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Detoxification of aflatoxin B^1 by bacteriocins and bacteriocinogenic lactic acid bacteria

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Abstract: The aim of this study was to investigate the effectiveness of lactic acid bacteria and their bacteriocins in detoxifying aflatoxins. Aflatoxin B_1 detoxification abilities of lactic acid bacteria both in liquid culture and as concentrated pellets, their bacteriocins, and mixtures of these 3 were evaluated. Mixed cultures of the 2 bacteria were also investigated. *Lactobacillus plantarum* and *Lactococcus lactis* were separately able to detoxify aflatoxin B_1 in solutions. *Lb. plantarum* had a better detoxification rate (46%) than *Lc. lactis* (27%). After heat treatment, only the groups that contained pellets released the bound toxin back into the solution. Although bacteria and their bacteriocins were effective individually at detoxification, their efficacy was increased when they were used together. When *Lc. lactis* and *Lb. plantarum* were incubated in separate tubes and then mixed, that group had a significantly increased ability to bind toxins (59%) compared to their use alone. When these 2 strains were incubated together in a single broth culture, the most successful detoxification rate (81%) was achieved. Within this co-culture group, the bacteriocins alone were the most effective (90%) at removing aflatoxin B_1 from solution.

Key words: *Lactobacillus plantarum*, *Lactococcus lactis*, bacteriocin, biological detoxification, aflatoxin B_1

1. Introduction

Aflatoxins are secondary metabolites produced by molds that have carcinogenic, mutagenic, and teratogenic effects on humans and animals (1–3). Among the aflatoxins, aflatoxin B_1 (AFB_1) is one of the strongest known hepatocarcinogens and is classified as a category 1A carcinogen by the International Agency for Research on Cancer (4). Due to their importance in showing these effects in the consumption of contaminated foods, there is a serious effort made by the food industry to make products safe. Moreover, considering the tendency of consumers to prefer natural products because of the worry about possibly hazardous food additives, both researchers and producers are taking responsibility to investigate new ways as an answer to the consumer expectations.

Although the different methods used at present are to some extent successful, they have major disadvantages with limited efficacy, possible losses of important nutrients, and normally high costs. Many workers in the field are of the opinion that the best solution for decontamination should be detoxification by biodegradation, giving a possibility for removal of mycotoxins under mild conditions without using harmful chemicals and without significant losses in nutritive value and palatability of decontaminated food and feed (5). A number of studies have found that some microorganisms, especially lactic acid bacteria, break down or bind to aflatoxins. Researchers have obtained different results in these studies conducted to investigate the in vitro aflatoxin binding/degradation effect of lactic acid bacteria (6–9). Zinedine et al. (10) indicated that the toxin binding level changes in some environmental conditions having different pH and temperature. Supporting this finding, Haskard et al. (7) also found that environmental conditions, acid and heating applications, and the structure of the bacteria had an important effect on the stability of the toxin–bacteria complex. In another study, Kabak (11) showed that the aflatoxin B_1 binding capability of probiotic lactic acid bacteria varied in vitro, and in food had 32%–46.5% of the in vitro toxin binding rate. Fuchs et al. (12) found that the effectiveness of lactic acid bacteria in detoxifying ochratoxin and patulin was affected by toxin concentration, cell density, pH of the environment, and whether the cells were viable. Based on these and similar results, Hernandez-Mendoza et al. (13) suggested that lactic acid bacteria bound the toxins to different extents depending on the environment and the amount of bile salt present.

Another challenge for researchers is achieving the same good results when lactic acid bacteria are applied to food. First of all, certain temperatures and pH levels must be provided; otherwise, the microorganisms would not show their effects. Probably, a higher number of bacteria would

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be needed. However, this may end up causing organoleptic changes in the food. Therefore, the use of bacteriocins, which are colorless and odorless components produced by lactic acid bacteria, seems very promising.

Bacteriocins are antimicrobial proteins or peptides that are synthesized by bacteria and secreted into their environment. Bacteriocins are secondary metabolite products that are secreted to inhibit the growth of similar and/or competitive bacterial strains (14). Bacteriocins have been tested as natural antimicrobials in food preservation and have been found to have strong antibacterial properties (15,16).

