Tackling mycotoxin dangers in human foods using *in vitro* and *in vivo* animal models: mitigation of aflatoxins and fumonisins

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List of abbreviations

ACCS100	Air Classified Calcium Silicate 100
ADME	Absorption, distribution, metabolism, and excretion
AF(s)	Aflatoxin(s)
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
AFP1	Aflatoxin P1
AC	Ascending colon
ACN	Acetonitrile
$AUC_{0\rightarrow t}$	Area under the concentration-time curve from time zero to time of last quantifiable concentration
BW	Body weight
C_{max}	Maximum observed concentration
COVID-19	Coronavirus Disease 2019
D	Day(s)
DC	Descending colon
DON	Deoxynivalenol
DON3G	Deoxynivalenol-3-glucoside
EC	European Commission
EFSA	European Food Safety Authority
ELEM	Equine leukoencephalomalacia
EU	European Union
FA	Formic acid
FAMHP - FAGG	Federal Agency for Medicines and Health Products
FASFC - FAVV	Federal Agency for the Safety of the Food Chain
FB	Fumonisin B series
FB1	Fumonisin B1
FDA	Food and Drug Administration

FPS	Federal Public Service
FUM(s)	Fumonisin(s)
GIT	Gastrointestinal tract
GRAS	Generally recognised as safe
Hct	Haematocrit
HFB1	Hydrolysed fumonisin B1
HIRUZ - CTU	Health, Innovation, and Research Institute - Clinical Trials Unit
HSRC REC	Human Sciences Research Council Research Ethics Committee
IARC	International Agency for Research on Cancer
ILVO	Flanders Research Institute for Agriculture, Fisheries, and Food
KEMRI-SERU	Kenya Medical Research Institute - Scientific and Ethics Review Unit
LEAP	Long-term EU-Africa Partnership
LOD	Limit of detection
LOQ	Limit of quantification
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MeOH	Methanol
NOAEL	No observed adverse effect level
NS	Novasil
OTA	Ochratoxin A
P.a.	Post administration
PCR	Polymerase chain reaction
pHFB1a	Partially hydrolysed fumonisin B1 a
pHFB1b	Partially hydrolysed fumonisin B1 b
PPE	Porcine pulmonary oedema
Sa	Sphinganine
SAHPRA	South African Health Products Regulatory Authority
SB	Sodium bentonite

SCFA(s)	Short chain fatty acid(s)
SD	Standard deviation
SEM	Standard error of the mean
SHIME	Simulator of human intestinal microbial ecosystem
So	Sphingosine
TC	Transverse colon
TDI	Tolerable daily intake
TK	Toxicokinetics
T_{max}	Time where maximum concentration is observed
U	Units
UHPLC-MS/MS	Ultra-high performance liquid chromatography-tandem mass spectrometry
USA	United States of America
VAMS	Volumetric absorptive microsampling
WHO	World Health Organisation
Y	Years
ZEN	Zearalenone
ZEN14G	Zearalenone-14-glucoside
°C	Degrees Celsius

1 General introduction

1.1 Exploring mycotoxins

1.1.1 Characterisation

Mycotoxins are small molecules produced as toxic secondary metabolites by toxigenic fungi. Mycotoxins are grouped together based on their frequent association to or causation of several diseases in both animals and humans, with death as a possible outcome [1]. Several subclassifications exist due to their diverse chemical structures, effects, and their production by several different fungal species. The uptake of mycotoxin-contaminated food can lead to acute or chronic toxicity, resulting in global food safety concerns [2]. More than 300 mycotoxins have been identified, with the following six groups or compounds being globally the most prevalent in food: aflatoxins (AFs), fumonisins (FUMs), trichothecenes, zearalenone (ZEN), ochratoxins, and patulin [2]. Furthermore, important food contaminating emerging mycotoxins are produced by *Alternaria* species [3]. Yet, while all mycotoxins are produced by fungi, not all fungal secondary metabolites are called mycotoxins [1]. Not all compounds are toxic for humans and animals, and some even have a pharmaceutical use. Therefore, one of the proposed classifications of the metabolites is based on the target species on which they exert their toxicological effects. Widely used and beneficial fungal metabolites for human and animal health are called antibiotics; these fungal products are toxic to bacteria. Furthermore, when plants are targeted, the metabolites are classified as phytotoxins [1,4]. Other subclassifications are based on the induced diseases in mankind and animals (e.g. human Balkan endemic nephropathy, equine leukoencephalomalacia (ELEM), porcine pulmonary oedema (PPE)), the target organs (e.g. liver, kidney, uterus, lung, heart), the chemical structure (e.g. coumarin, lactone), the biological effect (e.g. mutagenic, carcinogenic, teratogenic), the fungal species that produce them (e.g. *Aspergillus*, *Fusarium* species), and even when or where contamination of the crops occurs (e.g. pre- or post-harvest). Yet, mycotoxins are difficult to classify under only one category, one mycotoxin can possess several characteristics.

One official classification for mycotoxins exists and it is based on their carcinogenic potential for humans. Yet, there are only a few human epidemiological investigations performed that have explored the connection between the risk of cancer development and mycotoxin exposure [5]. Therefore, cancer risk assumptions are primarily based on experimental studies. The International Agency for Research on Cancer (IARC), the specialised

cancer agency part of the World Health Organisation (WHO), classifies agents in monographs based on scientific evidence obtained from both human and animal studies and other relevant data [6]. Its focus lies on the role of environmental and lifestyle risk factors in cancer development. This includes mycotoxins. The evaluated agents are divided into five groups, based on the scientific evidence derived from human and animal experimental studies (Table 1).

Table 1. The IARC classification [7], with mycotoxin examples mentioned in this thesis, based on their carcinogenic potential for humans.

IARC group	Carcinogenicity	Definition	Mycotoxin examples
Group 1	Carcinogenic to humans	There is sufficient evidence of carcinogenicity in humans	Aflatoxins B1, B2, and M1
Group 2A	Probably carcinogenic to humans	There is sufficient evidence of carcinogenicity in experimental animals and limited evidence of carcinogenicity in humans, but mechanistic data suggest otherwise	/
Group 2B	Possibly carcinogenic to humans	There is some evidence of carcinogenicity in humans and even less evidence of carcinogenicity in experimental animals, but mechanistic data and other relevant data support the evidence	Fumonisin B1 and B2, ochratoxin A
Group 3	Not classifiable as to its carcinogenicity to humans	There is limited evidence of carcinogenicity in humans and experimental animals, further research is needed	T2-toxin, patulin, deoxynivalenol, zearalenone
Group 4	Probably not carcinogenic to humans	There is strong evidence of no carcinogenicity in humans	/

The term “secondary metabolites” refers to metabolites produced for niche-specific functions and not required for the immediate survival or growth of the organism [8]. Whereas the goal of primary metabolism is to sustain the living state and growth directly. Yet, the reason behind mycotoxin production is not entirely clear. Speculations suggest it provides the fungus with an ecological advantage in adverse environments [9]. They have been referred to as “detoxification products”, although it is not specified which substances they detoxify. Researchers have noted these metabolites are formed when environmental conditions are no longer favourable for growth, such as a lack of nutrients. Others have mentioned that the process involved in the production of these metabolites plays a role in the metabolic activity in challenging environments [9]. Mycotoxins are reported to give a selective advantage in their natural state and Vining [10] divided their functionality into two categories:

- Extrinsic functions: those that impact growth and reproduction of other microorganisms in the immediate environment;
- Intrinsic functions: those that beneficially affect growth, physiology, and reproduction of the producer organism.

1.1.2 Discovery

Disease and death have been part of humankinds’ life since their existence. Several epidemics and even pandemics have tormented the human race throughout history. Yet, some of the causes still remain unknown up to date [11]. Illnesses caused by mycotoxins, also known as mycotoxicoses, were first recognised as animal diseases, before their significance in human health was acknowledged [12]. The prefix ‘myco-’ and the suffix ‘-toxin’ of the term mycotoxin are derived from ‘*múkēs*’ in Greek, meaning “fungus”, and ‘*toxicum*’ in Latin, signifying “poison”, respectively. Humans can be exposed through ingestion, contact, and inhalation [13]. Moreover, mycotoxins have been identified in human amniotic fluid of pregnant women, indicating prenatal exposure of the fetus [14]. The presence of mycotoxins dates back to more than 10,000 years ago, when the first human agricultural settlements were established [12,15]. Nomad lifestyle, with traditional hunter-gatherers, transformed into sedentary lifestyle, where the cultivation of crops and the farming of animals became important means for food security. The development of crop cultivation arised together with the necessary cereal storage, especially from one season to the next. The combination of grain

storage and the displacement of crop agriculture to new regions, differing from their adapted environment, climatically and geographically, lead to ideal growth conditions for mycotoxigenic fungi [12]. Thus, storage and in-field produced mycotoxins emerged. The destructive role of fungi and insects on stored grains was acknowledged as early as in Roman times. This was observed by how granaries were built. They were erected in such a manner as to prevent fungal and pest damage; raised floors to allow aeration, walled enclosures, and protective measures against insect and rodent infestation [12,16].

One of the oldest known human mycotoxicoses is ergotism or St. Anthony's fire [1]. The disease was common in Europe in the Middle Ages and it was accurately believed to be caused by the consumption of "bad food". In 1630, the disease was linked to the consumption of contaminated rye with ergot alkaloids [17]. These mycotoxins are produced by several species of *Claviceps*. Yet, the term mycotoxin was only conceived in 1962, after the incidence of a veterinary crisis called Turkey "X" disease. This was also the starting point for modern mycotoxicology [1,13]. In 1960, 100,000 healthy young turkeys, chickens, and ducks mysteriously acutely died [18]. The cause of the outbreak was linked to the imported feed originating from Brazil [12]. The feed contained peanut meal and resulted in the discovery of AFs [19]. In 1993, a reanalysis of the feed was performed and an additional mycotoxin was detected, namely cyclopiazonic acid [20]. In the years following the discovery of AFs, many advancements were made in mycotoxin research (named the mycotoxin gold rush), with the development of more sensitive analytical techniques, allowing the identification of more than 300 different mycotoxins [1]. Throughout history, many more mycotoxin outbreaks are known [12]. For example, in Japan in the 1600s, acute cardiac beriberi occurred due to consumption of contaminated rice with citreoviridin produced by *Penicillium* species, and in recent years this toxin re-emerged in Brazil. Balkan endemic nephropathy in the 1920s was thought to be caused by ochratoxin A intoxication, yet there is no evidence to support the assumption. In the 1930s, in Russia, alimentary toxic aleukia caused by T-2 toxin intoxication occurred, and thousands of horses died in the USA in the late 1800s and early 1900s due to FUMs resulting in ELEM. Yet, it was not until 1988 when the first isolation and therefore recognition of FUMs occurred [21,22], following an outbreak of ELEM in South Africa in 1970. Further investigation, in regions with high incidence of human oesophageal cancer, of home-grown corn, the staple diet, highlighted *F. verticillioides* to be the most prevalent fungus. Noteworthy of mentioning

and highlighting the contemporary dangers of mycotoxin consumption, is a more recent outbreak in 2021 of feline pancytopenia in cats in the UK, likely to be linked to the consumption of higher levels of the trichothecenes T2- and HT2-toxins in cat feed [23,24].

1.1.3 Prevalence

Every year, BIOMIN, now part of DSM, conducts a survey on the occurrence of prominent mycotoxins in various parts of the world, which is summed up in the DSM Mycotoxin Survey Report. In the first half of 2021 (January to June), 10,075 finished feed and raw commodity samples from 68 different countries were analysed on mycotoxins [25,26]. Together with the mycotoxins deoxynivalenol (DON) and ZEN, FUMs are the most abundant worldwide (Figure 1). From the total number of samples taken, FUMs were amongst the mycotoxins with the highest prevalence, especially in corn: 73% (North America), 98% (Central America), 83% (South America), 42% (Europe), 90% (Southeast and East Asia), 93% (Middle East) and 71% (sub-Saharan Africa) of the corn samples tested positive. Furthermore, AFs were found in 30% of the samples taken in South America. In Southeast Asia, AFs were present in 25% of the corn samples, and this number rises to a prevalence of 95% in the finished feed samples. South (81%) and Southeast Asia (52%), followed by sub-Saharan Africa (44%) had the highest prevalence of AFs worldwide in the samples collected in the first half of 2021. Of all the samples analysed in the first half of 2021, 65% were co-contaminated with more than one mycotoxin. Furthermore, compared to 2020, an increase in the abundance of the major mycotoxins was observed in Africa. For FUMs, there is a high risk in all parts of the world. Based on these findings, as well as on the fact that this doctoral thesis is part of a larger One Health project, namely MycoSafe-South, co-funded by EU ERA-Net Long-term EU-Africa Partnership (LEAP)-Agri, the focus of this thesis was on sub-Sahara African countries, although, mycotoxins are a global issue and need to be addressed effectively.

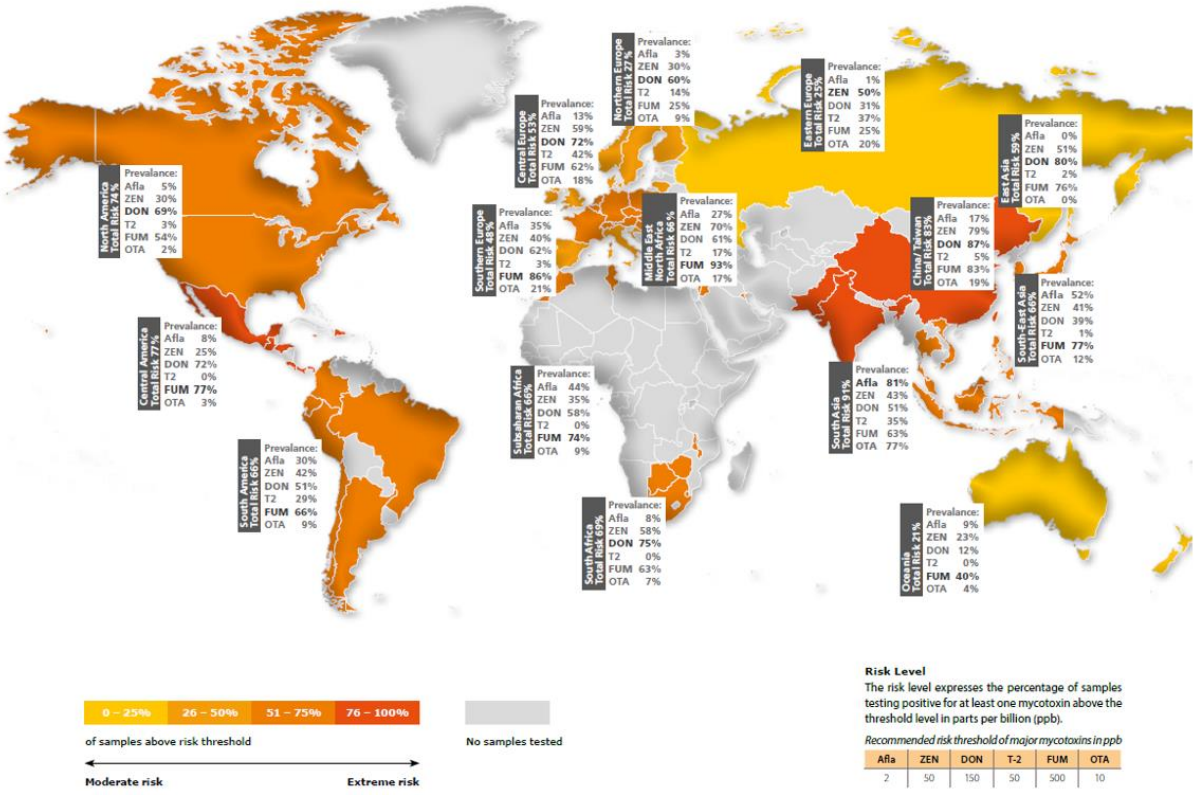

Figure 1. BIOMIN World Mycotoxin Survey January-June 2021 [25]. World map of mycotoxin prevalence and highlighted regions, from yellow to red, depending on risk level (% of samples above the recommended mycotoxin risk threshold levels). Recommended risk thresholds for aflatoxins (Afla) and fumonisins (FUM) are 2 and 500 µg/kg, respectively. Based on the analysed samples in the first half of 2021, the highest risk for Afla is in South(-East) Asia, followed by sub-Saharan Africa. For FUM, there is a high risk in all parts of the world.

As can be seen on Figure 1, Africa is largely coloured in grey, meaning that for the most parts "no samples were tested" [25]; the samples collected in Africa are limited (approximately *n* = 502 including the Middle East). This clearly indicates the need for more data collection in that continent. Noteworthy is that the small amount of "samples tested" in Africa in this survey can be attributed to a scarcity of mycotoxin occurrence reports available [27], possibly also due to a lack of transfer of data to the appropriate regulatory bodies [28].

In a study performed by Probst et al. [29], 339 maize samples were collected in 18 sub-Saharan countries in Africa. The mycotoxin analysis of these samples demonstrated that 65% were positive for AFs, 81% for FUMs, and 40% for DON. The per cent of samples exceeding the USA recommended limits for AFs (20 µg/kg), FUMs (2,000 µg/kg), and DON (1,000 µg/kg), was 47%, 49%, and 4%, respectively. In Kenya, in a study performed by Mutiga et al. [30], 985

milled maize samples were collected and analysed on AFs and FUMs. In 87% of the samples, FUMs were detected, with 50% exceeding the national legal limit of 1,000 µg/kg. In 41% of these samples, AFs were detected, with 4% over the Kenyan regulatory limit of 10 µg/kg for total AFs [30]. In a study performed in Ethiopia, by Worku et al. [31], 150 samples of stored maize were analysed. The samples were positive for AFs, FUMs, Ochratoxin A (OTA), and DON in 100%, 32.7%, 24%, and 7% of the cases, respectively. Co-occurrence of AFs and FUMs together was observed in 32.7% of the samples. The mycotoxin levels found in cereals in Africa highly vary as seen in Table 2.

Table 2. Mycotoxin concentration levels reported in cereals in Africa.

Mycotoxin	Foodstuffs	Concentration (range) (µg/kg)	References
AFs	Maize	<1.0 - 1,137	[22–28]
	Millet	<1.0 - 1,658	
	Sorghum	<1.0 - 3,419	
	Peanuts	0.7 - 622.1	
	Infant milk formula	9,796	
	Barley, teff, and wheat	0 - 26	
	Rice	28 -372	
FUMs	Maize	987 - 20,000	[22–28]
	Wheat	29 - 404	
	Sorghum	20.0 - 3,419	
OTA	Sorghum, barley, and wheat	54.1 - 2,106	[28]
	Rice	134 - 341	
DON	Sorghum	40 - 2,340	[22–28]
	Maize and wheat	100 - 11,022	
ZEN	Sorghum	32	[28]
	Sorghum beer	50	

AFs, aflatoxins; FUMs, fumonisins; OTA, ochratoxin A; DON, deoxynivalenol; ZEN, zearalenone

1.2 Mycotoxin occurrence and toxicity in humans, with a focus on children

A basic principle of toxicology is based on a quote attributed to Paracelsus "What is there that is not poison? All things are poison and nothing is without poison. Solely the dose determines that a thing is not a poison" ("*sola dosis facit venenum*", Paracelsus, 1538) [32]. Undoubtedly, this statement contains a truth, although other factors equally play a role in the extent of toxicity of a substance. Particularly for the onset of symptoms due to mycotoxin intoxication, besides the concentration, the type of mycotoxin, the route of exposure (oral uptake, inhalation, dermal contact), and the duration of exposure (acute, chronic) are important factors to consider [1]. Additionally, the age, gender, and health status of the exposed person play a role. Moreover, the interactions or synergistic effects of other factors, including genetic and non-genetic drivers (the exposome), such as diet and other toxins, cannot be excluded [33].

The mycotoxins that mainly cause concern for worldwide public health are predominantly produced by the fungal species *Aspergillus* (AFs, ochratoxin A (OTA)), *Fusarium* (FUMs, ZEN, trichothecenes), *Penicillium* (OTA), and *Alternaria* (alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), and altertoxins) [3,34,35]. Moreover, AFs, FUMs, trichothecenes, OTA, patulin, ZEN, and ergot alkaloids are important toxins affecting children's health (Table 2) [36]. In low and middle income countries, including countries in sub-Sahara Africa, climatic conditions and less than ideal agricultural practices contribute to favourable conditions for fungal growth on crops [37]. Furthermore, the lack of legislations and food processing inspections, unregulated local markets, and a dominant subsistence farmers' reliance for food production, increase the risk of mycotoxin contamination in foods. Therefore, in these regions, mycotoxin-induced adverse health effects are very contemporary after the consumption of these contaminated cereal-based foods. Particularly mycotoxins which contaminate cereals and groundnuts are of importance in human health [38]. Although mycotoxins are highly recognised in the scientific community, the general population, and frequently the people at risk, are not aware of their existence, let alone the related health risks they inflict [13]. This is especially concerning, as mycotoxins are presumed to be more toxic than pesticides.

Table 3. Selected mycotoxin-producing fungi of relevance to children's health with their respective mycotoxins and associated health effects [36].

Fungus	Mycotoxins	Associated health effects
Aspergilllus flavus, A. parasiticus	Aflatoxins	Vomiting, hepatitis, liver cancer, stunted growth
Fusarium verticillioides, F. proliferatum,	Fumonisins	Vomiting, neural tube defects, oesophageal cancer, stunted growth
Fusarium culmorum, F. graminearum	Deoxynivalenol	Vomiting
Fusarium sporotrichioides	T-2 toxin	Alimentary toxic aleukia, vomiting, haemorrhage
Aspergillus ochraceus, A. niger	Ochratoxins	Balkan nephropathy, renal cancer
Penicillium expansum	Patulin	Vomiting, cancer (suspect)
Fusarium graminearum	Zearalenone	Estrogenic effects, cervical cancer (suspect)
Claviceps purpurea	Ergot alkaloids	Ergotism
Alternaria alternata, A. tenuissima	Alternariol, alternariol monomethyl ether, tenuazonic acid	Mutagenic, esophageal cancer, haemorrhage

Both AFs and FUMs contaminate crops worldwide and both have severe consequences after human consumption [34]. Especially in sub-Saharan countries, the exposure to and the negative human health effects of these specific mycotoxins through the staple diet maize are observed [37,39–44]. Furthermore, co-contamination of cereals with aflatoxin B1 (AFB1) and fumonisin B1 (FB1) is of great concern [30].

Young individuals tend to be more vulnerable to mycotoxin exposure and thus more susceptible to mycotoxicoses than adults. This is due to metabolic and physiologic immaturity, with underdeveloped organ functions resulting in lower detoxification mechanisms [45]. Due

to their lower body mass and higher metabolic rate, they also have a relatively higher food intake [45–48]. Moreover, infant foods mainly consist of cereal-based ingredients, increasing the likelihood of mycotoxin exposure. Additionally, toddlers are exposed to an important and toxic metabolite of AFB1, namely aflatoxin M1 (AFM1), through the consumption of milk, creating an additional risk [44,49]. Furthermore, they are most vulnerable around the weaning period, when the diet shifts from fluids to solids, causing disruption of the intestinal barrier [50]. This leaky gut marks increased exposure to the consumed mycotoxins [47]. However, due to the scarcity of reliable mycotoxin exposure data in children, it is difficult to associate cause and health effects in children. Thus, the extent of the problem could be underestimated [38]. Many diseases in children can be ascribed to synergistic effects of mycotoxins with other mycotoxins, other toxins, and dietary aspects. Frequently, only chronic mycotoxin consumption results in visible disease. Furthermore, in low and middle income countries, the health systems lack capacity and the resources are limited to assess the cause of the diseases [37]. Therefore, it is not straightforward to link the symptoms with mycotoxin exposure.

A systematic literature review, performed by Tesfamariam et al. [51], consulted 86 peer-viewed articles, of which 30 were reported in this review, concerning associations between dietary mycotoxins exposure and child growth aged 5 years or younger. The authors noted that the evidence of association provided in studies found in literature are of low quality. However, a link between dietary exposure and malnutrition in children cannot be excluded, especially for AFs and FUMs. Malnutrition in this review was defined as wasting (low weight-for-height), stunting (low height-for-age), and underweight (low weight-for-age). The results found in the investigated studies were not consistent: negative, positive, and no associations were observed between dietary AF exposure and stunting in children. For dietary FUM exposure, negative and no associations were observed for child growth. Tesfamariam et al. concluded that more robust study designs are necessary, including sample sizes with an adequate power, the use of validated biomarkers, and the assessment of multi-mycotoxin occurrence and their synergistic effects.

1.2.1 Aflatoxin B1

Aflatoxin B1 is part of a group of mycotoxins (AFs) that are mainly produced by *Aspergillus flavus* and *A. parasiticus*. Aflatoxin B1 is the most prevalent and potent hepatotoxic

and hepatocarcinogenic of the AFs, belonging to the IARC group 1 human carcinogen. Therefore, the focus in this doctoral thesis lies on AFB1 specifically. After ingestion, it is quickly absorbed in the gastrointestinal tract due to its low molecular weight and lipophilic nature [52]. In the body, AFB1 is metabolised into a reactive aflatoxin 8,9-epoxide, among others, which can react further with DNA and form DNA adducts, such as AF-guanine adducts [53].

Aflatoxins are difuranocoumarin derivatives and fluoresce under ultraviolet light. Depending on the emitted colour, the name of the component was accorded (i.e. blue light, AFB1 and AFB2, and green light, AFG1 and AFG2). The name of AFM1 and AFM2 comes from the matrix it is excreted in, after hydroxylation of AFB1, namely milk. The chemical structure of AFB1 is presented in Figure 2.

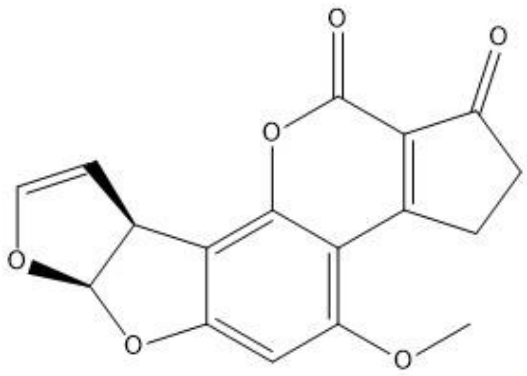

Figure 2. Chemical structure of aflatoxin B1.

Aflatoxin B1 occurs in a variety of agricultural produce, i.e. most commonly peanuts, maize, and their derived products. Moreover, these specific grains and nuts comprise the main ingredients of many children's foods [38]. Other affected crops include sorghum, rice, and wheat. Aflatoxins contaminate staple foods mainly where the climate is hot and humid, which is favourable for fungal growth.

Aflatoxins have been found to have carcinogenic, mutagenic, and teratogenic effects, with the liver as main target organ, followed by the kidneys. Stunting in children has also been connected to the presence of AFs in foods [21,54–56]. In Africa, in the 1930s, it was noted that where consumption of peanuts was high, there was a higher chance to be diagnosed with liver cancer [12]. Soon after, the link with AFs was established. To demonstrate the severity of AFs worldwide, in the 2015 published estimates of WHO [57], it was estimated that in 2010 AFs

were responsible for 22,000 of the 600 million foodborne illnesses and 19,000 of the 400,000 foodborne deaths.

Aflatoxin B1 can undergo four stages of phase I biotransformation in many animal species (Figure 3) [58]. This biotransformation takes place in the presence of microsomal cytochrome P450 enzymes and most frequently occurs in the liver. Aflatoxin B1 can undergo O-dealkylation to form the metabolite aflatoxin P1 (AFP1), keto-reduction to form aflatoxicol, epoxidation to AFB1 8,9-epoxide, and hydroxylation to form the metabolites AFM1, AFP1, aflatoxin Q1, or aflatoxin B2a. The most toxic metabolite is, as previously mentioned, the AFB1 8,9-epoxide which can bind to DNA or proteins (e.g. AF-lysine adduct), and results in mutations and cancer. Besides these phase I biotransformation reactions, the phase II reaction with glutathione is an important detoxification pathway for AFB1 8,9-epoxide and AFM1.

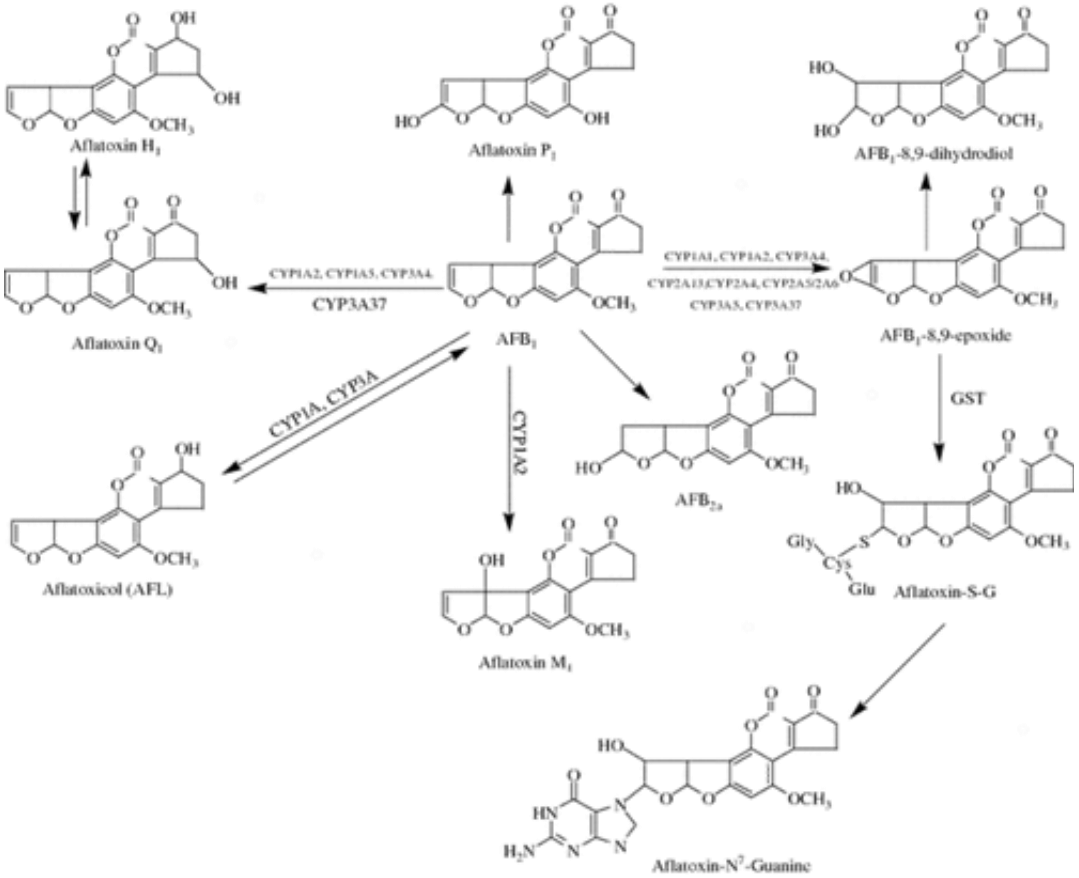

Figure 3. Chemical structure of the main metabolites formed after biotransformation of aflatoxin B1 in humans [59].

1.2.2 Fumonisin B1

Fumonisin B1 is the most prevalent member of the group of the fumonisin B (FB) series and is mainly produced by *Fusarium* fungi, such as *F. verticillioides* (previously known as *F. moniliforme*), *F. proliferatum*, and *F. fujikuroi* [21]. Maize is the most common crop contaminated with these mycotoxins, although other cereals can also be affected. Other major types of FBs found in foods are fumonisin B2 and B3. Fumonisins are formed by a diester of two molecules of propane-1,2,3-tricarboxylic acids with a 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyeicosane molecule, as shown in Figure 4.

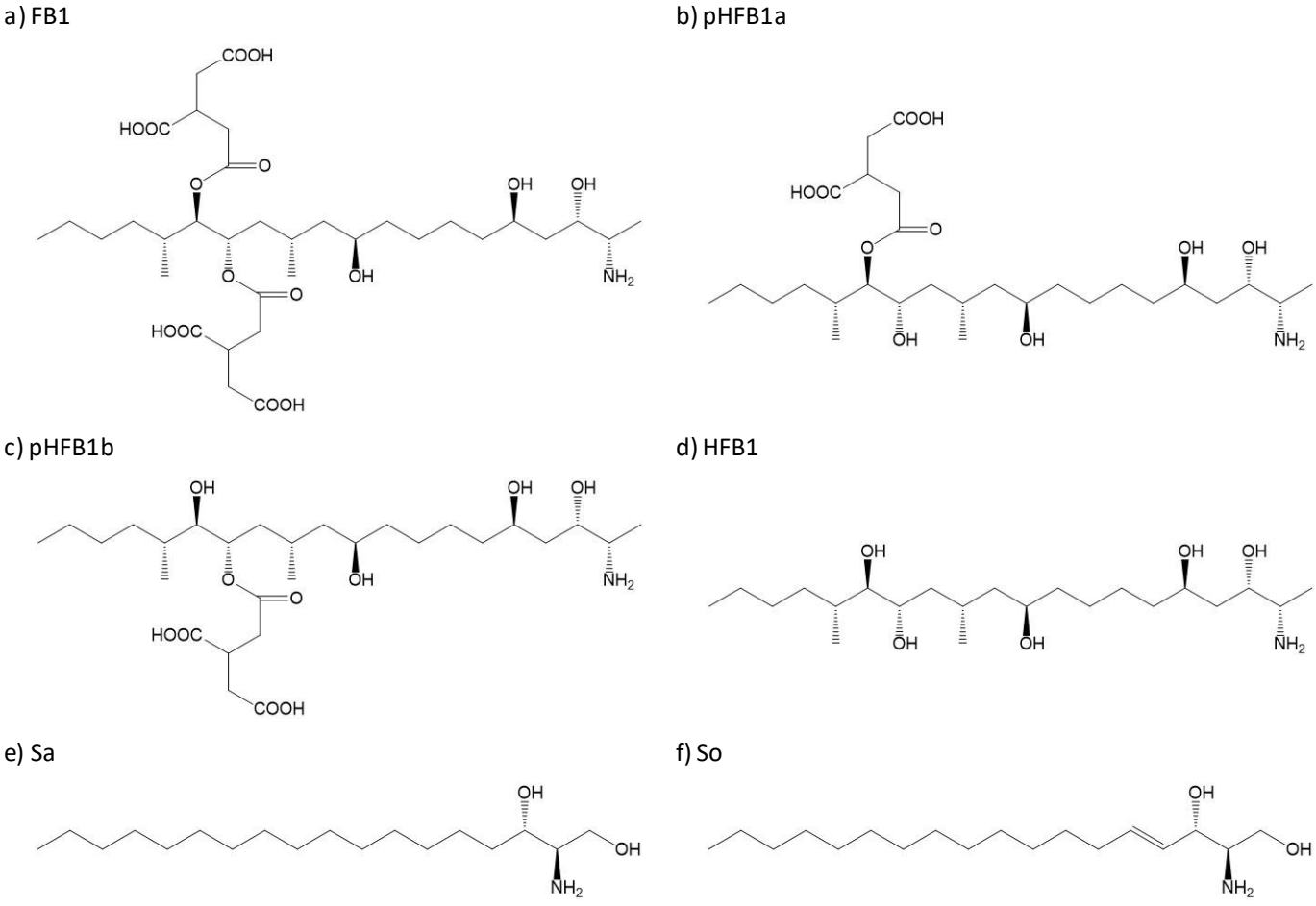

Figure 4. Chemical structures of a) fumonisin B1 (FB1), b) partially hydrolysed FB1a (pHFB1a), c) pHFB1b, d) hydrolysed FB1 (HFB1 or aminopentol), e) sphinganine (Sa) and f) sphingosine (So).

Following oral ingestion, FB1 absorption is very limited (4.07 ± 1.02%) in several animal species, and it is expected to be similar in humans [60]. Accumulation of FB1 occurs mainly after chronic exposure, with highest concentrations detected primarily in the liver, kidneys,

and spleen [61]. Intestinal microbiota have been noted to degrade FB1 into its hydrolysed metabolites, mainly into the partially hydrolysed forms; partially hydrolysed fumonisin B1a (pHFB1a) and pHFB1b. Complete hydrolysis of FB1 into hydrolysed fumonisin B1 (HFB1) was less frequently observed and only to limited amounts (<1%) (Figure 4) [61,62].

The toxicity of FBs is attributed to the alteration of the sphingolipid metabolism. The unsubstituted amino group at C2 and the tricarballylic side chains are suspected to play a role, as without these, no effects are observed *in vitro* and *in vivo* [63–65]. The hydrolysed FB1 forms are less potent ceramide synthase inhibitors, and therefore FB1 hydrolysis is regarded as a detoxification pathway. Fumonisin B1 competitively inhibits the N-acyltransferase or ceramide synthase in the sphingolipid biosynthesis pathway, resulting in the inhibition of *de novo* synthesis of ceramide [63] (Figure 5). This directly results in the accumulation of sphinganine (Sa) and, to a lesser extent, sphingosine (So) in tissues, serum, and urine (see Figure 4 for their chemical structures). Therefore, the detection of an increase in the Sa/So ratio is used as a FB1 biomarker of effect and thus exposure in several animal species. This Sa accumulation has been linked to apoptosis and mitosis in the liver and kidney. Furthermore, it is linked to disruption of folate transport.

In animals, consumption of FB1 contaminated feed can lead to PPE in pigs and ELEM in horses. Cancer and neural tube defects have also been observed in experimental animals [63,66]. In pregnant women, an increased risk of neural tube defects due to FUM exposure has been suggested in the unborn child following a 100% increase in the prevalence of the condition in 1990-1991 along the Texas-Mexico border [67]. In humans, FUM is classified as a group 2B IARC agent, referring to being possibly carcinogenic based on the current knowledge. A high incidence of oesophageal cancer has been observed in regions where FB1 contaminated corn is consumed [22]. Furthermore, the intake of FB1 in children has been correlated to stunting [21,56,68]. In many low and middle income countries, where FUM exposure is high, there is a lack of regulations or a lack of implementation of the regulations [69].

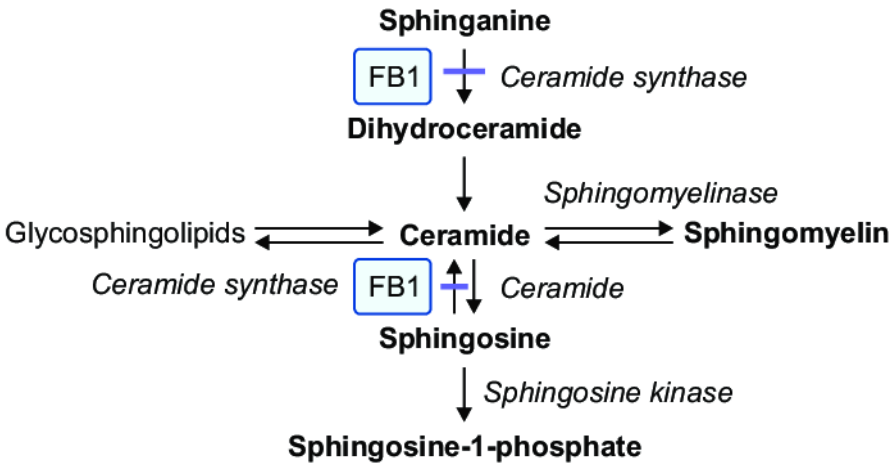

Figure 5. Fumonisin B1 (FB1) competitively inhibits N-acyltransferase or ceramide synthase in the sphingolipid biosynthesis pathway, resulting in the inhibition of *de novo* synthesis of ceramide [70].

HIGHLIGHTS

1. Maize and peanuts are part of the staple diet in many low and middle income countries, in particular sub-Saharan African countries.
2. Maize and peanuts are frequently contaminated with aflatoxins and fumonisins.
3. Children's food mainly consist of maize and peanut derived products.
4. Children are more vulnerable to mycotoxicoses.
5. In sub-Saharan African countries there is a lack of (implementation of) regulations and mycotoxin level monitoring in foods. Furthermore, there is a dominant subsistence farming culture, where the cereals are not subjected to any form of mycotoxin monitoring, nor pre- nor post-harvest.

1.3 Mycotoxin mitigation strategies

Toxigenic fungi and their food-borne mycotoxins pose major food safety and security risks worldwide, especially in low and middle income countries where they have serious consequences. Mycotoxins result in significant food and feed losses, as well as severely affect animal and human health. Furthermore, they have an economic impact, for example by preventing cereal export from Africa to the rest of the world [55].

