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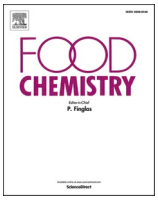

Role of red beetroot in bread for reducing mycotoxin risks: Bioavailability of beetroot polyphenols and betalains with ochratoxin a, aflatoxin B1 and zearalenone in Caco-2 cells

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ABSTRACT

The interaction between dietary bioactive compounds and mycotoxins in food safety is crucial due to the potential health risks raised by mycotoxins and the protective functions of bioactive substances. This study is focused on red beetroot (*Beta vulgaris*), a rich source of polyphenols and betalains, incorporated into a daily consumption food such as bread, to examine its effects on the bioavailability of mycotoxins using an *in vitro* Caco-2 cell model. This study investigates how these compounds affect the bioavailability of mycotoxins, specifically ochratoxin A (OTA), aflatoxin B1 (AFB1), and zearalenone (ZEA), which are known to compromise intestinal barrier function and nutrient absorption. Additionally, bioaccesibility and bioavailability of total betalains (betacyanins and betaxanthins) (TBC) and polyphenols (TPC) content was evaluated. The beetroot-enriched breads were subjected to an *in vitro* digestion process, followed by a transepithelial transport assay to assess the bioavailability in differentiated Caco-2 cells. Results indicate an increase in the bioaccesibility of TBC and TPC (up to 99 % and 27 %, respectively) during digestion, suggesting enhanced absorption and protective effects against mycotoxin-induced damage. The presence of beetroot bread polyphenols and betalains increased the bioavailability of mycotoxins, with complex interactions observed, particularly in triple mycotoxin's combination. The results highlight the complex interactions between dietary components and mycotoxins' bioavailability, underlining the importance of further research into their mechanisms of action and potential applications in food safety and nutrition.

1. Introduction

Food safety, particularly concerning the presence of toxic compounds like mycotoxins, is paramount for public health due to the potential adverse consequences upon exposure. Mycotoxins represent a significant risk and are produced by contaminating fungi in agricultural crops and in stored foods (Owolabi, Karoonuthaisiri, Elliott, & Petchkongkaew, 2023).

Currently, research spotlighting bioactive compounds in food, such as polyphenols, has expanded owing to their health-promoting properties (Llorens-Castelló et al., 2023; Pepe et al., 2015). Polyphenols, ubiquitously found in various foods, including fruits and vegetables, have garnered attention for their antioxidant, anti-inflammatory, and anticarcinogenic activities, among other bioactive properties (Yalcin & Çapar, 2017). It has been linked to positive effects in preventing chronic diseases and mitigating toxic effects of various substances (Elshama, Abdalla, & Mohamed, 2018; Liliana & Oana-Viorela, 2020). For example, it has been demonstrated that curcumin, a natural polyphenol, can inhibit the activation of key inflammatory pathways and reduce oxidative stress, thereby mitigating the toxic effects of various harmful agents (Aggarwal & Harikumar, 2009).

Red beetroot (*Beta vulgaris*) is a rich source of polyphenols, including flavonoids and phenolic compounds and mainly of betalains. These compounds have been linked to positive effects in preventing chronic

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diseases and mitigating toxic effects of various substances in the body (Costa et al., 2017; Liliana & Oana-Viorela, 2020; Murakami, 2014). In the realm of food toxicology, exploring whether beetroot polyphenols can attenuate the deleterious effects of mycotoxins, produced by contaminating fungi in food, is of interest. While polyphenols have demonstrated antioxidant and anti-inflammatory properties, suggesting a role in cellular protection against oxidative stress and mycotoxin-induced inflammation (Obafemi, Adedara, & Rocha, 2023; Penalva-Olcina, Juan, Fernández-Franzón, & Juan-García, 2022; Yalcin & Çapar, 2017), the interaction of beetroot polyphenols with mycotoxins, particularly in the intestinal tract, remains an underexplored domain.

Mycotoxins such as fumonisins (Yu, Zou, Zhao, & Zhu, 2023), ochratoxin A (Yoon, Shin, Park, Lee, & Lee, 2023) and aflatoxin B1 (Pan et al., 2024) can inflict damage to the intestinal epithelium and alter barrier function, thereby compromising nutrient absorption and gastrointestinal system integrity (Gonkowski, Gajęcka, & Makowska, 2020). The complexities of the interaction and bioavailability of beetroot polyphenols and mycotoxins within the gastrointestinal tract, especially in the context of these adverse effects, warrant further investigation. However, this interaction has been studied with other bioactive compounds, such as polyphenols or carotenoids from natural products (Juan et al., 2020), coffee products (Angeloni et al., 2021; Juan-García, Caprioli, Sagratini, Mañes, & Juan, 2021), *Lycium barbarum L.* (Juan-García, Montesano, Mañes, & Juan, 2019; Montesano, Juan-García, Mañes, & Juan, 2020) and garlic (Agahi, Penalva-Olcina, Font, & Juan-García, 2022). The three most detected and reported mycotoxins in the recent years by the EU 'Rapid Alert System or Food and Feed' (RASFF) are ochratoxin A (OTA), aflatoxins (AFs) and zearalenone (ZEA) and the most often detected and also more toxic is AFB1, a carcinogenic substance (IARC's group 1). Then, due to the diverse matrices where they can be present, it is possible to ingest them from different source at long of the day. Cereals can be the best matrix where mycotoxigenic fungi can grow and produce mycotoxins, and bread is the most consumed cereal product. Its elaboration permits to include not only flour but other ingredients such as seeds, raises, fiber or bioactive compounds.

Although the reciprocal effects of phenolic compounds and mycotoxins on each other's bioavailability are of interest; it is important to consider that the impact of mycotoxins on the uptake of phenolic compounds is also a significant area of investigation. Mycotoxins are known to compromise cellular functions, which could influence the absorption and effectiveness of beneficial dietary compounds. Understanding these interactions could delve into the specific mechanisms by which mycotoxins affect the transport and efficacy of phenolic compounds, potentially involving detailed analyses of membrane protein interactions and cellular uptake pathways which could be considered in the future.

Recent studies have shed light on the bioavailability of specific polyphenols and their protective roles in cellular models. For instance, polyphenols derived from Korean mint demonstrated notable bioavailability in both human plasma and Caco-2 cell monolayers, highlighting their potential as health-beneficial compounds (Lee et al., 2023). Furthermore, research involving the T-2 toxin in cereals and its impact on Caco-2 cell viability revealed that tyrosol, could potentially mitigate the toxic effects produced by T-2 exposure under certain conditions (Martínez-Alonso, Taroncher, Rodríguez-Carrasco, & Ruiz, 2023).

This study aims to investigate the interplay between dietary polyphenols and betalains found in red beetroot in homemade bread, and three mycotoxins (OTA, AFB1 and ZEA) in the context of their bioavailability, utilizing the Caco-2 cell model. Derived from a human colon adenocarcinoma cell line, this model, upon differentiation, closely resembles the intestinal epithelium, complete with relevant enzymes and transporters for the absorption and metabolism of nutrients and bioactive compounds. The beetroot-enriched breads were subjected to an *in vitro* digestion process. The focus is on evaluating the impact of beet polyphenols and betalains on the bioavailability of mycotoxins under various experimental conditions, including their individual presence and their combinations. Additionally, the study seeks to elucidate how these bioactive compounds from red beetroot-enriched breads are released from the food matrix and made available for absorption in the gastrointestinal tract (bioaccesibility), providing a comprehensive understanding of their potential protective roles against mycotoxin exposure.

