Aflatoxin M_1 Reduction by Probiotic Strains in Iranian Feta Cheese

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ABSTRACT: *Among the fermented products, cheese has a good potential to deliver probiotic microorganisms into the gastrointestinal system due to its high protein and fat contents. The contamination of milk with aflatoxin M deserves attention concerning cheese consumption due to the harmful effects on human health. In the present research, the reduction of aflatoxin M_1 (AFM_1) by two well-known probiotic strains was studied in artificially aflatoxin-contaminated Feta cheese. Changes in pH, the viability of the probiotic strains and the level of aflatoxin in the samples were analyzed during 60-day storage. The results showed that all samples containing probiotics dramatically reduced the AFM_1 levels. From both the health and economic aspects, the B. bifidum species at an inoculation level of 10^7 CFU/mL has proven to be the best treatment, due to the lowest cost of probiotics, highest survival rate, and 30.12% reduction of AFM_1.*

KEYWORDS: *Aflatoxin M_1, Feta cheese, probiotic.*

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INTRODUCTION

Cheese is one of the most popular fermented dairy products, globally available, and suitable for all age groups. Regardless of the economic and social level, there is a growing demand for cheese consumption in different countries over the last decade. The cheese ingredients market was projected to be valued at USD 87.08 Billion in 2017 and is expected to reach USD 102.14 Billion by 2022 [1].

One of the main concerns about cheese consumption is the contamination by aflatoxins [2-4], which are among the most common mycotoxins studied worldwide. They are mainly produced by *Aspergillus flavus, Aspergillus parasiticus,* and *Aspergillus nomius* [5-7]. A part of B_1 and B_2 aflatoxins that is swallowed by the dairy cows during feeding are hydroxylated in the animal's liver by the cytochrome P450 enzyme system after 12 hours and released into milk as Aflatoxin M_1 and M_2 [3, 8]. The affinity of aflatoxin M_1 (AFM_1) to the casein fraction in milk and the lack of absorption by milk fat has led to a concern about the presence of this toxin in cheese [9-11].

The technologies used for cheese making, including ultrafiltration, and acid and enzymatic coagulation are not effective for the removal of aflatoxin from the contaminated milk [9]. In addition, the amount of this mycotoxin does not change significantly during ripening and storage [5, 12]. The Food and Drug Administration of the United States and the European Union have established the maximum AFM_1 levels of 0.50 and 0.05 ppb in dairy products, respectively, while the Institute of Standards and Industrial Research of Iran (ISIRI) has established 0.25 ppb as the maximum AFM_1 levels [2, 3, 13]. Excessive aflatoxin levels are potentially hazardous to human health due to the toxic effects, including carcinogenesis, mutagenesis, defecation, and hepato-toxicity [8]. Therefore, extensive efforts must be made to reduce or eliminate the aflatoxin contamination of food. The physical and chemical detoxification methods developed for aflatoxin-contaminated food products have limited application due to several factors, including safety issues, possible reduction of nutrients, limited function, and high cost [14], thus, further studies including biological factors have been developed. At present, although there is a strong hypothesis about the reduction of aflatoxins in foods by binding to the cell wall components of probiotic microorganisms [15], little is known about the reduction of AFM_1 in cheese. The feasibility of reducing the amount of aflatoxin in cheese by probiotic bacteria is not only a harmLess and practical way but also enhances the importance of using probiotic products as functional foods [16]. Among the fermented products, cheese may be a suitable alternative as a probiotic carrier due to its high pH and high protein and fat contents, which can protect the microorganisms throughout the gastrointestinal system [17].

The viability of probiotics is defined until the moment of consumption of the product and the viability inside the human body. Although there is still no fixed and definitive standard for the viability of probiotics in food products, at least 10^6 CFU/mL of probiotic microorganisms per gram or milliliter of the product is generally accepted [18]. *Lactobacillus acidophilus, L. casei, Bifidobacterium bifidum, B. langum, B. infantis,* and *Enterococcus faecium* have been the most commercially species used in the food industry [15, 19], and *L.casei* and *B. Bifidum* are widely used to produce functional dairy products [20]. In this study, the effect of probiotic strains (*L. casei 431, B. bifidum BB-12*) and the inoculation level (10^7 CFU/mL, 10^9 CFU/mL) using pH as a basic factor to evaluate the biological conditions of the product was investigated. In addition, the reduction of the inoculated AFM_1 during 60-day storage as the ultimate goal was also assessed.

