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Impact of probiotics and β-glucan on the detoxification and gastrointestinal bioaccessibility of aflatoxin B1 in a cereal-based fermented beverage (boza)

Arife Macit[1]*, Sumeyra Sevim[2], Banu Sancak[3], Mevlude Kizil[4]

[1]Department of Nutrition and Dietetics, Faculty of Health Sciences, Munzur University, Tunceli, Turkey; [2]Department of Nutrition and Dietetics, Faculty of Health Sciences, Ankara Medipol University, Ankara, Turkey; [3]Department of Medical Microbiology, Faculty of Medicine, Hacettepe University, Ankara, Turkey; [4]Department of Nutrition and Dietetics, Faculty of Health Sciences, Hacettepe University, Ankara, Turkey

*Corresponding Author:** Arife Macit, Department of Nutrition and Dietetics, Faculty of Health Sciences, Munzur University, Tunceli, Turkey. Email: arifemacit@munzur.edu.tr

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Abstract

The aim of this study was to investigate the effects of *Lactobacillus leichmannii* as a probiotic bacteria, *Saccharomyces boulardii* as a yeast, and β-glucan as a prebiotic on the binding ability of aflatoxin B1 (AFB1) and its bioaccessibility in a cereal-based fermented beverage. For this purpose, nine study groups with their combinations were formed. The amount of unbound AFB1 was determined by high performance liquid chromatography (HPLC) after incubation and during the stomach, small intestine, and colon stages using an *in vitro* digestion model. After incubation, the highest percentage of AFB1 binding (27.79%) was obtained with *S. boulardii*. The lowest bioaccessibility was observed with *L. leichmannii* + *S. boulardii* at the stomach stage (41%), *L. leichmannii* + β-glucan at the small intestine stage (52%), and *L. leichmannii* + *S. boulardii* + β-glucan at the colon stage (35%). These findings suggest that probiotics and prebiotics reduce aflatoxin bioaccessibility, thereby limiting gastrointestinal absorption.

Keywords: aflatoxin B1, β-glucan, boza, *in vitro* digestion, *Lactobacillus leichmannii, Saccharomyces boulardii*

Introduction

Aflatoxins (AF) are highly toxic mycotoxins produced by certain *Aspergillus* (*A*) species, particularly *A. flavus, A. paraciticus,* and *A. nomius* (Ismail *et al.* 2018). Over 20 aflatoxin species have been identified, with aflatoxin B1 (AFB1) being the most prevalent in food products and the most harmful among these species (Afshar *et al.* 2020; Udomkun *et al.* 2017). AFB1 causes both acute and chronic effects in mammals and poultry, including carcinogenicity, DNA mutation, hepatotoxicity, and immunotoxicity (Cao *et al.* 2022; Ishikawa *et al.* 2017). Based on epidemiological, genetic, and experimental studies, AFB1 has been classified as a "Class 1: human carcinogen" by the International Agency for Research on Cancer (IARC 2002).

Boza is a traditional cereal-based fermented beverage widely consumed in Türkiye, particularly during the winter season (Arici & Daglioglu, 2002). It is

typically produced from cereals such as millet, maize, wheat, or rice, and fermentation is carried out by a mixed microbiota dominated by lactic acid bacteria and yeasts (Borcakli *et al*., 2018; Ray & Montet, 2017). The fermentation process generally occurs at 20–30 °C for 24–72 h, resulting in a slightly acidic and viscous product. During fermentation, microbial activity leads to the production of organic acids, vitamins, and bioactive compounds that may contribute to the nutritional properties of boza. Regular consumption of fermented products such as boza has been associated with improvements in lipid profiles, including reductions in triglyceride and very low-density lipoprotein (VLDL) levels (Gölünük *et al*., 2017). However, aflatoxins have been detected in various fermented cereal-based beverages, mainly due to contaminated raw materials such as cereals. Since aflatoxins are relatively stable compounds, they may persist during processing and pose a potential food safety risk (Namaumbo *et al*., 2023; Wacoo *et al*., 2019).

The utilization of chemical (e.g., ammoniation and ozonation) and physical (e.g., thermal treatment and irradiation) methods to reduce AFB1 levels in food products is considered ineffective and unsafe due to their potential to cause nutritional and organoleptic deficiencies in food products (Ismail *et al*. 2018). Therefore, there has been growing interest in biological approaches using microorganisms capable of binding aflatoxins (Macit *et al*., 2024). In particular, lactic acid bacteria such as *Lactobacillus* spp. (e.g., *L. rhamnosus*, *L. plantarum*) and yeasts such as *Saccharomyces cerevisiae* have been widely reported to bind AFB1 through interactions with cell wall components, including peptidoglycans and polysaccharides (Tajik & Sayadi, 2022; Wochner *et al*., 2019). This mechanism is primarily based on non-covalent interactions, which reduce the bioaccessible fraction of AFB1 in the gastrointestinal environment. Therefore, microbial binding has gained attention as a promising and relatively safe strategy to reduce AFB1 bioaccessibility in food systems (Serrano-Niño *et al*., 2015; Kabak & Ozbey, 2012; Afshar *et al*., 2020).

