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The Effect of *Lactobacillus rhamnosus* on AFM1 Detoxification in Ice Cream

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ABSTRACT

This study investigates the effectiveness of *Lactobacillus rhamnosus* in the detoxification of AFM1 contamination. Experiments conducted on two groups of ice cream samples measured an average AFM1 level of 0.0308 µg/kg in the control group and 0.0258 µg/kg in the group with *L. rhamnosus* added. The results indicate that *L. rhamnosus* significantly reduced AFM1 levels (46%) and was thus effective in the detoxification of AFM1. Our statistical results were found to be significant ($p<0.05$). These findings highlight the potential of *L. rhamnosus* to reduce AFM1 contamination in ice cream products, making a significant contribution to food safety. Controlling mycotoxins in food products is critical for protecting consumer health and enhancing food safety.

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Introduction

Ice cream is generally defined as "a dairy product obtained by processing a mixture of milk and dairy products (such as milk powder, cream), sweeteners, stabilizers, emulsifying agents, color and flavoring substances by adding air to them in freezers" (Türkmen and Gürsoy, 2017). Ice cream is a common consumer product, in addition to containing many bioavailable substances for the human body. It is known that its consumption is limited for some consumer groups (e.g. people with lactose intolerance and milk allergy) (Góral et al., 2018). It has been reported that the number of people with milk allergy and lactose intolerance is almost 80% of the world population (Pineli et al., 2015). At this point, beverages obtained from plant sources as an alternative to cow's milk and products produced using these beverages increase the variety of alternative products for consumer groups who have difficulty in daily nutrition (Góral et al., 2018).

Mycotoxins are secondary metabolic products produced by toxigenic molds and cause a disease called mycotoxicosis in humans and animals. (Miller, 2016). Mycotoxins are small molecular structures, usually aromatic compounds. Molds of the genera *Aspergillus, Penicillium, Fusarium* and *Alternaria* are among the most important species that synthesize mycotoxins and they are all from the hypomycetes class. Hundreds of mycotoxins have been identified today, but the most important in terms of their formation and toxicity are aflatoxins, fumonisins, trichothecenes, ochratoxins, patulin and zearalenone and their metabolites. There are many other toxicologically important but less studied mycotoxins such as ergot alkaloids, enniatins, alternaria toxins, moniliformin, citrinin, beauvericin, cyclopiazonic acid, roquefortine C, mycophenolic acid, penitrems (Kralj and Prosen, 2009). To date, approximately 400 different mycotoxins have been identified (Kowalska et al., 2017). Aflatoxins, Ochratoxin A, Patulin, Sterigmatocystin, Ergot alkaloids, Trichothecenes, Zearelenone, Citrinin, Penicillium, Fumonisin and Rubratoxin are among the mycotoxins of particular importance. Aflatoxins are the most studied group of mycotoxins due to their potential carcinogenic effects (Kowalska et al., 2017). Mycotoxins are also defined according to their effects on different tissues and organs or their mechanisms of action. Those that affect the liver are defined as "hepatotoxic", those that affect the nervous system are defined as "neurotoxic", those that affect the kidneys are defined as "nephrotoxic", those that affect the immune system are defined as "immunotoxic" and those that affect the embryo are defined as "embryotoxic" (Adhikari et al., 2017).

