American Journal of Food Science and Technology, 2018, Vol. 6, No. 2, 57-67
Available online at http://pubs.sciepub.com/ajfst/6/2/1

DOI:10.12691/ajfst-6-2-1

Combination Probiotic Supernatants Reduce Growth and Aflatoxin Production by *Aspergillus* spp in Food Contamination

Gamal M. Hamad[1,5,*], Marwa M Abu-Serie[2,5], Safwat H. Ali[3], Elsayed E. Hafez[4,5]

[1]Food Technology Department, Arid Land Cultivation Research Institute
[2]Medical Biotechnology Department, Genetic Engineering and Biotechnology Research Institute
[3]Department of Biochemistry Faculty of Agriculture Ain Shams University, Egypt
[4]Plant Protection and Biomolecular Diagnosis Department, Arid Land Cultivation Research Institute
[5]City of Scientific Research and Technology Applications (SRTA- CITY), New Borg El-Arab City, Egypt
*Corresponding author: hamad6751@yahoo.com

Abstract Antifungal proproties of the probiotic culture supernatant (PCS) of three of probiotic bacterial strains; Bifidobacterium bifidum, Lactobacillus acidophilus and Lactobacillus plantarum was tested as antifungal against two mycotoxin producing fungi; *Aspergillus flavus* and *Aspergillus parasiticus*. The results revealed that the probiotic culture supernatant showed high antifungal activity either on the fungal growth and aflatoxin (AF) production. Moreover, probiotic culture supernatant (PCS) at 1% concentration achieved high inhibition of AFB1 production by *Aspergillus flavus* by percentage reached to 76%. But this percentage was increased up to 77% in case of *Aspergillus parasiticus*. In more details, it was observed that probiotic culture supernatant (PCS) are capable to make complete inhibition for the synthesis of both AFG1 and AFG2 produced by *Aspergillus parasiticus*. In addition, the probiotic culture supernatant are rich with the antioxidant compounds which help in food control and induce the human immune system. It can conclude that the probiotic culture supernatant of the three examined probiotic strains could be used as food additives for controlling the food contamination and aflatoxin production.

Keywords: *Probiotic bacteria, Aspergilus spp, Aflatoxins, food contamination, food control*

Cite This Article: Gamal M. Hamad, Marwa M Abu-Serie, Safwat H. Ali, and Elsayed E. Hafez, "Combination Probiotic Supernatants Reduce Growth and Aflatoxin Production by *Aspergillus* spp in Food Contamination." *American Journal of Food Science and Technology*, vol. 6, no. 2 (2018): 57-67. doi: 10.12691/ajfst-6-2-1.

1. Introduction

Aflatoxins are secondary metabolites produced mainly by three filamentous fungal species: *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* [1,2]. The aflatoxins can be classified as B1, B2, G1, and G2 according to their fluorescence under ultraviolet light and molecular weight [3]. It well known that B1 aflatoxin is carcinogenic substance but B2, G1, and G2 are considred as possible carcinogenic substances [4].

Food contamination by aflatoxins is an important food safety concern for agricultural products. In order to identify and develop novel antifungal agents, several isolated compounds and plant extracts have been evaluated for their bioactivities [5]. Mycotoxins and their derivatives since their discoveries and until the present time are behind unspecified economic and medical damages [4]. Aflatoxins are in part responsible, of irreversible medical disasters that are not easily manageable such as cancer of the liver and kidneys, and consequently, showed losses in cereal products [6].

Indeed reference [7] used more than 120 lactic acid bacterial strains as antifungal against *Aspergillus fumigatus* and his finding that the lactic acid bacteria affected the fungal mycelia, biomass and reduced the toxins produced by this fungus. Reference [8] succeeded to isolate the lactic acid bacteria from *Nem chua* (food) and used the isolates to control nine different human pathogenic fungi. They reported that both *Lactobacillus plantarum* (P32B and V13A) and *Pediococcus pentosaceus* (P41A) showed high antifungal activity against the nine examined fungi. The same observation was obtained by Reference [9]. Reference [10] reported that they isolated 336 molds (mostly are *Asprgillus* spp) from dried corn, soaked corn and fermented corn paste and they found different *Lactobacillus spp* showed high antifungal activity especially the two strains *Lactobacillus brevis* G25 and *Lactobacillus cellobiosus*. Moreover, about 60 *Lactobacillus* sp. was used to antagonist the fungi; *Alternaria alternata*, *Alternaria brassicicola*, *Aspergillus niger*, *Fusarium latenicum*, *Geotrichum candidum*, and *Mucor hiemalis*) and yeasts (*Candida vini*). and it was postulated that Lactobacillus bacteria have high antifungal activity against molds and this activity varied

significantly and may affected by the type of used strain and the culture constituents [11].

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" FAO/WHO (*Food and agriculture organization/world health organization* 2002) [12]. In study of Reference [7] several Lactobacillus isolates mixture were screened for their inhibitory effect on mycelial production and good results were reported. The strain, which was identified as *L. casei* KC-324, showed the strongest antifungal activity on fungal growth and spore germination [7]. Lactic acid bacteria, especially Lactobacillus, are the most commonly used microorganisms as probiotics because of the perception that they are desirable members of the intestinal microflora and because these bacteria have "Generally Recognized As Safe" (GRAS) status [13].

Several methods and substances have been suggested from time to time to deal with this hazardous of mycotoxins. Few relevant studies were achieved on the effectiveness of probiotic culture media filterate against growth and aflatoxin production by *A. flavus & A. parasiticus*. Therefore, this study aimed to evaluate the efficiency of supernatant of three probiotic bacteria either to inhibit the growth and the afaltoxin production of two *Asprgillus* spp. The possible using the probiotic supernatnt as food additives as antifungal and/or antioxidant agents.

2. Material and Methods

2.1. Fungal Isolates and Fungal Inoculums Prepration

Aspergillus flavus strain (EMCC 274) and *Aspergillus parasiticus* (EMCC 886^T), were obtained from MERCEN, Ain Shames University, Cairo, Egypt and were kept on Potato-Dextrose-Agar (PDA) slant at 25°C for 10 days. Periodic transfers were done to keep the microorganism viable. The spores were harvested after establishing a good growth rate of each of the fungal cultures and were filtered with sterile cotton filter, to avoid the presence of conidia and mycelia. The spore's suspensions in PBS (pH - 7.0) were adjusted to the final concentrations in the range of 10^5-10^6 spores/ml (10^5 spores/ml A. *flavus* and 10^6 spores/ml *A. parasiticus*). [14,15]

2.2. Probiotic Bacteria and Their Culture Supernatant Preparation

Bifidobacterium bifidum (DSM 20082), *Lactobacillus acidophilus* (DSM 20079) and *Lactobacillus plantarum* (DSM 20174) were individually grown in 200 ml Man, Rogosa and Sharpe (MRS) broth and incubated at 37 °C for 2 days with shaking until OD at 600 nm was ranged from 0.4 to 1.6. After cultivation, the culture broth was centrifuged at 10,000 xg for 10 min. The supernatant was taken to a fresh new conical tube and stored at -70 °C deep freezer. Then, the culture supernatant was lyophilized at -50 °C (using lyophilizer Telstar Model 50, Spain) and the obtained powder was weighed.

2.3. Antifungal Activity of the Probiotic Culture Supernatant

The antifungal activity of the three prepared probiotic culture supernatant (*Bifidobacterium bifidum, Lactobacillus acidophilus* and *Lactobacillus plantarum*) were tested for their antifungal activity against *Aspergillus flavus* and *Aspergillus parasiticus* using agar well diffusion method according to [16,17] with sterile core borer of size 10.0 mm and with some modifications. The cultures of 48 hours old grown on potato dextrose agar (PDA) were used for inoculation of fungal strain on PDA plates. Then spread over the PDA plates using sterile cotton swabs. After dryness, the appropriate wells were made on agar plate by using cork borer. The culture supernantnt was loaded to each separate well (200 ul) and the plates were then kept at 4°C for 30 min. The plates were then incubated at 29°C for 3 days and were checked for clear zones formation. The formed clear zones were recorded and measured in millimeter and considered as antifungal activity of various supernantant. The antifungal potential of different supernatant was evaluated by comparing their zones inhibition.

