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Enzymatic Oxidation of Aflatoxin M_1 in Milk Using CotA Laccase

Yongpeng Guo [1], Hao Lv [1], Zhiyong Rao [1], Zhixiang Wang [1,*], Wei Zhang [1], Yu Tang [2] and Lihong Zhao [2,*]

[1] College of Animal Science and Technology, Henan Agricultural University, Zhengzhou 450046, China; guoyp@henau.edu.cn (Y.G.); lcxhau@163.com (H.L.); rzyhau@163.com (Z.R.); weizhang@henau.edu.cn (W.Z.)
[2] College of Animal Science and Technology, China Agricultural University, Beijing 100193, China; m17801115235@163.com
* Correspondence: wzxhau@henau.edu.cn (Z.W.); zhaolihongcau@cau.edu.cn (L.Z.)

Abstract: Aflatoxin M_1 (AFM_1) in milk poses a significant threat to human health. This study examined the capacity of *Bacillus licheniformis* CotA laccase to oxidize AFM_1. The optimal conditions for the CotA laccase-catalyzed AFM_1 oxidation were observed at pH 8.0 and 70 °C, achieving an AFM_1 oxidation rate of 86% in 30 min. The *K*m and Vmax values for CotA laccase with respect to AFM_1 were 18.91 μg mL^{-1} and 9.968 μg min^{-1} mg^{-1}, respectively. Computational analysis suggested that AFM_1 interacted with CotA laccase via hydrogen bonding and van der Waals interactions. Moreover, the oxidation products of AFM_1 mediated by CotA laccase were identified as the C3-hydroxy derivatives of AFM_1 by HPLC-FLD and UPLC-TOF/MS. Toxicological assessment revealed that the hepatotoxicity of AFM_1 was substantially reduced following oxidation by CotA laccase. The efficacy of CotA laccase in removing AFM_1 in milk was further tested, and the result showed that the enzyme agent achieved an AFM_1 removal rate of 83.5% in skim milk and 65.1% in whole milk. These findings suggested that CotA laccase was a novel AFM_1 oxidase capable of eliminating AFM_1 in milk. More effort is still needed to improve the AFM_1 oxidase activity of CotA laccase in order to shorten the processing time when applying the enzyme in the milk industry.

Keywords: aflatoxin M_1; CotA laccase; oxidation; milk

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1. Introduction

Milk is commonly recognized as a fundamental food product that provides a comprehensive range of readily accessible and bioavailable nutrients crucial for human growth, development, and health maintenance. Moreover, the consumption of milk products is correlated with a decreased risk of cardiovascular diseases, notably stroke [1]. However, the food hygiene of milk products is often compromised by aflatoxin M_1 (AFM_1), which has been a great concern for consumers. AFM_1, known as a milk toxin, is the hydroxylated form of aflatoxin B_1 (AFB_1), which is generated by the hepatic mitochondrial cytochrome P450 enzymes and excreted in the milk, feces, and urine of lactating animals after the consumption of an AFB_1-contaminated diet. AFM_1 appears in dairy cow milk within 12 h of the first AFB_1 administration and clears from milk after a 72 h AFB_1 withdrawal period [2,3]. The carry-over rate of dietary AFB_1 to milk AFM_1 in dairy cattle ranges between 0.3 and 6.2% depending on the health conditions, milk yield, feed type, and level of contamination [4]. AFM_1 remains stable during various milk processing methods, including sterilization, pasteurization, and fermentation, and its presence has been documented throughout the entire dairy supply chain, encompassing products such as milk powder, cheese, and yogurt [5]. The prevalence of AFM_1 in milk poses significant health risks, particularly for vulnerable populations such as infants and the elderly, who may be more susceptible to its harmful effects. Although AFM_1 has been found to be 10-fold less carcinogenic than AFB_1, IARC continues to classify it as a class 1 carcinogen [6]. Given the potential health risks of AFM_1, over 60 countries have developed guidelines for the maximum residue level of milk AFM_1. Among these, 34 countries, including China, have set the limit at 0.5 μg L^{-1} in fluid milk.

In contrast, the European Union has adopted a more stringent maximum level at 0.05 μg L^{-1} for AFM_1 in raw milk and milk intended for the production of dairy products.

The development of efficient and environmentally sustainable methods to reduce AFM_1 contamination in milk is urgently needed. Milk contamination can be reduced either indirectly, by preventing dairy animals' dietary exposure to AFB_1, or directly, by eliminating AFM_1 from contaminated milk. The implementation of good agricultural practices (GAPs), such as crop rotation, soil management, insect damage control, the selection of fungal-resistant crop varieties, and timely harvesting, is helpful for the inhibition of mold infection and AFB_1 generation [7]. Unfortunately, these pre-harvest prevention strategies are not consistently sufficient to produce AFB_1-free crops. To mitigate the carry-over of feed-derived AFB_1 to milk AFM_1, dairy farming commonly employs organic and inorganic adsorbents like yeast cell wall extracts [8], calcium montmorillonite clay [9], and hydrated sodium calcium aluminosilicates [10], which can bind AFB_1 within the gastrointestinal tract of animals. Moreover, several reports documenting the capacity of yeasts and lactic acid bacteria to bind AFM_1 in contaminated milk are available [11–13]. Despite favorable outcomes of microbial cells in milk AFM_1 elimination, the commercialization of adsorption-associated technologies still has limitations, like the instability of microbial cell–AFM_1 complexes, the increase in microbial loads, and the loss of nutritional value [14].

In recent years, research emphasis has gradually shifted toward the microbial and enzymatic degradation of aflatoxins in food commodities, which transforms aflatoxins into less harmful metabolites, while preserving the palatability and nutritional value of food and feed. A number of AFB_1-degrading fungal and bacterial strains have been identified, including *Pleurotus eryngii* [15], *Ganoderma sinense* [16], *Myroides odoratimimus* [17], *Pseudomonas aeruginosa* [18], *Cellulosimicrobium funkei* [19], and *Bacillus licheniformis* [20]. Several studies have also documented that AFB_1 can be degraded by laccase [21,22], peroxidase [23,24], dipeptidyl peptidase [25], and $F_{420}H_2$-dependent reductase [26]. However, the capacity and application potential of these microorganisms and enzymes for degrading AFM_1 have been rarely studied. In this study, we expanded upon previous findings that demonstrated the capacity of CotA laccase to directly oxidize AFB_1 without the need for redox mediators, resulting in the formation of aflatoxin Q_1 and epi-aflatoxin Q_1 [22]. We further investigated the enzymatic characteristics of CotA laccase in oxidizing AFM_1. CotA laccase-mediated AFM_1 oxidation products were identified. Additionally, we evaluated the toxicity of AFM_1 oxidation products using hepatocytes L-02. The interaction between CotA laccase and AFM_1 was investigated by molecular docking simulation. Furthermore, the efficacy of CotA laccase in removing AFM_1 in both skim milk and whole milk was evaluated for the first time. This work is expected to contribute to the advancement of enzyme-based strategies to mitigate AFM_1 contamination in milk products.

