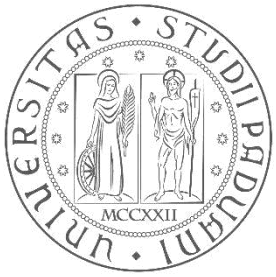

UNIVERSITÀ DEGLI STUDI DI PADOVA

Department of Land, Environment Agriculture and Forestry

Second Cycle Degree (MSc) in

Food and Health

Effectiveness of mineral adsorbents in reducing mycotoxins in chicken feed

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Preface

This thesis is the result of research conducted entirely at the Universitat de València, Spain. The work was carried out as part of a collaborative agreement between the Università degli Studi di Padova and the Universitat de València within the framework of Erasmus+ program.

I would like to express my sincere gratitude to the Universitat de València for providing me with the opportunity to conduct my research in its facilities and for the invaluable resources, support, and guidance offered throughout my stay. Special thanks go to María José and Cristina, for their mentorship and collaboration.

This experience has greatly enriched my academic and personal growth, allowing me to deepen my knowledge in the field of toxicology and to benefit from an international academic environment.

Finally, I extend my gratitude to Mauro Dacasto, for facilitating this research opportunity and for the continued support during this journey.

Elisa
Padua, 06/12/2024

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ABBREVIATIONS

AFB1: Aflatoxin B1

AFB2: Aflatoxin B2

AFG1: Aflatoxin G1

AFG2: Aflatoxin G2

AFM1: Aflatoxin M1

AFM2: Aflatoxin M2

AFs: Aflatoxins

$BMDL_{10}$: Benchmark dose level 10

DON: Deoxynivalenol

DNA: Deoxyribonucleic acid

EFSA: European Food Safety Authority (EFSA)

EU: European Union

FAO: Food and Agriculture Organization of the United Nations

FB1: Fumonisin B1

HACCP: Hazard analysis and critical control points

HBVG: Health based guidance values

HSCAS: Calcium sodium hydrate aluminosilicates

IARC: International Agency for Research on Cancer

LAB: Lactic Acid Bacteria

LD_{50}: Lethal dose 50

NOEL: No observed effect level

OTA: Ochratoxin A

PMTDI: Provisional maximum tolerable daily intake

PTWI: Provisional tolerable weekly intake

RASFF: Rapid Alert System for Feed and Food

RNA: Ribonucleic acid

SGF: Simulated gastric fluid

SIF: Simulated intestinal fluid

SSF: Simulated salivary fluid

TDI: Tolerable daily intake

ZEA: Zearalenone

ABSTRACT

The bioaccessibility of mycotoxin is an important factor that determines the possible toxicology risk in animals and human health. This term describes the fraction of a contaminant that is released from the food matrix during gastrointestinal digestion and is subsequently available for intestinal adsorption. In this work, the bioacessibility of the most relevant mycotoxins in chicken feed (AFB1, OTA, ZEA, DON, FB1) was investigated with INFOGEST method, to determine the mitigation capacity of asorbents.

Five different adsorbents (A1, A2, A3, A4, A5) were added to the chicken feed, previously fortified with the five mentioned mycotoxins, in order to determine and the concentration of them in each phase (oral, gastrointestinal, and intestinal).

The results showed that for mycotoxin AFB1, the most effective adsorbents with higher reduction percentages were A4 (95.3% ± 9.6 – 91.2% ± 7.9 – 49.2% ± 5.6) and A5 (95.8% ± 7.3 – 95.6% ± 8.7 – 82.0% ± 8.0) for oral, gastric and intestinal phases, respectively.

For OTA the most effective adsorbents were A1 (3.1% ± 0.8 – 32.5% ± 4.5) and A2 (1.6% ± 0.4 – 31.4% ± 5.6) for the oral and gastric phases, respectively.

The adsorbents that reduce DON in all the phases were A1, A2 and A3. For mycotoxin FB1, A2 was the most effective to reduce the mycotoxin concentration in all the three phases (82.1% ± 7.9 - 30.5% ± 6.1 – 72.2% ± 7.5, respectively) but the highest reduction rates were given by A3 and A4 in the oral phase. For the mycotoxin ZEN, A2 was the only adsorbent capable to reduce it in all the three phases, with the highest percentages (23.3 ± 2.5 – 23.0 ± 4.8 – 29.9 ± 4.1, respectively).

In conclusion, all the mineral adsorbents tested showed a mitigation capacity in reducing mycotoxins in chicken feed, but to reduce all the mycotoxins tested in chicken feed, a mixture of A2 and A4 adsorbents would be appropriate.

1. INTRODUCTION

1.1 Mycotoxins and Climate Change

Mycotoxins are becoming an increasing global problem due to Climate Change, causing a high economic impact, risks to food security, and a danger to human and animal health (Pappas *et al*, 2014; Casu *et al*, 2024). Mycotoxins transfer from animal feed to animal products, such as milk, animal tissues and eggs, providing a secondary route of human exposure through the consumption of animal products (Chhaya *et al*, 2022).

The health implications for animals exposed to mycotoxins include reduced feed intake, reduced productivity, fertility problems and reduced metabolic activities and growth rate, while humans may experience several health problems, including liver cancer, growth retardation, estrogenic problems, reduced immunity, and kidney problems (Chhaya *et al*, 2022). FAO estimates that 25% of agricultural crops are contaminated by mycotoxins every year (Mohammadi & Venkitasamy, 2023).

As can be seen in Figure 1, environmental factors including rainfall, temperature, humidity, and cultivation, harvesting, and storage practices, affect fungal proliferation and mycotoxin formation at any stage of crop production (Jallow *et al*, 2021).

Agricultural crops stored at temperatures above 20 degrees, humidity above 14% and with water activity between 0.80 and 0.82 are more sensitive to fungal growth, and therefore contamination of mycotoxins (Mohammadi & Venkitasamy, 2023).

Mycotoxin-producing fungi can contaminate crops both in the field, due to the significant presence in the field of pathogens, and after harvest, during storage, due to late drying. Mycotoxins can remain in the crop for a long time after the disappearance of fungi, so the absence of fungi does not guarantee the absence of mycotoxin contamination, just as the presence of fungi does not mean that there is contamination by mycotoxins. (Mohammadi & Venkitasamy, 2023).

Consequently, the increasing temperatures, the lower availability of water, the elevated frequency of fires, extreme rainfalls, and drought, potentially produced by Climate Change may induce a shift in the geographical distribution of mycotoxigenic fungi and the pattern of mycotoxin occurrence (Casu *et al.* 2024; Zingales *et al.*, 2022).

Currently the different mycotoxins and mycotoxin-producing fungi are found abundantly in certain geographical territories, such as in warm and humid areas of Central and South America, Africa, Middle East and South Asia where there have been

frequent cases of contamination of aflatoxins on food products such as corn, peanuts and spices (Chhaya *et al*, 2022).

Mycotoxins in food and feed is a serious problem throughout the food chain, from producers, processors, and consumers, and their presence is very difficult to avoid, in particular due to extreme environmental conditions, causing damage to both the economy and public health (Mohammadi & Venkitasamy, 2023).

The 2019 Rapid Alert System for Feed and Food (RASFF) report in Europe found that mycotoxins were among the top 10 reported hazards with foods from both EU and non-EU countries (Chhaya *et al*, 2022 – RASFF, 2019).

A report from the European Food Safety Authority (EFSA) described an emerging problem of contamination of corn, pistachio, and almond with aflatoxins in southern Europe due to the rise in average temperatures, identifying the Mediterranean areas as the most susceptible point to extreme climatic events, thus making it easier for aflatoxin contamination (Moretti *et al*, 2019 - EFSA, 2007).

The Figure 1 shows the intrinsic and extrinsic factors that affect the level of mycotoxins in the final product.

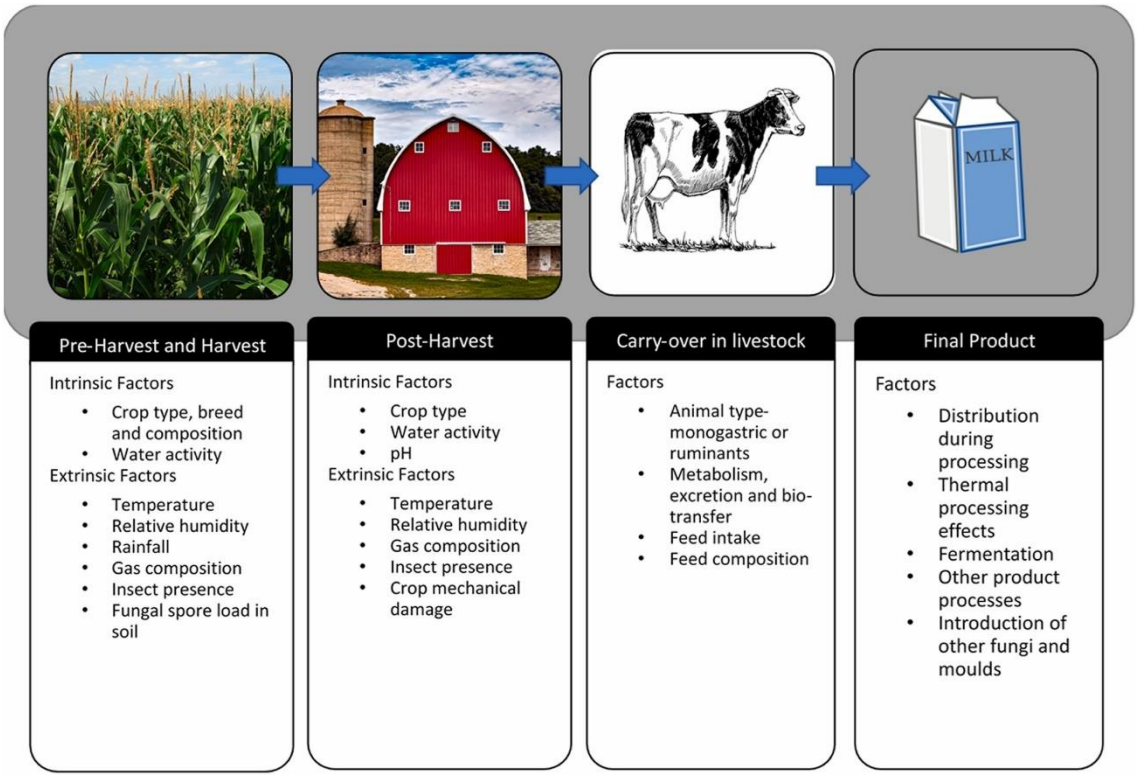


Figure 2. Intrinsic and extrinsic factors influencing mycotoxin level in final product. *Reference: Chhaya et al, 2022*.