Numerous studies have examined the use of probiotic microorganisms to decrease AFB1 levels and have demonstrated their ability to reduce aflatoxin concentrations in foods (Hamad *et al*. 2017; Pourmohammadi *et al*. 2021; Wacoo *et al*. 2019). Furthermore, prebiotics such as β-glucan and inulin have been reported to interact with AFB1 (Chaharaein *et al*. 2021; Vasconcelos *et al*. 2020; Wochner *et al*. 2019). β-Glucan is a natural polysaccharide found in cereal cell walls that can bind mycotoxins through physical adsorption and non-covalent interactions. This binding capacity is attributed to its high molecular weight and the presence of hydroxyl groups, which can interact with AFB1 molecules (Wang *et al*., 2025). Therefore, β-glucan has been considered a potential natural binding agent for reducing AFB1 bioaccessibility (Vasconcelos *et al*., 2020; Wochner *et al*., 2019). However, the exact binding mechanism is not fully understood, and the effect of natural binding substances on the bioaccessibility of aflatoxins in the gastrointestinal tract is still unclear. There are also limited studies on the effects of prebiotics on AFB1 bioaccessibility under the conditions of each gastrointestinal phase. Therefore, the aim of this study was to evaluate the effects of different combinations of *L. leichmannii* and *S. boulardii* with β-glucan on the binding capacity and bioaccessibility of AFB1 in a cereal-based fermented beverage (boza) under simulated gastrointestinal conditions. In addition, AFB1 levels were monitored across different stages of *in vitro* digestion, including the gastric, small intestinal, and colonic phases, each evaluated separately. It was hypothesized that the combined use of probiotics and β-glucan would enhance AFB1 binding and consequently reduce its bioaccessible fraction during digestion.

Materials and Methods

Chemicals and reagents

HPLC grade acetonitrile and methanol, calcium chloride ($CaCl_2$), monopotassium phosphate (KH_2PO_4), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH), monopotassium phosphate (KH_2PO_4), and urea were provided from ISOLAB (Eschau, Germany). Sodium chloride (NaCl), sodium sulfate (Na_2SO4), sodium phosphate (NaH_2PO_4), and ammonium chloride (NH_4Cl) were obtained from Merck (Darmstadt, Germany). Magnesium chloride ($MgCl_2$), potassium thiocyanate (KSCN), D-glucosamine hydrochloride, uric acid, bovine serum albumin (BSA), glucose, bile salts, α-amylase, pepsin, lipase, mucin, pancreatin, protease (P5147) and viscosyme® L (V2010) were supplied from Sigma-Aldrich (St Louis, USA).

Lactobacillus leichmannii (ATCC 10697) RSKK 06048 and *Saccharomyces boulardii* RSKK 06047 were obtained as lyophilized cultures from the Refik Saydam Laboratory Culture Collection in Ankara, Turkey. β-glucan from oats was supplied by Aksuvital in Turkey.

The AFB1 standard was supplied in a screw-cap bottle at a concentration of 25 μg/mL (TRILOGY TAS-M11LA1-10) in 10 mL of acetonitrile. AFB1 immunoaffinity column (R-Biopharm Aflaprep P07) containing AFB1-specific antibodies was used to ascertain AFB1 binding ability in the prepared samples.

The bulgur, corn flour, and wheat flour used in making boza were purchased from a local boza company in

Ankara, Turkey. The AFB1 level of boza made by the authors was analyzed by HPLC and AFB1 level in boza was determined below the detection limit.

Preparation of boza

To prepare boza, 1500 mL of water was brought to a boil (~100 °C) in a pot. Then, 80 g of bulgur was added and cooked for 1 h with continuous heating. Subsequently, 25 g of corn flour and 25 g of wheat flour were added together with an additional 1500 mL of water, and the mixture was boiled for a further 30 min. The mixture was then allowed to cool to room temperature (~25 °C). The cooked mixture was filtered through a sterile fine-mesh strainer (pore size approximately 0.1–0.5 mm) to remove coarse particles, resulting in a homogeneous liquid phase used for subsequent analyses.

Preparation of bacteria and yeast cultures

Lyophilized *L. leichmannii* was cultured in MRS broth (Merck, Darmstadt, Germany) and then subcultured on MRS agar (Condalab, Madrid, Spain). Microaerophilic conditions were maintained using the BD Gas Pack™ EZ Campy Container System (Becton, Dickinson and Company, USA), and cultures were incubated at 37 °C for 48–72 hours. Similarly, *lyophilized S. boulardii* was cultured in brain heart infusion broth and subsequently subcultured on Yeast Extract Peptone Dextrose (YPD) agar (Condalab, Madrid, Spain). Yeasts were incubated at 37 °C for 24–48 hours.

Preparation of the AFB1 contaminated samples

Aflatoxin B1 stock solution was diluted with acetonitrile to obtain a working standard solution at a concentration of 100 ng/mL. The prepared working solution was transferred to a sterile tube and then evaporated under nitrogen (N_2) gas to eliminate the acetonitrile. AFB1-contaminated boza samples were prepared by transferring the previously prepared boza, heated to 37 °C, into sterile tubes containing AFB1. In this study, nine different experimental groups were established, including positive and negative control groups. The characteristics of the study groups are detailed in Table 1. Each study group was prepared separately to assess AFB1 levels following incubation and at different stages of the *in vitro* digestion process (stomach, small intestine, and colon). The concentration of *L. leichmannii* was adjusted to 2×10^8 CFU/mL and that of *S. boulardii* to 2×10^6 CFU/mL using a turbidimetric method before addition to sterile tubes. The supernatants of bacterial and yeast suspensions centrifuged at 3000×g for 15 minutes were discarded, and the pellets were washed twice with distilled water. The pellets were suspended in AFB1-contaminated boza and mixed homogeneously. β-glucan, which has prebiotic properties, was added at a concentration of 1.5%. The amount added was determined to reveal the prebiotic effect of β-glucan and to be compatible with daily consumption (Basyigit Kilic & Akpinar Kankaya 2016; Kurtuldu & Ozcan 2018). All groups were prepared in triplicate. After incubation at 30 °C for 24 hours, samples not subjected to *in vitro* digestion were directly centrifuged at 3000×g and 4 °C for 15 minutes, and the supernatants were collected for AFB1 analysis. For samples subjected to *in vitro* digestion, the resulting digesta were centrifuged under the same conditions, and the supernatants were collected for AFB1 analysis.

