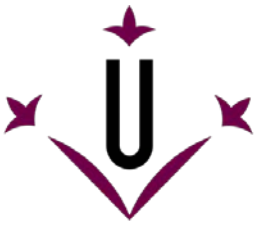

Mycotoxin bioaccessibility/absorption assessment using *in vitro* digestion models: a review.

Running head: *In vitro* mycotoxin bioaccessibility assessment.

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Abstract

In the evaluation of the oral bioavailability of a mycotoxin, the first step is the determination of its bioaccessibility, i.e., the percentage of mycotoxin released from the food matrix during digestion in the gastrointestinal (GI) tract that could be absorbed through the intestinal epithelium. As a first approximation to the problem, different *in vitro* digestion models have been recently used for bioaccessibility calculation, thereby avoiding the use of more complex cell culture techniques or the use of animals in expensive *in vivo* experiments.
In vitro methods offer an appealing alternative to human and animal studies. They usually are rapid, simple and reasonably low in cost, and can be used to perform simplified experiments under uniform and well-controlled conditions, providing insights not achievable in whole animal studies. The available *in vitro* methods for GI simulation differ in the design of the system, the composition of the physiological juices assayed, as well as in the use or not of intestinal microbiota. There are models that only simulate the upper part of the GI tract (mouth-stomach-small intestine), whereas other methods include the large intestine, so that the model chosen could have some influence on the bioaccessibility data obtained.
Bioaccessibility depends on the food matrix, as well as on the contamination level and the way the food/feed is contaminated (spiked or naturally).
This review focuses on the currently available data regarding the *in vitro* digestion models for the study of the bioaccessibility or absorption of mycotoxins, detailing the characteristics of each digestion step and the importance of the physiological juices employed during digestion. The effect that different factors play on the mycotoxin release from food matrix in the GI tract is also considered, and existing data on bioaccessibility of the main mycotoxins are given.

Keywords: masked mycotoxins, bioaccessibility, gastrointestinal simulation, intestinal absorption, *in vitro* models.

1. Introduction

Mycotoxins are a wide group of fungal secondary metabolites that exert multiple toxic effects on humans and animals. Some mycotoxins can cause autoimmune illnesses, have allergenic properties, and some of them are teratogenic, carcinogenic, mutagenic, nephrotoxic or estrogenic (CAST, 2003). Although hundreds of mycotoxins exist, the most important for public health are aflatoxins (AFs, aflatoxins AFB_1, AFB_2, AFG_1 and AFG_2 being the main ones), ochratoxin A (OTA), patulin (PAT), fumonisins (FBs) zearalenone (ZEA), and the trichothecene group, among them deoxynivalenol (DON), nivalenol (NIV), T-2 and HT-2 toxins being the most important. Recently, other mycotoxins - the so-called emerging mycotoxins, such as fusaproliferin, beauvericin (BEA), enniatins (ENs), moniliformin and the *Alternaria* toxins - are attracting the attention of researchers.

The major mycotoxin-producing fungal genera are *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* (Moss, 1992; Sweeney and Dobson, 1998). The growth of mycotoxigenic strains of these fungi on crops, either in the field or during storage, could lead to the accumulation of mycotoxins in a great variety of foods. Besides, the metabolism of ingested mycotoxins could result in modified mycotoxins, as happens when aflatoxin B_1 (AFB_1) is converted by hydroxylation to aflatoxin M_1 (AFM_1), mycotoxin mainly present in milk as a result of AFB_1 metabolism in cow and other mammals (Prandini *et al*., 2009).

Natural occurrence of mycotoxins in food has been broadly documented. Thus, mycotoxins have been widely detected in food of vegetal origin, mainly in cereals (barley, wheat, corn, oat, etc.) and their by-products (Marín *et al*., 2012; Rodrigues and Naehrer, 2012), as well as in nuts, dried fruits, spices, cocoa, coffee, beer, wine, and fruits, particularly apples (Bellí *et al*., 2002; Fernández-Cruz *et al*., 2010; Molyneux *et al*., 2007; Placinta *et al*. 1999; Santos *et al*., 2010; Turcotte *et al*., 2011). Mycotoxins also enter the human food chain via meat or other animal products such as eggs, milk and cheese as a result of contaminated livestock feed (Chen *et al*., 2012; Meyer *et al*., 2003).

This huge variety of food matrices in which mycotoxins occur can have a very significant effect on the bioavailability of mycotoxins, as complex and diverse reactions can occur between mycotoxin and the food matrix, which could interfere in the way these toxins are absorbed through the intestinal tract.

On the other hand, in the last years many structurally related compounds generated by plant metabolism or by food processing have been described in mycotoxin-contaminated commodities, which can co-exist together with the native toxins. These mycotoxin derivatives (named conjugated or “masked” mycotoxins) may have a very different chemical behaviour, thus they can easily escape routine analyses. Nevertheless, these forms could be hydrolysed to their precursors in the digestive tracts of animals or could exert toxic effects comparable to those imputable to free mycotoxins (De Saeger and van Egmond, 2012; Galaverna *et al*., 2009). This can make in some occasions very difficult to establish a clear relationship between the amount of ingested toxin and the toxic effects observed, and expected, for a given amount of mycotoxin. All these facts could help to explain the so-called “fumonisin paradox” (i.e. the fact that apparently low contaminated commodities induce severe toxic effects in animals), whereby the oral bioaccessibility of this mycotoxin could be affected by different factors, one of them the uptake of fumonisin B_1 (FB1) strongly conjugated to the food matrix or FB_1 derivatives with higher bioavailabilities (Shier, 2000). This could also happen with other mycotoxins.

So, knowing the amount of mycotoxin ingested may not be enough for exposure assessment. Knowing the amount of toxin that becomes available for absorption through the intestinal epithelium (which will be the measure of its bioaccessibility) is also required. Different factors, as pH changes, enzymatic activities, etc., play an important role during the gastrointestinal transit of mycotoxins and thus affect bioaccessibility.

To determine the bioaccessibility of mycotoxins (or in same cases, the absorption), and as a first approximation to the problem, different *in vitro* digestion models have been used, avoiding the use of more complex cell cultures or the ethically questionable use of animals in *in vivo* experiments.

This review focuses on the currently available data regarding the *in vitro* digestion models for the study of the bioaccessibility of mycotoxins, detailing the characteristics of each digestion step and the importance of the physiological juices employed during digestion. The effect that different factors play on the mycotoxin release from food matrix in the GI tract is also considered, and existing data on bioaccessibility of the main mycotoxins are given.

2. Bioaccessibility and bioavailability

The amount of mycotoxin consumed via food does not always reflect the amount of this compound that is available to exert its toxic action in a target organ of the body, as only a part of the ingested compound will be bioavailable. Thus the oral bioavailability (F) of a mycotoxin has been defined as the fraction of an orally ingested mycotoxin, in a certain food matrix, that finally reaches the systemic circulation and is distributed throughout the entire body to exert its toxic effect (Versantvoort, 2004). This definition assumes that toxicity is exerted by the parent compound and not by formed metabolites.

