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Original Article

Aflatoxin M1-Binding Ability of Selected Lactic Acid Bacteria Strains and *Saccharomyces boulardii* in the Experimentally Contaminated Milk Treated with Some Biophysical Factors

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ABSTRACT

There is a growing concern regarding the recurrent observation of aflatoxins (AFs) in the milk of lactating animals. Regarding this, the present study was conducted to assess the aflatoxin M1 (AFM1)-binding ability of three species, namely *Lactobacillus rhamnosus, L. plantarum,* and *Saccharomyces boulardii,* in AFM1-contaminated milk. The mentioned species were administered at the concentrations of 10^7 and 10^9 CFU/mL to skimmed milk contaminated with 0.5 and 0.75 ng/mL AFM1 within the incubation times of 30 and 90 min at 4°C and 37°C. *Lactobacillus rhamnosus* was found to have the best binding ability at the concentrations of 10^7 and 10^9 (CFU/ml), rendering 82% and 90% removal in the milk samples with 0.5 and 0.75 ng/ml AFM1, respectively. Accordingly, this value at 10^7 and 10^9 CFU/ml of *L. plantarum* was obtained 89% and 82% with 0.75 ng/ml of AFM1, respectively. For *S. boulardii* at 10^7 and 10^9 CFU/ml, the rates were respectively estimated at 75% and 90% with 0.75 ng/ml of AFM1. The best AFM1-binding levels for *L. rhamnosus, L. plantarum,* and *S. boulardii* were 91.82±10.9%, 89.33±0.58%, and 93.20±10.9, respectively, at the concentrations of 1×10^9, 1×10^7, and 1×10^7 CFU/ml at 37, 4, and 37°C, respectively. In this study, the maximum AFM1 binding (100.0±0.58) occurred while a combination of the aforementioned probiotics was employed at a concentration of 1×10^7 CFU/ml at 37°C with 0.5 ng/ml AFM1, followed by the combination of *L. rhamnosus* and *L. plantarum* (95.86±10.9) at a concentration of 1×10^9 CFU/ml at the same temperature with 0.75 ng/ml AFM1. It was concluded that the use of *S. boulardii* in combination with *Lactobacillus rhamnosus* and *L. plantarum,* which bind AFM1 in milk, can decrease the risk of AFM1 in dairy products.

Keywords: AFM1, Milk, decontamination, *Saccharomyces boulardii, Lactobacillus rhamnosus*, *Lactobacillus plantarum*

Capacité de Liaison À l'aflatoxine M1 présente dans le Lait Contaminé de Certaines Souches de Bactéries Lactiques Sélectionnées et de *Saccharomyces Boulardii* Expérimentalement Traité avec Certains Facteurs Biophysiques

Résumé: Il existe une préoccupation croissante concernant l'observation récurrente des aflatoxines (AF) dans le lait des animaux en lactation. L'obejectif de cette étude était d'évaluer la capacité de liaison de l'aflatoxine M1 (AFM1) de trois espèces, à savoir *Lactobacillus rhamnosus*, *L. plantarum* et *Saccharomyces boulardii*, dans le lait contaminé par FM1. Les espèces ont été administrées à des concentrations de 10^7 et 10^9 UFC/ mL dans du lait écrémé contaminé par 0.5 et 0.75 ng/mL d'AFM1 dans des temps d'incubation de 30 et 90 min à 4° C et 37° C. *Lactobacillus rhamnosus* s'est avéré avoir la meilleure capacité de liaison aux concentrations de 10^7 et 10^9

(UFC/ml). Cette bactérie lactique arespectivement éliminé 82% et 90% de l'AFM1 présente dans les échantillons de lait contenant 0.5 et 0.75 ng/ml d'aflatoxines. *L. plantarum* ajouté au lait à concentration de 10^7 et 10^9 UFC/ml s'est également avérée efficace et a diminué le contenu en AFM1 (0.75 ng/ml) de 89% et 82%, respectivement. Pour *S. boulardii* à 10^7 et 10^9 UFC/ml, les taux ont été respectivement estimés à 75% et 90% avec 0.75 ng/ml dAFM1. Les meilleurs niveaux de liaison à l'AFM1 ont été obtenus pour 1×10^9 UFC/ml de *L. rhamnosus*, 1×10^7 UFC/ml de *L. plantarum* et 1×10^7 UFC/ml de *S. boulardii* éliminant respectivement 91.82±10.9%, 89.33±0.58% et 93.20±10.9 dAFM1à 37, 4 et 37 °C. Dans cette étude, la liaison maximale à l'AFM1 (100.0±0.58%) s'est produite alors qu'une combinaison des probiotiques susmentionnés était utilisée à une concentration de 1×10^7 CFU/ml à 37°C avec 0.5 ng/ml d'AFM1. lL combinaison de *L. rhamnosus* et *L. plantarum* à une concentration de 1×10^9 UFC/ml à la même température avec 0.75 ng/ml d'AFM1s'est également montrée hautement efficace (95.86±10.9%). L'utilisation de *S. boulardii* en combinaison avec *Lactobacillus rhamnosus* et *L. plantarum*, peut considérablement réduire le risque de contamination a l'AFM1 dans les produits laitiers.
Mots-clés: AFM1, Lait, Décontamination, *Saccharomyces boulardii*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*