Mycotoxin contamination is mostly unavoidable. Therefore, in order to prevent or at least limit the extent of contamination in cereal-based foods and their derived products, pre-harvest and post-harvest mycotoxin mitigation techniques have to be applied. To achieve the best prevention or highest decontamination effect, both are important complementary strategies. Especially as post-harvest contamination is usually due to fungal contamination at pre-harvest stage [71]. To effectively apply preventive measures, it is important to understand which factors can influence mycotoxin production in agricultural products [72,73]. Intrinsic factors, such as moisture content, water activity, substrate or plant type, and nutrient composition, as well as extrinsic factors, including relative humidity, temperature, and oxygen levels, can play a role in mycotoxin production by toxigenic fungi. Furthermore, processing and storage factors, like drying, blending, addition of preservatives, atmosphere, or handling of grains, as well as other factors, such as the fungal strain, insect interaction, damage by plant disease, and the microbial ecosystem affect mycotoxin production. Figure 6 provides a visual overview of the different factors at different stages influencing AF production and reduction in maize.

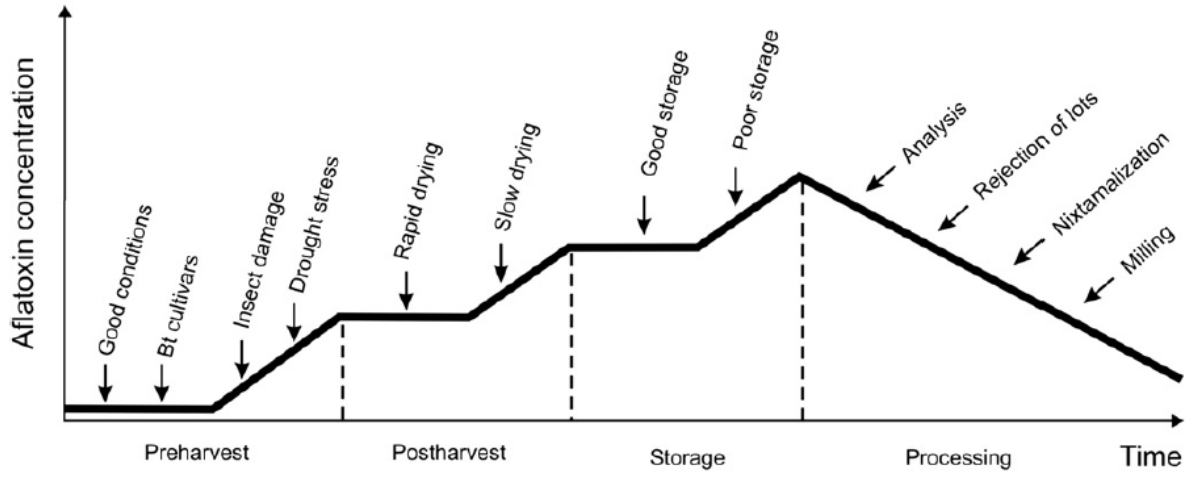

Figure 6. Important aspects during the production and processing of maize, influencing aflatoxin formation and reduction [74].

In developed countries strict food regulations achieve to reduce human exposure to AFs and FUMs [75]. Yet, in low and middle income countries, the mycotoxin problem remains. Additional preventive measures are necessary in these high risk countries to safeguard human health. These mitigation strategies need to be safe, inexpensive, environmentally friendly, practical to adopt, and are not allowed to compromise nutritional quality of the food.

1.3.1 Pre-harvest strategies

The first preventive measures can be adopted at pre-harvest stage, which refer to both during planting and in-field applications. Generally this stage starts at the choice of crops; the seeds must be pest- and disease-free and the use of (partially) resistant varieties against moulds and insects aids in growing healthy resilient crops, capable of withstanding fungal growth. Proper field and harvest management (Good Agricultural Practices, GAP), with or without the use of chemical or biological agents, are imperative to prevent mycotoxin contamination on crops [72]. It all begins with the choice of the time of year for sowing; this determines flowering date and prevailing weather conditions [76]. More frequent and severe spore infections are likely if flowering coincides with spore release [77]. Drought stress, caused by a lack of water, allows the plant to crack, allowing entry for fungal spores [72,76]. Crop rotation, removal of agricultural debris, and ploughing are effective strategies to prevent contamination of fungal spores from one cycle to the next or to prevent provision of an ideal fungal growth medium. Proper tillage, including digging, stirring, and overturning the soil prior to seeding will help decrease fungal spore contamination (Figure 7) [78].

Figure 7. Tillage using animal traction or ploughing [79].

The Codex Alimentarius [78] summarises good practical guidelines for farmers to prevent and reduce mycotoxin contamination. For example, crops of low susceptibility to toxigenic fungi such as clover, alfalfa, and other legumes are put forward as alternatives for maize and wheat, which are highly susceptible to *Fusarium* species, for use in a crop rotation system to reduce inocula in the field (Figure 8). The use of insecticides, fungicides, and pesticides can be useful when there is a risk of mycotoxin contamination [76]. Software

applications exist to help farmers predict the risk based on climatic parameters. Insects can act as fungal spore vectors and damage the crops, facilitating entry and colonisation of mycotoxin-producing fungi (Figure 9) [76,80].

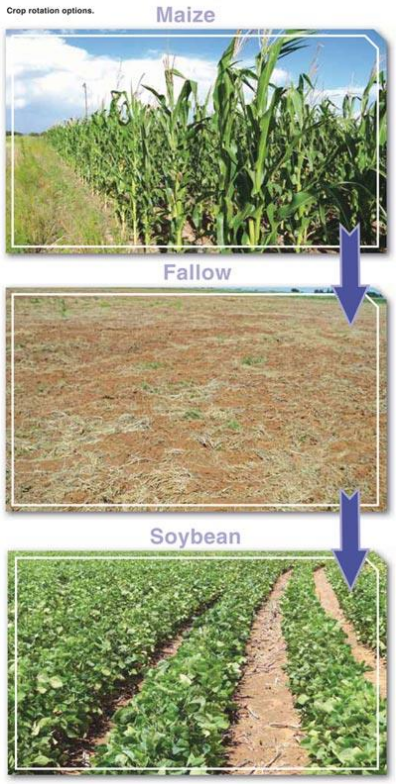

Figure 8. Crop rotation example in South Africa [81].

Figure 9. Ear rot on maize caused by *Fusarium verticillioides* (left) and *Aspergillus flavus* (right) after stem borer damage [82].

Biological mycotoxin control refers to the application of microorganisms or non-toxigenic strains as bio-control agents to compete with toxigenic strains. Their presence can result in competition for nutrients and space, parasitism of the pathogen, and secretion of antifungal compounds, among others [83,84]. In the USA, the application of atoxigenic strains of *A. flavus* and *A. parasiticus* have led to a nearly complete reduction of AFs in peanuts [72,85]. Furthermore, the atoxigenic *A. flavus* is commercialised in two products Afla-guard®

[86] and Aflasafe® [87] and results in an AFB1 reduction of more than 90%. Aflasafe is available in several African countries and had an application rate of 82% in lower Eastern Kenya in 2015 [88]. Aflasafe SN01 with atoxigenic *A. flavus* strains native to Senegal were used to significantly reduce AFs in peanuts and maize [89]. Furthermore, reducing plant stress, by avoiding high cropping density, preventing drought stress through proper irrigation, and applying proper fertilisation, can aid in preventing fungal and mycotoxin contamination [78].

1.3.2 Post-harvest strategies

Harvest should ideally take place when grains are at full maturity and contain a low moisture content. Later harvests of already fungal infected crops lead to an increased risk of higher mycotoxin levels, as well as a higher risk of exposure to pests [90]. Yet harvesting too early, when grains have not reached their maturity, the grains will contain a lower dry matter content, become weak upon drying, and be more susceptible to fungal infection during storage [71]. However, in low and middle income countries, early harvesting, unpredictable weather, labour constraint, a need for money, threat of thieves and rodents, push farmers to harvest at less than ideal times [72].

Following harvest, cereals are stored prior to or following further processing. Therefore, good storage conditions are critical in preventing mould growth in harvested cereals [76]. Various factors should be taken into account. Grains need to be properly and readily dried and stored with a moisture level of less than 15% and at a low temperature. Air circulation helps dry the grains before storage [78]. Mechanical drying is preferable, yet sun and open air drying on clean surfaces is acceptable (Figure 10). However, during this process, grains must be shielded from rain, dew, pests, and bird droppings. Following drying, cleaning the grains is necessary to remove possible moulds or mould spores, mechanically or manually, to remove damaged and immature kernels susceptible to infection, and other foreign plant materials. Winnowing and sorting methods can be helpful in cleaning the grains. Sorting of grains can be performed by visual inspection and removal of damaged, discoloured, moulded, small and shrivelled grains that may be heavily contaminated with mycotoxins (Figure 11) [91]. Besides sorting according to appearance, sorting can also be performed according to grain density; contaminated grain does not have the same density as safe grain [76]. Adjusting the atmosphere around stored crops, such as a low oxygen concentration and increased carbon

dioxide levels also help prevent mould growth [76]. The grains must be handled and stored with care so that they are not damaged, which would otherwise increase the chance of fungal development and mycotoxin production [78]. Preferably long-time storage should be avoided, as well as the mixing of grains, due to the chance of mixing highly mycotoxin contaminated with lower contaminated cereals. Ideally, all grains visibly affected by mould and all grains in the direct proximity should be destroyed (burned or buried). Storage facilities need to be clean (no dust, fungal spores, grain, crop residues, animal and insect excreta, soil, insects, foreign material), well ventilated (on raised platforms), yet provide protection from the outside environment and pests (insects, rodents, birds), and keep stored grains at a relatively constant temperature. Monitoring of the conditions during storage is essential [78].

Figure 10. Small-scale farmers in Uganda drying maize directly on the ground, which is not an ideal drying method and can increase the risk of fugal contamination [92].

Figure 11. Hand sorting of maize grains in Mozambique (left) [93] and removal of discoloured maize in Uganda (right) [92].

Good agricultural practices and proper storage conditions are required to limit the severity of mycotoxin contamination in food products. However, they do not remove all mycotoxins completely [94]. Several types of processing techniques have been found to be useful to further reduce the mycotoxin levels. These processes include among others, milling, washing, dehulling, extraction with solvents, heat treatment, ammoniation, and nixtamalisation [71,95]. Odukoya et al. [95] tested different nixtamalisation procedures (using calcium hydroxide, sodium hydroxide, potassium hydroxide, and calcium chloride) on the reduction of *Fusarium* mycotoxins in artificially contaminated maize and sorghum. Following the washing step, a reduction in FB1 of 73% and 49% was observed in maize and sorghum, respectively. After cooking, a reduction between 90% and 100% in FB1 levels in maize and sorghum was achieved, except for the calcium chloride treatment in sorghum, wherein only about a 50% reduction was obtained. The extent of mycotoxin degradation due to heat treatment depends both on the temperature as well as on the duration of heat exposure [94]. Adegoke et al. [96] reduced AFs by 68% and 81% in cereal pastes after boiling them for 30 and 60 min, respectively. Yet, roasting was found to reduce AFs in peanuts more than boiling. Another effective food processing technique for reducing mycotoxin contamination levels is fermentation [97,98]. This process is inexpensive, easy to implement, and enhances nutritional quality, making it a viable and sustainable process for low and middle income countries [97]. Fermentation transforms food with the help of metabolic activities of micro-organisms. Existing processes include acetic acid, alcoholic, lactic acid, and alkali fermentation. Fermentation results in the formation of metabolites, such as alcohol, carbon dioxide, acetic

acid, lactic acid, propionic acid, ammonia, and fatty acids [99]. These metabolites increase or decrease the pH, which enhances the performance of certain enzymes and suppress spoilage microorganisms [97]. Mycotoxin reduction through fermentation is owed to binding, biotransformation, or degradation [97].

Despite the existence of many post-harvest strategies and the large amount of research papers available describing these techniques, farmers, traders, processors, and consumers at risk lack the knowledge. Hence, the strategies described above are applied in an ideal world, but in Africa, the appropriate practices are currently not sufficiently implemented [100–102].

1.3.3 Detoxifying agents

A complete prevention of mycotoxins in finished food and feed is not achievable, despite the implementation of pre- and post-harvest strategies. Therefore, there is still a real risk of mycotoxin exposure and its consequential health implications. Furthermore, mycotoxin regulatory limits set in foods and feeds do not take mycotoxin co-contamination into account. Multi-mycotoxin contamination in cereals is very common, especially as most fungi can produce more than one mycotoxin [103]. Additionally, several fungi can contaminate the same crops at the same time. Some *in vivo* studies demonstrate that the effect on animal health of multiple mycotoxins combined can result in synergistic, additive, or antagonistic toxic effects when compared to single mycotoxin exposure [103,104]. Therefore, foods and feeds with mycotoxin levels below the maximum set limits can still pose health risks for human and animal consumers. Thus the application of mycotoxin detoxifying agents as food and feed additives provide an extra preventive measure to mitigate potential mycotoxin exposure. The maximum limits for AFM1, AFB1, and FB1 in human and infant foods are summarised in Table 4. In the feed industry, the addition of adsorbing or transforming agents, such as clays, enzymes, or microorganisms capable of detoxifying mycotoxins have been put forward as reliable mitigation techniques [76]. Mycotoxin detoxifiers are mixed in the feed and several are commercially available and commonly adopted. In 2009, a new functional group of feed additives was established in the EC regulation No 386/2009 "substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action" [105].

Table 4. Regulatory maximum levels for aflatoxin M1 (AFM1), aflatoxin B1 (AFB1), and fumonisin B1 (FB1) in human and infant foods, enforced by the European Union (EU), the Codex Alimentarius, the Food and Drug Administration (FDA) of the United States of America (USA), and some African countries.

Mycotoxin	Foodstuff	Maximum levels (µg/kg)	Source
AFM1	Liquid milk, raw milk, heat-treated milk and milk for the manufacture of milk-based products	0.05	EU [106] and Codex Alimentarius [107]
	Infant formulae and follow-on formulae, including infant milk and follow-on milk	0.025	EU [106]
	Fluid milk products	0.5	FDA, USA [108]
AFB1	Processed cereal-based foods and baby foods for infants and young children	0.1	EU [106]
	All cereals and all products derived from cereals, including processed cereal products	2	EU [106]
	Foods	5	African countries [109]
	Foods	20	FDA, USA [110]
FB1	Processed maize-based foods and baby foods for infants and young children	200	EU [106]
	Foods	2000	FDA, USA [111]
	Maize and wheat grain and flour	2000	South Africa [112]

Mycotoxin detoxifiers are divided into two groups, depending on their mode of action; the **mycotoxin-adsorbing agents** and the **mycotoxin-biotransforming agents** [113].

Mycotoxin binders, which are large molecular weight compounds, reduce exposure to mycotoxins by decreasing their bioavailability; they adsorb the mycotoxin and prevent it from being absorbed in the gastrointestinal tract. Therefore, the adsorbed mycotoxins do not reach the bloodstream, nor the intended target organs. The mycotoxin-adsorbing agent complexes are excreted through the faeces. Mycotoxin transforming detoxifiers degrade mycotoxins into less toxic or non-toxic metabolites. Bacteria, fungi, yeasts, and mycotoxin-degrading enzymes that they produce fall under the category of mycotoxin-biotransforming agents. Mycotoxin binders are used to detoxify feed with several mycotoxins, and are mainly considered to be non-specific [113]. Consequently, there is a possibility that the binder adsorbs vitamins, minerals, nutrients, or even medicinal products [114,115]. Furthermore, depending on the binder, there is no guarantee that the bond is not reversible; possibly the mycotoxins are released further in the intestines [116]. Contrarily to binders, enzymes tend to be mycotoxin specific, and are thus regarded as safer for the consumer [113]. The potential use of microorganisms as mycotoxin detoxifiers in humans has been suggested in previous studies [117]. Yet, up to date, mycotoxin detoxifiers are employed on a commercial level solely in animals. In humans, some detoxifiers have been applied in an experimental setting; this consisted of binders with a focus on mitigation of AFs, as indicated subsequently.

Following animal *in vivo* efficacy testing [118,119], an AF detoxifier, a calcium montmorillonite clay binder Novasil (NS), has been tested in humans. The safety of the product was determined in a 2-week study performed in 50 adult volunteers in Texas, USA, during the months of May through August 2004, by Wang et al. [120]. The participants were divided into two groups based on the product dosage administered; low dose (1.5 g/day) and high dose (3.0 g/day). Only mild gastrointestinal symptoms were reported, such as abdominal pain (6%), bloating (4%), constipation (2%), diarrhoea (2%), and flatulence (8%). Furthermore, levels of hematological parameters, liver and kidney function, electrolytes, vitamins A and E, and minerals were monitored and showed no significant changes between before and after detoxifier treatment. This product was then applied in an intervention study in Ghana performed from December 2005 to April 2006, by Afriyie-Gyawu et al. [121] and Wang et al. [122]. A 3-month placebo-controlled study was performed including 177 healthy volunteers, divided into three groups: low dose (1.5 g/day), high dose (3.0 g/day), and placebo (0 g/day). Novasil was administered in capsules and participants would ingest 3 capsules a day with

water, prior to the meals. Blood and urine samples were collected before and throughout the 3-month trial. The biomarkers for efficacy testing analysed in this study comprised serum AFB1-albumin adduct and urinary AFM1. A significant decrease in AFB1-albumin adduct (from a mean ± standard deviation of 1.20 ± 0.22 to 0.90 ± 0.16 pmol/mg albumin) was observed after 3 months in both the low and high dose groups compared to the placebo group. In the high dose group, a significant decrease (up to 58%) in the median level of AFM1 was found after 3 months compared to the median of the placebo group. The results suggest effective reduction in bioavailability of dietary AFs in humans through the administration of NS. In Kenya, this same product at 3 g/day was tested in a placebo controlled crossover intervention study with 50 participants during 7 days by Awuor et al. [123]. The same biomarkers for efficacy as mentioned above were analysed; AFB1-lysine adducts from serum albumin and urinary AFM1. The geometric mean for urinary AFM1 levels was lower during NS administration compared to the placebo ingestion. Blood was collected before and after the crossover trial (comprising both NS and placebo weeks, and the 5-day wash-out period) from 39 participants. The serum AFB1-albumin median levels significantly decreased from day 0 to day 20 (from 9.3 to 6.4 pg/mg albumin). The effective use of clay binders in humans at risk of aflatoxicosis seems promising, yet further research is necessary to determine its efficacy in preventing disease when there is a risk of high AF exposure [124]. Biomarkers of exposure and effect have been studied in animal experiments, and validated analysis methods have been described [125]. Especially urinary AFM1 is a well-established and scientifically recognised as a validated biomarker of AF exposure [126–132]. Furthermore, urinary AFB1-N7-guanine and serum AFB1-albumin are regarded as good biomarkers due to their strong correlation with AF intake [129,130,132–134].

The binder tested in this doctoral thesis is similar to NS. The product Mycofix® Secure is a bentonite clay with the main active substance being dioctahedral montmorillonite, which is a non-toxic, naturally occurring material authorised in the European Union as a technological feed additive [135]. Typical components of the bentonite are >70% smectite, <10% opal and feldspar, <4% quartz and calcite [136]. Furthermore, bentonite (E558) was also authorised as food additive until 2013 [137]. However, the authorisation was not prolonged due to the high intake of aluminium present in bentonite [138]. Despite that, according to the FDA, bentonite is still considered as a generally recognized as safe (GRAS) substance [139,140].

The second product tested in this doctoral thesis, FUM*zyme*®, contains fumonisin esterase as active substance and its efficacy in hydrolysing FB1 has been investigated in *in vitro* and *in vivo* animal experiments. No adverse health effects were observed in pigs [141]; the enzyme is very specific for cleaving the FUM side chains, resulting in the formation of less toxic FUM metabolites. Therefore no adverse health effects in humans are expected. This product is also evaluated by the European Food Safety Authority and is approved as feed additive by the European Commission (EC) [136,142]. The seemingly most promising human biomarker for FUM effect and thus exposure is the Sa/So ratio in blood or urine. However, an increase in the Sa levels or in the Sa/So ratio is difficult to correlate with FUM exposure in humans. The normal range varies widely and shifts over time [143–145]. Furthermore, the change in serum ratio occurs in a FB1 dose-dependent manner; the lower exposures to FUMs in practice compared to the higher exposures experimentally-induced, can make the detection of changes in these sphingoid bases or ratio more challenging in real life situations. The lack of reference values adds to the challenge. Therefore, further research in humans is necessary to determine the use of this biomarker in real life situations.

Several pig studies have been performed to test the efficacy of the enzyme. The efficacy is primarily demonstrated by measuring FB1 concentrations and associated biomarkers (Sa/So ratio, and hydrolysed FB1 derivatives) in serum, urine, and faeces [146]. Schwartz-Zimmermann et al. [147] observed a reduction of the FB1 concentration (30 mg/kg) of about 50% and more than 90% in pig faeces after addition of the enzyme during 7 days at a dose of 15 and 150 U/kg feed, respectively. Masching et al. [146] performed three experiments (*in vitro*, in turkeys, and in pigs) to determine the FB1 degrading potential of the same esterase. First, complete degradation of FB1 (300 µg per intestinal sample) into HFB1 was observed within two hours following inoculation with the enzyme (6 U per sample). Second, a decrease of approximately 46% and 77% in FB1 was observed in the excreta of turkeys after 7 and 14 days, respectively, following the consumption of FUM-contaminated feed (15,000 µg/kg) supplemented with the enzyme (15 U/kg feed). Third, in a pig trial, an average decrease of 72% in FB1 concentration in faeces was observed after 14, 28, and 42 days of feeding the piglets with FUMs (2,000 µg/kg) and fumonisin esterase (60 U/kg).

HIGHTLIGHTS AND RESEARCH GAPS

1. Several pre- and post-harvest mycotoxin mitigation strategies exist. Yet, mycotoxin contamination in finished foods in unavoidable.
2. Mycotoxin detoxifying agents (adsorbing and biotransforming agents) are commonly applied in animal feeds.
3. The application of mycotoxin detoxifiers could provide an extra preventive measure in humans at risk of mycotoxicoses.
4. Research about efficacy of mycotoxin detoxifiers in humans is not well investigated. *In vitro* and *in vivo* animal research as a first screening is necessary.
5. The efficacy of clay binders on AFs in humans is not well investigated.
6. The efficacy of the mycotoxin biotransforming agent fumonisin esterase on FUMs in humans is not well investigated.

1.4 Interactions between mycotoxins and human gastrointestinal microbiota

The differences in the composition of the microbial populations between animal species and their interaction with mycotoxins have partly been linked to susceptibility to mycotoxins among animal species [148]. The human gut microbiota is mainly comprised of four major phyla; *Firmicutes*, *Bacteriodetes*, *Actinobacteria*, and *Proteobacteria*, and their proportions vary in time and per gut segment [149]. The composition of the microbiota of an infant starts to resemble that of an adult by the age of 3 years [150]. Gut microbiota play an important role in nutrient, drug, and xenobiotic metabolism, as well as in the gut mucosal barrier integrity, immunomodulation, metabolic and neuroendocrine diseases, and in protection from pathogens [148,150,151]. The gut is usually the first site of contact with mycotoxins, as these are mostly present in food. Therefore, the gut is the first target for mycotoxin toxicity, and it is also the region responsible for absorption, leading to systemic mycotoxin exposure.

In the first few years of life, the development of gut microbiota is very important as it is implicated in early immune system development [152]. Studies have suggested that alterations in this development stage may have consequences later in life [153]. Development and maturation of the gut microbiota are highly dynamic processes influenced by several

external and internal factors. A difference in the gut microbiota is already observed between infants that are born through a caesarean section and those delivered naturally, and between breastfed and formula fed infants. Breastfed infants have a less diverse microbiota, yet it is composed of higher levels of *Bifidobacterium* species (*B. breve*, *B. bifidum*, and *B. longum*). This particular species thrives on human milk oligosaccharides. The composition of these carbohydrates is affected by the mother's genome, which thus has an effect on the infant's gut microbiota [154]. Interestingly, the subspecies *B. longum infantis*, which is specialised in human milk metabolism, varies among populations. It is present in about 10% of Finnish, 20% of Estonian, and 23% of Russian infants [152]. The shift from milk to solid food consumption marks an important change in the gut microbiota, transforming from a high-fat and low-carbohydrate milk diet to a low-fat and high-carbohydrate diet [155]. This solid food comes with a higher bacterial load and diversity [156,157], higher total short chain fatty acid levels, and a dominance of mature related phyla *Bacteroides* and *Firmicutes*, which are better in breaking down complex carbohydrates [157]. Infants have a lower bacterial alpha diversity, a lower functional complexity, and a higher degree of interpersonal variation in gut bacterial diversity between infants (beta diversity) than between adults [153]. The major differences between infants and adults are that infants have a lower alpha diversity or richness and more *Actinobacteria*, *Bacilli*, *Ruminococcaceae*, and *Bacteroidetes*, and less *Methanobacteriales* compared to adults [153].

Three phases in gut microbiota development are described, based on the most abundant phyla and changes in alpha diversity [158]. The first year of life (3 to 14 months) is considered developmental, in which the phyla (predominantly *Bifidobacterium* spp.) and alpha diversity gradually change, the second year (15 to 30 months) transitional, in which only *Bacteroidetes* and *Proteobacteria* continue to develop and alpha-diversity continues to change, and from year three, the microbiota stabilise and remain unchanged, with a higher diversity and consists predominantly of *Firmicutes* [158]. In children from 1 to 4 years, the predominant phyla are *Actinobacteria*, *Bacilli*, *Bacteroidetes*, and members of the *Clostridium* cluster IV [159]. *Clostridium* cluster XIVa is equally predominant in young children and adults and is thus considered to be established at an early age. However, some studies have recently suggested a continuous maturation of the microbiota beyond three years of age [153].

Differences in lifestyle, and westernisation in particular, strongly influence the composition of gut microbial populations in children, as already reported for adults [153]. In a study performed by Yatsunenko et al. [160], the differences in gut microbiota among human populations were examined by determining bacterial species in faecal samples from 531 individuals. The samples were collected from healthy children (<3 years and 3 to 17 years) and adults from the Amazonas of Venezuela, rural Malawi, and USA metropolitan areas and 16S rRNA sequencing was performed. Alpha diversity was lower in the children, over the age of 3, from the USA compared to the two other countries. The difference was explained by the more rural lifestyle, divergent environmental exposure, or difference in diet. In a study performed by Lin et al. [161], the faecal microbiota of healthy children (9 to 14 years) was compared between children living in an urban slum in Bangladesh with that of children of an upper-middle class suburban community in the USA. A higher diversity and enrichment in *Prevotella*, *Butyrivibrio*, and *Oscillospira*, and depletion in *Bacteroides* was observed in the Bangladeshi children.

There are two types of effects observed by the gut microbiota on mycotoxins; they can either biotransform or influence the absorption of mycotoxins [148]. The first refers to a change in structure of the parent compound into more, less or non-toxic metabolites. The latter refers to the possibility of the cell wall of the microbiota to bind to the mycotoxins, preventing their absorption into the bloodstream. The possible modifications to the ingested mycotoxins include hydroxylation, oxidation, hydrogenation, de-epoxidation, methylation, glycosylation and glucuronidation, esterification, hydrolysis, sulfation, demethylation, and deamination [162]. Mostly, these transformations are due to enzymes present in or secreted by the gut microbial cells [148].

The main observed effects of mycotoxins on intestinal microbiota are changes in the composition of the population; at phylum, genus, and species level [148]. These alterations can be a direct result of the antimicrobial properties of mycotoxins. However, they can also be secondary to the toxic effect of mycotoxins on the epithelial and immune cells in the gut, and the release of antimicrobial peptides by these cells. Furthermore, the toxic effect of mycotoxins can result in increased mucus and gut secretions, and diapedesis of immune cells in the affected region. These changes can lead to an altered and less favourable gut environment for bacterial growth. Furthermore, a disturbance in the bacterial population

equilibrium can cause dysbiosis, possibly resulting in the onset of disease. The toxic effects can also result in gut barrier dysfunction. An important consequence of the shift in microbiota after mycotoxin exposure, is the change in the amount and composition of the fatty acids and sphingolipids present in the digesta. As healthy gut microbiota is recognised to be linked to the host health, any disruptions can contribute to infectious and chronic diseases in humans, such as colorectal cancer and diabetes [148,150]. However, it is difficult to characterise the effects of mycotoxins on the microbiota as the concentration of the mycotoxins vary between the gut segments, due to differences in absorption and bile secretion, and also due to the effect of the microbiota on the mycotoxins. Depending on the experimental design of the study, results may vary considerably [163,164].

To investigate the composition, function, and dynamics of gut microbiota, especially when associated with health and disease, scientists analyse the microbial diversity [165]. Two important factors to take into account when assessing diversity are richness and evenness. Richness refers to the number of different species in a specific niche, but not the amount of individuals in each species. Evenness considers the distribution or uniformity of the species in the community [165]. Alpha diversity takes into account these two factors and describes the within habitat or intra-community diversity [166]. Beta diversity describes the in between habitat or inter-community diversity. To quantitatively measure diversity and determine changes in the microbial population composition, diversity indices are commonly calculated [165]. Several indices exist and there is no general agreement on which is the best to use. The Simpson diversity index is commonly used to determine alpha diversity based on operational taxonomic units (OTUs) [165]. Using the inverse of this index results in the higher the value of this index, the greater the diversity.

1.4.1 Aflatoxins and gut microbiota interactions

In contrast to the availability of many studies describing the pathogenesis of AFs, there are only few which describe the effect of AFs on gut microbiota [167]. A few studies demonstrating possible effects of AFB1 on gut microbiota are cited subsequently.

In a study performed on 20 rats by Wang et al. [168], AFB1 was administered at 5, 25, and 75 µg/kg BW. After 4 weeks, the faecal microbiota were analysed through 16S rRNA gene sequencing and the results were compared to the control group. An AFB1-dose dependent

decrease in alpha diversity was observed. At phylum level, there was no community shift detected, but some lactic acid bacteria were significantly reduced by AFB1. In a pig study [169], where the animals were fed with an AFB1 (320 µg/kg) contaminated diet for a month, an increase in the relative abundance of phylum *Bacteroidetes* and a decrease in *Firmicutes* were observed in the colon content. At genus level, compared to the control group, AFB1 had a negative impact on *Prevotella* (from 25% to 22%) and *Lactobacillus* (from 51% to 19%). Others seemed to be positively affected, such as the genera *Lachnospira* (from 2% to 5%) and *Campylobacter* (from 1% to 3%). In a broiler chicken study [170], a decrease in *Lactobacilli* in the cecal contents was equally observed following diet contamination with AFB1 at 1 µg/kg feed. Yet, with an AFB1 contamination level of 1.5 to 2 µg/kg feed, there was an increase of the *Lactobacilli* population. Furthermore, an AFB1 concentration of 2.5 µg/kg feed has been linked to an increase in short chain fatty acid production, which can be associated with a higher *Lactobacilli* prevalence in poultry [171]. In a study performed in Guatemala [172], faecal samples of 35 children were analysed to determine a link between AF exposure, stunted growth, and microbial dysbiosis. The results identified a higher chance of dysbiosis in children consuming an AF contaminated diet (>10 ng/kg BW). Furthermore, a significant difference in beta diversity between the shorter and taller children was observed. The children exposed to the higher AF levels had an increase in the genera *Bacteroides*, *Coprococcus*, and *Rikenellaceae*, and a decrease in *Streptococcus*. Based on all these studies, it is clear there is no golden thread that runs through the results. Aflatoxin B1 has demonstrated to affect gastrointestinal microbiota, but due to the complexity, more research is necessary, especially monitoring in humans at risk would provide more information.

Besides the mycotoxins having an effect on the composition of the gut microbiota, some bacteria, commonly referred to as probiotics, are administered to humans and have been observed to reduce the bioavailability of AFs. Most studies regarding AFs have focussed on bacteria belonging to the phyla *Actinobacteria* and *Proteobacteria*, and the genus *Lactobacillus* [173]. El-Nezami et al. [75] administered a *Lactobacillus rhamnosus* strain or a placebo for 5 weeks to 90 healthy male students in China, based on detectable urinary AFM1 concentrations. This specific strain had previously demonstrated its binding capacity to AFB1 (one bacterium could bind more than 10^7 AFB1 molecules) [174]. The binding is attributed to hydrophobic interactions between the AFB1 molecule and the bacterial cell wall. At week 3

and 5 of the intervention study, urine was collected and analysed for AFB-N^7-guanine, a degradation product of AFs. After 3 and 5 weeks of supplementation, a significant decrease was observed in the AF-guanine adduct by 36% and 55%, respectively. This study concluded that a probiotic food supplement can reduce the bioavailability of AFB1 and may offer an effective dietary approach to decrease the risk of liver cancer. In an intervention study performed in Malaysia by Mohd Redzwan et al. [175], 71 people were provided with a probiotic drink containing *Lactobacillus casei* Shirota for 4 weeks. In one group, a significant reduction in serum AFB1-lysine was observed after the probiotic treatment compared to the placebo administration (with baseline 6.24 ± 3.42 (SD) pg/mg albumin).

1.4.2 Fumonisins and gut microbiota interactions

Altering effects of FB1 on the intestinal microbiota has been observed in animal studies. Yet, it is unclear which mechanisms are involved and how FB1 exerts this effect. The shift in microbial composition is possibly due to antibacterial properties of FB1, or due to an indirect host-mediated response. However, Ali-Vehmas et al. [176] and Sondergaard et al. [177] observed no antibacterial properties of FB1 *in vitro*. Indirectly, mycotoxin consumption can lead to a decrease in appetite, which results in less available nutrients for the gut microbiota, resulting in a necessary adaption of the bacterial communities. In a pig trial performed by Le Floc'h et al. [178], it was observed that nutrient restriction can lead to a decrease in SCFA production and an increase in *Lactobacillus spp.* in faecal samples.

Mateos et al. [179] fed piglets a FB1-contaminated diet (12 mg/kg) during a month. The faecal microbiota were analysed through 16S rRNA gene sequencing and compared to the control group without the FB1-contaminated diet. A decrease in diversity and a disruption of the normal composition of the bacterial community were observed as soon as 15 days of FB1 exposure. Higher levels of *Lactobacillus* and lower levels of the *Lachnospiraceae* and *Veillonellaceae* families, and particularly operational taxonomic units (OTUs) of the genera *Mitsuokella*, *Faecalibacterium*, and *Roseburia* were detected. It was concluded that FB1 alters the age-related evolution of gut microbiota. Furthermore, in a study performed in piglets by Bracarense et al. [180], an indirect effect of FB1 on the microbiota was found, and alterations in the small intestine morphology and histology were seen. A decrease in goblet cells in the jejunum and ileum was observed which results in a decreased mucin production. Mucin plays

an important role in the gut barrier function; it provides attachment sites for intestinal microbiota as well as acts as an endogenous energy source. In an *in vitro* experiment with ruminal bacteria, performed by Srichana et al. [181], no effect on the SCFA concentrations was observed, but the cultures with FB1 did show a significantly lower acetate/propionate ratio compared to the control group during the entire study, suggesting a shift in microbiota.

In the case of AFs, the reduction in bioavailability is mainly based on binding with bacteria, FUMs are mainly hydrolysed into less toxic metabolites [148]. Biotransformation of FUMs occurs naturally to a limited extent in the gut of several animal species [62,182,183]. Furthermore, an enzyme, a recombinant carboxylesterase, from a bacterium *Sphingopyxis* sp. has demonstrated very effective hydrolysis capabilities for FB1 [182] in the gut of turkeys broilers, and swine (measured in the excreta) [146,184]. Noteworthy, the esterase in FUM*zyme*®, the product employed in this doctoral thesis, was initially identified and isolated from a soil bacterium *Sphingopyxis* sp. MTA144 [182,184,185]. Many studies suggest the main pathway of FB1 metabolism to be through hydrolysis, the removal of one or both tricarballylic side chains, resulting in partially or fully hydrolysed FB1 metabolites [62]. In an *in vitro* experiment with human faecal microbiota, Daud et al. [186] equally demonstrated the hydrolysis of FB1 into its metabolites. Yet, HFB1 was significantly present in only 2 out of the 5 cultures. Furthermore, *in vitro* studies show there is a discrepancy in the increased amount of metabolites and decrease of the parent molecule [187]. Therefore, there is a suspicion that there could also be a binding effect of certain unspecified bacteria to FUMs, like *Lactobacillus* for AFB1.

HIGHTLIGHTS AND RESEARCH GAPS

1. Effects of mycotoxins, including AFs and FUMs, on intestinal microbial composition have been observed. Yet, there is no consistency.
2. Effects of AFs on human intestinal microbiota are not well investigated.
3. Effects of FUMs on human intestinal microbiota are not well investigated.

2 Scientific aims

Mycotoxigenic fungi can grow on several crops both in the field and during storage. Therefore, their secondary metabolites, some of which are mycotoxins, can contaminate both feed and food worldwide. Mycotoxins are responsible for several negative health effects in both animals and humans. Consequently, the presence of mycotoxins endangers feed and food safety and security. Specifically in sub-Saharan Africa, the climate favours the growth of mycotoxin-producing fungi. Moreover, subsistence farming, the lack of regulations and control, further aggravate the risk of mycotoxicoses. The staple diet in this part of the African continent consists mainly of maize, which is frequently contaminated with AFs and FUMs. Therefore, the focus of this doctoral thesis was on the most prevalent and toxic mycotoxins of these groups, namely aflatoxin B1 (AFB1) and fumonisin B1 (FB1).

Several pre- and post-harvest mycotoxin mitigation techniques exist. Yet, the lack of knowledge on mycotoxins and the chronic nature of their effects, result in the ignorance of people at risk of mycotoxin intoxication. In the animal feed industry, mycotoxin detoxifiers can be mixed in the feed to prevent mycotoxin effects in animals. Binders, such as bentonite clay, and modifiers, such as enzymes, can be used to adsorb or hydrolyse mycotoxins, respectively. If these detoxifiers could also prevent disease related to mycotoxin exposure in humans, they could provide an easy-to-use option as additive in a cereal-based diet, at the pre-consumption stage.

The **general aim** of this doctoral thesis was to evaluate the efficacy of two European Food Safety Authority (EFSA)-evaluated and EU-approved mycotoxin detoxifier feed additives (an aflatoxin binder, Mycofix Secure®, and a fumonisin esterase, FUM*zyme*®), in humans, with focus on children. Two suitable and complementary human toddler models were employed to investigate their efficacy. First, a validated *in vitro* human child gut model, the simulator of the human intestinal microbial ecosystem (SHIME®) was used to determine the efficacy and effect on gut microbiota of both additives. Second, an *in vivo* piglet surrogate animal model was used to compare two candidate application methods of fumonisin esterase: intraoral and intragastric administrations. Ultimately, pilot human intervention studies with the two feed additives and naturally contaminated food were planned in Kenya and South Africa. Following complications with COVID-19 and ethical approval in these countries, a pilot study in Belgium was planned with fumonisin esterase following single FB1 intake. Therefore, a FB1 dose determination study in Belgian volunteers was performed.

The **specific aims** of this doctoral research are summarised in Figure 12 and are:

1. To evaluate the efficacy and effect on gut microbiota of two mycotoxin detoxifying animal feed additives in an *in vitro* SHIME model. The reduction of AFB1 and FB1 concentrations was investigated based on the mode of action of the additives, i.e. binding of AFB1 to an aflatoxin bentonite clay binder, and hydrolysis of FB1 into partially hydrolysed forms (pHFB1a and pHFB1b) and hydrolysed fumonisin B1 (HFB1) as a result of fumonisin esterase action, respectively. Additionally, the impact of the two detoxifiers on gut microbiota was examined through the monitoring of colonic bacteria communities and short-chain fatty acid (SCFA) concentrations (Chapter 1).
2. To compare the efficacy of two application modes of fumonisin esterase in an *in vivo* piglet model, to determine if capsule ingestion of this enzyme is effective in a suitable surrogate animal model for humans (children). The enzyme was administered intraorally (in-feed analogue) and intragastrically (capsule analogue). The change in biomarkers for FB1 exposure, namely FB1, HFB1, pHFB1a, and pHFB1b, were investigated both in serum and faeces. Additionally, the impact on the most widely used biomarker of effect, namely serum sphinganine/sphingosine (Sa/So) ratio, was determined (Chapter 2). Aflatoxin B1 was not included in this aim as previous studies in humans have already demonstrated the effect of bentonite clay binders through capsule ingestion or through drinking water.
3. To test the efficacy of the two detoxifiers in pilot human intervention studies, i.e. to reduce AFB1 and/or FB1 bioavailability in people at risk in affected areas in Kenya and South Africa. Due to COVID-19 and legal complications regarding the enzyme, the intervention studies in Africa were not performed. By adding bentonite clay in the participants' diet, the following biomarkers for AFB1 exposure and endpoints for efficacy testing would have been analysed; AFB1-lysine adduct and aflatoxin M1 (AFM1) concentration in blood and urine, respectively. By adding fumonisin esterase in the participants' diet, the following biomarkers and endpoints for FB1 would have been included; FB1 concentration in blood, urine, and faeces, Sa/So ratio in blood and urine, and pHFB1a, pHFB1b, and HFB1 metabolite concentration in blood and faeces (Chapter 3).
4. To determine a suitable single FB1 dose for humans necessary for quantification of FB1 and its metabolites pHFB1a, pHFB1b, and HFB1, in faeces (Chapter 3).

