Effect of some acid treated bacteria on reduction of impure aflatoxin B1 in ruminant gastrointestinal model

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Abstract: The occurrence of aflatoxin contamination is global, especially in tropical and subtropical countries. Lactic acid bacteria are of particular interest for reducing the bioavailability of aflatoxins. Bacterial aflatoxin B1 binding ability and aflatoxin B1- bacteria complex stability can be affected by acid treatment for a number of strains. In the present study, the ability of three strains of acid treated bacteria to bind high dosage of impure aflatoxin B1 in ruminant gastrointestinal model was investigated. All strains had significant impact on reducing aflatoxin B1 compared to the controls although there were no differences among bacteria significantly. Our results suggest that these treated strains have the ability to reducing high dosage of impure aflatoxin ruminant gastrointestinal model.
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1. Introduction

Aflatoxins are a group of secondary metabolite of *Aspergillus* fungi that grow, on a variety of food and feed commodities at any stage during growth, harvest, storage and transportation (8, 9). Aflatoxin B_1 is regarded as the most potent of the aflatoxins. Toxigenic fungal growth and aflatoxin contamination may occur in various food commodities (14). Aflatoxins are only partly degraded by the ruminal flora so, exposure to aflatoxins results in an impairment of liver function and reduced feed intake, which might also explain the reduced milk production in dairy cattle exposed to aflatoxins (4). When aflatoxin B1 contaminated feed is consumed, the toxins are metabolized to aflatoxin M1 and excreted into the tissues, biological fluids, and milk of lactating animals, including breast milk that have public health concern (4, 14).

The utilization of adsorbents capable of reducing the levels of free toxin available for gastrointestinal absorption is widely adopted for counteracting the biologically negative effects of aflatoxin in animal diets (12). Lactic acid bacteria due in large part to their GRAS status and use as probiotics are of particular interest for reducing the bioavailability of aflatoxins (8). Efficacy of aflatoxin binding was highly variable depending on the genus and strain of bacteria (6). Metabolic degradation of aflatoxin B1 by viable *Lactobacillus* strain has been excluded as a possible binding mechanism, since heat and acid killed remove aflatoxin B1 even more effectively than viable bacteria (11). The stability of the aflatoxin–bacteria complex is also a key consideration when evaluating a strains ability to reduce aflatoxin bioavailability, as aflatoxin release during gastric passage would have clear negative health implications (8) and previously reported that acid treatment being more effective than heat treatment in most cases (7).

Several *in vitro* methods have been proposed to screen different sequestering agents, but the results have not always been comparable to the *in vivo* responses in which the pH, aflatoxin B1: sequestering agent ratio, temperature, biological fluid, source of aflatoxins (aflatoxin B1 from standard or extracted from natural aflatoxin-contaminated feeds), dilution factor (aflatoxin: volume ratio, µg/mL) are important factors that affect this process. Also, authors did not consider the possible competition between aflatoxins and other biological molecules for sequestering agent binding sites in the gastrointestinal tract (12).

The present work was designed to study the ability of three strains of *Lactobacillus* in acid treated form to bind high dosage of impure aflatoxin B1 in ruminant gastrointestinal model.

2. Materials and methods

2.1. Bacterial strains, growth medium and cultural conditions

Lactobacilli strains were obtained as lyophilized powder from Persian Type Culture Collection. The strains used in this study included *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, and *Lactobacillus acidophilus*. Working cultures were prepared from lyophilized powder by two transfers in 25 mL of MRS broth (De Man, Rogosa

and Sharpe, Difco™) with inoculations at 0.3% (v/v) under aerobic condition and static incubation at 37 ºC for 24 h. Bacterial cells (10^9 CFU/mL) were enumerated using the pour-plate method (18) and the results are expressed as colony-forming units per milliliter (CFU/mL). After incubation, cells were collected by centrifugation (3000 × g, 10 min, 5 ºC) and washed twice with phosphate buffered saline (4 mL of PBS; pH 7.4, 0.01 M) (2,7). The culture of each flask (10^9 CFU/mL) was incubated in 4 mL of 2 M HCl for 1 h. Acid treated bacteria were then washed twice (4 mL of PBS), centrifuged, and the supernatant was removed prior to aflatoxin B1 binding assays (7).