2.3.1. MIC of the Three Probiotic Supernantant against *Aspergillus* spp

The combined mixture of culture supernatant that showed high antifungal activity against *Aspergillus flavus* and *Aspergillus parasiticus* were chosen, and their Minimum Inhibitory Concentration (MIC) was determined using descending concentrations of the mixed culture media filterates. The MIC of the mixed supernatant were diluted using sterile H_2O and were tested for their antifungal activity against *A. flavus* and *A. parasiticus* according to [18]. The different prepared concentrations were tested against the fungi strain using well diffusion assay as previously mentioned. The formed clear zones were measured and recorded and the MIC for each probiotic culture supernatant was determined.

2.4. Fungal Growth and Aflatoxin Production by Treated Fungi and Their Control

Fifteen ml of Yeast-Extract Sucrose (YES) broth medium, were put in a 250 ml flasks and then autoclaved at 120°C or 1.5 psi for 15 min. Inoculation was carried out by adding 1 ml of a suspension of spores (10^6 spores/ml) of toxigenic *A. flavus* and *A. parasiticus* strains without (control) or with 1%, 0.5% and 0.1% of one of the tested probiotic culture supernatant. The flasks were incubated in the dark for 7 days at 25°C. Then after incubation period, the growth of the mycotoxingenic fungi *A. flavus and A. parasiticus* in all flasks was visually examined.

2.4.1. Extraction of Aflatoxin from *A. flavus* and *A. parasiticus* Culture Filterates

Extraction of mycotoxins produced in the Yeast extract sucrose (YES) broth culture was carried out according to the method of [19]. Where, the mycelium of each flask contained Yeast extract sucrose (YES) broth was harvested by filtration through Whatman paper (No.4), and then extracted by 100 ml chloroform. Chloroform

extract was dried by addition of anhydrous sodium sulfate. The residue was transferred to eppendorff tube and evaporated off using a stream of nitrogen at temperature below 60°C. The dry film was used for the detection of aflatoxins by High Performance Liquid Chromatography (HPLC). The percentage of inhibition of fungal growth and aflatoxins were calculated using equation:

% inhibition = (control- treatment /control x100).

2.4.2. Determination of Aflatoxins by HPLC in the Fungal Culture Filtrate

Derivatization: the derivatives of tested samples and standards (control) were done as follow: Two hundred µl hexane were added to the clean up dry film of standard and tested samples followed by 50 µl Trifluoroacetic Acid (TFA) and mixed by vortex vigorously for 30 s. The mixture was let to stand for 5 min. To the mixture 450 ml water- acetonitrile (9 +1 v/v) by pipette were added and mixed well by vortex for 30 seconds, and the mixture was left to stand for 10 min. to form two separate layers.The lower aqueous layer was used for HPLC analysis [20,21]. The chromatographic system consisted of an automatic Agilent HewlettPackard brand n°1100 series, is managed by computer with the chemstation software. It is equipped with an auto-sampler (100 µl, injector loop), a Zorbax column with a Reverse-Phase C18 (4.6 x 250 mm, 5 µm) and a fluorescence detector. The detector was set at Ex= 360 nm, Em = 440 nm. The mobile phase was isocratic and composed of water - acetonitrile - methanol (6/2/3, v/v/v) with 120 mg of potassium bromide and 350 µl of 4 M nitric acid per liter of mobile phase. The flow rate was set at 1 ml/min. Each experiment was conducted in duplicate and aflatoxins contents were determined according to their corresponding standard curves. Calibration curves for each aflatoxin were determined, using a series of standard solutions prepared in methanol. Linear calibration graphs were obtained by plotting the peak area against the aflatoxin amount injected. Quantification of aflatoxins was performed by comparing the peaks areas with the calibration curves.

2.5. Determination the Cytotoxisity of the Probiotic Culture Supernatant (MTT) Assay

Peripheral blood is the available source of human normal cells for investigations of the toxicity of probiotic supernatant. Peripheral Blood mononuclear cells (PBMCs) were isolated according to the method was described by [22] using Ficoll-Hypaque density gradient centrifugation method.Fresh heparinized blood was mixed with an equal volume of PBS, slowly layered over equal volume of the Ficoll-Hypaque solution (density = 1.077 g/ml) and centrifuged for 30 min at 2000 rpm. The PBMCs at buffy layer was collected, resuspended in PBS and centrifuged for 5 min at 1650 rpm. Cells were resuspended in RPMI 1640 medium containing 10% FBS, counted, and viability was determined by staining of 50µl cells with 0.5% trypan blue and counting on a hemocytometer. The cytotoxicity assay was done according to [23]. To each well of the 96 well microtitre plate, $1x10^5$ mononuclear cells were seeded and treated with the serial dilutions of probiotic culture supernatant (6.25, 12.5, 25, 50 and 100 µg/ml). After 72 h incubation in 5% CO_2 incubator, 20 µl of the yellow water soluble substrate 3-(4,5-dimethyl thizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/ml in PBS, pH 7) was added to each well and incubated at 37°C for 4h. MTT solution was removed after centrifugation at 2000 rpm for 10 min and the insoluble blue formazan crystals trapped in cells were solubilized with 150 µl of 100% DMSO at 37°C for 10 min. The absorbance of each well was measured with a microplate reader at 570 nm. The half maximal Inhibitory Concentration (IC_{50}) and safe dose (EC100) values, as indices of toxicity and safety, were determined from the Graphpad Instat software using data that was calculated from the equation of cell viability.

2.6. Determination of the Antioxidant Activity of the Probiotic Supernatant by (DPPH) Assay

This assay measures the free radical scavenging capacity of the investigated probiotic culture supernatant (PCS) on the radical DPPH. In the presence of an antioxidant, which can donate an electron to DPPH, the purple color typical for free DPPH radical decays, and the absorbance change at λ = 517 nm is measured. Radical scavenging activity ofvarious concentrations (3.125, 6.25, 12.5, 25, and 50 µg/ml) of each probiotic culture supernatant (individual or mixed) was assayed according to [24] and similar concentrations of ascorbic acid were used as reference standard. The assay mixture contained in a total volume of 100 µl of prepared DPPH (0.004% in methanol) was added to 10 µl of the serial dilution of filterate or vitamin C (4.5 µg/ml). The plate was shaken and placed into the dark for 30 min. Then the decrease in absorbance was measured at 517 nm.

2.7. Determination of Total Phenolic Content in Mixed Probiotic Culture Supernatant

The total phenolic content of the probiotic culture supernatant was determined by Folin-Ciocalteu spectrophotometric method (26). A volume of 0.1 ml of Folin-Ciocalteu reagent was added to 2 ml (1%) of the probiotic culture supernatant. The mixture was allowed to stand for 15 min. Then, 3 ml of 2% sodium carbonate (Na_2CO_3) was added. The mixture was allowed to stand for 30 min at room temperature and the total phenolic content was determined spectrophotometrically (Labo America, USA) at 760 nm compared with Gallic acid as a standard. Total phenols were expressed in terms of mg of Gallic acid equivalent per gram of the sample using the linear regression equation obtained from the standard Gallic acid calibration curve y = 0.005x + 0.245. All samples were analyzed in triplicates [26].

2.8. Determination of Phenolic Compounds Contents in the Mixed Probiotic Supernatant by HPLC

The HPLC instrument conditions for analysis of phenolic compounds were Agilent1260 infinity HPLC

Series (Agilent, USA), equipped with Quaternary pump, a Zorbax Eclipse plus C18 column 100 mm x 4.6 mm i.d., (Agilent technologies, USA) and operated at 25°C. The separation was achieved using a ternary linear elution gradient with (A) HPLC grade water 0.2 % H_3PO_4 (v/v), (B) methanol and (C) acetonitrile. The injected volume was 20 µl. Detection: VWD detector set at 284 nm. This method was conducted according to Agilent Application Note, Publication number 5991-3801EN, 2016.

