An Acad Bras Cienc (2023) 95(Suppl. 1): e20220794 DOI 10.1590/0001-3765202320220794
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

MICROBIOLOGY

Detoxification of aflatoxin M1 in different milk types using probiotics

KUBRA SANALDI & AHMET Y. COBAN

Abstract: The aim of this study, research the potential use of probiotics in reducing the toxic effect of Aflatoxin M1 in cow milk, goat milk, sheep milk, and Phosphate-buffered saline (PBS). Milk and Phosphate-buffered saline were contaminated with Aflatoxin M1 at a concentration of 100 ppt. Then, various study groups were formed by adding *Lactobacillus acidophilus* DSMZ 20079, *Lactobacillus rhamnosus GG,* and *Bifidobacterium bifidum* DSMZ 20456 probiotic bacteria at a density of 10^8 CFU/ml. Then, working groups were stored for 1 day and Aflatoxin M1 levels were analyzed by an Enzyme-Linked Immunosorbent Assay kit. The binding level of Aflatoxin M1 by probiotic bacteria varies between 2.32-12.52% in Phosphate-buffered saline, 9.08-40.14% in cow milk, 15.01-38.01% in goat milk, and 32.49-42.90% in sheep milk. The highest binding level of Aflatoxin M1 was detected in sheep milk and the lowest in Phosphate-buffered saline. The binding ability of Aflatoxin M1 is ranked from highest to lowest in sheep milk, cow milk, and goat milk. The data obtained from this study is important because it is the first study to show that if sheep and goat milk is enriched with probiotics, it can reduce AFM1 exposure.

Key words: Probiotics, Aflatoxin M1, sheep milk, goat milk, cow milk.

INTRODUCTION

More than 400 types of mycotoxins produced by various fungal species have been identified, of which aflatoxins are the most well-known. Aflatoxin M1 (AFM1), a type of mycotoxin, is found in milk and has an important place in our diet. Aflatoxins are highly lipid-soluble compounds and readily pass into the circulation, usually via the gastrointestinal tract. They are transported from the blood to different tissues and to the liver, which is the main organ of the metabolism of xenobiotics. Aflatoxins are mainly metabolized by the liver to a reactive epoxide intermediate or hydroxylated to become the less harmful AFM1 (Murphy et al. 2006). AFM1, which is secreted into the milk of humans and mammals fed with contaminated food, has a high cytotoxic and carcinogenic effect. Therefore, it has been classified as belonging to group 1 carcinogen for humans by the International Agency for Research on Cancer (IARC) (IARC 2002).

AFM1 release is found in the milk of animals 12-24 hours after consuming contaminated feed and reaches high levels within a few days. AFM1 isn't found in milk approximately 24 hours after the removal of contaminated feed from the diet. Depending on the animal species, AFM1 levels in milk may differ. This rate varies between 0.35-3% in cows, 0.18-3% in goats, and 0.08-0.33% in sheep (Campagnollo et al. 2016). Milk and dairy products have an important place in our society and are frequently consumed especially by children. For this reason, it is necessary to frequently check the formation of aflatoxins in foods, especially in infant milk products. The upper limit for AFM1 in milk and dairy products varies between 0 and 1 µg/kg in international regulations (Iqbal et al. 2015).

The chemical composition of milk; is affected by various factors such as animal species, environmental conditions, lactation stage, or nutritional status of the animal. While cow's milk (83%) is the most frequently consumed milk type, other milk types such as goat (2.3%) and sheep (1.4%) are also preferred (Verduci et al. 2019). Among milk types, sheep milk can be distinguished from other milk types with its higher protein and fat content, while goat milk stands out with higher amounts of vitamins A and D. Goat and sheep milk are good sources of vitamin A (Pereira 2014).

Various physical, chemical, and biological processes are applied to remove mycotoxins such as aflatoxins from foods. However, some negative sides such as being expensive, low efficiency, and forming toxic secondary metabolites have limited physical and chemical applications. In recent years, there are studies on the biological detoxification of aflatoxins. In particular, the aflatoxin detoxification abilities of probiotic bacteria are being investigated. The most studied group of bacteria is lactic acid bacteria (LAB), which play a role in the fermentation of foods. Antifungal metabolites such as phenolic compounds, hydrogen peroxide, hydroxy fatty acids, and protein complexes released by lactic acid bacteria reduce or neutralize toxins. In addition, lactic acid produced as a result of the fermentation of lactose causes a rapid decrease in pH and thus gains importance in the control of pathogen contamination. Control of pathogen contamination in fermented dairy products largely depends on this fermentation procedure (Assaf et al. 2019).