INTRODUCTION

Aflatoxin (AF) is a kind of mycotoxin formed by a group of molds, including *Aspergillus flavus* and *A. parasiticus* (Sepahdari et al., 2010; Hassan et al., 2018). The greatest amount of AFs is present in the crops harvested and deposited in the warm regions throughout the world. According to the World Trade Institute, AFs are problematic not only for the countries producing agricultural products but also for the regions importing such products. Once AFB1 contaminates the feedstuff digested by lactating animals, it is converted into a 4-hydroxylated metabolite of AFM1 in the liver and detected in the blood within less than an hour (Fallah, 2010; Assaf et al., 2018). Accordingly, the AFM1 amount in dairy products is associated with the level of AFB1 in raw feedstuff. The AFM1 in milk can resist heat treatments, such as pasteurization; therefore, these treatments cannot decrease the level of mycotoxins (Galvano et al., 2001). Accordingly, there is a growing concern regarding the presence of mycotoxins in the milk and dairy products of lactating animals (Bahrami et al., 2016). According to the literature, AFM1 contamination in milk is a serious risk to human health (Galvano et al., 2001). Since milk is the main source of human nutrition, the safety of this product should be taken into account (Sarimehmetoğlu and Küplülü, 2004), especially for the sensitive-age groups who are high-risk consumers (Xiong et al., 2018). This issue highlights the importance of performing studies targeted toward the identification of efficient, affordable, and easy to use strategies for the degradation of AFM1 in milk (Lee et al., 2015). Contrary to the physical and chemical approaches, biodegradation is an effective and environment-friendly technique to eradicate or lower the level of AFM1 in milk or dairy products (Baranyi et al., 2015). Lactic acid bacteria (LAB), including some probiotic species, showing the ability to degrade AFM1 in milk, have been widely studied in this domain (Dalié et al., 2010; Sarlak et al., 2017). Accordingly, *Saccharomyces boulardii* is reported to be an effective probiotic to bind AFM1 (Corassin et al., 2013). However, LAB strains have shown different abilities for the mitigation of AFM1 in dairy products (Bovo et al., 2013; Ismail et al., 2017). In a study, the binding of AFM1 to the cell wall of *Lactobacillus rhamnosus* GG was observed to be moderately reversible, and AFM1 was reported to be detached in different conditions, such as repeated washes and heat treatments (Assaf et al., 2018). With this background in mind, the present study was carried out to determine the potential of two strains of LAB and *Saccharomyces bulardii,* alone or in combination, to degrade mycotoxins in skimmed milk artificially contaminated with AFM1.

MATERIAL AND METHODS

Reagents. For the purpose of the study, MRS broth (Agar and de Man, Rogosa, and Sharpe, 69966) and yeast nitrogen base (YNB; Y0626) were purchased from Sigma-Aldrich (MO, USA) to perform bacterial and yeast culturing. In addition, phosphate-buffered saline (PBS) was bought from Sinagene (Iran), and deionized water (DW) was prepared using a deionized system (GFL, Germany).