2.9. Statistical Analysis

Data were expressed as mean ± standard error (SE) by multiple comparisons one-way analysis of variance (ANOVA) using SPSS16 software program at probability (p) - values < 0.05 considered statistically significant.

3. Results

3.1. Antifungal Activity of Probiotic Culture Supernatant on Fungal Mycelium and/ Spors (Individual)

The effect of antifungal activity of each probiotic culture supernatant was assayed as shown in (Figure 1 and Figure 2) and (Table 1 and Table 2). It was observed that mycelium was completely inhibited (Figure 1) but the spores completely inhibited as shown in (Figure 2). The probiotic culture supernatant of concentrations (0.1, 0.5 & 1%) showed varied inhibitions zones on both mycelium and spores of the fungi (*A.flavus* and *A. parasiticus*). It was observed that concentration 1% showed high antifungal activity against the two examined fungi and this activity gave inhibition zones ranged from is showing from 0.6 to 4.0 cm. Additinally, *A. flavus* more sensitive for the three examined probiotic culture supernatant than *A. paraisticus*.

3.2. The Inhibition Effect of the Probiotic Culture Supernatant on Fungal Mycelium and Spores (Mixed)

The antifungal activity for the mixed probiotic culture supernatant was illustrated in (Figure 3) and (Table 3). The mixed probiotic culture supernatant (1%) showed inhibitions zones of mycelium growth and spores numbers of two examined fungus species; *A. flavus* and *A. parasiticus*. Results in (Table 3) show that twice combined probiotic culture supernatant *(Lactobacillus acidophilus & Bifidobacterium bifidum), (Lactobacillus acidophilus & Lactobacillus plantarum), (Lactobacillus plantarum & Bifidobacterium bifidum)* have higher inhibition zones with values 4.5, 4.4 & 4.2 cm in *Aspergillus flavus* compared to inhibition value 3.0, 3.2 & 2.9 cm in *Aspergillus parasiticus* for combined respectively. However, the triple mixed probiotic culture supernatant *(Lactobacillus acidophilus, Bifidobacterium bifidum* and *Lactobacillus plantarum*) was more effective as antifungal activity with inhibition zones 4.7 cm in *Aspergillus flavus* compared to 3.5 cm in *Aspergillus parasiticus.* Also the same trend was observed in inhibition mycelium and spores for binary probiotic culture supernatant *(Lactobacillus acidophilus & Bifidobacterium bifidum), (Lactobacillus acidophilus & Lactobacillus plantarum), (Lactobacillus plantarum & Bifidobacterium bifidum)* which give inhibition mycelium and spores (IMS) 0.4, 0.3, 0.2 cm compared to 2.3, 2.8 &2.6 cm in *Aspergillus flavus and Aspergillus parasiticus* respectively. In addition, the treatments (triple) probiotic culture supernatant (PCS) gave IMS in size 0.5cm with *Aspergillus flavus* compared to 3.1cm with *Aspergillus parasiticus* respectively. In general, it is noticed that mixed probiotic culture supernatant (PCS) was more effective as antifungal against *Aspergillus flavus and Aspergillus parasiticus* than the single or the binary (PCS).

Table 1. Inhibition of spores (clear zones in cm) for antifungal activities of single probiotic culture supernatant (PCS) against *Aspergillus flavus* and *Aspergillus parasiticus*

Strains of probiotic culture supernatant (PCFs)	Diameter of inhibition zone (cm)					
	A. flavus			*A. parasiticus*		
	Concentration (%)					
	0.1 %	0.5 %	1 %	0.1 %	0.5 %	1 %
L. acidophilus	3.7±0.42	3.8±1.2	4.0±1.1	ND*	2.0 ±0.6	2.8 ±0.3
B. bifidum	2.6 ±0.93	3.5 ±0.5	4.0 ±0.8	ND	1.4 ±0.12	2.2 ±0.23
L. plantarum	2.1 ±0.31	3.0 ±0.7	3.8 ±1.2	ND	ND	0.6 ±0.47

ND* not detected. All values were expressed as mean ± SE at p< 0.05

Table 2. Inhibition of mycelium and spores or spores (clear zones in cm) for antifungal activities of individual probiotic culture supernatant (PCS) against *Aspergillus flavus* and *Aspergillus parasiticus*

Strains of probiotic culture supernatant (PCFs)	Diameter of inhibition zone (cm)					
	A. flavus			*A. parasiticus*		
	Concentration (%)					
	0.1 %	0.5 %	1 %	0.1 %	0.5 %	1 %
L. acidophilus	ND	ND	0.4±0.22	ND*	0.9±0.47	2.2±0.36
B. bifidum	ND	ND	0.35±0.34	ND	0.4±0.38	1.8±0.37
L. plantarum	ND	ND	0.3±0.51	ND	0.4±0.49	0.8±0.64

ND* not detected. All values were expressed as mean ± SE at p< 0.05

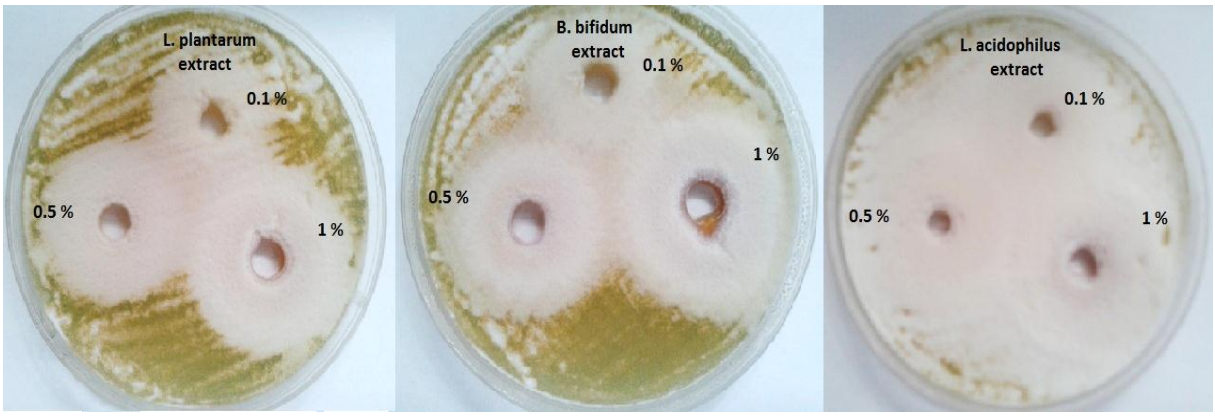

Figure 1. Clear zones of the antifungal activities of individual probiotic culture supernatant (PCS) of Lactobacillus acidophilus, Lactobacillus plantarum and Bifidobacterium bifidum against Aspergillus flavus

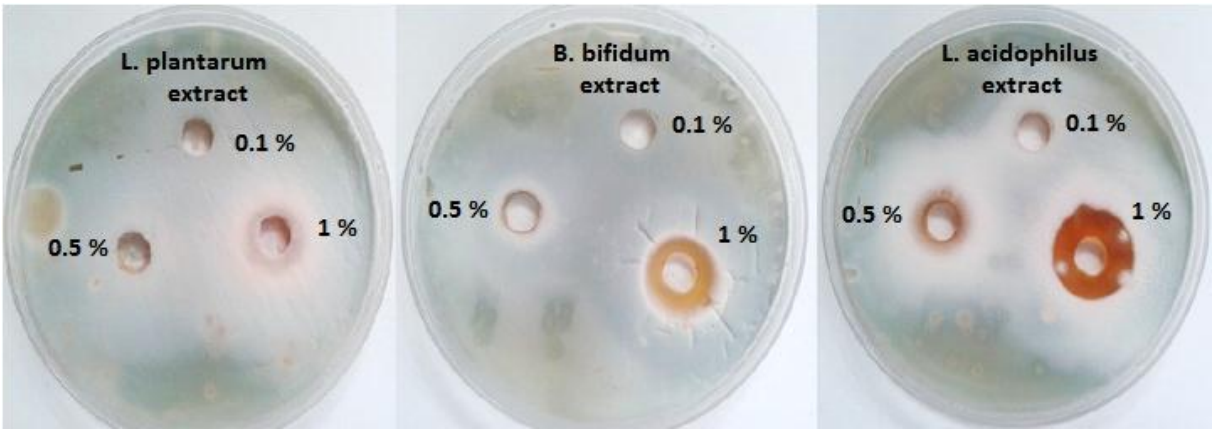

Figure 2. Clear zones of the antifungal activities of individual probiotic culture supernatant (PCS) of Lactobacillus acidophilus, Lactobacillus plantarum and Bifidobacterium bifidum against Aspergillus parasiticus

