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# Evaluation of the aflatoxin M1 retention capacity in a polysaccharide obtained by fermenting milk with kefir grains

# Evaluación de la capacidad de retención de la aflatoxina M1 en un polisacárido obtenido por la fermentación de leche con gránulos de kéfir

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## Abstract
Currently, it has been reported a high incidence of aflatoxin M1 (AFM1) in milk. This poses a risk to human health, because AFM1 is considered a class 1 carcinogen. Among the various strategies that have been studied to reduce AFM1 contamination in milk, the use of lactic acid bacteria has attracted considerable attention. This approach takes advantage of exopolysaccharides (EPS) present in bacterial cells walls that show affinity to the mycotoxin and can form an EPS-AFM1 complex. In addition, during milk fermentation with kefir grains an EPS called kefiran is produced which has a potential for applications in food industry, e.g., improving the rheological and functional properties of foods. In this study, the capacity of isolated kefiran to retain AFM1 from milk matrix and aqueous system was evaluated. The results demonstrated a decrease in AFM1 content by 81% in milk matrix added with EPS, while in aqueous system in the presence of EPS it was observed a 75% reduction of AFM1 content. This led to the conclusion that kefiran does have mycotoxin retention potential and that in milk, higher retention value of AFM1 could be attributed to the interactions with milk proteins.
*Keywords*: Aflatoxin M1, Exopolysaccharides, Kefir grains, Milk.

## Resumen
De acuerdo con reportes actuales, existe una alta incidencia de aflatoxina M1 (AFM1) en la leche. Este es un factor de riesgo para la salud humana, porque se considera un carcinógeno de clase 1. Entre las diversas estrategias que se han estudiado para reducir la presencia de AFM1 en la leche, se encuentra el uso de bacterias ácido-lácticas, ya que sus paredes celulares presentan exopolisacáridos (EPS), a los que se une la micotoxina. Además, los gránulos de kéfir fermentan la leche produciendo kefirano, un EPS con potencial aplicación en la industria alimentaria, debido a que mejora las propiedades reológicas y funcionales de los alimentos. En este estudio, se evaluó la capacidad de retención de AFM1 en el kefirano. Los resultados obtenidos demostraron que cuando el EPS se encuentra en la compleja matriz láctea, existe un efecto de disminución en la concentración de AFM1 del 81%, mientras que en un sistema acuoso libre de componentes de la leche fue del 75%. Esto llevó a la conclusión de que el kefirano tiene potencial de retención de micotoxinas y que en la leche también hubo un efecto de las proteínas presentes en la disminución de la concentración de AFM1.
*Palabras clave*: Aflatoxina M1, Exopolisacáridos, Granos de Kéfir, Leche.

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# 1 Introduction

Aflatoxins (AFs) are mycotoxins produced by fungi of the genus *Aspergillus* (Benkerroum, 2020; Bryden, 2019). Within this group, aflatoxin B1 (AFB1) is considered by the International Agency for Research on Cancer to be a group 1 carcinogen, i.e., that causes cancer in humans (IARC, 2012). Mycotoxigenic fungi are capable of growing on a wide variety of agricultural products, including forages that serve as livestock feed. When cows consume feed contaminated with AFB1, they biotransform this aflatoxin into its hydroxylated derivative aflatoxin M1 (AFM1), which is secreted into milk and urine (Benkerroum, 2020; Jiménez-Pérez *et al*., 2021). Unfortunately, even though heat treatments applied to food help preserve food safety, AFM1 is heat-resistant (Pankaj *et al*., 2018). According to the above, humans are exposed to these mycotoxins through the consumption of contaminated feed. Globally, a high incidence of AFM1 has been reported in milk and milk products (Muaz *et al*., 2022; Salari *et al*., 2020). In Mexico, several studies have found concentrations of AFM1 in milk and cheese even higher than 0.5 $\mu$g/kg which is the limit set by Mexican regulations (Jiménez-Pérez *et al*., 2021). In addition, it is known that AFM1 associates with milk proteins (Harshitha *et al*., 2023; Jiménez-Pérez *et al*., 2020).

