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DECONTAMINATION OF AFLATOXIN M_1 IN MILK THROUGH INTEGRATION OF MICROBIAL CELLS WITH SORBITAN MONOSTEARATE, ACTIVATED CARBON AND BENTONITE

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ABSTRACT

Aflatoxin M_1 (AFM_1) is a highly toxic milk contaminant that poses a grave threat to human health. Among various strategies proposed for AFM_1 decontamination, use of microbes have been considered to be one of the suitable techniques. However, the use of microbial cells alone had not proven to be efficient at higher AFM_1 levels. Moreover, the complex between microbial cell and AFM_1 had been suggested to be weaker and is dissociated soon after its exposure to washings. The objective of this study was to enhance the AFM_1 binding efficiencies of bacterial cells (C) belonging to *Lactobacillus paracasei* and *Bacillus coagulans* in the presence of activated carbon (CAC), bentonite (CBENT) and sorbitan monostearate (CSP60). The reduction of AFM_1 was found to be directly proportional to the concentration of microbial cells. Heat killed and acid treated *L. paracasei* successfully reduced AFM_1 in milk spiked at 0.2 µg/L to 89% and 100% in CBENT, 84% and 90% in CAC, 59% and 47% in CSP60 and, 51.5% and 42% in C, respectively. Among treatments involving *B. coagulans*, acid treated CSP60 proved to be least effective showing 44.6% reduction, while CBENT for both acid and heat treated along with acid treated CAC proved to be most effective by removing 100% AFM_1. CBENT and CAC (acid and heat killed) among both bacterial strains showed the formation of most stable complex with AFM_1 showing no release of detectable AFM_1 after couple of phosphate buffer saline (PBS) washings. Among other treatments, CSP60 of heat killed cells formed most stable complex for both *L. paracasei* and *B. coagulans* with 19% and 22% release of initially bound AFM_1, respectively. The results showed that the combination of microbial cells with activated carbon and bentonite may be used as an efficient and effective strategy to mitigate the problem of AFM_1 in milk.

Keywords: Aflatoxin M_1**;** decontamination**;** lactic acid bacteria; activated carbon; bentonite

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INTRODUCTION

Aflatoxins (AFs) are secondary metabolites of a few Aspergilli including *Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius, Aspergillus bombycis* and *Aspergillus pseudotamarii* (Cotty and Cardwell, 1999; Kurtzman *et al*., 1987; Mishra and Das, 2003; Peterson *et al*., 2001). Among all the AFs, aflatoxin B_1 (AFB_1) is known to be the most potent natural carcinogen to humans. Mammals fed on diet contaminated with AFB_1, tend to produce aflatoxin M_1 (AFM_1) through hydroxylation of tertiary carbon atom present in the difuran rings metabolized by cytochrome P450 enzyme system in the liver (Fallah *et al*., 2011). Although, AFM_1 has been shown to be 10 times less toxic as compared to AFB_1, its harmful effects are still a grave threat to immunologically vulnerable age groups particularly children and elderly due to consumption of milk in higher amounts (Maqbool *et al*., 2009; Munir *et al*., 1989). AFM_1 is known to possess carcinogenic, teratogenic, cytotoxic and genotoxic character (Neal *et al*., 1998; Shibahara *et al*., 1995). To cope with severe threats from presence of AFM_1 in milk and milk products, many countries have established regulations regarding its maximum permissible limits. The most widely accepted maximum limit for AFM_1 in milk is 0.05 µg/l set by European Union and 0.5 µg/l set by Codex Alimentarius as well as by FDA (Fallah, 2010).

The presence of AFM_1 in milk has been reported all round the world. However, the developing countries are especially at stake due to lack of awareness, poor monitoring system and insufficient regulatory infrastructure (Ismail *et al*., 2016). Moreover, absence of suitable practices for production and improper storage of animal feed augment the threat of these toxins. The paramount technique to avoid presence of AFM_1 in milk is to prevent cattle from consumption of feed contaminated with AFB_1, adopting good agricultural and good animal feeding practices. But, due to lack of suitable facilities, it may practically be impossible to prevent the contamination of feed with AFB_1 and subsequently AFM_1 in milk. Hence, there is a need to develop such novel techniques which are safe and cost effective to reduce levels of AFM_1 in milk. Amongst different strategies for decontamination of AFM_1, use of

probiotics and lactic acid bacteria (LAB) has been proposed to be effective (Bovo *et al*., 2013; Peltonen *et al*., 2000). Various levels of decontamination of AFM_1 from milk ranging up to 100% have been achieved using microbial cells (Corassin *et al*., 2013). However, the complex formed between microbial cell and AFM1, being reversible, disassociates releasing major amount of AFM_1 back into the medium during washing (Ismail *et al*., 2017; Serrano-Niño *et al*., 2013). The exact mechanism of AFM_1 binding with microbial cells is still not clear, however a few studies have proposed the involvement of hydrophobic interactions of AFM_1 with various components of cell walls of gram positive bacteria (Haskard *et al*., 2000; Lahtinen *et al*., 2004). Furthermore, the complex may not be stable due to the fact that aflatoxin can be retained in water due to hydrophilic interactions (Pierides *et al*., 2000). Nevertheless, there is a need for further studies to understand the exact mechanism and to identify certain compounds that can enhance the stability and efficiency of microbes-AFM_1. In this regard, certain surfactants may be helpful by forming a bridge between the cell wall and AFM_1 molecules. Sorbitan monostearate (Span 60) is a common hydrophobic surfactant used in food processing industries.

