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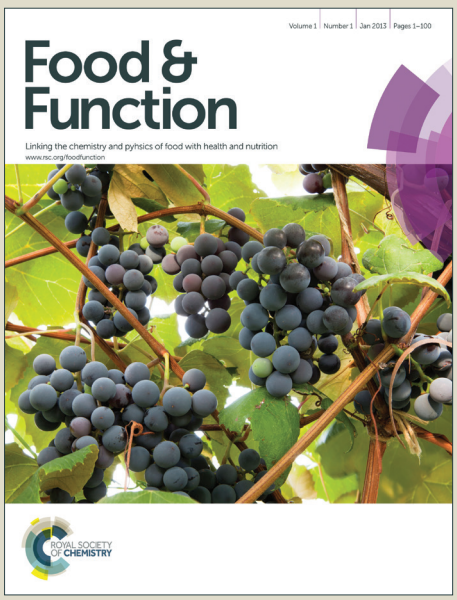

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Influence of prebiotics, probiotics and protein ingredients on mycotoxins bioaccessibility

M. Ferrer, L. Manyes, J. Mañes, G. Meca

Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

*Corresponding author: Giuseppe Meca

e-mail: giuseppe.meca@uv.es

Telephone: +34963544959

Fax: +3496354954

Abstract

The aim of this study was to investigate the influence of prebiotic compounds (cellulose and inulin), food ingredients (milk whey, β-lactoglobulin and calcium caseinate) and several probiotic microorganisms on the bioaccessibility of beauvericin (BEA), enniatins (ENs A, A1, B, B1), deoxynivalenol (DON) and zearalenone (ZEA) present in wheat crispy breads produced with wheat flour previously fermented with *F. tricinctum*, F. *culmorum* and *G. zeae*.

The bioaccessibility of mycotoxins was determined by a dynamic simulated gastrointestinal digestion system, imitating the human digestive physiological conditions until the gastroinetsinal tract. Mycotoxins were determined in the simulated intestinal fluids by liquid chromatography-tandem mass spectrometry (LC-MS/MS). ENs bioaccessibility ranged from 15.1 to 30.6%, whereas the values evidenced for the BEA ranged from 12 to 19%. DON showed bioaccessibility data ranged from 0.8 to 5.6% whereas for ZEA the data evidenced ranged from 26 to 44%. The bioaccessibility reduction evidenced using the probiotic microorganism for the mycotoxins studied ranged from 21 to 27.1% for ENs, from 29 to 39.7% for DON, from 41-57% for ZEA and from 6.6 to 10.5% for BEA.

The addition of prebiotic and bioactive microorganisms decreased the bioaccessibility of mycotoxins, with a concentration-dependent behavior, thus being a potential strategy to reduce human exposure to these minor mycotoxins.

Keywords: Mycotoxins, bioaccessibility, simulated gastrointestinal digestion, prebiotics, probiotics, LC-NS/MS.

1.0 Introduction

The Food and Agriculture Organization (FAO) estimates that as much as 25% of the world's animal feedstuff is contaminated by mycotoxins.[1] Several steps of the food production are susceptible by mold and mycotoxins contamination as: before harvesting, between harvesting and drying, and during storage. These bioactive compounds are persistent in the final products alone or in co-occurrence with other toxic compounds. [1,2] Enniatins (ENs) and beauvericin (BEA) are secondary fungal metabolites that were first isolated from *Fusarium oxysporum* by Gaumann *et al.*[3]. The natural occurrence of ENs was initially associated with plant diseases and therefore ENs were classified as phytotoxins. During the last decade, the presence of ENs, BEA and FUS in food commodities has been reported in some European countries (Finland, Norway, Spain, Slovakia, Croatia, Switzerland and Italy), USA, South Africa and Australia. Recently, our research group has reported the contamination of cereals (maize, wheat and barley) and cereal products (breakfast cereals) from Morocco and Spain.[4,5]

Deoxynivalenol (DON) is a mycotoxins mainly produced by *Fusarium graminearum* and affects animal and human health causing acute temporary nausea, vomiting, diarrhea, abdominal pain, headache, dizziness, and fever. Its worldwide surveillance confirms its occurrence in 57% of cereal samples like wheat.[2] Wheat is considered the most important cereal for human diet and European Union. According to EC 1881/2006 Regulation, the maximum levels of DON in cereal foods intended for direct human consumption is 200 and 750 $\mu g\ kg^{-1}$ for young children and adults, respectively.

Zearalenone (ZEA) is a contaminant of cereals and plant products[6] with average concentrations ranging between 5-50 $\mu g\ kg^{-1}$ and maximum concentrations from 120 to 180 $\mu g\ kg^{-1}$.[7]

The highest contamination has been detected in corn and corn products (3.1 μg kg^{-1} in Europe, 17.5 μg kg^{-1} in Africa, 9.83 μg kg^{-1} in South-America, 13.2 μg kg^{-1} in North-America, 16 μg kg^{-1} in Oceania), except for Asia, where the highest levels were found in wheat and rice (up to 600 μg kg^{-1}).[7]

Cereal-based products are a staple of the human diet but they are also susceptible to mycotoxin contamination. In particular, food- stuffs like wheat, maize, barley and cereal-based products such as breakfast cereals, bread and beer are frequently found contaminated by several mycotoxins as DON, ZEA, T-2, HT-2 and in some cases by minor *Fusarium* mycotoxins.[8] In addition, the presence of masked and parent mycotoxins in these matrices is also likely to occur, as described by several authors. [9,10,11,12]

In human health risk assessment, ingestion of food is considered a major route of exposure to many contaminants. The total amount of an ingested contaminant (intake) does not always reflect the amount that is available to the body, because only a smaller amount will be available for absorption.[13] 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.

