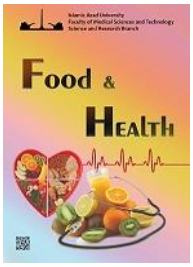

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Microbial methods effect on adsorption and reduction of Aflatoxin contamination in milk

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ABSTRACT

One of the best and most important foods in the human diet is milk. So, the presence of nutritional compounds and the absence of harmful components is very remarkable. Mycotoxins are an important toxin in food and feed, they are produced by molds. The presence of mycotoxins in food is an emerging issue in the world. Among different types of mycotoxins, aflatoxins are so considerable. Different methods are used to reduce them, but the usage of microbial methods such as Lactic acid bacteria, probiotics, and yeasts is a beneficial strategy. The most studied microorganisms are lactic acid bacteria due to their natural presence in milk and also are known as a GRAS (generally recognized as safe) substances. It is noteworthy to mention that the bacterial cell walls are so important in binding ability. Also, parameters such as pH, temperature of incubation, type of starter, which is used in the product, concentration of microorganisms in milk, and the level of toxin can affect the efficiency of microorganisms. Hence, this review was aimed to investigate the results of studies about the effectiveness of microbial methods on the adsorption and reduction of aflatoxin in milk. Also compares the effect of microorganisms, influencing factors, and mechanisms of these methods.

1. Introduction

One of the best and most important foods in our diet is milk. It is due to the presence of many nutritional compounds that are necessary for growth and human health, especially for children and infants. As a result, it is so important to deliver a healthy product to people. Mycotoxins are an important toxin in food and feed, they are produced by molds. Among different types of mycotoxins, aflatoxins are so considerable. The presence of mycotoxins in food is an emerging issue in the world (1). Aflatoxins are one of the most important mycotoxins and secondary metabolites produced by some *Aspergillus* species like *A. flavus*, *A. parasiticus*, and *A. numius*. These toxins exist in a variety of products, for example, peanut, spices, cottonseed, corn, rice, dried fruits, and cereals. Also, they produce during different levels like Growth period, harvest, post-harvest, and storage time (2). High humidity, warm weather, and plant injuries are suitable conditions to grow and aflatoxin production by molds (3). These toxins are known as compounds that have a notable effect on health like serious injuries to the liver, tumor induction, problems about immunosuppressive, and also mutagenic, teratogenic, and carcinogenic effects (4). Various types of aflatoxins, such as B_1, B_2, G_1, G_2, M_1, M_2, etc., were recognized but among them, the most common aflatoxins are B_1, B_2, G_1, G_2 in and aflatoxins M_1 and M_2 in milk. Aflatoxin B_1 (AFB_1) is classified as group 1 carcinogens by the International Agency for Research on Cancer (IARC) (5, 6). Aflatoxin M_1 (AFM_1) is a hydroxylated metabolite of AFB_1 (7). When AFB_1 in contaminated feed is eaten by animals like cows, it turns into AFM_1 in the liver and finally found in milk and dairy products (8). Unfortunately, AFM_1 is relatively stable during different steps of processing, for example pasteurization, cheese making, and storage time (9). The United States Food and Drug Administration published the levels for aflatoxin concentrations of 20 and 0.5 mg/kg for human food and milk, respectively. However, the Codex Alimentarius set the concentrations of 50 ng/kg and 0.025 mg/kg as the regulatory limit and for infant milk, respectively. On the other hand, the European Union limits the aflatoxin levels to no more than 20 mg/kg in feeds and 0.05 mg/kg in milk. The acceptable level of AFM_1 in milk and dairy products

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in Europe is approximately low due to their strict regulations (10). The toxicity level of AFM_1 is similar or a little milder than AFB_1 and its carcinogenic potential is ten times less than AFB_1 (11), but its cytotoxic, genotoxic, and carcinogenic effects have been proven (12). It is noteworthy to mention that milk and dairy products are widely consumed by different age groups of people. Ideally, one of the best ways to control these toxins in food and feed is prevention, but this cannot always happen, so various methods like physical, chemical, and biological have been used for detection and reduction of these toxins. Physical methods such as heating, cleaning, and washing are used but these methods are not readily applicable or so effective (13, 14). Also, different chemical compounds to detoxify these toxins have been used, but some of them have shown problems about safety and health. On the other hand, they can probably reduce the nutritional value of the product (15). Thus, biological methods widely investigated such as the usage of bacteria and yeasts to reduce the toxic effect of mycotoxins and also the prevention of using harmful chemical compounds. Hence, this review was aimed to investigate the results of studies about the effectiveness of microbial methods on the adsorption and reduction of aflatoxin in milk. Also compares the effect of microorganisms, influencing factors, and mechanisms of these methods.