Aflatoxins are toxic secondary metabolites produced by certain types of molds (*Aspergillus*, *Penicillium* and *Rhizopus*) such as *Aspergillus flavus* and *Aspergillus paraciticus*. Consumption of food or feed contaminated with aflatoxins can cause a disease called "aflatoxicosis" in humans and animals. An incident in England (1960) that resulted in the death of more than 100.000 turkeys caused aflatoxins to draw attention for the first time (Ağaoğlu et al., 2020). This disease, which also affected ducklings, was called "Unknown Turkey Disease" (Turkey-X-Disease) (Kowalska et al., 2017). Research has demonstrated that turkey feed containing moldy peanut meal imported from Brazil was contaminated with *Aspergillus flavus*. Consumption of these moldy feeds was determined to be the cause of poisoning cases seen in turkeys and ducks. The word aflatoxin originates from the letters "A" and "fla" of *Aspergillus flavus* and the suffix "toxin" (A-fla-toxin), which means poison (Kowalska et al., 2017, Ağaoğlu et al., 2020). Aflatoxins are compounds that have toxic effects on human and animal health. Depending on the amount of toxin ingested and the duration of the toxin's effect, acute and chronic poisoning can occur in humans and animals. In humans, vomiting, abdominal pain, jaundice, pulmonary edema, coma and convulsions are the most common symptoms of aflatoxin poisoning (Li et al., 2018). In chronic aflatoxicosis, immune suppression and liver damage occur. In addition to the carcinogenic, mutagenic, teratogenic, hepatotoxic and immunosuppressive properties of aflatoxins, they have also been reported to be effective in kidney damage and the formation of various organ tumors (lung, liver, breast, colon, rectum) (Li et al., 2018).

Mycotoxins are highly toxic substances produced by fungi that pose a serious threat to both human and animal health. Mycotoxin contamination in food and feed is a global problem that causes major economic losses every year (Biniş and Demir, 2023). As a result of years of research, various methods have been developed to render mycotoxins harmless, and among them, biological methods stand out with their great potential and advantages (Liu et al., 2022, Taşcı et al., 2022). Scientists are increasingly choosing biological transformation methods that aim to detoxify mycotoxins by breaking them down into less toxic compounds or enzymatically converting them. It has been shown that many microorganisms such as bacteria, molds and yeasts can biologically convert these toxins (Hathout and Aly, 2014). In recent years, biological detoxification approaches, especially those using microbial cells or enzymes, have proven to be highly effective in degrading mycotoxins to less harmful products with high specificity (Afsah-Hejri et al., 2020). Studies conducted on milk and dairy products have validated the success of probiotic-based detoxification to prevent aflatoxin contamination (Khamesipour et al., 2013, Godoy et al., 2024). Compared to physical and chemical methods, biological detoxification processes can be carried out with less loss of nutritional value of foods and under milder conditions (Zhu et al., 2017). Recently, there has been an increasing interest in the application of probiotics with high xenobiotic binding capacity, which can be used to protect against food contaminants, for example, to alleviate the acute and chronic toxicity of aflatoxins via *Lactobacillus* strains (Reddivari et al., 2017, Demir and Ağaoğlu, 2023). In vitro and in vivo experiments have shown that probiotic microorganisms can bind and/or metabolize various chemical contaminants such as organophosphorus pesticides, mycotoxins, and heavy metals (Feng et al., 2018, Godoy et al., 2024). Probiotics can directly bind certain xenobiotics to the cell wall, thereby reducing their bioactivity and toxicity (Alizadeh et al., 2022). Probiotic strains with xenobiotic binding capacity have been reported to be a simple and effective way to reduce the amount of contaminants absorbed from food in chemically contaminated areas worldwide (Biniş and Demir, 2023).

The aim of this study is to evaluate the potential of *Lactobacillus rhamnosus* to reduce Aflatoxin M1 (AFM1) levels in ice cream samples. Additionally, the study seeks to investigate the interaction mechanisms of this probiotic bacterium with AFM1, highlighting its potential as an effective biological solution for food safety.

Materials and Methods

Material

In the study, a total of 30 packaged ice cream samples in plain, chocolate and fruit varieties obtained from ice cream selling companies and local businesses in Sivas province were analyzed. These samples, taken under aseptic conditions and at different times, were brought to the laboratory of the Food Hygiene and Technology Department of the Faculty of Veterinary Medicine, Sivas Cumhuriyet University for analysis by applying a cold chain. The samples were stored in the freezer at -18°C until the analysis was completed.