The bioaccessibility and bioavailability of mycotoxins have been evaluated by many scientists. Avantaggiato, *et al.*[14,15] studied the intestinal absorption of zearalenone (ZEA), deoxynivalenol (DON) and nivalenol (NIV). Carolien *et al.*[16] and Kabak, *et al.*[17] described the suitability of an in vitro digestion model to measure the bioaccessibility of AFB1 and OTA from peanut slurry, buckwheat and infant formulas. Meca, *et al.*[18,19] studied the influence of different dietary fibers (used as capturing agents) on the bioaccessibility of *Fusarium* BEA and ENs.

The aim of this study was to evaluate as natural prebiotic compounds, probiotic microorganisms and also some protein ingredients reduce the bioaccessibility of the minor *Fusarium* mycotoxins ENs A, A1, B, B1, and BEA and also of the legislated mycotoxins ZEA and DON, using a dynamic *in vitro* simulated gastrointestinal digestion.

2.0 Materials and methods

2.1 Materials and reagents

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 ($CO(NH_2)_2$), Alpha-amylase (930 U/mg A3403), Hydrochloric acid (HCl), Sodium hydroxide (NaOH), Formic acid (HCOOH), Pepsin A (674 U/mg P7000), Pancreatin (762 U/mg P1750), Bile salts (B8631), Phosphate buffer saline (PBS, pH 7.5), and standard solutions of BEA, ENs (ENA, ENA_1, ENB and ENB_1), DON and ZEA were purchased from Sigma-Aldrich (Madrid, Spain). Acetonitrile, 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. The dietary fibers and the food ingredients used in this study as cellulose, inulin, milk whey, β-lactoglobulin, and calcium caseinate were gently donated by the Prof. Alberto Ritieni of the University of Naples "Federico II".

All stock solutions were prepared by dissolving 1 mg of the mycotoxins in 1 mL of pure methanol, obtaining a 1 mg/mL (1000 mg L^{-1}) solution. These stocks solutions were then diluted with pure methanol, in order to obtain suitable working solutions. All solutions were stored in darkness at 4ºC until the LC-MS/MS analysis.

2.2 Strain and culture conditions for mycotoxins production on solid wheat

Solid mediums of durum wheat were utilized in this study. The mediums were prepared weighting one kg of durum wheat in 2.5 L Erlenmeyer flasks and autoclaved at 121ºC during 20 min. The mediums were inoculated with 25 mL of a conidia suspension (10^6 conidia mL^{-1}) of *Fusarium tricinctum* CECT 20150 BEA and ENs producer, *Fusarium culmorum* CECT 2148 DON producer and with *Gibberella zeae* CECT 2150 ZEA producer.

The microorganisms were grown in a Potato dextrose broth (PDB) for BEA, ENs, DON and ZEA preinoculum. Conidial concentration was measured spectrophotometrically at 600 nm in sterile water and adjusted to 10^6 conidia/mL PDB, as reported by Kelly, *et al*.[20].

F. tricinctum, *F. culmorum* and *G.zeae* strains were obtained from the Spanish Type Culture (CECT Valencia, Spain), in sterile 18% glycerol. Fermentations were carried out at 25ºC on an orbital shaker (IKA Ks 260 basic, Staufen, Germany) in batch culture for 30 days. At the end of the fermentation, the solid cultures were autoclaved at 121ºC during 20 min to promote fungi inactivation, and the fermented cultures were finely grounded through a Oster Classic grinder (Oster, Valencia, Spain).

2.3 Bacterial strains and growth conditions

Thirteen commercial probiotic strains were used in the *in vitro* system to evaluate the capacity to degrade the mycotoxins during the simulated gastrointestinal digestion. In particular, *Lactobacillus johnsonii* CECT 289, *Lb. rhamnosus* CECT, *Lb. plantarum* CECT 220, *Lb. reuteri* CECT 725, *Lb. casei* CECT 475, *Bifidobacterium breve* CECT 4839T, *Bf. adolescentis* CECT 5781T, *Bf. bifidum* CECT 870T, *Bf. longum* CECT 4551, and, were obtained at the Spanish Type Culture Collection (CECT Valencia, Spain), in sterile 18%

glycerol. The bacterial strains were tested individually and were added in the simulated saliva before the gastric digestion step at $1.0x10^5$ UFC mL^{-1} to simulate the intake of a food enriched with probiotic microorganisms and consumed at the same time of the bioactive crispy bread produced in this study.

For longer survival and higher quantitative retrieval of the cultures, they were stored at -80ºC. When needed, recovery of strains was undertaken by two consecutive subcultures in appropriate media prior to use.[19]

2.4 Wheat crispy breads production

For the production of the wheat crispy breads with different fiber concentrations, 300 g of fermented wheat flour, 3 g of sucrose, and 6 g of NaCl, were mixed with prebiotic and food ingredients to obtain dough with 1% and 5% (w/w) of each compound employed. These mixtures were then blended with 180 mL of water during 5 min. No fermentation was done. The dough was divided into 10 g small, round portions and baked at 220ºC during 20 min.

2.5 *In vitro* dynamic digestion model

The gastrointestinal digestions in the *in vitro* dynamic model were carried out using 5 L bioreactors Infors (Bottmingen, Switzerland) (figure 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 100 rpm. Incubation temperature was maintained at 37ºC. Twenty grams of the crispy bread were mixed with 60 mL of artificial saliva (composed by: 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 pH of this solution was

taken to 6.8 with a 0.1 N NaOH solution). The mixture was placed in a plastic bag containing 1 L of water at 37ºC, homogenized with a Stomacher IUL Instruments (Barcelona, Spain) for 30 s and introduced in the fermenter vessel. Five g of pepsin (14,800 U) dissolved in 250 mL of 0.1N HCl were introduced into this mixture, through a fermenter insert. The pH of the mixture was taken to 2 with the addition of 0.5N HCl contained in a glass bottle, by means of a peristaltic pump. The incubation temperature was set at 37ºC, being transferred to the fermenter vessel through a heater plate. All the fermentation parameters were regulated through the software Iris 5.0 (Infors AG CH-4103, Bottmingen, Switzerland). The total incubation time was of 2h. An aliquot of 20 mL of gastric fluid was sampled for the determination of mycotoxinss gastric bioaccessibility.

