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Potential detoxification of aflatoxin B2 using *Kluyveromyces lactis* and *Saccharomyces cerevisiae* integrated nanofibers

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ABSTRACT: Current investigation has shown that human exposure to aflatoxins is not limited to the administration of contaminated cereals, but water is another possible source. This study was aimed to design easily applicable method to eliminate aflatoxin B2 (AFB2) from contaminated drinking water. Electrospinning has been used for preparation of probiotic-coated polyvinyl alcohol (PVA) and cellulose acetate (CA) nanofibers. Both of these hybrid nanofibers were studied by scanning electron microscopy (SEM) and Fourier-transformed infrared spectroscopy (FT-IR). SEM showed the proper coating of probiotic strains (*Kluyveromyces lactis CBS 2359 and Saccharomyces cerevisiae ATCC 9763*) on both nanofiber types. Different areas (1-5 cm^2) of the probiotic-nanofiber hybrid were used to enhance the removal of 20 ng/ml of aflatoxin B2 (AFB2) from prepared AFB2-contaminated water over time. Results revealed that a 5 cm^2 area of probiotic-coated PVA nanofibers can eliminate 97.5% of AFB2 as compared to 87.5%, 90.5%, 93.5%, and 95.5%, for 1 cm^2, 2 cm^2, 3 cm^2, and 4 cm^2, respectively, while probiotic-coated CA nanofibers were slightly less effective. Nevertheless, the cytotoxicity of probiotics-CA treated water on cultured human fibroblasts was almost 10 times lower than the cytotoxicity recorded in probiotics-PVA treated water. Therefore, results of the current research suggest that probiotics-polymer nanofiber membranes can be used as an extra stage in the water purification system for the treatment of AFB2-contaminated water.

INTRODUCTION

Mycotoxins have been primarily classified as aflatoxins (AF), zearalenone, fumonisins, trichothecenes, ochratoxin A, and ergot alkaloids (Var and Kabak, 2009). Aflatoxins are defined as secondary metabolites with low molecular weight that are produced by *Aspergillus flavus*, *Aspergillus parasiticus*, or *Aspergillus nomius* when they are growing on a variety of food or feed products. Mostly, they produce their toxins during the processing, transportation, and storage of food or feed commodities (Jiang *et al.*, 2005; Corassin *et al.*, 2013; Elsanhoty *et al.*, 2014). AFs are potent enough to provoke several adverse effects (mutagenicity, carcinogenicity, and teratogenicity) in both humans and animals (Lewis *et al.*, 2005). AFs have been mainly categorized as B1, B2, G1, and G2, in addition to M1and M2 AFs which are produced as metabolic products of the main aflatoxins (Richard, 2007).

Probiotics are identified as microorganisms which keep the balance of intestinal microflora on top of their ability to enhance the host health (Corassin *et al.*, 2013; Darsanaki *et al.*, 2014). They include different genera of microbes, such as: *Lactobacillus*, *Bifidobacterium*, some species of *Bacillus*

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or *Pediococcus*, in addition to some species of yeast (Soccol *et al.*, 2010). They can be classified as lactic acid bacteria and non-lactic acid bacteria; the former includes *Lactobacillus, Streptococcus, Enterococcus, Pediococcus* and *Bifidobacterium*, while the latter includes *Bacillus* and different species of yeast (Darsanaki *et al.*, 2014; Pfliegler *et al.*, 2015).

In addition to their ability of strongly binding the produced aflatoxins in contaminated media, probiotics have been reported as being effective candidates for aiding the inhibition of mold growth and the consequent aflatoxin production (Corassin *et al.*, 2013). At present, the mechanisms for the ability of probiotics to bind AFs are not clear, though it is proposed that the polysaccharides and peptidoglycans that compose the cell wall, are mainly responsible for the physical adhesion process (Shetty and Jespersen, 2006).

Electrospinning is a recent and important technique used for the production of nanofibers. It can produce light-weight nanofibers having a large specific surface area and porosity, which make them suitable for various potential applications, such as antimicrobial actions, supercapacitors (Miao *et al.*, 2016), tissue engineering (Eslah *et al.*, 2016), and as filtration membranes for water treatment applications. It can be formed from Nylon 6-6 (Vitchuli *et al.*, 2011), polyvinyl alcohol (PVA) (Eslah *et al.*, 2016), and cellulose acetate (CA) (Ma *et al.*, 2005). Among these polymers, cellulose is the most common and natural polymer that is characterized by its mechanical strength, extensive linearity, good flexibility, biodegradability, excellent durability, nontoxicity, chemical resistance, and low cost (Kim *et al.*, 2015). On the other hand, PVA, with relatively simple chemical structure (hydroxyl groups attached to the main chain), is a hydrophilic, biocompatible, and biodegradable polymer (Eslah *et al.*, 2016).