Method

AFM1 levels in ice cream samples were determined using ELISA (Enzyme-Linked Immunosorbent Assay) method. Shanghai Coon Koon Biotech Co CK-LAB KIT, CK-bio-20774 was used in the analyses. All analysis steps were carried out in accordance with the recommendations of the manufacturer. Additionally, the VITA-PRONIT probiotic microorganism commercial supplement (*Lactobacillus rhamnosus* 1.75 x 10^9 cfu/ml) was used for the detoxification of ice cream.

Kit Contents

Biotech Co CK-LAB KIT, CK-bio-20774 used in the analyses contains; 12x8 well strips (in foil), 6 standards (0, 5, 10, 25, 50 and 100 ppt), 15 ml AFM1 conjugate solution, 15 ml substrate solution, 15 ml stop solution and 15 ml wash solution (x20 concentrate). The detection limit of this kit is 5 ppt.

ELISA Kit Analysis Procedure

Samples and Aflatoxin M1 standards were added to antibody-coated microwells and AFM1 was allowed to bind to the antibody binding sites. Following a wash step, enzyme-bound aflatoxin was added, allowing antibodies to bind to the free binding sites. After a second wash step, enzyme substrate was added and the color change was observed. The color intensity is inversely proportional to the AFM1 concentration in the sample or standard. A stop solution was then added, changing the color from blue to yellow, and the absorbance of each well was measured at 450 nm and 630 nm. The measurement was completed within 10 min after the stop solution was added.

Preparation of Samples

Pretreatment of Ice Cream Samples

5 mL of the ice cream sample was left at room temperature and pipetted into a test tube and incubated at 4°C for 30 min. Then, the sample was centrifuged at 3500 rpm for 10 min. The oil layer formed after centrifugation was carefully removed with a Pasteur pipette and 100 µL of milk serum sample was separated from the layer below. After these procedures were completed, the samples were ready for analysis.

Test Protocol

Each standard (0, 5, 10, 25, 50 and 100 ppt) and 100 µL of samples were added to the antibody-coated wells using different pipette tips. The wells were incubated at room temperature for 45 min. After the incubation period, the microwell content was emptied into the waste container, and each microwell was filled with washing solution and emptied, washing a total of five times. Then, 100 µL of conjugate solution was added to each antibody-coated microwell, and the wells were incubated for another 15 min at room temperature. After incubation, the washing process was repeated as in the previous step. 100 µL of substrate solution was added to each microwell and incubated for 15 min at room temperature. Then, 100 µL of stop solution was added to each well, and a color change from blue to yellow was observed. Finally, the absorbance of each well was measured at 450 nm (reference wavelength 630 nm) with a microwell reader within 10 min.

Statistical Analysis

The descriptive statistics of AFM1 levels detected in the samples were analyzed, and the relationships between these values were evaluated using a paired samples t-test in SPSS version 23.00.

Results and Discussion

This study investigated the effect of *L. rhamnosus* on AFM1 levels in ice cream samples. The data obtained show variations in AFM1 levels across a total of 30 ice cream samples (Figure 1). The highest AFM1 level, measured before the addition of probiotics, was observed in sample 24 at 0.0467 µg/kg. This represents the highest level of AFM1 contamination in the ice cream. The lowest AFM1 level was detected in sample 19 after the addition of probiotics, measuring 0.0163 µg/kg. This indicates that probiotics significantly reduce AFM1 levels.

The average AFM1 levels were calculated as 0.0308 µg/kg before the addition of probiotics and 0.0258 µg/kg after the addition. This shows that *L. rhamnosus* reduced AFM1 levels by an average of 24.5%. The percentage reduction values observed among the ice cream samples exhibit significant differences. The highest percentage reduction was determined in sample 17 at 46.10%, reflecting the most effective condition for probiotic addition. Other notable reduction values were recorded in sample 16 at 40.05%, sample 18 at 34.60%, and sample 14 at 27.41%.