This doctoral thesis is part of a larger One Health project, namely MycoSafe-South, the "European–African partnership for safe and efficient use of mycotoxin-mitigation strategies in sub-Saharan Africa" (EU ERA-Net LEAP-Agri co-fund, 'A long term EU-Africa research and innovation partnership on food and nutrition security and sustainable agriculture'). The research focusses on post-harvest food processing techniques (by fellow doctoral researcher Julianah Odukoya), as well as reduction methods in the transmission of mycotoxins to dairy cattle (by fellow doctoral researcher David Kemboi), and poultry (by fellow doctoral researcher Phillis Ochieng), and their derived edible products, as well as to humans. The focus of this doctoral thesis lies on the human aspect.

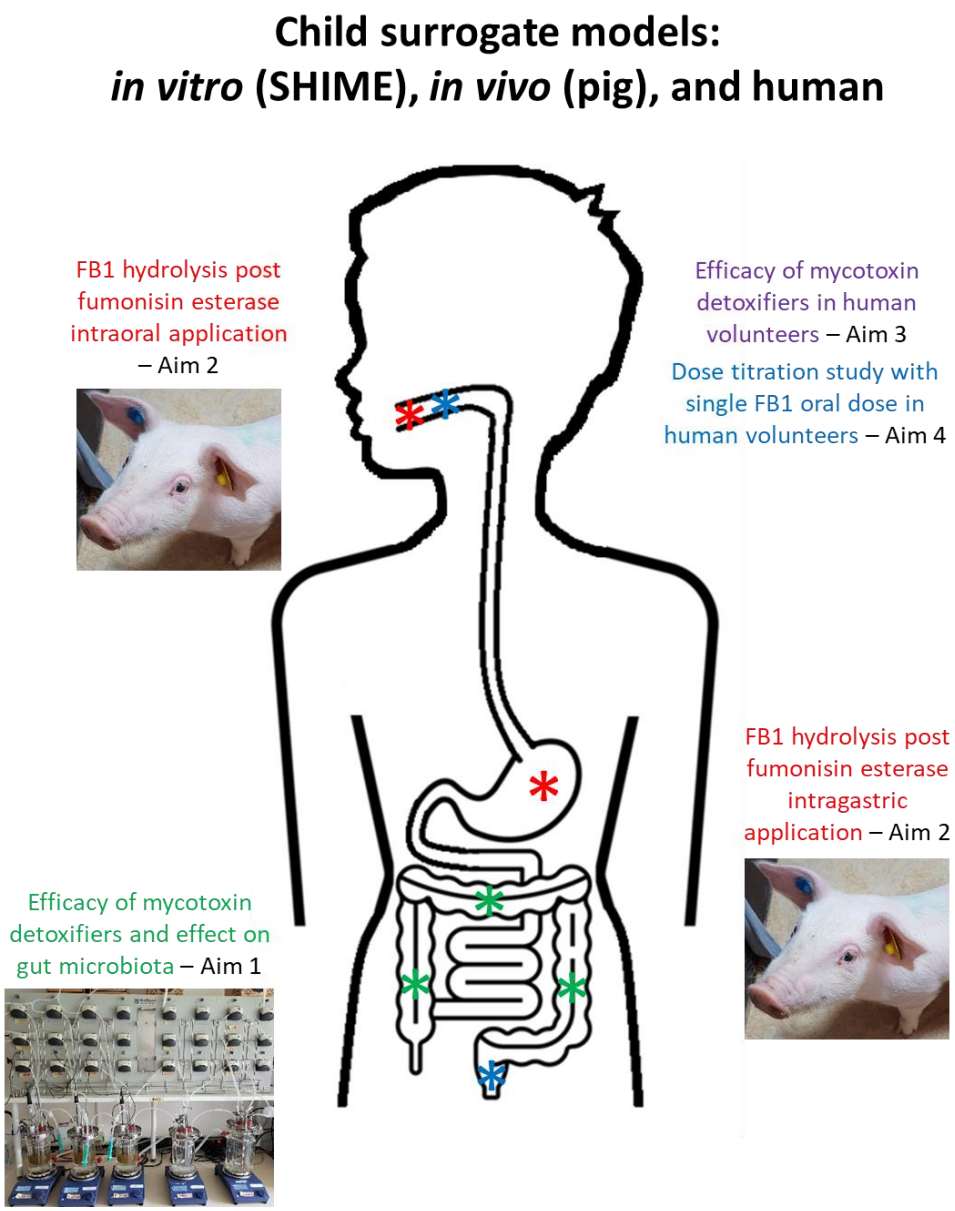

Figure 12. In this doctoral thesis, the efficacy and effect on gut microbiota of two mycotoxin detoxifiers (aflatoxin binder and fumonisin esterase) was assessed in a human *in vitro* (SHIME) model (aim 1). Next, the efficacy of two application modes (intraoral and intragastric) of fumonisin esterase was determined and compared in an *in vivo* piglet model (aim 2). Pilot human intervention studies including both detoxifiers were planned, but not performed (aim 3). A single oral FB1 dose for quantification in faecal samples was evaluated in humans (aim 4). The mycotoxins aflatoxin B1 (AFB1) and fumonisin B1 (FB1) were included. Efficacy of the mycotoxin detoxifiers was investigated by the analysis of AFB1, FB1, sphinganine/sphingosine (Sa/So) ratio, hydrolysed FB1 (HFB1), and partially hydrolysed FB1 (pHFB1a and pHFB1b). The coloured asterisks represent the administration site of the mycotoxins or detoxifiers, or the points of interest investigated in this thesis.

3 Chapter 1: The efficacy and effect on gut microbiota of an aflatoxin binder and a fumonisin esterase using an *in vitro* simulator of the human intestinal microbial ecosystem (SHIME®)

Adapted from

Neckermann, K.; Claus, G.*; De Baere, S.*; Antonissen, G.; Lebrun, S.; Gemmi, C.; Taminiau, B.; Douny, C.; Scippo, M-L.; Schatzmayr, D.; Gathumbi, J.; Uhlig, S.; Croubels, S. and Delcenserie, V. (*shared second author). The efficacy and effect on gut microbiota of an aflatoxin binder and a fumonisin esterase using an *in vitro* simulator of the human intestinal microbial ecosystem (SHIME®). *Food Research International*. **2021**, *145*, 110395. https://doi.org/10.1016/j.foodres.2021.110395

Sample collection (at ULiège) and mycotoxin analysis (at UGent) performed by Neckermann K. Short chain fatty analysis performed by Douny C (at ULiège). Metabarcoding analysis performed by Genalyse Partner (Herstal, Belgium).

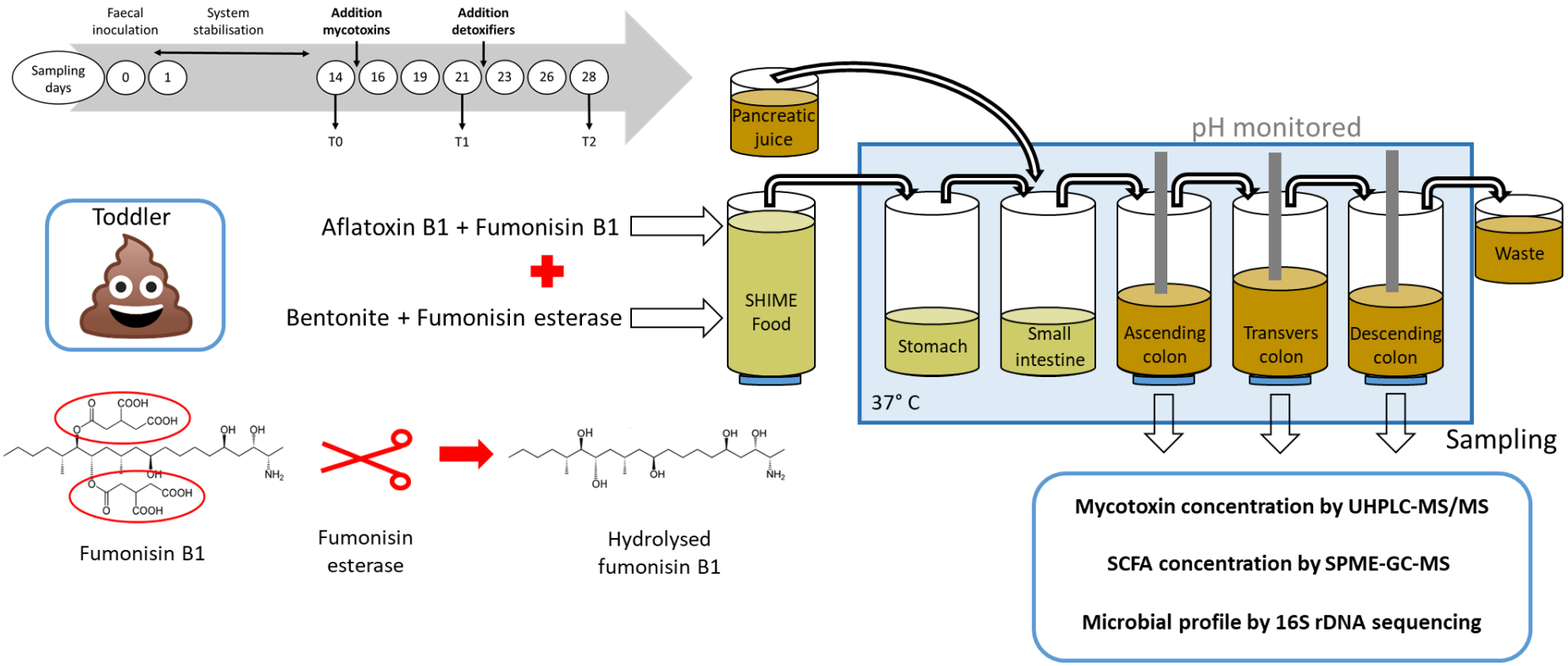

Figure 13. Graphical abstract of the scientific publication titled "The efficacy and effect on gut microbiota of an aflatoxin binder and a fumonisin esterase using an *in vitro* simulator of the human intestinal microbial ecosystem (SHIME®)", published in Food Research International (2021) [188].

3.1 Abstract

Mycotoxin intoxication is in general an acknowledged and tackled issue in animals. However, in several parts of the world, mycotoxicoses in humans still remain a relevant issue. The efficacy of two mycotoxin detoxifying animal feed additives, an aflatoxin bentonite clay binder and a fumonisin esterase, was investigated in a human child gut model, i.e. the *in vitro* Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). Additionally, the effect of the detoxifiers on gut microbiota was examined in the SHIME. A visualisation of the experiment setup performed in this chapter with the SHIME system is provided in Figure 13. After an initial two weeks of system stabilisation, aflatoxin B1 (AFB1) and fumonisin B1 (FB1) were added to the SHIME diet during one week. Next, the two detoxifiers and mycotoxins were added to the system for an additional week. The AFB1, FB1, hydrolysed FB1 (HFB1), partially hydrolysed FB1a (pHFB1a), and pFB1b concentrations were determined in SHIME samples using a validated ultra-high performance liquid chromatography-tandem mass spectrometry method. The short-chain fatty acid (SCFA) concentrations were determined by a validated gas chromatography–mass spectrometry method. Colonic bacterial communities were analysed using metabarcoding, targeting the hypervariable V1-V3 regions of the 16S rRNA genes. The mean AFB1 and FB1 concentrations significantly decreased 2 days after the addition of the detoxifiers; the mean AFB1 concentration (± SD) decreased from 24.60 ± 5.61 to 4.18 ± 4.35 µg/kg ($p < 0.001$) and the mean FB1 concentration (± SD) decreased from 872 ± 413 to 299 ± 271 µg/kg ($p < 0.01$). Likewise, the mean concentration of HFB1 (± SD) significantly ($p < 0.05$) increased from 299 ± 145 to 563.6 ± 65.2 µg/kg, 2 days after the administration of the detoxifiers. Concentrations of SCFAs remained generally stable throughout the experiment. No major changes in bacterial composition occurred during the experiment. The results demonstrate the promising effect of these detoxifiers in reducing AFB1 and FB1 concentrations in the human intestinal environment, without compromising the gastrointestinal microbiota.

Keywords

Mycotoxin; aflatoxin B1; fumonisin B1; bentonite binder; fumonisin esterase; fumonisin metabolites; detoxifiers; intestinal microbiota; human intestinal model; efficacy; *in vitro*; toddler SHIME

3.2 Introduction

Food security and safety continue to be one of the world's major challenges as world's population is expected to grow by another 2 billion people and is projected to reach 9.7 billion by 2050, as reported by the United Nations [189]. However, the rapidly augmenting population is not the only factor jeopardising food security. A universal concern for the safety of both food and feed is the presence of secondary fungal metabolites known as mycotoxins. Particularly in sub-Saharan Africa, where the highest prevalence of undernourishment prevails, cereal-based crops are spoiled by moulds and their toxins. Contamination with these toxicogenic fungi and their mycotoxins results in a decrease of the amount of edible food, jeopardising food security, and feed as well as in acute and chronic mycotoxicoses [190].

Aflatoxins (AFs) and fumonisins (FUMs) are mainly produced by *Aspergillus flavus* and *A. parasiticus*, and *Fusarium verticillioides* and *F. proliferatum* fungi, respectively, and can result in adverse health effects in both humans and animals [34]. Moreover, the notable persistence and ubiquitous nature of these toxins in sub-Saharan Africa are associated with large economic consequences. Food and feed spoilage directly affects smallholder farmers and families leading to a loss in resources and income. Subsistence farming is widespread in Africa and its produce is more prone to mycotoxin contamination [191]. Above all, while food security remains a problem [192,193], food safety is less regulated [109], sporadically leading to fatal mycotoxicoses [194]. In addition, mycotoxin contamination limits trade of agricultural products from sub-Saharan Africa to the rest of the world [41,42].

In Kenya, the exposure to aflatoxin B1 (AFB1) and fumonisin B1 (FB1) through the staple diet such as maize and groundnuts, including their derived products, is especially imminent [39–44]. Several outbreaks of aflatoxicosis, resulting in a large number of human casualties, have been reported in recent years in Kenya [195]. During the 2004 outbreak, a total of 317 cases of acute hepatic failure were documented, including 125 deaths after consuming locally grown maize stored under poor conditions. However, these numbers are most likely severely underestimated as hospitals are not always accessible for everybody and transportation is not always evident [196,197]. Aflatoxin B1 is carcinogenic to humans (International Agency for Research on Cancer - IARC Group 1), and is strongly correlated with liver cancer [198,199]. Co-contamination of cereals with AFB1 and FB1 is of great concern [30]. Fumonisin B1 is possibly

carcinogenic to humans and has been shown to cause oesophageal cancer in laboratory animals (IARC Group 2B) [198]. Chronic exposure to AFs and FUMs has also been linked to stunting in children [21,54–56]. Children are more vulnerable than adults; they have a lower detoxification capacity, grow rapidly, and have a high food intake per kg body weight. The metabolite AFM1 of AFB1 is excreted in milk, and is therefore an additional risk to child health [44,49]. Currently, Kenyans still consume grain and nut products exceeding the national set legal limits of mycotoxins [43]. Action must be urgently taken to further prevent mycotoxin outbreaks.

To reduce mycotoxin exposure to humans, both pre- and post-harvest strategies are equally important. Both need to be implemented collectively to prevent mycotoxicoses. Pre-harvest techniques are required in the field. These strategies include optimal planting density, crop rotation, tillage, plant stressor management, and chemical control [200]. Post-harvest techniques are of importance during the pre-consumption phase. These strategies include optimal storage management, with optimal temperature, moisture level, humidity, sorting, dehulling, and separation of spoiled grains. During food processing, nixtamalisation and fermentation are promising mitigation strategies [95,201].

Mycotoxin detoxifiers, such as binders and modifiers, are frequently applied as animal feed additives to prevent acute and chronic mycotoxin toxicity [202]. Two specific detoxifiers, a bentonite clay binder and a fumonisin esterase, have been evaluated by the European Food Safety Authority and approved as feed additives by the European Commission (EC) [136,142]. The AF binder, consisting of bentonite clay, binds AFB1 and other AFs, hence reducing their availability for gastrointestinal absorption. The FUM modifier consists of a purified enzyme that is specific for cleaving the FUM side chains, resulting in the formation of less toxic FUM metabolites (e.g. hydrolysed FB1 (HFB1) and partially hydrolysed FB1 (pHFB1a and pHFB1b) from the main analogue, FB1) in the gastrointestinal tract. The bentonite additive with identification code 1m558 (i.e. substance for the reduction of the contamination of feed by mycotoxins), has been approved for ruminants, poultry, and pigs, and the fumonisin esterase (1m03) for all poultry species and pigs [203]. Both products have been extensively tested in different types of *in vitro* and *in vivo* experimental studies and were found safe and effective in animals [136,142]. On the contrary, in humans, little information is available on the consumption of these detoxifiers. Bentonite has been reported to adsorb some minerals and

vitamins, such as Cu, Zn, Co, Mn, and vitamin B6 *in vitro* [204]. However, in animal trials, the effect of the same bentonite product adopted in this experiment was evaluated on the levels of vitamins in blood. No adverse effect of the binder was observed for vitamins such as A, D3, K, B1, B2, B6, and B12 in pigs after 7 days [205], in chickens after 35 days [206], and in dairy cattle after 7 days [207]. Furthermore, in a 2-week and 3-month human trial, performed by Wang et al. [120] in Texas and Afriyie-Gyawu et al. [132] in Ghana, respectively, the safety of a similar clay was tested, and no effects on several minerals, including Cu, Zn, and Mn, were observed, apart from an increase in Sr levels. Additionally, no bentonite-related differences were observed in haematology, liver and kidney functions and electrolytes. Furthermore, several studies reported by EFSA [136] showed a decrease in heavy metal (Cd, Pb, Cs, and Tl) uptake with the intake of bentonite.

To the authors' knowledge, no studies have been performed with the addition of detoxifiers to the Simulator of Human Intestinal Microbial Ecosystem (SHIME). In this respect, the SHIME is a promising *in vitro* technique to study the efficacy of such candidate detoxifiers as well as the effect of and on the microbiota. Biodegradation of ochratoxin A in a SHIME system has been studied [208]. A reduction in the beneficial species *Lactobacillus reuteri* was observed, and the descending colon was identified as being the main site of mycotoxin biodegradation.

Therefore, the current study was performed to investigate the potential detoxifying effect of an AF binder and a FUM modifier in humans, with focus on children. Instead of using human beings, this study benefits from the existence of the validated human gut model, to mimic the intestinal environment of a child. The goal of this study was to attain a post-harvest mitigation strategy, namely suitable detoxifiers that could be added to food in regions where contamination with AFs and FUMs is a food safety problem, and where the availability of food is limited, such as sub-Saharan Africa. Furthermore, this study investigated the effect of the treatment on the gut microbiota. With this toddler SHIME system, the effect of both the AF binder and FUM modifier on the mycotoxin concentrations and bacterial composition of the gut was investigated *in vitro*. Therefore, an analytical method to assess AFB1, FB1, as well as the FB1 hydrolysis products in SHIME medium samples was developed and validated. The impact of AFB1 and FB1 on the colonic microbiota was determined by measuring the short chain fatty acid (SCFA) production and by performing 16S metabarcoding analysis of bacterial

population. Recording SCFA production is useful to monitor the colonisation and adaptation of the gut microbiota in the *in vitro* system during the stabilisation period, and to study the effect of AFB1, FB1, and detoxifiers on human gut microbiota.

3.3 Materials and methods

3.3.1 Mycotoxins, detoxifiers, reagents, and materials

Analytical AFB1 and FB1 standards, and Biopure™ ^{13}C-labelled internal standards $^{13}C_{17}$-AFB1 and $^{13}C_{34}$-FB1 were purchased from Fermentek (Jerusalem, Israel) and Romer Labs (Tulln, Austria), respectively. Analytical standards of pHFB1a, pHFB1b, and HFB1, as well as the AFB1 binder, Mycofix® Secure (bentonite E558), and the FB1 modifier, FUM*zyme*® (Figure S1 in supplementary materials), were obtained from Biomin® (Tulln and Getzersdorf, Austria). Aflatoxin B1 stock and work solutions were prepared in acetonitrile (ACN) and stored at −20°C. Fumonisin B1 and metabolite solutions were prepared in water/ACN (50/50, v/v) and stored at 2 – 8 °C. Analytical grade formic acid, ACN, water, and methanol, as well as glycerol and sodium bicarbonate ($NaHCO_3$) were procured from VWR™ (Leuven, Belgium). Di- and tripotassium phosphate (K_2HPO_4 and K_3PO_4) were obtained from Chem-Lab NV (Zedelgem, Belgium) and hydrochloric acid (HCl) and sodium hydroxide (NaOH) from Sigma Aldrich BVBA (Overijse, Belgium). Formic acid, ammonium formate, and ACN used for the preparation of mobile phases were of ULC–MS grade and were acquired from Biosolve (Valkenswaard, The Netherlands). Oasis® PRiME HLB 96-well plates (30 mg sorbent per well) and 96-well sample collection plates were from Waters™ (Antwerp, Belgium).

3.3.2 Faecal Inoculum

The faeces used to inoculate the SHIME system were obtained from a 2.3 year-old male child of African descent living in Merelbeke, Belgium, as several factors such as dietary habits, genetics, and age have been shown to play a role in shaping the gut microbiome [209–211]. The child's diet reportedly consisted mainly of *ugali*, a typical African maize product, bread, Weetabix®, and spaghetti. The stool sample was collected, transported under cooled and anaerobic conditions, and processed upon arrival at the laboratory (< 3 h). A 20% (m/m) suspension of the faecal sample in phosphate buffer was prepared. The mixture was

homogenised and filtered in a stomacher bag with a mesh screen liner (80 microns pore size) (Biomérieux, Basingstoke, United Kingdom). An additional 20% of glycerol as cryoprotectant was added to the filtered substance. The sample was stored at −80°C until inoculation of the baby SHIME system.

3.3.3 Experimental design and sample collection

The toddler SHIME system consisted of five vessels in series, each in connection through tubes [212,213]. Each vessel represented a different part of the gastrointestinal tract. The stomach in the first vessel was followed by a duodenum and ileum compartment, and the three parts of the colon; the ascending (AC), transverse (TC), and descending (DC). Each vessel had two chambers; an inner one holding the gastrointestinal contents and an outer one with circulating warm water at 37°C.

The growth medium used in the toddler SHIME (ref PD-NM005) was provided by ProDigest (Ghent, Belgium) in powdered form, to be dissolved in water resulting in SHIME food for the system. Before connecting the feeding bottle to the SHIME system, the mixture was acidified with HCl (12 M) to obtain a pH of 3.8–4.0, corresponding to the pH of the stomach environment of a young child [213,214]. The pancreatic juice was prepared by adding 2.5 g/L of $NaHCO_3$, 4 g/L of bile salts (bovine), and 0.9 g/L of pancreatin (both provided by ProDigest) to distilled, sterilised water, and connected to the system. Furthermore, HCl (0.5 M) and NaOH (0.5 M) were connected to the colon compartments. Prior to the start-up of the experiment, 9, 14 and, 11 mL of faecal inoculum were added to the growth medium mixtures of 150, 240, and 180 mL present in the AC, TC, and DC compartments, respectively. In the colon compartments, stirrers rotated at 300 rpm continuously. The pH in the colon regions was automatically controlled through pH electrodes and maintained between 5.4–5.8, 6.0–6.3, and 6.3–6.5 in the AC, TC, and DC, respectively.

After inoculation, the system was left to stabilise for two weeks, to allow the bacteria time to adapt to the new environment. This was followed by the simultaneous addition of the two mycotoxins to 1 L of SHIME food for one week, i.e. AFB1 at a concentration of 81.6 µg/kg food and FB1 at a concentration of 2,000 µg/kg food. Afterwards, the mycotoxins were added during another week, together with addition of the two detoxifiers (Mycofix® Secure - 2.5 g/kg

food and FUM*zyme*® - 60 U/kg food). Food was pumped into the system three times a day (4 mL/min during 10.5 min).

Two repetitions were performed. An overview of the sampling days is presented in Figure 14. SHIME samples were collected from AC, TC, and DC on several days, i.e. one day after inoculation (day 1), immediately after the stabilisation period of 2 weeks (day 14 (T0)), during the first week with mycotoxins added (days 16, 19, and 21 (T1)), and during the second week with mycotoxins and detoxifiers added (days 23, 26, and 28 (T2)). A total of 12 mL was sampled from each colon compartment on each sampling occasion. All samples were stored at −20°C until further analyses.

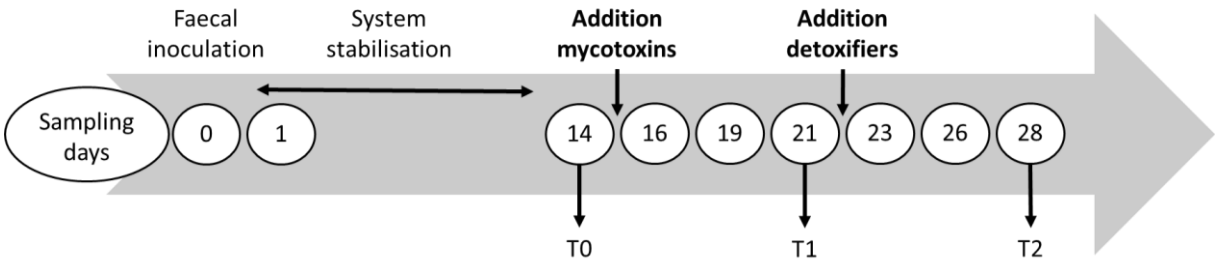

Figure 14. Timeline presenting the course of the experiment and the sampling days used for determination of mycotoxin concentration, short chain fatty acid concentration and metagenetic analysis. T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 µg/kg food) and fumonisin B1 (FB1) (2,000 µg/kg food); T2 = after one week of addition of AFB1 (81.6 µg/kg food) and FB1 (2,000 µg/kg food) with bentonite clay - Mycofix® Secure (2.5 g/kg food) and fumonisin esterase - FUM*zyme*® (60 U/kg food).

3.3.4 Mycotoxin analysis and method validation

Based on a validated multi-mycotoxin UHPLC-MS/MS method for porcine plasma [215], the analytical protocol to quantify the mycotoxins was adapted and fine-tuned for SHIME medium.

Samples were centrifuged (10 min, 2,851 × *g*, 4°C) to remove AFB1-binder bound compounds before transferring 250 µL of supernatant to a falcon tube. Next, 25 µL of an internal standard solution containing 100 ng/mL $^{13}C_{17}$-FB1 and 10 ng/mL $^{13}C_{34}$-AFB1 in ACN was added to the supernatant. The mixture was vortexed and allowed to equilibrate for 5 min

at room temperature. Deproteinisation was performed by the addition of 700 µL of 0.1% formic acid in ACN and vortexing. Mixing occurred on a vertical rotating shaker (Trayster digital, IKA®, Staufen, Germany) during 10 min at 80 rpm. Samples were centrifuged (10 min, 2,851 × *g*, 4°C) and supernatant was transferred to an Oasis PRiME HLB 96-well plate. The supernatant was allowed to slowly run through the sorbent before applying vacuum for 5 min. The eluate in the collector plate was dried under a nitrogen stream at 40°C. The residue was reconstituted in 250 µL of water/methanol (50/50, v/v) and vortexed. The collector plate was placed into an autosampler and an aliquot of 5 µL was analysed by the UHPLC-MS/MS instrument (Acquity H-Class UHPLC and Xevo® TQ-S MS, Waters, Milford, MA, USA). Mobile phase A was 0.3% formic acid and 10 mM ammonium formate in water, and mobile phase B was ACN. Chromatographic separation was achieved using an Acquity HSS T3 column (2.1 × 100 mm, 1.8 µm particles; Waters), thermostatted at 45°C.

The LC-MS/MS method was validated for AFB1, FB1, pHFB1a, pHFB1b, and HFB1 according to the protocol of De Baere et al. [216]. Blank SHIME medium, obtained after two weeks of stabilisation, was spiked with working solutions of AFB1, FB1, pHFB1a, pHFB1b, and HFB1. The validation requirements complied with the guidelines established by the EC [217]. The following parameters were assessed: method linearity, within- and between-day accuracy and precision, limit of detection (LOD), limit of quantification (LOQ), specificity, extraction recovery, matrix effect (signal suppression/enhancement). The results for each analyte are summarised in Tables S1 to S3.

According to literature, AFB1 (<1-1,000 µg/kg) and FB1 (<1-3,000 µg/kg) concentrations may be observed in a wide range in African crops [27,40,218–221]. Thus, AFB1 with a concentration of 81.6 µg/kg feed and FB1 with a concentration of 2,000 µg/kg feed were employed in the experiment to mimic high contaminations found under African circumstances.

3.3.4.1 Linearity

Linearity was assessed through preparation of three matrix-matched calibration curves. The correlation coefficients (r) and the goodness-of-fit coefficients (g) were calculated, and the acceptance criteria were set at ≥0.99 and ≤20%, respectively [222].

3.3.4.2 Within- and between-day precision and accuracy

The within-day precision and accuracy were evaluated by analysing six blank samples spiked at low (LOQ), medium, and high concentration levels per analyte. The same was performed for the between-day precision and accuracy, but repeated on three different days. The acceptance criteria for within-day and between-day accuracy were: -50% to +20%, -30% to +10%, and -20% to +10% for concentrations ≤1 µg/kg, 1-10 µg/kg, and ≥10 µg/kg, respectively. For the within-day precision, the relative standard deviation (RSD%) had to be lower than the maximum relative standard deviation (RSDmax), which was <25% and <15% for concentrations ≥1 to <10 µg/kg and ≥10 to <100 µg/kg, respectively [223]. For the between-day precision, the RSD% had to be lower than the RSDmax, which was defined by the Horwitz equation: $RSDmax = 2^{(1-0.5 \log Concentration\ (g/mL))}$ [224,225].

3.3.4.3 Limit of detection (LOD) and limit of quantification (LOQ)

The LOQ was the lowest concentration of the analyte for which the method was validated with an acceptable accuracy and precision according to the guidelines described above. The LOQ was also the lowest concentration of the calibration curves. The LOQ was determined by analysing different concentrations spiked in six-fold on the same day.

The LOD was determined by calculating the analyte concentration that corresponds with a signal-to-noise (S/N) ratio of 3/1, based on the S/N ratio of the analyte in the LOQ samples.

3.3.4.4 Extraction recovery and matrix effect

The extraction recovery (Re) and signal suppression/enhancement (SSE) due to matrix effects were calculated according to the method of Matuszewski et al. [226]. Three types of samples were prepared in triplicate:

- matrix-matched blank samples, spiked before extraction (=Spiked)
- matrix-matched blank samples, spiked after extraction (=SpikedExtract)
- standard solutions (=Standard)

The peak areas of these samples were compared to calculate the extraction recovery (Re) and signal suppression/enhancement (SSE) due to matrix effects:

- SSE = (peak area SpikedExtract/peak area Standard) x 100
- Re = (peak area Spiked/peak area SpikedExtract) x 100

3.3.5 Short-chain fatty acid analysis

The SCFA analysis was performed following the validated method for SHIME samples by Douny et al. [227]. To a 25 µL SHIME sample, 40 µL of 0.2 mg/mL methyl-valeric acid in water was added as internal standard, followed by the addition of 15 µL of 0.9 M H_2SO_4 in water and 920 µl of fresh SHIME medium to obtain 1 mL. This solution was then vortexed and analysed by solid phase micro extraction gas chromatography coupled to mass spectrometry (Thermo Fisher Scientific, Massachusetts, USA).

3.3.6 Metagenetic analysis – microbial community analysis

The metagenetic analysis or marker gene amplification of the microbial V1-V3 regions of 16S rRNA was performed following the guidelines for 16S metagenomic sequencing library preparation for Illumina® technology users [228] and the method described by Bondue et al. [229]. Microbial DNA was extracted from the pellet of SHIME samples using the PSP® Spin Stool DNA Plus Kit (Invitek, Berlin, Germany). The V1-V3 regions of 16S rRNA genes were amplified through PCR with the forward 5'-GAGAGTTTGATYMTGGCTCAG-3' and reverse 5'-ACCGCGGCTGCTGGCAC-3' primers. Clean-up and purification of the PCR products using the Agencourt AMPure XP beads kit (Beckman Coulter, Pasadena, CA, USA) was followed by a second round of PCR using the Nextera XT Index Kit, followed by purification. PCR products were quantified with Quant-IT PicoGreen (ThermoFisher Scientific, Waltham, MA, USA) followed by normalisation, pooling, and MiSeq sequencing using V3 reagents (Illumina, San Diego, CA, USA). Processing of sequence reads was carried out as described previously [229]. The obtained operational taxonomic units (OTUs) were used for the inverse Simpson diversity index calculation.

3.3.7 Statistical analysis

All statistical analyses for mycotoxin concentration, SCFA concentration and metagenetic analysis data were performed using the software package RStudio [230]. Additionally, statistical analysis regarding the alpha diversity between colon regions, using the inverse Simpson diversity index, was evaluated using AMOVA in Mothur [231].

Data from the three colon regions were analysed separately. In addition, SCFA concentrations of the three colon regions were summed to represent the gastrointestinal tract (GIT). For the metagenetic analysis, significant differences between the three colon regions were additionally explored.

Mycotoxin and SCFA concentration means of the three treatments and beta diversity were compared using a one-way ANOVA, provided the assumptions were met. Individual treatment means were then compared using Tukey's HSD test. Normality of the residuals was tested with the Shapiro-Wilk test, followed by a Levene's test for homoscedasticity. In case the residuals were not normally distributed, the non-parametric Kruskal-Wallis Rank Sum Test was performed, followed by the Pairwise Wilcoxon Rank Sum Test. In case the assumption of homoscedasticity was not met, a Welch's ANOVA was performed. The level of significance was set at 0.05.

3.4 Results and discussion

3.4.1 Statistical analysis

The SHIME samples collected before mycotoxin inoculation (day 1 and 14) contained traces of FB1 in all three colon regions of both repetitions, as shown in Figure 15. A mean (± standard deviation, SD) FB1 concentration of 2.93 ± 0.72 µg/kg and 0.93 ± 0.11 µg/kg (<LOQ of 1.0 µg/kg) was found in the day 1 and 14 samples, respectively. This finding could possibly be explained by the origin of the faecal inoculum or from the growth medium. The toddler consumed wheat- and maize-based products on a daily basis. Fumonisins have been found in these particular foodstuffs in Europe [232], although the levels found are mostly low. Based on international data from 2002, the estimated mean FB1 uptake in Europe was 0.2 µg/kg body weight per day [218]. In 2012, cereal samples collected in Northern Europe had levels for FB1 below the LOD of 100 µg/kg sample [233].

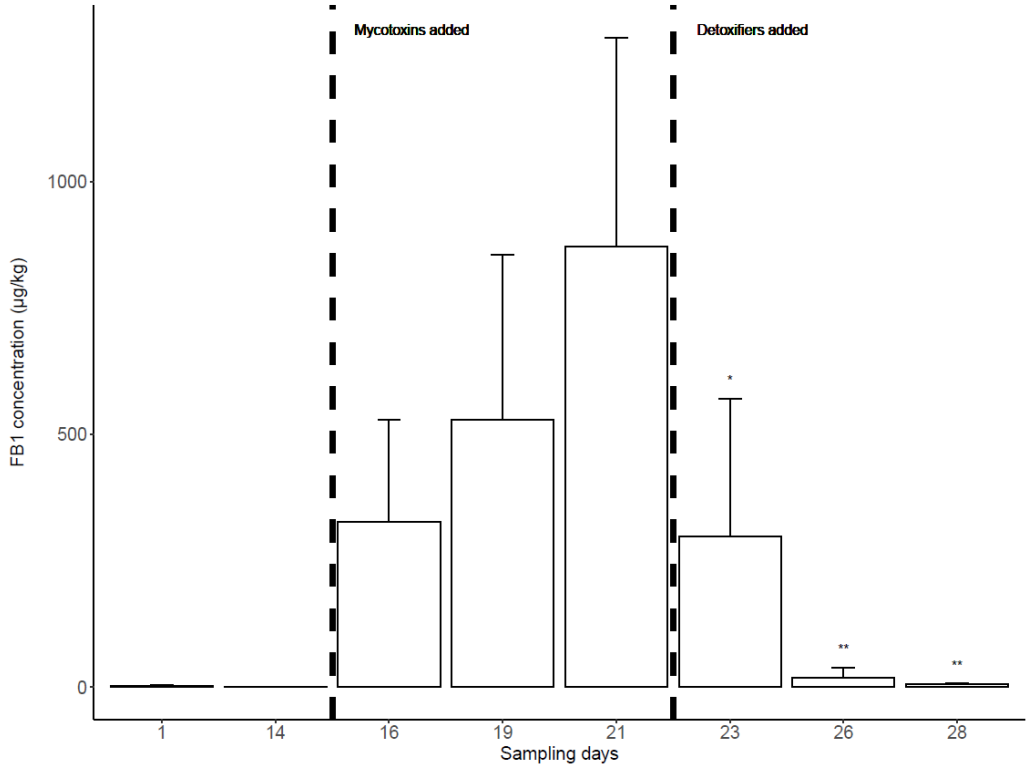

Figure 15. Mean concentration of fumonisin B1 (FB1) determined in the AC, TC, and DC compartment at different sampling days during the SHIME experiment (*n* = 2). Error bars represent the standard deviation. The dashed lines represent the addition of aflatoxin B1 (AFB1) (81.6 µg/kg food) and FB1 (2,000 µg/kg food), followed by addition of both mycotoxins and bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food). The asterisks represent the significant statistical difference (*, $p < 0.05$ and **, $p < 0.01$) compared to sampling day 21.

A clear effect of the enzyme was notable since the FB1 concentrations (± SD) were significantly reduced from 872 ± 413 µg/kg on day 21 to 299 ± 271 µg/kg ($p = 0.025$) on day 23, 18.8 ± 20.4 µg/kg ($p < 0.01$) on day 26 and 5.47 ± 2.57 µg/kg ($p < 0.01$) on day 28. This corresponds to a reduction in mean FB1 concentration by 65.0 ± 25.7%, 97.3 ± 3.1%, and 99.1 ± 0.7%, respectively. The cumulative increase in FB1 concentration from day 16 to 21 was due to the fact that FB1 was added to the food and subsequently pumped through the system three times per day.

In pig studies, the effect of the enzyme has previously been demonstrated by measuring FB1 concentrations and associated biomarkers (sphinganine to sphingosine (Sa/So) ratio, and hydrolysed FB1 derivatives) in serum, urine, and faeces [146]. A reduction of the FB1

concentration (30 mg/kg) of about 50% and more than 90% was observed in pig faeces 7 days after addition of the enzyme with a dose of 15 and 150 U/kg feed, respectively [147]. Masching et al. [146] performed three experiments to determine the FB1 degrading potential of the same esterase. First, within two hours, the enzyme (6 U per sample) degraded FB1 (300 µg per intestinal sample) completely into HFB1 in both jejunum and duodenum segments collected from a pig. Second, a decrease of approximately 46% and 77% in FB1 was observed in the excreta of turkeys after 7 and 14 days, respectively, following the consumption of FUM-contaminated feed (15,000 µg/kg) supplemented with the enzyme (15 U/kg) [146]. Third, in a pig trial, an average decrease of 72% in FB1 concentration in faeces was observed after 14, 28, and 42 days of feeding the piglets with FUMs (2,000 µg/kg) and fumonisin esterase (60 U/kg) [146]. Even though the SHIME system is a more complex multi-compartmental system, nearly 100% reduction was observed after 5 days (day 26), therefore inducing a higher FB1 reduction compared to the *in vivo* studies from Masching et al. [146]. Considering that the SHIME is an *in vitro* model, human *in vivo* studies will have to be performed to verify the efficiency of this enzyme in the human gut.

For HFB1, a significant increase in mean concentration was observed following addition of the detoxifiers (Figure 16). The mean concentration of HFB1 (± SD) increased from 299 ± 145 µg/kg on day 23, to 563.6 ± 65.2 µg/kg on day 26 and to 623.4 ± 71.0 µg/kg on day 28.