2. Material and methods

2.1. Sample preparation

Two beetroot by-product-enriched breads were prepared in the laboratory according to the recipe proposed by Llorens, Juan, Montesano, Mañes, & Juan-García, 2022, with slight modifications by adding dehydrated beetroot by-product provided by Agrosingularity S.L (Murcia, Spain) and to obtain 5 % and 10 % beetroot by-product-enriched breads (BB5% and BB10%). Identification and characterization of betalains from the beetroot by-product was also reported in that publication (Llorens-Castelló, Juan-García, Moltó-Cortés, Mañes-Vinuesa, & Juan-García, 2022). Fig. 1a and Fig. 1b reports chemical structure of principal betalains and polyphenols in beetroot by-products according to the chemical characterization previously done and reported by Llorens-Castelló et al. (2022) and Platosz, Sawicki, and Wiczkowski (2020). After mixing all the ingredients, doughs were homogenized manually, covered with a damp cloth and left 1 h to ferment at room temperature. After that, breads were baked at 200 °C for 30 min. Finally, breads were cooled at room temperature (1 h). Bread was cut in slices and in crumbs and kept cold before its use.

2.2. Chemicals and Reagents

All chemicals for the preparation of simulated digestion fluids: potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), sodium hydrogen carbonate ($NaHCO_3$), sodium chloride (NaCl), magnesium dichloride ($MgCl_2(H_2O)_6$), ammonium carbonate ($(NH_4)_2CO_3$), calcium chloride ($CaCl_2$), hydrogen chloride (HCl) and sodium hydroxide (NaOH) were from Merck (Darmstadt, Germany). α-amylase (930 U mg^{-1} A3403), pepsin from porcine gastric mucosa (674 U mg^{-1} P7000), pancreatin from porcine pancreas (762 U mg^{-1} P1750) and bile extract porcine (B8631) were purchased from Merck LifeScience S.L.U. (Madrid, Spain).

Folin-Ciocalteu's phenol reagent was purchased from Sigma-Aldrich (St. Louis, MO, USA). Gallic acid (3,4,5-Trihydroxybenzoic acid) ($C_7H_6O_5$) was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Sodium carbonate anhydrous, sodium hydrogen carbonate, sodium hydroxide and hydrochloric acid were from Merck KGaA (Darmstadt, Germany).

The chemical reagents and cell culture components used were Dulbecco's Modified Eagle's Medium (DMEM) (DMEM (1×) + GlutaMAX with 4.5 g/L D-Glucose and Pyruvate), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), penicillin, streptomycin, trypsin/EDTA solutions, phosphate buffer saline (PBS), Dimethyl sulfoxide (DMSO) were acquired from Thermo Fisher Scientific (MA, USA).

OTA, AFB1 and ZEA standard solutions (purity >99 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fig. 1c reports the chemical structure of mycotoxins studied. Stock solutions of OTA, AFB1 and ZEA were prepared in DMSO at appropriate working concentrations and maintained in the dark at −20 °C.

2.3. In vitro static digestion

In vitro digestion of breads enriched with beetroot (5 % and 10 %) was carried out in a static system according to the INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014) by triplicate simulation ($n = 3$). The simulation of gastrointestinal digestion entails the oral, gastric

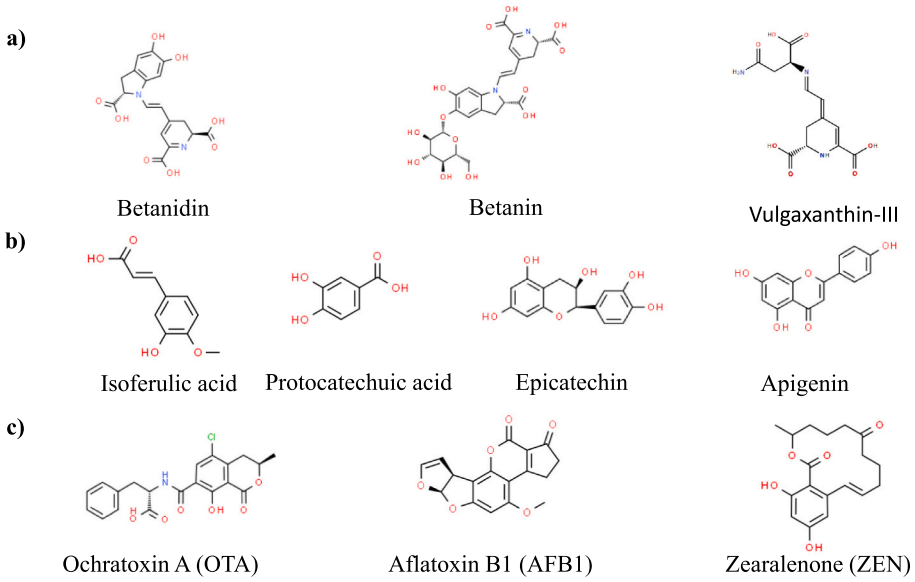

Fig. 1. Chemical structure of principal betalains (a) and polyphenols (b) present in beetroot extracts and mycotoxins studied (c).

and small intestine stages, and relies on the simulation of the conditions of pH, enzyme and bile concentrations, temperature, agitation and duration, among other variables. The quantities of sample and volumes used were previously estimated with the template for the harmonized *in vitro* digestion method from COST Infogest (http://www.proteomics.ch/IVD/). The formulated digestive fluids formulated for each phase comprise a mixture of electrolytes and enzymes which are specific to their respective stages. The simulated saliva (SSF) (pH 7), gastric (SGF) (pH 3) and intestinal (SIF) (pH 7) fluids were prepared fresh daily from stock solutions and the enzymatic activity of the enzymes was tested before each experiment (Brodkorb et al., 2019Minekus et al., 2014).

The digestion was carried out as follows: 2 mL of SSF at 37 °C were added to beetroot bread sample (2.5 g) to obtain a final volume ratio of 1:1 (*v*/v) and shaken manually 1 min, then $CaCl_2$ and α-amylase (75 U mL-1 per sample) was added. Volume was adjusted to 5 mL with distilled water. It was incubated for approximately 2 min at 37C°. For the gastric step, 4 mL of FGS were added and manually shaken for 1 min. $CaCl_2$ and pepsin (2000 U mL-1 per sample) were added and shaken 1 min. pH was adjusted to 3 with HCl 6 M. Distilled water was added to achieve a final volume of 10 mL and this mixture was incubated 2 h at 37C° in a bath with continuous agitation. Then, 4,5 mL of SIF were added, shaken 1 min, and $CaCl_2$, pancreatin (100 U mL-1 per sample) and bile salts (0.1 M per sample) were added and shaken 1 min. To inactivate enzyme activity pH was adjusted to 7 with NaOH 1 M and distilled water was added to obtain a final volume of 20 mL. This mixture was incubated 2 h at 37 °C in a bath with continuous agitation. Aliquots were extracted and subsequently placed into an ice bath. The separation of the liquid fraction from the residual undigested solids was achieved through centrifugation at 4000 rpm (5810R, Eppendorf, Hamburg, Germany) for 30 min at 4 °C to obtain the supernatant.