In vitro digestion system simulation

The *in vitro* digestion model simulates the physiological conditions of human gastrointestinal digestion. In

Table 1. Characteristics of study groups formed using boza.

	AFB1 (100 ng/mL)	*L. leichmannii* (10^8 CFU/mL)	*S. boulardii* (10^6 CFU/mL)	β-glucan (1.5%)
Positive control	+	-	-	-
Negative control	-	+	+	-
Test group 1	+	+	-	-
Test group 2	+	-	+	-
Test group 3	+	+	+	-
Test group 4	+	-	-	+
Test group 5	+	+	-	+
Test group 6	+	-	+	+
Test group 7	+	+	+	+

this regard, chemical and enzymatic solutions mimicking salivary, gastric, and duodenal fluids were prepared and sequentially added to the food. At each stage, the mixture was thoroughly mixed and incubated for specific periods. In this study, an *in vitro* digestion model designed by RIVM (Bilthoven, The Netherlands) was employed to assess the efficacy of natural binders in reducing AFB1 absorption in the gastrointestinal tract (Versantvoort *et al*. 2005). Additionally, to represent the entire gastrointestinal tract, the colon phase was simulated using enzymatic methods, as described in the literature (Papillo *et al*. 2014; Sevim *et al*. 2024). The components of the *in vitro* digestion model used are shown in Figure 1.

The *in vitro* digestion model was performed according to the method described by Versantvoort *et al*. (2005), with minor modifications. All digestion fluids were pre-heated to 37 °C prior to use. Briefly, 4.5 mL of sample was transferred into sterile tubes. The digestion process was carried out sequentially to simulate the oral, gastric, and intestinal phases. In the oral phase, 6 mL of simulated salivary fluid was added, and the mixture was incubated at 37 °C for 5 minutes under continuous mixing. Subsequently, 12 mL of gastric fluid was added, and the samples were incubated at 37 °C for 2 hours. Following gastric digestion, 12 mL of duodenal fluid, 2 mL of 1 M $NaHCO_3$, and 6 mL of bile solution were added simultaneously, and the samples were incubated at 37 °C for an additional 2 hours to simulate small intestinal conditions.

For the colon phase, bacterial enzyme activity was simulated using enzymatic treatments. After small intestinal digestion, 5 mL of protease solution (1 mg/mL, pH 8.0) was added, and the samples were incubated at 37 °C for 1 hour. Subsequently, 150 µL of Viscozyme® L was added to mimic microbial fermentation in the colon, and the samples were incubated at 37 °C for 16 hours (Papillo *et al*. 2014; Sevim *et al*. 2024). All steps were performed under controlled temperature conditions, and samples were mixed thoroughly at each stage to ensure homogeneity.

After the stomach, small intestine, and colon stages of *in vitro* digestion, the samples were centrifuged at 3000×g for 15 minutes, and the supernatant was separated. After AFB1 extraction, the amount of free AFB1 in the supernatant was determined by HPLC analysis. The *in vitro* digestion stages and AFB1 analysis process are shown in Figure 2. The bioaccessibility (%) was defined as the fraction of AFB1 released into the soluble phase after each stage of *in vitro* digestion (gastric, small intestinal, and colonic), representing the proportion potentially available for absorption. It was calculated by comparing the amount of AFB1 detected in the supernatant after digestion with the total AFB1 concentration before digestion (Tabatabaei-Moradi *et al*., 2024).