2. Materials and Methods

2.1. Materials and Reagents

CotA laccase was expressed and purified from *Escherichia coli* Rossta (DE3) bearing recombinant expression vector pET31b-CotA as described previously [22]. AFM_1 was purchased from Sigma-Aldrich (Shanghai, China). AFM_1 immune-affinity columns were obtained from Clover Technology Group, Inc. (Beijing, China). Ultrahigh-temperature-treated (UHT) whole milk and skim milk were purchased from a local supermarket.

2.2. Enzymatic Characteristics of CotA Laccase for Oxidizing AFM_1

The enzymatic characteristics of CotA laccase in the oxidation of AFM_1 were characterized. To assess the impact of pH on AFM_1 oxidation, a reaction mixture comprising 1 μg mL^{-1} of AFM_1 and 0.1 U mL^{-1} of CotA laccase was incubated at 37 °C across a range of pH conditions (pH 4.0 to 9.0) for 12 h. The impact of temperature on AFM_1 oxidation was assessed by incubating 1 μg mL^{-1} of AFM_1 with 0.1 U mL^{-1} of CotA laccase across a temperature range of 30 to 80 °C for 30 min. Additionally, the influence of metal ions on CotA laccase-mediated AFM_1 oxidation was evaluated by pre-incubating 0.5 U mL^{-1}

of CotA laccase with 10 mM of different metal ions at 37 °C and pH 7.0 for 10 min. Subsequently, AFM_1 (1 μg mL^{-1}) was introduced, and the mixture was incubated at 37 °C for an additional 30 min. The Michaelis–Menten kinetics of CotA laccase-mediated AFM_1 oxidation was analyzed at 37 °C and pH 7.0. Initial reaction velocities were determined by quantifying the reduction in AFM_1 concentration in 30 min following the introduction of 0.1 U mL^{-1} of CotA laccase. The experimental data were fitted to the Michaelis–Menten equation to derive the K_m and V_{max} values with Graphpad Prism 7.0.

2.3. AFM_1 Concentration Determination by HPLC

Chromatographic analysis was conducted utilizing a high-performance liquid chromatography (HPLC) system (Shimadzu LC-10 AT, Shimadzu, Tokyo, Japan), which was equipped with a post-column photochemical derivatization unit and an RF-20A fluorescence detector (Shimadzu, Tokyo, Japan). The separation process employed an isocratic method using a reverse-phase column (DIKMA, C18, 5 μm, 150 × 4.6 mm). The mobile phase consisted of acetonitrile, methanol, and water in a volumetric ratio of 24:8:68. The excitation and emission wavelengths were set at 365 nm and 435 nm, respectively. The flow rate was maintained at 1.0 mL min^{-1}, and the injection volume was 20 μL.

2.4. UPLC-TOF/MS Analysis of CotA Laccase-Mediated AFM_1 Oxidation Products

UPLC-TOF/MS was applied to identify CotA laccase-mediated AFM_1 oxidation products. Chromatographic separation was performed using an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm) (Waters, Milford, MA, USA). The mobile phase, delivered at a flow rate of 0.3 mL min^{-1}, comprised a binary solvent system of water and methanol. The elution gradient was as follows: 0–10 min, 5% to 50% methanol; 10–11 min, 50% to 95% methanol; 11–13 min, 95% methanol; and 13–15 min, 95% to 5% methanol. The sample injection volume was set at 2 μL. Mass spectral data were acquired using a Waters Xevo G2-XS QTOF mass spectrometer (Waters, Milford, MA, USA). The TOF-MS was conducted in ESI positive mode. The mass spectrometry parameters were set as follows: ion source temperature at 350 °C, nitrogen gas flow rate at 10 L min^{-1}, capillary voltage at 3000 V, and scan range from m/z 50 to 1000.

2.5. Homology Modeling and Molecular Docking

The three-dimensional structure of *B. licheniformis* ANSB821 CotA laccase was constructed with the X-ray crystal structure of *B. subtilis* 168 CotA laccase (PDB entry 2WSD) [27] as a template with the SWISS-MODEL sever. The three-dimensional structure of AFM_1 was generated using ChemBioDraw 2014. Molecular docking was conducted using MOE Dock in MOE v2014.0901. The docking procedure adhered to the "induced fit" protocol, which permitted the side chains of the receptor pocket to adjust in response to ligand conformations, while maintaining a positional constraint.

2.6. Cytotoxicity Evaluation of AFM_1 Oxidation Products

Human fetal hepatocyte cell line L-02 obtained from Tongpai Biotechnology Co., Ltd. (Shanghai, China) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 100 U mL^{-1} of penicillin and streptomycin. Cell viability was assessed using a CCK-8 assay kit (Biosharp, Beijing, China). L-02 cells were inoculated in a 96-well plate (1×10^4 cells each well), and treated with 100 μM of AFM_1 and CotA laccase-catalyzed AFM_1 oxidation products for 24 h. Afterwards, 10 μL of CCK-8 reagent was introduced into each well, followed by incubation at 37 °C for 1 h. The absorbance value at 450 nm was taken with a microplate reader to assess cell viability. The extracellular lactate dehydrogenase (LDH) activity and cell apoptosis rate of L-02 cells were also measured after exposure to 100 μM of AFM_1 and CotA laccase-catalyzed AFM_1 oxidation products for 24 h. LDH activity was determined using a commercially available kit (Biosharp, Beijing, China). The rate of cellular apoptosis was assessed utilizing the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Uelandy, Suzhou, China).

