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Natural Products for Preventing and Controlling Aflatoxin Contamination of Food

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Abstract

Aflatoxins are the most potent naturally occurring toxin and liver carcinogens known and their contamination of food is a significant risk factor for human health. Conventional chemical and physical approaches have been insufficient to eliminate aflatoxins from food, and the application of synthetic compounds can give rise to notable drug resistance and serious environmental and health problems. Awareness of these problems has led to an urgent need to identify safer alternative strategies. There are various natural compounds that influence aflatoxin contamination of food in different ways, including by inhibiting the growth of aflatoxigenic fungi, blocking aflatoxin biosynthesis, and removing or degrading aflatoxins. These inhibitors, many of which have shown great potentials for the control of aflatoxin contamination, have great promise for the development of new approaches to combatting aflatoxin contamination, and are capable of replacing or complementing conventional strategies. While more and more natural inhibitors are being identified, the modes of action of most of these are poorly understood. Further studies are necessary to better understand the mechanism of action of these compounds before their widespread commercial use. The objective of this chapter is to present the results of studies of the control of aflatoxin contamination using natural products.

Keywords: aflatoxin, natural products, antifungal, antiaflatoxigenic, detoxification

1. Introduction

Aflatoxins are a group of toxic secondary metabolites synthesized by fungi of the *Aspergillus* species, particularly *A. flavus* and *A. parasiticus* [1]. They are the most widely distributed soil-borne molds on earth and are capable of surviving on many organic nutrient sources, including stored grains and fodders, dead plants, insect and animal carcasses, and even immunocompromised humans and animals [2]. When grown under appropriate conditions, these fungi exist in the form of mycelia or conidia (asexual spores), while under adverse conditions such as a lack of nutrients or water, their fungal mycelium will transform to resistant structures known as sclerotia, which can survive in extremely harsh environmental conditions [3]. Aflatoxin-producing fungi affect many agricultural crops such as rice, corn, wheat, peanuts, and chilies. Pre- and post-harvest contamination of these crops with aflatoxins is common and annually causes great economic loss [4, 5].

Aflatoxins were first identified in 1960 in England as the cause of the Turkey X disease [6]. There are four major aflatoxins produced in nature: B1, B2, G1, and G2. They are named based on their fluorescence under ultraviolet light, and their relative mobility in thin-layer chromatography on silica gel. Most *A. flavus* produce aflatoxins B1 and B2, whereas *A. parasiticus* produce aflatoxins B1, B2, G1, and G2. Aflatoxin M1 is another frequently detected aflatoxin in nature; it is a hydroxylated derivative metabolized by cows from aflatoxin B1 and secreted in milk [7].

Aflatoxins are the most potent naturally occurring toxins and liver carcinogens known, and their contamination of food is a significant risk factor for human health, particularly in developing countries that lack detection, monitoring, and regulating measures to safeguard the food supply. It has been reported that approximately 4.5 billion people living in developing countries are chronically exposed to uncontrolled amounts of aflatoxins [7]. Long-term low-dose dietary exposure to aflatoxins is also a major risk for hepatocellular carcinoma. Aflatoxins have been designated as human liver carcinogens by the International Agency for Research on Cancer [8]. Therefore, the control and elimination of aflatoxigenic fungi and aflatoxins in food have great significance. To minimize potential exposure to aflatoxins, maximum levels of aflatoxins have been established by different countries [9]. The U.S. Food and Drug Administration specified a maximum of 20 ppb total aflatoxins for interstate trading of food and feedstuffs and 0.5 ppb aflatoxin M1 in milk. The European Commission has set the limits on cereals and derived products at 4 ppb for total aflatoxins and 2 ppb for aflatoxin B1, and for nuts and dried fruits subject to further processing at 10 ppb for total aflatoxins and 5 ppb for aflatoxin B1. The Korea Ministry of Food and Drug Safety imposed limits for aflatoxin B1 of 10 ppb and total aflatoxins of 15 ppb.

Chemical and physical approaches are widely used to minimize the risk of aflatoxin contamination of food. These are usually focused on inhibiting the development of spores and mycelia, and/or inactivation of aflatoxins by their transformation to nontoxic compounds. The most common methods include the use of synthetic fungicides, X-radiation, dehulling or cooking processes, and control of environmental factors during harvest and storage [10, 11]. These strategies are usually expensive, time-consuming, and inefficient. Some of them also

cause major changes in the physical properties of food and a serious loss of nutritive value and therefore are inappropriate to eliminate aflatoxins from food [12]. Synthetic chemicals are still the most widely used recourse to prevent fungal contamination of food crops. However, there are strict regulations on chemical compound use in food, and there is political pressure to remove hazardous chemicals from the market [13]. In addition to these limitations, the application of synthetic fungicides can also give rise to notable drug resistance and serious environmental and health problems [14]. Awareness of these problems has led to an urgent need to identify safer alternative strategies.

Natural products are chemical compounds or substances produced by a living organism, and their use as biocontrol agents provides an opportunity to avoid synthetic fungicides. Over the years, efforts have been made to identify new antifungal materials from natural sources for controlling aflatoxin contamination of food [15]. Many bacteria, fungi, and plants that share ecological niches with and encounter aflatoxigenic fungi have the ability to synthesize compounds that inhibit aflatoxin synthesis or remove aflatoxins from food without significant losses in nutritive value; they therefore could be used to replace or complement conventional strategies. Basically, there are three possible ways of using natural products to avoid the harmful effects of aflatoxin contamination of food and feed: (1) prevent and control aflatoxigenic fungus contamination (fungal growth inhibition), (2) inhibit aflatoxin biosynthesis (aflatoxin production inhibition), and (3) decontamination of aflatoxin-containing food and feed (aflatoxin detoxification). These microbial metabolites and plant constituents are natural products and therefore are desirable for use in food because they can be easily degraded in nature. A variety of naturally derived compounds have been studied for their antifungal and antiaflatoxigenic activities, many of which have shown great potential for controlling aflatoxin contamination. The objective of this chapter is to present the results of studies of the control of aflatoxin contamination using natural products from bacteria, fungi, and plants.

2. Fungal growth inhibition

Fungi of *Aspergillus* sp. are the only source of aflatoxin contamination in food. The production of aflatoxin is greatly dependent on the growth condition of *Aspergillus* fungi; thus, it can be suppressed effectively through inhibit fungal growth. Many compounds produced by bacteria, fungi, and plants are possessed of abilities to inhibit fungal growth at different levels, such as inhibit conidia production and germination, interrupt membrane formation, or damage cell membrane, and disrupt fungal mitochondria (**Table 1**).

The use of bacteria is a promising solution to alleviate fungal contamination in food. In recent years, the study and application of antifungal bacteria has received strong interest. Significant progress has been reported on the isolation and characterization of antifungal compounds. Various bacterial compounds including organic acids, phenyllactic acids, reuterin, and cyclic dipeptides, proteinaceous compounds, and fatty acids have been reported to be able to inhibit the growth of aflatoxigenic fungi (**Table 2**).