EXPERIMENTAL SECTION

Microbial strains and chemicals

Freeze-dried mesophilic and thermophilic starter bacteria and probiotic microorganisms (*L. casei 431, B. bifidum* BB-12) were purchased from Chr. Hansen (Denmark). The AFM_1 standard (≥98.00%), sodium citrate, and sodium chloride were purchased from Sigma co. (St. Louis, MO, USA). Other materials including peptone water, MRS medium, dichloromethane, methanol, n-hexane, and acetonitrile were obtained from Merck (Germany). Rennet was supplied by CSK Food Enrichment (Netherlands). All solutions were prepared with ultrapure water obtained with a PURELAB® Chorus 1 Type I Ultrapure Water System (ELGA LabWater, USA).

Production of AFM_1-contaminated probiotic cheeses

All samples were manufactured at Bell Dairy Factory (Qazvin, Iran). First, the boiling test was performed to detect the presence of detergents in milk, and the CMT test (Beta-star, USA) was used to detect the presence of antibiotics and sulfidic. To produce Feta cheese by the

ultrafiltration technique (Bacteriologically Acidified Feta (BAF)), the milk fat was separated using a fat separator and then milk was pasteurized by HTST method. The pasteurized milk was heated to 50 °C and concentrated 4.5 times by microfiltration followed by ultrafiltration. The residual milk (retentate) was pasteurized at 77 °C for 1 minute, homogenized, and cooled to 30°C. The AFM1 was added to the retentate until a final concentration of 250 ng/kg was reached, considering the initial AFM1 levels in raw milk.

Cheese starter containing mesophilic bacteria (*L. lactis* subtype of *Lactis* and *Lactococcus lactis* subsp. *Cremoris*) and thermophilic bacteria (*Streptococcus salivarius* subsp. *thermophilus*) was added at the concentration of 80 grams per ton of retentate. The probiotic cultures *L. casei* and *B. Bifidum* were added at the concentrations of 10^7 and 10^9 CFU/mL, respectively. After 2 minutes, after a slight decrease in pH, the retentate was poured into the molds, and microbial rennet was added at a concentration of 30 grams per ton of retentate, according to the manufacturer's instructions. The molds were kept for 20 minutes in a coagulation tunnel (37 °C) to coagulate. The parchment paper was then loaded onto the molds and added 2-3% salt (sodium chloride) to the paper. The molds were covered and incubated at 30-32 °C until reaching pH 4.7-4.8. Then, they were stored at a refrigerated temperature (4-6 °C). The cheese recipe was completed on the third day and was ready to consume.

For the experimental design, a control treatment (probiotic-free) and 5 treatments (in 3 replicates) were performed, totaling 15 samples, using the probiotic strains and the inoculation levels (10^7 CFU/mL, 10^9 CFU/mL) as the independent variables. To determine the variables, including the viability of probiotics, the pH values and the aflatoxin residues of the samples were determined in 6 stages (days 1, 3, 15, 30, 45, and 60 of refrigerated storage), and a total of 90 cheese units were produced. However, the pH of samples and the viability of probiotics were not measured on the first day of refrigerated storage. All molds were investigated for Coliform, molds, and yeast counts [21].

pH measurements

The pH of the samples was measured according to the National Iranian Standard N. 2852 using a pH meter (Ino Lab pH 720, Germany).