2.7. Performance of CotA Laccase in Degrading AFM_1 in Milk

The experiments regarding the elimination of AFM_1 in whole milk and skim milk by CotA laccase were carried out in 100 mL conical flasks. Milk samples were spiked with AFM_1 at a concentration of 2.0 ng mL^{-1}. To study the effect of the amount of CotA laccase on AFM_1 oxidation, CotA laccase was added to 20 mL of spiked milk to reach a final concentration ranging from 0.1 to 2.0 U mL^{-1}. The control was prepared with sodium phosphate buffer in place of CotA laccase. The samples were incubated at 37 °C for 12 h in a water bath. The effect of incubation time on AFM_1 oxidation was determined at 3, 6, 12, 18, and 24 h, respectively, with 1.0 U mL^{-1} of CotA laccase at 37 °C. All experiments were repeated three times. The concentration of AFM_1 in milk was measured with HPLC. Briefly, the milk samples were centrifuged at 6000 rpm for 20 min. Then, 10 mL of supernatant was collected and applied to an AFM_1 immune-affinity column at a steady flow rate of 1 mL min^{-1}. The column was washed twice with 10 mL of ultrapure water. AFM_1 was eluted from the column with 2 mL of methanol. The eluent was evaporated to dryness at 40 °C under a N_2 stream and reconstituted in 1 mL of ultrapure water before loading in the HPLC system. Validation parameters for the determination of AFM_1 concentration in milk with the HPLC-FLD method are summarized in Table S1.

3. Results and Discussion

3.1. Enzymatic Properties of CotA Laccase for Oxidizing AFM_1

Laccases, a class of multicopper oxidases, possess the ability to oxidize a diverse array of aromatic compounds [28]. Due to their versatility, stability, and broad substrate specificity, numerous applications of laccases have been developed within the food industry over recent decades, including roles in baking, fruit juice clarification, wine and beer stabilization, and sugar beet pectin gelation [29]. Our previous study found that CotA laccase could serve as a novel aflatoxin oxidase [22].

In the current study, we further characterized the enzymatic properties and kinetics of CotA laccase in the oxidation of AFM_1. The influence of pH, temperature, and metal ions on CotA laccase-catalyzed AFM_1 oxidation was initially examined. The optimal pH range for CotA laccase in oxidizing AFM_1 was observed at pH 7.0 to 9.0, with the oxidation rate remaining above 86%, and the highest AFM_1 oxidation rate of 94% was obtained at pH 8.0 (Figure 1A). The substrate-dependent optimal pH is a characteristic feature of laccases. Previously, the optimal pH values for *B. licheniformis* CotA laccase in the oxidation of 2, 2′-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), syringaldazine, 2,6-dimethoxyphenol, and AFB_1 were determined to be 4.2, 6.8, 7.2, and 8.0, respectively [22]. Therefore, it is essential to determine the optimal pH for the oxidation of various substrates using CotA laccase. The effect of temperature on the oxidation of AFM_1 by CotA laccase is illustrated in Figure 1B. The oxidation rate of AFM_1 increased from 22% to 86% as the temperature rose from 30 °C to 70 °C, and then dropped to 60% at 80 °C. This observation aligns with the known characteristics of *Bacillus* CotA laccases, which are recognized as thermophilic enzymes with optimal activity within a temperature range of 50 °C to 70 °C [30]. The influence of various metal ions on the CotA laccase-mediated oxidation of AFM_1 was examined, as depicted in Figure 1C. The presence of K^+, Li^+, and Ba^{2+} exhibited minimal impact on AFM_1 oxidation by CotA laccase, whereas Mn^{2+} and Ca^{2+} at 10 mM caused a more than 15% reduction in the AFM_1 oxidation rate. Moreover, Cu^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} could strongly inhibit the AFM_1-oxidizing activity of CotA laccase. Indeed, the inhibitory effects of Cu^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} are commonly observed in laccases, potentially due to their interaction with the electron transport system of the enzymes [31,32].

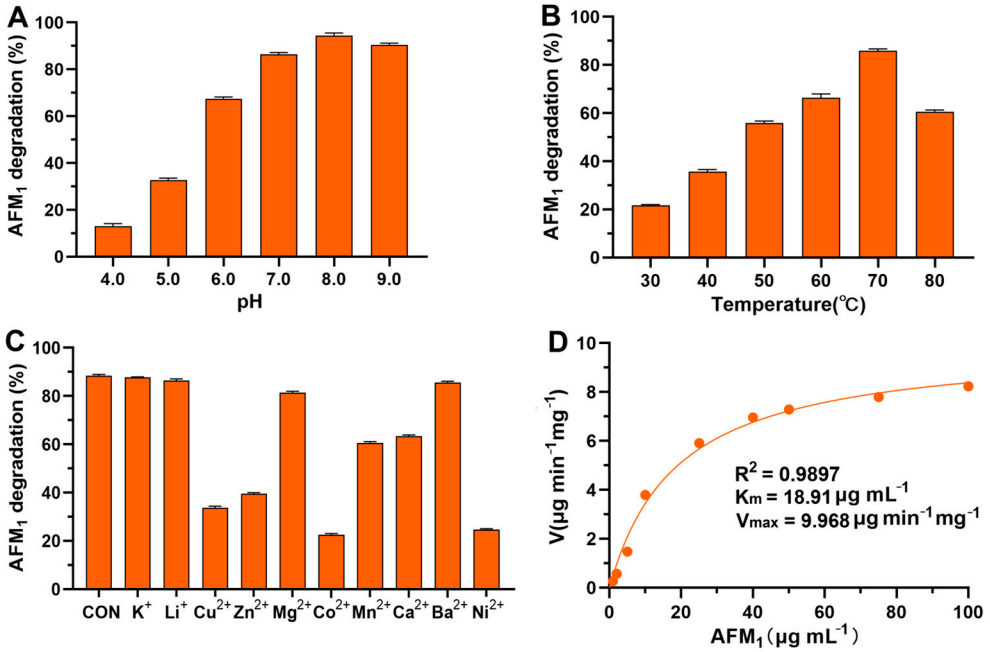

Figure 1. Enzymatic properties and kinetics of CotA laccase-mediated AFM_1 oxidation. Effect of pH (**A**), temperature (**B**), and metal ions (**C**) on CotA laccase-mediated AFM_1 oxidation. (**D**) Michaelis–Menten plot of CotA laccase-catalyzed AFM_1 oxidation.

The kinetic parameters of CotA laccase with respect to AFM_1 were determined by fitting the experimental data to the Michaelis–Menten plot (Figure 1D). The coefficient of determination (R^2) was 0.9897, indicating an excellent fit of the model to the data. The K_m and V_{max} values were calculated to be 18.91 μg mL^{-1} and 9.968 μg min^{-1} mg^{-1}, respectively. This strong correlation of the Michaelis–Menten model further implies that AFM_1 is a novel substrate of CotA laccase.