Target of action	Antifungal product	Source	Activity against	Reference	
Conidia production and/or germination	<i>Aegle marmelos</i> essential oil	<i>A. marmelos</i> (leaves)	<i>Alternaria</i> sp., <i>Colletotrichum</i> sp., <i>Curvularia</i> sp., <i>Ustilago</i> sp.	[16]	
	Aldehydes (C6)	Plants ¹	<i>Alternaria</i> sp., <i>Botrytis</i> sp.	[17]	
	(E)-Anethole, p-anisaldehyde, carvacrol, (-)-carvone, 1,8-cineole, (+)-limonene, myrcene, (±)- α -phellandrene, (±)- α -pinene	Plants	<i>Botrytis</i> sp., <i>Monilinia</i> sp., <i>Mucor</i> sp., <i>Penicillium</i> sp., <i>Rhizopus</i> sp.	[18]	
	Benzaldehyde	Plants	<i>Monilinia</i> sp., <i>Botrytis</i> sp.	[19]	
	Chitosan	Plants	<i>Botrytis</i> sp., <i>Rhizopus</i> sp.	[20]	
	Fusapyrone	<i>Fusarium semitectum</i>	<i>Botrytis</i> sp.	[21]	
	1-Octen-3-ol	<i>P. paneum</i>	<i>Penicillium</i> sp.	[22]	
	Terpenoid	<i>Nasutitermes</i> sp.	<i>Metarhizium</i> sp.	[23]	
	Membrane formation and/or integrity	Brefeldin A	<i>Eupenicillium brefeldianum</i>	<i>Pisolithus</i> sp.	[24]
		Carvacrol, thymol	Plants	<i>Candida</i> sp.	[25]
Clove essential oil		<i>Syzygium aromaticum</i>	<i>Candida</i> sp., <i>Aspergillus</i> sp. dermatophyte fungi	[26]	
Defensins		Plants	<i>Neurospora</i> sp., <i>Saccharomyces</i> sp.	[27]	
Eugenol, methyl eugenol		Plants	<i>Candida</i> sp.	[28]	
Geraniol		Plants	<i>Candida</i> sp., <i>Saccharomyces</i> sp.	[29]	
<i>Ocimum sanctum</i> essential oil		<i>O. sanctum</i>	<i>Candida</i> sp.	[30]	
Osmotin		Tobacco	<i>Aspergillus</i> sp., <i>Rhizoctonia</i> sp., <i>Macrophomina</i> sp., <i>Bipolaris</i> sp., <i>Fusarium</i> sp., <i>Phytophthora</i> sp., <i>Trichoderma</i> sp.	[31]	
Phytochemicals		<i>Thymus vulgaris</i> L.	<i>Rhizopus</i> sp.	[32]	
Zeamatin		<i>Zea mays</i>	<i>Candida</i> sp., <i>Neurospora</i> sp., <i>Trichoderma</i> sp.	[33]	

Target of action	Antifungal product	Source	Activity against	Reference
Cell organelles function	Cruentaren	<i>Byssovorax cruenta</i>	<i>Candida</i> sp., <i>Metschnikowia</i> sp., <i>Saccharomyces</i> sp., <i>Rhodotorula</i> sp., <i>Botrytis</i> sp., <i>Mucor</i> sp., <i>Rhizopus</i> sp.	[34]
	Defensin	Plants	<i>Candida</i> sp.	[35]
	<i>Anethum graveolens</i> essential oil	<i>A. graveolens</i> L.	<i>Aspergillus</i> sp.	[36]
	Haliangicin	<i>Haliangium luteum</i>	<i>Aspergillus</i> sp., <i>Botrytis</i> sp., <i>Fusarium</i> sp., <i>Mucor</i> sp., <i>Pythium</i> sp., <i>Saprolegnia</i> sp.	[37]
	Phytoalexins	<i>Musa acuminata</i>	<i>Cladosporium</i> sp., <i>Pyricularia</i> sp., <i>Plasmopara</i> sp., <i>Sphaeropsis</i> sp.	[38]
	Plagiochin E	<i>Marchantia polymorpha</i> L.	<i>Candida</i> sp.	[39]
	Pyrrolnitrin	<i>Burkholderia cepacia</i>	<i>Streptomyces</i> sp.	[40]
	<i>Tagetes patula</i> essential oil	<i>T. patula</i> L.	<i>Botrytis</i> sp., <i>Penicillium</i> sp.	[41]
	UK-2A, UK-3A	<i>Streptomyces</i> sp.	<i>Saccharomyces</i> sp.	[42]

¹ Natural product exists in different plants.

Table 1. Natural products against fungal growth.

Strain	Activity against	Inhibitory compound	Target of action	Reference
<i>Amorphophallus campanulatus</i> (Roxb)	<i>A. flavus</i>	Amblyone	Unknown	[43]
<i>Bacillus subtilis</i> AU195	<i>A. flavus</i>	Bacillomycin D	Conidial germination	[44]
<i>B. pumilus</i>	<i>A. parasiticus</i>	Cyclic polypeptide	Unknown	[45]
<i>B. subtilis</i> KS03	<i>A. flavus</i> , <i>A. parasiticus</i>	Iturin A	Cell surface hydrophobicity	[46]
<i>B. subtilis</i> YM 10-20	<i>A. flavus</i>	Iturin-like compound	Conidial germination	[47]
<i>Humicola fuscoatra</i> NRRL 22980	<i>A. flavus</i> .	Monorden, monocillin IV, cerebrosides	Unknown	[48]
<i>Lactobadillus casei</i> subsp. <i>pseudoplantarum</i>	<i>A. flavus</i>	Proteinaceous	Unknown	[49]
<i>Lactococcus lactis</i> ATCC 11454	<i>A. flavus</i>	Heat-stable low-molecular weight compounds	Unknown	[50]

Strain	Activity against	Inhibitory compound	Target of action	Reference
<i>Lb. casei</i> subsp. <i>pseudoplantarum</i>	<i>A. flavus</i>	Proteinaceous	Unknown	[51]
<i>Lb. casei</i> subsp. <i>pseudoplantarum</i> 371	<i>A. parasiticus</i>	Proteinaceous	Unknown	[52]
<i>Lc. lactis</i> subsp. <i>diacetylactis</i> DRC1	<i>A. flavus</i>	Proteinaceous	Conidial germination	[53]
<i>Lc. lactis</i> subsp. <i>lactis</i> CHD28.3	<i>A. flavus</i> , <i>A. parasiticus</i>	Proteinaceous	Unknown	[54]
<i>Pseudomonas aeruginosa</i> K-187	<i>A. flavus</i> , <i>A. parasiticus</i>	Pafungin	Hyphae lysis	[55]
<i>Streptomyces</i> sp. DPTB16	<i>A. flavus</i>	4'-Phenyl-1-naphthyl-phenyl acetamide	Unknown	[56]
<i>Streptomyces</i> sp. MRI 142	<i>A. parasiticus</i>	Aflastatin	Unknown	[57]
<i>S. albidoflavus</i> ANU 6277	<i>A. flavus</i>	3-Phenylpropionic acid	Unknown	[58]

Table 2. Antifungal compounds against aflatoxigenic fungi growth.