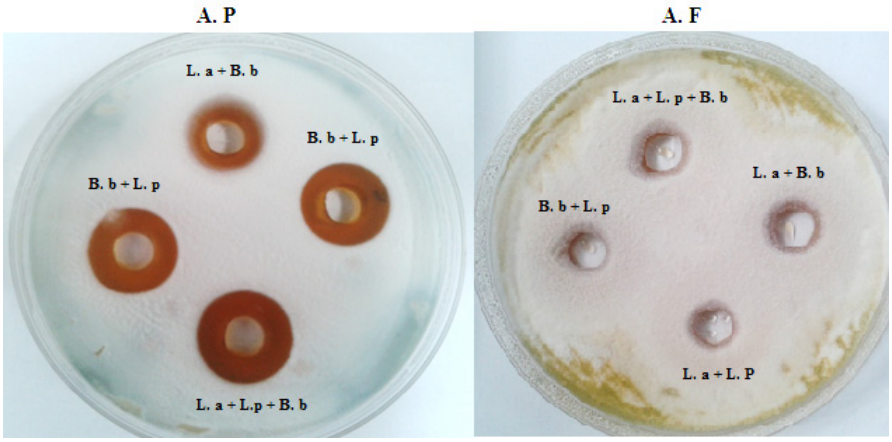

Figure 3. Clear zones of the antifungal activities of mix probiotic culture supernatant of different strains (Lactobacillus acidophilus (L.a.), Lactobacillus plantarum (L.p.) and Bifidobacterium bifidum (B.b.) against Aspergillus parasiticus (A.p.) and Aspergillus flavus (A.F)

Table 3. Inhibition of mycelium and spores or spores (Clear zones in cm) for antifungal activities of double and triple mixed probiotic culture supernatant (PCS) against *Aspergillus flavus* and *Aspergillus parasiticus*

Strains of probiotic culture supernatant (PCS)	Diameter of inhibition zone (cm)			
	A. flavus		*A. parasiticus*	
	Concentration (1%)			
	Inhibition of (IMS) diameter (cm)	Inhibition Spores (cm)	inhibition (IMS) diameter(cm)	inhibition Spores (cm)
L. acidophilus* + *B. bifidum	0.4±0.14	4.5±1.2	2.3±0.54	3.0±0.51
L. acidophilus* +*L. plantarum	0.3±0.07	4.4±1.6	2.8±0.83	3.2±1.1
B. bifidum* +*L. plantarum	0.2±0.05	4.2±.11	2.6±0.68	2.9±0.82
L. acidophilus* + *L. plantarum* + *B.bifidum	0.5±0.03	4.7±1.8	3.1±1.1	3.5±1.6

IMS: inhibition of mycelium and spores. All values were expressed as mean ± SE at $p < 0.05$

3.3. The MIC of Mixed Combination Probiotic Culture Supernatant against *A. flavus* and *A. parasiticus*.

Minimum Inhibitory Concentration (MIC) of probiotic culture supernatant (PCS) as antifungal activity descending gradient concentrations from 1% to 0.006% of mixed (PCS) at percentage 1.0, 0.5, 0.1, 0.05,0.025, 0.012, 0.006% (b) for mixed (PCS) of *(Lactobacillus plantarum, Lactobacillus acidophilus and Bifidobacterium bifidum).* against *A. flavus.* (a & b) and *A. parasiticus.* (c & d) were illustrated in (Figure 4) and Table (4). Results in (Table 4) show that MIC for 1% mixed probiotic culture supernatant (PCS) resulted inhibition zone 0.55 cm in *A. flavus* sample, while a remarkable inhibition zone (IZ) was 3.1 cm in *A. parasiticus* at the same concentration 1% of mixed PCFs. It is also observed that lower concentration than 1% of mixed (PCS) were not affected in sample treated with *A. flavus*. Meanwhile, inhibition zone (IZ) recorded 1.5, 0.3, & 0.15 cm were observed in sample contaminated with *A. parasitcus* and treated with 0.5, 0.1 & 0.05 % mixed (PCS). In contrary, lower concentration 0.006, 0.012 & 0.025 % of mixed PCs were not affected in sample treated with *A. parasiticus*. Generally, lower concentrations of mixed (PCS) have not induced detected inhibition as shown from (Figure 4) and (Table 4). Obviously from it was observed that spores inhibition was higherin the treated *A. flavus* with all the treatments but *A. parasiticus* not affected with low tretamens.

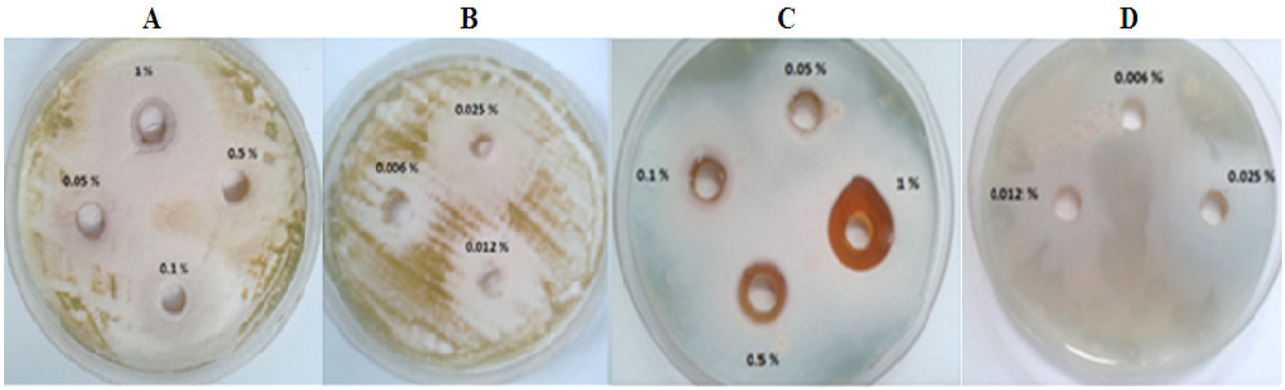

Figure 4. Minimum inhibitory (MIC) of antifungal activity of mixed probiotic culture supernatant (PCS) at descending gradient concentrations percentage 1.0, 0.5, 0.1, & 0.05%, (A); 0.025,0.012 & 0.006% (B) of mixed (PCS) of *Lactobacillus acidophilus, Lactobacillus plantarum and Bifidobacterium bifidum* (L.a. +L.p.+B.b). against *A.flavus*.(A & B) and *A.parasiticus*(C & D)

Table 4. Minimum inhibitory concentrations (MIC) of mixed probiotic culture supernatant (PCS) (L. acidophilus + L.plantarum +B. bifidum) against A. flavus and A. parasiticus (inhibition of mycelium and spores or spores)

Concentrations of mixed PCFs		Fungi			
		A. flavus		*A. parasiticus*	
Conc. of mixed PCS (%)	Conc. of mixed PCS (µg/ml)	inhibition zone diameter(cm)	inhibition spores (cm)	inhibition zone diameter(cm)	inhibition spores (cm)
1 %	10000 µg/ml	0.55±0.04	3.0±0.86	3.1±0.61	2.9±0.82
0.5 %	5000 µg/ml	ND	2.8±0.65	1.5±0.46	2.5±0.63
0.1 %	1000 µg/ml	ND	2.6±0.54	0.3±0.018	1.9±0.34
0.05 %	500 µg/ml	ND	2.1±0.49	0.15±0.07	1.4±0.14
0.025 %	250 µg/ml	ND	1.9±0.21	ND	ND
0.012 %	120 µg/ml	ND	1.5±0.38	ND	ND
0.006 %	60 µg/ml	ND	1.0±46	ND	ND