An aliquot from each digestion phases was collected to determine polyphenol and betalains from enriched bread and estimate the bioaccessibility value.

2.4. Bioavailability study

2.4.1. Cell culture

Human colon adenocarcinoma (Caco-2) cells were employed to evaluate bioavailability of OTA, AFB1, ZEA, polyphenols and betalains. To culture the Caco-2 cells, the method described by Juan et al., 2022, was followed. The cells were maintained in DMEM medium supplemented with 10 % fetal bovine serum (FBS), 1 % (*v*/v) HEPES buffer, 1 % (v/v) of NEAA, 100 U mL^{-1} penicillin, 100 U mL^{-1} streptomycin, and fungizone 0.1 %. It was incubated at 37 °C under 5 % CO_2 and 95 % air atmosphere at constant humidity. The medium was replaced every two days, and the cells were split with a trypsin-EDTA solution twice per week for cell counting and subculture. Cells were subculture routinely with only a small number of sub-passages (<20 subcultures) in order to maintain the genetic homogeneity.

2.4.2. Transepithelial transport assay

The transepithelial transport was evaluated to determine the bioavailability of the different compounds studied (OTA at 20 μM, AFB1 at 40 μM, ZEA at 20 μM, polyphenols and betalains) by triplicate ($n = 3$). It was conducted using differentiated Caco-2 cells, following the method described by Juan et al., 2022, with some modifications. Caco-2 cells have the ability to differentiate into an enterocyte-like phenotype, providing a valuable model for the human intestinal barrier. This characteristic makes them an ideal choice for investigating the transepithelial transport of nutrients and bioactive compounds, such as polyphenols, betalains and mycotoxins, from food sources, an objective of our study.

Specifically, Caco-2 cells were seeded at a density of 3.15×10^5 cells/well into 6-well transwell permeable inserts plates. With a diameter of 24 mm and a pore size of 0.4 mm (Corning, NY, USA). The medium was replaced every 2 days to facilitate cell growth, and transepithelial electrical resistance (TEER) was measured after each medium change. After 14 days, the Caco-2 cells were fully differentiated, with TEER values higher than 400 Ω/cm^2. On day 14, the apical (upper compartment) and basolateral (lower compartment) medium were removed. Differentiated Caco-2 cell monolayers were subjected to two gentle rinses using phosphate-buffered saline (PBS), and different conditions were assessed: Single, double and triple combinations of mycotoxins (20 μM of OTA and 40 μM for AFB1 and ZEA), both individually and concurrently in bread and red beetroot-enriched bread (10 % and 5 %) were performed.

The apical compartment was loaded with transport medium HBSS supplemented with HEPES (1 %) and the correspondent condition (1:1) (*v*/v) (1.5 mL), while fresh HBSS:HEPES was introduced into the basolateral compartment (1.5 mL). After 1 h, 2 h, and 3 h of incubation, the basolateral compartments were collected. In the fourth hour, both the apical and basolateral compartments were separately collected to evaluate the transepithelial transport of phenolic compounds, betalains and mycotoxins in beetroot-enriched breads. The cells were recovered from

the transwell supports, trypsin-EDTA was added and manually detached using a scraper. Each collected aliquot was stored at −20 °C for subsequent analysis.

2.5. Evaluation of bioavailable compounds

To assess the bioavailability percentage of mycotoxins, betalains and polyphenols, both the apical and basal compartments were analyzed. The calculation was devised to reflect the proportion of the total detected in the basolateral compartment in relation to the initial apical content, subsequently multiplied by 100 to express it as a percentage.

$$\text{Bioavailability (\%)} = \frac{\textit{Basal content}}{\textit{Initial apical content}} \times 100$$

2.6. Determination of mycotoxins

The quantification of mycotoxins in 5–10 % red beetroot-enriched bread was carried out using an Agilent 1290 Infinity UHPLC system interfaced with a 6460 Triple Quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was achieved on a 100 mm × 2.1 mm (ID), 2.7 μm, Agilent InfinityLab Poroshell 120 EC-C18 column, maintained at a constant temperature of 30 °C. The mobile phase consisted of water with 0.1 % formic acid and 5 mM ammonium formate (solvent A), and methanol with 0.1 % formic acid and 5 mM ammonium formate (solvent B). A 15-min gradient elution was employed at a flow rate of 0.3 mL/min. The gradient program was as follows: equilibration for 1 min at 70 % A, a linear gradient to 10 % A over 10 min, held at 10 % A for 2 min, returned to 70 % A in 0.5 min, and then re-equilibrated at 70 % A for 4.5 min. The injection volume was set to 3 μL.

Mass spectrometric detection was performed using an Agilent 6460 Triple Quadrupole equipped with an electrospray ionization (ESI) source utilizing Agilent Jet Stream Technology. The ESI source parameters were optimized and set to: sheath gas temperature at 340 °C, sheath gas flow at 10 L/min, drying gas temperature at 200 °C, gas flow at 8 L/min, nebulizer pressure at 40 psi, capillary voltage at 3500 V, and nozzle voltage at 500 V. The mass spectrometer was operated in dynamic multiple reaction monitoring (dMRM) mode, monitoring two specific mass transitions per toxin for accurate quantification and confirmation. The fragment voltage (FV), cell accelerator voltage (CAV), and collision energy (CE) for each mycotoxin were meticulously optimized using MassHunter Optimizer software, with the CAV parameter uniformly set at 7.

The analytical method was rigorously validated on beetroot breads to ensure its reliability, focusing on key analytical parameters. This included evaluating the linearity of response, assessing potential matrix interferences, and determining the method's sensitivity, specifically its limits of detection and quantification.

2.7. Total phenolic content (TPC)

The spectrophotometric determination of the total phenolic content (TPC) in digestion aliquots and samples from the bioavailability assay was performed in accordance with Singleton & Rossi, 1965, with slight modifications[12]. Specifically, Folin and Ciocalteu's reagent and water (1:5) were utilized, as well as 20 % Na_2CO_3 and. After 1 h of reaction, preserved from light, the absorbance was measured at $\lambda_{max} = 765$ nm (Synergy H1 Microplate reader, Bio Tek).

To create a calibration curve, gallic acid solutions of known concentrations were employed. Aliquots of the salival, gastric, and intestinal phases of the *in vitro* digestion and from the different hours (1, 2, 3 and 4 h) and compartments (apical and basolateral) of the bioavailability assay were taken and subjected to TPC analysis using the Folin-Ciocalteu method. Measurements were repeated three times for each sample. The precision of the measurements was assessed by calculating the standard deviation and the coefficient of variation.