Several strategies have been studied to reduce the incidence of this mycotoxin in milk. These approaches range from preventing cattle feed from being contaminated to decontaminating milk containing AFs. For example, a commonly studied strategy is the decontamination of livestock feed using non-nutritive adsorbents to which toxins could be attached. In this way, their bioavailability is decreased and thus prevented from being metabolized (Vila-Donat *et al*., 2018). However, there have been reports on the adverse effects of these materials on micronutrients present in the feed. Since micronutrients are also retained by adsorbent material, a decrease in nutritional value as well as cytotoxic effects have been reported in farm animals (Elliott *et al*., 2020). Other methodologies with proven efficacy in reducing AFs in dairy products are the structural modification of the mycotoxin by radiation, ozone application, cold plasma, among others (Pankaj *et al*., 2018). However, these methods can affect other components of the food matrix resulting in possible alteration of their nutritional properties, and consequently, they are not recommended. An alternative approach for the decontamination of milk is through the use of microorganisms, particularly lactic acid bacteria (LAB) and yeasts as it has been reported that AFM1 is adsorbed on the exopolysaccharides (EPS) present in the cell wall of LABs of the genus *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Streptococcus*, as well as yeasts of the genus *Saccharomyces*, *Kluyveromyces*, and *Candida*, in both viable and non-viable cells (Gentry *et al*., 2023). The drawback of this decontamination method is that it can affect the rheological and sensory properties of the milk, such as the increase in viscosity, the precipitation of proteins due to the change in pH, among others, so it can no longer be used for consumption as fluid milk.

On the other hand, the EPS are classified as heteropolysaccharides (HePs), composed of at least two different monosaccharide units, and homopolysaccharides (HoPs), formed by the same repeated unit of monosaccharide (Hernández-Rosas *et al*., 2021; Zannini *et al*., 2016). According to Hernández-Rosas *et al*. (2021), the composition of the medium where the LABs grow will greatly influence the yields of the EPS produced, as well as their structural composition as HoPs or HePs. The most studied EPS are from pure LAB strains (Contreras-López *et al*., 2021; Jimenez-Fernandez *et al*., 2021; Zannini *et al*., 2016), but they could be also obtained from microbial consortia, such as kefir granules. The latter are formed by LAB, acid-acetic bacteria, and yeasts symbiotically coexisting, which have been used for centuries to produce fermented milks (Taniguchi *et al*., 2001). This symbiont produces an EPS called kefiran, which is a HePs made up of equimolar amounts of glucose and galactose with a molecular weight (PM) of 1 kDa, as shown in Figure 1 (la Riviére *et al*., 1967; Marshall & Cole, 1985). Depending on the growing conditions and microbial composition of kefir granules, the MW of kefiran can range from 55 to 10,000 kDa (Exarhopoulos *et al*., 2018; Gentry *et al*., 2023).

According to different studies, kefiran has a potential for its use in the food industry because it is considered generally recognized as safe (GRAS) by the FDA. Among its most outstanding applications are as emulsifying agent, stabilizer, thickening agent, gelling agent, and it also presents antioxidant, antimicrobial, and antihypertensive properties (Blandón *et al*., 2018; Botelho *et al*., 2014; Maeda *et al*., 2004; Piermaria *et al*., 2008; Sabaghi *et al*., 2015). In addition, kefiran has been shown to possess prebiotic activity and to interact with whey proteins increasing the rheological characteristics of food derivatives. Finally, it was used in the preparation of biofilms that assist with the transport of probiotic bacteria, due to its resistance to digestive hydrolysis (Gagliarini *et al*., 2022; Piermaria *et al*., 2021).

Based on the above, the objective of this work was to produce and extract kefiran from fermented milk followed by the evaluation of the kefiran interaction with AFM1, both in milk and a buffered solution at a pH similar to that of milk artificially contaminated, in order to determine its decontamination properties.

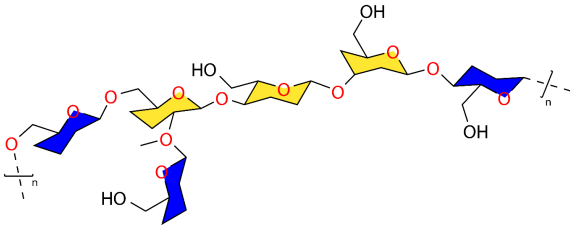


Figure 1. Chemical structure of the kefiran repeating subunit (Micheli *et al*., 1999). D-glucopyranose (blue) and D-galactopyranose (yellow). n/2 = [55-10,000] (Backbone [→ 6)$\beta$DGlcp(1 → 2,6)$\beta$DGalp(1 → 4)$\alpha$DGalp(1 → 3)$\beta$DGalp(1 → 4) $\beta$DGlcp(1 →], with a branched [$\beta$DGlcp(1 →)] to the first $\beta$DGalp residue).

