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Application of Kefir probiotics strains as aflatoxin B1 binder in culture medium, milk and simulated gastrointestinal conditions

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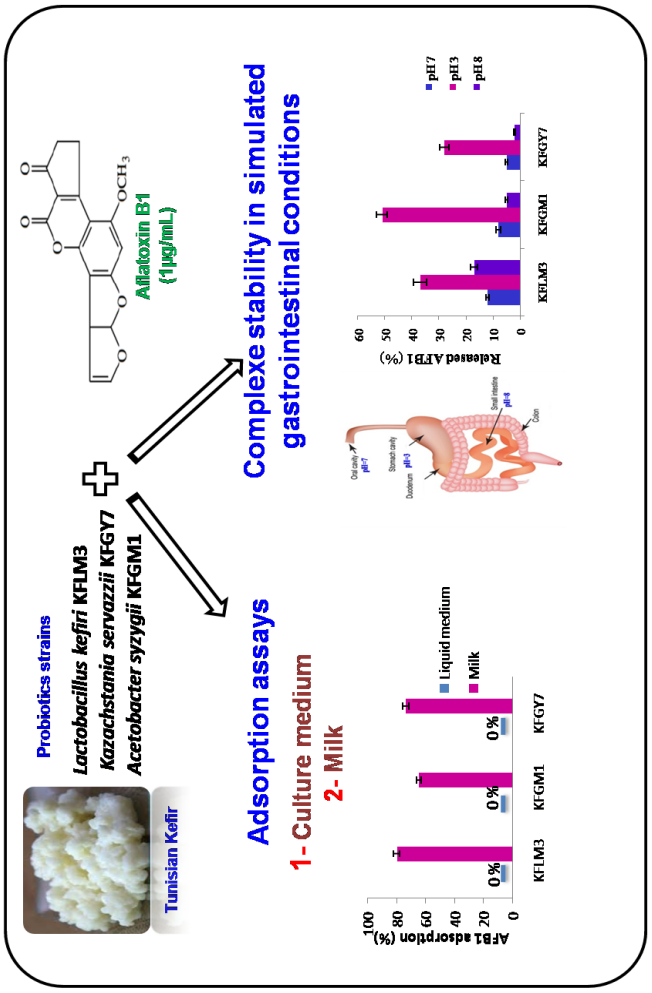
Graphical Abstract



Abstract.
Aflatoxin B1 (AFB1) contamination in food poses serious problems both for economic development and public health protection, thus leading to a focus on an effective approach to control it. In this context, probiotics strains (*Lactobacillus kefiri* KFLM3, *Kazachstania servazzii* KFGY7 and *Acetobacter syzygii* KFGM1) isolated from a Kefir culture were assessed for their AFB1 adsorption ability. The adsorption experiments were done in culture medium and in milk. The stability of microorganism/AFB1 complexes was evaluated using buffer solutions (pH=3, pH=7 and pH=8) to simulate the pH conditions in the gastrointestinal tract. Our results showed that strains binding assays for AFB1 in culture medium showed no effect (0%). However, the strain *L. kefiri* KFLM3 was the most active, adsorbing 80 % of AFB1 when cultivated in milk followed by *A. syzygii* KFGM1 (74%) and *K. servazzii* KFGY7 (65%). Nonetheless, the strain *K. servazzii* KFGY7 retained more AFB1 after the desorption experiments (65%). The present findings suggest that kefir isolated strains might be a promising candidate for exploitation in AFB1 detoxification in food and feed matrices.

Keywords: Probiotics; Adsorption; Aflatoxin B1; *Lactobacillus kefiri*; *Acetobacter syzygii*; *Kazachstania servazii.*

1. Introduction

Aflatoxins (AFs) are secondary metabolites of some strains of the molds *Aspergillus flavus* and *Aspergillus parasiticus* and can occur as natural contaminants of foods and feeds (Hathout et al. 2011). Aflatoxin B1 (AFB1) (Figure 1), the most toxic AF, is of particular interest because it is a frequent contaminant of many food products and one of the most potent naturally occurring mutagens and carcinogens known (IARC 2016). AFB1 has toxic, carcinogenic, mutagenic and teratogenic effects in laboratory animals (Paterson and Lima 2010).

Milk is the primary source of nutrition for growing infants. However, commercial milk and milk products as well as milk of nursing women can be contaminated with mycotoxins. Indeed, the carry-over into milk of aflatoxin M1 (AFM1), aflatoxicol, fumonisin B1 (FB1), ochratoxin A (OTA) and zearalenone (ZEA) have been reported (Fink-Gremmels 2008). Those mycotoxins are thermostable and are not destroyed by dairy processing methods; they remain in pasteurized milk and in fermented dairy products (Iha et al. 2013). Therefore, infants and young children are more sensitive to this serious problems than adults (Mohammadi 2011).

