

Efficiency of Selected Lactic Acid Bacteria Isolated from some Dairy Products on Aflatoxin B₁ and Ochratoxin A

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Abstract

Aflatoxin B₁ (AFB₁) and ochratoxin A (OTA) are two of the most important of mycotoxins associated with tropical and subtropical climate as in Egypt. Therefore, this study aimed to isolated lactic acid bacteria (LAB) and *Bifidobacteria* from some local dairy products from Egypt and using some strains to reduce/or remove AFB₁ and OTA toxins. Fifty samples, of milk and dairy products, including ten samples each, of Kareish, Damietta cheese (soft type), buffalo's milk, yoghurt (zabady), and naturally acidified milk (Rayeb) were screened for their load of *Lactobacilli*, *Lactococci* and *Bifidobacteria*, using MRS agar, M17 agar and (MRS-Cys) agar, respectively. The obtained data indicated that 38, 31 and 22 isolates belonged to *Lactobacillus*, *Lactococcus* and genus *Bifidobacterium*, respectively. Fourteen strains isolated from LAB and *Bifidobacterium* were tested on AFB₁ and OTA in PBs contaminated by 10 ppb with three times incubation periods (6, 12 and 36 hours) at 37°C. The results indicated that all tested strains were able to reduce AFB₁ at different rates ranging between 12.1 to 65.4% after incubation for 6 h. These rates increased to 78.8% and 89.9% after incubation at 37°C for 12 and 36h, respectively. The same results were with OTA, where percentages of reduction ranging from 81.4 to 80.4% were obtained when incubation with LAB cells was done at 37°C and prolonged to 36 h. According to the results, the percentage of reduction increased with the increasing time for each strain.

Keywords: Lactic acid bacteria (LAB), aflatoxin B₁ (AFB₁), ochratoxin A (OTA)

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INTRODUCTION

Mycotoxins can be defined as toxic secondary metabolites that are produced by many species of fungi in the field and /or during storage. Aflatoxin B₁ (AFB₁) and ochratoxin A (OTA) are two of the most important mycotoxins regarding their occurrence and toxicity¹. AFB₁ and OTA were classified by the International Agency for Research on Cancer (IARC) as human carcinogens that belong to Group 1 and Group 2B, respectively². Contamination by AFB₁ and OTA harms human and animal health and causes big economic losses that reach billions of dollars every year. Thus, getting rid of this toxin in food and feed by using certain strategies is needed urgently. Current methods to reduce mycotoxin contamination in food and feed can be classified into physical, chemical and biological methods^{3,4}. Many studies used the bacteria, yeasts and molds to reduce or degrade AFB₁ and OTA in foods and feeds^{5,6}. Lactic acid bacteria (LAB) are a good choice for control or reduce AFB₁ and OTA from a contaminated medium, generally considered to be safe according to USFDA; some of them also have many benefits on health, which are called probiotics bacteria⁷. Viable and non-viable cells of LAB have an adsorbent ability to bind toxins. Where it overlaps between the toxins and the functional groups of the cell surface happens, adsorption occurs. A lot of factors like temperature, pH, time of incubation, bacterial concentration and type of strain may affect the ability of LAB to bind toxins^{8,9}. Therefore, this study aimed to evaluate the ability of some strains LAB, isolated from dairy products, to reduce or degrade AFB₁ and OTA from liquid media at (6h, 12h and 36 h) period incubation.

MATERIALS AND METHODS

Samples from dairy and dairy products

Fifty samples were collected from milk and dairy products. The collected samples included ten samples from each of buffalo's milk, yoghurt, naturally acidified milk (Rayeb), Kareish and Domiati cheese (soft type). All samples were analyzed using three isolation media. De Man, Rogosa, Sharp agar (MRS) agar (Oxoid, UK) was used for isolation of *Lactobacilli*, MRS medium supplemented with L-cysteine (0.5 g/liter) (MRS-Cys) agar was used for isolation of *Bifidobacteria*,

however, M17 medium (Oxoid) agar was used for isolation of *Lactococci spp.*

Microbiological assessment of products

Twenty-five g/ml of each sample was aseptically transferred to conical flask 500 ml and homogenized in 225 ml of sterile tri-sodium citrate¹⁰. Ten-flods dilutions of homogenates were prepared and inoculated onto plates of MRS, MRS-Cys agar, which incubated in anaerobic condition using Co₂ generated kits (Anaero-Gen, Oxoid) and incubated at 37°C for 48 h, while M17 plates were incubated in aerobic conditions at 32°C. All plates were incubated for a period of three to five days^{11,12}.