Probiotics viability

To determine the viability of probiotic bacteria, 25 g sample was dissolved in 225 mL of 2% sodium citrate, and serial dilutions were performed in 0.15% peptone water [22]. The enumeration of probiotic bacteria was performed using MRS-bile agar medium by pour plate technique. The plates were incubated at 37°C for at least 72 hours [18, 23] under aerobic and anaerobic conditions for *L. casei* and *B. bifidum*, respectively. The anaerobic conditions were obtained using an anaerobic Jar and Type-A Gaspak. Mesophilic and thermophilic cheese starters did not grow in presence of bile salts in the control cultures [23].

Assessment of free AFM_1

The extraction, purification, and measurements of free AFM_1 in cheese samples were performed according to *Sarlak et al.* [15] with few modifications. In summary, 10 g of ground cheese, 10 g of elite, and 80 mL of dichloromethane were homogenized in an ultra turrax (Junke & Kunkel, Germany) for 3 minutes at 24000 rpm. To prevent solvent evaporation, the mixture was quickly passed through the Whatman filter paper Grade 1. Then, 45 mL of the filtrate was dried in a rotary evaporator (Heidolph, Germany) under a vacuum. The dried extract was dissolved in a 30:50:20 mixture of methanol, water, and n-hexane using an ultrasonic bath and vortex. The aqueous phase was separated using a separation funnel, and 35 mL of solution was passed through an immunoaffinity column (Aflaprep M, Scotland) containing monoclonal antibodies specific to aflatoxins. After the last drop of extract, the column was washed with 20 mL of distilled water in a two-step procedure. Passing 2.5 mL of acetonitrile from the column, the aflatoxin was isolated and collected in a vial. The contents of the vial were dried at 45 °C and re-dissolved using an ultrasonic bath and vortex using a 1 mL mobile phase (acetonitrile/methanol/ water, 20:20:60). Then, 200 µL of the final extract was injected into HPLC (Waters, USA) with a reversed-phase C_{18} column and fluorescence detector at a flow rate of 1 mL/min. The percentage of AFM_1 reduction in the samples (=% bonded-AFM_1) was calculated according to the following equation:

$$\text{AFM1 Reduction}(\%) = 1 - \frac{\text{Free AFM1 in the sample}}{\text{Initial AFM1 of the sample}} \times 100$$

Statistical analysis

The study was performed using a 2^2 completely randomized design, and all determinations were performed in 3 replicates. Data were analyzed by two-way analysis of variance (ANOVA) by applying Duncan's test, using the software SPSS version 24.0. Statistical significance was defined as $P < 0.05$.

RESULTS AND DISCUSSION

Changes in pH of the samples during storage

Fig. 1 shows the pH of AFM_1-contaminated cheeses during the refrigerated storage. Regardless of the minor differences, all treatments exhibited a pH decrease during the storage. All samples showed a lower pH at the end of the 60-day storage period when compared to the control, except for the treatment containing 10^7 CFU/mL *B. bifidum*, which indicates the effect of probiotic species on the pH reduction, as reported by other authors [24]. Although the pH value of the control was higher than those of the other samples during the storage period, no significant ($p>0.05$) differences were observed when compared with the treatment containing 10^7 CFU/mL of both *B. bifidum* and *L. casei* species. On the other hand, the treatments containing 10^9 CFU/mL were more effective for acid production for both species, with a significant difference when compared to the control ($p<0.05$), with the highest pH reduction on day 3. The pH reduction in the control sample, which contained only the starter culture, was slightly delayed, with a milder gradient at day 15. The lowest pH was observed on day 45 for the treatments containing 10^9 CFU/mL *B. bifidum* (pH=4.52) and *L. casei* (pH=4.43). This is despite the fact that the control sample has continued to reduce the pH until the final day of the storage. In general, samples containing *B. bifidium* have a higher pH than those containing *L. casei*. In the present study, the pH of the sample containing 10^7 CFU/mL *B. bifidium* was higher than the pH of the probiotic-free sample even after day 40. It is worth mentioning the similar pH reduction pattern of the bacterial species regardless of the inoculation level, as mentioned in previous studies [22, 24, 25], probably due to the unique biological ability of each strain [19]. The present results showed that the *Bifidobacterium* strains caused fewer changes in pH of the samples after day 15 of storage when compared to L. *casei*, which remained almost constant for the treatments containing 10^9 CFU/mL. Both species belong to the Lactic Acid Bacteria (LAB)