Microbial preparations. *Saccharomyces bulardii* (dry yeast from *Saccharomyces cerevisiae* HANSEN CBS 5926) as one of the probiotic species applied in this research was purchased (Ardeypharm Germany, Gmbh) in form of 250-mg capsules (2.5×10^9)). The yeasts were transferred to the Food Lab of the Veterinary Faculty of Islamic Azad University, Iran, and then extracted and cultivated. The yeast powder was cultured on Sabouraud dextrose agar (2% SDA) medium at 25-30 °C for 48 h. Subsequently, it was transferred from the SDA plate to the YNB medium to be incubated at 25 °C for 18 h to reach the logarithmic phase. *Lactobacillus rhamnosus* PTCC 1637 and *L. plantarum* PTCC 1745 were also studied for AFM1-binding ability in contaminated milk obtained from the Centre of Industrial Microorganisms Collection of Iran. These lyophilized bacteria were cultivated in the MRS broth under an aerobic condition at 37 °C for 48 h (Biernasiak et al., 2006). Well-grown colonies were kept on plates at the refrigerator temperature until requiring new cultures. These bacteria were repetitively cultivated until reaching a volume of 1×10^9 CFU/ml. Serial dilutions were then made for all probiotics to reach a concentration of 1×10^7 CFU/ml. The supernatants of the cultivated bacteria were discarded after refrigerated centrifugation at 3500×g for 10 min. Bacterial cells were then washed three times with 5 ml of sterile deionized water (DW) to avoid possible mistakes in the measurement of AFM1. A microbial suspension was prepared with PBS and adjusted to a 3-McFarland standard at the mentioned concentrations using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech Inc., USA). Furthermore, the turbidity was measured at 600 nm (Kahouli et al., 2017). The 1×10^9 CFU/ml solution was diluted to 100:1 to obtain a 1×107 CFU/ml dilution (Kirkpatrick et al., 2000), which was consequently inoculated to the skim milk.

Milk preparation. The fat-free skim milk (115363, Merck, Germany) was reconstituted by adding sterile DW (10:1 V/W) into a flask containing appropriate milk powder based on the instructions of the factory to obtain an applicable volume of solution for 240 samples (100 µl each), which were assessed via the enzyme-linked immunosorbent assay (ELISA) test.

Study design and aflatoxin solution. A standard dilution of AFM1 (10 µg/ml) suspended in acetonitrile was purchased (46319-U SUPELCO; Sigma-Aldrich) and thinned to 100 ng/ml using PBS. The ELISA test was performed using 240 Falcon tubes containing AFM1-contaminated skim milk at two practical concentrations (0.5 and 0.75 ng/ml), determined by a spectrophotometer (Ultrospec 2000). Three microorganisms were considered in this study for the implementation of five treatments. The treatments included *L. Rhamnosus, L. plantarum*, *S. bulardii*, *L. Rhamnosus+L. plantarum,* and *L. Rhamnosus+L. plantarum+S. Bulardii*. These groups were investigated at two concentrations (1×10^7 and 1×10^9 CFU/ml) and two temperatures of 4 °C and 37 °C during the incubation times of 30 and 90 min (Figure 1).

Quantification of aflatoxin M1 using enzyme-linked immunosorbent assay. For the purpose of the study, 1 ml of the probiotic suspension was added to Eppendorf microtubes and centrifuged at 3,500 rpm for 10 min. After the removal of the supernatant, the pellets were washed three times with normal saline. Subsequently, 1 ml of DW was transferred to the microtubes, which were added with 9 mL of reconstituted milk prepared as described earlier, and then heated (90 °C, 15 min) and chilled down to 37 °C and 4 °C for 30 and 90 min (Elsanhoty et al., 2014). In the next stage, 100 µL of the prepared supernatant was poured into a 96-well ELISA microplate that was coated with anti-AFM1 antibodies. The AFM1 working

solution (0.5 and 0.75 ng/ml) was then added to the wells in triplicate and incubated for 1 h at 20-25 ℃ in a dark room. The solution in each well was then discarded. After 10 min, each well was washed twice with 250 µL of buffer solution according to the kit instruction (Euro Proxima, Netherlands). Afterward, 100 µL of the conjugate solution (AFM1-HRP) was added to each well, incubated for 1 h at 20 ℃, and washed three times. Finally, 50 µL of the substrate and 50 µL of chromogen were added to each well, gently stirred, and incubated for 30 min. Then, 100 µL of the stop reagent was added to each well, stirred, and measured at 450 nm in the ELISA reader in order to measure the amount of unbound AFM1 according to the direct competitive ELISA technique (Euro Proxima, Netherlands) accomplished using a reader (model EL×808; BioTek, USA).