After the gastric digestion, pancreatic digestion was simulated increasing the pH to 6.5 with $NaHCO_3$ (0.5 N), which was contained in a glass bottle and introduced in 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 in the fermenter vessel and incubated at 100 rpm at 37ºC for 2 h. An aliquot of 20 mL of the duodenal fluid was sampled for the determination of mycotoxins duodenal bioaccessibility (figure 2).[21]

2.6 Mycotoxin extraction from wheat crispy bread

A modified version of the method suggested by Chelkowski *et al*.[22] for mycotoxins extraction was used. Briefly, BEA, ENs, DON and ZEA contained in 15 g of crispy breads were extracted with 100 mL of methanol:water mixture (75:25, v/v), by using an Ika T18 basic Ultraturrax (Staufen, Germany) for 5 min. The samples were filtered through Phenomenex No. 4 filter paper (Torrance, CA, USA) and the solvent was then removed

under reduced pressure using a rotary evaporator (Buchi, Switzerland) at 30ºC and 30 mbar pressure. The extract was redissolved in 5 mL of methanol, and filtered through a 0.22 µm filter (Phenomenex) before toxin identification and quantitation by LC-MS/MS.

2.8 Mycotoxin extraction from the simulated intestinal fluids

BEA, ENs, DON and ZEA contained in gastric, gastric+duodenal, fluids were extracted as follows. 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 4000 rpm for 10 min at 4ºC. The organic phases were completely evaporated with a rotary evaporator (Buchi, Switzerland) at 30ºC and 30 mbar pressure, resuspended in 1 mL of methanol and filtered with a 0.22 µM filter (Phenomenex, Madrid, Spain) before the LC-MS/MS analysis.[21]

2.9 LC-MS/MS mycotoxins analysis

BEA, ENs, DON and ZEA separation was achieved by an Agilent 1100 LC (Agilent Technologies, Santa Clara, California) coupled to an Applied Biosystems/MDS SCIEX Q TRAP TM linear ion trap mass spectrometer (Concord, Ontario, Canada). A Kinetex C18 (50 x 2.1 mm, 2.6 µm XB, 100Å) Phenomenex (Torrance, California) column was used.

As mobile phase, 5 mM ammonium formate and 0.1% formic acid in water (A) and 5 mM ammonium formate in methanol (B) were used. The gradient was as follows: at the start 10% of solvent B and after the percentage of solvent B was linearly increased to 100% in 6 min. The percentage of solvent B was kept for 6 min. Finally, the column was equilibrated to initial conditions for 2 min. The flow rate was 500 µl min^{-1} and the injection volume was

10 µL. The instrument was set in the positive ion electrospray mode, using the following parameters: cone voltage 40 V, capillary voltage 3.80 kV, source temperature 350ºC, desolvation temperature 270ºC and collision gas energy 5 eV. Multiple reactions monitoring (MRM) technique was employed for identification and quantification. The precursor ions and the products ions used for the detection of the mycotoxins studied are shown in Table 1.[21]

2.10. Statistical analysis

Statistical analysis of data were carried out using SPSS version 13 (SPSS, Chicago, IL, USA) statistical software package. Data were expressed as mean ± SD of three independent experiments. The statistical analysis of the results was performed by student's t-test for paired samples, and significantly different from the control were expressed as, $P \leq 0.05$ (*), $P \leq 0.001$ (**), $P \leq 0.0001$ (***).

3.0 Results and discussion

3.1 Influence of prebiotics and protein ingredients on mycotoxins bioaccessibility

In table 2 are summarized the results related to the mycotoxins bioaccessibility in the crispy breads treated with different prebiotic and protein ingredients.

In particular the mean gastric and duodenal bioaccessibility data for ENs in the control experiment (crispy bread without any prebiotic or ingredients) were of 39.6 and 33.4 % respectively. Employing the insoluble dietary fibers cellulose at 1 and 5%, the mean gastric and duodenal ENs bioaccessibility ranged from 29.7 and 22.4 and from 24.4 and 18.5 % respectively, with a bioaccessibility reduction calculated respect to the control in the duodenal compartment of 26.9 (1%) and 44.7% (5%) respectively ($P \leq 0.05$ and $P \leq 0.001$).

In the crispy breads produced with inulin at 1%, the gastric and duodenal bioaccessibility of ENs were of 30.4 and 26.6% respectively, whereas using inulin at 5% concentration the bioaccessibility data observed were of 30.5 and 30.6% respectively ($P \leq 0.05$).

Using the milk whey as protein food ingredient with ENs complexing property the highest reduction of the ENs bioaccessibility was detected at 5% concentration, with mean gastric and duodenal ENs bioaccessibility of 28.4 and 21.2% respectively with a reduction compared with the control of 28.3 and 36.6% respectively ($P \leq 0.05$ and $P \leq 0.001$).

Similar results were obtained in the crispy breads treated with β-lactoglobulin at 5% concentration where the mean reductions of the ENs bioaccessibility (gastric and duodenal) compared with the control were of 30.9 and 54.8% ($P \leq 0.05$ and $P \leq 0.001$).

The introduction of the calcium caseinate in the product preparation produced important reduction of the ENs bioaccessibility. The gastric bioaccessibility ranged from 28.7 and 20.3%, whereas the duodenal bioaccessibility varied from 24.0 to 17.0%. The ENs bioaccessibility reductions showed in the duodenal compartment compared with the controls were of 25.8 and 49.1% respectively ($P \leq 0.001$).