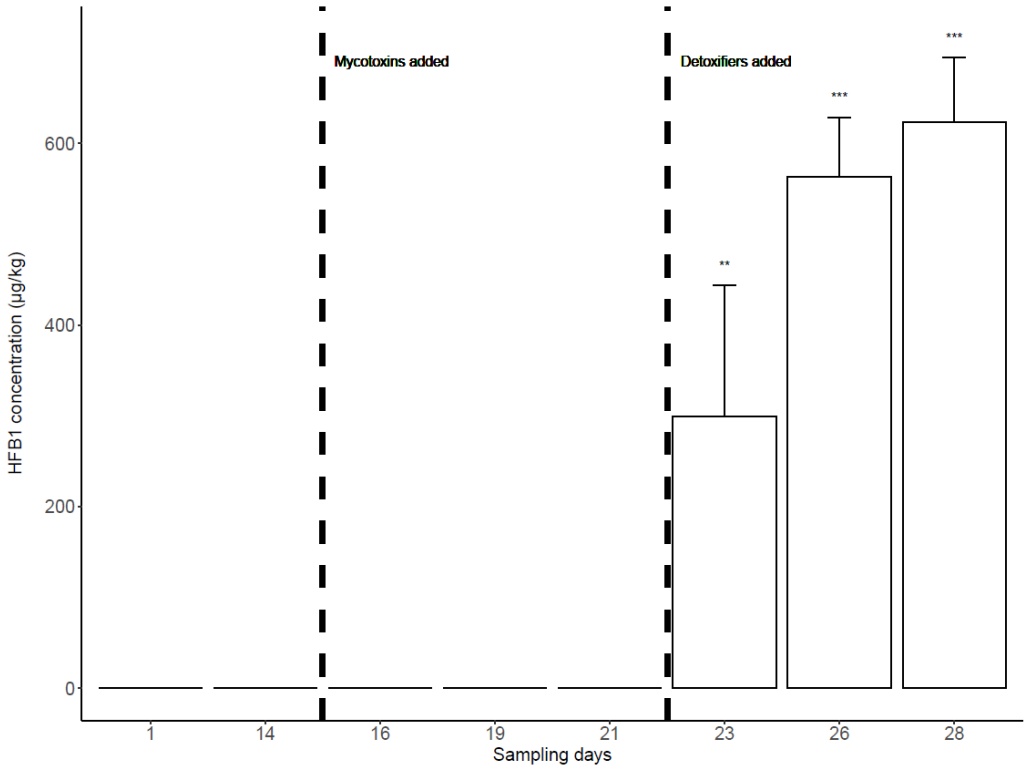

Figure 16. Mean concentration of hydrolysed fumonisin B1 (HFB1) determined in the AC, TC, and DC compartment at different sampling days during the SHIME experiment (n = 2). Error bars represent the standard deviation. The dashed lines represent the addition of aflatoxin B1 (81.6 µg/kg food) and fumonisin B1 (2,000 µg/kg food), followed by addition of both mycotoxins and bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food). The asterisks represent the significant statistical difference (**, $p < 0.01$ and ***, $p < 0.001$) compared to sampling day 21.

In other studies, it was noticed that the natural degrading capacity of the gut microbiota might contribute to FB1 hydrolysis; the hydrolysed forms amounting up to about 50% on average of the total excreted FUMs in faeces [141,147]. In this study, pHFB1a and pHFB1b were detected in samples before day 23 (Figure S2). However, no HFB1 was detected earlier than on day 23 (LOD = 0.31 µg/kg), supporting the evidence that gut bacteria do contribute to partial FB1 hydrolysis. The observation of decreasing FB1 concentrations (Figure 15) and the concurrent increase of HFB1 concentrations (Figure 16) following the addition of detoxifiers allowed us to conclude that the complete hydrolysis of FB1 into HFB1 in this experiment may solely be ascribed to the effect of the enzyme. This is similar to the study performed in the *ex vivo* pig gastrointestinal model of Masching et al. [146] where the degradation of FB1 by the

enzyme resulted in an increase in HFB1, yet no pHFB1a nor pHFB1b were detected. These hydrolysis products have been proven to be far less toxic compared to the parent toxin in mice, pigs, and rats. Grenier et al. [64] found that FB1 induced hepatotoxicity and affected the intestinal integrity and immune response through inhibition of ceramide synthase, whilst HFB1 only slightly altered the intestinal immune response. Furthermore, Voss et al. [234] observed that, in contrast to FB1, HFB1 did not induce neural tube defects in unborn mice. In both studies, HFB1 was also shown to only slightly disrupt the sphingolipid metabolism. Hahn et al. [235] did not detect any differences in Sa/So ratio between rats being fed pHFB1a and pHFB1b during three weeks and the negative control group. Therefore, the results of this study seem promising in reducing, or even eliminating the toxic effects of FB1 in exposed humans.

The AFB1 concentrations in the SHIME samples collected before mycotoxin inoculation were below the LOD of 0.002 µg/kg (Figure 17). On day 1, in one DC sample of one repetition a trace amount of AFB1 was detected with a concentration of 0.330 µg/kg. The finding of a low AFB1 concentration in a day 1 DC sample, and not in the AC nor TC, is most likely due to a contamination during sample processing and analysis in the laboratory. The cumulative increase in AFB1 concentration from day 16 to 21 has the same explanation as FB1; AFB1 was added to the food and was from there on pumped through the system three times per day.

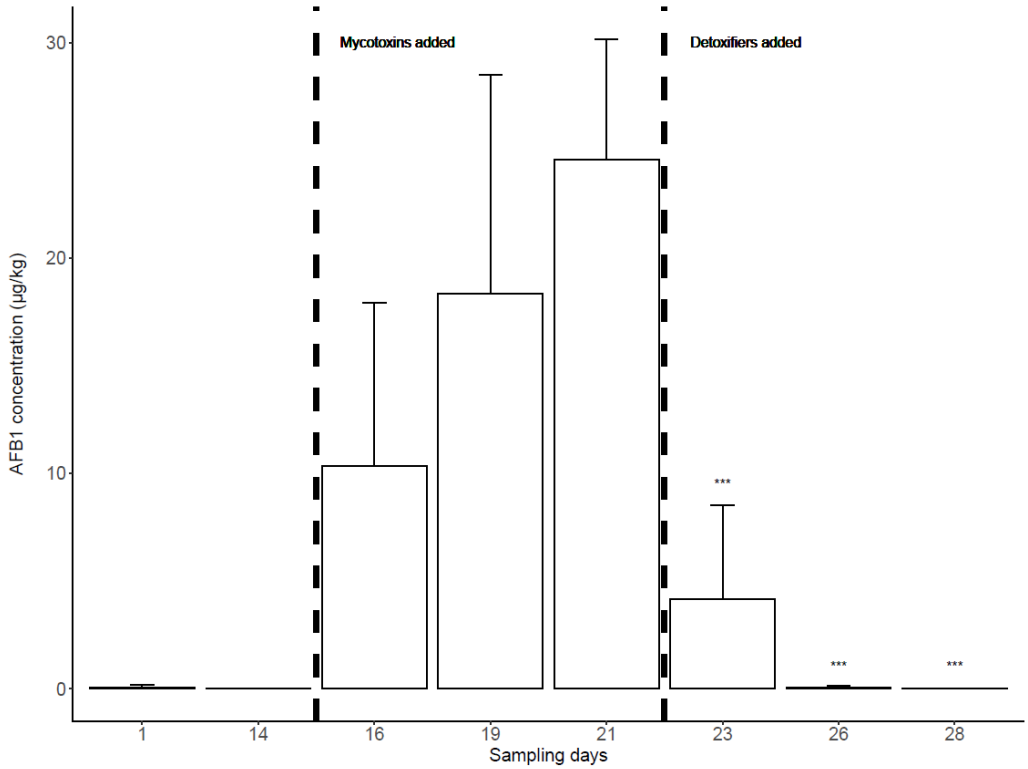

Figure 17. Mean concentration of aflatoxin B1 (AFB1) determined in the AC, TC, and DC compartment at the different sampling days during the SHIME experiment (*n* = 2). Error bars represent the standard deviation. The dashed lines represent the addition of AFB1 (81.6 µg/kg food) and fumonisin B1 (2,000 µg/kg food), followed by addition of both mycotoxins and bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food). The asterisks represent the significant statistical difference ($p < 0.001$) compared to sampling day 21.

When bentonite clay was administered together with the toxin, the AFB1 mean concentration ± SD significantly decreased about six-fold, i.e. from 24.60 ± 5.61 µg/kg on day 21 to 4.18 ± 4.35 µg/kg ($p < 0.001$) on day 23 (Figure 17). A decrease of 78.9 ± 23.9% on day 23, 99.7 ± 0.5% on day 26 and 100% on day 28 in mean AFB1 concentration was observed, when compared to day 21. This is in accordance with the reported AFB1-binding capacity of minimum 90% of bentonite E558 [236]. Bentonite clay has been proven to be safe and effective, and authorised as feed additive in binding AFB1 in animals (ruminants, poultry and pigs) [136,237]; a decrease in AFM1 excretion in milk of 55.0% to 68.0% and 17.3% to 21.3% was observed [238,239], and an *in vitro* study resulted in an AFB1 adsorption capacity from 90.0% to 95.3% [142]. The effectiveness for detoxification in humans was determined by measuring AFM1 in urine and the AFB1-lysine adduct in serum [120,123,240].

The adsorption of AFB1 to the binder in the GIT reduces the availability of AFB1 for absorption into the systemic circulation, hence reducing the oral bioavailability. Long-term *in vivo* studies will have to determine the effect of the binder in preventing further symptoms and diseases associated with acute and chronic AFB1 intoxications [122]. Furthermore, additional data of long-term *in vitro* or *in vivo* studies are necessary to monitor the effect of binder uptake during longer periods on the vitamin and mineral status.

3.4.2 Short-chain fatty acid (SCFA) analysis

Concentrations of nine SCFAs were investigated to determine and monitor the gut health during the trial in both repetitions. A variety of metabolites are formed during the microbial fermentation and the main end-products are SCFAs. Therefore, surveying the SCFA production is valuable in monitoring colonisation and adjustment of the gut microbiota during the stabilisation period of the system, as well as in assessing the effect of the mycotoxins and detoxifiers on the gut microbiota during the experiment [241]. The most abundant SCFA produced were acetate, followed by propionate and butyrate. These act primarily as energy source for colonocytes, making them vital to overall gastrointestinal health [242]. Additionally, isobutyrate and isovalerate were measured in the SHIME samples. Although other fatty acids, such as valerate, caproate, enanthate, and caprylate were also detected, their concentrations were found to be below the LOQ (48, 32, 32, and 32 mg/L, respectively). In all colon regions, the SCFA production was mostly similar in both repetitions. No significant differences were detected between the three treatments for individual SCFA concentrations in the AC, TC, DC (Figure 18), nor in the GIT (Figure S3). However, in DC, a decreasing trend in acetate was observed after administration of mycotoxins.

In our study, the mean SCFA proportions of the stabilised bacteria followed an acetate/propionate/butyrate molar ratio of approximately 71/20/9. Cinquin et al. [243] noticed a molar ratio of 75/19/6 in infant faeces and Cummings et al. [244] noted an average molar ratio of 60/24/16 in adult faeces. The SCFA ratio in our study approached the ratio found in infant faeces. The ratio in SCFA observed in the system at T0 in this study was comparable to other experiments performed in the SHIME with faecal material from a child (<2y old) [212,229].

Similar to the study of Bondue et al. [212], the SCFA concentration profile in the AC was evidently different from TC and DC (Figure 18), while it was nearly identical in TC and DC. This corresponds to what is commonly seen in the microbial profile, discussed later on. The lower pH in comparison to the TC and DC and availability of easily fermentable food in the AC will benefit some specific bacteria (mainly belonging to the phyla *Firmicutes* and *Proteobacteria*) that are readily able to consume these nutrients.

A decreasing trend in acetate concentration from treatments T0 to T1 in DC ($p = 0.061$) was observed (Figure 18). However, no significant difference in acetate concentration was established between T1 and T2 ($p = 0.62$). Gut microbial fermentation, and thus acetate producing bacteria, such as *Bifidobacteria*, *Lactobacillus*, *Akkermansia muciniphila*, *Prevotella*, and *Ruminococcus* [245], could be affected by the addition of mycotoxins. Yet, this effect was not observed, and no additional effect by the addition of detoxifiers was observed. Possibly, the slight decrease from T0 to T1 was due to natural fluctuations in time or method related variability. In the study of Zhou et al. [246], the oral exposure to AFB1 of rats to 5, 25, and 75 µg/kg BW, resulted in a decrease of acetate, propionate, and butyrate after 2 weeks onwards. Unfortunately, we implemented only one week of mycotoxin treatment. Our SHIME data are thus difficult to compare with these results. Acetate is formed by the decarboxylation of pyruvate by many bacterial groups present in the colon [241,247]. Although all SCFAs play a role in regulating several organ systems, it has been demonstrated that acetate in particular plays an important role in energy generation and substrate metabolism [248].

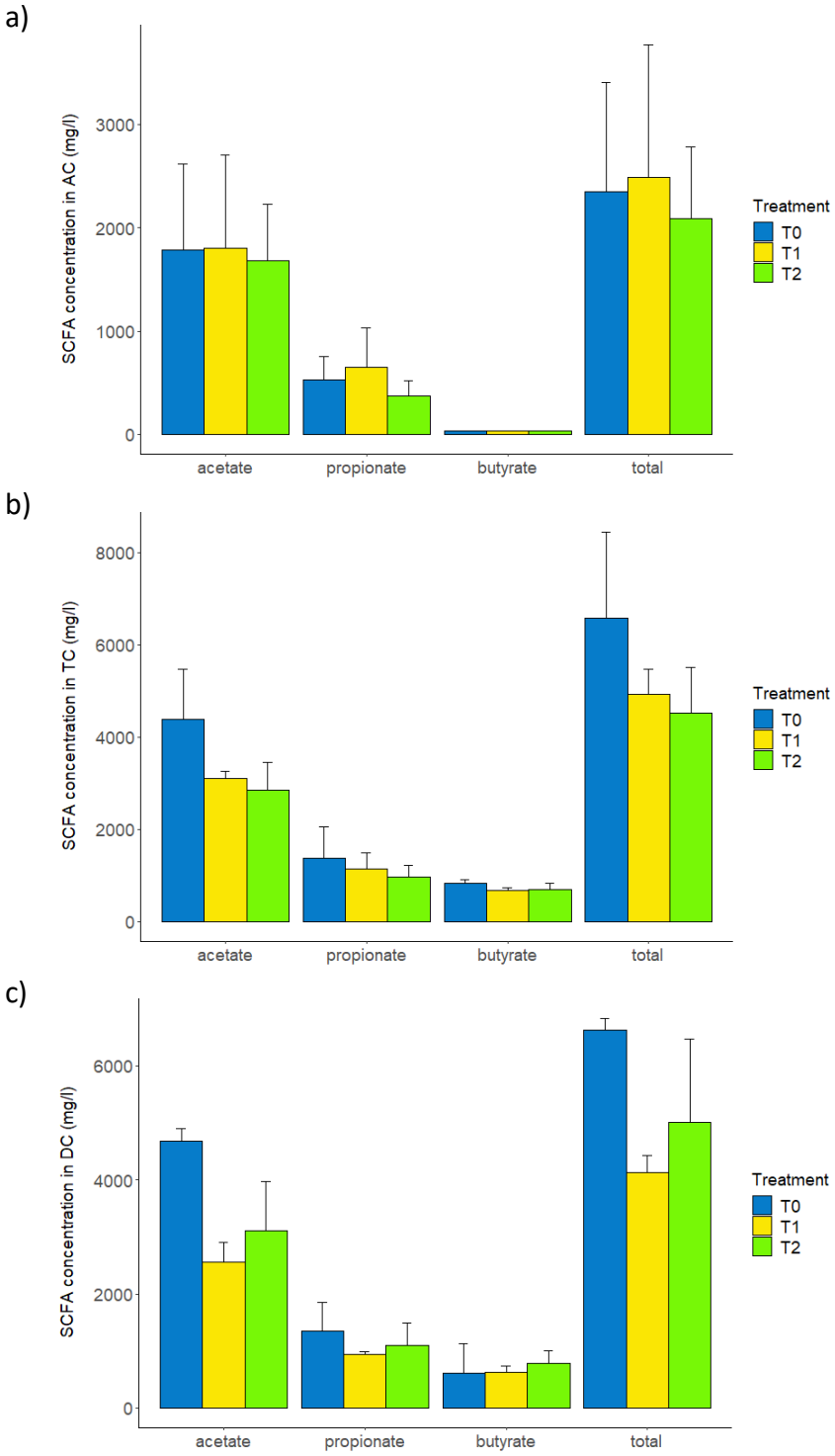

Figure 18. Mean concentrations of short-chain fatty acids (SCFAs) in the a) ascending (AC), b) transverse (TC) and c) descending colon (DC) (*n* = 2). Error bars represent the standard deviation. Total is the sum of acetate, propionate, butyrate, isobutyrate and isovalerate. T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 µg/kg food) and fumonisin B1 (FB1) (2,000 µg/kg food); T2 = after one week of addition of AFB1 (81.6 µg/kg food) and FB1 (2,000 µg/kg food), together with bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food).

Propionate concentrations were found to be rather similar in all three colon regions. It is mainly produced by *Bacteroidetes* spp., *Firmicutes*, *Lachnospiraceae*, and *Clostridium* cluster IX [213,243,249]. Butyrate was produced in a smaller amount than acetate and propionate. However, we found higher butyrate concentrations in both the TC and DC compared to the AC. This was in accordance with another study using the toddler SHIME model [229]. As for propionate, butyrate is produced by a restricted number of bacterial groups. Butyrate can be produced by two different pathways: the butyrate kinase pathway mainly used by *Clostridium* species and the butyryl-CoA:acetate-CoA transferase pathway that is preferred in presence of high acetate concentration [247]. Therefore, the production of butyrate occurs in the distal part of intestinal microbiota, where acetate concentration is high. The main butyrate producers are *Faecalibacterium prausnitzii* (*Clostridium* cluster IV), *Anaerostipes*, *Eubacterium*, and *Roseburia* spp. (*Clostridium* cluster XIVa) [250]. As those bacteria are strictly anaerobic bacteria, butyrate can also be considered as an internal control of the anaerobiosis of the model. Butyrate concentrations remained stable throughout the replicates, indicating anaerobic conditions were maintained in the system [229,251].

In general, the SCFA levels remained stable throughout the SHIME trial, indicating a healthy gut environment. The addition of solely the mycotoxins and in combination with detoxifiers did not negatively affect the SCFA producing capacity.

3.4.3 Metagenetic analysis

3.4.3.1 Alpha diversity

The microbial gut community was assessed using 16S rRNA sequencing and diversity was determined from the obtained OTUs. No statistically significant differences in the inverse Simpson diversity index were observed on genus level in AC, TC, DC, nor in the total GIT (Figures S4-7) after administration of mycotoxins and detoxifiers to the SHIME system. This is in contrast to the study of Mateos et al. [179], where six piglets were exposed to FUMs (10.2 mg FB1, 2.5 mg FB2 and 1.5 mg FB3 per kg feed), and after 15 days of exposure the inverse Simpson index significantly decreased compared to the control. For AFB1, Yang et al. [252] did not observe a significant difference in Simpson index between the control group and the group that received 20 µg/kg or 500 µg/kg AFB1 via their diet during 2 months in turbots.

Although no differences were observed between treatments within the colon regions, a significant difference ($p < 0.001$) in alpha diversity between the colon regions was determined (Figure 19). Alpha diversity in AC was significantly lower than in both TC and DC ($p < 0.01$). Due to the lower pH and the availability of highly fermentable substrate in the AC, only a few specific populations were present, limiting the bacterial diversity [212,213]. AC was dominated by the genus *Veillonella*, whereas TC and DC are mainly composed of *Lachnoclostridium*, *Bacteroides*, and unidentified genera from the *Lachnospiraceae* family.

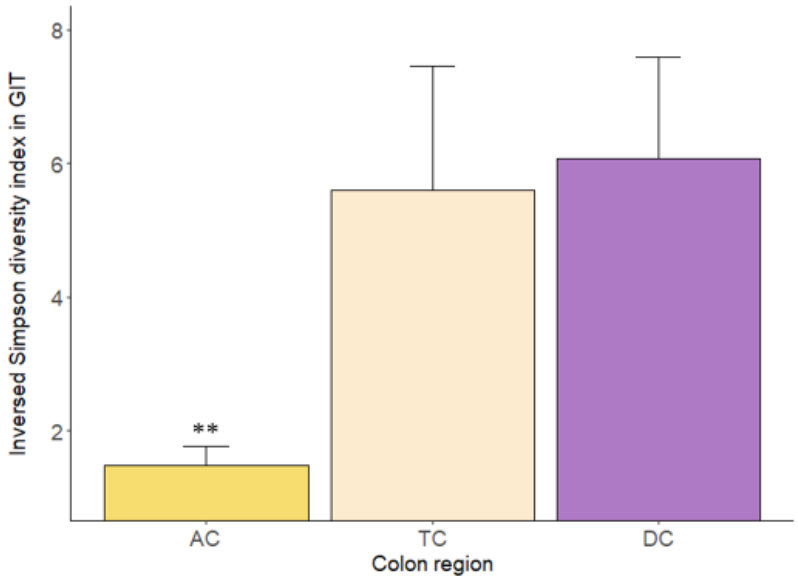

Figure 19. Inverse Simpson diversity index means per colon region in the gastrointestinal tract (GIT) on genus level (mean ± SD). The colon regions stand for ascending colon (AC), transverse colon (TC) and descending colon (DC). T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 µg/kg food) and fumonisin B1 (FB1) (2,000 µg/kg food); T2 = after one week of addition of AFB1 (81.6 µg/kg food) and FB1 (2,000 µg/kg food), together with bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food).

3.4.3.2 Beta diversity

Across all the phyla (Figure 20), families (Figure 21) and genera (Figures 22 and 23), no statistical significant differences in the relative abundance between the three treatments were found.

The main phyla present in the **faecal inoculum** consisted of *Firmicutes* (89.9%), *Bacteroidetes* (8.3%), and *Proteobacteria* (1.0%). These phyla were still present at **T0**, although at different proportions. On average, the bacterial population in AC constituted of *Proteobacteria* (64.1 ± 31.7%), *Firmicutes* (35.8 ± 31.6%), and *Bacteroidetes* (< 0.1%). In TC and DC, *Firmicutes* (62.8 ± 2.6% and 72.1 ± 5.3%, respectively) were followed by *Bacteroidetes* (30.7 ± 0.1% and 22.4 ± 4.1%, respectively), and *Proteobacteria* (6.2 ± 2.7% and 5.1 ± 1.3%, respectively). These relative abundances are similar to those found in pig faeces in the study of Mateos et al. [179], where *Firmicutes* (82%) were most abundant, followed by *Bacteroidetes* (14%), *Proteobacteria* (1.8%), *Spirochaetes* (1.5%), and *Actinobacteria* (0.7%). Similarly, Grosu et al. [169] found *Firmicutes* (58.3%), *Bacteroidetes* (34.5%), *Proteobacteria* (1.2%), *Spirochaetes*, and *Actinobacteria* (<0.01%) in the large intestines of piglets. At **T1**, in AC, *Proteobacteria* (54.2 ± 51.9%) remained present in the largest proportion, followed by *Firmicutes* (45.7 ± 52.8%). In TC and DC, the latter were present in the highest abundance (56.8 ± 14.7% and 72.0 ± 10.8%, respectively) as well as *Bacteroidetes* (38.6 ± 12.1% and 21.9 ± 12.7%, respectively). Although no difference was observed in the SHIME, in the study of Grosu et al. [169], after the addition of AFB1 to piglets' diet during 30 days at 320 µg/kg, *Bacteroidetes* (54.5%) statistically increased, whilst *Firmicutes* (33.2%) decreased when compared to the control group. The duration of the SHIME trial might be the constraining factor. At **T2**, in AC, the proportion of *Proteobacteria* (98.2 ± 2.0%) dominated and *Firmicutes* (1.8 ± 1.9%) decreased. In TC and DC, the percentage of *Firmicutes* (61.0 ± 18.4% and 64.5 ± 14.4%, respectively) and *Bacteroidetes* (33.1 ± 15.4% and 29.6 ± 13.0%, respectively) remained stable. The phyla ratios present in each individual repetition per treatment can be seen in Figure 20.

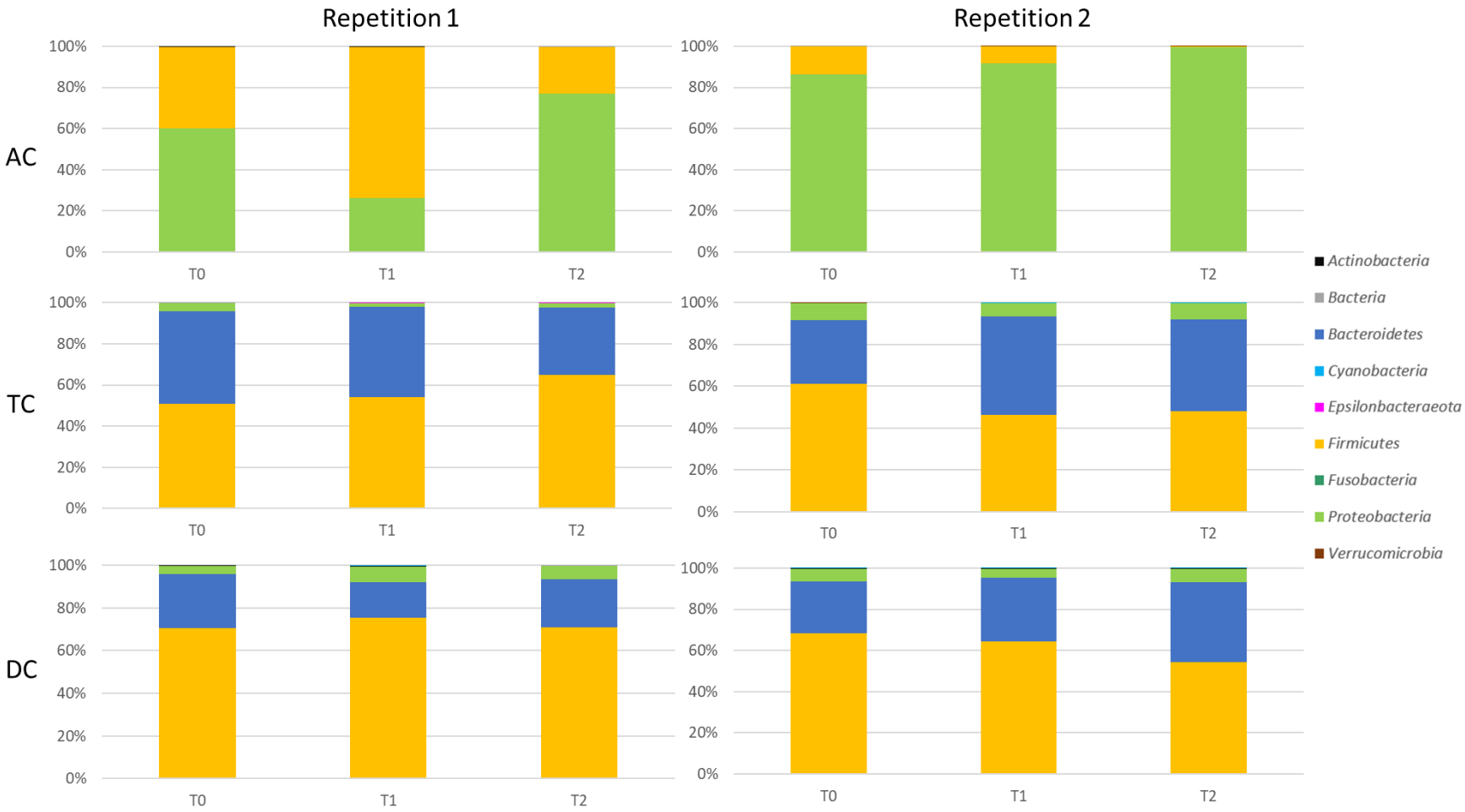

Figure 20. Microbial phyla present in the SHIME samples at T0, T1 and T2, in the AC, TC, and DC of both repetitions. T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 µg/kg food) and fumonisin B1 (FB1) (2,000 µg/kg food); T2 = after one week of addition of AFB1 (81.6 µg/kg food) and FB1 (2,000 µg/kg food), together with bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food); AC = ascending colon; TC = transverse colon; DC = descending colon.

Four major families analysed in the **faecal inoculum** were consecutively identified as *Ruminococcaceae* (42.7%), *Lachnospiraceae* (36.5%), *Veillonellaceae* (7.6%), and *Prevotellaceae* (6.6%). The first two families were also the main families found in the study of Bondue et al. [212]. Similar to the families found in piglet faeces by Mateos et al. [179], *Lachnospiraceae*, *Ruminococcaceae*, and *Prevotellaceae* were found above 10% and *Veillonellaceae* at 4.2%. Unlike in the latter study, no *Lactobacillaceae* were identified in the SHIME. At **T0**, in AC, mainly *Enterobacteriaceae* (63.6 ± 31.7%) and *Veillonellaceae* (35.6 ± 31.5%) were observed. In TC and DC, *Lachnospiraceae* (39.1 ± 5.1% and 47.1 ± 21.5%, respectively), *Bacteroidaceae* (23.7 ± 8.2% and 14.8 ± 9.7%, respectively), *Ruminococcaceae* (13.1 ± 3.1% and 6.6 ± 3.9%, respectively), and *Enterobacteriaceae* (5.5 ± 3.5% and 4.0 ± 1.8%, respectively) were detected. At **T1**, *Enterobacteriaceae* (53.6 ± 53.2%) and *Veillonellaceae* (45.2 ± 52.9%) remained stable in AC. In TC and DC, the proportions of *Lachnospiraceae* (42.7 ± 3.6% and 35.0 ± 4.2%, respectively), *Bacteroidaceae* (36.2 ± 12.1% and 17.8 ± 11.3%, respectively), *Ruminococcaceae* (4.6 ± 0.1% and 7.6 ± 0.7%, respectively), and *Enterobacteriaceae* (3.7 ± 3.3% and 4.0 ± 0.8%, respectively) remained equally steady. At **T2**, *Enterobacteriaceae* (95.8 ± 0.8%) dominated the AC, followed by *Veillonellaceae* (1.6 ± 1.8%). In TC and DC, the relative abundance of most families stayed similar; *Lachnospiraceae* (41.8 ± 9.7% and 36.8 ± 2.9%, respectively), *Bacteroidaceae* (27.7 ± 18.6% and 22.4 ± 18.4%, respectively), *Ruminococcaceae* (15.3 ± 5.8% and 8.5 ± 5.6%, respectively), and *Enterobacteriaceae* (4.9 ± 3.7% and 4.18 ± 2.0%, respectively).

In the study of Mateos et al. [179], a decrease in proportion of *Actinobacteria*, *Proteobacteria, Lachnospiraceae*, and *Veillonellaceae* was observed in piglets after an exposure period to a combination of fumonisin B1, B2, and B3 (10.2, 2.5, and 1.5 mg/kg, respectively) in the diet for 15 days. Oppositely to this study, these phyla and families remained stable in the SHIME model. Contrarily, to the study of Mateos et al. [179], the SHIME system, which was a human model, was exposed to AFB1 and FB1 only for a period of 7 days. Interestingly, in a study performed by Ishikawa et al. [253], a single oral AFB1 exposure increased the *Lachnospiraceae* family abundance in intestinal content of mice. However, the oral administration of AFB1 (63.4 µg/kg diet) to dairy cows during 5 days, did not affect the ruminal bacterial community on phylum, nor on family level [254]. Furthermore, the addition of a bentonite clay did not affect the bacterial community either.

It is difficult to analyse differences in results when comparing animal to human microbiota, as well as *in vitro* to *in vivo* studies, and this must be taken into consideration. Clearly, more studies are needed to analyse the impact of these mycotoxins on intestinal microbiota, especially on that of humans.

Figure 21. Microbial families present in the SHIME samples at T0, T1 and T2, in the AC, TC, and DC of both repetitions. T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 µg/kg food) and fumonisin B1 (FB1) (2,000 µg/kg food); T2 = after one week of addition of AFB1 (81.6 µg/kg food) and FB1 (2,000 µg/kg food), together with bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food); AC = ascending colon; TC = transverse colon; DC = descending colon.

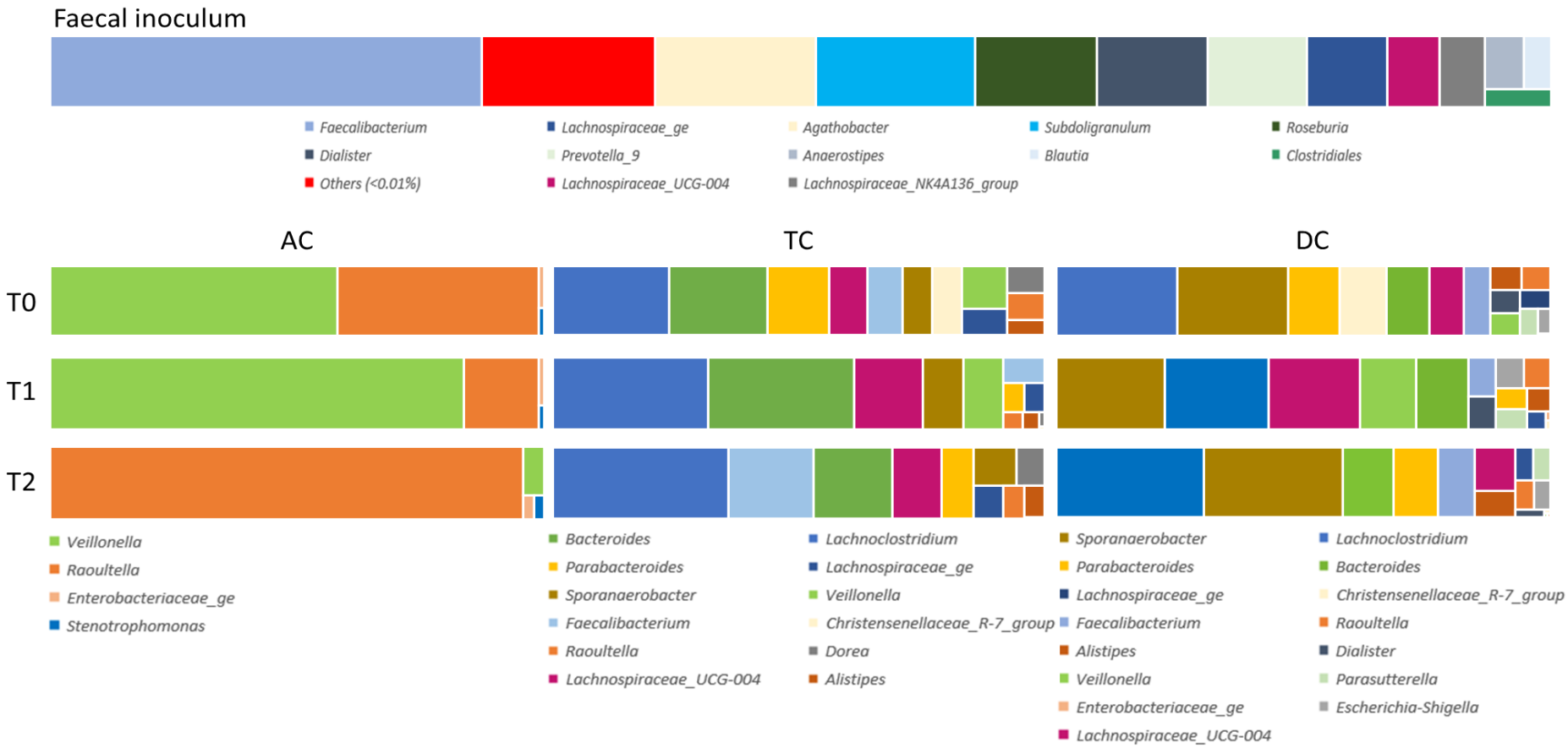

Figure 22. Tree map of microbial genera present (≥ 1%) in the faecal inoculum and in the first repetition in %, at T0, T1 and T2, in the AC, TC, and DC. T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 µg/kg food) and fumonisin B1 (FB1) (2,000 µg/kg food); T2 = after one week of addition of AFB1 (81.6 µg/kg food) and FB1 (2,000 µg/kg food), together with bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food); AC = ascending colon; TC = transverse colon; DC = descending colon.

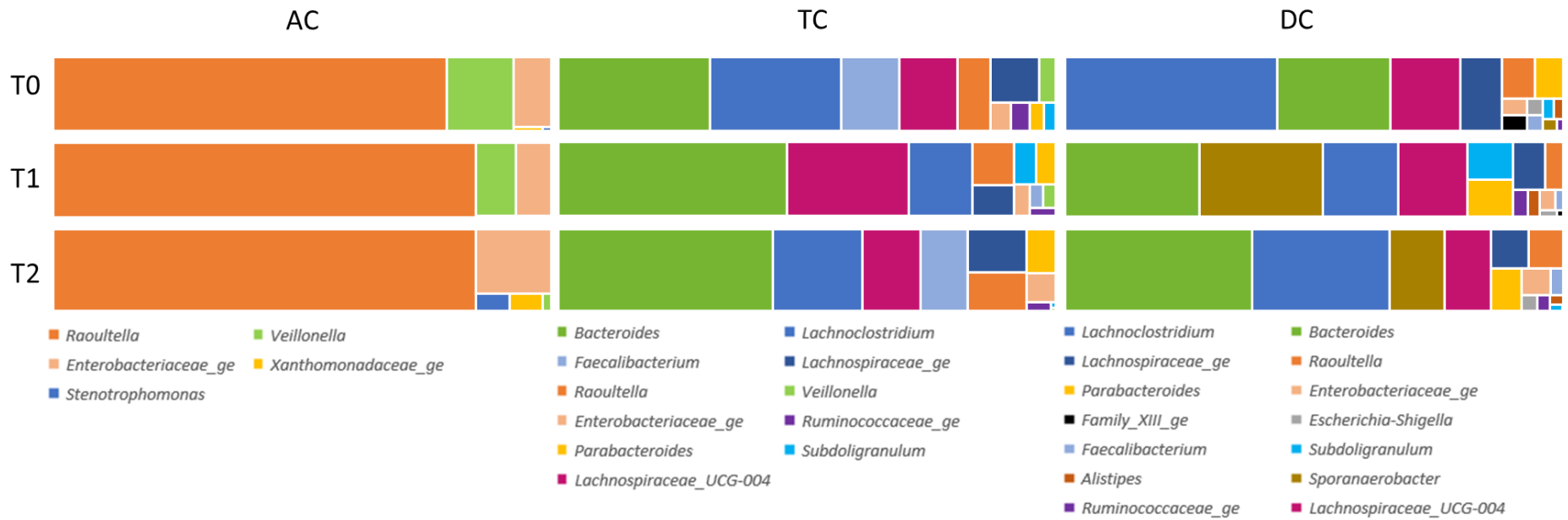

Figure 23. Tree map of microbial genera present (≥ 1%) in the second repetition in %, at T0, T1 and T2, in the AC, TC, and DC. T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 µg/kg food) and fumonisin B1 (FB1) (2,000 µg/kg food); T2 = after one week of addition of AFB1 (81.6 µg/kg food) and FB1 (2,000 µg/kg food), together with bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food); AC = ascending colon; TC = transverse colon; DC = descending colon.

3.5 Conclusions

Two detoxifiers, namely an aflatoxin binder and a fumonisin esterase, proved their effectiveness in the simulated human (toddler) gut environment. The addition of the detoxifiers resulted in a significant decrease in aflatoxin B1 (AFB1) and fumonisin B1 (FB1) concentrations. Approximately 100% of AFB1 and FB1 was removed in the system after five days, and bound to bentonite or hydrolysed to non-toxic variants, respectively. Our study provided strong evidence that the fumonisin esterase is responsible for the full hydrolysis of FUMs; the concentration of the fully hydrolysed FB1 – which is far less toxic compared to the parent molecule – only increased after the addition of fumonisin esterase. Future studies should include monitoring the effect of detoxifiers on nutritional status, namely the long-term safety of the products. The intestinal short-chain fatty acid profile, in terms of the acetate/propionate/butyrate ratio, resembled that of an infant. This confirmed the presence of healthy gut microbiota, which remained stable over the complete study period. The 16S metagenetic analysis gave further insights into the specific bacterial composition of the colon regions. No obvious changes in composition of the microbial communities were observed, again a confirmation of a suitable, healthy study environment and first evidence for the possibility to use these two detoxifiers in humans.

Funding

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The Laboratory of Pharmacology and Toxicology is part of the Ghent University expertise centre MSsmall. The authors are thankful for the cooperation of many colleagues at both Ghent and Liège University during sample collection and sample analysis.