However, some samples showed low percentage reductions despite the addition of probiotics, with sample 1 and sample 8 exhibiting reductions of only 2.60% and 3.30%, respectively. This suggests that the probiotic's efficacy (binding capacity) may not have been sufficient at the concentration added. In conclusion, this study demonstrates that *L. rhamnosus* has the potential to significantly reduce AFM1 levels in ice cream samples. The findings highlight the importance of probiotic applications in terms of food safety and indicate a need for further research.

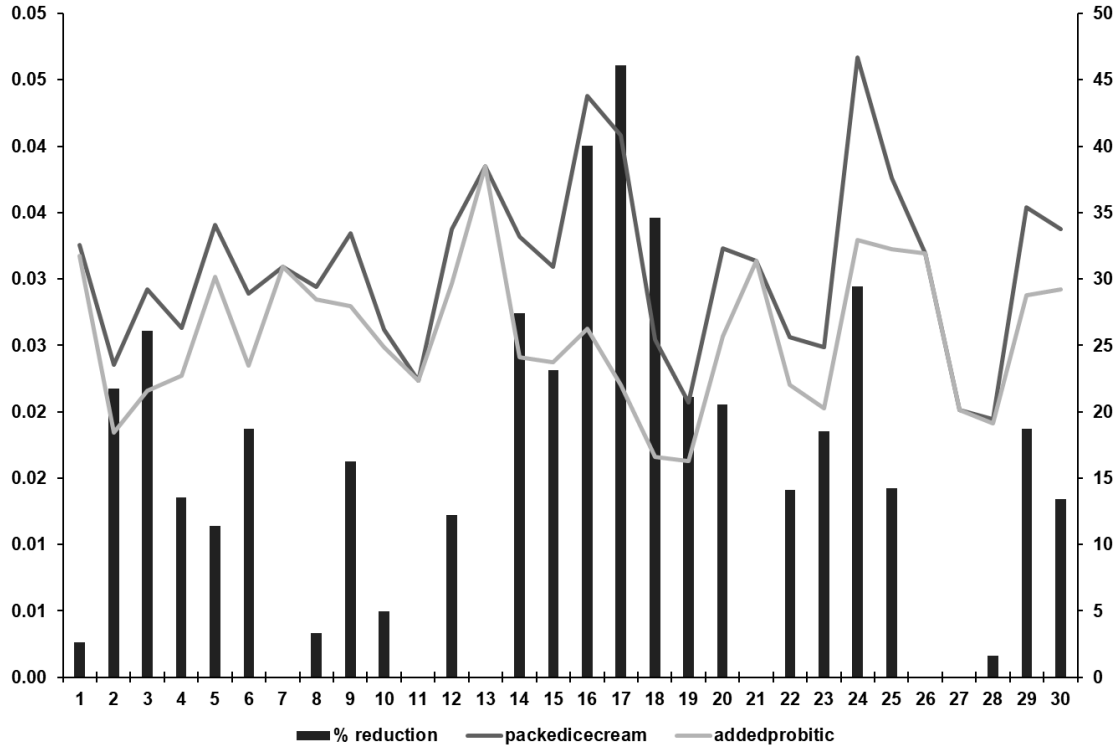

Figure 1. Comparison of AFM1 levels and % reduction in packed ice cream and ice cream with added *L. rhamnosus*.

In this study, AFM1 levels were measured before and after the addition of *L. rhamnosus* in 30 ice cream samples, and the difference between these two conditions was statistically analyzed. According to the Paired Samples Statistics table, the average AFM1 level before the addition of *L. rhamnosus* was 0.03076, while the average after addition was found to be 0.02579. This indicates that *L. rhamnosus* reduced the AFM1 level by an average of 0.004973 units. Additionally, the correlation coefficient between the measurements was 0.694, which is statistically significant ($p = 0.001$). According to the results of the Paired Samples Test, the average difference related to the reduction of AFM1 levels by *L. rhamnosus* is 0.004973, with a standard deviation of 0.004902. The obtained t-value is 5.557 and the p-value is 0.001. The p-value being less than 0.05 indicates that this reduction in AFM1 levels is statistically significant. Furthermore, based on the 95% confidence interval, it was observed that this difference ranges from 0.003143 to 0.006804. These results demonstrate that *L. rhamnosus* significantly reduces AFM1 levels, and this finding is statistically reliable (Table 1).

