are essential for on-site monitoring of aflatoxins. Constant developments to improve immunoassays and biosensors promise to bring new cost-effective, reliable, and straightforward methods to determine aflatoxins.

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Chapter 9
Removal and Detoxification of Aflatoxins

Zahid Rafiq Bhat and Khalid Rehman Hakeem

Abstract Contamination of various food items and crops including maize, groundnut, peanut, rice, wheat, barley, oilseed, and beverages is caused by well-known fungal carcinogenic mycotoxins produced by soil-borne *Aspergillus flavus* and *Aspergillus parasiticus*. The various aflatoxin compounds are aflatoxin B1 (AF-B1), B2 (AF-B2), G1 (AF-G1), and G2 (AF-G2) based on their blue and green fluorescence under UV light, while the metabolites of aflatoxin AF-B1 and AF-B2 are secreted into milk and are termed as aflatoxin M1 (AF-M1) and aflatoxin M2 (AF-M2), respectively. Among them, the most potent and toxic is AF-B1 associated with hepatocellular carcinoma (HCC). Several methods can detect aflatoxin in various food or crop products. Further, the exposure of human and animal subjects to such contaminated items can result into serious health problems to consumers. Thus, the detection, prevention, and degradation of aflatoxin in various food and crop items are of primary importance in terms of both health and economic indicators. In this chapter, we shall discuss the various traditional and new methods or technologies used to remove and decontaminate the food or crop products infected with *Aspergillus fungi*. Several traditional methods such as thermal and irradiation (physical), biological, and chemical treatments have been used to remove the molds containing aflatoxin. However, the technology involving traditional methods of decontamination don't suffice complete eradication of the mycotoxins. Therefore, novel strategies and technologies in the form of gamma and electron beam mediated irradiation microwave mediated heating, pulsed light, electrolyzed water treatment, cold plasma technique, and others have been explored to ensure complete and safe eradication of the aflatoxin-contaminated food items.

Z. R. Bhat
Laboratory of Epigenetics and Diseases, Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab, India

K. R. Hakeem (✉)
Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia

Princess Dr Najla Bint Saud Al- Saud Center for Excellence Research in Biotechnology, King Abdulaziz University, Jeddah, Saudi Arabia

K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_9

Keywords Aflatoxin · Detoxification · Toxicity · Physical · Chemical and biological methods

9.1 Introduction

The aflatoxin affecting food and crop items is a globally well-recognized significant problem that is associated with health hazards and huge economic losses to the food industry. Mycotoxins are defined as a class of secondary products of metabolism from certain molds which grow and produce mycotoxins especially in the crop varieties or while storing them particularly in tropical and subtropical climate zones (Kumar et al. 2017). These mycotoxins producing molds grow preferably on economically important crops and feed items like cereals, fruits, hazelnuts, almonds, seeds, fodder, etc. Interestingly, the humans get exposed to these toxins possibly due to intake of contaminated plant products or their metabolites in the animal-based foods like AF-M1 and AF-M2 (Agriopoulou et al. 2020). The term mycotoxins came into light in 1962 due to the sudden death of around 1 lakh turkey poultry due to the contaminated peanut feed. The AF-B1 is the most potent and highly toxic followed by AF-G1, AF-B2, and AF-G2. One of the very well-known carcinogenic etiological agents causing hepatocellular carcinoma (HCC) in both humans and animals is AF-B1 (Sarma et al. 2017). Undeniably, the most essential method of controlling contamination is to first apply preventive techniques due to high prevalence and incidence of AFB1 and other aflatoxin in food, feed, and crops. The affected foodstuffs can be subjected to the preharvest (at first place) and postharvest (later stage) treatments (Ismail et al. 2018). The preharvest treatment includes the timely rotation of crops, genetically improved and modified crops resistant to such aflatoxin-producing molds, environmental stress factors, proper use of pesticides, and timing of plantation (Dowd 2003; Rachaputi et al. 2002), while the postharvest treatment involves the drying of crops and proper packaging or storage with or without use of preservatives or pesticides (Neme and Mohammed 2017; Waliyar et al. 2015). All these traditional methods were unable to completely eradicate and contain the aflatoxin-affected crops and food products. Here, in this chapter, we shall discuss the novel technology to effectively remove and degrade the aflatoxin-producing mold contamination (Fig. 9.1).

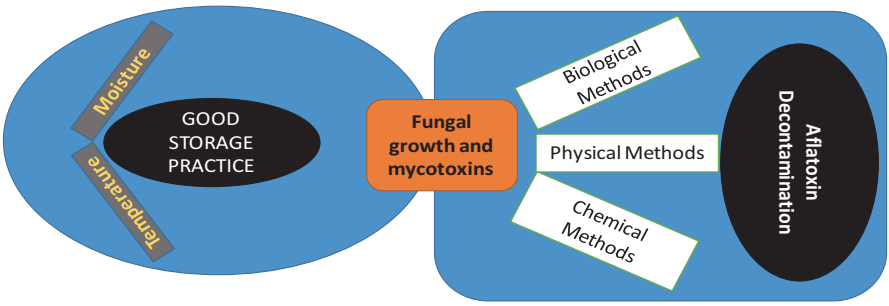

Fig. 9.1 Factors and methods to prevent and eliminate aflatoxins

9.2 Methods of Removal

9.2.1 Physical Methods

9.2.1.1 Physical Separation and Cleaning

The molds of *Aspergillus* are physically separated from the affected grains or feed by using the process of cleaning, sorting, and handpicking. This method is mostly used in developing parts of the world. It is the simplest method and does not involve any product changes (Park 2002).

9.2.1.2 Heating Treatment and Humidity

This treatment process can partially degrade the aflatoxin due to their thermostable nature. However, this method is still commonly adopted as heating can be carried out easily at low cost. Due to this reason, the food processing industry encourages the use of extrusion cooking particularly high temperature with short-time extrusion efficiently. Highly humid conditions have been recently reported to markedly enhance the degradation of aflatoxins (Rustom 1997).

9.2.1.3 Treatment by Thermal Microwave

This physical decontamination technique has proved as one of the best methods to contain mold contamination. Aflatoxin concentration has been reported to be significantly reduced by the process of microwave mediated thermal alkaline method in the Mexico food variety called tortillas (Basaran and Akhan 2010).

9.2.1.4 Irradiation Treatment

In this physical method, the high energy and penetrating gamma γ radiation is showered on major types of foodstuffs including beans, nuts, grains, palm juice, soybean, and animal/poultry feed. The effectiveness of this method of detoxification is moderate with an average decrease of 65% at high irradiation dose. The main advantage of this technology lies in its safe and economical decontamination of moderately mold- or aflatoxin-damaged feedstuffs (Rustom 1997).

9.2.1.5 Electrolyzed Water (EOW) Treatment

The major property of electrolyzed water (EOW) is the high levels of hydroxyl (OH) content, which makes the water very alkaline. This property of electrolyzed water gives its significant fungicidal activity against *Aspergillus flavus*. It is a

modern technology by which the aflatoxin including AFB1 content can be significantly reduced particularly with the neutral electrolyzed oxidizing water (NEW) treatment. Recent studies have reported that aflatoxin AFB1 was mostly degraded in 15 min by EOW treatment, and another study has shown that alkaline electrolyzed water (AIEW) could remove AFB1, and its best working condition was at pH 12.2. Interestingly, it was found that aflatoxin AFB1 removal could reach nearly 100% when 5.0 g peanut oil or olive oil with 10 ml AIEW with pH 12.2 along with the oscillation for 5 min at 20 °C (Jardon-Xicotencatl et al. 2015).

9.2.1.6 Pulsed Light Technology to Remove AFB1

The development of pulsed light technology is a nonthermal strategy to effectively decontaminate the surface of food and material by destroying bacteria, viruses, fungi, and spores. It was Moreau et al. who provided the first evidence of mycotoxins destruction and its efficiency calculated was around 98% by eight flashes of pulsed light (Moreau et al. 2013).

9.2.2 Chemical Methods to Remove Aflatoxin

The use of chemical additives upon the contaminated foods has been one popular method; especially the additives themselves would be used in the foods.

9.2.2.1 Ammonia Decontamination Treatment

It has been reported that the ammonization treatment of crops and feed products with gas from of ammonia (NH_3) or ammonium hydroxide (NH_4OH) at high temperature led to the significant and permanent removal of aflatoxin in an hour (Weng et al. 1994).

9.2.2.2 Hydrochloric Acid (HCl) Treatment

To evaluate the effect of HCl on AFB1, Aly and Hathout treated the corn gluten contaminated with aflatoxin molds under different concentrations. They found that the degradation of aflatoxin depends on the HCl concentration, temperature, and the time duration of treatment. It was found that when 1 mol/L HCl at 100 °C was added during the wet milling process, there occurred degradation of AFB1 by 27.6% in 4 h and 42.5% in 8 h. The complete degradation was obtained with 5 mol/L HCl in 4 h at 110 °C (Tabata et al. 1994).

9.2.2.3 Lactic Acid and Citric Acid Treatment

Some studies reported that the aflatoxin-contaminated foods are detoxified to a significant extent when treated with some organic acids like lactic acid and citric acid. Mendez-Albores et al. established that citric acid and lactic acid do degrade the aflatoxin. Interestingly, the percentage of aflatoxin degradation is proportional to the concentration of acids. Lee et al. showed that the percentage reduction of aflatoxin AFB1 is nearly 94.1 and 92.7%, by using 1.0 N citric acid and lactic acid for 18 h, respectively (Méndez-Albores et al. 2008).

9.2.2.4 Ozonation Treatment

Treatment with the ozone gas results in ozonolysis and degradation of all molds including *Aspergillus parasiticus* and *Aspergillus flavus* producing aflatoxin AFB1 at a concentration of 6–90 mg/L in short duration of 15 min. Study reported that aflatoxin-contaminated peanuts were very sensitive to the treatment with ozone (6.0 mg/L) at moisture content of 5% (w/w) and were sensitive to ozone and easily degraded in half an hour. The percentage degradation of the total aflatoxins and aflatoxin B1 (AFB1) were 65.8% and 65.9%, respectively. Another study also showed that 89.4% AFB1 in the peanuts was decomposed by ozone with a concentration at 50 mg/L, with flow rate 5 L/min for 60 h (Jr and King 2002).

9.2.3 Biological Methods to Remove Aflatoxin

One of the environmentally friendly and very effective methods to degrade the aflatoxin by using microbes or their enzyme products is the biological intervention. These methods are increasingly becoming popular as they are nature friendly, efficient, and specific to remove aflatoxin contamination from the foods or feed products.

9.2.3.1 Spectrum of Bacteria Present in Soil

Several soil bacteria such as *Flavobacterium aurantiacum* NRRL B-184, *Corynebacterium rubrum*, *Nocardia asteroides*, *Mycobacterium fluoranthenivorans*, *N. corynebacterioides* DSM20 151, and *Rhodococcus erythropolis* have the biological ability to degrade aflatoxins in a very effective and safe way (Eshelli et al. 2015). Biological degradation of aflatoxin is caused by M_1 by *Bacillus pumilus* E-1-1-1 (Gu et al. 2019). Soil- and waterborne *Flavobacterium aurantiacum* NRRL B-184 bacterium has been reported to detoxify aflatoxins with high efficiency. Ciegler et al. found that *F. aurantiacum* NRRL B-184 degraded the aflatoxin in milk, oil, peanut butter, peanuts, and corns and was partially removed from

soybeans contaminated with aflatoxin. The removal of aflatoxins was interestingly irreversible with no formation of any new toxic products. These bacteria can also increase AFB1 degradation by 10–15% in the presence of metal ions such as Cu^{2+}, Mn^{2+}, and Zn^{2+} suggesting the involvement of enzymatic activity system in aflatoxin AFB1 degradation by *F. aurantiacum* (D'Souza and Brackett 2001; Hao and Brackett 1988). Certain bacteria such as *Nocardia asteroides*, *Corynebacterium rubrum*, and *Mycobacterium fluoranthenivorans* sp. nov. DSM44556 are also able to detoxify aflatoxin. Interestingly, the cell-free extracts of *M. fluoranthenivorans* sp. nov. DSM44556 have shown significant degradation of AFB1 of more than 90% at high temperature in 4 h and were completely degraded in 8 h (Teniola et al. 2005). Thus the use of *F. aurantiacum*, *M. fluoranthenivorans*, and *N. corynebacterioides* could be a potential and promising application because of their potent efficient degradation of AFB1 in the food and feed process.

9.2.3.2 Fungi

Fungi can not only produce aflatoxins but also degrade aflatoxin. Such four fungal strains *Aspergillus niger*, *Eurotium herbariorum*, a *Rhizopus* sp., and non-aflatoxin-producing *A. flavus* were able to convert AFB1 to aflatoxicol-A (AFL-A); then AFL-A was converted to aflatoxicol-B (AFL-B) by the actions of medium components or organic acids produced from the fungi. Fungi *Penicillium raistrickii* NRRL 2038 could transform AFB1 to a new compound which is similar to AFB2. Kusumaningtyas et al. found that *Rhizopus oligosporus* was able to inhibit synthesis or to degrade AFB1 when cultured together with AFB1-producing fungi *A. flavus* (Wu et al. 2009; Zhao et al. 2020).

9.2.3.3 Yeasts and Lactic Acid Bacteria

The binding property of cell wall components of yeasts and lactic acid bacteria makes them able to bind and degrade the aflatoxins. Studies found that the mechanism of aflatoxin degradation is due to their adhesion to both yeasts and lactic acid bacteria in a unique and friendly manner. Evidences support that, during fermentation, brewing, yoghurt formation, or dairy product fermentation with yeast, the AFb1 concentration was significantly reduced. Relative hepatic weight, histopathological and biochemical parameters were improved and showed a positive protection in the process of drinking water with *S. cerevisiae* strain. However, reports also exist which state that the yeast and lactic acid bacteria have no degradation effect on aflatoxins. Peltonen has reported that several strains of lactic acid bacteria including 12 *Lactobacillus*, 5 *Bifidobacterium*, and 3 *Lactococcus* bacteria strains were able to bind AFB1. After a 72-h incubation period with the *Lactobacillus amylovorus* strains and one *Lactobacillus rhamnosus* strain, more than 50% of AFB1 were successfully removed rapidly. Another two lactic acid bacteria *Lactobacillus rhamnosus* strain GG (LBGG) and *L. rhamnosus* strain LC-705 (LC705) can significantly

and very quickly remove approximately 80% AFB1 from culture media in both temperature- and bacteria concentration-dependent manner (Rayes 2013; Wu et al. 2009).

9.2.3.4 Aflatoxin Degradation by Enzymes

Using microbial systems to isolate and purify some specific enzymes to degrade aflatoxin is highly advantageous in terms of retaining the nutritional value and flavor. Motomura et al. (2003) reported the ability of degrading AFB1 in cultured supernatants from 19 fungi and purified 1 enzyme with aflatoxin degradation activity by cleaving lactone ring from *P. ostreatus* supernatant. The enzyme kinetics showed that AFB degradation was optimum at 25 °C with a pH of 4.0–5.0. One of the reports suggest that an intracellular enzyme called aflatoxin-detoxifizyme, exhibited detoxification process on aflatoxin B1 and the optimum activity for the enzyme was at 35 °C with a pH of 6.8 (Liu et al. 2001).

9.2.3.5 Cold Plasma Technology to Remove AFB1

Earlier, cold plasma treatment was used to sterilize sensitive materials. Now, the technology has been used in the food industry to decontaminate the food articles as it has high efficiency, low impact on the quality of treated food products, short treatment time, and no residue (Schlüter et al. 2013). Moreover, the aflatoxin AFB1 removal and detoxification has been done by the treatment with microwave-induced argon plasma for 5 s (Park et al. 2007). Interestingly, about 88% of AFB1 has been degraded by the low temperature radio-frequency plasma within 10 min (Wang et al. 2015). Another high-voltage atmospheric cold plasma (HVACP) technology has been introduced as a novel nonthermal decontamination process that has the potential to be used in the food industry. HVACP can effectively degrade about 70% of the total aflatoxin in 12 min (Shi et al. 2017).

9.2.3.6 Sorbent Additives for Degradation of AFB1

Addition of sorbents can safely and effectively prevent aflatoxin contamination of foods. The unique mechanism in this process is that it doesn't degrade the aflatoxins in the foods or feeds but act as binding agent to prevent the absorption of aflatoxins like AFB1 from intestinal tract after ingestion. Addition of chlorophyllin as sorbent has reduced the formation of adducts of AFB1-DNA by 37% in rainbow trout, responsible for 77% reduction of tumor formation (Breinholt et al. 1995). In addition, it was also reported that chlorophyllin reduced the AFB1-DNA adducts, which augmented the favorable reduction of 65% of AFB1-album adducts and 90% reduction of urinary AFM1 (metabolite) in rats; interestingly, chlorophyll has also been

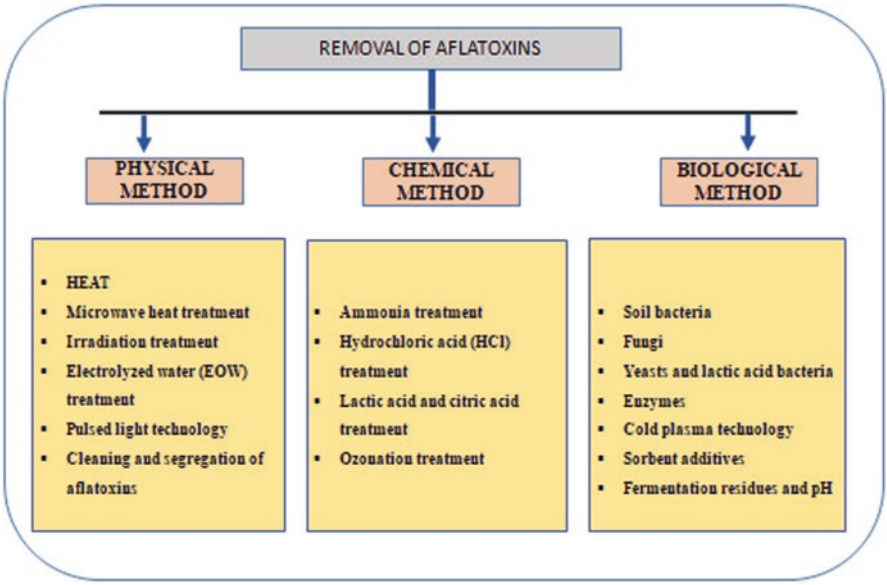

Fig. 9.2 Schematic representation of the methods involved in removal and detoxification of aflatoxins

able to reduce 55% of AFB1-DNA adducts, 51% of AFB1-album adducts, and 92% of urinary AFM1 levels, respectively (Simonich et al. 2007).

Like chlorophyllin and chlorophyll, clay also acts as a sorbent, and hence it is added to the aflatoxin-contaminated feeds; the formation of adducts markedly reduces the rate of absorption of AFB1 and consequently reduced the AFM1 level in milk (Phillips et al. 2008). The toxicity studies were performed on SD rats fed with NovaSil clay (NS) for a period of more than 6 months (Afriyie-Gyawu et al. 2005). In addition, different types of mineral clays have been tested for their capabilities to bind AF in animal feeds. These absorbents, such as activated carbon (charcoal), zeolite, and saponite-rich bentonite, reduced AFB1 absorption in the gastrointestinal tract (Giovati et al. 2015) (Fig. 9.2).

9.3 Conclusion

Among all mycotoxins, the group of aflatoxins has received much attention due to their severe impact on human and animal health. AFB1 is the most potent carcinogenic agent associated with hepatocellular carcinoma. And AFB1 can negatively affect nutrition absorption, growth and development, and immune system function. There has been a very significant development of methods and techniques to remove and prevent AFB1 contamination and degrade aflatoxins. The process of removal involves both the controlled storage conditions (good storage practices) and the

appropriate use of decontamination technologies. The simplest and most traditional, safe, and economical physical method is to separate the contaminated grains from the crop or food items. There are tremendous potential and challenges associated with the decontamination of aflatoxin in the food industry. One of the important challenges is to preserve the nutritional values or other important food qualities along with no added residues or new contaminates be produced. Most of the common and harsh physical and chemical strategies to detoxify aflatoxins might affect the nutritional values of the food items. Further they can also get unsafe for human or animal consumption. Interestingly, techniques like ozone treatment, gamma radiations, microwave heating, electron beam application, pulsed light application, electrolyzed water, and cold plasma treatment proved to have great potential and improved efficiency for future applications. Commercially, several techniques including inexpensive and comprehensive methods can be used to reduce aflatoxins as in the case of beer, wine, and beverage industry market. Biological techniques using bacteria and yeasts to remove or degrade aflatoxins seem to provide useful approaches. The mechanism by which a certain technique to remove and detoxify aflatoxins can be linked to determine the practical applications of these approaches in food or other products. Therefore, the efficiency and purpose of detoxification can be best achieved by combining both traditional and novel technologies.

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Chapter 10
Physical Decontamination and Degradation of Aflatoxins

Parisa Mostashari, Saber Amiri, Laya Rezazad Bari, Motahareh Hashemi Moosavi, and Amin Mousavi Khaneghah

Abstract Aflatoxins are significant mycotoxins produced by numerous fungi, particularly *Aspergillus flavus*, *A. parasiticus,* and *A. nomius*. Nuts, maize, dried fruits and spices, and meat and milk products are the significant sources of aflatoxins. Aflatoxins are potential human carcinogen agents with teratogenic, immunogenic, nephrotoxic, and genotoxic features. Aflatoxin decontamination has been an ongoing challenge for the food industry. However, their complete degradation and decontamination required further investigations. The present chapter delivers the roles of physical techniques used for aflatoxin degradation and decontamination in foodstuffs. Some aflatoxin decontamination physical techniques, including adsorption, thermal processing, radiations, cold plasma, electrolyzed water, ozonation, and

P. Mostashari
Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Research Center of Nutrition and Organic Products (RCNOP), Shahrekord Branch, Islamic Azad University, Shahrekord, Iran

S. Amiri
Department of Food Science and Technology, Faculty of Agriculture, Urmia University, Urmia, Iran
e-mail: sa.amiri@urmia.ac.ir

L. Rezazad Bari
Department of Horticultural Science, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran
e-mail: l.rezazad@uma.ac.ir

M. Hashemi Moosavi
Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

A. Mousavi Khaneghah (✉)
Department of Food Science and Nutrition, Faculty of Food Engineering, University of Campinas, Campinas, SP, Brazil
e-mail: mousavi@unicamp.br

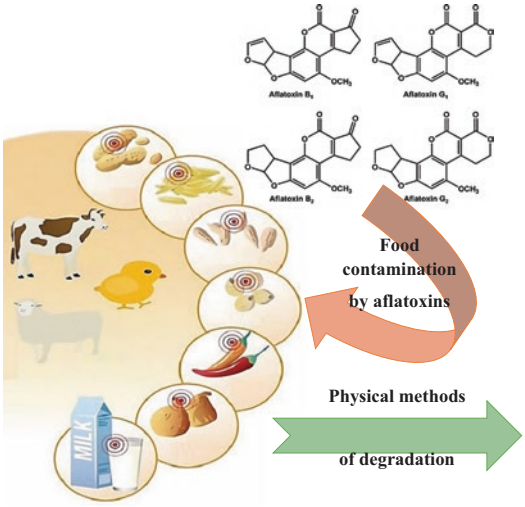
K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_10

pulsed electric field, are reviewed in detail. Decontamination mechanisms, degradation competence, advantages, and limitations of these physical techniques have been reviewed in this chapter. While thermal techniques cause aflatoxin degradation, they are not sufficient for comprehensive degradation in foodstuffs. Electrolyzed water, pulsed light, some radiations, and cold plasma techniques harbored higher aflatoxin degradation. However, further research should perform to evaluate degradant toxicology and its interaction with food components. It seems novel technologies, such as radiations, cold plasma, electron beam, pulsed light, electrolyzed water, ozonation, and pulsed electric field, have the significant potential for future applications in aflatoxin decontamination and degradation in the food industry.

Keywords Aflatoxins · Decontamination · Physical · Radiations · Cold plasma · Electrolyzed water · Ozonation · Electric field

10.1 Introduction

Aflatoxins are a group of mycotoxins primarily biosynthesized by *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* growing on livestock feed and foods in the field and throughout storage, transport, and processing. These fungal carcinogenic toxins are responsible for the occurrence of aflatoxicosis disease in humans and animals globally. Aflatoxicosis is mainly known for death and depression, vomiting, icterus, and hemorrhage in acute outbreaks. Subacute outbreaks are mostly known with occasional sudden deaths and weakness, thriftiness, anorexia, reduced growth, and feed efficiency (Khaneghah et al. 2017). Chronic outbreaks are usually caused by hepatotoxicity. Moreover, aflatoxins cause important economic burdens to agriculture owing to reduced crop yields and quality, reduced animal performance and production, and increased incidence of diseases (Kumar 2018). There are four distinct aflatoxin forms, including B_1, B_2, G_1, and G_2, and two derived products, namely, M_1 and M_2 (Nazhand et al. 2020). Main food products, including nuts, maize, and dried fruits and spices, along with meat and milk products, are the most susceptible foods for aflatoxin's production (Benkerroum 2019). Since aflatoxins are toxic, mutagenic, teratogenic, carcinogenic, and immunosuppressive to humans and animals, it is essential to prevent their products in food and feed and detoxify contaminated foods and feeds. Inhibition from aflatoxins production is not possible because of the primary nature of some products and the high costs of control. Thus, alternative actions should be implemented to decrease exposure risks to aflatoxins. Thermal and nonthermal operations in detoxification of aflatoxin-contaminated products are mainly suggested to reduce these toxins' risk in foods and feeds (Martinez-Miranda et al. 2019; Rushing and Selim 2019), as summarized in Fig. 10.1. This chapter focuses on an overview of the thermal and nonthermal methods used to detoxify foods after harvest.