2. The effect of lactic acid bacteria on Aflatoxins

Lactic acid bacteria are one of the most significant groups of organisms. They are commonly used in fermented products. In addition to their beneficial features, the ability of adsorption and reduction of aflatoxins has been considered (16). Probiotics can be described as: "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (17). The most studied microorganisms are lactic acid bacteria due to their natural presence in milk and also are known as GRAS (generally recognized as safe) substances (18). Also, some researchers reported that the inhibition of aflatoxin is due to the presence of lactic acid bacteria or their metabolites in milk. These metabolites are heat stable and low molecular weight compounds (19). It is noteworthy to mention that the bacterial cell walls are so important of binding ability. There is a difference in aflatoxins binding by various strains and it depends on the components in bacterial cell walls and cell structures. These toxins are bound onto the cell wall components by weak non-covalent interactions, also environmental conditions can be influential (20, 21). Therefore, the important elements which are responsible for the binding ability, including all kinds of Peptidoglycan layer, Polysaccharides, and Teichoic acids in the bacterial cell wall (21, 22). Martínez et al. (23) used probiotic bacteria in their study, which including *Pediococcus pentosaceus* and *Kluveromyces marxianus*. The results have shown the ability of these strains to degrade and adsorb AFM_1 to fewer toxic metabolites in milk. Also, a significant difference was observed about AFM_1 adsorption. As they reported, the difference between AFM_1 adsorption by these strains is related to the cell wall structure and cell membranes. The results of another research have shown that *Bacillus subtilis* isolated from the fish gut has a significant effect on AFM_1 adsorption. The adsorption percentages were 80.7%, 60%, and 81.5% for AFG_1, AFM_1, and AFB_1, respectively (24). Generally, the results of studies have shown that the binding ability of each microorganism has a direct relationship with the concentration of them (25). In research by Sadeghi et al. (26) the required bacterial population for *Lactobacillus brevis* to get the desired result in removing aflatoxins was 2×10^3 CFU/ml. Also, Fazeli et al. (27) reported 2×10^3 CFU/ml as the adequate amount to remove AFs for *Lactobacillus plantarum*, *L. fermentum*, and *L. casei*. In a study by Abdelmotilib et al. (28), the elimination effect of *Bifidobacterium bifidum* (57.68%) was more than *Lactobacillus acidophilus* (48.70%) and *Lactobacillus Plantarum* (36.90%). Moreover, the combination of three viable probiotic strains (*B. bifidum*, *L. Plantarum*, and *L. acidophilus*) at the concentration of 5×10^9 CFU/ml showed a higher percent of AFM_1 removal (64.62%) than the usage of one strain. In a study by Serrano-Nino et al. (29) five strains of probiotic bacteria were used to reduce the level of AFM_1 in vitro digestive model. According to their results, all strains demonstrated several degrees of aflatoxin binding, ranging from 19.95 to 25.43%. According to the findings of Kabak and Var (30), the minimum cell population (10^8 CFU/ml or gr) is needed for AFM_1 reduction in contaminated solutions, no need for long incubation time. In their study, *Bifidobacterium bifidum* was the best binder bacteria (approximately 25%) and *lactobacillus acidophilus* was the weakest removal bacteria. Also, the difference between the binding ability of bacteria at the concentration of 10^8 CFU/ml was not significant for 0 hours and 4 hours, except for 24 hours in all toxin concentrations. Line and Brackett (31) reported that viable bacteria with a cell population of 1×10^9 CFU/ml or greater are required for a considerable reduction of AFB_1. This amount was similar to the results of El-Nezami et al. (32) research. Their study is related to Trichothecene toxin which was bound to *Lactobacillus* and *Propionibacterium* strains. They described that for this purpose, a minimum cell population of 2×10^9 CFU/ml of viable cells is needed. Peltonen et al. (20) studied the binding ability of 12 *Lactobacillus*, five *Bifidobacterium*, and three *Lactococcus* strains to AFB_1 in PBS (Phosphate Buffer Saline). They reported that the range of the binding ability for *Lactobacillus*, *Bifidobacterium*, and *Lactococcus* strains was 17.3–59.7%, 18.0–48.7%, and 5.6–41.1%, respectively. Elsanhoty et al. (19) reported in their study that the amount of AFB_1 degradation depends on the type of starter. They used various strains of lactic acid bacteria in yogurt. The product containing 50% of yogurt starter culture (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) and 50% of *Lactobacillus plantarum* showed the highest reduction in the AFM_1 level at the end of the storage time. The result of another study showed that probiotic bacteria can bind to AFM_1 in a product called Doogh during fermentation and refrigerated storage time. AFM_1 binding ability of probiotic bacteria depends on the species. Both viable and non-viable (heat-killed) cells of *Lactobacillus acidophilus* had the binding

ability for removing AFM_1. Doogh with a final pH of 4.5 and 7 log CFU/mL of viable cells showed a fermented dairy drink with high probiotic viability and low level of AFM_1 (33). Furthermore, the results of various studies showed that probiotic bacteria can bind to AFM_1 in the intestine and reduce its bioaccessibility in the gut, so they could decrease the risk of mycotoxins in the human body (29).