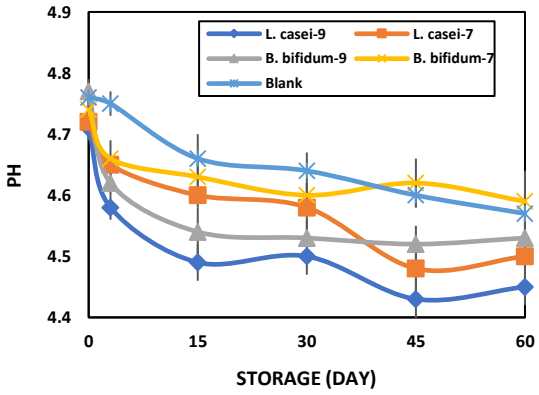

Fig. 1: pH values of treatments contaminated by aflatoxin M_1 during refrigerated storage.

family and are homofermentative. They consume lactose and produce lactic acid during fermentation, with different production rates. In general, *Bifidobacterium* strains produce 0.4-0.9% lactic acid, while *L. casei* strains produce 1.5-2% lactic acid by fermenting lactose [26]. Thus, this behavior can explain the difference in pH among the samples.

Survival of probiotic microorganisms during refrigerated storage

Table 1 shows the viability of the probiotic microorganisms in AFM_1-contaminated cheeses during refrigerated storage. The growing rate of probiotics in the cheese samples, decreased with the increase in storage time. Significant differences ($p<0.05$) were observed for the probiotics counts of all treatments on day 1 and day 3 when compared to the other storage periods. Although significant lower ($p<0.05$) probiotics counts were observed in the treatments containing 10^7 CFU/mL when compared to the treatments with 10^9 CFU/mL, similar inoculation levels had no significant effect on the probiotic viability during the refrigerated storage ($p>0.05$). The bioactivity of *L. casei* increased up to day 45 of refrigerated storage, with a significant decrease at day 60 ($p<0.05$). *Karimi et al.* [27] found similar results in ultrafiltrated Feta. Nevertheless, the viability of *B. bifidum* increased significantly in the treatments during 60 days of refrigerated storage ($p <0.05$). Similarly, Gomez and *Malikata* [19] reported good viability (>10^6 CFU/g) of *B. bifidum* strains in semi-hard cheese from goat's milk for a minimum of 9 weeks storage. *Corbo et al.* [28] also found

Table 1: Viability of probiotic microorganisms in AFM_1 contaminated cheeses during the storage period.

Treatments	Storage time (day)					
	0**	3	15	30	45	60
L. casei-9	9.09 ± 0.02^{aD}	9.22 ± 0.05^{aD}	9.82 ± 0.01^{bC}	10.04 ± 0.03^{aBC}	10.79 ± 0.07^{aA}	10.34 ± 0.06^{bB}
L. casei-7	7.16 ± 0.04^{bC}	7.37 ± 0.09^{bC}	8.52 ± 0.05^{cB}	8.34 ± 0.04^{cB}	9.14 ± 0.05^{bA}	8.71 ± 0.04^{dB}
B. bifidum-9	9.17 ± 0.03^{aC}	9.42 ± 0.07^{aC}	10.06 ± 0.09^{aB}	10.74 ± 0.03^{bA}	10.94 ± 0.03^{aA}	11.06 ± 0.08^{aA}
B. bifidum-7	7.31 ± 0.07^{bC}	7.47 ± 0.02^{bC}	8.39 ± 0.06^{cB}	8.56 ± 0.07^{cB}	8.64 ± 0.02^{cB}	9.11 ± 0.05^{cA}

** Means shown with different small and capital letters represent significant differences ($p < 0.05$) in the same Columns (among the treatments) and rows (between the days of storage), respectively.*

*** At the end of incubation*

Table 2: Free aflatoxin content in different treatments during glacial storage.