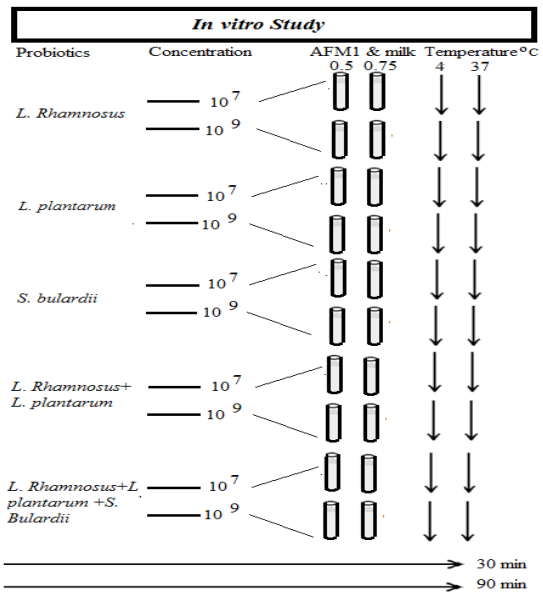

Figure 1. A schematic representation of in vitro experimentation on the exposure of some probiotics to AFM1-contaminated skim milk at different concentrations, temperatures, and incubation times.

All rinsing cycles were carried out three times. The drying phase of the wells was performed by turning the microtiter plate upside down on a paper towel (Wang et al., 2011). The percentage of AFM1 bound by the bacterial suspension was calculated using the following equation (Namvar Rad et al., 2018; Namvar Rad et al 2019):

$$\%\text{bound AFM1} = [1 - (\frac{\text{AFM1 peak area of sample}}{\text{AFM1 peak area of 0 day}}) \times 100]$$

Statistical analysis. The amount of AFM1 bound with five treatments of probiotics in the contaminated milk was analyzed in an in vitro study using a mixed model for repeated measures ANOVA through the general linear model procedure in SPSS, version 16 (SPSS Inc., IL, USA). A factorial arrangement was used to examine the microorganism species, microorganism concentration, concentrations of AFM1, incubation time, and temperature of milk solution as fixed factors. A p-value less than 0.05 was considered statistically significant. When significant p-values were obtained for each effect, the differences between the estimated marginal mean values were compared using a posthoc Tukey's HSD test.

RESULTS

The three strains applied in this study both individually and in combination with one another (Figure 1) were found to be effective in binding AFM1 in the skimmed milk. The AFM-binding capacity of the strains is demonstrated in tables 1 and 2. According to the results presented in Table 1, the type of microorganism is more important than microorganism concentration. The results revealed an increase in the percentage of AFM1 removal for *L. rhamnosus*, while the applied bacteria concentration level was augmented from 1×10^7 (91.57%) to 1×10^9 CFU/ml (57.55%) after 90 min of exposure. This pattern was not fitted to those observed for *L. plantarum, S.boulardii, L. rhamnosus+L. plantarum,* and *L. rhamnosus+L. plantarum+S.boulardii* treatments. According to the results obtained for *L. rhamnosus*, the maximum affinity for binding AFM1 occurred without any significant difference (P<0.05) in the absence (91.57%) and presence of temperature (91.82%). The results for *L. plantarum* showed that the maximum affinity for binding the AFM1 in the milk was obtained in the presence of temperature (89.33%) rather than in

absence of this variable (81.93%). This pattern was fitted for *S. boulardii*, indicating that the maximum percentage of AFM1 removal in the milk (90.66%) was greater in the absence of temperature in the model (83.17%). According to the model presented in Table 1 (including the type of microorganisms as an effective

Table 1. Estimated marginal mean values of aflatoxin M1 (%) removed due to the combined effect of microorganism type, microorganism concentration, aflatoxin M1 concentration, and time, using enzyme-linked immunosorbent assay (*n*=3)