Physical methods of aflatoxin degradation	Types		
Adsorption	Separation		
	Solvent extractions		
	Mineral adsorbents		
	Magnetic carbon		
Thermal processes	Cooking		
	Roasting		
	Baking		
	Extrusion		
	Microwave		
	Infrared		
Non-thermal processes	Radiation	Ionizing	X-rays
			Ultraviolet rays
			Gamma rays
			Electron beam
			Pulsed light
		Non-ionizing	Radio waves
	Cold Plasma		
	Electrolyzed Water		
	Ozonation		
	Pulsed electric field		

Fig. 10.1 Overview of physical methods for aflatoxin detoxification

10.2 Adsorption

10.2.1 Separation

Aflatoxins can survive during the heat and some physicochemical procedures performed in the food industry. However, it is essential to remove them from the food, and as a result, conventional procedures had been expanded, including irradiation, biodegradation, and physical separation, such as adsorption or biosorption, filtration, and extraction. Among these physical methods, adsorption is a common, easy, and economical way (Wang et al. 2020). The primary materials employed as adsorbents to mycotoxin include organic substances such as cell wall of yeasts and probiotic bacteria, activated carbon, and biopolymers (processed plant fibers) and inorganic materials such as zeolites, bentonites, and other clay minerals (Amiri et al. 2020; Čolović et al. 2019; Khiavi et al. 2020).

Processing of foods can affect aflatoxins in raw substance through (i) physical elimination, (ii) chemical conversion to metabolites with higher or lower toxicity, (iii) release from masked or entrapped forms to increase bioavailability, (iv) enzymatic decontamination, and (v) adsorption to solid surfaces (Muhialdin et al. 2020).

10.2.2 Solvent Extractions

Aflatoxin's physical degradation by solvent extraction with ethanol, hexane, and isopropyl alcohol is theoretically possible. These solvents mainly applied in the aflatoxin's removal procedure are recycled to extraction systems after distillation regeneration. Nevertheless, the distillation procedure is expensive, owing to high latent solvents and heat. Thus, adsorption techniques have been established to remove aflatoxins from solvents. Montmorillonite is an extremely operative compound for the removal of the adsorptive aflatoxin. Neutral alumina and silica are also operatives for the adsorption procedure. Ethanol and ethanol-based micelles are also useful for solvent extraction through the adsorption procedure (Asadi 2020; Endre et al. 2019; Heshmati et al. 2019; Rui et al. 2019).

The physical removal using solvent extraction is the possible option for decontaminated feed crops. Polar solvents such as pure ethanol or isopropyl alcohol can remove aflatoxins from feeds. In this method, solvents are usually regenerated through distillation and recycled into the extraction system (Dogan et al. 2017).

10.2.3 Mineral Adsorbents

Aflatoxin's molecule adsorption has been assessed in recent investigations. In adsorption procedures, toxin-absorbent binding in the digestive tract can reduce the content of aflatoxin, and the appropriate functional group's positioning and polarity are efficient in enhancing the adsorption of aflatoxin (Di Gregorio et al. 2014). Diverse neutral chemical agents, including indigestible carbohydrates (cellulose and polysaccharides of the bacteria's and yeast's cell walls such as peptidoglycans and glucomannans), synthetic polymers (polyvinylpyrrolidone and cholestyramine), vegetable fibers and humic acid, and synthetic silicates and clays, can be used for this purpose (Carraro et al. 2014; Elliott et al. 2020). Otherwise, synthetic polymers including cholestyramine, polyvinyl pyrrolidone, polysaccharides, cellulose, glucomannans, peptidoglycans, and alumino (hydrated sodium calcium aluminosilicate (HSCAS), clay, bentonite, sodium, and calcium aluminum silicates) are the main adsorbing components. Researchers reported that the adsorptive capacities of the bacteria's and yeast's cell walls were very high, and they were suitable substitutes to inorganic adsorbing agents (Solís-Cruz et al. 2017; Arak et al. 2019). Pyrrolidone is another agent that acts through physical adsorptive capacity and natural bridges to establish nitrogen and hydrogen in its structure. Nowadays, modified bacteria and yeast cell wall materials have been prepared with the high ability to adsorb a vast range of aflatoxins and even other mycotoxins (Campagnollo et al. 2020; Ghofrani Tabari et al. 2018).

Lately, industrial and agricultural wastes have been extensively used for the confiscation of mycotoxins. Agricultural wastes are attractive bio-sorbents as their significant constituents are lignin, cellulose, and hemicellulose, which harbor functional

groups including carbonyl, hydroxyl, sulfhydryl, esters, phenolic, and amino chains which carry dominant active sites for aflatoxin's capture. Hydrogen bonding, π–π interactions, secondary bond forces, and physicochemical interactions between the functional groups (phosphonates, carboxyl, sulfhydryl, amines, phosphates, and amides) on bio-sorbents and pollutants are the chemical procedures of the biosorption (Dai et al. 2018). Consequently, functional groups present the bio-agricultural wastes as promising compounds for the mycotoxin's adsorptive removal. The lignin- and cellulose-rich nature of bio-agricultural wastes caused high aflatoxin's adsorbent capacity.

Literature analysis showed that bentonites (montmorillonites), aluminosilicates, HSCAS, sepiolite, zeolites, activated carbons, and diatomite are the mineral adsorbents most commonly used in foods and feeds. However, aflatoxins may bind to adsorbents through diverse modes, including hydrogen bonding, hydrophobic binding, electrostatic repulsion and attraction, and coordination bonds. In keeping with this, many mineral components are active in aflatoxin adsorption (Di Gregorio et al. 2014). Reports showed that aluminosilicates are practical mineral adsorbents for aflatoxins and other low polar compounds, as aluminosilicates combine high selectivity, sympathy, and cation exchange capacity HSCAS as the most well-organized complex. Sepiolite, activated charcoal, and diatomite also display high capacities in aflatoxin's adsorption. Insertion of long organic chains amplified the adsorption efficiency of nonpolar compounds, including zearalenone and ochratoxin, and reduced the adsorption efficiency of polar compounds like aflatoxins. It seems that each adsorbent is specific to one type of and no adsorbent is specific to several mycotoxin's types. Finally, the adsorbent's ability to bind to aflatoxins is different because the clay minerals composition is also different, making it essential to use a set of various adsorbents when different aflatoxins are found in the same food (Rasheed et al. 2020).

10.2.4 Magnetic Carbon

There is substantial attention to magnetic particles, particularly magnetic carbon, technology owing to their application in mycotoxins' adsorption. Adsorption of aflatoxins from gaseous or aqueous effluents using magnetic adsorbents is mainly used in different studies. However, moderately small area surface and less adsorption capacity are two important disgraces of magnetic adsorbents that limited their application. Furthermore, their preparation needs several specific steps and expensive chemical reactions. Reversely, the high surface area, the absorbent structure, and the low cost of synthesizing magnetic carbon make it suitable for aflatoxin's adsorption. Additionally, the small iron oxide particles' presence on carbon's surface allows rapid adsorption of polar contaminants (Zahoor and Ali Khan 2016; Zahoor and Ali Khan 2016).

10.3 Thermal Processes

10.3.1 Cooking

Most aflatoxins are heat resistant within the typical food thermal processing (80–121 °C); consequently, little or no reduction in total toxin load happens after conventional cooking, including frying and boiling, even the following pasteurization. Diverse factors, including the primary contamination level, aflatoxin's type, and concentration, heating time and temperature, the amount of heat penetration, the pH and moisture's contents of food, and food ionic strength, play an essential role in the levels of aflatoxin degradation through the thermal processing. In keeping with this, high temperatures (237–306 °C) are considered as heating methods of aflatoxin's detoxification (Nazhand et al. 2020).

Aflatoxins are mainly broken down in high temperatures (237–306 °C). Although aflatoxins are extremely resistant toward high dry heat temperatures (melting temperature of 268–269 °C), some efforts have been performed to aflatoxin's inactivation in different food products. Reports revealed that aflatoxins partially occur mainly in temperatures above 150 °C (Zheng et al. 2017). Findings showed that conventional cooking caused up to 41% reduction in aflatoxins B1 and B2 contents. Additionally, maize grit's frying and boiling lead to a 34–53% and 28% average decrease in the aflatoxin B1 contents, respectively. Up to 89% reduction in the aflatoxin content of rice has been reported after conventional cooking. High moisture content in food facilitated the well opening of the lactone ring of the aflatoxin B1 and caused the terminal carboxylic acid formation, and as a result, heat-induced decarboxylation and destruction have occurred. Several findings showed that pressure cooking could effectively reduce the aflatoxin contents of food by up to 90% (Jalili 2016).

Alkaline cooking, which is also known as nixtamalization (cooking and soaking), is used to process some kinds of foods, particularly in Latin America. Reports showed that Alkaline cooking could well eliminate 50–85% of aflatoxin content of tortilla, tortilla chips, and maize chips. Another report showed that the alkaline-cooking procedure caused a 93–94% reduction in the levels of aflatoxin B1. Pasteurization (62 °C for 30 min) can decrease by about 32% of the aflatoxin M1 contents of naturally contaminated milk. Heating of milk, depending on the conditions employed, including time, temperature, and protein and fat contents of milk, can cause a decrease (10–35%) in the aflatoxin M1 content of milk samples (Jalili 2016; Schaarschmidt and Fauhl-Hassek 2019).

10.3.2 Roasting

Roasting is one of the most operative techniques for aflatoxin reduction in different kinds of commodities, including peanuts, pistachio, almonds, hazelnuts, walnuts, and coffee. This method is responsible for an average of 40–85% decrease in the aflatoxin contents of diverse kinds of nuts. Oven roasting of naturally contaminated nuts at 150 °C for 30 min caused a significant (up to 45%) decrease in the aflatoxin content. Rendering the time and temperature used for the roasting procedure and initial levels of mycotoxin contamination, roasting can reduce the aflatoxin content of nuts ranging from 15 to 65% (Rastegar et al. 2017). Reports revealed that ionic salts such as NaCl caused a significant increase in the aflatoxin degradation of nuts during the roasting procedure. Rendering the type of coffee and employed time and temperature, roasting caused a severe reduction in coffee beans' aflatoxin content (40–55%). Reports showed that the application of higher temperature in a shorter period caused higher aflatoxin destruction in roasted seeds than the lower temperature for a more extended period (Martins et al. 2017; Bakherad and Feizy 2018).

10.3.3 Baking

Baking is a word mainly used for dry cooking of flour-based foods, particularly bread, biscuits, cakes, and some kinds of pastries. The presence of aflatoxin in bakery products is undesirable as flour in all bakery products, and milk in some kinds of them is a source of aflatoxins. The most important factors affecting the efficacy of baking-induced aflatoxin degradation are the time and temperature used in the procedure (Milani et al. 2018; Karlovsky et al. 2016). Logically, high temperatures and a more extended period can reduce the aflatoxin content of bakery products significantly. However, the nutritional, sensory, and physicochemical properties of bakery products should also be considered. Noroozi et al. (2020) demonstrated that an increase in wheat flour's baking temperature to 280 °C for 15 min caused an effective decrease (53.9%) in the aflatoxin content of produced bread. Aflatoxins have boost breakdown temperatures ranging from 237 °C to 306 °C. Dry heating at temperatures below the thermal decomposition temperature of 267 °C has the lowest effects on aflatoxin degradation. Thus, using the baking procedure with one or more additional techniques used for aflatoxin degradation in bakery products is suggested.

10.3.4 Extrusion

Extrusion cooking is a short-term process at high temperatures in which raw materials are exposed to molecular transformations and chemical reactions caused by excessive shear. In the extrusion procedure, high temperature, high pressure, and severe shear forces are used. These synergetic procedures caused significant changes such as starch gelatinization, protein denaturation, food enzyme inhibition, and depletion of microbial counts and mycotoxins in the food matrix. This technique is mainly employed for wheat, corn, and rice. At the extruder processing, a dough-like mixture is driven into a stationary metal tube or barrel by a rotating screw shaft. Besides, heat can be applied in the steam form and is made through the mechanical energy of the turning screw and the barrel friction. Consequently, temperatures above 150 °C can be prepared, which caused aflatoxin destruction (Guo et al. 2020; Saalia and Phillips 2011).

There are two significant kinds of extruders in the food industry: single- and twin-screw extruders. Twin-screw extruders are frequently applied for extruding diverse raw materials since their elastic design permits a product's fast change. Twin-screw extruders are also appropriate for raw materials with a fat content of 18–22%. However, the material's fat content should not be higher than 12–17% in the single-screw extruders because the fat reduces the shear. As a result, the energy cannot be transformed into heat for cooking (Molla and Zegeye 2014).

Aflatoxin destruction through extrusion is mainly reliant on numerous issues, such as moisture content of the extrusion mixture, screw speed, extruder temperature, and the food residence time in the extruder. However, screw speed had a lesser effect under the same test circumstances. Additionally, using the mixing screws caused a somewhat higher aflatoxin reduction than non-mixing screws. The addition of sodium metabisulphite caused higher aflatoxin destruction in the extrusion procedure of foods. Furthermore, ammonium hydroxide and ammonium bicarbonate caused a significant reduction in the aflatoxin contents of food treated with extrusion procedure (Castells et al. 2005).

10.3.5 Microwave

Microwaves are 300 MHz–300 GHz electromagnetic waves with 1 m–1 mm wavelengths. Domestic microwave's frequency is 2450 MHz, whereas industrial microwave's frequency is either 915 or 2450 MHz. Microwave heating is an exclusive volumetric heating technique that converts electromagnetic into thermal energy by polarizing electromagnetic radiation. Microwave has been extensively utilized for heating, drying, cooking, and extraction of food (Guo et al. 2017). Additionally, microwave heating has been widely used in order to degrade aflatoxin in foods. Alkadi and Altal (2019) reported degradation of up to 67.7% of aflatoxin B1 in corn flour heated for 10 min in a microwave oven.

Khazaeli et al. (2017) investigated the effect of microwaves on the detoxification of pepper. The results showed that microwave power at 900 W for 30–240 s could significantly reduce the pepper samples' aflatoxin content. Degradation of aflatoxin B1 was significantly dependent on the temperature and time of the microwave process. This study showed that microwave heating could increase the detoxification rate of aflatoxins in comparison with standard heating methods. Patil et al. (2019) investigated the impact of microwave heating and gamma irradiation on decontamination of aflatoxin. For this purpose, peanuts (*Arachis hypogaea* L.) were contaminated with 300 µg/kg of aflatoxin B1 and individually treated with gamma irradiation (5, 7, and 9 kGy) and microwave heating (360, 480, and 600 W). According to the results, aflatoxin B1 decreased 20–43% and 59–67% by gamma irradiation and microwave heating, respectively. Treatment by 7 and 9 kGy of gamma irradiation and 360, 480, and 600 W microwave power had a synergistic effect on levels of aflatoxin B1 reduction and decreased >95% peanut contamination.

Findings of diverse research studies revealed that microwave heating caused moderate successful effects in reducing aflatoxin contents in foodstuffs. Microwave producers can modify the apparatus to precise uses and foodstuff types. Nevertheless, resolving the nonuniform distribution of temperature throughout microwave heating, which could induce hot and cold spot formation in treated food, has continuously posed a challenge (Menon et al. 2020). Cold spot formation imperfects aflatoxin detoxification, while hot spot formation because of overheating may cause nutritional and quality losses. Thus, supplementary surveys are essential to improve the efficiency of aflatoxin degradation, along with the clarity of the structure and the safety assessment of degradation products (Vearasilp et al. 2015).

10.3.6 Infrared

Infrared (IR) spectroscopy includes a comprehensive spectrum of wavelength (800 nm–1,000,000 nm). IR is part of the electromagnetic band located amid the visible and microwave regions with 0.5–100 µm wavelength ranges. IR rays are divided into three kinds of near-IR (NIR, wavelength ranges from 0.75 to 1.4 µm at temperatures below 400 °C), mid-IR (MIR, wavelength ranges from 1.4 to 3 µm at temperatures between 400 and 1000 °C), and far-IR radiation (wavelength ranges from 3 to 1000 µm at temperatures above 1000 °C) (Krishnamurthy et al. 2008). Their penetration into the foodstuffs causes vibrating movement and subsequent heating.

Heat induced by IR irradiation caused a reduction in fungal growth and subsequent aflatoxin production and structure destruction of produced aflatoxins. The effectiveness of microbial inactivation and mycotoxin destruction by IR heating relies on the IR power, peak wavelength, bandwidth of IR heating source, types of food materials, food sample temperature, food sample depth, food moisture content, and types of mycotoxins (Das and Das 2014). The IR-induced thermal process can damage DNA, RNA, ribosome, cell envelope, and proteins. In keeping with the high

advantages of IR irradiation, rare surveys have been conducted on its effects on foods' aflatoxin content (Aboud et al. 2019).

Jun and Irudayaraj (2003) used IR irradiation (5.88–6.66 μm wavelength) to inhibit *Aspergillus niger* and *Fusarium proliferatum* growth in corn flour. After 5 min of IR radiation exposure, 2.3 and 1.95 CFU/g logarithmic decreases were observed for *Aspergillus niger* and *Fusarium proliferatum*, respectively. Wilson (2019) reported that treatment of corn samples with different IR wavelength (3.2 μm) at product-to-emitter gap sizes (PEG) of 110 mm (intensity to 15.71 kW/m^2) for 60 s caused the highest reduction in the *Aspergillus flavus* load. Despite the high potency of IR irradiation, there is a significant demand for supplementary research conducted to assess this technique's effect on aflatoxin content of foods and its impacts on the sensory, nutritional properties, and physicochemical characters of targeted food samples.

10.4 Nonthermal Methods

10.4.1 Radiation

10.4.1.1 Ionizing

Gamma ray, X-ray, and ultraviolet rays are ionizing radiation. The irradiated molecules possibly change when exposed to a small increase in temperature. The irradiation causes a low increase in the temperature of materials. Notwithstanding the uncertainties about irradiated food safety, it has become a procedure for industrial-scale food product treatment decontamination and detoxification (Liu et al. 2016).

Food irradiation is a cold storage process that has been extensively studied over the past 45 years. Radiation sources that are authorized for food processing are:

1. Gamma rays produced from radioisotope cobalt-60 (1.17 and 1.33 MeV) and cesium-137 (0.662 MeV).
2. Electron beams produced by the machine (maximum energy 10 MeV).
3. X-ray (maximum energy 5 MeV) cobalt-60 in a nuclear reactor by neutron bombardment of completely pure plates.

X-rays

X-ray irradiation has not been extensively used for aflatoxin degradation in food samples. X-ray, produced by X-ray engine, may cause a reduction in the mycotoxin content of food through fungal elimination and toxin destruction. However, radiation processing of foods with X-rays (up to 7.5 MeV) can be applied without worry about induced radioactivity in food. In keeping with this, the numbers of research conducted to decrease the microbial load or mycotoxin content of foods through

X-ray treatment are very low (Aboud et al. 2019). This is partly due to the high cost of X-ray irradiation and the risk of radioactivity transmission into the foods. Byun et al. (2019) surveyed the effect of X-ray irradiation on the reduction of *Aspergillus flavus* load on red pepper powder. Their results showed that treatment of red pepper powder and gochujang with X-ray (3.5 kGy) caused under 2 log decrease in the count of *Aspergillus flavus* without any deleterious impacts on sensory qualities and physicochemical of products. They concluded that the X-ray was not completely effective in reducing the load of *Aspergillus flavus* in powdered foods.

Ultraviolet Rays

Ultraviolet (UV) irradiation has been recognized for an extended period of time as an operative technique for the aflatoxin destruction in foodstuffs. UV irradiation is more practical and economical without toxic consequences and contamination. Thus, it is hugely used for aflatoxin destruction. The aflatoxin B1 mainly absorbed UV rays at 222, 265, and 362 nm. Hydroxyl free radicals ($OH^{\cdot}$) formation during UV irradiation procedure caused the severe attack to the C8–C9 positions of aflatoxin B1. Figure 10.2 shows the aflatoxin B1 degradation pathway through UV irradiation (Jalili 2016).

Reports revealed that the cytotoxicity and mutagenicity of UV-treated aflatoxin decreased significantly (Guo et al. 2020; Mao et al. 2016). Intensity and radiation exposure length of UV are two key elements impacting the efficacy of aflatoxin destruction. Application of 800 μw cm^{-2} for 30 min UV irradiation was adequate to eliminate aflatoxin B1 in peanut, while the application of 200 and 400 μw cm^{-2} UV irradiations caused 79% and 85% aflatoxin reduction, respectively (Liu et al. 2011).

Depending on the application UV, the source devices used are different, but in general, they all have the same structure and are designed by creating an electric current between two electrodes. The dose UV or amount UV of energy applied to a surface over a period of time is calculated by the following formula:

$$\text{Dose } \mu w\left(-\frac{\text{sec}}{\text{cm2}}\right) = \text{Intensity} = \left(\frac{\mu w}{\text{cm2}} \times \text{sec}\right)$$

This intensity is determined by the power of the UV lamps and the processing time, and the exposure of the desired surface to UV. The antimicrobial properties of UV radiation are mainly due to DNA mutations and the absorption of light by these molecules. This mechanism leads to the inactivation or reduction of the microbial population in a sigmoidal cycle of microorganisms' growth. To achieve the antimicrobial activity, the amount of UV energy on each part of the product should be 400 J/m. Critical factors include beam rate, reactor arrangement and design, power, physical shape and effective wavelength, product flow pattern, and radiation path length. UV can also be used in combination with other technologies. For example, strong oxidizing agents such as ozone and oxygenated water, especially in the

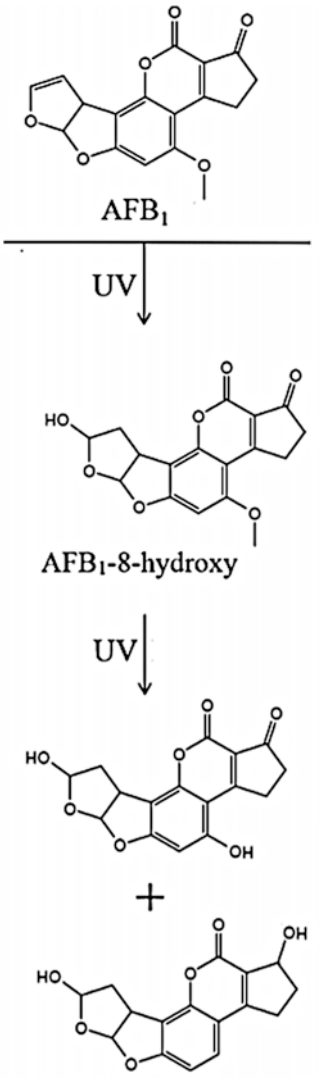


Fig. 10.2 Degradation pathway of UV-treated aflatoxin B_1

disinfection of water and surfaces in contact with food, are among the most important uses of UV in the food industry. Recently, attention has been paid to the use of UV to reduce the microbial load of juice.

Ghanghro et al. (2016) reported treatment of naturally contaminated wheat samples with short wave UV irradiation (254 nm for 160 min) caused more than 80% reduction in aflatoxin content. Previous surveys revealed that the application of moderate UV doses has not adversely impacted the sensory as well as physicochemical properties of food products (Delorme et al. 2020). Shen and Singh (2021) used the UV irradiation to detoxification of aflatoxins. The results showed that the

effect of UV irradiation was significantly increased once peanuts were rotated at 11 rpm in the UV chamber. Moreover, after treating with 2.3 mW/cm^2 UV-C for 2 h, the aflatoxin B1 detoxification rate increased from 60.8 ± 15.3 pmol/g h to 75.0 ± 10.9 pmol/g h in the rotated samples compared to not rotated peanuts. Li et al. (2020) contaminated peanuts with 500, 250, 250, and 250 μg/L of aflatoxin B1, B2, G1, and G2, respectively, and then irradiated with UV–ozone (3, 5, and 7 mg/L) and irradiation time (10, 20, 30, and 60 min). The decontamination rates of aflatoxin B1, B2, G1, and G2 increased by increasing ozone concentration and irradiation time. Aflatoxin B1 presented the maximum detoxification rate. Treating with 5 mg/L ozone combined with UV irradiation for 30 min, the detoxification rates of aflatoxin B1 were 79.01%.

Wanga et al. (2020) studied the quality of aflatoxin B1-refined groundnut kernel oils. Aflatoxin B1 detoxification in unrefined terrestrial oils was performed by UV. Ten samples of unrefined groundnut oil were treated with UV before measuring aflatoxin B1, fatty acid profile, and chemical characteristics. The 20-min treatment reduced aflatoxin B1 content by 99%. The amount of acid and peroxide in the oil samples showed slight changes after treating the samples with UV irradiation. The results showed that 21 types of fatty acids were identified in untreated oil samples. Minor changes in fatty acid levels were also observed after 20 min of treatment. The results showed that UV radiation effectively reduces the content of aflatoxin B1 in unrefined earthworm oils and maintains oil quality. This is a promising strategy for detoxifying aflatoxin B1 in groundnut oil. Mao et al. (2016) detoxified the produced aflatoxin B1 in peanut oil. The results showed that the possible photo degradation pathway of aflatoxin B1 in peanut oil is proposed. The human embryo hepatocyte viability assay showed that the cell toxicity of degradation products after UV irradiation was significantly lower than that of aflatoxin B1, which may be due to toxicological site breakage. Nonetheless, UV penetration into solid foods is low, which may eliminate its application in foods with high suspended solids content (Fan et al. 2017). Therefore, dense or granular foodstuffs should present in a thin layer form to better UV irradiation (Diao et al. 2015).

Gamma Rays

Gamma radiation is an operative method to preserve the quality of food products. Cobalt-59 and cesium-137 are formed by uranium cleavage or as a by-product of nuclear fuel processing. Both categories of high penetration gamma rays can be used in food processing in bulk or after packaging. Energetic photons produced by a gamma source, including cobalt-60 (^{60}Co), are extensively applied to destroy mycotoxins, particularly aflatoxins in foods. Some reports showed that aflatoxin content could be decreased with gamma irradiation, even in low doses (5–6 kGy) (Ghanem et al. 2008). The percentage of degradation by gamma rays decreases in aflatoxin B1 detoxification in different foods and different light sources with increasing concentration of aflatoxin or the dry and sedimentary state. However, other reports showed that aflatoxin degradation could be prepared only using gamma

irradiation in high doses. Sometimes reducing the dose of gamma rays by 100 kcal stimulates the production of aflatoxins in food products, and this is due to changes in the biochemical pathways of microorganisms and more production of mycotoxins (Hassanpour et al. 2019).

Kanapitsas et al. (2015) reported that the gamma irradiation (10 kGy) caused about 65% reduction in an initial aflatoxin B1 accumulation in raisin samples inoculated by *A. parasiticus*, compared to the control. Markov et al. (2015) also specified that gamma irradiation (10 kGy) of naturally contaminated maize samples caused a significant decrease in the aflatoxin B1 content. Higher doses (20–30 kGy) of gamma irradiation have also been used to decrease foodstuff aflatoxin contents. The results of various experiments indicate that doses of about 1 Mrd are sufficient to degrade aflatoxins in liquids, but higher doses are required in solid foods. Besides, the formation of other toxic substances due to the destruction of aflatoxin B1 by gamma rays has limited the use of this radiation.