ND not detected. All values were expressed as mean ± SE at p< 0.05

Table 5. Effect of different concentrations of mixed probiotic culture supernatant (PCS) (L. acidophilus + L. plantarum +B. bifidum) as antiaflatoxigenic activity againstA. parasiticus and A. flavus

Aflatoxins (ng/ml)	*A. flavus*				*Reductin%*	*A. parasiticus*				*Reductin %*
	Control	(0.1 %)	(0.5 %)	(1%)		Control	(0.1 %)	(0.5%)	(1 %)	
B1	75.265 ±5.12	68.295 ±3.21	47.132 ±4.23	18.248 ±3.15	57.017	168.249 ±6.36	124.846 ±5.24	85.635 ±3.81	40.423±2.47	127.826
B2	0.6247 ±0.14	0.458 ±0.046	0.274 ±0.026	0.092 ±0.0056	0.5327	1.634 ±0.41	1.245 ±0.086	0.783 ±0.13	0.405 ±0.035	1.229
G1	12.158 ±1.5	9.819 ±1.32	6.736 ±1.08	2.471 ±0.53	9.687	ND	ND	ND	ND	0
G2	0.0593 ±0.003	0.0416 ±0.005	0.0235 ±0.0015	0.0083 ±0.0021	0.051	ND	ND	ND	ND	0

ND not detected. All values were expressed as mean ± SE at p< 0.05.

3.4. Antiaflatoxigenic Effect of Mixed Probiotic Culture Supernatant (PCS) against Aflatoxin Production from *A. flavus* and *A. parasiticus*

Data in (Table 5) show the effect of different concentrations (0.1, 0.5 & 1 %) of mixed (PCS) *(Lactobacillus acidophilus, Lactobacillus plantarum and Bifidobacterium bifidum*) as antiaflatoxigenic activity against *A.flavus* and *A. parasiticus*. It was observed from (Table 5) *A.flavus* produces Aflatoxin lower than *A.parasiticus* 75 compared to 168 ng/ml in control samples respectively. Reduction in aflatoxin production Aflatoxin B1 from *A.flavus* were reduced from 75 in control sample to 68, 47, & 18 after treatment with 0.1, 0.5 and 1% concentration of mixed (PCS). At the same trend the production of Aflatoxin B1 was decreased from 168 ng/ml to 124, 85 & 40 ng/ml in *A.parasiticus* treated with 1% mixed (PCS) respectively. It is also observed that mixed (PCS) ssignificantly reduce aflatoxin production and the highest reduction was obtained with mixed (PCS) on *A.parasiticus* where the values were dramatically inhibited about four folded from 168 in control sample to 40 in sample treated with mixed (PCS) 1% concentration respectively. Generally all tested concentrations; 0.1, 0.5 & 1.0% of the mixed (PCS) capable inhibit AFtoxin production from both *A.flavus and A.parasiticus* in (Table 5). Moreover, proportional relationship found between the concentration of (PCS) and inhibition of the production of Aflatoxin in both tested fungi. Also, Aflatoxin G1 & G2 were inhibited due to (PCS) treatment in *A.flavus*., while it is absent and not detected in *A.parasiticus.* in control and treated samples.

3.5. Cytotoxicity Assay

The IC_{50} and EC_{100} were measured in the present study to evaluate the cytotoxicity effect of probiotic culture supernatant toward normal human peripheral blood mononuclear cells (PBMCs) using MTT assay. (Table 6) represents IC_{50} and EC_{100}µg/ml of single and combined probiotic culture supernatant. The maximum safe concentrations EC_{100}were 154.06, 139.22 &132.96 µg/ml for single *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Bifidobacterium bifidum*, respectively. The double and triple mixed probiotic culture supernatant *(Lactobacillus plantarum + Lactobacillus acidophilus) and (Lactobacillus plantarum + Lactobacillus acidophilus + Bifidobacterium bifidum)* gave the same effect as individual (PCS) with values between 168.9 and134.74 µg/ml respectively. Data in (Table 6) show that the highest values of IC_{50} were 312.53 & 367.9 µg/ml in single and double mixed (PCS) of *Lactobacillus acidophilus* only or along with *Lactobacillus plantarum* respectively. Meanwhile, no significant difference was observed between the doubleor triple combined probiotic culture supernatant (PCS). IC50 revealed that the maximum concentration recorded were 312.53, followed by 258.09, and 249.61, µg/ml for single probiotic culture supernatant of *(Lactobacillus plantarum, Lactobacillus acidophilus and Bifidobacterium bifidum)* respectively. In addition, the mixed (PCS) gave the same effect with value 367.9 and 360.83 indouble and triple mixed (PCS) *(Lactobacillus plantarum + Lactobacillus acidophilus) and (Lactobacillus plantarum + Lactobacillus acidophilus + Bifidobacterium bifidum)* are respectively. However, the highest IC_{50} reflect safety of both combined probiotic filterate.

Table 6. *IC_{50} and EC_{100} of* probiotic culture supernatant *against human PBMCs*

probiotic culture supernatant (PCS)	IC_{50} (µg/ml)	EC_{100} (µg/ml)
Lactobacillus acidophilus (L.a.)	312.53±19.44	154.06±8.54
Lactobacillus plantarum (L.p.)	258.09±6.11	139.22±2.46
Bifidobacterium bifidum (B.b.)	249.61±5.95	132.96±7.02
L. acidophilus +L. plantarum	367.9±5.9	168.9±2.8
L. acidophilus + L. plantarum + B.bifidum	360.83±6.6	134.74±4.94

All values were expressed as mean ± SE at p< 0.05

3.6. Antioxidant and Reducing Power of Probiotic Culture Supernatant (DPPH Assay)

Data in (Table 7) shows the results of antioxidant activity of probiotic culture supernatant on DPPH. Scavenging activity of probiotic culture supernatant on 1,1 Diphenyl-2-picrylhydrazyl (DPPH) is shown in (Table 7). Results revealed that the radicals scavenging activity of probiotic at IC50 were 34.19, 42.45 and 42.67 µg/ml as individual probiotic culture supernatant for *(Lactobacillus plantarum, Lactobacillus acidophilus and Bifidobacterium bifidum)* respectively. eanwhile, these values were 14.3 & 52.51 in twice mixed *(Lactobacillus plantarum, Lactobacillus acidophilus)* and triple mixed filterates *(Lactobacillus plantarum, Lactobacillus acidophilus and Bifidobacterium bifidum* compared to 4.46 for vit.C as reference standard control. Scavenging activity of *(Lactobacillus acidophilus* show highest potential than other filterates inthe present study. Not only mixed probiotic culture supernatant *(Lactobacillus plantarum, Lactobacillus acidophilus)* appeared higher potential scavenging power activity than all individual or triple mixed filterates with *B.b*.but, also, it have higher IC_{50} & EC_{100} as shown (Table 6). Accordingly, data show high potential antioxidant activity of these filterates against DPPH radicals. It is worth to mention that, both two combined probiotic culture supernatant represent three fold of vit.C scavenging activity and consequently, reflect the powerful of those both double mixed probiotic culture supernatant as a superior additive substance to food in biological defense system against oxidative stress.

Table 7. DPPH scavenging activity of single and mixed probiotic culture supernatant (PCS) (*L. acidophilus, L.plantarum and B. bifidum*)

Individual or mixed PCFs	IC50 (µg/ml)
Lactobacillus acidophilus (*L.a.*)	34.19±1.04
Lactobacillus plantarum (*L.p.*)	42.45±1.08
Bifidobacterium bifidum (*B.b.*)	42.67±0.46
L. acidophilus +L. plantarum	14.3±1.2
L. acidophilus + L. plantarum + B.bifidum	52.51±0.6
Vitamin C	4.46±0.38

All values were expressed as mean ± SE at p< 0.05

3.7. Total Phenolic Compounds in Probiotic Culture Supernatant (PCS)

Total phenolics concentration of probiotic culture supernatant was determined and reached 140.5 mg as Gallic acid/g mixed probiotic culture supernatant (PCS). The obtained results revealed higher antioxidant capacity of these probiotic culture supernatant which reflect its abilityto play a promising role as scavengerof free radicals and considered as a good benefit scavenger when added to food, as food additive.

