Original Research Article

Novel Combination contains probiotic bacterial and Yeast strains to reduce of Aflatoxin M1 in Milk

ABSTRACT

Milk and milk products are important contribution to the human diet especially children. However, the presence of aflatoxins as AFM1 in milk and milk products are considered undesirables due to their health risks in consumer's body. For that reason this study aimed to assess the ability of some microbial species on aflatoxin removal especially the AFM1 in the milk. AFM1 residue was determined by HPLC after different incubation period (12h, 24h, 48h and 72h) of the probiotic bacteria and/or yeasts in PBS as model for AFM1 determination. This study was performed during the period between 2015 and 2017.The combination of nonviable probiotic bacterial and yeast species (*Lactobacillus plantarum, Lactobacillus acidophilus, Bifidobacterium bifidum, Kluyveromyces lactis* and *Saccharomyces cerevisiae*) succeeded to reduce AFM1 from 50 (ng/ml) during the incubation periods; 12h, 24h, 48h and 72h, into 9.72±1.31, 6.68±0.55, 5.70±0.33 and 4.56±0.15 ng ml-1, respectively. The highest AFM1 removal % was recorded as; 80.56%, 86.64%, 88.60% and 90.88% in the treated milk samples in respective manner. Sensor evaluation was carried out using Yoghurt as model in sample size 50 gm for each sample.

Conclusion: This study concluded that the combination consisting of probiotic bacteria and yeasts could be used in reducing the concentration of the AFM1 in aflatoxin contaminated milk.

Keywords: Probiotic bacteria, Yeasts, Aflatoxin M1, Contamination Milk.

1. INTRODUCTION

Aflatoxins are a group of mycotoxins which are considered as the most potent carcinogens. Aflatoxins can not only be found as contaminants in the stable diet (cereal grains) but also are found in milk when the dairy animals ingest contaminated feed with aflatoxin B1 and B2 [1-3]. Aflatoxin B1 (AFB1) is converted by the normal metabolism process to aflatoxin M1 but aflatoxin B2 (AFB2) is converted to aflatoxin M2 and then aflatoxin M1 and M2 are excreted and occurred in milk so AFM1 and AFM2 are considered as hepatic hydroxlated metabolites of aflatoxin B1 and B2 [4-6]. Milk and dairy products are considered as very important part of human diet food habit in every home with high rate of consumption for all age because milk is high in nutritional value which maintains the human health. However; it may be act as a

vehicle of contaminants such as aflatoxins which cause various physiological risks effects in human consumers especially the children who are considered more group susceptible than adults to aflatoxins effects as growth retardation, stunning and liver cancer [7-8]. Aflatoxin M1 (a member of aflatoxins) may be found in breast milk, animal milk and different dairy products. AFM1 has linear relationship with the aflatoxin B1 in animal feed that is ingested by dairy animals. AFM1 is stable in raw milk and different processed products from milk which does not destroyed by pasteurization or heat treatments. Cream separation from milk has a small effect on AFM1 amount in skim milk because AFM1 prefers the binding with the casein (milk protein). The maximum concentration acceptable limit of aflatoxin M1 permitted in milk consumption by humans is 0.5 ppb (parts per billion) that is established by Egyptian standard specification (E.S.S) and European standard regulation [9-11].

AFM1 is more specifically a problem of food safety than a problem of hygiene which cause different risks and pathogens in human health. For these reasons, there are strategies or innovative solutions for reducing and inhibiting health risks of aflatoxin and overlook the issue of aflatoxin exposure by using certain probiotic strains which can bind with aflatoxin to form the complex probiotic-aflatoxin and then improve elimination of this complex from the gut through feces. Therefore, this biological strategy prevent the absorption of these aflatoxin in human and animal bodies through gastrointestinal tract, improve aflatoxin decontaminating from body and minimize potential risks of aflatoxin [12-14].

Usage of the probiotics in milk is considered important step which can minimize the toxins in the diet, lower the risks and enhance the health. These biological methods use to sequestrate the aflatoxin M1 without affecting the nutritional value, taste of the milk products. Some probiotic strains like *Bifidobacterium Bifidum*, *Lactobacillus plantarum*, *Lactobacillus acidophilus* have the ability to minimize risks of aflatoxin M1 and also some types of yeast as *Saccharomyces cerevisiae* and *Kluyveromyces lactis* have the ability to sequestrate aflatoxin M1 from milk and milk products [15-18]. For that reasons this study aimed to find a microbial combination which able to reduced and control the toxicity resulted from aflatoxins in contaminated milk especially the AFM1.

2. MATERIAL AND METHODS

MICROBIAL STRAINS COLLECTION

All the bacterial strains and yeasts were kindly obtained from microbiological resources centers (Cairo MIRCEN, Egypt)

2.1 Standard aflatoxin M1 (AFM1) solutions

Standard solution of AFM1 (10 µg/ml) was obtained from Sigma-Aldrich (St. Louis, MO, USA). A stock standard solution of AFM1 was prepared by dissolving standard in benzene: acetonitrile (98:2, v/v) until used in the test quantitative measurement of aflatoxin M1 in milk and dairy products as described by AOAC (2000) [19-20]. Another stock standard solution of AFM1 was prepared by dissolving standard in PBS at concentration 50 ng ml^{-1} till used in test of the evaluation of the ability of some probiotic strains on aflatoxin M1 reduction. AFM1 stock standard solution was packed in amber vials to protect the work concentration from the light and then stored at 4 °C in refrigerator.

2.2 Evaluation the ability of some probiotic viable species (*Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum)* on aflatoxin M1 reduction

Lactobacillus plantarum, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* are some of probiotic viable which were selected based on their use as probiotic cultures in dairy industry on available information concerning their effects on reduction of aflatoxins in aqueous solution. Several types of lactic acid bacteria (LAB) have binding ability with AFM1 in liquid media and milk solution [21].

2.2.1 Preparation of probiotic bacterial strains

Each probiotic bacterial strain (*Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum)* was cultivated individually in De-Man-Rogosa-Sharpe broth (MRS) supplemented with 0.05% L-cysteine at pH 6.5 and incubated at anaerobic conditions at 37 °C for 24 h in anaerobic shaker incubator at 200 rpm with 5% CO_2 [22]. Each probiotic bacterial strain was placed in centrifuge at (4000 rpm, 4 °C and 15 min) to harvest its cells in pellet then washed by phosphate buffer saline (PBS) twice. The pellet of each strain was suspended in PBS at pH 6.8 to determine optical density (OD) by using spectrophotometer at wavelength of 600 nm. Then the suspension were adjusted into different starting concentration treatment at OD_{600} 0.72± 0.03 equal 1×10^9 CFU ml^{-1}, OD_{600} 2.16±0.03 equal 3×10^9 CFU ml^{-1} and OD_{600} 3.6±0.035 equal 5×10^9 CFU ml^{-1}. The suspension was diluted with PBS until reaching the required concentration treatment. Also, the treatment dose of combination probiotic bacterial strains was prepared by taken equal amount from each bacterial strain at 5×10^9 CFU ml^{-1} to give 1ml PBS had three probiotic bacterial strains (*Bifidobacterium bifidum* DSM 20082*, Lactobacillus plantarum* DSM 20174 and *Lactobacillus acidophilus* DSM 20079) [23-25].