1.2 Principal feed mycotoxins

Mycotoxins are non-enzymatic metabolites, dangerous for organisms, produced by some species of fungi such as *Aspergillus* spp., *Penicillium* spp., or *Fusarium* spp. (Mohammadi & Venkitasamy, 2023).

Aspergillus and *Penicillium* species, for instance, frequently develop on food and feed during storage, while *Fusarium* spp. often infect growing crops in the field such as wheat, barley, and corn (Alshannaq & Yu, 2017). Aflatoxin B1 (AFB1), Deoxynivalenol (DON), Zearalenone (ZEA), Ochratoxin A (OTA) and Fumonisin B1 (FB1) are considered the most relevant mycotoxins both from the economic point of view and for their prevalence and Figure 2 shows the chemical structure of the main mycotoxins in feed.

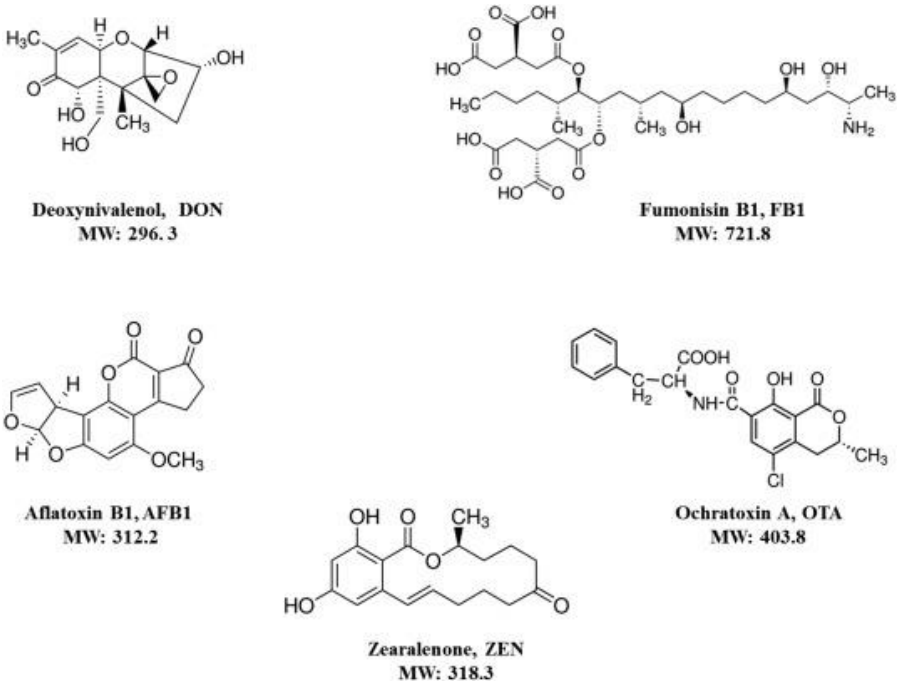


Figure 2. Chemical structures of major mycotoxins in animal feed. *References: Vila-Donat P. et al, 2018.*

The Tables 1 and 2 show a summary of the most relevant mycotoxins that produce a risk on livestock, their commodity, climate occurrence, regulation guidance (Tab.1) and the main toxicological effects that mycotoxins pose to mammals (Tab. 2).

Table 5: The major mycotoxins in food and feed, the main commodity affected, climate occurrence and European Union guidance in feed. *Reference: Chhaya et al, 2022. https://doi.org/10.1016/j.tifs.2021.07.040*

Mycotoxin	Fungi	Commodity	Climate occurrence	Regulation / Guidance (EU) in feed
Aflatoxin B1, B2, G1, G2	*Aspergillus flavus, Aspergillus parasiticus*	Maize, nuts, rice, wheat	Temperate, tropical and sub-tropical	AFB_1 in all feed materials with moisture content of 12%: 0.02 mg kg^{-1}
Ochratoxin A	*Aspergillus ochraceus, Aspergillus carbonarius, Penicillum verrucosum*	Cereals, coffee, wine, Infant Formula	Cool- temperate to tropical regions	Feed materials with 12% moisture content in cereals and cereal products: 0.25 mg kg^{-1} Compound feed for poultry: 0.1 mg kg^{-1}
Fumonisin	*Fusarium verticillioides, Fusarium proliferatum*	Maize, wheat	Hot- temperate regions, ubiquitous	Maize and maize products: 60 mg kg^{-1}
Zearalenone	*Fusarium graminearum, Fusarium culmorum*	Maize, wheat, oats	Northern temperate regions	Guidance value in cereal and cereal products except maize by-products with moisture content of 12%: 2 mg kg^{-1} Maize by-products: 3 mg kg^{-1}
Deoxynivalenol	*Fusarium graminearum, Fusarium culmorum, Fusarium acuminatum*	Maize, wheat, oats	Northern temperate regions	Guidance value in cereal and cereal products except maize by-products with moisture content of 12%: 8 mg kg^{-1} Maize by-products: 12 mg kg^{-1} Compound feed: 5 mg kg^{-1}

AFB_1 = Aflatoxin B1

Table 6: The major mycotoxins in feed, toxic effects, classification by IARC, Health Based Guidance Values. *Reference: Chhaya et al, 2022. https://doi.org/10.1016/j.tifs.2021.07.040*

Mycotoxin	IARC classification	Toxic effects in mammals	Health Based Guidance Values (HBVG)
Aflatoxin B1, B2, G1, G2	Group 1: Carcinogenic to humans with sufficient evidence	Vomiting, abdominal pain, coma, liver cancer	AFB_1 $BMDL_{10}$ for hepatocellular cancer: 0.4 µg kg^{-1} bw day^{-1} AFB_1 NOEL: 0.75 µg kg^{-1} bw day^{-1} (rat)
Ochratoxin A	Group 2B: possibly carcinogenic	Affects productivity in animals, body weight gain, kidney damage	PTWI: 120 ng kg^{-1} bw $week^{-1}$ NOEL: 150 µg kg^{-1} bw day^{-1} (mouse)
Fumonisin	Group 2B: possibly carcinogenic	Kidney and liver damage	$BMDL_{10}$: 0.1 mg kg^{-1} bw day^{-1} TDI: 1.0 µg kg^{-1} bw day^{-1}
Zearalenone	Group 3: not classifiable, not enough evidence to classify as a carcinogen	Infertility in pigs, mice, rats, cattle, reduced milk production	PMTDI: 0.5 µg kg^{-1} bw day^{-1} NOEL: 40 µg kg^{-1} bw day^{-1} (pigs) TDI: 0.25 µg kg^{-1} bw day^{-1}
Deoxynivalenol	Group 3: not classifiable, not enough evidence to classify as a carcinogen	Slow growth and low milk production in cattle, known as vomitoxin for humans, abdominal pain, headache, dizziness and fever	PMTDI: 1 µg kg^{-1} bw day^{-1} TDI: 1.2 µg kg^{-1} bw day^{-1}

AFB_1 = Aflatoxin B1, $BMDL_{10}$ = Benchmark dose level (associated with a 10% response adjusted for background), HBVG = Health based guidance values, IARC = International Agency for Research on Cancer, NOEL = No observed effect level, PMTDI = Provisional maximum tolerable daily intake, PTWI = Provisional tolerable weekly intake, TDI = Tolerable daily intake

Further characteristics of mycotoxins in feed are explained below, concerning:

- Growth factors
- Toxic effects in mammals
- Occurrence in food from animals that can affects consumer health

1.2.1 Aflatoxins

Aflatoxins are the most toxic and the best-known mycotoxins, produced by moulds of the genus *Aspergillus*. These mycotoxins grow at temperatures between 26°C and 38°C and moisture levels above 18%. There are six forms of aflatoxins: AFB1, AFB2, AFG1, AFG2, AFM1, AFM2 (Marc, 2022). The lethal dose (LD_{50}) value varies from 0.5 to 10 mg/kg body weight in different animal species and humans. Acute aflatoxicosis is characterized by vomiting, abdominal pain, pulmonary and cerebral edema, coma, seizures, and even death. In animals, symptoms of gastrointestinal dysfunction, reproduction problems, reduction of feed conversion and efficiency, and decrease in milk and egg production (Alshannaq & Yu, 2017).

1.2.2 Ochratoxins

Ochratoxins are mycotoxins produced by molds of the genus *Aspergillu*s and *Penicillium*, and the most important is ochratoxin A (OTA). This toxin synthesis occurs when crop moisture is over 16% and the temperature is between 20 and 25 ºC (Marc., 2022). OTA is acutely nephrotoxic and hepatotoxic, and the LD_{50} of this mycotoxin ranges between 0.2 and 58.3 mg/kg, being chickens more sensitive than species such as mice and rats (López de Cerain, 2000). Its toxicological effects include immunotoxicity, genotoxicity, neurotoxicity, teratogenicity and embryotoxicity in both humans and animals. (Alshannaq & Yu, 2017).

1.2.3 Zearalenone

The mycotoxin zearalenone (ZEA) is produced by moulds of the species *Fusarium*. This mycotoxin is found in contaminated cereals, mainly maize, but also in wheat, barley, sorghum, rye, and corn silage (Buszewska-Forajta, 2020). The contamination with this mycotoxin increases especially in humid and slightly colder climates, with temperatures of 10-15, and the most toxic form is α-zearalenone (Marc R.A., 2022). ZEA is stable at normal cooking temperatures yet is partially eliminated from high temperatures (Alshannaq & Yu, 2017).