The mechanism of probiotic bacteria in the detoxification of mycotoxins isn't yet fully known. There are two kinds of assumptions. The first of these is the reduction of intestinal absorption through cell wall binding between probiotic bacteria and mycotoxins. The second is the breakdown of carcinogen-containing compounds through specific metabolic products. Cell wall components of bacteria, such as peptidoglycan, are sites responsible for the binding of mycotoxins. Besides peptidoglycan, other components such as polysaccharides and teichoic acid also play a role in the binding process. The aflatoxin binding capacity of probiotic bacteria may vary depending on the strain type, mutagen concentration, ambient pH, and incubation time (Fashandi et al. 2018).

In a study, the AFM1 binding abilities of yogurt starter bacteria were compared in milk and PBS. Binding was much greater in milk when compared to milk and Phosphate-buffered saline (PBS). This situation suggested that AFM1 can bind to milk casein (El Khoury et al. 2011). To determine whether AFM1 binds to casein, contaminated milk was treated with a proteolytic enzyme. It was found that proteins in processed milk bind 30.7% more AFM1 on average, and casein binds AFM1 in the range of 17.9-55.3µg per gram (Brackett & Marth 1982).

Goat and sheep milk also affects the functional properties of probiotics such as survival in the gastrointestinal tract, adhesion to the intestinal epithelium, immunomodulation, and antimutagenic properties. Goat milk is considered the most important probiotic carrier among non-beef dairy products. Goat milk and sheep milk can help preserve the viability of probiotics during storage (Ranadheera et al. 2016).

It is reported that to see the beneficial effects of probiotics on health, there should be at least $1x10^8$ CFU/ml live microorganisms in yogurt or fermented milk (Rezac et al. 2018). Since it is close to the values specified in the regulations, the study was carried out using $1x10^8$ CFU/ml live microorganisms.

In the literature, it has been shown that probiotics can be effective in reducing

aflatoxin concentration in cow milk and PBS environments. However, no study was found on AFM1 detoxification by different animal milk. Therefore, this study aims to investigate the effect of probiotic bacteria on AFM1 levels in different animal milk. Inulin, which has prebiotic properties, was expected to support the development of probiotics, and the indirect effect of inulin on the reduction of AFM1 by probiotics was also investigated.

MATERIALS AND METHODS

Microorganisms and chemicals

L. acidophilus DSM 20079*, L. rhamnosus* GG*, B. bifidum* DSM 20456 strains belonging to Bifidobacterium and Lactobacillus genus, which have been added to dairy products recently, were used in the study. *Lactobacillus acidophilus* DSMZ 20079*, Lactobacillus rhamnosus* GG*, Bifidobacterium bifidum* DSMZ 20456 strains from the German Collection of Microorganisms and Cell Cultures were used in the study. AFM1 standard was used in powder form obtained from *Aspergillus flavus*, with at least 98% purity and 50 µg (Sigma-Aldrich; St. Louis, USA). MRS Broth for growth of test bacteria (Merck; Germany), inulin from chicory (Sigma-Aldrich; St. Louis, USA), and PBS in tablet form (Sigma-Aldrich; St. Louis, USA) were used. AgraQuant® Aflatoxin M1 High Sensitivity (5/100) assay kit with 96 wells, 100 ppt reading limit was used (Romer Labs; Austria). Cow milk, goat milk, and sheep milk were obtained raw from a local producer.

Preparation of cultured bacteria and bacterial cell

Stock strains stored at -80˚C were expected to dissolve at room temperature. Then, it was transferred to 10 ml MRS broth containing %0.05 L-cysteine HCl. It was incubated under anaerobic conditions for 3 days. Thus, the bacteria are activated. To adjust the number of bacteria to be added to the study groups, bacterial cultures were centrifuged at 3000 g for 15 minutes and the remaining liquid medium was removed. Then, it was washed with deionized water and centrifuged again at 3000 g for 15 minutes. For each bacteria to be added to the study groups by removing the deionized water, Mc Farland adjustment was made with preliminary trials to have a density of 10^8 CFU/ml.

Preparation of milk samples

Cow milk, goat milk, and sheep milk fat were separated (<0.01) and heat treated at 90˚C for 5 minutes.

Preparation of aflatoxin M1 solution and contamination

Studies using AFM1 were carried out under a chemical cabinet, using a chemical mask (Isolab; Germany) and nitrile gloves. 50 µg AFM1 standard was dissolved and diluted with acetonitrile. Then, acetonitrile was removed under N_2 gas with the nitrogen evaporator. In the study groups, 10 ml milk and PBS were contaminated with a final AFM1 concentration of 100 ppt (Sevim et al. 2019, Zamberlin & Samaržija 2017). After it was vortexed for 1 minute to ensure toxin dispersion. Bacteria and 2% inulin were added to AFM1 containing cow milk, goat milk, sheep milk, and PBS medium in various combinations. Probiotic bacteria were single and combinations were made as *L. acidophilus + B. Bifidum, L. rhamnosus + B. Bifidum*. The specified strains were added to PBS and 3 different types of milk (cow's milk, goat's milk, and sheep's milk) individually and in combinations.

