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ORIGINAL ARTICLE

Investigating the ability of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* on the reduction of aflatoxin B_1, ochratoxin A, and zearalenone in dough and toast Bread

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ABSTRACT

Background and Objectives: Wheat and its derived products are high-risk commodities for aflatoxin contamination. The objective of this study was to investigate the effect of using *Saccharomyces cerevisiae, Lactobacillus plantarum,* and the dough fermentation and baking periods on reducing aflatoxin B_1 (AFB_1), ochratoxin A (OTA), and zearalenone (ZEA) toxins.
Materials and Methods: Toast bread flour contaminated with AFB_1, OTA and ZEA (10,10 and 400 ng/g) were separately treated with *S. cerevisiae* and *L. plantarum* (at a concentration of 10^8 CFU/g). The reduction of mycotoxins was examined immediately after dough preparation, at the end of fermentation, and after baking.
Results: The type of microorganism, fermentation and baking significantly affected the reduction of mycotoxins (AFB_1, OTA, and ZEA). After baking, neither AFB_1 nor OTA were detected in any of the toast bread samples, with a 100% reduction observed in all treatments. In contrast, the percentage reduction of ZEA after baking compared with immediately after dough preparation ranged from 98.90% to 100%, and the percentage reduction of ZEA at the end of fermentation compared with immediately after dough preparation ranged from 97.80% to 99.57%.
Conclusion: The findings of this study suggest that *L. plantarum* and *S. cerevisiae* can be used as additives or processing agents to decrease mycotoxins in fermented wheat foods.

Keywords: Aflatoxin B_1; Ochratoxin A; Zearalenone; *Saccharomyces cerevisiae*; *Lactobacillus plantarum*; Bread

INTRODUCTION

Nowadays, the demand of consumers to use healthy products has increased (1-3). Wheat bread, as a staple food, provides a significant portion of daily energy, protein, minerals, and B-group vitamins required by people (4). Mycotoxins are secondary metabolites produced by filamentous fungi in food, particularly agricultural products, and can cause issues such as mycotoxicosis in humans and other animals (5). The term "mycotoxin" is derived from the words "myco" and "toxin," originating from the Greek words "mykes" and "toxikon," meaning "mold" and "poison" produced by a living organism. Mycotoxins are low molecular weight molecules (Mw<700) and are toxic even at low concentrations (6).

Mycotoxins can be found, especially in grains and grain-based products, which can become contaminated in the field or during storage. Due to secondary contamination, mycotoxins can often be present in animal-origin food products exposed to mycotoxins. Milk and dairy products, meat and meat products

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may also be contaminated. The Food and Agriculture Organization estimates that approximately 25% of grains produced worldwide are contaminated with mycotoxins, but this figure is likely closer to 50%. They are most commonly found in food and feed include aflatoxins, ochratoxin A, deoxynivalenol (DON), HT-2 and T-2 toxins, zearalenone, and fumonisins (7).

Aflatoxins (A-flavus-toxins) are considered the most well-known and toxic mycotoxins, produced by Aspergillus mold species. Their growth particularly increases at temperatures between 26 to 38°C and humidity above 18%. *Aspergillus flavus* is responsible for producing aflatoxins B_1 and B_2 in grains, while *Aspergillus parasiticus* may produce aflatoxins in stored oilseeds (8). Among the types of aflatoxins, AFB_1 is the most toxic and has been classified as a Group I carcinogen by the International Agency for Research on Cancer (IARC) (9, 10). The maximum permissible residue limits for aflatoxins set by the European Union are 2 ng/g for AFB1 and 4 ng/g for total AF (G_2, G_1, B_2, B_1) in nuts, dried fruits, and grains for human consumption (11). It is worth noting that the maximum permissible amount of aflatoxin B_1 in food for human consumption, according to global standards, is 1-20 ng/g (12).

The ochratoxin (OT) group includes OTA, OTB and OTC. The OT molecule is composed of dihydro-isocoumarin and L-β-phenylalanine (13). The most toxic representative of this group is OTA, which is isolated from the mold *Aspergillus ochraceus*. This toxin is primarily produced by *Aspergillus ochraceus* members. The production of this toxin occurs over a wide temperature range, with optimal conditions for its synthesis being temperatures between 20 to 25°C and 16% humidity (14). Based on carcinogenic indices, IARC classifies this toxin as a Group B_2 possible human carcinogen (7). OTA primarily targets the kidneys, liver, and cardiovascular system (15). In the European Union, the maximum permissible limits for OTA in grains, grain-based foods, and dried fruits are 5, 3, and 10 ng/kg, respectively, and 0.5 ng/kg in children's food (16).

Zearalenone (ZEA) mycotoxin was named after *Giberella zeae* mold, from which it was isolated in 1962. The major producers of zearalenone include molds such as *Fusarium graminearum, F. roseum, F. culmorum,* and *F. tricinctum.* Zearalenone is a non-steroidal estrogenic mycotoxin with a resorcylic acid-lactone chemical structure (17). Zearalenone primarily contaminates barley, wheat, corn, and rice, and to a lesser extent, fruits and vegetables. The EFSA (European Food Safety Authority) has declared a daily intake of 0.25 µg/kg body weight for adults to be safe. The maximum permissible limit of zearalenone in unprocessed grains is 100-200 µg/kg, while for processed grains, this limit is reduced to 75 µg/kg (18). As the biological detoxification of zearalenone is studied more deeply, likely, new probiotic strains (*Bacillus, Lactobacillus,* and yeast) and degrading enzymes (lactonase, peroxidase) will likely be increasingly discovered (19).

