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Aflatoxin M1 binding by dairy strains of lactic acid bacteria

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Abstract

The aim of this study is to determine the ability of specific lactic acid bacteria strains to remove aflatoxin M1 from liquid media. Six dairy strains of lactic acid bacteria **were tested for their ability to remove aflatoxin M1 from liquid medium. Both viable and dead bacteria from the same population were tested. Two lactic acid bacterial strains which exhibited the best AFM1 removal abilities were also tested using contaminated skimmed and full cream milk. Both skimmed milk and full cream milk were used with both viable and heat-killed bacteria assessed. All strains, both viable and heat-killed, could reduce the AFM1 content of a liquid medium. From the results we can conclude that specific dairy strains of lactic acid bacteria can offer means of decontaminating aflatoxin M1 from milk.**

Introduction

Aflatoxin belongs to a group of fungal toxins known as mycotoxins, and is widespread in agricultural products and food. Aflatoxin is associated with both acute and chronic toxicity in animals and humans, including acute liver damage, liver cirrhosis and liver cancer. Aflatoxins are predominantly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, but may also be produced by other strains, such as *Aspergillus nomius*, *Aspergillus tamarii*, and *Aspergillus pseudotamarii*. **Aflatoxin M1 (AFM1) is a highly toxic compound found in milk. Aflatoxin M1 is considered as a 'milk toxin'. Presence of aflatoxin M1 in milk is a public health hazard. Growing children are more sensitive than adults as milk is one of their main sources of nutrients.**

METHODS

- Bacterial strains and culture conditions.

Six dairy strains of lactic acid bacteria, *Lactobacillus acidophilus* strain LA1, *Lactobacillus gasseri* strain ATCC 33323, *Lactobacillus rhamnosus* strain GG (ATCC 53013), *Lactobacillus rhamnosus* strain LC-705, *Lactobacillus rhamnosus* strain 1/3, and *Lactococcus lactis* ssp. *cremoris* strain ARH74, were tested for their ability to remove aflatoxin M1 from liquid medium. A 15 mL overnight starter cultures were inoculated to 150 mL growth media and incubated in a 5 % CO_2 (95 % air) atmosphere at +37°C for 20 hours.

- AFM1 removal assay.

Solid AFM1 from *Aspergillus flavus* (Sigma Chemical Company) was suspended in benzene-acetonitrile (97:3, vol:vol) in order to make a 10 µg/mL stock solution.

The concentration of the stock was verified by recording a UV/VIS spectrum of a diluted AFM1 sample (Hitachi U-2000) and calculating its actual value from the Lambert-Beer equation $A = \varepsilon cl$. A solution equivalent to 0.15 µg AFM1/mL phosphate buffered saline was prepared for the assay. The benzene-acetonitrile derived from the stock was evaporated by heating in a hot water bath (+80°C) until the visible benzene-acetonitrile droplets disappeared (5-10 min).

After 20 hours incubation, the bacterial growth was stopped by placing the culture in an ice water bath. The cell concentration of a bacterial culture was determined using flow cytometry (FCM). The results obtained from FCM were compared with the results of traditional plate counting representing only the viable bacteria of the culture. A volume of the culture broth corresponding to 1.5×10^{10} cells was centrifuged at 3000-3500 g for 10-15 minutes depending how easily the bacteria formed a pellet. The collected cells were washed with MilliQ (MQ) water and suspended in 1.5 mL PBS contaminated with AFM1. The bacterial suspensions were incubated overnight (15-16 hours) at +37°C and centrifuged on the next day. The AFM1 content of the supernatant was determined by a high pressure liquid chromatographic (HPLC) method.