Determination of AFM1 binding capability of bacteria

After the samples were incubated for 4 hours at 37C, they were stored at +4˚C for 1 day. AFM1

analysis of milk and PBS samples was performed following the study protocol specified in the AgraQuant® Aflatoxin M1 test kit. According to this protocol, samples were centrifuged at 3000 g for 10 minutes before analysis. 100 ppt, 50 ppt, 25 ppt, 10 ppt, 5 ppt, and 0 ppt standards were added as 100 µl to the first 6 wells. Then, the samples were added to 100 and incubated for 45 minutes at room temperature. At the end of the period, 5 washing processes were carried out with the washing solution. Then, 100 µl of the conjugate solution was added to each well and incubated for 15 minutes at room temperature. Washing was repeated 5 times. After washing, 100 µl of substrate solution was added to each well and incubated for 15 minutes at room temperature. At the end of the time, the reaction was stopped by adding 100 µl of stop solution. All the experiments were performed in triplicate and the absorbance was measured using a microplate reader (Allsheng, China) at 450 nm. The amount of AFM1 corresponding to the absorbance values was calculated. The limit of detection (LOD) was 4.2 ppt and recovery of AFM1 was found to be between 95.7-79.8%.

Statistical analysis

Statistical comparisons were made using One way ANOVA, Tukey's test, and Dunnett's Multiple Comparison post-test by Instat-3 statistical software (GraphPad Software Inc., San Diego, CA, USA). The results are considered to be statistically different at $p < 0.05$.

RESULTS

Composition of different types of milk used in the study

Among the cow milk, goat milk, and sheep milk used in the study, the highest protein content was found in sheep milk with 5.68%. The lowest protein content was found in cow milk with 2.59%. The protein content of goat milk was found as 3.04%.

Effect of probiotics on AFM1 binding ability in PBS

The percentage of AFM1 binding in PBS of study groups numbered 1 to 10 is given in Figure 1a-d. The lowest AFM1 binding ability in PBS was found in the group containing *L. rhamnosus+B. bifidum*+inulin with a rate of 2.32±0.96% ($p>0.05$). The highest AFM1 binding ability was found in the group containing *L. acidophilus+B. bifidum* with a rate of 12.52±1.19% ($p<0.01$). AFM1 binding ability of 12.52±1.19% in the group containing *L. acidophilus+B. bifidum* ($p<0.01$), 9.64±2.23% of AFM1 binding ability in the group containing *B. bifidum* ($p<0.05$) and 8.75±3.10% in the group containing *L. acidophilus+B. bifidum*+inulin ($p<0.05$) were found to be significant compared to the initial. The AFM1 binding abilities of the other study groups weren't significant compared to the initial ($p>0.05$).

Among the groups without inulin, the lowest AFM1 binding ability was found in the group containing *L. rhamnosus* with a rate of 2.56±2.02%, and the highest AFM1 binding ability was found in the group containing *L. acidophilus+B. bifidum* with a rate of 12.52±1.19%. A significant difference was found between *L. rhamnosus* and *L. acidophilus+B. bifidum* ($p<0.01$), between *L. acidophilus* and *L. acidophilus+B. bifidum* ($p<0.01$), between *L. rhamnosus* and *B. bifidum* ($p<0.05$). There was no significant difference between the other study groups without inulin ($p>0.05$).

Among the inulin-added groups, the lowest AFM1 binding ability was found in the group containing *L. rhamnosus+B. bifidum* with a rate of 2.32±0.96% and the highest AFM1 binding ability was found in the group containing *L. acidophilus+B. bifidum* with a rate of 8.75±3.10%. A significant difference was found between *B.*