According to Iran's national standard NO.5925, the permissible limits on bread are 5 ng/g for aflatoxin B_1, 5 ng/g for ochratoxin A, and 200 ng/g for zearalenone (20). Pre- and post-harvest aflatoxin contamination of products can be somewhat controlled by implementing Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs), and Good Storage Practices (GSPs). Additionally, new processing technologies such as microwaves, UV, pulsed light, electrolyzed water, cold plasma, ozone, electron beam, or gamma radiation, combined with biological, physical, chemical, or genetic engineering methods have the potential to improve the efficiency of aflatoxin elimination and overcome the limitations of each specific technology. However, understanding the mechanisms of aflatoxin detoxification is crucial to ensure that no aflatoxin residues remain when these methods are applied to food and feed samples. Therefore, the use of new technologies along with increasing public awareness of the implementation of GAPs, GMPs, and GSPs to control aflatoxin contamination in food and feed is vital for ensuring food safety and security and maintaining human and animal health (21).

Many microorganisms, such as bacteria, yeasts, molds, and actinomycetes are capable of reducing the presence of mycotoxins in food and feed (7). However, in most cases, the exact mechanism of their action remains unknown. Among microorganisms, lactic acid bacteria (LABs) and yeasts are a unique group that are widely used in the production and preservation of fermented foods. They are able to remove mycotoxins from food by converting them into other products or by binding to the cell surface (22). Therefore, they can be used as effective agents to reduce mycotoxin levels in contaminated food, by biological control (23).

To eliminate or reduce mycotoxins in animal feed,

various physical, chemical, and biological methods have been used, but the results related to physical and chemical reductions have not been very satisfactory. Today, the use of microbes and enzymes produced by them as biological methods has received more attention. One method of reducing mycotoxins is the use of yeasts (24). Yeasts can detoxify mycotoxins in various ways: biodegradation, Biosorption, or inhibition of mycotoxin production (25). The complex interactions of yeasts with mycotoxins suggest that the structural integrity of the cell wall, physical and morphological structure, and chemical components all play significant roles in the adsorption process. Therefore, future approaches may rely on a combination of different microorganisms to provide complementary benefits in the adsorption of mycotoxins by yeast (26).

Considering that one of the natural contaminants of grains is mycotoxins, and mycotoxins can be detoxified biologically, various studies have been conducted, such as estimating the amount of OTA, ZEA, and AFB_1 through the consumption of flour and bakery products (27), examining the concentration of ZEA after 6 hours of incubation with *Lactobacillus* and *S. cerevisiae* strains (28), the bioavailability of OTA and AFB_1 in bread enriched with fermented whey/pumpkin (29), the impact of bread processing conditions on the ZEA levels in contaminated wheat flour (30), and the effect of five LAB strains (*L. rhamnosus, L. plantarum, Bifidobacterium bifidum, Streptococcus thermophilus,* and *L. reuteri*) on reducing AFB_1 during the production of Fino bread (31) have been conducted, the aim of the present study was to investigate the effect of using *S. cerevisiae* and *L. plantarum* in the production of toast bread dough to reduce OTA, ZEA, and AFB_1 toxins during fermentation and baking of toast bread. Additionally, the impact of using *S. cerevisiae* and *L. plantarum* on the sensory and textural properties of the bread, in three samples of toast bread without contamination by toxins and containing the mentioned probiotics (LAB and yeast), was checked.

MATERIALS AND METHODS

Materials. Wheat flour (Setareh Company, Iran), sugar (Shahde Ghand Company, Iran), salt (Golha Food Industries, Iran), sodium hydroxide, phenolphthalein, sulfuric acid, phosphate-buffered saline, hexane, standard mycotoxins (zearalenone, ochratoxin A and aflatoxin B_1), MRS culture medium and nutrient agar medium (Merck, Germany) were prepared. *Saccharomyces cerevisiae* PTCC 5269, *Lactobacillus plantarum* PTCC 1058 were purchased in pure and lyophilized form from the collection of the Scientific and Industrial Research Organization of Iran.

Preparation of probiotic bacteria. The preparation of probiotic bacteria was done according to the methof of Assaf et al. (32), with slight modifications.

Saccharomyces cerevisiae and *Lactobacillus plantarum* were inoculated into the MRS broth culture medium and incubated until reaching the logarithmic phase at 37°C. After 24 hours of cultivation, the cells were centrifuged for 15 minutes at 3000 rpm at 4°C. The resulting precipitate was washed 3 times with PBS (Phosphate-buffered saline). Finally, using sodium phosphate buffer solution, the turbidity was measured by spectrophotometer at a wavelength of 625 nm and the absorbance value was about half McFarland, which is equivalent to 10^8 cfu/ml bacteria (32). Also, for certainty, the number of bacteria was determined using standard plate counting with the help of MRS agar culture medium.

Preparation method of toast bread. To prepare the control toast bread, initially, 1 kg of flour, 11 g of sugar, 11 g of salt, 11 g of baking yeast, and 1 g of dough improver were mixed in a mixer. Then, semi-warm water at a temperature of 32-35°C (51% of the dry weight) was added, followed by 12 g of liquid oil after some initial mixing.

The mixture was then kneaded in the mixer for 2 min at low speed for 5-6 min at high speed. The dough was allowed to rest for 11 min. Subsequently, the dough was portioned into pieces weighing about 100 g each and shaped into cubes. This was followed by the final fermentation in a warm chamber at 37°C for 12 h. The dough was then baked in an oven at 280°C for 11 min. After cooling, the bread was packaged in polyethylene bags and stored at 20°C for testing (33).

To prepare probiotic toast bread, 108 probiotic bacteria (*Lactobacillus plantarum* and *Saccharomyces cerevisiae*) (Table 1) were added to the control toast bread material along with adding semi-warm water in the method of preparation toast bread control sample.