Identification of LAB and *Bifidobacterium*

All the selected strains (120) isolates were activated twice in MRS-Cys, MRS and M17 broth at 37°C for 24 h. The selected isolates were observed by optical microscopy to determine their morphology and Gram staining results¹³. Additionally, they were tested for catalase¹⁴, oxidase, nitrate reductase, gelatinase activities, production of indol, and production of gas from glucose¹⁵. The API-50CHL identification system (BioMerieux, Lyon, France) was used for *Lactobacilli* and *Bifidobacterium*, whereas the API- 20 STREP identification system (Bio-Merieux, Lyon, France) was used for *Lactococci*.

Preparation of culture cells

The strains of lactic acid bacteria were activated overnight (16 h) in 100 ml MRS, MRS-Cys and M17 broth. A cell free solution was obtained by centrifugation of culture at 5000 rpm at 4°C for 20 min.

Preparation of AFB₁ and OTA spiked solution

AFB₁ and OTA standards were purchased from Sigma, chemical Co. (St. Louis, MO, U.S.A). Stock solutions and standards were prepared and assayed according to AOAC Method 971.22, 2005¹⁶. Standards separately were added by 10 ppb to 100 mL phosphate buffered saline (PBS).¹⁷All solvents were of HPLC grade. The water was double distilled with Millipore water purification system (Bedford, M A, USA).

AFB₁ and OTA binding assay *in vitro*

One hundred milligrams of bacterial cells were added to 100 ml of PBs contaminated by 10 ppb of AFB₁ and OTA both are separate from each other. Samples were incubated at 37°C for 6, 12

and 36h for testability of strains for binding of toxins. Then in each incubation period; filtration for discarding bacterial cells was done through filtration then extraction and determination of AFB₁ or OTA in PBs (100 mL) with three replicates for each incubation time.

Extraction and determination of AFB₁ using HPLC

AFB₁ was extracted and cleanup using immunoaffinity column (IAC) Aflatest[®]-p affinity to obtained the dry film, then determination AFB₁ in samples after derivatives process by adding 100µl of trifluoroacetic acid (TFA) to samples and mixed well for 30 s and the mixture stands for 15 min, finally measurement by HPLC¹⁷

Extraction and determination of OTA using HPLC

OTA was extracted and cleaned up using the method described in AOAC, (2007)¹⁸ using the IAC (Ochra Test[®]-p affinity column), then concentrations of OTA were determined by High- Performance Liquid Chromatography (HPLC) system¹⁹.

Statistical analyses

All data were statistically analyzed using the General Linear Model procedure of the SPSS ver. 18 (IBM Corp, NY). The significance of the differences among treatment groups was determined by Waller–Duncan k-ratio. All statements of significance were based on the probability of P- value ≤0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria and Bifidobacterium

A total of fifty samples of milk and dairy products were investigated for their load of LAB and *Bifidobacterium* spp. This included 10 samples, each of buffalo’s and Rayeb milk, yoghurt, Kareish and Domiati cheese. The results showed in (Fig. 1) as an average log of a colony-forming unit (cfu) of LAB and *Bifidobacterium* spp.

Yoghurt was the highest product in log count among all other products, for example the

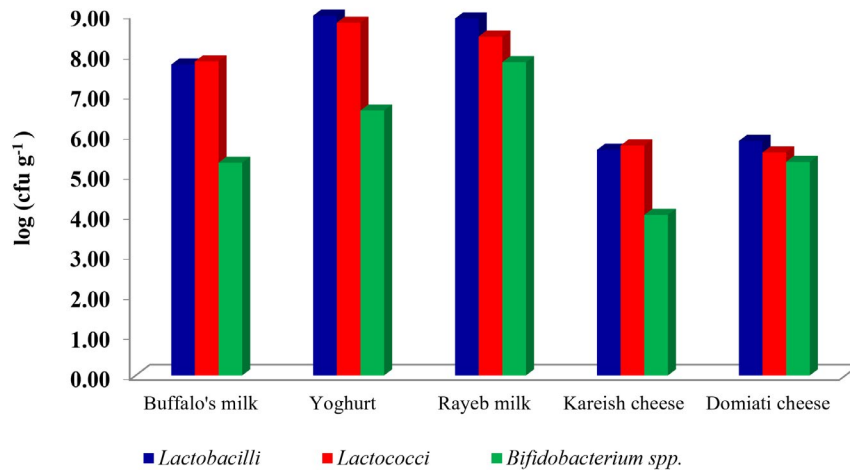


Fig. 1. Log (cfu g/ml-1) of LAB and *Bifidobacterium* in some dairy and dairy products.