In fact, the oral bioavailability comprises three different and sequential processes (Brandon *et al.*, 2006):

a) the release of the mycotoxin from the food matrix during digestion in the GI tract. In this step we are measuring the bioaccessible mycotoxin (F_B).

b) the absorption of the bioaccessible mycotoxin through the intestinal epithelial cells of the GI tract (F_A), being transported to the blood (or lymph) stream.

c) the metabolism of the mycotoxin previous to systemic circulation (i.e., the biotransformation and excretion by the intestinal epithelium or the liver), the so-called first pass effect (F_M).

So, the equation that defines the bioavailable fraction of an ingested mycotoxin, that is, the fraction that reaches the systemic circulation, is defined by:

$$F = F_B \times F_A \times F_M \quad (1)$$

Bioaccessibility (B) has become important because it represents the amount of the mycotoxin that can reach the blood after intestinal absorption. It is worth to mention that bioaccessibility has been calculated only in *in vitro* systems. This concept is only applicable to oral exposure. Other routes of exposure do not depend on the process described above. Bioaccessibility is given in percentages and calculated with the following formula:

$$B\ (\%) = [\text{mycotoxin}_{\text{chyme}}] \text{ after GI digestion} / [\text{mycotoxin}_{\text{food matrix}}] \text{ before GI digestion} \quad (2)$$

Physiologically speaking, bioaccessibility refers to the amount of toxin that is liberated from the food matrix in the stomach and is available for absorption in the small intestine (Peijnenburg and Jager, 2003; Ruby *et al.*, 1996). Bioaccessibility (some times called *digestibility*) involves all the events that occur before intestinal and hepatic presystemic metabolism, and that take place during the digestion of food until the macronutrients and micronutrients can be assimilated into the cells of the intestinal epithelium (Fernandez-García *et al.*, 2009), and can be considered as an indicator for the maximal oral bioavailability of the toxin, which can be used for realistic worst case risk assessment of the toxin in a consumer product (Brandon *et al.*, 2006).

For a mycotoxin administered in solution, as it is with drinking water, the bioaccessibility of the toxin is assumed to be 100%. The toxin does not need to be mobilized from the matrix as it is already in solution and, thereby, available for absorption in the intestine. After ingestion of other matrices such as feed or food, the toxin may be partially or totally released from their matrix during digestion in the GI tract. Only the bioaccessible fraction is available for transport across the intestinal epithelium and can contribute to the internal exposure (Versantvoort *et al.*, 2004).

The food matrix mainly affects the bioaccessibility, whereas absorption and metabolism depend more on the toxin specific properties and on the animal physiology and, therefore, the food matrix is expected to have less influence on theses processes (Brandon *et al.*, 2006). Thus the bioaccessibility of a given mycotoxin can differ according to the considered food, as

has recently been demonstrated in the case of the bioaccessibilities of DON in different types of Italian pasta (Raiola *et al.*, 2012b), of BEA in wheat crispy breads with different fiber concentrations (Meca *et al.*, 2012b) and of PAT in different apple products (Raiola *et al.*, 2012a).

3. *In vitro* digestion models

As described before, the bioaccessibility depends on the mycotoxin and the food matrix considered. This implies that for health risk assessment of the more important mycotoxin-contaminated foods, it would be convenient to obtain food-specific results of oral bioavailability in order to better adjust the legal limits of different food groups. Thus, *in vitro* digestion models based on human or animal physiology have been developed, not only for mycotoxins, but also for other areas of application. Most researches have been devoted to investigation of bioaccessibility of food components and contaminants, but also of toxics on soils (Avantaggiato *et al.*, 2003; Boisen and Eggum, 1991; Brandon *et al.*, 2006; Döll *et al.*, 2004; Dominy *et al.*, 2004; Garret *et al.*, 1999; Gil-Izquierdo *et al.*, 2002; Larson *et al.*, 1997; Miller *et al.*, 1981; Minekus, 2005; Minekus *et al.*, 1999, Oomen *et al.*, 2003; Ortega *et al.*, 2009; Ruby *et al.*, 1999; Savoie, 1994; Versantvoort *et al.*, 2004, 2005).

Most of the *in vitro* digestion models simulate, in a simplified manner, the digestion processes in mouth, stomach, and small intestine, often obviating some physiological processes that occur during digestion, such as peristalsis or the existence of intestinal microbiota.

Digestion is a well known process in which the breaking down of food into smaller components that can be absorbed by the bloodstream and distributed throughout the body takes place. Briefly, in humans, and in general in the monogastric animals, digestion is a sequential process that begins with a mechanical and chemical digestion in the mouth, where food is chewed and mixed with saliva (rich in amylases) and where many polysaccharides are breaking down. Then, the stomach continues smashing the food and breaking food constituents mechanically and chemically with the aid of pepsine and some gastric lipases; mainly protein and peptide degradation takes place, although some lipolysis also occurs (Forte, 1996). In the small intestine, where absorption of nutrients is mainly conducted, the presence of lipids in the duodenum stimulates the secretion of bile salts, phosphatidylcholine, and cholesterol from the gall bladder and pancreatic fluids (containing pancreatic lipase/colipase, etc.) from the pancreas. Water and minerals are reabsorbed back into the blood in the colon (large intestine), together with some vitamins, such as biotin and vitamin K produced by bacteria (Conigrave and Young, 1996).

The *in vitro* digestion models try to mimic this layout, especially in the first three compartments of the GI tract (because mycotoxin absorption takes place mainly in the small intestine). Main parameters to control are temperature (although if simulates human GI tract all reactions are carried out at 37 ºC), time in each compartment, pH changes, ionic strength, gastric/intestinal juice composition and enzymatic activities.

The main features that are requested to an optimum *in vitro* dynamic digestion model are (Minekus, 2005; Versantvoort *et al.*, 2004;):

1) The model must be similar and representative of the physiological processes in the human body (or in the considered animal).
2) Digestion must be a dynamic process which helps to food disintegration and absorption. Biochemical reactions, flow (hydrodynamics) and mechanical forces must be in accordance with the kinetics of digestion. The rate of release (emptying) should be controlled for a quick or prolonged release.
3) The system should allow simulating fasted or fed conditions.
4) The model should include anaerobic conditions and presence of typical GI microbiota.

5) The methodology should be easy and applicable, robust and reproducible.

Most of the designed models attempt to fulfill with the first four requirements, the latter being the more difficult to achieve due to methodological complications related to the anaerobic assay conditions.

With regard to the type of models used, most models are static GI models which simulate the transit through the digestive tract by sequential (compartmentalized) exposure of the food to simulated mouth, gastric and small intestinal conditions. These models are a good first approximation to the problem, as they are of easy performance, and generally allow rapid processing of a large number of samples, but represent in a lesser extent the GI physiological reality.

On the other hand, dynamic GI models mimic the gradual transit of ingested compounds through the simulated compartments of the GI tract, giving a more realistic simulation. In these models, successive physiological conditions in the stomach and segments of the intestines of humans and animals are closely simulated. Digestion products and other small molecules are absorbed from the different intestinal compartments by dialysis. These models usually take into account factors as gastric emptying patterns, GI transit times in combination with changing pH values, variable concentration of electrolytes, enzymes and bile salts, absorption of water and, in some cases, microbial activity during passage of the food through the entire GI tract (Zeijdner *et al.*, 2004).

