Journal of Food Composition and Analysis xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

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

# Influence of probiotic microorganisms on aflatoxins $B_1$ and $B_2$ bioaccessibility evaluated with a simulated gastrointestinal digestion

F. Saladino[a], E. Posarelli[b], C. Luz[a], F.B. Luciano[c], M.T. Rodriguez-Estrada[b], J. Mañes[a], G. Meca[a,*]

[a] Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia,Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain
[b] Dipartimento di Scienze e Tecnologie Agro-Alimentari, Alma Mater Studiorum-Università di Bologna, Viale G. Fanin 40, 40127 Bologna, BO, Italy
[c] School of Life Sciences, Pontifícia Universidade Católica do Paraná, Rua Imaculada Conceição 1155, 80215-901 Curitiba, Brazil

ARTICLE INFO

*Article history:*
Received 7 October 2016
Received in revised form 12 January 2017
Accepted 18 January 2017
Available online xxx

*Keywords:*
Aflatoxins
Bioaccessibility
Probiotic bacteria
*Lactobacillus*
*Bifidobacterium*

ABSTRACT

Aflatoxins (AFs) are produced mainly by the molds *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin $B_1$ ($AFB_1$) is classified as carcinogenic to humans. The aim of this study was to evaluate the capacity of different strains of *Lactobacilli* (*Lb.*) and *Bifidobacteria* (*Bf.*) to reduce the bioaccessibility of $AFB_1$ and aflatoxin $B_2$ ($AFB_2$), spiked in loaf bread, using a dynamic *in vitro* simulated gastrointestinal digestion system. Aliquots of 20 mL of gastric and duodenal fluids were sampled for the determination of the mycotoxins gastric and duodenal bioaccessibility respectively, by liquid-chromatography coupled to the mass spectrometry in tandem (LC–MS/MS). A reduction of AFs bioaccessibility compared to the control (digestion without bacterial strains) was evidenced. The strains that evidenced the highest gastric and duodenal bioaccessibility reductions of $AFB_1$ and $AFB_2$ were *Lb. johnsoni* CECT 289, *Lb. reuteri* CECT 725, *Lb. plantarum* CECT 220 and *Lb. casei* CECT 4180, with values ranging from 76.38 to 98.34% for $AFB_1$ and from 77.14 to 98.66% for $AFB_2$. These results suggest that a food enriched with specific probiotic microorganisms and consumed at the same time as food contaminated with AFs, could reduce the risk associated to the intake of these toxic compounds contained in food.



## 1. Introduction

Aflatoxins (AFs), a group of structurally related toxic secondary metabolites of fungi, are primarily produced by *Aspergillus flavus* and *Aspergillus parasiticus*, groups of fungi that could be found in corn, nuts, peanuts, coconut, dried fruits and beer (Frenich et al., 2009; Li et al., 2009a,b; Williams et al., 2004). There are four major AFs named as $B_1$, $B_2$, $G_1$ and $G_2$. Among them, aflatoxin $B_1$ ($AFB_1$) is classified as a carcinogenic substance of group 1 by the International Agency for Research on Cancer (IARC) as it may interfere with the inductive of specific enzymes and forbid the synthesis of RNA 5 (IARC, 2012; Merrick et al., 2013; Wild and Montesano, 2009). Owing to the highly resistance to degradation during food processing, $AFB_1$ could enter the food chain and provide a threat to human health (Castells et al., 2007). Therefore, the regulatory limits for AFs ($B_1 + B_2 + G_1 + G_2$), even for $AFB_1$, have been established in several countries. The European Commission has set strict limits for the maximum allowed levels (MAL) of $AFB_1$ in ground-nuts, dried fruits and their products, in which the MAL of $AFB_1$ could not be greater than 2 $\mu g\,kg^{-1}$ for Retail Ready Foods (Van Egmond, 1995). It has been proved that the intake of $AFB_1$ over a long time may be dangerous even at a very low concentration. Therefore, the assays with high sensitivity and specificity are required to determine AFs at trace level in foods and agricultural products.

Even though the consumption of food contaminated with AFs should be strictly avoided due to its toxicity and carcinogenic effect, several studies show presence of AFs in different cereal products (Saladino et al., 2017; Iqbal et al., 2014; Serrano et al., 2012), sometimes above the limits enforced by the European legislation. For this reason different strategies have been developed to prevent the growth of mycotoxin producing fungi on food and feed, as well as to decontaminate and/or detoxify mycotoxin-contaminated products. One of the most used strategy to reduce the mycotoxins bioaccessibility during the gastrointestinal digestion is the employment of probiotic bacteria. *Lb.* and *Bf.* have shown AF-binding ability. This mechanism is unclear but it is suggested that is a physical phenomenon associated with bacterial cell wall

* Corresponding author.
*E-mail address:* giuseppe.meca@uv.es (G. Meca).

http://dx.doi.org/10.1016/j.jfca.2017.01.010
0889-1575/

structure. Peptidoglycans and polysaccharides have been suggested to be the two most important elements responsible for the binding (Kabak et al., 2009).

In human health risk assessment, ingestion of food is considered a major route for exposure to many contaminants. The total amount of an ingested contaminant (intake) does not always reflect the quantity that is available to the body, because only a smaller amount will be available for absorption. As a consequence, bioaccessibility, defined as the amount of contaminant released through the gastrointestinal tract from the food matrix and then potentially absorbable, can be considered a measure for the assessment of mycotoxin bioavailability in food (Versantvoort et al., 2005).