Many bacteria produce organic acids such as lactic, acetic, and propionic acids. The production of these weak organic acids results in an acidic environment that generally restricts the growth of both bacteria and fungi [59]. Phenyllactic acid has been widely reported to have antifungal activities, and its broad-spectrum antibacterial and antifungal action makes it one of the most extensively studied antifungal organic acids derived from bacteria. Over the last decade, a number of studies have identified phenyllactic acid as the causative agent of antifungal activity. Its lack of toxicity to both animals and humans body, and its lack of any smell make phenyllactic acid a potential candidate for the control of food spoilage [60]. In addition, phenyllactic acid can also play a synergistic role with other metabolites [61, 62]. Reuterin is another antifungal compound produced by bacteria. This low-molecular-weight compound has also been reported to possess broad-spectrum antimicrobial activity. It has been demonstrated to be capable of inhibiting the growth of a wide range of molds including *A. flavus* [63]. Some fatty acids produced by bacterial strains have also received great attention for their antifungal properties. For example, 2-hydroxy-4-methylpentanoic acid produced by *Lactobacillus plantarum* VE56 and *Weissella paramesenteroides* LC11 is thought to act in synergy with other inhibitory metabolites and was shown to cause growth arrest in *Aspergillus* species [64]. Peptides inhibiting fungal growth have also been isolated from some bacterial strains. For example, Garofalo et al. demonstrated the existence of a series of peptides responsible for the antifungal activity of *Lb. rossiae* LD108. These peptides induced a clear delay in fungal growth on different bakery products, and were shown by Matrix assisted laser desorption/ionization time-of-flight mass spectrometric analysis to cause gluten proteolysis [65].

Fungal metabolites have also been used to reduce aflatoxin contamination in various crops. A recent study showed that culture filtrates of *Trichoderma* spp. at 200 mL/kg showed 72–93% inhibition of mycelial growth of *A. flavus* [66]. Nakaya reported the production of a small basic

antifungal protein by the mold *A. giganteus* [67], and thoroughly characterized the structure of this protein as a highly twisted β -barrel stabilized by four internal disulfide bridges, which resembles the structure of some antifungal polypeptides produced by plants, such as defensins and thionins [68]. Similar proteins with high sequence homology have been described in other fungi, such as *Aspergillus niger* and *Penicillium chrysogenum* [69]. It is possible that the production of these antifungal proteins provides the producer with a competitive advantage against other fungal strains in the same environment.

Plants lack an immune system and must depend on other mechanisms to defend themselves against fungal invaders. One such mechanism is the synthesis of bioactive compounds that act specifically to inhibit fungal growth. Many plant extracts, particularly essential oils, have been reported to possess significant antifungal activity. An extract of *Azadirachta indica* was observed to be a good inhibitor of the growth of both *A. flavus* and *A. parasiticus in vitro* [70], and the oil from *Ocimum canum* exhibited activity against a broad range of fungi, including aflatoxin-producing fungi [71]. Several peptides and proteins are also associated with the antifungal activity of plants. Huang et al. reported that the grains of Tex6 wheat contain zeamatin, a thaumatin-like protein belonging to the PR5 group of pathogen related proteins, which inhibits the growth of *A. flavus* [72]. Chen et al. identified a 14-kDa protein that was present in resistant maize genotypes but in only a very low concentration in susceptible genotypes. This protein was identified as a trypsin inhibitor that also inhibited conidial germination and hyphal growth of *A. flavus*. Further studies showed that this protein inhibits the α -amylase from *A. flavus* [73]. Chitin is a common constituent of fungal cell walls. All organisms that contain chitin also contain chitinases (EC 3.2.1.14), which are presumably required for morphogenesis of cell walls [74]. Other organisms that do not contain chitin may produce chitinases to degrade the polymer for food. Plants have also been found to contain chitinase. Because plants do not contain chitin in their cell walls, it has been postulated that they produce chitinase to protect themselves from chitin-containing parasites including fungi [75]. Roberts and Selitrennikoff reported the isolation of a chitinase from barley grain that acting alone could inhibit fungal growth [76]. The antifungal activity of bacterial chitinases was also investigated, because plant and bacterial chitinases differ markedly in their antifungal activity, and this difference in biological activity correlates with differences in their substrate specificities [77]. Seeds of many plants contain high concentrations of chitinases, glucanases, and ribosome-inactivating proteins that may help protect seeds and seedlings from fungal infection [78]. One study showed that a maize chitinase preparation was highly active and caused a 100-fold reduction in the minimum dose of nikkomycin required to inhibit fungal growth [79]. Careful analysis of the maize preparation revealed several proteins and several enzyme activities. Further study revealed that zeamatin, a 22-kDa protein, is responsible for this synergistic activity, and showed that zeamatin exerts its antifungal effects by damaging fungal membranes [33].

Antifungal peptides and proteins have also been found in insects. Cecropins, originally isolated from the immune hemolymph of the *Cecropia moth*, are a key component of the immune response in insects. They have been shown to possess strong inhibitory activity against fungal growth. It has been reported that the structural features of the cecropins include a strongly basic N-terminus, an intermediate hinge region containing glycine and/or proline, and a

hydrophobic C-terminus, which are all necessary for its lethal activity. Studies suggested that the fungicidal activity of cecropins is mediated by the formation of pores across cell membranes that lead to leakage of cytoplasmic contents and ultimately to cell death [80]. In addition, Powell et al. found that one kind of cecropin, cecropin B, can inhibit fungal growth by suppressing the germination of fungal conidia [81]. Consequently, these peptides have been studied for engineering fungal disease resistance in plants.

3. Aflatoxin production inhibition

One important side effect of fungal growth inhibition is the rapid spread of resistant strains. Therefore, inhibitors of aflatoxin production may be a better choice for control and prevention of aflatoxin contamination of food. Current methods to control aflatoxin contamination are mainly based on chemical strategies (pesticides and fungicides). However, the excessive use of chemical treatments has many undesirable consequences: (1) marked pollution of the environment, (2) an increase in resistant pathogen populations, and (3) the presence of chemical residues in food. Specific microbial metabolites and plant constituents have been shown to be effective inhibitors of aflatoxin production without significantly affecting fungal growth; in fact, numerous compounds and extracts possessing inhibitory activity for aflatoxin biosynthesis have been reported. However, tools and techniques have only recently become available to investigate the molecular mechanisms by which these inhibitors regulate aflatoxin biosynthesis [82].

Microbially derived inhibitors of aflatoxin production are of practical use because of their strong activity and the possibility of large-scale production. For example, a number of *Lentinula edodes* isolates are able to inhibit aflatoxin production and the isolate CF42 shows significant inhibitory activity. This effect is probably the result of the presence in the extracts of a number of different compounds with different inhibitory strategies. This is supported by the results obtained with fractionation of *L. edodes* CF42 filtrates, which leads to a decrease in their inhibitory effect but not to a complete loss of effect. Reverberi and coworkers also reported that culture filtrates of *L. edodes* isolate CF42 are able to inhibit aflatoxin production. *L. edodes* is able to release and accumulate lentinans and other β -glucans in the culture media. A recent study reported a direct relationship between aflatoxin inhibition and the β -glucan content of lyophilized *L. edodes* filtrates, suggesting that β -glucans could be amongst the factors responsible for their inhibitory effect on aflatoxins [83]. The ability of fungal β -glucans to act as free radical scavengers was recently shown in animal models [84]. *In vivo* research showed that glucans and glycoproteins extracted from fungi protect macrophages from the damage caused by lipoperoxide accumulation, mainly by activating the transcription of genes related to the macrophage antioxidant system [85]. Because the molecular analyses carried out on *A. parasiticus* mycelia treated with CF42 filtrates showed a significant activation of hsf2-like transcription factors of the fungal antioxidant system, a similar effect on macrophages could be hypothesized. It could be suggested that culture filtrates of *L. edodes* interfere with the cascade of signals that allows aflatoxin biosynthesis. It has also been hypothesized that accumulated β -glucans in the culture filtrates of *L. edodes* are able to inhibit aflatoxin production by *A. parasiticus* through the enhancement of the internal antioxidant system.