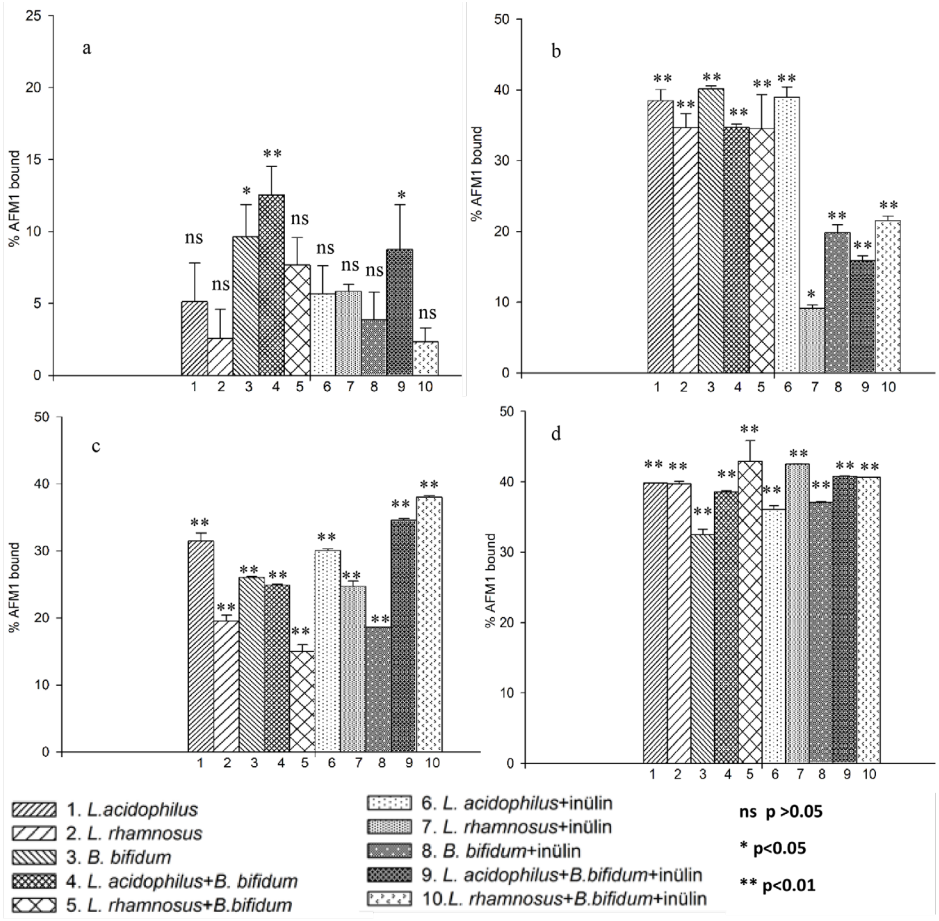

Figure 1. (a) Investigation of the effect of probiotic bacteria on AFM1 binding ability in PBS. (b) Investigation of the effect of probiotic bacteria on AFM1 binding ability in cow milk. (c) Investigation of the effect of probiotic bacteria on AFM1 binding ability in goat milk. (d) Investigation of the effect of probiotic bacteria on AFM1 binding ability in sheep milk.

bifidum and *L. acidophilus+B. bifidum* (p<0.05), between *L. acidophilus+B. bifidum* and *L. rhamnosus+B. bifidum* (p<0.05). There was no significant difference between the other study groups with inulin (p>0.05).

Effect of probiotics on AFM1 binding ability in cow milk

The percentage of AFM1 binding in cow milk of study groups numbered 1 to 10 is given in Figure 1a-d. The lowest AFM1 binding ability in cow milk was found in the group *L. rhamnosus*+inulin with a rate of 9.08±0.50% (p<0.05). The highest AFM1 binding ability was found in the group containing *B. bifidum* with a rate of 40.14±0.38% (p<0.01). AFM1 binding ability was found significant in *L. rhamnosus*+inulin group (p<0.05) and in other groups (p<0.01).

Among the groups without inulin, the lowest AFM1 binding ability was found in the group containing *L. rhamnosus+B. bifidum* with a rate of 34.50±4.83% and the highest AFM1 binding ability was found in the group containing *B. bifidum* with a rate of 40.14±0.38%. There was no significant difference between the study groups without inulin (p>0.05).

Among the inulin-added groups, the lowest AFM1 binding ability was found in the group containing *L. rhamnosus* with a rate of 9.08±0.50%, and the highest AFM1 binding ability was found in the group containing *L. acidophilus* with a rate of 38.94±1.45%. There wasn't a significant difference between *B. bifidum* and *L. acidophilus+B. bifidum* (p<0.05), *B. bifidum* and *L. rhamnosus+B. bifidum* (p>0.05). A significant difference was found between *L. acidophilus+B.*

bifidum and *L. rhamnosus+B. bifidum* (p<0.05), between *L. rhamnosus* and *L. acidophilus+B. bifidum* (p<0.01) and among others between two groups with inulin (p<0.001).

Effect of probiotics on AFM1 binding ability in goat milk

The percentage of AFM1 binding in goat milk of study groups numbered 1 to 10 is given in Figure 1a-d. The lowest AFM1 binding ability in goat milk was found in the group *L. rhamnosus+B. bifidum* with a rate of 15.01±0.97% (p<0.01). The highest AFM1 binding ability was found in the group containing *L. rhamnosus+B. bifidum*+inulin with a rate of 38.01±0.21% (p<0.01). AFM1 binding ability of all study groups in goat milk was significant compared to the initial (p<0.01).