3.2. Identification of CotA Laccase-Mediated AFM_1 Oxidation Products

CotA laccase-mediated AFM_1 oxidation products in sodium phosphate buffer were characterized by HPLC-FLD and UPLC-TOF/MS analysis. The oxidation of AFM_1 resulted in the formation of two products with retention times earlier than that of AFM_1 (Figure 2A). The oxidation products were further characterized by MS analysis, as shown in Figure 2B and Figure S1. The two products showed the same molecular ion peak at m/z 345.03 ($[M + H]^+$), m/z 367.01 ($[M + Na]^+$) and m/z 382.98 ($[M + K]^+$), corresponding to the formula of $C_{17}H_{12}O_8$, which suggested the addition of a single oxygen to AFM_1 ($C_{17}H_{12}O_7$). Aflatoxins can be degraded by several mechanisms, such as epoxidation, hydroxylation, dehydrogenation, and reduction. Research by Loi et al. [21] suggested that treating aflatoxins with Lac2 laccase led to cleavage of the lactone ring, whereas Alberts et al. [33] found that pure laccase of *T. versicolor* changed the double bond of the furan ring. Moreover, Liu et al. [34] reported that both the furan ring and lactone ring were abolished after treatment by *Bacillus pumilus* superoxide dismutase.

Our previous study confirmed that CotA laccase catalyzed the C3-hydroxylation of AFB_1, leading to the formation of a pair of epimers, AFQ_1 and epi-AFQ_1 [22]. Based on HPLC-FLD and UPLC-TOF/MS analysis in this study, the same degradation pathway was proposed for AFM_1, as shown in Figure 2C. The two transformation products were named aflatoxin N_1 (AFN_1, 3*S*) and epi-aflatoxin N_1 (epi-AFN_1, 3*R*). Further study is necessary to confirm their chemical structures using nuclear magnetic resonance spectroscopy.

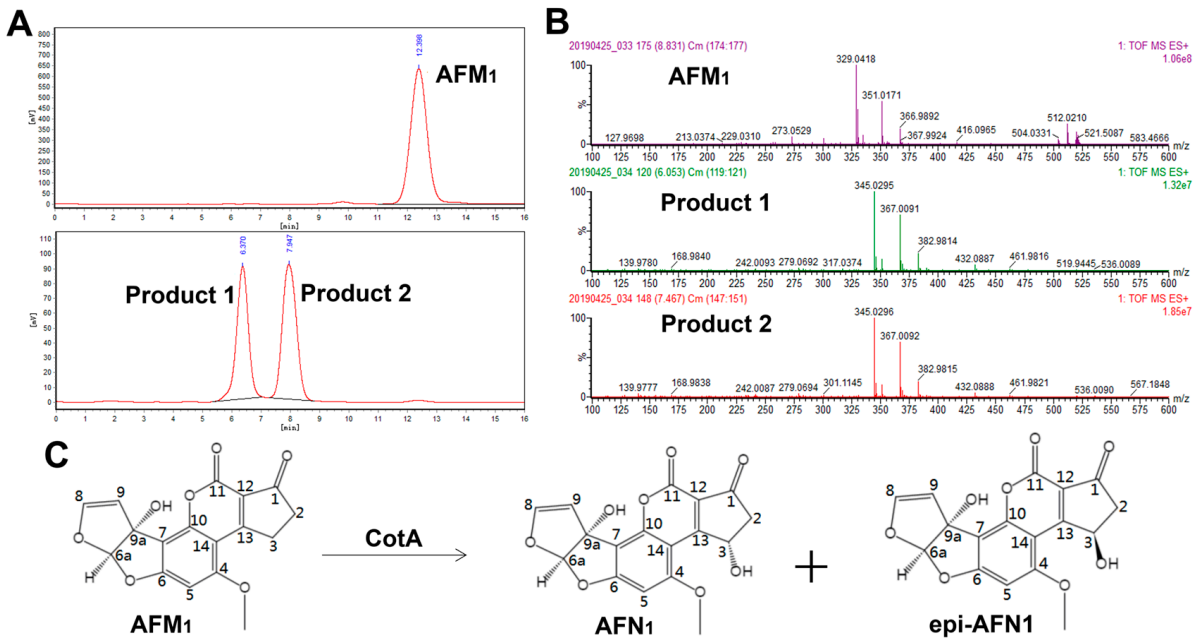

Figure 2. Identification of CotA laccase-mediated AFM_1 oxidation products. (**A**) HPLC chromatograms of AFM_1 and CotA laccase-mediated AFM_1 oxidation products. (**B**) Mass spectra analysis of AFM_1 and CotA laccase-mediated AFM_1 oxidation products. (**C**) The reaction scheme for AFM_1 oxidation by CotA laccase.

3.3. Interaction of AFM_1 with CotA Laccase by Molecular Docking

A docking simulation study was conducted to examine the interaction of AFM_1 with CotA laccase. The docking score for the binding mode of AFM_1 with CotA laccase was calculated to be -5.50 kcal mol^{-1} (Figure 3). The binding free energy was negative, reflecting that AFM_1 oxidation by CotA laccase was spontaneous. The main interaction observed between AFM_1 and CotA laccase was hydrogen bonding. Specifically, the two carbonyl oxygen atoms of the lactone group in AFM_1, acting as hydrogen bond acceptors, formed hydrogen bonds with the backbone of Gly321 and Gly322, respectively. Additionally, AFM_1 provided good van der Waals interaction with Ile260, Ile318, Cys320, and Ile417. Liu et al. [35] also reported that hydrogen bonding was the primary driving force for the binding between aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2) and *Trametes* sp. C30 laccase. The in silico investigation conducted by Dellafiora et al. [36] indicated distinct binding configurations of AFB_1 and AFM_1 within the catalytic sites of laccases derived from *Trametes versicolor*. The hydroxyl group on the difuran moiety of AFM_1 enabled a more profound penetration into the catalytic pocket of *T. versicolor* laccases, attributed to the establishment of an additional hydrogen bond within the AFM_1–laccase complex. However, we found that the simulated binding model of AFM_1 to CotA laccase was the same as the AFB_1-CotA interaction model [22], which was in agreement with the finding in this study that AFB_1 and AFM_1 underwent same molecular modification by CotA laccase.