Lyophilized filtrates of *L. edodes* could be applied alone or in association with other food-grade compounds, to prevent aflatoxin production in food and feed [86]. In addition, the polysaccharides of this basidiomycete have low cytotoxicity for animal cells and could contribute to the nutritive value of the food or feed supplemented with these extracts [87].

Plant-derived inhibitors of aflatoxin production have great potential because not only are they highly effective but also the genes responsible for their biosynthesis could be transferred into susceptible host plants to create transgenic plants that resist aflatoxin contamination by *in situ* production of aflatoxin production inhibitors. For example, gallic acid is an effective plant-derived compound that inhibits aflatoxin production by *A. flavus* and disrupts expression of early and late aflatoxin biosynthesis genes. Evidence suggests that its aflatoxin production inhibitory activity may correlate with its strong antioxidant activity [82]. Transgenic plants with elevated levels of gallic acid that suppress aflatoxin production have been created [88]. Many essential oils obtained from parts of higher plants have also been shown to possess antiaflatoxic properties [89]. Various individual and combined plant extracts have been evaluated for their efficacy against aflatoxin production *in vitro*. For example, *Satureja hortensis* L. essential oil was found to inhibit production of aflatoxin B1 and G1 by *A. parasiticus*. The aflatoxin-production inhibitors were separated using reverse-phase high-performance liquid chromatography and finally identified as carvacrol and thymol. Further testing revealed that both carvacrol and thymol were able to effectively inhibit production of aflatoxin B1 and G1 in a dose-dependent manner [90].

In principle, there are three possible ways to inhibit aflatoxin biosynthesis (**Figure 1**). First, there can be alteration of the physiological environment or disturbance of the signaling inputs perceived by the fungus. For example, eugenol is a major phenolic component of essential oils extracted from cloves, cinnamon, and nutmeg. It has been shown in multiple experiments to inhibit aflatoxin biosynthesis. Evidence suggests that eugenol inhibits aflatoxin biosynthesis by lowering the physiological requirement for the enzymes activities involved in responding to oxidative stress. Eugenol treatment of fungi growing on Potato Dextrose Agar plates has been shown to result in the reduction of enzyme activities (glutathione peroxidase, microsomal reductases, superoxide dismutase, and xanthine oxidase) involved in responding to oxidative stress, concomitant with the inhibition of aflatoxin production by up to 50% [91]. Zingerone is another plant-derived aflatoxin inhibitor isolated from certain parts of *Zingiber officinale* or *Amomum melegueta*. Zingerone has a greater effect on aflatoxin biosynthesis than on fungal growth. Kim et al. found that zingerone, at a concentration of 5 mM, reduced aflatoxin production to 11% of the control with little reduction in fungal growth. They also found that yeast mutants with increased sensitivity to mitochondrial oxidative stress were more susceptible to combined H₂O₂ and zingerone treatment than a wild-type strain [92]. This result indicates that the antiaflatoxic activity of zingerone may be attributed to its alteration of the mitochondrial function in aflatoxin-producing fungi. Flavonoids and isoflavonoids are also inhibitory to aflatoxin production, but most are active only at high concentrations. In an early study, flavonoids (eriodictyol and luteolin) isolated from peanut shells were tested for their effects on aflatoxin production by *A. flavus* and *A. parasiticus*. Eriodictyol showed considerable inhibition of aflatoxin production with minimal influence on fungal growth, while luteolin was much more potent against *A. parasiticus* (IC₅₀ < 0.35 mM) than against *A. flavus*

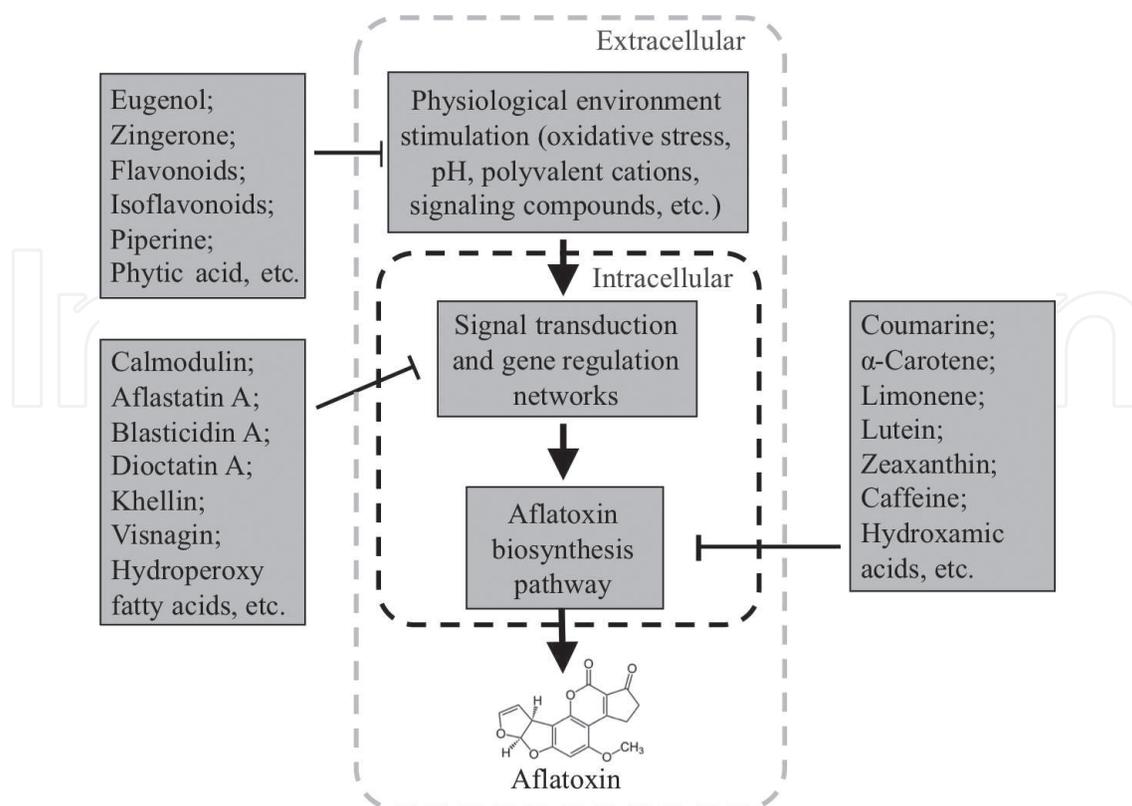


Figure 1. Schematic representation of aflatoxin production inhibition by natural products at different levels.