Both viable and dead bacteria from the same population were tested. In order to test dead bacteria the washed pellets were resuspended in 4 mL of PBS and the suspensions were placed in a boiling water bath for 1 hour. The dead bacteria were pelleted again and subjected to AFM1 as described above. In addition to this, both precultured and lyophilized *Lactobacillus rhamnosus* strain GG (LBGG) and *Lactobacillus rhamnosus* strain LC-705 (LC-705) were tested. After sterile weighing of 0.1 g (~1×1010 cells/mL) lyophilized bacteria, 4 mL PBS was added and cells to be viable were incubated for 1 hour at +37°C, whereas cells to be heat-killed were boiled for 1 hour.

All assays were performed in triplicates and both positive (cell-free PBS contaminated with AFM1) and strain related negative controls (bacteria suspended in pure PBS) were included.

- AFM1 removal assay with contaminated test milk.

This step was carried out with two lactic acid bacterial strains which exhibited the best AFM1 removal abilities in PBS. Both skimmed milk and full cream milk were used with both viable and heat-killed bacteria assessed.

Skimmed milk and full cream milk powders were suspended into MQ water (0.2 g/mL) and a portion of the reconstituted milks was separated for artificial AFM1 contamination. The rest were used for negative controls.

Precultured bacteria were harvested and treated, but instead of contaminated PBS, the bacterial pellets were suspended in spiked skimmed or full cream milk. All removal assays were carried out in triplicates, so a total of 12 tubes with cells subjected to 1.5 mL milk containing 0.15 µg/mL AFM1 was needed per bacterial strain.

Additionally, when two 1.5 mL cell-free positive controls (skimmed and full cream milk) and a 1.05 mL AFM1 standard were taken into account, 3.3 µg (or 330 µL) AFM1 (per strain) was required from the benzene-acetonitrile stock.

Because evaporation of benzene-acetonitrile from milk was considered to be uncertain, the calculated volume of stock solution was evaporated to dryness under a smooth N2 stream in a dry block heater (+80°C).

The AFM1 residue was redissolved in 1.05 mL methanol to obtain a solution with 3.14 µg/mL AFM1. A volume of 0.5 mL was transferred to 10 mL of milk (skimmed or full cream) resulting in 10.5 mL contaminated milk containing 0.15 µg/mL AFM1. The rest (0.05 mL) of the contaminated methanol was diluted to same concentration by adding 1 mL pure methanol.

After an overnight incubation in 5 % CO_2 atmosphere at +37°C, the suspensions were centrifuged and 0.5 mL supernatant samples collected. Uncontaminated milk preparations with and without bacteria were used as negative controls. Spiked skimmed and full cream milk without bacteria served as the positive controls from which duplicate samples (2×0.5 mL) were taken for further measures.

The method for extraction of AFM1 from collected milk samples was of El-Nezami *et al.* and it applies a solid phase extraction.

The milk samples were diluted into 5 mL in MQ water and tempered in water bath at +40°C for 1 hour in order to facilitate the passage through Sep-pak® C18 cartridge. A conditioning step is required for reversed phase C18 and the stationary phase was moistened with 10 mL acetonitrile. This was followed by an equilibration step using 10 mL of MQ water. The 5 mL sample was loaded onto the cartridge and passed through. Interfering components were removed by sequentially washing the cartridge with 10 mL of MQ water, 10 mL of basic 10 % acetonitrile (ammonium hydroxide (conc. 29.5 %):acetonitrile:water, 1:10:90) and 10 mL of acidic 10 % acetonitrile (acetic acid:acetonitrile:water, 1:10:90). AFM1 was eluted with 5 mL of acidic 40 % acetonitrile (acetic acid:acetonitrile:water, 1:40:60) and the eluent was vortexed with 2 mL of dichloromethane for 1 minute. After 15 min centrifugation at 3000 r.p.m, the dichloromethane fraction was removed and the remaining acetonitrile fraction was extracted a second time with dichloromethane. The two dichloromethane fractions were pooled and evaporated dry under N2 stream in a heating block (+70°C). The residue was redissolved in 0.5 mL methanol and the sample analyzed with HPLC.