Recently is increasing the interest in the use of microorganisms to reduce the absorption of mycotoxins, present in food and feed, in the gastrointestinal tract. In particular, Kabak and Ozbey (2012a) studied the effectiveness of some probiotic bacteria to reduce the amount available for intestinal absorption of AFs from different contaminated food materials obtaining reductions in the bioaccessibility up to 35.6% for $AFB_1$, 35.5% for $AFB_2$, 31.9% for $AFG_1$ and 33.6% for $AFG_2$. Kabak and Ozbey (2012b) obtained a reduction between 15.5% and 31.6% in $AFM_1$ bioaccessibility (in milk) in the presence of probiotic bacteria and Serrano-Niño et al. (2013) showed reduction of $AFM_1$'s bioaccessibility in phosphate buffer saline (PBS) from 22.72 to 45.17% using five different probiotic strains.

This is the first report in which is evaluated the effect of the intake of a simulated food enriched with probiotic microorganisms on reducing AFs bioaccessibility if consumed at the same time as contaminated loaf bread.

The aim of this study was to evaluate the capacity of probiotic microorganisms to reduce the bioaccessibility of $AFB_1$ and $AFB_2$ using a dynamic *in vitro* simulated gastrointestinal digestion system.

## 2. Materials and methods

### 2.1. Chemicals

Potassium chloride (KCl), potassium thiocyanate (KSCN), sodium dihydrogen phosphate ($NaH_2PO_4$), sodium sulfate ($Na_2SO_4$), sodium chloride (NaCl), sodium hydrogen carbonate ($NaHCO_3$), urea ($CO(NH_2)_2$), α-amylase (930 U $mg^{-1}$ A3403), hydrochloric acid (HCl), sodium hydroxide (NaOH), formic acid (HCOOH), pepsin A (674 U $mg^{-1}$ P7000), pancreatin (762 U $mg^{-1}$ P1750), bile salts (B8631), phosphate buffer saline (PBS, pH 7.5) and standard solutions of $AFB_1$ and $AFB_2$ (≥98% purity), were purchased from Sigma-Aldrich (Madrid, Spain). Methanol and ethyl acetate were supplied by Fisher Scientific (Madrid, Spain). Deionized water was purchased from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath.

### 2.2. Bacterial strains and growth conditions

Fifteen commercial probiotic strains were used in the *in vitro* system to evaluate the capacity to reduce AFs bioaccesibility during simulated gastrointestinal digestion. In particular, *Lb. rhamnosus* CECT 278T (1), *Lb. ruminis* CECT 4061T, *Lb. casei* CECT 475 (1), *Lb. rhamnosus* CECT 288 (2), *Lb. johnsonii* CECT 289, *Lb. casei* CECT 4180 (2), *Lb. plantarum* CECT 220, *Lb. reuteri* CECT 725, *Lb. bulgaricus* CECT 4005, *Lb. paracasei* CECT 277, *Lb. salivarus* CECT 4062, *Bifidobacterium Longum* CECT 4551, *Bf. bifidum* CECT 870T, *Bf. breve* CECT 4839T, and *Bf. adolescentis* CECT 5781T were obtained from the Spanish Type Culture Collection (CECT Valencia, Spain), in sterile 18% glycerol. The bacterial strains were tested individually