2.3 Binding ability of the viable species of (*Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum)* with aflatoxin M1

The adjusted inoculum concentration of collected cells were suspended as viable in eppendroff tube containing 1 ml of phosphate buffered saline (PBS) contaminated with aflatoxin M1 at concentration of 0.05 ug ml^{-1} (50 ng ml^{-1}). The three different concentration of each inoculum strain (1×10^9 CFU ml^{-1}, 3×10^9 CFU ml^{-1} and 5×10^9 CFU ml^{-1}) in table (1) were mixed with 1 ml PBS supplemented with 50 ng ml^{-1} of aflatoxin M1 followed by incubation at 37°C for different times (12h, 24h, 48h and 72h).

Table 1. Viable and Nonviable probiotic species and inoculum dose of treatment

Viable and Nonviable probiotic strains	Inoculum dose of treatment
Lactobacillus acidophilus DSM 20079 (A)	1×10^9 CFU ml^{-1}
	3×10^9 CFU ml^{-1}
	5×10^9 CFU ml^{-1}
Lactobacillus plantarum DSM 20174 (B)	1×10^9 CFU ml^{-1}
	3×10^9 CFU ml^{-1}
	5×10^9 CFU ml^{-1}
Bifidobacterium bifidum DSM 20082 (C)	1×10^9 CFU ml^{-1}
	3×10^9 CFU ml^{-1}

	5×10^{9} CFU ml^{-1}
Combination of probiotic strains (A+ B+C)	5×10^{9} CFU ml^{-1}
+ ve control	PBS + AFM1
- ve control	PBS+ Strain (A) without AFM1
	PBS+ Strain (B) without AFM1
	PBS+ Strain (C) without AFM1
	PBS+ Combination of probiotic strains without AFM

Combination of probiotic strains (A+ B+C) = 333.33 µl of each strain at 5×10^{9} CFU ml^{-1}.

2.3.1 Measurement of aflatoxin M1

Each sample was centrifuged to separate the cells of probiotic strains from the supernatant fluid for analysis by HPLC. The ability of each strain and the combination of strains *(Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum)* to be adsorbed or metabolized aflatoxin M1,then the remaining aflatoxin M1 was be determined by HPLC which was unbounded to the probiotic bacterial strains after the different incubation times (12h, 24h, 48h and 72h). Then the result of remaining aflatoxin M1 amount compared to the positive control and the negative control to evaluate the ability of each strain individually on aflatoxin M1 reduction and to investigate the potential of the interaction or combination of the three strains on aflatoxin M1 reduction [26].

2.3.2 Derivtization of sample

A 100 µl triflour acetic acid with 200 µl N-hexane were added to each sample residues, followed by shaking with vortex for 30 second and samples were left for 15 min at room temperature. Then 900 µl (Water: Acetonitrile, 9:1) were added and mixed well using vortex. The haxane layer was removed and samples were subjected for HPLC analysis.

2.3.3 HPLC-FLD Fluorescence detector analysis and Chromatographic conditions

Determination of aflatoxins (AF) were carried out according to [27] using HPLC system (Model 6000) a solvent delivery system (Model 720) system controller equipped with Fluorescence detector (Model 274) at 360 Ex, and 450 EM. The separation was achieved with a symmetry column, (150x 4.6 mm i.d), 5µm at a flow rate of l ml min^{-1} with an isocratic system composed of 1 % acetic acid: Methanol: Acetonitriel (55: 35:10).

2.4 Assessment of the potential of nonviable probiotic bacterial and yeast strains on sequestration of AFM1

2.4.1 Evaluation of the efficiency of nonviable probiotic bacterial strains *(Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum)* on reduction of aflatoxin M1

The probiotic bacterial strain (*Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum)* were centrifuged at 6,000 rpm for 15 min and the pellets were re-suspended in 10 ml PBS buffer followed by heat treatment through autoclaving (121 ºC and 1.5 psi for 20 min) to become nonviable by heat treatment (the viability was tested by the

culturing the heated microbes and the samples showed no growth were selected). Pellets were further centrifuged at 6,000 rpm for 15 min, washed twice with distilled water, re-suspended in PBS (pH 6.8) and the optical densities were measured at 600 nm to adjust the three different concentrations (1×10^9 CFU ml^{-1} , 3×10^9 CFU ml^{-1} and 5×10^9 CFU ml^{-1}). The experiment was carried out as described in table 2.

Table 2. Probiotic bacterial strains (*Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum*) and inoculum dose of treatment

Nonviable probiotic strains	Inoculum dose of treatment
Lactobacillus acidophilus DSM 20079 (A)	1×10^9 CFU ml^{-1}
	3×10^9 CFU ml^{-1}
	5×10^9 CFU ml^{-1}
Lactobacillus plantarum DSM 20174 (B)	1×10^9 CFU ml^{-1}
	3×10^9 CFU ml^{-1}
	5×10^9 CFU ml^{-1}
Bifidobacterium bifidum DSM 20082 (C)	1×10^9 CFU ml^{-1}
	3×10^9 CFU ml^{-1}
	5×10^9 CFU ml^{-1}
Combination of 3 probiotic strains (A, B &C)*	5×10^9 CFU ml^{-1}
+ ve control	PBS + AFM1
-ve control	PBS+ Strain (A) without AFM1
	PBS+ Strain (B) without AFM1
	PBS+ Strain (C) without AFM1
	PBS+ 3 probiotic strains without AFM1

*Combination of 3 probiotic strains (A, B &C) = 333.33 ul of each strain at 5×10 9 CFU ml^{-1}.

2.5 Evaluation the efficiency of nonviable yeast strains (*Kluyveromyces lactis* and *Saccharomyces cerevisiae*)

The yeast strains (*Kluyveromyces lactis* and *Saccharomyces cerevisiae*) were used as nonviable strains by heating 10 min in autoclave in three different concentrations (1×10^9 CFU ml^{-1}, 3×10^9 CFU ml^{-1} and 5×10^9 CFU ml^{-1}) to assess the potential of these nonviable strains on sequestration of aflatoxin M1. The inoculum strains were mixed with 1 ml PBS supplemented with 50 ng ml^{-1} l of aflatoxin M1 followed by incubation at 37°C for different

times (12h, 24h, 48h and 72h). The experiment was carried out as described in table 3 [28-32].