Public health concerns over ZEA are associated with its structural similarity with natural oestrogens; therefore, ZEA can compete with the natural hormone of humans and animals by reducing the activity of oestrogens, changing metabolic pathways, and

influencing oestrogen receptor synthesis. Consequently, the long-term exposure to this mycotoxin can damage the reproductive organs and cause the development of breast and prostate cancer (Alshannaq & Yu, 2017, Buszewska-Forajta, 2020). According to the legislation of the EU, the maximum levels established of ZEA are vary between 20 and 100 ppb in different foodstuffs (EC 1126/2007).

1.2.4 Fumonisins

Fumonisins are produced by molds of the genus *Fusarium* and include fumonisins B1, B2, B3, and B4. The most toxic and most frequent fumonisin in human food is FB1, representing 70-80% of the fumonisin family (Marc, 2022; Alshannaq & Yu, 2017). FB1 commonly contaminates corn grains, but it can also be found in sorghum, wheat, barley, soybeans, asparagus, figs, black tea and medicinal plants (Alshannaq & Yu, 2017). The main target of fumonisins is the liver and kidneys, and the maximum tolerable daily intake set by the WHO is 2 µg/kg body weight. (Alshannaq & Yu, 2017).

1.2.5 Trichothecenes

The trichothecenes are the most chemically diversified family of mycotoxins, produced by the genus *Fusarium* which mainly contaminates the plants cultivated in the fields. Among these, deoxynivalenol (DON) is the most common and studied mycotoxin, as well as the most widely distributed in Japan, Korea, Europe, South Africa, and Australia. It is mainly found in maize, wheat and oats, and presents an LD_{50} of 46-78 mg/kg (Alshannaq & Yu, 2017).

The most common symptoms following human exposure to DON-contaminated cereals are nausea, vomiting, diarrhea, abdominal pain, headache, dizziness, and fever. In animals, consumption of DON-contaminated feed produces slow growth, reduced milk and egg production, refusal of feed, and reduction of immune response. (Alshannaq & Yu, 2017). Moreover, trichothecenes react with DNA, RNA, and cellular organelles by easily penetrating the cell membrane, inhibiting protein synthesis, and consequently interrupting DNA and RNA synthesis (Alshannaq & Yu, 2017).

1.3 Mycotoxins in chicken feed

Animal feed is one of the most important components to produce safe, affordable and sustainable animal protein, but are exposed to a wide range of environmental contaminants that may pose a serious risk to animal and human health. Long-term exposure of farm animals to feed contaminated with mycotoxins may lead to the

deposition of residues of mycotoxins in animal tissues and organs, and to transfer to animal products such as meat, milk and eggs, and consequently indirect human intake of mycotoxins. Feed and food products are often contaminated with multiple mycotoxins, both regulated and unregulated, at higher levels than food, as they are made from a mixture of cereals and soybeans, which are more susceptible to fungal infections (Kolawole *et al*, 2020).

The European Union (EU) has established maximum permitted levels of 0.02 mg/kg for aflatoxin B1 (AFB1) and recommended values of 5, 0.25, 0.25, 0.1 and 20 mg/kg respectively for deoxynivalenol (DON), zearalenone (ZEN), T-2, ochratoxin A (OTA) and fumonisin (FB1+FB2) in poultry feed (Kolawole *et al*, 2020). The exposure of broilers to a diet contaminated with 6,5 mg/kg DON, 0,47 mg/kg 15-acetyl-DON and 0,73 mg/kg ZEN showed significantly reduced feed intake, while a diet for broilers containing up to 0,4 mg/kg AFB1, 0,2 mg/kg OTA and 0,3 mg/kg DON caused a reduction in feed intake and food efficiency (Kolawole *et al*, 2020).

A decrease in feed intake of 35 % and body weight of 50 % was observed in broilers exposed to 2 mg/kg AFB1 and 100 mg/kg FB1. A diet containing a mixture of T-2 (0.2-2.2 mg/kg) and DON (4.9-24.9 mg/kg) reduced lipid peroxidation and induced oxidative stress in the liver of the chicken. In the production of broilers, feed contaminated with mycotoxins may cause a significant economic loss for poultry farmers, causing adverse effects on animal health and performance and increasing production costs (Kolawole *et al*, 2020).

1.4 Mycotoxin reduction and detoxification strategies in animal feed

The prevention of fungal contamination, and mycotoxins formation must be performed throughout the food production chain, from plant growth to distribution by implementing good manufacturing practices and the HACCP system developed by the *Codex Alimentarius* (Vila Donat *et al*, 2018).

Pre-harvest measurements include using resistant varieties of cereals, the selection of high-quality seeds, avoiding high plant densities, preventive management of insect infestations, crop rotation, soil cultivation, irrigation, and fertilization. Post-harvest measurements include the control of moisture levels of stored grains (less than 15%), the maintenance at low temperatures, and the preservation of the integrity of grains. Postharvest approaches to prevent the growth of mycotoxin-producing fungi and detoxify mycotoxins from contaminated food include chemical, biological, and physical treatments (Vila Donat *et al*, 2018).

1.4.1 Chemical treatments

Alkaline treatments: Ammonia, sodium hydroxide, potassium hydroxide and sodium carbonate, etc., have been used for the destruction of various mycotoxins in the moldy feedstuffs Ozone treatment. AFs, DON, ZEN and FB1 have been shown to be effectively degraded by ozone (Vila Donat *et al*, 2018).

1.4.2 Biological treatments

a) Yeast cell wall: *Saccharomyces cerevisiae* is used as a starter culture in fermented food and beverages, and mainly consists of proteins, lipids, and polysaccharides, with glucans and mannans. Yeast cell wall exhibits a great variety of accessible mycotoxin adsorption loci as well as different binding mechanisms (hydrogen bonds, ionic or hydrophobic interactions) (Vila Donat *et al*, 2018).

b) Lactic acid bacteria (LAB): Some LAB strains such as *Lactobacillus rhamnosus* display the ability to bind certain compounds in the small intestine with cell wall peptidoglycans, polysaccharides, and teichoic acid proposed as crucial elements in that process (Vila Donat *et al*, 2018).

c) Micronized fibres and bio-sorbent: Micronized fibers can be obtained from different plant materials such as cereals or legumes (wheat, barley, alfalfa, oat, and pea hulls). They consist mainly of cellulose, hemicellulose, and lignin, and they have been utilized as mycotoxin adsorbents due to favorable gut adsorption and enhanced fecal excretion. Regarding bio-sorbents, red wine waste such as dehydrated grape pomace (rich in phenolic compounds) has recently been demonstrated to be an excellent adsorbent for simultaneously removing several mycotoxins from a liquid medium (Vila Donat *et al*, 2018).

d) Activated carbon: s a non-soluble powder, produced by the pyrolysis of several organic compounds, followed by its chemical or physical activation aimed to developing a highly porous structure (Vila Donat *et al*, 2018).

1.4.3 Physical treatments

- Selection and separation*:* The uneven distribution of mycotoxins in cereals and their presence in mouldy or broken parts allows the isolation of feed contaminated by mycotoxins through sieving, aspiration, gravity, or photoelectric separation (Liu *et al*, 2022).

- Washing and solvent extraction*:* Methanol, ethanol, hexane, acetonitrile, isopropanol, and aqueous acetone are the most used for mycotoxin extraction. This methodology has the disadvantage of nutrient loss and the high cost of the management of toxic extracts, and for this reason its application on a large scale is limited (Liu *et al*, 2022).

- Heating: The efficiency of this method is related to the chemical structure, mycotoxin concentration, and from process conditions such as temperature, durability, moisture content, pH, and ion concentration (Liu *et al*, 2022).

- Irradiation: It could be viable to removing mycotoxins from food on an industrial scale. It is classified into ionizing (X-ray, γ-ray, and electron beam) and non-ionizing (ultraviolet, infrared, and microwave) radiations. Their safety issues such as microorganisms' mutagenesis and the nutritional value degradation of feedstuffs require further studies (Liu *et al*, 2022).

- Adsorption: Adsorbent ligands can form a complex with mycotoxins, thus preventing the passage of mycotoxins from the gastrointestinal tract to the blood and organs of animals. Ideally, absorbents should possess a high adsorption capacity against mycotoxins, a low non-specific nutrient bond, high safety, stability, and palatability (Liu et al, 2022).

The main types of mycotoxin adsorbents are aluminosilicate minerals.

Aluminosilicate minerals

Those are the most widely applied and studied minerals in the decontamination of mycotoxins. They include bentonite, montmorillonite, zeolite, calcium sodium aluminosilicate hydrate, kaolin, and illite. The binding efficiency of these adsorbents is significantly related to the surface area, charge distribution, and pore size of the

adsorption ligands and the charge distribution, polarity, and shape of the mycotoxin (Liu *et al*, 2022). The main aluminosilicate minerals used as adsorbents are:

a) Calcium sodium hydrate aluminosilicates (HSCAS): commonly used in animal feed as anti-caking agent. This aluminosilicate has been shown to act as an enterosorbent that securely and selectively binds aflatoxins in the gastrointestinal tract of animals, reducing their bioavailability and associated toxicity. (Vila Donat *et al*, 2018).

b) Bentonites: a phyllosilicate clays with a layered crystalline microstructure of variable composition. The safety and efficacy of bentonite as a feed additive have been evaluated by the EFSA, which concluded that this clay is not absorbed in the gastrointestinal tract and does not show genotoxic potential, hence providing no direct toxicological risk for the human and animal health (Vila Donat *et al*, 2018).

c) Zeolites: The zeolite structure consists of an assemblage of SiO_4 and AlO_4 tetrahedra joined in various regular arrangements through shared oxygen atoms to form an infinite three-dimensional cage-like structure. Zeolites have a large internal surface, associated with its elevated cation exchange capacity and with the adsorption of polar molecules (Vila Donat *et al*, 2018).

d) Other clays: Diatomite, a mineral formed by the accumulation and fossilisation of diatomaceous algae shells in lacustrine and marine environments, and sepiolite, a complex magnesium silicate belonging to the group of hormites. They have been demonstrated adequate sorbent activity for some polar molecules due to their negative charges associated with a high specific surface area (Vila Donat *et al*, 2018).