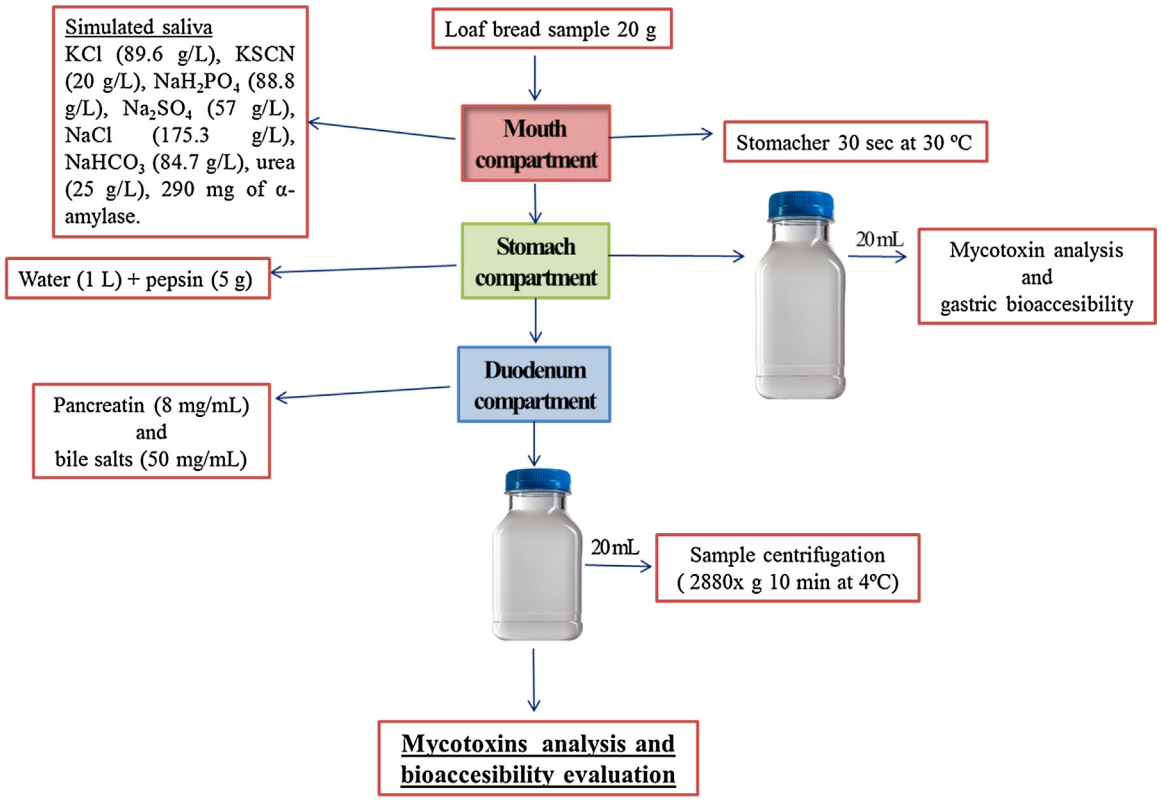


**Fig. 1.** Schematic representation of the *in vitro* simulated gastrointestinal digestion system used for the digestion of the loaf bread spiked with $AFB_1$ and $AFB_2$.

Please cite this article in press as: F. Saladino, et al., Influence of probiotic microorganisms on aflatoxins $B_1$ and $B_2$ bioaccessibility evaluated with a simulated gastrointestinal digestion, J. Food Compos. Anal. (2017), http://dx.doi.org/10.1016/j.jfca.2017.01.010

and were added in the simulated saliva before the gastric digestion step at $\sim 10^5$ CFU $mL^{-1}$ to simulate the intake of a food enriched with probiotic microorganisms and consumed at the same time as spiked loaf bread produced in this study.

For longer survival and higher quantitative retrieval of the cultures, they were stored at −80 °C. When needed, the recovery of strains was undertaken by two consecutive subcultures in appropriate media prior to use.

### 2.3. Loaf bread preparation

The recipe for loaf bread preparation was composed by: 600 g of wheat flour, 20 g of sucrose, 10 g of NaCl, 40 g of yeast for bakery products (Levital, Spain) and 350 mL of water. The ingredients were kneaded manually for 5 min and the dough produced was left rising for 1 h at room temperature. Baking was performed at 200 °C for 40 min in a deck oven (MIWE, Arnstein, Germany). The oven was presteamed (300 mL of water) before loading. The breads were kept for 30 min on cooling racks at room temperature. Twenty grams of loaf bread were spiked with 90 μL $AFB_1$ and $AFB_2$ at 14.79 and 6.14 mg $kg^{-1}$, in 9 spot of 10 μL each to cover significatively the bread loaf surface respectively, using a stock methanolic solution (1000 μM) of each AFs. After 12 h contact at room temperature to completely remove the solvent, the bread was used for the *in vitro* dynamic digestion.

### 2.4. In vitro *dynamic digestion model*

Gastrointestinal digestion in the *in vitro* dynamic model was carried out using 5 L bioreactors Infors (Bottmingen, Switzerland) (Fig. 1) with a working volume of 4 L. For agitation, two Rushton turbines (Ø = 45 mm) were used. The agitation rate during all the gastrointestinal digestion steps was set at 2 g. The incubation temperature was maintained at 37 °C.

Twenty grams of spiked loaf bread were mixed with 60 mL of artificial saliva (composed of: 10 mL of KCl (89.6 g $L^{-1}$), 10 mL of KSCN (20 g $L^{-1}$), 10 mL of $NaH_2PO_4$ (88.8 g $L^{-1}$), 10 mL of $Na_2SO_4$ (57 g $L^{-1}$), 1.7 mL of NaCl (175.3 g $L^{-1}$), 20 mL $NaHCO_3$ (84.7 g $L^{-1}$), 8 mL of urea (25 g $L^{-1}$), and 290 mg of α-amylase). The bacterial strains were also added individually at $\sim 10^5$ CFU $mL^{-1}$ to simulate the intake of a food enriched with probiotic microorganisms and consumed at the same time as contaminated loaf bread. The pH of this solution was increased to 6.8 with a 0.1 N NaOH solution. The mixture was placed in a plastic bag containing 1 L of water at 37 °C, homogenized with a Stomacher IUL Instrument (Barcelona, Spain) for 30 s and introduced in the fermenter vessel. Five g of pepsin (14 800 U) dissolved in 250 mL of 0.1 N HCl was introduced into this mixture, through a fermenter insert. The pH of the mixture was decreased to 2 with the addition of 0.5 N HCl contained in a glass bottle, by means of a peristaltic pump. The incubation temperature was set at 37 °C, by transferring the mixture to the fermenter vessel through a heater plate. All fermentation parameters were regulated through the software Iris 5.0 (Infors AG CH-4103, Bottmingen, Switzerland). The total incubation time was 2 h. An aliquot of 20 mL of gastric fluid was sampled for the determination of the mycotoxins' gastric bioaccessibility.