Moreover, there have been several studies aiming decontamination of aflatoxins using activated carbon and clay minerals such as bentonite among which a limited number have been focused upon AFM_1 (Carraro *et al*., 2014; Di Natale *et al*., 2009). Activated carbon is a processed carbon having low volume pores which increases its surface area for adsorption, while clay minerals are small particles existing on Earth's surface consisting mainly of silica, alumina or magnesia. Activated carbon and bentonite have also not been reported to pose any potential threat even through direct contact with food stuff (Wang *et al*., 2005). The use of bentonite in food is mentioned in USA regulations (FDA, 2018) listing bentonite as GRAS (generally recognized as safe) ingredient that can be used human food. But the use of these adsorbents in higher concentrations for treatment of milk to decontaminate AFM_1 can affect the milk quality (Carraro *et al*., 2014). Although the use of microbial cells and these adsorbents have been carried out individually to reduce AFM_1 levels in milk, but the combination of these agents have not been tested. Since these agents are already being used in food processing industries, hence their application in milk industry for decontamination of AFM_1 is justified.

Due to the capabilities of probiotics to reduce AFM_1 in milk, two of the probiotic strains which previously had not been tested for their AFM_1 binding abilities, namely *Lactobacillus paracasei* and *Bacillus coagulans*, were selected for this study. Both the strains exhibit probiotic properties and have been regarded safe for long term human exposure (Endres *et al*., 2009; Jia *et al*., 2011; Phillips *et al*., 2006). The objectives of this study was to enhance the AFM_1 binding efficiency of selected bacterial strains in the presence of sorbitan monostearate, activated carbon and bentonite. Moreover, the stability of microbial-AFM_1 complex among all the treatments was analyzed through phosphate buffer saline (PBS) solution washings.

MATERIALS AND METHODS

The experiment involved the assessment for impact of heat and acid treatment on different concentrations of bacterial cells (10^7, 10^8 and 10^9 cfu/ml) and their combination with different agents (Span 60, activated carbon and bentonite) on the reduction of AFM_1 in milk spiked at three different levels of AFM_1 (0.05, 0.1 and 0.2 µg/L). Two bacterial strains were studied independently during the analysis. The extent of initially bound AFM_1 released after two PBS washings was also assessed during the study.

Materials: Two bacterial strains namely *Lactobacillus paracasei* (*L. paracasei*) CECT 4022 and *Bacillus coagulans* (*B. coagulans*) CECT 12 were obtained in lyophilized form directly from Spanish Type Culture Collection. AFM_1 standard solution of 500 µg/L was obtained from Romer Labs Diagnostic GmbH (Art # 002030). Activated carbon (SKU 18001), bentonite (SKU 285234) and sorbitan monostearate (SKU S7010) were purchased from Sigma Aldrich, USA. The ELISA kits used for quantification of AFM_1 in the samples were obtained from Romer Labs Inc. (AgraQuant® Aflatoxin M1 Sensitive COKAQ7100).

Preparation of Bacterial Culture: *L. paracasei* CECT 4022 and *B. coagulans* CECT 12 were activated in MRS Broth and Nutrient Broth, respectively at 30°C for 24 hours. Subsequently, both of the cultures were streaked on their respective solid media and incubated for 24 hours at 30°C. Afterwards, isolated colonies of both the strains were used for estimation of bacterial concentration through turbidimetric method as described by Bovo *et al*. (2013) and Ismail *et al*. (2017). The growth curves were constructed by correlation of colony count through spread plate method and corresponding absorbance observed at 600 nm using spectrophotometer (UV 3000 Vis Spectrophotometer, ORI Germany). The volumes of culture broth containing 10^7, 10^8 and 10^9 cfu of *L. paracasei* CECT 4022 and *B. coagulans* CECT 12 were centrifuged at 3000×*g* for 15 minutes to obtain pellets of respective cells.

Preparation of Heat and Acid Killed Bacterial Cells: The heat killed microbial cells were obtained after re-suspending the *L. paracasei* CECT 4022 and *B. coagulans* CECT 12 pellets separately in PBS and placing in water bath at 90°C for 1 hour. Acid treated

bacteria were prepared by re-suspending the *L. paracasei* CECT 4022 and *B. coagulans* CECT 12 cells in 2 M HCl followed by incubation at 37°C for 1 hour. Acid treated bacterial cells were washed twice with PBS to remove any residues of acid in order to prevent any chemical denaturation of AFM_1. Both acid and heat killed treatments were centrifuged at 3000×*g* for 15 minutes to obtain pallets of bacterial cells (El-Nezami *et al*., 1998b; Ismail *et al*., 2017).

Preparation of Spiked Milk Samples: Raw cow milk was purchased from the local farmer and pasteurized at 63°C for 30 minutes to prevent any deteriorative changes from microbial activity. Afterwards, the fat was removed through centrifugation at 3000×*g* for 5 minutes in order to prevent any matrix interferences in further analysis. The presence of AFM_1 in milk below detection limits was ensured. The final concentrations of 0.05, 0.1 and 0.2 µg/L of AFM_1 spiked milk samples were prepared by adding 10 µl, 20 µl and 40 µl of AFM_1 standard separately in 100 ml skim milk for further investigations.

Decontamination of AFM_1 through Bacterial Cells and their Combination with Activated Carbon, Bentonite and Sorbitan Monostearate: The nonviable bacterial cells (C), both heat killed and acid treated, having $10^{7,}$ 10^8 and 10^9 cfu were separately re-suspended in 1 ml of spiked milk samples. For treatments combined with activated carbon (CAC) or bentonite (CBENT), the suspension of bacterial cells in milk was added with activated carbon and bentonite (2% each). Span 60 treatment (CSP60) was prepared by suspending the bacterial cells in 1 ml solution of 0.2% span 60 followed by centrifugation at 3000×*g* for 15 minutes and re-suspending in 1 ml of AFM_1 spiked milk samples. All of the treatments were incubated at 37°C for 1 hour followed by centrifugation at 3000×*g* for 15 minutes to assess the decontamination of AFM_1.