3.4. Hepatotoxicity Evaluation of AFM_1 Oxidation Products

The liver, being the main organ for mycotoxin metabolism, is primarily impacted by AFM_1 toxicity. A previous study has documented that AFM_1 exposure results in liver damage, characterized by poor cytoplasmic integrity, inflammatory infiltration, sinusoidal dilation, and nuclear irregularities [37]. Thus, this study assessed the cytotoxicity of AFM_1 oxidation products using hepatocytes L-02. As shown in Figure 4A, the viability reduced remarkably following exposure to 100 μM of AFM_1 for 24 h. Conversely, treatment with 100 μM of CotA laccase-mediated AFM_1 oxidation products did not notably impact cell viability. The detrimental impact of AFM_1 on L-02 cells was corroborated by a notable increase in the extracellular LDH activity of the AFM_1 group (Figure 4B). LDH, a cytoplasmic

enzyme, is rapidly released into the culture medium upon cytomembrane leakage, serving as an indicator of cell damage. Consistent with the result of cell viability, no significant difference was observed in extracellular LDH activity between the group treated with AFM_1 oxidation products and the control group. Furthermore, flow cytometry analysis indicated a significant increase in cell apoptosis rate in the AFM_1 group compared to the control, whereas the AFM_1 oxidation products did not induce cell apoptosis (Figure 4C,D). Collectively, these findings suggest that the CotA laccase-mediated oxidation of AFM_1 effectively detoxifies its hepatotoxic effects.

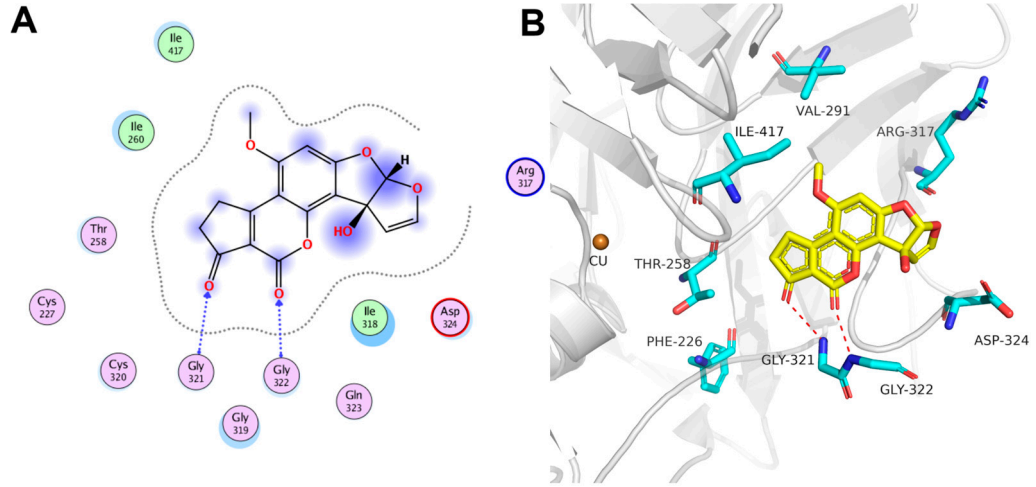

Figure 3. Molecular docking analysis of AFM_1 with CotA laccase. (**A**) The two-dimensional interaction model of AFM_1 with CotA laccase. (**B**) The three-dimensional interaction model of AFM_1 with CotA laccase.

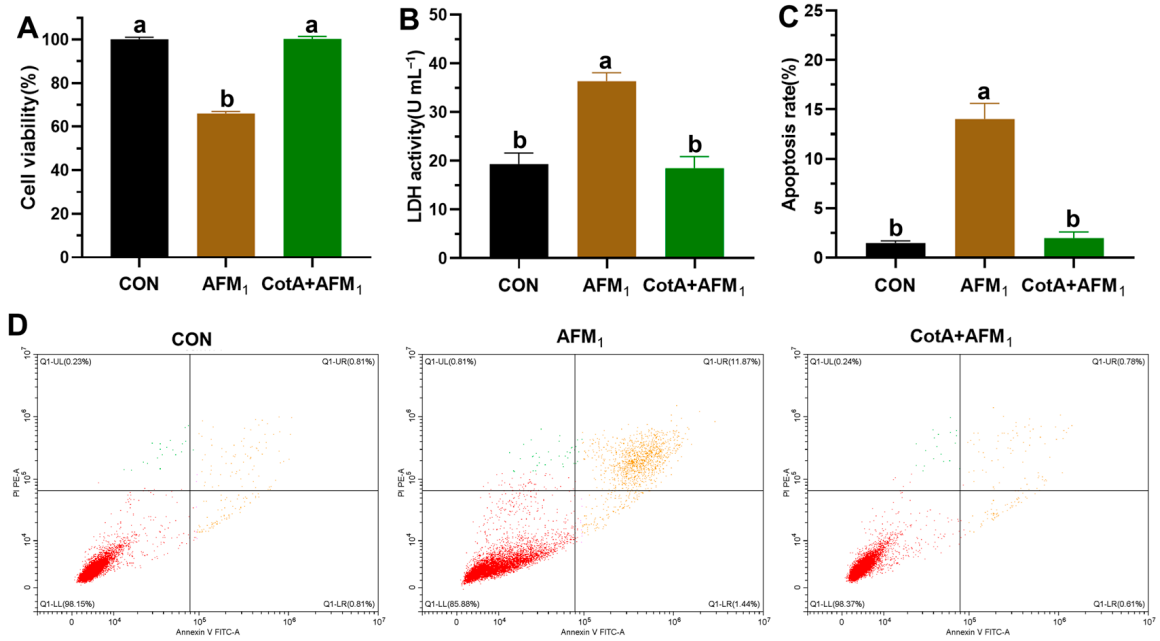

Figure 4. Evaluation of the cytotoxic effects of AFM_1 and its oxidation products. (**A**) Viability of L-02 cells following exposure to 100 μM of AFM_1 and CotA laccase-catalyzed AFM_1 oxidation products. (**B**) LDH activity. (**C**,**D**) Apoptosis rate of L-02 cells. Different letters denote statistically significant differences between groups ($p < 0.05$).

3.5. Performance of CotA Laccase to Degrade AFM$_1$ in Milk

The performance of CotA laccase to eliminate AFM$_1$ in milk was further investigated. Whole milk and skim milk spiked with AFM$_1$ at a concentration of 2 ng mL^{-1} were subjected to treatment with CotA laccase. The impact of the amount of CotA laccase on AFM$_1$ elimination is shown in Figure 5A. The AFM$_1$ removal percentage increased with the increase in CotA laccase from 0.1 to 2.0 U mL^{-1}, and the maximum removal rates were 83.5% and 65.1% for skim milk and whole milk, respectively.