Table 1. LAB isolated from some dairy and dairy products samples

Products	<i>Lactobacilli</i>		<i>Lactococci</i>		<i>Bifidobacterium</i> spp.	
	Isolates	Strain	Isolates	Strain	Isolates	Strain
buffalo's milk	14	6	15	4	13	4
Yoghurt	22	9	21	8	17	5
Rayeb milk	24	10	25	7	21	6
Kareishcheese	13	6	14	5	8	3
Domiati cheese	16	7	17	7	10	4
Total	89	38	92	31	69	22

count of *Lactobacilli* was 8.97 cfu g⁻¹, and 8.79 cfu g⁻¹ for *Lactococci*, whereas kareish cheese was the lowest product in log count among all other products. For example, the count of *Lactobacilli* was 5.62cfu g⁻¹ and 4.00 for *Bifidobacterium*. The same point of view was adapted by El-Kholy et al., (2016)¹⁰ who reported that the highest counts of *L. reuteri* were recorded for functional Tallaga cheese made with 3, 5 and 10% of mushroom. However, the results of this study were similar to Hussein et al., (2017)²⁰, who reported that, counts of *Lb. bulgaricus* and *S. thermophilus* increased until day 3 in all yoghurt treatments (p>0.05), then declined thereafter. The obtained data in Table (1) showed that 250 isolates were picked up from the specific selective media of *Lactobacilli*, *Lactococci* and *Bifidobacterium*. All the selected isolates of LAB and *Bifidobacterium* were Catalase-

negative and Gram-positive. Ninety-two isolates were spherical; however, the other 158 isolates were rod shaped. These isolates were taken to be identified according to their morphological, cultural characteristics according to Sneath et al., (2009)²¹ and 50CHL API- 20STREP identification system (BioMerieux).

Among the identified 91 LAB and *Bifidobacterium* strains, 31 isolates belong to genus *Lactococcus*, while the other 38 isolates belong to genus *Lactobacillus* and 22 isolates belong to genus *Bifidobacterium*. The species which represent the total cocci were identified as (9 strains) *Lactococcuslactis* sub sp. *Lactis*, (4 strains) *Leuconostoc mesenteroides*, (11 strains) *Lactococcus cremoris* and (7 strains) *Streptococcus thermophilus*. On the other hand, the identified strains belonging to genus *Lactobacillus*, 8 strains

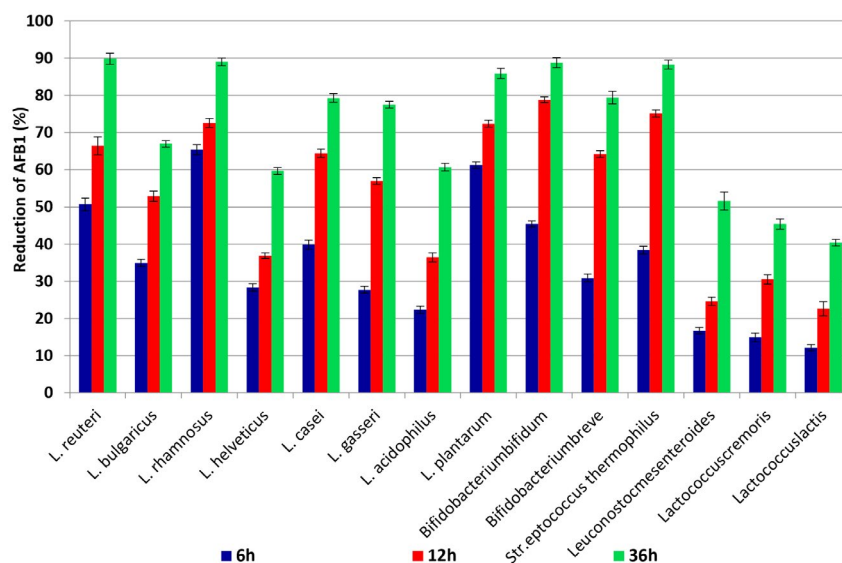


Fig. 2. The percentages of reduction AFB1 after incubation for 6h, 12h and 36h at 37°C with LAB cells.