In order to contaminate the toast bread samples with mycotoxins according to the Table 1, AFB_1

Table 1. Treatments of current research

Treatment	Zearalenone (ng/g)	Aflatoxin B_1 (ng/g)	Ochratoxin A (ng/g)	*Saccharomyces cerevisiae* (CFU/g)	*Lactobacillus plantarum* (CFU/g)
Toast dough containing regular yeast	-	-	10	-	10^8
Toast dough containing regular yeast	-	10	-	-	10^8
Toast dough containing regular yeast	400	-	-	-	10^8
Toast dough containing regular yeast	-	-	10	10^8	-
Toast dough containing regular yeast	-	10	-	10^8	-
Toast dough containing regular yeast	400	-	-	10^8	-
Toast dough containing regular yeast (Control)	-	-	-	-	-

(10 ng/g), OTA (10 ng/g), and ZEA (400 ng/g) were added to the flour used in the toast bread control. It should be noted that the amount of mycotoxins in toast is twice the permissible limits stated in the national standard NO-5925. The reduction levels of these toxins were examined immediately after dough preparation, at the end of the fermentation period (12 h) at 37°C, and after baking (in an oven at 280°C) to determine the effects of fermentation and the microorganisms on toxin reduction. The permissible limits for OTA and AFB_1 are 5 ng/g, and for ZEA, it is 200 ng/g (20).

Measurement of aflatoxin B_1, ochratoxin A, and zearalenone. Aflatoxin B_1, ochratoxin, and zearalenone were tested by high-performance liquid chromatography (HPLC) (Waters Company- USA) equipped with an autosampler and fluorescence detector, and purification with an immunoaffinity column (Libios Company- USA). The experiment was carried out in 3 stages: extraction, purification and determination of the toxin amount. In the extraction stage, first, to activate the columns, PBS solution was passed through them and then each sample was mixed well and then 10 g of each sample was weighed with an error of 0.1 g was also weighed for the Spike sample with an error of 0.1 and 50 µl of standard mycotoxins (zearalenone, ochratoxin A and aflatoxin B_1) with a concentration of 1000 ng/g were added at different places in the sample. The steps were as follows: First, 10 ± 0.1 g of the sample was weighed and then one gram of NaCl and 100 ml of extraction solvent (MeOH: H2O= 80: 20) were added to it and mixed with a blender for three minutes and filtered with ordinary filter paper and then 5 ml of the filtered solution was added to 25 ml of PBS solution and shaken vigorously. The mixture was passed through glass fiber filters (GFF) and finally all the diluted extract was removed while bringing the column temperature to laboratory temperature and passed through 20 ml of PBS solution. In the concentration step, 36 ml of diluted extract was passed through the column and the column was washed with 10 ml of PBS solution. Then, the column was dried by passing gentle air pressure for 10-15 seconds and then 500 µl of MeOH:AOCH (98:02) was added to the column and collected in a vial. After a one-minute stop, 1000 µl of MeOH:AOCH (98:02) was added to the column and collected in a vial. Then 1500 µl of water was added to it and after mixing with a vortex, the HPLC column was washed with 20 ml of PBS solution and 100 µl of it was injected into the HPLC column. In the quantification stage, detection was performed with a fluorescence detector with λme=333, λxe=460, Gain=1000, and Attn=16, and quantification was performed by comparing the area under the curve of each sample and the standard, considering the dilution factor (34). An example of a standard chromatogram of OTA, ZEA, AFB1 is shown in Fig. 1.

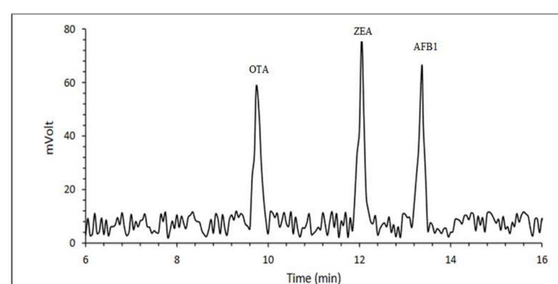

Fig. 1. Standard HPLC chromatogram of OTA, ZEA, AFB_1

HPLC method validation. Limit of detection (LOD) and limit of quantification (LOQ) are defined as the lowest concentration of an analyte in a sample that can be identified and determined with suitable precision and accuracy (35).

Sensitivity and mycotoxins recovery, LOD and LOQ detection used for aflatoxin, ochratoxin A and zearalenone were determined. 5-point standards calibration curves ranges of mycotoxins for ochratoxin and aflatoxin were 0.05-10 ng/g, and for zearalenone it was 100-500 ng/g.

Evaluation of textural and sensory properties of toast bread. To assess the impact of using *S. cerevisiae* and *L. plantarum* on the sensory and textural properties of the bread, three samples of toast bread without toxin contamination containing *S. cerevisiae* and *L. plantarum* were prepared, and specific volume and sensory evaluations (taste, color, freshness [less staling], texture, and overall acceptance) were performed on the samples.

Specific volume evaluation after baking. The specific volume of the bread was measured after cooling for 31 minutes at room temperature using the rapeseed displacement method. Each bread loaf was weighed and placed in a graduated container, filled with rapeseed until the total volume of the container was reached. The bread was then removed, and the volume of the seeds was noted. The volume of the bread was calculated by subtracting the volume of the seeds from the initial volume of the container (36).

Sensory evaluation. Sensory evaluation was conducted over 7 days of storage (days 1, 3 and 7). The sensory characteristics of the samples were evaluated using a five-point hedonic scale. In a laboratory setting, individuals (10 semi-trained panelists) with characteristics such as non-smokers, non-alcoholic drinkers, aged 20-35 years, and without illness were selected. Each panelist read the question and, based on their preference and opinion, chose an appropriate response from the hedonic scale. The prepared toast bread, coded with three-digit numbers, was presented to the trained panelists along with a questionnaire. Evaluators were asked to rate the bread based on overall quality, including attributes like color, taste, freshness (less staling), texture, and overall acceptance, from 1 to 5, where 5 indicated the best quality and 1 indicated the lowest quality (36).

Data analysis. The single and interaction effect of independent variables on dependent variables were analyzed by Minitab 21 software using one-way and two-way analysis of variance. All tests were conducted in triplicate, and results were reported as mean ± standard deviation with 95% confidence.