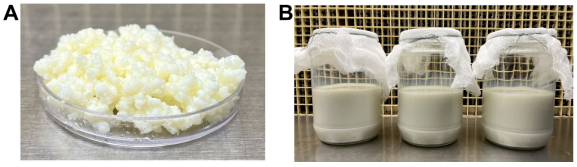


Figure 2. (A) Kefir grains. (B) Jars covered with a sterile sky-blanket.

## 2 Methodology

### 2.1 *Kefiran production*

500 mL of reconstituted milk was prepared as follows: 60 g of powdered milk (Alpura®) were dissolved in 450 mL of sterile water, then pasteurized at 60 °C for 30 min, and then 100 g (wet mass) of kefir grains (Figure 2A) were added to glass jars that had been previously sterilized by steaming for 15 min. The jars were covered with a sterile sky-blanket (Figure 2B) and placed in an incubator at 30 °C for 24 h. After the fermentation time had elapsed, kefir granules were removed from the fermented milk by filtration under sterile conditions. The pH of the milk was recorded before and after fermentation. This procedure was done in triplicate.

### 2.2 *Kefiran extraction*

The extraction was made from fermented milk according to the following steps:

1. The first step was protein precipitation, for which a solution of trichloroacetic acid (TCA) (Meyer, Mexico City, Mexico) at 80% (w/v) was added to fermented milk (Figure 3A) in a ratio of 16% (v/v). This mixture was homogenized by stirring and then centrifuged at 10,000 rpm for 20 min at 4 °C (Avanti J-E centrifugal, Beckman Coulter).

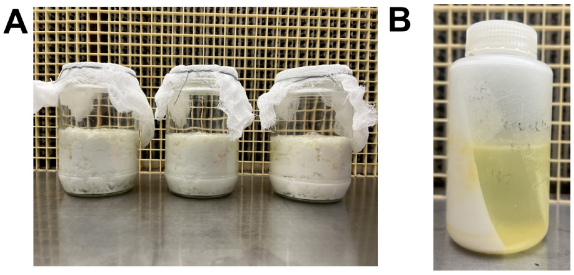


Figure 3. (A) Milk fermented with kefir grains. (B) Proteins precipitated after acid treatment and centrifugation.

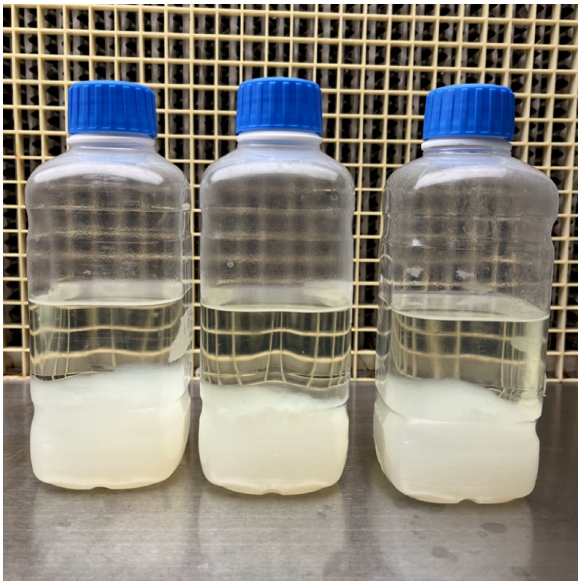

Figure 4. Kefiran precipitated by the action of absolute ethanol.

2. Subsequently to avoid EPS hydrolysis, the supernatant (Figure 3B) was recovered and adjusted to pH 7.0 with NaOH 4N (JT-Baker, New Jersey, USA).

3. The neutralized solution was added with three volumes of absolute ethanol, leaving it to rest overnight at 4 °C for the EPS to precipitate, as can be seen in Figure 4. Afterwards, it was centrifuged at 4000 rpm for 20 min at 4 °C.