1.5 INFOGEST

Bioaccessibility is the fraction of food or feed that, following consumption, is released from the food matrix during digestion and is accessible for absorption in the small intestine or for biotransformation by the intestinal microbiota. Bioavailability is the amount of food or feed that, after the digestion, is absorbed and reaches target tissues in an intact form, metabolized to perform its bioactivity or to be stored (Bobrowski Rodrigues *et al*, 2022)

The *in vitro* digestion model INFOGEST simulates the digestion conditions in the three gastrointestinal regions for food digestion: mouth, stomach, and small intestine. For each

gastrointestinal region, temperature, incubation time, mechanical forces, pH value, mineral composition, enzymatic activities, mucin level and bile salt levels are established, standardizing the compositions of simulated saliva, gastric fluids and small intestine. (Zhou *et al*, 2023).

INFOGEST is a standardized protocol based on an international consensus developed by COST INFOGEST network. The method is designed to be used with standard laboratory equipment; it is a static digestion method that uses constant flows to digestive fluids at constant pH for each stage of digestion. Food samples are subjected to sequential oral, gastric and intestinal management analyses while parameters such as electrolytes, enzymes, bile, dilution, pH and digestion time are based on available physiological data (Brodkorb *et al*, 2019).

The digestion procedure, summarized in Figure 3, is divided into three phases: preparation, digestion procedure and treatment of the sample with subsequent analysis. For the preparation of the in vitro digestion sample, the activities of all digestive enzymes and bile salt concentrations should be determined experimentally, using the recommended standardized tests for amylase, pepsin, lipase (both gastric and pancreatic), trypsin and chyotripsin (Brodkorb *et al*, 2019).

Digestion involves the exposure of the food to three successive digestive phases, with constant experimental conditions during each phase: oral, gastric and intestinal. The oral phase involves the dilution of food with simulated salivary fluid (SSF), with or without salivary amylase, and for solids or semi-solids, simulated chewing of food. When used, food exposure to salivary amylase is limited to 2 min at pH 7 (Brodkorb *et al*, 2019).

The oral phase should be included in all simulated digestion procedures, regardless of the state of the food (liquid or solid) to ensure dilution consistency. The oral bolus is then diluted with simulated gastric fluid (SGF) and gastric enzymes (pepsin and gastric lipase) and incubated under stirring at pH 3,0 for 2 hours. The gastric chyme is then simulated intestinal fluid (SIF), bile salts and pancreatic enzymes and incubated at pH 7 for another 2 hours. The last step of the digestion procedure involves sampling, sample treatment, storage and subsequent analysis of samples (Brodkorb *et al*, 2019).

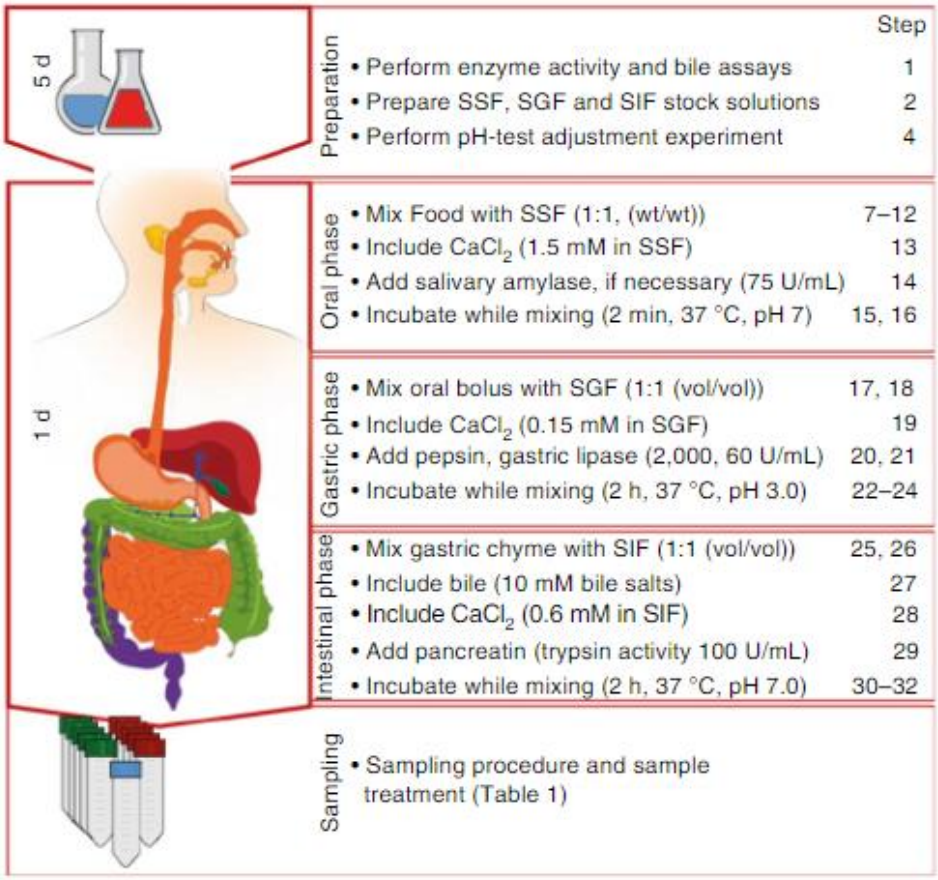


Figure 3. Flow diagram of the INFOGEST 2.0 digestion method. *Reference: Brodkorb et al, 2019.*

2 OBJECTIVES OF THE THESIS

The general aim of this master's thesis was to evaluate the efficacy of different mineral adsorbents to reduce the mycotoxin content in chicken feed.

To achieve this general goal, 5 partial objectives were proposed:

1. To review of the main mycotoxin in feed and the different adsorbents used to reduce mycotoxin concentration in feed.

2. To determine the efficacy of different concentrations of individual adsorbents in reducing the mycotoxin content in chicken feed samples during the **oral phase** of an *in vitro* digestion process.

3. To determine the efficacy of different concentrations of individual adsorbents in reducing the mycotoxin content in chicken feed samples during the **gastric phase** of an *in vitro* digestion process.

4. To determine the efficacy of different concentrations of individual adsorbents in reducing the mycotoxin content in chicken feed samples during the **intestinal phase** of an *in vitro* digestion process.

5. To evaluate the data obtained to determine the best adsorbent to reduce mycotoxins in chicken feed.

3 RESEARCH METHODOLOGY

3.1. Material and Reagents

3.1.1. Feed sample

The chicken feed was provided by a mining and clay company. The sample was homogenized using a grinder and was subsequently stored in dry conditions at room temperature.

3.1.2. Mycotoxins

A mixture of 5 mycotoxins at different concentrations was used to fortify the feed samples:

- Aflatoxin B1 (AFB1): 20 ppb. Ref. Sigma-Aldrich A6636
- Deoxynivalenol (DON): 5 ppm. Ref. Sigma-Aldrich D0156
- Fumonisin B1 (FB1): 50 ppm. Ref Sigma-Aldrich F1147
- Ochratoxin A (OTA): 100 ppb. Ref Sigma-Aldrich O1877
- Zearalenon (ZEA): 500 ppb. Ref Sigma-Aldrich Z2125

3.1.3. Adsorbents

A total of 12 adsorbents were tested in different concentrations, individually or in combination. In particular, 6 absorbents (samples 1 to 6) were examined individually at a 0.2% (w/w) final concentration, 1 absorbent (sample 7) was analyzed at a 0.1% (w/w) final concentration, and 9 combinations of absorbents were studied at different concentrations (Table 3).

Actually, not all the results are available as they have not been processed yet, therefore only the results processed have been shown, that are those for the 5 adsorbents individually tested.

Table 7. Adsorbents tested in different concentrations.

Adsorbent	Concentration (%, w/w)
A1 A2 A3 A4	0,2

A5 A6	
A6	0,1
A7+ A8 + A9	A6 (0,2%) + A7 (0,2%) + A8 (0,1%)
A7 + A8 + A10	A6 (0,2%) + A7 (0,2%) + A9 (0,1%)
A7 + A8 + A11	A6 (0,2%) + A7 (0,2%) + A10 (0,1%)
A7+ A8 + A12	A6 (0,2%) + A7 (0,2%) + A11 (0,1%)
A6 + A9	A6 (0,2%) + A9 (0,1%)
A7 + A9	A7 (0,2%) + A9 (0,1%)
A7 + A10	A7 (0,2%) + A10 (0,1%)
A7 + A7	A7 (0,2%) + A7 (0,1%)
A7 + A7	A7 (0,2%) + A7 (0,2%)

3.1.4. Reagents needed for the *in vitro* digestion and the extraction method

- 6-hydrate magnesium chloride ($MgCl_2(H2O)_6$) 0.15 M
- Bile salts
- Calcium chloride ($CaCl_2$) 0.3 M
- Carbonato de amono $(NH_4)_2CO_3$
- Ethyl acetate
- Human salivary alpha-amylase
- Hydrochloric acid (HCl) 6 M
- Methanol
- Monopotassium phosphate (KH_2PO_4) 0.5 M
- Pancreatin
- Pepsin
- Potassium chloride (KCL) 0.5 M
- Simulated fluids
- Sodium bicarbonate ($NAHCO_3$) 1 M
- Sodium chloride (NaCl) 2 M
- Sodium hydroxide (NaOH) 1 M

3.1.5. Preparation of simulated fluids

Preparation of simulated fluids was performed by addiction of KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$ and H_2O at different concentrations to achieve a FSS at pH 6., SGF at pH 3 and SIF at 6.4. The pH of the fluids was adapted to the conditions of

the gastrointestinal tract of chickens. Every day of the experiment, the pH of the solutions was adjusted with HCl (6 M) or NaOH (1 M).

3.1.6. Laboratory equipment and consumables

- Analytical balance
- Centrifuge
- Falcon centrifuge tubes: 15 and 50 mL
- Freezer
- Laboratory fumed hood
- Laminar flow cabinet
- Micropipettes: 2 µL, 20 µL, 200 µL, 1 mL, and 10 mL
- Orbital stirrer
- pHmeter
- Plastic pipettes
- Refrigerator
- Thermostatic bath
- UHPLC-MS/MS
- Vortex mixer

3.1.7 Enzyme preparation

Alpha-amylase: (1 mg in 10 ml)

1 mg of alpha-amylase was weighed in a 15 mL falcon and 10 mL of distilled water was added. The mixture was vortexed for 30 s until the enzyme dissolution.