RESULTS

Validation of the mycotoxins. Limit of detection (LOD) for total aflatoxin B_1, ochratoxin A and zearalenone were 0.02, 0.01 and 0.1 ng/g, respectively. Limits of quantification (LOQ) for total aflatoxin B_1, ochratoxin A and zearalenone were 0.03, 0.02 and 0.2 ng/g, respectively. The average recovery for all mycotoxins were 94.4 to 97.8%. The LOD and LOQ obtained in this study were very low so the identifications were highly accurate.

Results of Ochratoxin A (OTA). According to Table 2, the analysis of variance for OTA levels showed that the type of treatment, fermentation, and their interaction had a significant effect ($p<0.01$) on OTA levels in the toast bread dough immediately after preparation, after 12 hours of fermentation, and after baking. Additionally, the OTA levels in the toast bread dough samples immediately after preparation, at the end of the fermentation period (12 hours), and after baking are presented in Table 4. Immediately after dough preparation and after 12 hours of fermentation, the highest OTA levels (10.37 and 8.31 ng/g, respectively) were found in the sample containing 10^8 CFU/g *L. plantarum* and 10 ng/g OTA ($p \leq 0.05$). Furthermore, the results indicated that after baking, OTA was not detected in any of the toast bread samples, and it reached zero.

The percentage reduction of OTA was calculated by subtracting the OTA levels at the end of fermentation from the levels immediately after dough preparation and then expressed as a percentage. According to Table 3, the analysis of variance for the percentage reduction of OTA showed that the type of microorganism (presence or absence of *S. cerevisiae* and *L. plantarum*), toxin level, and their interaction (type of microorganism and toxin level) had a significant effect ($p<0.01$) on the reduction of OTA in the toast bread dough. The treatment containing *S. cerevisiae* showed significantly ($p \leq 0.05$) better performance in reducing OTA compared to the treatment containing *L. planta-*

Table 2. Analysis of variance of aflatoxin B_1, zearalenone and ochratoxin A

Sources of variation	AFB_1		Zearalenone		Ochratoxin	
	p	F	p	F	p	F
Type of treatment (A)	0.000**	38397.559	0.000**	11377423.535	0.000**	2926.744
Fermentation time (B)	0.000**	73194.059	0.000**	45395876.827	0.000**	642.647
Interaction effect (type of treatment × fermentation time)	0.000**	10334.649	0.000**	11117167.267	0.000**	230.017
R^2	0.998		0.998		0.997	

The sign ** indicates significance at the 1% level.

Table 3. Analysis of variance of percentage reduction of aflatoxin B_1, zearalenone and ochratoxin A

Sources of variation	Percentage reduction of aflatoxin B_1		Percent reduction of Zearalenone		Ochratoxin A reduction percentage	
	p	F	p	F	p	F
Type of microorganism	0.000**	6766.839	0.000**	1137.628	0.000**	435968339.275
Amount of toxin	0.000**	2585.005	0.000**	903.182	0.000**	2354081133.683
Interaction effect (type of microorganism × amount of toxin)	0.000**	1749.765	0.000**	26.178	0.000**	435968339.275
R^2	0.999		0.967		0.988	

The sign ** indicates significance at the 1% level.

Table 4. Changes in OTA levels in toast dough containing *Lactobacillus plantarum* and *Saccharomyces cerevisiae* immediately after dough preparation, at the end of the fermentation period (12 hours), and after baking, compared with the control

Treatments (toast formulation)			The amount of OTA (ng/g)		
Ochratoxin A (ng/g)	*Lactobacillus plantarum* (CFU/g)	*Saccharomyces cerevisiae* (CFU/g)	Immediately after the dough is ready	At the end of the fermentation period (12 hours)	After baking the toast
10	10^8	-	10.37 ± 0.21^{aA}	8.31 ± 0.45^{aB}	0.00 ± 0.00^{aC}
10	-	10^8	8.46 ± 0.32^{bA}	2.70 ± 0.15^{bB}	0.00 ± 0.00^{aC}
(control)	-	-	0.39 ± 0.02^{cA}	0.36 ± 0.02^{cA}	0.00 ± 0.00^{aB}

- The results are reported as mean ± standard deviation.
- Unsimilar lowercase letters indicate a significant difference in the column (p<0.05).
- Unsimilar capital letters indicate significant differences in the row (p<0.05).

rum. Since OTA was not present in any of the samples after baking, the percentage reduction of this mycotoxin was 100% in all treatments.

Results of Zearalenone (ZEA). According to Table 2, the analysis of variance for ZEA levels showed that the type of treatment, fermentation time, and their interaction had a significant effect (p<0.01) on ZEA levels in the toast bread dough immediately after preparation and after 12 hours of fermentation. Additionally, the ZEA levels in the toast bread dough samples immediately after preparation, at the end of the fermentation period (12 hours), and after baking are presented in Table 5. The results showed that immediately after dough preparation and at the end of the fermentation period, the highest ZEA levels (421.30 and 3.84 ng/g, respectively) were found in the sample containing 10^8 CFU/g *S. cerevisiae* and 400 ng/g ZEA and the sample containing 10^8 CFU/g *L. plantarum* and 400 ng/g ZEA ($p \leq 0.05$). Overall, there was a significant reduc-

Table 5. Changes in ZEA content in the toast bread dough containing *Lactobacillus plantarum* and *Saccharomyces cerevisiae* immediately after the preparation of the dough, at the end of the fermentation period (12 hours) and after baking the bread and comparing it with the control

Treatments (toast formulation)			The amount of ZEA (ng/g)		
Zearalenone (ng/g)	*Lactobacillus plantarum* (CFU/g)	*Saccharomyces cerevisiae* (CFU/g)	Immediately after the dough is ready	At the end of the fermentation period (12 hours)	After baking the toast
400	10^8		406.05 ± 5.23^{bA}	3.84 ± 0.04^{aB}	1.47 ± 0.02^{aC}
400		10^8	421.30 ± 7.05^{aA}	1.77 ± 0.07^{bB}	0.84 ± 0.01^{bC}
(Control)	-	-	36.67 ± 0.84^{cA}	0.80 ± 0.08^{cB}	0.37 ± 0.01^{cC}

