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Removal of Aflatoxin B_1 from Contaminated Liquid Media by Dairy Lactic Acid Bacteria

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ABSTRACT

In the present study the ability of some dairy strains of lactic acid bacteria to remove aflatoxin B_1 from contaminated media was investigated. Twelve out of forty two isolates from yogourt, raw milk and karisk cheese have shown different levels to bind aflatoxin B_1 (AFB_1). The highest isolates for binding AFB_1 were identified as *Lactococcus lacti*s and *Sterptococcus thermophilus*. Dead cells (by boiling) of *L. lacti*s and *S. thermophilus* bind 86.1 and 100% of the AFB_1 (2 µg) added to the phosphate buffer solution, respectively while the viable cells of both strains bind only 54.35 and 81.0%, respectively. Washing the cells of *L. lacti*s pellet binding the AFB_1 by buffer, methanol and chloroform released 13.13, 20.0 and 27.5% of the toxin, respectively. Buffer solution and methanol could not released the AFB_1 complex formed with the living or dead cells of *S. thermophlilus*, while the chloroform released 39.5, 17.0 and 28.12 of AFB_1 from living cells, boiling cells and autoclaving cells, respectively. Dead cells pellet of *L. lacti*s removed 100% of AFB_1 (2 µg) contaminating, maize, sunflower and soybean oils, while dead cells of *S. thermophilus* pellet removed 96.8, 81.0 and 96% of the toxin contaminated previous oils, respectively.

Key Words: Removal of aflatoxin; Contaminated liquid; Acid bacteria

INTRODUCTION

Mycotoxins are toxic substances produced by filamentous fungi that grow on agricultural products in the field before harvest or during storage (Pitt & Hocking, 1997; Arafa *et al.*, 2000; Aziz *et al.*, 2004). Aflatoxins and other toxic metabolites have been found in edible tissues, milk and eggs for human use after ingestion of contaminated feed by farm animals (Rustom, 1997; Pittet, 1998). *Aspergillus flavus* and *A. parasiticus* are the primary fungal species that produce aflatoxins in food and feed commodities (Gourama & Bullerman, 1995; Aziz *et al.*, 2002; Aziz *et al.*, 2004). Aflatoxin B_1 (AFB_1) is considered at present to be one of the most dangerous contaminants in food and feed (Saleh & Aziz, 1996; Aziz & Youssef, 2002; Aziz *et al.*, 2004; Aziz & Mahrous, 2004). Aflatoxins are hepatocarcinogens and inhalation of aflatoxin can also impair human health (Eaton & Gallagher, 1994).

The ubiquity of the aflatoxin-producing fungi and the potent biological activity of their mycotoxins at very low concentrations have stimulated much research on different aspects to control toxic production (Aziz & Shahin, 1997). Aflatoxins are also of industrial importance due to the economic loss resulting from the contaminated crop, (Pittet, 1998). Consequently there is a great demand for novel strategies to prevent both the formation of aflatoxin in foods and feeds and the impact of existing aflatoxin contamination (El-Nezami *et al.*, 2000). Previous studies have shown that two probiotic strains *L. rhamnosus* strain GG (ATCC5103) and *L. rhamnosus* strain LC-705 (DSM 7061), efficiently remove AFB_1 from solution (El-Nezami *et al.*, 1996 & 1998a; Haskard *et al.*, 2001). Similar mutagen-binding abilities have been reported for viable and non-viable (heat-treated) bacteria (Zhang & Ohta, 1990; Orrhage *et al.*, 1994; Thyagaraja & Hosono, 1994; Sreekuman & Hosono, 1998).

Non-viable (heat & acid-treated) strains GG and Lc-705 bind AFB_1 as effectively as viable bacteria (El-Nezami *et al.*, 1998b).

The present work was designed to study the ability of viable and non-viable (heat-treated) specific bacterial strains isolated from dairy products for binding AFB_1 from liquid media and the influence of continual washing on the stability of AFB_1 complex formed with bacterial strains. The capability of the selected strains for removing AFB_1 from certain economic vegetable oils was also studied.