4. The supernatant was discarded, and the precipitate dissolved in 40 mL of deionized water. This solution was dialyzed using a cellulose membrane (30 kDa pore, Sigma-Aldrich, Darmstadt, Germany) in 500 mL of deionized water for 48 h, changing water four times at 3, 6, 12, and 24 h (Figure 5).

5. Finally, the dialyzed kefiran was dehydrated in a freeze dryer at -65 °C (Scientz-10N, Ningbo Scientz Biotechnology).

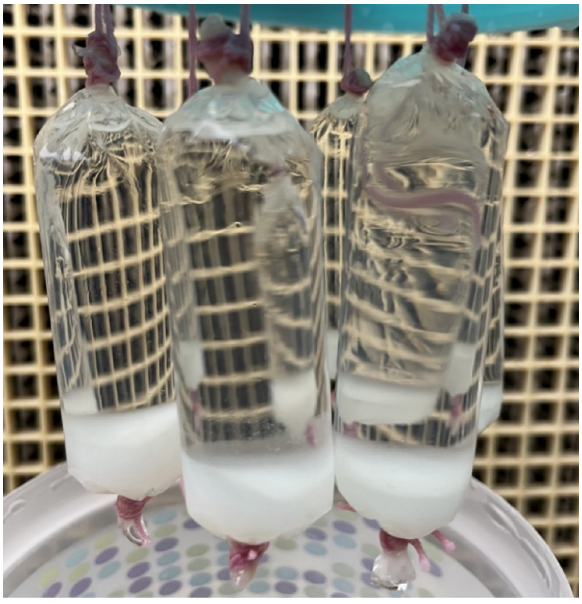

Figure 5. Dialysis tubes with the extracted kefiran.

## 2.3 Chemical characterization of kefiran

### 2.3.1 Total sugars

To determine the total sugar content in lyophilized kefiran, the phenol-sulfuric method was used (Dubois *et al*., 1956). Shortly, in a 1.5 mL Eppendorf® microtube, 1 mg of kefiran was dissolved in 1 mL of deionized water. Subsequently, this solution was transferred to a 15 mL test tube containing 1 mL of a 5% (w/v) phenol solution (Sigma-Aldrich, Darmstadt, Germany) and homogenized with a vortex at maximum velocity for 30 s. Once the above mixture was homogenized, 5 mL of concentrated sulfuric acid (JT-Baker, New Jersey, USA) was slowly added and gently shaken. The mixture was left to sit in the extraction hood until it reached room temperature. The absorbance was then measured at a wavelength of 490 nm (Shimadzu UV-1800 spectrophotometer). To estimate the concentration of sugars, a standard glucose curve was previously prepared (Sigma-Aldrich, Darmstadt, Germany) with different concentrations from 0 to 100 $\mu$g/mL.

### 2.3.2 Protein

The Bradford micro assay method (Bradford, 1976) was used to determine the protein content of lyophilized kefiran. In a 1.5 mL Eppendorf® microtube, 1 mg of kefiran was dissolved in 1 mL of deionized water. Subsequently, an aliquot of the solution (100 $\mu$L) and 1 mL of Bradford's reagent (Sigma-Aldrich, Darmstadt, Germany) were mixed in another microtube. This mixture was homogenized by inversion (5 times) and kept at rest in darkness for 5 min. The absorbance was then read at a wavelength of 595 nm. To estimate the protein concentration, a standard curve of bovine serum albumin with concentrations from 0 to 100 $\mu$g/mL was previously performed.

## 2.4 Quantification of kefiran

Quantification was performed in step 3 of section 2.2, prior to the dialysis process. The concentration was determined by spectrophotometry at a wavelength of 720 nm, interpolating into a standard dextran curve (PM 9400 Da; Sigma-Aldrich, Darmstadt, Germany) at concentrations ranging from 2 to 10 mg/mL.

## 2.5 Evaluation of the interaction of kefiran and AFM1

In order to evaluate the interaction between kefiran and AFM1, artificial contamination of milk with the toxin was carried out as follows: 100 $\mu$L of AFM1 (1 $\mu$g/mL) (Enzo Life Sciences, Inc., New York, USA) was added to 5 mL of reconstituted milk (as described in section 2.1) to reach a concentration of 0.02 mg of AFM1/mL of milk. Additionally, 10 mg of kefiran were dissolved in 1 mL of a phosphate buffer solution (PBS) at pH 6.8 and 50 $\mu$L of the kefiran solution was added to the contaminated milk to reach a concentration of 0.1 mg/mL, to avoid any rheological change in the milk due to the effect of kefiran. The mixture was vortexed for 1 min to homogenized. Subsequently, kefiran and AFM1 were allowed to interact for 6 h at 4 °C with radial shaking at 200 rpm. In addition, a control sample was established that consisted of substituting milk for PBS to evaluate the possible interaction of the components of the milk.