Related to the mycotoxin DON, the gastric and duodenal bioaccessibility in the control experiments were of 12.6 and 11.0 % respectively, lower data if compared with the mean bioaccessibility detected for ENs. Using the fiber cellulose at 1 and 5% concentrations the bioaccessibility reduction of this mycotoxin was 2.0 fold highest respects to the data evidenced in the control experiment ($P \leq 0.05$). An important reduction of the DON gastric and duodenal bioaccessibility was produced using the soluble fiber inulin at 5% with data of 1.5 and 0.8% respectively, and with a reduction compared to the control of 88.1 and 92.7% ($P \leq 0.05$). Also employing milk whey and calcium caseinate at 5% concentration the reduction of DON bioaccessibility ranged from 90.0 to 92.7% ($P \leq 0.0001$).

Analyzing the data related to the mycotoxin ZEA (table 1), the gastric and duodenal bioaccessibility in the control experiment were of 54.6 and 44.3% respectively. Among the dietary fibers used the highest bioaccessibility reduction was observed using cellulose at 5% concentration obtaining gastric and duodenal bioaccessibility data of 39.8 and 29.0% respectively, with a bioaccessibility reduction compared with the control experiment of 34.6% ($P \leq 0.001$). Also an important reduction of the ZEA bioaccessibility was obtained using calcium caseinate at 5% concentration, where the gastric and duodenal bioaccessibility data were of 28.7 and 26.0% respectively, with a reduction compared with the control of 41.4% ($P \leq 0.0001$).

The mycotoxin BEA, presented data of gastric and duodenal bioaccessibility of 28.4 and 19.6% respectively. The highest reduction of the gastric and duodenal BEA bioaccessibility were detected using cellulose at 5% concentration with 15.3 and 12.0% respectively ($P \leq 0.001$), whereas using milk whey (5%) the data observed were of 18.1 and 12.1% respectively. The reductions of the BEA bioaccessibility evidenced using these two compounds were of 38.0% ($P \leq 0.001$).

The differences detected in the bioaccessibility data of the mycotoxins studied can be related to many factors:

(a) Food composition: usually the bioactive compounds mycotoxins presents in food included the toxic compounds as the mycotoxins are complexed to the food matrix. The formation of this complex is related to the amount of the micro and macronutrients contained in the food.[17] Different bioaccessibility values were also evidenced by Carolien et al.16 comparing the bioavailability of the same compounds contained in a liquid or solid food.

b) Influence of the prebiotic and ingredients used: usually the insoluble fibers have the property to form more stable complexes with the bioactive compounds in food presents included the mycotoxins reducing its bioaccessibility as demonstrated by a previous study of Mallebrera et al.[23] The fibers as the cellulose and derivate are very resistant to the digestion process mediated by the gastrointestinal enzymes reducing the possibility that the mycotoxins included in the network formed with the fiber could return to be bioaccessible.

c) Mycotoxins structure: the mycotoxins with no protein structure have a bioaccessibility lower than other compounds as the ENs that are composed by a cyclical peptides.

For the first time the bioaccessibility reduction of 7 different mycotoxins employing soluble, insoluble and protein ingredients (sequestering materials) was studied employing a simulated gastrointestinal digestion system that mimics the physiological condition of the human digestion. Anyway in the scientific literature are available articles that describe the reduction of mycotoxins bioaccessibility with sequestering materials for animal nutrition using a multimycotoxin approach. In particular Avantaggiato et al.[14] , studied the intestinal absorption of FB_1 and FB_2, OTA, DON, AFB_1 and ZEA contained in different feeds, in the presence or not of sequestering materials as aluminum silicates, actives carbons and other similar structures, using a laboratory model that mimics the metabolic processes of the gastrointestinal tract of healthy pigs. When the sequestering materials were added to the feed (control), the total intestinal absorptions of mycotoxins (corresponding to the mycotoxin amounts measured in jejunal plus ileal dialysate fluids) were 105% for FB_1, 89% for FB_2, 87% for OTA, 74% for DON, 44% for AFB_1, and 25% for ZEA, with a mean value of 70%. The adsorbent materials used by the authors, are authorized for animal nutrition and could not be employed for human nutrition. Comparing the bioaccessibility data with the values produced in our study the bioaccessibility of DON is considerably

higher and the bioaccessibility of ZEA is 2 times lower, possibly due to the difference affinity between the complexing compounds structures and the mycotoxins, compared with the compounds used in our study.

Related to the study of the minor *Fusarium* mycotoxins Bioaccessibility Manzini et al.[21], investigated the bioaccessibility of beauvericin (BEA) and enniatins (ENs) present in wheat crispy breads adding inulin and fructooligosaccharides (FOS). The bioaccessibility of mycotoxins was determined by a dynamic simulated gastrointestinal digestion system, imitating the digestive physiological conditions until the colonic compartment. BEA and ENs bioaccessibility detected in the processed samples ranged from 23 to 93%. The data obtained by the authors are comparable with those obtained in our study. Also Meca et al.[19] investigated the bioaccessibility of the ENs in wheat crispy breads produced with three different inulin concentrations (1, 5 and 10%). The mean bioaccessibility data of the four ENs (A, A_1, B and B_1) ranged from 68.67% to 84.67 in the experiments carried out without inulin, whereas the data ranged from 51.00 to 74.00% in the experiments carried out with the wheat crispy bread produced with 5 and 10% of the inulin.