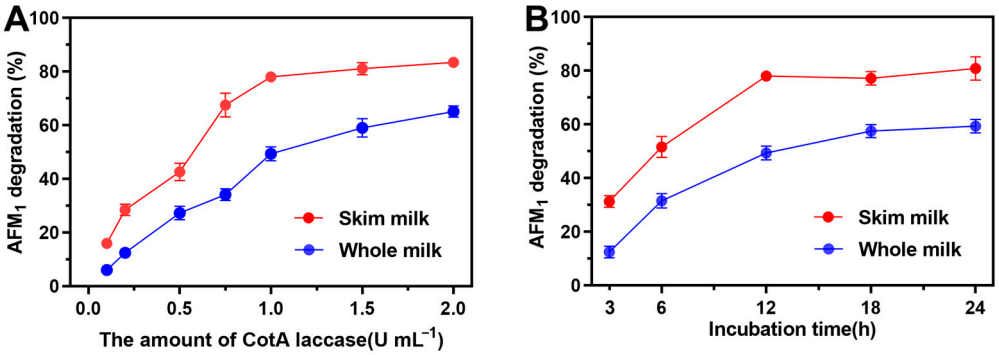

Figure 5. Elimination of AFM$_1$ in milk by CotA laccase. Effect of CotA laccase amount (**A**) and incubation time (**B**) on AFM$_1$ degradation rate in skim milk and whole milk.

As summarized in Table 1, several research efforts have been dedicated to the enzymatic elimination of AFM$_1$ in milk. Marimón Sibaja et al. [38] reported that commercial horseradish peroxidase (HRP) could reduce AFM$_1$ content in UTH milk by 65.0%. Kerstner et al. [39] used crude extract of peroxidase from rice bran to eliminate AFM$_1$ in milk and achieved the removal percentage of 71.2%. However, H_2O_2 was required for the oxidation of AFM$_1$ by peroxidases. With respect to large-scale milk processing, CotA laccase has the advantage of directly oxidizing AFM$_1$ by using O_2 as an electron acceptor. The food matrix can exert a significant influence on the performance of enzymes in food processing. The AFM$_1$ elimination efficiency of CotA laccase was significantly reduced by fat and fat-soluble vitamins in whole milk.

Table 1. Summary of enzymatic elimination of AFM$_1$ in milk.

Enzyme	Origin	Reaction Conditions	Elimination Rate	Reference
HRP	*Amoracia rusticana*	0.015 U mL^{-1} HRP, 0.08% H_2O_2, 5 ng mL^{-1} AFM$_1$, 30 °C for 8 h	65.0%	[38]
RBP	Rice bran	0.015 U mL^{-1} HRP, 0.08% H_2O_2, 5 ng mL^{-1} AFM$_1$, 4 °C for 24 h	71.2%	[39]
SOD	*Bacillus pumilus*	1 U mL^{-1} SOD, 2 μg mL^{-1} AFM$_1$, 40 °C for 24 h	26.0%	[34]
CAT	*Bacillus pumilus*	1 U mL^{-1} CAT, 2 μg mL^{-1} AFM$_1$, 40 °C for 12 h	47.2%	[40]
POD1	*Bacillus pumilus*	1 U mL^{-1} POD1, 2 μg mL^{-1} AFM$_1$, 35 °C for 12 h	22.4%	[41]
POD2	*Bacillus pumilus*	1 U mL^{-1} POD2, 2 μg mL^{-1} AFM$_1$, 35 °C for 12 h	25.6%	
POD3	*Bacillus pumilus*	1 U mL^{-1} POD3, 2 μg mL^{-1} AFM$_1$, 35 °C for 24 h	24.3%	
Lac	*Trametes versicolor*	20 mg mL^{-1} Lac, 0.5 ng mL^{-1} AFM1, 25 °C for 80 min	32.0%	[42]
CotA	*Bacillus licheniformis*	2 U mL^{-1} CotA, 2 ng mL^{-1} AFM$_1$, 37 °C for 12 h	83.5% for skim milk; 65.1% for whole milk	This study

The impact of incubation time on the elimination of AFM_1 is given in Figure 5B. The removal percentage of AFM_1 in milk by CotA laccase increased with increasing incubation time, which reached 80.9% and 59.3% for skim milk and whole milk, respectively, at 37 °C after 24 h. Procedures possessing high applicable potential for the elimination of AFM_1 in milk should meet a set of criteria including safety, rapidity, and selectivity [43]. The negative impact of CotA laccase treatment on the nutritive value, organoleptic profiles, and technological properties of milk should be further investigated.

4. Conclusions

This study presents the first characterization of the enzymatic properties and kinetics of CotA laccase in oxidizing AFM_1. The optimal oxidation of AFM_1 by CotA laccase was observed at pH 8.0 and 70 °C. Molecular docking simulations suggested that AFM_1 could interact with CotA laccase via hydrogen bonding and van der Waals interactions. The CotA-AFM_1 complex formed two hydrogen bonds via the carbonyl oxygen atoms and the Gly321 and Gly322 residues. Moreover, HPLC-FLD and UPLC-TOF/MS analysis indicated that CotA laccase oxidized AFM_1 to its C3-hyhroxy derivatives AFN_1 and epi-AFN_1. Furthermore, the oxidation of AFM_1 catalyzed by CotA laccase was observed to significantly reduce its cytotoxicity in hepatocytes. Subsequently, CotA laccase was employed to eliminate AFM_1 in milk, with the removal percentage being influenced by factors such as milk type, CotA laccase concentration, and incubation duration. It was shown that 83.5% of AFM_1 in skim milk and 65.1% of AFM_1 in whole milk were eliminated by 2 U mL^{-1} of CotA laccase at 37 °C after 12 h. This study highlights the AFM_1 oxidase activity of CotA laccase. Nonetheless, further research is necessary to increase the AFM_1 removal efficiency of CotA laccase in milk by enzyme engineering technology.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods13223702/s1, Figure S1: (**A**) Selected ion chromatography of AFM_1; (**B**) Selected ion chromatography of CotA laccase-mediated AFM_1 oxidation products. Table S1. Validation parameters for the determination of AFM_1 concentration in milk with HPLC-FLD method.