Pepsin: (32 mg in 2 ml)

32 mg of pepsin was weighed in a 15 mL falcon tube and 2 mL of distilled water was added. The mixture was vortexed for 30 s until the enzyme was completely dissolved.

Pancreatin: (1.4 g in 10 ml)

1.4 mg of pancreatin was weighed in a 15 mL falcon tube and 10 mL of distilled water was added. The mixture was vortexed for 30 s until the enzyme was completely dissolved.

Bile salts (0.3270 g in 5 ml)

0.3270 g of bile salts were weighed in a 15 mL falcon tube and 5 mL of distilled water was added. The mixture was vortexed for 1 min until the enzyme was completely dissolved.

3.2 *In vitro* digestion

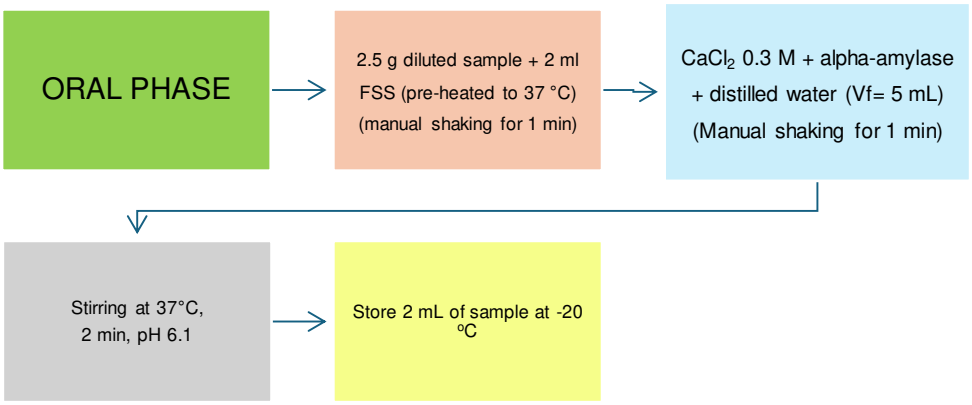
A static *in vitro* digestion model was used to simulate the digestion of the feed samples by the gastrointestinal tract. The *in vitro* digestion method was based on the INFOGEST standardized protocol with slight modifications (Brodkorb et al., 2019; Minekus et al., 2014). This model consists of three steps to mimic the physiological conditions of the human gastrointestinal tract: saliva, gastric, and small intestine digestion. In this study, the conditions of each phase were modified to adapt to the gastrointestinal tract of chickens. In this study, 12 adsorbent conditions were tested at each of the three gastrointestinal phases: salivary, gastric, and intestinal. Control samples without adsorbents were included, both, negative (feed) and positive (feed and the mycotoxins mixture). All the samples were determined by triplicate. Consequently, a total of 193 samples were analyzed.

To perform the *in vitro* digestion of the samples, first, 2.5 g of feed was weighed and mixed with 2.5 mL of pre-heated (37°C) SSF, 2.4 mL of distilled water, and 100 µL of the mycotoxin mixture. For negative controls that do not contain the mycotoxin mixture, 2.5 mL of distilled water were added.

For the oral phase, 2.5 mg of this sample mixture was weighted in a 50 mL falcon tube, and mixed with 2 mL of SSF preheated to 37°, by manual shaking for 1 min. Then, $CaCl_2$ 0.3 M, alpha-amylase and distilled water were added, following by manual shaking for 5 seconds and shaking in the bath at 37°.

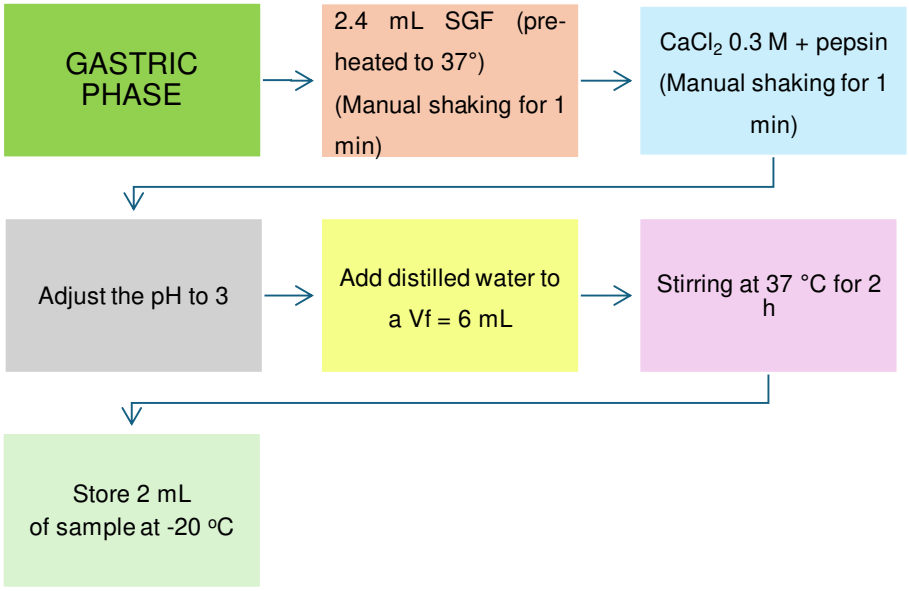
After 2 min shaking at 37 °C, 2 mL of the liquid phase were collected from each tube and frozen at -20 °C until use (Figure 4). The rest of the sample was used to continue with the gastric phase.

Figure 4. Schematic representation of the oral phase according to INFOGEST method.



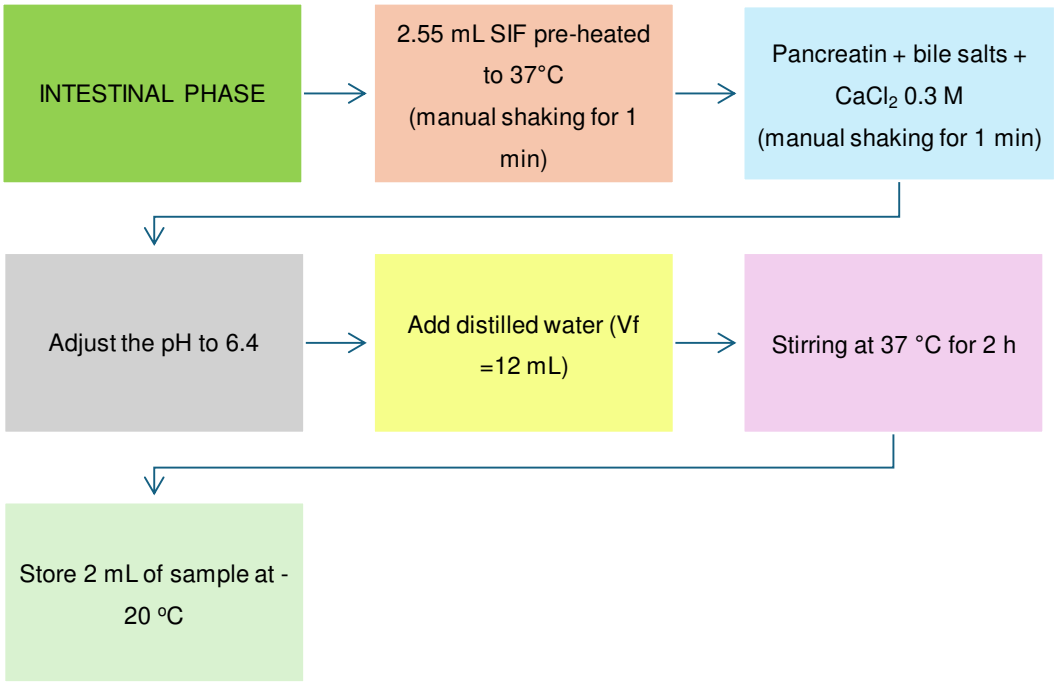
To simulate the gastric phase, 2.4 mL of preheated SSG (37°C) was added to the sample tubes. After manually shaking for 1 min, $CaCl_2$ 0.3 M and of pepsin were added, and the mixture was stirred again. Next, the pH was adjusted to 3 by adding HCl 6 M, and then, distilled water was added to a final sample. After 2 h at 37 °C under stirring, 2 mL of the liquid phase of the samples was collected and stored in the freezer at -20°C until use (Figure 5). The rest of the sample was used to continue with the intestinal phase.

Figure 5. Schematic representation of the gastric phase according to INFOGEST method



For the intestinal phase, 2,55 mL of SIF pre-heated at 37 ºC were added to each sample, followed by manual stirring for 1 min. Then pancreatin, 750 bile salts, and $CaCl_2$ 0.3 M were added, and the mixture shaken for 1 min. The pH was adjusted to 6.4 with NaOH 1M and distilled water was added up to 12 mL final volume. And the samples maintained for 2 h at 37 ºC under stirring. Finally, 2 mL of the liquid phase was collected and frozen at -20 ºC until use (Figure 6).

Figure 6. Schematic representation of the intestinal phase according to INFOGEST method.



3.3 Extraction method

Before the chromatographic analysis, an extraction process was performed to extract the mycotoxins from the digested feed samples. For this purpose, a liquid-liquid extraction method was used according to the protocol described by Llorens et al. (2022) with slight modifications. Briefly, the tubes containing the digestion tubes at the different phases were thawed and then centrifuged at 4000 rpm and 4 ºC for 20 min. Next, 1.8 mL of sample was transferred to a 15 ml falcon tube and 2 mL of ethyl acetate were added. The tubes were mixed with a vortex for 1 min and then the mixture was centrifuged at 4000 rpm and 4 ºC for 10 min and the organic phase was collected. This procedure was repeated three times. Finally, the organic phase was brought to dryness at 40°C by nitrogen flow (TurboVap). The dried residue was resuspended in 0.2 mL of

MeOH/H20 (70/30, v/v) kept in the vortex for 1 min, filtered with a 0.2 µm filter and finally, vialized until analysis by UHPLC-MS/MS.