3.8. HPLC Analysis of Phenolic Compounds for Mixed Probiotic Culture Supernatant

HPLC analysis for mixed probiotic culture supernatant (PCS) has been used to qualitative and quantitative analysis of different phenolic compounds found in (PCS). As shown in (Figure 5) and Table 8, forty peaks of phenolic compounds have been appeared in chromatogram (Figure 5). On one side, fourteen phenolic compounds were detected and identified while,the rest twenty six compound detected but were unknown as shown in chromatogram (Figure 5). The obtained results revealed that, the highest phenolic compound was p-hydroxy benzoic acid with concentration 16.13 mg/100g. While the lowest phenolic compound was p-coumaric acid with concentration 0.04 mg/100g in mixed (PCS). The rest fourteen compounds showed a different concentrations and their range was variable and ranged between the two mentioned values. Fractionated phenolic compounds found in (Figure 5) have antifungal properties like benzoic acid, Salicylic acid, Gallic acid o-Coumaric acid and Gallic acid which found in 1.83, 3.40, 1.20, 0.50, 1.20 mg/100g (PCS) respectively. Most of the (PCS) phenolic compounds have antifungal activity against both of *A. flavus*. and and *A. parasticus*.

Table 8. Qualitative and quantitative fractionated phenolic compounds of mixed probiotic culture supernatant (PCS) (mg/100 g) from HPLC chromatogram

Number	Retention time	Phenolic compound	Conc. (mg %)
6	4.116	Gallic acid	1.20±0.084
10	8.542	Catechol	0.49±0.061
12	10.32	p- Hydroxy benzoic acid	16.13±1.82
13	10.341	Caffeine	0.28±0.051
14	11.463	Vanillic acid	0.46±0.15
15	11.850	Caffeic acid	0.06±0.021
18	13.451	Syringic acid	ND
19	7.764	Vanillin	1.13±0.27
20	14.598	p- Coumaric acid	0.04±0.014
22	15.723	Ferulic acid	0.07±0.012
26	17.163	Ellagic acid	0.44±0.016
27	17.900	Benzoic acid	1.83±0.36
28	18.495	o- Coumaric acid	0.50±0.0.41
31	20.063.	Salicylic acid	3.40±0.24
34	23.388	Cinnamic acid	0.15±0.013

All values were expressed as mean ± SE at p< 0.05

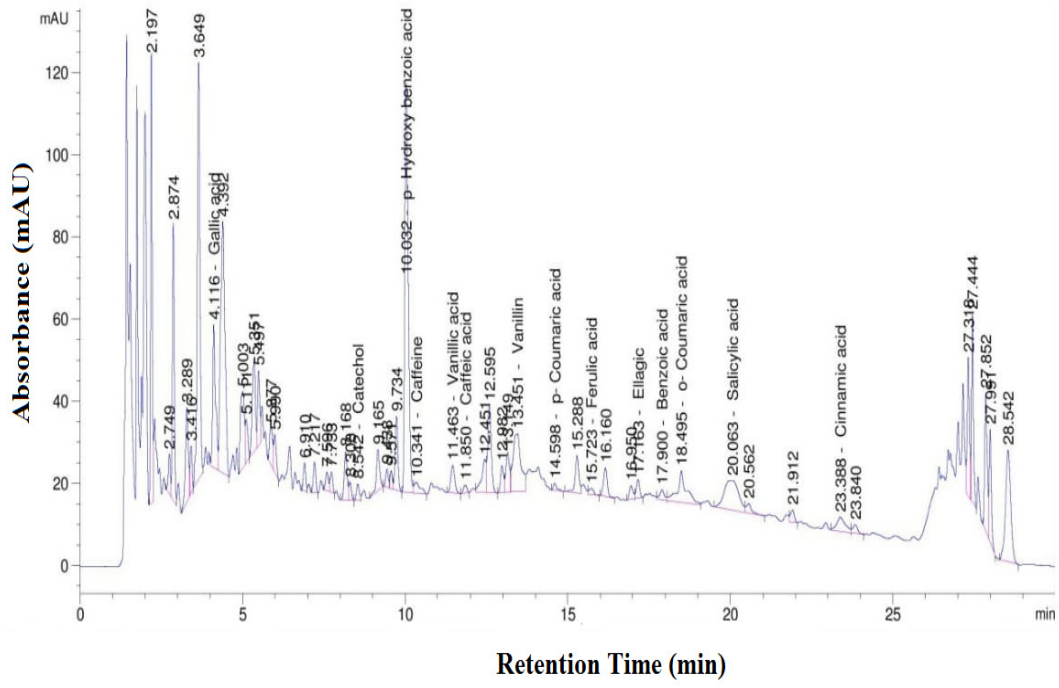

Figure 5. Chromatogram of HPLC separation profile of Phenolic compounds

4. Discussion

Probiotic culture supernatant of microorganisms of probiotic bacteria *L. acidophilus and L. plantarum and B.bifidum* were evaluated as natural probiotic culture supernatant to control food contamination and as food additive agents to prevent or reduce aflatoxin production by *Aspergillus flavus and Aspergillus parasiticus*. All probiotic culture supernatant significantly inhibit and reduce the growth of *Aspergillus flavus and Aspergillus parasiticus* (Table 1, Table 2, Table 3, Table 4 & Table 5) and (Figure 4). However, the good results were obtained when the filterates of the three probiotic bacterial strains *(Lactobacillus plantarum, Lactobacillus acidophilus and Bifidobacterium bifidum*) were mixed with concentration of 1%. Abbaszadeh *et al*. [27] reported that lactic acid bacteria showed high antifungal activity against; *Aspriglus flavus, A. niger, A. parasiticus* and *Penicillum chrysogenum* [7,11,28]. Moreover, both *Bifidobacterium Bifidum* and *Lactobacillus fermentum* showed high antifungal activity and suppersion the aflatoxin production by *A. parasiticus* [29]. It was reported that a wide range of species and subspecies include; *Lactococcus lactis*, *L. cremoris*, *L. diacetylactis*, *L. acidophilus*, *L. plantarum* and *L. curvatus* are cabaple to inhibit the growth of some pathogenic fungi. The inhibition was performed through proteins and polypeptides produced by these bacterial species [30]. Hassan and Bullerman 2008 [31], indicated that the probiotic bacteria which used as starters in the food in the past, produce different active bicompounds which able to resist the actvivity of the microbal toxins and they reported that among these active metabolites are; fatty acids, organic acids, aroma compounds, hydrogen peroxide and bacteriocins.