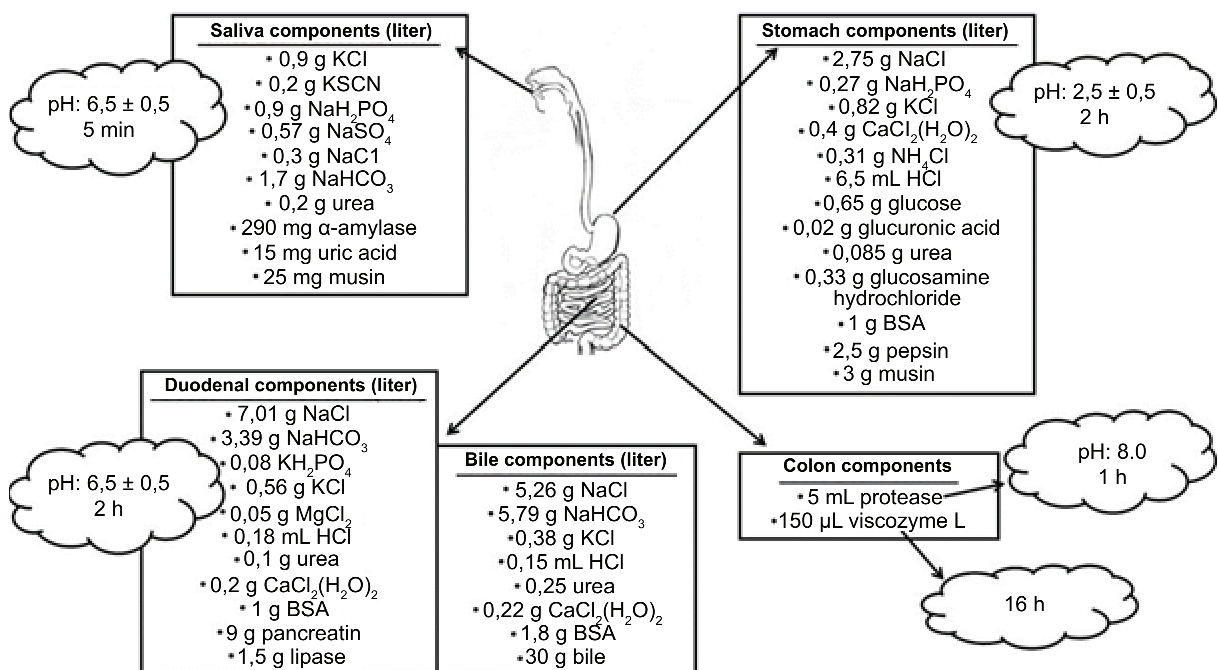

Figure 1. ***In vitro* digestion components.**

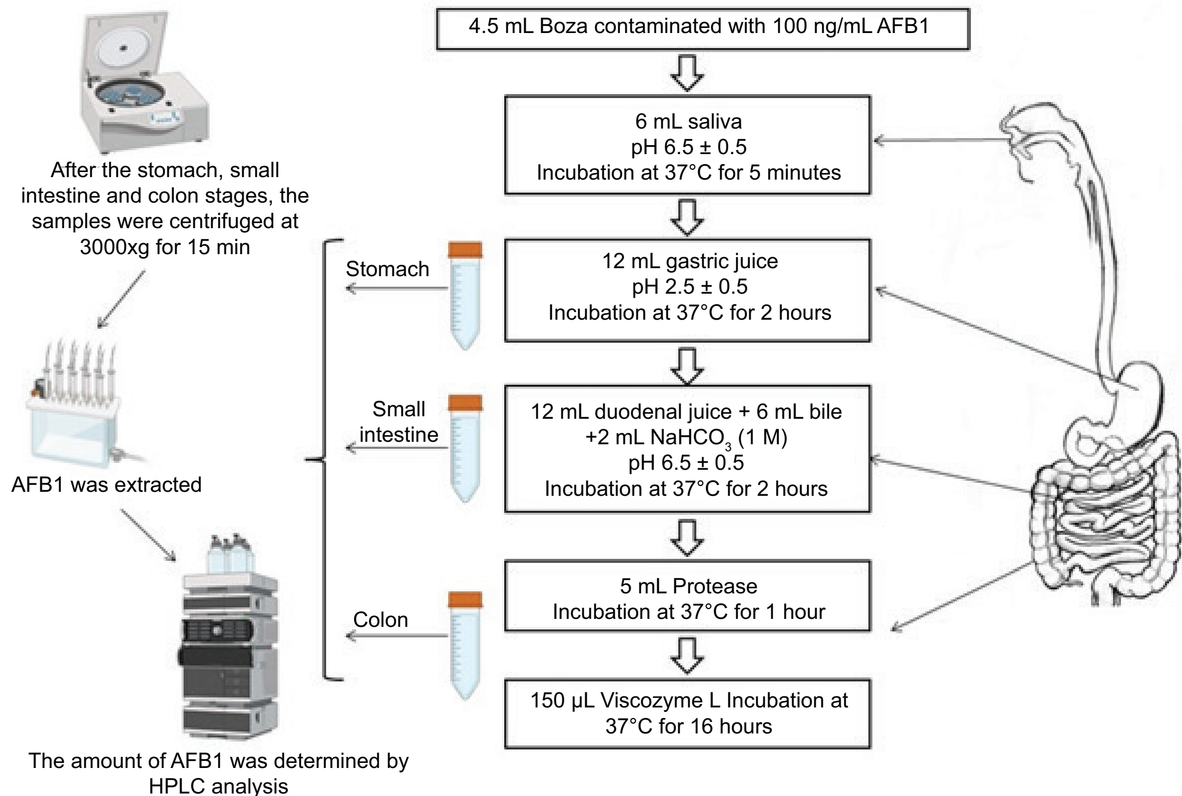

Figure 2. ***In vitro*** **digestion stages and AFB1 analysis process.**

$$\%\ AFB1\ Bioaccessibility = \left(\frac{Amount\ of\ AFB1\ in\ vitro\ digested\ sample}{Amount\ of\ AFB1\ before\ digestion} \right) \times 100$$

In contrast, AFB1 binding (%) was defined as the proportion of AFB1 associated with bacterial cells, reflecting the reduction in free (unbound) toxin in the supernatant. Binding was calculated based on the decrease in the AFB1 peak area in the sample relative to the positive control (Sevim *et al*., 2024).

$$\%\ AFB1\ Binding = \left(1 - \frac{peak\ area\ of\ AFB1\ in\ the\ sample\ supernatant}{peak\ area\ of\ AFB1\ in\ the\ positive\ control} \right) \times 100$$

Although bacterial binding may influence AFB1 bioaccessibility by reducing the freely available fraction of the toxin, these two parameters represent distinct analytical outcomes and were therefore evaluated separately in this study.

Analysis of aflatoxin B1

Free AFB1 in the supernatant obtained from all samples was extracted using an R-Biopharm (Darmstadt, Germany) immunoaffinity column with a vacuum manifold system. The extracted samples were collected in vials, wrapped in aluminum foil to prevent light exposure, and preserved at 4 °C prior to HPLC analysis.

The amounts of unbound AFB1 were assessed using an HPLC instrument equipped with a fluorescence detector. An analytical C18 column (5 µm, 250 × 4.6 mm) was employed in the study. The mobile phase was prepared from a mixture of ultrapure water/methanol/acetonitrile (6:3:2, v/v/v), to which 132 mg potassium bromide (KBr) and 110 µL 65% nitric acid (HNO_3) were added per liter. The mobile phase was filtered through 0.45 µm filter paper and prepared for use. The column temperature was set at 25 °C, the excitation wavelength at 360 nm, the emission wavelength at 440 nm, and the pump flow rate at 1 mL/min. HPLC analysis was conducted by

injecting 100 μL of each sample, and the results were recorded. The proportion of AFB1 bound to bacteria was determined using the following method (Sevim *et al*. 2024).