2.2. Preparation of Aflatoxin B1 working solution

Aflatoxin was produced via fermentation of rice as described by Shotwell *et al. (*1966). Inoculum was prepared by inoculating tubes of Czapek agar with spores of *Aspergillus parasiticus.*

At the end of fermentation process, aflatoxins were extracted by soaking rice in chloroform overnight at room temperature for three times (16). Finally, for preparing aflatoxins working solution, chloroform evaporated in vacuo and then suspended in methanol and differential analysis for aflatoxin B1, B2, G1, G2 was measured by HPLC method. Of the total aflatoxins content in final solution, 84.64 % was aflatoxin B1 and 15.36% was aflatoxin G1. Aflatoxin B2 and aflatoxin G2 were not detected.

2.3. AFB1 binding assay

A solution of high dosage of aflatoxin B1 (18 μg/mL) was prepared in PBS (pH 6.5) and the methanol was evaporated by heating in a water bath (80°C, 15 min). Bacterial pellets were suspended in 1.5 mL of the working solution of aflatoxin B1 (18 μg/mL) and incubated in simulated ruminant gastrointestinal situation based on Calsamiglia and Stern. (1995) and Gargallo *et al.* (2006) methods by some modification.

Briefly, for simulating rumen situation, samples were incubated for 16 h in PBS that have similar pH (6.5) to rumen of high producing dairy cows that consume high concentrate ration. Then by adding 1 N HCl solution containing pepsin (10 g/l) to each sample, pH adjusted to 1.9 and were incubated for 1 h to mimicking abomasums situation. After this incubation phase, pH was neutralized with 10 N NaOH and then a buffer-pancreatine solution (5 M phosphate solution, pH 7.8, containing 30 g/L pancreatine) were added and incubated for 24 h to simulate small intestine situation. All incubated were carried out with constant rotation at 39 ºC.

All assays were performed in triplicate and a bacterial control (bacteria suspended in PBS) and an aflatoxin B1 control (18 μg/mL of aflatoxin B1 in PBS) were also incubated for all treatments (3, 7, 8, 13, 14).

2.4. Quantification of unbound aflatoxin B1 by ELISA

The bacteria were pelleted (3000 × *g*, 10 min, 5°C) and the supernatant fluid containing unbound aflatoxin B1 were collected and analyzed by microtitre plate Enzyme linked immunosorbent assay (ELISA) method. Sample preparation was performed by diluting supernatant fluid (12,000 times) containing unbound aflatoxin by methanol (33%). This diluting was done for reaching the aflatoxin B1 concentration of samples between standards aflatoxin B1 concentration in ELISA kit (Euroclone S.p.A, Italy). Procedure of aflatoxin B1 quantification were done according to test kit manual. The percentage of aflatoxin B1 bound by the bacterial suspension was calculated using the following formula (1, 3, 7, 8, 14, 17):

$$\text{AFB1 \%} = \left[1 - \frac{\text{amount of aflatoxin B1 in samples}}{\text{amount of aflatoxin B1 in toxin control}}\right] \times 100$$

2.5. Statistical analysis

All experiments and analyses were performed in triplicate. Data analysis was carried out by ANOVA and Duncan's mean comparison tests using the SAS statistical package v. 9.1 to identify significant differences between bacterial strains. P values ≤ 0.05 were considered to be significant (15).

3. Results

The results of remained amount of aflatoxin B1 in supernatant of sample liquid tests after incubation in ruminant gastrointestinal model was ranging from 12.8 to 14.7 μg/mL. Amount of aflatoxin B1 remained shows that 18.3 to 28.8 percentage of aflatoxin B1 in each sample test tube (18 μg/mL) bound to the bacteria (Table 1).