The results obtained, are evaluated by measuring the mycelial growth and quantification of aflatoxin B1, B2, G1 & G2 production. Data revealed that soluble biomolecules compounds secretedin mixed probiotic culture supernatant (PCS) was able to block the aflatoxin biosynthesis pathway at concentration as shown in (Table 5). In addition, B1 decreased from 75.265 to 18.248 ng/ml in *Aspergillus flavus* and from 168.249 to 40.423 ng/ml in *Aspergillus parasiticus* with a variable significant effect on mycelium growth with values 0.5, 4.7 and 3.1, 3.5 inhibition zones of mycelium and spores or spores depending the concentration percentage of mixed proproties culture supernatant (PCS) utilized in the present study. The study confirms that probiotic culture supernatant (PCS) compounds have antifungal activity against *Aspergillus flavus and Aspergillus parasiticus*. However, analyzing and fractionation of this effective mixed probiotic culture supernatant (PCS) by HPLC technique detected and identified 16 phenolic compounds that interpret the potential antioxidant properties and antifungal activity of probiotic culture supernatant (PCS) may be due to mainly to those bioactive molecules founds in these probiotic culture supernatant. Reference [32,33] found that 20 isolates of *A. parasiticus* all of them are able to produce aflatoxins; G1, B1, G2, and B2 at concentration ranges between 1.7-18.2 and 0-8.2 ng/g and succeded to reduce the aflatoxin production by lactic acid bacteria. Ghazvini *et al*. [29] reported that lactic acid bacteria succeded to reduce the aflatoxin production by 99% for the treated *A. parasiticus*. In addition, the reduction was observed in the fractions of aflatoxins; B1, G1 and G2. These results agree with the results obtained in this study that the reduction reached to 127%,

The present work proved that the mixed probiotic culture supernatant of *Lactobacillus acidophilus, Lactobacillus plantarum* and *Bifidobacterium.bifidium* succeded high antifungal activity. Besides, the inhibition growth of *Aspergillus flavus* and *Aspergillus parasiticus* by probiotic culture supernatant (PCS) was confirmed using antimicrobial tests and HPLC analysis which revealed the presence of phenolic compounds presented in identical chromatogram (Figure 5). Some phenolic compounds found in higher percentage and other not, this may be interpreting the potential power of phenolic compounds as antioxidants capacity and antifungal activity. Also, probiotic culture supernatant show phenolic compounds like benzoic acid and salicylic acid which responsible for antifungal activity. In addition, Gallic acid plays a principal role in protecting the probiotic organisms from fungal invasion. This result are in accordance with Reference [6] who reported that soluble phenolic compounds have different resistance to infection and the most inhibitory effect was produced by T-cinnamic acid followed by p-hydroxy benzoic acid, vanillin and salicylic acid. Moreover, Reference [34] reported that the active fractions of phenolic compounds include p-benzoic acid. These compounds in concentrations of 10 ppm inhibit growth of the test organism by 10-15% when acting separately, but 100% when all mixed were applied. The inhibition was 40% by *Lactobacillus plantarum* alone. The inhibition was 10-15% by separate culture supernatant in concentrations of 10 ppm and maximally 20% in combinations. Fungal growth was inhibited by unfractionated *Lactobacillus plantarum* culture supernatant was 37%. In addition, benzoic acid which detected in proproties culture supernatant (PCS) is one of the oldest chemical preservatives used in the cosmetic, drug and food industries. Benzoic acid has GRAS (Generally Recognized As Safe) status and sodium benzoate was the first chemical preservative approved by the US Food and Drug Administration for use in foods [35]. The activity of benzoic acid (pKa 4·19) is greatest at low pH values. Most yeasts and fungi are inhibited by 0·05-0·1% of the undissociated acid [36].

Lactobacillus acidophilus culture supernatant is the best culture supernatant among those used even individual or mixed with *Lactobacillus plantarum* The antimicrobial activity of proproties culture supernatant against *Aspergillus flavus* fungi revealed that those filterate possess varied medicinal properties and antioxidant activity that was effective in decreasing and lowering cell cytotoxicity (Table 6). In addition, those obtained culture supernatant 1% proproties culture supernatant (PCS) induced inhibition of aflatoxins production from either *Aspergillus flavus* or *Aspergillus. Parasiticus*. *A. parasiticus* with values 18.24 ng/ml and 40.42% in treated samples compared to 75.26 & 168.24 in AFB1 in control and as shown in level of AFB2, AFG1 & AFG2 (Table 4) which reflect the antitoxigenic properties of those culture supernatant as inhibitors agents for fungi to secret aflatoxins.This results are in accordance with Reference [37] found that some linoleic acid derivatives are able to

inhibit toxin synthesis in *Aspergillus* spp. Accordingly to antioxidant analysis probiotic possess and exhibit the highest antioxidant activity, this could be related with their content of natural compounds in culture supernatant synthesis by probiotic bacteria. Also, the presence and abundance of detected compounds could be associated with a general status of the physiology of the probiotic organism. On other words, all components detected could be involved in fungus defense system.

In the present study, crude culture supernatant of probiotic bacteria *(Lactobacillus plantarum, Lactobacillus acidophilus and Bifidobacterium bifidum*) were tested individual or in mixed as inhibitors for the growth of aflatoxins fungi *Aspergillus flavus Aspergillus parasiticus*, besides, the production of aflatoxins. Thus, these filterates offer very interesting opportunity as alternative safe agents against oxidative stress and protective the body from hazardous of aflatoxins antifungal which related to cancer induction, like nephrotoxicity, hepatotoxicity mutagenic, teratogenic and carcinogenic effects. The combined culture filterates of probiotic found to be highly effective antioxidant rich with phenolic compounds as proved from HPLC analysis improving and enhanced antioxidant enzymes and protect cell from damage. Not only that, but also, lowered aflatoxins production from toxic fungi Aspergillus Species. The same observation was demonstrated by Reference [38], they used a mix of probiotic culture supernatant against different strains of asprigillus. The obtained results in this results are agree with the results obtained by [39,10,40,41,42], all of these scientists examined the activity of different probiotics bacteria as antifungal and they reported that the metabolites produced by different bacteria could react as antitoxic and as growth inhbitors for different types of food borne fungi.

5. Conclusion

In the present study, proproties culture supernatant of probiotic bacteria *Lactobacillus plantarum* (L.p), *Lactobacillus acidophilus* (L.a), *Bifidobacterium bifidum* (B.b). were tested individual or mixed as inhibitors for the growth of fungi *Aspergillus Flavus* and *Aspergillus parasiticus*, and the production of Aflatoxins as well. Our results revealed that the mixed probiotic culture supernatant represent as alternative safe therapeutic agents and as food additive to inhibition fungal growth and aflatoxin production and protecting us from the hazardous effects resulted from aflatoxin. Moreover, the result obtained also suggested that mixed probiotic culture supernatant could be consider as promising and potential antitoxin product as food additive to control food contamination and as a protective new natural substance agent.

Acknowledgements

The The authors listed in this manuscript deeply appreciated to Arid Lands Cultivation Research Institute (ALCRI), City of Scientific Research and Technological Applications (SRTA-City) on their valiuable help in this work.

References

[1] Oliveira M. S, Prado G, Abrantes F. M,. Santos L. G, Veloso T. Incid˜encia de aflatoxinas, desoxinivalenol e zearalenona em produtos comercializados em cidades do estado de Minas Gerais no per´ıodo de 1998-2000. *Revista do Instituto Adolfo Lutz*. 2002; 61: 1-6.

[2] Rodriguez-Amaya D. B, Sabino M. Mycotoxin research in Brazil: the last decade in review. Brazilian Journal of Microbiology. 2002; 33: 1-18.

[3] Kwiatkowski A, Alves A. P. F. Importancia da detecc¸aoe do controle de aflatoxinas em alimentos. SaBios Revista de Sa´ude e Biologia. 2007; 2: 45-54.

[4] Arzandeh S, Selamat J, Lioe H. Aflatoxin in raw peanut kernels marketed in Malaysia. Journal of Food and Drug Analysis. 2010; 18: 44-50.

[5] Yazdani D, Zainal Abidin M, Tan Y H, Indu B J, Sahar S. Inhibition of aflatoxin biosynthesis in Aspergillus flavus by phenolic compounds extracted of Piper betle L. Iranian Journal of Microbiology. 2013; 5: 428-433.

[6] Candela M E, Alcazer, Espin D., Egea A. C, Almeda L. Soluble phenolic acids in *Capsicum annuum* stems infected with Phytophthora capsici. Plant Pathology. 1995; 44: 116.

[7] Kim Jeong-Dong. Antifungal Activity of Lactic Acid Bacteria Isolated from Kimchi Against Aspergillus fumigatus. Mycobiology. 2005; 33: 210-214.

[8] Phong H X, Hong Van T, Thanh N N, Dang Long B H, Dung N Thi P. Antifungal Activity of Lactic Acid Bacteria Isolated from Nem Chua. Chemical and Biomolecular Engineering. 2016; 1: 49-54.

[9] Magnusson J, Strom K, Roos S, Sjogren J, Schnurer J. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiology Letters. 2003; 219: 129-135.