Aflatoxins B1, B2, G1, and G2 are sensitive to gamma rays which change their structure. Radiation at low doses reduces the toxin while high doses stop the production of aflatoxins. According to experiments, the use of 100 kg of radiation after ten days prevents about 60% of fungal growth and spore production, but in doses of 200–400 kg of gamma rays, *Aspergillus flavus* was very little in the environment (Hassanpour et al. 2019). Milk and dairy products are the most important foods that are exposed to potential risks of aflatoxin M1. Hassanpour et al. (2019) used gamma radiation to decrease the toxin in pasteurized milk to a level lower than the European Commission Codex Alimentarius standard. They exposed pasteurized milk containing aflatoxin M1 to low-level gamma radiation and compared the results with control sample values. The results showed a decrease in aflatoxin in milk by different doses of low-level gamma radiation. According to the control sample, the results revealed that 51.5% of aflatoxin M1 was reduced in pasteurized milk after 4 days and 99% decrease after 8 days. The dose of low-level gamma radiation applied to milk was 0.39 mGy per day. They reported that this dose did not greatly impair the milk's sensory quality and chemical content but could improve its shelf life and provide healthier milk.

Jalili et al. (2012) investigated the effect of gamma radiation (cobalt-60) on the remaining disinfectant aflatoxin B1, B2, G1, and G2 in infected black pepper. The pepper samples' moisture content was set at 12% or 18% and the irradiated gamma ray (5 to 30 kGy) per day. Gamma radiation and moisture content showed significant effects in reducing aflatoxins. Maximum reductions of aflatoxins, at 18% moisture content and by 30 kGy, were 50.6%, 39.2%, 47.7%, and 42.9% for aflatoxin B1, B2, G1, and G2, respectively. Iqbal et al. (2013) investigated the impact of gamma radiation on aflatoxin B1 content in chili pepper samples. Applied on chili samples were 2, 4, and 6 kGy per day of gamma radiation. The results showed that 6 kGy had the highest effect on reducing aflatoxin B1 and total aflatoxins decreased by 1–2 log.

Electron Beam

Electron beam machines use linear accelerators and accelerate electron beams to very high speeds close to light speed. The main advantage of using such a radiation system is that it can be turned on and off like a bubble and has nothing to do with the nuclear industry. However, unlike gamma radiation from cobalt-60, these high-energy electron beams have limited penetration power and are therefore suitable for relatively thin foods (5–10 cm). They can be used to irradiate foods such as white meats and legumes. Electron beams can be converted to X-rays, in which case they have a higher penetration power than gamma rays from cobalt-60 and cesium-137 sources (Yang et al. 2019).

Electron beam irradiation has also recognized the capability for aflatoxin destruction in foodstuffs. Additionally, it has some advantages, including low equipment costs, short processing time, and dosage control. Reports showed that the aflatoxin B1's electron beam irradiation in an acetonitrile solution caused $C_{14}H_{12}O_5$ and $C_{17}H_{14}O_5$ formation (Khaneghah et al. 2020). In an aqueous solution, aflatoxin B1 elimination through the electron beam procedure caused the production of five by-products, with four of them losing the double bond in the terminal furan ring (Liu et al. 2016). Ames test and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide revealed a significant decrease in cytotoxicity mutagenicity of derived products after the electron beam procedure. However, the efficiency of the electron beam decontamination procedure becomes lower than either gamma irradiation. Assuncao et al. (2015) conveyed that treatment of different nuts with gamma irradiation (5–10 kGy) caused 70.6–84.2% reduction in the aflatoxin content, while their treatment with the same doses of electron beam procedure caused 53.3–65.7% reduction. Liu et al. (2018) reported that the electron beam procedure was not effective in aflatoxin degradation in peanuts. Detoxification of aflatoxin B1 by electron beam irradiation has been shown well to follow first-order reaction kinetics ($R^2 > 0.95$). Ames and cytotoxicity tests were used to determine the residual toxicity of aflatoxin B1 degrading products in aqueous solution, and the results showed that the mutagenicity and cytotoxicity of electron beam irradiation-treated samples were significantly reduced compared to untreated samples (Li et al. 2015; Yim et al. 2015).

Pulsed Light

Another nonthermal technology used for aflatoxin degradation is pulsed light. Pulsed light produces short, high-intensity flashes of broad-spectrum white light (100–1100 nm). Produced light's intensity is around 20,000 times greater than direct sunlight. The synergy between full spectra of UV, visible, and infrared light caused adequate destruction of microorganism's nucleic acid and cell wall and aflatoxin degradation in foods (Abuagela et al. 2018; Wang et al. 2016). Wang et al. (2016) used pulsed light (0.52 J cm^{-1} per pulse) to reduce the aflatoxin content of rough rice. They showed that rough rice treatment for 80 s decreased aflatoxins B1

and B2 to 75.0% and 39.2%, respectively, even though 15 s treatment decreased aflatoxins B1 and B2 by 90.3% and 86.7%, respectively.

Reports showed that treatment of foods with pulsed light caused inactivation of cytotoxicity and mutagenicity effects of aflatoxins B1 and B2 (Wang et al. 2016). Abuagela et al. (2019) conducted a study to investigate the combined effect of citric acid and pulsed light therapies on B-aflatoxins' inactivation. Aflatoxin levels (B_1 and B_2) in peanuts were measured after citric acid + pulsed light treatment and compared with control peanuts. The results showed that the total amount of aflatoxins decreased to 98.2 ± 1.03% from the combined treatment of citric acid + pulsed light, and besides, the results confirmed that the combined treatment of citric acid + pulsed light reduces aflatoxins B_1 and B_2 to 98.9 ± 0.8% and 98.1 ± 1.1%, respectively. Abuagela et al. (2018) reported a 91% decrease in aflatoxin contents of peanuts treated with pulsed light (0.4 J cm^{-1} per pulse). Reports showed that pulsed light treatments did not affect treated foods' chemical qualities, including fatty acid content and peroxide and acidity values. However, some changes have been reported in the color parameters of nuts treated with pulsed light. Additionally, the application of pulsed light to aflatoxin destruction is not economic in some cases. Moreau et al. (2013) demonstrated that the pulsed light could detoxify 92.7 ± 0.8% of aflatoxin B1 in the food system. Furthermore, they showed the same treatment for aflatoxin B1 had complete removal of these aflatoxins' mutagenic ability.

10.4.1.2 Nonionizing

Radio waves, microwave, infrared waves, and visible light in high intensity are nonionizing radiation. These irradiations can significantly increase the temperature of materials. Radio wave irradiation, also known as radiofrequency (RF), is a new treatment technique at a frequency range from 3 kHz to 300 MHz to decrease the aflatoxin contents of foodstuffs. RF technique has been established to be effective when applied for pre-storage sanitization of agricultural products. RF waves are usually absorbed by fats, water, and sugars in the food matrix and then converted straight into atomic motion heat, which causes some degradation in the aflatoxin content (Wang et al. 2015). Vearasilp et al. (2015) applied the RF to decrease aflatoxin B1 in *Perilla frutescens* L. highland oilseed. They described that *Aspergillus niger*, *Aspergillus flavus* loads, and aflatoxin B1 contents in seeds with an initial moisture content of 18% were highly decreased using RF treatment at 90 °C for 7 min. RF technology has some operative advantageous such as low cost, rapid heating, deep thermal penetration, and the possibility of better quality control (Guo et al. 2020). Despite the high importance of RF technology, scarce data are available about its application in aflatoxin destruction in foodstuffs.

10.4.2 Cold Plasma

Plasma is an extremely animated ionized gas that comprises ions, electrons, UV light, and reactive neutral species, including reactive oxygen and nitrogen species (ROS and RNS, respectively). Rendering the temperature, plasma is divided into cold (nonthermal) and thermal types. Cold plasma is mainly produced through electrical releases in gases at atmospheric pressure of 30–60 °C (Hertwig et al. 2018). Dielectric barrier discharge (DBD), radiofrequency plasma (RFP), corona discharge (CD), and atmospheric pressure plasma jets (APPJ) can also generate cold plasma. The cold plasma technique has a high ability to induce rapid aflatoxin detoxification in room conditions (Misra et al. 2019; Siciliano et al. 2016). DBD-derived nitrogen plasma was applied by Siciliano et al. (2016) to decrease the aflatoxin concentration of hazelnuts. The technique mentioned above caused 70% degradation in aflatoxin B1 during 1150 W plasma treatment for 12 min.

Reports showed that aflatoxins B1 and G1 were more sensitive to DBD-derived nitrogen plasma treatment (Siciliano et al. 2016). Plasma frequency and processing time affected aflatoxin degradation during the cold plasma procedure (Sakudo et al. 2017). Shi et al. (2017) evaluated the effects of air and modified atmosphere gas containing 65% O_2, 30% CO_2, and 5% N_2, as a carrier gas and relative humidity (RH) (5, 40, and 80%) in aflatoxin B1 decontamination reinforced by high voltage atmospheric cold plasma. The highest aflatoxin degradation was obtained in 40% RH modified atmosphere gas. Puligundla et al. (2020) showed that CD plasma jet treatment for 30 min caused 95, 56.6, and 45.7% reduction in aflatoxin B1 content of glass slides, rice, and wheat, respectively.

The aflatoxin degradation ability of the cold plasma technique is hugely dependent on the exposure time, type of plasma system, applied operating parameters such as moisture, carrier gas, and energy input, and type of foodstuffs (Hertwig et al. 2018). Furthermore, the negative influences of cold plasma procedure on the nutritional value and physicochemical and organoleptic characteristics of foodstuffs should be studied in further investigations (Hertwig et al. 2018). Devi et al. (2017) aimed to study the inactivation of *A. fungus*, *A. parasiticus*, and aflatoxins B1, B2, G1, and G2 in peanuts using cold plasma. Contaminated peanuts with *A. flavus* and *A. parasiticus* were treated by cold plasma for 60 W, 12 min, and 40 W, 15 min. To facilitate spore germination, the moisture content of plasma-treated samples was increased to more than 10%. Control and untreated samples were incubated for 5 days before aflatoxin analysis at 30 °C. The total number of bacteria in plasma-treated peanuts decreased sharply compared to untreated samples. With increasing power consumption and treatment time, inactivation efficiency increases. The 99.9 and 99.5% reductions were obtained for total counts of yeast and mold at 60 W and 15 min of plasma treatment, respectively. On the other hand, in air plasma, oxygen-based radicals provide an aggressive oxidative environment in which spore-covering proteins can be destroyed. As the coating is lost, the spore nucleus is exposed to radicals produced in the plasma.

Another mechanism that causes fatal damage in spores can be the accumulation of charged particles on the surface of the spores and the constant bombardment of free radicals on the spores' surface, which causes the cell wall to rupture. Relative humidity can also play an essential role in microbial inactivation. Water molecules are converted to OH radicals by plasma. These radicals have a high oxidation ability and increase inactivation efficiency. In a series of reactions, the decomposition of water leads to the formation of H_2O_2 molecules, which has a synergistic effect on inactivation.

Air plasma inactivation efficiency is more affected by the applied power and time. Air plasma is the source of reactive oxygen and reactive nitrogen species, resulting in greater inactivation efficiency by these species' presence. The toxicity, carcinogenicity, and mutagenic potential of aflatoxins are classified in B1 > G1 > B2 > G2. Aflatoxin B1 can be metabolized by cellular enzymes and is thought to be associated with harmful and carcinogenic effects. Aflatoxin synthesize in plasma-treated and untreated samples was determined. At 40 W for 15 min and 60 W for 12 min, over 70% and a 90% decrease in aflatoxin B1 concentration were detected. The time required to degrade aflatoxins using cold plasma is significantly less than that of UV and gamma rays. Plasma has the ability to degrade mycotoxins that can be used effectively in the food industry (Gavahian and Khaneghah 2020; Liao et al. 2019).

10.4.3 Electrolyzed Water

Electrolyzed water (EW) is mostly created during the passage across an electrolytic chamber of the dilute salt solution (~ 1% NaCl), in which the cathode and anode are segregated using a membrane. Two distinct types of EW including neutral electrolyzed water (NEW, with an oxidation-reduction potential (ORP) of 800–900 mV, pH of 5.0–6.5, and high dissolved oxygen (DO)) and acidic electrolyzed water (AEW ORP >1000 mV, pH < 3.0, and high DO) are mainly applied in the food industry. EW has revealed talented capability for aflatoxin detoxification in foods and agricultural products (Pankaj et al. 2018). The special issue presented and the current experimental use of innovative mitigation strategies to control mycotoxins in field settings have been reported. Gómez-Espinosa et al. (2017) used NEW to prevent aflatoxin production in turkey poultry. They reported that serum biochemical composition changes, enzyme activity, relative organ weights, and morphological changes associated with aflatoxins were all reduced using NEW decontamination (Gómez-Espinosa et al. 2017). Zhang et al. (2012) reported an 85% decrease in aflatoxin B1 content of peanuts after 15 min soaking in AEW without any harmful effects on peanuts' nutritional and organoleptic characteristics.

Reports showed that the high content of available chlorine concentration (ACC) in EW was the main factor accountable for the degradation of aflatoxin B1. Jardon-Xicotencatl et al. (2015) stated that soaking of corn in NEW for 15 min did not decrease the aflatoxin content. Nevertheless, genotoxicity and cytotoxicity of

aflatoxins were significantly decreased after NEW soaking (Gómez-Espinosa et al. 2017). Escobedo-González et al. (2016) demonstrated that the addition of –Cl and –OH groups to the aflatoxin B1 C8 and C9 positions was the primary way that caused aflatoxin detoxification. Economic basis, high efficiency, and the wide range of applicability are the main advantages of the EW-induced aflatoxin decontamination technique (Rahman et al. 2016).

10.4.4 Ozonation

Ozone is a specific oxidizing gas with 2.07 V redox potential capacity with high detoxifying abilities toward mycotoxins in foods (Pandiselvam et al. 2019). UV irradiation, water electrolysis, and oxygen electrical discharge are standard techniques used for ozone production. Ozone is generally recognized as a safe component that caused aflatoxin degradation without the formation of dangerous derived residues. Ozone-induced aflatoxin degradation was tested on different foodstuffs, particularly wheat, corn, peanuts, and pepper. A significant decrease in the aflatoxin nephrotoxicity and hepatotoxicity was observed in ozone-induced treatments (Diao et al. 2013).

Aflatoxin destruction induced by ozone mainly occurs through an electrophilic attack on the double bond in difuran ring mediety, which caused primary ozonide formation, followed by reorganization into molozonide by-products, including ketones, aldehydes, and organic acids (Fig. 10.3) (Jalili 2016). Food moisture content, ozone concentration, and exposure time are the most critical factors that affected the ozone-induced aflatoxin destruction. High moisture content is an inhibitory factor for ozone-induced aflatoxin destruction. Some reports showed that ozone changed the fatty acid profile and protein contents of treated foods (Brodowska et al. 2018; Ianni et al. 2019). Thus, it is essential to assess all aspects of the ozonation procedure on a laboratory scale and in the commercial application of this technology in diverse kinds of foods.

10.4.5 Pulsed Electric Field

Pulsed electric field (PEF) is one of the nonthermal processing techniques used for mycotoxins destruction in foodstuffs. PEF uses short electricity pulses for microbial inactivation with the lowest harmful effect on food quality. Otherwise, PEF technology includes using electrical treatments of diverse electric field strength (1–40 kV/cm) for short periods to a product placed amid two electrodes (Pallares et al. 2020). The technique is mainly used for mycotoxin decontamination in diverse foods, particularly fruit juices, vegetables, liquid eggs, and milk (Misra et al. 2018).

The possible application of PEF on liquid foods has been well ascertained, wherein its application on solid matrix has become the current area of research

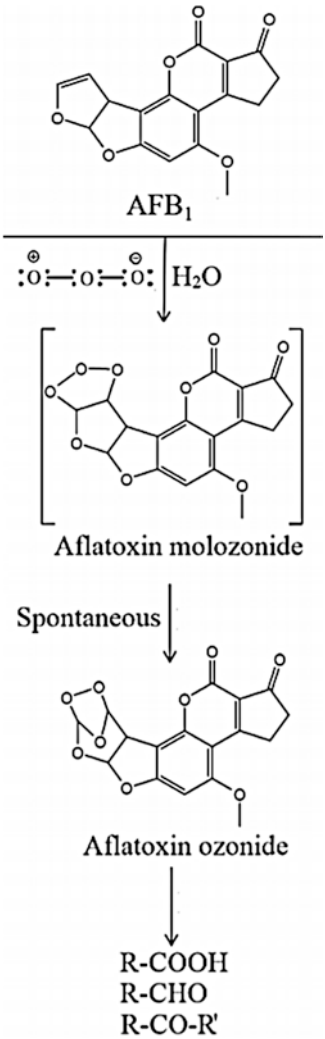


Fig. 10.3 Ozone-induced degradation mechanisms of aflatoxin B_1

(Gabrić et al. 2018). Nevertheless, little research is accessible up to now about the effects of PEF on aflatoxin contents of foods (Subramanian et al. 2017; Vijayalakshmi et al. 2018). Bulut et al. (2020) reported that PEF (0.97–17.28 J energy) caused the uppermost decreases of peroxide value and acidity number of 67.4 and 85.7%, respectively, and did not change color parameters of a treated sesame seed. Additionally, a 60% decrease of *Aspergillus parasiticus* counts occurred at the maximum PEF energy. Furthermore, concentrations of aflatoxins B1, B2, G1, and G2 reduced by 86.9, 98.7%, and 94.7, 92.7%, respectively. Vijayalakshmi et al. (2018) focused on analyzing the effect of the PEF process on reducing toxin content. Process parameters of different pH model system (potato dextrose agar) artificially spelt with aflatoxin mixture standard were optimized using the response surface

methodology. Optimization of PEF process affects aflatoxin B1 response and total reduction of aflatoxins by pH (4–10), pulse width (10–26), and output voltage (20–65%). The numerical optimization performed showed that the predicted and actual values are the same, proving the installed models' adequacy. They also demonstrate the potential use of PEF in reducing toxins.

Khoori et al. (2020) applied the PEF methods to total removal of aflatoxins and aflatoxin M1 in probiotic milk was investigated. The results showed that there is a significant and synergistic effect on all independent variables in reducing the values of aflatoxin M1 and total aflatoxins in acidophilus milk. The optimized parameter was 13.15 microseconds for pulse duration. Dairy products are an important part of people's daily diet. Aflatoxin M1 is derived from the 4-hydroxy acids aflatoxin B1 and aflatoxin B1 and milk-infected animals. This study investigated the use of hurdle (UV and PEF) technologies to reduce aflatoxins in milk containing probiotic bacteria. The results showed a significant and synergistic effect on all independent variables in reducing aflatoxin M1 values and total aflatoxins which is present in acidophilus milk, which also reduces the viability of bacteria. However, further research should be performed to obtain more information about the effects of PEF technology on aflatoxin content and nutritional, sensory, and physicochemical properties of targeted foods.

10.5 Conclusion

Aflatoxin decontamination remains an important challenge of the food industry. Physical methods of aflatoxin degradation, including adsorption, thermal process, and nonthermal methods, have been assessed in this chapter. Among them, more novel techniques, such as electron beam, UV, microwave heating, gamma irradiation, pulsed light, cold plasma, and electrolyzed water, can efficiently cause aflatoxin decontamination and degradation in some kinds of foodstuffs. These methods presented substantial promise for future application. However, aflatoxin decontamination efficiency relies on several factors, including food conditions (pH and moisture contents and food constituents), their application's accuracy, and decontamination circumstances. Additionally, different methods should assess the food matrix, food nutritional values, and human health. Moreover, it is essential to ensure no residue be left or new contaminants are produced in the aflatoxin degradation and decontamination procedure. The authors of the present chapter recommended combining these technologies to improve decontamination efficiency and overcome some specific methods' limitations. However, further research should address to obtain comprehensive findings on this area.

Acknowledgments Amin Mousavi Khaneghah would like to thank the support of Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Grant #2018/15432-7).

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Chapter 11
Chemical Degradation of Aflatoxins

Otniel Freitas-Silva, Caroline Corrêa de Souza Coelho, Felipe Machado Trombete, Renata Regina Pereira da Conceição, and Regiane Ribeiro-Santos

Abstract The removal of toxigenic fungi species from the genus *Aspergillus* and their highly toxic aflatoxins from contaminated foodstuffs is a difficult task, in view of their high resistance to heat treatments and solubility only in intermediate polar solvents. In this regard, chemical methods have been proposed for decontamination of aflatoxins (AFs) in foodstuffs, including ozonation, application of organic acids, hydrogen peroxide, and plant extracts, among other compounds. However, the production and use of chemical compounds are subjected to strong legislative pressure. This chapter presents the state of the art on the control of mycotoxins regarding the application of chemicals for the reduction of fungi and degradation of AFs, as well as corresponding regulatory and food safety issues. One of the most relevant chemical methods is the ozonated water which, in addition to being effective in AF detoxification, also eliminates pathogenic agents and microorganisms, contributing to food safety. However, the choice of the chemical product, concentration, time, and way of application generally has a large impact on the sensory attributes and nutri-

O. Freitas-Silva (✉)
Embrapa Food Agroindustry, Brazilian Agricultural Research Corporation, Rio de Janeiro, Brazil
e-mail: otniel.freitas@embrapa.br

C. C. de Souza Coelho
Food and Nutrition Graduate Program—Federal University of State of Rio de Janeiro, UNIRIO, Rio de Janeiro, Brazil
e-mail: caroline.coelho@unirio.br

F. M. Trombete
Department of Food Engineering, Federal University of São João del-Rei, Sete Lagoas, MG, Brazil
e-mail: trombete@ufsj.edu.br

R. R. P. da Conceição
Federal University of Minas Gerais, Institute of Biological Sciences, Avenida Antônio Carlos, Belo Horizonte, MG, Brazil

R. Ribeiro-Santos
Federal Institute of Education, Science and Technology of Pernambuco—IFPE, Vitória de Santo Antão, PE, Brazil

K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_11

tional values of the treated foods. In addition, residues from chemical used for aflatoxin decontamination can cause direct damage to human and animal health or induce negative effects through interaction with other nutrients. Further studies are necessary to better understand the mechanisms of detoxification by chemical compounds aiming at their application on an industrial scale.

Keywords Chemical detoxification · Aflatoxins · Ozone · Ammonization · Organic acids · Plant extracts

11.1 Introduction

The exposure to chemical compounds is a key regulatory aspect in the food area, due to the potential harmful effects to humans and the environment (Crawford et al. 2017). To meet these requirements, researchers are studying chemical treatments that are safe and nonaggressive to the environment and foods, in particular the degradation of aflatoxins (AFs) and reduction of the presence of aflatoxigenic fungi (Bhatnagar-Mathur et al. 2015).

The widely studied chemical methods in this respect are ozone, acid, and alkaline treatment and application of hydrogen peroxide, chlorine dioxide, and plant extracts and their phytochemicals (Afsah-Hejri et al. 2020; Brodowska et al. 2018; Dickson 2019; Jubeen et al. 2020; Makhuvele et al. 2020). Nevertheless, there is still an absence of knowledge concerning the advantages and limitations of respective technology when applied to food processing. The efficiency of the processes my vary according to combinations of food matrix, type of contaminant, nature of the process, and the target fresh characteristics of the product (Freitas-Silva and Souza 2016).

The removal of AFs from contaminated foodstuffs is a difficult task, in view of the their high resistance to heat treatments and solubility only in intermediate polar solvents (Gibellato et al. 2021; Karlovsky et al. 2016). There is also a great concern since the chemical control methods can generate some undesirable by-products, due to the breakdown of the target molecules. These can also be toxic, also causing undesirable changes in food characteristics, such as sensory properties and nutrient levels (Afsah-Hejri et al. 2020; Bhatnagar-Mathur et al. 2015; Karlovsky et al. 2016).

This chapter provides a multidisciplinary overview of the state of the art of the chemicals potentially used for the degradation of fungi and their AFs and the corresponding regulatory and food safety issues.

11.2 Toxicity and Safety Aspects

AFs are highly toxic secondary metabolites that can cause various diseases in humans and animals. In addition to toxicity, the stability of these mycotoxins in different conditions poses health risks and significant financial losses (Peng et al.

2018). They are reportedly the most potent mycotoxins in terms of carcinogenic, mutagenic, teratogenic, hepatotoxic, nephrotoxic, and immunosuppressive effects (Haque et al. 2020; Ji et al. 2016).

Aflatoxicosis can be classified as acute, when there is a quick and evident toxic response, or chronic, involving exposure to food contaminated with low doses for a long period, with irreversible effects, being considered the most worrying (Conte et al. 2020). AFs have strong acute toxicological and chronic hepatocarcinogenic effects on the liver, an organ considered highly susceptible to the effects of this mycotoxin (Conte et al. 2020; Ji et al. 2016). Several reports describe chronic aflatoxicosis with cellular hepatocarcinoma (HCC) and acute toxicity, with abdominal pain, vomiting, edema, and death (Haque et al. 2020). Figure 11.1 shows the AF occurrence and contamination in the human and livestock food chain and its effect on human and animal health (Table 11.1).