Paster et al. (17) showed that propionic acid and nisin together inhibited the growth of toxin-producing molds. However, the authors emphasized that while nisin and propionic acid together completely inhibited mold growth and spore development, nisin alone was not effective. On the basis of this result, they reported that even though it is an effective antibacterial, nisin alone would not be recommended as an antifungal agent in the food industry. On the other hand, Yang and Chang (18) declared that the metabolites produced by *Lactobacillus plantarum* could be successfully used for the same purpose as an alternative antifungal to chemical preservatives. Although there are several studies investigating the effect of bacteriocins on mold growth and/or their toxin production, we could not find any studies determining the usage possibility of these proteins for aflatoxin detoxification. There is a pressing need for a natural detoxifying agent against aflatoxins. In this study we have investigated the viability of using bacteriocins to detoxify aflatoxins.

2. Materials and methods

2.1. Test groups

Cultures of *Lb. plantarum* and *Lactococcus lactis* were tested either separately or together for their ability to detoxify aflatoxin B_1 in phosphate buffered saline (PBS). The antimicrobial chemicals propionic acid and benzoic acid were also tested. The test groups and subgroups are shown below and in Table 1.

- Group plant: A culture of *Lb. plantarum*.
- Group lactis: A culture of *Lc. lactis*.
- Group mix-sub: Mixed culture formed by combining *Lc. lactis* and *Lb. plantarum* cultures after they had been allowed to grow separately in MRS broth (mixed subsequently).
- Group mix-int: Mixed culture formed by incubating *Lc. lactis* and *Lb. plantarum* together in MRS broth (mixed initially).
- Group Prp: Propionic acid (Merck, 800605) solution that was prepared at 0.1% concentration using distilled water at 65 °C.
- Group Bzc: Benzoic acid (Merck, 100136) solution that was prepared at 0.1% concentration using distilled water at 65 °C.

Aflatoxin B_1 was included where indicated at a final concentration of 0.05 µg/mL (50 ppb). All groups were incubated at 30 °C. Aflatoxin levels in the samples were measured at 6, 24, and 36 h during the incubation. After 36 h, the reactions were sterilized by autoclaving at 121 °C for 30 min, and the amount of toxin was measured with the enzyme-linked immunosorbent assay (ELISA).

2.2. Microorganisms

Lactococcus lactis subsp. *lactis* and *Lactobacillus plantarum* were used as bacteriocinogenic lactic acid bacteria strains. These 2 bacteria had been isolated previously in our laboratory and identified using conventional culture techniques (15). During this investigation their identities were confirmed by polymerase chain reaction (PCR). The characteristics of the bacteriocins produced by the 2 species were also investigated in the earlier study. These bacteriocins remain active at high temperatures and at both high and low pH levels. They exhibit a strong antimicrobial effect and are highly resistant to autoclave treatment when produced by test microorganisms incubated together in the same medium (15).

2.3. Preparation of the stock aflatoxin solution

Aflatoxin B_1 (Biopure, BRM 002017) at 2.02 µg/mL was added to 5 mL of acetonitrile. The solution was placed in a water bath for 5–10 min at 70 °C to evaporate the acetonitrile. One milliliter of 0.1 M PBS (pH 6.8) was added to the aflatoxin and the tube was shaken carefully to prepare the stock aflatoxin solution (10.10 µg/mL).

2.4. Activation of bacteriocinogenic lactic acid bacteria

Lactic acid bacteria were incubated at 30 °C for 18 h in MRS broth (Oxoid, CM 0359). After incubation, modified Chalmers agar (19) plates were streaked. After incubation at 30 °C, bacterial purity was checked and they were stored in brain-heart infusion agar slants (Oxoid, CM 1136) at 4 °C for the duration of the study.

2.5. Production of bacteriocins

Lactic acid bacteria were inoculated in MRS broth and incubated at 30 °C for 18 h. The cultures were centrifuged at 10.000 × *g* for 10 min at 4 °C. The supernatants were collected and sterilized by filtering through a 0.22-µm filter (Millipore, SLGV 033RS). The pH of the sterile supernatant was adjusted to 6.5–7 using 5 N NaOH or 5 N HCl, and 1 mg/mL of catalase (Sigma, C 9322) was added to remove any hydrogen peroxide. The supernatants were incubated at 37 °C for 2 h and then placed in a 60 °C water bath for 10 min to inhibit any enzymes. The supernatants were concentrated in an evaporator at 50 °C for 12 h. To precipitate the supernatant proteins, ammonium sulfate (Merck, 31119) was added to a concentration of 70%, and the solution was stirred for 18 h at 4 °C using a magnetic stirrer. The samples were centrifuged at 15,000 × *g* for 30 min. The protein precipitate was collected and diluted 10-

Table 1. Test groups.