3.4 Liquid chromatography coupled to tandem mass spectrometry

The determination of mycotoxins AFB1, DON, ZEA, OTA, FB1 was carried out using UHPLC-MS/MS, with a Sciex TRIPLE QUAD 6500+ (Sciex, Concord, Ontario, Canada) equipped with electro-spray ionization (ESI) (TurboV) coupled to a UHPLC Agilent 1260 HPLC system (degasser, quaternary pump and column oven) with an Eksigent ULC 100 HTC-xt autosampler, equipped with a BEH® C18 Column (1.7 µm 100 Å, Column LC 50 x 2.1 mm, Waters).

The mobile phases were (A) H_2O with 5 mM ammonium formate and 0.1% formic acid, and (B) methanol (0.1% formic acid). The initial gradient was 95% phase A which was maintained for 2 min and then the gradient gradually decreased by 11 minutes to 0% phase A; then it was maintained 2 minutes at 0% of phase A and to finish the gradient it grew linearly until 95% of phase A being maintained until minute 20 at 95% of phase A for the rebalancing of the column.

The injection volume was 5 µL and constant temperature of 30 °C in the column. The flow was 0.35 mL/min. The mass spectrometer was used in positive ionization mode and in multiple selection mode of monitored reactions (SRM), with an ionization source turbo Spray Ion Drive, with the following conditions: curtain gas (CUR) 30 psi, ion spray voltage (IS) at 4.5 kV, temperature 300°C, and ion source gas (GS) 1 and 5 to 55 psi. Table 4 shows the parameters of UHPLC-MS/MS for the determination of mycotoxins AFB1, DON, ZEA, OTA, FB1.

Table 8. Parameters of the UHPLC-MS/MS S for the determination of the studied mycotoxins.

Mycotoxin	RT (min)	Precursor ion	Product ion	CE (eV)	DP (V)	EP (V)	CXP (V)
AFB1	12.2	313.1	285.2[Q]	20	30	10	15
	12.2	313.1	241.1[q]	30	30	10	15
OTA	13.5	404.0	239.0[Q]	25	30	10	15
	13.5	404.0	341.1[q]	30	30	10	15
FB1	12.90	722.0	352.0[Q]	40	30	10	15
	12.90	722.0	406.0[q]	25	30	10	15
DON	7.82	297.0	231.0[Q]	20	30	10	15
	7.82	297.0	249.1[q]	30	30	10	15
ZEN	13.49	317.1	131.1[Q]	35	30	10	15
	13.49	317.1	175.2[q]	40	30	10	15

Q: quantifier *m/z*; q: qualifier *m/z*; AFB1: aflatoxin B1; OTA: ochratoxin A; FB1: fumonisin B1; DON: deoxynivalenol; ZEN: zearalenone; RT = retention time; CE = collision energy; DP = declustering potential; EP = entrance potentional; CXP = collision cell exit potential; eV = electronvolt; V = volt.

4 RESULTS AND DISCUSSION

For the study of the bio accessibility of the mycotoxin mixture a sample of chicken feed was fortified at a concentration of 20 ppb of AFB1, 5 ppm of DON, 50 ppm of FB1, 100 ppb of OTA and 500 ppb of ZEA and its digestion was performed in vitro following the protocol INFOGEST 2.0.

The digestions of the fortified samples were performed in triplicate and an aliquot was collected after the completion of each of the phases (oral, gastric and intestinal). In parallel, an in vitro digestion of the same unsaturated sample (control sample) was performed, also collecting an aliquot from each stage of the digestion process. The aliquots of each of the phases were extracted as described in point 3.3 and they were analyzed through UHPLC-MS/MS.

The chromatograms UHPLC-MS/MS of the digested (intestinal phase) samples from a feed sample fortified with AFB1, OTA, ZEN, FB1 and DON are shown in Figure 7.

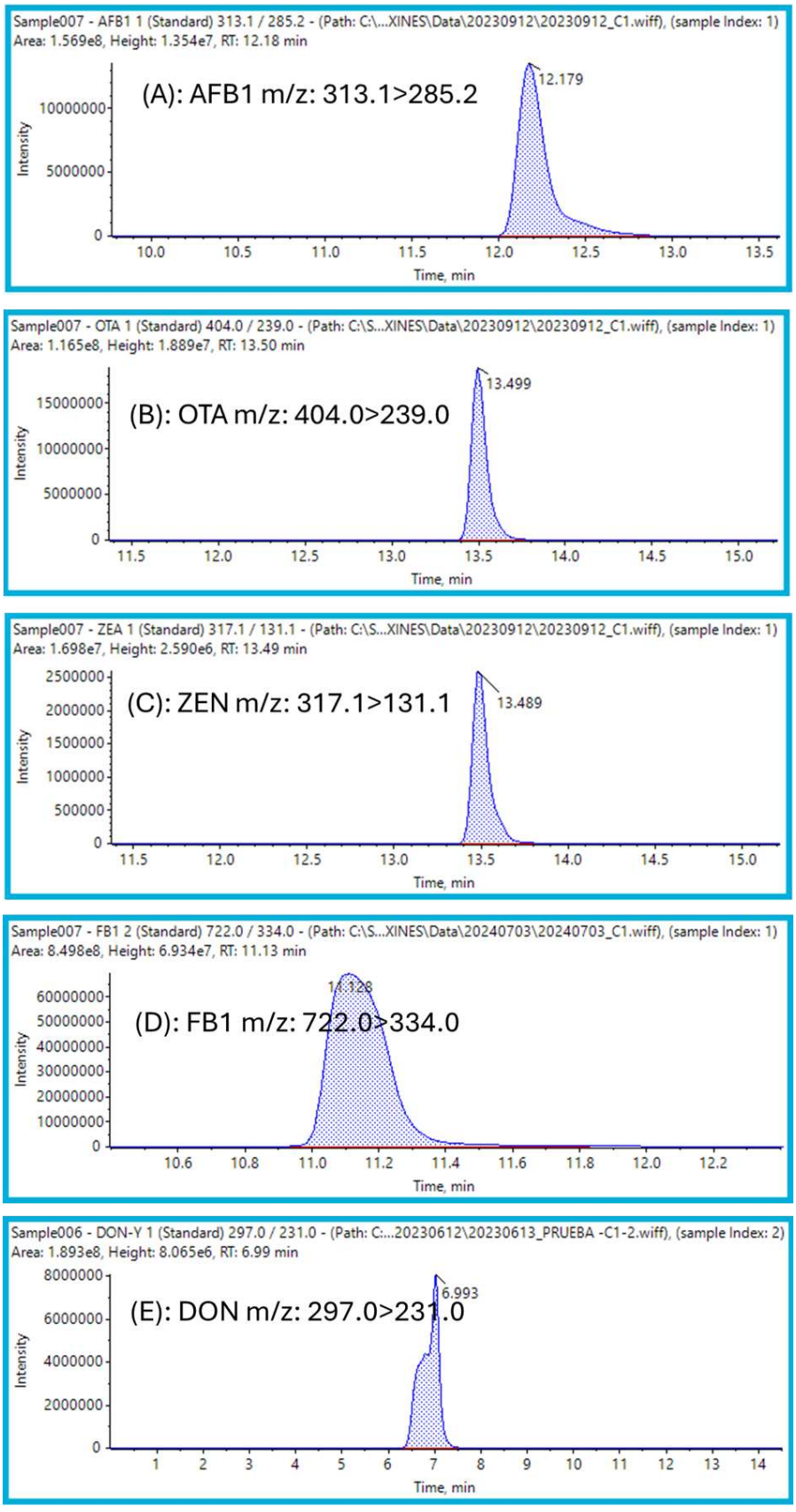


Figure 7. UHPLC-MS/MS chromatogram corresponding to: (A) aflatoxin B1, (B) ochatoxin A, (C) zearalenone, (D) fumonisin B1 and (E) deoxynivalenol

For the quantification of AFB1, a 6-point calibration line was constructed (concentration range: 0 to 0,016 ppm) (Figure 8). Good linearity was obtained in the working interval by the correlation coefficient obtained (R2 = 0,9870).

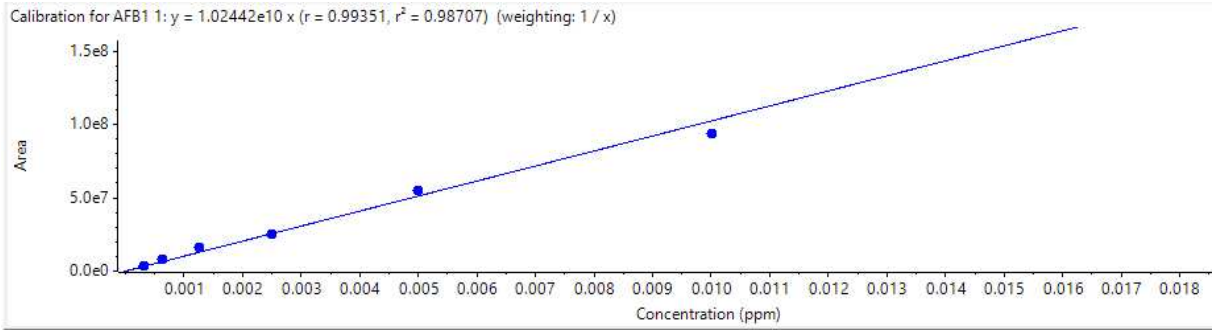


Figure 8. Calibration curve of AFB1.

For the quantification of OTA, a 6-point calibration line was constructed (concentration range: 0 to 0,09 ppm) (Figure 9). Good linearity was obtained in the working interval by the correlation coefficient obtained (R2 = 0,9950).