## 2.6 AFM1 extraction

Each 5 mL sample (milk or control) was placed in a water bath at 60 °C for 5 min, then centrifuged at 6000 rpm for 5 min at 4 °C. If the formation of a layer of fat was observed, it was discarded. The supernatant was transferred to a 15 mL Falcon tube and 1 g of NaCl and 3.8 mL of HPLC grade acetonitrile (ACN) were added (Sigma-Aldrich, Darmstadt, Germany). The mixture was stirred manually for 10 s and centrifuged at 6000 rpm for 5 min at 4 °C. The supernatant was recovered from the previous step to which 1.5 mL of chloroform and 5 mL of deionized water were added, mixed again manually for 10 s, and centrifuged at 6000 rpm for 15 min at 4 °C. The resulting organic phase was collected in a ball flask and vacuum distilled using a rotary evaporator (HS-2001NS, Hahnshin Scientific Co.). After complete solvent evaporation, the residue (AFM1) was recovered using 300 $\mu$L of mobile phase, which is a mixture of ACN and deionized water (40:60, v/v). After vigorously shaking the flask with the sample to dissolve all the AFM1 present, the solution was filtered with through a 0.2

$\mu$m nylon membrane (Titan 3 HPLC filterpurple) and refrigerated at 4 °C in amber vials until analysis for quantification.

### *2.7 Quantification of AFM1*

AFM1 was quantified through high-performance liquid chromatography (HPLC). A chromatograph (Manager 5050, pump 1050; Knauer) with fluorescence detector (RF-20A, Shimadzu) was used at an excitation (254 nm) and emission (365 nm) wavelengths, and a reversed phase column (Jupiter 300Å 5$\mu$ C18, 250 x 4.6 mm, Phenomenex). Isocratic runs were performed for 20 min using the mobile phase (section 2.6). The flow rate was 0.4 mL/min and the injection volume was 50 $\mu$L. The liquid from the interaction tests (milk and control) was filtered through a 0.2 $\mu$m nylon membrane before being injected into the HPLC. Chromatograms were recorded using ClarityChrom software (Version 3.07). The AFM1 working standards for the standard curve were calculated to reach concentrations on a range of 0.05 to 1.0 $\mu$g/mL.

### *2.8 Statistical analysis*

Comparative studies of the mean values obtained in AFM1 concentration were carried out using the Tukey test of multiple comparisons, with a significance level of 95%, using the Minitab17 statistical program.

## 3 Results and discussion

### *3.1 Kefiran production*

The fermentation of milk with the kefir granules took place within the analyzed period, as the pH decreased from 6.8 to 4.5 after 24 h. The pH change showed that the microbiota was fermenting lactose and producing lactic acid. In addition, an increase in the biomass weight of 50 ± 2% was recorded, indicating that there was kefiran production since it is associated with the formation of the granules (Gentry *et al*., 2023).

According to the spectrophotometric quantification method, a kefiran concentration of 4.471 ± 0.012 mg/mL was obtained, having a higher production compared to that reported by Carrero-Puentes *et al*. (2022), as a concentration of 0.276 ± 0.042 mg EPS/mL was achieved when fermenting whey with *Lactobacillus delbrueckii* ssp. *bulgaricus* NCFB 2772. This strain is usually presented in the microbiota of kefir granules. On the other hand, a freeze-dried kefiran production of 810 ± 60 mg/L was also determined which corresponded to a production yield of 83%. Based on these results, it can be inferred that the symbiotic effect of the various microorganisms that make up the kefir granules generates an increase in the production of kefiran. For example, Cheirsilp & Radchabut (2011) reported kefiran production of 938 mg/L when fermenting modified MRS medium, with two isolated strains of *Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae*, the former being the largest producer of EPS while the yeast helps decrease the acid produced to prevent inhibition of bacterial growth.