As a solution to this serious problem, numerous physical, chemical and biological strategies have been reported for mycotoxin detoxification. Recently, several microorganisms were investigated for mycotoxin degradation or adsorption, such as lactic acid bacteria (Abrunhosa et al. 2014, Elsanhoty et al. 2013), yeasts (Petruzzi et al. 2015, Zhang et al. 2016), and other bacteria (Harkai et al. 2016).

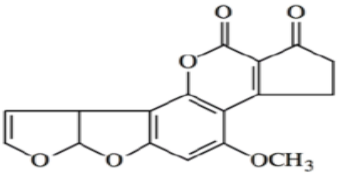


Figure 1: Structure of AFB1

Kefir is a fermented milk drink with an acidic taste and creamy consistency produced by lactic-alcoholic fermentation of milk by gelatinous irregular grains, which range from 0.3 to 3.5 cm in diameter. The kefir microbial community encompasses a complex mixture of lactic acid bacteria (LAB) (*Leuconostocs, Lactobacilli, Streptococci, lactococci, Enterobacter, Acinetobacter, Enterococcus, and Pseudomonas spp.*), acetic acid bacteria and yeasts (*Kluyveromyces, Candida, Torulopsis, Saccharomyces, Rhodotorula* and *Zygosaccharomyces*) that cohabitate in a protein and polysaccharide (kefiran) matrix (Garofalo et al. 2015).

Microbial fermentation produces several bioactive compounds, such as peptides, amino acids, bacteriocins, ethanol, CO_2, acetaldehyde, acetoin, diacetyl, exopolysaccharides, folic acid, calcium and vitamins B1 and B12, as well as lactic and acetic acid (Garofalo et al. 2015). Due to its high nutritional value and content of natural probiotics, Kefir possesses numerous health benefits (Satir and Guzel-Seydim 2015), including modulating the immune system and enhancing digestive health, as well as antimicrobial, anti-tumoral and antioxidant activities (Ahmed et al. 2013, Vinderola et al. 2005). As far as we know, the effect of Kefir probiotics strains on AFB1 has not previously been investigated.

The main of the present study was to investigate AFB1 adsorption properties of Kefir isolate bacteria and yeast in both culture medium and milk. Additionally, the stability of the microorganism/ AFB1 complexes was tested by simulating a pH change in the gastrointestinal tract.

2. Material and methods

2.1. Probiotics strains preparation

Bacteria (*Lactobacillus kefiri* KFLM3 and *Acetobacter syzygii* KFGM1) and yeasts (*Kazachstania servazzii* KFGY7) (Table 1) isolated from Kefir were sub cultured twice in Man Rogosa Sharpe agar (MRS) and in YPD (20 g/L of bacteriological peptone, 10 g/L of yeast extract, 20 g/L of glucose and 20 g/L of bacteriological agar) before experimental use. Bacteria were incubated at 30 °C for 5 days under anaerobic conditions and yeast was incubated at 25 °C for 3 days.

Table 1

Lactic acid bacteria used in this study

Strains	Origin	Identification	Accession number
KFLM3	Kefir Milk	*Lactobacillus kefiri*	KX987247
KFGM1	Kefir grain	*Acetobacter syzygii*	KX987248
KFGY7	Kefir grain	*Kazachstania servazii*	KX987246

2.2. Evaluation of the AFB1 binding properties of strains isolated from Kefir

The microorganisms isolated from Kefir were investigated for AFB1 adsorption properties using culture media and milk. The strains were propagated respectively in 10 mL of MRS or YPD broth at 30 °C for 3 days. Optical density (O.D.) was determined at 600 nm and adjusted to 2.0 with sterile distilled water. Stock standard solutions of AFB1 (Sigma) was prepared in methanol at 1 mg/mL and stored at -20 °C until use.

Commercial UHT cows' milk was artificially contaminated with 1 µg/mL of AFB1 by adding the appropriate amount of stock standards. Falcon tubes containing 5 mL of AFB1-contaminated milk were prepared in triplicate to study the adsorption properties of strains. Similarly, MRS broth and YPD broth supplemented with 1 µg/mL of AFB1 was prepared. Strains were inoculated (1%, w/v) in MRS broth, yeasts in YPD broth and both were tested in milk containing AFB1 as described previously. The tubes were mixed and incubated aerobically at 25 °C for 24 h. Three non-inoculated tubes containing (MRS, YPD or milk) contaminated with AFB1 were also prepared and incubated to be used as negative controls.