MATERIALS AND METHODS

Microorganisms. Forty-two bacterial isolates were isolated from the dairy products: Yogourt, Raw milk and Karish Cheese using MRS agar medium (Oxoid, 1982). Twenty-seven isolates were found to belong to genus Lactococcus and fifteen isolates belonging to genus Streptococcus according to Buchaman and Gibbons (1984). The 42 isolates were tested for their capability for binding AFB_1 each isolate was cultured in 25 mL of MRS broth medium in conical flask, incubated for 24 h at 37°C. Therefore known amount of AFB_1 (2 µg) was added to each flask and after 30 min of incubation all bacterial samples were centrifuged. The remainder AFB_1 in the media was detected

by TLC. Twelve from the previous isolates were found to be positive for binding AFB_1 with different levels. The highest isolates binding AFB_1 were completely identified as *L. lactis* and *S. thermophilus.*

Binding assay. *Lactococcus lactis* and *Strephococcus thermophilus* were cultured in 25 mL of MRS broth medium for 24 h at 37°C, in conical flasks. The flasks of each strains were devided into three groups. The culture of each flask (10^7 - 10^8 CFU/mL) of the first group was centrifuged (viable cells), while the second group was boiled at 100°C for 30 min and the third group was autoclaved at 121°C for 15 min (dead cells) before the centrifugation process for obtaining the pellets of the strains.

The bacterial pellets of each strain either viable or non-viable were suspended in 4 mL of phosphate buffer saline (PBS) containing 2 µg of AFB_1 for ½ h then centrifuged at 5000 rpm/min for 10 min. The remainder AFB_1 in the solution was quantified by TLC and the binding AFB_1 was calculated.

Complex stability. In this experiment the AFB_1 complex stability with the bacterial cells was evaluated. The pellets obtained from the previous experiment, were washed by 4 mL of PBS, methanol or chloroform. After centrifugation, the released AFB_1 was quantified by TLC and the AFB_1 remaining in the pellets were calculated.

Removing of AFB_1 contaminated some vegatable oils. Viable and non-viable bacterial pellets obtained from 25 mL MRS broth medium were mixed with 4 mL of vegetable oils (maize, sunflower or soyabean) containing 2 µg AFB_1 for ½ h. Thereafter all samples were centrifuged then the released AFB_1 in oils were determined subsequently the remained binding with bacterial cells were calculated.

Aflatoxin B_1 assay. AFB_1 in all previous experiments was determined according to the method described by (AOAC, 1984).

Statistical analysis. All data were statistically analysed according to Sendecor and Cochran (1980), last significant difference (LSD) was used for comparing treatment means.

RESULTS AND DISCUSSION

Various food commodities may be contaminated with aflatoxins, which even in small quantities have been detrimental effects on human and animal health. This study assessed the binding of aflatoxin B_1 (AFB_1) from contaminated solution by 27 strains of *Lactococcus* sp. and 15 strains of *Streptococcus* sp. isolated from yogourt, raw milk and karish cheese, (Table I).

Twelve out of the forty two previous isolates have shown different levels to bind AFB_1. The highest isolates for binding AFB_1 were identified as *Lactococcus lactis* and *Sterptococcus thermophilus,* where they bind 54.85% and 81.0% of the toxin, respectively (Table II). Peltonen *et al.* (2001) studied the binding of aflatoxin B_1 (AFB_1) from contaminated solution by 12 *Lactobacillus,* five *Bifidobacterium* and three *Lactococcus* strains and they revealed that two *Lactobacillus amylovorus* and one *Lactobacillus rhamnosus* strains removed more than 50% AFB_1. Also, El-Nezami *et al.* (1998a) reported that *Lactobacillus rhamnosus* strain GG (LB GG) and *L. rhamnosus* strain LC (LC 705) can significantly remove AFB_1 when compared with other strains of either Gram-positive or Gram-negative bacteria and the removal of AFB_1 by these two strains was both temperature and bacterial concentration dependent.

The data in (Table III) revealed that the two tested strains were efficient in binding AFB_1. It is clear from the table that dead cells by boiling of *L. lactis* and *S. thermophilus* bind 86.1 and 100% of AFB_1 (2 µg) added to the phosphate buffer, respectively compairing with 80 and 83% in case of dead cells by autoclaving, while the viable cells of both strains bind only 54.85 and 81% of AFB_1, respectively. Pierides *et al.* (2000) studied a safe and practical decontamination method with specific lactic bacteria strains to remove aflatoxin M_1 (AFM_1) from liquid media and the authors reported that all strains whether viable or heat-killed, could reduce the AFM_1 content of the liquid medium. Furthermore, Haskard *et al.* (2001) revealed that *Lactobacillus rhamnosus* strain GG (A53103) and *L. rhamnosus* strain LC-705 (DSM 7061) removed AFB_1 most efficiently and the non-viable (heat-or acid-treated) bacteria retained the highest amount of AFB_1. The protective effect of bacterial strains against aflatoxins has been reported by several workers (El-Nezami *et al.,* 1998b; Kankaanpaa *et al.,* 2000; Pierides *et al.,* 2000; Peltonen *et al.,* 2001), who revealed that physical binding has been proposed as one mechanism of toxin removal.