2.8. Total Betalain Content (TBC)

To determine the total betalain content (TBC) of aliquots taken during the digestion phases, a spectrophotometric method was employed. This method involved measuring the absorbance at two different wavelengths, namely 470 nm for betaxanthins and 535 nm for betacyanins. In order to achieve optimal absorption values of $0.8 \leq A \leq 1.0$ at their respective absorption maxima, the samples were diluted with either distilled water or HBSS. Subsequently, the content of betacyanin and betaxanthin was calculated according to the formula (Stintzing et al., 2005):

$$BC\left(\frac{mg}{L}\right) = \frac{A \cdot DF \cdot MW \cdot 1000}{\varepsilon \cdot L}$$

In the aforementioned formula, the variable "A" denotes the absorption values of betacyanin and betaxanthin measured at 470 and 535 nm, respectively. "DF" represents the dilution factor of the samples, while "MW" stands for the molecular weight of the compounds (550.5 g/mol for betacyanin and 358.3 g/mol for betaxanthin). The parameter "L" denotes the path-length in the spectrophotometer that depends on the volume used, measured in centimeters, and "ε" represents the extinction coefficient of the compounds ($60{,}000\,L\,mol^{-1}\,cm^{-1}$ at $\lambda = 535$ nm for betacyanin and $48{,}000\,L\,mol^{-1}\,cm^{-1}$ at $\lambda = 470$ nm for betaxanthin).

Duplicate measurements were conducted using a UV–visible spectrophotometer Synergy H1 Microplate reader, Bio Tek). The precision of the measurements was assessed by calculating the standard deviation and the coefficient of variation.

2.9. Statistical analysis

Experiments were executed in duplicate, and the results were expressed as mean ± standard deviation (SD). The data were analyzed using the SPSS software (Version 28; IBM Chicago, IL, USA). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons to determine the significant differences between: betalains/poplyphenols and digestion phases in different breads and TPC and TBC in combinations of mycotoxins. Bars labeled with different letters represent means that are statistically different at the *0.05* significance level by One-way ANOVA. By student's *t*-test for independent samples were performed to determine differences between BB5%, BB10% and control in the TPC, TBC and mycotoxin bioavailability. A difference between means was considered significant if $p\text{-value} \leq 0.05$.

3. Results & discussion

3.1. Bioaccessibility and bioavailability of polyphenols

3.1.1. Bioaccessibility

Understanding the bioaccessibility of polyphenols is fundamental to optimizing their beneficial health effects, as it determines the degree to which these bioactive compounds can be released from the food matrix and absorbed into the bloodstream. The bioaccessibility of total polyphenols in studied red beetroot-enriched breads (BB5% and BB10%) across oral, gastric, and intestinal phases was evaluated and reported in Fig. 2. The values are expressed in percentage (%) because it is compared the initial quantity of TPC (mg gallic acid/g extract) and the quantity observed in each phase (oral, gastric and intestinal). The study revealed a notable difference in bioaccessibility between beetroot-enriched breads, showing higher polyphenol bioaccessibility in BB10% (27 % ± 1.5 in the intestinal phase).

The bioaccessibility of total polyphenols shows a significant increase from the oral to the intestinal phase (Fig. 2), but with significant differences between BB5% and BB10% in the gastric and intestinal phase.

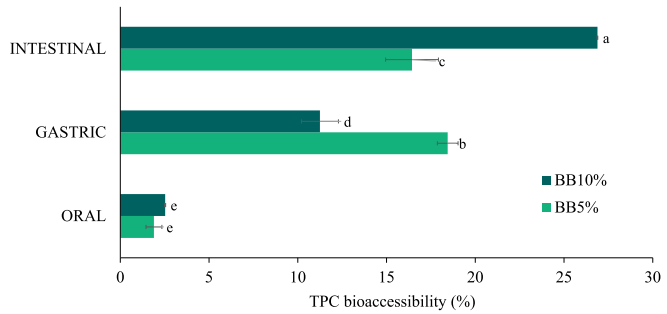

Fig. 2. Bioaccesibility of total polyphenol content (TPC, %) in intestinal, gastric and oral phases during the digestion process of BB5% and BB10 % breads. Letters in bars correspond to differences among groups (<0.05).

The higher increase in BB10% during the intestinal phase suggests that a higher concentration of red beetroot may enhance the stability and absorption of polyphenols (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013). The observed increase aligns with other study that evaluated the TPC in fresh beetroot (Wang et al., 2020). They observed an increase during the gastric phase, as in the case of BB5%, and a decrease in the intestinal phase. Furthermore, they observed a reduction in the intestinal phase. This reduction, as in the case of BB5%, may be due to the interaction with enzymes such as α-amylase. On the other hand, in the gastric phase, the release of polyphenols increases due to the action of enzymes and the reduction in pH, which release phenolic compounds that were linked to carbohydrates (Cassani, Gerbino, Moreira, & Gomez-Zavaglia, 2018). Moreover, the interaction of phenolic compounds with sugars and various dietary components throughout the digestion process may modify the concentration of these compounds. This change impacts their solubility and the likelihood of their absorption into the body (Ortega, Macià, Romero, Reguant, & Motilva, 2011).

3.1.2. *Bioavailability*

Different analyses were conducted to assess the bioavailability of mycotoxins (OTA, AFB1 and ZEA) and bioactive compounds (polyphenols and betalains) through transepithelial transport. The used concentrations of OTA (20 μM), AFB1 (40 μM) and ZEA (40 μM) were according to previous studies, Minekus et al., 2014; Cano-Sancho, González-Arias, Ramos, Sanchis, & Fernández-Cruz, 2015, which stablished these concentrations below the DL_{50}. These values that are below the IC_{50} as previously established in our lab and also coinciding with studies that have reported IC_{50} values in Caco-2 cells for these three mycotoxins (Berger et al., 2003; Ji et al., 2019). Furthermore, in our previous study it was evaluated the bioaccessibility of fortified bread with all three mycotoxins with all the identification and characterization of betalatins from the beetroot by-product (Llorens-Castelló et al., 2022) and the following values were observed: 16 ± 2 % for AFB1, 16 ± 1 % for OTA and 26 ± 4 % for ZEA when fortified at 100 ng/g (a concentration according the recently occurrence review of mycotoxins in food commodities reported by Tran, Viktorová, & Ruml, 2020. Bibliography was revised on previous studies which investigated the bioavailability of these three mycotoxins by Caco-2 cells, the ranges reported are 1–100 μM for OTA, 1–25 μM for AFB1 and 25 μM for ZEA. Hence, with this study it is trying to complete this information and known if it is possible to modulate mycotoxin bioavailability individually or combined with other components or food with biofunctional activity.

Bioavailability, results are reported in Fig. 3 and the purpose is to understand their absorption and potential bioefficacy once ingested. Polyphenols and betalains are known for their antioxidant and anti-inflammatory properties, contributing to the prevention of chronic diseases. By evaluating their bioavailability, the aim is to elucidate how effectively these compounds can be absorbed by the intestinal epithelium and become available for physiological functions. This knowledge is important for the development of functional foods and nutraceuticals designed to deliver health benefits by enhancing the bioaccessibility and bioavailability of these beneficial antioxidant compounds. Moreover, the interplay with the mycotoxins results of interest when combined. The objective is to discern the absorption dynamics and potential interactions *in vitro* between these bioactive and toxic compounds within the gastrointestinal tract. Understanding the transepithelial transport of polyphenols and betalains and polyphenols is vital for assessing their protective roles against oxidative stress and inflammation. Additionally, evaluating the transport of mycotoxins is essential for risk assessment and the development of dietary strategies to mitigate their absorption and adverse health effects. The experiment aimed to evaluate the impact of various parameters on betalain and polyphenol concentrations in beetroot-enriched breads. The parameters included the percentage of beetroot by-product (BB5 % and BB10 %) and the presence of different mycotoxins— OTA (20 μM), AFB1 (40 μM), and ZEA (40 μM) (Fig. 3). Furthermore, combinations of these mycotoxins were also assessed (Fig. 4).