Because AFs are highly toxic and have carcinogenic effects, there is a concern about the control and/or elimination of mycotoxin-producing fungi, as well as AFs

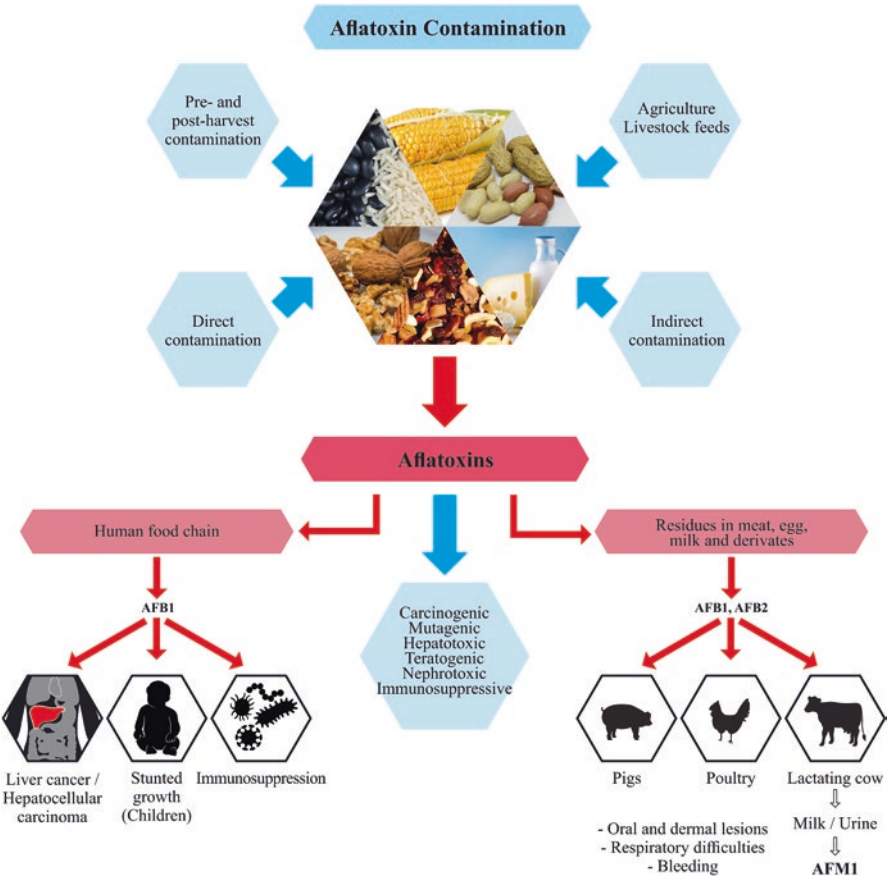

Fig. 11.1 AFs' effects on humans and animals. Adapted from Haque et al. (2020)

Table 11.1 Toxic effects of AFs in humans and animals

Exposed species	AF type	Foodstuff	Clinical-pathological effects	References
Bullfrogs	Total AF	Feed mixed with rice bran	Severe and progressive liver lesions with structural collapse, increased hepatocyte, and bile duct cell proliferation, the appearance of basophilic hepatocytes, and diffuse scarring	Grassi et al. (2007)
Chickens and turkeys	AFB1	Contaminated feed	Reduced feed intake, lower weight gain, decreased efficiency of nutrient usage	Pandey and Chauhan (2007)
Chinchillas	Total AF	Contaminated feed	Hepatic enlargement with pale-yellowish coloration, diffuse cytoplasmic vacuolation, the appearance of cytoplasmic vacuoles in the hepatocytes, and death	González Pereyra et al. (2008)
Rabbits	AFB1	Corn	Suppressed immunity, oxidative stress, and hepatic And renal ailment, oligospermia, teratospermia, and asthenozoospermia	Hanafi et al. (2010)
Humans (children)	Total AF	Eggs, milk	Malnutrition, liver cancer associated with HBV	Tchana et al. (2010)
Dogs			Icterus, gastro-enterorrhagia, hepatosis, fatty hepatosis, and bile duct proliferation	Arnot et al. (2012)
Calves	Total AF	Diet consisting of alfalfa hay and Sunflower cake	General unthriftiness, diarrhea, hydrothorax, lymphopenia, monocytosis, megalocytosis, hepatic congestion with necrosis and mortality	Kaleibar and Helan (2013)
Buffaloes	AFB1	Contaminated feed	Reduction of average daily feed intake and hematological parameters, along with elevation of serum biochemical parameters	Akhtar et al. (2014)
Poultry	AFB1	Contaminated feed	Immune tissue atrophy, reduced weights of the bursa, spleen, and thymus, histopathological lesions	Chen et al. (2014a)
Buffaloes	AFB1	Contaminated cottonseed cake	Compromised metabolism and increased protein levels in affected animals	Aslam et al. (2014)
Cattle	AFB1	Corn rich forage	Anorexia, depression, photosensitization, diarrhea, hemorrhages of viscera, blood exudation from natural orifices, prolapse, and death	Umar et al. (2015)
Camels	AFB1	_	Pathological changes in the liver, fatty degeneration with variable areas of petechial hemorrhages, congestion, fibrosis, and large whitish focus of necrosis	Al-Hizab et al. (2015)

(continued)

Table 11.1 (continued)

Exposed species	AF type	Foodstuff	Clinical-pathological effects	References
Pigs	Total AF	Corn	Fever, weight loss, tachycardia, tachypnea, lethargy, muscle tremors, muscle weakness, diarrhea, ascites, hydropericardium syndrome, petechial hemorrhages in the mesentery, subcutaneous edema, and mesocolon	Gomes Olinda et al. (2016)
Cattle cells	AFB1	Contaminated feed	Affected the cell transcriptome, the majority of significant genes being associated with cancer, cell damage and apoptosis, inflammation, bioactivation, and detoxification pathways	Pauletto et al. (2020)

*Adapted from Iqbal et al. (2019)

in food and animal feed (Velazhahan et al. 2010). Prevention is the main control alternative, but different methods can be used for AF decontamination/detoxification, such as the physical, chemical, and biological methods (Haque et al. 2020; Kumar 2018). These methods have been developed so that the process does not reduce the nutritional value, quality, and palatability of the food, while removing/destroying the spores and mycelia of *Aspergillus* sp. to avoid new contamination through the production of new mycotoxins, in addition to not producing new toxic substances (Kumar 2018). However, physical and chemical methods can result in loss of nutritional parameters and higher biosafety risk, without considering the high cost of equipment. Hence, the search for biological methods that can decontaminate/detoxify products is increasing (Gao et al. 2011).

Several chemical agents can be used for AF decontamination of human and animal food, such as oxidizing agents (H_2O_2 and O_3), bases (ammonia and hydrated oxide), organic acids (citric acid, lactic acid, propionic acid), among others (Grenier et al. 2014; Haque et al. 2020). These decontamination agents convert mycotoxins into less toxic compounds, but they can also impair food quality, have negative environmental impacts, and leave residues in food products. Furthermore, they can convert the structure of mycotoxins into other compounds with unknown structures, with potentially deleterious effects on human and animal health (Afsah-Hejri et al. 2020; Haque et al. 2020a; Mir et al. 2021).

The removal of AFs in food and feed is complex. However, the risk of exposure to these mycotoxins can be mitigated by establishing tolerated limits and monitoring products (Giovati et al. 2015).

The implementation of regulatory and monitoring measures is necessary because of potential effects of mycotoxins on human and animal health. AFs are involved in the development of liver cancer, and maximum tolerable limits in food have been established to assure safety (Marroquín-Cardona et al. 2014). Regulations on AFs exist in many countries, mainly regarding AFB1, AFB2, AFG1, AFG2, and AFM1, with their maximum tolerated limits established based on their varying toxicities (ANVISA 2011; European Commission 2007).

11.3 Use of Chemical Methods for Microbial Inactivation

Aspergillus spp. can grow on virtually any organic substance, where they are able to produce several metabolites commonly reported in agricultural products (Roohi et al. 2020). *Aspergillus* spp. decrease the nutritional values of foods, besides changing their appearance, leading to economic losses (Dronavalli and Kang 2019). There are more than 18 different AFs, of which AFB1, AFB2, AFG1, and AFG2 are considered carcinogens by the International Agency for Research on Cancer (IARC 2012).

The biosynthesis of AF is optimized at moderately high temperatures. Consequently, high contamination rates are found in regions with warm and humid climates. Expansion of *A. flavus* and AFs contamination of crops due to global warming has been projected by recent studies. According to Medina et al. (2017), mycotoxins are considered one of the most important food safety hazards affected by climate change. They pointed out that changes in temperature, precipitation, and atmospheric CO_2 concentration are expected to cause an increased risk of mycotoxigenic fungal contamination of cereal crops.

Battilani et al. (2016) also predicted the same scenario and reported that within the next 100 years, under a +2 °C scenario, AFB1 will become a food safety issue in maize in Eastern Europe, the Balkan Peninsula, and Mediterranean regions. This is explained because *A. flavus* grows well in warm and dry weather and can spread easily from southern Europe and other regions. Bailly et al. (2018) reported for the first time the contamination of French maize kernels with an AF associated with *A. flavus*, along with the presence of other species like *A. parasiticus*.

Considering this scenario of increased contamination of cereals and other raw materials by AFs, several chemical methods have been studied and aimed at the removal of mycotoxigenic fungi from food surfaces, consequently preventing the formation of mycotoxins in food. Ferreira et al. (2020) studied the effect of ozonation on *A. flavus* contamination in Brazil nuts, using 13.0 mg/L, at 25 °C for 120 min. The *A. flavus* count was reduced by 1.25 and 1.28 log cycles at pH levels of 7.1 and 3.0, respectively. The ozone treatment was efficient to control *A. flavus* in Brazil nuts and can be applied for surface decontamination of different materials, such as corn grits (Porto et al. 2019), wheat grains (Trombete et al. 2017), coffee beans (Akbar et al. 2020), and many others, as reviewed by Wen et al. (2020).

Natural antimicrobials in foodstuffs have gained importance due to their safety perceived by consumers. Nazareth et al. (2019) evaluated the efficacy of allyl isothiocyanate (AITC) in preventing the growth of *Penicillium verrucosum* and the consequent production of ochratoxin A (OTA) in barley during storage for 90 d. Isothiocyanates are aliphatic and aromatic compounds found in cruciferous plants belonging to the families *Brassicaceae*, *Capparaceae*, and *Caricaceae*. The authors reported that the population of *P. verrucosum* was significantly reduced after 24 h of AITC exposure. After 90 d, the untreated control group reached a fungal population of 8.3 log CFU/g while the treated samples showed no fungal presence.

Lopes et al. (2018) also demonstrated the effects of isothiocyanate to inhibit the growth of *Aspergillus parasiticus* on Brazil nuts inoculated with 10^4 spores/g under controlled relative humidity (RH = 95 or 85%). Samples treated with up to 2.5 μL/L of gaseous AITC reduced the fungal population to undetectable levels after 30 days' storage. Lorini et al. (2018) studied the antifungal effect of different types of propolis from the stingless bee *Scaptotrigona polysticta* and two types produced by *Apis mellifera* (red and brown) against *Aspergillus flavus*. No extract was effective in inhibiting mycelial growth and sporulation of *A. flavus*, but the red extract inhibited spore germination. The red propolis had the highest contents of total phenolics and total flavonoids (5.38 and 2.77 g/100 g), which can be associated with the inhibition of spore germination.

Loi et al. (2020) reviewed different strategies applied to reduce fungal contamination and AF production in human and animal food. Among them, the use of natural plant-derived compounds was considered a promising strategy to control *Aspergillus* spoilage in integrated pre- and postharvest management. The antifungal effects are related to the high levels of phenols, aldehydes, and terpenes extracted from medicinal plants, spices, and fruits, which have been classified as generally recognized as safe (GRAS). The authors stated that the application of plant extracts in pre- and postharvest management could be a potential strategy to control aflatoxigenic fungi and prevent AF contamination.

11.4 The Use of Chemical Methods for AF Degradation

AF contamination is a global concern in the stages of production of crops and distribution and consumption of processed foods due to its toxic, mutagenic, and carcinogenic properties (Fouché et al. 2020). AF can occur in human and animal food products before and after harvesting (Nazhand et al. 2020), and several technologies have been studied to eliminate or reduce these contaminations.

Since their discovery in the 1960s, AFs have been found to have a considerable impact on the health of humans and animals, and over the years various procedures have been employed around the world to minimize their contamination of crops and exposure of humans and animals (Tumukunde et al. 2020). Among these, numerous chemical methods have been investigated for decontaminating food products containing AFs, but there is still no single method having the necessary efficiency and safety without changing other characteristics of the food.

Hence, further research is required to develop effective methods for degrading AFs. AF detoxification can occur through the degradation of its structure, and many chemical methods can be used, such as ozone (O_3) and hydrogen peroxide (H_2O_2), which oxidize the molecule, or aldehydes, bases, or acids that can open the lactone rings of AFs, forming a water-soluble compound called beta-keto acid (Nazhand et al. 2020). The treated food must be free of the chemicals used, and the nutritional value should not be altered (Makhuvele et al. 2020). These are important limiting factors for the use of acids or alkalis in contaminated foods.

For this reason, methods such as the use of reducing agents like sodium bisulfite and ammonia have been studied and applied for food mycotoxin detoxification. Sodium bisulfite has the affinity to react with AFs and the mechanisms of action include the formation of sulfonate derivatives, while peroxide and heat enhance the destruction of AFs (Colović et al. 2019).

Ammonization was regarded as the most economically practicable technique to detoxify AFs in foodstuffs in the 1990s, and many countries approved this process for AF-contaminated crops. There is evidence indicating that typical signs of aflatoxicosis were significantly reduced or eliminated after replacing AF-contaminated feed with ammonia-treated feed (Peng et al. 2018). As reviewed by Makhuvele et al. (2020), treatment of cereals such as corn with ammonia gas can be used to reduce the contamination by AF. However, the authors also highlighted that this method could impair food quality and cause deterioration due to excessive ammonia levels.

Regardless the decontamination technique applied, the degradation of AFs can be modeled as a reaction with zero-, first-, or second-order kinetics, and based on the order of reaction, which determines the number of molecules participating in a reaction, the rate of AF degradation can be determined, as mentioned by Roohi et al. (2020). AFs are highly stable chemical compounds with decomposition temperatures around 237–306 °C, so they are not destroyed by regular thermal processing or cooking (Marshall et al. 2020).

11.4.1 Ozone

Ozone gas (O_3) was discovered by Swiss chemist Christian Friedrich Schönbein in the middle of the nineteenth century. In 1848, Hunt concluded that ozone was the allotropic form of oxygen, and a decade later its O_3 triatomic composition was identified (Silva et al. 2011). At room temperature and in low concentrations, it appears as a colorless gas; in high concentrations, it acquires a bluish color (Porto et al. 2019; Freitas-Silva et al. 2013). Ozone gas has a penetrating odor and is easily detectable at very low concentrations (0.01–0.05 ppm) (Coelho et al. 2015).

Ozone was used, for the first time as a food preservative in 1909, in cold meat storage chambers. However, at that time its use as a disinfectant did not reach greater proportions in the food industry due mainly to its cost concerning other substances, such as chlorine (Chiattone et al. 2008). Only in 1982 O_3 was declared as a GRAS substance by the FDA (Food and Drug Administration) with the use permitted only as a sanitizer for bottled water. Some years later its use was extended to food (Freitas-Silva and Venâncio 2010). Since then, there has been a growing interest in the application of ozone in food processing, and the use of its gaseous form in sanitization has emerged as an alternative to traditional chlorine-based treatment due to its effectiveness in low concentrations, short contact time, and decomposition into nontoxic products (Freitas-Silva and Souza 2016).

Compared with other oxidizing agents, ozone stands out for being the sanitizer with a high oxidation potential that can come into contact with food (2.07 mV).

Ozone is the second most powerful oxidizing agent second only to fluoride (3.06 mV) (Coelho et al. 2015).

O_3 is frequently applied for disinfection of fruits, meats, and poultry, as well as a sterilizing agent in plant-based foods and milk products. Other applications of O_3 include the surfaces of silos, storage chambers, and tanks aiming at increased preservation of fruits, grains, cheeses, poultry products, and meats. The direct application of O_3 during the storage, due to its high oxidation power, keeps the environment clean and sterile to ensure hygiene, as well as improving the color, visual appearance, and smell of products. In this way, O_3 gives a high capacity for disinfection and sterilization, allowing the sanitizing action to occur in less time of contact and concentration. This generally increases the storage time of products and processed foods (Brodowska et al. 2018; Silva et al. 2011).

Several studies have reported the use of O_3 as a microbicide for the decontamination of surfaces and indoor environments along with food preservation and water treatment. Because the oxygen is the final product of O_3 decomposition, there is no residue formed after the decontamination process.

There are also evidence that O_3 is a strong agent for degradation of mycotoxins (Freitas-Silva and Souza 2016; Freitas-Silva and Venâncio 2010; Trombete et al. 2016a, b). On the other hand, O_3 can also cause undesirable effects in the food matrix, such as oxidative degradation of chemical constituents, resulting in loss of color, flavor changes and other modifications in the food quality (Pandiselvam et al. 2018). Therefore, the use of O_3 in the food industry has required extensive research to improve existing techniques or develop new approaches to effectively improve the quality of food while preventing changes in sensory properties of food products.

11.4.2 Acid and Alkaline Treatment

Several organic and inorganic acids are used in food processing, some of which can reduce the AF levels of contaminated food. Some acids, such as lactic, citric, tartaric, propionic, and hydrochloric, are more effective at degrading AFs than others, such as succinic, acetic, ascorbic, and formic, which are only marginally successful, as reviewed by Sipos et al. (2021). Jalili et al. (2011) studied the effects of 18 different acids and alkalis on the levels of AF detoxification of black and white pepper, resulting in 18–51% efficacy using chloride and phosphoric acids, as well as sodium, potassium, or calcium hydroxide, and certain sodium salts (bicarbonate, bisulfite, hydrosulfite, chloride, and sulfate).

Jubeen et al. (2020) studied the effects of three organic acids in aqueous solutions with 1–9% concentration aiming to detoxify AFs in almonds, peanuts, pistachios, and walnuts. Citric and lactic acids caused conversion of AFB1 into a less toxic product, identified as AFD1, via hydrolysis of the lactone ring. The authors reported that solutions containing 9% citric, lactic, or propionic acid during 15 min promoted AF reductions higher than 96%.

Bordin et al. (2014) reported the relevance of AFs in edible oils and the effects of the alkaline solution commonly used to process crude oils on the reduction of AF levels in peanut, corn, soybean, groundnut, and sunflower oils. Mahoney & Molyneux (2010) reported that refined vegetable oils are free from AFs, but Shephard et al. (2012) showed that in unpurified or crude vegetable oils, AFs can be found.

AFB1 in peanut oil seriously threatens the health of consumers, but as demonstrated by Ji et al. (2016), mixing and centrifugation with an alkaline solution transfer the mycotoxins to the sediment phase, indicating that alkali refining is an effective method for removing AFB1 in peanut oil. AFB1 is often detected in peanut oil. Ji et al. (2016) studied an alkali refining method to mitigate this contamination. The results showed that AFB1 declined by 98.94% after the oil was refined with NaOH under the optimum detoxifying conditions (23.4% NaOH solution, excess alkali equal to 0.30%, and final temperature of 77 °C). Although the initial and final temperatures of alkali refining were not significant, the concentration of NaOH solution markedly affected the efficiency of AFB detoxification in peanut oil.

11.4.3 Hydrogen Peroxide

Treatment with hydrogen peroxide (H_2O_2) is considered to have potential detoxifying AFs due to its oxidation ability when combined with other detoxification methods (Shen and Singh 2021). Depending on the concentrations applied, H_2O_2 can have an impact on sensory attributes and nutritional values of treated foods. Jalili et al. (2011) reported that 20 g/L of H_2O_2 effectively reduced AFB1 by 44% in black pepper at room temperature after 2 h. However, the addition of hydrogen peroxide bleached the color of the white pepper and damaged the skin of the black pepper.

Dickson (2019) investigated the use of food-grade H_2O_2 to detoxify AFB1 in groundnuts and peanut butter. The effect of different concentrations (0.5–2.5%) of H_2O_2 was investigated. The AFB1 removal efficiency increased with the increase of H_2O_2 concentration up to 1% and the removal was temperature-dependent. Also, the pH at 6.5 was best for AFB1 removal from peanut samples. The highest detoxification efficiency (99.32%) was found at 1% H_2O_2, and acidic pH was more suitable for this process.

Jalili et al. (2011) evaluated the effect of hydrogen peroxide and 17 other acidic, salt, and alkaline compounds on the reduction of AFB1, AFB2, AFG1, and AFG2 in black and white pepper. The results showed a significant reduction of all mycotoxins. The lowest and highest reductions using H_2O_2 were 32.3% and 43.8%, respectively, in black pepper, and 34.8% and 47.3%, respectively, in white pepper. According to Shen and Singh (2021), future studies applying H_2O_2 should focus on promoting the oxidation process while maintaining an acceptable quality of foods.

11.4.4 Chlorine Dioxide Gas

Yu et al. (2020) applied chlorine dioxide gas (ClO_2 gas) to detoxify AFB1 in corn for the first time and found a reduction of 90.0% of AFB1 levels. The authors described that structurally, the biological activity of AFB1 was removed due to the disappearance of the C8–C9 double bond in the furan ring and the modification of cyclopentanone and methoxy after the treatment. Also, the cell viability assay with human embryo hepatocytes confirmed the low toxicity of the degradation products.

The efficacy of ClO_2 for the elimination of AFB1 in artificially contaminated animal feed samples was also evaluated by (Fajri et al. 2017). The authors applied 200 mg/L of gaseous ClO_2 for 24 h and reported the reduction of AFB1 by 59.81%.

11.4.5 Plant Extracts and their Phytochemicals

Plant extracts and their phytochemicals have also been applied as antifungal agents for detoxification of AF contamination, as recently reviewed by Makhuvele et al. (2020). Negera and Washe (2019) demonstrated that in various spices evaluated, garlic caused a 61.7% reduction of AF in maize, followed by lemon at 60.6%.

Ponzilacqua et al. (2019) observed a time-dependent capacity of aqueous extracts of sweet passion fruit (*Passiflora alata*), araçá (*Psidium cattleianum*), rosemary (*Rosmarinus officinalis*), and oregano (*Origanum vulgare*) to degrade AFB1 in vitro. Rosemary extract had the highest percentage of AFB1 reduction, followed by oregano and araçá. Ginger (*Zingiber officinale*) essential oil was tested as an antifungal and antiaflatoxigenic agent for the treatment of stored maize grains (Nerilo et al. 2020). The main compounds in essential oil reported by the authors were α-zingiberene (23.85%) and geranial (14.16%). The results showed the essential oils inhibited AF production (AFB1 and AFB2) at concentrations of 25 and 50 μg/g and controlled the fungal growth of *Aspergillus flavus*.

11.4.6 Chemical Methods Applied for AFM1 Degradation

AFM_1 and AFM_2 are mycotoxins present in milk and derivatives and are formed from the metabolism of AFB_1 and AFB_2, respectively. The presence of AFM_1 in milk and milk products has been recognized as a problem for over 30 years. Thus, preventive measures to avoid fungal contamination of cattle feed are the best control method for AFM_1 and AFM_2 in milk. However, this is hard to avoid in some countries (Nguyen et al. 2020). Treating milk containing AFM_1 using chemical methods has also been studied, such as the use of hydrogen peroxide and O_3. These studies are limited since most of the techniques studied leave residues in the treated food, making it impossible to consume.

The effect of different concentrations of H_2O_2 (from 0.02 to 0.1%) with heat treatments in milk artificially contaminated with AFM1 was studied by Motawee et al. (2006). The authors observed that the increase of H_2O_2 led to an increase in AFM1 degradation. Complete degradation occurred by using sterilization at 121 °C for 5 min with 0.1% H_2O_2.

Mohammadi et al. (2017) observed that milk samples containing 0.56 μg/kg of AFM1 when exposed to gaseous O_3 (80 mg/min) for 5 min exhibited detoxification of around 50%. Also, the longest exposure time to O_3 was most efficient in reducing the AFM1 levels without causing oxidation in lipids or changes in the milk pH.

11.5 Effects of Chemical Methods on Human and Animal Foods

Contamination with AF, the strongest known natural carcinogenic compound, is found in cereals, nuts, and spices and is considered a major safety concern of food products for human and animal consumption (Jalili et al. 2011). The reduction in the risk of human and animal exposure to AF is directly associated with the need for degradation of this mycotoxin or its removal from food. However, although many studies have been carried out on decontamination of food products, by physical, chemical, or microbiological means (Gibellato et al. 2021; Ji et al. 2016; Nazhand et al. 2020; Shen and Singh 2021), little information is available on the effect of these treatments on product quality and health effects.

Since the methods used to mitigate AF in food products can cause damage to other food components, thus inducing an undesirable change in the quality of the treated foods, either visual damage or negative effect on the nutritional profile, it is recommended that the final products be subjected to quality analysis (de Oliveira et al. 2020). Thus, if the product undergoes a change in quality during processing, the technique used should not be considered suitable for use on foods for humans or animals (Marshall et al. 2020).

The possibility of removing mycotoxins by applying chemical compounds has been the subject of much discussion (Marimón et al. 2019; Pankaj et al. 2018). According to Jalili et al. (2011), AFs can react with different chemicals and be converted into nontoxic or less toxic and mutagenic compounds. These chemicals include bases (Méndez-Albores et al. 2013; Vidal et al. 2018), acids (Basaran 2011; Jubeen et al. 2020; Méndez-Albores et al. 2007), oxidizing agents (Ribeiro et al. 2020; Shen and Singh 2021), salts (Jalili et al. 2011), gases (Lee et al. 2020; Yu et al. 2020), O_3 (Ismail et al. 2018; Jubeen et al. 2020; de Oliveira et al. 2020; Porto et al. 2019; Trombete et al. 2016a, b, 2017), and plant extracts (Loi et al. 2020; Makhuvele et al. 2020).

Although numerous chemical control and detoxification methods have been developed to prevent contamination by AFs, some are not allowed by the US Food and Drug Administration (FDA) due to the potential toxicity of the reaction products

as well as losses in nutritional quality and palatability of products. Thus, it is essential to develop appropriate mitigation methods to guarantee safety for animal and human consumption (Haque et al. 2020). Table 11.2 summarizes the different chemical detoxifying agents of AFs, as well as their results and food quality parameters. In this topic, we discuss below the impact of applying the main chemical methods on food quality.

11.5.1 Ozonation

Ozonation is a chemical method that is effective in controlling fungal growth and mycotoxin contamination (Ismail et al. 2018; Jubeen et al. 2020; de Oliveira et al. 2020). Its potential to prevent the synthesis of AFs is directly related to its antimicrobial action. Its high oxidative potential makes it capable of oxidizing glycolipids, glycoproteins, and amino acids of the cell wall and membrane, in addition to other cell constituents (de Oliveira et al. 2020)

However, the concentration and time of application are factors that must be taken into account when applying O_3 to food products, since high doses and times can cause damage to the nutritional properties of the product. Some studies have demonstrated the application of a low O_3 concentration and short treatment time as ideal parameters to mitigate AF contamination and reduce the impact on the nutritional value of peanuts (Atakan and Caner 2021; Chen et al. 2014a, b). Thus, when applying ozonation to control mycotoxin contamination, it is important to consider the need for physical-chemical assessment of food for human and animal consumption.