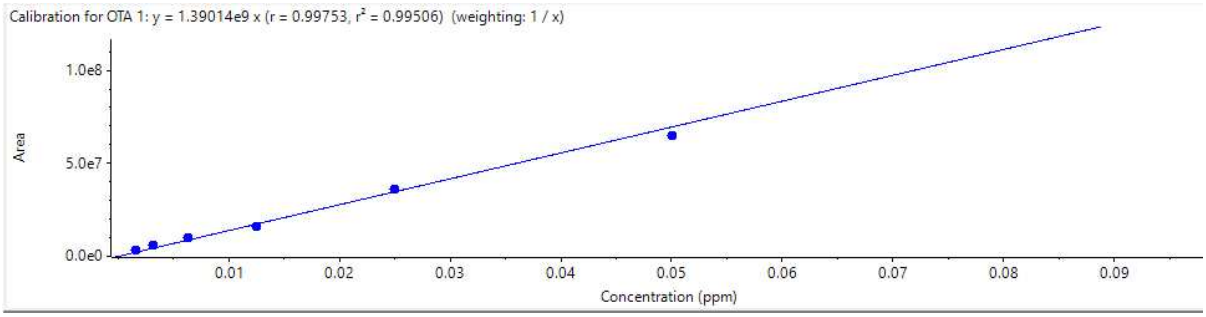


Figure 9. Calibration curve of OTA.

For the quantification of ZEN, a 6-point calibration line was constructed (concentration range: 0 to 0,40 ppm) (Figure 10). Good linearity was obtained in the working interval by the correlation coefficient obtained (R2 = 0,9970).

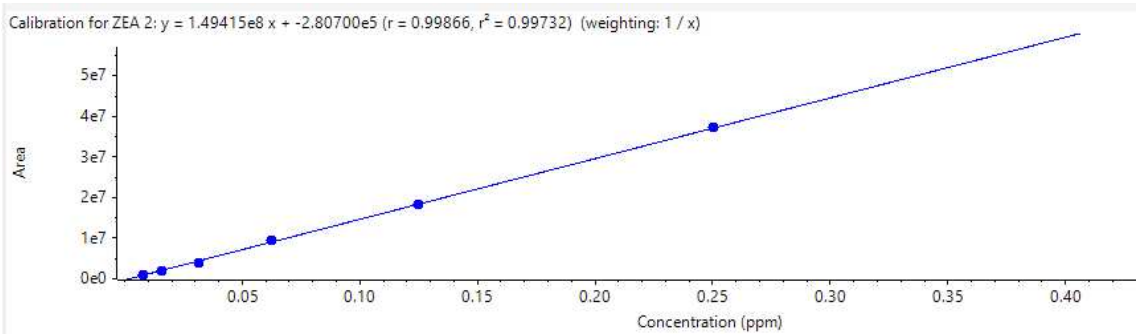


Figure 10. Calibration curve of ZEN.

For the quantification of FB1, a 6-point calibration line was constructed (concentration range: 0 to 0,12 ppm) (Figure 11). Good linearity was obtained in the working interval by the correlation coefficient obtained (R2 = 0,9689).

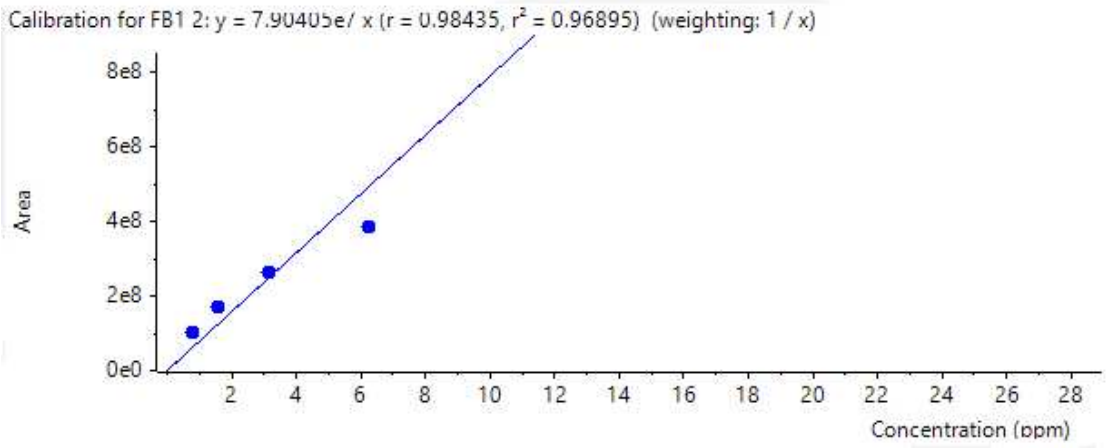


Figure 11. Calibration curve of FB1.

For the quantification of DON, a 6-point calibration line was constructed (concentration range: 0 to 3.8 ppm) (Figure 12). Good linearity was obtained in the working interval by the correlation coefficient obtained (R2 = 0,969).

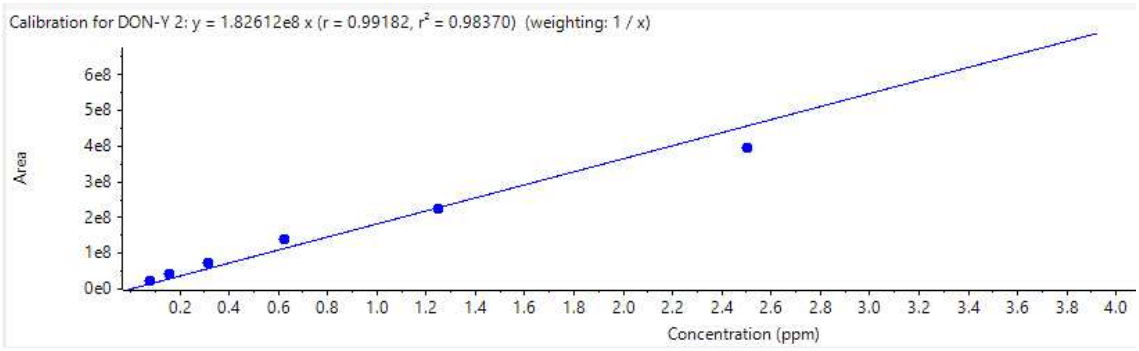


Figure 12. Calibration curve of DON.

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In the UHPLC-MS/MS determination process, the lowest point of the calibration line (LCP) for each of the mycotoxins tested corresponds to the following concentration:

- AFB1: 2.50 ng/g
- DON: 625 ng/g
- FB1: 3.1 ng/g
- OTA: 12.5 ng/g
- ZEN: 62.5 ng/g

As observed in Figure 13, the UHPLC-MS/MS analysis of chicken feed revealed a natural contamination of AFB1 (4,9 ± 1,3 ng/g) at Rt = 11.987, OTA (16,3 ± 5,6 ng/g) at Rt = 13.438 y ZEN (90,0 ± 16,0 ng/g) at Rt = 13.427 (Figure 13).

Figure 13. UHPLC-MS/MS chromatogram corresponding to a feed sample naturally contaminated by (A) aflatoxin B1, (B) ochatoxin A, and (C) zearalenone.

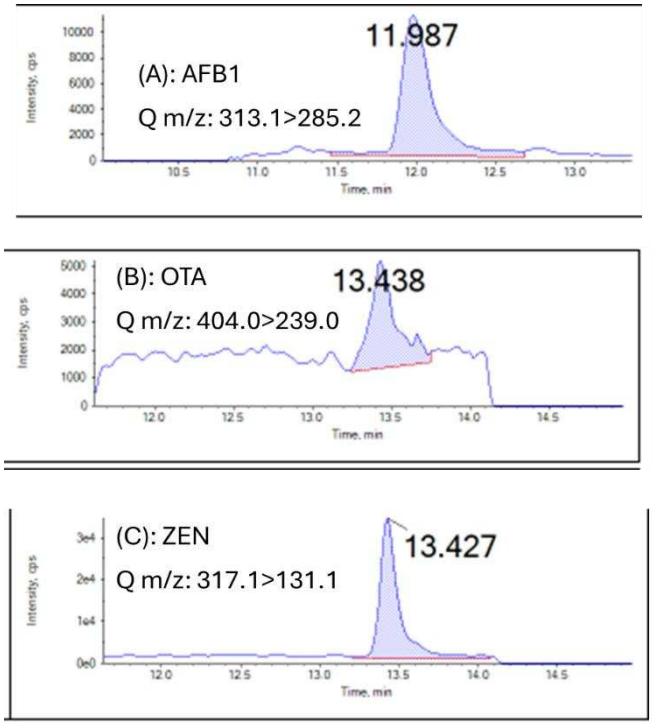

Table 5 shows the results for chicken feed samples added with different mineral adsorbent, in order to know if the samples are naturally contaminated by some mycotoxins.

Table 5. Quantification of naturally occurring mycotoxins (ng/g) in chicken feed supplemented with adsorbent minerals submitted to in vitro simulated digestion.

Adsorbent	Contamination (ng/g)				
	AFB1	OTA	DON	FB1	ZEN
Tipo 1	2,6 ± 0,12	n.d.	n.d.	n.d.	n.d.
Tipo 2	2,9 ± 0,92	n.d.	n.d.	n.d.	<LCP
Tipo 3	4,6 ± 1,4	n.d.	n.d.	n.d.	n.d.
Tipo 4	<LCP	<LCP	n.d.	n.d.	71,0 ± 6,2
Tipo 5	<LCP	<LCP	n.d.	n.d.	n.d.

To evaluate the reduction of mycotoxins due to different adsorbent minerals, the chicken feed samples added with the adsorbent minerals were fortified with a mixture of AFB1, OTA, DON, FB1 y ZEN.

Tables 6 to 10 show the levels of each mycotoxin present in feed after the three steps of digestion, following the addition of the mix of mycotoxins and different adsorbent. 3 tests were carried out for each type of absorbent and for each phase. The results are expressed in µg/kg.

Table 6. Quantification of AFB1 (µg/kg) fortified in chicken feed added with adsorbent minerals during simulated in vitro digestion.

Adsorbent	Contamination AFB1(µg/kg)		
	Oral phase	Gastric phase	Intestinal phase
A1	9,32	8,41	11,84
	10,15	11,34	10,73
	7,84	9,95	13,26
A2	5,22	14,12	14,12
	4,46	9,93	12,11
	5,53	11,92	12,93
A3	0,72	0,74	19,98
	0,69	0,85	18,15
	0,89	0,90	16,52
A4	0,72	1,84	8,93
	1,15	1,73	9,94
	0,93	1,68	11,32
A5	0,73	0,87	2,70
	0,92	0,86	4,32
	0,83	0,94	3,90

Table 7. Quantification of OTA (µg/kg) fortified in chicken feed added with adsorbent minerals during simulated in vitro digestion.