Related to the bioaccessibility of the mycotoxins DON in samples destined for human nutrition, Raiola et al.[24] evaluated the contamination level of 27 samples of dried pasta for young children consumption. The samples that showed the highest amounts of the mycotoxin were cooked for 10 min, digested with an in vitro gastrointestinal protocol and bioaccessibility values were calculated. Seven of the 27 samples exceeded from 120% to 225% the legal limit of 200 µg/kg for DON fixed for processed cereal-based baby foods by a European Regulation; and the mean value of gastric bioaccessibility verified for the DON resulted of 23.1%, whereas mean duodenal bioaccessibility was 12.1%. The results

obtained by the authors are comparable with those obtained in our study and in particular in the control experiments.

3.2 Influence of probiotics microorganisms on mycotoxins bioaccessibility

In table 3 are evidenced the data related to the bioaccessibility of the mycotoxins present in the crispy breads produced with different probiotic microorganisms. The mean gastric and duodenal bioaccessibility of ENs detected in the control experiment were of 39.6 and 33.4% respectively. The strains that produced a significance reduction of the ENs bioaccessibility were *Bf. bifidum*, and *L. johnsonii*, with a reduction at duodenal level of 25.7% ($P \leq 0.001$). Using the strain of *L. johnsonii*, the gastric and duodenal ENs bioaccessibility data were of 21.2 and 27.8% respectively with a reduction of the duodenal ENs bioaccessibility respect to the control experiment of 36.6%.

Related to the mycotoxin DON, the data of the gastric and duodenal bioaccessibility evidenced in the control experiment was of 48.3 and 59.3% respectively. The strain that produced the highest reduction of the DON bioaccessibility was *L. johnsonii* with a 48.3% compared with the control experiment ($P \leq 0.0001$). The other strains tested, produced duodenal bioaccessibility data variable from 34 to 38% with reductions compared to the control ranged from 21.4 and 29.6 to %.

ZEA bioaccessibility (gastric and duodenal) calculated in the control experiment was of 60.2 and 71.9% respectively, with an important reduction expressed by *Bf. adolescentis*, (41.1% of duodenal bioaccessibility) of 31.8% ($P \leq 0.0001$). Another strain that reduce significance ZEA bioaccessibility was *L. reuteri* showing a duodenal bioaccessibility of 46.2% with a reduction compared to the control of 23.3% ($P \leq 0.0001$).

Related to the bioactive compound BEA, the highest bioaccessibility reductions were showed by *Bf. bifidum* and *L. johnsonii* strains. In particular the gastric and duodenal bioaccessibility were of 10 and 7.8% respectively, with a reduction compared to the control of 31.0 and 41.6% respectively ($P \leq 0.001$ and $P \leq 0.0001$)). The other strains used in this study did not show a significance reduction of the BEA bioaccessibility.

In the scientific literature only few studies are available on the relation between the probiotic microorganisms contained in food and the modulation of mycotoxins bioaccessibility.

In particular Kabak et al.[17] studied the release of aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) from different food products in the gastro-intestinal 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 varying binding capacity to AFB_1 and OTA depending on the bacterial strain, toxin studied, 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.

Mallebrera et al.[23] investigated the influence of several dietary fibers (galactomanan, glucomannan, citrus fiber, bamboo fiber, carrot fiber, pie fiber, b-glucan, xilan, and cellulose) and probiotic strains (*Lactobacillus animalis*, *Lb. casei*, *Lb. plantarum*, *Lb. rhuminis*, *Lb. casei casei*, *Bifidobacterium breve*, *Bf. adolescentis*, *Bf. bifidum*, *Corynebacterium vitaeruminis*, *Streptococcus faecalis*, *Eubacterium crispatus*, and *Saccharomyces cerevisiae*) on the minor *Fusarium* mycotoxin BEA bioaccessibility employing a model solution. The reduction of BEA bioaccessibility in the experiments

carried out using the prebiotic compounds ranged from 60 to 80%, whereas in the trials carried out using the probiotic strains the bioaccessibility observed ranged from 30 to 85%. A BEA degradation product produced by colonic fermentation was identified using the technique of LC–MS-LIT. The data evidenced in this study are different compared with those produced in our investigation; in particular the BEA bioaccesibility reduction is higher due that the experiments performed by the authors were carried out using a liquid model solution in which the properties of the probiotic microorganisms to ferment and metabolize the BEA are higher respect to the fermentation of a solid food used in our study.

Conclusions

The bioaccessibility of the mycotoxins BEA and ENs A, A_1, B, B_1, DON and ZEA in presence of different prebiotic, food ingredients and probiotic microorganisms was studied using a dynamic simulated gastrointestinal digestion system. The addition of the dietary fiber inulin and cellulose and of the food ingredient β-lactoglobulin to the crispy breads prepared, reduced significatively the bioaccessibility of the mycotoxins studied. The bioaccessibility of the mycotoxins ENs and ZEA ranged from 12 to 30.6%, whereas the data showed for DON ranged from 0.8 to 5.6%. The data detected for ZEA were the highest and varied from 26 to 44%. The probiotic microorganisms that produced the highest reduction of the mycotoxins bioaccessibility were *Bf. Bifidum* and *L. johnsonii* with reduction data ranged from 10.5% for BEA to 39.7% for DON.

In conclusion, several components used in this study as bioactive compounds or microorganisms were able to bind/degrade the mycotoxins up. The results help to explain how some natural components present in food can reduce the risk associated to the intake

401 of some toxic compounds presents in food, representing a potential strategy to reduce
402 human exposure to these mycotoxins.
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Legend of figures and tables

Figure 1. Figure of the dynamic lab fermenter used to simulate the physiological steps of the gastrointestinal digestion

Figura 2. Schematic representation of the *in vitro* simulated gastrointestinal digestion system used.

Table 1. Precursor ion and product ions of the mycotoxins BEA, EN A, A_1, B, B_1, DON and ZEA used for the detection of these bioactive compounds by LC/MS/MS.