There are many biological conditions which differ from *in vitro* and *in vivo* systems. The best approximation to the composition of digestive juices ensures better mimicked digestion in the GI tract and the results may be approximate to those observed *in vivo*. However, in some cases the results do not agree with those observed in *in vivo*, as will be seen below for FB_1 *in vitro* simulation.

3.1. Main physiological components of the in vitro models.

3.1.1. Saliva.

Digestion is a physiological process that starts in the mouth with a mechanic action during which salivary fluids initiate the hydrolytic processes, the central nervous system is stimulated and the cephalic phase of digestion is initiated. Salivary fluid is an exocrine secretion consisting of approximately 99% water, containing a variety of electrolytes (sodium, potassium, calcium, chloride, magnesium, bicarbonate, phosphate) and proteins, including enzymes (mainly amylases and, in a minor extent, lipases), immunoglobulins and other antimicrobial factors (lysozyme, peroxidase systems, lactoferrin, histatins and agglutinins), hormones, mucosal glycoproteins, traces of albumin and some polypeptides and oligopeptides of importance to oral health. There are also glucose and nitrogenous products, such as urea and ammonia (Rantonen, 2003).

Saliva production is stimulated by visual and olfactory stimuli, sour taste, chewing and the presence of food particles in the mouth. Composition of saliva depends on the flow rate: at higher flow rates, sodium, calcium, chloride, bicarbonate and amylases increase, whilst phosphate concentrations and mucin decrease, and the potassium concentration show little change (Versantvoort *et al.*, 2004).

The salivary α-amylase, which acts as an endoglycosidase, hydrolyzes starch and related R-1,4-linked polysaccharides starting the starch digestion and its transformation to oligosaccharides and monosaccharides.

The lingual lipase is a triacylglycerol lipase that hydrolyzes dietary lipids on the carboxylic ester to produce diglycerols. This hydrolysis continues in the stomach by the gastric lipase,

but the activity of lingual lipase has been described between pH 2 and 6.4, indicating that the lingual lipase is active from the mouth to the small intestine where the activity decreases at pH 6.9, while gastric lipase is still active in the intestine (Liao *et al.*, 1984).
Mucin lubricates oral surfaces, provides a protective barrier between underlying hard and soft tissues and the external environment, and aid in mastication, speech and swallowing.
Saliva behaves as a buffer system to protect the mouth. Urea acts as a buffer present in total salivary fluid; it is a product of aminoacid and protein catabolism that causes a rapid increase in biofilm pH by releasing ammonia and carbon dioxide when hydrolyzed by bacterial ureases.
In the main *in vitro* models, simulated saliva consists of a simplified version of this complex biological fluid, containing electrolytes (KCl, KSCN, NaH_2PO_4, $NaSO_4$, NaCl and $NaHCO_3$), urea and α-amylase (Gil-Izquierdo *et al.*, 2002), whereas other models also uses uric acid and mucin (Versantvoort *et al.*, 2005). Generally, the pH value used in this fluid is around 6.8.
However, salivary digestion is omitted in some *in vitro* models, as the TIM-1 dynamic gastrointestinal model (Minekus *et al.*, 1995), because it is considered that it does not represent great changes in matrices or components of interest.

3.1.2. Gastric juices

The gastric phase is activated when the acid secretion begins and finishes when the stomach contents reach the duodenum to start the intestinal phase. Gastric juice is secreted by the gastric glands of the stomach, and its production is regulated through specific neural and hormonal pathways, by the eating act and by the presence of food in the stomach. In the adult human, the stomach typically secretes about 2-3 liters of gastric juice per day. The three major constituents of gastric juice are the mucus, the enzymes and the aqueous component, the production of hydrochloric acid being a key factor as produces a significant drop in pH values (Forte, 1996).
Gastric mucin is a large glycoprotein which is thought to play, together with $NaHCO_3$ secretion, a major role in the protection of the gastrointestinal tract from acid, proteases, pathogenic microorganisms, and mechanical trauma.
The main enzyme in gastric juice is pepsin, although other enzymes like the gastric lipase are also present. Pepsin is actually a heterogeneous group of endoproteases responsible for the proteolytic activity of gastric juice (Forte, 1996). Gastric juice lowers the pH of the gastric content, due to secretion of hydrochloric acid. Gastric pH values have been reported between 1 and 3 (Allen and Flemström, 2005; Jolliffe *et al.*, 2009; Kong and Singh, 2009), while during digestion the pH can increase up to 7.5 (Kong and Singh, 2009). This pH change is important for the activation of pepsinogens precursors (Vertzoni *et al.*, 2005) and to stabilize pepsin that has optimal proteolytic activity in the same pH range (i.e., pH 1-3).
When gastric juice is neutralized as it passes into the duodenum, pepsin is denatured and thus eliminated from further digestive function.
The lipids are emulsified and micellizated in the stomach and the small intestine, respectively (Carey *et al.*, 1983). The acidic pH optimum for lipolysis is from 3.5 to 6.0 and lipase activity achieves a wide range of pH which allows the enzyme to act in the stomach where the postprandial pH is from 4.5 to 5.5. In the small intestine the pH range is between 5.0 and 6.5 and lipase activity is proportional to the bile concentration after ingestion (Liao *et al.*, 1984).
In the *in vitro* models, gastric juice is often simulated only with a strong decrease of the pH, but more complete systems include gastric juices containing pepsin (Gil-Izquierdo *et al.*, 2002), and also several electrolytes (NaCl, KCl, $CaCl_2$, $NaHCO_3$), a lipase and bovine trypsin (Versantvoort *et al.*, 2005). The gastric juice used in the TIM-1 dynamic gastrointestinal model (Minekus *et al.*, 1995), one of the more complete, includes pepsin, mucin, glucose,

glucuronic acid, urea, glucoseamine hydrochloride, BSA and several salts (NaCl, NaH_2PO_4, KCl, $CaCl_2 \cdot 2H_2O$, NH_4Cl and HCl).

The pH value of the gastric juice during the *in vitro* GI simulation varies between models. In some cases the pH is a constant value, usually low (1.3-2) (Gil-Izquierdo *et al.*, 2002; Versantvoort *et al.*, 2004), but in other models pH values decrease during simulation from higher values (7-5) to lower values at the end (3-2), in a gradual or discontinuous way (Döll *et al.*, 2004; Minekus *et al.*, 1995). The gastric pH value is a crucial factor in the bioaccessibility determination as it is essential for the activity of pepsin, enzyme that can contribute to the release of hidden mycotoxins from proteins of the food matrix (Dall'Asta *et al.*, 2009). However, some conjugated mycotoxins, as the DON-3-glucoside are resistant to acidic conditions, thus it is extremely unlikely that this compound can be hydrolyzed into DON in the stomach (Berthiller *et al.*, 2011).