Treatments	Storage time (day)						
	0**	1	3	15	30	45	60
L. casei-9	562 ± 1.12^{bA}	534 ± 1.36^{abB}	510 ± 1.35^{bC}	185 ± 1.34^{dF}	281 ± 1.88^{cE}	301 ± 1.48^{cD}	305 ± 3.52^{dD}
L. casei-7	566 ± 3.09^{bA}	530 ± 2.22^{bB}	515 ± 2.86^{bB}	203 ± 2.42^{cF}	308 ± 1.62^{bE}	365 ± 2.88^{bD}	441 ± 1.72^{bC}
B. bifidum-9	560 ± 4.52^{bA}	529 ± 1.02^{bB}	464 ± 3.12^{cC}	167 ± 1.33^{eF}	252 ± 3.09^{dE}	278 ± 3.06^{dD}	289 ± 4.62^{eD}
B. bifidum-7	571 ± 1.34^{bA}	535 ± 2.19^{abB}	529 ± 5.30^{aB}	219 ± 3.08^{bE}	205 ± 2.46^{eE}	371 ± 1.32^{bD}	399 ± 2.47^{cC}
Blank	583 ± 1.72^{aA}	545 ± 1.08^{aB}	540 ± 1.42^{aB}	498 ± 3.07^{aD}	522 ± 1.72^{aC}	520 ± 3.17^{aC}	525 ± 3.04^{aC}

* Means shown with different small and capital letters represent significant differences ($p < 0.05$) in the same columns (among the treatments) and rows (between the days of storage), respectively.

** At the end of incubation

higher viability of *B. bifidum* in Italian cheese when compared to other probiotic species during 56 days of storage.

The survival of probiotics in fermented dairy products during fermentation and refrigerated storage is affected by unfavorable conditions, such as low pH and high acidity. It has been reported that the number of *Bifidobacterium* in yogurt is reduced by about two logarithms after 28 days of storage. *Bifidobacterium* is sensitive to pH 4.6 thus, unlike yogurt, it can survive in cheese.

Amount of free and bonded AFM_1 during storage

As shown in Table 2, different free AFM_1 levels were observed in the probiotic and control samples during storage, which is calculated using relation 2.1. Although some of the initial aflatoxins have been removed during syneresis [13], the AFM_1 levels in all samples at day 0 were more than double the initial toxin levels inoculated into the retentate. This result may be due to the tendency of toxins to be absorbed into cheese curd rather than whey, and the curd structure can trap well the toxin inside, as reported by some researchers [5, 10, 13]. The concentration of 500 ppb of toxin at day 0 of storage was considered 100% of the initial aflatoxin (see Table 2) for each treatment, which was considered as the basis for calculating the subsequent reductions.

Subsequently, the free aflatoxin decreased until day 15 for all samples and reached the lowest level throughout the entire storage period, except for the treatment containing 10^7 CFU/mL *B. Bifidium*. The most successful reductions of free AFM_1 occurred in the treatments *B. bifidum-9*, *L. casei-9*, and *L. casei-7*, with a reduction of 70.17%, 67.08%, and 64.13%, respectively (Table 3). The lowest free AFM1 level for the treatment *B. bifidum-7* was observed with a slight delay on day 30 (64.09% reduction). Significant differences were observed between the pairwise treatments (*B. bifidum-9 and B. bifidum-7*) and (*L.casei-9* and *L.casei-7*) ($p<0.05$), with no differences for the other pair comparisons (including *B. bifidum-9 and*

Table 3: Decrease of free AFM_1 levels (%) in different treatments during storage.

Treatments	Storage time (day)						
	0**	1	3	15	30	45	60
L. casei-9	0.00	4.98	9.25	67.08	50.14	46.44	45.72
L. casei-7	0.00	6.36	9.01	64.13	45.58	35.51	22.08
B. bifidum-9	0.00	5.53	17.14	70.17	55.33	50.35	48.39
B. bifidum-7	0.00	6.30	7.35	61.64	64.09	35.02	30.12
Blank	0.00	6.51	7.37	14.57	10.46	10.8	9.94

L. casei-9) ($p>0.05$). However, there was a significant decrease in the amount of free-AFM_1 in all probiotic treatments when compared to the control sample, with the best percentage of 14.57% observed on day 15.