Supplementary materials

Table S1. Results of the linearity (coefficient of determination r^2, $1/x^2$ weighted), extraction recovery, limit of detection (LOD), limit of quantification (LOQ) and signal suppression/enhancement (SSE) for the UHPLC-MS/MS method validation of aflatoxin B1 (AFB1), fumonisin B1 (FB1), hydrolysed fumonisin B1 (HFB1), partially hydrolysed fumonisin B1a (pHFB1a) and partially hydrolysed fumonisin B1b (pHFB1b) in Simulator of Human Intestinal Microbial Ecosystem (SHIME) medium.

	R^2	Extraction Recovery, %	LOD[1], µg/kg	LOQ, µg/kg	SSE, %
AFB1	0.998	55.7	0.002	0.050	45.5
FB1	0.996	91.1	0.299	1.000	152.0
HFB1	0.997	89.0	0.013	0.500	159.0
pHFB1a	0.997*	116.0	0.045	0.530	135.0
pHFB1b	0.998	109.0	0.049	0.730	129.0

[1] Calculated based on the signal-to-noise ratio

* 1/x weighted

Table S2. Results of the within-day and between-day precision and accuracy for the UHPLC-MS/MS validation of aflatoxin B1 (AFB1), fumonisin B1 (FB1), hydrolysed fumonisin B1 (HFB1), partially hydrolysed fumonisin B1a (pHFB1a) and partially hydrolysed fumonisin B1b (pHFB1b) in Simulator of Human Intestinal Microbial Ecosystem (SHIME) medium.

Analyte	Theoretical concentration, µg/kg	Mean concentration ± SD (*n* = 6)	Precision, RSD %		Accuracy, %	
			within-day (*n* = 6)	between-day (*n* = 6)	within-day (*n* = 6)	between-day (*n* = 6)
AFB1	0.05	0.05 ± 0.00	2.1	6.9	-2.5	4.2
	0.10	1.00 ± 0.05	2.3	4.7	4.4	-0.2
	10.00	10.40 ± 0.27	1.0	2.6	5.8	3.6
FB1	1.00	1.02 ± 0.06	3.9	5.7	-0.3	1.6
	20.00	18.40 ± 1.50	1.7	8.1	-4.9	-7.9
	200.00	214.30 ± 5.43	3.1	2.5	3.5	7.1
HFB1	0.50	0.48 ± 0.05	6.0	9.9	2.6	-5.0
	10.00	10.50 ± 0.37	3.8	3.5	1.2	5.1
	100.00	102.30 ± 6.28	4.4	6.1	-5.7	2.3
pHFB1a	0.53	0.56 ± 0.05	3.3	9.8	11.8	5.2
	2.65	2.39 ± 0.20	2.1	8.4	-10.8	-9.9
	10.60	10.60 ± 0.88	8.5	8.3	-8.0	0.1
pHFB1b	0.73	0.74 ± 0.03	3.1	3.8	0.3	1.6
	3.66	3.51 ± 0.29	1.8	8.2	4.2	-4.2
	14.63	15.60 ± 0.60	4.5	3.8	3.3	6.3

Table S3. Overview of compound specific MS/MS parameters, measured in ESI positive mode.

Analyte	Precursor ion (*m/z*)	Quantifier ion (*m/z*)	Qualifier ion (*m/z*)	Cone voltage (V)	Collision energy (eV) (a-b)	Retention time (min)
AFB1	313.0	285.1	241.1	35.0	23.0-34.0	4.94
FB1	722.2	334.2	352.2	40.0	37.0-37.0	4.48
HFB1	406.3	352.3	334.2	40.0	25.0-25.0	4.38
pHFB1a	564.3	352.3	334.3	40.0	37.0-37.0	4.37
pHFB1b	564.3	352.3	334.3	40.0	37.0-37.0	4.46
$^{13}C_{-17}$-AFB1	330.1	255.1	301.0	20.0	35.0-28.0	4.94
$^{13}C_{34}$-FB1	756.5	374.3	356.2	15.0	35.0-40.0	4.48

Note: *m/z* = mass-to-charge ratio; (a-b): collision energy for the quantifier (a) and qualifier ion (b), respectively

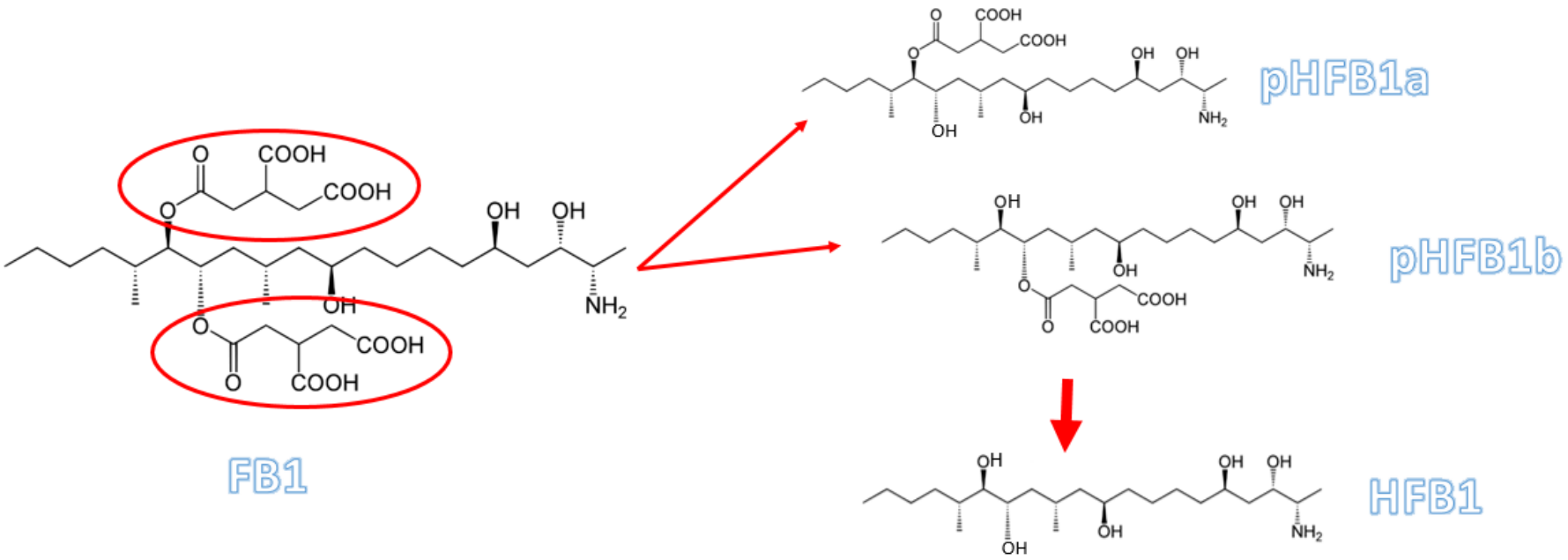


Figure S1. Cleavage of one (partially hydrolysed fumonisin B1a and b, pHFB1a and b) or both side chains (hydrolysed FB1, HFB1) of fumonisin B1 (FB1) due to fumonisin esterase.

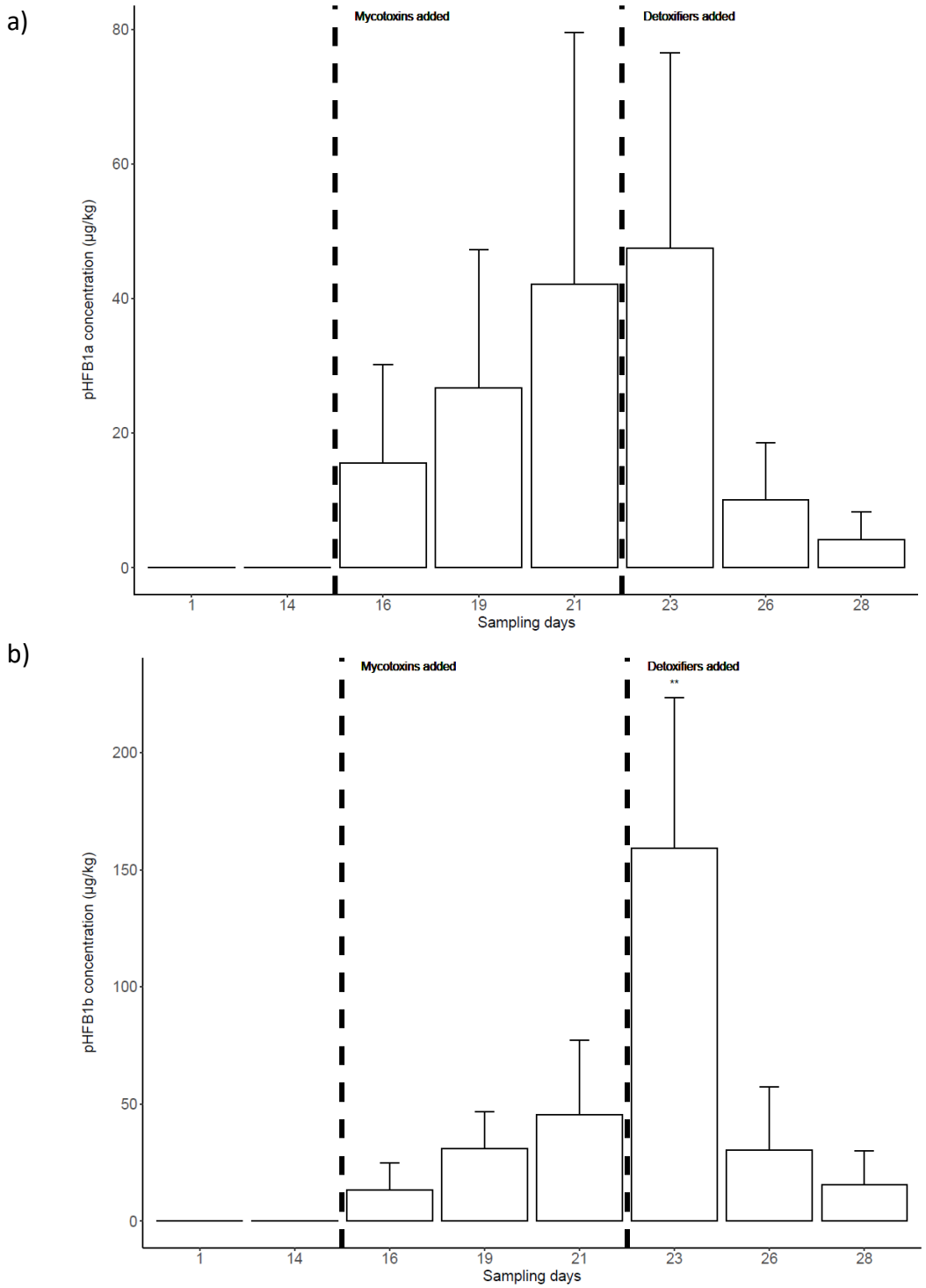


Figure S2. Mean concentration of a) partially hydrolysed fumonisin B1a and b) partially hydrolysed fumonisin B1b (pHFB1a and pHFB1b) determined in the AC, TC, and DC compartment at different sampling days during the SHIME experiment (n = 2). Error bars represent the standard deviation. The dashed lines represent the addition of aflatoxin B1 (81.6 µg/kg food) and fumonisin B1 (2,000 µg/kg food), followed by addition of both mycotoxins and bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food). The asterisks represent the significant statistical difference ($p < 0.01$) compared to sampling day 21.

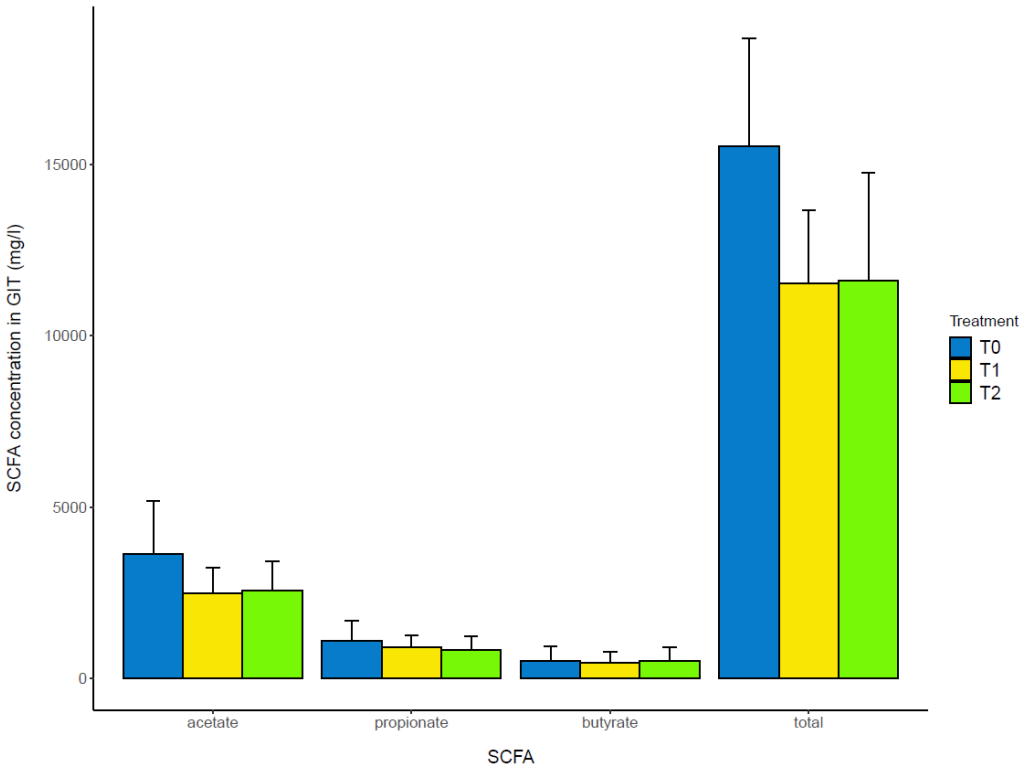
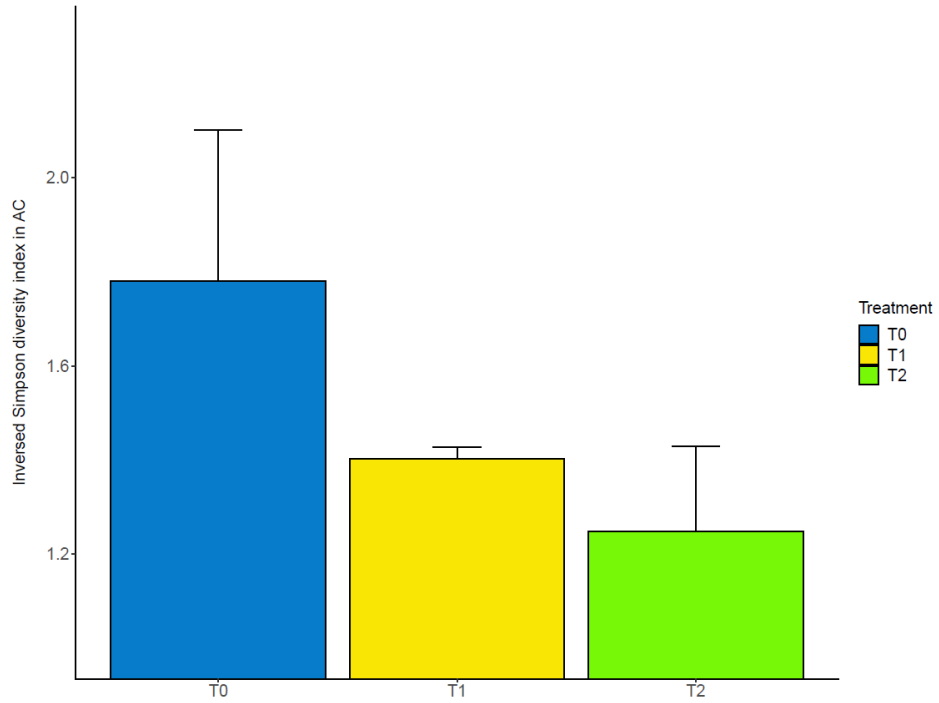
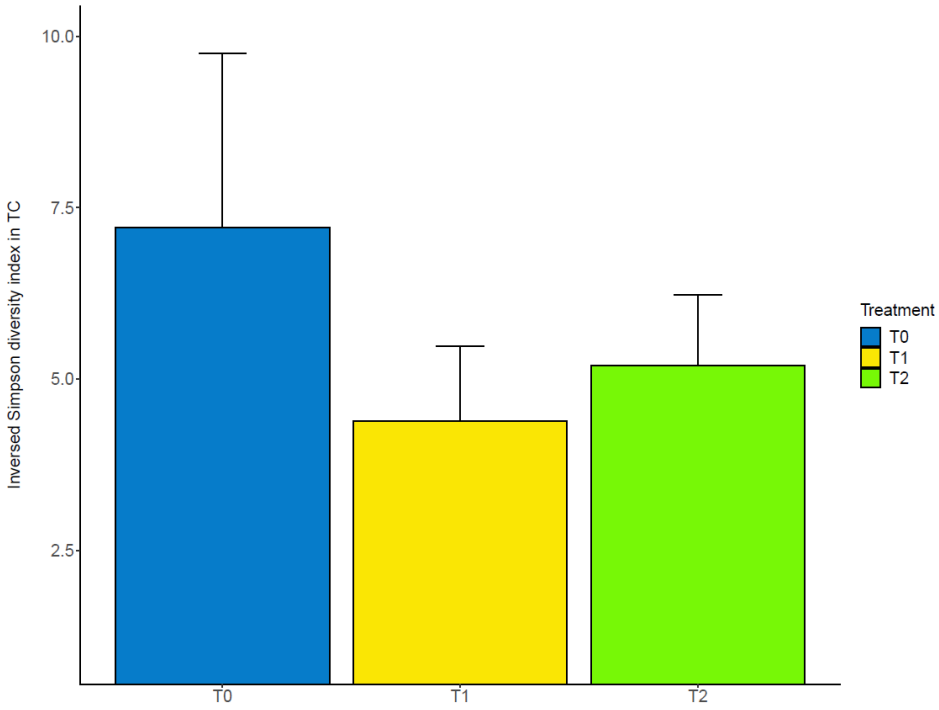


Figure S3. Mean concentrations of short-chain fatty acids (SCFA) in the gastrointestinal tract (GIT) (n = 6). Error bars represent the standard deviation. Total is the sum of acetate, propionate, butyrate, isobutyrate and isovalerate. T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 µg/kg food) and fumonisin B1 (FB1) (2,000 µg/kg food); T2 = after one week of addition of AFB1 (81.6 µg/kg food) and FB1 (2,000 µg/kg food), together with bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food).

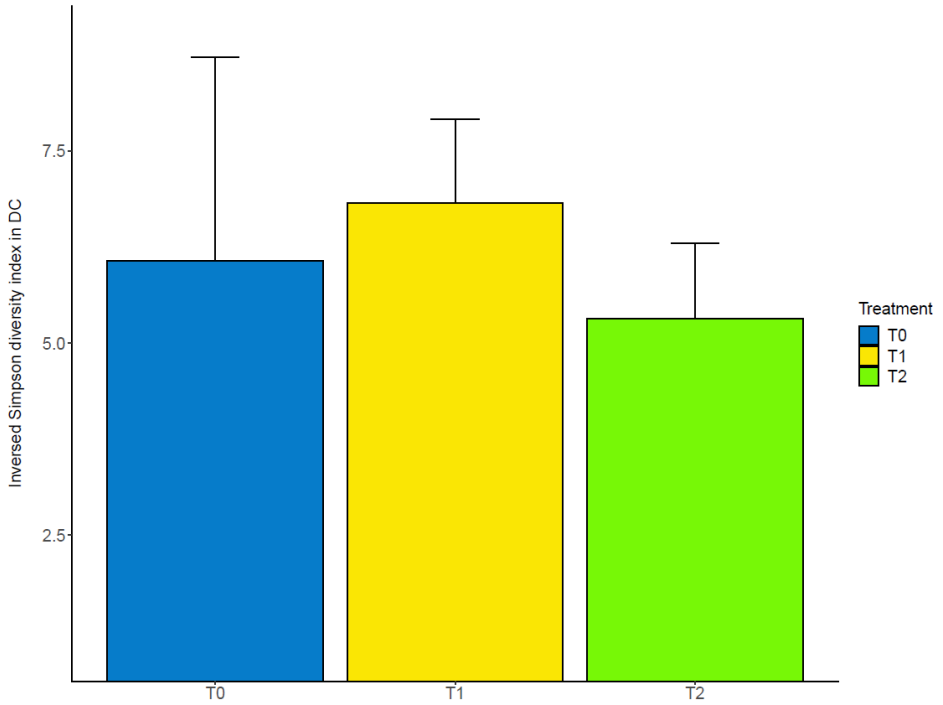
S4)


Inversed Simpson diversity index in AC
2.0
1.6
1.2
T0
T1
T2
Treatment
T0
T1
T2

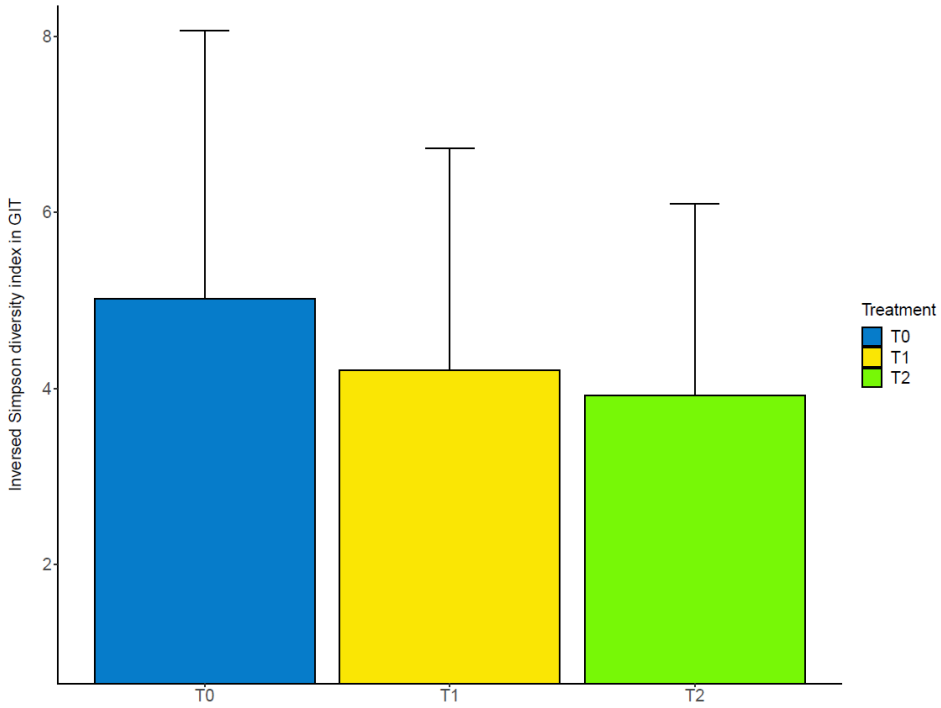
S5)


Inversed Simpson diversity index in TC
10.0
7.5
5.0
2.5
T0
T1
T2
Treatment
T0
T1
T2

S6)



S7)



Figures S4-7. Inverse Simpson diversity index means per treatment (T0, T1 and T2) in the ascending (AC), transverse (TC), and descending colon (DC), and in the gastrointestinal tract (GIT) on genus level (mean ± SD). T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 µg/kg food) and fumonisin B1 (FB1) (2,000 µg/kg food); T2 = after one week of addition of AFB1 (81.6 µg/kg food) and FB1 (2,000 µg/kg food), together with bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food).

4 Chapter 2: Efficacy of fumonisin esterase in piglets as animal model for fumonisin detoxification in humans: pilot study comparing intraoral to intragastric administration

Adapted from

Neckermann, K.; Antonissen, G.; Doupovec, B.; Schatzmayr, D.; Gathumbi, J.; Delcenserie, V.; Uhlig, S. and Croubels, S. Efficacy of Fumonisin Esterase in Piglets as Animal Model for Fumonisin Detoxification in Humans: Pilot Study Comparing Intraoral to Intragastric Administration. *Toxins*. **2022**, *14*, 136. https://doi.org/10.3390/toxins14020136

Sample collection performed by Neckermann K (at UGent). Mycotoxin analysis performed by Biomin (Tulln, Austria).

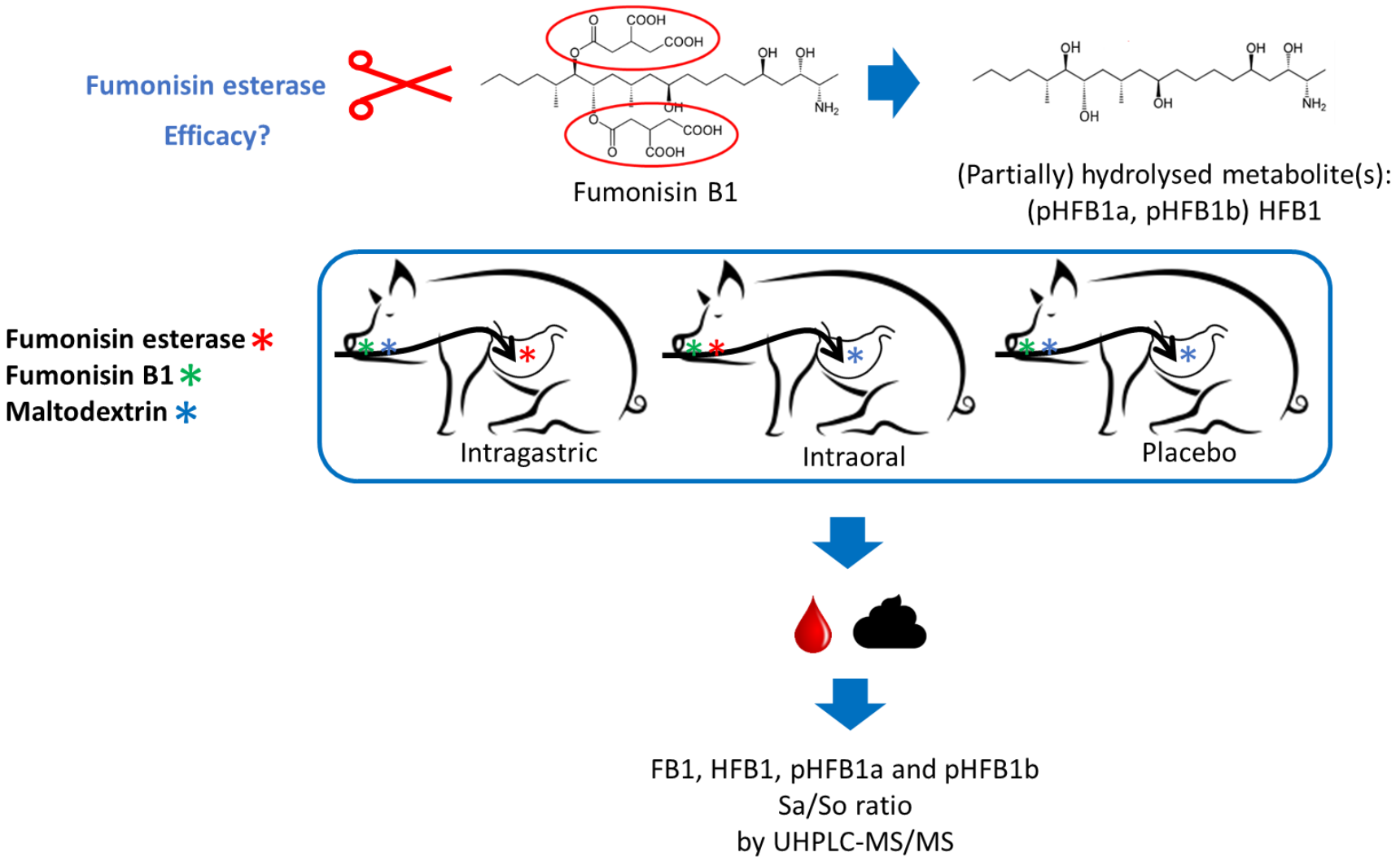


Figure 24. Graphical abstract of the scientific publication titled "Efficacy of Fumonisin Esterase in Piglets as Animal Model for Fumonisin Detoxification in Humans: Pilot Study Comparing Intraoral to Intragastric Administration", published in Toxins (2022) [255].

4.1 Abstract

Fumonisins (FUMs), a group of highly prevalent and toxic mycotoxins, are suspected to be causal agents of several diseases in animals and humans. In the animal feed industry, fumonisin esterase is used as feed additive to prevent mycotoxicosis caused by FUMs. In humans, a popular dosage form for dietary supplements, with high patient acceptance for oral intake, is capsule ingestion. Thus, fumonisin esterase provided in a capsule could be an effective strategy against FUM intoxication in humans. To determine the efficacy of fumonisin esterase through capsule ingestion, two modes of application were compared using piglets in a small-scale preliminary study. The enzyme was administered intraorally (in-feed analogue) or intragastrically (capsule analogue), in combination with fumonisin B1 (FB1). A visualisation of the experiment performed in this chapter in piglets is provided in Figure 24. Biomarkers for FB1 exposure; namely FB1, hydrolysed FB1 (HFB1), and partially hydrolysed forms (pHFB1a and pHFB1b), were measured both in serum and faeces using a validated liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method, and toxicokinetic parameters were calculated. Additionally, the serum sphinganine/sphingosine (Sa/So) ratio, a biomarker of effect, was determined using LC-MS/MS. A significantly higher Sa/So ratio was shown in the placebo group compared to both esterase treatments, from 6 h and 12 h onwards, compared to that of the intraoral and intragastric treatments, respectively; demonstrating the efficacy of the esterase. Moreover, a significant decrease in serum FB1 area under the concentration-time curve (AUC), from an AUC (± SEM) of 128 ± 15.5 to 51.4 ± 17.0 h x ng/mL, and an increase of faecal HFB1 AUC, from an AUC (± SEM) of 795 ± 323 to 1,745 ± 289 h x µg/g, were observed after intraoral esterase administration compared to the placebo group. However, these effects were not observed with statistical significance after intragastric esterase administration with the current sample size.

Keywords

Biomarkers; detoxifier administration route; efficacy; fumonisin B1; fumonisin esterase; human model; mycotoxin; pig; Sa/So ratio; toxicokinetics

4.2 Introduction

Worldwide, 25% of food and feed is contaminated with toxic fungal metabolites with levels above the EU and Codex established limits [256]. However, the extent of the mycotoxin prevalence is underestimated as the aforementioned percentage neglects to take into account the amount of agricultural products that are contaminated with levels above detectable analytical levels, but below established limits [256]. The consumption of mycotoxin contaminated cereals, cereal derived products, or animal derived products is related to various types of diseases, or mycotoxicoses [257,258]. The disease can manifest itself acutely or chronically, such as the development of cancers and immune deficiency [257]. Of the more than 500 described mycotoxins, some are more prominent and/or more toxic compared to others and pose a greater risk to human and animal health. Fumonisins (FUMs), produced by several species of *Fusarium* fungi, are one of the groups of mycotoxins with major importance; fumonisin B1 (FB1) being the most toxic and prevalent congener in nature (Figure 25) [34,258]. It is predominantly present in maize and maize-based products, which is the preferred staple food in most low and middle income countries, especially in Latin America and Africa [259]. Fumonisin B1 has been classified as a group 2B 'possibly carcinogenic to humans' by the International Agency for Research on Cancer (IARC). In animals, FB1 intoxication has resulted in equine leukoencephalomalacia, a fatal brain disease in horses, porcine pulmonary oedema in pigs, neural tube defects in mice, and additionally hepatotoxic and nephrotoxic effects have been observed experimentally [55,234,260]. In humans, FB1 has been associated with an increased risk of oesophageal cancer, birth defects, and adverse effects in liver and kidneys [21,55,66,261]. Furthermore, FB1 intake has been correlated to stunting in children [9,12,13] and has been shown to disrupt the proper functioning of the intestinal barrier [262]. Toxicity caused by FB1 and other FUMs is plausibly linked to their inhibitory activity on sphinganine and sphingosine N-acyltransferase. Disruption of the sphingolipid biosynthesis results in a build-up of sphingoid bases and a decline of complex sphingolipids [262,263]. Sphingolipids are membrane lipids and play an important role in the regulation of fundamental cellular processes, i.e., cell division, differentiation, and apoptosis [264,265]. Hence, the sphinganine/sphingosine (Sa/So) ratio in blood or urine has been proposed as a reliable biomarker to evaluate FUM exposure and to demonstrate an adverse effect both after acute and chronic FUM exposure [141,146]. In animals, the Sa/So ratio in serum has been proven to

be a reliable method to assess exposure [147]. In humans, the use of this ratio in urine or blood has been questioned [143]. Yet, the Sa/So ratio was noted to be useful when the FB1 contamination is high [266]. Despite the high level of variability even under controlled circumstances [267], the analysis of FB1 in urine is put forward as a valuable biomarker in humans [268]. Due to poor oral absorption, FB1 and its metabolites are predominantly excreted through the faeces (ranging from 52% to 94%) [61], making faecal sampling and analysis valuable. Furthermore, in addition to FB1 itself, its hydrolysed metabolites, i.e., partially hydrolysed FB1a (pHFB1a), pHFB1b, and hydrolysed FB1 (HFB1), were proposed to be useful as short-term biomarkers (Figure 25) [269].

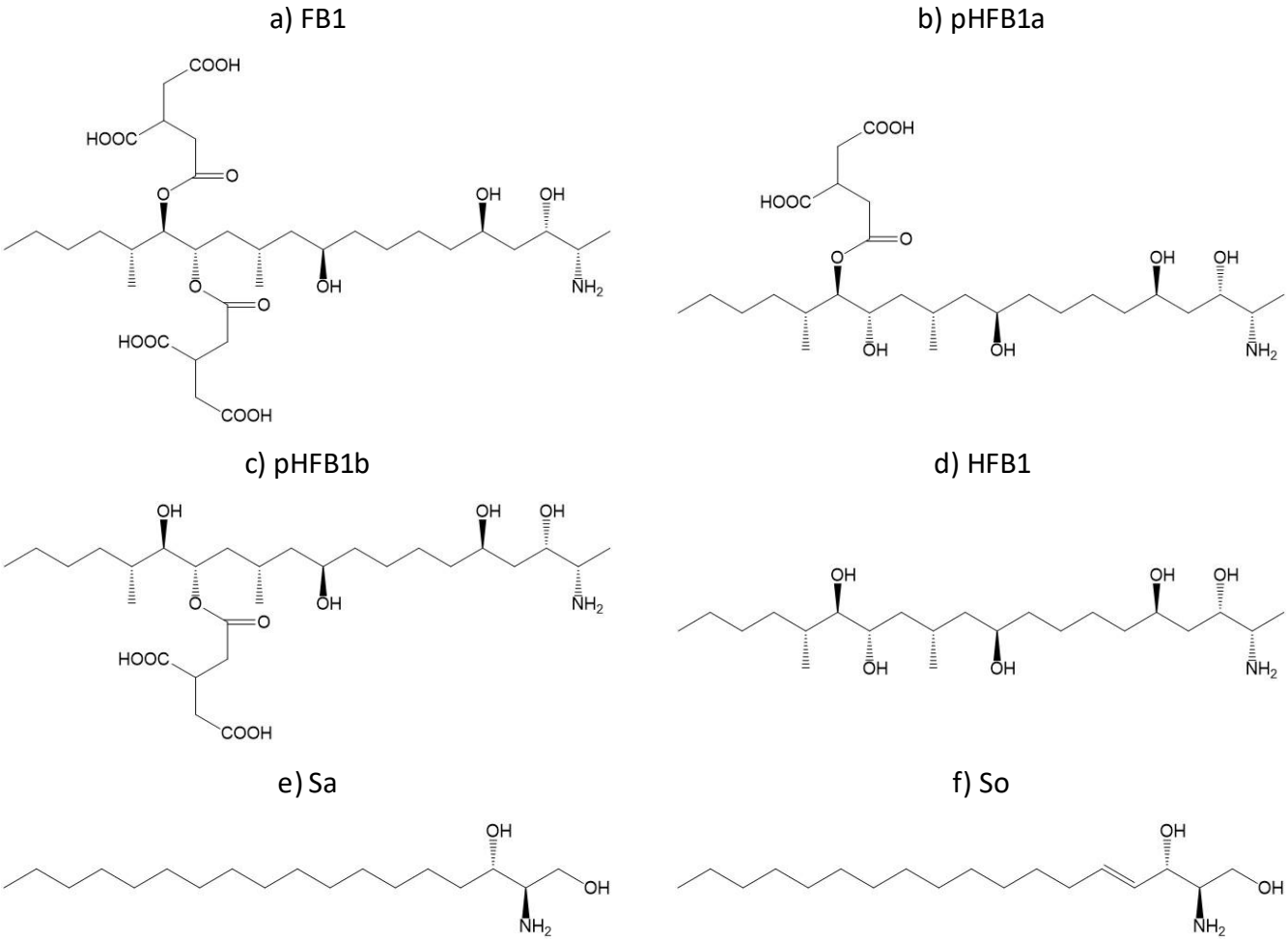


Figure 25. Structures of a) fumonisin B1 (FB1), b) partially hydrolysed FB1a (pHFB1a), c) pHFB1b, d) hydrolysed FB1 (HFB1 or aminopentol), e) sphinganine (Sa), and f) sphingosine (So).

To prevent acute and chronic mycotoxicosis in animals and humans, several pre- and post-harvest intervention strategies are implemented [200,270]. Pre-harvest mitigation strategies include, among others, breeding of crops with enhanced resistance against disease

and infection with mycotoxigenic fungi, and implementing optimal agricultural practices in the field [200,271]. Post-harvest techniques include correct drying, sorting, and shelling practices of maize, followed by proper storage and pest prevention [270]. Furthermore, some types of food processing, e.g., nixtamalisation and fermentation, have shown promising results [95,201]. Other mitigation strategies are the employment of detoxifiers as feed additives, namely mycotoxin binders or modifiers. Application of mycotoxin detoxifiers can decrease the amount of mycotoxins absorbed from the gut, thereby resulting in reduction or prevention of adverse health effects. The specificity of binders such as clay minerals is contested, although some tend to have a greater affinity for certain mycotoxins [272]. Modifiers change mycotoxin molecules into less toxic variants through conjugation with functional groups, ring cleavage, hydrolysis, deamination, or decarboxylation [202,258]. Biological detoxifiers include application of certain microorganisms and their enzymes, i.e., bacteria, fungi, and yeasts; isolated from different sources, such as soil, animal gastrointestinal flora, and water. The advantage of enzymes for mycotoxin detoxification is their high specificity [258]. One such enzyme for mycotoxin detoxification is an esterase, specifically designed to cleave the ester bonds in FUM side chains, releasing tricarballylic acid(s), and resulting in partially or fully hydrolysed FB1 (aminopentol) [185]. These metabolites have been demonstrated to be less toxic than the parent FB1 molecule [7,34,35]. In the human pharmaceutical industry, capsules as dosage form is widely adopted due to its many advantages. The capsule is self-administrative, odourless, tasteless, easy-to-swallow, and can be manufactured in a variety of attractive colours [273]. Furthermore, it can be opened and its contents mixed in food. These characteristics would make a detoxifier enclosed in a capsule, an attractive form for human consumption.

In this study, a European Food Safety Authority (EFSA) evaluated and approved as feed additive by the European Commission, fumonisin esterase, was administered to piglets to compare the efficacy of two different administration routes, i.e., intraoral and intragastric [185]. In order to simulate the effect in humans as closely as possible, the pig was put forward as a suitable animal model. Pigs are physiologically and anatomically similar to humans regarding the gastrointestinal tract, liver, kidneys, and cardiovascular organs [61,274]. The juvenile pig was used as model for the toddler; as young children are at a higher risk of mycotoxicoses due to higher exposure (a higher food intake/kg body weight (BW) and a higher

consumption of cereal-based products), and a lower detoxification capacity due to metabolic and physiological immaturity [46–48]. In a previous experiment, this esterase demonstrated a promising effect in reducing FB1 concentrations in the human intestinal environment, using a toddler Simulator of the Human Intestinal Microbial Ecosystem [188]. A nearly complete reduction in FB1 concentration was achieved following addition of the enzyme.

The aim of this study was to investigate whether the FB1-degrading enzyme is able to reduce the exposure of FB1 in piglets through administration of the enzyme either intragastric (capsule imitation) or intraoral. The latter method of enzyme administration corresponds to the current employed standard route of in-feed administration of the commercial product. To the authors' knowledge, no studies have been performed to investigate the possible efficacy of the enzyme when bypassing the mouth. The efficacy of the enzyme was determined by analysing relevant biomarkers for FB1 exposure and effect, and selected toxicokinetic parameters, and by comparing these parameters of the two administration routes to each other as well as to a placebo.