Stability Testing of Microbial Cell-AFM_1 Complex: The stability of microbial cell-AFM_1 complex for all the treatments (C, CAC, CBENT & CSP60) was analyzed by washing the pellets twice with PBS as described by Ismail *et al*. (2017). The pellets were re-suspended in PBS and mixed thoroughly using vortex mixer for 20 seconds. The cells were centrifuged at 3000×*g* for 15 min to collect the supernatant for quantification of released AFM_1 after each washing.

Quantification of AFM_1: Supernatants (0.4 ml) from centrifuged samples of C, CAC, CBENT & CSP60 were mixed separately with 0.1 ml of methanol (Sigma Aldrich) to quantify AFM_1 through ELISA. The mixture (100 µl each) was added to 200 µl of conjugate solution in dilution wells and mixed thoroughly. Afterwards, 100 µl from each dilution well was transferred to respective antibody coated micro well followed by incubation at room temperature for 1 hour. The microwells were then drained off, washed with washing buffer and dried by tapping the microwells on several layers of adsorbent paper at flat surface followed by addition of 100 µl substrate to each microwell and incubation for 20 minutes at room temperature in dark. Stop solution (100 µl) was added to each microwell to quench the reaction. The absorbance was measured at 450 nm by placing the micro well plate in ELISA reader (Bio-Tek ELx800, Indonesia) as recommended by the manufacturer.

The absorption intensity was observed to be inversely proportional to the AFM_1 concentration. The log–logit AFM_1 sheet supplied with the kit was used to generate a standard curve and calculate the concentration of AFM1 in the samples. Furthermore, the ELISA method was validated by analyzing the recovery percentages of AFM_1 in milk spiked at 0.025, 0.05, 0.1 and 0.2 µg/L.

Statistical Analysis: All the treatments were analyzed in triplicates and the results were expressed in mean ± SD. The statistical analysis of data was performed using three way ANOVA and LSD using Statistix 8.1 to analyze statistical significant differences among treatments within milk samples spiked with same AFM_1 levels. The probability level of <0.05 was considered to be statistically significant.

RESULTS

ELISA Method Validation: The standard curve is shown in Figure. 1. The efficiency of ELISA method is presented in Table 1. The recovery of spiked milk samples were found in the ranges of 94.7% to 97%. The limit of detection provided by the manufacturer for ELISA kits used in current study was 0.018 µg/L.

Decontamination of AFM_1 through Lactobacillus paracasei Combined with Activated Carbon, Bentonite and Sorbitan Monostearate: Significant differences were observed among the treatments of spiked milk with different levels of AFM_1 having various concentrations of microbial cells (Table. 2). Among 0.05 µg/L AFM_1 spiked milk samples, residual levels below detection limits revealed that maximum reduction of the toxin was exhibited by CAC and CBENT for both acid treated and heat killed samples. The minimum AFM_1 reduction was exhibited by the acid treated C with 27%, 42% and 60% AFM_1 removal against 10^7, 10^8 and 10^9 cfu/ml, respectively. Higher amounts of AFM_1 were removed from spiked milk by heat killed treatments C and CSP60 as compared to their corresponding acid treatments. The amount of AFM_1 reduced through heat treated C was 32% and 55% in correspondence to 10^7 and 10^8 cfu/ml, respectively. Moreover, reduction of 42% was observed against 10^7 cfu/ml and no residual AFM_1 was detected in treatment with 10^8 cfu/ml heat killed cells treated with CSP60. Furthermore, all the treatments involving 10^9

cfu/ml microbial cells reduced the AFM_1 below detection limits except for acid treated C.

Among milk samples spiked with 0.1 µg/L AFM_1, no detectable amounts of AFM_1 were observed in CAC and CBENT for both acid treated and heat killed treatments. The reduction rate of AFM_1 was observed to be less for acid treated than heat treated cells (C treatment). Next to CAC and CBENT, AFM_1 was observed to be reduced maximum in heat killed CSP60 samples in order of $10^7<10^8<10^9$ cfu/ml showing 40%, 64% and 76% reduction, respectively. Minimum reduction among all treatments was observed in acid treated cells (C treatment) showing decrease of 25%, 47% and 62% in AFM_1 against 10^7, 10^8 and 10^9 cfu/ml, respectively.

Similar trend was observed for milk samples spiked with 0.2 µg/L AFM_1. The heat killed treatments except for CAC and CBENT reduced more AFM_1 as compared to acid treated treatments. The levels of AFM_1 reduced by CAC with 10^7, 10^8 and 10^9 cfu/ml were 81%, 82% and 82% for heat killed treatments while 84%, 88% and 90 % for acid treated treatments, respectively. Higher reduction was observed for 10^9 cfu/ml cells between all the treatments spiked with 0.2 µg/L AFM_1, amongst which acid treated CBENT and C were found to bind the highest (<LOD) and lowest (42%) amounts of AFM_1, respectively.

Decontamination of AFM_1 through Bacillus coagulans combined with Activated Carbon, Bentonite and Sorbitan Monostearate: The decontamination of AFM_1 through *B. coagulans* combined with activated carbon, bentonite and sorbitan monostearate in milk samples spiked with 0.05 µg/L AFM_1 was observed to be highest (<LOD) by CAC and CBENT among both heat killed and acid treated bacterial cells (Table. 3). Among all the treatments spiked with 0.05 µg/L AFM_1, minimum reduction was observed for acid treated CSP60 with removal of 28%, 44% and 60% AFM_1 against 10^7, 10^8 and 10^9 cfu/ml of *B. coagulans*, respectively. In contrast, the acid treated C treatment reduced AFM_1 levels by 50% and 62% through 10^7 and 10^8 cfu/ml, respectively, while no detectable amounts of AFM_1 were observed in C treatment with 10^9 cfu/ml bacterial cells. The reduction achieved through heat killed treatment C was observed to be 38% and 48% against 10^7 and 10^8 cfu/ml. The heat treated CSP60 reduced 52% AFM_1 against 10^7 cfu/ml. No detectable amounts were observed for heat treated C involving 10^9 cfu/ml and CSP60 involving 10^9 and 10^8 cfu/ml *B. coagulans*.