Table IV shows the effect of washing with buffer, methanol and chloroform on releasing of AFB_1 remaining bound with viable and non-viable (heat-treated) *L. lactis* and *S. thermophilus.* After three washes with phosphate buffer and methanol did not release any detectable AFB_1 from viable or non-viable *S. thermophilus* cells and 39.51, 17.0 and 28.29% of the AFB_1 released after three washes by chloroform. On the other hand the data show that 6.4 and 13.13% of the AFB_1 released after washing with phosphate buffer for boiling and autoclaving *L. lactis,* respectively while 6.89 and 20% of AFB1 released after three washes with methanol and 13.95 and 27.50 for chloroform, respectively.

Haskard *et al.* (2001) studied the stability of AFB_1 complex formed with 12 bacterial strains in both viable and non-viable heat-or acid treated forms by repetitive aqueous extraction and the authors reported that by the fifth extraction up to 71% of the total AFB_1 remained bound and non-viable bacteria retained the highest amount of AFB_1. The authors concluded that the binding is of a reversible nature, but the stability of the complexes formed depends on strain, treatment and environmental conditions. The stability of complex formed between AFB_1 and heat-treated *S. thermophilus* in the present study is similar to that of heat-treated *Bifidobacterium* strains (Oatley *et al.,* 2000).

Table I. Screening of bacterial isolates from dairy products for binding aflatoxin B_1 (AFB_1)

Substrate	No. isolates	Genus	Level of binding toxin	Substrate	No. isolates	Genus	Level of binding toxin
Yogourt	1	Strep. spp	++	Karish cheese	28	Lac. spp	-
	2	Strep. spp	-		29	Lac. spp	-
	3	Strep. spp	+		30	Lac. spp	-
	4	Strep. spp	-		31	Lac. spp	+
	5	Strep. spp	++		32	Lac. spp	-
	6	Strep. spp	+		33	Lac. spp	-
	7	Strep. spp	-		34	Lac. spp	-
	8	Strep. spp	-		35	Lac. spp	-
	9	Strep. spp	+		36	Lac. spp	-
	10	Strep. spp	-		37	Lac. spp	-
	11	Lac. spp	-		38	Lac. spp	-
	12	Lac. spp	-		39	Lac. spp	-
	13	Lac. spp	++		40	Lac. spp	-
	14	Lac. spp	-		41	Lac. spp	-
	15	Lac. spp	+++		42	Lac. spp	-
	16	Lac. spp	-				
	17	Lac. spp	-				
	18	Lac. spp	-				
	19	Lac. spp	+				
	20	Lac. spp	-				
	21	Lac. spp	-				
Raw milk	22	Lac. spp	++				
	23	Strep. spp	-				
	24	Strep. spp	++				
	25	Strep. spp	-				
	26	Strep. spp	+				
	27	Strep. spp	-				

-: Negative +: Weak
++: Medium +++: Hight
*: Initial AFB1 = 2 µg

Table II. Ability of *Lactococcus lactis* and *Streptococcus thermophilus* for binding AFB_1

Strain	Con. of AFB_1 (2µg)	*Remaining of AFB_1 (µg)	**Binding of AFB_1 (µg)	Percentage of binding AFB_1 (%)
Lactococcus lactis	2	0.913	1.087	54.85
Streptococcus thermophilus	2	0.380	1.62	81.0

*detected in buffer **Binding by bacterial cells

TableIII. Comparison between viable and dead cells of *Lactococcus lactis* and *Streptococcus thermophilus* for binding AFB_1

Treatments	Lactococcus lactis				Streptococcus thermophilus			
	*Concentration of AFB_1 remainder (µg)	%	*Concentration of AFB_1 bound (µg)	%	*Concentration xx of AFB_1 remainder (µg)	%	*Concentration of AFB_1 bound (µg)	%
Living cells (viable cells)	0.913	45.65	1.087a	54.35	0.38	19.0	1.62a	81.0
Dead cells by boiling	0.278	13.90	1.720b	86.10	0.00	0.00	2.00b	100.0
Dead cells by autoclaving	0.400	20.00	1.60c	80.00	0.34	17.00	1.66c	83.0

* Initial AFB_1 concentration = 2µg Values are mean of three replicates a, b, c: indicates statistical differences in mean ($P \leq 0.05$)

However other studies (Haskard *et al.*, 2000 & 2001), have shown that the relative amounts of AFB_1 removed by viable and heat and acid treated bacteria depend on initial AFB_1 concentrations.