The basal polyphenol concentrations in BB5 % ranged from 4.63 % to 17.46 % across different hours, being the third hour the one with highest values, while the bioavailability was 51.6 ± 0.5 % in the BB5 % (Fig. 3). The bioavailability was higher in the presence of AFB1 (66.5 ± 0.8 %), followed by OTA (60 ± 1.2 %) showing significant differences between BB5% and BB10 % in all cases. The presence of ZEA, seemed to reduce

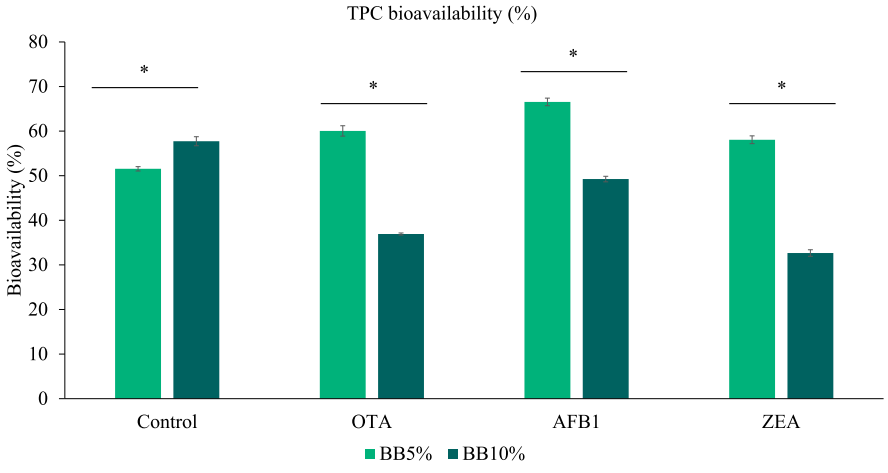

Fig. 3. Influence of OTA (20 μM), AFB1 (40 μM) and ZEA (40 μM) in the bioavailability of TPC in BB5% and BB10 % breads. *Denotes significant differences between BB5% and BB10 % ($p < 0.05$).

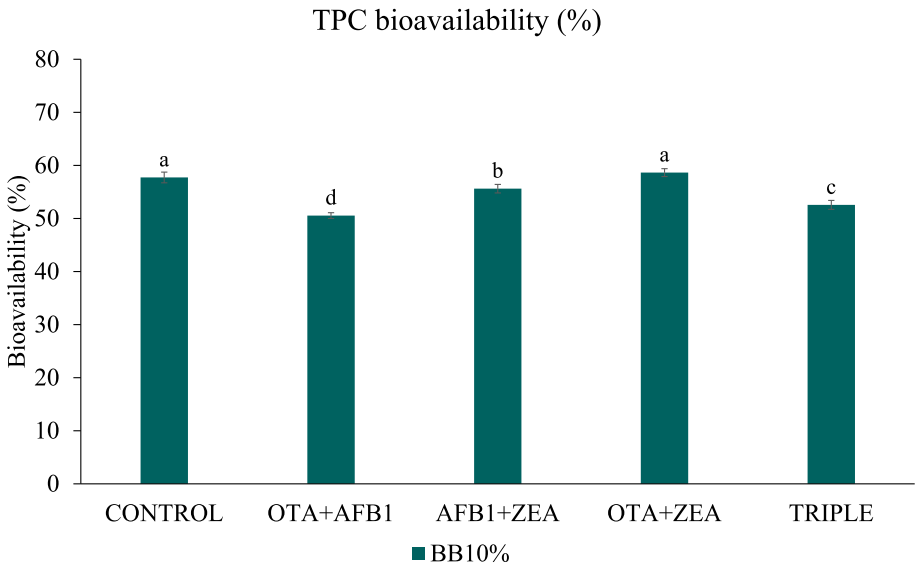

Fig. 4. Influence of mycotoxin combinations (binary and triple at OTA (20 μM), AFB1 (40 μM) and ZEA (40 μM)) in the bioavailability of TPC in 10 % enriched beetroot bread (BB10 %). Letters in bars correspond to differences among groups (*<0.05*).

the bioavailability (32.7 ± 0.7 %), suggesting a potential antagonistic interaction affecting the permeability or stability of the polyphenols. Significant differences (*p-value < 0.05*) were observed in polyphenols concentrations when dual and triple combinations of mycotoxins were present showing higher bioavailability in the OTA + ZEA combination (58.6 ± 0.7 %), followed by AFB1 + ZEA (55.6 ± 0.8 %) and OTA + AFB1 (50.5 ± 0.5%) (Fig. 4). While the polyphenol bioavailability in the control BB10% was 57.7 ± 1.0%. The presence of the triple mycotoxin combination (OTA + AFB1 + ZEA) resulted in lower polyphenols' bioavailability compared to the control, with values around 52.6 ± 1.3 %.

Mycotoxins like OTA, AFB1, and ZEA are known to compromise the integrity of the intestinal barrier, and their biotransformation could influence the bioavailability of co-occurring compounds (Noonong et al., 2022). The bioavailability of polyphenols and their interactions with mycotoxins in Caco-2 cells is a complex area requiring further research. Prior research has demonstrated variability in the bioavailability of polyphenols (5–25 %) and their inhibitory effects on certain enzymes and transporters in Caco-2 cells (Di Domenico et al., 2020; Kan et al., 2021; Konishi, Kobayashi, & Shimizu, 2003).

In this study, a noteworthy level of bioavailability was observed for the polyphenols present in beetroot-enriched breads, indicating that these compounds have the capability to traverse the Caco-2 layer with a high permeability. The results of this study are slightly higher than those reported by Di Domenico et al. (2020), which reached levels around 25 % for determinate polyphenols, and those obtained by Konishi et al., 2003 for *p*-coumaric acid and gallic acid (~11 %).

3.2. Bioaccessibility and bioavailability of betalains

3.2.1. Bioaccessibility

The bioaccessibility of two groups of betalains (betacyanins and betaxanthins) in red beetroot-enriched breads (BB5% and BB10%) across oral, gastric, and intestinal phases were evaluated. This provides insight into the digestive stability and potential for absorption of these compounds. In general, the bioaccessibility of betalains was higher than that of total polyphenols (99 % and 55 % for each beetroot-enriched bread).