Among the groups without inulin, the lowest AFM1 binding ability was found in the group containing *L. rhamnosus+B. bifidum* with a rate of 15.01±0.97% and the highest AFM1 binding ability was found in the group containing *L. acidophilus* with a rate of 31.45±1.20%. There wasn't a significant difference between *B. bifidum* and *L. acidophilus+B. bifidum* in terms of AFM1 binding ability (p>0.05). A significant difference was found between *L. rhamnosus* and *L. rhamnosus+B. bifidum* (p<0.05), between *L. acidophilus* and *B. bifidum* (p<0.01), and between *L. acidophilus* and *L. acidophilus+B. bifidum* (p<0.01), between *L. rhamnosus* and *B. bifidum* (p<0.01), and between *L. rhamnosus* and *L. acidophilus+B. bifidum* (p<0.01) and among others between two groups without inulin (p<0.001).

Among the inulin-added groups, the lowest AFM1 binding ability was found in the group containing *B. bifidum* with a rate of 18.59±0.05%, and the highest AFM1 binding ability was found in the group containing *L. rhamnosus+B. bifidum* with a rate of 38.01±0.21%. A significant difference was found between *L. acidophilus+B. bifidum* and *L. rhamnosus+B. bifidum* (p<0.01) and among others between two groups with inulin (p<0.001).

Effect of probiotics on AFM1 binding ability in sheep milk

The percentage of AFM1 binding in sheep milk of study groups numbered 1 to 10 is given in Figure 1a-d. The lowest AFM1 binding ability in sheep milk was found in group *B. bifidum* with a rate of 32.49±0.78% (p<0.01). The highest AFM1 binding ability was found in the group containing *L. rhamnosus+B. bifidum* with a rate of 42.90±2.92% (p<0.01). AFM1 binding ability of all study groups in goat milk was significant compared to the initial (p<0.01). AFM1 binding ability of all study groups in sheep milk was significant compared to the initial (p<0.01).

Among the groups without inulin, the lowest AFM1 binding ability was found in the group containing *B. bifidum* with a rate of 32.49±0.78%, and the highest AFM1 binding ability was found in the group containing *L. rhamnosus+B. bifidum* with a rate of 42.90±2.92%. A significant difference was found between *B. bifidum* and *L. acidophilus* (p<0.05), between *B. bifidum* and *L. rhamnosus* (p<0.05), and between *B. bifidum* and *L. rhamnosus+B. bifidum* (p<0.01). There was no significant difference between the other groups without inulin (p>0.05).

Among the inulin-added groups, the lowest AFM1 binding ability was found in the group containing *L. acidophilus* with a rate of 36.07±0.47%, and the highest AFM1 binding ability was found in the group containing *L. rhamnosus* with a rate of 42.51±0.08%. A significant difference was found between *L. acidophilus* and *L. rhamnosus*, and between *L. acidophilus* and *L. acidophilus+B. bifidum*, between *L. acidophilus and L. rhamnosus+B. bifidum,* between *L. rhamnosus* and *B. bifidum*

($p<0.05$). There was no significant difference between the other groups with inulin ($p>0.05$).

The AFM1 binding ability of probiotics in PBS and different types of milk is summarized in Table I and Figure 1.

DISCUSSION

In our study, *L. acidophilus* 5.11-39.81%, *L. rhamnosus* 2.56-39.71%, *B. bifidum* 9.64-40.14%, *L. acidophilus+B. bifidum* 12.52-38.55%, *L. rhamnosus+B. bifidum* showed the ability to bind AFM1 7.65-42.90%. The probiotic bacteria strain with the highest AFM1 binding ability was determined as *L. acidophilus+B. bifidum* in PBS, *B. bifidum* in cow milk, *L. acidophilus* in goat milk, and *L. rhamnosus+B. bifidum* in sheep milk ($p<0.01$). The highest AFM1 binding ability was achieved by different probiotic bacteria in a different mediums. Therefore, a single effective strain doesn't come forward.

Although the mechanism of binding of probiotic bacteria to aflatoxin isn't yet clear, it is associated with components such as polysaccharides and peptidoglycan in the bacterial cell wall. Therefore, the integrity of bacterial cell wall components becomes very important in aflatoxin detoxification. A study reported that the aflatoxin binding capacity is significantly reduced in case of complete or partial damage to the cell wall (Hernandez-Mendoza et al. 2009). In our study, the difference in the ability of probiotic bacterial species to bind AFM1 may be due to different cell wall structures. In addition, the interactions of bacteria with each other during the storage process may also have affected AFM1 levels.

The ability of *L. plantarum* to bind AFM1 in milk medium at a density of 10^9 CFU/ml was investigated at different toxin concentrations (0,5,1,2,5,10 ppb). The highest binding ability was determined as 61.33% at 5 ppb AFM1 concentration. Increasing the AFM1 concentration from 5 ppb to 10 ppb resulted in a decrease in the percent binding. This is explained by the limited binding site of the microorganism and the saturation of this binding site at high toxin concentrations. Binding has been associated with physical adsorption (Yuksel & Albayrak Bulut 2020).

