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Binding ability of aflatoxin M_1 to yoghurt bacteria

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Summary: Aflatoxin M_1 (AFM_1) is a highly toxic compound found in milk. Several microorganisms have been previously reported to bind or degrade AFM_1 from liquid media. This study was performed to assess the binding of AFM_1 in contaminated phosphate buffered saline (PBS). *Lactobacillus delbrueckii subsp. bulgaricus* CH-2 and *Streptococcus thermophilus* ST-36 were used for this purpose. Removal activities of two strains were also assessed using contaminated reconstituted milk and contaminated yoghurt made from reconstituted milk. ELISA procedure was used in this study *Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2 bound in PBS at 18.70% and in milk at 27.56% while *Streptococcus thermophilus* ST-36 bound in PBS at 29.42% and in milk at 39.16%. AFM_1 was bound at the level of merely 14.82% in yogurt. The results indicated that binding ability of *Streptococcus thermophilus* ST-36 was higher than that of *Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2 in both PBS and reconstituted milk. Both of microorganisms bound higher in milk than in PBS. Also, AFM_1 binding levels were at least level in yoghurt (%14.82). These findings supported that specific yoghurt bacteria used in this study can offer decontaminating AFM_1 from milk.

Key words: Aflatoxin M_1, binding, yoghurt bacteria

Aflatoksin M_1'in yoğurt bakterilerine bağlanma yeteneği

Özet: Bu çalışmada, kontamine PBS'de AFM_1'in bağlanma yeteneği araştırıldı. Bu amaçla, *Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2 ve *Streptococcus thermophilus* ST-36 bakterileri kullanıldı. AFM_1'in bu iki bakteriye bağlanma yeteneği, aynı zamanda kontamine sütte ve kontamine sütten yapılan yoğurtta da araştırıldı. Çalışmada, ELISA yöntemi kullanıldı. *Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2'nin AFM_1'i PBS'de % 18.70, sütte % 27.56; *Streptococcus thermophilus* ST-36'nın ise PBS'de % 29.42 ve sütte % 39.16 düzeylerinde bağlama yeteneğinde olduğu belirlendi. Yoğurtta ise bağlanma en düşük düzeyde saptandı(% 14.82). Sonuç olarak, gerek PBS'de gerekse sütte *Streptococcus thermophilus*'un bağlama yeteneği, *Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2'den daha yüksek bulundu. Aynı zamanda her iki bakterinin de PBS'e göre sütte daha fazla bağlama yeteneği olduğu saptandı. Elde edilen bulgulara göre, bu çalışmada kullanılan spesifik yoğurt bakterilerinin AFM_1'i sütten uzaklaştırma yeteneğinde olduğu belirlendi.

Anahtar sözcükler: Aflatoksin M_1, bağlanma, yoğurt bakterileri

Introduction

Aflatoxins are the most potent toxic, mutagenic, teratogenic and carcinogenic metabolites produced by the species of *Aspergillus flavus, Aspergillus flavus* subsp. *parasiticus* and *Aspergillus nomius* on food and feed materials. There are four main toxins which have been divided into B and G groups (B_1, B_2, G_1 and G_2). Of these, aflatoxin B_1 is most toxic and most carcinogenic. Aflatoxin M_1, a hydroxylated metabolite of aflatoxin B_1 is an important toxin present in the milk of lactating animals fed with aflatoxin B_1 contaminated feeds. Presence of aflatoxin M_1 in milk is a public health hazard. There is a general consensus that approximately 1-3% of the aflatoxin B_1 initially present in the animal feedstuff appears as aflatoxin M_1 in milk (5,8,18,23). Evidence of potential hazardous human exposure to AFM_1 from dairy products arises from many studies on the occurrence of AFM_1 in dairy products (15,27). Since milk has the greatest demonstrated potential for introducing aflatoxins residues from foods of animal origin into the human diet and is also the main nutrient for infants and children, the occurrence of aflatoxin M_1 in milk and dairy products is a concern (9,10,12,21,22). The best way to control the presence of AFB_1 in foods and feeds is to prevent their formation. Various physical, chemical and biological agents have been used to detoxify aflatoxins from food and feed materials (2,11,20). But there are currently no acceptable chemical, physical or biological methods to counteract the AFM_1 problem in milk (24). Thus, a practical and effective method is needed to be developed for the detoxification of AFM_1 contaminated milk.

Some strains of lactic acid bacteria have been reported to be effective in removing AFB_1 and AFM_1 from contaminated liquid media and milk(1, 14, 16, 17). For this purpose, this study was carried out in order to investigate the ability of *Lactobacillus delbrueckii* subsp.

bulgaricus and *Streptococcus thermophilus* to remove AFM_1 from contaminated phosphate buffered saline (PBS) and reconstituted skim milk. Removal activities of these strains were also assessed in fermented milk product such as yoghurt because of symbiotic relationship.

Materials and Methods

Standard of AFM_1

Solid AFM_1 (Sigma) was suspended in benzene-acetonitrile (97/3, vol/vol) to obtain an AFM_1 concentration of 1 μg/ml and 5 μg/ml.