4.3 Materials and methods

4.3.1 Animals

Twenty-four 4-week old piglets (12 males, 12 females) were obtained from Ra-Se Genetics® (Ooigem, Belgium). Upon arrival, the pigs were housed in groups of three in standard pig stables at the Faculty of Veterinary Medicine, Ghent University (Merelbeke, Belgium). Piglets received *ad libitum* access to water and feed (Biggistart Opti® flour, AVEVE Lammens Filip, Massemen, Belgium), and were provided with varying stable enrichment (rubber and rope-like chew toys, balls and towels) throughout the entire trial. The starter feed was tested for possible contamination with mycotoxins prior to the start of the experiment by a validated multi-mycotoxin liquid chromatography-tandem mass spectrometry (LC-MS/MS) method at Primoris (Zwijnaarde, Belgium). The feed contained low levels (143 µg/kg) of deoxynivalenol, below the recommended guidance value of the EU (2006/576/EC) [275]. After an acclimatisation period of 6 and 8 days, half of the piglets were divided into 3 treatment groups, respectively. One male piglet was euthanised before the start of the trial due to lameness and arthritis in both hind legs and a fever, and the administration of nonsteroidal

anti-inflammatory drugs had no effect. All animals were weighed daily until the day prior to treatment administration.

The piglet trial was assessed and approved (on 20 June 2019) by the Ethical Committee of the Faculty of Veterinary Medicine and the Faculty of Bioscience Engineering of Ghent University with case number EC2019-37.

4.3.2 Experimental design

To ensure sobriety and possible interaction, 12 h prior to and up to 4 h p.a., the animals were fasted, and the piglets were separated individually by wooden boards in each pen. All animals (mean BW ± SD, 8.38 ± 0.70 kg) received a single intraoral dose of FB1 (2 mg/kg BW). The pigs were allocated to three different treatments (4 males, 4 females per group). In the placebo group, the pigs (*n* = 7 (3 males, 4 females), 8.56 ± 0.50 kg) received maltodextrin (300 mg/kg BW) both intraorally and intragastrically through gavage. The intraoral-treatment group (*n* = 8, 8.33 ± 1.00 kg) received fumonisin esterase (3 U/kg BW) intraorally and maltodextrin (300 mg/kg BW) through gavage (Table 5). The intragastric-treatment group (*n* = 8, 8.29 ± 0.51 kg) received fumonisin esterase (3 U/kg BW) through gavage and maltodextrin (300 mg/kg BW) intraorally (Table 5).

Table 5. Overview of the administered products, doses per kg body weight (BW) and the administration route per treatment group.

Treatment Groups	Products Administered	Dose (/kg BW)	Administration Route
Placebo (*n* = 7)	Fumonisin B1	2 mg	Intraoral
	Fumonisin esterase	-	-
	Maltodextrin	300 mg	Intraoral + Intragastric
Intraoral (*n* = 8)	Fumonisin B1	2 mg	Intraoral
	Fumonisin esterase	3 U	Intraoral
	Maltodextrin	300 mg	Intragastric
Intragastric (*n* = 8)	Fumonisin B1	2 mg	Intraoral
	Fumonisin esterase	3 U	Intragastric
	Maltodextrin	300 mg	Intraoral

Eleven blood samples (2 mL each) per pig were collected in serum clot activator tubes (Vacutest Kima, Novolab, Geraardsbergen, Belgium) through venipuncture from the *vena jugularis* according to the time points presented in Figure 26. The collected serum tubes were placed upright and allowed to clot at room temperature during at least 30 min. The samples were centrifuged (10 min, 2851× *g*, 4 °C), and serum was stored at −20 °C until further analyses. All faeces were collected throughout the trial from the floor of each individual pen until 72 h p.a. Fresh faeces (collected within 5min after production) were collected at seven occasions (Figure 26). These samples were lyophilised for approximately 48 h, ground (with mortar and pestle) and stored at −20 °C until further analysis.

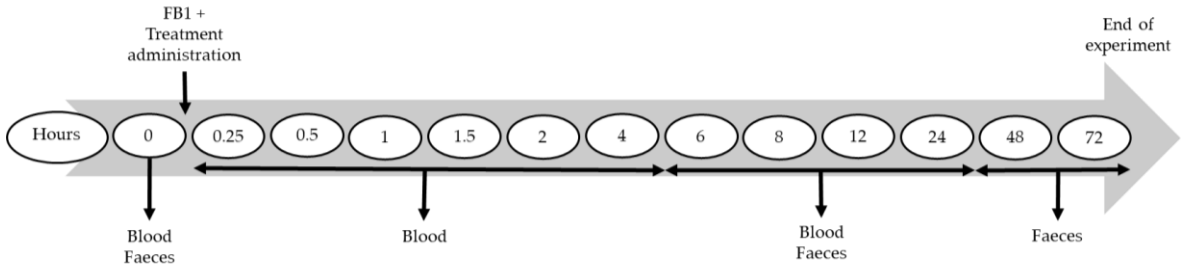


Figure 26. Sampling points in hours after fumonisin B1 (FB1) and treatment administration. Blood and faeces were collected at the different times indicated. Treatment consisted of fumonisin esterase administration intraorally or intragastrically, or maltodextrin (a placebo).

In the majority of the piglets, salivation or gagging with small amounts of regurgitation was observed on average at 1.8 h following FB1 and treatment administration. Most likely, this was the result of aversion to the taste of FB1, which was provided as a culture material of *Fusarium verticillioides*. Due to fasting of the piglets and treatment administration in liquid form, passage through the stomach was deemed to have been mostly attained.

4.3.3 Products, treatment preparation, and administration

The FB1 culture material of *F. verticillioides* (containing 8.60 mg/g FB1) was obtained from Romer Labs (Tulln, Austria). The *F. verticillioides* strain M-3125 was cultured on rice, homogenised, and lyophilised [184,276]. Maltodextrin (placebo) and fumonisin esterase (10 U/g, FUM*zyme*®) were obtained from BIOMIN Holding GmbH (Tulln and Getzersdorf, Austria). The enzyme was initially identified and isolated from a soil bacterium *Sphingopyxis* sp. MTA144 [33,55,57]. The genes encoding the enzymatic activity were used to transform the

yeast *Komagataella pastoris* into a fumonisin esterase secreting recombinant strain (*K. pastoris* DSM 26643) [184,185]. Maltodextrin was used as a carrier to produce the final product FUM*zyme*®.

All piglets received the FB1 culture material in powdered form directly administered into the mouth. For the intraoral administrations of maltodextrin and fumonisin esterase, the products were left in powdered form and administered, after homogenisation, via a 15 mL falcon tube (VWR, Leuven, Belgium) into the mouth. The mouth was held closed until swallowing occurred. For the intragastric administrations, the powders were dissolved in 20 mL of water and mixed vigorously, prior to intragastric administration with the help of a gavage tube.

4.3.4 Biomarker analysis

Analysis of the Sa/So ratio in serum was carried out as described previously by Schwartz-Zimmermann et al. [147]. Serum aliquots (200 µL) were shaken with 600 µL of methanol/acetonitrile (50/50, *v*/*v*) for 30 min, followed by centrifugation at 14,000× *g*. Pellet extraction was performed with 300 µL of methanol/water (80/20, *v*/*v*), followed by centrifugation. The supernatant was dried and the residue reconstituted in 300 µL of acetonitrile/water (30/70, *v*/*v*). The solutions were centrifuged prior to LC-MS/MS analysis. The limit of quantification (LOQ) of Sa and So is 1.5 ng/mL in serum.

Quantification of FB1 and its metabolites in serum was carried out as previously described by Schertz et al. [277]. A ^{13}C-labelled internal standard of FB1 was added to the thawed serum aliquots (300 µL), followed by the addition of 900 µL of methanol/acetonitrile (50/50, *v*/*v*). Samples were shaken (30 min, room temperature) and centrifuged (2800× *g*). Extraction of the pellets was performed twice with 200 µL of acetonitrile/water/formic acid (50/49/1, *v*/*v*/*v*), followed by centrifugation prior to analysis of the supernatant. The LOQs for FB1, HFB1, pHFB1a and pHFB1b analysis in serum are 0.39, 0.67, 0.14 and 0.21 ng/mL, respectively.

Fumonisin B1 and its metabolites, HFB1, pHFB1a, and pHFB1b, were analysed in faeces as specified by Schwartz-Zimmermann et al. [147]. Briefly, extraction of the freeze-dried faeces aliquots (300 µg) was performed three times with acetonitrile/water/formic acid

(74/25/1, *v*/*v*/*v*). The samples were centrifuged at 14,000× *g*, and the supernatant diluted 1 + 1 (v + v) with acetonitrile/water (30/70, *v*/*v*) prior to LC-MS/MS analysis. The LOQs are 0.74, 0.70, 0.81, and 1.0 µg/g for FB1, HFB1, pHFB1a, and pHFB1b, respectively.

All serum and faecal concentrations below the LOQ were excluded for the toxicokinetic and statistical analysis.

4.3.5 Toxicokinetic and statistical analysis

One pig from the intragastric-treatment group was excluded from all datasets in serum and faeces, as its serum Sa/So ratio was abnormally high before the start of the treatment. Additionally, the high Sa/So ratio for this individual was identified as outlier at different time points using a Q-Q plot, and subsequently confirmed with a Bonferroni outlier test.

For statistical analysis of the Sa/So ratio in serum, a linear mixed effects (lme) model (with treatments as fixed effects and pigs as random effects) was applied using the software package RStudio [230]. Due to inherent non-linearity in the data, the prerequisite of residuals being normally distributed was not met, and therefore, the data were log transformed (log(Ratio)~Treatment*Time) to meet this criterium.

Data obtained from the serum and faecal samples were analysed with regard to the following toxicokinetic parameters using non-compartmental analysis (Phoenix, version 8.1, Princeton, NJ, USA): maximum observed concentration (C_{max}), time where maximum concentration was observed (T_{max}), area under the concentration-time curve from time zero to time of last quatifiable concentration ($AUC_{0\rightarrow t}$). The AUC was calculated with the linear-up/log-down trapezoidal method. The AUC was used to determine the efficacy of fumonisin esterase by comparing the two routes of administration (intraoral and intragastric) to placebo.

The detoxifier was considered effective when the AUC of FB1 for the control group (placebo) was significantly higher than the AUC of FB1 following esterase treatment. Furthermore, the detoxifier was equally regarded effective when the AUC of HFB1 for the control group (placebo) was significantly lower than the HFB1 AUC following esterase treatment.

The effect of the treatment, based on the AUC, was calculated as follows, and expressed as per cent:

$$\frac{\text{Treatment AUC} - \text{Control AUC}}{\text{Control AUC}} . 100\ [\%]$$

Statistical analysis was performed in RStudio with a two-sided t-test with the level of significance set at 0.05.

4.4 Results

To determine the effect of fumonisin esterase in relation to its route of administration, the biomarkers for FB1 exposure and effect in pigs listed in Table 6 were analysed in serum and faeces.

Table 6. Biomarkers for fumonisin B1 (FB1) exposure and effect measured in serum and faeces after administration of FB1 and fumonisin esterase to pigs.

Serum	Faeces
Sa/So ratio FB1 HFB1, pHFB1a, pHFB1b	FB1 HFB1, pHFB1a, pHFB1b

Sa/So (sphinganine/sphingosine) ratio, FB1 (fumonisin B1), HFB1 (hydrolysed fumonisin B1 or aminopentol), pHFB1a, and pHFB1b (partially hydrolysed fumonisin B1a and B1b)

4.4.1 Biomarkers in serum

Before the administration of FB1 to 22 piglets, the mean Sa/So ratio (± standard deviation, SD) was determined as 0.15 ± 0.02. Following administration of FB1, a significantly higher Sa/So ratio was observed in the placebo group compared to that of the intraoral and intragastric esterase treatment ($p < 0.05$) from 6 h and 12 h onwards, respectively (Figure 27). No significant difference was demonstrated between the intraoral and intragastric treatments. Throughout the 24 h trial, the Sa/So ratio in the intraoral treatment group remained stable. The highest mean Sa/So ratio was observed 24 h post treatments, and was 0.41 ± 0.09, 0.17 ± 0.03 and 0.23 ± 0.12 in the placebo, intraoral and intragastric groups,

respectively. The observed increase in the Sa/So ratio can be attributed to an increase in Sa concentration rather than a decrease in So concentration.

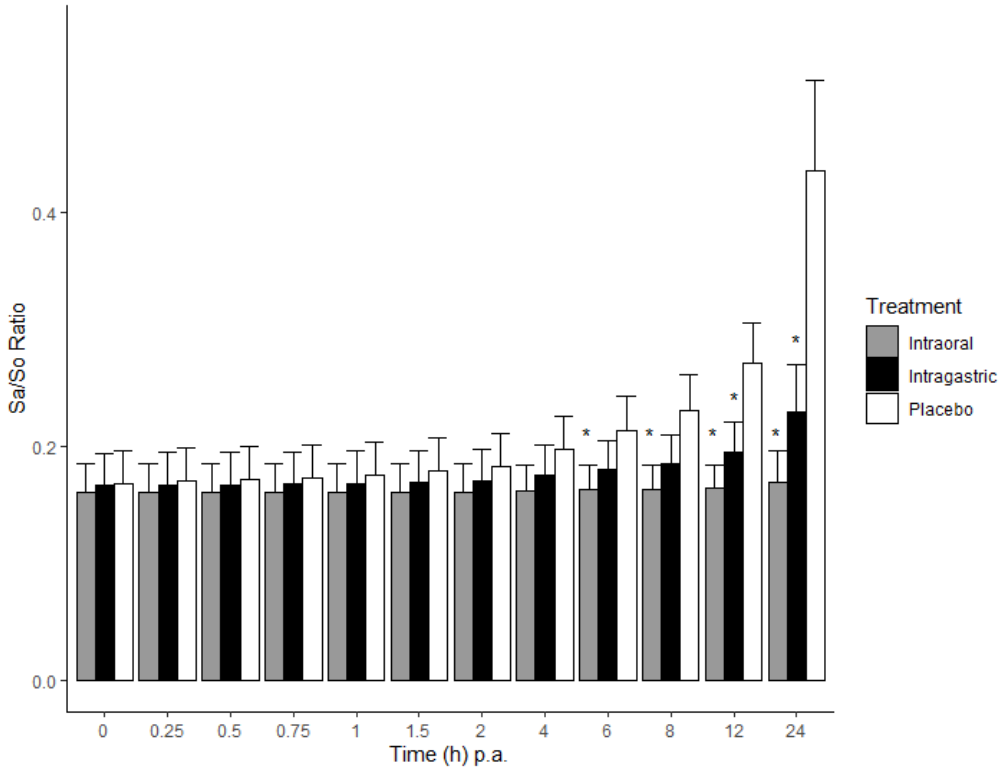


Figure 27. Mean sphinganine/sphingosine ratio (Sa/So) determined in pig serum during 24 h post-administration (p.a.) of a single intraoral administration of fumonisin B1 (2 mg/kg BW), either with a placebo (control group, $n = 7$), or with fumonisin esterase intraoral ($n = 8$) or intragastric ($n = 7$) administration. Error bars are the 95% confidence intervals. The asterisks represent a significant statistical difference ($p < 0.05$) compared to the placebo group.

Following FB1 administration to pigs, FB1 and pHFB1b were measured in serum as early as 15 min p.a. in all three treatment groups (Figure 28). Whereas HFB1 and pHFB1a were only measured as early as 15 min p.a. in the intraoral treatment group. Hydrolysed FB1 was observed at levels above LOQ at all sampling time points in the intraoral group from 15 min up to 24 h p.a., with a peak at 30 min. This was not the case in the intragastric group, where HFB1 was only detected at concentrations above the LOQ from 4 h p.a. and onwards. In the placebo group, it took up to 8 h p.a. to detect levels of HFB1.

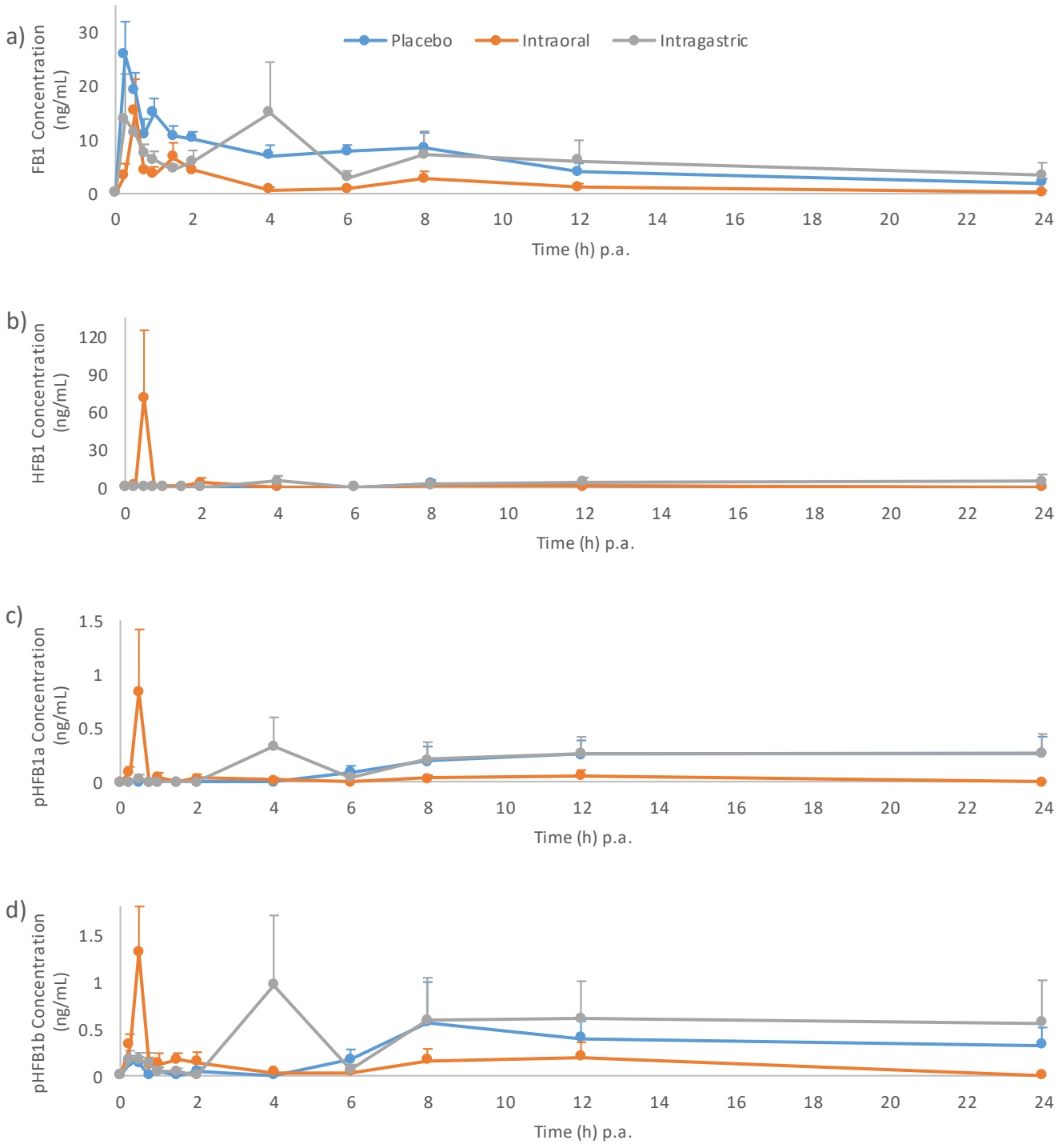


Figure 28. Mean concentration-time curve (+standard error of the mean, SEM) of a) fumonisin B1 (FB1), b) hydrolysed fumonisin B1 (HFB1), c) partially hydrolysed fumonisin B1a (pHFB1a), and d) pHFB1b, determined in pig serum after a single intraoral administration of FB1 (2 mg/kg BW), either with a placebo (control group, $n = 7$, blue curve), with fumonisin esterase intraoral ($n = 8$, orange curve) or intragastric ($n = 7$, gray curve) administration. The scale of the y-axis is different for individual plots.

An overview of the mean (± standard error of the mean, SEM) toxicokinetic parameters is presented in Table 7. A statistically significant ($p < 0.01$) decrease of 59.8% in the FB1 area under the concentration-time curve ($AUC_{0\rightarrow t}$), from time zero p.a. to time of last quantifiable concentration, in the intraoral treatment group compared to the placebo was observed. On the contrary, this effect was not observed in the intragastric treatment group when compared to the placebo.

Table 7. Mean ± standard error of the mean (SEM) of toxicokinetic parameters for fumonisin B1 (FB1) and its hydrolysed variants determined in serum after single oral administration of FB1 (2 mg/kg BW) to pigs, combined with either a placebo (control group, $n = 7$), or fumonisin esterase administered intraorally ($n = 8$) or intragastrically ($n = 7$).

Mycotoxin	Treatment	Maximum Observed Concentration (C_{max}) (ng/mL) ± SEM	Time of Observed Maximum Concentration (T_{max}) (h) ± SEM	Area under the Concentration-Time Curve Time Zero to Time Last Quantifiable Concentration ($AUC_{0\rightarrow t}$) (h x ng/mL) ± SEM	Difference in $AUC_{0\rightarrow t}$ between Control (Placebo) and Treated (Intraoral or Intragastric) Groups in %
FB1	Placebo	23 ± 3.8	2.2 ± 1.1	128 ± 15.5	
	Intraoral	10 ± 2.8	1.9 ± 0.9	51.4 ± 17.0 **	−59.8 **
	Intragastric	22 ± 7.8	7.0 ± 3.1	148 ± 51.7	+15.6
HFB1	Placebo	6.4 ± 4.8	5.6 ± 2.7	36.5 ± 31.0	
	Intraoral	33 ± 21	3.2 ± 1.2	49.8 ± 24.3	+36.4
	Intragastric	23 ± 6.9	11 ± 4.7	141 ± 54.4	+286
pHFB1a	Placebo	0.7 ± 0.2	18 ± 3.5	6.80 ± 2.18	
	Intraoral	0.6 ± 0.3	2.3 ± 1.5	2.12 ± 1.04	−68.8
	Intragastric	0.8 ± 0.3	13 ± 3.7	7.33 ± 2.82	+7.79
pHFB1b	Placebo	0.9 ± 0.4	9.9 ± 4.0	7.60 ± 2.43	
	Intraoral	0.9 ± 0.2	2.5 ± 1.2	3.07 ± 1.14	−59.6
	Intragastric	1.8 ± 0.7	11 ± 3.4	15.7 ± 6.60	+107

** $p < 0.01$, significantly different from placebo.

4.4.2 Biomarkers in faeces

Intragastric administration of fumonisin esterase resulted in a delayed FB1 peak (48 h p.a.), relative to placebo (Figure 29). Furthermore, there was a tendency to an earlier gradual increase in HFB1 concentration in the intraoral group compared to the other two treatment

groups, while its concentration peaked at 48 h p.a. in both the placebo and intragastric groups (Figure 29). Similar to what was observed in serum, the (partially) hydrolysed metabolites of FB1 were also detected when no enzyme (placebo) was administered. Based on the toxicokinetic parameters determined in faeces, no statistically significant differences in the FB1 AUC from time zero to time of last quantifiable concentration at 72 h ($AUC_{0\rightarrow t}$) (h x µg/g) between the three treatments were observed (Table 8). However, for HFB1, a significant increase of 119% in AUC between the placebo and intraoral ($p < 0.05$) treatments was observed, as well as a significant difference in AUC between intraoral and intragastric ($p < 0.05$). Furthermore, a significant decrease in AUC of both pHFB1a (by 79.0%) and pHFB1b (by 60.0%) from placebo to the intraoral treatment was calculated.

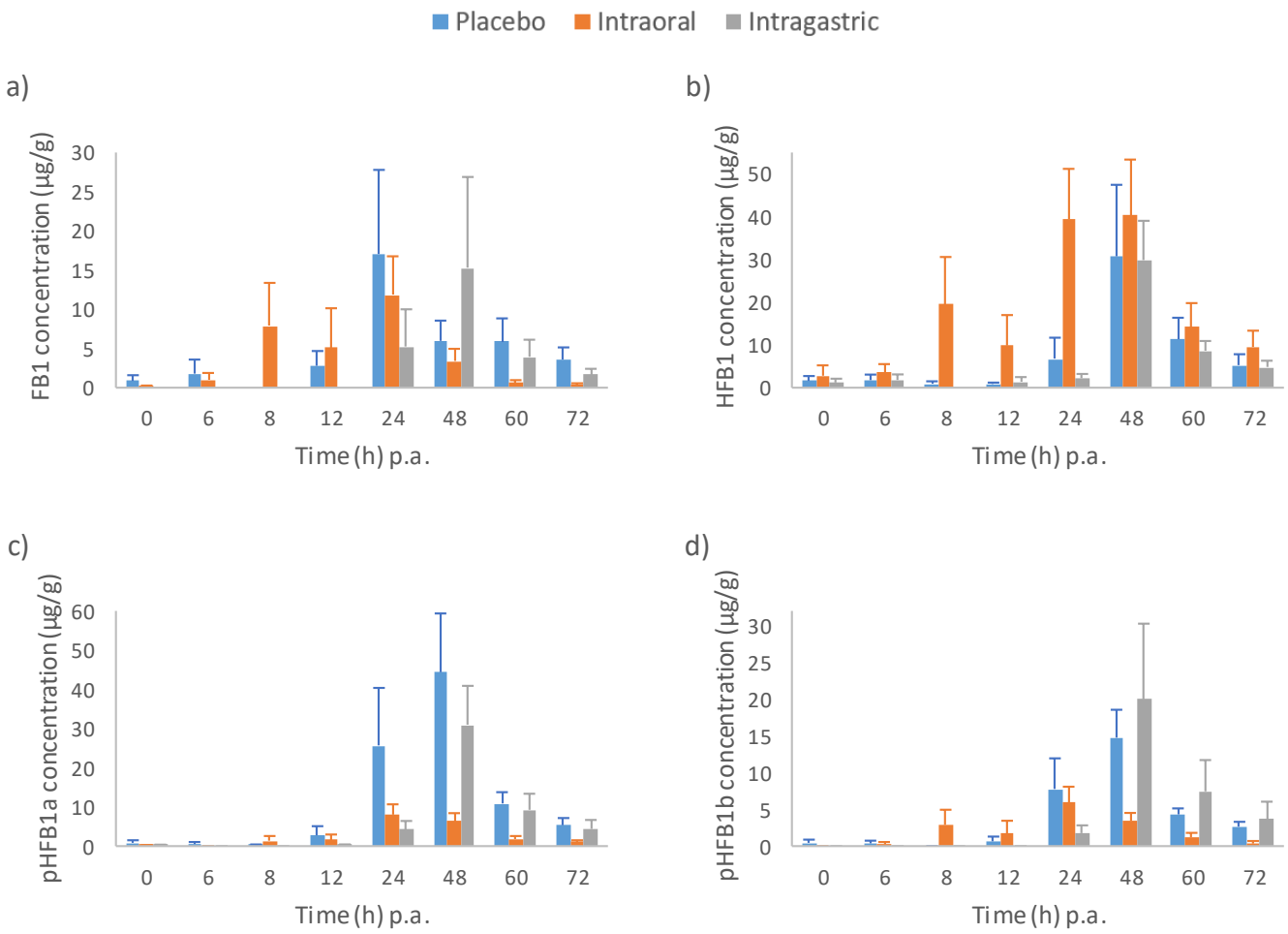


Figure 29. Mean concentration-time bar chart (+ standard error of the mean, SEM) of a) fumonisin B1 (FB1), b) hydrolysed fumonisin B1 (HFB1), c) partially hydrolysed fumonisin B1 a (pHFB1a), and d) pHFB1b, determined in pig faeces post-administration (p.a.) of a single intraoral administration of FB1 (2 mg/kg BW) either with a placebo (control group, *n* = 7, blue bar), or with fumonisin esterase intraoral (*n* = 8, orange bar) or intragastric (*n* = 7, gray bar) administration. The scale of the y-axis is different for individual plots.

Table 8. Mean ± standard error of the mean (SEM) of toxicokinetic parameters for fumonisin B1 (FB1) and its hydrolysed variants determined in faeces after single oral administration of FB1 (2 mg/kg BW) to pigs, combined with either a placebo (control group, *n* = 7), or fumonisin esterase administered intraorally (*n* = 8) or intragastrically (*n* = 7).

Mycotoxin	Treatment	Maximum Observed Concentration (C_{max}) (µg/g) ± SEM	Time of Observed Maximum Concentration (T_{max}) (h) ± SEM	Area under the Concentration-Time Curve Time Zero to Time Last Quantifiable Concentration ($AUC_{0\rightarrow t}$) (h x µg/g) ± SEM	Difference in $AUC_{0\rightarrow t}$ between Control (Placebo) and Treated (Intraoral or Intragastric) Groups in %
FB1	Placebo	20 ± 10	39 ± 5.7	617 ± 305	
	Intraoral	16 ± 4.5	31 ± 4.4	391 ± 125	–36.6
	Intragastric	18 ± 13	52 ± 2.5	531 ± 384	–13.9
HFB1	Placebo	28 ± 15	33 ± 7.7	795 ± 323	
	Intraoral	66 ± 11	34 ± 5.6	1,745 ± 289 *	+119 *
	Intragastric	30 ± 9.2	48 ± 0.0	796 ± 185 $^{\Delta}$	+0.13 $^{\Delta}$
pHFB1a	Placebo	56 ± 15	34 ± 4.8	1,512 ± 333	
	Intraoral	11 ± 2.1	38 ± 5.3	318 ± 73.0 *	–79.0 *
	Intragastric	31 ± 9.9	48 ± 0.0	977 ± 335 ^{a}	–35.4 ^{a}
pHFB1b	Placebo	17 ± 3.9	38 ± 4.8	552 ± 109	
	Intraoral	8.2 ± 1.7	29 ± 6.0	221 ± 43.8 *	–60.0 *
	Intragastric	23 ± 12	48 ± 0.0	777 ± 420	+40.8

* $p < 0.05$, significantly different from placebo; $^{\Delta}$ $p < 0.05$, significantly different from intraoral; ^{a} $p < 0.1$, trend towards a intraoral-intragastric difference.

4.5 Discussion

The aim of this study was to determine the efficacy of fumonisin esterase for hydrolytic breakdown of FB1, when administered intragastrically and intraorally, and to compare it to placebo application. The rationale of the study was to imitate capsule ingestion, which is one of the most popular dosage forms on the market for pharmaceuticals and food supplements [273]. The evaluation of the efficacy was carried out by analysing relevant biomarkers of FB1 exposure and effect as reported in previous studies [278], as well as by investigating and comparing certain toxicokinetic parameters.

Fumonisin B1 disrupts the *de novo* synthesis of sphingolipids due to inhibition of the crucial ceramide synthase enzyme, resulting mainly in a build-up of Sa, causing a time-dependent increase in the Sa/So ratio [70,279]. This correlates to the findings in this study, where the increase of the ratio in the placebo group was attributed to an increase in Sa. While the Sa/So ratio in human serum and urine is a contested biomarker for FB1 exposure, most likely due to relatively low FB1 exposure levels, it has been shown to be reliable in animals, such as pigs, horses, rodents, rabbits, chickens, ducks, monkeys, and trouts [6,23,45]. Furthermore, in humans, the range of normal Sa and So levels is large and levels within one individual vary over time [143]. Schwartz-Zimmermann et al. [269] acclaimed this biomarker in serum to be the most reliable when compared to other recognised biomarkers and matrices. Furthermore, the Sa/So ratio was suggested to be the preferred choice to measure enzyme efficacy for FUM detoxification. However, to assess the effectiveness of this specific detoxifier, it has been pointed out that either high FUM uptake levels, or long-term studies including a large number of animals are required. Our study confirms the Sa/So ratio to be a relevant biomarker for high acute FB1 exposure (2 mg FB1/kg BW), which was also concluded by Schwartz-Zimmermann et al. [269] in a pig trial, and similarly described in a human study performed by Qiu and Liu [266].

In our study, one pig from the intragastric-treatment group was excluded from the datasets. It had a Sa/So ratio of 0.56, which was 3.6 times higher than the average ratio (0.15 ± 0.02) found in the other 22 pigs. Moreover, Schertz et al. [141] reported an average Sa/So ratio between 0.10 and 0.15 at time point 0 h, which is similar to the average of the remaining 22 pigs. In our study we show that without the supplementation of the hydrolysing enzyme (placebo group), the Sa/So ratio significantly increased when compared to both groups that received the enzyme. No statistical difference in the ratio was observed between the groups that received the enzyme either intraorally or intragastrically. This indicates the efficacy of the enzyme, independent of the route of administration. However, a gradual increase (although not statistically significant) in this Sa/So ratio for the intragastric group towards 24 h p.a. is visible compared to the intraoral treatment. More information on the longer-term effect on the Sa/So ratio could have been obtained if serum was collected beyond 24 h p.a. Schertz et al. showed the continued increase of the Sa/So ratio beyond 24 h in the placebo group relative to intraoral fumonisin esterase treatment following a similar single-dose exposure with FB1

(at 2.47 mg/kg BW) [141]. While the intragastric administration of fumonisin esterase has so far not been tested, it is noteworthy that we achieved a measurable statistical difference in the Sa/So ratio from the placebo as early as 12 h post-treatment (6 h for the intraoral treatment). Although this indicates a faster effect of the enzyme when administered intraorally, compared to intragastric administration, the intragastric treatment clearly prevented a significant increase of the ratio.

In a previous piglet feeding study by Masching et al. [146], fumonisin esterase was mixed in the feed and administered over 42 days (d). The feed contained 2 mg FB1/kg feed (roughly 0.84 mg/d, estimated based on a consumption of 50 g of feed per day). In their study, Masching et al. could not observe an effect of FB1 on the Sa/So ratio even after 14 d. A first significant difference between the FB1 and the enzyme administered groups was observed after 28 d. The Sa/So ratio significantly increased to 0.26 ± 0.08 on day 28 and further increased to 0.39 ± 0.02 on day 42. In contrast, in a study where the administered single FB1 dose (2.47 mg/kg BW) was slightly higher as in our study, a significant difference to placebo was only observed after 24 h [141]. This indicates an effect related to how the animals were exposed to FB1, i.e., either being mixed in the diet versus administered as culture material on an empty stomach. Previous studies have confirmed the uptake of FB1 being much more rapid in fasted animals [280,281].

The observed effect of the fumonisin esterase on the Sa/So ratio was not reflected in the concentrations of FB1 and its hydrolytic metabolites in the serum and faecal samples. There was no statistical significant difference in AUC for FB1 or FB1-metabolites between the placebo and intragastric groups. Nevertheless, a significant difference in the FB1 AUC between intraoral enzyme administration and placebo further confirmed the efficacy of the enzyme. A significant decrease in the AUC of FB1 of nearly 60% in the intraoral treatment compared to the placebo group was observed in serum. This reduction is less than the 90% reduction in AUC of FB1 in serum that was observed in a similar single-dose treatment study performed by Schertz et al. [277], using the same detoxifier. The administration method of both FB1 and the enzyme, as well as the feeding state of the animals were the main differences to our study. While FB1 and the enzyme were both administered intraorally to fasted pigs in this study, they were mixed in the basal diet of fed pigs, in the aforementioned study. To our knowledge, all previous studies evaluating the efficacy of fumonisin esterase were carried out following the

instructions on the leaflet, namely mixing the enzymatic product in the feed. The manufacturer claims the enzyme to be activated in the saliva (combination of moisture and pH) as a result of chewing, and it is assumed to perform most of its activity in the mouth.

The conversion of FB1 to the completely hydrolysed product HFB1 as result of the enzyme activity has been observed in previous studies [19,20,48]. This was confirmed in our study in the faecal samples by a significant increase of nearly 120% in the AUC of HFB1 in the intraoral group compared to the placebo. Accordingly, serum HFB1 levels above LOQ were measured in all samples from the intraoral group. Additionally, a significant decrease in the partially hydrolysed forms of FB1 (pHFB1a and pHFB1b) in the faeces of the intraoral group relative to placebo was observed. These results concur with what was observed in other studies [269,277] where the fumonisin esterase has been shown to cleave both side chains of FB1, rather than only a single one. This results in a decrease in the partially hydrolysed metabolites in favour of an increase of the fully hydrolysed metabolite HFB1. The natural gastrointestinal degradation of FB1 into its partially hydrolysed metabolites attributed to microbial hydrolysis, was previously observed both *in vitro* [146,187] and *in vivo* [20,48,50] experiments. Such microbial hydrolysis was also detected in this study, as levels of pHFB1a and pHFB1b were measured in both the serum and faecal samples in the placebo group (no enzyme administered). Even though most studies report a predominant formation of partially hydrolysed metabolites [146,188], HFB1 was equally observed at low concentrations in the placebo group where exclusively FB1 was administered, suggesting further degradation of the partially hydrolysed forms, most likely due to activity of the bacterial microbiota [282]. Furthermore, these findings were reflected in the serum in our study, where HFB1 levels were also observed in the placebo group.

The toxicokinetic analysis showed a maximum serum FB1 level in the placebo group at 2.2 h (T_{max}) p.a. The serum T_{max} for the hydrolytic metabolites was observed later, at 5.6 h, 18 h and 9.9 h p.a. for HFB1, pHFB1a, and pHFB1b, respectively. Our observed FB1 serum T_{max} was comparable to what was observed by Prelusky et al. [60] and Dilkin et al. [283] in pigs, i.e., between 60 and 90 min and after 2 h, respectively. In their studies, FB1 was applied intragastrically with a single dose. In contrast, Dilkin et al. observed maximum FB1 excretion in faeces already between 8 and 24 h p.a. [283], while in our study this was detected between 24 and 48 h p.a. According to literature data, when pigs were non-fasted and FB1 was applied

with the feed, the FB1 faecal peak occurred later, after 48 h [277]. Although the maximum FB1 concentration in serum was observed earlier in this study compared to that by Schertz et al. [277] (9.5 h p.a.), at least in part explained by the difference in fasted vs fed state, respectively, Schertz et al. also reported delayed maximum concentrations for the metabolites. The importance of the feeding status of the experimental animals on the oral absorption and faecal excretion of the toxin has previously been pointed out [277], and was confirmed in this study. Furthermore, the feeding status can also negatively affect the effect of the enzyme. Consumption of the enzyme in capsule form might prevent full contact to FB1 contaminated food present in the gastrointestinal tract. Therefore, the possibility exists that the enzyme would not be able to perform or reach its full detoxifying potential. Further research would also be needed regarding the optimal dose of the enzyme to reach its maximum effect.

In this study, we observed an early peak in FB1 serum metabolite concentrations (HFB1, pHFB1a, and pHFB1b) following intraoral application of fumonisin esterase, compared to the intragastric group. These findings could indicate a delay in absorption or enzyme effect. However, similar delayed maximum concentrations were observed in the faecal samples following intragastric application of the fumonisin esterase, possibly indicating a slowed activity of the enzyme when administered intragastrically compared to intraorally. The hydrolysed FB1 metabolite profile after intragastric administration was somewhat comparable to the placebo group, whereby more distal microbiota is responsible for the metabolite formation. The lack of statistical significance could be ascribed to the large inter-individual variability between the pigs.

Considering the impact of FB1 on human health, including the higher vulnerability of children, the preliminary data obtained in this study in piglets, as animal model for humans, show that the consumption of fumonisin esterase mixed in the food might reduce FB1 exposure and hence prevent its deleterious effects. However, further testing in humans would be necessary to confirm this hypothesis. Likewise, to determine the efficacy of the intragastric administration of the enzyme, more research is necessary, preferably involving a larger sample size and a longer period of sampling (>24 h p.a. blood sampling). Furthermore, even though the Sa/So ratio in serum had been identified as the most reliable biomarker for confirming fumonisin esterase efficacy [147], it would be advised to continue analysing both several FB1 biomarkers for exposure and effect to determine the efficacy of fumonisin esterase. The

analysis of a combination of several biomarkers seems to remain relevant for future FB1 detoxification studies.

4.6 Conclusions

From the preliminary data obtained in this study, the hydrolysis efficacy of fumonisin esterase to degrade FB1 into its less toxic metabolites HFB1, pHFB1a and pHFB1b was evident. A significant increase of the Sa/So ratio was prevented by both intragastric and intraoral administration of the fumonisin esterase enzyme when compared to the placebo group. However, the efficacy of the enzyme when administered intragastrically was not reflected in the FB1, HFB1, pHFB1a, and pHFB1b levels in serum and faecal samples, while this was observed in the intraoral group. Based on these results, for human use of this enzyme , capsule ingestion cannot be recommended; it can be advised to thoroughly mix fumonisin esterase in the food prior to consumption.