Table 3. Nonviable yeast species **(*Kluyveromyces lactis* and *Saccharomyces cerevisiae*) and inoculum dose of treatment**

Nonviable yeast strains	Inoculum dose of treatment
Kluyveromyces lactis (CBS2359) (D)	1×10^9 CFU ml^{-1}
	3×10^9 CFU ml^{-1}
	5×10^9 CFU ml^{-1}
Saccharomyces cerevisiae (ATCC 64712) (E	1×10^9 CFU ml^{-1}
	3×10^9 CFU ml^{-1}
	5×10^9 CFU ml^{-1}
Combination of yeast strains (D &E)*	5×10^9 CFU ml^{-1}
+ ve control	PBS + AFM1
- ve control	PBS+ Strain (D) without AFM1
	PBS+ Strain (E) without AFM1
	PBS+ Combination of yrast strains without AF

* Combination of yeast strains (D &E) = 500 µl of each strain at 5×10^9 CFU ml^{-1}.

2.6 Evaluation of the potential of the combination of nonviable probiotic and yeast strains on aflatoxin M1 reduction in PBS

The combination of nonviable probiotic bacterial and yeast strains (5×109 CFU ml-1) were used in concentration of 5×10^9 CFU ml^{-1} at equal volume to evaluate the efficiency of this combination on binding of aflatoxin M1. The experiment was carried out as described in table 4. The inoculum strains were mixed with 1 ml PBS supplemented with 50 ng ml^{-1} of aflatoxin M1 followed by incubation at 37°C for different times (12h, 24h, 48h and 72h) [29,30].

Table 4. Nonviable probiotic bacterial and yeast strains in PBS

Nonviable microbial strains	Inoculum dose of treatment
Combination of probiotic strains (A, B & C)	5×10^9 CFU ml^{-1}
+ yeast strains (D &E) *	
+ ve control	PBS + AFM1

-ve control	PBS + probiotic strains (A, B &C) + yeast strains (D &E) without AFM1

* Combination of probiotic strains (A, B &C) + yeast strains (D &E): The cells were mixed in equal volumes in 1ml of PBS media.

2.7 Evaluation of the potential of the combination of nonviable probiotic bacterial and yeast strains on aflatoxin M1 reduction in skim milk sample

The combination of nonviable probiotic bacterial and yeast strains (5×10^9 CFU ml^{-1}) were used and incubated in skim milk contaminated with aflatoxin M1 at 50 ng ml^{-1} to evaluate their sequestration effect after different time (12, 24, 48 and 72 hour) of incubation as described in table 5. The skim milk was evaluated previously to detect its freedom from AFM1 before being used in the test. After the binding times occurred, the tubes of the milk test were centrifuged to separate the milk layer in supernatant than the pellets of microbial strains were taken for analysis of AFM1 residues and to determine the removal of aflatoxin M1 in milk by the nonviable combination of probiotic [31-33].

Table 5. Dose culture of nonviable combination of probiotic bacterial and yeast strains on aflatoxin M1 in milk

Nonviable microbial strains	Inoculum dose of treatment
Combination of probiotic strains (A, B &C) + yeas strains (D &E) *	5×10^9 CFU ml^{-1}
+ ve control	Milk + AFM1
-ve control	Milk + probiotic strains (A, B &C) + yeast strains (D &E) without AFM1

* Combination of probiotic strains (A, B &C) + yeast strains (D &E): The cells were mixed in equal volumes in 1ml of milk.

2.8 Scanning Electron Microscope analysis (SEM)

Scanning Electron Microscope analysis was used to detect the characterization of the cell walls of the nonviable probiotic bacterial strains (*Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum*), the yeast strains (*kluyveromyces lactis* and *Saccharomyces cerevisiae*) and the combination of bacterial and yeast strains using Energy-Dispersive Analysis X-ray (Joel Jsm 6360LA, Japan). The combination strains in each group (mixed probiotics strains, mixed yeast strains and the combination of bacterial & yeast strains) were mixed in equal volume in 1ml PBS media contaminated with AFM1 (50 ng/ml) and incubated for 72 h at room temperature as treated sample and without AFM1 as untreated sample. Each combination from mixed probiotics, mixed yeast and the combination of bacterial & yeast strains were separately spread over a clean glass slide, coated with gold particles and photographed using scanning electron microscope (SEM) [30].

2.9 Sensory evaluation of treatment yoghurt sample by the best efficient combination of probiotic bacterial and yeast strains on aflatoxin M1 sequestration

Yoghurt was mixed with the combination of nonviable probiotic bacterial and yeast species (treatment).Whenever, the control sample was only Yoghurt without any microbes. The size of each sample was about 50 gm. Yoghurt was prepared from total milk fat obtained from reputable large milk and dairy products supermarket then was boiled for 20 min (to avoid the presence of another microbes in the raw milk). Further, that the milk kept to cool to 43ºC before addition of yorghurt starter cultures (*S. thermophiles* and *L. bulgaricus*) obtained from the same source of milk with shaking to distribute the starter culture in the milk (control sample). Also, milk was inoculated with the combination of nonviable probiotic bacterial and yeast strains (*Lactobacillus plantarum*, *Lactobacillus acidophilus, Bifidobacterium bifidum*, Kluyveromyces *lactis* and *Saccharomyces cerevisiae*) at an inoculum dose of treatment at 5×10^9 CFU ml^{-1} so we used as (treatment sample). Then the inoculated milk for yoghurt preparation was incubated at 43ºC for 5h after that the samples were cooled in refrigerator 4ºC until the sensory evaluation. The panel persons of sensory evaluation included 32 members from Food Technology Department, Animal and Fish Production Department and other departments, Arid Lands Cultivation Research Institute (ALCRI), City of Scientific Research and Technological Applications (SRTA-City). The yoghurt samples (control and treatment samples) were evaluated for appearance, texture, tenderness, flavor and taste and overall acceptance according to scores from 1-7 whereas 1= Very poor, 2= Poor, 3= Fair, 4=Medium, 5=Good, 6= Very good and 7= Excellent was the best score [34,35].

2.10 Statistical analysis

The results were performed by SPSS (Statistical package for social science) software program version 16 for Statistical analysis.