- The results are reported as mean ± standard deviation.
- Unsimilar lowercase letters indicate a significant difference in the column ($p<0.05$).
- Unsimilar capital letters indicate significant differences in the row ($p<0.05$).

tion ($p \leq 0.05$) in ZEA levels in all treatments at the end of the fermentation period compared to immediately after dough preparation. According to Iran's national standard NO.5925, the permissible limits on bread are 200 ng/g for zearalenone (20). According to the results obtained in this study, the ZEA levels were below this limit after 12 hours and at the end of the fermentation period, as well as after baking, in all treatments.

The percentage reduction of ZEA was calculated by subtracting the ZEA levels at the end of fermentation from the levels immediately after dough preparation and after baking and then expressed as a percentage. According to Table 3, the analysis of variance for the percentage reduction of ZEA showed that the type of microorganism (presence or absence of *S. cerevisiae* and *L. plantarum*), toxin, and their interaction (type of microorganism and toxin) had a significant effect ($p<0.01$) on the reduction of ZEA in the toast bread dough. The highest percentage reduction of ZEA (99.57%) was observed in the toast bread dough containing 10^8 CFU/g *S. cerevisiae* and 400 ng/g ZEA, which was significantly different from other treatments ($p \leq 0.05$). Overall, the treatment containing *S. cerevisiae* showed significantly ($p \leq 0.05$) better performance in reducing ZEA compared to the treatment containing *L. plantarum*. Furthermore, the ZEA levels after baking showed a significant reduction ($p \leq 0.05$), with a significant difference ($p \leq 0.05$) between the ZEA levels immediately after dough preparation, at the end of the fermentation period, and after baking. The percentage reduction of ZEA after baking compared to immediately after dough preparation was 98.90-100%, and the percentage reduction of ZEA at the end of fermentation compared to immediately after dough preparation was 97.80-99.57%.

Results of Aflatoxin B_1 (AFB_1). According to Table 2, the analysis of variance for changes in AFB_1 levels revealed that the type of treatment, fermentation time, and the interaction of these two factors significantly affected AFB_1 levels in toast bread dough immediately after preparation and after 12 hours of fermentation ($p<0.01$). The AFB_1 levels in the toast bread dough samples immediately after preparation, at the end of the fermentation period (12 hours), and in the baked toast bread are presented in Table 6. The results showed that the highest AFB1 levels immediately after dough preparation and after the fermentation period were 11.75 and 5.56 ng/g, respectively, in the sample containing 10^8 CFU/g *L. plantarum* and 10 ng/g AFB_1 ($p<0.05$). In all treatments, there was a significant reduction ($p<0.05$) in AFB_1 levels at the end of the fermentation period compared to immediately after dough preparation. The standard AFB_1 level in bread was 25 ng/g (20). Based on the results, the AFB_1 levels after 12 hours of fermentation and baking in all treatments were lower than this standard.

The percentage reduction of AFB_1 was calculated by subtracting the AFB_1 levels at the end of fermentation from the levels immediately after dough preparation and then expressed as a percentage. According to Table 3, the analysis of variance for the percentage reduction of AFB_1 showed that the type of microorganism (presence or absence of *S. cerevisiae* and *L. plantarum*), toxin level, and their interaction (type of microorganism and toxin level) significantly affected the reduction of AFB_1 in the toast bread dough ($p<0.01$). The lowest reduction (36.67%) was observed in the

control sample. Furthermore, the results indicated that after baking, AFB_1 was not detected in any of the toast bread samples, and the percentage reduction of this mycotoxin was 100% in all treatments. Additionally, the treatment containing *S. cerevisiae* showed significantly better performance in reducing AFB_1 compared to the treatment containing *L. plantarum* ($p<0.05$).

Results of specific volume toast bread. The results shown in Table 7 indicated that in the toast bread samples, the type of treatment (presence or absence of *S. cerevisiae* and *L. plantarum*) significantly affected the specific volume ($p<0.01$). The highest specific volume was observed in the toast bread sample containing *S. cerevisiae* (3.87 cm^3/g), and the lowest specific volume (2.62 cm^3/g) was found in the control sample.

Results of sensory evaluation. The mean sensory evaluation scores of the toast bread samples containing *S. cerevisiae, L. plantarum*, and the control during the storage period are presented in Fig. 2. The results shown in Fig. 2 indicated that in the toast bread samples, the type of treatment (presence or absence of *S. cerevisiae* and *L. plantarum*) and storage time significantly affected the sensory evaluation scores (taste, color, freshness, texture, overall acceptance) ($p<0.01$).

The taste sensory evaluation score of the treatments containing *S. cerevisiae* and *L. plantarum* decreased significantly ($p\leq0.05$) during the storage time from the 1th to the 7th day. There was no significant difference between the 3th and 7th days of storage in the control sample ($p>0.05$). In this sample, a significant decrease in the taste sensory evaluation score ($p\leq0.05$) was observed on the mentioned days compared to the first day.