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References

1. Thorning, T.K.; Raben, A.; Tholstrup, T.; Soedamah-Muthu, S.S.; Givens, I.; Astrup, A. Milk and dairy products: Good or bad for human health? An assessment of the totality of scientific evidence. *Food Nutr. Res.* **2016**, *60*, 32527. [CrossRef] [PubMed]
2. Guo, Y.; Zhang, Y.; Wei, C.; Ma, Q.; Ji, C.; Zhang, J.; Zhao, L. Efficacy of *Bacillus subtilis* ANSB060 biodegradation product for the reduction of the milk aflatoxin M_1 content of dairy cows exposed to aflatoxin B_1. *Toxins* **2019**, *11*, 161. [CrossRef] [PubMed]
3. Ogunade, I.M.; Arriola, K.G.; Jiang, Y.; Driver, J.P.; Staples, C.R.; Adesogan, A.T. Effects of 3 sequestering agents on milk aflatoxin M_1 concentration and the performance and immune status of dairy cows fed diets artificially contaminated with aflatoxin B_1. *J. Dairy Sci.* **2016**, *99*, 6263–6273. [CrossRef]
4. Veldman, A.; Meijs, J.A.C.; Borggreve, G.J.; Heeres-van der Tol, J.J. Carry-over of aflatoxin from cows' food to milk. *Anim. Sci.* **1992**, *55*, 163–168. [CrossRef]

5. Ismail, A.; Akhtar, S.; Levin, R.E.; Ismail, T.; Riaz, M.; Amir, M. Aflatoxin M1: Prevalence and decontamination strategies in milk and milk products. *Crit. Rev. Microbiol.* **2016**, *42*, 418–427. [CrossRef]
6. Ostry, V.; Malir, F.; Toman, J.; Grosse, Y. Mycotoxins as human carcinogens-the IARC monographs classification. *Mycotoxin Res.* **2017**, *33*, 65–73. [CrossRef]
7. Zhu, Y.; Hassan, Y.I.; Lepp, D.; Shao, S.; Zhou, T. Strategies and methodologies for developing microbial detoxification systems to mitigate mycotoxins. *Toxins* **2017**, *9*, 130. [CrossRef] [PubMed]
8. Firmin, S.; Morgavi, D.P.; Yiannikouris, A.; Boudra, H. Effectiveness of modified yeast cell wall extracts to reduce aflatoxin B_1 absorption in dairy ewes. *J. Dairy Sci.* **2011**, *94*, 5611–5619. [CrossRef]
9. Maki, C.R.; Thomas, A.D.; Elmore, S.E.; Romoser, A.A.; Harvey, R.B.; Ramirez-Ramirez, H.A.; Phillips, T.D. Effects of calcium montmorillonite clay and aflatoxin exposure on dry matter intake, milk production, and milk composition. *J. Dairy Sci.* **2016**, *99*, 1039–1046. [CrossRef]
10. Queiroz, O.C.M.; Han, J.H.; Staples, C.R.; Adesogan, A.T. Effect of adding a mycotoxin-sequestering agent on milk aflatoxin M_1 concentration and the performance and immune response of dairy cattle fed an aflatoxin B_1-contaminated diet. *J. Dairy Sci.* **2012**, *95*, 5901–5908. [CrossRef]
11. Corassin, C.H.; Bovo, F.; Rosim, R.E.; Oliveira, C.A.F. Efficiency of *Saccharomyces cerevisiae* and lactic acid bacteria strains to bind aflatoxin M_1 in UHT skim milk. *Food Control* **2013**, *31*, 80–83. [CrossRef]
12. Elsanhoty, R.M.; Salam, S.A.; Ramadan, M.F.; Badr, F.H. Detoxification of aflatoxin M_1 in yoghurt using probiotics and lactic acid bacteria. *Food Control* **2014**, *43*, 129–134. [CrossRef]
13. Foroughi, M.; Sarabi Jamab, M.; Keramat, J.; Foroughi, M. Immobilization of *Saccharomyces cerevisiae* on perlite beads for the decontamination of aflatoxin M_1 in Milk. *J. Food Sci.* **2018**, *83*, 2008–2013. [CrossRef] [PubMed]
14. Nguyen, T.; Flint, S.; Palmer, J. Control of aflatoxin M1 in milk by novel methods: A review. *Food Chem.* **2020**, *311*, 125984. [CrossRef]
15. Brana, M.T.; Cimmarusti, M.T.; Haidukowski, M.; Logrieco, A.F.; Altomare, C. Bioremediation of aflatoxin B_1-contaminated maize by king oyster mushroom (*Pleurotus eryngii*). *PLoS ONE* **2017**, *12*, e0182574. [CrossRef]
16. Lou, H.; Yang, C.; Li, Y.; Li, Y.; Li, Y.; Zhao, R. Optimization of aflatoxin B_1 degradation in corn by *Ganoderma sinense* through solid-state fermentation. *LWT* **2023**, *183*, 114959. [CrossRef]
17. Mwakinyali, S.E.; Ming, Z.; Xie, H.; Zhang, Q.; Li, P. Investigation and characterization of *Myroides odoratimimus* strain 3J2MO aflatoxin B_1 degradation. *J. Agric. Food Chem.* **2019**, *67*, 4595–4602. [CrossRef]
18. Sangare, L.; Zhao, Y.; Folly, Y.M.; Chang, J.; Li, J.; Selvaraj, J.N.; Xing, F.; Zhou, L.; Wang, Y.; Liu, Y. Aflatoxin B_1 degradation by a *Pseudomonas* strain. *Toxins* **2014**, *6*, 3028–3040. [CrossRef]
19. Sun, L.H.; Zhang, N.Y.; Sun, R.R.; Gao, X.; Gu, C.; Krumm, C.S.; Qi, D.S. A novel strain of *Cellulosimicrobium funkei* can biologically detoxify aflatoxin B_1 in ducklings. *Microb. Biotechnol.* **2015**, *8*, 490–498. [CrossRef]
20. Zhang, F.-L.; Ma, H.-H.; Dong, P.-Y.; Yan, Y.-M.C.; Chen, Y.; Yang, G.-M.; Shen, W.; Zhang, X.-F. *Bacillus licheniformis* ameliorates aflatoxin B_1-induced testicular damage by improving the gut-metabolism-testis axis. *J. Hazard. Mater.* **2024**, *468*, 133836. [CrossRef]
21. Loi, M.; Fanelli, F.; Zucca, P.; Liuzzi, V.C.; Quintieri, L.; Cimmarusti, M.T.; Monaci, L.; Haidukowski, M.; Logrieco, A.F.; Sanjust, E.; et al. Aflatoxin B_1 and M_1 degradation by Lac2 from *Pleurotus pulmonarius* and Redox Mediators. *Toxins* **2016**, *8*, 245. [CrossRef] [PubMed]
22. Guo, Y.; Qin, X.; Tang, Y.; Ma, Q.; Zhang, J.; Zhao, L. CotA laccase, a novel aflatoxin oxidase from *Bacillus licheniformis*, transforms aflatoxin B_1 to aflatoxin Q_1 and epi-aflatoxin Q_1. *Food Chem.* **2020**, *325*, 126877. [CrossRef] [PubMed]
23. Hu, S.; Xu, C.; Lu, P.; Wu, M.; Chen, A.; Zhang, M.; Xie, Y.; Han, G. Widespread distribution of the DyP-carrying bacteria involved in the aflatoxin B_1 biotransformation in *Proteobacteria* and *Actinobacteria*. *J. Hazard. Mater.* **2024**, *478*, 135493. [CrossRef] [PubMed]
24. Adegoke, T.V.; Yang, B.; Tian, X.; Yang, S.; Gao, Y.; Ma, J.; Wang, G.; Si, P.; Li, R.; Xing, F. Simultaneous degradation of aflatoxin B_1 and zearalenone by porin and peroxiredoxin enzymes cloned from *Acinetobacter nosocomialis* Y1. *J. Hazard. Mater.* **2023**, *459*, 132105. [CrossRef] [PubMed]
25. Zhang, H.; Cui, L.; Xie, Y.; Li, X.; Zhao, R.; Yang, Y.; Sun, S.; Li, Q.; Ma, W.; Jia, H. Characterization, Mechanism, and application of dipeptidyl peptidase III: An aflatoxin B_1-degrading enzyme from *Aspergillus terreus* HNGD-TM15. *J. Agric. Food Chem.* **2024**, *72*, 15998–16009. [CrossRef]
26. Graham, D.E. A new role for coenzyme F420 in aflatoxin reduction by soil mycobacteria. *Mol. Microbiol.* **2010**, *78*, 533–536. [CrossRef]
27. Enguita, F.J.; Martins, L.O.; Henriques, A.O.; Carrondo, M.A. Crystal structure of a bacterial endospore coat component. A laccase with enhanced thermostability properties. *J. Biol. Chem.* **2003**, *278*, 19416–19425. [CrossRef]
28. Martins, L.O.; Durao, P.; Brissos, V.; Lindley, P.F. Laccases of prokaryotic origin: Enzymes at the interface of protein science and protein technology. *Cell. Mol. Life Sci.* **2015**, *72*, 911–922. [CrossRef]
29. Mayolo-Deloisa, K.; Gonzalez-Gonzalez, M.; Rito-Palomares, M. Laccases in food industry: Bioprocessing, potential industrial and biotechnological applications. *Front. Bioeng. Biotechnol.* **2020**, *8*, 222. [CrossRef]
30. Chauhan, P.S.; Goradia, B.; Saxena, A. Bacterial laccase: Recent update on production, properties and industrial applications. *3 Biotech* **2017**, *7*, 323. [CrossRef]
31. Sun, F.; Yu, D.; Zhou, H.; Lin, H.; Yan, Z.; Wu, A. CotA laccase from *Bacillus licheniformis* ZOM-1 effectively degrades zearalenone, aflatoxin B_1 and alternariol. *Food Control* **2023**, *145*, 109472. [CrossRef]