In a study investigating the effect of different microbial densities (10^7, 10^8, 10^9, and 10^{10} CFU/ml) on AFM1 binding, heat-killed *S. cerevisiae* and 3 LAB strains were evaluated individually and in combination at the end of one hour. It has been determined that the ability of microorganisms

Table I. Summary of the ability of probiotics to bind AFM1 in different types of milk and PBS.

	% Binding of AFM1 (mean ± SD)									
Medium	*L. acidophilus*	*L. rhamnosus*	*B. bifidum*	*L. acidophilus* + *B. bifidum*	*L. rhamnosus* + *B. bifidum*	*L. acidophilus* + inülin	*L. rhamnosus* + inülin	*B. bifidum* + inülin	*L. acidophilus* + *B. bifidum* + inülin	*L. rhamnosus* + *B. bifidum* + inülin
PBS	5.11±2.69	2.56±2.02	9.64±2.23	12.52±1.19	7.65±1.90	5.64±1.96	5.84±0.47	3.84±1.93	8.75±3.10	2.32±0.96
Cow milk	38.50±1.50	34.63±2.01	40.14±0.38	34.68±0.47	34.50±4.83	38.94±1.45	9.08±0.50	19.78±1.16	15.79±0.72	21.50±0.66
Goat milk	31.45±1.20	19.56±0.82	26.03±0.10	24.87±0.14	15.01±0.97	30.07±0.22	24.70±0.78	18.59±0.05	34.57±0.23	38.01±0.21
Sheep milk	39.81±0.03	39.71±0.34	32.49±0.78	38.55±0.18	42.90±2.92	36.07±0.47	42.51±0.08	37.09±0.09	40.77±0.05	40.64±0.03

to bind to AFM1 is directly proportional to the density of microorganisms, that is, the highest AFM1 binding is 10^{10} CFU/ml and the lowest is 10^{7} CFU/ml (Ismail et al. 2017).

In a study, the binding levels of *L. acidophilus, L. rhamnosus,* and *B. bifidum* strains at a density of 10^{8} CFU/ml to AFM1 in PBS and milk medium were investigated after 4 hours. *B. bifidum* has the highest AFM1 reduction level in PBS (25.02%) and milk (25.94%) (Fashandi et al. 2018). In another study, the AFM1 reduction level of *L. acidophilus* was found to be 15.9%, while the AFM1 reduction level of *L. rhamnosus* was found to be 16.7% in PBS. The reduction level of AFM1 was found to be 27.8% in the combination of *L. acidophilus* with yogurt starter bacteria. It has been shown that there was a decrease in AFM1 levels with a decrease in pH in the study groups. It was thought that the heat and acidity formed during fermentation increased the ability of microorganisms to bind AFM1 (Elsanhoty et al. 2014).

Studies have indicated that the connection between aflatoxin and microorganisms is a rapid process that occurs in the first minutes of contact (Bovo et al. 2013, Corassin et al. 2013, Serrano-Nino et al. 2013). For this reason, one day was considered sufficient in our study.

Heat-killed cells are preferred to avoid possible effects of fermentation and to achieve higher binding rates. Killing the bacteria to be used in the study by heat treatment may affect the bonding through the formation of some reaction products between the cell wall components. Depending on the heat treatment time, type, and temperature, reversible or irreversible denaturation events occur. A reversible denaturation of proteins or other cell wall components can cause renaturations after heating (Assaf et al. 2019). In our study, heat treatment was not applied to bacteria, and it was studied with living cells.

In a study investigating the stability of AFM1 during probiotic yogurt production and cold storage, a probiotic culture containing *L. acidophilus, B. lactis, S. thermophilus,* and *L. bulgaricus* was added to the milk sample and it was contaminated with 100 ng/l AFM1. Then the milk was stored at +4˚C for 21 days. It was found that the decrease in AFM1 level at the end of storage was approximately 41% (Montaseri et al. 2014). The level of AFM1 reduction depends on many variables such as the type of bacteria used, toxin concentration, incubation temperature, incubation time, and analysis method. Therefore, it is expected that the data obtained from our study will be different from previous studies.

Mechanical and thermal treatments didn't have a significant effect on the AFM1 concentration in milk. In a study, the effect of milk fat and different heat treatments on AFM1 distribution was investigated. Milk samples were first contaminated with AFM1 at the maximum legally permissible concentration of 50 ng/kg, then the fat of the milk samples were separated and different pasteurization processes (65˚C / 30 min, 73˚C / 20 seconds, 95˚C / 15 minutes) were applied. According to the results of the study, no significant difference was found in AFM1 concentrations between whole-fat, skim, and pasteurized milk samples (Barukčić et al. 2018).