Microorganisms	Microorganism concentration (CFU/ml)	AFM1 (ng/ml)	Time (min)	Mean± SE %	95% Confidence interval	
					Lower bound	Upper bound
L. rhamnosus	10^7	0.5	30	71.83±0.41[a]	71.01	72.65
			90	84.77±7.77[b]	69.30	100.25
		0.75	30	51.82±0.41[a]	51.00	52.64
			90	57.55±7.77[b]	42.08	73.03
	10^9	0.5	30	62.10±0.41[a]	61.27	62.92
			90	52.20±7.77[a]	36.72	67.67
		0.75	30	89.86±0.41[a]	89.04	90.68
			90	91.57±7.77[a]	76.10	107.05
L. plantarum	10^7	0.5	30	30.23±0.41[a]	29.41	31.05
			90	53.96±7.77[b]	38.49	69.44
		0.75	30	81.88±0.41[a]	81.06	82.71
			90	81.93±7.77[a]	66.45	97.40
	10^9	0.5	30	73.10±0.41[a]	72.27	73.92
			90	43.53±7.77[b]	28.05	59.00
		0.75	30	62.15±0.41[a]	61.33	62.97
			90	78.84±7.77[b]	63.36	94.32
S.boulardii	10^7	0.5	30	62.53±0.41[a]	61.71	63.35
			90	80.63±7.77[b]	65.15	96.10
		0.75	30	80.02±0.41[a]	79.20	80.84
			90	83.17±7.77[b]	99.32	98.65
	10^9	0.5	30	72.23±0.41[a]	71.41	73.05
			90	83.83±7.77[b]	68.35	99.30
		0.75	30	81.93±0.41[a]	81.11	82.75
			90	83.02±7.77[a]	67.54	98.49
L. rhamnosus+ *L. plantarum*	10^7	0.5	30	72.53±0.41[a]	71.71	73.35
			90	84.53±7.77[a]	69.05	100.0
		0.75	30	83.64±0.41[a]	82.82	84.46
			90	83.42±7.77[a]	67.94	98.89
	10^9	0.5	30	81.76±0.41[a]	80.94	82.58
			90	75.46±7.77[b]	59.99	90.94
		0.75	30	92.08±0.41[a]	91.26	92.91
			90	84.93±7.77[b]	69.45	100.40
L. rhamnosus+ *L. plantarum*+ *S.boulardii*	10^7	0.5	30	82.26±0.41[a]	81.44	83.08
			90	70.26±7.77[b]	99.47	85.74
		0.75	30	86.97±0.41[a]	86.15	87.80
			90	93.57±7.77[b]	78.10	109.05
	10^9	0.5	30	72.66±0.41[a]	71.84	73.48
			90	84.56±7.77[b]	69.09	100.04
		0.75	30	75.62±0.41[a]	74.80	76.44
			90	82.95±7.77[b]	67.48	98.43

(At each row, different superscripts show a significant difference between the values for the time of exposure at the significance level of 0.05.)

factor), the threshold of AFM1 binding by probiotics was observed in the treatment of *L. rhamnosus+L. plantarum+S. boulardii* at a concentration of 10^7 CFU/ml, while the AFM1 concentration was initially set at 0.75 ng/ml. However, for all the microorganism types tested in this study, the affinity to bind AFM1 was significantly (P<0.05) greater than that measured for other treatments. Accordingly, the minimum

Table 2. Estimated marginal mean values of aflatoxin M1 (%) removed due to the combined effect of microorganisms types, microorganism concentration, aflatoxin M1 concentration, storage temperature, and time, using enzyme-linked immunosorbent assay (n=3)

Microorganis ms	MC	Temp. (°C)	AFM1(ng/ml)	Time (min)	Mean± SE %	95% Confidence interval	
						Lower bound	Upper bound
L. rhamnosus	10^7	4	0.5	30	82.73±0.58[a]	81.57	83.89
				90	83.75±10.9[a]	69.30	105.63
			0.75	30	40.88±0.58[a]	39.72	42.05
				90	51.55±10.9[b]	29.67	73.44
		37	0.5	30	60.93±0.58[a]	59.77	62.09
				90	85.80±10.9[b]	63.91	107.68
			0.75	30	62.75±0.58[a]	61.59	63.91
				90	63.55±10.9[a]	41.67	85.44
	10^9	4	0.5	30	61.06±0.58[a]	59.90	62.22
				90	61.13±10.9[a]	39.24	83.01
			0.75	30	90.26±0.58[a]	89.10	91.42
				90	91.33±10.9[a]	69.44	113.21
		37	0.5	30	63.13±0.58[a]	61.97	64.29
				90	43.26±10.9[b]	21.38	65.15
			0.75	30	89.46±0.58[a]	88.30	90.62
				90	91.82±10.9[b]	69.93	113.70
L. plantarum	10^7	4	0.5	30	35.60±0.58[a]	34.43	36.76
				90	44.80±10.9[b]	22.91	66.68
			0.75	30	89.33±0.58[a]	88.17	90.49
				90	79.24±10.9[b]	57.35	101.13
		37	0.5	30	24.86±0.58[a]	23.70	26.02
				90	63.13±10.9[b]	41.24	85.01
			0.75	30	74.44±0.58[a]	73.28	75.60
				90	84.62±10.9[b]	62.73	106.50
	10^9	4	0.5	30	62.46±0.58[a]	61.30	63.62
				90	34.46±10.9[b]	12.58	56.35
			0.75	30	75.20±0.58[a]	74.03	76.36
				90	82.53±10.9[b]	60.64	104.41
		37	0.5	30	83.73±0.58[a]	82.57	84.89
				90	52.605±10.9[b]	30.71	74.48
			0.75	30	49.11±0.58[a]	47.94	50.27
				90	75.15±10.9[b]	53.27	97.04
S. boulardii	10^7	4	0.5	30	62.26±0.58[a]	61.10	63.42
				90	68.06±10.9[b]	46.18	89.95
			0.75	30	75.60±0.58[a]	74.43	76.76
				90	75.68±10.9[a]	53.80	97.57
		37	0.5	30	62.80±0.58[a]	61.63	63.96
				90	93.20±10.9[a]	71.31	115.08
			0.75	30	84.44±0.58[a]	83.28	85.60
				90	90.66±10.9[a]	68.78	112.55
	10^9	4	0.5	30	61.46±0.58[a]	60.30	62.62
				90	83.60±10.9[a]	61.71	105.48
			0.75	30	89.06±0.58[a]	87.90	90.22
				90	90.62±10.9[a]	68.73	112.50
		37	0.5	30	83.00±0.58[a]	81.83	84.16
				90	84.06±10.9[a]	62.18	105.95
			0.75	30	74.80±0.58[a]	73.63	75.96
				90	75.42±10.9[a]	53.53	97.30

