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# Detoxification effect of Bifidobacterium bifidum against aflatoxin M1 in weaning baby food

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**ABSTRACT**— Aflatoxin M1 (AFM1) concerns potent hazard in baby food for infant and young children. Bacterial detoxification is a promising method to reduce mycotoxins in food matrix. Bifidobacterium bifidum investigate the binding capacity towards AFM1 in phosphate buffered saline (PBS) and prepared baby food. The binding ability was evaluated regarding to bacterial population (107, 108 and 109 cfu/g), incubation intervals (0, 6, 12 and 24 hrs), viable and heat treated cells against AFM1. The unbound AFM1 was quantitative analyzed by Competitive Direct ELISA method. The experimental use of several concentrations of B. bifidum showed gradual AFM1 decrease forming binding AFM1-bacteria complex by incubation time. The AFM1 reduction percent/24hrs of B. bifidum cells ranged from 34.15 to 56.66% in PBS and from 35.77 to 64.10% in baby food samples. High bacterial concentration, nonviable bacteria and increase storage intervals are factors had significant ($p<0.05$) AFM1-bacteria binding ability to reduce AFM1 in spiked baby food samples contained 1x109 cfu/g of nonviable bacterial cells to be 17.72±0.68 ng/kg. The high bacterial concentration of nonviable cells shows significant high detoxification effect in spiked samples, when compared with PBS ($p<0.05$) and may comply with the acceptable limits in the Egyptian standards. It could be concluded that, the bacterial population, heat-treated cells and time of incubation have a positive binding effect toward AFM1 in baby food.

**KEYWORDS:** Aflatoxin M1, Detoxification, Bifidobacterium bifidum, baby food.

## 1. INTRODUCTION

Infant and young children are the most vulnerable toward the adverse effect of mycotoxins three times higher than adults. It is due to detoxify the toxicants and the high metabolic rate [1]. Multiple ingredients present including milk powder, fruits drive baby food contamination to be inevitable, which conceder risk to children depends on the frequency and magnitude of exposure [2,3]. Aflatoxins are mycotoxins produced as hazardous secondary metabolites produced by certain members from *Aspergillus sp*, i.e. *Aspergillus flavus, Aspergillus parasiticus*, etc., causing carcinogenic, teratogenic and mutagenic effect [4]. Aflatoxin M1 (a carcinogenic mycotoxin produced in the liver) is the 4-hydroxy derivative of aflatoxin B1 which excreted in milk of lactating animals. Heat treatment such as sterilization or pasteurization cannot remove aflatoxin M1 from infants and baby food. To remove AFM1 from milk, Physical and chemical methods are limited because of safety issues, quality losses, poor efficiency, and high cost [5]. The prevalence of AFM1 was detected in 49% of raw milk samples ranged between 53 and 207 ng/kg with mean±standard deviation of 100.3±0.8ng/kg [6]. The Egyptian standards coined the maximum aflatoxin M1 level in infant and baby food should not exceed 25 ng/kg [7]. It is indispensable to keep mycotoxins within acceptable levels [2]. Several investigations have demonstrated the probiotic bacterial i.e. *Bifidobacterium bifidum,* potential to reduce and remove of AFM1 using in vivo and in vitro model systems [8,9]. Bifidobacteria are the predominant microorganisms in the breast fed infants large intestine contains about 99% of the cultivatable flora. Several health benefits have

been claimed for these live probiotic bacteria such as inhibition of certain diseases, immunity system promoting, reduction of lactose intolerance, reduction of cholesterol level and production of vitamins mainly the B group [10]. Bifidobacteria is probiotic, live nonpathogenic and able to survive in the digestive tract microbiota when consumed in adequate amount, can confer a health effect. It is a component of breast milk and consequent breastfed infant gut, while once soled food introduced to its weaning diet this dominant bacterium decreased [11]. It is applied as Lactic Acid Bacteria (LAB) whereas has specific sugar fermentation and phylogenetically unrelated [12]. Promising studies showed binding capability of probiotics as *Bifidobacterium sp* against aflatoxins in different food matrix [13,14]. Both viable and nonviable lactic acid bacteria and bifidobacterial cells can remove AFM1 by noncovalent binding the bacterial cell wall chemical components and the toxins [15]. This binding effect of AFM1 may range 21 to 92% and 26 to 94% against viable and nonviable cells, respectively [5]. AFM1 binding assay are conducted in phosphate buffered saline (PBS) as a buffer solution to assess the ability without interference with the effect of food matrix [16]. The aim of the present study was to investigate the potential of *Bifidobacterium bifidum* to reduce aflatoxin M1 and its application in baby food.