Goat milk is an important probiotic carrier among non-bovine milk products. In addition, sheep milk can help protect the viability of probiotics during storage (Ranadheera et al. 2016). In a study, it was determined that fermented goat milk containing *L. acidophilus* could maintain its viability at a sufficient level (>10^{7} CFU/ml) during 21 days of storage at +4˚C (Ranadheera et al. 2016). In another study, standard yogurt culture and a probiotic strain, *L. rhamnosus*, were used for the production of yogurt from sheep milk. After 21 days of storage at +4˚C, the viability of yogurt culture bacteria

and *L. rhamnosus* was found to be sufficient (>10^6 CFU/ml) (Zamberlin & Samaržija 2017).

In our study, the binding ability of AFM1 was found to be higher than PBS in milk. Similarly in a study, *L. bulgaricus* and *S. thermophilus* showed 18.7% and 29.42% AFM1 reduction levels respectively in PBS. In milk, it was determined that AFM1 decreased levels increased by 27.56% and 39.16% respectively. When yogurt was formed by adding *L. bulgaricus* and *S. thermophilus* to milk, a decrease of 14.82% AFM1 was observed, and it was suggested that this might be due to the decrease in the bond established between protein-AFM1 due to the acceleration of the fermentation process and increasing proteolytic activity in yogurt (Sarımehmetoğlu & Küplülü 2004). In a study by Brackett and Marth, it was reported that there was an average of 30.75 more AFM1 in milk treated with proteolytic enzyme than in unprocessed milk, and it was suggested that AFM1 binds to milk protein. This confirmed the interaction of aflatoxin with milk proteins and showed that as the number of proteins increases, the amount of AFM1 bound will also increase (Brackett & Marth 1982).

In the literature, it has been determined that studies affecting the level of AFM1 reduction were carried out in PBS and cow milk environments, but there weren't any studies in goat and sheep milk. In this study, in which the effect of probiotic bacteria on AFM1 was investigated in different milk types, 10 study groups were formed. In 8 of 10 study groups, the highest AFM1 binding ability was observed in sheep milk. The lowest AFM1 binding ability was observed in the PBS medium in all study groups. Goat milk followed the lowest AFM1 binding ability after PBS with 7 study groups. When the study groups without inulin and the study groups with inulin were investigated, it was determined that the highest AFM1 binding ability was in sheep milk, followed by cow milk, goat milk, and PBS respectively. In addition, the binding ability of AFM1 is 2.32-12.52% in PBS, 9.08-40.14% in cow milk, 15.01-38.01% in goat milk, and 32.49-42.90% in sheep milk.

Based on all this data, it can be said that the ability of probiotic bacteria to bind AFM1 among different types of milk occurs in sheep milk, cow milk, and goat milk, respectively, from highest to lowest.

Protein percentages of the milk used in our study were listed as sheep milk (5.68%)>goat milk (3.04%)>cow milk (2.59%) from highest to lowest. Based on the interaction of aflatoxins with milk proteins, the binding abilities of AFM1 were expected to be ranked from highest to lowest as sheep milk>goat milk>cow milk. As expected in the results of our study, the highest AFM1 binding was observed in sheep milk with a protein percentage of 5.68%. However, while higher AFM1 binding was expected in goat milk than in cow milk, contrary, higher AFM1 binding was observed in cow milk. This can be explained by the fact that the protein values of cow milk (2.59%) and goat milk (3.04%) are close.

The decrease in AFM1 levels in fermented milk is attributed to factors such as low pH, organic acid formation, or other fermentation byproducts. Low pH during fermentation alters the structure of casein from milk proteins and leads to clot formation. Changes in the casein structure during fermented milk production affect the relation of AFM1 with this protein (Govaris et al. 2002). In our study, the relationship between the number of milk proteins and AFM1 was investigated. In the future, studies are needed to investigate the number of proteins in different types of milk, as well as the relationship between casein amounts and AFM1.

The symbiotic use of probiotics and prebiotics is attracting the attention of both consumers and the dairy industry due to their potential beneficial effects. The inclusion

of symbiotic foods consisting of probiotics, prebiotics, or both in the diet is seen as one of the effective biological methods for the removal of food-borne mutagens (Kearney & Gibbons 2018). The synergistic effect of probiotics and prebiotics in aflatoxin detoxification may be due to the role of prebiotics in increasing the viability of probiotics as a fermentable energy source and by forming soluble fiber (Wochner et al. 2019). Thus, a higher reduction in intestinal absorption of mycotoxins can be achieved.

In a study, 1%, 2%, and 3% concentrations of inulin were added to yogurt containing *S. thermophilus, L. acidophilus,* and *Bifidobacterium* sp. and stored at +4 C for 21 days. Inulin showed a stimulating effect on the growth of *L. acidophilus* and *Bifidobacterium sp*. While %2 inulin showed the highest effect on the growth of *L. acidophilus* within 1-14 days, this period was determined as 1-7 days for *Bifidobacterium sp*. 2% inulin showed the highest effect on the growth of *L. acidophilus* on day 7, while on day 1 for *Bifidobacterium* sp. At the end of the study, lactic acid bacteria numbers were found to be sufficient in 97% of the samples (Gustaw et al. 2011).