Figure 2 shows that the scatterplot matrix consists of four panels illustrating the relationships between the variables "packedicecream" (packed ice cream) and "addedprobiotic" (probiotic added). The positive trend observed in the graph indicates a strong correlation between the two groups, with AFM1 levels being relatively close to each other. However, there is an overall decrease in AFM1 levels following the addition of probiotics, reflecting the differences between the two groups. Both the correlation analysis and the results of the preceding t-test support the conclusion that the addition of probiotics significantly reduces AFM1 levels.

Aflatoxins are toxic metabolites that pose a significant threat to food safety. Liu et al. (2020) emphasized in their study that the biological decontamination of aflatoxins is particularly achieved through the use of lactic acid bacteria (LAB).

Table 1. Statistical Analyses

Paired Samples Statistics

Pair	Mean	N	Std. Deviation	Std. Error Mean
Packed Ice Cream	0.03076	30	0.006717	0.001226
Added Probiotic	0.02579	30	0.005483	0.001001

Paired Samples Test

Pair	Mean Difference	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference	t	df	Sig. (2-tailed)
Packed Ice Cream - Added Probiotic	0.004973	0.004902	0.000895	Lower: 0.003143, Upper: 0.006804	5.557	29	0.001

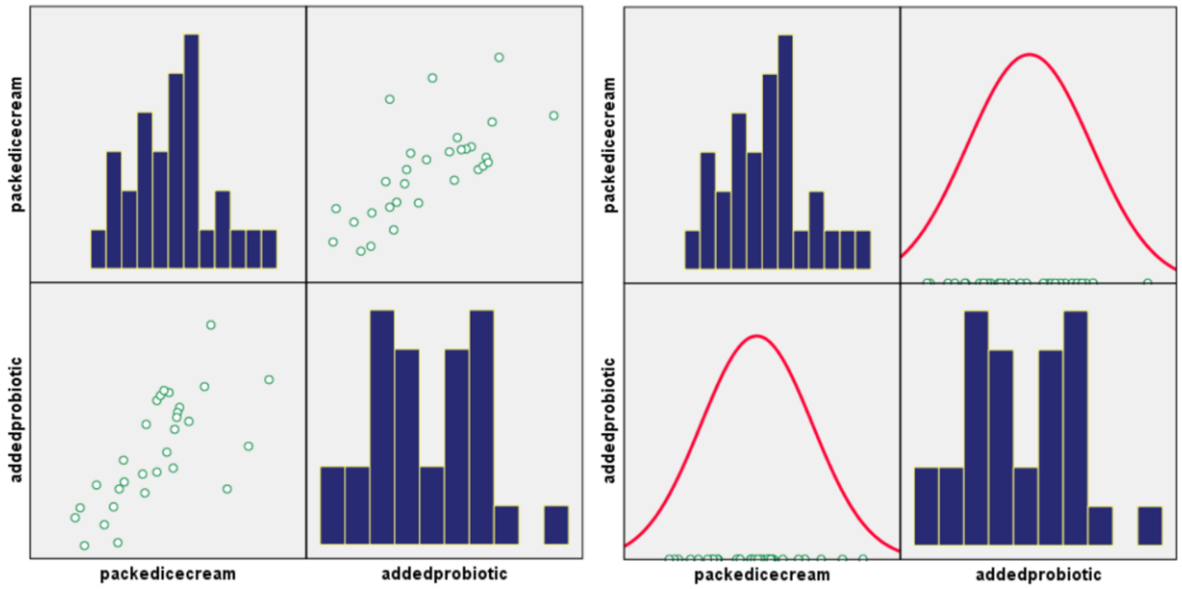

Figure 2. Scatterplot matrix comparing AFM1 levels in packed ice cream and ice cream with added *L. rhamnosus*