Statistical analysis

Data analysis was carried out using SPSS 22.0 statistical software. Results are presented as mean ± standard deviation (SD). Differences in AFB1 bioaccessibility and binding levels between study groups following incubation and *in vitro* digestion (stomach, small intestine, and colon) were evaluated using one-way ANOVA. Group comparisons for significant differences were further analyzed using the Tukey post hoc test, and statistical significance was accepted at $p < 0.05$.

Results and Discussion

Method validation

For method validation, the standard addition method was utilized to assess the recovery levels of AFB1. Before extraction, the AFB1 stock solution was prepared at concentrations of 25, 50, and 100 ng/mL and then spiked into the samples. The mean recovery rate of AFB1 was determined to be 99.69%. The Relative Standard Deviation (RSD), Limit of Detection (LOD), and Limit of Quantification (LOQ) were calculated based on the calibration curve of the AFB1 standard solution analyzed in this study and are presented in Table 2. The findings align with those reported in the literature (Kabak & Ozbey 2012; Romero-Sánchez *et al.* 2024; Vasconcelos *et al.* 2020). Additionally, HPLC chromatograms for the positive control, negative control, and *S. boulardii*-treated samples following incubation are shown in Figure 3.

AFB1 binding capacity of β-glucan and probiotics in Boza

In this research, β-glucan and selected probiotic strains were used as natural binding agents to reduce AFB1 levels in boza. The use of natural compounds such as β-glucan and microorganisms for AFB1 removal is of particular interest because of their potential application in the development of functional food products; however, their effectiveness should be carefully evaluated. To the best of our knowledge, although the AFM1 binding capacity of *S. boulardii* in food matrices has been investigated in previous studies (Khadivi *et al*. 2020; Martinez *et al*. 2019), its ability to bind AFB1 in food has been evaluated for the first time in the present study. Likewise, this study is the first to investigate the AFB1 binding capacity of *L. leichmannii*. The percentage of AFB1 binding by β-glucan, probiotic yeast, and bacteria is presented in Table 3. After incubation, AFB1 binding capacities ranged from 12.73% to 27.79%, with *S. boulardii* showing the highest binding efficiency and *L. leichmannii* + *S. boulardii* + β-glucan showing the lowest. The disparity between these two groups was found to be statistically significant ($p < 0.001$). These results indicate that AFB1 reduction in boza is dependent on the type of microorganism and the combinations applied. Similarly, Martinez *et al*. (2019) reported that the *S. boulardii* strain RC009 had a 25% AFM1 binding capacity, and Khadivi *et al*. (2020) showed that a different *S. boulardii* strain had an AFM1 binding capacity ranging from 61.46% to 93.20% in milk. These findings reveal that *S. boulardii* strains exhibit significant differences in aflatoxin binding capacity.

The AFB1 binding ability of microorganisms is thought to depend on their cell wall composition (Macit *et al*. 2024). *S. boulardii* cell walls are composed of β-glucan structures that provide numerous physical interaction sites for toxin binding (Pereyra *et al*. 2018). The primary cell wall constituents of lactic acid bacteria that participate in aflatoxin binding include peptidoglycans, polysaccharides, teichoic acid, and lipoteichoic acid (Sarlak *et al*. 2017; Serrano-Niño *et al*. 2015; Vasconcelos *et al*. 2020; Wochner *et al*. 2019). The AFB1 binding capacity of samples containing *L. leichmannii* (26.54%) showed results similar to those of samples containing *S. boulardii*. Furthermore, Zinedine *et al*. (2005) stated that the AFB1 binding rates of *L. lactis* Lb5 and Lb8 strains were 16.81% and 20.26%, respectively. However, the AFB1 binding capacity obtained using the binary combination of probiotic yeast and bacteria (21.72 ± 0.41%) was lower than the binding rate provided by yeast or bacteria alone. This result indicates that the combination of *S. boulardii* and *L. leichmannii* did not produce a synergistic effect. In addition, no significant interaction effect between β-glucan and the

Table 2. Recovery (%), relative standard deviation (RSD), limit of detection (LOD) and limit of quantification values of analyzed AFB1 (n = 3).

	Spiking level (ng/ml)	Recovery (%)	RSD (%)	LOD (ng/ml)	LOQ (ng/ml)	R^2
AFB1	25	99.27	2.72	0.1	0.3	0.9982
	50	101.19	1.30			
	100	98.62	2.35			