3.1.3. Intestinal juices.

Following gastric digestion, the stomach releases food into the duodenum through the pyloric sphincter. Duodenum receives pancreatic enzymes from the pancreas and bile from the liver and gallbladder. These fluids are important in aiding digestion and absorption. Peristalsis also aids digestion and absorption by churning up food and mixing it with intestinal secretions. The rest of the small intestine, located below the duodenum, consists of the jejunum and the ileum. These parts of the small intestine are largely responsible for the absorption of fats and other nutrients. The intestinal wall releases mucus, which lubricates the intestinal contents, and water, which helps dissolve the digested fragments. The main components of all the biological fluids implied in the intestinal digestion of food are:

- The exocrine pancreatic secretions, which mainly contain pancreatic enzymes as proteases (in zymogenic form, including trypsinogen, chymotrypsinogen, procarboxypeptidase), lipases (that degrade triglycerides into fatty acids and glycerol), cholesterol esterase, phospholipase, nucleases and amylase.
- The liver secretes bile and bicarbonate into the small intestine. The bile secretion contains bile salts, lecithin, cholesterol and bilirubin.
- The small intestine secretes watery mucus (that protects the intestinal mucosa from auto-digestion by proteases and acid) and hormones (like secretin, somastotin, motilin, cholecystokinin and the gastric inhibitory peptide). In the small intestine there are numerous "brush border" enzymes whose function is to further cleave the already-broken-down products of digestion into absorbable particles. Some of these enzymes include: sucrose, lactase, maltase and other disaccharides.
- The large intestine secretes mucus (for lubrication and mechanical protection), and bicarbonate and potassium ions (for protection from bacterial acid).

Given this high number of chemical compounds forming part of the intestinal secretions, it is understandable that *in vitro* models must simplify the components of the intestinal tract to the main components, especially those related with enzymatic properties. Thus *in vitro* small intestine simulation basically includes the main duodenal electrolytes (among which the calcium salts are very important), pancreatin (a mixture of several digestive enzymes produced by the exocrine cells of the pancreas: amylase, lipase and protease) and bile salts (Gil-Izquierdo *et al.*, 2002; Minekus *et al.*, 1995). Complementarily, some models add other components as mucin, lipases, BSA and urea (Versantvoort *et al.*, 2005).

Calcium ion (Ca^{2+}) acts as a co-factor required for enzymatic activity at a low concentration, as 40 mM is enough to make, for example, lipolysis to increase (Brownlee *et al.*, 2010; Kimura *et al.*, 1982). This ion is usually added in simulated intestine digestion systems as $CaCl_2$, at concentrations ranging from 0.3 to 22.2 g/L (see Table 1).

Gastrointestinal enzymes generally have a greater resistance to irreversible denaturation, but in the different parts of the intestine of healthy subjects there can be a broad pH range, which can vary from pH 5.9 to pH 9 (Brownlee *et al.,* 2010). pH values employed for the *in vitro* intestinal fluids used with mycotoxins have ranged from 6 to 8.2.

Bile salts have a severe impact on bioaccessibility, as they have the capacity of surfacting and decreasing the surface tension, consequently, creating an apolar environment in the interior of bile salt micelles for hydrophobic contaminants and thereby increase their solubility (Oomen *et al.,* 2004).

3.1.4. Microbial interactions.

The interaction of digestive microbiota with mycotoxins has been predominantly studied in models simulating the rumen of ruminants. Thus, *in vitro* metabolism of different mycotoxins (AFB_1, AFG_2, DON, diacetoxyscirpenol and T-2 toxin) by bacterial, protozoal and ovine ruminal fluid preparations has been determined by Westlake *et al.* (1989). Mobashar *et al.* (2012) have recently studied the contribution of the different microbial populations of rumen on OTA degradation using the *in vitro* Hohenheim gas incubator system. Authors have found that in contrast to the opinions in many publications, the bacterial (and not the protozoal) community played the dominant role in ruminal OTA degradation. Similarly, *in vitro* ruminal degradation of AFB1 has been described (Jiang *et al.*, 2012).

However, the most commonly *in vitro* models used for determination of bioaccessibility of mycotoxins in human or monogastric systems ignore what happen in the large intestine, as it is in the small intestine where absorption of mycotoxins occurs. Besides, simulation of the large intestine, for its implications in fermentation processes that take place on this location, necessarily involves the use of microbiota of human or animal origin, under anaerobic conditions, making more difficult the operating procedure.

Laboratory systems to simulate the large intestine have been used successfully (Minekus, 2005), but they generally do not combine physiological concentrations of metabolites with physiological numbers of fecal microorganisms. The TIM-2 large intestinal system developed at the TNO Nutrition and Food Research Center (The Netherlands) simulates human and monogastric large intestine, and use a complex, metabolic active microbiota of human origin. The fecal inoculum used contains a mixture of total anaerobes, facultative anaerobes and methanogenic bacteria, as well as Enterobacteriaceae, *Bacteroides*, *Bifidobacterium* and *Lactobacillus*. This model is kept always under anaerobic conditions by flushing with nitrogen (Minekus *et al.,* 1999). At the moment, and to our knowledge, this system has not been used with mycotoxins, unlike what happened with its brother system, the TIM-1, which simulates the small intestine.

The effect of different probiotic bacteria on the bioaccessibility of AFB_1 and OTA (Kabak *et al.*, 2009) and the main four AFs (Kabak and Ozbey, 2012b) has been studied using the *in vitr*o model developed by Versantvoort *et al.* (2005), but in this case the bacteria used (different *Lactobacillus* and *Bifidobacterium* strains) were employed as mycotoxin adsorbents in a system that only simulates the stomach and small intestine. Thus no physiological interactions were expected and anaerobic conditions were not employed.

3.1.5. Other factors: temperature, peristalsis and transit (incubation) time.

Temperature used for *in vitro* bioaccessibility assays of mycotoxins always simulates the physiological temperature of the human being, so 37 ºC has been the selected temperature for all the studies.

Regarding gastrointestinal movements, the mechanical action and hydrodynamic flow created by the contraction waves of the stomach muscles also play a critical role in gastric digestion. Movements in the stomach are agitation mechanics that help to mix the food with the components of gastric juice to obtain a homogeneous bolo. Stomach contractions generate a fluid flow of the gastric contents that cause a shearing effect on the food surface. Similarly, in the small intestine peristaltic waves not only move food along the intestine, but also mix the food chyme to help in the digestive process. In the *in vitro* models usually used with mycotoxins these physiological movements are simulated in different ways, as the use of orbital shakers (Gil-Izquierdo *et al.*, 2002), head-over-heels rotation (Versantvoort *et al.*, 2004), movement of tubes in a water bath (Döll *et al.*, 2004) or the employment of peristaltic valve pumps (Minekus *et al*., 1995). The influence of the movement system selected has not been properly evaluated in the bioaccessibility assays conducted with mycotoxins.

The gastric emptying is regulated by different factors as the volume of the meal, its osmotic pressure and the caloric content of the food. Gastric emptying is also the final expression of complex hormonal interactions and digestive electrical signal driven by the neuronal net and juice secretions. The half-emptying time in healthy subjects occurred around 90 minutes (in a range from 60 to 277 minutes), although there are great differences between solid and fluid meals (Versantvoort *et al.*, 2004). However, after a heavy meal completely emptying of the stomach can take up to 16 hours (Davenport, 1984). Incubation time in the stomach conditions usually used in *in vitro* models ranged from 2 to 4 hours.

With regard to residence time in the small intestine, the mean transit time is of the order of 3 hours (range 1 to 6 hours), 2-3 hours being the time usually employed in the *in vitro* models.

