International Journal of Food Microbiology 251 (2017) 1–7

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Adsorption of aflatoxin B1, zearalenone and ochratoxin A by microorganisms isolated from Kefir grains

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ARTICLE INFO

Article history:
Received 2 January 2017
Received in revised form 11 March 2017
Accepted 27 March 2017
Available online 28 March 2017

Keywords:
Kefir
Adsorption
Mycotoxins
Lactobacillus kefiri
Acetobacter syzygii
Kazachstania servazzii

ABSTRACT

A strategy to reduce the deleterious effects of mycotoxins is to use dietary supplements that contain microorganisms that bind mycotoxins and decrease their gastrointestinal absorption. Novel strains were isolated from a Kefir culture and assessed for their mycotoxin adsorption and biotransformation ability. The most active strains were identified using DNA sequencing, and the stability of microorganism/mycotoxin complexes was evaluated using buffer solutions to simulate the pH conditions in the gastrointestinal tract. Our results showed that the microorganism consortium of Kefir grains adsorbed 82 to 100% of aflatoxin B1 (AFB1), zearalenone (ZEA) and ochratoxin A (OTA) when cultivated in milk. The main strains that were capable of mycotoxin adsorption were identified as *Lactobacillus kefiri*, *Kazachstania servazzii* and *Acetobacter syzygii*. The strain *L. kefiri* KFLM3 was the most active, adsorbing 80 to 100% of the studied mycotoxins when cultivated in milk. Nonetheless, the strain *K. servazzii* KFGY7 retained more mycotoxin after the desorption experiments (65, 69 and 67% for AFB1, OTA and ZEA, respectively). These findings suggest that Kefir consumption may help to reduce gastrointestinal absorption of these mycotoxins and consequently reduce their toxic effects. The isolated strains may be of interest for the development of fermented dairy products for human consumption that have a new probiotic characteristic, the adsorption of mycotoxins.

1. Introduction

Mycotoxins are ubiquitous secondary metabolites that are produced by filamentous fungi mainly belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium*. Mycotoxins can be found in food and animal feed around the world (Monbaliu et al., 2009). The Food and Agriculture Organization (FAO) estimated that approximately 25% of food production is contaminated with at least one mycotoxin (CAST, 1989). Humans are directly exposed to mycotoxins through consumption of contaminated plant-derived foods when agricultural commodities are colonized by mycotoxigenic fungi or indirectly by consumption of animal-derived products when livestock are fed mycotoxin-containing feed (Zain, 2011). A typical example is the carry-over of mycotoxins and their derivatives into milk. In ruminant animals, ingested mycotoxins can be metabolized by the rumen microbiota or animal organism and excreted in milk. The carry-over into milk of aflatoxin M1 (AFM1), aflatoxicol, cyclopiazonic acid, fumonisin B1 (FB1), ochratoxin A (OTA), T-2 toxin, deoxynivalenol and their de-epoxy-forms, zearalenone (ZEA) and its derivative α-zearalenol, 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).

Ingestion of mycotoxins may cause acute mycotoxicosis and several chronic adverse effects, such as mutagenic, carcinogenic, teratogenic, estrogenic and immunosuppressive effects (Paterson and Lima, 2010). The International Agency for Research on Cancer (IARC) classified aflatoxin B1 (AFB1) in group 1 (carcinogenic to humans), OTA and FB1 in group 2B (possibly carcinogenic to humans) and ZEA in group 3 (not classifiable as to its carcinogenicity to humans) (IARC, 2016).

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. Therefore, infants and young children are more sensitive to these serious problems than adults (Mohammadi, 2011). Studies have reported an association between AFB1 exposure and stunted foetal, infant and child growth in West African countries (Gong et al., 2004). Moreover, diagnosis of nephropathic patients from Tunisia showed that the presence of OTA was linked to a chronic interstitial nephropathy (Zaied et al., 2011). In consideration of the health risks and to ensure food safety, the Joint FAO/WHO Expert

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http://dx.doi.org/10.1016/j.ijfoodmicro.2017.03.021
0168-1605/

Committee on Food Additives (JECFA) sets limits for mycotoxin daily intake as follows: ZEA - 0.5 μg/kg body weight per day and OTA - 112 ng/kg body weight per week, and no limits have been indicated regarding tolerable intake for AFB1 because of its carcinogenic effects (Bol et al., 2016).

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).