(At each row, different superscripts show a significant difference between the values for the time of exposure at the significance level of 0.05.).

average percentage of AFM1 reduction was observed in *L. plantarum,* which was 30.23±0.41 in the absence of "temperature" in the model (Table 1) and 24.86±0.58 in the presence of this variable (Table 2). Given that the maximum ability to remove aflatoxin was observed in the model described in Table 2, it was attempted to explain this model in more detail. Among the effects presented in tables 1 and 2, the model showed more outrageous data given in Table 2. The lowest and the greatest extents of binding affinity were observed for *L. plantarum* (24.86%) and *L. rhamnosus+L. plantarum+S. boulardii* (100.0%) treatments at 10^7 CFU/ml and 37 °C when the tested milk was introduced to 0.5 ng/ml AFM1 and at the same concentration at 4 °C with 0.75 ng/ml AFM1, respectively.Variable amounts of AFM1 remained bound to the microbial cells (Table 2) in AFM1-contaminated milk after 30 and 90 min. At 4 °C, the best binding ability was obtained for *L. rhamnosus* at 10^7 and 10^9 CFU/ml with 82% and 90% removal when the milk was contaminated with 0.5 and 0.75 ng/ml AFM1, respectively. Accordingly, this value at 10^7 and

Table 2 (continued). Estimated marginal mean values of aflatoxin M1 (%) removed due to the combined effect of microorganisms types, microorganism concentration, aflatoxin M1 concentration, storage temperature, and time, using enzyme-linked immunosorbent assay (*n*=3)

Microorganisms	MC	Temp.(°C)	AFM1(ng/ml)	Time(min)	Mean± SE %	95% Confidence interval	
						Lower bound	Upper bound
L. rhamnosus+ L. plantarum	10^7	4	0.5	30	83.46±0.58[a]	82.30	84.62
				90	85.80±10.9[b]	63.91	107.68
			0.75	30	82.71±0.58[a]	81.54	83.87
				90	75.46±10.9[b]	53.58	97.35
		37	0.5	30	61.60±0.58[a]	60.43	62.76
				90	83.26±10.9[b]	61.38	105.15
			0.75	30	84.57±0.58[a]	83.41	85.74
				90	91.37±10.9[b]	69.49	113.26
	10^9	4	0.5	30	81.66±0.58[a]	80.50	82.82
				90	63.60±10.9[b]	41.71	85.48
			0.75	30	89.20±0.58[a]	88.03	90.36
				90	74.00±10.9[b]	52.11	95.88
		37	0.5	30	81.86±0.58[a]	80.70	83.02
				90	87.33±10.9[b]	65.44	109.21
			0.75	30	94.97±0.58[a]	93.81	96.14
				90	95.86±10.9[a]	73.98	117.75
L. rhamnosus+ L. plantarum+ S. boulardii	10^7	4	0.5	30	82.40±0.58[a]	81.23	83.56
				90	87.00±10.9[b]	65.11	108.88
			0.75	30	100.0±0.58[a]	98.83	101.16
				90	91.73±10.9[b]	69.84	113.61
		37	0.5	30	82.13±0.58[a]	80.97	83.29
				90	53.55±10.9[b]	31.64	75.41
			0.75	30	73.95±0.58[a]	72.79	75.11
				90	95.42±10.9[b]	73.53	117.30
	10^9	4	0.5	30	83.73±0.58[a]	82.57	84.89
				90	93.33±10.9[b]	71.44	115.21
			0.75	30	68.71±0.58[a]	67.54	69.87
				90	75.73±10.9[b]	53.84	97.61
		37	0.5	30	61.60±0.58[a]	60.43	62.76
				90	75.80±10.9[b]	53.91	97.68
			0.75	0.75	82.53±0.58[a]	81.37	83.69
				90	90.17±10.9[b]	68.29	112.06