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5 Chapter 3: *In vivo* human pilot intervention study for efficacy testing of aflatoxin and fumonisin detoxifiers: planned study designs and single fumonisin B1 dose determination and analysis in faeces of volunteers

Mycotoxin analysis performed by Biomin (Tulln, Austria).

5.1 Abstract

Aflatoxins and fumonisins play an important role in disease development in both animals and humans. To prevent mycotoxicosis in animals, mycotoxin detoxifier feed additives are administered. This effective mitigation strategy could be extrapolated to humans. Three designs were worked out for human intervention studies in Kenya, South Africa, and Belgium. Both a bentonite aflatoxin binder and a fumonisin esterase were planned to be administered to humans. Due to COVID-19 and complications with the legal status of the enzyme, these studies could not be performed. Thus, an alternative study was performed to determine the single fumonisin B1 (FB1) dose necessary to quantify FB1 or its metabolites, partially hydrolysed FB1a (pHFB1a), pHFB1b, and HFB1, in faeces. A single FB1 dose (1 or 2 µg/kg BW) was administered to four volunteers in Belgium. Faeces were analysed with a validated liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method. A single dose of 72.3 and 87.5 µg FB1 (1 µg/kg BW) each resulted in one faecal sample with levels above the LOQ (160 ng/g). On the other hand, a single dose of 2 µg FB1/kg BW resulted in FB1 levels above the LOQ in several faecal samples. Hydrolysed FB1 was not quantified in any samples. The legal status and safety of fumonisin esterase for human use must be determined prior to the execution of future intervention studies. A dose of 2 µg FB1/kg BW is advised for future fumonisin esterase efficacy studies.

Keywords

Aflatoxin B1; bentonite; biomarkers; detoxifier administration; dose determination; efficacy; fumonisin B1; fumonisin esterase; mycotoxin

5.2 Introduction

Mycotoxins are toxic secondary metabolites produced by fungi present on agricultural produce such as cereals and cereal-based products. Crops can be contaminated both in the field and during storage [261]. These toxins present an important risk factor in both the food and feed chain [190]. Aflatoxins (AFs) and fumonisins (FUMs) are two groups of mycotoxins, represented by aflatoxin B1 (AFB1) and fumonisin B1 (FB1), and are mainly produced by *Aspergillus* and *Fusarium* fungi, respectively [260,284]. They commonly contaminate maize, which is the preferred grain in Africa, Central America and Mexico [285]. Besides maize, groundnuts are a major source of AFB1 intake in humans [68]. Specifically in sub-Saharan Africa, where the general practice of subsistence farming is employed, the risk of AFB1 and FB1 intoxication is further aggravated by a lack of regulations and food safety inspections [37,40,43]. Additionally, the consumption of an unbalanced, maize-rich diet further contributes to the cumulative AFB1 and FB1 exposure, increasing the risk of adverse health effects [286]. Maize is considered an important food source in countries where consumption is higher than 50 g/person/day [285]. In Africa, consumption ranges from 52 to 328 g/person/day [285]. Stronger yet, Shephard et al. [37] reported an excessive maize consumption of 400 to 500 g/person/day in African diets. According to an annual market report of Grain SA in 2017 [287], between 67% and 83% of South Africans consume maize at an estimated amount of more than 600 g per person per day. In contrast, in Europe consumption ranges from 58 to 181 g maize/person/day [285]. Probst et al. [29] showed that 81% of the maize samples collected from 18 different African countries were positive for FUMs, with 49% of the samples containing levels above the USA recommended limit of 2,000 µg/kg [111]. The EU limit for FUMs in maize intended for direct human consumption is set at 1,000 µg/kg [106]. Likewise, in a study performed in Kenya by Mutiga et al. [30], FUMs were detected in 87% of the collected maize samples from local millers, with 50% exceeding the national legal limit of 1,000 µg/kg. In 41% of these samples, AFs were detected, with 4% over the Kenyan regulatory limit of 10 µg/kg for total AFs. Co-contamination of cereals with AFB1 and FB1 is evident in Kenya [30].

Mycotoxins have been shown to result in various diseases in animals. Chronic exposure to FB1 causes neural tube defects in unborn mice, equine leukoencephalomalacia in horses, porcine pulmonary oedema in pigs, and experimental studies have shown hepatotoxic and

nephrotoxic effects [55,234,260]. Although the causal association between the FB1 uptake and disease has not yet been demonstrated in humans, long-term FB1 uptake potentially contributes to an increased risk of developing oesophageal cancer, intestinal barrier dysfunction, as well as birth, kidney, and liver defects [21,55,66,198,261,262]. Therefore, FB1 is classified in the International Agency for Research on Cancer (IARC) group 2B as 'possibly carcinogenic to humans' [198]. In animals, AFB1 intoxication has resulted in death as well as in chronic diseases negatively affecting liver function, reproduction, immunity, and milk and egg production. Additionally, produced milk can contain transformed AFs, i.e. aflatoxin M1 and M2 (AFM1 and AFM2) [284,288]. In humans, AFB1 intake is strongly correlated with liver cancer and is classified in the IARC group 1 'carcinogenic to humans' [198,199]. Acute AFB1 intoxication outbreaks have occurred with several casualties due to fatal liver damage. The 2004 outbreak in Kenya, with a total of 317 cases, with 125 deaths, following the consumption of locally grown maize stored under poor conditions, is the most severe one documented [196,197]. Eastern Kenya, and particularly Makueni and Kitui districts have been hit by other AF outbreaks, such as in 1981 and in 2005/2006, resulting in both human and animal deaths [289,290]. In 2016, an outbreak of acute human aflatoxicosis occurred in Tanzania resulting in a reported total of 68 cases with a 30% fatality rate [291]. Kenyans and South Africans continue to be exposed to grain and nut products exceeding the set standards of mycotoxins [43,292,293]. Additionally, chronic intake of AFs and FUMs have been suggested by the WHO and others to play a role in stunting in children [21,55,56,68]. A provisional maximum tolerable daily intake (TDI) for FUMs in humans was established [294,295]. The European Commission has designated 2 µg/kg body weight (BW) as TDI for FB1 [296]. This figure is based on the total NOAEL (no observed adverse effect level) from subchronic toxicity studies and long-term toxicity/carcinogenicity studies in rats equivalent to 200 and 250 µg/kg BW/day, respectively, and applying a safety factor of 100 [297,298]. Although 2 µg/kg BW is listed as TDI on the website of the European Union, the European Food Safety Authority (EFSA) recently lowered the TDI of FB1 to 1 µg/kg BW [299], based on an increased incidence of megacytic hepatocytes found in a chronic study in mice.

Although several pre- and post-harvest mycotoxin mitigation techniques exist, mycotoxin intoxication remains a present-day hazard in low and middle income countries. Especially in countries where a large portion of the population relies mainly on self-grown

cereals and unregulated markets, the consequences of AFB1 and FB1 intoxication remain significant [37]. To prevent exposure and disease, with particular focus on Africa, implementation of effective mitigation strategies is needed; preferably collectively both pre- and post-harvest complementary approaches.

The aim of our study was to reduce AFB1 and FB1 exposure in adult volunteers by evaluating the efficacy of two mycotoxin detoxifiers. Bentonite clay is a EC-approved technological feed additive used as pellet binder, anti-caking agent, and coagulant. The bentonite product evaluated in this doctoral thesis, Mycofix® Secure, is additionally authorised for the use as AF-binder in feed. Currently, its classification is a "generally recognised as safe (GRAS) substance" according to the Food and Drug Administration (FDA) [140]. Additionally, bentonite (E558) was authorised as a food additive until 2013 [137]. Fumonisin esterase, another EU-approved feed additive detoxifier for all animal species [300], is an enzyme which specifically modifies FUMs into non-toxic metabolites, and has been registered under the name FUM*zyme*®. Providing detoxifiers to humans that are at risk of being exposed to contaminated food can decrease the amount of mycotoxins absorbed from the gastro-intestinal tract into the blood, potentially resulting in less or absence of adverse health effects. This can enhance individual, as well as communal, and even national social and economic development.

Most mycotoxin detoxifiers are added to feed, food, or (experimentally) to animals [301]. There are only a few studies where the detoxifiers were administered directly to humans [258]. Moreover, previously performed intervention studies in humans mainly focussed on the mitigation of AFs. In a cross-over study performed by Awuor et al. [123] in Kenya, a calcium montmorillonite clay (Air Classified Calcium Silicate 100 or ACCS100) or a calcium carbonate placebo was consumed by 50 participants in a glass of water during 7 days, each. Daily collected urine samples were analysed for AFM1. Blood samples were collected at the beginning and end of the trial (after 20 days – following 7 d treatment, 5 d wash-out, and 7 d treatment) and assessed for AFB1-lysine adducts from serum albumin. Urinary AFM1 concentrations were significantly lower during treatment compared to placebo (geometric mean of 7.4 and 10 versus 16 and 18 pg AFM1/mg creatinine, respectively). Pollock et al. [240] performed a 3-month intervention study with a placebo or two doses (1.5 or 3 g) of ACCS100 daily in 234 volunteers in Texas. At month 3, the level of the AFB1-lysine adduct in serum

decreased in both treatments, yet only the low dose mean (± SD) (2.71 ± 1.35 pg/mg albumin) was significantly different from the placebo (3.22 ± 1.85 pg/mg albumin). In Ghana, NovaSil (NS), a calcium montmorillonite clay, in capsule form was provided in a low (1.5 g) and high (3 g) dose three times a day to 507 volunteers during three months [122,132]. Urine and blood samples were collected after one, two, and three months of NS treatment, and one month after the end of NS treatment. At 3 months, the AFB1-albumin adduct mean (± SD) significantly decreased in both treatment groups (0.90 ± 0.16 pmol/mg albumin) compared to the placebo (1.20 ± 0.22 pmol/mg albumin). The median urinary AFM1 level significantly decreased in the high dose (21.61 pg/mg creatinine) treatment group compared to the placebo (52.38 pg/mg creatinine).

At first, our aim was to conduct a pilot intervention study in humans in Kenya, followed by an attempt in South Africa, and eventually in Belgium. Adult volunteers were to consume AFB1 and/or FB1 naturally contaminated food (their normal diet) along with one of the two detoxifiers. Several repetitive biological samples (blood, urine, and faeces) were to be collected and analysed for AFB1, AFB1-lysine serum adduct, FB1, and its degradation products (hydrolysed FB1 (HFB1) and partially hydrolysed FB1, pHFB1a and pHFB1b) as established biomarkers for mycotoxin exposure, and to evaluate the efficacy of the detoxifiers. Furthermore, FB1-related biomarkers of effect, i.e. the serum and urine sphinganine/sphingosine (Sa/So) ratio, would be evaluated. Unfortunately, complications with Ethical Committee approvals in Kenya, South Africa and Belgium prevented the intervention studies from being performed within the timeframe of the MycoSafe-South project.

Nevertheless, in an attempt to contribute to research for future studies, a FB1 dose determination study was carried out on 4 adult volunteers in Belgium. In this controlled pilot study, the lowest single FB1 dose that results in quantifiable (>limit of quantification or LOQ) concentrations of FB1 and/or its metabolites in human stool samples, was evaluated. Considering that FB1 is poorly absorbed in the digestive tract (<6% oral bioavailability, based on animal data [60–62]), the majority of the mycotoxin is excreted in the faeces. Therefore, stool is an appropriate matrix to measure FB1 and its degradation products (pHFB1a, pHFB1b, and HFB1). As is observed in animals, human urinary excretion of FB1 after oral FB1 administration is also reported to be low, an average of 0.5 ± 0.24% of the dose (2.94 ± 0.55

µg FB1/kg BW) was recovered from 8 individuals that consumed contaminated diets [61,302]. A dose of 1 µg/kg FB1/BW (TDI) and 2 µg/kg FB1/BW was provided to two individuals each. Based on the results obtained in this preliminary study, more extensive and controlled studies to assess the efficacy and safety of fumonisin esterase, should be performed. Provided that its effectiveness and safety are demonstrated, further research could lead to the approval of this product as a food additive.

5.3 Materials and methods

5.3.1 Fumonisin B1 dose determination for analysis in human faeces

Participants

To determine the required single FB1 dose for quantification in faeces, four (self-described) healthy adults (2 males and 2 females) from two different age groups were recruited in Belgium. The volunteers were briefed thoroughly on the goal and the expectations during the trial. Prior to participation, an informed consent form (Appendix 1) was provided in Dutch (mother tongue of all participants), and signed. A socio-demographic questionnaire was filled out to determine participation eligibility (Appendix 2). All consenting participants were inquired about their health status (medication intake or medical condition), age, height, weight and type of diet consumed in a small questionnaire. Furthermore, prior to the start of the experiment, participants received information on frequent maize and rice containing foods. Information on how to collect representative stool samples and apply correct labelling was also provided (Appendix 3). The average age of the participants was 44.8 years (y) (28, 29, 59, and 63 y), the average height and weight was 177 cm (172, 186, 170, and 180 cm) and 77.5 kg (61.5, 87.5, 72.3, and 88.5 kg), respectively.

Experimental design, treatment preparation, and administration

The study consisted of 8 consecutive days, during which the participants were asked to abstain from eating any maize and rice containing products, and to keep a detailed written food diary. On day 4, participants were weighed at the Laboratory of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ghent University, and were presented with a wheat-based cookie (snack cups, HAUST, obtained from Colruyt, Wetteren, Belgium) spiked with FB1 (1 or 2 µg/kg BW) to be consumed on an empty stomach. Participants were asked to

chew the cookie for 30 s before swallowing. Chewing was necessary to mimic future efficacy studies with fumonisin esterase for proper enzyme activation and effect. Participants were asked to remain sober until 3 hours post FB1 administration (p.a.). From days 2 to 8, the participants were instructed to collect a representative stool sample during every toilet visit with the help of faecal collection paper (Fe-Col®, VWR™, Leuven, Belgium). This stool collection method is based on the every two years' screening for colorectal cancer in the 50+ year-old Flemish population [303]. The samples were labelled appropriately with a personal code, date, and time, and stored in the freezer (–18°C) at the participants' home. An overview of the followed procedure is provided in Figure 30. At the end of the trial, all stool samples were collected at the laboratory and stored at –20°C until further processing.

Analytical FB1 standard was purchased from Fermentek (Jerusalem, Israel). A FB1 solution was prepared in water/ethanol (75/25, *v*/*v*) and stored at –20°C. Ethanol (96%) was purchased from a local pharmacy (Goed Apotheek, Wetteren, Belgium). This FB1 solution was used to spike the cookie. Two people received a dose of 2 µg/kg FB1/BW (person A and B, female and male) and the other two a dose of 1 µg/kg FB1/BW (person C and D, female and male). Three participants were blinded to the dose.

Biomarker analysis

The frozen stool samples were lyophilised for 48 h at the Laboratory of Chemical Analysis, Faculty of Veterinary Medicine, Ghent University. The samples were ground and homogenised, using mortar and pestle, and stored at −20°C until further analysis. Biomarker analysis (FB1, pHFB1a, pHFB1b, and HFB1) was performed at BIOMIN Research Center, Tulln, following the method previously described by Schwartz-Zimmermann et al. [269]. Aliquots of 300 mg of freeze-dried faeces samples were extracted three times with acetonitrile (ACN)/water/formic acid (FA) (74/25/1, *v*/*v*/*v*), followed by centrifugation at 14,000 x *g*. The supernatant was diluted 1+1 (*v*+*v*) with ACN/water (30/70, *v*/*v*) prior to LC-MS/MS analysis. The limits of quantification (LOQ) were set at 160, 480, 64, and 48 ng/g for FB1, HFB1, pHFB1a, and pHFB1b, respectively.

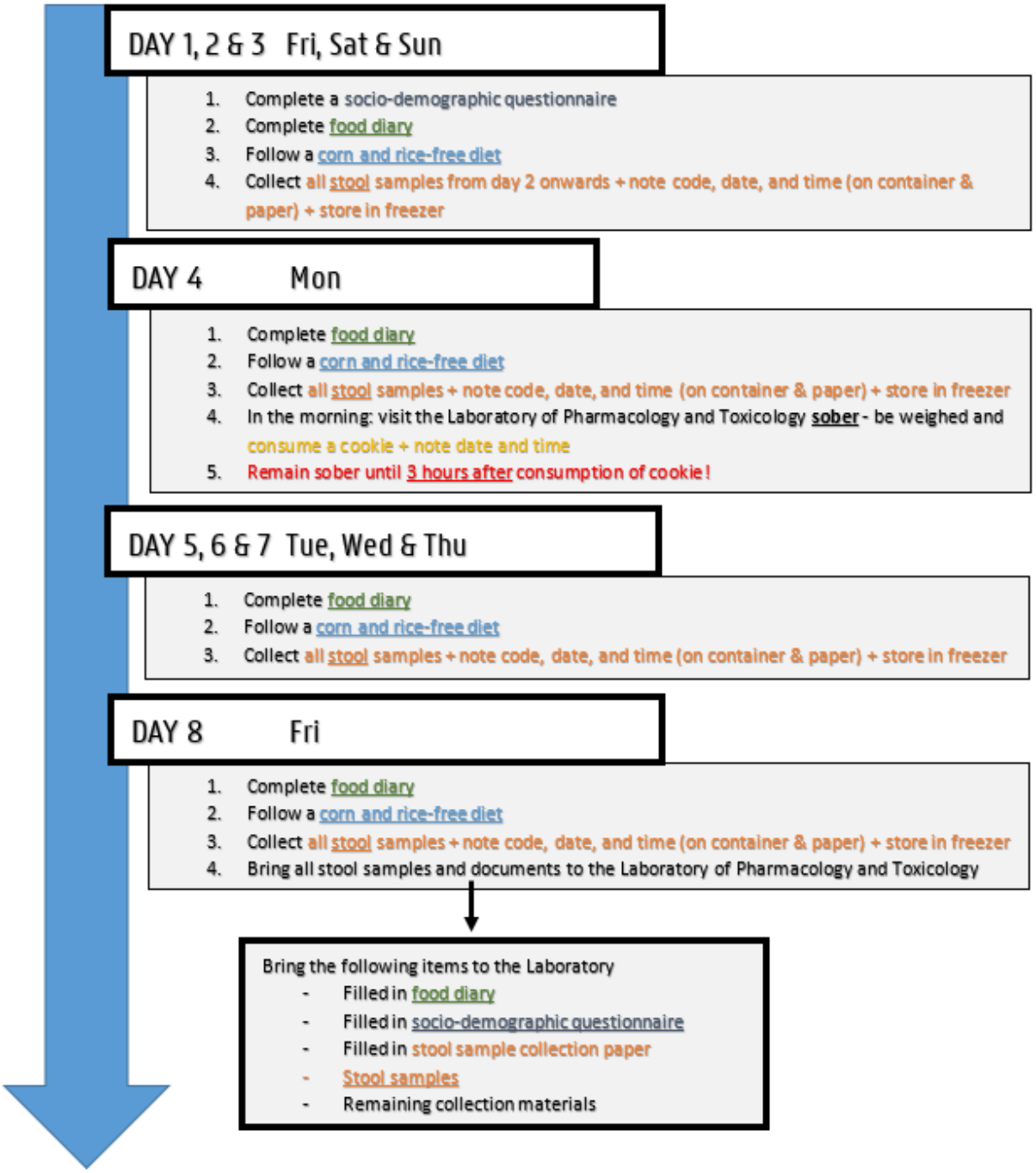


Figure 30. Preliminary trial procedure for participants.

5.3.2 Planned human intervention studies – Kenya, South Africa, and Belgium

Study proposal applications for Ethical Committee consideration were prepared to conduct pilot human intervention studies in Kenya, South Africa, and Belgium. In this section, the design of these trials is described.

5.3.2.1 Kenya

Participants

Sixty healthy adult (≥ 18 y) volunteers per region (Makueni County and Siaya County) would be recruited to participate in a cross-over trial. The participants' health would be assessed by medical personnel through physical examination and blood analysis as part of the inclusion process. Furthermore, urine screening for AFB1 or FB1 biomarker presence (levels > LOQ) would be performed to exclusively include people at risk; i.e. AFB1 or FB1 consumption in daily life. Validated urinary biomarkers for AFB1 and FB1 exposure are AFM1 [130] and FB1 [268], respectively. The required inclusion criteria for trial participation are summarised in Table 9.

Table 9. Inclusion criteria for participation in intervention study.

Inclusion criterium	Explanation
Healthy	HIV negative, hepatitis B and C negative, no liver, thyroid, heart, lung, gastrointestinal, nor kidney disease (incl. non-diabetic), no chronic medication, not alcoholic
Consumption of grain and nut products	At least 4 times per week, e.g. maize, wheat, sorghum, and groundnuts
50/50 female/male	To achieve proper representation of the population
Not pregnant	For the safety of the baby
AFM1 or FB1 concentration in urine	Above the limit of quantification (LOQ)
Informed consent form	Understood and signed

Experimental design, treatment preparation, and administration

Prior to execution of the intervention study, AFB1 and FB1 contamination levels in foods would be screened at two sites; Makueni County for AFB1 and Siaya County for FB1. Cereals such as maize, wheat, millet, and sorghum, as well as peanuts, would be sampled at different locations within the sites. Farmers, mills, and markets would be included to identify sites at risk and provide an idea of the magnitude of the problem.

A single-blinded cross-over trial would take place in both regions, at locations at risk, based on the previous assessment. Half of the participants would receive a placebo during 7 days (d) and the other half would receive bentonite clay (for AFB1 in Makueni County, Mycofix® Secure, Biomin, Tulln, Austria) or fumonisin esterase (for FB1 in Siaya County, FUM*zyme*®, Biomin, Tulln, Austria) during 7 d. Following these 7 d, a wash-out period of 5 d would be implemented, during which the participants would consume neither detoxifiers, nor placebo. Subsequently, the treatment for both groups would be switched, or crossed over.

Calcium carbonate and maltodextrin would be used as the placebos for bentonite and fumonisin esterase, respectively. Maltodextrin is a starch derivative, and is often applied as a food additive in several food products, as well as used in drugs as a binding agent. The USA Food and Drug Administration (FDA) has declared maltodextrin a GRAS-compliant food additive (https://www.fda.gov/food/food-additives-petitions/food-additive-status-list). It is almost flavourless, easily dissolved, and digested. Additionally, maltodextrin is the carrier of the enzyme. Therefore, both the enzyme and maltodextrin are not expected to have a negative effect on the taste of the meal. Calcium carbonate is a common dietary supplement, to complement calcium deficient diets [304]. Both detoxifiers and both placebos would be provided by BIOMIN Holding GmbH (Tulln and Getzersdorf, Austria).

The detoxifiers and placebos would be distributed in powdered form in sachets. One sachet would have to be sprinkled on and thoroughly mixed in each ready-to-eat meal. During the trial, participants would continue to consume their normal diets including the detoxifier or placebo with all three main meals of the day. Each sachet would contain 1 g of bentonite, or 1 g of calcium carbonate; or 180 U of fumonisin esterase (10 U/g), or 18 g of maltodextrin. The bentonite and its corresponding placebo dose is based on the effective dose implemented in the study of Awuor et al. [123]. The fumonisin esterase and maltodextrin doses are

calculated according to the dose of 3 U/kg BW used in a previously performed pig study (see Chapter 2.) [255], and based on an average BW of 60 kg [305–307]. At the end of the trial, a brief questionnaire regarding the acceptability and palatability of the detoxifiers would be provided.

At the start and end of the treatment weeks, blood sampled in two serum clot activator tubes (Vacutest Kima, Novolab, Geraardsbergen, Belgium) of 5 ml each, would be collected from the *vena cephalica* by medical staff. The serum tubes would be placed upright and allowed to clot at room temperature for at least 30 min before centrifugation (10 min, 2,851 × *g*, 4°C). Throughout the trial, in a medical facility, every morning between 7 and 9 am, clinical officers would perform a finger prick to collect one capillary blood drop of the participants via a 10 µL Volumetric Absorptive Microsampling (VAMS) device (Mitra®) (Figure 31). The VAMS devices would be obtained from Neoteryx (Torrance, CA, USA). In addition, a urine and a stool sample would be collected every day (Figure 32). Participants would receive two sterile sample collection bottles in a cooler box to collect their first urine and stool sample of the day. The VAMS devices would be stored at room temperature. Urine, faeces, and serum samples would be stored at −20°C until further analyses.

Figure 31. A Volumetric Absorptive Microsampling (VAMS) device. A blood drop is collected from the finger using a VAMS device.

Besides the human-derived samples, a 100 g grain and 50 g nut sample, would be collected at the beginning of both treatment weeks, as depicted in Figure 32. Furthermore, the amount consumed per day would be inquired to estimate the daily mycotoxin intake.

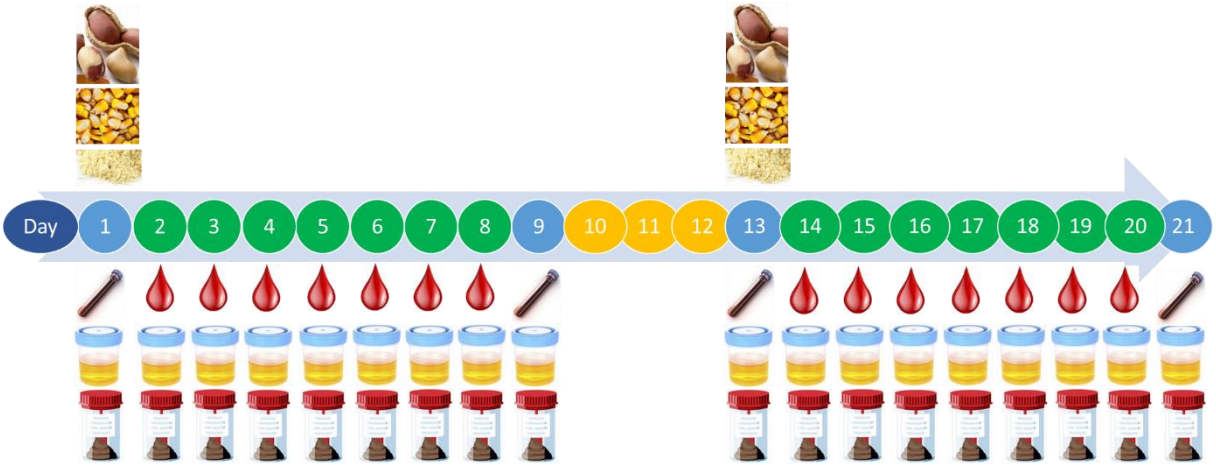


Figure 32. Sampling timeline throughout the entire 3-week trial. Days 1 and 9, 13 and 21 represent the start and end of the treatment weeks, respectively. The days 2 to 8 and 14 to 20 indicated in green represent treatment days, during which bentonite/fumonisin esterase or placebo would be consumed 3 times per day with the main meals. The blood tubes on the days indicated in blue represent the blood that would be sampled from the cephalic arm vein, the containers were to be used for urine and stool sample collection, and the drop of blood shows the finger prick blood sampling via a Volumetric Absorptive Microsampling device. Additionally, a grain/flour and nut sample present at the participants' household would be collected on the day prior to the start of the treatment weeks, namely days 1 and 13. Days 9 to 13 would count as the wash-out period.

Mycotoxin biomarker analysis

Fumonisin B1 and its metabolites, HFB1, pHFB1a and pHFB1b, would be analysed in faeces and urine as specified by Schwartz-Zimmermann et al. [147]. Analysis in faeces was briefly explained above in a) Fumonisin B1 dose determination for analytical quantification in human faeces. To 400 µL urine samples, 20 µL of ACN/water (30/70, v/v) containing 13C-labelled internal standards of FB1 and HFB1, and 1.2 mL of methanol (MeOH)/ACN (50/50, v/v) would be added together and shaken for 30 min. Next, the samples would be centrifuged, and the pellets and supernatants separated. The pellet would be re-extracted with 0.3 mL of MeOH/water (80/20, v/v). The supernatants would be combined, evaporated, reconstituted in 300 µL of ACN/water (30/70, v/v) and centrifuged. Finally, 13C-labelled internal standards of pHFB1a and pHFB1b would be added to a 100 µL aliquot prior to LC-MS/MS analysis.

The serum Sa/So ratio, which is an established biomarker of effect for FB1 exposure [255], would be inferred as described by Schwartz-Zimmermann et al. [147]. Prior to centrifugation (14,000 x *g*), 300 µL of MeOH/ACN (50/50, v/v) would be shaken with 200 µL of serum aliquots for 30 min. Pellet extraction would be performed with 300 mL of MeOH/water (80/20, *v*/*v*), followed by centrifugation. Next, the supernatant would be dried and the residue reconstituted in 300 µL of ACN/water (30/70, v/v), followed by centrifugation, and LC-MS/MS analysis.

In serum, FB1 and metabolites analysis would be carried out as previously described by Schertz et al. [277]. Serum aliquots of 300 µL would be left to thaw, before adding a 13C-labelled internal standard of FB1, followed by the addition of 900 µL of MeOH/ACN (50/50, *v*/*v*). Samples would be shaken (30 min, room temperature) and centrifuged (2,800 *x g*). Extraction of the pellets would be performed twice with 200 µL of ACN/water/FA (50/49/1, *v*/*v*/*v*), followed by centrifugation prior to analysis of the supernatant.

Quantification of FB1, AFB1, and AFM1 in the VAMS would be performed based on the analysis method previously described by Vidal et al. [308]. Extraction of the dried tips (with 10.4 µL of blood) would be achieved with 250 µL of ACN/water/acetic acid (59/40/1, *v*/*v*/*v*), with 13C-labelled internal standards of AFB1 or FB1, followed by ultra-sonication (20 min) and shaking (30 min). The tips would be removed and the solution would be evaporated, followed by reconstitution in 50 µL of MeOH/water (60/40, *v*/*v*). The samples would be vortexed and centrifuged (10 min at 5,000 *x g*) prior to LC-MS/MS analysis.

Aflatoxin M1 and AFB1 in urine would be analysed based on the method previously described by Šarkanj et al. [131]. Thawed urine aliquots of 500 µL would be centrifuged (3 min at 5,600 *x g*), and vortexed following the addition of 500 µL of water. Subsequently, the samples would be passed through Oasis PRiME® HLB SPE columns (30 mg). The elution of the AFs would occur with 600 µL of ACN, preceded by washing the columns twice with 500 µL of water. The eluted samples would be evaporated to dryness under a stream of nitrogen gas at 40 °C. Finally, the residue would be dissolved in 500 µL of 25% MeOH.

Additionally, a method sensitive enough to determine AFB1-lysine in serum with a nano-liquid chromatography - high resolution mass spectrometry (nLC-HRMS) method would be developed at the Toxinology Research Group, Norwegian Veterinary Institute in Oslo, Norway.

5.3.2.2 South Africa

A study proposal for Ethical Committee consideration in South Africa, with a similar study design and procedure as the one prepared for Kenya, was drafted and submitted. Slight modifications were made to the plan of conduct and are reported subsequently.

Study sites highly affected by both AFB1 and FB1-food contamination would be identified. Maize, sorghum, and peanut samples in two regions, Soweto and Alexandra, would be screened for AFB1 and FB1 contamination. The area(s) at risk of mycotoxin exposure would be included in the study. After local authority approval, collaborative information sessions (with community workers) on negative health effects caused by AFB1 and FB1 would be organised in these affected areas. These sessions would be used as recruitment opportunities. For the interested parties, information on the detoxifying agents and on the trial setup would be provided. All interested volunteers would go through the same clinical examination procedure and would have to comply with the inclusion criteria as mentioned in Table 9.

A total of 90 participants from different households would be required. The trial would consist of a single-blinded parallel design instead of a cross-over, as presented in Figure 33. During one week, 30 adult participants would consume placebos (calcium carbonate (1 g) and maltodextrin (18 g) per meal), another 30 participants bentonite (1 g per meal, Mycofix® Secure, Biomin, Tulln, Austria), and the last 30 participants fumonisin esterase (180 U per meal, FUM*zyme*®, Biomin, Tulln, Austria). Sampling of grains/nuts, blood, urine, and faeces would be similar to the Kenyan cross-over trial.

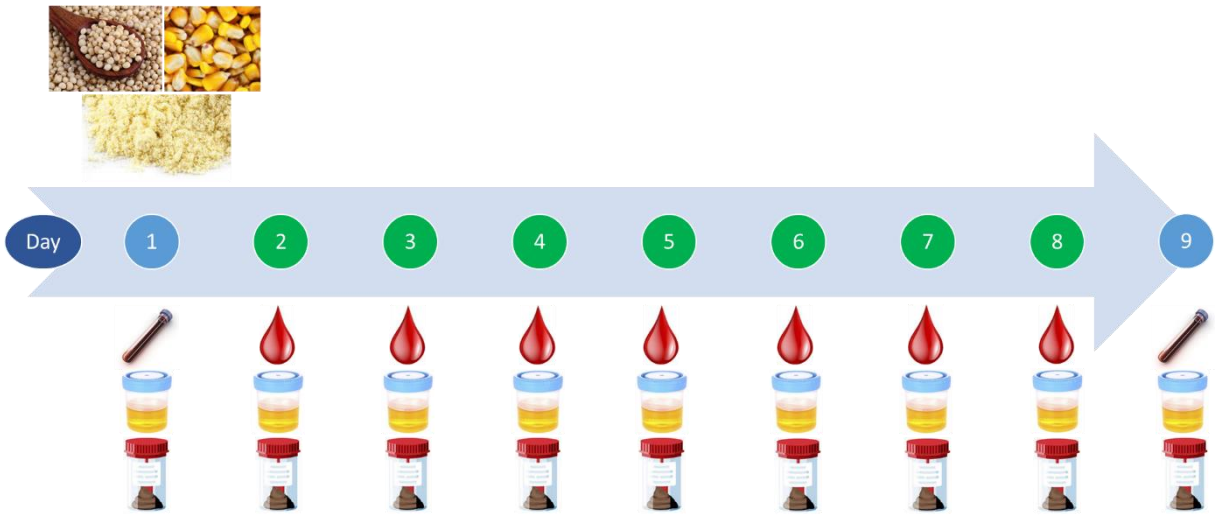


Figure 33. Sampling timeline throughout the trial. Days 1 and 9 represent the start and end of the treatment week, respectively. The days 2 to 8 indicated in green represent the treatment days, during which bentonite/fumonisin esterase or placebo would be consumed 3 times per day with the main meals. The blood tubes on the days indicated in blue represent the blood that would be collected from the cephalic arm vein, the pots stand for a urine and stool sample collection and the drop of blood shows the finger prick to collect a blood drop via a Volumetric Absorptive Microsampling device. Additionally, a grain/flour and nut sample present at the participants' household would be collected on the day prior to the start of the treatment week, i.e. day 1.

5.3.2.3 Belgium

In Belgium, food is regularly monitored for mycotoxin levels. Thus, people consume food that is likely to contain AFB1 and FB1 at levels well below the set maximum limits. In order to examine the efficacy of the detoxifiers, mycotoxin excretion levels should be high enough to establish a significant difference between the effect of a placebo and a detoxifier. As AFB1 is a group 1 IARC compound classified as 'carcinogenic to humans', it cannot be administered to people. FB1 is a group 2 IARC classified compound; the causal link between FB1 consumption and human disease has not yet been established. Yet, FB1 has been suggested to cause negative health effects after chronic uptake. Therefore, an intervention study providing a single low dose of FB1 to participants was planned. By administering a known amount of FB1 together with an enzyme or placebo, in combination with a controlled diet, the amount of (hydrolysed) FB1 (metabolites) can be determined in faeces under controlled conditions.

Twenty self-described healthy adult volunteers (10 females and 10 males), would be recruited for a single-blinded parallel study. All volunteers would have to meet the inclusion criteria of being between 18 and 65 years old, not pregnant, have no liver, bile, or kidney disorders, or are not taking any medication affecting the functioning of these organs. Fumonisin B1 (2 µg/kg BW) would be administered orally in combination with fumonisin esterase (treatment, 3 U/kg BW, *n* = 10) or with maltodextrin (placebo, 0.3 g/kg BW, *n* = 10). The intervention study would last 8 days (Figure 30), during which all volunteers would be required to follow a corn- and rice-free diet. Furthermore, a food diary of all foodstuffs consumed during the study would be kept. In the morning of day 4, participants would be invited to the Laboratory of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ghent University, sober. The BW of each volunteer would be determined, to correctly dose both FB1 and the detoxifier. The volunteer would receive a spiked wheat-based cookie to consume completely. It should be chewed on for 30 seconds so that the enzyme has time to be activated in the saliva. This cookie would be contaminated with 2 µg of FB1/kg BW in combination with either the fumonisin esterase (FUM*zyme*®) (treatment, 3 U/kg BW), or with maltodextrin (placebo, 0.3 g/kg BW). Maltodextrin is the carrier of the enzyme, which is also present in the fumonisin esterase test product. The same procedure as the one presented in Figure 30 would be followed. From days 2 to 8, participants would have to collect a representative stool sample of all produced faeces, using faecal collection paper (Fe-Col®, VWR™, Leuven, Belgium). Collection date and time would be noted. All samples would be stored in a freezer (–18°C) at the participants' home during the trial. At the laboratory, all samples would be stored at –20°C until further processing. A detailed description of both the processing and analytical methods are provided in section a) Fumonisin B1 dose determination for analytical quantification in human faeces.

5.4 Results

5.4.1 Fumonisin B1 dose determination for analysis in human faeces

Person B reported to have lost a few samples due to the tearing of faecal collection papers. The food diaries indicated that all participants kept themselves to the restricted diet. Following a single oral dose of 1 µg/kg FB1/BW, FB1 could be detected above LOQ in one sample from both participants (person C after 24.6 h and person D after 102 h) (Figure 34). Conversely, FB1 could be detected above LOQ in several samples following the administration

of the double dose of 2 µg/kg BW in persons A and B (Figure 34). A first and highest FB1 concentration of 7.84 x 10^3 ng/g was quantified as early as 13.8 h p.a. in person A. In person B, the highest concentration measured was 5.33 x 10^3 ng/g after 25.9 h p.a. The last quantified FB1 concentration was at 54.9 h p.a. (234 ng/g, person A) and 102 h p.a. (486 ng/g, person D) following 2 and 1 µg FB1/kg BW administration, respectively.

HFB1 could not be detected above LOQ in any samples from persons that received either of the two administered FB1 doses.

On the other hand, both pHFB1a and pHFB1b levels were observed in the samples of one person of each administered dose. A pHFB1a concentration of 168 ng/g was determined (>LOQ) as soon as 13.8 h p.a. and of 484 ng/g at 24.6 h p.a. in person A and C, respectively. The last levels measured (>LOQ) were 65 ng/g at 47.3 h and 135 ng/g at 50.3 h p.a. in person A and C, respectively. For pHFB1b, in person A, a concentration of 49 and 32.2 ng/g was observed at 28.6 h and 47.3 h p.a., respectively. In person C, pHFB1b could be quantified at concentration of 144 ng/g at 24.6 h p.a. and the latest quantifiable concentration of 58.9 ng/g was observed at 50.3 h p.a.