Group plant	
Bacteriocin	*Lb. plantarum* bacteriocin + PBS + AFB_1
Pellet	*Lb. plantarum* pellet + PBS + AFB_1
Liquid culture	*Lb. plantarum* liquid culture + AFB_1
All	*Lb. plantarum* bacteriocin + liquid culture + pellet + AFB_1
Group lactis	
Bacteriocin	*Lc. lactis* bacteriocin + PBS + AFB_1
Pellet	*Lc. lactis* pellet + PBS + AFB_1
Liquid culture	*Lc. lactis* liquid culture + AFB_1
All	*Lc. lactis* bacteriocin + liquid culture + pellet + AFB_1
Group mix-sub (mixed subsequently)	
Bacteriocin	*Lc. lactis* bacteriocin + *Lb. plantarum* bacteriocin + PBS + AFB_1
Pellet	*Lc. lactis* pellet + *Lb. plantarum* pellet + PBS + AFB_1
Liquid culture	*Lc. lactis* liquid culture + *Lb. plantarum* liquid culture + AFB_1
All	*Lc. lactis* (bacteriocin + liquid culture + pellet) + *Lb. plantarum* (bacteriocin + liquid culture + pellet) + AFB_1
Group mix-int (mixed initially)	
Bacteriocin	Combined bacteriocin + PBS + AFB_1
Pellet	Combined pellet + PBS + AFB_1
Liquid culture	Combined liquid culture + AFB_1
All	Combined bacteriocin + combined liquid culture + combined pellet + AFB_1
Group Prp	
Propionic acid	0.1% propionic acid + PBS + AFB_1
Group Bzc	
Benzoic acid	0.1% benzoic acid + PBS + AFB_1
Control groups	
Positive control	AFB1 + PBS
*Negative control 1	Lactic acid bacteria pellet + PBS
*Negative control 2	Lactic acid bacteria liquid culture
*Negative control 3	Crude bacteriocin + PBS
Negative control 4	0.1% Propionic acid + PBS
Negative control 5	0.1% Benzoic acid + PBS

*: Prepared for each bacteria group.

fold with sterile 0.1 M PBS (pH 6.8), and the ammonium sulfate was removed by dialysis against sterile 0.1 M PBS (pH 6.8) using a 1.000-Da molecular weight dialysis membrane (Spectra/Por 7 - 132104) (15,16). The dialysate, which contained partially purified protein, was used as a crude bacteriocin solution in all tests.

2.6. Preparation of lactic acid bacteria cultures and pellets

Active cultures of lactic acid bacteria were prepared by incubating them at 30 °C for 18 h in MRS broth. The concentration of the bacterial cultures was determined by spectrophotometry at 600 nm (approximately 1×10^{10} CFU/mL). Pellets were obtained by centrifuging the bacterial culture at 10,000 × *g* for 5 min at 4 °C. The bacteria pellets were resuspended in 0.1 M PBS (pH 6.8) before use.

2.7. Determination of aflatoxin levels

The degree of aflatoxin detoxification in each experiment was based on measurements of the amount of AFB_1 present before incubation and after 6, 24, and 36 h of incubation. Measurements were taken at 450 nm in an ELISA reader using an AFLA B1 ELISA test kit (Tecna Celer, MA 220). The sensitivity limit of the kit is rated at 1 ppb (µg/L). The

results were calculated according to the manufacturer's instructions.

The method used in this study could measure the level of the aflatoxin in the test environment. We calculated the bound aflatoxin level by subtracting the final amount from the amount that we had added at the beginning of the experiment.

2.8. Confirmation of the lactic acid bacteria's identity

2.8.1. *Lb. plantarum*

Genomic DNA was extracted from bacteria using a DNA extraction mini kit (QIAGEN, QIAamp-51304). The PCR assay was performed according to Torriani et al. (20) with some modifications. Primers for *Lb. plantarum* were planF (5'- CCG TTT ATG CGG AAC ACC TA - 3') and pREV (5'- TCG GGA TTA CCA AAC ATC AC - 3'). Both primers are *rec A* gene-based. The amplicon size was 318 bp. *Lb. plantarum* NRRL - B 4496 (ARS culture collection; United States Department of Agriculture) was used as a positive control during PCR.