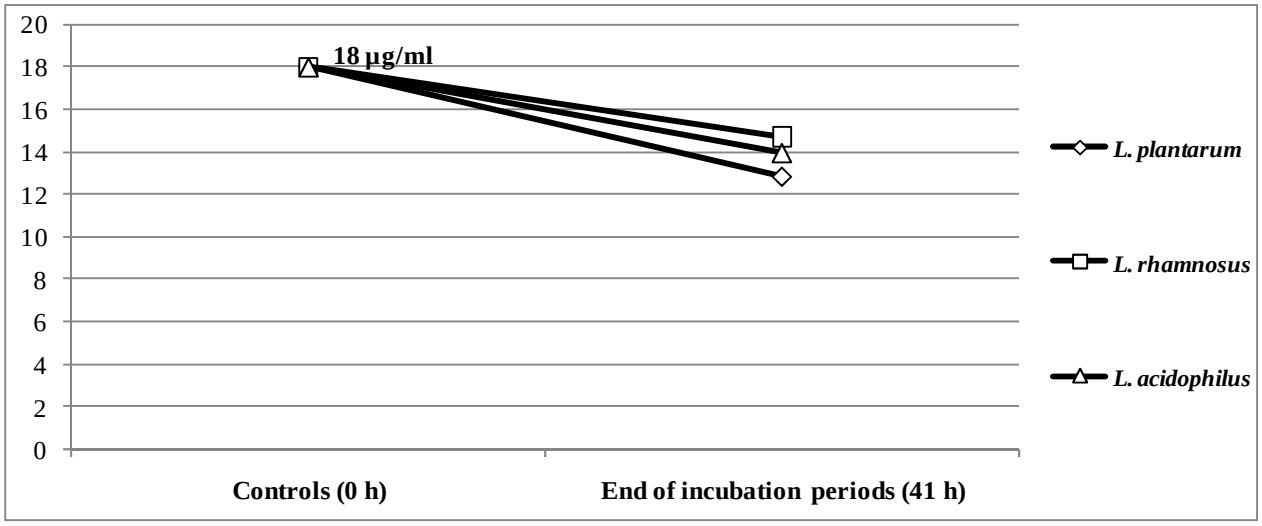
Our results indicated that three strains of lactic acid bacteria used in this experiment exhibited aflatoxin B1 binding properties and using acid treated bacteria reduced aflatoxin B1 compared to the controls significantly although there were no differences among bacteria (Figure 1).

TABLE 1. Effect of acid treated bacteria on remained amount (μg/mL) and percentage of bound (%) aflatoxin B1 in supernatant fluid after incubation in ruminant gastrointestinal tract situation.

	Treated lactobacilli [a]			P-value
	L. plantarum	*L. rhamnosus*	*L. acidophilus*	
Remained aflatoxin B1 [b] (µg/mL)	12.811 ± 2.113	14.697 ± 0.987	13.965 ± 1.639	0.520
Bound aflatoxin B1[c] (%)	28.829 ± 11.740	18.347 ± 5.485	22.418 ± 9.106	

[a] Bacteria were incubated in 2 M HCl at 37°C for 1 h.
[b] Amount of aflatoxin B1 remained (µg/mL) after 10^9 treated bacteria were incubated with aflatoxin B1.
[c] Percentage of aflatoxin B1 removed after 10^9 treated bacteria were incubated with aflatoxin B1.

Figure 1. Effect of acid treated bacteria on remained aflatoxin B1 (μg/mL) in supernatant fluids after 41 h incubation in ruminant gastrointestinal situation



4. Discussion

Lactic acid bacteria and intestinal bacteria have been previously reported to bind various dietary contaminants such as aflatoxins (14). While it is difficult to compare results of aflatoxin B1 binding levels from different studies, due to the possible impact of procedural differences. The results observed in the current study are in the range of those reported previously. Previous investigations into levels of aflatoxin B1 binding by lactobacilli have reported values ranging from 5% to 84% (8).