Degradation and adsorption are the main mechanisms for reducing aflatoxins using a biocontrol agent. Some microorganisms such as *Bacillus* species can degrade aflatoxin into non-toxic metabolites by secreting the enzyme laccase that is able to cleave the lactone ring of the aflatoxin. However, probiotic strains have the ability to adsorb aflatoxins to the cell wall components such as oligomannans, thus no degradation was reported for them [7].

The decrease in free aflatoxin content in the control sample (probiotic-free) may be probably due to both the incomplete extraction from the complex cheese structure and the effect of the fermentation process. Some researchers believe that the species with a higher fermentation rate are more effective in reducing aflatoxin. *Perides et al.* [29] stated that the ability to further AFM_1 bonding by *L. rhamnosus LC-705* when compared to *L. rhamnosus GG* is due to its faster fermentation. In contrast, Hernandez and Mendoza [20] have found no significant difference in binding between five probiotic species and aflatoxin at different incubation periods. On the other hand, the reduction of pH during fermentation, which occurred on a steep slope until the 15^{th} day, led to a change in the structure of milk proteins and cheese curd [30, 31], which probably results in AFM_1 binding or retention within the clot structure. Also, *Sarimehmet et al.* [31] and *Sarlak et al.* [15] reported that traditional cheese starters, like probiotics, are effective in reducing aflatoxin.

As shown in Table 3, the probiotic samples were more effective to reduce the free AFM_1 levels when compared to the control, throughout the storage period. *Kabok* and *Var* [16] studied the bonding of six *Lactobacillus* and *Bifidobacterium* strains in a buffer medium and found no effect of the storage time on the AFM_1 level. Other studies have also shown that the concentrations of AFM_1 in white and Kashar cheese did not change significantly during storage [32], while *Peltonen* [33] observed that *Lactobacillus amylovorus* strains can decrease the amount of aflatoxin over time.

Studies have shown that the viability of a bacterium is not a necessary condition for binding to aflatoxin [15, 33]. Some researchers have attributed the bacterial removal of aflatoxins to their ability of acid production and lower pH [33]. The sustainability of the aflatoxin-lactic acid bacteria (AF-LAB) complex in a wide range of pH is an important factor for using those microorganisms to remove aflatoxins from foods. It is also noteworthy that the pH dependence varies among lactic acid bacteria species. *Fuchs et al.* [34], contrary to the previous research, showed that the pH level was effective on LAB survival for binding patulin and Ochratoxin A, which contrasts with other studies regarding the pH dependence for the removal of aflatoxin B_1 and zearalenone [35, 36].

It is worth mentioning the further increase in free AFM_1 in the samples of the present study, which occurred for most treatments after 15 days of storage. It has been proven that the removal of aflatoxin by LAB is not a chemical process and is based on the physical connection between bacteria and toxins. The remaining AFM_1 in cheese during the storage period indicated that this physical connection may be lost during the storage and part of the aflatoxin returned to cheese. The binding mechanism between toxin and bacteria is fast and reversible [37], and the strength of the complex formed between the bacteria and aflatoxin is also related to the species, environmental conditions during the complex formation, and the processes used to determine the resistance (e.g., the use of acid and heat or dead microorganisms).

In the mycotoxin reversibility phenomenon, both the species and the bacterial concentration are important, while the LAB viability is not necessarily effective [14]. The reversibility of binding and discontinuation of aflatoxin-bacteria is higher at lower microorganism concentrations (10^7CFU/mL level). The lowest amount of free aflatoxin is related to the level of inoculation of 10^9 CFU/mL *B.bifidum*, probably due to the highest live probiotics logarithm in these species. In this regard, Shah and wu [38] reported that *B.bifidum* exhibited the least amount of toxin reversibility. *Hernandez Mendoza* [20] investigated the binding between aflatoxin B_1 and eight *Lactobacillus casei* species and found that the released aflatoxin ranged from 0 to 9.2%. A possible explanation for this variation is the different binding sites of the different species [20]. The decrease in aflatoxin absorption during the storage period, in addition to the complex reversibility, can also be attributed to the reduction of probiotic levels due to the competitive relationship between the starter cultures and the probiotic species.