2.8.2. *Lc. lactis*

Genomic DNA of bacteria was extracted by using a DNA extraction mini kit (QIAGEN, QIAamp-51304). The PCR assay was performed according to Pu et al. (21) with some modifications. Primers for *Lc. lactis* were LacreR (19 - GGGATCATCTTTGAGTGAT) and LacF (19 - GTACTTGTACCGACTGGAT). The amplicon size was 161 bp. *Lactococcus lactis* spp. *lactis* CECT 4432 (ARS culture collection) was used as a positive control during PCR.

2.9. Statistical analysis

Every test was performed 3 times independently. In order to evaluate the results statistically, Minitab 12.1 for Windows was used. The ANOVA one-way test was applied to compare the groups and the Tukey test was utilized to determine the importance levels of differences between groups.

3. Results

The identities of the 2 lactic acid bacteria used in this study were confirmed by PCR. Figure 1 shows the bands for *Lb. plantarum* and *Lc. lactis*. The efficacy of lactic acid bacteria and crude bacteriocins in detoxifying AFB_1 solutions was determined by measuring AFB_1 concentration using an AFLA B1 ELISA test kit. The standard curve for aflatoxin B1 is shown in Figure 2.

The length of the incubation period was not statistically important for AFB_1 detoxification ($P > 0.05$). Toxin binding was nearly complete after 6 h and did not change during longer incubations. All of the groups inactivated the toxin throughout the incubation and did not release toxin back into the solution. Moreover, after autoclaving, only those groups containing pellets released the bound toxin back into the solution. The average results over the 3 trials showing the effects of incubation time and heat (autoclaving) on AFB_1 detoxification are shown in Table 2.

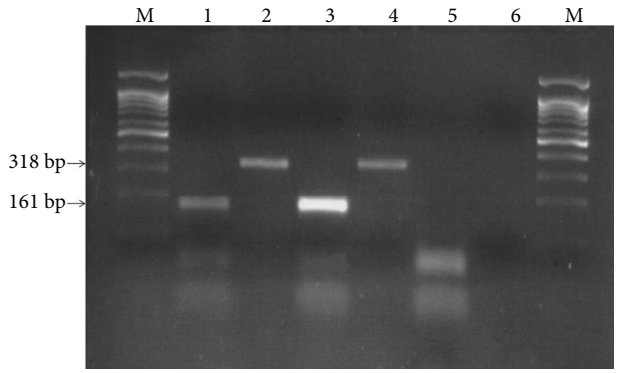

Figure 1. Amplification products obtained from PCR. Lanes: Lane M contained a 100-bp DNA ladder (Fermentas - SM0241); lanes 1 and 2, PCR amplification products from *Lc. lactis* subsp. *lactis* and *Lb. plantarum*, respectively; lanes 3 and 4, positive controls for *Lc. lactis* subsp. *lactis* and *Lb. plantarum*, respectively; lanes 5 and 6, DNA-free PCR mixture as negative controls for *Lc. lactis* subsp. *lactis* and *Lb. plantarum*, respectively. Sizes (bp) of PCR products are indicated.

The effectiveness of each subgroup at detoxifying the AFB_1 solution was compared at 36 h of incubation. Significant differences were found among groups ($P < 0.05$; Table 2). In Group plant, the mixture of liquid culture, pellet, and bacteriocin was the most successful at detoxification (46%; Table 3). The bacteriocin of *Lb. plantarum* was more effective ($P < 0.05$) than either a pellet or liquid culture of the bacteria. In contrast, a liquid culture of *Lc. lactis* (Group lactis) was more effective ($P < 0.05$) than bacteriocin at detoxifying the AFB_1 solution. The most successful subgroup in Group lactis was the mixture of liquid culture, pellet, and bacteriocin (27%), as it was in Group plant.