Akbas and Ozdemir (2006) demonstrated the efficiency of gaseous O_3 for the degradation of AFs in pistachio grains, where exposure time of 420 min and the concentration of 9.0 mg/L provided caused the highest levels of degradation of AF total (24%) and AFB1 (23%), normally predominant and the most toxic among the AF forms. In ground pistachios, there was a reduction of only 5% in the level of AF total and AFB1 when ozonated under the same conditions. Considering these outcomes, the authors also evaluated the possible effects of different O_3 treatments on pistachio grains and observed there were no significant changes concerning the level of peroxide (often used as an indicator of the primary products of lipid oxidation), color, pH, and moisture. The authors also evaluated the sensorial quality attributes of pistachio samples treated with and without O_3. For this, ten experienced individuals analyzed the samples for sweetness, rancidity, overall taste, appearance, and general palatability. For consumers, the samples of ozonated and non-ozonated pistachio grains showed no difference regarding sweetness, rancidity, flavor, appearance, and general palatability scores. However, O_3 treatments with longer times and higher concentrations negatively affected the organoleptic properties of ground pistachio grains, possibly due to the larger surface area of the samples and consequently greater contact of the samples with the ozone (Akbas and Ozdemir 2006).

Table 11.2 Effect of different chemical agents to detoxify AFs on food quality parameters

Chemical methods	Food/feed type*	Conditions	Outcome	
			Decontamination	Quality parameters
Gaseous ozone	Pistachio kernels [a]	5.0, 7.0, and 9.0 mg/L O_3 concentrations for 140 and 420 min at 20 °C and 70% RH	AFB1 and AF total could be reduced by 23 and 24%, respectively, when ozonated at 9.0 mg/L O_3 concentration for 420 min	No significant changes occurred in pH, color, moisture content, free fatty acid values, and fatty acid compositions. No significant changes were observed in sweetness, rancidity, overall flavor, appearance, and overall palatability of ozonated and non-ozonated pistachio kernels
	Ground pistachios[a]	5.0, 7.0, and 9.0 mg/L O_3 concentrations for 140 and 420 min at 20 °C and 70% RH	AFB1 and AF total could be reduced by 5% when ozonated at 9.0 mg/L O_3 concentration for 420 min	Longer exposure times and concentrations of O_3 caused negative effects on the sensorial properties of ground pistachio grains, concerning sweetness, flavor, appearance, and general palatability
	Brazil nuts/oil[b]	2.42, 4.38, 8.88, and 13.24 mg/L for 0, 60, 120, 180, and 240 min at 25 °C	At a concentration of 8.88 mg/L for 240 min, the gas was able to reduce the count of *Aspergillus flavus* greater than 3.10 log cycles	Ozonation was not able to cause oxidation and to alter the lipid profile of the Brazil nuts oil
	Hazelnuts[c]	5, 10, and 20 ppm for 10 and 20 min	The highest degradation for AFB1 (38.96%) and AF total (31.35%) were obtained with the use of 20 ppm/20 min ozonation. In the 5 μg/kg contaminated group, the AFB1 reduction rate was 19.88% after 5 min and 32.23% after 20 min	Increases were noted in tocopherol and peroxides, especially with 20 ppm ozonation. The PV and tocopherol levels changed significantly, which led to the loss of nutritional quality of nuts during extended storage. A loss in the color of the nuts was more evident after 20 ppm/20 min

	Wheat grains[d,e]	10 to 60 mg/L, from 2 to 5 h of exposure, grain mass from 2 to 5 kg	The concentration and exposure time positively influenced the reductions of AFB1, AFB2, AFG1, and AFG2. The most significant reduction was obtained with the use of 60 mg/L of O_3 for 5 h, with samples weighing 2 kg	60 mg/L positively affected the tenacity and the drop number of flour. Alveography and farinography showed that the gluten content, chemical composition, mineral, and sensory profiles were not affected by ozonation. Gaseous ozonation, when applied in the conditions of 10 to 60 mg/L, from 2 to 5 h of exposure, grain mass of 2–5 kg, did not harm the wheat quality
Acidic treatment	Black and white pepper[f]	0.02 g/mL for 2 h. chemicals: Sulfuric acid, chloride acid, phosphoric acid, benzoic acid, citric acid, and acetic acid	All treatments reduced AF. The most effective acid was citric acid, which showed a detoxification effect of 28% for AFB1 in white pepper	The authors reported no damage to the surface of the white and black pepper
Alkaline treatment	Black and white pepper[f]	0.02 g/mL for 2 h. chemicals: Ammonia, ammonium acetate, sodium bicarbonate, sodium bisulfite, sodium hydrosulfite, sodium chloride, and sodium sulfate	Among the basic compounds, the greatest reduction was obtained with the treatment with sodium hydroxide, which ranged from 52.0 ± 2.4% for AFG1 in black pepper to 54.5 ± 2.7 for AFB1 in white pepper	Black or brown spots were observed on the surface of the grains of white and black pepper
	Cocoa liquor	250 °C for 15 min; alkalinization of cocoa (NaOH, ca $(OH)_2$ and KOH; concentrations: 10, 20, and 30 g/kg)	Significant effect on AF degradation. The three alkaline agents were equally effective, with 98% reduction, when the cocoa liquor was subjected to treatment with 30 g/kg	With the increase in the concentration of alkali, lower values of protein and crude fats were recorded
Hydrogen peroxide	Black and white pepper[f]	0.02 g/mL hydrogen peroxide concentration for 2 h	Reduced contamination from 32.3% ± 4.2% for AFB2 to 45.0% ± 3.3% for AFB1	There was an improvement in the color of the white pepper due to its whitening effect, but there was damage to the outer skin of the black pepper

*[a](Akbas and Ozdemir 2006); [b](de Oliveira et al. 2020); [c](Atakan and Caner 2021); [d](Trombete et al. 2017); [e](Trombete et al. 2016a, b); [f](Jalili et al. 2011); [g](Méndez-Albores et al. 2013)

In Brazil nuts, de Oliveira et al. (2020) evaluated the application of O_3 gas to preserve the nuts, its efficiency in mitigating contamination caused by *Aspergillus flavus*, and the possible effects on the color and quality of crude oil extracted from ozonated nuts. Regarding the effect of O_3 on *A. flavus*, the authors reported that the concentrations of 8.88 and 13.24 mg/L for 240 min were efficient in inactivating potentially aflatoxigenic microorganisms in the Brazil nuts. They also observed a greenish color in non-ozonated nuts, characteristic of the colonies of *A. flavus*, in contrast to nuts treated with a concentration of 13.24 mg/L, where the color of the fungal colonies was lighter. According to Shier et al. (2005), the color change in colonies of *A. flavus* is attributed to the oxidation of anthraquinone, an intermediate pigment in AF synthesis.

Due to the high oxidative potential of O_3, the quality of the oil extracted from the nuts was a fundamental aspect analyzed by the authors, since the Brazil nut is composed of approximately 66.0 g/100 g of lipids, of which unsaturated fatty acids predominate (Freitas and Naves 2010; Thomson 2011). When oxidized, unsaturated fatty acids are susceptible to changes in odor and taste, altering the sensorial characteristics of the Brazil nut oil. Under the adopted conditions, although O_3 had high oxidative potential, ozonation was unable to cause sufficient oxidation to alter the oil's lipid profile (de Oliveira et al. 2020).

11.5.2 Acid and Alkaline Treatment

Treatments with acidic and basic chemical compounds are widely used in the food industry (Basaran 2011; Sipos et al. 2021; Guo et al. 2021; Méndez-Albores et al. 2013; Vidal et al. 2018; Méndez-Albores et al. 2007; Basaran 2011; Jubeen et al. 2020).

In black and white pepper grains, the effect of different chemical treatments during the washing step was evaluated by (Jalili et al. 2011). They investigated 18 different chemicals, including acidic compounds (sulfuric acid, hydrochloric acid, phosphoric acid, benzoic acid, citric acid, acetic acid) and alkaline compounds (ammonia, sodium bicarbonate, sodium hydroxide, potassium hydroxide, calcium hydroxide), as a potential strategy to eliminate AFB1, AFB2, AFG1, and AFG2. The chemicals were diluted to a concentration of 2%, and the grains were immersed for 2 h, washed to neutral pH, and dried. All treatments were effective for AF reduction. However, basic substances showed better results than acidic substances. Among the basic compounds, the greatest reduction was obtained via the treatment with sodium hydroxide, which ranged from 52.0 ± 2.4% for AFG1 in black pepper to 54.5 ± 2.7 for AFB1 in white pepper. However, in most samples, there were no significant differences when comparing treatments with sodium hydroxide, calcium hydroxide, potassium hydroxide, and ammonia. Despite the promising effects of the alkaline compounds, black or brown spots were observed on the surface of the grains. Besides the lower reduction of AFs with acid treatments, the authors did not report damage to the surface of the grains (Jalili et al. 2011).

Méndez-Albores et al. (2013) evaluated the effect of the roasting process (250 °C for 15 min) and cocoa alkalinization (NaOH, Ca (OH) $_2$ and KOH, concentrations: 10, 20, and 30 g/kg) on the stability of AFB1 + AFB2. Roasting caused a reduction of up to 71% in the AF content. The alkalinization process had a significant effect on the AF degradation, where the three chemical methods applied were equally effective, with a reduction of 98% when the cocoa liquor was subjected to the treatment with 30 g/kg. The physical-chemical parameters were evaluated, and the main results showed that in general, increased alkali concentration was associated with lower values of protein and crude fats. According to the authors, this might have been associated with the oxidative destruction of proteins by deamination and the hydrolysis and saponification of triglycerides, with the consequent formation of salts. Further according to the authors, these results are important because the excessive addition of alkalis to perform Dutching, a necessary action to reduce the level of cocoa acidity, can lead to the generation of an undesirable soapy flavor in chocolate.

11.5.3 Hydrogen Peroxide

In milk and its derivatives, contamination by AFM1 is a common problem. The main control is to prevent contamination of livestock feed. However, the treatment of milk contaminated with AFM1 is an alternative control measure. Unfortunately, selecting a treatment method that is effective but does not affect the organoleptic quality of milk has been a challenge to researchers and dairy farmers (Nguyen et al. 2020).

H_2O_2 is an example of an oxidizing agent that can be used to treat contaminated milk, by degrading AFM1. Moreover, its use in food processing instead of pasteurization, when milk is transformed into certain types of cheese, was reported by Doyle et al. (1982). After 2000, there was declining interest in studies of the AF degradation by H_2O_2, so most studies associated with AF degradation by hydrogen peroxide were published more than 20 years ago (Shen and Singh 2021).

According to (Ismail et al. 2018), although effective, the use of H_2O_2 in some cases leaves residues higher than the permitted level in food and therefore poses a risk to human health. The regulations issued by the FDA state that the permitted concentration of H_2O_2 is 0.5 mg/L in processed foods and a final concentration of 0.05% by weight of milk in the end product (Abbas et al. 2010; Nguyen et al. 2020).

The efficiency of H_2O_2 in mitigating AFB1, AFB2, AFG1, and AFG2, in grains of black and white pepper, was evaluated by Jalili et al. (2011). The grains were washed with 2% H_2O_2. According to the authors, the treatment was efficient in reducing contamination, ranging from 32.3% ± 4.2% for AFB2 to 45.0% ± 3.3% for AFB1. The authors also reported an improvement in the color of white pepper due to the whitening effect. However, there was damage to the outer skin of the black pepper grains.

11.5.4 Ammoniation

Most grains are harvested and stored in silos for medium or long terms until they are sent for processing, and the quality is influenced by several interacting abiotic and biotic factors. The contamination of these products by fungi and mycotoxins results in nutritional losses and the quality of grains, in addition to representing a significant danger to the food chain. In this context, the use of methods to mitigate the formation of mycotoxins during storage to maintain postharvest quality is essential (Magan and Aldred 2007).

Ammoniation was one of the first chemical methods studied and was widely used in the 1990s for the decontamination of mycotoxins in food since it was considered effective and economically viable at that time (Peng et al. 2018). This method is now mainly used for animal feed processing (Pankaj et al. 2018). According to Ismail et al. (2018), although ammonia can effectively modify or destroy AFs, its use can pose safety problems due to the formation of toxic derivatives, as well as impair quality and sensory parameters.

11.6 Nanotechnological Methods for AF Mitigation

To minimize problems related to the contamination of food and feed by mycotoxin-producing fungi, some traditional strategies are available, such as the use of natural chemical, physical, biological, and phytochemical methods (Peng et al. 2018). The drawbacks of some applications due to low fungicidal effects, alteration of the sensory and nutritional quality of food, and possibility of toxicity caused by-products, generating risks to human and animal health, require the search for new methods, such as the use of nanoparticles (NPs) (Jogee and Rai 2019).

The use of nanotechnological methods is a new, promising, and inexpensive strategy that can be ecologically correct for the control of mycotoxigenic fungi and mycotoxins, both when applied directly in agriculture and the food industry (Haque et al. 2020). Nanomaterials are structures with at least one dimension of nanometric size (Coelho et al. 2020; Pacaphol et al. 2019; Siqueira et al. 2010). Several studies have been conducted to investigate the preparation techniques for the synthesis of NPs, where the substrate used, time, and form of preparation are considered. In this context, the use of biological systems or chemical and physical methods are the main ways of obtaining NPs (Coelho et al. 2018, 2020; Pereira and Arantes 2020).

Metallic NPs have been widely analyzed by researchers due to the wide applications for the control of toxigenic fungi and the production of mycotoxins or pathogens in food and feed. These are named according to the metal used for synthesis, for example, zinc NPs (ZnNPs), silver NPs (AgNPs), copper NPs (CuNPs), sulfur NPs (SNPs), and zinc oxide NPs (ZnONPs), among others (Abd-Elsalam et al. 2017; Guo et al. 2021; Jogee and Rai 2019; Kazemi et al. 2020).

The potential use of AgNPs against aflatoxigenic fungi and AF accumulation in a corn-based culture medium was investigated by Gómez et al. (2019). Among the results reported by the authors was the effectiveness of AgNPs in controlling the growth of the main aflatoxigenic species that affect food and the production of PAs. The target species and their associated mycotoxins were *Aspergillus flavus* (AFB1 and AFB2) and *A. parasiticus* (AFB1, AFB2, AFG1, and AFG2). The spore suspensions were supplemented with doses of 0–45 μg/mL of AgNPs and incubated for 2–30 h. Aliquots were removed and cultivated in a corn-based medium (MBM) for 10 d. The authors reported that the efficiency of the doses applied was better for larger doses together with the longer exposure time for *A. flavus* and *A. parasiticus*. A dose of 15 μg of AgNPs/mL applied for 30 h killed 100% of the spores of *Aspergillus flavus*, when the evaluation was carried out for 8 h, it was necessary to apply 30 μgAgNPs/mL to mitigate 100% of the spores of *A. flavus*. For *A. parasiticus*, a dose of 30 μgAgNPs/mL applied for 30 h killed 100% of the spores, but when the evaluation was carried out for 20 h, it was necessary to apply 45 μgAgNPs/mL to kill 100% of the spores. The monitoring of mycotoxin production by aflatoxigenic species was also evaluated. For *A. flavus*, a dose of 15 μgAgNPs/mL applied for 30 h eliminated 100% of the mycotoxins AFB1 and AFB2. For *A. parasiticus*, only with the dose of 30 μg AgNPs/mL applied for 30 h was it possible to eliminate 100% of the mycotoxins AFB1, AFB2, AFG1, and AFG2.

Asghar et al. (2018) evaluated the antifungal activity of different concentrations of iron (Fe), copper (Cu), and silver (Ag) (10, 25, 50, and 100 μg/mL), synthesized from green and black tea leaf extracts, against the fungal species *Aspergillus flavus* and *A. parasiticus*, as well as the adsorbent capacity of AFB1 in solution. AgNPs showed superior antifungal activities and reduced the AF production compared to FeNPs and CuNPs. For *A. flavus*, the inhibition rates were 100, 77.2, and 43.5%, while for *A. parasiticus* they were 100, 83.1, and 51.6% (100 μg/mL AgNPs, CuNPs, and FeNPs, respectively). The adsorption capacity of all NPs regarding AFB1 contamination was in the order Fe-NPs > Cu-NPs > Ag-NPs.

Khalil et al. (2019) investigated the synthesis of silver NPs (AgNPs) from cell-free cultures (CFF) of the fungi *Fusarium chlamydosporum* NG30 and *Penicillium chrysogenum* NG85 (FAgNPs and PAgNPs, respectively). The authors demonstrated the antifungal activity and potency in preventing the production of mycotoxins by AgNPs. The minimum inhibitory concentrations (MICs) of *Aspergillus flavus* were 48 and 45 mg/mL for FAgNPs and PAgNPs, respectively, while the MIC values against AF total production by *A. flavus* were 5.9 and 5.6 mg/mL for FAgNPs and PAgNPs, respectively.

Among metallic NPs, ZnO has gained more attention due to its promising properties, such as low cost, nontoxicity, stability, biosafety, and biocompatibility, along with its antibacterial, antifungal, and antiviral activities. The use of these NPs in plant protection systems, such as nanofertilizers and fungicides, has been of great interest to farmer. In the food industry, different zinc compounds are used in different forms as human dietary supplements and also are authorized for food fortification (citrate, acetate, chloride, gluconate, lactate, zinc oxide, carbonate, and sulfate), which are considered GRAS (Abd-Elsalam et al. 2017).

Some researchers have reported the action of Zn NPs against different fungi. ZnONPs were synthesized from solutions of zinc chloride (ZnCl2) and sodium hydroxide (NaOH) by Hassan et al. (2013). The authors investigated the antifungal effect of different concentrations of ZnONPs (0, 2, 4, 6, 8, and 10 μg/mL) against the growth of mycotoxigenic fungi and mycotoxin production in food. The authors reported that as the concentration of ZnONPs increased, the levels of mycotoxins produced decreased. The concentration of 8 μg/mL of ZnONPs inhibited the growth of aflatoxigenic fungi. The antifungal effect of ZnONPs was observed as damage to the membrane of *Aspergillus* conidial cells and some spots on intercellular components, leading to leakage and finally cell death (Hassan et al. 2013).

Another nano-based approach is the use in formulations for the production of nanocomposites with antimicrobial potential for food preservation. Kazemi et al. (2020) investigated the use of packaging containing additives such as cinnamon essential oil, sprayed ZnONPs, and nitrogen-modified atmosphere (MAP), as well as the combination of the three additives, in increasing pistachios' useful life. The rancidity rate and the browning reactions resulting from the lipid oxidation in the pistachio seeds were lower in the samples that were stored in packages containing the three factors, indicating their combination acted as a barrier system and inhibited oxidation. Sensory analysis revealed that the presence of ZnONPs improved the quality of the packaged sample concerning odor, texture, and color, keeping the seeds firmer and with an attractive color for at least 4 weeks. Packaged pistachio samples tasted better, which might have been associated with the antioxidant properties of the essential oil and the ability of ZnONPs to maintain the quality of fresh pistachios. The microbiological results showed that in pistachios stored in packages without additives, colonies of *Aspergillus spp*. were found, as well as AFB1, unlike those stored in modified packaging, which was effective in inhibiting the growth of fungi and the production of mycotoxins.

The use of nanomaterials for coatings was studied by Tatlisu et al. (2019). Nanofibers from polyvinyl alcohol and whey protein loaded with thymol (TLNs), an antimicrobial compound, were evaluated as a Kashar cheese coating. The authors demonstrated that TLNs were more efficient against the mycelial growth of *A. parasiticus* than the free thymol compound alone. Cheeses coated with TLNs did not show growth of *A. parasiticus* until the seventh day of storage.

11.7 Concluding Remarks and Trends

AF contamination in food and feed is still a major problem that affects human and animal health, also causing economic losses. Chemical treatments have increasingly attracted the attention of researchers and food companies and have been widely applied to mitigating mycotoxins. However, the drawbacks of each method must be evaluated, since the choice of the chemical product, concentration, time, and way of application generally has a large impact on the sensory attributes and nutritional

values of the treated foods, and their residues can cause direct damage to human and animal health or induce negative effects through interaction with other nutrients.

Nanoscale materials are used in different fields of activity, among which is the production of agrochemicals (nanochemicals), drugs (nanocarriers), and food products (nanosensors, nanocomposites, nanoemulsions, etc.). Their use as antimicrobial agents has been studied and they are effective against some AF-producing fungi. Their analysis as antifungal agents has produced promising results, with NPs being able to limit the growth of mycotoxigenic fungi in several food matrices during storage.

Therefore, AF detoxification by chemical methods should be studied more to better understand the mechanisms of detoxification and consequently to apply these methods on an industrial scale. Also, local governments and research institutes should invest more in the transfer of technologies to small farmers and agribusinesses, especially considering clean and widely studied technologies. This includes the use of ozonated water, which in addition to being effective in AF detoxification also eliminates pathogenic agents and microorganisms, contributing to food safety.

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Chapter 12
Biological Decontamination of Aflatoxins

Carlos Augusto Fernandes de Oliveira, Marisa Matias de França, Carlos Humberto Corassin, and Khurram Muaz

Abstract Aflatoxins (AFs) are secondary metabolites that can be produced by filamentous fungi from the genus *Aspergillus*, mainly *A. flavus*, *A. parasiticus* and *A. nomius*. AFs are considered a risk to human health due to exposure both to the consumption of food of plant origin and the consumption of residues in food of animal origin, such as meat, milk, and eggs. Because of the high thermal stability of AFs, decontamination methods have been proposed to degrade or reduce these toxins without changing the characteristics of the food. Biological methods based on the use of several selected microorganisms including bacteria, fungi, yeasts, and algae have attracted great scientific attention. AFs detoxification occurs through binding (adsorption) by components of the microorganism's cell wall or by biodegradation from enzymes, with several hypotheses being formed about specific mechanisms of action and there are several factors that influence the success of this process for each one major mycotoxins. In this chapter, the available literature on the use of lactic acid bacteria (LAB) and yeasts in the AFs decontamination is discussed along with their proposed mechanisms of action.

Keywords Adsorption · Biotransformation · Lactic acid bacteria · Yeasts · Enzymes

C. A. F. de Oliveira (✉) · M. M. de França · C. H. Corassin
Department of Food Engineering, School of Animal Science and Food Engineering, University of São Paulo, Pirassununga, SP, Brazil
e-mail: carlosaf@usp.br

K. Muaz
Institute of Food Science & Nutrition, Bahauddin Zakariya University, Multan, Punjab, Pakistan

K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_12

12.1 Introduction

Filamentous fungi develop naturally in foods such as grains and cereals in the presence of favorable conditions such as water activity (a_w), temperature, aeration, physical damages to seeds, and microbial interactions. Some species of filamentous fungi, during their development, can produce a range of toxic secondary metabolites, called mycotoxins (Ismail et al. 2018). The most important genera of potentially toxigenic fungi causing damage in animals and humans are *Aspergillus*, the main producer of the nominated aflatoxins (AFs); *Fusarium* which produces fumonisins (FB), zearalenone (ZEN), and trichothecenes such as deoxynivalenol (DON) and T-2 toxin; and the genus *Penicillium* which produces ochratoxin A (OTA), though this toxin may be also produced by some *Aspergillus* species (Buszewska-Forajta 2020).

The damage caused by fungi and mycotoxin production is not only limited to economic losses and health effects but also affects the human food security, as mycotoxin residues can be detected in products of animal origin as well (Muhialdin et al. 2020). The toxic effects of mycotoxins are mostly related to their carcinogenic potential. Such mechanisms are well elucidated for some mycotoxins, such as AFs, and less clarified for OTA and FB_1 (Ostry et al. 2017). Among these mycotoxins, the most common and toxic compounds are the AFs. These can be produced during the pre- and postharvest periods of the grains, influenced by multiple factors, which is why their universal control is complex. Good practices in agriculture, storage, and transportation of grains can be used to mitigate aflatoxin levels. Crop practices such as insect control, use of antifungal agents and maintenance of seed integrity and cleaning, humidity, and temperature control during storage are points to be considered (Ismail et al. 2018).

Good agricultural practices are considered as the primary strategy for controlling the presence of fungi and mycotoxins such as the AFs. However, they do not guarantee total prevention of such toxins in crops because of difficulties in the application of controlling practices for AFs in the field. In addition, the destruction of AFs during the processing of food products is not feasible due to the high thermal stability of these compounds (Muhialdin et al. 2020) Thus, innovative decontamination techniques are required to achieve the destruction, removal, or formation of compounds of reduced toxicity, while maintaining the physical, sensorial, and nutritional characteristics of foods. These methods are classified into physical, chemical, or biological, each one providing decontamination effects based on the removal of AFs from raw materials and/or finished products, or detoxification through modification of the chemical structure of the molecule which reduced the toxicity (Karlovsky et al. 2016).

The physical methods are represented by thermal inactivation, irradiation with gamma rays, microwaves or ultraviolet light, and solvent extraction. Among the existing chemical methods, there are ozonation and adsorbents of different classes of origin, such as aluminosilicates (Di Gregorio et al. 2014). Biological methods are based on the use of selected microorganisms such as bacteria, yeasts, filamentous

fungi, and algae (Bovo et al. 2013) and enzymes produced by microorganisms (Taheur et al. 2019). Methods of detoxification by microorganisms have proven to be promising and can increase the quality of food both for animal and for human consumption. Considering the diversity of biological strategies and microorganisms studied, this chapter provides a literature review on the efficiency and mechanisms of action of lactic acid bacteria (LAB) and yeasts in the decontamination of AFs in food products.

12.2 Biological Approaches for Aflatoxin Decontamination

Biological detoxification methods for AFs in foods are based on a wide range of microorganisms and their metabolites that can be selected, which must be able to act on the toxin by mechanisms that result in nontoxic compounds or, at least, less toxicity (Muhialdin et al. 2020). Figure 12.1 presents an overview of microorganisms that have exhibited decontamination effects against AFs. However, the selected microorganisms must meet the characteristics that guarantee the safety of the detoxification process in food, for example, they are not pathogenic, and they produce known and stable nonreversible compounds, in addition to the ease of cultivation in the laboratory and stability in the food without changing its nutritional value or physical, chemical, and sensory properties.

In general, the decontamination process of AFs by microorganisms occurs through a binding process (adsorption) or by biotransformation of the toxic compounds. The adsorption of AFs by microbial cells may be influenced by several factors, including the incubation conditions and variations in cell wall components among different species, which ultimately modify the affinity with the AFs. Regarding the biodegradation of AFs by microbial cells, the mechanisms proposed include numerous reactions such as hydrolysis, acetylation, oxygenation, and deepoxidation. In addition, several factors may influence the success of microbiological detoxification, such as the concentration of agents and the incubation time and temperature (Taheur et al. 2019).