Table 2. ANOVA of the effect of different incubation time and type of LAB strains on reduction AFB1

Source	SS	df	MS	F	P
Intercept	360767.631	1	360767.6	59956.35	0.000000
LAB	35011.1	13	2693.161	447.579	0.000000
Time	28302.06	2	14151.03	2351.774	0.000000
LAB*time	2778.899	26	106.881	17.763	0.000000
Error	505.442	84	6.017		
Total	427365.1	126			

SS: Sum of Squares, df: degree of freedom, MS: mean square, P: probability at confidence 0.95

of them belonged to *Lactobacillus acidophilus*, 3 strains of them belonged to *Lactobacillus casei* sub sp. *ramnosus*, 5 strains of them belonged to *Lactobacillus plantarum*, 4 strains of them belonged to *Lactobacillus helveticus*, 3 strains of them belonged to *Lactobacillus gasseri*, 4 strains of them belonged to *Lactobacillus reuteri*, 6 strains of them belonged to *Lactobacillus delbrueckii* sub sp. *bulgaricus* and 5 strains of them belonged to *Lactobacillus casei* sub sp. *casei*. Furthermore, the identified strains belonging to genus *Bifidobacterium* 9 strains of them belonged to *Bifidobacterium bifidum* and 13 strains of them belonged to *Bifidobacterium breve*.

Evaluation of efficacy of some LAB strains to reduce AFB₁

The results indicated that using *L. rhamnosus* gave the highest reduction for AFB₁

after incubation at 37°C for 6, 12 and 36 h, where 65.4%, 72.6% and 89.0%, respectively. When using *L. plantarum* for removing or degradation of AFB₁, the increase in removal with increased time of incubation was 61.3%, 72.4 and 85.9% after 6, 12 and 36h, respectively. While *Bifidobacterium bifidum* and *Streptococcus thermophilus* reduced AFB₁ 88.8 and 88.3% after 36h of incubation time, respectively. Generally, the minimum reduction percentages of AFB₁ were 12.1, 22.6 and 40.4% after incubation for 6h, 12h and 36h, respectively with *Lactococcus lactis*. ANOVA analysis of the effect of incubation time and type of LAB strains on the content of AFB₁ in liquid media is shown in (Table 2), which shows that there is a significant effect due to the incubation period as well as LAB strain. The obtained data shown in (Fig. 2) reflected that bind of AFB₁

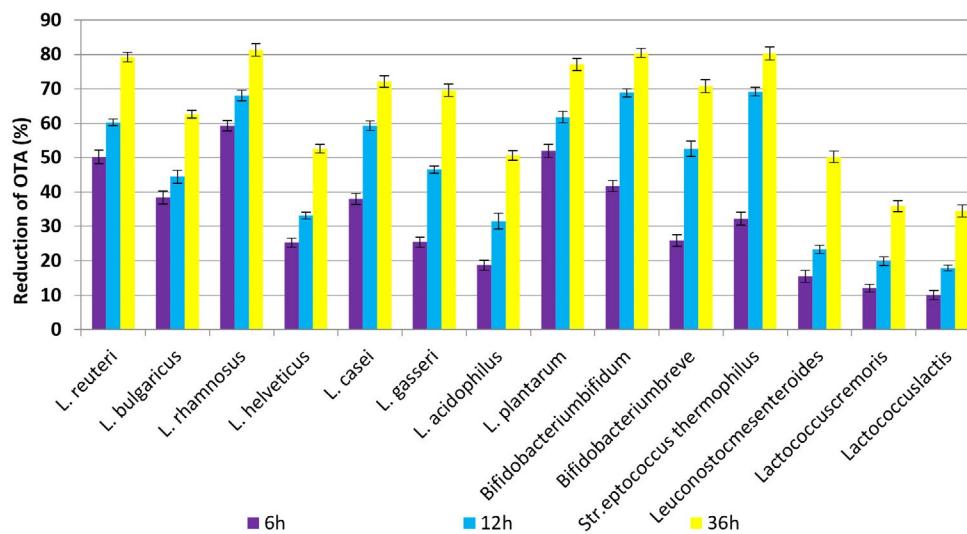


Fig. 3. The percentages of reduction OTA after incubation for 6h, 12h and 36h at 37°C with LAB cells.

Table 3. ANOVA for effect of incubation time and type of LAB strains on reduction OTA

Source	SS	df	MS	F	P
Intercept	284572.571	1	284572.571	8399.328	0.000000
LAB	31173.733	13	2397.979	70.77779	0.000000
Time	17536.946	2	8768.473	258.8067	0.000000
LAB*time	5172.956	26	198.960	5.872418	0.000000
Error	2845.953	84	33.880		
Total	341302.160	126			

SS: Sum of Squares, df: degree of freedom, MS: mean square, P: probability at confidence 0.95.