32. Li, T.; Huang, L.; Li, Y.; Xu, Z.; Ge, X.; Zhang, Y.; Wang, N.; Wang, S.; Yang, W.; Lu, F.; et al. The heterologous expression, characterization, and application of a novel laccase from *Bacillus velezensis*. *Sci. Total Environ.* **2020**, *713*, 136713. [CrossRef]
33. Alberts, J.F.; Gelderblom, W.C.; Botha, A.; van Zyl, W.H. Degradation of aflatoxin B_1 by fungal laccase enzymes. *Int. J. Food Microbiol.* **2009**, *135*, 47–52. [CrossRef]
34. Liu, X.; Zhao, F.; Wang, X.; Sang, Y. Superoxide dismutase, a novel aflatoxin oxidase from *Bacillus pumilus* E-1-1-1: Study on the degradation mechanism of aflatoxin M_1 and its application in milk and beer. *Food Control* **2024**, *161*, 110372. [CrossRef]
35. Liu, Y.; Mao, H.; Hu, C.; Tron, T.; Lin, J.; Wang, J.; Sun, B. Molecular docking studies and in vitro degradation of four aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2) by a recombinant laccase from *Saccharomyces cerevisiae*. *J. Food Sci.* **2020**, *85*, 1353–1360. [CrossRef]
36. Dellafiora, L.; Galaverna, G.; Reverberi, M.; Dall'Asta, C. Degradation of aflatoxins by means of laccases from *Trametes versicolor*: An in silico insight. *Toxins* **2017**, *9*, 17. [CrossRef]
37. Gao, Y.-N.; Wu, C.-Q.; Wang, J.-Q.; Zheng, N. Metabolomic analysis reveals the mechanisms of hepatotoxicity induced by aflatoxin M_1 and Ochratoxin A. *Toxins* **2022**, *14*, 141. [CrossRef]
38. Marimón Sibaja, K.V.; de Oliveira Garcia, S.; Feltrin, A.C.P.; Diaz Remedi, R.; Cerqueira, M.B.R.; Badiale-Furlong, E.; Garda-Buffon, J. Aflatoxin biotransformation by commercial peroxidase and its application in contaminated food. *J. Chem. Technol. Biotechnol.* **2019**, *94*, 1187–1194. [CrossRef]
39. Kerstner, F.; Cerqueira, M.B.R.; Treichel, H.; Santos, L.O.; Garda Buffon, J. Strategies for aflatoxins B_1 and M_1 degradation in milk: Enhancing peroxidase activity by physical treatments. *Food Control* **2024**, *166*, 110750. [CrossRef]
40. Liu, X.; Zhao, F.; Wang, X.; Peng, K.; Kang, C.; Sang, Y. Degradation mechanism of aflatoxin M_1 by recombinant catalase from *Bacillus pumilus* E-1-1-1: Food Applications in Milk and Beer. *Foods* **2024**, *13*, 888. [CrossRef]
41. Liu, X.; Zhao, F.; Chitrakar, B.; Wei, G.; Wang, X.; Sang, Y. Three recombinant peroxidases as a degradation agent of aflatoxin M_1 applied in milk and beer. *Food Res. Int.* **2023**, *166*, 112352. [CrossRef] [PubMed]
42. Rezagholizade-shirvan, A.; Ghasemi, A.; Mazaheri, Y.; Shokri, S.; Fallahizadeh, S.; Alizadeh Sani, M.; Mohtashami, M.; Mahmoudzadeh, M.; Sarafraz, M.; Darroudi, M. Removal of aflatoxin M_1 in milk using magnetic laccase/MoS2/chitosan nanocomposite as an efficient sorbent. *Chemosphere* **2024**, *365*, 143334. [CrossRef] [PubMed]
43. Kolarič, L.; Minarovičová, L.; Lauková, M.; Kohajdová, Z.; Šimko, P. Elimination of aflatoxin M_1 from milk: Current status, and potential outline of applicable mitigation procedures. *Trends Food Sci. Technol.* **2024**, *150*, 104603. [CrossRef]