## 2. MATERIALS AND METHODS

### *2.1 Samples*

Weaning baby food was prepared in triplicate according to Bahlol et al. [17], where its formulas was chosen regarding to the most acceptable. Baby food formula (percent) was as follow: Papaya (45%), Potato (10%), Carrot (5%), Skim milk (5%), Sugar (5%), apricot (10%), mango (10%) and guava (10%). Samples were prepared as control and contaminated with AFM1 (50 ng/kg) and treated with viable or nonviable *Bifidobacterium bifidum* (T1=$10^7$, T2=$10^8$ and T3=$10^9$ cfu/g).

### *2.2 Bacterial strain*

Lyophilized *Bifidobacterium bifidum* EMCC1537 was obtained from Microbiological Resources Center (MIRCEN, Ain Shams University, Cairo, Egypt) in order to evaluate its binding ability. The strain was reactivated by subculturing on De Man Regosa and Sharp medium (MRS) broth at 37ºC/24 hrs. The resultant suspension was centrifuged (Centrifuge K2015, Centurion Scientific, UK) at 4000 rpm/15 min, the pellet was washed twice with phosphate buffered saline (PBS) then adjusted to the final concentrations [9]. The bacteria were used as either viable (active cells) or nonviable (heat treated cells at 90ºC/1 hr) cells [16].

### *2.3 Aflatoxin M1working solutions*

Aflatoxin M1 standard solution (10 µg/ml) in acetonitrile was supplied from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile was evaporated in water-bath (45ºC) then the dried AFM1 was suspended in PBS to form the final dilution and kept in amber vial at 4ºC until use in binding assay and contaminating baby food samples [18].

### *2.4 Aflatoxin M1 binding assay*

Active *Bifidobacterium bifidum* cultures (1 ml) contained T1=$10^7$, T2=$10^8$ and T3=$10^9$ cfu/ml were prepared in 1.5 ml eppendorf microtubes, centrifuged (Micro Centrifuge 5415C, Eppendorf, Germany) at 5000 rpm/10 min then the bacterial pellets were washed twice with 1 ml of sterile distilled water. The final bacterial pellets were resuspended in 1 ml of PBS spiked with 50 ng/kg of aflatoxin M1, shaked with vortex for 15 seconds then incubated at 37ºC/0, 6, 12, 24 hrs. The aflatoxin M1 binding effect at different concentrations of *Bifidobacterium bifidum* serial dilutions during the incubation periods were examined by centrifuging at 5000

rpm/10 min and the unbound aflatoxin M1 was determined in the supernatant using ELISA. Positive control contained PBS spiked with Aflatoxin M1 (50 ng/kg) and negative control contained non-spiked PBS with *Bifidobacterium bifidum* cells [14].

### *2.5 Aflatoxin M1 analysis*

Competetive Direct-Enzyme Linked Immunosorbent Assay (CD-ELISA) method was carried out for quantitative AFM1 detection using RIDASCREEN Aflatoxin M1 kit (Art. 1121, R-Biopharm AG, Darmstadt, Germany) according to its individual provided protocol [19]. Its microplate wells were subjected to microplate reader (MRX, Dynatech, UK) and the absorbance was measured at *k*= 450 nm with version 1.2 Software to obtain values in ng/kg.

### *2.6 Statistical analysis*

All measures were carried out in triplicates, in which all values were presented in as Average±Standard Deviation and Removal percent /24 hrs.