(At each row, different superscripts show a significant difference between the values for the time of exposure at the significance level of 0.05.)

MC: microorganism concentration (CFU/ml), AFM1: aflatoxin M1, Temp: temperature

10^9 CFU/ml of *L. plantarum* was obtained as 89% and 82% with 0.75 ng/ml AFM1, respectively. For *S. boulardii* at 10^7 and 10^9 (CFU/ml), this rate was found to be respectively 75% and 90% with 0.75 ng/ml of AFM1. In addition, these values at the same concentration for *L. rhamnosus*+*L. plantarum* with 0.5 and 0.75 ng/ml of AFM1 were respectively 85% and 89%. Eventually, this rate for *L. rhamnosus*+*L. plantarum*+*S. boulardii* reached to 100% and 93% with 0.75 and 0. 5 ng/ml of AFM1, respectively.

DISCUSSION

Our study was aimed to assess the ability of some LAB bacteria and *S. boulardii* to mitigate AFM1 in milk. There are globally extended physical and chemical methods for the removal of AFM1. Heating and ozone application are among the physical methods applied for detoxification. Furthermore, AF can be biodegraded via bacteria and other probiotics (Mohammadi et al., 2017). In another study, the AFM1 value was reported to significantly reduce (53%) concurrent to an upturn in the contact time (10 min) to ozone. The value obtained for the complex of AFM1/probiotics could be based on some physicochemical parameters, such as temperature and the concentrations of AFM1 and probiotics (Peltonen et al., 2001; Bovo et al., 2013; Bhatnagar-Mathur et al., 2015). In the current study, the maximum percentage of AFM1 reduction was greater in the model including more physicochemical factors, compared to those of the models containing fewer physicochemical factors (Table 1). As the results indicated, the highest affinity for AFM1 binding occurred when mycotoxin was set at 0.75 ng/ml rather than at 0.5 ng/ml (Table 2). In the previous studies, *L. rhamnosus* in exposure to AFM1 at the concentrations of 20 and 150 ng/ml could reduce this mycotoxin by 24.7-26.3% (Kabak and Var, 2004) and 50.7% (Pierides et al., 2000), respectively. The AFM1 level removed from spiked skim milk samples (0.5 ng/ml) treated with LAB pool, including *L. rhamnosus* cells, was lower (12%), compared with those removed from the samples only treated with *S. boulardii* after 30 and 60 min of exposure (90.3% and 92.7%, respectively). However, *S. boulardii* in combination with the LAB pool could remove more than 99% of AFM1 (Corassin et al., 2013), which is consistent with the findings of a study performed by Gonçalves et al. (2015). This finding is relatively in accordance with our results showing that the maximum rates of AFM1 degraded by LAB pools (i.e., *L. rhamnosus*+*L. plantarum* and *L. rhamnosus*+*L. plantarum*+*S. boulardii*) were 95.86% within 90 min and 100.0% after 30 min. These results imply that LAB pool bacteria can degrade AFM1 to a lower extent in comparison to *S. boulardii* combined with *the* LAB pool. The maximum AFM1-binding levels for *L. rhamnosus* and *L. plantarum* in the reconstituted milk were obtained as 95.1% and 78.6%, respectively. In line with our findings, Ismail et al. (2017) reported that the percentage of AFM1 removal from skimmed milk increased with the elevation of probiotic concentration. They also reported that the maximum ability values of 28%/73% and 13%/40% for *S. boulardii* and *L. plantarum* at the concentrations of 10^7 and 10^9 CFU/ml for AFM1 binding in skimmed milk, respectively. Contrarily, in the current study, this value for *L. plantarum* at the concentrations of 10^7 and 10^9 CFU/ml was estimated at 89.33% and 83.73%, respectively. With regard to *S. boulardii,* these values were 93.20% and 90.62%, respectively. These findings revealed a decrease in the binding affinity of AFM1 for these two probiotics in milk with the increase of their concentrations from 10^7 and 10^9 CFU/ml. In previous studies, the optimum concentration of LAB was reported to occur at 10^8 CFU/ml for binding AFM1 in a contaminated media (Sarimehmetoğlu and Küplülü, 2004; Kabak and Var, 2008). This finding is consistent with those reported in the previous study (Abbes et al., 2013), evidently pointing out the efficiency of *L. rhamnosus* for binding AFM1 at a concentration of 10^8 CFU/ml in contaminated reconstituted milk. Furthermore, *L. plantarum* MON03 and *L. rhamnosus* GAF01 were reported to show the maximum AFM1-binding ability at 10^8 CFU/ml at each incubation time