($IC_{50} \sim 6$ mM). The authors suggested that the differences in the responses of *A. flavus* and *A. parasiticus* to luteolin may be caused either by differences between the two fungi or by the culture conditions [93]. In fact, *A. flavus* and *A. parasiticus* are known to respond differently to oxidative stress. It has been reported that some oxidizing agents such as cumene hydroperoxide stimulate aflatoxin biosynthesis in *A. parasiticus* but not in *A. flavus* [94]. Glyceollin is a soybean isoflavonoid that has promise for engineering aflatoxin resistance in plants. It has been reported that 20 μ M glyceollin strongly inhibited aflatoxin production in a high-glucose liquid medium, and the authors also speculated that glyceollin contributes to the resistance of soybean to aflatoxin contamination [95], although the mechanism by which glyceollin inhibits aflatoxin biosynthesis is uncertain. However, as a natural plant defense compound with a known biosynthetic pathway, glyceollin is particularly promising for the construction of aflatoxin resistant plants. Other biflavonoids have also been tested for their antiaflatoxic activity. Gonçalez et al. found that some of the major biflavonoids isolated from *Ouratea* species had excellent inhibitory activity at micromolar concentrations, reducing aflatoxin B1 production to <30% of the control at approximately 9 μ M [96]. The antiaflatoxic effects of flavonoids and isoflavonoids might result from their antioxidant activity, because recent studies showed that aflatoxin production was closely related to the peroxidation of the fungal cell and several antioxidants have been reported to strongly inhibit aflatoxin production [97]. More recently, a study further underlined the importance of the role played by oxidative stress in the fungal cell in aflatoxin biosynthesis [98]. However, it has not been definitively demonstrated whether antioxidants work by direct interaction with reactive species or

by the stimulation of the fungal cell antioxidant system. Furthermore, the molecular basis of the relationship between cell antioxidant defenses and aflatoxin formation is not yet fully understood. Information about the intracellular mechanism that leads to aflatoxin synthesis could be useful to achieve control over aflatoxin production. Piperine, a natural constituent found in many pepper species, inhibited aflatoxin production by *A. parasiticus* without obvious reduction in fungal growth. Piperine possesses direct antioxidant activity against various free radicals which may be related to its antiaflatoxic activity [99]. Phytic acid is an abundant component of seeds that can act as a chelator of polyvalent cations, especially zinc. The effect of phytic acid on aflatoxin production by *A. parasiticus* strongly depends on the pH of the medium: it was reported that 14.3 mM did not inhibit aflatoxin production at $\text{pH} \leq 4.5$, but when the pH was about 6.6, phytic acid strongly inhibited aflatoxin production [100]. Regulation of aflatoxin production by phytic acid is attributed to its chelation of zinc and other polyvalent cations. It is also a natural antioxidant, and this antioxidant activity may also contribute to its antiaflatoxic properties by inhibiting iron-catalyzed free radical production and lipid peroxidation [101].

The second way to inhibit aflatoxin biosynthesis is to interfere with the signal transduction networks or gene expression regulation of aflatoxin biosynthesis by, for example, using calmodulin inhibitors, most of which are alkaloid and peptide compounds that have been isolated from a wide variety of natural sources, including many plant species [102]. Multiple lines of evidence support the idea that calcium-dependent signaling plays an important role in the regulation of aflatoxin biosynthesis [103]. Calmodulin-binding domains have been identified in the primary sequences of aflatoxin pathway transcriptional regulators (AflR and AflJ) and biosynthetic enzymes, presenting the possibility that calmodulin may influence transcriptional regulation of the aflatoxin biosynthesis gene cluster [104]. Aflastatin A and blasticidin A are well-known microbial-derived aflatoxin inhibitors. They are structurally related compounds produced by *Streptomyces* sp. that strongly inhibit aflatoxin production in *A. parasiticus* [57]. It has been reported that aflastatin A and blasticidin A inhibit the biosynthesis of important intermediates of aflatoxins (e.g., norsolorinic acid) and the transcription of aflatoxin biosynthetic genes [105]. Even though their mode of action is unknown, it was suggested that this inhibition is probably a result of perturbations in primary metabolism [106]. It has been reported that aflastatin A significantly enhances glucose utilization and the accumulation of ethanol in fungal cells. The level of transcription of genes for aldehyde dehydrogenase and acetyl-CoA synthetase, which are involved in ethanol utilization, was also suppressed by aflastatin A [105]. Diocatin A is another antibiotic isolated from *Streptomyces* sp. that inhibits both conidiation and aflatoxin biosynthesis in *A. parasiticus*. Diocatin A treatment also reduced the expression of AflR and aflatoxin biosynthesis genes. The molecular target of diocatin A has not yet been identified, although it was suggested that diocatin A inhibits aflatoxin biosynthesis through the FadA heterotrimeric G-protein signaling cascade [107]. Khellin and visnagin, products of the plant *Ammi visnaga*, were tested on *A. flavus*. Both showed potent inhibitory activity ($\text{IC}_{50} < 0.1 \text{ mM}$) for aflatoxin production [108]. Khellin and visnagin are pharmacological agents that can inhibit cyclic adenosine monophosphate (cAMP) phosphodiesterases inhibitory activity, and cAMP has been shown to influence aflatoxin production [109]. Hydroperoxy fatty acids from plants, including methyl jasmonate

(MeJA), 9S-hydroperoxy-trans-10,cis-12-octadecadienoic acid (9S-HPODE), 13S-hydroperoxy-cis-9,trans-11-octadecadienoic acid (13S-HPODE), and 13S-hydroperoxy-cis-9,trans-11,cis-15-octadecatrienoic acid (13S-HPOTE), can mimic fungal signal factors and could potentially interact with G-protein-coupled receptor complexes upstream of the heterotrimeric G-protein complex that has been shown to regulate aflatoxin production [110]. Plant hydroperoxy fatty acids have varied effects on aflatoxin production. For example, MeJA significantly inhibited aflatoxin production by *A. flavus* on agar plates with an $IC_{50} < 10$ nM, but stimulated aflatoxin production by *A. parasiticus* after 7-day incubation in YES medium [111]. 13S-HPODE and 13S-HPOTE, at 100 μ M, eliminated aflatoxin production by *A. parasiticus* in A&M medium, whereas 9S-HPODE slightly increased or decreased aflatoxin production, depending on the concentration tested [111]. Burow and coworkers reported that, in *in vitro* experiments, 13S-hydroperoxy fatty acids at concentrations of 10 and 100 μ M repressed aflatoxin pathway gene expression and significantly reduced aflatoxin production in *A. parasiticus*. It has also been reported that treatment with 1 μ M 13S-hydroperoxy linoleic acid significantly decreased aflatoxin production when it was repeatedly added to growth media at 24-h intervals. However, the same concentrations of 9S-hydroperoxy linoleic acid did not reduce aflatoxin production. These results suggested that specific seed lipoxygenase activity could provide resistance to mycotoxin contamination by *Aspergillus* sp. [112].

The third way to inhibit aflatoxin biosynthesis is to block the activity of aflatoxin biosynthesis-related enzymes. For example, coumarins have been found to strongly inhibit aflatoxin production without causing significant reductions in fungal growth [108]. It has been suggested that structural similarities between these coumarone and aflatoxins may result in competitive inhibition of biosynthetic enzymes. Terpenoids are a major class of natural products synthesized in plants through the mevalonic acid pathway. There are reports that different terpenoids, including camphene, α -carotene, limonene, lutein, and zeaxanthin, are inhibitory to aflatoxin biosynthesis in solid or liquid media [113]. The inhibition by terpenoids may occur at the level of whole-pathway regulation. For example, α -carotene was found to be able to block the synthesis of norsolorinic acid, the first stable aflatoxin precursor, thereby preventing the accumulation of subsequent pathway intermediates. Caffeine is another well-studied inhibitor of aflatoxin production, with studies showing that decaffeinated coffee beans and powder support higher aflatoxin production than normal beans and powder, and that incorporation of coffee into growth medium at concentrations of 1% (w/v) inhibits total aflatoxin production by 25%, with no significant reduction in fungal growth. The inhibitory effect of caffeine on glucose uptake is considered to be the possible mode of action for its antiaflatoxigenic activity [114]. Hydroxamic acids, such as 6-methoxy-benzoxazolin-2-one (MBOA), are also strong inhibitors of aflatoxin biosynthesis. MBOA significantly inhibits α -amylase induction [115]. It was suggested that the perturbation of sugar utilization by MBOA might be the major reason for its antiaflatoxigenic activity.