The treatments CAC and CBENT for both acid treated and heat killed cells decreased the residual AFM_1 levels to below detection limits (<LOD) at all microbial concentrations in milk samples spiked with 0.1 µg/L AFM_1. Minimum reduction was observed by the acid treated CSP60 treatment exhibiting 31%, 46% and 64% for 10^7, 10^8 and 10^9 cfu/ml bacterial cells, respectively. The treatment C for acid treated cells was found to be most efficient in AFM_1 removal after CAC and CBENT in milk samples added with 0.1 µg/L AFM_1 showing 48.37%, 63.3% and 78.56% reduction through 10^7, 10^8 and 10^9 cfu/ml, respectively.

For samples spiked with 0.2 µg/L AFM_1, maximum reduction was observed for CBENT among both heat killed and acid treated, while for CAC in only acid treated samples with residual AFM_1 levels <LOD against 10^9 cfu/ml bacterial cells. Higher reduction was observed for heat killed CBENT (85% and 89%) as compared to acid treated CAC and CBENT (83% and 87% for both treatments) against 10^7 and 10^8 cfu/ml, respectively. Minimum reduction of AFM_1 among samples spiked with 0.2 µg/L was observed in acid treated CSP60 with 14%, 22% and 45% removal against 10^7, 10^8 and 10^9 cfu/ml bacterial cells, respectively. Among remaining treatments, highest reductions were observed through heat killed CSP60 treatment showing 25%, 47% and 59% removal of AFM_1 for 10^7, 10^8 and 10^9 cfu/ml cells, respectively. Furthermore, acid treated C showed higher efficiency for AFM_1 removal (24%, 33% and 57%) as compared to heat treated (21%, 29% and 53%) *B. coagulans* at 10^7, 10^8 and 10^9 cfu/ml, respectively.

Stability Testing of Microbial Cell-AFM_1 Complex: The treatments CAC and CBENT for both *L. paracasei* and *B. coagulans* did not release any AFM_1 in detectable amounts showing a stable complex formation. Furthermore, no detectable amounts of AFM_1 were released after 2 washings with PBS solution for any treatment at 0.05 µg/L and 0.1 µg/L. While at 0.2 µg/L, detectable amounts were released by both heat killed and acid treated microbes at 10^9 cfu/ml in treatments C and CSP60. For *L. paracasei*, among the treatments spiked with 0.2 µg/L AFM_1, C and CSP60 having 10^9 cfu/ml released detectable amounts of AFM_1 during the washing treatments amounting up to 53% and 19% in heat killed cells while 30% and 61% for acid treated cells after two washings, respectively (Figure. 2. a).

For *B. coagulans*, lower amounts were released by heat killed CSP60 with 22% release of initially bound AFM_1 during first washing and no release of detectable amounts after second washing. While, maximum amounts were released by acid treated CSP60 releasing 33% and 22% of initially bound AFM_1 during first and second washing, respectively (Figure. 2. b).

Table 1. ELISA Method Efficiency and Validation for AFM_1.

Spiked AFM1 in milk (µg/L)	AFM1 Detected (µg/L)	Recovery %	Variation Coefficient
0.025	0.0237	94.7	2.44
0.050	0.0480	96.0	2.08
0.100	0.0967	96.7	1.58
0.200	0.1940	97.0	1.55

Table. 2 Residual levels of AFM_1 (µg/L) after decontamination of milk spiked at different concentrations of AFM_1 with heat killed and acid treated *L. paracasei* cells in combination with sorbitan monostearate, activated carbon and bentonite.