In this experiment we tested aflatoxin B1 binding process after expose these bacteria-aflatoxin B1 complexes to ruminant gastrointestinal situation that consist of different pH (6.5, 1.9, 7.0 and 7.8) and enzymes (pepsin and pancreatin) continuously. This situation selected because of the reason that the aflatoxin B1 binding by the strains studied can be varied significantly during the incubation period.

This means that some aflatoxin B1-bacteria complexes were not bound strongly and during passing through gastrointestinal tract, aflatoxin B1 could be released back into the solution from the lactobacilli-aflatoxin B1 complex (14). Haskard *et al.* (2001) reported that around 10% of bound aflatoxin B1 is released by aqueous solutions of pH 2 to 10 representative of the gastrointestinal tract pH range and suggesting that a cation-exchange mechanism is not operating in binding mechanisms (7).

In this experiment we used acid treated bacteria based on the previous observations that bacterial viability is not a prerequisite for efficient aflatoxin B1 removal and this finding that treatments (heat and acid) markedly increased the bacterial aflatoxin B1 binding ability (14) and acid treatment being more effective than heat treatment in most cases (7). So for binding capacity determination of

acid treated lactobacilli we used definite amount of bacterial population density and toxin. The results of most studies suggested that animals are more susceptible to naturally contaminated feed compared with purified mycotoxins because of synergistic and additive interactions, the presence of undetectable conjugated forms, and changes in feedstuffs induced by fungi. So, despite of other research that used pure aflatoxin B1 for *in vitro* assessing binding capacity of lactobacilli, we used impure aflatoxin B1 extracted from naturally contaminated rice (10).

Cell wall polysaccharide and peptidoglycan are the two main elements responsible for the binding of aflatoxins to lactobacilli. Both of these components are expected to be greatly affected by acid treatment. Acid may break the glycosidic linkages in polysaccharides resulting in the observed compromised structural integrity. Although the peptidoglycan layer is quite thick in these organisms, there may be a decrease in thickness, reduction in cross-links, and/or increase in pore size. This perturbation of the bacterial cell wall may allow aflatoxin B1 to bind to cell wall and plasma membrane constituents that are not available when the bacterial cell is intact.

Heat and acid treatments of bacteria significantly enhanced the stability of the complex formed with aflatoxin B1 for a number of strains, and no significant change in stability for other strain. Haskard *et al.* (2001) reported that acid treatment being more effective than heat treatment in most cases because the most of the aflatoxin B1 is bound to bacterial surfaces; however, these surfaces may be intracellular in the case of acid treated bacteria rather than extracellular. So, stability of the complexes formed between lactobacilli and aflatoxin B1 was strain specific but we couldn't find this specificity in this experiment likely due to similarity in types, numbers, availability of aflatoxin binding sites for B1 and G1 types, high dosage of toxin, bacterial cell wall and cell envelope structures of used bacteria (14).

5. Conclusion

The results of present study show that three strains of acid treated *Lactobacillus* used in this experiment had the ability to bind high dosage of impure aflatoxin B1 in ruminant gastrointestinal model although there were no differences between strains. So, further investigation is needed for assessing the aflatoxin B1 binding ability of these bacteria in ruminant gastrointestinal model.