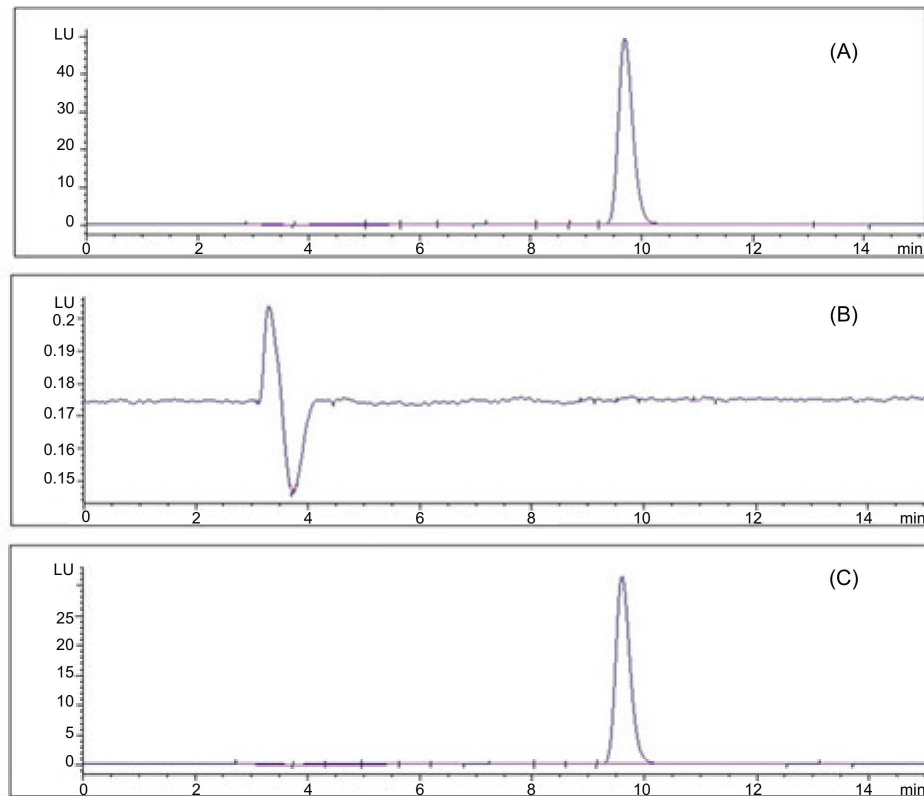

Figure 3. HPLC chromatograms of the positive control (A), negative control (B), and *S. boulardii*-treated sample (C) after incubation.

Table 3. AFB1 binding rates measured after incubation and *in vitro* digestion model in the study groups.

	% AFB1 binding			
	After incubation	After stomach	After small intestine	After colon
L. leichmannii	26,54 ± 0,59[a]	9,20 ± 1,95[a]	11,59 ± 5,40[ad]	22,36 ± 4,68[a]
S. boulardii	27,79 ± 1,95[a]	13,17 ± 2,04[a]	11,86 ± 4,32[ad]	16,69 ± 1,70[a]
L. leichmannii + *S. boulardii*	21,72 ± 0,41[b]	55,17 ± 2,31[b]	19,46 ± 5,07[a]	51,17 ± 6,42[bc]
β-glucan	20,89 ± 0,78[b]	35,25 ± 6,94[c]	20,95 ± 5,73[a]	41,02 ± 1,60[b]
L. leichmannii + *β*-glucan	18,27 ± 0,61[b]	34,96 ± 3,21[c]	41,10 ± 1,25[b]	19,58 ± 4,18[a]
S. boulardii + *β*-glucan	18,35 ± 1,89[b]	34,86 ± 3,21[c]	20,69 ± 7,98[a]	12,34 ± 4,37[a]
L. leichmannii + *S. boulardii* + *β*-glucan	12,73 ± 1,51[c]	39,69 ± 4,73[c]	4,08 ± 3,20[cd]	55,46 ± 6,81[c]
p	<0,001	<0,001	<0,001	<0,001

Data are presented as mean ± standard deviation.
AFB1 binding percentages were analyzed using one-way ANOVA. Values with different letters in the same column indicate statistical significance according to the Tukey post hoc test ($p < 0.05$).
The initial AFB1 concentration was 100 ng/mL.

tested microbial strains was observed in terms of AFB1 binding efficiency. Therefore, although these natural binders show potential for reducing AFB1 levels, their practical application in food systems should be interpreted with caution.

While AFB1 binding to bacterial cells may contribute to a reduction in its bioaccessible fraction, binding efficiency should not be directly interpreted as an equivalent measure of bioaccessibility, as these parameters reflect different aspects of toxin behavior during digestion.

Although the maximum binding observed in this study (approximately 27–28%) can be considered moderate, its practical relevance should be interpreted within the context of the experimental conditions. The boza matrix, characterized by its complex composition, including carbohydrates, proteins, and fermentation-derived metabolites (Borcakli *et al.* 2018; Mostafa *et al.* 2024), may influence binding efficiency by competing with microbial binding sites or altering toxin availability. In addition, bacterial cell concentration and preparation methods are known to play a critical role in mycotoxin binding capacity (Macit *et al.* 2024). Variations in cell density, viability, and surface properties may affect the number of available binding sites and thus the overall binding efficiency. The selected AFB1 concentration may also influence binding behavior, as higher toxin levels can lead to partial saturation of binding sites, thereby limiting relative binding percentages (Sahebghalam *et al.* 2018). From a practical perspective, although the observed binding levels are moderate, even partial binding may contribute to reducing the free and potentially bioaccessible fraction of AFB1 in the gastrointestinal environment. Therefore, microbial binding should be considered a complementary mitigation strategy rather than a standalone detoxification approach.

Effect of β-glucan and probiotics on AFB1 bioaccessibility

After incubation, it was observed that AFB1 levels in the boza decreased because of the binding capacity of the probiotic yeasts and bacteria studied. However, studies conducted in different food matrices have suggested that aflatoxins can form stable complexes with microbial cell wall components, and these complexes may persist during gastrointestinal digestion (Kabak *et al.* 2009; Wacoo *et al.* 2019; Serrano-Niño *et al.* 2013). Based on these findings, a similar interaction may also occur in cereal-based fermented products such as boza. Accordingly, AFB1 may be present in both free and bound forms in the gastrointestinal tract. Some researchers have indicated that probiotic microorganisms can bind aflatoxins and reduce their absorption and bioaccessibility during digestion (Vasconcelos *et al.* 2020; Tajik & Sayadi 2022; Kabak & Ozbey 2012). It has also been suggested that the aflatoxin–microorganism complex may be excreted via feces; however, this mechanism has not yet been fully elucidated (Tajik & Sayadi 2022; Tian *et al.* 2022). To fully understand this mechanism, it is crucial to assess alterations in the levels of the microorganism–aflatoxin complex that reaches the colon after enzymatic digestion in the colonic phase. In addition, the evaluation of post-gastric processes appears to be limited in the literature, and only one study has addressed this subject (Saladino *et al.* 2018). Therefore, it is essential to examine the behavior of the microorganism–aflatoxin complex throughout the digestive tract, including the gastric and colonic phases. Moreover, AFB1 binding variability across digestion phases reflects the dynamic interaction between toxins and binding agents under changing gastrointestinal conditions. Bile salts and digestive enzymes in the intestinal phase weaken non-covalent interactions, causing partial AFB1 desorption (Serrano-Niño *et al.* 2013; Romero-Sánchez *et al.* 2024). Enzymatic activity may also modify bacterial cell wall components, thereby reducing binding site accessibility (Serrano-Niño *et al.* 2013). pH changes from acidic gastric to near-neutral intestinal environments alter the surface charge and structural conformation of bacterial cell walls and β-glucan, affecting binding affinity (Serrano-Niño *et al.* 2015; Kabak *et al.* 2009; Saladino *et al.* 2018). Additionally, β-glucan undergoes physicochemical changes during digestion that influence AFB1 interaction (Wang *et al.* 2025; Chaharein *et al.* 2021). These findings confirm that AFB1 binding is reversible, as aflatoxin–microorganism interactions are primarily non-covalent and environmentally sensitive (Serrano-Niño *et al.* 2015; Kabak *et al.* 2009). The observed binding fluctuations across phases highlight the importance of evaluating binding stability in a phase-dependent manner.