Similarly, the mixture of liquid culture, pellet, and bacteriocin had the strongest effect (59%) in Group

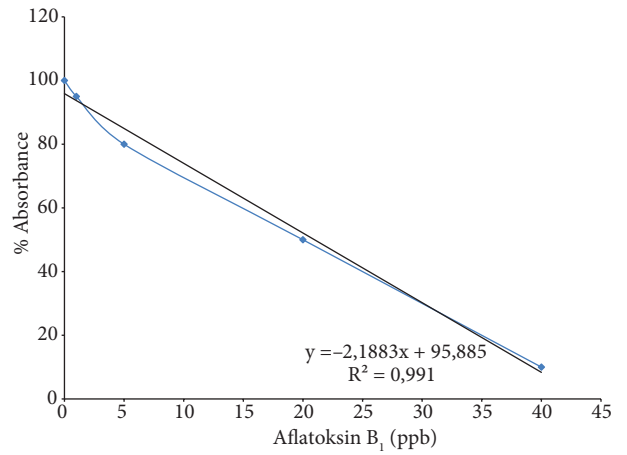

Figure 2. Standard curve for aflatoxin B_1.

Table 2. The effect of time and heat treatment on AFB_1 reduction by detoxifying agents.

Groups	AFB_1 % reduction rate (mean ± SD, n = 3)			
	6 h	24 h	36 h*	After autoclaving
Lb. plantarum (Group plant)				
Pellet	32.88 ± 2.84	33.92 ± 2.86	33.43 ± 1.54^{I}	32.08 ± 1.63
Bacteriocin	43.19 ± 1.08	43.58 ± 1.30	43.33 ± 1.48^{G}	43.75 ± 1.27
Liquid culture	38.14 ± 0.22	38.22 ± 0.21	38.72 ± 0.48^{H}	38.27 ± 0.89
All	46.57 ± 2.04	46.13 ± 2.66	46.19 ± 2.69^{F}	45.83 ± 2.97
Lc. lactis (Group lactis)				
Pellet	18.40 ± 1.04^{a}	18.83 ± 1.55^{a}	18.69 ± 0.44^{aKL}	15.08 ± 0.54^{b}
Bacteriocin	15.39 ± 0.52	16.18 ± 1.11	16.61 ± 0.68^{L}	14.88 ± 0.95
Liquid culture	21.15 ± 0.50	21.89 ± 1.12	22.12 ± 0.97^{K}	20.66 ± 0.29
All	27.15 ± 1.74	27.41 ± 1.30	27.45 ± 1.87^{J}	26.62 ± 0.93
Mixed subsequently (Group mix-sub)				
Pellet	47.03 ± 1.28^{a}	46.79 ± 0.89^{a}	48.10 ± 1.23^{aF}	41.64 ± 0.75^{b}
Bacteriocin	42.95 ± 0.39	43.49 ± 1.56	44.08 ± 2.23^{FG}	42.47 ± 0.55
Liquid culture	55.21 ± 1.88	55.64 ± 1.45	56.18 ± 0.95^{E}	54.40 ± 1.44
All	58.68 ± 1.12	58.86 ± 1.04	59.06 ± 0.84^{DE}	58.27 ± 0.28
Mixed initially (Group mix-int)				
Pellet	60.16 ± 0.31^{b}	60.90 ± 0.23^{ab}	61.94 ± 0.72^{aD}	61.85 ± 1.03^{ab}
Bacteriocin	89.51 ± 0.50	89.75 ± 0.37	90.10 ± 0.31^{A}	89.71 ± 0.66
Liquid culture	65.75 ± 1.06	66.59 ± 1.14	66.70 ± 0.76^{C}	65.49 ± 0.42
All	80.27 ± 0.33	80.95 ± 0.91	81.67 ± 1.03^{B}	80.13 ± 0.20
Benzoic acid (Bzc)	39.11 ± 0.86	38.44 ± 1.12	38.61 ± 0.49^{H}	38.91 ± 0.24
Propionic acid (Prp)	5.54 ± 1.68	6.24 ± 1.07	6.62 ± 1.13^{M}	5.75 ± 1.54

$^{a, b, c}$…: Values with different letters are significantly different (P < 0.05) within each row.
$^{A, B, C}$…: Values with different letters are significantly different (P < 0.05) within each column.
*: Comparison of all subgroups after 36 h.

mix-sub, followed by liquid culture alone, pellets alone, and bacteriocin. When pure forms of the culture were compared with combined forms, the most effective group was Group mix-int at detoxification. The effect of Group mix-sub (2 bacteria incubated separately and then mixed in a single tube) was also much higher than the effect of individual cultures. Moreover, the results indicated that the most effective group was the bacteriocins (90%) when bacteria were incubated together (Group mix-int), followed by the mixed group, then liquid culture and lastly pellets.