3.2. In vitro models used in bioaccessibility or absorption studies on mycotoxins.

Related to mycotoxins, one of the sequential discontinuous models most frequently used has been that developed by Gil-Izquierdo *et al.* (2002), a modification of a previous method described by Miller *et al.* (1981) to study iron bioavailability. This method, with slight modifications (Meca *et al.*, 2012ab; Raiola *et al.*, 2012ab), consists of two sequential steps: an initial saliva/pepsin/HCl digestion for 2h at 37 ºC to simulate the mouth and the gastric conditions, and a second digestion with bile salts/pancreatin for 2.5h at 37ºC to simulate duodenal digestion.

Similarly, the method proposed by Versantvoort *et al.* (2004), and known as RIVM model, has been widely used. This system comprises a three-step procedure simulating digestive processes in mouth, stomach and small intestine. In each compartment digestion takes place after addition of simulated physiological juices and incubation at 37 ºC for a time relevant for the considered compartment. The system has been employed mainly to simulate human GI tract (Brandon *et al.*, 2006; De Nijs *et al.*, 2012; Kabak *et al.*, 2009, 2012ab; Motta and Scott, 2009; Versantvoort *et al.*, 2004, 2005). A very similar compartmentalized approach, but simpler in terms of composition of simulated physiological juices, has been described by Gawlik-Dziki *et al.* (2009), and has been used for AFB1 bioaccessibility calculations (Simla *et al.*, 2009).

Döll *et al.* (2004) developed an *in vitro* model to simulate the effect of the GI tract on DON and ZEA and some potential detoxifying agents. The *in vivo* conditions (pH, temperature and transit time) mimic the porcine GI tract as reviewed by Dänicke *et al.* (1999) and Clemens *et al.* (1999). The system consists in a sequential incubation of the mycotoxins in (0.1 M, , pH 5) phosphate-citrate buffer during 2 h followed by a pH decrease to 3.0 using 300 µL of ortho-phosphoric acid (85%), pH value that is maintained during 2 h (4 h in all, simulates gastric digestion). Thereafter, the pH is increased to 6.0 by adding 600 µL NaOH (12 M) and

incubation for 3 h after adjusting the pH to 7.0 with 400 µL NaOh (12 M). All the incubations are carried out in a water bath regulated at 37 ºC.

The dynamic *in vitro* gastrointestinal model developed at the TNO, known as TIM model has been widely used in the determination of absorption of mycotoxins and other contaminants (Minekus, 1998; Minekus *et al*, 1995, 1999). TIM model is a multi compartmental, continuous, dynamic, computer-controlled system which closely simulates the *in vivo* conditions of the stomach and small intestine (TIM-1) and large intestine (TIM-2) of humans and monogastric animals. The system simulates the peristaltic movements in the GI tract, mixing and moving the contents gradually through the stomach and the intestine. This system allows the simulation of the gastric empyting and intestinal transit times, and a computer-controlled system introduces the simulated salivary, gastric, biliary and pancreatic secretions at the appropriate moment. Absorption of water and digested food compounds from the small intestinal compartment is achieved by the use of hollow fiber membrane systems that are hooked up at the middle and end part of the small intestine simulating the jejunum and ileum (Zeijdner *et al.*, 2004). In the large intestinal model (TIM-2) a complex metabolic active microbiota of human origin performs the fermentation of undigested food components (Minekus, 2005). The system has been used simulating the human situation for babies, young adults and elderly people (Dominy *et al.*, 2004; Minekus, 1998; Oomen *et al.*, 2002) and, on the other hand, the dog, pig and calves situation (Avantaggiato *et al.*, 2003, 2004, 2005, 2007; Minekus, 1998; Smeets-Peeter *et al.*, 1998, 1999). This system seems to be one of the more complete and realistic of those available, but it has also some limitations as there are no mucosal cells inside the model, there is no immune system and there are no real feed back mechanisms, except for pH and intestinal water absorption (Zeijdner *et al.*, 2004).

Table 1 shows the main methods used to evaluate the bioaccessibility or absorption of mycotoxins, as well as their main characteristics.

[Insert Table 1 in this place, if possible]

4. *In vitro* bioaccessibility/absorption data of mycotoxins

Although simulated gastric and intestinal fluids have been used extensively in the evaluation of the stability of the adsorbent-mycotoxin union (Ramos, 1996a; Scheideler, 1989), it was not until the development of the *in vitro* GI models described previously that effective evaluation of the bioaccessibility/absorption of mycotoxins in different food matrices has begun. In the next sections, available data on bioaccessibility or absorption of mycotoxins, calculated using the *in vitro* models previously explained, will be described (Tables 2 and 3).

[Insert Table 2 in this place, if possible]

4.1. Aflatoxins.

In 2003, Zeijdner *et al.* using the TIM-1 GI *in vitro* model tested the absorption of AFB_1 in an experiment designed for the evaluation of the efficacy of a natural magnesium smectite in pig GI tract. Considering only the data from the control, without adsorbent, it could be observed that in a contaminated feed with 20 µg/kg AFB_1 (65% natural contamination), the amount of mycotoxin absorbed was of 47% (Zeijdner *et al.*, 2003, 2004). This result was confirmed by Avantaggiato *et al.* (2007), using the same *in vitro* model trying to assess the efficacy of a carbon/aluminosilicate-based product on a multicontaminated feed containing AFB_1, FB_1, FB_2, DON, ZEA and OTA, as a 46% absorption for AFB_1 was observed, although the initial level of contamination was almost 10-fold higher (193 µg/kg). The AFB_1 absorption occurred

mainly (approx. 75%) at the upper part of the system (simulating jejunum) and less at the ileum.

Shortly after, Versantvoort *et al.* (2004), in the first document where the term "bioaccessibility" is applied in the field of mycotoxins, described the bioaccessibility of AFB_1 from peanut slurries using the RIVM model for GI human tract simulation. Data obtained using a low amount of AFB_1 (3 ng AFB_1, from 0.5 g of a peanut slurry contaminated at 6 µg/kg) or a higher amount (27 ng AFB_1, from 4.5 g of a peanut slurry containing 6 µg/kg) showed similar results of bioaccessibility, ranging between 80 and 81%, considerably higher than the previous works. The bioaccessibility of AFB_1 from 9 peanut slurries ranging from 0.6 to 14 µg/kg in the chyme (1.5 to 36 µg/kg contamination level of peanuts) was determined and it was observed that a more or less constant bioaccessibility percentage of 90% at each contamination level was found. The bioaccessibility rose to 104-111% when AFB_1 was in a food mix containing a standard meal plus the peanut slurry (1 and 3 ng AFB_1 in the digestion model). To test the robustness of the digestion model, some changes were applied, as the decrease of the pH in the small intestinal compartment, the prolongation of the incubation time in the small intestine section (4h instead 2h), or doubling the bile concentration, but none of these variations affected the bioaccessibility of AFB_1 from the food mix. This data were confirmed with very slight modifications (83-84% bioaccessibility in peanut slurries, 108-115% in food mix) shortly thereafter by the same authors (Versantvoort *et al.*, 2005), and by Simla *et al.* (2009) in ground corn (92.5-98.1% bioaccessibility) and peanut (91.2-97.0% bioaccessibility).