Figure 4 shows the impacts of various test groups on AFB1 bioaccessibility under simulated digestive conditions. In this study, AFB1 bioaccessibility in boza was 92% at the end of gastric digestion. Following gastric digestion, AFB1 bioaccessibility varied between 41% and 84% among different test groups formed by adding probiotic microorganisms and prebiotics. The highest AFB1 binding after *in vitro* gastric digestion was observed in the group containing *L. leichmannii* + *S. boulardii*, with AFB1 bioaccessibility decreasing by 55.17 ± 2.31% in this group (Table 3). These findings are consistent with those reported by Saladino *et al.* (2018), who observed a reduction in post-gastric AFB1 bioaccessibility ranging from 15.41% to 98.09% using fifteen different probiotic strains.

Upon completion of digestion in the small intestine, the bioaccessibility of AFB1 was determined to be 88% (Figure 4). Similarly, Kabak *et al.* (2009) reported that AFB1 bioaccessibility ranged between 86% and 94% in different contaminated foods. Moreover, another study determined that AFB1 bioaccessibility in different contaminated foods ranged from 85.1% to 98.1% (Kabak & Ozbey 2012). As shown in Figure 4, AFB1 bioaccessibility after small intestinal digestion ranged from 52% to 84% among different test groups containing prebiotics and probiotics. After small intestinal digestion, the highest AFB1 binding was observed in the group containing *L. leichmannii* + β-glucan, and AFB1 bioaccessibility decreased by 41.10 ± 1.25% in

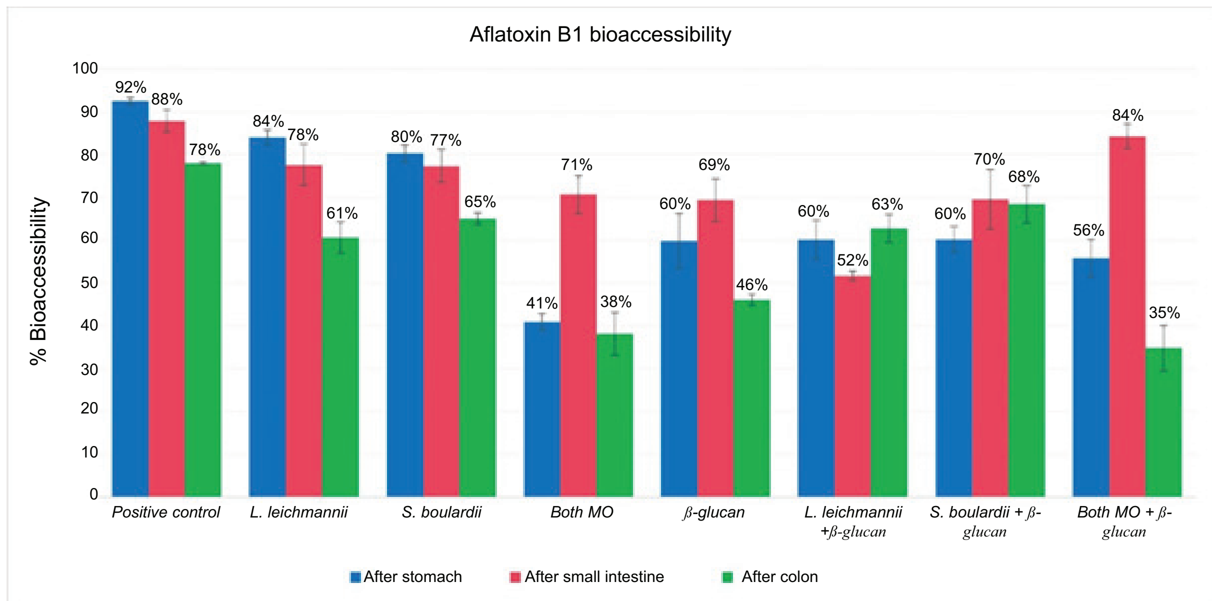

Figure 4. AFB1 bioaccessibility levels after *in vitro* digestion model. Values are expressed as mean ± standard deviation (SD). Error bars represent SD.

this group (Table 3). Tajik and Sayadi (2022) showed that the probiotic bacteria *L. acidophilus* ATCC 4356 and *L. casei* ATCC 39392 reduced AFB1 bioaccessibility by 24.3% to 70.0%. Likewise, Saladino *et al.* (2018) found that fifteen different probiotic bacteria reduced AFB1 bioaccessibility by 30.39% to 96.73%. Similar results have been reported in several other investigations, indicating that probiotic strains diminish the bioaccessibility of AFB1 in food (Vasconcelos *et al.* 2020; Wochner *et al.* 2019).