To test adsorption, after the incubation period, tubes were first centrifuged at 9000 RCF for 20 min, the clear liquid fraction transferred to clean tubes and an equal volume of the earlier organic solution was added. After being strongly vortexed for 1 min, they were also left to stand overnight. Thereafter, all samples were filtered into clean 2-mL vials using a syringe filter (0.2 µm, Nylon) and preserved at -20 °C until HPLC analysis.

2.3. Stability of the microorganism/mycotoxin complexes at different pHs

To determine the stability of the microorganism/AFB1 complexes, pellets of the Kefir strains with bound AFB1 were sequentially washed with buffer solutions at pH 7, 3 and 8 to simulate the pH conditions of the gastrointestinal tract. Experiments were started by adding 5 mL of 0.1 M phosphate buffer at pH 7 to pellets and by incubating under rotary agitation at 37 °C for 5 min to simulate the time spent in the mouth compartment.

Thereafter, cells were centrifuged (6500 g, 10 min, 4 °C) and the supernatant was collected to evaluate the amount of mycotoxins released. Then, the pellets were re-suspended in 5 mL of 0.1 M

citrate/phosphate buffer at pH 3, and the tubes were incubated under the same conditions for 2 h to simulate the conditions of the stomach. Supernatant recuperation was performed as described previously, and pellets were re-suspended in 5 mL of 0.1 M phosphate buffer pH 8 and incubated in the same way for 2 h to simulate the small intestine compartment. Tubes were centrifuged a final time, and the three supernatants obtained were extracted separately as previously described in section 2.2.

The amount of released AFB1 was quantified by HPLC, and the percentage of AFB1 bound was calculated.

2.4. Mycotoxin analysis

Quantification of mycotoxins was performed by high performance liquid chromatography (HPLC) with fluorescence detection. The HPLC system was equipped with a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, a Jasco FP-920 fluorescence detector and a Jones Chromatography 7971 column heater that was maintained at 30 °C.

The instrument and chromatographic data were managed by a Varian 850-MIB data system interface and a Galaxie chromatography data system, respectively. An analytical C18 reversed-phase YMC-Pack ODS-AQ column (250 × 4.6 mm I.D., 5 µm) connected to a pre-column with the same stationary phase was used. For AFB1 determination, a mixture of deionized water/acetonitrile/methanol (3:1:1, v/v/v) was used as the mobile phase at a flow rate of 1.0 mL/min.

The excitation (ex) and emission (em) wavelengths were set at 365 and 435 nm, respectively (Soares et al. 2010). Mobile phase was filtered and degassed with a 0.2-µm membrane filter (GHP, Gelman). An aliquot of 50 µL was injected for analysis. The retention time of AFB1 was approximately 20.3 min. AFB1 was identified by comparing the retention time of the peak samples with the standard curves.

3. Results and discussion

3.1. Evaluation of the AFB1 binding properties of strains isolated from Kefir

Two bacterial strains, *Lactobacillus kefiri* KFLM3 and *Acetobacter syzygii* KFGM1 and one yeast (*Kazachstania servazzii* KFGY7) were isolated from Kefir (grains and milk). Data showed that the adsorption of AFB1 was strain and culture medium dependent (Figure 2).

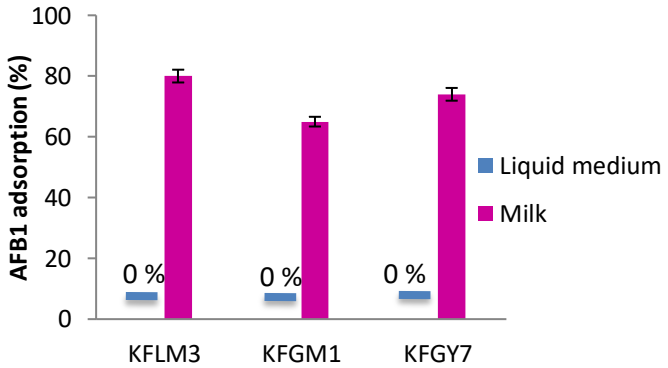


Figure 2: Percentage of AFB1 eliminated by the Kefir strains in adsorption experiments conducted in liquid medium (MRS for KFLM3, KFGM1 and YPD for KFGY7) and milk.

According to Figure 2, the strains binding assays for AFB1 in MRS showed no effect (0%). These data are in disagreement with data obtained by other authors, who reported the ability of *Lactobacillus rhamnosus* strains to bind 77-95% AFB1 (Turbic et al. 2002).

At this point, it was observed that the isolated strains added a limited adsorption capacity in culture medium. In addition, experiments were carried on the isolated strains out in milk to verify whether the composition of the medium could have influenced the binding capability of the isolates.

Remarkably, the Kefir isolates modified their binding behaviour and showed a higher adsorption potential for the tested mycotoxin in milk than in MRS or YPD medium. In milk, the amount of AFB1 removed in milk reached 80% for AFB1 depending on the strain (Figure 2).