Microbial Cells (Cfu/ml)	Heat/Acid Treatment	Treatment	Residual AFM_1 in µg/L (Reduction %)		
			0.05	0.1	0.2
10^7	Heat killed	C[a]	0.0339 ± 0.0015^{B} (32.2)	0.0662 ± 0.0024^{B} (33.8)	0.1632 ± 0.0026^{B} (18.4)
		CSP60[b]	0.0289 ± 0.0018^{D} (42.2)	0.0596 ± 0.0006^{C} (40.4)	0.1501 ± 0.0011^{D} (25)
		CAC[c]	$<LOD^{G}$	$<LOD^{K}$	0.0381 ± 0.0012^{K} (81)
		CBENT[d]	$<LOD^{G}$	$<LOD^{K}$	0.0279 ± 0.0013^{N} (86.05)
	Acid treated	C	0.0366 ± 0.0012^{A} (26.8)	0.0747 ± 0.0013^{A} (25.3)	0.1734 ± 0.0017^{A} (13.3)
		CSP60	0.0306 ± 0.0014^{C} (38.8)	0.0662 ± 0.0007^{B} (33.8)	0.1561 ± 0.0011^{C} (21.95)
		CAC	$<LOD^{G}$	$<LOD^{K}$	0.0314 ± 0.0011^{M} (84.3)
		CBENT	$<LOD^{G}$	$<LOD^{K}$	0.0243 ± 0.0012^{O} (87.85)
10^8	Heat killed	C	0.0227 ± 0.0015^{E} (54.6)	0.0396 ± 0.0024^{F} (60.4)	0.1302 ± 0.0029^{E} (34.9)
		CSP60	$<LOD^{G}$	0.0362 ± 0.0007^{G} (63.8)	0.1092 ± 0.0015^{G} (45.4)
		CAC	$<LOD^{G}$	$<LOD^{K}$	0.0354 ± 0.0006^{L} (82.3)
		CBENT	$<LOD^{G}$	$<LOD^{K}$	0.024 ± 0.0013^{O} (88)
	Acid treated	C	0.0291 ± 0.0013^{D} (41.8)	0.0527 ± 0.0025^{D} (47.3)	0.1562 ± 0.0013^{C} (21.9)
		CSP60	0.0239 ± 0.0003^{E} (52.2)	0.0421 ± 0.001^{E} (57.9)	0.1308 ± 0.0013^{E} (34.6)
		CAC	$<LOD^{G}$	$<LOD^{K}$	0.0236 ± 0.0008^{OP} (88.2)
		CBENT	$<LOD^{G}$	$<LOD^{K}$	0.0214 ± 0.0007^{PQ} (89.3)
10^9	Heat killed	C	$<LOD^{G}$	0.0276 ± 0.0027^{I} (72.4)	0.0970 ± 0.0019^{I} (51.5)
		CSP60	$<LOD^{G}$	0.0238 ± 0.0019^{J} (76.2)	0.0821 ± 0.0016^{J} (58.95)
		CAC	$<LOD^{G}$	$<LOD^{K}$	0.0318 ± 0.0016^{M} (84.1)
		CBENT	$<LOD^{G}$	$<LOD^{K}$	0.0218 ± 0.0007^{OPQ} (89.1)
	Acid treated	C	0.0198 ± 0.0011^{F} (60.4)	0.0383 ± 0.001^{F} (61.7)	0.1159 ± 0.0024^{F} (42.05)
		CSP60	$<LOD^{G}$	0.0311 ± 0.0016^{H} (68.9)	0.1060 ± 0.0019^{H} (47)
		CAC	$<LOD^{G}$	$<LOD^{K}$	0.0200 ± 0.0017^{Q} (90)
		CBENT	$<LOD^{G}$	$<LOD^{K}$	$<LOD^{R}$

[a]C: Cells only, [b]CSP60: Cells treated with sorbitan monostearate (Span 60), [c]CAC: Cells in combination with activated carbon
[d]CBENT: Cells in combination with bentonite
Different capital letters within same column show significant difference ($p<0.05$)

Table. 3 Residual levels of AFM_1 (µg/L) after decontamination of milk spiked at different concentrations of AFM_1 with heat killed and acid treated *B. coagulans* cells in combination with Sorbitan Monostearate, Activated Carbon and Bentonite.

Microbial Cells (Cfu/ml)	Heat/Acid Treatment	Treatment	Residual AFM_1 in µg/L (Reduction %)		
			0.05	0.1	0.2
10^7	Heat killed	C[a]	0.0310 ± 0.0017^{B} (38)	0.0592 ± 0.0026^{B} (40.8)	0.1582 ± 0.0030^{B} (20.9)
		CSP60[b]	0.0238 ± 0.0008^{E} (52.4)	0.0539 ± 0.0021^{C} (46.1)	0.1500 ± 0.0015^{C} (25)
		CAC[c]	$<LOD^{G}$	$<LOD^{J}$	0.0426 ± 0.0008^{K} (78.7)
		CBENT[d]	$<LOD^{G}$	$<LOD^{J}$	0.0298 ± 0.0019^{N} (85.1)
	Acid	C	0.0248 ± 0.0012^{DE} (50.4)	0.0516 ± 0.0015^{D} (48.4)	0.1527 ± 0.0018^{C} (23.7)

	treated	CSP60	0.0358±0.0015^{A} (28.4)	0.0686±0.0018^{A} (31.4)	0.1711±0.0022^{A} (14.5)
		CAC	<LOD^{G}	<LOD^{J}	0.0327±0.0016^{LM} (83.7)
		CBENT	<LOD^{G}	<LOD^{J}	0.0343±0.0020^{L} (82.9)
10^{8}	Heat killed	C	0.0260±0.0018^{D} (48)	0.0422±0.0008^{E} (57.8)	0.1418±0.0015^{D} (29.1)
		CSP60	<LOD^{G}	0.0321±0.0026^{G} (67.9)	0.1058±0.0011^{G} (47.1)
		CAC	<LOD^{G}	<LOD^{J}	0.0308±0.0020^{MN} (84.6)
		CBENT	<LOD^{G}	<LOD^{J}	0.0225±0.0006^{P} (88.8)
	Acid treated	C	0.0191±0.0008^{F} (61.8)	0.0367±0.0017^{F} (63.3)	0.1332±0.0017^{E} (33.4)
		CSP60	0.0280±0.0010^{C} (44)	0.0536±0.0017^{CD} (46.4)	0.1569±0.0019^{B} (21.6)
		CAC	<LOD^{G}	<LOD^{J}	0.0257±0.0014^{O} (87.2)
		CBENT	<LOD^{G}	<LOD^{J}	0.0266±0.0012^{O} (86.7)
10^{9}	Heat killed	C	<LOD^{G}	0.0263±0.0024^{H} (73.7)	0.0949±0.0023^{H} (52.6)
		CSP60	<LOD^{G}	0.0203±0.0010^{I} (79.7)	0.0822±0.0014^{J} (58.9)
		CAC	<LOD^{G}	<LOD^{J}	0.0209±0.0016^{P} (89.6)
		CBENT	<LOD^{G}	<LOD^{J}	<LOD^{Q}
	Acid treated	C	<LOD^{G}	0.0214±0.0010^{I} (78.6)	0.0855±0.0018^{I} (57.3)
		CSP60	0.0201±0.0014^{F} (59.8)	0.0359±0.0022^{F} (64.1)	0.1108±0.0019^{F} (44.6)
		CAC	<LOD^{G}	<LOD^{J}	<LOD^{Q}
		CBENT	<LOD^{G}	<LOD^{J}	<LOD^{Q}