3. Results and discussion

3.1 Evaluation the ability of viable probiotic strains on aflatoxin M1 reduction

Results presented in table 6 shows the effect of different concentration of viable probiotic strains in removal of AFM1 (50 ng ml^{-1}) residues along 72h. It can be seen from the table that *Lactobacillus plantarum* at 1×10^9 CFU ml^{-1} had removal effect on AFM1 (50 ng/ ml^{-1}) to 40.14±1.23, 38.24±1.44, 36.73±11.56 and 33.64±1.25 ng ml^{-1} during different time 12h, 24h, 48h and 72h, respectively. When the inoculum concentration was increased to 3×10^9 CFU ml^{-1}, the removal effect of AFM1 was increased from 32.72% to 34.10% with AFM1 residual at 39±11.07, 37.22±1.64, 35.74±1.32 and 32.95±1.62 ng ml^{-1}, respectively during the different times. The highest concentration of this strain (5×10^9 CFU ml^{-1}) with the highest incubation time (72h) produced the highest removal effect on AFM1 (50 ng ml^{-1}) to 36.90% with AFM1 residual at 50.23±1.36, 38.95±1.24, 35.78±1.24, 33.69±1.41 and 31.55±1.22 ng ml^{-1}.

Lactobacillus acidophilus at 3×10^9 CFU ml^{-1} had removal effect of AFM1 (50 ng ml^{-1}) to 34.26±1.53, 30.78±1.62, 29.02±1.35 and 26.53±1.27 ng ml^{-1} during different time 12h, 24h, 48h and 72h, respectively. However, the concentration of 5×10^9 CFU ml^{-1} produced more AFM1 reduction from 50 to 25.65±1.76 ng ml^{-1}. Also, it clear from the table that *Bifidobacterium bifidum* was reduced AFM1 concentration to (50 ng ml^{-1}) after 72h of incubation period to 27.47±1.36, 24.71±1.31 and 21.16±0.87 ng ml^{-1} at 1×10^9 CFU ml^{-1}, 3×10^9 CFU ml^{-1} and 5×10^9 CFU ml^{-1},respectively so when the probiotic concentration and incubation time were increased, the effect

of removal AFM1 was increased from 45.06% at 1×10^9CFU ml^{-1} to 57.68% at 5×10^9 CFU ml^{-1} after 72h. The removal effect of *Bifidobacterium bifidum* (57.68%) was more than *Lactobacillus plantarum* (36.90%) and *Lactobacillus acidophilus* (48.70%) which was considered the highest viable probiotic strain between other strains.

Moreover, the combination between the three viable probiotic strains (*Bifidobacterium bifidum*, *Lactobacillus plantarum* and *Lactobacillus acidophilus*) at concentration 5×10^9 CFU ml^{-1} produced higher removal AFM1 percent (64.62%) than each individual strain. The combination of different probiotic strains had sequestrate effect with AFM1 (50 ng ml^{-1}) in BPS media to became 17.69±1.24 ng ml^{-1}. Some research reported results in agree with results obtained in this study concerning to the binding effect of some bacterial and yeast strains in PBS media, milk and in yoghurt sample.

These results agree with findings by [24] whereby three strains of lactic acid bacteria; *Lactobacillus delbrueckii* spp. bulgaricus, *Lactobacillus rhamnosus* and *Bifidobacterium lactis* had removal effects of AFM1 in skim milk. This removal was ranged from 0.5 to 0.442±0.022 and to 0.442± 0.022 ng ml^{-1} during 30 and 60 min of incubation respectively. Similalrly, findings by [23] reported that reported that five strains of LAB and bifidobacteria to remove aflatoxin M1(AFM1) from yoghurt. *Lactobacillus plantrium* was the highest strain capable of removing AFM1. Yoghurt fermented by 50% yoghurt culture (*Streptococcus* thermophilus and *Lactobacillus bulgaricus*) and 50% *Lactobacillus plantrium* recorded the highest reduction in the level of AFM1 at the end of storage period. Using a different combination of strains of LAB including *Lactobacillus casei sp*. (ATCC 15088), *Lactobacillus acidophilus* (ATCC 11975), similar results were obtained by [36].The reduction level by these strains ranged from 26.2% to 34.0%, depending upon the bacterial isolates. Studies by [37] on the ability of Lb bulgariscus to reduce AFMI from PBS and yorghurt established a 40% binding after 2h PBS incubation and a further increase to 87.6% afetr 14h. In yogurt the AFM1 binding reached up to 60% after 6 h yogurt incubation. Sarimehmetoğlu and Küplülü (2004) [38] analyzed commonly used yogurt bacteria, *Lactobacillus delbrueckii subsp*. bulgaricus for its binding ability of AFM1 in PBS and in milk. Binding was better in milk (27.6%) than in PBS (18.7%) after 4 h incubation at 37 °C.

Table 6: Effect of different concentration viable of probiotic strains in removal of AFM1 (50 ng/ml) by detection AFM1 residual during different time and removal % after 72h.

Type of strain	Inoculum concentration	0 h	12 h	24 h	48 h	72 h	Removal % after 72h
Lactobacillus plantarum	1×10^9 CFU ml^{-1}	50.17±1.15	40.14±1.23	38.24±1.44	36.73±11.56	33.64±1.25	32.72%
	3×10^9 CFU ml^{-1}	50.04±1.42	39±11.07	37.22±1.64	35.74±1.32	32.95±1.62	34.10%
	5×10^9 CFU ml^{-1}	50.23±1.36	38.95±1.24	35.78±1.24	33.69±1.41	31.55±1.22	36.90%
Lactobacillus acidophilus	1×10^9 CFU ml^{-1}	50.26±0.56	35±1.10	32.71±1.64	30.95±1.52	29.02±1.29	41.96%
	3×10^9 CFU ml^{-1}	50.15±0.66	34.26±1.53	30.78±1.62	29.02±1.35	26.53±1.27	46.94%
	5×10^9 CFU ml^{-1}	50.16±0.90	33.72±1.28	30.29±1.27	27.26±1.43	25.65±1.76	48.70%
Bifidobacterium bifidum	1×10^9 CFU ml^{-1}	50.34±0.78	34.61±1.51	31.952±1.25	31.84±1.24	27.47±1.36	45.06%

	3×10^9 CFU ml^{-1}	50.20±0.56	31.84±1.71	27.59±1.62	26.74±1.38	24.71±1.31	50.58%
	5×10^9 CFU ml^{-1}	50.22±0.65	26.84±1.58	25.29±1.20	23.07±1.43	21.16±0.87	57.68%
CPS-V	5×10^9 CFU ml^{-1}	50.22±1.36	22.93±1.14	20.06±1.25	18.56±1.23	17.69±1.24	64.62%
+ve control	BPS + AFM1	50	49.99	49.98	49.88	49.85	0.00%
-ve control	BPS +P	0.00	0.00	0.00	0.00	0.00	0.00%

CPS-V: Combination probiotic strain viable (*B. bifidum*+ *L. acidophilus* + *L. plantarum*).
Mean and SD of AFM1residual