3. The effect of yeasts on Aflatoxins

In different studies by researchers, the usage of yeasts has been considered. *Saccharomyces cerevisiae* is the most effective yeast to bind aflatoxin (34). Moreover, nonviable cells do not lose their ability to adsorb and reduce toxins (35). The probable mechanism for the binding of AFM_1 is the adsorption with components on the cell wall. Yeasts can adsorb different compounds such as toxins on their cell wall and it is confirmed that a covalent bond formation between the toxin and cell wall can reduce the level of them in products (6). The presence of a component called mannan in the cell wall plays an important role in aflatoxin binding by *S. cerevisiae* (36). The combination of yeasts and probiotic bacteria could be appropriate to reduce AFM_1 in milk (28). In a study by Salim et al. (37), *S. cerevisiae* and lactic acid bacteria strains were used and the percentage of AFM_1 reduction level was increased in samples. Foroughi et al. (38) reported that immobilized cells of *S. cerevisiae* can be used to detoxify the level of AFM_1 in contaminated milk. According to their descriptions, two types of ceramics (activated alumina and alumina silicate) bind to considerable amounts of AFM_1 in liquid foods and can be a sufficient platform for immobilized yeast cells. In a study by Khiavi et al. (8), yeast cell walls were disrupted and separated by the thermal shock ultrasonication method and the usage of sodium dodecyl sulfate (SDS) caused protein denaturation and change cell surface characteristics, these features expose additional binding sites and increase binding capacity. The results of their research showed that the most effective method for the disruption of yeasts cells walls is thermal shock ultrasonication. Also, they reported that the adsorption of AFM_1 is probably due to mannan and β-D glucan present in the cell wall of yeasts. If yeast cell walls are used in the form of a mixture at the contact time of 24 hours, the AFM_1 level will decrease by about 83%, but at contact time of 15 minutes, this reduction was approximately lower. The most reduction in AFM_1 level was related to the use of calcium alginate beads at contact time of 15 minutes (8). When contacting time increases, more aflatoxin will adsorb by microorganisms. These findings were consistent with the results of Corassin et al. study (39). It has shown that the sample containing immobilized yeasts cell walls on silica nanoparticles entrapped in alginate gel, significantly decrease the AFM_1 content (86%) at the contact time of 24 hours (8, 40).

4. The effect of different treatments on Aflatoxins

It has been shown that the bacterial aflatoxin binding capacity increased by heat and acid treatments and can be stable under different temperatures (4–37°C) and pH (3, 9, 15, 16, 41). El-Deeb et al. (42) reported that the use of microorganisms, enzymes, and particularly the presence of organic acids can reduce the AFM_1 level in milk. Also, some researches showed that heat treatment significantly increased the bacteria's ability to reduce AFM_1. So, the presence of viable bacteria is not a prerequisite to adsorb or reduce aflatoxin (19, 20, 30, 43, 44). Bovo et al. (41) reported in their study that only the heat-killed cells were used for AFM_1 binding and their reason refers to avoid the possible fermentation problems in milk. A higher binding ability of dead microbial cells than viable cells was observed. Also, the heat-killed bacterial cells that were bound to AFM_1 may excrete without absorbing from the human body. The results of Pierides (45) study showed an increase in the AFM_1 binding ability of 8 *Lactobacillus* strains by heat inactivation. The binding capacities of heat-killed bacteria were found to range from 14.04% to 28.97%. Actually, it depends on the concentration of toxin and incubation conditions (temperature and duration). Also, a small difference ($p<0.05$) about the reduction of AFM_1 at the concentration of 5 ng/ml between viable and heat-killed cells of *L. acidophilus* (16.05% and 24.08%, respectively) at 0 hour was recognized in Kabak and Var (30) study. Another study reported that heat-killed dairy strains (lactic acid bacteria) can remove aflatoxin as same as viable cells (43). In addition, using viable or acid and heat-killed bacterial cells to reduce and adsorb aflatoxin in products depends on initial concentrations of toxin (20, 44). Hernandez-Mendoza et al. (46) reported that when *Lactobacillus* strains were exposed to bile, the binding ability increases. Also, other studies suggested that the binding ability will improve if bacteria expose to bile, which can alter cells (21). Furthermore, the presence of bile may change the conformation of cell wall compounds (e.g. proteins, phospholipids, and glycolipids), it can induce new aflatoxin binding sites or enhance the already existing sites and eventually increase aflatoxin binding ability (47). Most experiments reported that the bile could enhance bacterial binding ability with two mechanisms: (I) altering the expression of bacterial genes, which results in the encoding of more new proteins on cell walls, (II) altering the structure of bacterial cell walls as well as proteins and phospholipids, actually, it causes the new binding sites (47-51). It must be mentioned that in a study by Hernandez-Mendoza et al. (46), a significant reduction in removal capacity was observed when the loss or destruction of the cell wall (total or partial) occurred in response to enzymatic treatments. More studies are needed to figure out the effect of each parameter on the binding ability. For example, incubation conditions like temperature and time or bacterial and aflatoxin concentration. Although the best temperature and incubation time was suggested at 25-30°C and 48 hours (52), different studies have used various incubation time and temperature. In a study by Fazeli et al. (27), LABs incubated in the presence of AFB_1 for 72 hours at 37 °C. According to their result, the percent of AFB_1 elimination was variable and it depends on different incubation time. Also, a higher AFB_1 removal was shown in 72 hours vs. 24. The