Removal percent/24 hrs was calculated following equation according to Khadivi et al. [20].

$$\text{Removal percent /24 hrs} = \left(1 - \frac{\text{AFM1/24 hrs}}{\text{AFM1/0 hrs}}\right) x100$$

Statistical analysis was performed by one-way analysis of variance (ANOVA) using SPSS 20.0 software package program (SPSS Inc., Chicago, IL, USA, 2011). The five analyzed factors were the *Bifidobacterium bifidum* population, the viability of bacterial strain (active or inactive), Aflatoxin M1 content and incubation period. Results considered statistical significant showed $p<0.05$ [16]. The results were expressed in line and bar charts.

## 3. RESULTS AND DISCUSSION

AFM1, which pose a serious impact on infant and children health [21], was investigated for its degradation in baby food (with skim milk component) by treatments contained concentrations of viable and nonviable *B. bifidum* stored at 4ºC for several intervals up to 24 hrs. *B. bifidum* was the added bacterial strain to baby food samples where it is the most abundant microflora in the gut of the healthy breast-fed infants [22]. The results in Figure (1: a, b and c) express the non-bound percent of AFM1 to reflect the potential of *B. bifidum* decrease the mycotoxin content in PBS. Gradual removal percent reveals the binding efficiency of the bacterial strain at 24 hrs. Highest bacterial concentration shows decrease of AFM1as well as time of storage which shows further decline the mycotoxin values. AFM1 content was the highest in treatments contained all of the bacterial concentrations of either viable or nonviable bacterial cells at the first storage interval (0 hr) ranged between 50.14±0.07 and 50.50±0.26 ng/kg, with no significant difference ($p>0.05$). The values of AFM1 were decreased by the time to be the lowest within 24 hrs storage interval of viable cells (31.62±1.14, 27.57±1.40 and 25.53±0.88 ng/kg) and nonviable cells (23.20±2.10, 20.59±0.40 and 17.72±0.68 ng/kg) due to the binding effect of the bacterial cells in the concentrations $10^7$, $10^8$ and $10^9$ cfu/g, respectively. Also, significant decrease was observed by prolong the storage interval ($p<0.05$). The highest removal percent showed the binding capacity of high concentration (1x$10^9$ cfu/g) of nonviable cells at the end of the storage interval (24 hrs) was 64.68%, while the lowest was 37.38 % with the lowest bacterial concentration (1x$10^7$ cfu/g). Positive and negative controls showed zero removal percent/24 hrs.