[10] Sorensen K, Kim K.-H, Takemoto J.Y. In vitro antifungal and fungicidal activities and erythrocytes toxicities of Pseudomonas syringae pv. Syringae. Antimicrob Agents Chemother. 1996; 40: 2710-2713.

[11] LipiNska L, Klewicki R, Klewicka E, Bodziejczyk K. K, Sójka M B, Nowak A. Antifungal Activity of *Lactobacillus* sp. Bacteria in the Presence of Xylitol and Galactosyl-Xylitol. BioMed Research International. 2016; 2016: 1-8.

[12] FAO/WHO (Food and Agriculture Organization/World Health Organization)Working Group, Guidelines for the Evaluation of Probiotics in Food, FAO/WHO, London, UK, 2002.

[13] Shokryazdan, P., C.C.R. Sieo, J.B. Kalavathy, N. B. Liang, M. F. Alitheen, Y.W.Ho. Jahromi. 2014. Probiotic Potential of Lactobacillus Strains with Antimicrobial Activity against Some Human Pathogenic Strains. *BioMed Research Int*. 1-116.

[14] Ignatova-Ivanova Ts, Ivelina Stefanova, Ismail E. Ismailov, Ivaylo K. Ivanov, Valerij Ch. Christov. 2015. In Vitro Studies of Antifungal Activity of a Bifunctionalized Allene Ethanol Extracts. International Journal of Research Studies in Biosciences. 3, 2,PP 39-44.

[15] Deabes M. M, Abou El-Soud N. H, Abou El-Kassem L. T. In Vitro Inhibition of Growth and Aflatoxin B1 Production of Aspergillus Flavus Strain (ATCC 16872) by Various Medicinal Plant Essential Oils. Macedonian Journal of Medical Sciences. 2011; 4: 345-350.

[16] Perez C, Paul M., Bezique P. An Antibiotic assay by the agar well diffusion method. Alta Biomed. Group Experiences.1990; 15:113.

[17] Varaprasad B, Prasanth K K, Chandrasekr N, Somasekhar P. Antifungal activity of selected plant extracts against phytopathogenic fungi Aspergillus niger F2723. Indian Journal of Science and Technology.2009; 2: 87-90.

[18] Kadaikunnan S, Rejiniemon T. S, Khaled J. M, Alharbiand N. S, Mothana R. In-vitro antibacterial, antifungal, antioxidant andfunctional properties of Bacillus amyloliquefaciens. Annals of Clinical Microbiology and Antimicrobials. 2015; 14:9.

[19] Munimbazi C, Bullerman LB. Isolation and partial characterization of antifungal metabolites of *Bacillus pumilus*. *Journal of Applied Microbiology*. 1998; 84: 959-969.

[20] Pane B. O, Philippe A. N., Nicolas B., Aly S., Alfred S. T. Aflatoxigenic potential of *Aspergillus* spp. isolated from groundnut seeds, in Burkina Faso. West Africa African Journal of Microbiology Research. 2012; 6: 2603-2609.

[21] Hamad G. M, Zahran E., Hafez E. E. The efficacy of bacterial and yeasts strains and their combination to bind aflatoxin B1 and B2 in artificially contaminated infant's food. Journal of food safety. 2017; 37: 1-9.

[22] Boyum A, Isolation of mononuclear cells and granulocytes from human blood. Scand. Journal of Clinical and Laboratory Investigation. 1968. 21: 77-89.

[23] Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods. 1983; 65: 55-63.

[24] Braca A, Tommasi N. D, Bari L.D, Pizza C, Politi M, Morelli I. Antioxidant principles from Bauhinia terapotensis. *Journal of Natural Products*. 2001; 64: 892-895.

[25] Mcdonald S, Prenzler P. D, Autolovich M, Robards K, "Phenolic Content and Antioxidant Activity of Olive Oil Extracts. Food Chemistry. 2001; 73: 73-84.

[26] Hamad G. M, Taha T.H, El-Deeb N.M., Alshehri A.M.A. Advanced trends in controlling Helicobacter pylori infections using functional and therapeutically supplements in baby milk. J Food Sci Technol. 2015; 52: 8156-8163.

[27] Abbaszadeh S, Tavakoli R, Sharifzadeh A, Shokri H. Lactic acid bacteria as functional probiotic isolates for inhibiting the growth of *Aspergillus flavus* , *A. parasiticus* , *A. niger* and *Penicillium chrysogenum.* Journal de Mycologie Médicale. 2015; 25: 263-267.

[28] Tatsadjieu L N, Tchikoua R, Funtong C M M. Antifungal Activity of Lactic Acid Bacteria against Molds Isolated from Corn and Fermented Corn Paste. American Journal of Microbiological Research. 2016; 4: 90-100.

[29] Ghazvini, R. D, Kouhsari E, Zibafar E, Hashemi S J, Amini A, Niknejad F. Antifungal Activity and Aflatoxin Degradation of *Bifidobacterium Bifidum* and *Lactobacillus Fermentum* Against Toxigenic *Aspergillus Parasiticus.* Open Microbiol J. 2016; 10: 197-201.

[30] Batish VK, Roy U, Lal R., Grover S. Antifungal attributes of lactic acid bacteria--a review. Crit Rev Biotechnol. 1997; 17: 209-25.

[31] Hassan YI, Bullerman LB. Antifungal activity of Lactobacillus paracasei ssp. tolerans isolated from a sourdough bread culture. International Journal of Food Microbiology 2008; 121: 112-115.

[32] Habibipour R, Tamandegani PR, Farmany A. Monitoring of aflatoxin G1, B1, G2, and B2 occurrence in some samples of walnut. Environmental monitoring and assessment 2016; 188: 669.

[33] Delie D.K, Deschamps A.M, Richard-Forget F. Lactic acid bacteria-Potential for control of mound growth and mycotoxins: A review. J Food Control. 2010; 21:370-380.

[34] Niku-Paavola M.-L, Laitila A, Mattila-Sandholm T, Haikara A. New types of antimicrobial compounds produced by Lactobacillus plantarum. Journal of Applied Microbiology. 1999; 86: 29-35.

[35] Jay J.M, Food preservation with chemicals. In Modern Food Microbiology, 4th edn, ed. Jay, J.M. 1992; 251-289. New York: Chapman & Hall.

[36] Chipley J.R, Sodium benzoate and benzoic acid. In Antimicrobialsin Foods ed. Davidson, P.M. and Branen, A.L. 1993; pp. 11-48. New York: Marcel Dekker.

[37] Christensen SA, Kolomiets MV. The lipid language of plant fungal interactions. *Fungal Genetics and Biology.* 2011; 48:4-14.

[38] Yadav V, Gupta J, Mandhan R, Chhillar A.K, Dabur R, Singh D.D, Sharma G.L. Investigations on anti-Aspergillus properties of bacterial products. Letters in Applied Microbiology. 2005; 41: 309-314.

[39] Lee C.H, Kim S.H, Hyun B.C, Suh J.W, Yon C, Kim C.O, Lim Y.A, Kim C.S. Cepacidine A. a novel antifungal antibiotic produced by Pseudomonas cepacia. I. Taxonomy, production, isolation, and biological activity. J Antibiot, 1994; 47: 1402-1405.

[40] Kim K.K, Kang J.G, Moon S.S, Kang K.Y. Isolation and identification of antifungal N-Butylbenzenesulphonamide produced by Pseudomonas sp AB2. J Antibiot. 1999; 53: 131-136.

[41] Furtado N.A.J.C, Said S, Ito I.Y, Bastos J.K. The antimicrobial activity of Aspergillus fumigatus is enhanced by a pool of bacteria. *Microbiol Res*. 2002; 157: 207-211.

[42] Woo J.H, Kitamura E, Myuoga H, Yuto K. An antifungal protein from the marine bacterium Streptomyces species strain AP 77 is specific for Pythium Porphyrae, a causative agent of red rot disease in Porphyra spp. Appl Environ Microbiol. 2002; 68: 2666- 2675.