At day 30, lower concentrations of free aflatoxin were observed for all treatments containing 10^9 CFU/mL when compared to the treatments with 10^7 CFU/mL, except for the treatment containing 10^7 CFU/mL *B. bifidum*. Several factors can contribute to the removal of aflatoxin by probiotic microorganisms, including the temperature, incubation time, competitive flora, cell viability and permeability of microorganisms, probiotic species, the concentration of microorganisms, and aflatoxin levels, growth medium, aflatoxin binding time, and pH [39]. In this study, only the effect of some variables on the reduction of AFM_1 was investigated. Previous studies have shown the effectiveness of the bacteria population in reducing aflatoxin, which has a greater effect on the AFM_1 elimination by both probiotic species used in this study at the inoculation level of 10^9 CFU/mL when compared to the 10^7 CFU/mL inoculation level. *Linnaeus* and *Bracthe* reported that the number of live bacterial cells to be contacted with aflatoxin should be at least 10^9 CFU/ mL. *Kabak* and *Var* [40] also expressed the factor of the bacterial cell population as a critical point in influencing the rate of aflatoxin reduction. Although higher bacterial concentration leads to an increase in aflatoxin binding, it is never enough to bind all toxins in the environment. *Sarlak et al.* [15] also found that the amount of aflatoxin removed increased with the increase in the bacterial concentration, but the percentage of this elimination was not significant. In addition, the excessive use of microorganisms, despite its higher effectiveness in aflatoxin removal, may also result in undesired organoleptic changes in the final product [41]. Therefore, the selection of appropriate concentrations of probiotic species is important from the economic and commercial point of view, and the inoculation of probiotic bacteria should not result in poor product quality, with a negative effect on the texture and the sensory properties of probiotic cheese. According to this study, the higher inoculation level (10^9 CFU/mL) was more effective to reduce the amount of aflatoxin; however, it was not selected as the optimal treatment.

Overall, the results confirmed that the *Bifidum* species was more effective than *L. Casei* in reducing free AFM_1 and a lower final AFM_1 level was found at the end of the storage (Table 3). The ability of aflatoxin to bind to different bacterial species can be due to both their unique chemical properties and the organization of their cell wall structure. The better performance of *B. bifidum* in this study was consistent with the findings of *Fang et al.* [25], who investigated the differences between two *Lactobacillus* and *Bifidobacterium* species, and found that *Bifidobacterium* exhibited a better anticancer activity when compared to *Lactobacillus* strains due to the differences in the cell wall. Little is known about the structure of the compounds that are effective in binding aflatoxin to the bacterial wall. However, since protein-rich carbohydrate compounds or cell wall glycans are responsible for aflatoxin binding to the bacterial surface, lowering the optimal growth temperature can reduce the growth of the cell wall and its mannan and β-glucan molecules leading to a reduction of the aflatoxin absorption. The reduction of toxin binding in the present study may be due to the low temperature of the product during storage (4-6 °C) when compared to the optimum growth temperature (37 °C) for the two existing species.

CONCLUSIONS

Few studies have been conducted on the reduction of aflatoxin levels in cheese by probiotics during refrigerated storage, with effective results observed in the present study. The type of probiotic strains had a significant effect on the biochemical changes and the AFM_1 reduction ($p < 0.05$). From both the health and economic aspects, the *B. bifidum* species with 10^7 CFU/mL inoculation level had the highest survival rate and 30.12% reduction of AFM_1 at day 60, thus it has proven to be the best treatment.

Studies on other factors affecting the binding of the toxin to bacteria and the behavior of other probiotic species should be performed.

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