Table 2. Bioaccessibility of ENs, DON, ZEA and BEA present in wheat crispy bread, produced with prebiotic and food ingredients. Significantly different from the control, $P \leq 0.05$ (*), $P \leq 0.001$ (**), $P \leq 0.0001$ (***).

Table 3. Bioaccessibility of ENs, DON, ZEA and BEA present in wheat crispy bread, produced with probiotic microorganisms. Significantly different from the control, $P \leq 0.05$ (*), $P \leq 0.001$ (**), $P \leq 0.0001$ (***).

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499 **Figure 1**.

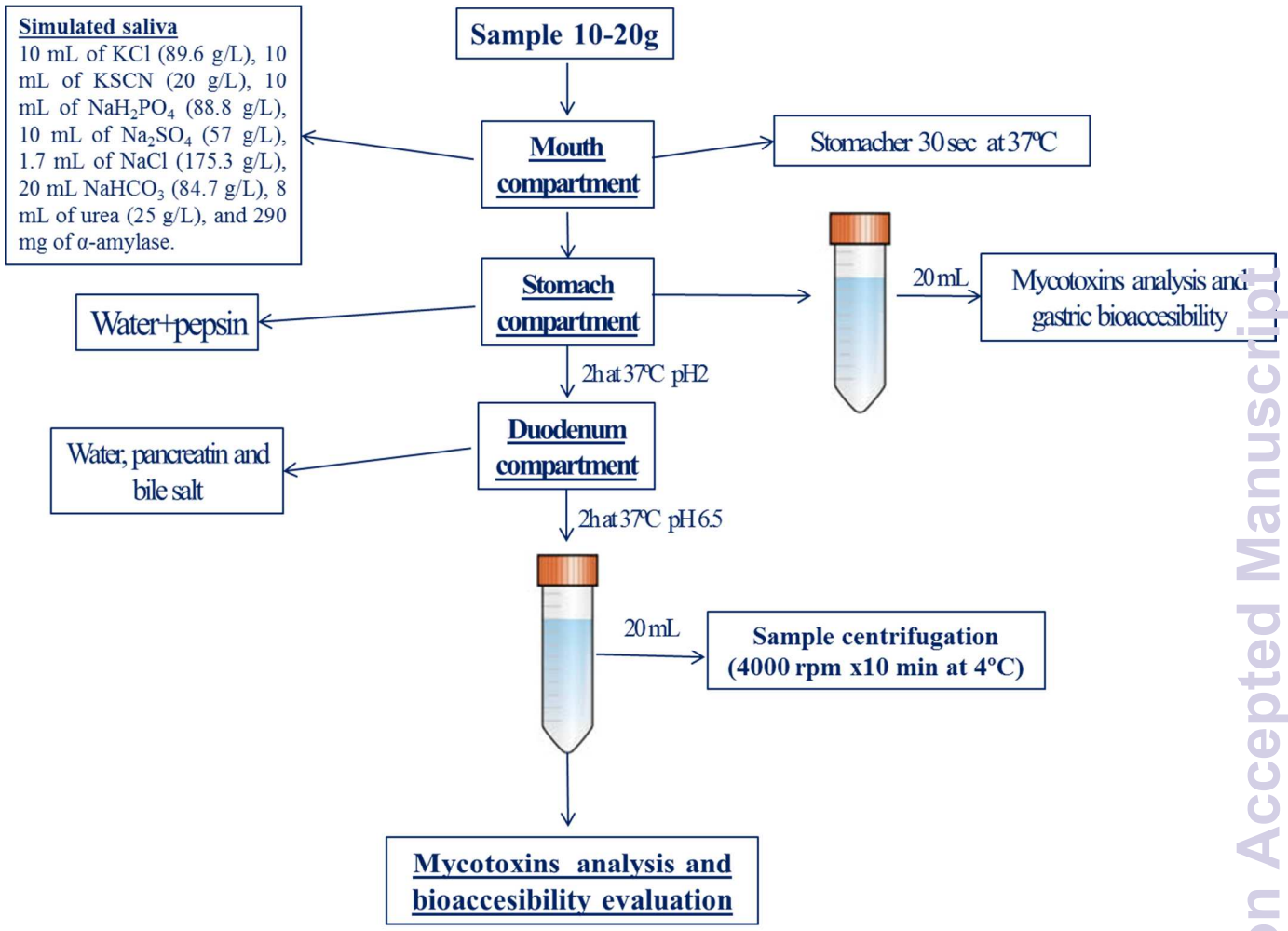

Figura 2.

521 **Table 1.**
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Mycotoxins	Precursor ion (m/z)	Product ions (m/z)
BEA	784.5 $(M+H)^+$	244.2-251.2
EN B	640.0 $(M+H)^+$	196.0-527.0
EN B_1	654.3 $(M+H)^+$	196.4-228.1
EN A	682.9 $(M+H)^+$	210.3-555.4
EN A_1	668.5 $(M+H)^+$	210.5-541.7
DON	297.0 $(M+H)^+$	175.1-115.1
ZEA	319.0 $(M+H)^+$	187.0-185.0

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Table 2.