In this study, the binding mechanisms of LAB with aflatoxins were examined in detail, highlighting the potential of these bacteria for the decontamination of harmful substances in food and agriculture. When compared to the data obtained in this study, it suggests that lactic acid bacteria may play an important role in the effective reduction of aflatoxins. In this study, the results obtained from measuring AFM1 levels in 30 ice cream

samples demonstrate how the addition of LAB affects aflatoxin levels. For example, the highest reduction of 46.10% supports the interaction of LAB with aflatoxins and how this interaction binds aflatoxins. This finding is consistent with the results from Liu et al. (2020), which also emphasized the importance of LAB's capacity to bind aflatoxins.

Moreover, the highest reduction rate of 46.10% noted in this study clearly demonstrates the effectiveness of LAB in aflatoxin decontamination. Liu et al. (2020) stated that LAB can bind aflatoxins, thereby removing them from food systems. This situation aligns with the highest reduction of 46.10% observed in this study, indicating that the use of LAB can effectively reduce aflatoxins to safe levels. Consequently, the data obtained suggest that lactic acid bacteria could be an effective strategy for reducing aflatoxin contamination in food products. The information presented in Liu et al. (2020) and similar studies further strengthens the potential of these microorganisms in food safety applications. Future studies could contribute to the knowledge base in this field by examining the interaction mechanisms of LAB with aflatoxins in greater detail (Liu et al., 2020).

In the study conducted by Rezasoltani et al. (2021), the detoxification effects of the probiotics *Saccharomyces boulardii*, *Lactobacillus casei*, and *Lactobacillus acidophilus* on AFM1 in reconstituted milk were investigated. The research results indicated that the highest AFM1 removal rate was achieved with a concentration of *S. boulardii* at 10^9 cfu/ml for 90 minutes at 37°C with a toxin concentration of 0.75 ng/ml, yielding a cleaning rate of 96.88±3.79. Additionally, a significant cleaning rate of 71.46±3.79 was found with *L. acidophilus* at a concentration of 10^7 cfu/ml for 90 minutes at 4°C with the same toxin concentration. Similarly, the binding percentage achieved with *L. casei* at a concentration of 10^7 cfu/ml was determined to be 64.31±3.79. These findings support the notion that probiotics can be an effective strategy for the detoxification of harmful compounds like AFM1. When compared to the data in our study, similar results were obtained using *L. rhamnosus* in samples with the highest aflatoxin reduction. This suggests that probiotics offer a potential solution for food safety (Rezasoltani et al., 2021).

In the study conducted by Abbès et al. (2013), the in vitro binding capacity of *L. rhamnosus* GAF01 to AFM1 and its protective role against the immunotoxic effects of AFM1 were examined. The study found that *L. rhamnosus* GAF01 exhibited a binding ability of AFM1 ranging from 15.3% to 95.1% in reconstituted milk samples containing 0.05, 0.10, and 0.20 µg AFM1/ml, with this binding occurring as a stable complex formation between the bacteria and AFM1. Additionally, it was noted that *L. rhamnosus* GAF01 provided a protective effect on the total white and red blood cells and lymphocyte subtypes by preventing the adverse effects of AFM1 on the immune system after a 15-day treatment. These findings indicate that probiotics could play a significant role in food safety and may be effective in the detoxification of AFM1. When compared with in this study on the interaction of *L. rhamnosus* GAF01 with AFM1 reveal that this probiotic strain holds substantial potential for the detoxification of harmful compounds. For instance, our experiments similarly found a high binding capacity of *L. rhamnosus* to AFM1, highlighting its potential to reduce aflatoxin levels in food products. These studies support the notion that probiotics could be a reliable and effective method for the detoxification of AFM1 in food products (Abbès et al., 2013).