The color sensory evaluation score of the treatment containing *L. plantarum* decreased significantly ($p\leq0.05$) during the storage time from the 1st to 7th day. There was no significant difference between the samples containing *S. cerevisiae* and the control between the 3th and 7th days of storage ($p>0.05$). In these samples, a significant decrease in the color sensory evaluation score ($p\leq0.05$) was observed on the mentioned

Table 6. Changes in the amount of AFB_1 in toast bread dough containing *Lactobacillus plantarum* and *Saccharomyces cerevisiae* immediately after preparing the dough, at the end of the fermentation period (12 hours) and after baking the bread and comparing it with the control

Treatments (toast formulation)			**The amount of AFB_1 (ng/g)**		
Aflatoxin B_1 (ng/g)	***Lactobacillus plantarum* (CFU/g)**	***Saccharomyces cerevisiae* (CFU/g)**	**Immediately after the dough is ready**	**At the end of the fermentation period (12 hours)**	**After baking the toast**
10	10^8		11.75 ± 0.00^{aA}	5.56 ± 0.18^{aB}	1.47 ± 0.02^{aC}
10		10^8	9.93 ± 0.00^{bA}	0.93 ± 0.06^{cB}	0.84 ± 0.01^{bC}
(Control)	-	-	2.07 ± 0.00^{cA}	1.31 ± 0.05^{bB}	0.37 ± 0.01^{cC}

- The results are reported as mean ± standard deviation.
- Unsimilar lowercase letters indicate a significant difference in the column ($p<0.05$).
- Unsimilar capital letters indicate significant differences in the row ($p<0.05$).

Table 7. Specific volume of toast bread samples containing *Saccharomyces cerevisiae, Lactobacillus plantarum* and control

Treatment	***Lactobacillus plantarum* (CFU/g)**	***Saccharomyces cerevisiae* (CFU/g)**	**Special volume (cm^3/g)**
T^1	10^8		3.57 ± 0.04^b
T^2		10^8	3.87 ± 0.15^a
T^3(Control)	-----	------	2.62 ± 0.06^c

- The results are reported as mean ± standard deviation.
- Unsimilar lowercase letters indicate a significant difference in the column ($p<0.05$).

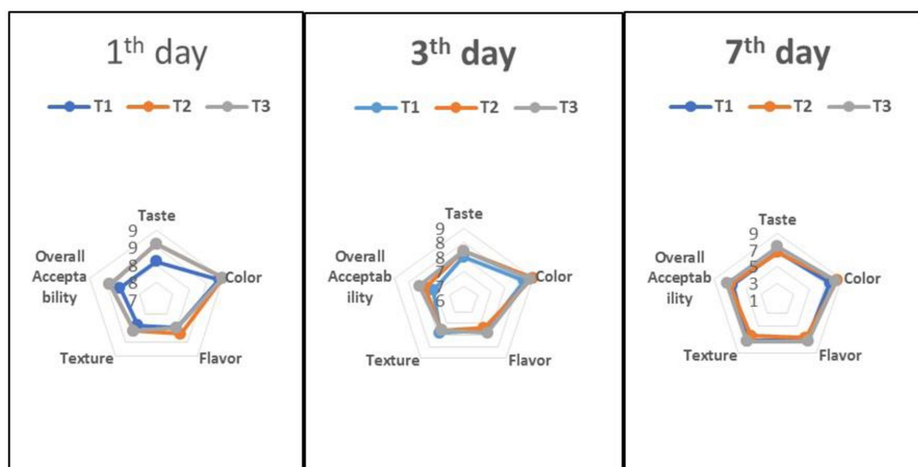

Fig. 2. Comparison of average sensory evaluation of toast bread samples containing *Saccharomyces cerevisiae, Lactobacillus plantarum* and control during storage time

T[1] = toast containing 10^8 cfu/g *Lactobacillus plantarum,* T[2] = toast containing 10^8 cfu/g *Saccharomyces cerevisiae* and T3 = control test (without microbial strain)

- Unsimilar lowercase letters indicate a significant difference between the treatments on the specific day of the test ($p < 0.05$).
- Unsimilar capital letters indicate the significant difference of each treatment during the storage time ($p < 0.05$).

days compared to the 1st day.

The freshness sensory evaluation score of the treatment containing *S. cerevisiae* decreased significantly ($p \leq 0.05$) during the storage period from the 1st to 7th day. There was no significant difference between the samples containing *L. plantarum* and the control between the 3th and 7th days of storage ($p > 0.05$). In these samples, a significant decrease in the freshness sensory evaluation score ($p \leq 0.05$) was observed on the mentioned days compared to the 1st day.

The texture sensory evaluation score of the treatment containing *S. cerevisiae* decreased significantly ($p \leq 0.05$) during the storage period from the 1st to 7th day. There was no significant difference between the samples containing *L. plantarum* and the control between the third and seventh days of storage ($p > 0.05$). In these samples, a significant decrease in the texture sensory evaluation score ($p \leq 0.05$) was observed on the mentioned days compared to the 1st day.

The overall sensory acceptance score of treatments containing *S. cerevisiae, L. plantarum* and control decreased significantly ($p \leq 0.05$) during storage from the 1st to 3th day. There was no significant difference between the 3th and 7th days of storage ($p > 0.05$).

The sensory evaluation scores (taste, color, freshness, texture, overall acceptance) of the treatments containing *S. cerevisiae* and *L. plantarum* significantly decreased during the 7-day storage period compared to the first day ($p < 0.05$).

DISCUSSION

Ochratoxin A (OTA). Based on the results obtained from this study, only the OTA level in the sample containing 10^8 CFU/g *L. plantarum* and 10 ng/g OTA was higher than the standard level after 12 hours (at the end of fermentation), and in other treatments, this level was observed to be lower. Some reports exist regarding the reduction of mycotoxins during fermentation (37).

Researchers in their study showed higher percentages of OTA reduction in the production of cake (90%), bread (80%), and biscuits (85%) for OTA, with lower reduction levels of 65% for pasta, indicating that the consumption of these products could account for 30.5% of the weekly tolerable intake of OTA (27). Additionally, Mohammad & Hashemi (38) reported high OTA removal (32-58%) by *L. plantarum* strains in cream after 24 hours of fermentation, which was similar to the results of this study. Badji et al. (39) stated that tested LAB strains were capable of reducing OTA levels under laboratory conditions. This result suggests that these LAB strains could be used as additives or processing agents to reduce mycotoxin levels in fermented wheat foods such as sourdough bread. The results obtained were in line with the findings of this study.