Kabak *et al.* (2009), who also used the RIVM in vitro model found similar data, with an AFB_1 bioaccessibility that ranged from the 86% in naturally contaminated pistachio nuts, to the 88-94% in artificially contaminated infant food. These authors also found that the bioaccessibility of AFB_1 could be reduced in a 37% by the addition of a probiotic bacteria (*Lactobacillus acidophilus* NCC12). Although other probiotics were used (from *Lactobacillus* and *Bifidobacterium* genera), results were not conclusive when the standard deviations were taken into account.

These authors extended their studies on AFs bioaccessibility to the four main Aflatoxins (Kabak and Ozbey, 2012) and to AFM_1 (Kabak and Ozbey, 2012a). In the first case, a wide range of artificially contaminated food matrices were assayed (peanut, pistachio, hazelnut, dried figs, paprika, wheat and corn) finding bioaccessibilities that ranged from 85.1-98.1% for AFB_1, 83.3-91.8% for AFB_2, 85.3-95.1 for AFG_1 and 80.7-91.2% for AFG_2. The bioaccessibilities of all four toxins were independent of the 3 spiking levels (2, 5 and 10 µg/kg for AFB_1 and AFG_1, and 0.6, 1.5 and 3 µg/kg for AFB_2 and AFG_2) and the 7 different food matrices assayed. The highest AFB_1 bioaccessibility value was reported in dried figs (average 94.4%), whereas the lowest was detected in wheat samples (average 87.2%). Again, these authors studied the effect of the addition of probiotic bacteria (from *Lactobacillus* and *Bifidobacterium* genera), finding a bioaccessibility reduction that could reach in the best situation a 35.6% reduction.

With regard to AFM_1, results found in spiked and naturally contaminated UHT milk, with contamination levels ranging from 0.011 to 0.939 µg/L, showed bioaccessibilities around 80.5-86.3% (Kabak *et al.*, 2012a). There were no significant differences among the spiked and naturally contaminated milk samples.

Assuming that the bioaccessibility of AFs, including AFM_1, would be around 80-90%, depending on the matrices and contamination levels considered; it can be concluded that AFs are almost completely released from the food matrices during digestion, implying a high toxicological risk.

4.2. Ochratoxin A.

At present, there are few studies on the bioaccessibility of OTA and its relation to different food matrices. Versantvoort *et al.* (2004), using the RIVM model, determined the OTA bioaccessibility in two lots of buckwheat, finding that a considerable amount of OTA, above 45%, was released from the food matrix. Bioaccessibilities seemed to be not dependent of the amount of food in the digestion model, the presence of other components in the food matrix or some variations in parameters of the *in vitro* model (as lowering the intestinal pH, prolongation of the transit time or doubling the bile concentration). Values obtained ranged from 45% (in a buckwheat batch contaminated at 20 µg/kg) to 84% (in a buckwheat batch contaminated at 7 µg/kg).

However, in 2005, the same authors using the same experimental design reported bioaccessibilities of OTA considerably higher. Thus, values ranging from 86 to 116% were described, although in this second report the amount of mycotoxin used in the digestion model was lower (Versantvoort *et al.*, 2005).

Those data agree with the results later reported by Avantaggiato *et al.* (2007) who found, using the TIM-1 model with an artificially OTA contaminated corn feed, absorptions near 88%. Most of the OTA was released from the food matrix in the jejunal dialysate, showing a high absorption in the upper part of the small intestine, whereas in the ileal dialysate absorption of the ingested OTA was limited to approximately 15%.

However, Kabak *et al.* described again in 2009 much lower values for OTA bioaccessibility (22-32%) using the RIVM model with naturally OTA contaminated buckwheat or with low- and high-contaminated spiked infant foods. Bioaccessibilities found seemed not to be contamination level dependent, but it seemed that bioaccessibility increased if spiked contamination was employed, as in this case the toxin may not be tightly bound to the food matrix. Besides, the authors suggested that the difference found between the bioaccessibility of AFB_1 and OTA (AFB_1:OTA ratio at 3:1, according to their data) should be taken into account in risk assessment, as AFB_1 presents a higher toxicological risk.

It seems obvious that more data are required to more accurately assess OTA bioaccessibility. The effect that OTA contamination level, type of contamination (natural *vs.* spiked) and food matrix exerts in bioaccessibility must be more profoundly studied in this mycotoxin.

4.3. Patulin.

Raiola *et al.* (2012a) determined the bioaccessibility of PAT in apple juices and purees by using an *in vitro* model based on the sequential steps of GI digestion defined by Gil-Izquierdo *et al.* (2002) (gastric and duodenal digestion), pepsin, pancreatin and bile extract used were demineralised using a Chelex-100 resin, as described by Jovaní *et al.* (2004). Unlike the original Gil-Izquierdo method, a brief salivary digestion was added and no dialysis of the pepsin digest was carried out. Commercial PAT-free apple products were contaminated with 50 µg PAT/L (juices) or 25 µg PAT/kg (purees) and digested. A higher bioaccessibility was observed in apple juices with pulp (67.3-70.9%), followed by puree samples (55.7-58.2%). Apple and pear nectar (38.9%) and clarified apple juices (25.3-28.6%) showed much lower bioaccessibilities. The authors suggested that these data should be taken into account in the risk assessment of this toxin, as high bioaccessibilities could be found in apple juices, frequently ingested by children over 3 years.

Similar high PAT bioaccessibilities were found by Brandon *et al.* (2012) in apple products using the RIVM digestion model. The bioaccessibility of PAT was assayed using two different amounts of food per digestion tube (2.0 and 4.5 g, representing half a meal and a normal amount of dinner). Home-made apple sauce made from apples contaminated with *Penicillium expansum*, and spiked apple sauce and baby fruit were assayed alone or in

combination with other foods as yoghurt, biscuits or a standard Dutch dinner. Level of PAT contamination ranged from 99.8 (spiked foods) to 110-485 µg/kg (naturally contaminated). Results showed that bioaccessibility was similar for the two amounts of food and that bioaccessibility values were high, ranging between 55 and 100%.

[Insert Table 3 in this place, if possible]

4.4. Fusarium mycotoxins.

4.4.1. Zearalenone.

Avantaggiato *et al.* (2003) using the TIM-1 model that simulates the porcine gastrointestinal tract determined the GI absorption of ZEA. Contaminated feed (820 µg ZEA/kg), containing in its composition artificially contaminated wheat, was pumped into the GI-model. Intestinal absorption of the food-released ZEA was measured analysing the dialysates at the jejunal and ileal locations of the system. A 32% absorption was observed at the jejunal+ileal compartment, although absorption occurred mainly in the jejunal part of the model (22%). Absorption at the stomach+duodenal compartment was considerably lower (4%). Authors considered that almost all the ZEA released from the food matrix during digestion was rapidly absorbed, a result consistent with the *in vivo* data obtained by Ramos *et al.* (1996b) in rats that showed a high rate of ZEA absorption through the intestinal lumen by a passive process.
These results were confirmed by Zeijdner *et al.* (2004) using a naturally contaminated feed (100 µg ZEA/kg). Feed was digested in a TIM-1 model during six hours, obtaining again a 22% of absorption in the jejunal compartment and a 10% in the ileal compartment.
Avantaggiato *et al.* (2007) reported an absorption of 25% (16% jejunum; 9% ileum) using a naturally contaminated feed containing 1.3 mg ZEA/kg. The authors concluded that neither the level of ZEA in the feed nor the origin of contamination (natural or spiked) has an effect on the intestinal absorption of ZEA, setting an average value of 28% of the matrix-released mycotoxin. On the other hand, absorption of ZEA in the TIM-1 model was lower and slower than the observed for other mycotoxins as FBs, DON, OTA or AFB_1. Consequently, ZEA absorption took place in the distal part of the small intestine, with a higher absorption in the ileum compartment compared with the rest of mycotoxins.