After gastric digestion, pancreatic digestion was simulated by increasing the pH to 6.5 with $NaHCO_3$ (0.5 N), which was contained in a glass bottle and introduced into the fermenter vessel through a peristaltic pump. Thereafter, 25 mL of pancreatin (8 mg $mL^{-1}$) and 25 mL of bile salts (50 mg $mL^{-1}$) dissolved in 200 mL of water, were introduced into the fermenter vessel and incubated at 2 g at 37 °C for 2 h. An aliquot of 20 mL of the duodenal fluid was sampled for the determination of the mycotoxins' duodenal bioaccessibility (Manzini et al., 2015).

### 2.5. Mycotoxin extraction from the simulated intestinal fluids

AFs $B_1$ and $B_2$ contained in gastric and gastric + duodenal fluids were extracted as follows (Tafuri et al., 2008). Five milliliters of each mixture were placed in a 14 mL plastic test tube, and extracted three times with 5 mL of ethyl acetate using a vortex VWR International (Barcelona, Spain) for 1 min. The mixtures were then centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 2880g for 10 min at 4 °C. The organic phases were completely evaporated with a rotary evaporator (Buchi, Switzerland) at 30 °C and 30 mbar pressure, resuspended in 1 mL of methanol and filtered with a 0.22 μM filter (Phenomenex, Madrid, Spain) before being analyzed by liquid-chromatography coupled to the mass spectrometry in tandem (LC–MS/MS).

### 2.6. LC–MS/MS aflatoxin identification and quantification

The liquid-chromatography system consisted of a binary LC-20AD pump, a SIL–20AC homoeothermic auto-sampler, a CTO-20A column oven and a CMB-20A controller (Agilent, Santa Clara, USA) an Analyst Software 1.5.2 (Applied Biosystems, Foster City, USA) was used for data acquisition and processing. The separation of AFs was performed on a Gemini NX C18 column (150 × 2.0 mm I.D, 3.0 mm; Phenomenex, CA, USA) at room temperature (20 °C).

The mobile phase was composed of solvents A (5 mM ammonium formate and 0.1% formic acid in water) and B (5 mM ammonium formate and 0.1% formic acid in methanol) at a flow rate of 0.25 mL/min. The elution gradient was established initially with 10% eluent B, increased to 80% in 1.5 min, then kept constant from 1.5 to 4 min, increased to 90% from 4 to 10 min, increased again to 100% from 10 to 14 min and finally return to the initial conditions and requilibrate during 10 min. The injection volumen was 20 mL. An API-4000 triple-quadruple MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with ESI interface in positive mode was used for detection in multiple reactions monitoring (MRM) mode. The main MS parameters were optimized and finally set as follows: nebulizer gas (GS1), 55 psi; auxiliary gas (GS2), 50 psi; curtain gas (CUR) 15 psi; capillary temperature 550 °C; ion spray voltage (IS) 5500 V. Nitrogen was used as the nebulizer, heater, curtain and collision gas. The precursor-to-product ion transitions were *m/z* 313.1/241.3-284.9 and *m/z* 315.1/259.0-286.9 for $AFB_1$ and $AFB_2$, respectively. Quantification of AFs was carried out by comparing peak areas of investigated samples with the calibration curve performed with standards (concentrations ranging from 0.1 to 100 μg $L^{-1}$).

### 2.7. Statistical analysis of data

Graphpad Prism version 6.0 (Graphpad Software Inc., La Jolla, CA, USA) was used for the statistical analysis of data. Differences between groups were carried out using analysis of variance ANOVA followed by Dunnet's multiple comparison tests. Differences were considered significant if $p \leq 0.05$.

## 3. Results and discussion

### 3.1. Method performance

Mean recoveries were operated on the fortified intestinal fluids (free from contamination of AFs) (n = 3) at levels of AFs (0.1–100 μg $L^{-1}$). The recoveries evidenced for $AFB_1$ and $AFB_2$ were 88.3 ± 3.4% and 83.6 ± 4.2%, respectively. Intra-day (n = 3) and interday (3 different days) variation values ranged between 2.6 and 4.2%. The detection limit (LOD) and the limit of quantification (LOQ) values were calculated according to *s/n* = 3 and s/n = 10,

Please cite this article in press as: F. Saladino, et al., Influence of probiotic microorganisms on aflatoxins $B_1$ and $B_2$ bioaccessibility evaluated with a simulated gastrointestinal digestion, J. Food Compos. Anal. (2017), http://dx.doi.org/10.1016/j.jfca.2017.01.010

respectively. The LODs and the LOQs of AFs were 0.04 and 0.15 μg $L^{-1}$ for $AFB_1$ and 0.21 and 0.72 μg $L^{-1}$ for $AFB_2$, respectively.

### 3.2. Evaluation of the AFs contained in the gastric and duodenal fluids (bioaccessibility)

Tables 1 and 2 show the bioaccessibility data of the AFs $B_1$ and $B_2$ present in loaf bread spiked with these two contaminants (the initial concentrations present in the food matrix were 14.79 and 6.14 mg $kg^{-1}$ for $AFB_1$ and $AFB_2$, respectively), after being digested using an *in vitro* method to mimic the conditions of human gastrointestinal digestion in presence of different probiotic strains. In particular, in the control experiment (consisting of a spiked loaf bread digested without probiotic strains), the $AFB_1$ bioaccessibility of the stomach and the duodenal digestion were 53.89 and 25.76%, respectively. All tests performed with the probiotic strains, evidenced a reduction of the $AFB_1$ bioaccessibility compared with the control experiments. The mean $AFB_1$ bioaccessibility (considering both stomach and duodenal compartments) ranged from 0.94% (with *Lb. johnsoni*) to 30.71% (with *Lb. bulgaricus*). The lowest $AFB_1$ bioaccessibility was obtained with *Lb. johnsoni*, displaying gastric and duodenal bioaccessibility reductions of 98.09 and 96.73%, respectively. In the treated samples, the mean $AFB_1$ bioaccessibility reduction was equal to 59.12%. The strains that evidenced the highest $AFB_1$ bioaccessibility reductions were *Lb. johnsoni*, *Lb.reuteri*, *Lb. plantarum* and *Lb. casei* (2), with values ranging from 76.38 to 98.34%.