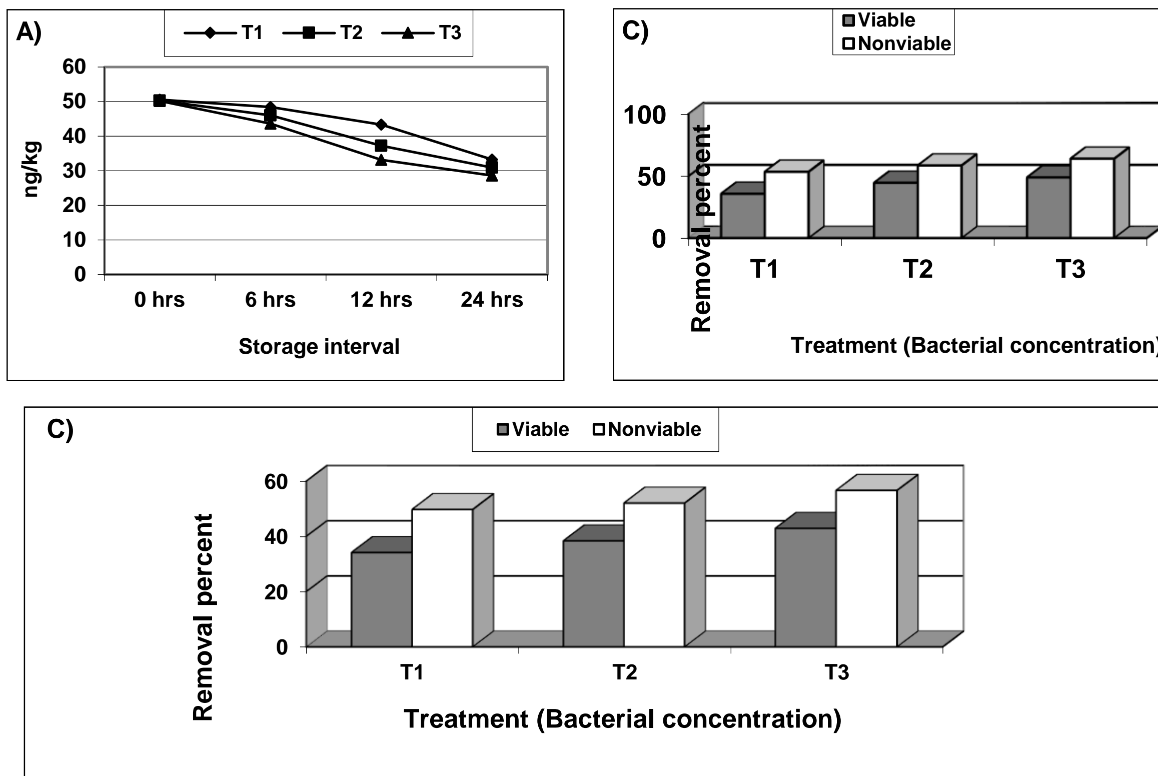


**Figure (1):** Binding efficiency of Bifidobacterium bifidum against AFM1 in PBS.

* T1=$10^7$, T2=$10^8$ and T3=$10^9$ cfu/g
** Significant reduction was observed between control and treatment samples ($p<0.05$).

A) Viable bacteria.
B) Nonviable bacteria.
C) Removal percent/24 hrs.

Figure (2: a, b and c) showed the baby food samples spiked with 50 ng/kg of AFM1. Detoxification of AFM1 was determined in different bacterial concentrations, during storage intervals up to 24 hrs at 4ºC and viability of B. bifidum bacterial cells. Significant reduction ($p<0.05$) was observed in samples contained nonviable bacterial cells compared with those contained viable cells expressed as removal percent/24 hrs were 53.76 to 64.68% and 37.38 to 49.08%, respectively. These values varied due to the bacterial cells concentrations in which the low (1x107 cfu/g) removed AFM1 to be 37.38 and 53.76% while the high (1x109 cfu/g) removed 49.08 and 64.68% in the presence of viable and nonviable bacterial cells, respectively. The storage interval showed AFM1 decrease with ranges between 49.23±2.11 to 49.36±1.65 and 33.25±1.56 to 21.73±2.09 ng/kg in 0 and 24 hrs, respectively. Concerning probiotic bacterial effect on reducing AFM1, the above mentioned results are consistent with those reported by Khadivi et al. [20], who found an extensive reduction from 82 to 90% with bacterial concentrations from 107 to 108 cfu/g in skimmed milk. Probiotic bacteria should be ≥ 108-109 cfu/g in food matrix to obtain significant binding effect [23]. Also, Rabie et al. [24] found AFM1 reduction ranged from 31.3 to 100% as a function of storage period, bacterial strain and combination of bacterial genera.