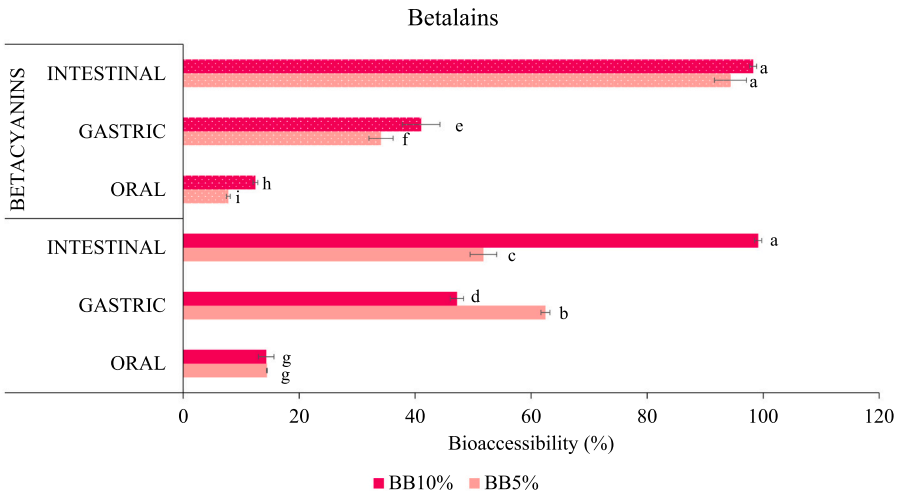

Fig. 5. Bioaccessibility (%) of betalains (betacyanins and betaxanthins) in in intestinal, gastric and oral phases during the digestion process of BB5% and BB10 % breads. Letters in bars correspond to differences among groups (*<0.05*).

The bioaccessibility of betacyanins (Fig. 5) significantly increases from oral to intestinal phase in both BB5% and BB10% and it was was above 98 % in both types of breads with no significant differences between them. This suggests that betacyanins are relatively stable during the digestive process, especially in higher concentrations of red beetroot. The significant increase in the intestinal phase indicates that betacyanins are likely absorbed more efficiently in the latter stages of digestion. Moreover, it has been shown the stability and enhanced absorption of anthocyanins and ellagitannins in the small intestine (González-Barrio, Edwards, & Crozier, 2011; Tesoriere et al., 2012).

The bioaccessibility of betaxanthins, showed a bioaccesibility in the BB10% intestinal phase of 99 ± 0.6 %. In contrast BB5%, showed significantly different values (*p-value* < *0.05*) with 52 ± 2.2 %. While there was an increase from the oral to the gastric phase, the intestinal phase for BB5% shows a significant decrease, contrary to BB10%, where it significantly increases to nearly 100 %. This discrepancy could be due to the matrix effect of the bread, which may influence the release and subsequent absorption of betaxanthins (Tesoriere, Fazzari, Angileri, Gentile, & Maria, 2008).

An earlier study by Llorens-Castelló et al., 2022 focused specifically on the bioaccessibility of betanin, betanidin, and vulgaxanthin-III, rather than a broad range of betalains reporting the identification and characterization of betalains´ profile from the beetroot-by-product. This distinction is essential as betalains constitute a diverse group of compounds with varying stabilities, and not all betalains may behave similarly during digestion. Indeed, the decreased bioaccessibility of the three specific betalains studied aligns with the notion that certain betalains might be more susceptible to degradation throughout the digestive process (Beisl et al., 2021). Furthermore, the present study, which aimed to include the assessment of TBC, in contrast to the limited scope of the previous study, underlines our objective to provide a more complete understanding of the bioaccessibility of total betalains.

The observed increase in bioaccessibility of betalains in beetroot-enriched breads during digestion agrees with Wang et al. (2020) that in the bioaccessibility of total betalains, they obtained higher values than for polyphenols, reaching 50 % in the oral phase and close to 100 % in the gastric phase, although they observed a decrease in the intestinal phase. On the other hand, in the gastric phase, the release of betalains increases due to the action of enzymes and the reduction in pH, which release phenolic compounds that were linked to carbohydrates, as in the case of polyphenols.[17]

3.2.2. *Bioavailability*

The determination of TBC was performed in the samples collected from the transepithelial assay. For breads BB5% and BB10%, the betalain bioavailability varied over time. For betaxanthins, the BB5% showed absorption values ranging from 12.37 % to 13.21 % during the 4-h assay, whereas, for the BB10%, it had values between 10.06 % and 14.59 %. For betacyanins, the BB5 % exhibited bioavailabilities ranging between 10.63 % to 12.24 %, while the BB10%, ranged from 8.44 % to 14.92 %.

Then, the total observed bioavailability was very similar with no significant differences in both prepared breads obtaining of 46 ± 0.7 % and 45 ± 0.7 % for betacyanins in BB5 % and BB10%, respectively (Fig. 6a); and 51 ± 0.4 and 48 ± 1.9 % for betaxanthins in BB5% and BB10%, respectively (Fig. 6b).

Regarding betacyanin bioavailability in presence of mycotoxins, the bioavailability increased with the single mycotoxins (OTA, AFB1 and ZEA) in BB10% up to 49 ± 2.2 % (Fig. 6a) with significant differences between BB5% and BB10% in the case of OTA and AFB1.

When considering binary combinations of mycotoxins (OTA + AFB1, AFB1 + ZEA, OTA + ZEA) along with BB10% (Fig. 7), an increase of bioavailability was observed for the OTA + AFB1 combination (47 ± 0.3 %) and OTA + ZEA (46 ± 0.2 %). On the other hand, OTA + ZEA and AFB1 + ZEA, bioavailability decreased compared to ZEA alone with BB10%, from 49 ± 0.4 % to 46 ± 0.2 % (in OTA + ZEA) and to 45 ± 0.3 % (in AFB1 + ZEA). The lower bioavailability in BB5% with triple mycotoxins could be indicative of an antagonistic interaction between the compounds, affecting the betalain levels. This phenomenon aligns with the findings of Beisl et al. (2021), who reported altered intestinal barrier function and biotransformation in the presence of mycotoxin mixtures, indicating that mycotoxin interactions can significantly impact bioactive compound bioavailability.

Regarding betaxanthins, they exhibited the same trend as betacyanins when mycotoxins were present. Their bioavailability in BB5% was slightly but significantly higher than BB10% for the majority of cases where mycotoxins were present, except for ZEA (Fig. 6 b). When considering betaxanthin bioavailability in dual and triple combinations of mycotoxins in BB10% (Fig. 7), an increase of bioavailability was observed for all double combinations compared to control, especially the OTA + AFB1 combination (53 ± 0.3 %) in comparison with the 47 ± 0.1 % and 50 ± 0.2 % of single OTA and AFB1. The triple combination exhibited highest bioavailability of betaxanthins in BB10% (55 ± 2.5% for BB10%).