Taheur et al. (2019) reviewed the results from in vivo and in vitro studies on the application of microorganisms and enzymes for detoxification of AFs and concluded that bacteria, fungi, and yeasts have been widely researched in foods intended for animals and humans. Main examples include *Lacticaseibacillus casei* (formerly *Lactobacillus paracasei*) LOCK 0920, *L. brevis* LOCK 0944, *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) LOCK 0945, *Bacillus pumilus* (Slizewska and Smulikowska 2011), *Mycobacterium fluoranthenivorans* (Sangi et al. 2011), *Bacillus subtilis* ANSB060 (Gao et al. 2011), *Lentilactobacillus kefiri* (formerly *Lactobacillus kefiri*), *Kazachstania servazzii, Acetobacter syzygii* (Taheur et al. 2017), *Rhodococcus erythropolis* (Teniola et al. 2005), *Pseudomonas putida* (Samuel et al. 2014), *Streptomyces lividans* TK 24 (Eshelli et al. 2015), *Saccharomyces cerevisiae* (Zhu et al. 2015), and *Pichia anomala* (Ruiyu 2012).

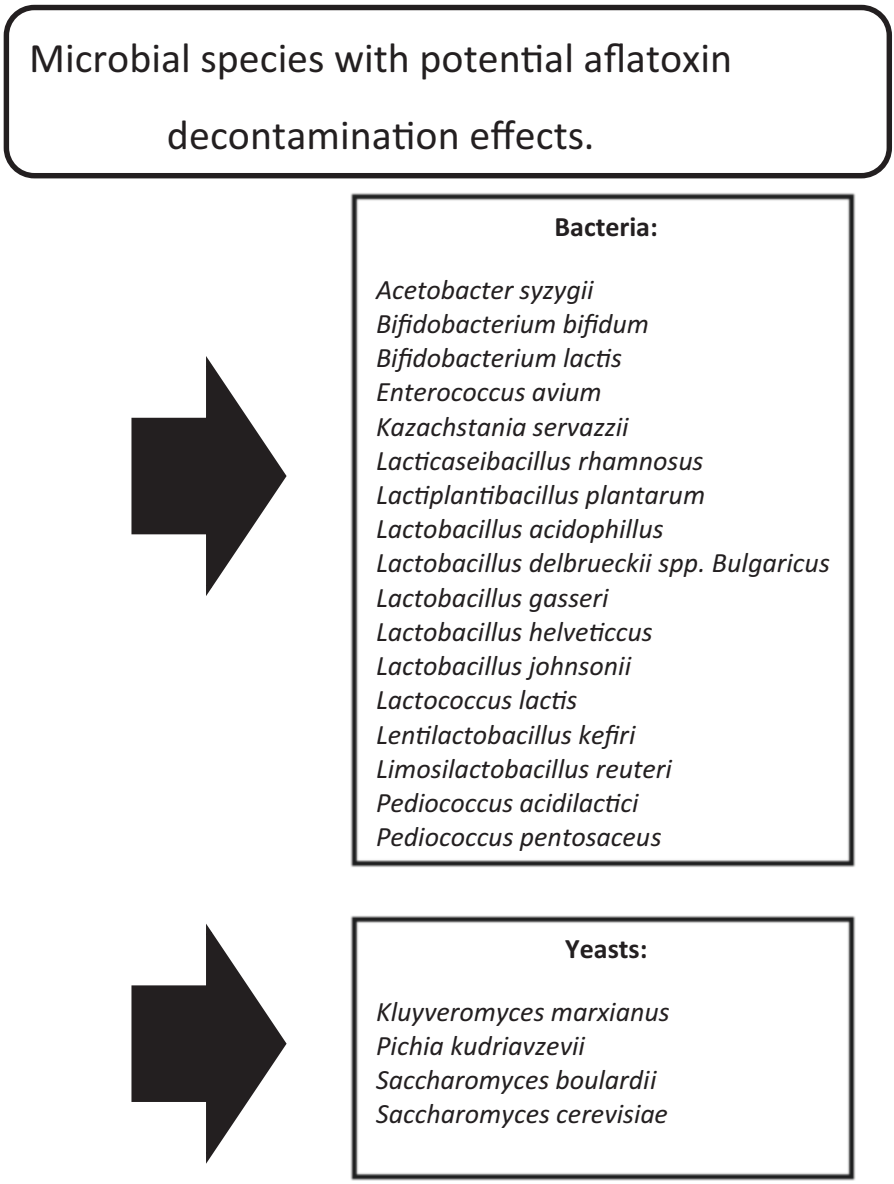

Fig. 12.1 Microbial species with potential aflatoxin decontamination effects

The selected microorganisms with detoxification capacity may have different origins, including starter cultures used in the food industry as probiotics or isolated from different nonfood sources such as the soil (e.g., *B. licheniformis*) and rumen (e.g., *L. mucosae*) or even from the intestines of birds, such as *Lysinibacillus* sp. (Taheur et al. 2019).

12.3 Aflatoxin Decontamination by Microbial Adsorption

12.3.1 Lactic Acid Bacteria

The group of lactic acid bacteria includes a variety of gram-positive genera, in general, not sporulated, catalase negative, which in their metabolic processes can ferment carbohydrates source of glucose with the production of lactic acid as a single or one of the final products. *Streptococcus, Lactobacillus, Leuconostoc*, and *Lactococcus* are prominent representatives of this group. Lactic acid bacteria are widely used in human diets in the form of functional foods, which have shown health benefits (Mahmood et al. 2019). The use of LAB in food commodities for various benefits further provides the basis for the possibility of their application as a safety feature for use in other activities such as mycotoxin detoxification; thus, they represent one of the main groups used for this purpose. In addition, they are more adaptable to use as food additives since they naturally colonize the digestive tract of humans and most species are easily cultivated in the laboratory (Muhialdin et al. 2020; Wu et al. 2009).

LAB are producers of several proteases that can hydrolyze cell wall proteins, among other enzymes that have strong influence in biological detoxification (Juodeikiene et al. 2011; Wu et al. 2009). In addition, LAB are capable of producing bioactive compounds such as peptides, acids, carbon dioxide, and hydrogen peroxide, which can act as a inhibitor of fungi growth or to decontaminate mycotoxins. Although the enzymatic activity is very expressive, the LAB adsorption capacity, due to cell wall of peptidoglycans and polysaccharides, is also a proposed mechanism for AF decontamination (Chapot-Chartier and Kulakauskas 2014). Thus, the mycotoxin detoxification process involves three different propositions: the adsorption action by the cell wall, the enzymatic biodegradation, and the process of interaction of the bioactive metabolites with mycotoxins (Muhialdin et al. 2020). The LAB may be used as decontaminants as both whole cells, viable or nonviable, and through the application of purified enzymes. According to Luz et al. (2018) and Muhialdin et al. (2020), the success of the biological decontamination process based on adsorption by lactic acid bacteria depends on factors such as the mycotoxins concentration in the study, bacterial cells concentration, and reaction conditions such as time and incubation temperature, hence making it a multifactorial process.

Table 12.1 presents the outcomes from some recent studies on the use of LAB for biological detoxification of AFs. In a study involving *B. subtilis* ANSB060, Gao

Table 12.1 Summary of the literature that used lactic acid bacteria in studies of biological detoxification of aflatoxins

Type of lactic acid bacteria	Main outcomes	Reference
Lactiplantibacillus plantarum, *enterococcus avium*, *Pediococcus pentosaceus*, *lactobacillus delbrueckii* spp. *bulgaricus*, *Lacticaseibacillus rhamnosus*, *Bifidobacterium lactis, and L. gasseri*	Reductions of up to 46% of AFM_1, varying according to contact times (15 min–24 h) and cell viability (viable and nonviable) in PBS	Bovo et al. (2013)
B. bifidum, L. johnsonii, Limosilactobacillus reuteri, and Lacticaseibacillus rhamnosus	Reductions of 24–45% of AFM_1 in milk	Serrano-Niño et al. (2013)
Lacticaseibacillus rhamnosus, L. delbrueckii spp. *Bulgaricus, and B. lactis*	Reductions of 11.5–11.7% of AFM_1, varying according to contact times (30–60 min) in UHT skim milk	Corassin et al. (2013)
Lactiplantibacillus plantarum and *Lacticaseibacillus rhamnosus*	Reductions of 69–95% of AFM_1, varying according to contact times (360–1440 min) in skim milk	Abbes et al. (2013)
Lactobacillus acidophilus and B. lactis	Reductions of 30–96% of AFM_1, varying according to contact times (1440–2880 min) in milk	El-Kest et al. (2016)
Lentilactobacillus kefiri, *Kazachstania servazzii,* and *Acetobacter syzygii* (from kefir grains)	Reductions of 82–100% of AFB_1 in milk	Taheur et al. (2017)
L. helveticus	Reductions of 92% of AFM_1 in milk	Ismail et al. (2017)
Mixed cultures of LAB from dairy products	Reductions of 58% of AFM_1 in fermented milk	Barukcic et al. (2018)
Lactiplantibacillus plantarum	Reductions of 94.5% of AFM_1 in milk, using heat-treated cells	Kuharic et al. (2018)
Lacticaseibacillus rhamnosus	Reductions of 18–26% of AFM_1, varying according to contact times (960 min) and cell viability (viable and nonviable) in skim milk	Abdallah et al. (2018)
Lacticaseibacillus rhamnosus, *P. acidilactici,* and *P. pentosaceus*	Reductions of 26–61% of AFM_1 in milk after 48 h of contact time	Martínez et al. (2019)
Lacticaseibacillus rhamnosus and *Lactococcus lactis*	Reductions of 94% of AFM_1 in cheese at day 10 of storage	Gonçalves et al. (2020)

AFM_1 aflatoxin M_1, *AFB_1* aflatoxin B_1

et al. (2011) evaluated the removal capacity of AFB_1, AFM_1, and AFG_1, obtaining a reduction of 81.5% for AFB_1, 60% for AFM_1, and 80.7% for AFG_1. The authors highlighted that *B. subtilis* ANSB060 has potential as an AF detoxifier in industry, in addition to its antibacterial properties against certain pathogens.

Strains isolated from Kefir grains were used by Taheur et al. (2017) to evaluate their detoxification capacity of AFB_1. The authors reported that the Kefir microbiota grown in milk adsorbed AFB_1 at percentages ranging from 82 to 100%, with the most active strains being *Lentilactobacillus kefiri, Kazachstania servazzii*, and *Acetobacter syzygii*. Therefore, the consumption of Kefir can help to reduce the gastrointestinal absorption and thereby reduce the toxic effects of AFB_1.

The LAB detoxification of AFM_1, a metabolite from biotransformation of AFB_1 in the liver, has also been evaluated in several studies. Bovo et al. (2013) determined the ability of different lactic acid strains to remove AFM_1. The authors obtained a mean removal in ranges of 5.60 ± 0.45% to 45.67 ± 1.65%, with heat-killed cells of *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*), *Lactobacillus delbrueckii* spp. *Bulgaricus*, and *Bifidobacterium lactis* having the highest percentage (>33%) of AFM_1 removal. In addition, removal by inactive cells was often higher among the strains studied, indicating that LAB have potential for application in reducing AFM_1 in milk and its products. In an attempt to select microorganisms capable of reducing the availability of AFM_1 in milk and dairy products, Martinez et al. (2019) tested the adsorption capacity of probiotic lactic acid bacteria, *Lacticaseibacillus rhamnosus* RC007, *Pediococcus acidilactici* RC005, and *P. pentosaceus* RC006. All strains adsorbed AFM_1 significantly in milk after 24 and 48 hours, with *Lacticaseibacillus rhamnosus* RC007, *P. acidilactici* RC005, and *P. pentosaceus* RC006 showing adsorption percentages of 61%, 34%, and 26%, respectively.

The use of LABs in the detoxification of AFM_1 directly in dairy products was evaluated by Barukcic et al. (2018). In this study, the authors observed that the adsorption efficiency of probiotics were higher than non-probiotics cultures in different dairy products prepared from fresh milk experimentally contaminated with toxin. *Lacticaseibacillus casei* (formerly *Lactobacillus casei*) in fermented milk exhibited 58% of AFM_1 removal, while the most efficient non-probiotic culture was YC with 41% of AFM_1 removal. From this research, it can be concluded that LAB cultures provide an interesting option for AFM_1 removal from dairy products involving bacterial cultures.

12.3.2 Yeasts

According to Jespersen (2003), yeasts have been used for food fermentation for centuries, with the main purpose of product preservation. Although many species participate in the food fermentation process, the most used species is *Saccharomyces cerevisiae.* Like LAB, yeasts have also been extensively evaluated for their ability of detoxifying mycotoxins (Campagnollo et al. 2020). Thus, considerable research has been conducted in order to elucidate the mechanisms of detoxification and related factors, in order to provide basis for their practical application along with LAB in decontamination of AFs in food products (Hathout and Aly 2014).

The proposed mechanisms of action of yeasts also include adsorption, due to the presence of oligosaccharide mannans, such as glucomannans, in the yeast cell wall (Hathout and Aly 2014; Raju and Devegowda 2000) and biotransformation into less toxic compounds by enzyme complex. The results from some recent studies on the use of yeast cells for biological detoxification of AFs are presented in Table 12.2. The decontamination of aflatoxin B_1 by *S. cerevisiae* via adsorption was tested by Shetty, Hald, and Jespersen (2007), who reported AFB_1 removal of more than 40%. In cultivation at temperatures between 20 °C and 37 °C, the adsorption phenomenon occurred in an expected way; however, it was affected at 15 °C, a fact that may indicate that the temperature may also play an influential role in the process. The

Table 12.2 Summary of the literature that used yeasts in studies of biological detoxification of aflatoxins

Type of yeasts and lactic acid bacteria	Main outcomes	Reference
Saccharomyces cerevisiae	Reductions of 90–92% of AFM_1, varying according to contact times (30 to 60 min) in UHT skim milk	Corassin et al. (2013)
S. cerevisiae	Reductions of 90–99% of AFB_1, varying according to contact times (5 to 30 min) in PBS	Gonçalves et al. (2015)
S. cerevisiae	Reductions of 71–84% of AFM_1, varying according to contact times (1 to 21 days) and treatment (viable, acid, heat, ultrasound) in yogurt	Karazhiyan et al. (2016)
S. cerevisiae	Reductions of 92–100% of AFM_1 in milk	Ismail et al. (2017)
S. cerevisiae	Reduction of 50–89% in the AFM_1 excretion in milk from dairy cows fed diets containing 480 μg AFB_1/per day	Goncalves et al. (2017)
S. cerevisiae	Reductions of 6–46% of AFB_1, varying according to contact times (30–1440 min) in PBS	Aazami et al. (2018)
S. cerevisiae	Reductions of 45–73% of AFB1, varying according to cell viability (viable and nonviable) in PBS	Tabari et al. (2018)
Kluyveromyces marxianus and *Pichia kudriavzevii*	Reduction of up 85% of AFB_1	Intanoo et al. (2018)
S. cerevisiae	Reductions of 81–100% of AFM_1, varying according to contact times (40 to 80 min) in milk	Foroughi and Sarabi (2019)
K. Marxianus, S. boulardii and *S. cerevisiae*	Reductions of 36%, 25% and 19% of AFM_1 in milk	Martínez et al. (2019)
S. cerevisiae	Reduction of toxic effects of AFB_1 in quail fed diets containing 5000 μg AFB_1/kg	Mahmood et al. (2019)
K. Marxianus and *P. kudriavzevii*	Reduction of 72% in the AFM_1 excretion in milk from dairy cows fed diets containing 22.28 μg AFB_1/kg	Intanoo et al. (2020)
S. cerevisiae	Reductions of 100% of AFM_1 in cheese at day 20 of storage	Gonçalves et al. (2020)

AFM_1 aflatoxin M_1, *AFB_1* aflatoxin B_1

adsorption of toxin with cell components of the cell wall was confirmed as the authors reported greater adsorption by cells that received either thermal or chemical treatment, compared to viable incubated cells.

In an attempt to select microorganisms that are capable of reducing the availability of AFM_1 in milk and dairy products, Martinez et al. (2019) tested the yeast adsorption capacity using *S. boulardii* RC009, *K. marxianus* VM003, and *S. cerevisiae* RC016. All of the strains showed adsorption, with highest AFM_1 removal of 36% by *K. marxianus* VM003, followed by 25% removal through *Saccharomyces boulardii* RC009 and 19% by *S. cerevisiae* RC016. *S. boulardii* RC009 and *K. marxianus* VM003 were able to degrade AFM_1 more efficiently and in less time.

Intanoo et al. (2018) isolated 3 yeast strains (two *Kluyveromyces marxianus* and one *Pichia kudriavzevii*) from bovine ruminal fluid with the ability to detoxify up to 85% AFB_1 in cattle feed spiked with 200, 400, 600, 800, and 1000 ng/mL of AFB_1. The authors proposed that these yeasts can be used as potential additives in the feeding of dairy cattle because of their detoxification capacity as well as their adaptability to anaerobic and aerobic conditions, which can help to survive the rumen environment. Subsequently, the authors performed an in vivo test to evaluate the effect of dietary supplementation of dairy cows with these two yeasts on the bioconversion of AFB_1 from the diet into AFM_1 excreted in milk. Dairy cows were submitted to four types of diets spiked with 22.28 μg of AFB_1/kg, one control without yeast supplementation and three treatments with the addition of *K. marxianus* CPY1 (K1Y), *K. marxianus* RSY5 (K2Y), or *P. kudriavzevii* YSY2 (PY). The amount of biomass inclusion was 2 g per day in a concentration of 1×10^9 CFU/g of yeast or cornmeal biomass in the control group. The inclusion of 2 g of yeast biomass per day reduced the biotransformation of AFB_1 into AFM_1 and its subsequent excretion in milk by 72.1%, in addition to improving milk composition.

The detoxification effect of AFB_1 by *S. cerevisiae* was tested in vivo, using quails (*Coturnix japonica*) fed with diets containing 0.5 mg/kg of AFB_1 and different levels of *S. cerevisiae* (0.5, 1 and 2 mg/kg of feed) and basal diet as the control. The use of *S. cerevisiae* significantly reduced the deleterious effects of AFB_1, demonstrating that the addition of *S. cerevisiae* can be an alternative detoxification method for AFB_1 in quails (Mahmood et al. 2019).

12.4 Aflatoxin Decontamination by Microbial Degradation

Although the aflatoxin decontamination by LAB and yeast strains have been shown to occur predominantly through adsorption mechanism, many studies have revealed that a number of microbial strains are capable to decontaminate aflatoxins through degradation, as reviewed by Guo et al. (2020b) and Guan et al. (2021). This mechanism involves the conversion of AFs in to nontoxic or less toxic compound as compared to its parent compound by the action of metabolites produced during the microbial life activities. The substances produced by microorganisms that cause AFs degradation mainly include enzymes. In contrast to the adsorption process,

degradation involves structural changes in aflatoxin, so the toxicity of the resultant compound is an important aspect to be considered while deciding the applicability of any enzyme for this purpose.

The AFs structurally contain furan rings in which the presence of double bond acts as a major site leading to mutagenic, carcinogenic, and teratogenic effects (Samuel et al. 2013). In addition, the main toxic structure in AFs is the coumarin lactone ring, which may be readily hydrolyzed (Theumer et al. 2018, Zhao et al. 2016). Samuel et al. (2014) revealed through gas chromatography mass spectrometry (GC–MS) and Fourier transform infrared spectroscopy (FT-IR) analyses that *Pseudomonas putida* MTCC 1274 and 2445 were able to modify AFB_1 by breaking the furan and lactone rings, consequently converting AFB_1 into three derivatives, namely, AFD_1 ($C_{16}H_{14}O_5$), AFD_2 ($C_{17}H_{14}O_6$), and AFD_3 ($C_8H_4O_3$), which exhibit none or lower toxicity levels as compared to AFB_1. Similarly, another study by Cserhati et al. (2013) showed degradation of AFB_1 into non-genotoxic products by *Rhodococcus* strains. The cleavage of AFB_1 lactone group by *R. erythropolis* was confirmed through thin-layer chromatography (TLC) analysis (Eshelli et al. 2015). Various other strains including *Bacillus velezensis*, *Lysinibacillus fusiformis*, *Staphylococcus warneri*, and non-toxigenic *A. niger* have also shown AFs degradation into substances exhibiting lower toxicity levels (Adebo et al. 2016; Shu et al. 2018; Zhang et al. 2014).

Several studies have attempted to isolate and identify different AF degrading enzymes from various microorganisms, including laccases, peroxidases, oxidases, and reductases. Laccase is an extracellular multicopper enzyme produced widely by bacteria, fungi, higher plants, and insects. Laccases have the ability to oxidize a variety of phenolic and non-phenolic aromatic compounds while reducing molecular oxygen in water. Zeinvand-Lorestani et al. (2015) evaluated the AFB_1 degradation by pure laccase from *Trametes versicolor*, observing a 59% reduction in the prooxidative properties of the toxin and complete elimination of its genotoxicity. Furthermore, it has been shown that laccase enzyme activity can be improved greatly in the presence of redox mediators (Loi et al. 2018). The redox mediators carry electrons between laccase and the target substrates. Loi et al. (2016) showed a significant increase in AFB_1 degradation from 23% to 99% by applying Lac2 laccase from *Pleurotus pulmonarius* with addition of 10 mM acetosyringone (AS) as a redox mediator. The use of both fungal and bacterial laccases has been applied for AF degradation. In comparison with fungal laccases, the bacterial laccases proved to exhibit higher thermostability, wider pH range, and broader substrate spectrum making them more suitable for degradation of xenobiotics such as the AFs (Guan et al. 2018). It has been shown that AFB_1 is able to interact with CotA laccase through hydrogen bonding and van der Waals interactions, resulting in its catalysis to C3-hydroxylation. The resultant biotransformed products include aflatoxin Q_1 and epi-aflatoxin Q_1, which are nontoxic to human liver cells L-02 (Guo et al. 2020a).

Peroxidases are a large group of oxidoreductase enzymes produced by several organisms ranging from plants and humans to bacteria. These enzymes are involved in oxidation of different substrates with subsequent breakdown of hydrogen

peroxide. Many studies regarding the degradation of AFs through peroxidases obtained from different sources have been conducted (Tripathi and Mishra 2011; Marimón Sibaja et al. 2019). The study involving manganese peroxidase (MnP) from a white-rot fungus, *Phanerochaete sordida* YK-624, resulted in 86% degradation of AFB_1 and 69.2% reduction of its mutagenic activity (Wang et al. 2011). The mechanism involved in degradation of AFB_1 by MnP included the oxidation of AFB_1 to AFB_1–8,9-epoxide followed by hydrolysis to form AFB_1–8,9-dihydrodiol with H_2O_2 addition (Wang et al. 2019). Moreover Loi et al. (2020) reported degradation of 96% AFB_1 into AFQ_1 by type B dye decolorizing peroxidase (Rh_DypB) from *Rhodococcus jostii*.

Aflatoxin oxidase (AFO), an intracellular enzyme previously known as aflatoxin detoxifizyme (ADTZ), was extracted from a mushroom *Armillariella tabescens* (Liu et al. 2001). This enzyme was observed to have 42% amino acid sequence similarity with dipeptidyl peptidase III (DPP III) enzyme family (Wu et al. 2015). The potential site of action of this enzyme for AFB_1 degradation is the double bond in difuran ring moiety of AFB_1. Consequently, H_2O_2 is produced with water as the hydrogen donor (Wu et al. 2015). Another category of AF degrading enzymes includes $F_{420}H_2$-dependent reductases (FDRs), which have been divided into two classes (FDR-A and FDR-B). The FDR-A enzymes have been observed to possess up to 100 times more AF degrading activity than the FDA-B class (Lapalikar et al. 2012).

The use of enzymes for AFs degradation can be favorable in many cases as their application can prevent degradation by microorganisms and avoid any drastic changes in organoleptic properties of food commodities. However, the commercial application of enzymes is based on a multistep complex procedure. The initial step involves screening of microorganisms capable to degrade AFs, followed by isolation and purification of AF degrading enzymes. Next, an integrated approach is applied for the elucidation of AF degraded product structures and their safety evaluation. Finally, the degrading effectiveness of these enzymes is evaluated in the target food matrix and in animal models. Although many enzymes have proven to be effective for AFs degradation in those steps, there are several obstacles limiting their commercial applications. One of the major hurdles is the low production yields of these enzymes, making it uneconomical to produce them through conventional techniques. However, new methodologies such as recombinant DNA techniques may be helpful in addressing this issue. Another constraint is the efficiency and stability of enzymes in various food commodities under diverse processing conditions, as the enzymes may behave differently under different environmental conditions (Brana et al. 2020). Further studies regarding effectiveness of enzymes in reducing the bioavailability of AFs in digestive tract can provide a helpful solution. The safety evaluation of intermediate products formed during the AFs degradation is also mandatory to pave a way for this technique towards commercial use.

12.5 Concluding Remarks and Future Perspectives

The biological detoxification of AFs by LAB and yeasts is a promising strategy for application in the food industry. However, the successful application of these microorganisms to adsorb or degrade AFs depends on several factors such as the intrinsic characteristics of the strain used and the reaction conditions such as temperature and incubation time. Therefore, investigations are still required to clarify the mechanisms of action of these microorganisms to optimize the decontamination process and designate strategies aiming at practical applications in foods. Microbial enzyme-based approaches have been successfully applied for AFs detoxification. However, there is a need for the development of more viable techniques for large-scale production of efficient microbial enzymes for AFs degradation, to make this mode of decontamination economically feasible. In addition, little is known about the toxicity of the possible detoxification metabolites formed during the detoxification processes of AFs by LAB or yeasts. This assessment should be considered as essential in further microbiological detoxification studies, to ensure its safety and, consequently, the potential for their practical use in the food industry.

Acknowledgments The authors thank São Paulo Research of Foundation (FAPESP) for the fellowship (Grant # 2017/20081-6) and the Brazilian National Council for Scientific and Technological Development, CNPq [Grant # 302195/2018-1].

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Chapter 13
Climate Change and the Impact on Aflatoxin Contamination in Foods: Where Are We and What Should be Expected?