The current work was aimed to design novel and easy-to-handle hybrid probiotic-polymeric nanofibers that would improve AF removal from contaminated water. This new design is strongly recommended for use as an extra stage in drinking water purification systems. Also, the study was extended to characterize the fabricated nanofibers, with/without probiotic-coating, and to determine the cytotoxicity of treated and untreated AF-contaminated water on a normal human cell line.

MATERIALS AND METHODS

Materials

The polymers used were: 1. Polyvinyl alcohol(PVA) with an average MW of 72,000 g/mol (Merck, Germany); 2. Cellulose acetate (CA) (acetyl group 29−44 %) (Loba Chemie, India). Acetone (99%) was from POCH Chemicals, Poland.

The yeast strains (*K. lactis* CBS 2359 and *S. cerevisiae* ATCC 9763) were kindly provided by the Food Technology Department of ALCRI, SRTA-City. Aflatoxin B2 was purchased from Sigma-Aldrich, and all of the high-grade broth culture components were purchased from Oxoid, UK.

Detection of AFB2 existence in different water types

Three different water types (Tap, Distilled, and Milli-Q) were examined for the existence of AFB2, using HPLC analysis.

Yeast strains and culture conditions

Two different probiotic strains (*K. lactis* CBS 2359 and *S. cerevisiae* ATCC 9763) were cultured in YPD broth (Bacteriological peptone, 20 g/l; yeast extract, 10 g/l; glucose, 20 g/l) with subsequent incubation at 30°C and 200 rpm for 24 hours. After incubation, the cells were collected by centrifugation for 15 minutes at 6000 rpm, followed by washing twice using PBS buffer (pH=7.2) and once using distilled water, according to (El-Nezami *et al.*, 1998). Thereafter, the pellets were re-suspended in 0.1 M PBS (pH=6.8) followed by heat-acid treatment.

Heat-acid treatment of probiotic cultures

K. lactis and *S. cerevisiae* cell probiotic suspensions were autoclaved at 121°C, for 20 minutes. Then, the cultures were centrifuged for 15 minutes at 6000 rpm, washed twice with distilled water and treated with 10 ml of 2M HCl, and followed by incubation at 37°C and 200 rpm for 90 minutes. Moreover, the cells were centrifuged at 6000 rpm for 15 minutes, washed twice with distilled water, re-suspended in the PBS buffer (pH=6.8); the OD was measured at 600 nm and adjusted to 0.72±0.03 (El-Nezami *et al.*, 1998; Shetty and Jespersen, 2006; Rahaie *et al.*, 2012).

Electrospinning fabrication of hybrid probiotic-polymer nanofibers

Preparation of probiotic-polymer solutions

Electrospinning was performed after dissolving either PVA or CA in the suitable solvent with simultaneous combination comprising a specific amount of probiotic bacteria. For PVA, 1 gm was dissolved in 10 ml distilled water at 80°C under moderate stirring in order to form a homogeneous solution. After the dissolving was completed, 0.5 ml of *K. lactis* CBS 2359 and *S. cerevisiae* (OD_{600nm} 0.72±0.03) was added to the PVA solution with continuous stirring. For CA, 1 gm in 10ml acetone at room temperature (RT), under moderate and continuous stirring, to form a homogeneous solution with the same probiotic suspensions.

Preparation of nanofibers

Either probiotic-PVA and probiotic-CA liquids were supplied through a 20 mm NORM-JECT LuerLok syringe of plastic tip, through an 18-gauge stainless-steel needle and 90 blunt end. Each liquid was introduced to a nanofiber collector composed of aluminum foil that overlaid a laboratory-produced roller having a diameter of 10 cm. Then, the collector was positioned at a 15 cm tip to the collector distance (TCD). Throughout the electrospinning process, 18 kV of positive high voltage was utilized by the needle and the solution feed rate of 0.4 mL/h was run by using a KDS 200 syringe pump (Holliston, MA).

Characterization of the prepared hybrid probiotic-polymeric nanofibers

Scanning electron microscopy (SEM)

The surface of the prepared PVA and CA nanofibers, with

or without probiotic coating) was observed using SEM (JSM 6360LA, Jeol, Japan).