**Table 1**
Gastric and duodenal bioaccessibility reduction of $AFB_1$ present in loaf bread, subjected to digestion with probiotic microorganisms. Significantly different from the control, $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***).

| Samples | Aflatoxin $B_1$ | | | |
|---|---|---|---|---|
| | Concentration (ppm) | Bioacc. (%) | Mean | Bioacc. Red. (%) |
| Control[a] S[b] | 7.97 ± 0.40 | 53.89 | 39.82 | |
| Control D[c] | 3.81 ± 0.10 | 25.76 | | |
| *Bf. longum S* | 3.97 ± 0.20 | 26.81 | 17.80 | 50.25*** |
| *Bf. longum D* | 1.30 ± 0.030 | 8.79 | | 65.88*** |
| *Bf. bifidum S* | 4.82 ± 0.20 | 32.60 | 22.38 | 39.51*** |
| *Bf. bifidum D* | 1.80 ± 0.01 | 12.17 | | 52.76*** |
| *Bf. breve S* | 3.18 ± 0.20 | 21.49 | 16.53 | 60.12*** |
| *Bf. breve D* | 1.70 ± 0.08 | 11.56 | | 55.11*** |
| *Bf. adolescentis S* | 5.45 ± 0.40 | 36.88 | 26.05 | 31.57** |
| *Bf. adolescentis D* | 2.25 ± 0.20 | 15.21 | | 40.94*** |
| *Lb. rhamnosus* (1) *S* | 3.20 ± 0.40 | 40.15 | 25.82 | 25.49** |
| *Lb. rhamnosus* (1) *D* | 1.70 ± 0.03 | 11.49 | | 55.38*** |
| *Lb. ruminis S* | 3.31 ± 0.03 | 22.35 | 16.58 | 58.58*** |
| *Lb. ruminis D* | 1.60 ± 0.01 | 10.82 | | 58.01*** |
| *Lb. casei* (1) *S* | 2.44 ± 0.02 | 16.47 | 12.29 | 69.43*** |
| *Lb. casei* (1) *D* | 1.20 ± 0.03 | 8.11 | | 68.50*** |
| *Lb. rhamnosus* (2) *S* | 3.45 ± 0.40 | 23.30 | 16.31 | 56.73*** |
| *Lb. rhamnosus* (2) *D* | 1.38 ± 0.07 | 9.30 | | 63.89*** |
| *Lb. johnsoni S* | 0.15 ± 0.01 | 1.03 | 0.94 | 98.09*** |
| *Lb. johnsoni D* | 0.12 ± 0.02 | 0.84 | | 96.73*** |
| *Lb. casei* (2) *S* | 0.46 ± 0.03 | 3.12 | 2.72 | 94.21*** |
| *Lb. casei* (2) *D* | 0.34 ± 0.03 | 2.31 | | 91.02*** |
| *Lb. plantarum S* | 0.13 ± 0.01 | 0.90 | 0.98 | 98.34*** |
| *Lb. plantarum D* | 0.16 ± 0.01 | 1.06 | | 95.90*** |
| *Lb. reuteri S* | 1.43 ± 0.03 | 9.67 | 7.88 | 82.06*** |
| *Lb. reuteri D* | 0.90 ± 0.02 | 6.09 | | 76.38*** |
| *Lb. bulgaricus S* | 6.74 ± 0.40 | 45.58 | 30.71 | 15.41* |
| *Lb. bulgaricus D* | 2.34 ± 0.03 | 15.84 | | 38.49** |
| *Lb. paracasei S* | 5.26 ± 0.40 | 35.57 | 25.25 | 34.00** |
| *Lb. paracasei D* | 2.20 ± 0.05 | 14.93 | | 42.06*** |
| *Lb. salivarus S* | 5.69 ± 0.70 | 38.50 | 28.22 | 28.55** |
| *Lb. salivarus D* | 2.64 ± 0.30 | 17.93 | | 30.39** |

[a] The control consisted of spiked loaf bread digested without probiotic strains.
[b] Stomach.
[c] Duodenum.