Results

TABLE 1. Comparison of bacterial concentrations quantitated by flow cytometry (FCM) and plate counting methods (CFU).

Strain	FCM (counts/mL)[a]	Plate Counting (CFU/mL)[b]
Lactobacillus acidophilus strainLA1	1.6×10^9	1.8×10^9
Lactobacillus gasseri (ATCC 33323)	5.6×10^8	3.7×10^8
Lactobacillus rhamnosus strain GG	6.9×10^8	5.2×10^8
Lactobacillus rhamnosus strain LC-705	8.3×10^8	5.2×10^8
Lactobacillus rhamnosus strain 1/3	6.2×10^8	9.6×10^8
Lactococcus lactis ssp. *cremoris* strain ARH74	1.0×10^9	2.6×10^8

[a] FCM results are the means of two to four measurements.
[b] Plate counting results are the means of two plate assays.

TABLE 2. Removal of AFM1 from PBS by viable and heat-killed bacteria. Each value is a mean ± STD of three samples. The results for lyophilized *L. rhamnosus* strain GG (LBGG) and *L. rhamnosus* strain LC-705 (LC-705) are included. The bacteria are listed in order from best to worst viable remover.

Strain		% AFM1 removed in 15-16 hr	% AFB1 removed in 4 hr.	in 24 hr.
L. rhamnosus strain GG (precultured)	Viable	50.8 ± 2.1	77.0 ± 0.4 6	5.2 ± 1.4
	Heat-killed	57.9 ± 3.2		
L. rhamnosus strain GG (lyophilized)	Viable	53.7 ± 1.2	64.0 ± 2.0	
	Heat-killed 5	6.3 ± 2.7		
L. rhamnosus strain LC-705 (precultured)	Viable	46.4 ± 2.7	75.2 ± 1.3	76.2 ± 4.4
	Heat-killed	51.8 ± 3.0		
L. rhamnosus strain LC-705 (lyophilized)	Viable	45.9 ± 1.6	51.0 ± 1.0	
	Heat-killed	56.4 ± 2.3		
Lc. lactis ssp. *cremoris* strain ARH74	Viable	40.4 ± 2.6		
	Heat-killed	38.7 ± 2.6		
L. gasseri (ATCC 33323)	Viable	30.7 ± 5.7	51.2 ± 1.8	48.3 ± 0.2
	Heat-killed	61.6 ± 0.6		
L. acidophilus strain LA1	Viable	18.4 ± 4.0		
	Heat-killed	25.6 ± 4.7		
L. rhamnosus strain 1/3	Viable	18.4 ± 1.4		
	Heat-killed	39.7 ± 0.8		

TABLE 3. Removal of AFM1 from skimmed and full cream milk by viable and heat-killed *L. rhamnosus* strain GG and *L. rhamnosus* strain LC-705. Each value is a mean ± STD of three samples.

Strain		% AFM1 removed from Skimmed Milk	% AFM1 removed from Full Cream Milk
L. rhamnosus strain GG	Viable	18.9 ± 1.8	26.0 ± 1.5
	Heat-killed	26.9 ± 3.3	36.6 ± 1.1
L. rhamnosus strain LC-705	Viable	68.6 ± 0.8	63.7 ± 0.9
	Heat-killed	27.3 ± 4.7	31.3 ± 1.8

Conclusions

* Viable LBGG and LC-705 were the most effective of the tested strains in removing AFM1. Surprisingly, *L. rhamnosus* 1/3 showed the poorest capacity in taking up AFM1 even though this strain is considered genetically relatively similar to LBGG. Thus, even closely related strains may have different biological activities.
* No differences in the AFM1 removal ability were observed between precultured and lyophilized cells of LBGG or LC-705.
* Heat-treated LBGG and LC-705 bound similar amounts of AFM1 in skimmed milk, whereas LBGG removed AFM1 slightly better in full cream milk.