Adsorbent	Contamination OTA (mg/kg)		
	Oral phase	Gastric phase	Intestinal phase
A1	95,28	58,46	-
	95,93	73,94	-
	99,29	69,66	-
A2	99,42	61,59	-
	97,31	73,87	-
	98,67	69,19	-
A3	-	79,64	-
	-	96,61	-
	-	86,72	-
A4	-	-	73,64
	-	-	84,91
	-	-	91,65
A5	-	-	84,61
	-	-	94,87
	-	-	77,19

Table 8. Quantification of DON (mg/kg) fortified in chicken feed added with adsorbent minerals during simulated digestion in vitro.

Adsorbent	Contamination DON (mg/kg)		
	Oral phase	Gastric phase	Intestinal phase
A1	2,75	2,48	2,59
	3,01	2,39	2,91
	2,86	2,69	2,84
A2	2,73	2,04	2,57
	3,11	2,13	2,71
	2,87	2,09	2,80
A3	2,81	2,47	2,88
	3,12	2,57	2,67
	2,95	2,51	3,03
A4	3,94	3,29	3,27
	3,76	3,38	3,63
	3,69	3,45	3,58
A5	-	-	-
	-	-	-
	-	-	-

Table 9. Quantification of FB1 (mg/kg) fortified in chicken feed added with adsorbent minerals during simulated in vitro digestion.

Adsorbent	Contamination FB1 (mg/kg)		
	Oral phase	Gastric phase	Intestinal phase
A1	33,94	-	33,62
	37,74	-	41,45
	41,72	-	35,83
A2	8,65	34,64	11,97
	10,21	32,53	12,65
	7,98	37,17	17,25
A3	0,76	-	-
	0,89	-	-
	0,89	-	-
A4	5,05	-	-
	5,44	-	-
	5,29	-	-
A5	10,02	-	-
	11,21	-	-
	10,38	-	-

Table 10. Quantification of ZEN (µg/kg) fortified in chicken feed added with adsorbent minerals during simulated in vitro digestion.

Adsorbent	Contamination ZEN (mg/kg)		
	Oral phase	Gastric phase	Intestinal phase
A1	493,8	-	-
	496,7	-	-
	492,9	-	-
A2	381,6	372,4	337,2
	374,1	395,3	347,8
	394,8	388,8	367,1
A3	454,3	499,3	-
	458,7	498,1	-
	462,1	493,2	-
A4	430,6	452,6	424,2
	451,9	469,7	419,7
	450,7	479,5	410,1
A5	-	-	-
	-	-	-
	-	-	-

Tables 11 to 15 show the percentage of reduction of each mycotoxin, after the addition of different mineral adsorbent, after each phase of simulated digestion (oral, gastric and intestinal).

Table 11. Reduction of AFB1 (%) fortified in chicken feed added with adsorbent minerals during simulated digestion in vitro. Fortification level of AFB1: 20 µg/kg

Adsorbent	Reduction of AFB1 (%)		
	Oral phase	Gastric phase	Intestinal phase
A1	54,6 ± 4,7	50,7 ± 4,1	40,3 ± 6,1
A2	74,8 ± 6,7	40,2 ± 5,0	34,7 ± 5,3
A3	96,1 ± 8,4	95,9 ± 9,3	9,1 ± 1,3
A4	95,3 ± 9,6	91,2 ± 7,9	49,2 ± 5,6
A5	95,8 ± 7,3	95,6 ± 8,7	82,0 ± 8,0

Table 11 shows that A3, A4 and A5 reduce AFB1 very well, especially in the oral and gastric phase. Adsorbents A1 and A2 have lower reduction rates than the other three. In general, the oral and gastric phases are those in which AFB1 is better reduced, while in the intestinal phase is less reduced. For this mycotoxin, the most effective adsorbents is A5.

Table 12. Reduction of OTA (%) fortified in chicken feed added with adsorbent minerals during simulated digestion in vitro. OTA fortification level: 100 µg/Kg.

Adsorbent	Reduction of OTA (%)		
	Oral phase	Gastric phase	Intestinal phase
A1	3,1 ± 0,8	32,5 ± 4,5	n.o.
A2	1,6 ± 0,4	31,4 ± 5,6	n.o.
A3	n.o.	12,0 ± 3,1	n.o.
A4	n.o.	n.o.	16,5 ± 4,1
A5	n.o.	n.o.	14,2 ± 3,7

n.o.: no reduction observed

Table 12 shows that A1 and A2 reduce OTA only in the oral and gastric phases, but at low levels. A3 reduces mycotoxin only in the gastric phase, with a low percentage, while A4 and A5 reduced mycotoxin only in the intestinal phase. The gastric phase is the

one in which OTA is more reduced, while the oral phase is the one in which it less reduced. For this mycotoxin, similar effective reduction was observed with A1 and A2.

Table 13. Reduction of DON content (%) fortified in chicken feed added with adsorbent minerals during simulated digestion in vitro. DON fortification level: 5 mg/Kg

Adsorbent	Reduction of DON (%)		
	Oral phase	Gastric phase	Intestinal phase
A1	42,0 ± 4,3	49,6 ± 5,1	44,4 ± 3,4
A2	42,1 ± 5,4	58,1 ± 6,2	46,1 ± 5,1
A3	42,5 ± 4,9	49,4 ± 4,0	42,9 ± 5,6
A4	24,2 ± 3,8	32,6 ± 4,8	30,2 ± 4,1
A5	n.o.	n.o.	n.o.

n.o.: no reduction observed

Table 13 shows that DON is reduced by A1, A2, A3 and A4 at all three digestion phases but is not reduced by A5. Among the 4 adsorbents, A1, A2 and A3 are those with the highest percentages of reduction, while A4 showed lower percentages. It can be said that in all three phases the mycotoxin was reduced in similar percentages by the adsorbents A1, A2 and A3, therefore they are the most effective adsorbents for DON.

Table 14. Reduction of FB1 (%) fortified in chicken feed added with adsorbent minerals during simulated digestion in vitro. Fortification level of FB1: 50 mg/kg

Adsorbent	Reduction of FB1 (%)		
	Oral phase	Gastric phase	Intestinal phase
A1	24,5 ± 3,6	n.o.	26,1 ± 5,2
A2	82,1 ± 7,9	30,5 ± 6,1	72,2 ± 7,5
A3	98,3 ± 8,9	n.o.	n.o.
A4	89,5 ± 9,4	n.o.	n.o.
A5	78,9 ± 8,4	n.o.	n.o.

n.o.: no reduction observed

Table 14 shows that A1 reduces FB1 in the oral and intestinal phase, but not in the gastric phase, A2 reduces mycotoxin in all three phases, whereas A3, A4 and A5 reduce mycotoxin only in the oral phase.

The oral phase is the one in which the mycotoxin concentration is reduced by all the adsorbents. The most effective adsorbent to reduce FB1 in all three phases is A2, while A3 and A4 are the most effective for the oral phase.

Table 15. Reduction of the content of ZEN (%) fortified in chicken feed added with adsorbent minerals during simulated digestion in vitro. Fortification level of ZEN: 500 µg/Kg

Adsorbent	Reduction of ZEN (%)		
	Oral phase	Gastric phase	Intestinal phase
A1	1,1 ± 0,3	n.o.	n.o.
A2	23,3 ± 2,5	23,0 ± 4,8	29,9 ± 4,1
A3	8,4 ± 1,4	0,6 ± 0,2	n.o.
A4	11,2 ± 2,3	6,5 ± 1,2	16,4 ± 3,6
A5	n.o.	n.o.	n.o.

n.o.: no reduction observed

Table 15 shows that ZEN is reduced only by A1, A2, A3 and A4, but not by A5. In particular, A2 and A4 reduce DON in all the three phases. In the intestinal and gastric phases, the highest reduction percentage observed was after the addition of A2, so he most effective adsorbent in reducing mycotoxin in all the phases is A2.

In conclusion, for mycotoxin AFB1, the most effective adsorbents with higher reduction percentages were A4 and A5. For OTA the most effective adsorbents were A1 and A2 in the gastric phase, and A4 and A5 in the intestinal phase. The adsorbents that reduce DON in the oral, gastric and intestinal phases were A1, A2 and A3. For mycotoxin FB1, A2 is the most effective in reducing the mycotoxin concentration in the three phases and for mycotoxin ZEN, A2 is the only adsorbent capable to reducing it in all the three phases, with higher percentages than the other adsorbents.

Therefore, if a single adsorbent had to be chosen to be added to a commercial chicken feed, A2 would be chosen. However, AFB1 could not be reduced with A2. As AFB1 is the only regulated mycotoxin in feed, it would be appropriate to add a mixture of mineral adsorbent A2 and A4, in order to achieve the highest possible reducing effect with this type of substances present in the chicken feed.

5. CONCLUSIONS

The general aim of this master's thesis was to evaluate the efficacy of different mineral adsorbents to reduce the mycotoxin content in chicken feed. In accordance with the objectives of this work, the following conclusions were found:

1. In the literature reviewed, the main mycotoxins in feed are AFB1, DON, ZEA, OTA and FB1, and the different adsorbent used to reduce mycotoxin concentration in feed are calcium sodium hydrate aluminosilicates (HSCAS), bentonites, zeolites, diatomite, sepiolite.
2. For mycotoxin AFB1, the most effective mineral adsorbents tested with higher reduction percentages were A4 and A5.
3. For OTA the most effective adsorbents were A1 and A2, although they reduce it only in the oral and gastric phases.
4. The adsorbents that reduce DON both in the oral, gastric and intestinal phase are A1, A2 and A3.
5. For mycotoxin FB1, A2 is the most effective at reducing the level of mycotoxin in the three stages, but the highest reduction rates are given by A3 and A4, although they act only in the oral phase.
6. For the mycotoxin ZEN, A2 is the only adsorbent capable of reducing it in all the three phases, with higher percentages than the other adsorbents.
7. To reduce all the mycotoxins tested in chicken feed, a mixture of A2 and A4 adsorbents would be appropriate.

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