Paula Alvito and Ricardo Assunção

Abstract Climate change (CC) is nowadays unquestionable and governmental institutions, scientific community, and citizens are becoming more and more aware about this reality. Mycotoxins are one of the most important food safety hazards due to its high toxicity and associated health effects. Among mycotoxins, aflatoxins are those calling for a special attention due to its carcinogenic effects, high number of associated notifications in food and feed products, and mainly the possibility to become a future food safety issue due to CC, especially in the Mediterranean region. Temperature raise and extreme changes in rain fall/drought episodes could be responsible for *Aspergillus flavus* growth and aflatoxin contamination in crops, which could impact their productivity and affect food availability in the future, as well as food safety. Predictive modelling could establish important contributions to anticipate the impact of future CC scenarios on aflatoxin contamination, and consequently on health. This chapter gives an overview on the effects of CC on mycotoxin occurrence in foods, focused on aflatoxins, its associated health consequences and available legislation, fungal growth conditions and aflatoxin production, and associated episodes in Europe. Additionally, this presents a review on the models used to predict consequences of CC in aflatoxins production and direct losses. Gathering all this information, this chapter will contribute to increase awareness

P. Alvito (✉)
Food and Nutrition Department, National Institute of Health Dr. Ricardo Jorge (INSA), Lisbon, Portugal

Centre for Environmental and Marine Studies (CESAM), University of Aveiro, Aveiro, Portugal
e-mail: paula.alvito@insa.min-saude.pt

R. Assunção
Centre for Environmental and Marine Studies (CESAM), University of Aveiro, Aveiro, Portugal

Comprehensive Health Research Center (CHRC), Lisbon, Portugal

K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_13

about the impact of CC on mycotoxin contamination in foods, providing a particular focus on the aflatoxin consequences.

Keywords Aflatoxins · Climate change · *Aspergillus flavus* · Health · Food safety

13.1 Climate Change and Mycotoxins

Global warming due to climate change is becoming more certain and accepted. As stated by the Intergovernmental Panel on Climate Change,

> "Human influence on the climate system is clear, and recent anthropogenic emissions of greenhouse gases are the highest in history. Warming of the climate system is unequivocal, and since the 1950s, many of the observed changes are unprecedented over decades to millennia. The atmosphere and ocean have warmed, the amounts of snow and ice have diminished, and sea level has rise" (IPCC 2014).

At the Conference of Parties (COP21), which occurred in December 2015 in Paris, negotiators from 195 countries agreed to "pursue efforts to limit the (global average) temperature increase to 1.5 °C above pre-industrial levels," recognizing that this would significantly reduce the risks and impacts of climate change (UNFCCC. Conference of the Parties (COP) 2015).

Projected climate change effects will undoubtedly influence primary agricultural systems, including animal and plant production, and thus food availability (Van Der Fels-Klerx et al. 2016). Global warming and changes in rainfall amount and distribution will probably bring about shifts in the onset and length of growing and in the geographical range of certain crops (Thornton et al. 2014). In Europe, advancements of sowing date, flowering, and maturity of cereals by 1–3 weeks have been projected in response to global warming for 2031–2050, the changes being largest in northern Europe (Olesen et al. 2012). Poleward areas in Asia, Europe, and North America are projected to exhibit an increase climatic suitability for maize production, while a decrease suitability in South America, Africa, and Oceania (Ramirez-Cabral et al. 2017). Within Europe, some crops prevalent in southern regions, due to their temperature requirements, could become viable and productive further north and at higher altitude (Gornall et al. 2010).

Under this context, the European Food Safety Authority (EFSA) has studied the potential impact of climate change (CC) in Europe. EFSA reported that the CC effects will have a regional and detrimental or advantageous depending on geographical region (Battilani et al. 2017). This suggests that in northern Europe the effects may be positive since no significant changes in weather conditions are expected, while the Mediterranean basin may be a hot spot where many effects will be negative, with extreme changes in rainfall/drought, elevated temperatures, and CO_2 impacting on food production. In fact, it has been suggested that CC may be responsible for up to a 1/3 of yield variability in key staple commodities on a global basis (Ray et al. 2015).

The large impacts of global warming projected on crops worldwide will influence not only food security, by reducing yields and thus food availability, but also food and feed safety. From all the potential food safety hazards that could be affected by CC, food contamination by mycotoxins is considered one of the most important factors (Miraglia et al. 2009). Future changes in climate, i.e., temperature and precipitation, and/or in the atmospheric CO_2 concentration, are expected to carry along an increased risk of mycotoxin contamination of cereal crops in the field and might have an impact on the geographical distribution of certain cereals, mycotoxigenic fungi, and their mycotoxins (Medina et al. 2017).

Mycotoxigenic fungi have their own requirements of temperature and humidity for crop infection, mycotoxin production, and survival, which reflects their geographical distribution and determines a gradient of mycotoxin contamination worldwide (Van Der Fels-Klerx et al. 2016). In response to changes in climate as the global warming, some species might shift their geographical distribution, conducting to changes in the pattern of mycotoxin occurrence and crop infection. Occurrence data from international organizations such as the Food and Agriculture Organization (FAO), European Food Safety Authority (EFSA), or World Health Organization (WHO) databases are mainly related to processed food and feed samples and have contributed to risk assessment studies aimed at evaluating risks of human and animal exposure to mycotoxins. With new climatic conditions, new risk maps due to climatic change will need to be elaborated to monitor human exposure derived from CC (Perrone et al. 2020) and prevent associated diseases (Fig. 13.1).

Battilani et al. (2016) predicted that, within the next 100 years, aflatoxin B_1 will become a food safety issue in maize in Eastern Europe, Balkan Peninsula, and the Mediterranean regions, especially under a scenario of +2 °C in air temperature. This would be related to the geographical shift of *A. flavus*, which grows well under warm and dry weather and would move from southern Europe, where those environmental conditions can now sometimes be met, to more northern and eastern regions

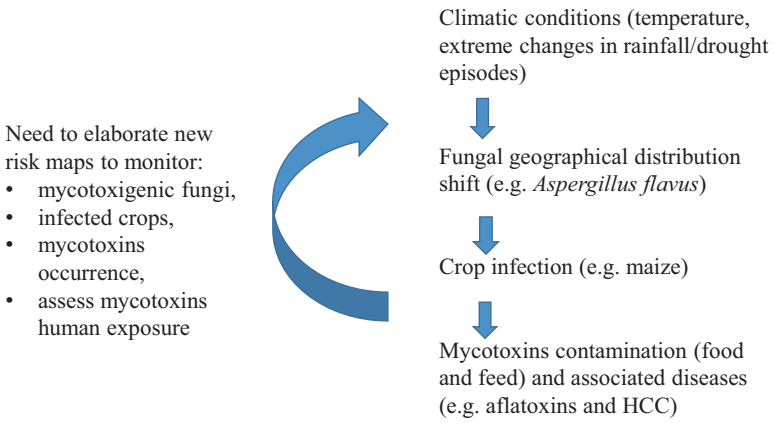

Fig. 13.1 Impact of new climatic conditions on fungal geographical distribution, crop infection, and mycotoxin contamination

below the 45° North latitude, as reported by Medina et al. (2017). It is expected that in the future the number of DALYs (disability-adjusted life years) and the associated cases of disease (viz., hepatocellular carcinoma, HCC) due to aflatoxins exposure, the most hepatotoxic compounds known, will increase due to climate change (Assunção et al. 2018). Human biomonitoring of mycotoxin biomarkers will also be particularly useful to monitor human exposure, assess risks, and identify relationships between diseases and mycotoxins, under CC scenarios (Arce-López et al. 2020; Habschied et al. 2021).

13.2 Impact of Climate on Aflatoxin Production and Food Contamination

13.2.1 Health Consequences of Exposure to Aflatoxins

The groups of mycotoxins of greatest concern to food and feed safety include the aflatoxins (AFs), fumonisins (FBs), ochratoxins (OA), trichothecenes (TCT), and zearalenone (ZEA), which are produced by several genera of filamentous fungi, namely, *Aspergillus*, *Fusarium*, and *Penicillium*. Their associated diseases range from cancers to acute toxicities and to developmental effects (Bennett and Klich 2003; Wu et al. 2014).

AFs have the highest acute and chronic toxicity of all mycotoxins and assume particular importance if considered the potential impact of climate and the health consequences that could be associated (Benkerroum 2019; Dövényi-Nagy et al. 2020; Fouché et al. 2020; Serraino et al. 2019; Taniwaki et al. 2019; Valencia-Quintana et al. 2020). Aflatoxins (AFs) B_1, B_2, G_1, and G_2 are four naturally occurring AFs produced by various strains of *Aspergillus*, mainly by *Aspergillus flavus and A. parasiticus*, but also less frequently in *A. bombycis, A. ochraceoroseus, A. nomius,* and *A. pseudotamari*. AFs are difuranocoumarin derivatives with immunotoxic, mutagenic, and carcinogenic effects and the toxicity is mainly caused by the lactone and the difuran ring. Aflatoxin B_1 (AFB_1) is the most carcinogenic and best-studied aflatoxin. Aflatoxin M_1 (AFM_1) is the 4-hydroxy derivative of AFB_1, formed in the liver and excreted in the milk and the mammary glands of both humans and lactating animals that have been fed with AFB_1 contaminated diet (International Agency for Research on Cancer 2012). In the light of changing weather conditions, it is necessary to continuously monitor the presence of AFs producers in food and feed matrices as well as in the indoor environments where the food/feed is stored, in order to prevent human health deleterious effects related to ingestion of contaminated food and feed (Valencia-Quintana et al. 2020).

13.2.2 Aflatoxins' Worldwide Legislation and Notifications

Recognizing the toxicity of AFs and the threat to human health, legal regulatory limits have been established (JEFCA 2008, 2017). In the EU particularly, the content of AFB_1 is limited to 2 μg/kg in food intended for direct consumption and 5 μg/kg if it is intended for further processing; in Australia, the regulatory limit is 15 μg/kg and applies to total AFB_1 in peanuts and tree nuts; in the USA, AFB_1 in all food crops is limited to 20 μg/kg, while in Brazil to 30 μg/kg; in Japan, AFB_1 is limited to 10 μg/kg (Eskola et al. 2020; JEFCA 2008). Regarding these limits, there are several problems including the fact that they are not harmonized among the countries; the regulation does not apply to the same food/food products (e.g., USA and Japan establish AFs legislation to all food crops, and Australia only for peanuts and tree nuts) and the limits apply to AFB_1 and/or total AFs (e.g., USA sets limits only for AFB_1 and Australia for aflatoxins) (Eskola et al. 2020).

The established legislation reflects the different contamination patterns usually found in specific foods from particular regions. One of the most frequently and heavily contaminated food products are pistachios where the estimated mean concentration of AFs in the time of the last comprehensive evaluation was 54 μg/kg (JEFCA 2008). As an example, food products, e.g., different nuts, spices, cocoa, and dried fruits, from South America and Middle East, often contain AFB_1/AFs; however, the importing countries, especially in the EU, have stricter regulatory limits than the exporting countries, which usually represents a reason of concern. In the latest risk assessment of AFs conducted by EFSA, it was concluded that pistachios, peanuts, other legumes, and seeds should be continuously monitored as food where the contamination with AFB_1 and total AFs is expected (Schrenk et al. 2020).

Mycotoxin contamination results in more notifications than any other chemical hazard in the Rapid Alert System for Food and Feed (RASFF), and the foremost toxins that have been associated with the notifications are AFs, especially in the nuts and nut products (Alshannaq and Yu 2017). RASFF reported 5045 and 439 notifications for mycotoxin contamination in food and feed products, respectively, exported to EU countries from around the world during the years 2010–2019. Among food notifications, 89% (n = 4487) of notifications for mycotoxin contamination were attributed to AFs contamination. The top 10 countries linked to 80% of RASFF mycotoxin notifications on food products were Turkey (32.7%), China (15.1%), India (12.2%), USA (10.7%), Iran (9.5%), Argentina (8.0%), Egypt (4.8%), Brazil (2.6%), Pakistan (1.7%), Nigeria (1.5%), and Ghana (1.3%) (Alshannaq and Yu 2017).

13.2.3 *Aflatoxin Producers and Impact of Environmental Factors*

To understand the impact of CC on mycotoxins production, it is important to understand the optimal conditions for fungal growth and the circumstances under which the mycotoxins are produced. Most of the *Aspergillus* species grow above the 25° latitude north and south, with a high occurrence between 26W° and 35° latitudes, while it is uncommon in latitudes above 45° (Klich 2007). *A. flavus,* main aflatoxin fungi producer, germinates under a wider range of temperature and water activity. Temperatures suitable for growth of *A. flavus* are varying with a minimum from 10 to 12.8 °C, a maximum between 43 and 48.8 °C and an optimum near 33.8 °C were stated. Proper water activity (a_w) for growth are, for instance, 0.82 at 25.8 °C, 0.81 at 30.8 °C, and 0.80 at 37.8 °C (Pitt and Hocking 2009). Optimal temperature and water activity for *A. flavus* growth and AFB_1 production are summarized in Table 13.1 (Sanchís and Magan 2004).

Germination, growth, infection, and toxin production by *Aspergillus* species are determined by relatively few measurable parameters. According to Medina et al., the main driving force of AF production is a combination of three environmental factors: ambient temperature, water activity, and elevated concentration of CO_2 (Medina et al. 2014). While three-way interactions between CO_2 concentration, temperature, and water activity do not have a significant effect on the growth of *A. flavus*, they do stimulate the biosynthesis of phenotypic AFB_1 (Medina et al. 2015). Sudden changes in rainfall/drought patterns and a consecutive humidity in addition to temperature and CO_2 increase directly affect expression of regulatory (aflR) and structural genes (aflD) involved in AF biosynthesis (Medina et al. 2014).

It was also found that the diversity of *Aspergillus* strains is strongly linked to the amount of rainfall and, accordingly, soil wetness and soil temperature (Fouché et al. 2020). Soil temperature and moisture strongly affect soil microbial activity including the growth and distribution of the mycotoxigenic fungi, but they can also modify the host resistance and host-pathogen interactions (Moretti et al. 2019).

Table 13.1 Optimal temperature and water activity (a_w) for *A. flavus* growth and AFB_1 production (Sanchís and Magan 2004)

Fungi	Climatic conditions	Growth	AFB_1 production
A. flavus	Temperature (°C)	35	33
	Water activity (a_w)	0.95	0.99

13.2.4 Impact of Climate Change on Aflatoxin Contamination: Examples of Concern

Aflatoxin outbreaks are most severe in tropical and subtropical areas around the world, with temperate regions also favoring aflatoxin contamination. Until 2004, aflatoxin contamination in Europe was only confined to imported foods such as peanut cake, palm kernel, copra, and corn gluten meal (EFSA Panel on Contaminants in the Food Chain 2004). However, a big survey conducted by the EFSA established the emerging issue of potential aflatoxin contamination of corn, almonds, and pistachios grown in areas of southern Europe due to the subtropical climate which had occurred in the recent years (EFSA Panel on Contaminants in the Food Chain 2007). A shift in traditional occurrence areas for aflatoxins is therefore to be expected due to the increasing average temperatures. In this respect, the Mediterranean area has been identified as a climate change hot spot where extreme changes in temperature, CO_2 levels, and rainfall patterns are predicted. Regarding AFs, their contamination events are more prevalent during times of high heat and drought, which may stress the host plant and thereby facilitate *A. flavus* infection (Marasas et al. 2008). In fact, until a few years ago, aflatoxins had not been signaled as a matter of concern for primary production in Europe. However, in 2003, 2012, and 2015 several reports from Southern-Eastern Europe countries identified an alarming contamination in maize (Battilani et al. 2017; Logrieco 2008).

High AF contamination in maize is often associated with weather conditions, as the preceding period was extremely warm and dry and was characterized by a very low average rainfall. Significant colonization by *Aspergillus* section *Flavi* and widespread contamination by AFs in maize was recorded for the first time in Italy in 2003, and consequently, in milk (Giorni et al. 2007; Piva et al. 2006). As reported at Table 13.2, during the years 1996, 1999, and 2003, and considering 3 sites in northern Italy (eastern, central, and western areas), temperatures ranged from 19 to 23 °C,

Table 13.2 Meteorological data and AFB_1 maize grains contamination (μg/kg) reported in Northern Italy region, associated with *A. flavus* episode in 2003 (adapted from (Piva et al. 2006; Camardo Leggieri et al. 2015))

EU region	Year	1996	1999	2003	2009	2010	2011	Reference
Northern Italy	**Meteorological data (summer)**							Piva et al. (2006), Camardo Leggieri et al. (2015)
	Mean T (°C)	19	21	23	26	23	25	
	Mean R (mm)	235	235	98	27/4[a]	74/69[a]	0	
	AFB_1 contamination							
	% Pos	57		75	96	77	59	
	Mean	1.7		4.4	35	16	10	
	Max	>20		154.5	560	213	335	

T (temperature), R (total rainfall), % Pos (% positives), Max (maximum)
[a]Data from different regions in northern Italy

precipitation was 235 mm for 1996–1999 and less than half in 2003, and AFB_1 contamination was detected in 57% of grain samples in 1996–1999 and in 75% in 2003 (Piva et al. 2006). Later, during the period from 2009 to 2011, mean temperatures increased (23–26 °C), mean rainfall decreased (74–0 mm), and AFB_1 contamination increased (59–96% positive samples), with a maximum of 560 μg/kg in 2009 (Camardo Leggieri et al. 2015).

During 2003 episode, *A. flavus* was able to colonize ripening maize by outcompeting the more common *Fusarium* species (Giorni et al. 2008; Magan et al. 2011). Drought and extreme elevated temperatures (>35 °C) resulted in a change from *Fusarium verticillioides* and contamination with fumonisins of maize, to *A. flavus* and AFs (Giorni et al. 2007). Reduced sporulation occurred at dry conditions of ≤0.90 water activity (aw) and *A. flavus* can even grow at 0.73 aw and produce aflatoxins at 0.85 aw, while *F. verticillioides* growth is low at 0.90 aw and fumonisins produced at >0.93 aw (Medina et al. 2015).

Due to the extreme weather conditions in 2012 in Central Europe, aflatoxin contamination of maize and milk caused serious problems in Serbia (Kos et al. 2013), Croatia (Pleadin et al. 2014), and Macedonia (Dimitrieska-Stojković et al. 2016). AFs were also detected in maize kernels in Hungary after harvest in 2012 (Dobolyi et al. 2013). In 2013, a large shipment of maize from East Europe, which was distributed to feed producers in Germany and the Netherlands, was found to be contaminated with AFB_1. Dairy farms in Germany and the Netherlands were affected as well, since AFM_1 was found in the milk at farms that used compound feed produced from the AFB_1 contaminated maize, and a major recall started (Van der Fels-Klerx et al. 2019).

13.3 Modelling the Effects of Climate Change Future Scenarios on Mycotoxin Contamination

The prediction of the consequences of CC on the mycotoxin contamination contribute with data that could anticipate the associated challenges of the future climate scenarios. Mathematical modelling, through, e.g., predictive modelling of fungal growth or mycotoxin production under specific conditions, despite important, has not been widely explored, needing further work and research. Concerning aflatoxins, Table 13.3 summarizes the reported exercises already performed concerning the modelling of the future consequences that climatic changes will uncover.

The main efforts already performed were dedicated to explore the effects that different atmospheric conditions, i.e., air temperature, humidity, rain, and precipitation, could exert on the fungal growth and mycotoxin production (Abdel-Hadi et al. 2012; Battilani et al. 2016; Camardo et al. 2019; Milicevic et al. 2019). Different approaches in terms of mathematical modelling were applied, including the multiple linear and nonlinear regression models. The mechanistic model AFLA-maize (Battilani et al. 2016) was developed specifically to predict the *Aspergillus flavus*

Table 13.3 Models used to predict consequences of climate change in aflatoxins production and direct losses

Type of modelling	Applied model	Modelled factors	Model output	Reference
Predictive modelling – Contamination levels	Mixed-growth-associated product formation model	Temperature Water activity Growth rate	Aflatoxin production	Abdel-Hadi et al. (2012)
Predictive modelling – Contamination levels	Mechanistic model, AFLA-maize	Air temperature Relative humidity Rain Maize growth stage	Aflatoxin index (probability of overcoming the threshold of 5 μg of toxin per kg of kernels at harvest)	Battilani et al. (2016)
Predictive modelling – Contamination levels	Multiple linear regression analysis	Precipitation on a monthly level, average monthly temperature and humidity	Aflatoxin M_1 prevalence	Milićević et al. (2019)
Predictive modelling – Fungal growth and contamination levels	Nonlinear regression model of Bete-Analytis	Temperature (minimum, maximum)	Fungal growth/ mycotoxin production	Camardo et al. (2019)
Model chain	Forecasting model AFLA-maize (simplified)	Maize flowering and harvest dates: Estimated through Temperature sum Weather data (maximum air temperature (°C), minimum air temperature (°C), mean air temperature (°C), sum of precipitation (mm/day) and vapor pressure (hPa))	Aflatoxin level in maize	Van der Fels-Klerx et al. (2019)
	Carryover model	Aflatoxin level in maize Aflatoxin levels in compound feed ingredients (except maize) Compound feed composition Milk yield	Aflatoxin level in milk	

(continued)

Table 13.3 (continued)

Type of modelling	Applied model	Modelled factors	Model output	Reference
Economic impact estimation	Model 1: Accounting for variability in type 1 (false positive) and type 2 (false negative) errors associated with mycotoxin testing	Model 1: Amount of bushels of corn harvested; ratio of corn within a specified USFDA regulatory range; probability of acceptance; state of interest; USFDA action level range of interest	Direct losses (incurred by the farmers and grain elevators; not include estimates of losses to the livestock industry or costs incurred for mycotoxin prevention)	Mitchell et al. (2016)
	Model 2: Accounting for a partial equilibrium economic analysis assuming no type 1 or type 2 errors	Model 2: Production; amount of bushels of corn harvested; ratio of corn within a specified USFDA regulatory range; average price received per bushel of corn; amount discounted from the base price; state of interest; USFDA action level range of interest		

growth and aflatoxin production in maize, using weather data as input. This model was also linked to a crop phenology prediction module, which considers temperature sums, focusing the crucial stages of flowering and ripening and the date of harvest. As output, AFLA-maize calculates the Aflatoxin Risk Index (ARI) which corresponds to the probability of overcoming the threshold of 5 μg of toxin per kg of kernels at harvest (Battilani et al. 2016). This approach was additionally applied as a forecasting model under the model chain developed by Van der Fels-Klerx (2019). This model considers a sequence of data and models that are chained together (forecasting model – AFLA-maize – and carryover model) in which the outputs obtained in the model will be the input to the other model. Taking into account the inputs variables, climate conditions were considered to anticipate the contamination of maize by AFs (forecasting model), and consequently these data were used to predict the AFM_1 concentrations in milk in the Netherlands (carryover model).

In addition to the predictive modelling, economic impact estimation was also performed, anticipating the direct losses incurred by the farmers and grain elevators due to market loss resulting from aflatoxin contamination (Mitchell et al. 2016). The considered model did not include the estimates of livestock industry losses or costs incurred for mycotoxin prevention. The probability of acceptance based on the operating characteristics curves for aflatoxin sampling and testing and the partial equilibrium economic analysis, assuming Type 1 or Type 2 errors, to estimate losses due to aflatoxins levels above allowed limits, were applied under this context.

Unrelated with the type of models or the variables used as inputs, these exercises unanimously concluded that expected CC will impact the food contamination by AFs, presenting a wide array of consequences, affecting directly humans and animals which will be exposed to and indirectly the economy, which will be affected due to (direct and indirect) market losses. Thus, modelling exercises constitute important tools that should be further developed and implemented, contributing to define adequate mitigation and adaptation strategies in order to tackle the main consequences of CC.

13.4 Conclusions and Future Perspectives

If in the past the CC phenomena and their impact posed some doubts and questions, currently this problem is unquestionable and could not be ignored anymore. The impact of CC on health due to the reduction of access to safe foods should be adequately tackled, and mitigation and adaptation strategies have been identified and are being implemented, despite needing extra efforts and urgent actions.

Under the context of mycotoxins present in foods, ideally, the food contamination by these toxins must be avoided as much as possible. However, complete prevention is not feasible since mycotoxins are natural contaminants of foods, which pose particular difficulties to avoid completely contaminating foods. Consequently, measures of surveillance and monitoring of human and animal exposure, the contamination of foods, and the environment, including the atmospheric indicators that could provide conditions to fungi growth and mycotoxins production, require particular attention from governments and scientists. Some tools, e.g., predictive modelling, could establish important contributions to anticipate the impact of future CC scenarios. Good storage and agricultural practices should be defined, communicated, and implemented to reduce the probability of contamination, and consumers should be engaged and informed about these often neglected food hazards, supported by robust scientific evidence.

This is a moment for action to avoid the consequences of CC. Mycotoxins present in foods represent a significant and worrying problem that should not be neglected; otherwise the health of current and next generations will be significantly compromised.

Acknowledgments Work co-funded through Portuguese national funds: FCT/MCTES through CESAM (UIDP/50017/2020 + UIDB/50017/2020) and earlyMYCO (PTDC/MED-TOX/28762/2017).

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Chapter 14
Aflatoxins: A Brief Summary

Zubair Ismail, Kashif Akram, Iqra Naeem, Carlos Augusto Fernandes de Oliveira, Amir Ismail, Ambreen Fazal, and Arif Shahzad

Abstract Aflatoxins are the highly toxic secondary metabolites of fungal species of *Aspergillus* origin, particularly of *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins are present in a number of food commodities, especially cereals, spices, dry fruits, and milk and milk products. Aflatoxins are classified as group 1 category carcinogenic compound by the International Agency for Research on Cancer (IARC) and are also hepatotoxic, immunosuppressant, growth retardant, teratogenic, and mutagenic. Based on the severe health implications of aflatoxins, countries across the world have established maximum permissible limits/regulations for aflatoxins in different foodstuffs. Prevalence of aflatoxins in a number of food commodities and its highly toxic nature have compelled the researchers across the world to explore safe, reliable, and commercially implementable methods for the removal/degradation of aflatoxins.