depends heavily on incubation time, where the highest binding occurred after incubation for 36h using *L. reuteri* and *L. rhamnosus* 89.9 and 89.0%, respectively, moreover *Bifidobacterium bifidum* and *Stre. thermophilus* removed 88.8% and 88.3% from AFB₁ at the same time, respectively. Many previous studies reported that the binding of AFB₁ by LAB depends on several factors such as incubation time, concentration of toxin, bacterial counts, temperature and pH. *L. rhamnosus* and *L. acidophilus* successfully got rid of nearly 80 and 60% of AFB₁; respectively in PBS. They also noticed a rapid toxin binding average (80%) in 1 h by LAB²². Peltonen et al., (2001)²³, reported that *L. rhamnosus* could successfully bind >50% of AFB₁ content in PBs at 37°C within 24 h. On the other hand, Kankaanpaa et al., (2000)²⁴, noticed that the ability of AFB₁ to bind *in vitro* and *in vivo* depended on LAB strain. Though the complex formed between AFB₁ and bacteria was proved to be stable, the mechanism of binding between AFB₁ and LAB was not illustrated yet. It was thought that AFB₁ binds to cell wall components, including both polysaccharides and peptidoglycans. It was identified before that both hydrogen bonds and Van der Waals interactions were involved in AFB₁ binding²⁵⁻²⁷. AFB₁ binds by the LAB cell wall during processes namely adsorption and desorption, primarily, the AFB₁ molecule is trapped inside the single helix of the (1 → 3)-β-D-glucan chain. Then, the AFB₁ molecule is covered by the branched (1 → 6)-β-D-glucan chain, where it is preserved inside the helix. It also showed some involvement with both electrostatic interactions and hydrogen bonds between AFB₁ molecules and the bacterial cell wall. Furthermore, AFB₁ can be bound to the bacterial cell wall through weak non-covalent interactions such as associating with hydrophobic pockets on the bacterial surface. The differences in AFB₁ binding by the strains are probably due to the different bacterial cell wall and cell envelope structure^{28, 29}.

Impact of LAB on the content of OTA in contaminated PBs medium

The obtained results indicated that the highest reduction percentage of OTA after incubation for 6h was 59.3, 52.0 and 50.2% after incubation with cells from *L. rhamnosus*, *L. plantarum* and *L. reuteri*, respectively. Besides,

the lowest reduction was 10.5, 12.1 and 15.5% in samples incubated with *Lactococcus lactis*, *Lactococcus cremoris* and *Leuconostoc mesenteroides*, respectively. Incubation with *L. rhamnosus* for 12 and 36h removed 68.1 and 81.4% from OTA, respectively. On the other hand *Str. thermophilus* and *Bifidobacterium bifidum* cells removed 80.3 and 80.4% from OTA after incubation for 36h at 37°C. The ANOVA analysis in (Table 3) showed significant differences between reduction percentages of OTA after incubation with different LAB cells as well as significant differences in incubation time. These results indicated that the incubation time was one of the important factors for the efficiency of LAB cells to bind OTA in PBs. Increasing the incubation time to 12 and 36h led to increasing the reduction of OTA in contaminated PBs as shown in Fig. 3. These results are consistent with many studies conducted in this field²⁹, where Małgorzata, (2014)³⁰ found that in the case of live bacterial cells, OTA decrease ranged from 16.9% to 35% in MRS medium and from 14.8% to 26.4% in PBS after 12 h, while thermally inactivated bacterial biomass gave the higher binding to AFB₁ (46.2% to 59.8%). Furthermore, Del Prete et al. (2007)³¹ investigated the fifteen strains belonging to five relevant oenological LAB species that were grown in broth synthetic culture medium spiked with OTA. The portion of OTA removed during the bacterial growth was 8 to 28%. The OTA was removed from the supernatants between 31 to 57% by the bacterial pellet. The most effective mechanism to remove OTA is binding with the bacterial cell wall, where it contained peptidoglycan matrices, polysaccharides, as well as teichoic and lipoteichoic acid, and a protein S- layer. However, binding is based on the adsorption capacity of toxins to the cells and not on enzyme activity. This is where peptidoglycan and exopolysaccharides play an important role. LAB which is thermally inactivated gives higher removal capacity may due to changes on the cell surface. Another proposed hypothesis, namely binding of toxin by bacterial cells is also influenced by electron donor-acceptor and Lewis acid-base interactions, this may due to that the cells of LAB consider as strong electron donors and weak electron acceptors, which is supported by the hydrophilic nature of their surface^{32,33}.

CONCLUSION

Incubation of LAB cells with PBs contaminated with AFB₁ and OTA might reduce the toxin contamination. The obtained results indicated that all strains were able to reduce AFB₁ and OTA but at different rates which ranged between 10 to 50% with incubation for 6h and increased to 75 % after incubation for 12h. Moreover, increasing the incubation time with LAB cells to 36h led to removing 80 and more than 88% from AFB₁ and OTA in contaminated PBs.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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