Fourier transform infrared spectroscopy (FTIR)
It was performed using a Shimadzu FTIR8400 S (Japan), to examine the presence of functional groups in selected PVA and CA fibers as well as to confirm their chemical structure.

Detoxification of AFB2 from water using probiotic-coated PVA and CA nanofibers
Different areas (1, 2, 3, 4, and 5 cm^2) of the probiotic-coated PVA and CA nanofibers, in addition to probiotic-free nanofibers, were prepared and tested for their capability of eliminating 20 ng/ml of AFB2 from 5 ml of Milli-Q water. The positive control tubes were prepared using nanofiber-free water amended with 20 ng/ml AFB2 while the negative control tubes were prepared using toxin-free tubes. Then, the prepared tubes were incubated at 30°C and 150 rpm for 24, 48, and 72 hours respectively. After the incubation, the residual toxin was measured using the HPLC instrument and the percentage of elimination was estimated.

Determination of AFB2 residue using HPLC
Sample preparation, derivatization, fluorescence detector analysis, and chromatographic conditions were done according to preceding authors (Guan *et al.*, 2011; Serrano-Niño *et al.*, 2013; Hamad *et al.*, 2017).

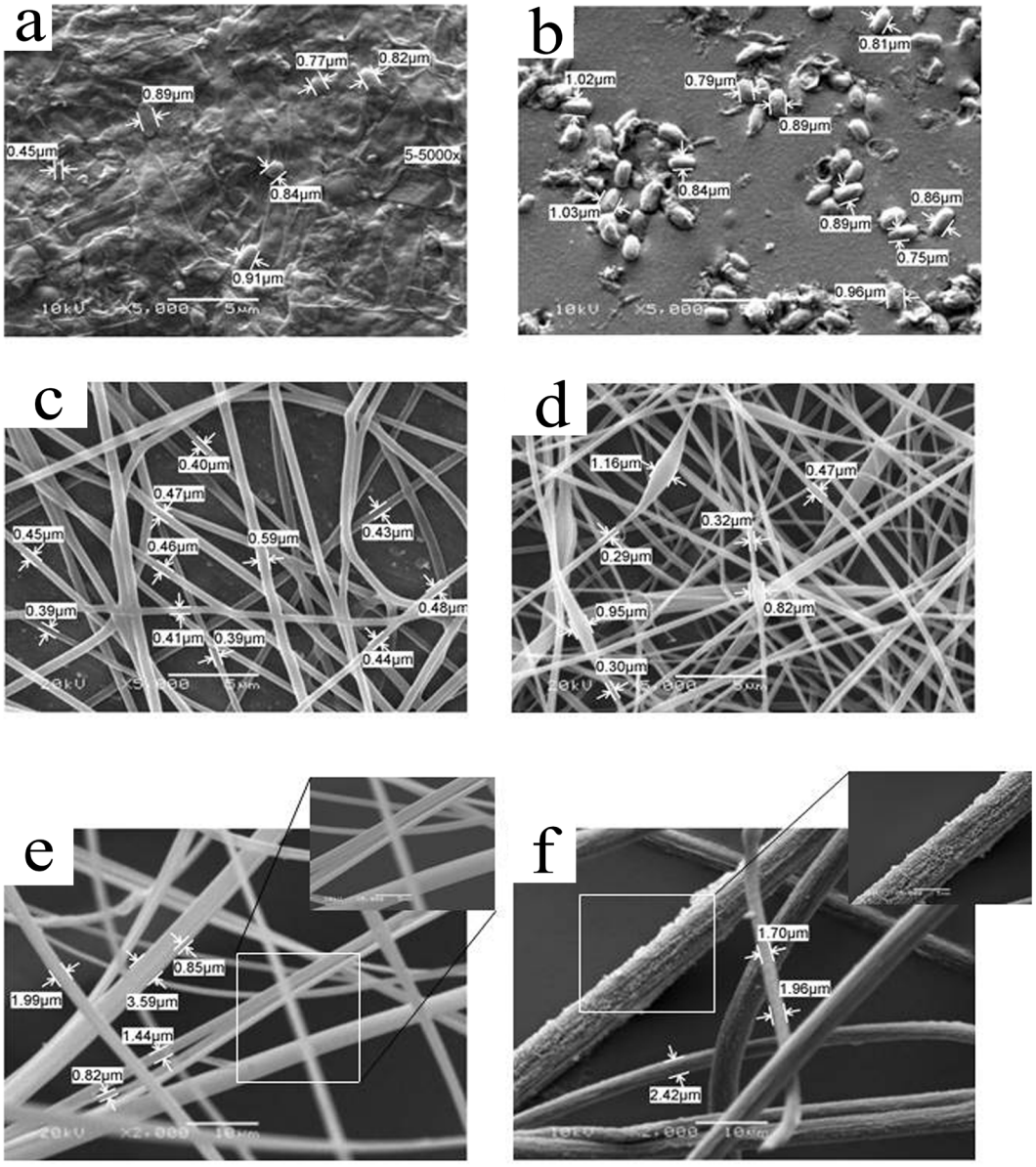

FIGURE 1. SEM microscopy. a. *K. lactis CBS 2359;* b. *S. cerevisiae* ATCC 9763; c. free PVA nanofibers; d. hybrid probiotics-PVA nanofibers; e. free CA nanofibers; f. hybrid probiotics-CA nanofibers

Human fibroblast cells

Human skin fibroblasts were used for cytotoxicity determination and were cultured on DMEM medium, supplemented with 10% fetal bovine serum and 200 mM L-glutamine.

Cytotoxicity assay

The safety pattern of the treated and untreated samples was quantitatively evaluated on human fibroblast cells, using the neutral red assay protocol (Borenfreund and Puerner, 1985). Briefly, about 100 µl of cell suspension (6×10^4 cell/ml) were cultured on a flat bottom 96-well plate. After 24 hours, the cultivated cells were incubated at 5% CO_2 at 37°C; thereafter, aliquots of 100 µl of each of the serially diluted, treated and untreated AFB2-contaminated water samples were incubated with the cells, under the above mentioned conditions, for 72 hours. Thereafter, 100 µl