(i.e., 0, 6, and 24 h) and AFM1 concentration. In another study, the maximum affinity values of *L. rhamnosus* for removing AFM1 from milk were reported as 19.70% and 24.46% at 4 °C and 37 °C, respectively (Bovo et al., 2013). In the present study, a lower degree of temperature (4 °C) increased the microorganism ability (100.0±0.58%) to bind AFM1. In this regard, the maximum ability of *L. rhamnosus* to bind AFM1 was observed at a concentration of 10^9 CFU/ml (91.33% and 91.82% at 4°C and 37 °C, respectively) which was more than those of *L. plantarum,* observed at a concentration of 10^7 CFU/ml (89.33% and 84.62%). This could be due to the thick layer of peptidoglycan in the cell wall of *L. rhamnosus*.

The achievement of different results by the microbes suggests that various binding sites are associated with different strains and binding terminals in each probiotic. Plasma membrane polysaccharide and peptidoglycan are the key components accountable for the physical removal of AF by probiotics (Haskard et al., 2001). The AFM1-binding ability of probiotics is reported to be changeable and reversible. Moreover, the attachment value may vary depending on the time of exposure and loose noncovalent adhesion (Hernandez-Mendoza et al., 2009). This value might also differ from species to species due to alteration in the probiotic cell wall, alignment of the cell pocket, and outcome of diverse attachment sites (Peltonen et al., 2001; Bovo et al., 2013). Viable *Flavobacterium aurantiacum* decreases AF irreversibly from media by metabolic degradation (Haskard et al., 2001). On the other hand, the complex of AFM1/probiotic cell walls is described as a rapid procedure and the optimum attachment occurs within the first minutes of the exposure (El-Nezami et al., 1998; Bovo et al., 2015). The levels of the complex of AFM1/*L. rhamnosus* exposed in the PBS medium reached 79.2% and 71.6% after 4 and 8 h of incubation, respectively (Elsanhoty et al., 2014). In a study, the immobilized *S. boulardii* could completely degrade AFM1 (at an initial concentration of 80 ng/ml) in milk within 40 min (Foroughi et al., 2018). Our study showed that the removal of AFM1 from milk was not stable from 30 to 90 min. In this regard, the results obtained from the LAB pool with *S. boulardii* treatment (Table 2) indicated a reduction from 100% to 91.7%, respectively. Similarly, *L. rhamnosus* has the potential to form complex AF/cell wall, which varied significantly within the incubation periods of 0, 24, 48, and 72 h (Hernandez-Mendoza et al., 2009). In the present study, the best AFM1 binding affinity took place at 4 °C at a concentration of 10^7 CFU/ml. It was concluded that *S. boulardii* in combination with *L. rhamnosus* and *L. plantarum* would bind AFM1 in reconstituted artificially contaminated milk and decrease its carcinogenic potential in humans. In addition, the best AFM1-mitigation value was gained for the aforementioned probiotics while their concentrations were adjusted at 1×10^8 CFU/ml at the early phase of incubation time. Regarding this, a combination of *L. rhamnosus, L. plantarum,* and *S. boulardii* can be considered a good probiotic candidate for use in daily milk.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contribution

Study concept and design: Razavilar, V.,
Acquisition of data: Akbari -Adergani, B.
Analysis and interpretation of data: Razavilar, V.,
Drafting of the manuscript: Khadivi R.
Critical revision of the manuscript for important intellectual content: Khadivi R.
Statistical analysis: Amir Ali Anvar
Administrative, technical, and material support: Amir Ali Anvar

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