In recent years, prebiotics have emerged as a promising approach for researchers exploring alternative methods to mitigate aflatoxins (Chaharaein *et al.* 2021; Sevim *et al.* 2019; Vasconcelos *et al.* 2020). Wochner *et al.* (2019) demonstrated that the incorporation of prebiotics such as inulin, β-glucan, and polydextrose resulted in a reduction in AFB1 bioaccessibility in milk, with reductions ranging from 23.68% to 72.67%. In the current study, AFB1 bioaccessibility following the gastric and small intestinal phases, with the addition of β-glucan alone, was observed to be 60% and 69%, respectively. Among prebiotics, β-glucan has been shown to enhance the viability and functionality of microorganisms such as *L. acidophilus*, *L. plantarum*, and *B. animalis* subsp. *lactis* in various *in vitro* studies (Basyigit Kilic & Akpinar Kankaya 2016; Kurtuldu & Ozcan 2018; Rosburg *et al.* 2010). Several studies have further reported that the combination of β-glucan with probiotic microorganisms effectively reduces AFB1 bioaccessibility (Vasconcelos *et al.* 2020; Wochner *et al.* 2019). Similarly, this study demonstrated that β-glucan alone led to a 20.95 ± 5.73% reduction in AFB1 bioaccessibility, while the combination of *L. leichmannii* and β-glucan resulted in a 41.10 ± 1.25% reduction after the small intestinal stage (Table 3).

To the best of our knowledge, research on AFB1 bioaccessibility has predominantly focused on the small intestinal digestion stage, with limited studies evaluating the colonic phase. In the present study, AFB1 bioaccessibility at the colonic stage was assessed using the enzymatic colonic digestion method developed by Papillo *et al.* (2014). Sevim *et al.* (2024) reported that the bioaccessibility of AFM1 in milk reached 81.40% following the colon stage. Likewise, the current study found that AFB1 bioaccessibility in boza was 78% after the colon phase, with differential effects of β-glucan and probiotics on colonic bioaccessibility rates (Figure 4). AFB1 bioaccessibility after the colonic stage varied between 35% and 68% among different test groups containing prebiotics and probiotics. At the conclusion of the colonic phase, the greatest reduction in AFB1 bioaccessibility was observed in the group containing *L. leichmannii* + *S. boulardii* + β-glucan (55.46%), while the smallest reduction was observed in the group containing *S. boulardii* + β-glucan (12.34%) (Table 3).

As shown in Figure 4, the test groups containing *L. leichmannii* and *S. boulardii* exhibited a more stable

structure against digestive fluids, whereas the other test groups were more sensitive to the effects of enzymatic complexes in these fluids. This suggests that aflatoxin binding may be reversible and that the binding process is affected by both enzyme complexes and experimental conditions. Moreover, some researchers have indicated that the physical binding of natural binders such as probiotics to aflatoxins can lead to reversible binding (Kabak & Ozbey 2012; Sevim *et al.* 2024). This is thought to occur because aflatoxin–microorganism interaction usually takes place via physical adsorption or cell wall components (Afshar *et al.* 2020; Corassin *et al.* 2013; Macit *et al.* 2024). Although the results are promising, potential scale-up challenges such as matrix variability, processing conditions, and the stability of microorganism–aflatoxin interactions should be considered when translating these findings to industrial applications.

This study has several limitations that should be considered when interpreting the results. First, the *in vitro* digestion model used in this study provides valuable insights into AFB1 bioaccessibility; however, it does not fully replicate *in vivo* conditions. In particular, *in vitro* bioaccessibility does not necessarily correspond to *in vivo* bioaccessibility, as physiological processes such as intestinal absorption, metabolism, and systemic distribution are not represented. In addition, the colon phase simulation does not incorporate the complex and dynamic interactions of the gut microbiota. *In vivo*, microbial competition, metabolism, and enzymatic activity may significantly influence both AFB1 binding and release. Therefore, while the findings provide important mechanistic insights, further *in vivo* studies are needed to confirm the biological relevance of AFB1 binding and bioaccessibility under physiological conditions.

Conclusions

In this study, the effects of probiotics (*L. leichmannii* and *S. boulardii*) and the prebiotic β-glucan on the binding capacity and bioaccessibility of AFB1 in boza, a cereal-based fermented beverage, were systematically investigated using an *in vitro* digestion model. The results indicated that the tested compounds reduced the bioaccessibility of AFB1 to varying extents. The lowest bioaccessibility was observed after the gastric phase (41%) with *L. leichmannii* + *S. boulardii*, after the small intestinal phase (52%) with *L. leichmannii* + β-glucan, and after the colon phase (35%) with *L. leichmannii* + *S. boulardii* + β-glucan. These findings demonstrate that probiotics and prebiotics may exert differential effects on AFB1 binding and bioaccessibility under simulated gastrointestinal conditions. While the results suggest that *L. leichmannii*, *S. boulardii*, and β-glucan are promising candidates for reducing AFB1 bioaccessibility, these findings are limited to *in vitro* conditions. Therefore, further *in vivo* and food system-based studies are needed to confirm their practical applicability and effectiveness in real-life scenarios.

Data Availability

The data will be made available on request.

Mandatory Disclosure on Use of Artificial Intelligence

No AI-assisted technologies were used in the preparation of this manuscript.

Authors Contribution

A.M.: conceptualization, methodology, formal analysis, investigation, writing – original draft, visualization. S.S.: formal analysis, methodology, writing – original draft, investigation. B.S.: investigation, formal analysis, review & editing. M.K.: conceptualization, methodology, writing – review & editing, supervision, funding acquisition, project administration.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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