Kefir is a fermented dairy product originating from the Caucasus Mountains. The word Kefir "keyif" comes from the Turkish language and means "good feeling" owing to the refreshing nature of this beverage (Leite et al., 2013). It is obtained from lactic-alcoholic fermentation of milk by gelatinous irregular grains, which range from 0.3 to 3.5 cm in diameter. The colour of the grains varies from white to light yellow, and they resemble tiny florets of cauliflower (Hamet et al., 2013). Kefir grains are a complex symbiotic association of lactic acid bacteria (*Lactobacillus kefiri*, *Lactobacillus kefiranofaciens*, *Lactobacillus kefirgranum*, *Lactobacillus parakefiri*, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus helveticus*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, and *Lactobacillus gasseri*), yeasts (*Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Candida kefir*, *Pichia fermentans*, *Kazachstania unispora*, and *Kazachstania exigua*) and acetic acid bacteria 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). A few studies regarding mycotoxin decontamination by Kefir have reported the ability of Kefir grains to reduce 96.8% of AFG1 at a concentration of 20 μg/kg in pistachio nuts after a 6 h contact time at 30 °C (Ansari et al., 2015). In addition, the ability of Kefir grains to bind 91.9% of AFM1 (0.5 μg/L) in milk has been reported (Isakhani et al., 2014). However, as far as we know, the effect of Kefir microbiota on other mycotoxins has not previously been investigated, nor has any active strain been isolated from it.

The objective of the current study was to investigate the microbiota characteristics of Kefir from Tunisia and to isolate bacteria and/or yeasts with biodegradation and/or adsorption properties for AFB1, OTA and ZEA. Additionally, the main strains involved in the adsorption of mycotoxins studied were identified by DNA sequencing, and the stability of the microorganism/mycotoxin complexes was tested by simulating a pH change in the gastrointestinal tract.

2. Materials and methods

2.1. Kefir production

Kefir used in this study was a traditional culture and original grain from Monastir, Tunisia. The preparation of Kefir was as follow: grains (10% w/v) were activated in commercial Ultra-high temperature cows' milk (protein, 3.4%; fat, 1.6%; carbohydrate, 5.1%) and incubated at 25 °C for 24 h. The grains were then retrieved by sieving from the cultured milk and rinsed with mild sterile distilled water to remove the clotted milk. Afterwards, the grains were re-inoculated into fresh sterile milk and incubated under the same conditions. The milk was changed every day during the one week incubation period. This step was repeated twice to obtain grains well acclimated to milk and ready for use as starter inocula in the following experiments.

2.2. Microbiological analysis of Kefir

Enumeration and isolation of microorganisms from both fermented milk and Kefir grains were performed. After an incubation period of activated grains at 25 °C for 24 h in milk, ten millilitres of Kefir fermented milk was homogenized with 90 mL of a sterile sodium thiosulfate solution (0.2% w/v) for 1 min using a Stomacher. Then, ten grams of Kefir grains were suspended in 90 mL of sterile saline (0.85%) and homogenized with a Stomacher for 20 min. Serial decimal dilutions were prepared in the same diluent, and 0.1 mL was inoculated in triplicate by surface spreading on specific solid media. Lactic acid bacteria (LAB) were isolated on de Man Rogosa Sharpe agar (MRS) and incubated at 30 °C under anaerobic conditions for 5 days. Yeasts were isolated on yeast extract peptone dextrose (YPD) agar at 25 °C for 3 days. MRS agar (Oxoid, Hampshire, UK), and YPD was prepared with 20 g/L of bacteriological peptone (Himedia, Mumbai, India), 10 g/L of yeast extract (Himedia, Mumbai, India), 20 g/L of glucose (Fisher Chemical, Porto Salvo, Portugal) and 20 g/L of bacteriological agar (Oxoid, Hampshire, UK). LAB and yeasts were counted, and some strains were isolated, streak-plate purified and examined microscopically. The results of the viable counts were expressed as the means of colony forming units (CFU) per gram or per mL of Kefir ± standard deviation. Isolated colonies were grown in MRS broth supplemented with 20% glycerol and preserved at −80 °C until further analyses.

2.3. Evaluation of mycotoxin-detoxifying properties of Kefir

The ability of Kefir cultures to biotransform or adsorb mycotoxins in milk was first evaluated. Stock standard solutions of AFB1 (Sigma), OTA (Panreac) and ZEA (Sigma) were 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 each mycotoxin (AFB1, OTA and ZEA) by adding the appropriate amount of stock standards. Falcon tubes containing 5 mL of mycotoxin-contaminated milk were prepared in triplicate to study the biotransformation and adsorption properties of Kefir. Kefir grains (10%, w/v) were added to tubes containing five millilitres of mycotoxin-contaminated milk. The tubes were mixed and incubated aerobically at 25 °C for 24 h. Three non-inoculated tubes containing milk contaminated with mycotoxins were also prepared and incubated to be used as negative controls.

After the incubation period, for the determination of mycotoxins, tubes intended to test biotransformation were treated differently from those intended to test adsorption. To test biotransformation, 5 mL of acetonitrile/methanol/acetic acid (78/20/2, v/v/v) was directly added to tubes, and after a strong vortex mixing for 1 min, they were left to stand overnight at room temperature. Negative controls were also treated in this way. To test adsorption, 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.

2.4. Evaluation of the mycotoxin-detoxifying properties of strains isolated from Kefir

After streak-plate purification and microscopic inspection, some of the microorganisms isolated from Kefir were investigated for mycotoxin biotransformation and adsorption properties using culture media and milk. Bacteria and yeasts 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. MRS broth, YPD broth and milk supplemented with 1 μg/mL of each mycotoxin