**Table 2**
Gastric and duodenal bioaccessibility reduction of $AFB_2$ present in loaf bread, subjected to digestion with probiotic microorganisms. Significantly different from the control, $p \leq 0.05$ (*), $p \leq 0.001$ (**), $p \leq 0.0001$ (***).

| Samples | Aflatoxin $B_2$ | | | |
|---|---|---|---|---|
| | Concentration (ppm) | Bioac. (%) | Mean | Bioac. Red (%) |
| Control[a] S[b] | 3.50 ± 0.2 | 57.00 | 46.74 | |
| Control D[c] | 2.24 ± 0.3 | 36.48 | | |
| *Bf. longum S* | 3.00 ± 0.1 | 48.86 | 36.64 | 14.29* |
| *Bf. longum D* | 1.50 ± 0.3 | 24.43 | 33.04** | |
| *Bf. bifidum S* | 3.10 ± 0.2 | 50.49 | 39.90 | 11.43* |
| *Bf. bifidum D* | 1.80 ± 0.1 | 29.31 | 19.64* | |
| *Bf. breve S* | 2.20 ± 0.3 | 35.83 | 24.43 | 37.14** |
| *Bf. breve D* | 0.80 ± 0.1 | 13.03 | 64.29*** | |
| *Bf. adolescentis S* | 2.80 ± 0.4 | 45.60 | 33.39 | 20.00** |
| *Bf. adolescentis D* | 1.30 ± 0.2 | 21.17 | 41.96** | |
| *Lb. rhamnosus* (1) *S* | 2.10 ± 0.2 | 34.20 | 24.43 | 40.00** |
| *Lb. rhamnosus* (1) *D* | 0.90 ± 0.1 | 14.66 | 59.82*** | |
| *Lb. ruminis S* | 2.20 ± 0.3 | 35.83 | 24.43 | 37.14*** |
| *Lb. ruminis D* | 0.80 ± 0.2 | 13.03 | 64.29*** | |
| *Lb. casei* (1) *S* | 2.00 ± 0.3 | 32.57 | 21.99 | 42.86*** |
| *Lb. casei* (1) *D* | 0.70 ± 0.1 | 11.40 | 68.75*** | |
| *Lb. rhamnosus* (2) *S* | 2.40 ± 0.2 | 39.09 | 31.76 | 31.43** |
| *Lb. rhamnosus* (2) *D* | 1.50 ± 0.3 | 24.43 | | 33.04** |
| *Lb. johnsoni S* | 0.80 ± 0.1 | 1303 | 8.96 | 77.14*** |
| *Lb. johnsoni D* | 0.30 ± 0.08 | 4.89 | | 86.61*** |
| *Lb. casei* (2) *S* | 0.50 ± 0.2 | 8.14 | 4.89 | 85.71*** |
| *Lb. casei* (2) *D* | 0.10 ± 0.02 | 1.63 | | 95.54*** |
| *Lb. plantarum S* | 0.40 ± 0.1 | 6.51 | 3.66 | 88.57*** |
| *Lb. plantarum D* | 0.05 ± 0.01 | 0.81 | | 97.77*** |
| *Lb. reuteri S* | 0.35 ± 0.06 | 5.70 | 3.09 | 90.00*** |
| *Lb. reuteri D* | 0.03 ± 0.01 | 0.49 | | 98.66*** |
| *Lb. bulgaricus S* | 2.30 ± 0.08 | 37.46 | 27.69 | 34.29*** |
| *Lb. bulgaricus D* | 1.10 ± 0.3 | 17.91 | | 50.89*** |
| *Lb. paracasei S* | 2.20 ± 0.2 | 35.83 | 26.06 | 37.14** |
| *Lb. paracasei D* | 1.00 ± 0.2 | 16.29 | | 55.36*** |
| *Lb. salivarus S* | 2.30 ± 0.3 | 37.46 | 31.76 | 34.29** |
| *Lb. salivarus D* | 1.60 ± 0.2 | 26.06 | | 28.57** |

[a] The control consisted of spiked loaf bread digested without probiotic strains.
[b] Stomach.
[c] Duodenum.

Regarding $AFB_2$, its bioaccessibility at gastric and duodenal levels were 57% and 36.48%, respectively. In the samples treated with the probiotic strains during the gastrointestinal digestion, the mean lower and higher bioaccessibility for this contaminant were detected in samples treated with *L. reuteri* (3.09%) and *Bf. bifidum* (39.90%). When comparing the data of the treated samples with those of the control ones, a mean $AFB_2$ bioaccessibility reduction of 52.65% was noted. The strains that showed the highest bioaccessibility reductions of $AFB_1$ (*Lb. johnsoni*, *Lb. reuteri*, *Lb. plantarum* and *Lb. casei* (2)) also showed the highest $AFB_2$ bioaccessibility reductions (77.14-98.66%). In particular, as can be observed in Tables 1 and 2, the relation between bioaccessibility reductions of $AFB_1$ and $AFB_2$ is rather similar among the same probiotic strain and also among all the strains.

The observed differences among probiotic strains in reducing AFs bioaccesibility is unclear, however it has been speculated that cell surface hydrophobicity can be related to AF-binding (Oatley et al., 2000). It is thought that AF molecules are bound on the cell wall components of specific bacteria so that the different efficacy of the bacteria might be due to completely different binding sites present in different strains or minor differences in similar binding sites that varies in a strain dependent manner (Hernandez-Mendoza et al., 2009). In previous studies have been found that some strains of *Lb.* and *Bf.* have AF-binding ability. El-Nezami et al. (1998) showed that within 24 h cultures of *Lb. rhamnosus* strain GG and *Lb. rhamnosus* strain LC-705 were able to remove approximately 80% of the $AFB_1$. In other research by Peltonen et al. (2000) the binding of $AFB_1$ by *Lb. paracasei* F19, *Bf. lactis* Bb-12, *Lb. crispatus*