of neutral red stain was added to each well. Only living cells are permeable to neutral red and can fuse it into liposomes, affording a quantitative estimation of the cytotoxic effects. The intensity of the stain was evaluated by using an automated ELISA microplate reader suited at 540 nm (reference filters=620 nm). It is worth mentioning that the treated water samples were obtained after the removal of 20 ng/ml of AFB2 using 5 cm^2 of either probiotic-coated PVA or CA nanofibers.

RESULTS AND DISCUSSION

SEM examination

The morphological appearance of free probiotic yeasts and probiotic-coated nanofibers was photographed using SEM. As shown in Fig. 1a, the bulk size of the *K. lactis* strain ranged from 450 nm to 910 nm, with shapes ranging from oval to round structures, while the bulk size of the *S. cerevisiae* strain ranged from 750 nm to 1000 nm with a predominately oval appearance (Fig. 1b). Variation in the size of yeast cells might be related to the differentiation among un-synchronized budding cells and the growth rates of the examined cells (Johnston *et al.*, 1979). On the other hand, PVA-free nanofibers showed smooth, longitudinal fibers, with diameters ranging from 390 nm to 590 nm (Fig. 1c). However, the hybrid solution of PVA with probiotic strains showed an increase in diameter in some positions along the formed fibers, indicating the proper incorporation of the strains inside the fibers. As shown in Fig. 1d, the incorporation of the probiotic strains inside the fibers succeeded in increasing the diameter of the fibers-ranging between 820 nm to 1160 nm-which almost matches the previously measured probiotic size. Moreover, there was a remarkable variation between free CA nanofibers and hybrid probiotic-CA nanofibers. Fig. 1e illustrates the usual smooth longitudinal formation of nanofibers with a diameter range ~800 nm. This morphological appearance became completely different after the incorporation of probiotics. As illustrated in Fig. 1f, the diameter of the CA nanofibers increased to comprise a range between 177 nm and 2420 nm with surface roughness.

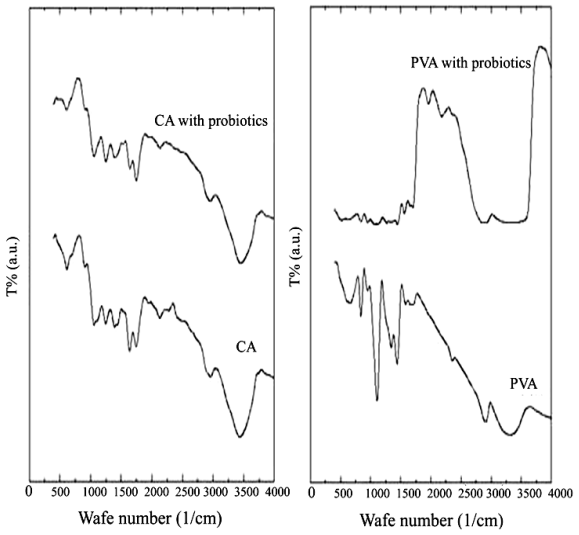

FIGURE 2. FTIR of PVA and CA nanofibers with and without probiotics.