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References

1. Ardic. M.; Karakaya, Y.; Atasever, M. and Durmaz, H. (2008). Determination of aflatoxin B1 level in deep-red ground pepper (isot) using immunoaffinity column combined with ELISA. J. Food. Chem Tox. 48: 1596-1599.
2. Calsamiglia, S. and Stern, M. D. (1995). A three-step in vitro procedure for estimating intestinal digestion of protein in ruminants. J. Anim. Sci. 73:1459-1465.
3. El-Nezami, H.; Polychronaki, N.; Salminen, S. and Mykkanen, H. (2002). Binding rather than metabolism may explain the interaction of two food-grade *Lactobacillus* strains with zearalenon and its derivative alpha–zearalenol. J. Appl. Environ. Microbio. 67: 3545-3549.
4. Fink-Gremmels, J. (2008). Mycotoxins in cattle feeds and carry-over to dairy milk: A review. Food Additives and Contaminants, 25 (2), 172-180.
5. Gargallo, S.; Calsamiglia, S. and Ferret, A. (2006). Technical note: A modified three-step in vitro procedure to determine intestinal digestion of proteins. J. Anim. Sci. 84: 2163-2167.
6. Gratz, S.; Wu, Q. K..; El-nezami, H.; Juvonen, R. O.; Mykkanen, H. and Turner, P. C. (2007). *Lactobacillus rhamnosus* strain GG seduces aflatoxin B1 transport, metabolism, and toxicity in Caco-2 cells. J. Appl. Environ. Microbio. 73: 3958-3964.
7. Haskard, C. A.; El-Nezami, H. S.; Kankaanpaa, P. E.; Salminen, S. and Ahokas, J. T. (2001). Surface binding of aflatoxin B1 by lactic acid bacteria. J. Appl. Environ. Microbio. 67: 3086-3091.
8. Hernandez-Mendoza, A.; Garcia, H. S. and Steele, J. L. (2009). Screening of *Lactobacillus casei* strains for their ability to bind aflatoxin B1. J. Food. Chem Tox. 47:1064-1068.
9. Huwing, A.; Freimund, S.; Kappeli, O. and Dutler, H. (2001). Mycotoxin detoxication of animal feed by different adsorbents. J. Toxlet. 122:179-188.
10. Korosteleva, S. N.; Smith, T. K. and Boermans, H. J. (2007). Effects of feedborne *Fusarium* mycotoxins on the performance, metabolism, and immunity of dairy cows. J. Dairy Sci. 90: 3867-3873.

11. Lahtinen, S. J.; Haskard, C. A.; Ouwehand, A. C.; Salminen, S. J. and Ahokas, J. T. (2004). Binding of aflatoxin B1 to cell wall components of *Lactobacillus rhamnosus* strain GG. Food Additives and Contaminants, 21, 158–164.
12. Moschini, M.; Gallo, A.; Piva, G. and Masoero, F. (2008). The effects of rumen fluid on the *in vitro* aflatoxin binding capacity of different sequestering agents and *in vivo* release of the sequestered toxin. Anim. Feed Sci. Technol. 148, 292-309.
13. Niderkon, V.; Morgavi, D. P.; Aboab, B.; Lemaire, M. and Boudra, H. (2009). Cell wall component and mycotoxin moieties involved in the binding of fumonisin B1 and B2 by lactic acid bacteria. J. Appl Microbiol. 106: 977-985.
14. Peltonen, K.; El-Nezami, H.; Haskard, C.; Ahokas, J. and Salminen, S. J. (2001). Aflatoxin B1 binding by dairy strains of lactic acid bacteria. J. Dairy Sci. 84:2152–2156.
15. SAS Institute. 2004. SAS User's Guide: Statistics. Version 9.1 ed. SAS Institute Inc., Cary, NC.
16. Shotwell, O. L.; Hesseltine, C. W.; Stubblefield, R. D. and Sorenson, W. G. (1966). Production of aflatoxin on rice. J. Appl Microbiol. 106: 977-985 .
17. Total aflatoxin ELISA kit for the quantative analysis of total aflatoxin in grains, nuts, cottonseeds, cereals and feeds. 2011. Available: http://www.biomedica.hu/fileadmin/img/HU/hu_news_021110/Total_AFLATOXIN.pdf/.
18. Vinderola, C. G.; Bailo, N. and Reinheimer, J. A. (2000). Survival of probiotic microflora in Argentinian yoghurts during refrigerated storage. Food Res. Int. 33, 97– 102.

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