4. Aflatoxin detoxification

Aflatoxins are extremely stable under most conditions encountered during food storage, handling, and processing. Therefore, preventing contamination with aflatoxigenic fungi is the most

rational and economic approach for controlling aflatoxin contamination of food. However, detoxification of aflatoxin is required for food already contaminated with aflatoxin. Although various methods have been described for detoxification of aflatoxins in foods, the most commonly used physical and chemical approaches are usually high cost or complex processes, and many also result in nutrient loss and food safety issues.

Biological detoxification of aflatoxins by employing natural products has been shown to be very effective in removing aflatoxin from food. In principle, there are four possible biological approaches to avoid the toxic effects of aflatoxins on the human body: (1) remove aflatoxins through surface adsorption to bacterial or fungal cells; (2) transform aflatoxins into nontoxic compounds by enzymatic degradation; (3) introduce aflatoxin adsorbents into contaminated food and feed to bind the toxins and inhibit their absorption from the gastrointestinal tract; and (4) metabolize aflatoxin into relatively nontoxic compounds via different metabolic pathways (**Figure 2**).

Aflatoxin detoxifying microorganisms were first demonstrated in 1996, when Ciegler et al. identified a *Flavobacterium aurantiacum* strain. In their research, they also found that both growing and resting cells of *F. aurantiacum* could remove aflatoxin from contaminated milk, oil, peanut butter, peanuts, corn, and soybeans. The detoxification was found to be irreversible with no new toxic products being formed [116]. Lillehoj et al. found that while both living and dead cells of *F. aurantiacum* were capable of removing aflatoxin from solution, aflatoxins removed by living cells could not be recovered while toxins removed by dead cells were simply adsorbed to the cell walls [117]. Line and Brackett also found that the degradation of aflatoxin B1 by *F. aurantiacum* was independent of the nutrients in the culture medium, suggesting that this organism can be used for detoxification in different fermentation processes [118].

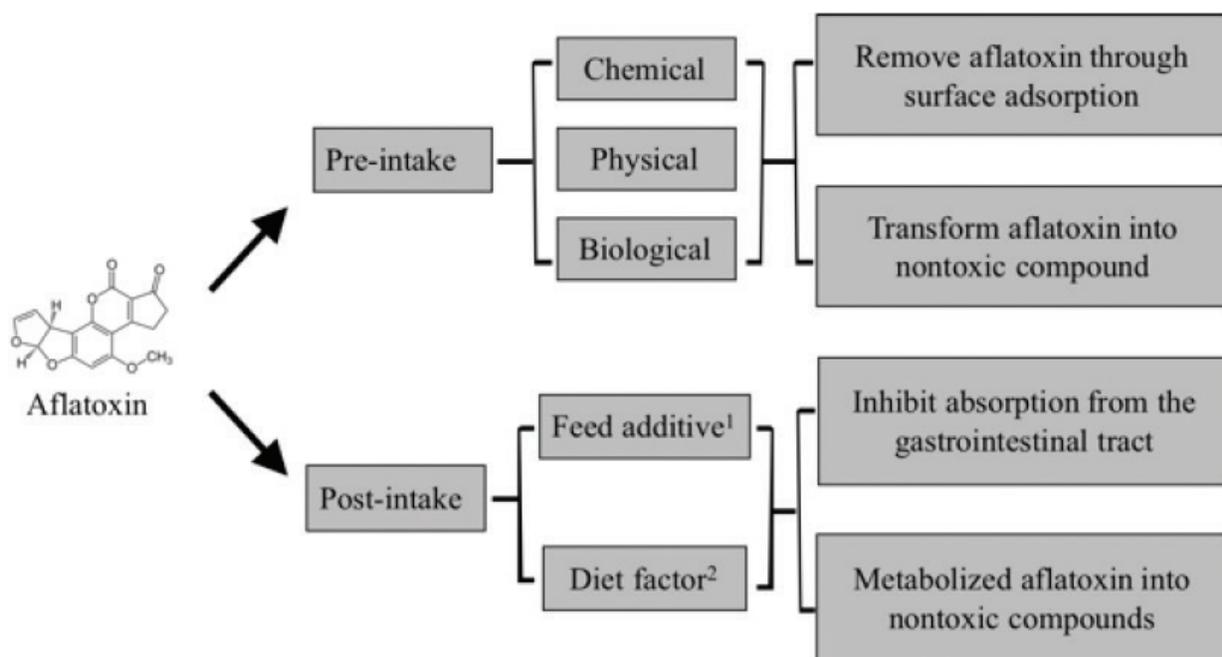


Figure 2. Schematic representation of aflatoxin reduction with different mode of action. ¹ Detoxification in animal body; ² Detoxification in human body.

Over the last few decades, considerable literature has accumulated that describes methods for removing aflatoxins using different microorganisms. Pure cultures of bacteria and fungi that detoxify aflatoxins, which include *Rhizopus* sp., *Aspergillus* sp., *Candida* sp., *Corynebacterium* sp., *Trichoderma* sp., *Mucor* sp., *Neurospora* sp., and *Rhodococcus* sp., have been isolated from complex microbial populations by screening and enrichment culture techniques [119, 120]. Among these, *Rhizopus* sp. was reported to be specifically suitable for large-scale detoxification of food and feed by solid-state fermentation. However, when used in food, viable microorganisms must be controlled to avoid undesired fermentation and undesirable compounds [121]. Among the different potentially decontaminating microorganisms, yeasts and lactic acid bacteria (LAB) have been widely used as starter cultures in the food and beverage industry for thousands of years. Therefore, yeasts and LAB have enormous potential as tools for tackling the problem of aflatoxin contamination of foods and feed [122].

Many reports state that the use of brewer's yeast cells as an animal feed additive resulted in a reduction in the toxic effects of aflatoxins [123]. In an early study, some yeast strains isolated from West African maize were found to be able to bind 15–60% (w/w) of aflatoxin B1 and this toxin binding was highly strain specific [124]. Yeast cells of *Saccharomyces cerevisiae*, which are generally used as performance promoters in poultry feeds, have also been shown to have beneficial effects against aflatoxin B1 exposure [125]. In fact, there have been many reports of yeasts and yeast cell components providing in varying degrees of protection of animals from aflatoxins in feed [126]. Baptista et al. reported that the addition of dried yeast and yeast cell walls to rat rations along with aflatoxin B1 resulted in a significant reduction of aflatoxin toxicity [127]. An *in vitro* study showed that modified mannan-oligosaccharides derived from *S. cerevisiae* showed a dose-dependent binding of aflatoxin as high as 95% (w/w) [128]. A later study confirmed that glucomannans from yeast cells have protective effects against the toxicity of aflatoxins in broiler chickens [129]. However, Baptista et al. found in a feeding experiment that mannan-oligosaccharides did not show significant suppressive effects on aflatoxin-induced damage in rats [127]. Unfortunately, no explanation could be given for this difference. It is well known that yeast cells bind sterols from the medium via cell wall mannan [130]. According to some of the studies reported, dead yeast cells still have this binding ability [127]. Therefore, it is likely that the removal of aflatoxin is not by covalent binding or metabolism, but by means of adhesion to cell wall components. It has been reported that the mannan components of the cell wall play a major role in aflatoxin binding by *S. cerevisiae* [128]. However, more kinetic studies are needed to assess the role of different components of the cell wall in aflatoxin binding.