Several yeast species' cell walls can also bind mycotoxins from agricultural goods, successfully sani-

tizing them. Yeasts also have other beneficial properties, such as breaking poisons into less harmful or even non-toxic forms (40).

The interaction between mycotoxins and probiotic cells is influenced by the cellular wall's integrity, which is responsible for the absorption capacity (26). Probiotics can bind mutagens and carcinogens, such as aflatoxins (41). Fermentation, antibiosis, and the capacity of the microbial cell wall to attach to the toxin are factors in these microorganisms' decontaminant action (42). Yeast and lactic acid bacteria (LAB) mycotoxin involve fighting binding aflatoxins (43). Several yeast species' cell walls can also bind mycotoxins from agricultural goods, successfully sanitizing them. Yeasts also have other beneficial properties, such as breaking toxins into less harmful or even non-toxic forms (44).

Zearalenone (ZEA). The results showed that the treatment containing *S. cerevisiae* significantly ($p<0.05$) reduced ZEA more effectively compared to the treatment containing *L. plantarum*. Additionally, the ZEA levels after baking showed a significant reduction ($p<0.05$), with significant differences ($p<0.05$) between the ZEA levels immediately after dough preparation, at the end of the fermentation period, and after baking.

Similar results were obtained by other researchers. Bol et al. (27) examined the ZEA levels through the consumption of bakery and pasta products considering the effects of food processing. They reported the highest percentage reduction of ZEA in cake production (95%), followed by biscuits (90%), bread (89%), and pasta (75%). In line with the results of this study, Chlebicz & Slizewska (28) investigated the detoxification properties of probiotic *Lactobacillus* sp. bacteria (12 strains) and Scerevisiae yeast (6 strains) against mycotoxins, such as ZEA, which are frequently implicated as feed contaminants. They reported that ZEA was reduced by 57% and 65% by lactobacilli and yeasts after 6 hours, respectively, and suggested that these strains could potentially be used as food and feed additives for the detoxification of food contaminated with mycotoxins after further investigation, which are potential threats to human and animal health.

A result of study Taheri et al., showed that the ZEA concentration in dough decreased by 13.27%, 23.52%, and 35.27% after 30, 60, and 90 minutes of fermentation, respectively and a slight reduction in ZEA was observed in the baked product at 150°C, but heating at 220°C had a significant impact on ZEA reduction. A 63.23% reduction in ZEA content was observed during 90 minutes of fermentation and baking at 220°C. Optimal bread preparation conditions significantly impacted the reduction of ZEA levels in bread produced from contaminated wheat flour. Longer fermentation times (30 to 90 minutes) were more effective in reducing ZEA content in baked bread than baking at 150°C. However, higher baking temperatures (185°C or higher) were more effective than longer fermentation times (30), which were consistent with the results of this study.

Aflatoxin B_1 (AFB_1). Given the ubiquitous presence of aflatoxins, preventive and corrective measures, including detoxification techniques, are essential. Physical and chemical anti-contamination strategies are undesirable, so biological methods can be used. The use of lactic acid bacteria, which have previously proven to be generally recognized as safe (GRAS), should be considered as a bioremediation agent for aflatoxins (45). On the other hand, wheat is a high-risk commodity for aflatoxin contamination. During bread production, many processes can affect aflatoxin stability (46).

Milani et al. (46) examined the impact of bread-making processes on aflatoxin (AF) levels. The results showed that the maximum reduction in aflatoxin levels was observed during the first resting stage, and the minimum reduction was observed during baking. The order of AF reduction during the bread-making process was $AFB_1 > AFB_2 > AFG_1$, which was similar to the results of this study.

Bol et al. (27) investigated the effect of cooking on AFB_1 levels and exposure through the consumption of pasta and bakery products. Percentages of AFB_1 reduction were observed in cake (70%), biscuits (40%), Bread (36%) and pasta (10%). Therefore, the consumption of these products could account for 24.6% of the maximum tolerable daily intake of AFB_1.

Probiotic bacteria (*L. plantarum* and *L. paracasei*) and their cell-free extracts produced under optimal conditions showed antifungal and anti-mycotoxin effects, provided that these bacteria were screened and selected before application (47).

A study examined the bioaccessibility of AFB_1 in bread enriched with whey protein/pumpkin powder. The results showed that pumpkin powder was

the most effective substance, showing a significant reduction in AFB_1 bioaccessibility up to 74%. Whey, fermented whey, and the combination of fermented whey with pumpkin showed a reduction in intestinal bioaccessibility between 57% and 68% for AFB_1 (29).

Similar results were obtained by other researchers. Badji et al. (39) stated that the reference strain *L. plantarum* (LP R1096) and two strains (Lab-L4/al and Lab-L1) belonging to the genus Enterococcus were capable of reducing AFB1 from contaminated CPB under tested conditions with the highest efficiency for AFB_1.

Many reports have shown the potential of *Lactobacillus* strains to remove mycotoxins from contaminated liquid environments (28, 48). The ability of *L. plantarum* to reduce AFB_1 concentration (11.69%) was also reported by Damayanti et al. in 2017 (49).

Milani et al. (46) stated that the processing of wheat flour into bread could significantly reduce aflatoxin levels. The highest reduction in all stages of the process could be observed between the flour and initial dough (first resting stage), as it was longer than the final dough and carried out at a temperature closer to the optimum for *S. cerevisiae,* i.e., 37°C. Baking showed the highest reduction, probably due to the thermal sensitivity of aflatoxins. Baking at a certain temperature had little effect on AF degradation. The highest reduction in AFB_1 during the bread-making process was observed because AFB_1 had the lowest molecular weight and the smallest molecular size, and was likely more affected by degrading factors (46).