4.4.2. Deoxynivalenol and nivalenol.

Simultaneous absorption of DON and NIV was evaluated using the porcine TIM-1 model by Avantaggiato *et al.* (2004). A meal containing DON and NIV spiked ground wheat was employed for absorption determination. Contamination level of the meal was 560 µg DON/kg and 760 µg NIV/kg. Results showed that both toxins were simultaneously absorbed in the small intestine, DON absorption being 2.4 times higher that NIV uptake. A total absorption of 51% was observed for DON, 44% of which takes place in the jejunum and the rest in the ileum compartment. On the other hand, NIV showed a 21% absorption, 18% of which corresponding to jejunum. The authors suggested that the higher absorption of DON with regard to NIV (ratio 5:2) may be due to its higher hydrophobicity.
However, using the same *in vitro* model, higher values for DON absorption (68% at jejunum; 6% at ileum) were found later using 10-fold naturally contaminated grains (Avantaggiato *et al.*, 2007).
Recently, Raiola *et al.* (2012b) determined the bioaccessibility of DON in pasta samples using the human *in vitro* model described by Gil-Izquierdo *et al.* (2002), slightly modified. In most of the samples, the *in vitro* model was adjusted to simulate child digestion (higher gastric pH,

reduced amount of pepsin, pancreatin and bile salts) and in this case the mean value for DON bioaccessibility was 19.5% (ranging from 2.12% to 38.41%) for the gastric compartment and 9.7% for the duodenal compartment (range 1.11-17.91%). In one sample where adult digestion was simulated, the bioaccessibilities found were considerably higher (32.81% average; 41.49% for gastric and 24.13% for duodenal compartments). Although bioaccessibilities found in the adult model were higher, authors postulate that, considering the small dimension of the child intestinal tract, the released DON present in this place could probably produce more damage to the intestinal enterocytes respect to that in an adult.

Regarding masked mycotoxins, a conjugated form of DON, the DON-3-ß-D-glucoside (DON-3-G) could be present in DON-contaminated plants, as glucosylation represents a major route to detoxify xenobiotics for plants. In fact, it has been proven that in some cases mean DON-3-G contamination exceeded the DON contamination (Sasanya *et al.*, 2008). A major concern is the hydrolysis of the DON-3-G conjugate back to its toxic precursor DON during gastrointestinal passage. This fact could clearly influence the real bioaccessibility value of this toxin in a food from which only DON contamination is known. For this reason DON reversion from DON-3-G was determined using an *in vitro* experiment that mimics the digestion conditions at the GI tract (acid hydrolysis of DON-3-G using 0.02-0.2 M HCl, pH 1.7-0.7; artificial stomach juice containing pepsin; artificial non-microbial gut juice containing pancreatin) (Berthiller *et al.*, 2011). Results showed that neither the acidic hydrolysis nor the GI enzymatic simulations resulted in DON regeneration. However, metabolic activity of some usual GI bacteria could produce the hydrolysis of DON-3-G to DON. Several *Enterococcus* species (*E. durans*, *E. faecium* and *E. mundtii*), but also *Enterobacter cloacae*, *Lactobacillus plantarum* and *Bifidobacterium adolescentis* were able to cleave DON-3-G in the semi-anaerobic conditions assayed, releasing up to values from 6 to 62% of DON after 8h, depending of the considered species. Similarly the hydrolytic enzyme cellobiase produces a 73% DON-3-G hydrolysis in 18h, suggesting that DON- 3-G could be cleaved and DON released in the GI tract of plant-based cellulose-foraging ruminants (Berthiller *et al.*, 2011).

Once the possibility of a GI reversion of DON-3-G was stated, De Nijs *et al.* (2012) calculated the possible transformation of this masked mycotoxin to DON using the RIVM *in vitro* digestion model. Thus, an infant formula with 2778 µg DON-3-G/kg was introduced into the system, but DON was not detected in the chyme of the digested samples (this means that, in theory, less than 5% of DON-3-G was hydrolysed to DON). The level of DON-3-G detected after digestion was 55% (that is, the bioaccessible fraction). When samples were only spiked with DON (2222 µg/kg) the amount of DON detected after digestion was 65%, and DON-3-G was not detected. These results confirm the previous data, but as this model does not consider the existence of intestinal microbiota, the question of whether the DON-3-G hydrolysis at this location would lead to an increase in the DON uptake remains unanswered.

4.4.3. Fumonisins.

Absorption of FB_1 and FB_2 were determined by Avantaggiato *et al.* (2007) in a naturally contaminated blend of grains by using the TIM-1 *in vitro* model. Absorption of both mycotoxins takes place mainly in the simulated jejunum (87% FB_1, 75% FB_2) and in a lesser extent in the ileum (17.3% FB_1, 13.9% FB_2). Thus, average intestinal absorption was 104% for FB_1 and 89% for FB_2, indicating that release of FBs from the food matrix and, consequently, absorption in the simulated intestinal tract is an almost complete fact and that absorption of bioaccessible fumonisins would take place mainly in the upper part of the small intestine. On the other hand, maximum absorption occurred in the first 2 hours of digestion. These results do not agree with those from *in vivo* experiments, as it is known that FB_1 has

very species-specific toxicity and a very low bioavailability when administered orally, resulting from low uptake coupled with efficient biliary excretion (Shier, 2000). Low oral FB_1 bioavailability has been demonstrated in swine (3-6%) (Prelusky *et al.*, 1994) and laying hens (0.7%) (Vudathala *et al.*, 1994), whereas in cows no FB_1 or known metabolites were found in the plasma of orally administered cows, indicating no or very limited bioavailability in ruminants (Prelusky *et al.*, 1995). These discrepancies between the results obtained *in vitro* and highlight the limitations of *in vitro* models.

It has been hypothesized that the totally hydrolyzed FB_1 metabolite HFB_1 is the real molecule involved in fumonisin toxicity, is more polar than FB_1 and *in vivo* studies demonstrated its higher absorption in rats (Hopmans *et al.*, 1997). However, recent *in vivo* studies wih piglets have shown that FB1 hydrolysis strongly reduces its toxicity both in the GI tract and the liver (Grenier et al., 2012) these findings suggest that HFB1, rather than being the molecule implicated in FB1 toxicity, represents a mechanism for detoxification. Effects of HFB1 on the intestine have been poorly studied, and most of the data have been obtained from in vitro studies with intestinal cell lines.

It has been demonstrated that although HFB_1 could be absorbed in a dose-dependent manner by the human colon adenocarcinoma Caco-2 cells, while FB_1 was not absorbed by human colon adenocarcinoma Caco-2 monolayer of cells. In spite of this, HFB1 was not accumalated inside the cells, and no viability alterations or barrier damage was observed (De Angelis et al., 2005). Similarly, in vivo HFB1 ingestion assays showed that this compound did not induce toxic effect in the piglet intestine, as intestinal integrity was not altered and intestinal immunity was not compromised (Grenier et al., 2012).