[a]C: Cells only, [b]CSP60: Cells treated with sorbitan monostearate (Span 60), [c]CAC: Cells in combination with activated carbon, [d]CBENT: Cells in combination with bentonite

Different capital letters within same column show significant difference ($p<0.05$)

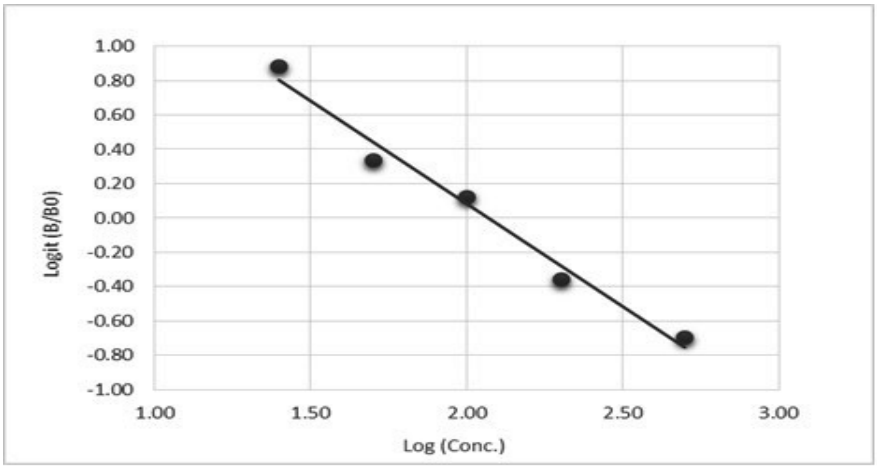
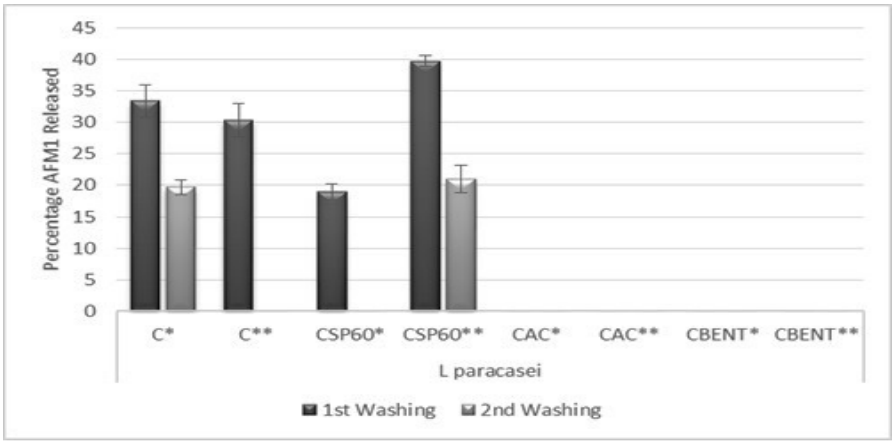


Figure. 1 Calibration curve of aflatoxin M_1. B/B0 represents absorbance at 450 nm for the sample, or the standard divided by absorbance at the same wavelength for the control.



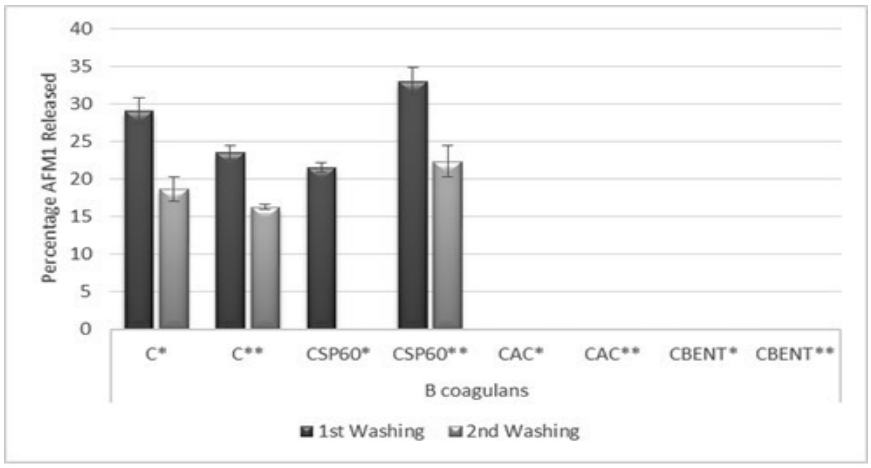


Figure. 2 Percentage of initially bound AFM_1 released after 2 washings with PBS for treatments involving (a) *L. paracasei* and (b) *B. coagulans*

C: Cells only, CSP0: Cells treated with sorbitan monostearate (Span 60), CAC: Cells in combination with activated carbon, CBENT: Cells in Combination with bentonite

*Treatments involving heat killed cells

** Treatments involving acid treated cell

DISCUSSION

The GRAS (Generally Recognized As Safe) status of LAB and probiotics along with their natural occurrence in variety of food commodities have encouraged the researchers to explore their AFM_1 reducing potentials (Elsanhoty *et al*., 2014; Serrano-Niño *et al*., 2013). The removal of AFM_1 through binding with bacterial cell wall involving non covalent interactions and subsequent removal by centrifugation has been reported to be an efficient method for its decontamination (Bovo *et al*., 2013; Serrano-Niño *et al*., 2013). The current study was also based upon utilization of two bacteria belonging to the said categories which had not been reported earlier for AFM_1 decontamination. Furthermore, the novelty of this study was the application of the bacterial strains in combination with span 60, activated carbon and bentonite for AFM_1 reduction in milk. These combinations, to the best of our knowledge, have not been studied before for AFM_1 decontamination in any media.