The decrease in pH during fermentation due to organic acids produced by yeast likely led to the reduction or degradation of AFs (50). Many food processing operations (such as roasting, baking, frying, and cooking) involve heat treatments. Therefore, thermal inactivation of mycotoxins has been extensively studied. Mycotoxins vary in stability under heat treatments. Aflatoxins are resistant to thermal inactivation and are only destroyed at temperatures around 250°C (46). However, fermentation can make them susceptible due to the production of enzymes, ethanol, and CO_2. The effects of various treatments, including washing, heating, and steaming, on aflatoxin reduction were examined. It was concluded that AFB_1 concentration decreased more with heating than washing (51). Aflatoxin reduction during thermal processing depends on the moisture content of the product. Moisture content is an important factor in the inactivation of aflatoxins, especially for AFB_1. The presence of water helps to open the lactone ring in AFB_1. Based on data collected from several studies on the effect of moisture content on aflatoxin degradation, it can be concluded that degradation increases with higher moisture content (52).

Moisture is an important factor for aflatoxin reduction by heating as moisture is necessary for the hydrolysis of the aflatoxin lactone ring during baking. Previous studies showed that increased moisture content significantly affected aflatoxin reduction, confirming our findings. As bread dough contains high moisture content and since it is baked at temperatures above 250°C, aflatoxin levels can be reduced (53). Chlebicz & Slizewska (28) stated that AFB_1 reduction by *Lactobacillus* (60%) and yeast (65%) was observed in vitro. The highest reduction in AFB_1 was observed after 6 hours of incubation. However, a reduction in concentration was also observed after 24 hours of incubation.

Specific volume. The highest specific volume was observed in the toast bread sample containing *S. cerevisiae* (3.87 cm^3/g), and the lowest specific volume (2.62 cm^3/g) was found in the control sample. Zare et al. (54) stated that the specific volume and porosity of the product (toast bread by sourdough containing kombucha beverage and soybean milk and *L. fermentum* and *L. plantarum*) increased insignificantly when sourdough containing *L. fermentum* starter was used compared to the *L. plantarum* starter, which seems to be due to the heterofermentative nature of *L. fermentum* starter and the production of carbon dioxide gas during fermentation activity.

Sensory evaluation. The results obtained from the sensory evaluation (considering the specific volume evaluation results of the toast bread samples) were not unexpected. It is worth mentioning that the evaluators did not find significant differences between the sensory properties of samples prepared with *L. plantarum* and *S. cerevisiae* after seven days of storage, and the control sample (containing regular yeast) received lower scores. In this regard, Alian et al. (55) examined the effect of using sourdough containing various probiotic bacteria on the quality and shelf life of Egyptian bread, and the sensory evaluation results did not show significant differences between the use of sourdough containing *L. plantarum, L. helveticus, Bifidobacterium, Leuconostoc mesen-*

teroides, and the control sample, and gave similar color scores to the product containing these starters. Gharekhani et al. (56) also stated that samples prepared from sourdough containing *L. plantarum* and *L. sanfranciscensis* starters received similar sensory evaluation scores.

Fouad et al. (31) reported that adding LAB cells (*L. rhamnosus, L. plantarum, Bifidobacterium bifidum, Streptococcus thermophilus,* and *L. reuteri*) did not cause significant differences in taste and color for the final Fino bread. This was similar to the results of this study, which showed that there were no significant differences in the sensory evaluation scores of taste and color between the toast bread sample containing *L. plantarum* and the control sample.

CONCLUSION

The results showed that the type of treatment, fermentation time, and their interaction significantly affected mycotoxin levels (OTA, ZEA, and AFB_1) in toast bread dough immediately after preparation and after 12 hours of fermentation ($p<0.01$). Significant reductions in mycotoxin levels (OTA, ZEA, and AFB_1) were observed in all treatments except the control at the end of the fermentation period (12 hours) compared to immediately after dough preparation. Additionally, the results indicated that the type of microorganism (presence or absence of *S. cerevisiae* and *L. plantarum*), type of toxin, and the interaction of these two factors (type of microorganism and type of toxin) significantly affected the reduction percentages of mycotoxins (OTA, ZEA, and AFB_1) in the toast bread dough ($p<0.01$). After baking and preparation of the toast bread, mycotoxins (OTA, ZEA, and AFB_1) were not detected in any of the toast bread samples, and the reduction percentages of OTA and AFB_1 after baking were 100% in all treatments, while the reduction percentages of ZEA after baking compared to immediately after dough preparation were 98.90-100%, and compared to the end of the fermentation period they were 50-100%. Additionally, the reduction percentages of ZEA at the end of fermentation compared to immediately after dough preparation were 97.80-99.57%. In the prepared toast bread samples (without mycotoxins), the results showed that the addition of *S. cerevisiae* and *L. plantarum* significantly affected the specific volume ($p<0.01$). Sensory evaluation results showed that the presence or absence of *S. cerevisiae* and *L. plantarum* and storage time significantly affected the sensory evaluation scores (taste, color, freshness, texture, overall acceptance) ($p<0.01$). The sensory evaluation scores (taste, color, freshness, texture, overall acceptance) significantly decreased during the 7-day storage period in treatments containing *S. cerevisiae* and *L. plantarum* ($p<0.05$), but remained higher than the control treatment. Therefore, the results of this study indicate that *L. plantarum* and *S. cerevisiae* can be used as additives or processing agents to reduce mycotoxin levels in fermented wheat foods such as bread. Additionally, these strains could potentially be used as food and feed additives for detoxifying mycotoxin-contaminated food after further investigation, which poses potential threats to human and animal health. Future research should evaluate the impact of *L. plantarum* and *S.cerevisiae* along with other nutritional compounds such as protein (like whey protein) and carbohydrates (such as barley, corn, or fiber) on reducing mycotoxin levels in toast bread, the effect of *L. plantarum* and *S. cerevisiae* on reducing mycotoxin levels in other fermented products like dairy products, and the effect of other probiotic strains like *L. casei, L. acidophilus,* and *S. cerevisiae* on reducing mycotoxin levels in various types of leavened bread.

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