3.2 Evaluation the efficiency of nonviable

3.2.1. Evaluation the efficiency of nonviable probiotic strains on reduction of aflatoxin M1

Non viable L. Plantarum was found to reduce AFMI from50 ng/ml to 33.54±1.44, 26.15±1.64 and 24.13±0.95 at 1×10^9 CFU ml^{-1}, 3×10^9 CFU $ml^{-1}$and 5×10^9 CFU ml^{-1}, respectively after 72h (Table 7). *Lactobacillus plantarum* had the sequestration effect of AFM1 which produced removal % at 51.74%. On the other hand, nonviable *Lactobacillus acidophilus* at 3×10^9 CFU ml^{-1} reduced concentration of AFM1 from 50 to 17.51±1.28 ng ml^{-1}. However, the concentration at 5×10^9 CFU ml^{-1} had reduction effect on AFM1 concentration to 22.65±1.37, 20.76±1.11, 17.89±1.33 and 16.04±1.00 ng ml^{-1} during different times 12h, 24h, 48h and 72h, respectively. The highest concentration of *Lactobacillus acidophilus* at 5×10^9 CFU ml^{-1} gave 67.92% removal effect.

Bifidobacterium bifidum was considered higher probiotic effect than other two strains on AFM1 sequestration, which had AFM1 removal % at 70.62% to AFM1 removal %. AFM1 reduced to 21.00±1.43, 18.37±1.34, 16.67±1.64 and 14.69±1.62 ng ml^{-1} during 12h, 24h, 48h and 72h, respectively at 1×10^9 CFU ml^{-1} of nonviable *Bifidobacterium bifidum*. When the concentration and the incubation time increased the effect of *Bifidobacterium bifidum* was increased to 21.00±1.31, 18.37±1.37, 16.67±1.27 and 14.69±0.93 ng ml^{-1} during different incubation period. However, the highest reduction effect of nonviable probiotic appeared by combination, these strains to give removal effect to 79.66% and AFM1 concentration residual became 10.17±1.03 ng ml^{-1} after 72h.

Assessed that probiotic-yeast coctile; *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Kluyveromyces lactis* and *Saccharomyce cerevisiae,* had the highest effect of aflatoxins (B1, B2, G1 and G2) removal after 72h (95.59%) in PBS media and when applied in contaminated Cerelac with aflatoxins, the removal percentage was increased by time 6, 12, 24, 48 and 72h to 8.17, 36.12, 44.75, 64.72 and 93.21%, respectively. Also, when these probiotic-yeast coctile were applied in vivo study had a high effective role in the reduction of aflatoxins (B1, B2, G1 and G2) in mother serum rat and also reduction aflatoxins metabolites (M1 and M2) in babies' serum rat serum [39].

Lactobacillus gasseri was tested by [39] for ita ability to remove AFBI from liquid PBS. Heat killed bacteria had a better AFM1 binding ability than the viable bacteria, 61.5% and 30.8%, respectively and studied the abilities of *Lactobacillus rhamnosus* GG (ATCC 53013), *Lactobacillus rhamnosus* LC-705 and *Lactobacillus rhamnosus* 1/3 to bind AFM1 from PBS. *Lactobacillus rhamnosus* GG bound over 50% of the AFM1 in PBS in all tested forms (precultured, freeze dried, viable and heat killed). Viable *Lactobacillus rhamnosus* LC705 bound around 45–46% and the heat-killed more than 50%. The heat killed *Lactobacillus* rhamnosus 1/3 strain bound 40% and the viable 18% of the added AFM1. *Lactobacillus rhamnosus* GG and LC-705 were further tested in skim milk and in

full cream milk. *Lactobacillus rhamnosus* GG bound with limitations: viable cells bound 19% of AFM1 in skim milk and 26% in full cream milk. The heat killed *Lactobacillus rhamnosus* GG bound 27% of AFM1 in skim milk and 37% in full cream milk. The viable *Lactobacillus rhamnosus* LC-705 bound over 60% of the AFM1 in skim and full cream milk when the binding share of heat-treated cells remained at around 30%. While Viable and heat killed *Lactobacillus lactis ssp*. *cremoris* (ARH74) strain removed 40.4% and 38.9% of AFM1, respectively, from PBS [40].

Table 7: Effect of different concentration nonviable probiotic strains in removal of AFM1 (50 ng ml^{-1}) by detection AFM1 residual during different time and removal % after 72h.

Type of strain	Inoculum concentration	0 h	12 h	24 h	48 h	72 h	Removal % after 72h
Lactobacillus plantarum	1×10^9 CFU ml^{-1}	50.00±0.21	39.86±0.31	37.42±1.40	34.52±1.23	33.54±1.44	32.92%
	3×10^9 CFU ml^{-1}	50.02±0.62	37.41±0.58	31.65±1.66	28.02±1.34	26.15±1.64	47.70%
	5×10^9 CFU ml^{-1}	50.10±1.42	34.63±1.63	28.41±1.41	26.69±1.77	24.13±0.95	51.74%
Lactobacillus acidophilus	1×10^9 CFU ml^{-1}	50.20±0.23	29.81±1.52	26.53±1.34	23.55±1.31	20.17±1.32	58.98%
	3×10^9 CFU ml^{-1}	50.11±0.34	28.99±1.26	21.96±1.44	18.99±1.06	17.51±1.28	64.98%
	5×10^9 CFU ml^{-1}	50.12±0.24	22.65±1.37	20.76±1.11	17.89±1.33	16.04±1.00	67.92%
Bifidobacterium bifidum	1×10^9 CFU ml^{-1}	50.09±0.56	21.00±1.43	18.37±1.34	16.67±1.64	14.69±1.62	62.44%
	3×10^9 CFU ml^{-1}	50.15±1.10	26.59±1.61	21.07±1.23	18.19±1.18	15.94±1.16	68.12%
	5×10^9 CFU ml^{-1}	50.10±0.44	21.00±1.31	18.37±1.37	16.67±1.27	14.69±0.93	70.62%
CPS-NV	5×10^9 CFU ml^{-1}	50±0.62	19.81±1.53	16.53±1.34	13.55±1.47	10.17±1.03	79.66%
+ve control	BPS + AFM1	50.22±0.61	50.22±0.61	50.22±1.53	49.90±1.34	49.80±1.47	0%
-ve control	BPS +P	0.00	0.00	0.00	0.00	0.00	0%

CPS-NV: Combination probiotic strain nonviable (*B. bifidum*+ *L. acidophilus* + *L. plantarum*).