The use of LAB in food fermentation dates back several centuries. Early studies showed that different LABs, including *Lactobacillus rhamnosus*, *L. acidophilus*, *L. gasseri*, and *L. casei* Shirota, could effectively remove up to 80% of aflatoxin B1 from contaminated culture media [131]. Among these, *L. rhamnosus* strains GG and LC705 showed similar aflatoxin B1 binding, even though they showed differences with respect to other metabolites. Later, more strains of LAB were found to be capable of binding aflatoxins in a strain-specific manner [132]. Several studies also indicated that the aflatoxin binding ability of LAB is highly strain specific [133]. Haskard et al. studied the mechanism of aflatoxin binding to *L. rhamnosus* using enzyme treatments and showed that the binding is predominantly to carbohydrate and some protein components of the cell wall [134].

Urea treatment decreased the binding significantly, indicating that hydrophobic interactions play an important role. Recent studies have shown that peptidoglycan is most likely the carbohydrate involved in the aflatoxin B1 binding process [135]. Haskard et al. found that the binding of aflatoxins to the cell surface of *L. rhamnosus* strains LGG and LC105 is considerably strong [136]. Living cell retained 38 and 50% (w/w) of bound aflatoxin after repeated washings with water. Even stronger binding was found in heat- and acid-treated cells which retained 66–71% (w/w) of the toxin, indicating a higher stability binding complex. This stronger binding was attributed to better access of aflatoxins to the treated cells. In addition, it has also been noted that autoclaving and sonication did not release any detectable toxin from prewashed cells, indicating the high stability of the complex. Binding of aflatoxins was also found to be unaffected by pH but could be easily disrupted with organic solvents, suggesting that hydrophobic interactions rather than cation exchange are the major mechanism of binding [134].

Enzymatic inactivation of aflatoxins is another attractive strategy for food decontamination. Several microorganisms can transform aflatoxin B1 to aflatoxicol and other less toxic or non-toxic compounds. It has been reported that aflatoxin B1 can be detoxified into aflatoxin B2a during yoghurt fermentation [137]; that aflatoxin B1 is detoxified during fermentation of milk by lactic bacteria [138]; and that *Armillariella tabescens* produces detoxification enzymes that show AFB1 detoxification activity [139]. A crude enzyme preparation isolated from *Stenotrophomonas* also showed strong aflatoxin-degrading activity and could degrade 85.7% of aflatoxin B1 [140]. Several other microbes, including *Corynebacterium rubrum*, *Aspergillus repens*, *Trichoderma viride*, *Mucor griseocyanus*, *Dactylium denroides*, *Mucor alternans*, *Rhizopus arrhizus*, *Rhizopus oryzae*, and *Rhizopus stolonifer*, have been reported to possess aflatoxin-degrading enzymes [141]. However, the degradation of aflatoxins is generally slow and incomplete: it was reported that *D. dendroides*, *A. repens*, and *M. griseocyanus* take 3–4 days to transform 60% of aflatoxin B1 to aflatoxicol [142].

Mycotoxin-producing fungi are also able to degrade or transform aflatoxins and possibly use them as a source of energy under suitable conditions. Several investigators have observed that aflatoxigenic strains produce large amounts of aflatoxins that usually decrease during continued incubation of the cultures [143]. *A. parasiticus* and *A. flavus* have been demonstrated to be able to degrade aflatoxins in a process that is strongly affected by the mycelia and culture conditions (pH, temperature, and inoculum, etc.), and probably involves peroxidases and P450 monooxygenases [144]. Detoxification of aflatoxin B1 by a cell-free enzyme preparation from *Armillariella tabescens*, an edible fungus used in Chinese traditional medicine, has also been reported [139]. Ames tests revealed a complete loss of mutagenicity and infrared spectroscopy of the product purified by thin-layer chromatography indicated that the difuran ring skeleton had opened; however, the structure of the product remains to be determined.

Another practical approach to reducing the toxicity of aflatoxin to humans and animals is the addition of non-nutritional inert adsorbents to the diet. These adsorbents sequester the aflatoxins in the gastrointestinal tract, thereby minimizing their toxic effects. Various adsorbents have been tested, including activated carbon, bentonite, cholestyramine, hydrated sodium calcium aluminosilicate, and zeolite, and produced promising results with respect to aflatoxin binding [145]. However, their application in food is limited because of their negative impacts such as reducing

nutrient utilization. Therefore, the use of microorganisms and other natural products has become increasingly attractive as a reliable alternative to chemical adsorbents in the gastrointestinal tract.

The potential application of natural products as aflatoxin binders in human foods and animal feeds depends on their stability and the residence time of the complex in the gastrointestinal tract. The adhesion of aflatoxin-binding microorganisms to intestinal cells appears to be highly strain specific. Yeast cells generally show very low adhesion to the intestine but are capable of withstanding the harsh environment of the gastrointestinal tract [146]. Animal feeding experiments have shown that the addition of whole cells or cell walls of *S. cerevisiae* to the diet resulted in a significant reduction of aflatoxin toxicity, indicating the possible stability of the yeast--aflatoxin complex during its passage through the gastrointestinal tract [125]. A recent study in mice showed that *S. cerevisiae* over a 6-week period improved weight gain and reduced the genotoxicity produced by aflatoxin B1 [147]. Yeast cell wall components have also been evaluated as aflatoxin adsorbents. An *in vitro* study showed that esterified glucomannan from yeast cells displayed a very high capacity (97%) to adsorb aflatoxin B1 from aqueous solutions [148]. The addition of esterified glucomannan (0.1%) to chicken feed containing aflatoxins (2 mg/kg) significantly reduced the potentially adverse effects of the aflatoxins on hematological parameters, total protein, albumin values, and aspartate amino-transferase activity in broiler chickens. Other *in vivo* studies also showed that esterified glucomannan decreased the number and severity of pathological changes caused by aflatoxin treatment [129].

LAB cells usually show considerably higher adhesion to intestinal cells compared with that of yeast cells. However, it has been reported that aflatoxin binding considerably reduced the adhesive properties of some LAB strains and resulted in the faster excretion of immobilized aflatoxin B1 [149]. Gratz et al. also found that pre-exposure of the cells of a *L. rhamnosus* strain to aflatoxin B1 reduced its binding to intestinal mucus and thus resulted in the faster removal of bound aflatoxin [150]. An *in vitro* study using the chicken duodenum loop technique showed that a *L. rhamnosus* strain removed up to 54% (w/w) of the added aflatoxin B1 and reduced as much as 73% of intestinal adsorption [151]. Some researchers have suggested that the aflatoxin molecules bind to bacterial cell wall components such as polysaccharide and peptidoglycan [152]. Bacterial cell surface hydrophobicity may also play an important role in the binding of aflatoxins.