In the context of mycotoxin interaction with bioactive compounds, a study by Martínez-Alonso et al. (2023) explored the bioaccessible fraction of T-2 toxin and its effect on the viability of Caco-2 cells in the presence of tyrosol, a phenol. This study agrees with our findings where

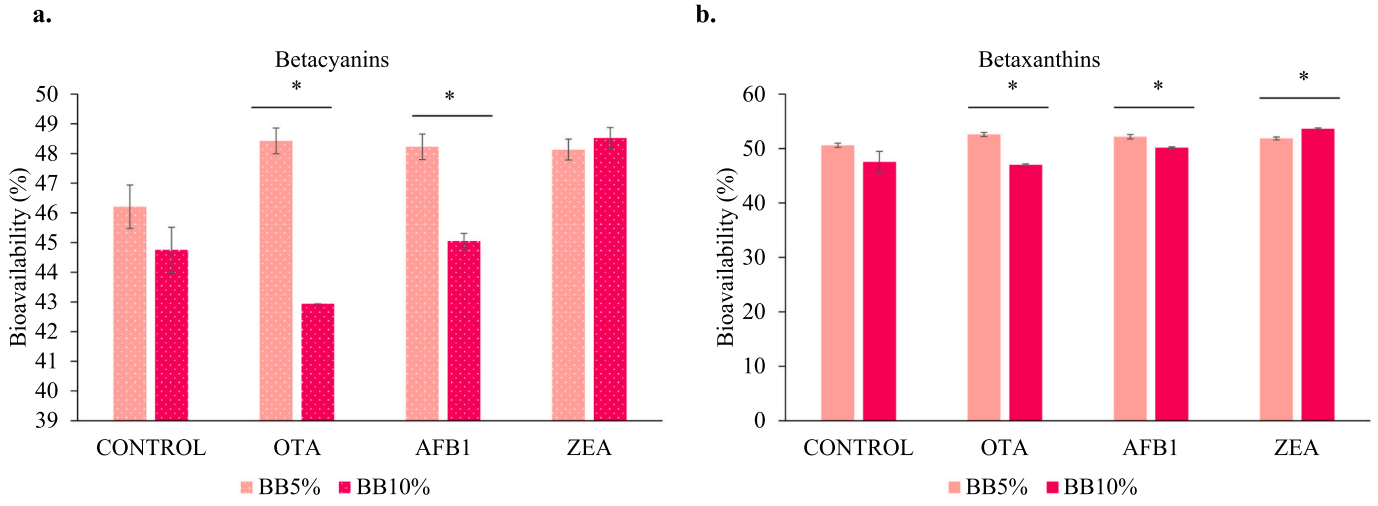

Fig. 6. Influence of OTA (20 μM), AFB1 (40 μM) and ZEA (40 μM) in the bioavailability of betacyanins (a) and betaxanthin (b) in BB5% and BB10% breads. *Denotes significant differences between BB5% and BB10% ($p < 0.05$).

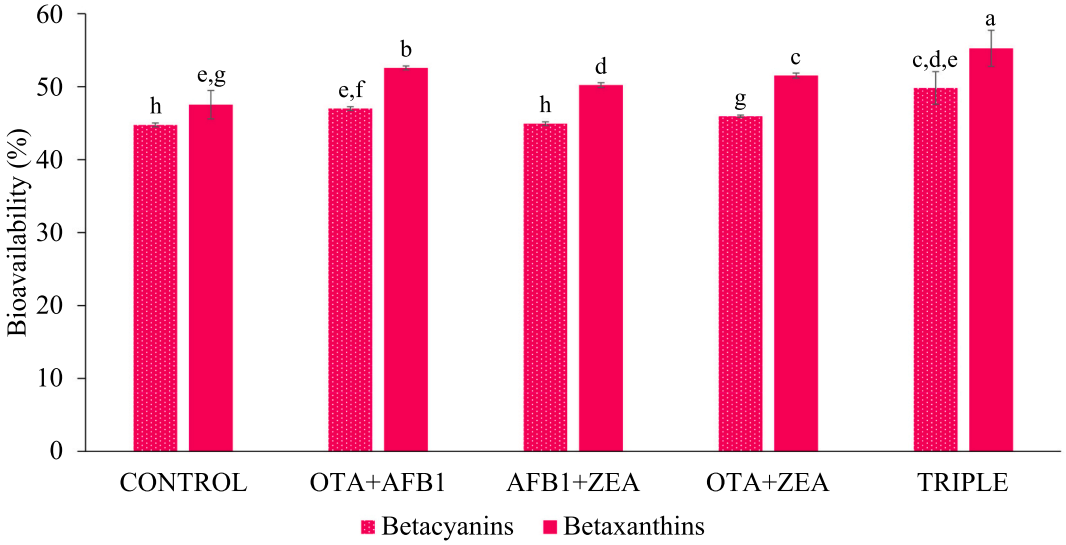

Fig. 7. Influence of the combinations of mycotoxins (binary and triple at 20 μM for OTA and 40 μM for AFB1 and ZEA) in the bioavailability of betacyanins and betaxanthins in 10% enriched beetroot bread (BB10%).Letters in bars correspond to differences among groups (*<0.05*).

the presence of multiple mycotoxins seemed to influence the betalain concentrations, suggesting a possible interaction between the mycotoxins and the betalains.

Interestingly, the BB10% did not consistently exhibit higher betalain bioavailability compared to the BB5%. This could be attributed to a complex interplay of factors such as the cellular uptake mechanisms and the intrinsic stability of betalains under different mycotoxin influences, as also suggested by Saber, Abedimanesh, Somi, Khosroushahi, & Moradi, 2023, who studied the antiproliferative and apoptotic effects of red beetroot and betanin on human colorectal cancer cell lines. The presence of single mycotoxins, especially in BB10 %, appeared to lower the betacyanin bioavailability. This could be indicative of an antagonistic interaction between the mycotoxins and betalains, possibly affecting the stability and bioavailability of the betalains within the Caco-2 cellular environment. Moreover, a study by Tran et al., 2020, emphasized the significance of assessing the bioavailability and biotransformation of mycotoxins in understanding their *in vivo* cytotoxicity. The authors utilized Caco-2 cells to evaluate the permeability, intestinal transport, and metabolism of mycotoxins, highlighting the utility of this cellular model in bioavailability assessments.

3.3. Bioavailability of mycotoxins

3.3.1. Bioavailability of OTA

The bioavailability of OTA exhibited a significant increase across (*p-value < 0.05*) all tested conditions in control and BB10 % (Fig. 8a). Notably, a significant rise in bioavailability was observed in the triple combination, where it increased from 17.7 ± 1.8 % to 90.9 ± 1.1 %. In other conditions, the bioavailability of OTA remained below 16 % in all instances. This pattern highlights the strong impact of interactions between mycotoxins and food matrix components on OTA bioavailability, especially in the case of more complex mixtures.