Fourier transform infrared spectroscopy

FTIR spectroscopy was performed to detect the existed functional groups involved in the formation of PVA and CA fibers. Fig. 2 shows the FTIR spectra of PVA fibers with and without the yeast strains. PVA exhibited a characteristic broad-band OH group signal at 3568–3037 cm^{-1}. The bands at 2914 cm^{-1} were ascribed to symmetric stretching of CH2 (Rosi *et al.*, 2014; Abu-Saied *et al.*, 2017), while the bands at 1236 cm^{-1} and 1159 cm^{-1} were due to CH2 and CH wagging vibrations, respectively.

The IR spectrum, that relates to CA fibers with and without yeast strains, indicated some characteristic functional groups. The C=O functional group of CA was assigned at band 1753 cm^{-1}, and the peak at 1428 cm^{-1}-was attributed to the CH2 vibration (Fig. 2). In addition, the sharp absorption peak at 1631 cm^{-1} was assigned to C=C stretching, and the peak for C=O was at 1041cm^{-1}. Moreover, the peak at 3477 cm^{-1} is related to O=H stretching (Ibrahim *et al.*, 2015), appears much larger when compared to the same peak in the spectrum for CA with yeast strains.

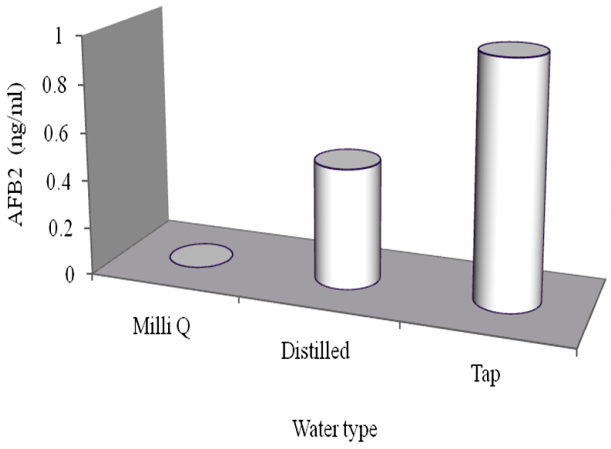

FIGURE 3. Determination of AFB2 concentrations in different water types.

Detection of AFB2 in water

At the beginning of our study, we were targeting to prepare a specific concentration of AFB2 for both the control and the treatment trials. We were surprised to find out AFB2 contamination in tap and distilled water (Fig. 3). It was somewhat surprising for us to find that most of the surrounding water was contaminated with aflatoxins. As shown in Fig. 3, AFB2 was detected in both tap and distilled water (1 ng/ml and 0.5 ng/ml respectively). However, Milli-Q water was the only variant that was free of AFB2; hence, it was chosen for furher use. Aflatoxins were often detected in cereals, cereal products, milk, and fruits (Stoloff *et al.*, 1991), but the detection of aflatoxins in water has been unfrequently reported (e.g., Paterson *et al.*, 1997). In a particular case, AFB2 was detected in a cold-water storage tank (1.7 µg/l and 0.2 µg/l in two sequential samples, while AFB1 was undetectable in both samples) (Paterson *et al.*, 1997).

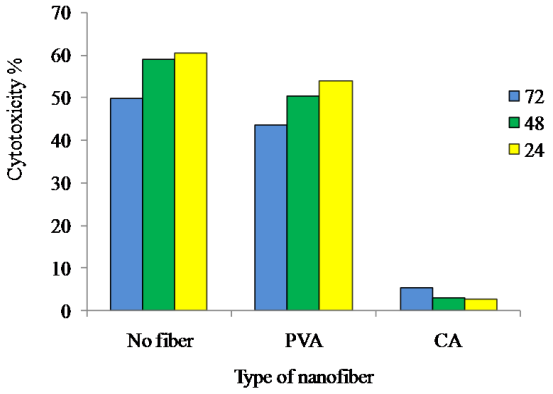

FIGURE 4. Cytotoxicity percentages on fibroblast cells exposed for 24, 48 and 72 h to AFB2 containing water samples, which were either untreated or treated with probiotics-coated PVA and CA nanofibers.