3.3 Evaluation the efficiency of some nonviable yeast strains *kluyveromyces lactis* and *Saccharomyces cerevisiae)* on reduction of aflatoxin M1

Non-viable yeast strains was found effective in the removal of AFM1 after 72h (Table 8). It can be seen from table (8) that *Kluyveromyces lactis* at 1×10^9 CFU ml^{-1} had removal effect on AFM1 (50 ng ml^{-1}) to 25.01±1.06, 22.36±1.27, 20.34±1.33 and 19.93±1.25 ng ml^{-1} during different time 12h, 24h, 48h and 72h, respectively. on the other hand at 3×10^9 CFU ml^{-1}, the AFM1 residues became 24.39±1.52, 21.08±1.42, 18.97±1.02 and 16.20±1.64 ng ml^{-1}, respectively during the different times (12h, 24h, 48h and 72h, respectively). However, *Kluyveromyces lactis* at 5×10^9 CFU ml^{-1} reduced AFM1 to 22.48±1.39, 18.86±1.64, 16.67±1.92 and 15.43±1.15 ng ml^{-1}, respectively during the different times which was more removal effect than low concentration.

On the other hand, nonviable *Saccharomyces cerevisiae* reduced AFM1 (50 ng ml^{-1}) to 24.30±1.54, 22.61±1.14, 21.73±1.34 and 17.74±1.35 ng ml^{-1} during 12h, 24h, 48h and 72h, respectively at 1×10^9 CFU ml^{-1}. The effect of *Saccharomyces cerevisiae* was increased to 20.76±1.27, 19.63±1.75, 16.96±1.61 and 13.32±1.28ng/ml at 3×10^9 CFU ml^{-1}. This removal effect of *Saccharomyces cerevisiae* was more increased to 16.81±1.61, 13.59±1.56, 12.32±1.27 and 10.63±1.01 ng ml^{-1} at 5×10^9 CFU ml^{-1} during different incubation time12h, 24h, 48h and 72h, respectively. Also, the results showed that the removal effect of *Saccharomyces cerevisiae* was higher than *Kluyveromyces lactis*.

The combination of nonviable yeast strains (*Kluyveromyces lactis* and *Saccharomyces cerevisiae*) had a higher removal effect at 5×10^9 CFU ml^{-1} of concentration with 72h incubation period (85.68%) on AFM1 (50 ng ml^{-1}) than using each yeast strain separately (69.14% for *Kluyveromyces lactis* and 78.74% for *Saccharomyces cerevisiae*). Findings by [28] on the use of *Saccharomyces cerevisiae* are in agreement with findings from the current study which established it as the most effective species in AFM1 removal. However, when used *Saccharomyces cerevisiae* with LAB strains, the AFM1 removal percentage was increased in the milk sample. Also, the researcher detected the increased of incubation time effect positively on the removal percentage which near to the results of the present study. The highests AFM1 reduction when yeasts were used was in the range 65.33-68.89% [41].

Table 8. Effect of different concentration nonviable yeast strains in removal of AFM1 (50 ng/ml) by detection AFM1 residual during different time and removal % of AFM1 after 72h.

Type of strain	Inoculum concentration	0 h	12 h	24 h	48 h	72 h	Removal % after 72h
kluyveromyces lactis	1×10^9 CFU ml^{-1}	50.21 ±1.0	25.01±1.06	22.36±1.27	20.34±1.33	19.93±1.25	60.14%
	3×10^9 CFU ml^{-1}	50.09±0.88	24.39±1.52	21.08±1.42	18.97±1.02	16.20±1.64	67.60%
	5×10^9 CFU ml^{-1}	50.19±1.30	22.48±1.39	18.86±1.64	16.67±1.92	15.43±1.15	69.14%
Saccharomyces cerevisiae	1×10^9 CFU ml^{-1}	50.23±1.62	24.30±1.54	22.61±1.14	21.73±1.34	17.74±1.35	64.52%
	3×10^9 CFU ml^{-1}	50.32±1.42	20.76±1.27	19.63±1.75	16.96±1.61	13.32±1.28	73.36%
	5×10^9 CFU ml^{-1}	50.14±1.22	16.81±1.61	13.59±1.56	12.32±1.27	10.63±1.01	78.74%
CYS-NV	5×10^9 CFU ml^{-1}	50.19±1.06	14.34±1.47	13.65±1.63	10.46±1.83	7.16±0.90	85.68%
+ve control	PBS + AFM1	50.25±1.21	50.20±1.46	49.98±1.36	49.88±0.98	49.85±1.13	0%
-v econtrol	PBS +P	0.00	0.00	0.00	0.00	0.00	0%

CYS-NV: Combination yeast strains non-viable (*S. cerevisiae +k. lactis*).

3.4 Evaluation the efficiency of some nonviable bacterial and yeast strains *(Lactobacillus plantarum, Lactobacillus acidophilus, Bifidobacterium bifidum, Kluyveromyces lactis* and *Saccharomyces cerevisiae)* on reduction of aflatoxin M1 in PBS

Data presented in table (9) revealed that the combination of probiotic (*Lactobacillus plantarum, Lactobacillus acidophilus* and *Bifidobacterium bifidum*) and yeast strains (*Kluyveromyces lactis* and *Saccharomyces cerevisiae*) had the highest removal effect of AFM1 (87.92%) after 72h of

incubation. Also, the table shows the AFM1 residues to 13.98±1.34, 10.53±1.26, 8.49±0.63 and 6.04±0.15 during different incubation period at 12h, 24h, 48h and 72h, respectively. Another research by [42] reported that *Lactobacillus Casei* TD4 had AFM1 reduction percentage (91.91%), *Lactobacillus bulgaricus* had 87.6% and *Streptococcus thermophilus* had 70% removal of AFM1 however, the efficiency of removal was increased by using the yeast with the bacterial strain. [43] reported that *Bifidobacterium bifidum, Lactobacillus spp.* and *Lactobacillus spp.* had binding ability with AFM1 in solution media. [44] mentioned that probiotic strains in yoghurt had removal effect (49%) of AFM1 at the end of storage period. [45] evaluated that Lactobacillus acidophilus removed 90% of aflatoxin M1 contaminated in yoghurt samples during the first day then the removal increased by the storage time. [38] used a yogurt mixture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*) to study the AFM1 binding during yogurt fermentation. The mixture bound only 15% of the AFM1 added to the yogurt. [36] studied the ability of yogurt culture mixture *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*) to remove AFM1 from PBS and yogurt. In both matrices binding increased during 6 h incubation and reached approximately 45% of AFM1 removal level. In PBS the incubation was continued up to 14 h and the binding share of the mixture reached almost 65%.

Table 9. Effect of nonviable combination of probiotic bacterial and yeast strains in PBS to removal of AFM1 (50 ng ml^{-1}) during different time and removal % of AFM1 after 72h.