3.3.2. Bioavailability of AFB1

The study revealed distinct variations in the bioavailability of AFB1 with BB10% digest. When AFB1 was present alone, its bioavailability was measured at 0.13 ± 0.04 % (Fig. 8b). This contrasted markedly with its bioavailability in double (14.5 ± 0.5–18.5 ± 2.8 %) and triple combinations (95.7 ± 0.9 %). Furthermore, the introduction of beetroot bread digest significantly influenced AFB1 bioavailability across all combinations. In the presence of this digest, bioavailability increased to 5.9 ± 3 % for AFB1 alone, ranged from 23.8 ± 3.3–29.8 ± 0.6 % in

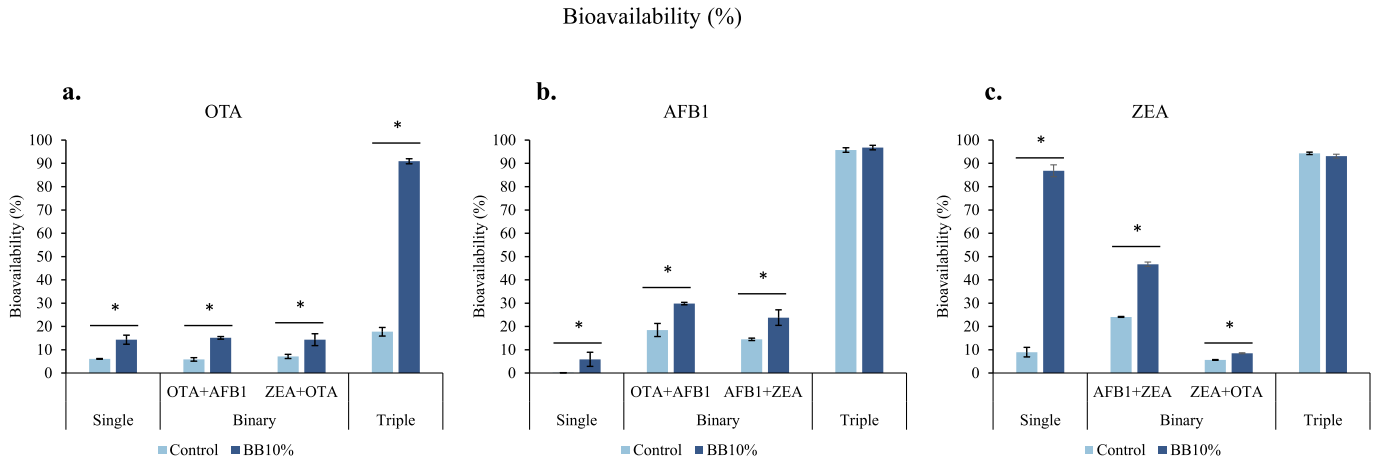

Fig. 8. Bioavailability of studied mycotoxins (20 μM of OTA, 40 μM for AFB1 and ZEA) and their combinations (binary and triple) in 10% enriched beetroot bread (BB10%). *Denotes significant differences between control and BB10% (*p < 0.05*).

double combinations, and reached 96.8 ± 1 % in the triple combination with no significant differences in the latter combination compared to the control. This phenomenon is attributable to the interaction of beetroot bread components, such as fibers and complex carbohydrates, with AFB1, potentially enhancing its release and absorption.

3.3.3. Bioavailability of ZEA

ZEA displayed an increase in bioavailability when present alone (Fig. 8c), significantly increasing from 8.9 ± 2 % to 86.8 ± 2.6 % with the addition of 10% beetroot bread. In double combinations, the bioavailability varied, showing 24 ± 0.2–46.7 ± 0.9 % for the AFB1 + ZEA combination and 5.6 ± 0.2–8.5 % for the ZEA + OTA combination in the control and BB10%, respectively. However, in the triple combination, there was a slight, but not statistically significant decrease in bioavailability from 94.3 ± 0.5 % to 93.1 ± 0.8 %. This data suggests a complex interaction between ZEA and other mycotoxins or food matrix components, influencing its bioavailability.

These results demonstrate a significant influence of other compounds on the bioavailability of the studied mycotoxins. This observation is critical, as it highlights either synergistic or antagonistic interactions of mycotoxins with other substances. In BB10% there was an increase in the bioavailability. This increase can be attributed to molecular interactions that alter the solubility, stability, or metabolism of the mycotoxin, thereby facilitating its absorption. These findings align with previous studies suggesting that the interaction of mycotoxins with other compounds can modify their toxicokinetic profile (Lo Dico, Croubels, Carcelén, & Haranczyk, 2022).

In addition, it has been suggested that polyphenols and betalains may interfere with the absorption and bioavailability of mycotoxins in the gastrointestinal tract, decreasing their potential toxicity (Ling, Wan, El-Nezami, & Wang, 2016). Nevertheless, it is important to note that the interaction between beet polyphenols and mycotoxins can be complex and may depend on several factors, such as the concentration and type of polyphenols and mycotoxins, as well as specific experimental and biological conditions. Therefore, further research is needed to further understand the underlying mechanisms and the efficacy of beet polyphenols in mitigating the toxic effects of mycotoxins.

This study shows that the bioactive compounds in beetroot-enriched bread (TBC and TPC) increase both their absorption and the bioavailability of mycotoxins in Caco-2 cells. This indicates a dual interaction: while these compounds may protect against mycotoxin-induced damage, they could also enhance the bioavailability of the mycotoxins. In an *in vivo* context, this duality could have significant implications. The increased bioavailability of mycotoxins could potentially heighten their toxicity, although the presence of TBC and TPC might partially mitigate these effects through their antioxidant and anti-inflammatory properties. However, the complexity of metabolic interactions and biotransformation within a whole organism could result in a dynamic that differs from that observed *in vitro*. According to this, it can be suggested that, *in vivo*, the protective capacity of bioactive compounds against damage might depend on several factors, including dosage, food matrix, gut microbiota, and the efficiency of hepatic detoxification mechanisms. To better understand these interactions, future studies should focus on *in vivo* research evaluating the bioavailability and toxic effects of mycotoxins in the presence of beetroot-enriched foods. Animal models, or eventually clinical studies, could provide data on how these interactions affect mycotoxin toxicity and the effectiveness of bioactive compounds in protecting against their effects.

4. Conclusions

The findings from this study provide valuable insights into the bioavailability and bioaccessibility of mycotoxins (OTA at 20 μM, AFB1 and ZEA at 40 μM) and bioactive compounds, as well as for, polyphenols and betalains in beetroot-enriched breads. The increase in the bioavailability of AFB1, ZEA, and OTA in the presence of red beetroot-enriched bread suggests that dietary components found in red beetroot, can significantly influence the solubility and absorption of these mycotoxins, especially in triple combinations of mycotoxins. This highlights the potential synergistic or antagonistic interactions that can occur between different mycotoxins and dietary constituents.

The study also sheds light on the increased bioavailability of polyphenols and betalains, which could lead to protective effects of these compounds against the detrimental effects of mycotoxins. Moreover, the investigation into the TPC and TBC in beetroot-enriched breads highlighted the impact of red beetroot concentration and the presence of mycotoxins on the bioavailability of these compounds. The higher bioavailability observed in BB10% bread suggests that increasing the concentration of red beetroot may enhance the stability and absorption of polyphenols and betalains during digestion. However, the results also highlight the need for further research to understand this interaction.

Finally, a more detailed study of the interaction between foodstuffs and mycotoxins is needed in order to provide appropriate dietary guidelines.

CRediT authorship contribution statement

Llorens Paula: Investigation, Formal analysis, Data curation. **Juan-García Ana:** Writing – review & editing, Visualization, Project administration, Funding acquisition. **Pakkanen Hannu:** Validation, Methodology, Data curation. **Moltó Juan Carlos:** Supervision. **Vehniäinen Eeva-Riikka:** Validation, Supervision, Methodology, Data curation. **Juan Cristina:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The authors do not have permission to share data.

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