The results of the study showed a direct relation of bacterial cells concentration with amounts of AFM_1 removed (Table 2 and 3). Higher bacterial cell concentrations were able to reduce AFM_1 to a larger extent from milk samples spiked with AFM_1. This clearly depicted that degree of AFM_1 removal was dependent upon cell concentration as reported previously by Ismail *et al*. (2017) who showed reduction ranging between 73% to 100%, 41% to 74%, 27% to 54% and 13% to 42% using 10^{10}, 10^9, 10^8 and 10^7 cfu/ml bacterial cells, respectively depending upon initial levels of AFM_1 in milk. Kabak and Var (2008) also observed a decrease in AFM_1 removal when the bacterial cell concentrations were reduced from 10^8 cfu/ml to 10^7 cfu/ml with AFM_1 removal ranging between 10.22 to 26.65% and 0 to 5.02%, respectively. Another study by El-Nezami *et al*. (1998a) suggested that cell concentrations of lactic acid bacteria in excess of 10^9 cfu/ml were required for efficient removal of aflatoxins. Although the removal of AFM_1 was in linear relation with cell concentration, the percentage reduction in AFM_1 decreased when the initial AFM_1 levels were increased beyond a certain point among similar cell concentrations. The study by Ismail *et al*. (2017) also revealed if bacterial cell concentration was kept constant, the percentage of AFM_1 removal decreased when initial AFM_1 levels were increased. Line and Brackett (1995) proposed a similar trend showing decrease in percentage decontamination after the milk was spiked with aflatoxins beyond a certain limit. This indicated that there were limited binding sites on bacterial cell walls at which AFM_1 could form a complex. As the cell concentrations increased, the amount of available binding sites also increased, but as the AFM_1 concentration was increased, the percentage removal started to decrease when the binding assay reached a saturation point. Although Kabak and Var (2008) showed no effect of AFM_1 concentration on the percent removal efficiencies of bacterial cells showing 23.48%, 26.62% and 24.77% AFM_1 removal at 5, 10 and 20 µg/L, respectively but the amounts of AFM_1 removed increased with increase in AFM_1 concentration which might have occurred due to lack in satisfaction of bacterial cell binding sites at lower concentrations of spiking with AFM_1.

Since many of the previous studies have ruled out the role of metabolic degradation of aflatoxins through bacterial cells on the basis of higher binding efficiencies by nonviable cells as compared to those

shown by viable cells (Bovo *et al*., 2013; Corassin *et al*., 2013; El-Nezami *et al*., 1998a, b; Elsanhoty *et al*., 2014; Pierides *et al*., 2000), heat killed and acid treated cells were utilized for AFM_1 removal in the current study. Furthermore, their usage might also not result in any undesirable fermentative changes in milk that could have been caused by in case of viable cells. Due to the capability of nonviable cells to remove aflatoxins, it had been suggested that components of bacterial cell wall, including peptidoglycans and polysaccharides, were majorly involved in toxin removal through formation of a non-covalent complex with aflatoxins (Lahtinen *et al*., 2004; Shetty *et al*., 2007). The acid and heat treatments may have drastic effect on bacterial cell wall peptidoglycans and polysaccharides. The heat treatment may cause denaturation of cell wall proteins resulting changes in interactions or may form maillard reaction products as well. Likewise, acid treatment may affect the cell wall by breaking the glycosidic linkages between polysaccharides causing formation of monomers which may convert into aldehydes or by hydrolysis of proteins into smaller peptides and amino acids. Furthermore, the acid treatment may compromise the structural integrity of cell wall by breaking the peptidoglycan, consequently reducing the thickness of this layer and increasing the pore size by decreasing cross linkages (Haskard *et al*., 2001). These changes might expose the sites of microbial cell which were previously unavailable towards binding with aflatoxins (El-Nezami *et al*., 1998b; Haskard *et al*., 2000). The AFM_1 reduction patterns of acid and heat treatments of C for both the strains tested in this study were in contrast from each other. The acid treated C of *B. coagulans* had higher binding efficiencies than heat killed C. In contrast, *L. Paracasei* showed less removal of AFM_1 through acid treated C in comparison to heat killed C. This might have occurred due to differences in cell wall structures of both the bacteria resulting in varying exposures of binding sites as a result of HCl treatment. Furthermore, the study revealed various binding capacities for both the strains tested. The binding properties might have been different due to varying degree of changes among the structure and the composition of cell wall (Piuri *et al*., 2005; Vinderola *et al*., 2000).

Haskard *et al*. (2000) suggested that the protein denaturation due to acid and heat treatment results in formation of hydrophobic surfaces which further act as binding sites for aflatoxins. In an attempt to increase the binding sites of bacterial cell wall, the cells were treated with sorbitan monostearate (Span 60). Sorbitan monostearate (SP60) has low Hydrophile-Lipophile Balance (HLB) value and is used for water in oil emulsions (Gadhave, 2014). Furthermore, SP60 is used as an emulsifier in various food commodities (E 491). The ADI for sorbitan esters either alone or in combination is 10 mg /kg body weight per day and the SP60 has been shown to have no observed adverse effect level (NOAEL) up to 2,600 mg /kg body weight per day (EFSA, 2017). The results of our study showed higher levels of decontamination through acid and heat killed CSP60 of *L. paracasei* in comparison to its C counterparts. This might have happened due to binding of SP60 with relatively higher hydrophobic sites of bacterial cell wall which were previously vacant as AFM_1 could not bind at these sites due to large differences in polarity. This phenomenon might have further exposed the relatively less hydrophobic regions of sorbitan monostearate to form a complex with AFM_1, consequently increasing the binding efficiency of the cells. For *B. coagulans*, higher levels of AFM_1 removal were achieved for heat killed CSP60 than heat killed C treatment, while lower levels of AFM_1 removal were achieved in case of acid treated CSP60 as compared to acid treated C treatment. This can be explained in light of varying structure and composition of cell wall resulting in different effect of acid treatment on different bacteria (Piuri *et al*., 2005; Vinderola *et al*., 2000). The heat treatment might have exposed larger number of relatively higher hydrophobic sites favoring the binding of AFM_1 through SP60 complex, while the acid treatment may have exposed more binding sites favoring formation of AFM_1 complex directly with bacterial cell wall.