Type of strain	Inoculum concentration	0 h	12 h	24 h	48 h	72 h	Removal % after 72h
CPYS-NV	5×10^{9} CFU ml^{-1}	50.23±1.42	13.98±1.34	10.53±1.26	8.49±0.63	6.04±0.15	87.92%
+ve control	BPS + AFM1	50.00±1.16	50.00±1.30	49.95±1.11	49.77±1.08	49.30±0.81	0%
-v econtrol	BPS +P+Y	0	0	0	0	0	0%

CPYS-NV: combination non-viable strains (*B. bifidum+L. acidophilus+L. plantarum +S. cerevisiae+ k. lactis*).

3.5 Evaluation potential of the combination of nonviable probiotic bacterial and yeast strains on aflatoxin M1 reduction in milk

The effect of the highest effective combination in PBS (combination of probiotic bacterial and yeast strains nonviable) for sequestration of AFM1 (50 ng ml^{-1}) in milk as experimental media and distribution the removal % of AFM1 during different times (0h, 12h, 24h, 24h, 48h and 72h) is demonstrated in Table 10.

It shows from the table that the combination of nonviable probiotic bacterial and yeast strains sequestrate of AFM1 (50 ng ml^{-1}) during different times (12h, 24h, 24h, 48h and 72h) with low AFM1 residues as 9.72±1.31, 6.68±0.55, 5.70±0.33 and 4.56±0.15ng ml^{-1}, respectively and with high removal % of AFM1 to 80.56%, 86.64%, 88.60% and 90.88%, respectively in milk sample. [24] when used three strains of lactic acid bacteria (*Lactobacillus delbrueckii spp. bulgaricus*, *Lactobacillus rhamnosus* and *Bifidobacterium lactis*) with *Saccharomyces cerevisiae* (killed by heat), the AFM1 residues decreased to 0.042± 0.003 ng ml^{-1} during 30 while during 60 min there was no AFM1 residues detected (0 ng ml^{-1}). when these LAB strains used with *Saccharomyces cerevisiae* (killed by heat) the AFM1 residues decreased to 0.042± 0.003 ng ml^{-1} during 30 while during 60 min there was no AFM1 residues detected (0 ng ml^{-1}).

Table (10): Effect of the highest effective combination of (probiotic bacterial and yeast strains nonviable) for sequestration of AFM1 (50 ng ml^{-1}) in milk as experimental media and distribution the removal % of AFM1 during different times (0h, 12h, 24h, 24h, 48h and 72h).

Type of strain	Inoculum concentration	0 h	12 h	24 h	48 h	72 h
CPYS-NV in Milk	5×10^{9} CFU ml^{-1}	50.10±1.10	9.72±1.31	6.68±0.55	5.70±0.33	4.56±0.15
+ve control	Milk + AFM1	50.21±0.32	49.90±1.14	49.87±1.05	49.76±1.16	49.33±1.21
-ve control	Milk + CPYS	0	0	0	0	0
Removal %	5×109 CFU/ml	0%	80.56%	86.64%	88.60%	90.88%

CPYS-NV: Total combination non-viable strains (*B. bifidum+L. acidophilus+L. plantarum +S. cerevisiae+ k. lactis*).

3.6 Scanning Electron Microscope (SEM) of different combination from different probiotic bacterial and yeast strains with AFM1

Scanning Electron Microscopy (SEM) results of nonviable combination of probiotic bacterial strains control and treatment are illustrated in Fig1(P). It is clear from the figure the difference in the cell wall of probiotic bacterial strains *(Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum*) in control sample and in treatment one which had spots on their cell wall after adsorption of AFM1 in these spots of cell wall. **Figure 1 (Y)** shows scanning Electron Microscope (SEM) of nonviable combination of yeast strains control (yeast strains without AFM1) and treatment (yeast strains with AFM1) by using magnification at 500 x. It is clear from the figure the difference in the cell wall of yeast strains Kluyveromyces *lactis* and *Saccharomyces cerevisiae*) of the control sample to the treatment yeast sample which had spots on their cell wall after sequestration with AFM1 in these spots on the cell wall. **Figure 1 (P+Y)** shows scanning Electron Microscope (SEM) of nonviable combination of probiotic bacterial and yeast strains (control and treatment) by using magnification at 500 x. It is clear from the figure that the cell wall of both probiotic bacterial and yeast strains *(Lactobacillus plantarum*, *Lactobacillus acidophilus, Bifidobacterium bifidum*, *kluyveromyces lactis* and *Saccharomyces cerevisiae*) in the cell wall in the control sample appeared without this spots on their cell wall while the gical reduction of AFM1. The probiotic-aflatoxin complex and also, yeast-aflatoxin complex imtreatment sample bind or sequestrate with AFM1 in their cell wall spots which act as a good bioloproved the reduction of aflatoxin M1 higher than using probiotic bacterial or yeast strains individually because sequestration sites were became more in the using case of probiotic bacterial with yeast strains.