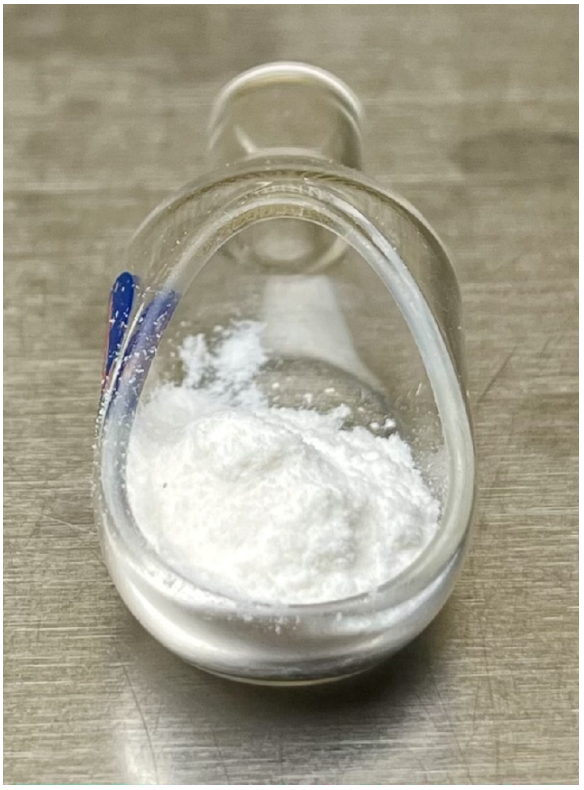

Figure 6. Lyophilized kefiran.

### *3.2 Characterization and quantification of kefiran*

The freeze-dried kefiran obtained by fermenting 500 mL of milk (Figure 6) was analyzed for its sugar and protein composition. A total sugar concentration of 21.46 ± 0.5 mg kefiran/mL was determined, which was sensibly higher than previously reported by Rimada & Abraham (2003). These authors evaluated different polysaccharides extraction methods, and in the method that was like the one used in the present work, a concentration of 0.645 mg EPS/mL was reported. As for the protein content, it was determined at 1.44 ± 0.04 mg protein/mL, giving a carbohydrate: protein ratio of 15:1 (93.8 and 6.2% w/v, respectively). Even though these results presented a rather high concentration of proteins, they are still in accordance with those reported by Exarhopoulos *et al*. (2018), who determined that the dry-based concentration of carbohydrates was considerably higher than that of protein in kefiran

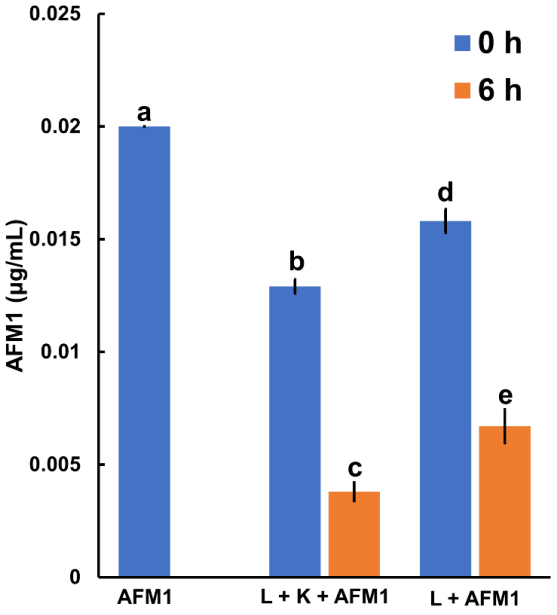


Figure 7. Effect of milk matrix (L) and kefiran (K) on AFM1 concentration at 0 and 6 h. AFM1 column represents the AFM1 initial concentration prior to the assays. The mean values that do not share the same letter are significantly different (Tukey test, 0.05).