In our study, there are study groups in which the addition of 2% inulin has a positive effect on AFM1 binding. An increase in AFM1 binding was observed in 2 groups with the addition of inulin. This is an increase from 24.87±0.14% to 34.57±0.23% in goat milk containing *L. acidophilus+B. bifidum* and from 15.01±0.97% to 38.01±0.21% in goat milk containing *L. rhamnosus+B. bifidum* ($p<0.001$). In a study using an in vitro digestion model, the effect of *L. acidophilus* with different combinations of inulin, oligofructose, β-glucan, and polydextrose on AFM1 detoxification in whole milk was investigated. At the end of the study, the level of reduction of AFM1 by *L. acidophilus* alone was higher than when prebiotics was added. *L. acidophilus*, alone and in combination with prebiotics, has also been shown to reduce AFM1 bioavailability (Wochner et al. 2019).

In our study, the prebiotic effect of inulin differed among probiotic bacteria. This is due to the specific response of probiotic bacteria strains to prebiotics. The positive effect of the addition of inulin on the binding of AFM1 was observed significantly in the *L. acidophilus+B. bifidum* and *L. rhamnosus+B. bifidum* study groups in which probiotics were combined rather than in the study groups in which they were used alone ($p<0.001$). Based on this, in our study, it can be said that the addition of inulin has a positive effect on the AFM1 level when probiotics are in combination. In a similar study, AFM1 binding abilities of *L. plantarum, B. bifidum, and B. animalis* were investigated by using yogurt starter cultures with the addition of inulin alone or in combinations. At the end of 1-day storage, the addition of inulin negatively affected the AFM1 reduction level of *B. bifidum+B. animalis*, while it positively affected the AFM1 reduction level of *L. plantarum+B. bifidum* and *L. plantarum+B. animalis* (Sevim et al. 2019).

As a result of our study, it was found that *L. acidophilus, L. rhamnosus,* and *B. bifidum* probiotic bacteria, which are frequently used in the dairy industry, are effective in reducing AFM1 levels. Therefore, probiotic bacteria appear to play an important role and can be used as a biological agent to reduce the toxic effect of AFM1. Regular consumption of probiotics can contribute to the improvement of health status.

In our study, the decreased level of AFM1 was found to be higher in milk than in PBS. This confirmed the interaction of aflatoxin with milk proteins. The addition of inulin, on the other hand, negatively affected the level of AFM1 reduction in general. In our study, sheep milk was found to be the medium with the highest AFM1 reductions. Milk-containing probiotic bacteria may be preferred to reduce AFM1

exposure directly to milk consumption. Among the milk types, sheep milk can be given priority.

Acknowledgments

Authors appreciate Serhat Bozkurt, Orhan Kocak, Kubra Yildirim, and Ece Simsek for providing support to this research. This study was funded by Akdeniz University Scientific Research Projects Coordination Unit (Project number: TYL-2020-5406). The Origin of an article: Thesis.

Abbreviations

Abbreviation	Full name
AFM1	Aflatoxin M1
L. acidophilus DSMZ	*Lactobacillus acidophilus* DSMZ
L. rhamnosus GG	*Lactobacillus rhamnosus GG*
B. bifidum DSMZ	*Bifidobacterium bifidum* DSMZ
LAB	lactic acid bacteria
IARC	International Agency for Research on Cancer
ELISA	Enzyme-Linked Immunosorbent Assay (Enzyme-Linked Immunosorbent Assay
PBS	Phosphate-buffered saline
µg	Microgram
ng	Nanogram
CFU	Colony-forming unit

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How to cite

SANALDI K & COBAN AY. 2023. Detoxification of aflatoxin M1 in different milk types using probiotics. An Acad Bras Cienc 95: e20220794. DOI 10.1590/0001-3765202320220794.

Manuscript received on September 30, 2022;
accepted for publication on January 23, 2023

KUBRA SANALDI[1,2,3]
https://orcid.org/0000-0002-4399-3693

AHMET YILMAZ COBAN[1]
https://orcid.org/0000-0002-8815-6063

[1]Akdeniz University, Faculty of Health Sciences, Department of Nutrition and Dietetics, Dumlupinar Bulvarı 07058 Kampus, Antalya, Turkey

[2]Akdeniz University, Tuberculosis Research Center, Dumlupinar Bulvarı 07058 Kampus, Antalya, Turkey

[3]Akdeniz University, Department of Medical Biotechnology, Institute of Health Sciences, Dumlupinar Bulvarı 07058 Kampus, Antalya, Turkey

Correspondence to: **Ahmet Yilmaz Coban**
E-mail: cobanay2003@gmail.com

Author contributions

A.Y.C. designed the experiment. K.S. performed all the laboratory analyses and wrote the manuscript. K.S. and A.Y.C. read and approved the final manuscript.