(Morotomi & Mutai, 1986; Tanabe *et al.*, 1991; Rajendran & Ohta, 1998) reported that the cell wall polysaccharide and peptidoglycan are the two main elements responsible for the binding of mutagens to lactic acid bacteria. This perturbation of the bacterial cell wall may allow AFB_1 to bind to cell wall and plasma membrane constituents that are not available when the bacterial cell is intact. The effective removal of AFB_1 by all non-viable bacteria suggests that binding rather than metabolism is involved in all cases.

In a previous study Haskard *et al.* (2001) revealed that autoclaving and sonication did not release any detectable AFB_1 from bacterial pellets that had been washed five times with water and the authors concluded that binding of AFB_1 appears to be predominantly extracellur for viable and heat-treated bacteria. They added that denaturation by high temperatures does not cause the most strongly bound AFB_1 to be released and that the AFB_1 is not bound to loosely attached bacterial components.

Haskard *et al.* (2001) observed that the organic

Table IV. Effect of washing bacterial pellets with buffer, methanol and chloroform on releasing AFB_1 binding with viable and nonviable *L. lactis* and *S. thermophilus

Treatments		Buffer		Methanol		Chloroform	
	Concentration of AFB_1 in pellet (µg)	Released AFB_1 (µg)	%	Released AFB_1 (µg)	%	Released AFB_1 (µg)	%
			Lactococcus lactis				
Living cells	1.087	0.45 a	41.4	0.57 a	52.4	0.61 a	56.12
Dead cells by boiling	1.720	0.11 b	6.4	0.12 b	6.98	0.24 b	13.95
Dead cells by autoclaving	1.60	0.21 c	13.13	0.32 c	20.00	0.44 c	27.50
			Streptococcus thermophilus				
Living cells	1.62	0.0	0.0	0.0	0.0	0.64 a	39.51
Dead cells by boiling	2.00	0.0	0.0	0.0	0.0	0.34 b	17.00
Dead cells by autoclaving	1.66	0.0	0.0	0.0	0.0	0.48 c	28.92

*Values are mean of three replicates a, b, c: indicates statistical differences in mean ($P \leq 0.05$)

Table V. Capability of *L. lactis* and *S. thermophilus* (viable or dead cells) for removing AFB_1 contaminated vegetable oils.

Strains	Oils	Living cells			Dead cells*		
		Remainder AFB_1 (µg)	Bound AFB_1 (µg)	%	Remainder AFB_1 (µg)	Bound AFB_1 (µg)	%
L. lactis	Maize	0.266	1.734 a	86.7 a	0.00	2.0 a	100 a
	Sunflower	0.354	1.646 b	82.3 b	0.00	2.0 b	100 a
	Soybean	0.580	1.420 c	71.0 c	0.00	2.0 c	100 a
S.thermophilus	Maize	0.170 a	1.830 a	91.5 a	0.065	1.935 a	96.8 a
	Sunflower	0.187 a	1.813 a	90.7 a	0.366	1.634 b	81.7 b
	Soybean	0.670 b	1.330 b	66.5 b	0.080	1.920 a	96.0 c

Initial AFB_1 concentration (2 µg) Values are mean of three replicates

* Dead cell: by boiling a, b, c: indicates statistical differences in mean ($P \leq 0.05$)

solvents released almost all AFB_1 bound to *L. rhamnosus* G.G and *L. rhamnosus* Lc 705 and the authors noticed that the order of effectiveness of extraction methanol < acetonitrile = benzyne < chloroform, does not match the order of decreasing polarity and finally, this may be because the hydrophobicily of the AFB_1 molecule most closely and these results show that the hydrophobic interactions play a major role in the binding mechanism. These observation were previously documented by Haskard *et al.* (2000), who revealed that chloroform extraction does not exposure the intracellular hypobilic membrane, but rather only extracts extracellular components. Theoretical calculations by Oatley *et al.* (2000) demonstrate that AFB_1 removal does not aries solely from trapping of the toxin in the bacterial pellet during centrifugation.

Table V reveals the percentage of AFB_1 bound with viable and heat-treated bacteria and the remaining bound using different vegetable oils. It is clear that 71 to 86.7% and 100% of the AFB_1 remaining bound for viable and non-viable *L. lactis* and 66.5 to 91.5% and 81.7 to 96.8% of the AFB_1 remained bound for both viable and non-viable *S. thermophilus*, respectively in the different types of vegetable oils. Rasic *et al.* (1991) added AFB_1 to the yoghurt and acidified milk at concentrations of 1000 and 1400 µg/Kg. They found that the AFB_1 reduced in yoghurts (pH 4.0) by 97.8 and 90%, respectively. The maximum decrease of AFB_1 was occurred during the milk fermentation. They also found that the decrease of AFB_1 (conc. 1000 µg/Kg) in milk acidified with citric, lactic and acetic acids (pH 4.0) was 90, 84 and 73%, respectively.

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