Please cite this article in press as: F. Saladino, et al., Influence of probiotic microorganisms on aflatoxins $B_1$ and $B_2$ bioaccessibility evaluated with a simulated gastrointestinal digestion, J. Food Compos. Anal. (2017), http://dx.doi.org/10.1016/j.jfca.2017.01.010

M247 and MU5, *Lb. salivarius* LM2-118 and *Lb. johnsonii* LJ-1 was found to range from 5.8 to 31.3%. *Lb johnsonii* LJ-1 and *Lb paracasei* F19 were the best binders with approximately 30% binding. Kabak and Var (2004) determined that the ability of *Lb. acidophilus* NCC12, *Lb. acidophilus* NCC36, *Lb. acidophilus* NCC68, *Bf. bifidum* Bb13, *Bf. bifidum* NCC3881 and *Lb. rhamnosus* to bind $AFM_1$ ranged between 25.7–32.5% and 21.2–29.3% in phosphate-buffered saline and skimmed milk, respectively.

Kabak et al. (2009) studied the release of $AFB_1$ and ochratoxin A (OTA) from different food products in the gastrointestinal tract in the absence and presence of probiotics, as possible adsorbents. The average bioaccessibility of $AFB_1$ and OTA without probiotics was about 90% and 30%, respectively, depending on several factors such as food product, contamination level, compound and type of contamination (spiked versus naturally contaminated). The six probiotic bacteria showed a variable $AFB_1$ and OTA binding capacities, which depended on the bacterial strain, toxin, type of food and contamination level. A reduction of 37% and 73% was observed for the $AFB_1$ and OTA bioaccessibility in the presence of probiotic bacteria, respectively.

Raiola et al. (2012) analyzed 27 samples of dried pasta characterized by size, packaging and marketing intended for young children consumption, by liquid chromatography (LC) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) for Deoxynivalenol (DON), OTA and $AFB_1$ determination. The samples that showed the highest amounts of one of the mycotoxins were cooked for 10 min, digested with an *in vitro* gastrointestinal protocol and bioaccessibility values were calculated. Seven of the 27 samples exceeded by 120–225% the European legal limit set for DON in processed cereal-based baby foods (Commission Regulation (EC) 1126/2007). The mean value of gastric bioaccessibility of DON was 23.1%, whereas the mean duodenal bioaccessibility was equal to 12.1%.

Kabak and Ozbey (2012) investigated the bioaccessibility of AFs from various spiked food matrices (peanut, pistachio, hazelnut, dried figs, paprika, wheat and maize) and evaluated the effectiveness of six probiotic bacteria in reducing AF bioaccessibility using an *in vitro* digestion model. The bioaccessibility of AFs from seven food matrices ranged from 85.1% to 98.1% for $AFB_1$, 83.3% to 91.8% for $AFB_2$, 85.3% to 95.1% for AFG1 and 80.7% to 91.2% for $AFG_2$. The bioaccessibilities of all four compounds were independent of the spiking level and food matrix. The inclusion of probiotic bacteria showed a significant ($p < 0.05$) reduction in the bioaccessibility of AFs: up to 35.6% for AFB1, 35.5% for AFB2, 31.9% for AFG1 and 33.6% for AFG2. AF-binding activity of probiotic bacteria in simulated gastrointestinal conditions was reversible, and 10.3–39.8% of bound AFs were released back into the digestion juices from the bacteria–AF complexes.

## 4. Conclusions

The present study showed the capacity of probiotic bacteria to reduce the bioaccessibility of $AFB_1$ and $AFB_2$ in spiked loaf bread. In particular, the highest bioaccesibility AFs reduction was obtained when the spiked loaf bread was digested together with *Lb. johnsoni* CECT 289, *Lb. casei* CECT 4180, *Lb. plantarum* CECT 220 and *Lb. reuteri* CECT 725, reaching reduction up to 98.66%.

Results from this study suggest that a food enriched with specific probiotic microorganisms and consumed at the same time as food contaminated with AFs, could reduce the risk associated to the intake of these toxic compounds contained in food.

## Acknowledgements

The research was supported by the project for emerging research groups GV-2016-106 from the Generalitat Valenciana, by the pre PhD program of University of Valencia "Santiago Grisolia" and by the European Project (H2020-Research and Innovation Action) MycoKey "Integrated and innovative key actions for mycotoxin management in the food and feed chain" GA 678781. E. Posarelli is grateful for the traineeship granted by the Erasmus + Mobility program of the University of Bologna (Italy).