Detoxification of AFB2 using probiotics-coated nanofibers

Both of free and probiotic-coated PVA or CA nanofibers were tested for their capacity to remove AFB2 from a 20 ng/ml solution (Tab. 1). Detoxification of AFB2 from water was dependent on both time and nanofiber size area. The probiotic-coated PVA nanofibers showed a higher efficiency for AFB2 removal than the probiotic-coated CA nanofibers. After 72 hours of incubation, 5 cm^2 of the probiotic-coated PVA nanofibers was able to remove 97.5% of AFB2 as compared with 80.8% for the probiotic-coated CA nanofibers. On the other hand, the ability of probiotics-free nanofibers to remove AFB2 was negligible. This indicates that the detoxification process is mainly dependent upon the presence of probiotics. Moreover, the remaining AFB2 residue ranged from 0.5 to 2.5 ng/ml after probiotic-coated PVA nanofiber treatments as compared with 3.84 to 6.12 ng/ml after probiotic-coated CA nanofibers treatments, after three days of incubation and using 1-5 cm^2 area of each nanofiber. These residual quantities were detected at higher concentrations during shorter incubation time periods (24 hours and 48 hours). Recent studies have used probiotic strains for the detoxification of aflatoxin from food and dairy products (Guan *et al.*, 2011; Serrano-Niño *et al.*, 2013; Hamad *et al.*, 2017) but, to our knowledge, this is the first report of the use of probiotic-coated nanofibers for the detoxification of aflatoxins from water.

Cytotoxicity

The cytotoxicity of treated and untreated water samples was determined against a human fibroblast cells. As shown in Fig. 4, the cytotoxicity percentage of untreated water samples containing 20 ng/ml of AFB2 was higher than the percentage in the presence of either probiotics-coated PVA or CA nanofibers, at three experimental time periods.

The obtained data revealed that the probiotics-coated CA nanofibers were better for AFB2 removal than the corresponding PVA nanofibers. Furthermore, the cytotoxicity

TABLE 1

Bioremediation of AFB2 at presence/absence of nanofibers enveloped probiotics within limited time intervals.

Specifications		Incubation time (hours)/ AFB2 residue (ng/ml)					
		24 h		48 h		72 h	
		PVA	CA	PVA	CA	PVA	CA
Treatments	(-Ve control)	0.000		0.000		0.000	
	*(+Ve control)	20		19.6		19.8	
	*Probiotics free nanofiber	19.2	18.9	18.7	19	19.2	19.1
	*1 cm^2 of probiotics containing nanofiber	12	7.40	5.3	6.56	2.5	6.12
	*2 cm^2 of probiotics containing nanofiber	10	7.32	4.6	5.80	1.9	4.68
	*3 cm^2 of probiotics containing nanofiber	7.5	7.12	3.5	5.24	1.3	4.96
	*4 cm^2 of probiotics containing nanofiber	6.3	5.56	3.1	4.72	0.9	3.44
	*5 cm^2 of probiotics containing nanofiber	4.8	5.04	2	3.96	0.5	3.84

* Treatments that pre-amended with 20 ng/ml AFB2

of probiotics-CA treated water was almost 10 times lower than the cytotoxicity recorded for probiotics-PVA treated water. When compared with the negative control of fibroblast cells, probiotics-CA treated water showed a cytotoxicity pattern ranging from 2.8%-5.2%, in contrast with the 43.6%-53.9% that was recorded for probiotics-PVA treated water. As shown in Tab. 1, the residue of AFB2 was 0.5 ng/ml after using 5 cm^2 of probiotics-PVA nanofiber, as compared with the probiotics-CA nanofiber which showed 3.84 ng/ml of AFB2 residue after 72 hours of incubation time. These results were surprising, because they are oppositely matched with the cytotoxicity results. We attribute the cytotoxicity of probiotics-PVA treated water to the expected leakage of PVA molecules which might be responsible for the observed cytotoxic effect on fibroblast cells. This assumption is supported by El-Fawal *et al.* (2017) findings, who reported that PVA molecules have a cytotoxic effect against fibroblast cell lines and the addition of κ-carrageenan to PVA could increase its safety against fibroblast cells.

In conclusion, novel designs for the fabrication of probiotics-coated PVA and CA nanofibers have been recently fabricated, and their efficiency for remediating AFB2 from contaminated water has been shown. Water that was treated by probiotics-coated CA nanofibers has been proved safer than water treated with probiotics-PVA nanofibers, when tested on the cell viability of human fibroblasts. Therefore, probiotics-coated CA nanofibers can be recommended for used as an extra stage in water purification machines for the treatment of AFB2-contaminated water.

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