It is evident that much better results were obtained when *Lc. lactis* and *Lb. plantarum* were incubated in the same liquid medium. In particular, bacteriocin of this group was the most active one among the others (P < 0.05).

With a reduction rate of 38%, benzoic acid was better able to detoxify the AFB_1 solution than either *Lc. lactis* or propionic acid (Table 2). Propionic acid had the lowest reduction rate at 6%.

4. Discussion

This study investigated whether bacteriocinogenic lactic acid bacteria isolated from fermented foods and their bacteriocins could be used for the detoxification of aflatoxins. The findings showed that *Lb. plantarum* had greater toxin-binding ability, with a toxin reduction rate of 46%, than *Lc. lactis* did (27%). Supporting our result, Oluwafemi et al. (22) also found that *Lb. plantarum* was quite effective in detoxifying toxins and noted that the binding increased in proportion to the amount of toxin. However, after investigating AFB_1 detoxification using *Lc. lactis*, *Lb. plantarum*, and several other lactic acid bacteria strains, Zinedine et al. (10) determined that the binding rate varied between 1.80% and 44.89%. In contrast to our

Table 3. Comparative evaluation of the subgroups' detoxification effects.

The effects of each group were compared after 36 h	Pellet	Bacteriocin	Liquid culture	All
Lb. plantarum (Group plant)	33.43 ± 1.54^{cC}	43.33 ± 1.48^{aB}	38.72 ± 0.48^{bC}	46.19 ± 2.69^{aC}
Lc. lactis (Group lactis)	18.69 ± 0.44^{cD}	16.61 ± 0.68^{cC}	22.12 ± 0.97^{bD}	27.45 ± 1.87^{aD}
Mixed subsequently (Group mix-sub)	48.10 ± 1.23^{bB}	44.08 ± 2.23^{cB}	56.18 ± 0.95^{aB}	59.06 ± 0.84^{aB}
Mixed initially (Group mix-int)	61.94 ± 0.72^{dA}	90.10 ± 0.31^{aA}	66.70 ± 0.76^{cA}	81.67 ± 1.03^{bA}

$^{a, b, c}$…: Values with different letters are significantly different ($P < 0.05$) within each row.
$^{A, B, C}$…: Values with different letters are significantly different ($P < 0.05$) within each column.

study, their rate for *Lc. lactis* was 16%, and only 2.14% for *Lb. plantarum*. Haskard et al. (7) found that *Lc. lactis* bound AFB_1 more strongly (at a rate of 59%) than *Lb. plantarum* performed (binding to toxin at a rate of 29.9%). Several studies have conjectured that the interstrain difference in toxin binding ability could be attributed to the species of bacteria, cell density in bacteria cultures, the structure of the bacterial cell wall, whether the bacteria cells were heat-treated, the type of toxin, the length of the incubation period of the toxin–bacteria complex, the incubation environment, the temperature, and the pH (6,7,23–25).

Researchers pointed out that cell wall structure and specifically cell wall polysaccharide and peptidoglycan were the main elements responsible for the binding of mutagens to lactic acid bacteria (6,7). They also explained that heat-treated bacteria had the same ability to remove AFB_1 as viable bacteria, and so metabolic degradation of AFB_1 by viable bacteria has not been a possible mechanism.

In this study, results indicated that toxin binding was almost complete in the first 6 h. There was no statistically significant difference between the degree of toxin reduction after 24 and 36 h ($P > 0.05$). Others have reported similar findings, indicating that the incubation period did not make a significant difference on AFB_1 binding (6,8). El Nezami et al. (6) reported a rapid toxin binding rate of 80% in 1 h by lactic acid bacteria. They found that the concentration of bacteria was very important in achieving this binding. However, Khanafari et al. (26) showed that *Lb. plantarum* bound AFB_1 at the rate of 45% in 1 h and they observed total binding after 90 h. These authors emphasized that time was quite important in achieving such a high binding rate. They reported that during the growth phase, due to changes on the surface of the bacteria, the use of live cultures of *Lactobacillus* and long incubation periods had a significant effect on toxin binding.