Number of studies have shown the aflatoxin removal capacities for bentonite and activated carbon by addition in animal diet (Diaz *et al*., 2004; Jaynes *et al*., 2007), however the direct exposure of milk with these adsorbents for AFM_1 removal have been studied in very few cases (Applebaum & Marth, 1982). Although bentonite and activated carbon showed high AFM_1 removal efficiencies in milk during previous studies but they also showed some changes in milk properties (Carraro *et al*., 2014; Di Natale *et al*., 2009). This study utilized less amounts of adsorbents (2%) as compared to up to 5% of activated carbon and bentonite by Di Natale *et al*. (2009) and up to 10 % bentonite by Carraro *et al*. (2014), additionally, the adsorbents were analyzed in combination with bacterial cells. The combination of cells with activated carbon and bentonite (CAC and CBENT) showed significantly higher removal of aflatoxins as compared to other treatments which might have been due to availability of more binding sites in the presence of both the bacterial cells and the adsorbent. In addition, higher affinities of these treatments towards AFM_1 can be attributed to larger surface area and highly porous structure of activated carbon and bentonite (Di Natale *et al*. 2009). Furthermore, as the aflatoxins are mainly present in milk serum and casein (Ottaviani, 1991) and the milk serum mainly constitutes of water, the AFM_1 hydrophobicity can promote its binding to surface of carbon which has more affinity towards AFM_1. Similarly, formation of hydrogen bonding among aflatoxins molecules and bentonite edges might have

resulted in efficient removal of AFM_1 from spiked milk samples (Desheng *et al*., 2005). The variations among AFM_1 binding abilities of these two agents can be due to variations in pore size and in chemical affinities between the surface functional groups of the adsorbent and toxin (Alfarra *et al*., 2004). Among the treatments involving activated carbon and bentonite, the bentonite treatment was found to be more effective as compared to activated carbon. These results were in contrast to the studies of Di Natale *et al*. (2009) who showed higher AFM_1 binding ability of activated carbon as compared to bentonite. However, this variation can be attributed to the presence of microbial cells in the medium which could have adsorbed on the activated carbons consequently decreasing the porosity and AFM_1 adsorption (Rivera *et al*., 2001). In addition, the simultaneous presence of bacterial cells and bentonite in a medium may result in formation of bacteria-bentonite clusters which may trap the AFM_1 increasing the efficiency of the treatment (Yang *et al*., 2012).

The complex of CAC and CBENT with AFM_1 for both heat and acid treatments was found to be most stable followed by heat killed CSP60. The results were almost in line with the study of Ismail *et al*. (2017), who showed release of 19.5% to 69.8% of initially bound AFM_1 by various lactobacillus strains and their mixture. Bovo *et al*. (2013) reported the release of 40.57% to 87.37% of initially bound AFM_1 by lactobacillus strains after washings with PBS solution. The presence of weak non covalent hydrophobic interactions between microbial cells and AFM_1 were suggested to be the cause of low AFM_1 retention by microbial cells (Serrano-Niño *et al*. 2013). In contrast, Kabak and Var (2008) revealed the release of only 5.62–8.54% of bound AFM_1 by using various *Lactobacillus* and *Bifidobacterium* strains. The variations among different strains treated with heat and acid in addition to SP60 can be attributed to differences in binding sites of various strains as a result of denaturation or hydrolysis of cell wall components. Lower levels of release for AFM_1 might have been due to interactions among AFM_1 molecules with cell walls of adjacent bacterium, consequently developing a cross-linked matrix to prevent aflatoxin release due to washings with PBS (Hernandez-Mendoza *et al*., 2009). Although, higher stability levels were achieved in our study, but there is still a need to study in detail the exact mechanism for release of AFM_1 bound to microbial cells under various conditions in order to decrease their release in different environments.

The two bacterial cells *L. paracasei* and *B. coagulans* were found to be effective at higher concentrations in reducing AFM_1 levels. Furthermore, the combinations of bacterial cells with activated carbon and bentonite proved to be more efficient as the removal of up to 100% AFM_1 was achieved. Moreover, microbial cell–AFM_1 complex in the presence of these combinations proved to be more stable as compared to cells alone. The addition of sorbitan monostearate also increased the binding capacity of heat killed and acid treated *L. paracasei* cells and heat treated *B. coagulans* cells. The stability of microbial cell–AFM_1 complex due to treatment with SP60 was also enhanced but it was less than that achieved by addition of bentonite and activated carbon. In addition, treatment of cells with SP60 also resulted in higher AFM_1 removal efficiency which can be attributed to exposure of new binding sites due to role of SP60 as a bridge between highly hydrophobic sites of cell wall and hydrophilic AFM_1. The utilization of the adsorbents analyzed in this study in combination with microbial cells can prove to be highly efficient in terms of reducing AFM_1 to below permissible limits and its daily intake among humans without posing any probable side effects. Hence, the addition of an operation such as filtration using composite materials based on agents used in the study for milk processing can be explored further. Although, this might add another processing step, but the milk having AFM_1 above maximum limits can be utilized for dairy products, if not for direct consumption, after processing instead of being wasted. Nevertheless, future studies must be focused on the effect of these materials on the properties of milk and further optimization of the process.

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