results of a study indicated that there was a reduction in the level of AFM_1 during different storage time and pH development about all treatments. Also, the amount of AFM_1 was reduced by a decrease in pH. Generally, a relationship was found between the reduction in pH values and the reduction in AFM_1, wherein the more decrease in pH values, the more reduction in AFM_1 contents (19). Maryamma et al. (53) achieved the same result when they used different strains of LAB including *Lactobacillus acidophilus*, *L. casei*, *L. rhamnosus* and *L. rhamnosus*. The reduction level of toxin by these strains ranged from 26.2% to 34.0%, depending upon the bacterial isolates. However, Megalla and Hafez (54) stated that the complete elimination of AFB_1 is caused by its transformation into AFB_2 by non-enzymatic process, i.e., the acid present in yogurt. One of the reasons for AFM_1 reduction in yogurt during storage time may be due to the oxidation of glucose by glucose oxidase. According to the results of Yousef and Marth (55) study, the oxidation of glucose produces gluconolactone and these products will be distributed in yogurt. Hydrogen peroxide can turn into singlet oxygen, which is more reactive ($H_2O_2 \rightarrow H_2O+O$). This reactive oxygen may react with the AFM_1 molecule and adsorb it. Also, gluconic acid can be made by the hydrolysis of gluconolactone and it may decrease the amount of pH (3.9) in yogurt, so AFM_1 can be reduced. In another study, Govaris et al. (56) studied the stability of AFM_1 in yogurt, which artificially contaminated with concentrations of 0.05 and 0.1 µg/L during storage time at 4°C for 4 weeks at pH values of 4.0 and 4.6. They reported that there was no significant difference ($p>0.01$) in AFM_1 level at pH 4.6 but in the yogurt, at pH 4.0 the AFM_1 level reduced significantly ($p<0.01$) after the third and fourth weeks of storage at both concentrations. So, this reduction in AFM_1 may be a function of low pH. It had been found that the aflatoxin binding to bacterial cell walls was reversible and it depends on the conditions utilized for recovery. The results of Kabak and Var (30) study indicated that a small amount of AFM_1 was released back into the solution (34). Serrano-Niño et al. (29) reported in their study that at least a small proportion of AFM_1 was bound to the bacteria in a reversible form. Actually, the stability of this binding depends on the kind of strain and environmental condition during the formation process (57). Also, another study reported that although the binding process of AFB_1 is efficient, it could be partially reversible. All in all, the researchers concluded that different parameters can affect the reduction of aflatoxin levels, such as low pH, the presence of organic acids, metabolites produced during the fermentation by the microorganism. Also, other factors including the concentration of toxin, the concentration of microorganisms, and incubation conditions (temperature and duration) may change the efficiency of microorganisms to reduce and adsorb aflatoxin from different products (41).

5. Conclusion

The results of various studies have shown that the presence of aflatoxin in milk and dairy products is a serious issue that can make different problems for human health. Also, the increase of AFM_1 level in milk and dairy products exceeding of Codex limit may have a bad effect on international trade in dairy products in global markets. Ideally, one of the best ways to control Contaminants in food and feed is prevention, but this cannot always happen. The different method has used to reduce the level of mycotoxins, but according to the results of studies, one of the best ways for this purpose is the biological method, for example, the usage of lactic acid bacteria, probiotics, and yeasts. It is noteworthy to mention that the bacterial cell walls play an important role in binding ability. Also, parameters such as pH, temperature of incubation, type of starter, which is used in the product, concentration of microorganisms in milk, and the level of toxin can affect the efficiency of microorganisms. The results of various studies showed that appropriate strains can adsorb and reduce aflatoxin in milk. Generally, if food organizations implement regulations strictly or improve analytical facilities and harvesting practices, these natural contaminants will reduce in milk and dairy products as human food. Although more details about AFM_1 absorption in the intestine and the impact of probiotics in relevant animal models are needed.

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