Sample	ENs bioacc. (%)				Mean	DON bioacc. (%)	ZEA bioacc. (%)	BEA bioacc. (%)
	A	A1	B	B1				
Control								
Stomach	25.7±2.2	30.3±3.2	65.1±4.1	37.3±2.2	**39.6**	12.6±1.0	54.6±3.2	28.4±2.2
Duodenum	18.2±2.6	25.1±2.5	62.9±5.8	27.4±4.1	**33.4**	11.0±0.8	44.3±2.5	19.6±1.9
Cellulose 1%								
Stomach	8.0±0.6	28.8±2.2	64.8±4.2	17.0±1.1	**29.7***	7.1±0.4*	50.7±4.0	25.8±2.6
Duodenum	6.8±1.2	18.6±1.9	60.4±3.6	11.7±0.8	**24.4***	5.6±0.7*	44.3±3.7	19.7±2.7
Cellulose 5%								
Stomach	7.1±0.6	19.3±1.5	54.1±3.6	9.1±0.8	**22.4***	6.4±0.3*	39.8±3.5**	15.3±1.6**
Duodenum	3.2±0.2	13.9±0.4	49.3±5.1	7.5±0.4	**18.5****	5.0±0.2*	29.0±4.0**	12.0±1.9**
Inulin 1%								
Stomach	15.3±2.2	29.4±2.2	63.6±5.1	13.3±0.4	**30.4***	9.7±1.0*	53.9±2.9	27.6±2.8
Duodenum	11.4±1.1	23.1±3.1	61.1±2.2	10.8±0.8	**26.6****	1.8±0.05***	40.3±4.5	17.7±2.2
Inulin 5%								
Stomach	16.2±0.5	28.9±1.2	64.7±4.8	12.1±2.0	**30.5***	1.5±0.2***	45.2±5.0*	21.5±1.9**
Duodenum	13.4±0.6	24.0±2.0	58.6±5.1	9.3±1.6	**30.6***	0.8±0.01***	38.4±3.9*	13.2±1.0**
Milk way 1%								
Stomach	13.6±0.6	30.1±3.1	63.3±2.0	15.7±1.1	**30.7***	5.4±0.6**	52.1±4.8	26.4±1.7
Duodenum	5.6±0.4	18,8±2.0	58.9±3.1	9.4±0.6	**23.2****	1.9±0.3**	33.7±3.0**	17.3±1.5
Milk way 5%								
Stomach	11.9±1.0	29.2±2.3	57.9±3.4	14.5±2.0	**28.4***	1.0±0.1***	49.3±4.9*	18.1±1.4**
Duodenum	5.3±0.3	16.3±1.1	54.3±4.3	8.8±0.7	**21.2****	0.8±0.2***	41.6±6.1*	12.1±1.6**
β-lactoglobulin 1%								
Stomach	14.2±0.4	34.6±2.6	63.8±5.8	15.3±1.0	**32.0***	5.1±0.2**	52.3±2.9	26.3±2.8
Duodenum	6.5±0.3	17.0±0.4	59.5±4.0	9.3±0.7	**23.1****	0.9±0.07***	36.8±4.1*	16.2±1.9
β-lactoglobulin 5%								
Stomach	13.6±0.8	22.4±2.0	63.4±5.3	10.8±1.0	**27.6***	3.2±0.3**	46.4±4.6**	20.0±2.0*
Duodenum	5.4±0.2	7.0±0.5	40.9±3.9	7.1±0.5	**15.1****	2.9±0.4**	32.3±5.0**	14.9±1.6*
Calcium caseinate 1%								
Stomach	9.4±1.5	24.7±1.6	65.0±4.0	13.2±0.6	**15.8****	3.0±0.2**	45.2±3.7**	23.7±1.9*
Duodenum	9.1±1.0	19.6±1.0	54.6±5.3	12.9±1.0	**24.0****	2.8±0.3**	32.6±5.4**	17.0±1.4*
Calcium caseinate 5%								
Stomach	4.6±0.4	14.7±0.8	56.1±4.8	5.7±0.3	**20.3****	1.5±0.1***	28.7±4.1***	17.8±2.4*
Duodenum	3.0±0.1	8.7±1.1	48.3±3.8	8.0±0.9	**17.0****	1.1±0.2***	26.0±5.3***	12.1±1.3*

Table 3.

Sample	ENs bioacc. (%)				Mean	DON bioacc. (%)	ZEA bioacc. (%)	BEA bioacc. (%)
	A	A1	B	B1				
Control								
Stomach	25.7±1.5	30.3±3.5	65.1±4.1	37.3±3.5	**39.6**	59.3±4.1	71.9±4.5	15.8±1.1
Duodenum	18.2±1.8	25.1±3.8	62.9±5.0	27.4±4.3	**33.4**	48.3±5.3	60.2±5.6	11.3±0.8
Bf. longum S	12.0±1.3	31.5±2.7	62.5±5.1	11.7±1.3	**29.4****	51.2±4.6*	66.4±3.8*	14.3±2.0
Bf. longum D	8.3±0.6	26.2±2.9	60.4±5.9	11.5±1.0	**26.6****	38.7±3.8**	49.2±4.9**	10.5±1.3
Bf. bifidum S	8.4±0.9	25.4±3.4	58.0±4.6	11.1±0.9	**25.8****	50.8±3.8*	62.6±4.8*	10.0±0.7**
Bf. bifidum D	7.4±0.4	24.2±3.8	56.7±4.7	10.6±0.7	**24.8****	33.4±4.2**	47.2±6.4**	7.8±0.6**
Bf. breve S	11.7±1.6	32.1±2.9	61.6±4.6	15.2±1.6	**30.1***	48.4±4.9*	67.3±5.3	15.1±0.9
Bf. breve D	10.1±1.5	27.5±3.4	56.5±5.8	11.9±1.2	**26.5****	36.7±3.6*	57.0±3.9	10.5±1.3
Bf. adolescentis S	10.2±0.9	33.2±3.3	65.4±5.3	13.8±1.5	**30.7***	53.1±3.7*	57.2±4.6**	12.9±0.8*
*Bf. adolescentis*D	9.1±0.7	25.8±1.9	60.6±4.1	12.7±1.4	**27.1****	37.4±4.3*	41.1±5.8***	9.9±0.8*
L. rhamnosus S	12.1±1.0	31.0±4.4	63.4±4.4	15.0±1.7	**30.4***	56.9±5.0	65.3±4.9*	11.8±1.2*
L. rhamnosus D	9.3±0.8	28.5±2.2	60.3±3.8	12.7±1.3	**27.7****	34.1±4.0**	49.1±4.9**	9.3±0.8*
L. johnsonii S	11.9±1.7	29.4±3.4	60.1±6.1	9.6±0.6	**27.8****	39.6±4.9**	63.1±6.2*	10.1±1.0*
L. johnsonii D	8.0±0.9	21.5±2.8	47.1±4.3	8,3±0.7	**21.2*****	29.1±0.9***	54.2±5.7*	6.6±0.3***
L. casei S	10.4±2.2	33.9±4.1	59.7±4.2	14.8±1.0	**29.7****	40.3±2.9**	65.9±3.9	15.4±1.5
L. casei D	8.8±0.7	28.3±2.9	55.7±3.9	12.3±1.3	**26.2****	34.1±3.4**	58.3±4.9	10.3±0.4
L. plantarum S	10.4±1.5	33.8±2.8	65.2±5.4	14.9±1.3	**31.0***	51.4±5.3*	64.1±5.5	12.5±1.0*
L. plantarum D	9.2±0.6	24.2±1.7	62.5±4.9	11.1±1.2	**26.7****	39.7±2.9*	52.3±5.5*	10.8±0.6*
L. reuteri S	11.4±0.7	27.1±2.9	63.0±2.9	15.3±1.3	**29.2****	59.2±3.7*	59.8±4.9**	11.6±1.9*
L. reuteri D	8.6±0.6	22.6±3.7	61.4±4.6	12.0±1.4	**26.1****	35.9±3.7*	46.2±2.8***	9.9±0.7*