Keywords Aflatoxins · Food commodities · Carcinogenic · Regulations · Safe · Removal · Degradation

Z. Ismail · I. Naeem · A. Ismail (✉) · A. Fazal · A. Shahzad
Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan, Pakistan
e-mail: amirismail@bzu.edu.pk

K. Akram
Department of Food Science, Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Pakistan

C. A. F. de Oliveira
Department of Food Engineering, School of Animal Science and Food Engineering, University of São Paulo, Pirassununga, SP, Brazil

K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_14

14.1 Aflatoxins: A Brief Summary

The availability, quality, and safety of food are the burning issues of modern life, especially due to the rapid increase in the world population and uncertainty in world food production due to climate changes. Mycotoxins are the toxic secondary metabolites naturally produced by certain fungal species that contaminate a wide array of feed and food crops. Food and feed contamination gives rise to severe health implications for both humans and animals and has the potential to negatively affect the world economy. The Food and Agriculture Organization of the United Nation (FAO) estimated that mycotoxins may cause contamination of around 25% of the world's crop; however, this figure has been recently shown to rise up to 60–80% (Eskola et al. 2020).

Aflatoxins are the most important mycotoxins that are produced by fungal strains of the *Aspergillus* genus, particularly *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*. For the first time, aflatoxins were discovered after an epidemic known as "Turkey X syndrome" in which more than 0.1 million turkeys in England unexpectedly died in the year 1960. The turkeys unveiled symptoms of severe poisoning and the death of these animals happened within days or weeks. Later, it was discovered that the source of this toxic exposure was peanut meal contaminated with metabolites of *A. flavus* which were then named "aflatoxins" (toxins from *A. flavus*). Members of three sections of *Aspergillus* genus, namely, *Flavi*, *Ochraceorosei*, and *Nidulantes*, have been reported to produce aflatoxins (Richard 2008). More than twenty different types of aflatoxins have been identified so far, and among them aflatoxin B_1 (AFB_1), aflatoxin G_1 (AFG_1), aflatoxin B_2 (AFB_2), and aflatoxin G_2 (AFG_2) are the most toxic as these compounds significantly contributed to the contamination of food and feed consumed by humans and animals in the various regions of the world (Pildain et al. 2008). The International Agency for Research on Cancer (IARC) has categorized these toxins, particularly AFB_1 and the mixture of these four aflatoxin's types (termed as total aflatoxins) as carcinogens of group 1 category. Besides carcinogenicity, aflatoxins have also been reported to be teratogenic, immunosuppressant, growth retardants, and embryotoxic (IARC 2012; Ismail et al. 2018).

Chemically, aflatoxins are difuranocoumarins comprised of a coumarin nucleus attached to furan and lactone and are categorized into two groups, namely, difurancoumarin-cyclo-pentanones (consists of AFB_1, AFB_2, AFM_1, and AFM_2), and difuran-coumarin lactones (consists of AFG_1 and AFG_2). The members of the B series fluoresce blue under ultraviolet (UV) light, while the members of the G series fluoresce green under UV light with the fluorescence emitted at around 400 nm and 500–550 nm, respectively. While, on the other hand, AFM_1 and AFM_2 are derived from B types of aflatoxins through various metabolic processes and are commonly found in the milk of animals and humans. Aflatoxins are soluble in organic solvents such as chloroform, methanol, and benzene. These are stable at a temperature higher than 100 °C but are susceptible to UV radiation (Benkerroum 2020; Cole and Kirksey 1971; Ferreira et al. 2019).

The toxicological properties of aflatoxins have been well studied in both humans and animals. The health impacts of aflatoxins are affected by various factors including dose and duration of exposure, age, gender, ethnic group, species, individual's health status, and exposure to other toxic substances. The toxic potencies order of four major types of aflatoxins is $AFB_1 > AFG_1 > AFB_2 > AFG_2$. Aflatoxin B_1 is the most toxic aflatoxin of which toxicity is 68 times of arsenic and 10 times of potassium cyanide (Liao et al. 2020; Li et al. 2009). AFM_1 is ten times less toxic than AFB_1; however, it has also been categorized as a carcinogen of group 1 category by IARC (Nasir et al. 2021).

The biosynthesis of aflatoxins is structured by at least 53 genes comprising of 12 negative regulatory genes, 39 positive regulatory genes, and two other regulatory genes (afrmtA and nmrA). Among 53 regulatory genes, two genes (aflR and aflS) are present in the aflatoxin gene cluster, while the remaining genes are physically unrelated. Among thirty-nine positive regulatory genes, aflS and aflR encode the pathway-specific regulators while the thirty-seven positive regulatory genes and 12 negative regulatory genes encode some proteins which are engaged in signal transduction, transcriptional regulation, development and morphogenesis, and post-translational modification (Chang et al. 2011; Liao et al. 2020; Roze et al. 2013). The biosynthesis of aflatoxins consists of several enzymatic steps. In the beginning, nine acetyl units of acetyl CoA and malonyl CoA are converted into hexanoyl CoA by the action of fatty acid synthase. The product is then converted into norsolorinic acid (1st stable precursor in aflatoxin biosynthesis) by polyketide synthase. After that, a series of enzymatic conversions results in the formation of averantine, hydroxy averantine, averufanin, averufin, versiconal hemiacetal acetate, versiconal, and versicolorin B. By this process, versicolorin B is converted into dihydro-dimethyl sterigmatocystin by O-methyl transferase enzyme and later converted into O-methyl sterigmatocystin and dihydro-O-methyl sterigmatocystin and ultimately AFB_1 (Liao et al. 2020; Trail et al. 1995).

The biosynthesis of aflatoxins is affected by various biological factors (such as cultivar, soil type, viable fungal species in the soil, and plant metabolites), physiological factors (such as culture pH, developmental stage of crop, and oxidative stress), nutritional sources (such as amino acids, carbon, nitrogen, lipids, and trace elements), environmental factors (such as topography, climate, weather, temperature, water activity, drought, rainfall), and agricultural factors (such as sowing time, tillage, crop rotation, irrigation, and application of fertilizers) (Iqbal et al. 2019).

The toxicity of aflatoxins in both humans and animals depends on bioactivation and detoxification. In the liver, AFB_1 is bioactivated to short-lived highly reactive and electrophilic exo- and endo-8,9 epoxide (AFBO) by the liver cytochrome p450 enzyme system. AFBO covalently reacts with DNA and protein to form adducts that mediate the acute and chronic toxicity of cells and cause DNA mutation leading to tumor development. Elseways, AFBO can be metabolically detoxified by a phase II detoxifying enzyme known as glutathione S transferases (GSTs) which catalyze the conjugation of AFBO with glutathione (GSH). Vulnerability to AFB_1 induced toxicity is linked with the metabolic fate of AFB_1. AFB_1 metabolism is divided into four major pathways, i.e., epoxidation to the most toxic AFBO, hydroxylation to a mildly

toxic AFM_1, and relatively nontoxic AFQ_1 or AFB_{2a}, keto-reduction to moderately toxic aflatoxicol (AFL), and demethylation to nearly nontoxic AFP_1. These different AFB_1 metabolic pathways primarily depend on various CYP450 isozymes which vary among different species. The toxicity to AFB_1 within the species also depends on the species-specific concentration of liver GST isozymes, and in humans, it is linked with polymorphisms of genes encoding these enzymes (Deng et al. 2018).

Aflatoxins can be found in a variety of food and feed commodities worldwide, particularly in cereals, animal forage, oilseeds, spices, nuts, and dried fruits. Furthermore, the toxin and its derivatives have also been found in milk, meat, and eggs of animals fed on aflatoxin-contaminated diets (Ismail et al. 2018). Aflatoxin production and contamination of food with aflatoxigenic fungi can occur in any step of the food production chain such as in the field, during processing, transportation, and storage. Groundnuts and maize are the major sources of aflatoxins exposure in humans and are consumed in substantial quantities by a large number of people worldwide, mainly in low- and middle-income countries in tropical or subtropical regions (Chen et al. 2018).

Humans are exposed to aflatoxins through consumption of aflatoxin-contaminated agricultural commodities or animal products such as milk and meat from animals previously fed on aflatoxin-contaminated diet. Agricultural workers and farmers are also exposed to aflatoxins through inhalation and dermal route; however, food is the major route of exposure to aflatoxins (Phillips et al. 2008; Turner et al. 2003). Human exposure to aflatoxins can be estimated by measuring both external and internal exposure; however, estimation of external exposure by analytical quantification of aflatoxin in the diet does not represent the exact exposure as the amount detected in the raw food commodities is not certainly equivalent to the quantity ingested. Therefore, human biomonitoring is the best approach to more precisely assess the degree of aflatoxin exposure as the biomarkers used in biomonitoring are non-subjective and can better estimate the internal and biologically effective dose (Alves et al. 2014; Qian et al. 1994). Aflatoxin biomarkers that are presently in use include aflatoxin-albumin adducts (AF-alb) or aflatoxin-lysine (AF-lys) adducts in plasma or serum (with a half-life of around 2 months allowing assessment of chronic aflatoxin exposure), aflatoxin N^7 guanine adduct (AF-N^7 gua) in urine (reflecting the over previous 24-hour exposure), and aflatoxin M_1 (primarily in breast milk and urine reflecting the previous day exposure) (Kensler et al. 2011; Leong et al. 2012).

Aflatoxins are related to various illnesses, such as aflatoxicosis in both humans and animals around the globe, and are considered to be mainly detrimental to health as they have mutagenic, carcinogenic, immunosuppressive, and teratogenic effects. Acute aflatoxicosis in humans is symptomized as abdominal cramps, vomiting, pulmonary edema, liver necrosis, fatty liver, convulsions, and even death in severe cases (Dhanasekaran et al. 2011). It is estimated that more than 4.5 billion people of the world are under the burden of aflatoxin exposure (Williams et al. 2004). Chronic dietary aflatoxin exposure has been associated with the development of hepatocellular carcinoma or liver cancer which is the 3rd leading cause of cancer-related deaths worldwide. Studies have reported that aflatoxin exposure interacts synergistically with hepatitis B and C viral infection leading to development of tumor due to

alteration at codon 249 in regions of T53 gene. T53 gene is the chief site for AF-DNA adduct formation and is accountable for the development of liver cancer (Cai et al. 2020; Zhang et al. 2017). In children, aflatoxin exposure is linked with low birth weight, neonatal jaundice, growth retardation, suppression of the immune system, as well as mental illnesses. Besides posing serious public health issues, aflatoxin contamination of food also presents a significant economic hurdle in low- and middle-income countries, particularly in Africa and Asia whose trade balance is dependent on cereals such as maize, rice, and peanuts (Ladeira et al. 2017).

Considering the toxicological aspects, various countries have established tolerable limits for aflatoxins in food and animal feed commodities. These set limits of aflatoxins in foods vary based on the type of food, type of aflatoxin, and technical feasibility, and a country's economic status, ranging from 0.025 to 100 ppb. Whereas the maximum permissible limit for aflatoxins in animal feed is much higher, reaching beyond 300 ppb. Owing to the genotoxic and carcinogenic potency of aflatoxins, the most acceptable scale in the formulations of standards for aflatoxins, as proposed by FAO, is set up based on "As Lowest as Reasonable Acceptable" (ALARA) concept. Food that does not meet the set regulations for human consumption is either utilized for less profitable purposes (animal feed), or, in case of too high levels, it may be discarded completely, thus leading to higher annual losses in the agricultural sector (Ismail et al. 2018; Rushing and Selim 2019).

In developed countries, human and animal exposure to aflatoxins is controlled primarily through monitoring and surveillance. Various analytical procedures have been established to identify and quantify the levels of aflatoxins in various food commodities. The key steps include extraction, cleanup, and quantification. To lessen the quantification error, two different procedures can be applied to quantify the same toxin, or collaborative testing procedures can be operated which need extensive planning in terms of trial design, the matrix type, the contamination level of the toxin of interest, and the number of samples. Generally, analysis of aflatoxins in food and feed samples is performed using enzyme-linked immunosorbent assays (ELISA), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), fluorescence spectrophotometry (FS), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Omara et al. 2020). However, these methods are laborious, expensive, and time-taking and cannot be employed for on-time detection of aflatoxin contamination. Nowadays, emerging technologies such as optical-based methods are gaining interests owing to their rapid and nondestructive methods of sensing the contamination of aflatoxin or aflatoxigenic fungi and are suitable for online and real-time application. Optical-based measurement techniques are based on hyperspectral imaging (HSI), fluorescence spectroscopy (FS), and near-infrared spectroscopy (NIRS) and have provided promising outcomes for the assessment of aflatoxin and fungal contamination in varieties of food commodities (Tao et al. 2018).

Since aflatoxin exposure can result in chronic, acute, and subacute toxicity in both humans and animals, considerable emphasis has been engrossed on the control or removal of aflatoxigenic fungi and/or their toxic metabolic products. Aflatoxin control can be aimed at two levels along the food supply chain, including preharvest

and postharvest levels. Preharvest control mainly focuses on the prevention of toxin production by eliminating or preventing the growth of aflatoxigenic fungi. Preharvest aflatoxin mitigation strategies consist of good agricultural practices such as pest control, correct application of fungicides, implementation of crop rotation program, proper treatment of the seedbed, use of genetically resistant cultivars, control of seed feeding and pod insects, avoiding mechanical damage during and after crop harvesting, and biological control. Biological control is a novel preharvest measure in which non-toxigenic strains of fungi are applied to the field crop that competes and displaces the toxigenic fungal strains thus resulting in aflatoxin reduction. Postharvest mitigation strategies focus on rapid postharvest drying and controlled environmental conditions during storage and transportation (Marshall et al. 2020). The combination of GAPs and the maintenance of proper storage conditions is used to reduce the potential for aflatoxin contamination; however, these approaches have been shown to assure the complete removal of aflatoxigenic fungi and/or aflatoxins. Also, most of the prevention strategies are only applicable in economically developed countries and cannot be adopted in resource-poor countries because of some economic constraints and unfavorable climatic conditions. Therefore, the techniques to decontaminate or detoxify the aflatoxins are further required to control the risk of aflatoxins in food and feed. Physical, chemical, and biological methods are the three major approaches for the decontamination of aflatoxins. All of these approaches, by modulating and disrupting the structure, can inhibit or reduce its transfer to the digestive system and could minimize their availability to the target site (Peles et al. 2021).

Physical methods for aflatoxins decontamination aimed at either eliminating the toxin or infected part from the food commodities and/or degrading the toxin within the food matrix. The removal of aflatoxins at initial postharvest stages can be achieved by separation of infected kernels mostly based on detection of fluorescence, discoloration, or presence of fungus on the surface of contaminated grain. Aflatoxins from oilseeds or their flour can be physically eliminated by the solvent extraction method by the use of a various combinations of organic solvents such as methanol, water, acetonitrile, acetone, isopropanol, and hexane. Physical removal of aflatoxin can also be achieved by the use of adsorbents, which can bind and inactivate the toxin. The most commonly used adsorbent materials are organic adsorbents (such as bacterial and yeast cell wall extracts and plant fibers) and mineral adsorbents (including activated charcoal, clay, diatomaceous earth, hydrated sodium calcium aluminosilicate, bentonite, zeolites, fuller's earth,). Physical methods for degradation of aflatoxins involves heat treatments (extrusion cooking, roasting, high-pressure cooking), and irradiation treatment such as UV radiation, gamma irradiation, near-infrared irradiation, pulsed electric field, pulsed light treatment, nonthermal or cold plasma treatment, use of electrolyzed water, and ultrasound waves (Ismail et al. 2018; Pankaj et al. 2018; Peles et al. 2021).

Chemical methods of aflatoxin degradation are based on the degradation of its molecular structure through chemical compounds including oxidizing agents (such

as hydrogen peroxide and ozone), hydrolytic agents (such as aldehydes, acids, and bases), plant extracts, and different types of gases. Among organic and inorganic acids, lactic acid, citric acid, propionic acid, tartaric acid, and hydrochloric acids proved to be more effective than ascorbic acid, acetic acid, succinic acid, and formic acid that were reported to be slightly successful. Among bases, sodium hydrosulfite, sodium hydroxide, sodium hypochlorite, potassium hydroxide, calcium hydroxide, and sodium sulphate have been reported to be useful chemicals in the degradation of aflatoxins (Ismail et al. 2018; Peng et al. 2018). Among various gases, the use of ozone is reported to be the most successful method for degradation of aflatoxins, mainly AFB_1 and AFG_1, as there is a double bond at the C8–C9 position in their chemical structure which is highly sensitive to ozonation. Chemical treatment of food contaminated with aflatoxins resulted in the conversion of aflatoxin into its less toxic form. For example, treatment with lactic acid resulted in the conversion of AFB_1 into AFD_1, due to hydrolysis of terminal lactone ring (Jubeen et al. 2020). Similarly, citric acid treatment of AFB_1 resulted in the formation of β-keto acid followed by the formation of AFD_1 due to decarboxylation of the lactone ring. Toxicity assessment revealed 18-fold lesser mutagenic activity of AFD_1 than AFB_1 (Méndez-Albores et al. 2005). However, the strategy may leave the residues of chemical substances in the food matrix, therefore questioning the safety of chemical procedures for aflatoxin's degradation.

Biological methods are gaining popularity among researchers and consumers because of successful outcomes presenting efficient decontamination of aflatoxins. The use of biological agents such as yeast, molds, bacterial strains, and their enzymes is a cost-effective and eco-friendly approach that does not leave any undesirable residues in the food. Moreover, biodegradation methods do not affect the nutritional and sensory properties of food (Mir et al. 2021; Peles et al. 2021). Several bacterial strains such as *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus rhamnosus, Lactobacillus reuteri, Lactobacillus casei,* (Chlebicz and Śliżewska 2020; Tajalli et al. 2016), *Streptomyces cacaoi, Streptomyces luteogriseus, and Streptomyces rimosus* (Harkai et al. 2016); yeast cells such as *Saccharomyces cerevisiae* (Bueno et al. 2007), *Candida utilis* (Jakopović et al. 2018), *Phanerochaete chrysosporium, and Pleurotus ostreatus* (Wang et al. 2011); and microbial enzymatic metabolites such as lactoperoxidase (Karim and Kamkar 2000), peroxidase (Zaid 2017), reductase (Li et al. 2019), and manganese peroxidase (Yehia 2014) have been reported to biologically degrade or adsorb the aflatoxins. The degradation of mycotoxins through microorganisms follows two pathways: first, mycotoxins may adsorb through structures in the cell wall (such as peptidoglycans and polysaccharides) of the microorganism, thus leading to detoxification. Secondly, microorganisms metabolize mycotoxins and convert them into less toxic metabolites (Ismail et al. 2018; Mahato et al. 2021). The adsorption potential of some microorganisms can be used for enterosorption to reduce aflatoxin bioavailability in the intestinal tract of animals (Fochesato et al. 2019).

14.2 Recommendations for Management of Aflatoxin Control and Related Health Risks

The prime objective of scientific research on aflatoxins is to develop effective means to reduce the health risk they cause to humans and animals. Despite massive work that has been done to alleviate the occurrence of these toxins in the food and feed items since their discovery, they are extensively distributed at high concentrations in nature and continue to elevate serious public health issues and economic losses. Besides, aflatoxins are one of the major hurdles to international trade of agricultural commodities, particularly between developing and industrialized nations. Measurements to improve the situation can be initiated either at the food and feed levels by lowering their contamination and so the dietary exposure (primary prevention) and/or at the consumer level by implementing strategies to hinder or prevent the onset of illnesses particularly in individuals or populations at high risk (secondary and tertiary prevention) (Benkerroum 2020).

Effective management of aflatoxin contamination of food and feed to minimize human exposure to aflatoxin or mitigate aflatoxin-related health risk is a complicated process that needs multi-sectoral, integrated, scalable, and adequately resourced control programs including preharvest and postharvest prevention and/or reduction strategies, post-contamination management of aflatoxins, proper monitoring and surveillance, and awareness of aflatoxins. Sourcing information from various research and review articles, this section of the book provides an extensive review of the intervention strategies to mitigate the aflatoxin-induced health risks, and the need for further investment in the area of aflatoxin research.

Primary prevention involves preharvest intervention strategies aimed at avoiding or preventing fungal growth and aflatoxin production. This prevention level needs considerable and effective planning for reducing the production of aflatoxins while keeping the conditions unfavorable for the growth of aflatoxigenic fungi. The practices must include development of cultivars genetically resistant to fungal infestation, timely harvesting, avoiding drought stress, providing sufficient plant nutrition, ensuring adequate weed control, controlling the attack of insects and pests, providing enough plant nutrition, seedbed treatment, tillage and ploughing, using biological control agents such as preservatives and fungicides against fungal growth, managing proper crop rotation, and following appropriate harvesting practices (Bediako et al. 2019; Mannaa and Kim 2017).

Secondary and tertiary preventions are required if the fungal contamination of agricultural commodities starts at the early stages. The measures adopted (postharvest interventions) aimed at either completely removing the aflatoxigenic fungi and aflatoxins from the commodities or reducing them to the level that cannot cause any health hazards to the consumers. Such measures must include removal of contaminated grains or seeds; maintaining postharvest environmental conditions such as optimum temperature and humidity; re-drying the commodities after harvesting; protecting the stored commodities from any conditions favorable for continuing fungal growth; application of proper food processing practices such as washing,

dehulling, crushing, and milling as per recommendations of international food safety standards; and inactivation or decontamination of aflatoxins in aflatoxin-contaminated foods (Bediako et al. 2019; Mannaa and Kim 2017).

The role of regulatory bodies in the development of functioning food safety system is significant and cannot be neglected. The regulatory authorities must make sure that the aflatoxin content in the food commodities available in the market is within the set regulatory limits. For this purpose, proper monitoring and surveillance system should be maintained at both the national and international levels. Such information may help in evaluating control efforts and the development of climate models to enhance the better and earlier estimation of aflatoxin levels (Achaglinkame et al. 2017; Kademi et al. 2019).

Aflatoxin management requires creating awareness across the board from "farm to folk" about reducing aflatoxin exposure and its health concerns by targeting agricultural, dietary/nutritional, and health education mainly in the at-risk population. Researchers, agriculturalists, and medical professionals should work actively and collaboratively to create public awareness on the health risks of aflatoxins and their control measures in order to reduce the aflatoxins' prevalence and incidence of disease (Achaglinkame et al. 2017). The public awareness campaigns will also be helpful in extending the scientific information to the general public for vast personal and national development. In addition to the agricultural interventions described above, dietary/nutritional interventions are also significant, particularly in resource-poor countries where aflatoxin contamination is widespread due to poor infrastructure of the agriculture sector as well as favorable climatic conditions for fungal growth and aflatoxin production, and controlling and/or eradicating aflatoxin production is not possible at initial stages of crop production and processing. Moreover, the implementation of expensive technologies for decontamination of aflatoxin is difficult in such countries. Therefore, dietary modifications are the only option for saving the health of people from at-risk communities. Such practices may include promoting consumption of diversified foods; choosing food rich in probiotics and chemopreventive agents such as fermented foods, fruits, and vegetable; selecting those staples which are relatively at lower risk of aflatoxin contamination (such as millet, oats, rice, sorghum, beans, etc.) instead of those that are more prone to the attack of aflatoxigenic fungi and toxin production (such as maize and groundnut); and optimal food practices (such as washing before cooking, boiling/cooking in plenty of water, extrusion/high-pressure cooking, roasting, baking, and nixtamalization/lime treatment) (Negash 2018; Visser et al. 2020).

14.3 Future Perspectives

The regulation of aflatoxin production is an intricate process and is still far from being understood completely. Currently, it is recognized that regulation of aflatoxin biosynthesis is not only based on pathway-specific genes but also on the global regulators that are capable of controlling aflatoxin biosynthesis due to modulation

of gene expression at a transcriptional level. Therefore, the knowledge of these regulatory pathways will be an opportunity for the development of cost-effective and efficient control methods to reduce aflatoxin contamination in food commodities (Liao et al. 2020). Future research should focus on biocontrol strategies to reduce the levels of aflatoxins in food and feed at both preharvest and postharvest, while more effort should be made on the production of cultivars that are genetically resistant to the infection of *Aspergillus* species and aflatoxin production.

Most of the chemical and physical methods for aflatoxins degradation (discussed in this book) such as the use of chemical agents and irradiation treatment show good perspectives for minimizing the dietary aflatoxins exposure; however, these may affect the nutritional composition of food. The biological methods for aflatoxins decontamination also showed good perspectives, but practical implementation of these methods is still in infancy, and at present, these methods cannot be adopted for food and feed on a commercial level. Therefore, studies to understand the mechanism behind the biological detoxification of aflatoxins are required. Also, practical aspects of the application of these procedures in food items, primarily pertaining to their effect on sensory attributes of foods, need to be explored. Aflatoxin decontamination strategies when applied to feed and food products must be multidimensional as there is no single approach that can be applied universally to deal with the emerging problem. Also, these strategies must be proven not to cause any changes to the nutritional and organoleptic properties of food. Moreover, appropriate toxicity testing of the treated products should be performed to ensure that any secondary degradation products formed are harmless to human and animal health (Ismail et al. 2018; Mwakinyali et al. 2019).

The conventional methods for detection and quantification of aflatoxins based on immunology and chromatographic principles are widely studied and used but are time-taking and costly and require skilled personnel to be employed. Therefore, these methods are not easily reachable to developing nations where the incidence of aflatoxins is high. Hence, the development of low-cost, easy-to-use, and reliable procedures to meet the requirements of small and medium enterprises of food and feed processors and smallholder farmers is urgently required in developing countries so that the information generated can be used for determination of exposure and performing health risk assessment at both individual and population levels (Tumukunde et al. 2020). Moreover, the analytical procedures that can simultaneously identify and quantify a broad number of mycotoxins, their metabolites, and their degradation products in food with low limits of detection and quantification are required to minimize the operational costs and to allow more recurrent evaluation of mycotoxins in food (Iqbal et al. 2015).

Understanding the role and diversity of the key metabolic isozymes involved in the metabolism of aflatoxins will facilitate in establishing novel nutritional interventions to prevent aflatoxin-attributable health risks. Researchers should also focus on developing the mathematical models that can be used in assessing the removal of aflatoxins in various food commodities and to analyze the effectiveness of different control measures mainly designed for detoxification of aflatoxins from the food chain (Deng et al. 2018).

Most of the studies suggesting various intervention strategies to reduce the dietary exposure of aflatoxin have not reported the feasibility and cost-effectiveness of these interventions. Hence, studies focusing on feasibility and cost of various aflatoxin control interventions (both at pre- and postharvest levels) are urgently needed to assist public health decision makers and other stakeholders to appreciably administer resources in low- and middle-income countries (Visser et al. 2020). In conclusion, it is the need for the hour to adopt the novel aflatoxin decontamination and detection techniques to embark on the vision of achieving food and feed safety and security around the world.

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