Aflatoxins absorbed into the bodies of humans or animals may also be metabolized into relatively nontoxic compounds via different metabolic pathways. The process of detoxification of aflatoxins usually involves removing the double bond of the terminal furan ring or opening the lactone ring. Once the lactone ring is opened, further reactions can occur to alter their binding properties to DNA and proteins [153]. The main reactions of aflatoxin metabolism in humans and animals are hydroxylation, oxidation, and demethylation. There are numerous studies concerning the metabolism of aflatoxin *in vitro* and *in vivo*. Salhab and Edwards found that the liver preparations of rabbit and trout were able to metabolize aflatoxin B1 into aflatoxicol by reducing the cyclopentenone carbonyl of aflatoxin B1 in an *in vitro* experiment [154]. An *in vivo* study by Roebuck and Wogan also found that aflatoxicol is the major metabolite

of aflatoxin B1 in duck liver, whereas aflatoxin B1 was mainly converted into aflatoxin P1 and aflatoxin Q1 (relatively nontoxic) in human and monkey livers [155]. In fact, there is a great diversity among different animal species in the metabolism of aflatoxins. For example, aflatoxin B1 was able to convert into aflatoxin M1 in ducks, rats, and monkeys but not in humans [156], while in chicken liver, aflatoxin B1 was metabolized into a peptide conjugate of aflatoxin B2a and a glucuronide conjugate of aflatoxin M1 [157]. Donnelly et al. found that lipoxygenase and prostaglandin H synthase were the main enzymes responsible for the biotransformation of aflatoxin B1 in human lung, while in rat liver, aflatoxin B1 is transformed by a mixed-function monooxygenase [158]. In addition, various forms of cytochromes were found to have different biotransformation capacities for aflatoxins. Yoshizawa et al. reported that, in rat liver, transformation of aflatoxin M1 was strictly mediated by cytochrome P448, while transformation of aflatoxin Q1 was catalyzed by both cytochrome P450 and P448 [159]. In human liver, the cytochrome P450-dependent polysubstrate monooxygenase system is the major isoform involved in aflatoxin transformation [158].

5. Application of natural inhibitors

The preferred strategy for reducing the concentrations of aflatoxins in foods is prevention of aflatoxin formation during preharvest and postharvest of the various susceptible crops. In this context, non-aflatoxigenic *Aspergillus* strains have been used to prevent preharvest aflatoxin contamination of crops, such as peanuts, maize, and cottonseed, and have shown great potential. Recent advancements in the use of biocontrol strategies involving microorganisms should soon lead to increased practical applications for the benefit of the food industry. Some microorganisms such as *R. stolonifer* and *A. fumigatus*, which have been used in aflatoxin removal experiments, are not likely to be used in the field because of their potential to cause infection of the plants. However, these strains could still be used to provide natural compounds for prevention of aflatoxin formation. Alternatively, the genes responsible for their antiaflatoxigenic activity could possibly be incorporated into the host plant genome to produce crops resistant to aflatoxin contamination. The use of metabolites from microorganisms and plants as natural agents to control aflatoxin contamination has received much attention in recent years. Although the use of natural metabolites has shown promising results under controlled conditions in *in vitro* experiments, these studies need to be extended *in situ* to systems involving foods or feeds. More work is required to further characterize the antifungal and antiaflatoxigenic mechanisms involved.

To achieve effective control of aflatoxin contamination in food, high concentrations of natural compounds are generally needed. The incorporation of natural compounds into packaging materials can be a useful strategy to solve this problem. In the last decade, there have been plentiful studies of the development of active packaging materials. Because the introduction of protective agents in packaging materials can be used to protect food without direct addition of new chemicals, it has received great interest from both the food industry and academic communities. Many natural extracts, such as essential oils and their constituents, are categorized as flavorings

in Europe and are categorized as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration. For this reason, they have often been proposed for and used in active packaging. For the purpose of the design of active packaging, it is necessary to establish which compounds have antiaflatoxigenic properties and what concentration is required to obtain maximum inhibition. In addition, because the volatile nature of some components, the release rate of the encapsulated compounds from their polymer matrix should be controlled, thereby magnifying their antiaflatoxigenic action on the product by both direct contact and through the head space of the packaging. The processes of encapsulating natural aflatoxin inhibitors into the polymer matrix should also be carefully controlled. Previous studies have shown notable losses of the active compounds during the film formation step of the casting technique [160].

Nanotechnology-based systems associated with natural compounds are also a good option. There are many well-known benefits of associating natural compounds with nanotechnological drug-delivery systems [161]. One good example is a nanoemulsion: in an emulsified form, natural compounds may be applied as an aqueous-based treatment. In fact, fine droplets may improve the delivery of inhibitory compounds to cereals because they may be able to penetrate into the cracks and crevices on the cereal surfaces. Nanoemulsions are emulsion droplets with a radius below 100 nm, which can be formed using both high-energy and low-energy methods [162]. High-energy methods require specialized mechanical devices, such as high pressure valve homogenizers, sonicators and microfluidizers. These devices are capable of generating intense mechanical forces that can intermingle and disrupt the oil and water phases. Low-energy methods rely on the spontaneous formation of nano-sized oil droplets, which is a physicochemical process that occurs under appropriate conditions with certain combinations of surfactant, oil, and water. The spontaneous emulsification method has recently been reported to be suitable for application in the food industry for fabricating effective antimicrobial nanoemulsions from essential oils [163].

As mentioned previously, genetic engineering is another way to utilize these compounds. Host crop species can be engineered to gain resistance to aflatoxin contamination by incorporation of the genes for biosynthesis of natural inhibitors. There are likely hundreds, if not thousands, of natural compounds that influence aflatoxin biosynthesis at concentrations ranging from submicromolar to millimolar. Unfortunately, many of these inhibitors are not suitable for genetic engineering. The complexity of altering plant natural product pathways makes it difficult to engineer crop species resistant to aflatoxin contamination. In addition, the majority of aflatoxin inhibitors reported so far were tested *in vitro* in media that do not approximate the conditions on the host plant. The tissue specificity and/or inducible expression of inhibitors are also important considerations [164]. Therefore, it is critical to identify the most promising candidates before attempting to engineer aflatoxin-resistant plants. Compared with the production of exogenous inhibitors, the development of plants that already possess aflatoxin inhibitors might be easier, because the biosynthetic pathways are already present in the host, and an increased inhibitor concentration can be achieved by upregulating endogenous genes. Most genetic and molecular approaches aimed at preventing aflatoxin biosynthesis have not yet reached commercial application in the field and require substantial further development.

6. Conclusions

In summary, there are various natural compounds that influence aflatoxin contamination in food through different ways, including inhibition of the growth of aflatoxigenic fungi, blocking aflatoxin biosynthesis, and removal or degradation of aflatoxin. These inhibitors are highly promising for the development of new approaches to fighting aflatoxin contamination in food and have the capability to replace or complement conventional strategies. A common feature of many inhibitors is their antioxidant activity; yet, the relationship of antiaflatoxigenic activity and antioxidant activity is unknown. Some inhibitors of aflatoxin production are specifically targeted to the biosynthesis of aflatoxin without affecting the development of the fungal cells. However, most inhibitors also inhibit fungal growth at higher concentrations. This may indicate that secondary metabolism (aflatoxin) is sensitive to stress resulting from low concentrations of growth-inhibitory compounds. The production of norsolorinic acid, the first stable intermediate in the aflatoxin biosynthetic pathway, was inhibited in parallel with aflatoxin production at the regulatory level of biosynthesis rather than at specific steps within the pathway, indicating the importance of this intermediate. More and more natural inhibitors are being identified, yet the modes of action of most are poorly understood. Further studies are necessary to better understand the mechanisms of action of those compounds before they can be widely used commercially. Using new biological approaches, researchers are now combining datasets from profiling of transcripts, proteins, and metabolites generated using inhibitory compounds with different modes of action, which will provide useful information for dissecting different facets of aflatoxin regulation.

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