Dall'Asta *et al.* (2009) addressed the problem created by the presence of hidden (masked) FBs in the food matrix, as we explained before regarding DON-3-G. With regard to extractable FBs (expressed as the sum of FB_1, FB_2 and FB_3) an increase of 30-50% in FB concentration was observed after *in vitro* digestion of naturally contaminated corn flours using the RIVM model, which greatly affects the bioaccessibility determination. The authors suggested that the binding mechanism of FBs in raw corn is mainly due to an association with macromolecules such as starch and proteins. Additionally, no hydrolyzed or partially hydrolyzed FBs were found in the chyme. Besides, the total FBs found after digestion are slightly higher than those obtained after an alkaline hydrolysis, suggesting that masked FBs are native forms, which can be released under conditions found in the GI tract. As a clear example of how the presence of masked mycotoxins could affect bioaccessibility measurements, in a reference material with a declared FBs contamination of 3036 µg/kg, an amount of 8010 µg/kg of free FBs was detected after the *in vitro* digestion (Dall'Asta *et al.*, 2009).

Similarly, Motta and Scott (2009) studied the bioaccessibility of total bound FB_1 (TB FB_1) in naturally contaminated corn flakes using the RIVM model. Corn flake samples assayed had a low level (23 µg/kg TB FB_1) or high level (92 µg/kg TB FB_1) of contamination. Results showed that in the case of the high level contaminated corn flakes a 51% (range 37-64%) bioaccessibility of TB FB_1 was found, showing that masked mycotoxins could be bioaccessible for absorption at the small intestine. In addition, authors suggested that TB FB_1 could be a substrate for the intestinal bacteria and could be also hydrolyzed to regenerate the FB_1 or to create other hydrolyzed metabolites, increasing the possibility of exposure to this group of toxins after ingestion.

4.4.4. Emerging *Fusarium* mycotoxins.

Fusarium species are also responsible for the production of another group of bioactive compounds considered as "minor" mycotoxins. This group includes enniatins (ENA, ENA1,

ENB and ENB1), fusaproliferin and beauvericin (Meca *et al.*, 2010). All of these toxins belong to the group of "emerging" mycotoxins, which are neither routinely determined, nor legislatively regulated; however, the evidence of their incidence is rapidly increasing.

Enniatins represent an emerging food safety issue because of their extensive incidence, documented in recent decades, in various small grain cereals (Santini *et al.*, 2012). Bioaccessibility of ENNs has been evaluated by the *in vitro* method of Gil-Izquierdo *et al.* (2002) on spiked wheat crispy breads (Meca *et al.*, 2012a). Thus, breads were contaminated with ENA, ENA1, ENB and ENB1 at two concentrations each (1.5 and 3.0 µmol/g) and gastric and duodenal bioaccessibilities were calculated. At the gastric compartment, bioaccessibility ranged between 69.0% (ENA1 at 1.5 µmol/g) and 91.0% (ENA at 3 µmol/g), whereas at duodenal compartment the range was between 68.6% (ENB at 1.5 µmol/g) and 87.3% (ENA at 3 µmol/g). The enniatin that showed the highest bioaccessibilities was the ENA, and the mean bioaccessibility considering the four compounds analyzed resulted in 80%. Given the fact that cereals are frequently contaminated, that it has been demonstrated that ENs can exert toxic activity at low micromolar concentrations in mammalian cells (Meca *et al.*, 2011), and that high bioaccessibilities were found these data must be taken into account in future legislative actions.

A similar study was conducted by the same group with beauvericin (Meca *et al.*, 2012b). BEA has also shown to be toxic in *in vitro* studies with human cell lines, including the intestinal Caco-2 cells (Prosperini *et al.*, 2012). Gil-Izquierdo *et al.* (2002) model was used, with slight modifications; a simulation of the large intestine (colonic digestion) was employed introducing a high inoculum (10^{14} cfu/mL) of several characteristic bacteria of the GI tract after the duodenal digestion. Wheat crispy breads were spiked with 5 or 25 mg BEA/kg; bioaccessibility was around 40% until duodenal digestion and around 30% until duodenal+colonic digestion, with higher bioaccessibilities at the lower BEA dose employed. When crispy breads were produced with different concentrations of soluble alimentary dietary fibers, bioaccessibilities were drastically reduced at the duodenal digestion, but results were quite variable when considered the joint duodenal+colonic digestion. Thus, some fibers as chitosan medium MW and galattomannan considerably reduced BEA bioaccessibility when used at 5%. On the contrary, the use of fructooligosaccharides, inulin or pectin increases the BEA bioaccessibility after duodenal+colonic digestion, mainly at low BEA levels (5 mg/kg).

5. Conclusions

According to the reviewed studies, it can be concluded that bioaccessibility of mycotoxins greatly depends on the considered toxin, as well as on the food matrix in which experiments are carried out. As a general rule it can be established that bioaccessibility of AFs, FBs and ENNs shows high values, generally ranging between 70 and 100%, while PAT, DON and BEA show intermediate values, around 30-70%, and ZEA presents lower values. The bioaccessibility of OTA has proven to be very variable, with values near 100% in some cases but below 30% in others.

The great variability of results between different mycotoxins and, for the same mycotoxin among the different studied matrices, highlights the need for further studies on bioaccessibility of these fungal metabolites, increasing the number and types of studied foods. The true knowledge of the percentage of mycotoxin that can be absorbed in the small intestine, from the initially present in food, would enable a more accurate risk assessment.

It is also necessary to increase the number of studies with naturally contaminated foods, as the mycotoxin-matrix binding has shown to be stronger, which could affect the bioaccessibility values. On the other hand, when working with spiked samples it is necessary that the mycotoxin contamination level fits the natural contamination found in foods. In the same

sense, a special attention has to be paid to masked mycotoxins, as an overestimation of bioaccessibility could occur due to an incorrect estimation of the bioaccessible toxin after the digestive process, in relation to the initially known amount of toxin.

Determination of the internal exposure is a good approach to improve the risk assessment of a mycotoxin. *In vitro* methods offer an appealing alternative to human and animal studies. They can be rapid, simple and reasonably low in cost, can be used to perform simplified experiments under uniform and well-controlled conditions, and may provide insights not achievable in whole animal studies, but they are not free of some weaknesses. In one hand, in many cases they do not take into account important physiological factors as the lack of intestinal mucosa, enterohepatic cycling, and immune system and, in most cases they do not take into account the existence of an intestinal microbiota which possesses enzymatic capabilities that can decisively influence the release of the mycotoxin from the food matrix or the bioconversion of these toxins in the GI tract. On the other hand, these models usually do not take the large intestine into account, as absorption of toxins mainly takes place in the small intestine.

Combined use of these *in vitro* digestion models with other techniques that employ intestinal cell lines, as *in vitro* intestinal absorption models that use Caco-2 cells (De Nijs *et al.*, 2012), conducting for instance transepithelial transport studies (Meca *et al.*, 2012a, Prosperini *et al.*, 2012) may offer a more complete picture of what happens in the intestinal tract during intestinal digestion.

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