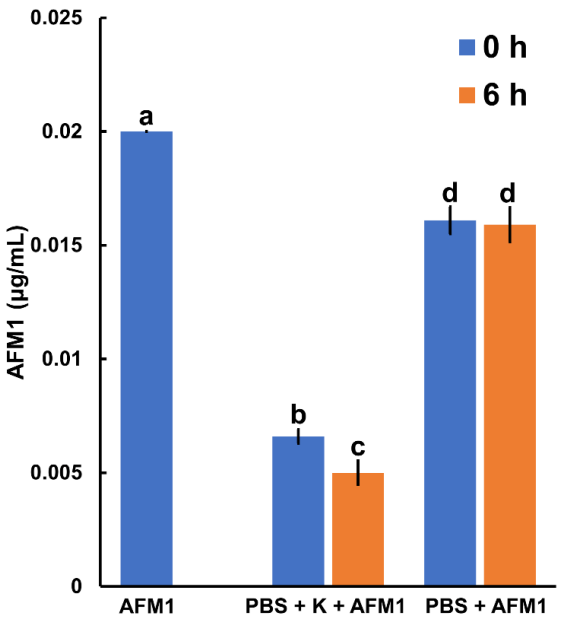


Figure 8. Effect of phosphate buffer solution (PBS) and kefiran (K) on AFM1 concentration at 0 and 6 h. AFM1 column represents the AFM1 initial concentration prior to the assays. The mean values that do not share the same letter are significantly different (Tukey test, 0.05).

(99.5 and 0.1%, respectively). However, it should be noted that these authors performed the solvation process with ethanol in triplicate, which may explain the very low concentration of proteins. Although a significant proportion of protein is still observed in this work, the results show that the extraction of kefiran was adequate, since most of the carbohydrates were preserved. The carbohydrate: protein ratio is critical for affinity studies, as it has been shown that milk proteins tend to associate with AFM1 (Harshitha *et al*., 2023; Jiménez-Pérez *et al*., 2020), which may interfere with the assays of the kefiran interactions with mycotoxin.

### *3.3 Interaction between kefiran and AFM1*

Results obtained in the interaction tests in milk are presented in Figure 7. An effect of EPS on AFM1 retention can be observed from the onset of the interaction (0 h), where there was a 35.5% decrease in mycotoxin content. As the interaction continued, the concentration of AFM1 decreased by 81% after 6 h. On the other hand, a similar behavior was observed in the test without kefiran (control), with a decrease in mycotoxin concentration of 21% (0 h) and 66.5% (6 h). Despite the fact that a reduction in AFM1 content was observed in the control trial, the greatest retention of this mycotoxin was recorded in the presence of kefiran. The effect observed in the control sample (without kefiran) could be attributed to the interaction between proteins and AFM1, which coincides with other reports that mycotoxin has formed complexes with milk proteins (Harshitha *et al*., 2023; Jiménez-Pérez *et al*., 2020).

To determine the interference of milk proteins in the AFM1 retention by kefiran, the experiment was carried out in PBS at pH 6.8, as shown in Figure 8. As can be seen in the first column (AFM1), the initial concentration was 0.02 $\mu$g AFM1/mL. When kefiran was added to the AFM1 buffer solution, a decrease of 67% was observed for the mycotoxin which concentration was further reduced by 75% after 6 h. For control sample (PBS + AFM1), a constant decrease of approximately 20% was observed at both time intervals that can be explained by the loss of mycotoxin during extraction process (also seen in Figure 7, AFM1+M at 0h). Therefore, it can be assumed that kefiran can act as AFM1 adsorbent material. Likewise, it was confirmed that milk proteins contribute to the reduction of the mycotoxin content with or without the presence of kefiran. The coexistence of kefiran with proteins in general had been studied and was focused on the biofilms production by combining kefiran and whey proteins. The latter work to encapsulate probiotic bacteria or even antibiotics by acting as a vehicle. In fact, kefiran had been shown to resist hydrolysis by digestive enzymes (Gagliarini *et al*., 2019; Gentry *et al*., 2023; Moradi & Kalanpour, 2019).

## Conclusion

Based on the results obtained in this work, it has been demonstrated that the interaction of kefiran with AFM1 has a strong potential to be used as a vehicle to decontaminate milk. It was shown that addition of 0.1 mg EPS/mL to a complex matrix such as artificially contaminated milk, it is possible bind free AFM1 and reduce its concentration in milk. This opens the possibility of further research to achieve a system adequate for the detoxification of milk contaminated with mycotoxin without altering the nutritional and rheological properties of the milk due to low concentration of kefiran used. Likewise, it is important to look for an alternative method for the extraction of EPS where there is a better removal of proteins, in order to better understand the interactions between this matrix and the toxin.

### *Acknowledgment*

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