Culture preparation

Lactobacillus delbrueckii subsp. *bulgaricus* CH-2 and *Streptococcus thermophilus* ST-36 were originally obtained from Chr. Hansen's Lab (Denmark*). Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2 was cultivated in 25 ml MRS broth (Oxoid CM 359) at 37°C for 24 h. *Streptococcus thermophilus* ST-36 was cultivated in 25 ml M17 broth (Oxoid CM817) at 37°C for 24 h. The bacterial growth was determined at MRS agar (Oxoid CM361) for *Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2 and M17 agar (Oxoid CM785) for *Streptococcus thermophilus* ST-36 after 24 hours incubation at 37°C using traditional plate counting. At the same time, cultivation broths were centrifuged at 3500 x g for 15 min. The bacterial pellets were washed with PBS (Oxoid BR14a) twice.

Contamination with AFM_1 in PBS

A solution of 10 ng AFM_1/ml PBS was prepared for the assay. The benzene/acetonitrile derived from the stock was evaporated by heating in a water bath at 80°C. Bacterial pellets were suspended in 1.5 ml PBS contaminated with AFM_1 and incubated at 37°C for 4 h. Bacterial suspensions were then centrifuged at 3500 x g for 10 min. Unbound AFM_1 content in the supernatant was determined by ELISA. Each sample for the ELISA analysis was diluted 1:125 in PBS. ELISA procedure was performed according to R-biopharm GmbH recommendations. Binding of AFM_1 by *Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2 and *Streptococcus thermophilus* ST-36 cells was analysed according to Pierides et al (17). Cell-free PBS contaminated with AFM_1 was used for positive control. Bacteria suspended in PBS were used for negative control. All assays were performed at control groups, too.

Milk contamination with AFM_1

Reconstituted milk with 12% nonfat dry matter was prepared from skim milk powder (easy soluble skim milk powder, PINAR) in distilled water. A portion of the reconstituted milk was used for artificial AFM_1 contamination. The rest was used for negative control.

Bacterial pellets were collected as described earlier, but bacterial pellets were suspended in contaminated nonfat reconstituted milks. Stock solution (1 μg AFM_1/ 1 ml benzene/acetonitrile) was evaporated to dryness under a smooth N_2 stream. The AFM_1 residue was redissolved in 1 ml methanol. A volume of 0.01 ml was transferred from the contaminated methanol to 1.5 ml of reconstituted skim milk, resulting in milk containing 10 ng/ml AFM_1. Bacterial pellets was suspended in reconstituted milk contaminated with AFM_1 and incubated at 37°C for 4 h. After incubation period, suspensions were centrifuged. Unbound AFM_1 content in the supernatant was determined by ELISA (14). Each sample for the ELISA analysis was diluted 1:125 in PBS.

Cell-free reconstituted milk contaminated with AFM_1 was used for positive control. Bacteria suspended in reconstituted milk were used for negative control. All assays were performed at control groups, too. Procedure of contamination with AFM_1 in reconstituted milk was that of Pierides et al (17).

Contamination of reconstituted skim milk and yoghurt production

Yoghurt was made from the reconstituted skim milk presented 12% nonfat dry matter. Prepared skim milk was heated at 90°C for 5 min and then cooled to 42°C.

Stock solution (5 μg/ml AFM_1 in benzene/acetonitrile) was collected as described earlier. But AFM_1 residue was redissolved in 2 ml methanol. A volume of 0.08 ml was transferred from the contaminated methanol to 20 ml of skim milk, resulting in milk containing 10 ng/ml AFM_1. After that, 20 ml milk was inoculated with 2% starter cultures.The ratio of *Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2 : *Streptococcus thermophilus* ST-36 was 1:1.

Cell-free reconstituted milk contaminated with AFM_1 was used for positive control, yoghurt made from reconstituted milk and uncontaminated as negative control. All groups were incubated at 42°C for 4 h. Yoghurt was centrifuged at the end of incubation and unbound AFM_1 content in the supernatant was determined by ELISA. Each sample for the ELISA analysis was diluted 1:125 in PBS. ELISA procedure was performed according to R- biopharm GmbH recommendations.

In this study, all assays were performed five times and both positive and negative controls were included.

Statistical analysis

The variance analysis (with two factors) was done for determining the difference as binding amount of aflatoxin M_1 in two medium of two bacteria. In addition, one-way ANOVA variance analysis was also done for comparison of binding in yoghurt. DUNCAN test was used for determining the different groups after the one-way variance analysis.

Results

In this study, in vitro binding ability of AFM_1 to *Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2 and *Streptococcus thermophilus* ST-36 was investigated in the liquid medium (PBS), reconstituted milk and yoghurt comparatively.

Comparing two strains for statistical analysis, *Streptococcus thermophilus* ST-36 showed significantly high ($p< 0.01$) percentage of AFM_1 binding ability according to *Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2 in PBS and milk (Table 1). On the other hand the percentage of removal activity of both *Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2 and *Streptococcus thermophilus* ST-36 in PBS showed significant differences ($p< 0.01$) according to milk. At the same time the differences between milk and yoghurt were found statistically important ($P< 0.01$) (Table 1).