Zhang et al. (2019) investigated the binding capacity of *L. rhamnosus* LC-4 to AFM1. The study demonstrated that this probiotic strain could effectively bind AFM1 and exhibited high binding efficiency even in acidic environments. The critical role of peptidoglycans in the AFM1 binding process underscores the potential of LAB in food safety. The detoxification ability of *L. rhamnosus* in yogurt suggests that the use of such bacteria as food additives could be an important strategy (Zhang et al., 2019).

Salem-Bekhit et al. (2023) evaluated the detoxification of AFM1 in milk using *L. rhamnosus* and *Saccharomyces cerevisiae*. In this study, optimal conditions were determined using a Box-Behnken design, achieving a remarkable 98.4% detoxification of AFM1. This impressive result highlights the impact of probiotics on milk contamination (Salem-Bekhit et al., 2023). On the other hand, Elsanhoty et al. (2014) investigated the removal of AFM1 in yogurt production using various LAB and bifidobacterial strains. The results indicated that *Lactobacillus plantarum* was the most effective strain, binding AFM1 at the highest rate and reducing AFM1 levels throughout the storage period of the yogurts. These findings support the significant role of probiotics in the detoxification of AFM1 in food products (Elsanhoty et al., 2014). While these two studies highlight the effectiveness of *Lactobacillus* species in reducing AFM1, our hypothesis to lower AFM1 levels using *L. rhamnosus* in our study are aligned with these findings. The potential of probiotics to ensure food safety and reduce the bioavailability of toxins underscores the importance of such applications in industrial settings when combined with your research on dairy products.

Kuharić et al. (2019) explored the use of local LAB in milk and dairy products to reduce AFM1 levels. In this study, ten local LAB species were isolated, and their binding efficacy to AFM1 was evaluated. The binding efficiency varied between 21% and 94% after incubating viable and heat-treated LAB cells in AFM1-enriched milk samples for 0, 2, 4, and 24 hours. *L. plantarum* KM, which exhibited the highest binding activity, was used for the removal of AFM1 from milk. A combination of heating, filtration, and centrifugation achieved up to 96% removal of AFM1 (Kuharić et al., 2019).

Assaf et al. (2019) examined the use of microbial-derived adsorbents to reduce AFM1 contamination in milk. Different strategies were evaluated regarding the binding stability of AFM1 and the efficacy of microbial adsorbents. The abilities of biological adsorbents, such as bacteria and yeast, to bind AFM1 in complex form within milk were emphasized. These findings present promising alternatives for the biological removal of AFM1 (Assaf et al., 2019).

These studies, along with our data on the effectiveness of *L. rhamnosus* in reducing AFM1 levels, suggest that microorganisms play an important role in the removal of aflatoxin, with various LAB species exhibiting different binding efficiencies. In particular, the use of *L. rhamnosus*

consistently demonstrates its capability to effectively reduce AFM1, aligning with results obtained in previous studies. This study evaluated the effectiveness of *L. rhamnosus* in reducing AFM1 levels. Previous research has shown that local LAB have a significantly high binding capacity for AFM1 (Kuharić et al., 2019; Assaf et al., 2019). In particular, the interaction of *L. plantarum* KM with AFM1 has demonstrated up to 96% removal efficiency. These results suggest that *L. rhamnosus* could be used as an effective biological solution in milk contaminated with AFM1. Furthermore, the potential of combining heating, filtration, and centrifugation in the removal of AFM1 enhances the applicability of these methods. As a result, such biological approaches present promising alternatives for improving the safety of dairy products and reducing consumer exposure to aflatoxins.

Conclusion

Future studies should investigate the effects of different LAB species on AFM1 more comprehensively. In addition, larger-scale and long-term experiments should be conducted to determine the most effective conditions for the removal of AFM1. Moreover, field studies should be conducted to understand how the results obtained in laboratory settings can be translated into practical applications. By doing so, feasible strategies can be developed to reduce AFM1 contamination.

Declarations

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