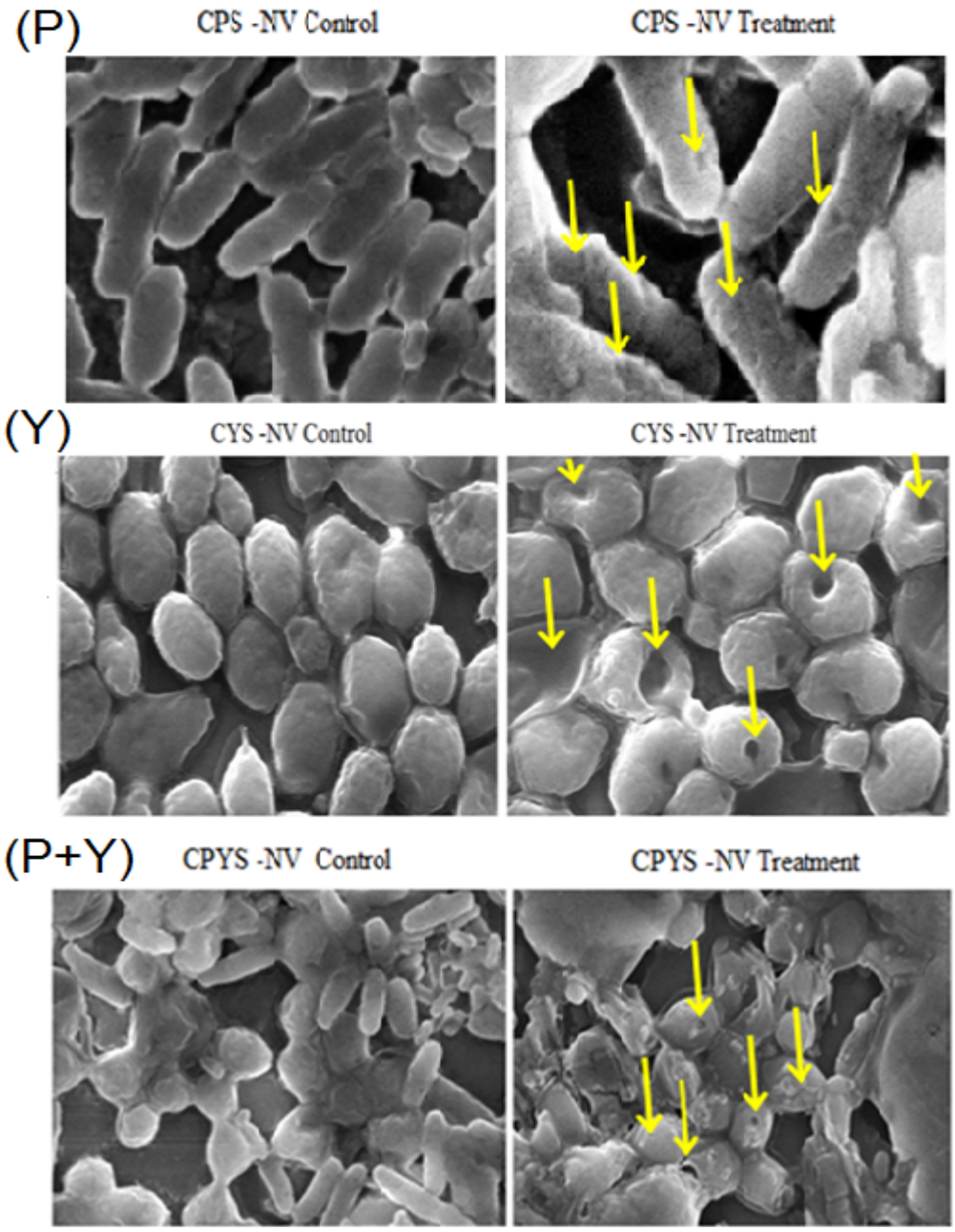

Figure 1. Scanning Electron Microscope (SEM) showing a nonviable combination, control and treatment by using magnification at 500 x.

3.7 Sensory evaluation the best efficient combination of strains on aflatoxin M1 sequestration applied in yoghurt

The mean and standard deviation of sensory evaluation scores of yoghurt was treated with the combination of nonviable probiotic bacterial and yeast strains are illustrated in Table 11. It is clear from the table that control yoghurt sample was taken scores 6.15±0.76, 6.18±0.64, 6.00±0.91,

6.00±0.87 and 5.93±0.87 while inoculated yoghurt sample (inoculated with combination of nonviable probiotic bacterial and yeast strains) (*B. bifidum+L. acidophilus+L. plantarum +S. cerevisiae+ k. lactis*) was taken scores 5.84±1.11, 5.75±1.16, 5.84±1.11, 5.96±1.33 and 5.96±1.23 (good score) regarding to appearance, texture, tenderness, flavor (odour & taste) and overall acceptance, respectively.

Table 11. Sensory evaluation scores of treatment yoghurt sample.

Sensory evaluation parameter	Control yoghurt sample	Treatment yoghurt sample
Appearance	6.15±0.76	5.84±1.11
Texture	6.18±0.64	5.75±1.16
Tenderness	6.00±0.91	5.84±1.11
Flavour (odour & taste)	6.00±0.87	5.96±1.33
Overall acceptance	5.93±0.87 (Good score)	5.96±1.23 (Good score)

32 panel members Maximum score = 7.

The results on the sensory variables of yoghurt with nonviable combination of probiotic bacterial and yeast strains (Treatment yoghurt sample) or without (Control yoghurt sample) are illustrated in Figure 2. Treatment yoghurt sample prepared with nonviable combination of probiotic bacterial and yeast strains *(Lactobacillus plantarum*, *Lactobacillus acidophilus, Bifidobacterium bifidum*, *Kluyveromyces lactis* and *Saccharomyces cerevisiae*) to compare to the control yoghurt sample prepared without these strains in appearance, texture, tenderness, flavour and overall acceptance.

Figure 2. Yoghurt models prepared by nonviable combination compared with the control yoghurt.

The distribution of sensory evaluation scores for yoghurt sample was treated with a nonviable combination of probiotic bacterial and yeast strains are illustrated in Table 12. Treatment yoghurt sample was excellent (score 7) in overall acceptance of (46.87%) of the samples, in appearance (34%), texture (29.41%), tenderness (46.87%) and flavor (50%) by the panel members. On the other hand, the control yoghurt sample was excellent in overall acceptance of (28.12%) with (34%), (29.41%), (28.12%) and (29.41%) in appearance, texture, tenderness and flavor, respectively.

Table 12. Sensory evaluation scores for yoghurt sample treated either by nonviable combination of probiotic bacterial or yeast strains.

Sensory evaluation parameter	Appearance		Texture		Tenderness		Flavor (odour & taste		Overall acceptance	
	C	T	C	T	C	T	C	T	C	T
Excellent (7)	11 34%	11 34%	10 29.41%	10 29.41%	9 28.12%	15 46.87%	10 29.41%	16 50%	9 28.12%	15 46.87%
Very good (6)	16 50%	10 29.41%	18 56.25%	9 28.12%	17 53.12%	6 18.75%	14 43.75%	7 21.87%	14 43.75%	6 18.75%
Good (5)	4 12.5%	7 21.87%	4 12.5%	10 29.41%	4 12.5%	4 12.5%	6 18.75%	3 9.37%	7 21.87%	8 25%
Medium (4)	1 3.12%	3 9.37%	ND	2 6.25%	1 3.12%	6 18.75%	2 6.25%	5 15.62%	2 6.25%	2 6.25%
Fair (3)	ND	ND	ND	ND	1 3.12%	1 3.12%	ND	ND	ND	ND
Poor (2)	ND	1 3.12%	ND	ND	ND	ND	ND	1 3.12%	ND	1 3.12%
Very poor (1)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

C = Control sample of yoghurt

T = Treatment inoculated sample of yoghurt with *B. bifidum+L. acidophilus+L. plantarum +S. cerevisiae+ K. lactis* (nonviable combination of probiotic bacterial and yeast strains) (CPYS).

4. CONCLUSION

In conclusion, probiotic bacteria and yeast strains are able to make detoxification for aflatoxin M1 in contaminated milk. But a combination from probiotic bacteria and yeast could be good for removal and elimination of aflatoxins M1 from milk. Moreover, probiotic bacteria and yeast could be used as food additives to reduce the bioavailability of the aflatoxins in dairy products.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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