Table 1. Comparison of strains-media and yoghurt
Tablo 1. Mikroorganizma-ortam ve yoğurdun karşılaştırılması

Strain-Media	X± SX
Lactobacillus delbrueckii subsp. *bulgaricus* CH-2	^{C}18.7± 0.582
PBS	
Lactobacillus delbrueckii subsp. *bulgaricus* CH-2	^{B}27.56±0.699
Milk	
Streptococcus thermophilus ST-36	^{B}29.42± 0.601
PBS	
Streptococcus thermophilus ST-36	^{A}39.16± 0.459
Milk	
Yoghurt	^{D}14.82± 0.558

Discussion

It was determined that *Streptococcus thermophilus* ST-36 has a more binding ability in comparison *Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2 both in PBS and reconstituted milk (Table 1).

Lactic acid bacteria are known to bind aflatoxins. Recently, heat-killed bifidobacteria have been reported to bind aflatoxin B_1 in PBS (14). *Lactobacillus rhamnosus* GG, *Bifidobacterium bifidum* BGN4, *Bifidobacterium sp*. JO3, *Bifidobacterium longum* JR 20 and *Bifidobacterium* sp. CH4 bound to AFB_1 by 37±1%, 46±4%, 41±3%, 37±3% and 37±1%, respectively. Pierides et al (17) reported that viable *L. rhamnosus* GG bound to AFM_1 by 77±0.4%, *L. rhamnosus* LC-705 bound by 75.2±1.2% and *L. gasseri* (ATCC 33323) bound by 51.4±1.9% after 4 h incubation in PBS. However the binding ability of heat-killed same bacteria was determined as 57.8±3.3%, 51.6±3.0% and 61.5±0.7%, respectively after the 15-16 h incubation in PBS. In the same study, binding of AFM_1 to both viable and heat-killed *L. rhamnosus* GG was reported as 18.8±1.9% and 26.6±3.2%; *L rhamnosus* LC-705 was reported as 69.6±0.9% and 27.4±4.8% in skim milk. Peltonen et al (16) investigated the AFB_1 binding ability of 12 *Lactobacillus*, 5 *Bifidobacterium* and 3 *Lactococcus* in PBS. In their study, *Lactobacillus* strains bound by 17.3-59.7% AFB_1, *Bifidobacterium* strains by 18.0-48.7% and *Lactococcus* strains by 5.6-41.1% AFB_1. El-Nezami et al (7) observed that *L. rhamnosus* GG and *L. rhamnosus* LC 705 bound to AFB_1 by 80% in 24 h. These studies suggested that significantly different binding abilities of lactic acid bacteria were due to different cell-wall structure. Thus, in this study binding ability of yogurt cultures examined were found different. Also, Pierides et al (17) reported that *L. rhamnosus* 1/3 presented a less binding ability than *L. rhamnosus* GG in spite of the same genetic structure, and they presumed that this was caused by different biological activities of the strain.

When the binding ability of yoghurt cultures in PBS and reconstituted milk were compared, the binding was much greater in milk (Table 1). The principal reason of that may be due to the binding properties of aflatoxin to casein. So, Brackett and Marth (3) reported that an average of 30.7% more AFM_1 was found in milk treated with proteolytic enzyme than in untreated milk and they suggested that AFM_1 is bound by milk protein. Also, the same authors (4) reported that AFM_1 did not display a homogeneous distribution in milk and a part of AFM_1 could not be extracted from milk. Tabata et al (25) reported that milk concentrations had an effect on AFM_1. Pierides et al (17) reported that contrarly to this study, binding ability of AFM_1 to *L. rhamnosus* GG and *L. rhamnosus* LC-705 was less in milk.

It was seen that the binding after yoghurt manufacturing was less than that in milk, examined separately (Table 1). This may be caused by fermentation, which is greater in yogurt than in milk and by the fact that AFM_1, which is bound to casein is extracted better than milk(19). Van Egmond et al (26) found AFM_1 was recovered in slightly greater amounts from yoghurt than from the original milk. They believe the increased AFM_1 content in yoghurt possibly results from a more complete recovery of AFM_1 from yoghurt than milk. Munksgaard et al (13) found the level of AFM_1 during production of yoghurt to be increased on average by 9%. They explained that AFM_1 is extracted better from cultured products. At the same time, the binding abilities may be decreased because of synergetic reproduction in yoghurt, although it is reported by El-Nezami (6) that the binding abilities increased in acid treatment in PBS experimentally . In fact the binding determined in yoghurt, was found to be even less than the bindings determined separately in PBS.

In this study it was determined that both *Lactobacillus delbrueckii* subsp. *bulgaricus* CH-2 and *Streptococcus thermophilus* ST-36 have binding abilities during yoghurt manufacturing. Thus, it could be suggested that yoghurt cultures could be used in the removal of AFM_1

from food and feed. Still, more research is required, e.g. using different incubation times, temperatures, aflatoxin amounts and dry-matter amounts. Conducting more experiment particularly in a food medium would be useful in the protection from aflatoxins, a major public health problem. In addition, conducting the experiments in vivo will play an important role in determining the binding properties of bacteria.

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