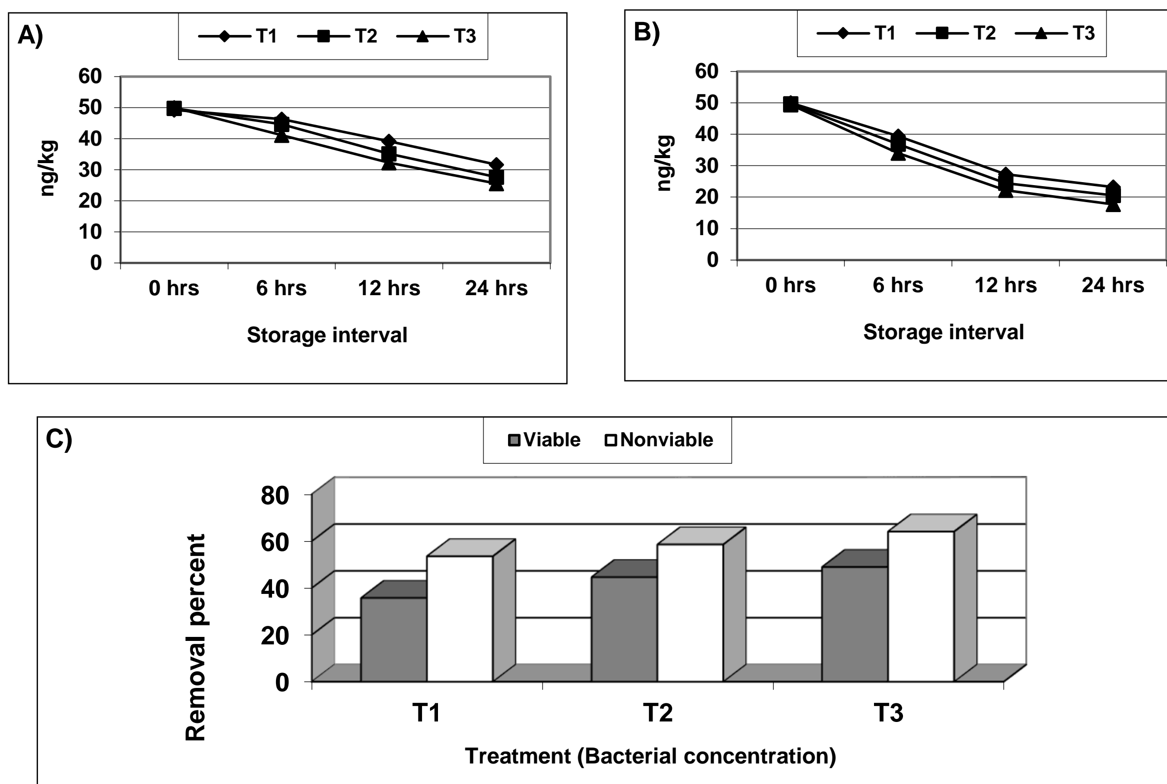


**Figure (2):** Binding efficiency of Bifidobacterium bifidum against AFM1 in spiked baby food.

* Treatments represent T1=107, T2=108 and T3=109 cfu/g.

** Significant reduction was observed between control and treatment samples ($p<0.05$).

A) Viable bacteria.

B) Nonviable bacteria.

C) Removal percent/24 hrs.

Products contained milk had higher reduction percent attribute to the binding effect of toxin and casein [23]. Elevation of probiotic bacterial cells concentration increases the removal percent of AFM1 in skimmed milk [25]. The ability to obtain AFM1-probiotic complex are based on physicochemical parameters as AFM1 and probiotics via temperature. The treatments contained more physicochemical factors had the maximum AFM1 reduction percentage [20]. Bacteria facilitate its binding capacity by captions with negative surface charge [26]. Without needing bacterial activity, cell wall binding eliminate AFM1 by its adhesiveness element, i.e. peptidoglycans, polysaccharides and teichoic acid [12]. Polysaccharides and peptidoglycans are able to be affected by heating which denature proteins, increase hydrophobic nature of surface and form Maillard reaction. These reactions let binding complex of aflatoxin to the plasmatic membrane and bacterial cell wall which are absent in intact cell wall [18]. Probiotics have the potential to inactivate toxins with surface binding with the great adhesive action of S-layer proteins in the bacterial cell wall. Heating treatment causes surface protein denaturation and partial peptidoglycan breakdown which lead to new binding sites [26]. Alignment of the probiotic cell pocket, alteration in the cell wall and diverse attachment sites differ the binding values between species [20]. The same results were reported in which probiotic bacteria has the potential to mitigate AFM1 and its toxic effect in food samples which enhance its food safety [9,14,16]. Whereas, partial reversible binding and release of AFM1 was found by Assaf et al. [16] with repeating washes, no such findings were observed in current study which is consistent with those reported by Panwar et al. [14] who

stated that, B. bifidum produce the most stable AFM1-bacterial complex. Also, many advantages of adding probiotic bacteria to food as supporting the immune system, anticarcinogenic activity, maintain normal flora in intestines and decrease gastrointestinal pathogens population [6].

## 4. CONCLUSION

Bifidobacterium bifidum has biological impact against AFM1 in weaning baby food matrix. The formed AFM1-bacteria complex is effective to produce safe milk contained baby food products. Bacterial population, viability (heat-treated cells) and time of incubation have positive effect toward bacterial-mycotoxin binding ability and consequence reducing AFM1. These results can be applied in commercial and economical opportunity to detoxify AFM1 content in weaning baby food through adding B. bifidum which is predominant in infant intestines.

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