The strains *L. kefiri* KFLM3 and *K. servazzii* KFGY7 showed the highest AFB1 binding ability, with removal percentages of 80% and 74%, respectively followed by *A. syzygii* KFGM1 (65%). These observations suggest that the binding capacity is dependent on the particular characteristics of each strain but also that the culture medium used to grow the strains is the most determinant factor.

A variation in the binding ability between strains was also reported by Peltonen et al. (2001), which considered that dissimilarities in the bacterial cell wall composition were the reason for the different AFB1-binding capabilities of the tested strains. The number of binding sites available probably differs from strain to strain. The growth of microorganisms in milk was probably faster than in MRS and YPD, leading to the formation of more biomass and, consecutively, to higher binding percentages. In addition to growth, it is also possible that the nutritional composition of milk could had favour the biosynthesis of cell wall components involved in the binding of the mycotoxins.

3.2. Stability of the microorganism/AFB1 complexes at different pHs

The evaluation of the stability of the microorganism/AFB1 complex is important for predicting the release of AFB1 during gastrointestinal passage and consequently for estimating the real absorption potential of AFB1. Thus, after the binding assays were performed in milk, the obtained pellets were assessed for complex stability by successive treatment with buffers at pH 7, 3 and 8 to simulate the pH of the gastrointestinal tract.

The percentage of AFB1 released was determined after each treatment and is presented in Figure 3. Concerning the strains isolated from Kefir, the results show that the microorganism/ AFB1 complexes are more sensitive at pH 3 and more stable at pH 7 and 8. The strain *L. kefiri* KFLM3 released, after the three treatments, a considerable amount of AFB1 (66%).

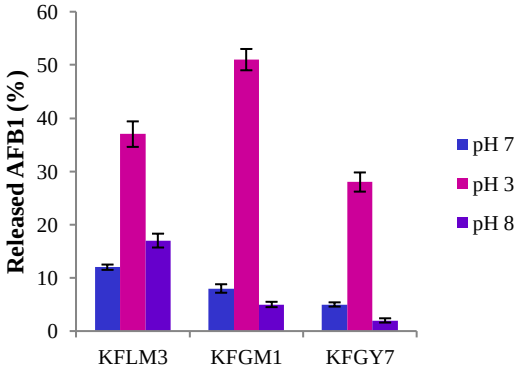


Figure 3: Percentage of AFB1 recovered from microorganism cells after incubation with buffer solutions at pH 7, 3 and 8.

Similarly, strain *A. syzygii* KFGM1 released 64% of AFB1. Compared to bacteria, the yeast *K. servazii* KFGY7 showed the most stable microorganism/ AFB1 complexes. As presented in Table 2, *K.*

servazii KFGY7 only released 35% of AFB1. In summary, KFGY7 was the most effective among the three tested strains.

Table 2
Percentage of total AFB1 recovered from microorganism cells after incubation with different buffer solutions

	KFLM3	KFGM1	KFGY7
Total of released AFB1(%)	66±4.1	64± 3.1	35± 2.6

Our findings are also in accordance with those of Haskard et al. (2001), who reported reversible binding of aflatoxins to probiotic bacteria. Other authors reported partially reversible AFB1 binding with *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Lactobacillus amylovorus* (Peltonen et al. 2001, Hernandez-Mendoza et al. 2009). By contrast, a stable AFM1-LAB (*L. rhamnosus* and *L. plantarum*) complex was reported by Elsanhoty et al. (2014).

In summary, the AFB1 binding process of the Kefir isolates was partially reversible, showing a moderate non-covalent interaction between toxins and microorganisms. Moreover, as described by Bevilacqua et al. (2014), the amount of mycotoxin released into the medium was proportional to the number of treatments performed. The percentage of mycotoxins released was also dependent on the strain, buffer pH and mycotoxin type. The binding characteristics of a strain are possibly dependent on the exopolysaccharides produced by the microorganisms or on their cell wall composition.

4. Conclusions

In this research, Kefir isolates (*L. Kefiri* KFLM3, *A. syzygii* KFGM1 and *K. servazii*KFGY7) are able to bind considerable amounts of AFB1 in milk. However, the AFB1 binding process was reversible, and different quantities of toxin were released after exposure to successive pH treatments. Even so, on average, the yeast *K. servazii*KFGY7 was able to retain approx. 52% of the mycotoxins added to milk, *Lactobacillus kefiri* KFLM3 retained approx. 34% and *Acetobacter syzygii* KFGM1 retained 23%. Thus, our findings support that the isolated strains may be of interest for biotechnological applications in the dairy and feed industries as a novel source of AFB1-adsorbing microorganisms.

Acknowledgments

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