## References

Castells, M., Ramos, A.J., Sanchis, V., Marin, S., 2007. Distribution of total aflatoxins in milled fractions of hulled rice? J. Agric. Food Chem. 55 (7), 2760–2764.
Commission Regulation (EC) 1126/2007. Off. J. Eur. Union L 255/14, 20 August 2008.
El-Nezami, H., Kankaanpaa, P., Salminen, S., Ahokas, J., 1998. Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen: aflatoxin B1. Food Chem. Toxicol. 36, 321–326.
Frenich, G., Vidal, J., Romero-Gonzalez, L.M.R., Aguilera-Luiz, M., 2009. Simple and high-throughput method for the multimycotoxin analysis in cereals and related foods by ultra-high performance liquid chromatography/tandem mass spectrometry. Food Chem. 117, 705–712.
Hernandez-Mendoza, A., Garcia, H.S., Steele, J.L., 2009. Screening of Lactobacillus casei strains for their ability to bind aflatoxin B1. Food Chem. Toxicol. 47, 1064–1068.
IARC, 2012. Chemical agents and related occupations. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 100F. , pp. 1–599 (PMID:23189753).
Iqbal, S.Z., Rabbani, T., Asi, M.R., Jinap, S.1, 2014. Assessment of aflatoxins, ochratoxin A and zearalenone in breakfast cereals. Food Chem. 157, 257–262.
Kabak, B., Ozbey, F., 2012a. Assessment of the bioaccessibility of aflatoxins from various food matrices using an in vitro digestion model, and the efficacy of probiotic bacteria in reducing bioaccessibility. J. Food Compos. Anal. 27, 21–31.
Kabak, B., Ozbey, F., 2012b. Aflatoxin M1 in UHT milk consumed in Turkey and first assessment of its bioaccessibility using an in vitro digestion model. Food Control 28, 338–344.
Kabak, B., Var, I., 2004. Binding of aflatoxin M1 by *Lactobacillus* and *Bifidobacterium* strains. Milchwissenschaft 59, 301–303.
Kabak, B., Brandon, E.F.A., Var, I., Blokland, M., Sips, A.J.A.M., 2009. Effects of probiotic bacteria on the bioaccessibility of aflatoxin B1 and ochratoxin A using an in vitro digestion model under fed conditions. J. Environ. Sci. Health 44, 472–480.
Li, P.W., Zhang, Q., Zhang, W.1, 2009a. Immunoassays for aflatoxins. Trac-Trends Anal. Chem. 28, 1115–1126.
Li, P., Zhang, Q., Zhang, W., Zhang, J., Chen, X., Jiang, J., et al., 2009b. Development of a class-specific monoclonal antibody-based ELISA for aflatoxins in peanut. Food Chem. 115, 313–317.
Manzini, M., Rodriguez-Estrada, M.T., Meca, G., Mañes, J., 2015. Reduction of beauvericin and enniatins bioaccessibility by prebiotic compounds: evaluated in static and dynamic simulated gastrointestinal digestion. Food Control 47, 203–211.
Merrick, B.A., Phadke, D.P., Auerbach, S.S., Mav, D., Stiegelmeyer, S.M., Shah, R.R., et al., 2013. RNA-Seq profiling reveals novel hepatic gene expression pattern in aflatoxin B1 treated rats. PLoS One 8 (4), e61768.
Oatley, J.T., Rarick, M.D., Ji, G.E., Linz, J.E., 2000. Binding of aflatoxin B1 to bifidobacteria in vitro. J. Food Prot. 63, 1133–1136.
Peltonen, K., El-Nezami, H., Pierides, M., Salminen, S., Ahokas, J.T., 2000. Binding of aflatoxin B1 by probiotic bacteria. J. Sci. Food Agric. 80, 1942–1945.
Raiola, A., Meca, G., Mañes, J., Ritieni, A., 2012. Bioaccessibility of deoxynivalenol and its natural co-occurrence with ochratoxin A and aflatoxin B1 in Italian commercial pasta. Food Chem. Toxicol. 50, 280–287.
Saladino, F., Quiles, J.M., Mañes, J., Fernández-Franzón, M., Bittencourt, F.L., Meca, G., 2017. Dietary exposure to mycotoxins through the consumption of commercial bread loaf in Valencia, Spain. LWT – Food Sci. Technol. 75, 697–701.
Serrano, A.B., Font, G., Ruiz, M.J., Ferrer, E., 2012. Co-occurrence and risk assessment of mycotoxins in food and diet from Mediterranean area. Food Chem. 135, 423–429.
Serrano-Niño, J.C., Cavazos-Garduño, A., Hernandez-Mendoza, A., Applegate, B., Ferruzzi, M.G., San Martin-González, M.F., García, H.S., 2013. Assessment of probiotic strains ability to reduce the bioaccessibility of aflatoxin M1 in artificially contaminated milk using an in vitro digestive model. Food Control 31, 202–207.
Tafuri, A., Meca, G., Ritieni, A., 2008. A rapid high-performance liquid chromatography with fluorescence detection method developed to analyze ochratoxin A in wine. J. Food Prot. 71, 2133–2137.
Van Egmond, H.P., 1995. Mycotoxins: regulations: quality assurance and reference materials. Food Addit. Contam. 12 (3), 321–330.
Versantvoort, C., Oomen, A., Van de Kamp, E., Rompelberg, C., Sips, A., 2005. Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. Food Chem. Toxicol. 43, 31–40.
Wild, C.P., Montesano, R., 2009. A model of interaction: aflatoxins and hepatitis viruses in liver cancer aetiology and prevention. Cancer Lett. 286 (1), 22–28.
Williams, J.H., Phillips, T.D., Jolly, P.E., Stiles, J.K., Jolly, C.M., Aggarwal, D., 2004. Human aflatoxicosis in developing countries: a review of toxicology exposure, potential health consequences, and interventions. Am. J. Clin. Nutr. 80 (5), 1106–1122.

Please cite this article in press as: F. Saladino, et al., Influence of probiotic microorganisms on aflatoxins $B_1$ and $B_2$ bioaccessibility evaluated with a simulated gastrointestinal digestion, J. Food Compos. Anal. (2017), http://dx.doi.org/10.1016/j.jfca.2017.01.010