In our study we investigated whether the toxin would be released into the environment by applying heat treatment (121 °C for 30 min after 36 h incubation). Interestingly, it was observed that autoclaving had little effect on toxin release except in the groups containing pellets in the lactis and mix-sub groups. These results could be attributed to various metabolites in the liquid culture. However, Haskard et al. (7) reported that autoclaving did not release any detectable AFB_1 from the lactic acid bacteria pellets, which they attributed to the fact that denaturation by high temperatures does not cause the most strongly bound AFB_1 to be released and that this AFB_1 is not bound to loosely attached bacterial components.

In this study, while reduced toxin binding ability was observed in some pellet groups after autoclaving, toxin binding remained stable in the bacteriocin groups. This could be due to heat resistance of the bacteriocins produced by *Lc. lactis* and *Lb. plantarum* (15). This interesting finding showed that these 2 bacteriocins had a greater toxin-binding ability than their antimicrobial effect, as we reported previously (15).

It has been observed that although the bacteria and their metabolites that we tested had detoxifying effects on their own, their efficacy was increased when the bacteria cultures were grown separately and then mixed together. Moreover, these strains were even more effective at detoxifying AFB_1 solutions when the 2 types of bacteria were cultured together. In this latter case, the bacteriocins were the most effective in toxin reduction (90%; $P < 0.05$). Toxin reduction capacity is increased in mixed cultures, but it is not certain why incubating the cultures together yielded the most efficacious results. Perhaps co-culturing of the bacteria increased the concentration of each culture. If that were true, then El Nezami et al. (6) would have a point. In their paper they reported that bacteria concentration significantly affected toxin binding by lactic acid bacteria. Similarly, there are several studies showing that using higher bacteria concentrations causes a higher toxin binding level. In one of these investigations, Kabak (25) reported that cell concentration had a dramatic effect on toxin binding and suggested the presence of cells at or above the level of 10^8 CFU/mL for better results. Other researchers have emphasized that the toxin has been bound using weak covalent bonds depending on the structure of the bacterial cell and they also confirmed the correlation between concentration and the toxin binding effect (6,7,27,28). However, in this study, only one bacterial

concentration was tested and it remained the same both when cultured together or individually. Therefore, culture concentration is not the cause of the toxin binding differences that we report. In contrast to our findings, Oluwafemi et al. (22) reported that combined cultures gave the same results as individual cultures for lactic acid bacteria detoxification. Although more studies are needed to accurately interpret this situation, our data lead us to conclude that each bacterial strain is releasing proteins that have an antagonistic effect on the other strain. Bacteriocins have strong toxin-binding ability. Alternatively, the 2 types of bacteria have a synergistic effect on one another, achieving successful detoxification through much stronger growth and more robust metabolites.

Lactic acid bacteria are found in many foods as natural flora and are being used as starter cultures and probiotic cultures in many foods. These microorganisms, which are considered to be natural additives, and the bacteriocins that they produce detoxify aflatoxin B_1 very effectively. We have determined that the bacteriocin proteins, which were resistant to heat treatment, consistently detoxified AFB_1. These proteins, which are colorless, odorless, and work at neutral pH levels, can be used in the food industry as an alternative to chemical preservatives for removing aflatoxins.

Although many in vitro studies about the aflatoxin detoxification effect of lactic acid bacteria have been cited in the literature, we could not find any for bacteriocins. In this study, the in vitro detoxifying effect of bacteriocins has been examined. However, it is possible to obtain different results using them in the food environment. Supporting this idea, some researchers have reported that lactic acid bacteria detoxified the toxins at different levels in PBS and milk products. It has been observed that the binding effect was higher specifically in milk products, and this finding was attributed to the toxin binding effect of casein (9,29,30).

In the other studies, toxin–bacteria complex stability has been tested after heat treatment, washing, and acid application in PBS environments. However, the findings are not sufficient to evaluate the possible results in food. The researchers emphasized that there should be at least 10^8–10^9 CFU/g of bacteria in food in order to obtain good results and they also reported that this level of bacteria was normally found in probiotic foods (9,29,30).

In order to evaluate the success of in vitro trials in the frame of food environment, further studies must be conducted and the factors having effects on detoxification must be investigated in detail. Specifically, the stability of the toxin–bacteriocin and/or toxin–bacteria complex, sufficient bacteria and/or bacteriocin concentration, and required time period for toxin binding should be determined in food models. During these investigations, possible toxin presence in food should also be considered.

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