Sample	ENs bioacc. (%)				Mean	DON bioacc. (%)	ZEA bioacc. (%)	BEA bioacc. (%)
	A	A1	B	B1				
Control								
Stomach	25.7±2.2	30.3±3.2	65.1±4.1	37.3±2.2	**39.6**	12.6±1.0	54.6±3.2	28.4±2.2
Duodenum	18.2±2.6	25.1±2.5	62.9±5.8	27.4±4.1	**33.4**	11.0±0.8	44.3±2.5	19.6±1.9
Cellulose 1%								
Stomach	8.0±0.6	28.8±2.2	64.8±4.2	17.0±1.1	**29.7***	7.1±0.4*	50.7±4.0	25.8±2.6
Duodenum	6.8±1.2	18.6±1.9	60.4±3.6	11.7±0.8	**24.4***	5.6±0.7*	44.3±3.7	19.7±2.7
Cellulose 5%								
Stomach	7.1±0.6	19.3±1.5	54.1±3.6	9.1±0.8	**22.4***	6.4±0.3*	39.8±3.5**	15.3±1.6**
Duodenum	3.2±0.2	13.9±0.4	49.3±5.1	7.5±0.4	**18.5****	5.0±0.2*	29.0±4.0**	12.0±1.9**
Inulin 1%								
Stomach	15.3±2.2	29.4±2.2	63.6±5.1	13.3±0.4	**30.4***	9.7±1.0*	53.9±2.9	27.6±2.8
Duodenum	11.4±1.1	23.1±3.1	61.1±2.2	10.8±0.8	**26.6****	1.8±0.05***	40.3±4.5	17.7±2.2
Inulin 5%								
Stomach	16.2±0.5	28.9±1.2	64.7±4.8	12.1±2.0	**30.5***	1.5±0.2***	45.2±5.0*	21.5±1.9**
Duodenum	13.4±0.6	24.0±2.0	58.6±5.1	9.3±1.6	**30.6***	0.8±0.01***	38.4±3.9*	13.2±1.0**
Milk way 1%								
Stomach	13.6±0.6	30.1±3.1	63.3±2.0	15.7±1.1	**30.7***	5.4±0.6**	52.1±4.8	26.4±1.7
Duodenum	5.6±0.4	18,8±2.0	58.9±3.1	9.4±0.6	**23.2****	1.9±0.3**	33.7±3.0**	17.3±1.5
Milk way 5%								
Stomach	11.9±1.0	29.2±2.3	57.9±3.4	14.5±2.0	**28.4***	1.0±0.1***	49.3±4.9*	18.1±1.4**
Duodenum	5.3±0.3	16.3±1.1	54.3±4.3	8.8±0.7	**21.2****	0.8±0.2***	41.6±6.1*	12.1±1.6**
β-lactoglobulin 1%								
Stomach	14.2±0.4	34.6±2.6	63.8±5.8	15.3±1.0	**32.0***	5.1±0.2**	52.3±2.9	26.3±2.8
Duodenum	6.5±0.3	17.0±0.4	59.5±4.0	9.3±0.7	**23.1****	0.9±0.07***	36.8±4.1*	16.2±1.9
β-lactoglobulin 5%								
Stomach	13.6±0.8	22.4±2.0	63.4±5.3	10.8±1.0	**27.6***	3.2±0.3**	46.4±4.6**	20.0±2.0*
Duodenum	5.4±0.2	7.0±0.5	40.9±3.9	7.1±0.5	**15.1****	2.9±0.4**	32.3±5.0**	14.9±1.6*
Calcium caseinate 1%								
Stomach	9.4±1.5	24.7±1.6	65.0±4.0	13.2±0.6	**15.8****	3.0±0.2**	45.2±3.7**	23.7±1.9*
Duodenum	9.1±1.0	19.6±1.0	54.6±5.3	12.9±1.0	**24.0****	2.8±0.3**	32.6±5.4**	17.0±1.4*
Calcium caseinate 5%								
Stomach	4.6±0.4	14.7±0.8	56.1±4.8	5.7±0.3	**20.3****	1.5±0.1***	28.7±4.1***	17.8±2.4*
Duodenum	3.0±0.1	8.7±1.1	48.3±3.8	8.0±0.9	**17.0****	1.1±0.2***	26.0±5.3***	12.1±1.3*