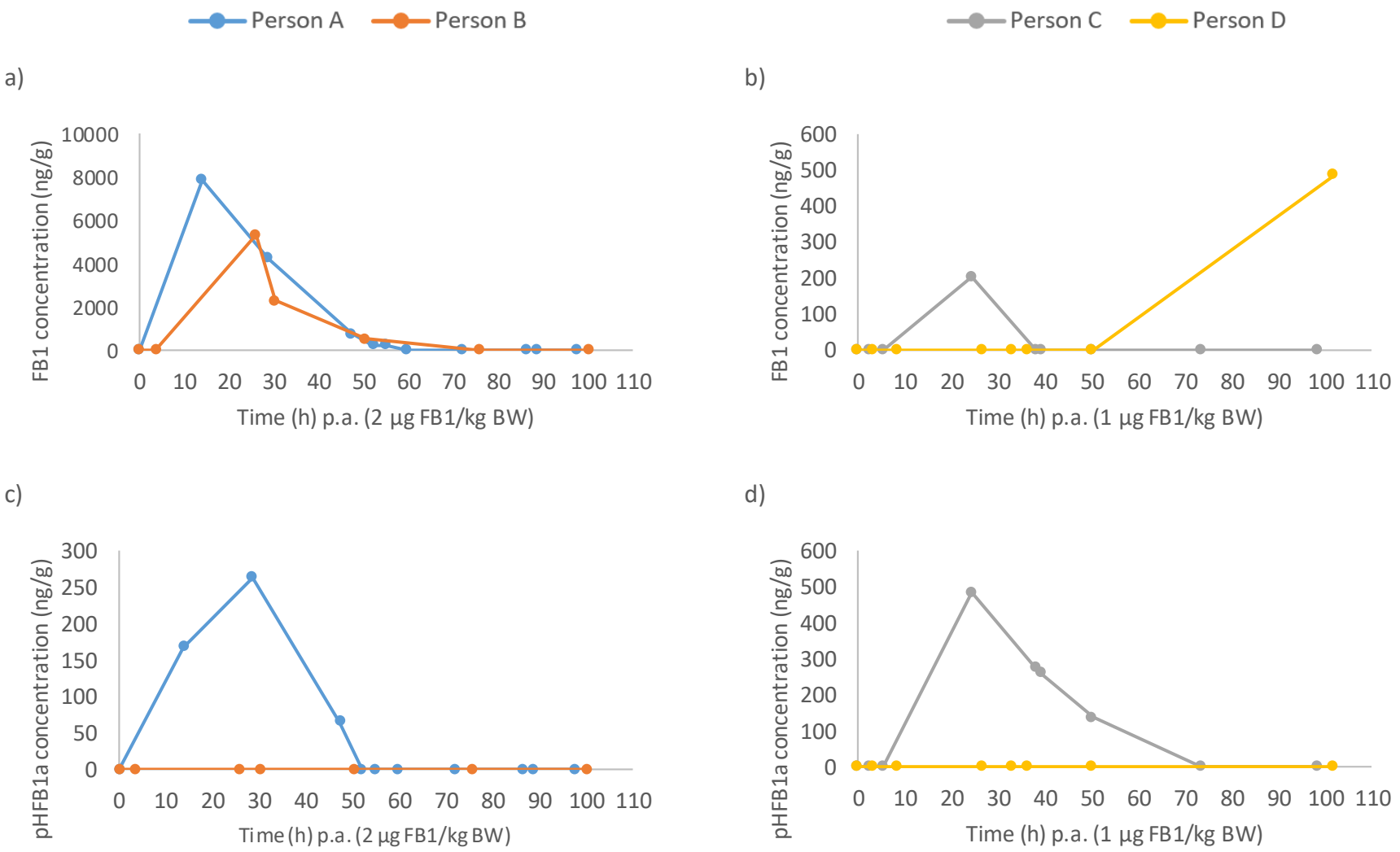
5.4.2 Planned human intervention studies – Kenya, South Africa, and Belgium

In collaboration with an experienced medical epidemiologist, employed at the Division of Global Health Protection of the Kenya Medical Research Institute (KEMRI, Nairobi), an intervention study was planned to be conducted in Kenya. A project proposal for the Scientific and Ethics Review Unit (SERU) of KEMRI was drafted. However, at the beginning of the COVID-19 pandemic, KEMRI put all non-essential research projects on hold. Later that year, due to fear of unintentional COVID-19 exposure of participants and staff, intervention trial applications were no longer accepted when close human contact was required.

A similar human intervention trial was to be executed in South Africa as part of the MycoSafe-South project. Therefore, the study plans were redirected to South Africa (Johannesburg). In cooperation with an experienced member of the Human Sciences Research Council (HSRC), some adjustments were made and an ethical application was submitted to the HSRC Research Ethics Committee (REC). The Committee disapproved the application as they reported not to be qualified to assess this type of trial. The REC noted this trial to be a “clinical

trial which refers to a systematic study involving human participants that aims to answer specific questions about the safety or efficacy of a medicine or method of treatment." However, the HSRC REC focuses on social science research. Additionally, more information would have to be provided about the detoxifiers, the underlying chemistry and pharmacology, pre-clinical safety studies, animal data, and the results of phase 1 and 2 human studies. Approval of the South African Health Products Regulatory Authority (SAHPRA) was also necessary. Furthermore, at this point the South African variant of COVID-19 was circulating and increasing in magnitude. Due to the close contact that would be necessary for the trial, it was not advisable for the safety of both the participants, the medical staff, and co-workers in the field.

Alternatively, a small scale study involving the administration of FB1 and fumonisin esterase in Belgian volunteers was prepared. With the assistance of an expert in clinical trials regarding the exposure to mycotoxins in humans, an ethical dossier was drafted for the Health, Innovation, and Research Institute (HIRUZ) - Clinical Trials Unit (CTU). However, issues arose regarding the nature of fumonisin esterase. The question remained whether it was to be classified as a food additive or a medicinal drug. For guidance, HIRUZ - CTU referred us to the UGent Faculty of Bioscience Engineering. We were advised to analyse the feed additive on its composition (mineral, protein, and vitamin content, and microbiological profile) and contact medical doctors to estimate the safety of the product. Despite these efforts, we were further referred to the Federal Agency for Medicines and Health Products (FAMHP - FAGG) and to the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO). The Head of Technology and Food Department at ILVO replied that a feed additive cannot be administered to humans, unless perhaps for research purposes. In this case, an Ethical Committee would have to give approval. From the FAMHP's point of view, and with the current information available, the detoxifier was rather regarded as a medicinal drug. To establish the definitive nature of fumonisin esterase for human use, more discussions between experts of several committees (FAMHP, Federal Agency for the Safety of the Food Chain or FASFC, Federal Public Service Health or FPS Health, Commission for Medical Ethics UZGent) would have to take place. Due to the complexity of the situation and a lack of time, this process was not pursued during this PhD research.


Person A
Person B
a)
FB1 concentration (ng/g)
10000
8000
6000
4000
2000
0
0 10 20 30 40 50 60 70 80 90 100 110
Time (h) p.a. (2 µg FB1/kg BW)
Person C
Person D
b)
FB1 concentration (ng/g)
600
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80 90 100 110
Time (h) p.a. (1 µg FB1/kg BW)
c)
pHFB1a concentration (ng/g)
300
250
200
150
100
50
0
0 10 20 30 40 50 60 70 80 90 100 110
Time (h) p.a. (2 µg FB1/kg BW)
d)
pHFB1a concentration (ng/g)
600
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80 90 100 110
Time (h) p.a. (1 µg FB1/kg BW)

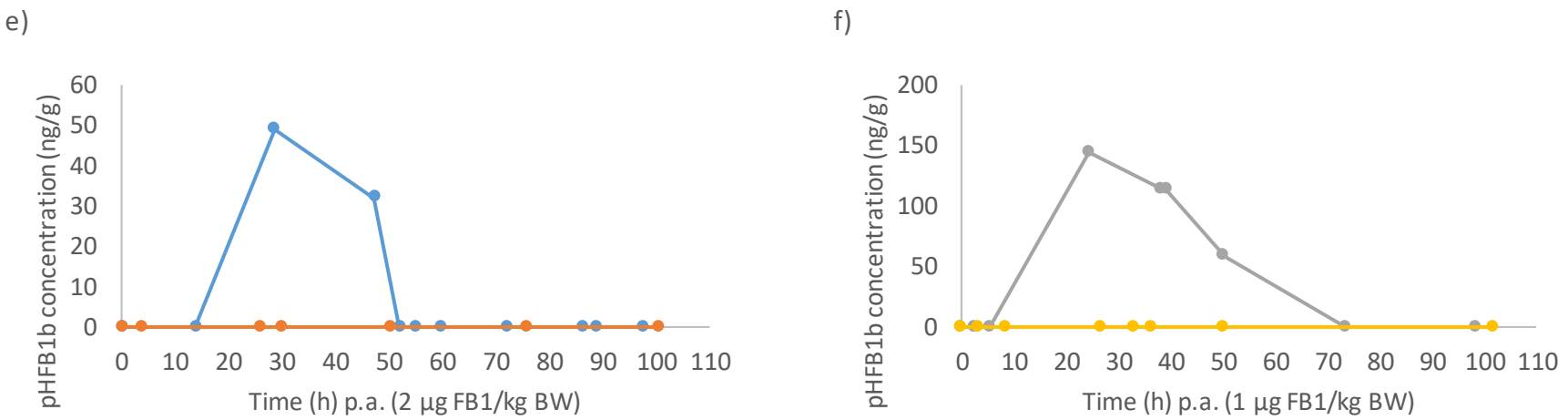


Figure 34. Concentration-time curves of a) and b) fumonisin B1 (FB1), c) and d) partially hydrolysed FB1a (pHFB1a) and e) and f) partially hydrolysed FB1b (pHFB1b) in faeces following a single dose administration of FB1 to four adult volunteers, 2 µg/kg body weight (BW) in a), c), and e) (*n* = 2) and 1 µg/kg BW in b), d), and f) (*n* = 2).

5.5 Discussion

The aim of the planned studies was to determine the efficacy of fumonisin esterase and bentonite to mitigate exposure to FUMs and AFs, respectively, in humans at risk through contaminated food. The evaluation of the efficacy would be carried out by analysing biomarkers of exposure and effect of AFB1 and FB1 in several biological matrices. Previously performed human intervention studies to decrease AF exposure comprised a cross-over study [123] and two parallel studies [122,132,240]. All three studies investigated the efficacy of a calcium montmorillonite (bentonite) clay by analysing AFB1-lysine adducts in serum and urinary AFM1 levels. Based on the significant decrease in the levels of the biomarkers when compared to the placebo group, measuring both AFB1-lysine and AFM1 would be valuable to assess the efficacy of an AF binder. Unfortunately, due to complications including legislative requirements, ethical considerations, and also COVID-19, an intervention trial with any of the mycotoxin detoxifiers in Kenya, South Africa, or Belgium was not possible. The first step for future research on the applicability of fumonisin esterase as mycotoxin detoxifier in humans would be to determine to which product category or classification it belongs, in order to establish the legislative requirements needed. It can either be classified as a technological food additive, dietary supplement, or as a medicinal product. Furthermore, it should be investigated and clarified whether its detoxification effect fully or partially occurs in food, prior to consumption, or if the enzyme is active in the orogastrointestinal tract. Different legislations apply depending on where the effect of the enzyme takes place. If the detoxifier is solely active in food, it could be classified as a technological food additive (EC Regulation 1332/2008). If it is active following ingestion, it could be classified as a dietary supplement (novel food legislation) (R&D Department FAMHP, personal communication, 22/09/2021). While bentonite (E558) was an authorised human food additive in the EU until 2013 [137], fumonisin esterase has only been accepted as a feed additive for animals. Thus, prior to efficacy testing of fumonisin esterase in human volunteers, safety testing of the product should be considered.

The aim of our preliminary *in vivo* experiment was to determine the lowest single oral FB1 dose to humans, 1 or 2 µg/kg BW, resulting in quantifiable levels of FB1 and/or its metabolites in faeces. Establishing such a dose can be useful for future efficacy studies

involving FB1 detoxifiers, such as fumonisin esterase. To control and prevent FUM uptake through food consumption, four participants were asked to follow a corn- and rice-free diet during the study. Worldwide, FUMs have been found as maize contaminants [309]. However, other agricultural products can also be potentially contaminated, but only limited and outdated research on the presence of FUMs in foodstuffs in Europe is available in the literature. Sporadically, FUMs have been identified in cereals such as sorghum and rice [218]. Furthermore, in Germany, these contaminants were also detected in rice [309,310]. In the 2020 annual survey report of Biomin [311], other cereals besides corn were analysed; wheat, oats, rice, rye, and barley were included. Out of the 883 tested cereal samples not including corn, 11% were contaminated with FUMs (mean of 264 µg/kg), whereas 71% of the 733 corn samples tested positive (mean of 1,153 µg/kg). Based on these above-mentioned studies, both maize and rice were excluded from the diet, while wheat was not. Daily, the majority of the Belgian population (82.3%) consumes bread and cereals (mean of 133 ± 59 g/person/day) [312]. These products are mainly wheat-based. Although this food type might be a source of FUM uptake in Europe, more data is needed. In Europe, wheat is especially a source of the mycotoxin deoxynivalenol (DON) [313]. Furthermore, for the convenience of the participants, this cereal type was not restricted.

Previous studies with human subjects analysed FB1 and its metabolites in urine; this matrix was presented as an adapted matrix to determine biomarkers of exposure in humans [69]. Riley et al. [302] experimentally administered a known amount of FB1 to humans in the USA through consumption of tortillas and maize-based biscuits. An average concentration (± SD) of 2.94 ± 0.55 µg/kg FB1/(BW*d) was administered for 3 consecutive days to 8 volunteers. Likewise, 4 µg/kg FB1/(BW*d) for 3 and 6 consecutive days was consumed by one volunteer each. In these aforementioned studies, only urine was collected and analysed; and the total urinary excretion of FB1 was less than 1% of the cumulative dose. Since FB1 is poorly absorbed (<6% oral bioavailability, based on animal data) [60–62], the majority of the absorbed amount is excreted in faeces. Furthermore, a low urinary excretion of FB1 and a high inter-individual variation, make faeces a more reliable matrix.

A single dose of 72.3 µg FB1 (equivalent to 1 µg/kg BW) given to person D was not sufficient to measure FB1 nor its metabolites in faeces prior to 100 h p.a. In one sample of this particular person, 486 µg/g of FB1 was quantified at 4.25 days (d) p.a. A possible explanation

is that contamination occurred during sample processing or analysis of this particular sample. However, gut motility differs between individuals and even within the same individual [314,315]. This person mentioned (self-described) having a slow bowel transit (defecation less than daily). Although, a frequency of 3 times per day to 3 times per week is a generally accepted norm [316]. Data in the literature concerning total gastrointestinal transit time varies from 18 to 100 h (4.2 d), although a transit of 72 h is still considered normal [314,315,317]. Thus, another possible explanation is a delayed FB1 excretion due to slower gut motility.

Person C, who received a dose of 87.5 µg (1 µg/kg BW), produced only one sample (at 1 d p.a.) with quantifiable FB1 levels (204 µg/g). Yet, the other samples from this person contained partially hydrolysed metabolites, pHFB1a and pHFB1b, without quantifiable FB1. As was observed in several previously performed (*in vitro* and *in vivo*) experiments, partial (and complete) FB1 hydrolysis has been causally linked to gut microbiota (see Chapter 1) [141,188,269]. Interestingly, in our study, no HFB1 was observed in either of the volunteers. Therefore, this biomarker could be useful to measure in faeces when investigating the efficacy of fumonisin esterase in humans. The partially hydrolysed forms (pHFB1a and pHFB1b) were detected above LOQ in two of the four participants' faecal samples, while FB1 was detected above LOQ in samples from three of the volunteers. An interesting finding was that in the case of person B, FB1 was quantified in the faeces, but no quantifiable levels of pHFB1a or pHFB1b were found. Further research could include the investigation of the gut microbiota to explore the differences in bacteria profiles to explain why no hydrolysed products of FB1 were quantified.

A single dose of 2 µg/kg BW resulted in multiple faecal samples with FB1 levels above LOQ in both volunteers, with highest concentration reaching 7.84 x 10^3 and 5.33 x 10^3 ng/g. Although a dose of 1 µg/kg BW resulted in one faecal sample per person with FB1 levels above LOQ, much lower FB1 concentrations of 204 and 486 ng/g were recorded. Based on these results, a suggested single dose of 2 µg/kg BW is put forward for future studies to obtain multiple faecal samples with FB1 levels above LOQ per person. However, our study was only performed on two volunteers with two dosages. Although the obtained results give an indication, a study with a larger sample size and with other dosage levels between 1 and 2 µg/kg BW could provide a more accurate optimal single FB1 dose for analytical

quantification in faeces. Additionally, future studies with collection of human stool samples are advised to be performed with a more robust faecal collection method.

5.6 Conclusions

The legal status and categorisation of fumonisin esterase, either as a technological food additive, dietary supplement, or as a medicinal product, should be further investigated and determined before a human intervention study can be performed. Furthermore, the safety of the enzyme in humans should be investigated before the implementation of an efficacy study. A single FB1 dose of 1 µg/kg BW (72.3 and 87.5 µg FB1 each) resulted in one FB1 positive (>LOQ) faecal sample. A single FB1 dose of 2 µg/kg BW resulted in FB1 levels above LOQ (160 ng/g) in several faecal samples. Quantification of FB1 in multiple samples is preferable for future fumonisin esterase efficacy studies. Furthermore, HFB1 could not be quantified in any sample, possibly making this metabolite a relevant biomarker in human faeces to determine fumonisin esterase efficacy.

Funding: The research that led to these results was conducted within the ERA-NET LEAP-Agri MycoSafe-South project funded by the Belgian Federal Science Policy Office (BELSPO) (contract number BL/02/LeapAgri 01), the Belgian National Fund for Scientific Research (NFSR), the Research Council of Norway (grant number 290459), the Kenyan Ministry of Education, Science and Technology (MoEST) and BIOMIN Holding GmbH. The detoxifier FUM*zyme*® was provided by BIOMIN Research Center, Tulln, Austria.

Appendix 1

Informatiebrief voor de deelnemers aan een experiment

Titel van de studie: Onderzoek naar de afbraak van fumonisine B1 door het fumonisine-esterase via orale ingestie, door opsporing van fumonisine en diens metabolieten in fecale biospecimens

Beste,

U wordt uitgenodigd om deel te nemen aan een klinische studie. Neem, voor u beslist deel te nemen aan deze studie, voldoende tijd om deze informatiebrief aandachtig te lezen en dit te bespreken met de onderzoeker of zijn/haar vertegenwoordiger, of met andere personen. Neem ook de tijd om vragen te stellen indien er onduidelijkheden zijn of indien u bijkomende informatie wenst. Dit proces wordt 'informed consent' of 'geïnformeerde toestemming' genoemd. Eens u beslist heeft om deel te nemen aan de studie zal men u vragen om het toestemmingsformulier achteraan deze bundel te ondertekenen.

1) Wat is het doel van de studie?

Wij nodigen u uit om deel te nemen aan een klinische interventiestudie met als doel de doeltreffendheid van een specifiek gericht enzym in de mens te beoordelen. Het doel van deze studie is dan ook om de concentratie van fumonisine B1 en diens afbraakproducten of metabolieten te bepalen in stoelgang na orale inname van dit mycotoxine via een tarwekoekje. Deze studie gebeurt in het kader van het Europees-Afrikaans LEAP-Agri project 'MycoSafe-South' (gefinancierd door de Belgian Federal Science Policy Office (BELSPO) en BIOMIN Holding GmbH) en wordt uitgevoerd door de Universiteit Gent (Vakgroep Pathobiologie, Farmacologie en Bijzondere Dieren). Met behulp van de resultaten uit deze studie kunnen de overheden in risicolanden, indien nodig, overgaan tot het nemen van extra maatregelen betreffende regelgeving, controle, preventie en vermindering van de blootstelling aan mycotoxines in de voeding, en dus bijdragen tot een verhoogde voedselveiligheid. Uw deelname aan dit onderzoek biedt een grote meerwaarde voor vele risicolanden, voornamelijk in Afrika en Zuid-Amerika, waar maïs het hoofdingrediënt is in de voeding van zowel mens als dier. Wij hopen dan ook dat we op uw medewerking kunnen rekenen en willen u daarvoor nu reeds bedanken.

2) Wat houdt deelname aan de studie in voor u?

Mycotoxines zijn stoffen die geproduceerd worden door schimmels. Ze kunnen voorkomen in plantaardige grondstoffen voor voedingsproducten zoals graangewassen. Gewassen kunnen zowel op het veld als tijdens het bewaren gecontamineerd worden met mycotoxines en deze kunnen hierdoor in de voedselketen terechtkomen. Het is aangetoond dat verschillende mycotoxines (synergistisch) de oorzaak kunnen zijn van verschillende ziekteverschijnselen bij de mens. Ze kunnen voornamelijk een gezondheidsrisico inhouden bij chronische blootstelling. Om de humane blootstelling aan fumonisines in Afrika te verminderen en de ziektevers chijnselen te voorkomen, moet dringend ingegrepen worden. Er bestaan zowel voor- als na-oogststrategieën en deze zijn belangrijke complementaire benaderingen. Het doel van deze studie is ingrijpen op de naoogst of pre-consumptiefase, teneinde de orale absorptie van fumonisine B1 te verminderen of volledig te voorkomen. Door

de werking van dit enzym in de mens na te gaan, kan men, samen met onze partners in Afrika, overgaan tot een extra preventieve stap richting het verminderen of volledig verhinderen van de blootstelling aan fumonisine B1 in de mens. Voor deze studie zijn we op zoek naar 20 Belgische vrijwilligers; 10 mannen en 10 vrouwen.

De experimentele kant van deze studie is de collectie van representatieve stalen van al uw geproduceerde stoelgang gedurende de periode van de studie. De stalen zullen onderworpen worden aan een gespecialiseerde techniek, namelijk vloeistofchromatografie gekoppeld aan massaspectrometrie om het profiel van fumonisine B1 en diens afbraakproducten in de stalen te identificeren.

3) Hoeveel patiënten zullen aan deze studie deelnemen?

Voor dit onderzoek zijn we op zoek naar 20 gezonde volwassenen (10 mannen en 10 vrouwen) tussen de 20 en 65 jaar oud in België.

4) Wat is de duur van deze studie?

De verwachte totale duur van uw deelname aan de studie is 8 dagen.

Uw deelname aan de studie omvat 3 bezoeken aan het laboratorium van Farmacologie en Toxicologie op de faculteit van de Diergeneeskunde (1 - ophalen collectie materiaal en de nodige documenten, 2 - gewicht bepaling en opname gecontamineerd tarwekoekje, 3 - deponeren van de stalen, de ingevulde documenten en het resterende collectie materiaal).

5) Wat wordt verwacht van de DEELNEMER?

Voor het welslagen van de studie, is het uitermate belangrijk dat u volledig meewerkt met de onderzoeker en dat u de instructies nauwlettend opvolgt.

Bovendien moet u onderstaande voorwaarden respecteren:

U kan **NIET** deelnemen indien u een (ernstige) aandoening heeft aan nieren, lever of galblaas en wanneer u bepaalde geneesmiddelen inneemt die een invloed hebben op de werking van de nieren, de galblaas of de lever. Alsook mag u niet zwanger zijn.

U zal gecontacteerd worden door de studiecoördinator om een tijdstip af te spreken voor het uitvoeren van de studie. De studie duurt 8 dagen. Van dag 1 t.e.m. dag 8 mag u geen maïs- of rijst bevattende producten eten. U wordt gevraagd om een voedseldagboek bij te houden tijdens deze 8 dagen. Van dag 2 t.e.m. dag 8 collecteert u een representatief staal van elk geproduceerde stoelgang. Welke producten u niet mag eten en hoe u gedurende 7 dagen de stalen moet collecteren, vindt u in de documenten die u zullen bezorgd worden bij de start van de studie. Op dag 4 komt u nuchter naar het laboratorium, wordt u gewogen en vervolgens krijgt u een tarwekoekje met een waterige oplossing met het mycotoxine fumonisine B1 en/of het enzym/placebo. De collectie van de stoelgang en het invullen van het voedseldagboek gebeurt thuis/op het werk. De stoelgangstalen en het voedseldagboek dienen afgegeven te worden op het laboratorium van Farmacologie en Toxicologie (Hoogbouw, ingang 24, 2de verdieping) in de week na afloop van de studie.

U kan weigeren deel te nemen aan deze studie en u kan zich op elk moment terugtrekken uit de studie zonder dat u hiervoor een reden moet opgeven en zonder dat dit op enigerlei wijze een invloed zal hebben.

6) WELKE procedures vinden tijdens de studie plaats?

a) Procedures:

VOEDSELDAGBOEK BIJHOUDEN

Gedurende 8 dagen (van 3 dagen voor tot 4 dagen na de dag van behandeling) noteert u alles wat u consumeert in het voedseldagboek.

STOELGANG COLLECTIE

Gedurende dag 2 tot en met dag 8 collecteert u uw stoelgang in de voorziene staalcontainers. Markeer het staal, en noteer datum en tijdstip ook telkens op 'uw schema'.

b) Studieverloop:

Indien u besluit deel te nemen aan de studie, en aan alle voorwaarden voor deelname voldoet, zal u onderstaande testen en onderzoeken doorlopen:

U zal gecontacteerd worden door de studiecoördinator om een tijdstip af te spreken voor het uitvoeren van de studie. De studie duurt 8 dagen. Van dag 1 t.e.m. dag 8 mag u geen maïs- of rijst bevattende producten eten. Van dag 2 t.e.m. dag 8 collecteert u een representatief staal van elke geproduceerde stoelgang. Welke producten u niet mag eten en hoe u gedurende 7 dagen de stalen moet collecteren, vindt u in de documenten die u zullen bezorgd worden bij de start van de studie. Op dag 4 komt u nuchter naar het laboratorium, wordt u gewogen en vervolgens krijgt u een tarwekoekje met een waterige oplossing met het mycotoxine fumonisine B1 en/of het enzym/placebo. Verder moet u een voedseldagboek bijhouden tijdens deze 8 dagen. De collectie van de stoelgang en het invullen van het voedseldagboek gebeurt thuis/op het werk en deze dienen afgegeven te worden op het laboratorium van Farmacologie en Toxicologie (Hoogbouw, ingang 24, 2de verdieping) in de week na afloop van de studie.

De stoelgangstalen zullen tijdelijk in de -20 °C diepvries bewaard worden in het laboratorium van Farmacologie en Toxicologie tot aan de verdere verwerking en analyse. Uw stalen die in het kader van deze studie worden afgenomen en geanalyseerd, zullen steeds geanonimiseerd worden na afname.

Resterende stalen worden bewaard in de prospectieve research biobank (**MYTOX**). Een biobank is een faciliteit waar menselijk lichaamsmateriaal (zoals bloed, urine, weefselstalen,…) samen met bijkomende gegevens die betrekking hebben tot dit materiaal, wordt bewaard. Uw stalen zullen worden bewaard voor een periode van 10 jaar en zullen gebruikt worden om studie-specifieke analyses op uit te voeren. Na afloop van deze periode, zullen uw stalen verder bewaard worden in de prospectieve research biobank voor toekomstig wetenschappelijk onderzoek uitsluitend in het kader van deze ziekte/pathologie of behandeling. Dergelijke nieuwe studie dient steeds ingediend en goedgekeurd te worden door het ethisch comité.

De medisch beheerder van deze biobank is Prof. Dr. Van der Straeten (directeur HIRUZ, catherine.vanderstraeten@uzgent.be). U blijft echter "eigenaar" van uw lichaamsmateriaal. Dat betekent dat u steeds kan eisen dat de biobank uw opgeslagen stalen vernietigt. U moet hiervoor contact opnemen met de bovengenoemde arts, die er dan voor zorgt dat uw opgeslagen lichaamsmateriaal wordt vernietigd.

7) Wat zijn uw rechten bij deelname aan deze studie?

De deelname aan deze studie is volledig vrijwillig, er kan op geen enkele manier sprake zijn van dwang. U kunt weigeren om deel te nemen aan de studie en u kunt zich op elk ogenblik terugtrekken uit de studie zonder dat u hiervoor een reden moet opgeven en zonder dat dit op enige wijze een invloed zal hebben op uw behandeling of de verdere relatie met de onderzoeker. Dit zal ook geen negatieve invloed hebben op de kwaliteit van de zorgen en uw verdere opvolging.

Uw deelname aan deze studie zal beëindigd worden als de medisch beheerderende arts van deze studie (Dr. Katrien Vanslambrouck) meent dat dit in uw belang is. U kan ook voortijdig uit de studie teruggetrokken worden door de onderzoeker als u de in deze informatiebrief beschreven procedures niet goed opvolgt of u de beschreven voorwaarden niet respecteert.

Indien u uit de studie gehaald wordt, zullen de reeds verzamelde geanonimiseerde gegevens in de databank blijven voor analyse, maar zal geen nieuwe data toegevoegd worden.

Deze studie werd voorafgoedgekeurd door een onafhankelijke Commissie voor Medische Ethiek verbonden aan het Universitair Ziekenhuis van Gent en de Universiteit Gent. De studie wordt uitgevoerd volgens de richtlijnen voor de goede klinische praktijk (ICH/GCP) en de verklaring van Helsinki opgesteld ter bescherming van mensen deelnemend aan klinische studies. In geen geval dient u de goedkeuring door de Commissie voor Medische Ethiek te beschouwen als een aanzet tot deelname aan deze studie.

a) Vertrouwelijkheid

In overeenstemming met de Algemene Verordening Gegevensbescherming (of GDPR) (EU) 2016/679 van 27 april 2016 (die vanaf 25 mei 2018 in voege is) en de Belgische wet van 30 juli 2018, betreffende de bescherming van natuurlijke personen in verband met de verwerking van persoonsgegevens en betreffende het vrije verkeer van die gegevens, zal uw persoonlijke levenssfeer worden gerespecteerd en kan u toegang krijgen tot de verzamelde gegevens. Elk onjuist gegeven kan op uw verzoek gecorrigeerd worden.

Uw toestemming om deel te nemen aan de studie betekent dat we gegevens van u verwerken voor het doel van de klinische studie. Deze verwerking van gegevens is wettelijk voorzien op basis van artikel 6, § 1 a en artikel 9, § 2(j) van de Algemene Verordening Gegevensbescherming.

Alle informatie die tijdens deze studie verzameld wordt, zal geanonimiseerd worden (hierbij is er totaal geen terugkoppeling meer mogelijk naar uw persoonlijk dossier). In deze studie kunnen ook gegevens verzameld worden via een vragenlijst. Daartoe zal u gevraagd worden een persoonlijk email-adres te bezorgen waarop u deze vragenlijst wenst te ontvangen. Enkel de geanonimiseerde gegevens zullen gebruikt worden voor analyse van de gegevens, en in documentatie, rapporten of publicaties (in medische tijdschriften of congressen) over de

studie. Na het afwerken van de questionnaire zal uw email adres worden verwijderd. Vertrouwelijkheid van uw gegevens wordt dus steeds gegarandeerd. Zowel persoonlijke gegevens als gegevens aangaande uw gezondheid zullen verwerkt en bewaard worden gedurende minstens 20 jaar. De verwerkingsverantwoordelijke van de gegevens is de instelling van de hoofdonderzoeker, Prof. Marthe De Boevre (UGent, FFW). Het onderzoeksteam van de lokale (hoofd)onderzoeker, Prof. Marthe De Boevre, zal toegang krijgen tot uw persoonsgegevens. In het kader van de gegevensbescherming zullen de gegevens verwerkt worden door personen behorend tot het onderzoeksteam, inclusief interne medewerkers met een niet-gezondheidszorgberoep.

De Data Protection Officer kan u desgewenst meer informatie verschaffen over de bescherming van uw persoonsgegevens. Contactgegevens: Hanne Elsen, privacy@ugent.be.

Vertegenwoordigers van de opdrachtgever, auditoren, de Commissie voor Medische Ethiek en de bevoegde overheden, allen gebonden door het beroepsgeheim, hebben rechtstreeks toegang tot uw medische dossiers om de procedures van de studie en/of de gegevens te controleren, zonder de vertrouwelijkheid te schenden. Dit kan enkel binnen de grenzen die door de betreffende wetten zijn toegestaan. Door het toestemmingsformulier, na voorafgaande uitleg, te ondertekenen, stemt u in met deze toegang.

De Belgische toezichthoudende instantie die verantwoordelijk is voor het handhaven van de wetgeving inzake gegevensbescherming is bereikbaar via onderstaande contactgegevens:

Gegevensbeschermingsautoriteit (GBA)
Drukpersstraat 35 – 1000 Brussel
Tel. +32 2 274 48 00
e-mail: contact@apd-gba.be
Website: www.gegevensbeschermingsautoriteit.be

b) Verzekering

De opdrachtgever voorziet in een vergoeding en/of medische behandeling in het geval van schade en/of letsel ten gevolge van deelname aan deze klinische studie. Voor dit doeleinde is een verzekering afgesloten met foutloze aansprakelijkheid conform de wet inzake experimenten op de menselijke persoon van 7 mei 2004 (KBC Insurance – Polisnummer W8/28963726/0100). Indien de onderzoeker van mening is dat er verband met de studie mogelijk is (er is geen verband met de studie bij schade ten gevolge van het natuurlijke verloop van de ziekte of ten gevolge van gekende bijwerkingen van de standaardbehandeling), zal hij/zij de aangifteprocedure bij de verzekering starten. Op dat ogenblik kunnen uw gegevens doorgegeven worden aan de verzekeraar. In het geval van onenigheid met de onderzoeker of met de door de verzekeringsmaatschappij aangestelde expert, en steeds wanneer u dit nodig acht, kunnen u, of in geval van overlijden uw rechthebbenden, de verzekeraar rechtstreeks in België dagvaarden (KBC Insurance NV; Professor Roger Van Overstraetenplein 2, 3000 Leuven; Tel: +32 16 24 55 81).

8) Wat zijn de risico's en verwachte voordelen bij deelname aan deze studie?

Deelname aan deze studie brengt voor u waarschijnlijk geen onmiddellijk therapeutisch voordeel. Uw deelname in de studie kan wel helpen om in de toekomst patiënten of personen met een verhoogd blootstellingsrisico beter te kunnen helpen.

De waarschijnlijkheid dat u door deelname aan deze studie enige schade ondervindt, is extreem laag. Wel is het mogelijk dat zich andere risico's en ongemakken voordoen die op dit moment nog onbekend zijn. Het is daarom van groot belang om elke nieuwe gezondheidsklacht zo snel mogelijk aan de onderzoeker te melden, ongeacht of de klacht volgens u te maken heeft met de studie of niet.

U hebt het recht op elk ogenblik vragen te stellen over de mogelijke en/of gekende risico's van deze studie. Als er in het verloop van de studie gegevens aan het licht komen die een invloed zouden kunnen hebben op uw bereidheid om te blijven deelnemen aan deze studie, zult u daarvan op de hoogte worden gebracht. Mocht u door uw deelname aan de studie toch enig nadeel ondervinden, zal u een gepaste behandeling krijgen.

9) Zijn er kosten verbonden aan de deelname aan deze studie?

Uw deelname aan deze studie brengt geen extra kosten mee voor u.

10) Is een vergoeding voorzien bij deelname aan deze studie?

Neen, er is geen monetaire vergoeding voorzien.

11) Tot wie kunt u zich richten in het geval van problemen of indien u vragen heeft?

Als er een letsel optreedt ten gevolge van de studie, of als u aanvullende informatie wenst over de studie of over uw rechten en plichten, kunt u in de loop van de studie op elk ogenblik contact opnemen met de onderzoeker of een medewerker van haar team:

Naam: Prof. Marthe De Boevre

Adres: Ottergemsesteenweg 460, 9000 Gent

Telefoonnummer: 092648117 of 047441614

TOESTEMMINGSFORMULIER VOOR DE DEELNEMERS AAN EEN EXPERIMENT

Referentienummer van de deelnemer voor deze studie	

Aankruisen door de deelnemer indien akkoord

Ik heb het document "Informatiebrief voor de deelnemers aan een experiment" pagina 1 tot en met 6 gelezen en begrepen en ik heb er een kopij van gekregen. Ik heb uitleg gekregen over de aard, het doel, de duur, de te voorziene effecten van de studie en over wat men van mij verwacht. Ik heb uitleg gekregen over de mogelijke risico's en voordelen van de studie. Men heeft me de gelegenheid en voldoende tijd gegeven om vragen te stellen over de studie en ik heb op al mijn vragen een bevredigend antwoord gekregen, ook op medische vragen.
Ik begrijp dat deelname aan de studie vrijwillig is en dat ik mij op elk ogenblik uit de studie mag terugtrekken zonder een reden voor deze beslissing op te geven en zonder dat dit op enigerlei wijze een invloed zal hebben op mijn verdere behandeling.
Ik begrijp dat auditors, vertegenwoordigers van de opdrachtgever, de Commissie voor Medische Ethiek of bevoegde overheden, mijn gegevens mogelijks willen inspecteren om de verzamelde informatie te controleren. Bovendien ben ik op de hoogte dat bepaalde gegevens doorgegeven worden aan de opdrachtgever van de studie. Te allen tijde zal mijn privacy gerespecteerd worden.
Ik ben me ervan bewust dat deze studie werd goedgekeurd door een onafhankelijke Commissie voor Medische Ethiek verbonden aan het UZ Gent en de Universiteit Gent en dat deze studie zal uitgevoerd worden volgens de richtlijnen voor de goede klinische praktijk (ICH/GCP) en de verklaring van Helsinki, opgesteld ter bescherming van mensen deelnemend aan experimenten. Deze goedkeuring was in geen geval de aanzet om te beslissen om deel te nemen aan deze studie.
Men heeft mij ingelicht dat zowel persoonlijke gegevens als gegevens aangaande mijn gezondheid worden verwerkt en bewaard gedurende minstens 20 jaar. Ik ben op de hoogte dat ik recht heb op toegang en op verbetering van deze gegevens. Aangezien deze gegevens verwerkt worden in het kader van medisch-wetenschappelijke doeleinden, begrijp ik dat de toegang tot mijn gegevens kan uitgesteld worden tot na beëindiging van het onderzoek. Indien ik toegang wil tot mijn gegevens, zal ik mij richten tot de onderzoeker die verantwoordelijk is voor de verwerking ervan.

Ik stem in om deel te nemen aan de volgende delen van de studie:

1) Ik stem ermee in om volledig samen te werken met de onderzoeker. Ik zal haar op de hoogte brengen als ik onverwachte of ongebruikelijke symptomen ervaar.	
2) Ik ben akkoord dat mijn stoelgang wordt gecollecteerd.	
3) Ik ben akkoord dat mijn ingevoerde data in de vragenlijsten worden gebruikt.	
4) Ik stem ermee in dat mijn email adres gebruikt wordt voor het versturen van vragenlijsten.	
5) Ik stem ermee in dat mijn stalen na afloop van de studie overgebracht worden naar een prospectieve research biobank voor toekomstig wetenschappelijk onderzoek uitsluitend in het kader van mijn ziekte/pathologie of behandeling. Dergelijke nieuwe studie dient steeds ingediend en goedgekeurd te worden door het ethisch comité.	

Naam en voornaam van de deelnemer	Handtekening	Datum
Naam en voornaam van de onderzoeker*	Handtekening	Datum

2 kopieën dienen te worden vervolledigd. Het origineel wordt door de onderzoeker bewaard in het ziekenhuis gedurende 20 jaar, de kopie wordt aan de deelnemer gegeven.

* Aankruisen door de onderzoeker indien akkoord

Ik verklaar de benodigde informatie inzake deze studie (de aard, het doel, en de te voorziene effecten) mondeling te hebben verstrekt evenals een exemplaar van het informatiedocument aan de deelnemer te hebben verstrekt.	
Ik bevestig dat geen enkele druk op de deelnemer is uitgeoefend om hem/haar te doen toestemmen tot deelname aan de studie en ik ben bereid om op alle eventuele bijkomende vragen te antwoorden.	

Appendix 2

Socio-demografische vragenlijst

Toelichting en instructies bij het invullen van de vragenlijst.

Deze korte vragenlijst peilt naar enkele algemene zaken. Wij hebben deze gegevens nodig om de resultaten op een juiste manier te kunnen interpreteren. De gegevens die wij met deze vragenlijst verzamelen zijn alleen ten behoeve van het wetenschappelijk onderzoek naar de afbraak en excretie van fumonisine B1 in stoelgang door een enzym bij mensen en zullen vertrouwelijk behandeld worden. Dit betekent dat alleen het onderzoeksteam dat betrokken is bij deze studie inzage in de gegevens heeft en dat deze niet aan derden worden doorgegeven. Bovendien wordt alles gecodeerd en hoeft u nergens uw naam te vermelden. Gelieve deze vragenlijst ingevuld mee te brengen samen met uw stoelgangstalen en bijhorende voedseldagboek en dit af te geven aan één van onze medewerkers op een vooraf afgesproken tijdstip.

Identificatienummer/Code: ……

Geboortedatum (dd/mm/jjjj) ……

Geslacht

- Man
- Vrouw

Lengte

……m……cm

Gewicht

……kg……g

Heeft u een ziekte of aandoening met betrekking tot … ?

- Lever
- Gal
- Nieren

Neemt u regelmatig geneesmiddelen en/of voedingssupplementen? Welke dosis?

Geneesmiddel/ Voedingssupplement	**Dosis + Frequentie (Dagelijks/ 1x/week…)**

Bent u zwanger of bestaat er een kans dat u zwanger bent?

- Ja
- Neen

Geeft u borstvoeding?

- Ja
- Neen

Volgt u momenteel een dieet waarbij bepaalde voedingsmiddelen niet mogen gegeten worden dit omwille van ziekte, allergie of zonder enige reden?

- Neen
- Ja, namelijk
    - Glutenvrij
    - Lactose-beperkt
    - Eiwit-beperkt
    - Energiebeperkt (vermageren)
    - Vet- of cholesterolarm
    - Zoutarm (hoge bloeddruk)
    - Diabetes
    - Andere …….

Welk eetpatroon volgt u?

- West-Europees
- Mediterraan
- Oosters

- Halal
- Kosjer
- Weinig vlees
- Vegetarisch
- Veganistisch
- Andere …….

Appendix 3

STOELGANG COLLECTIE

Gedurende dag 2 tot en met dag 8 collecteert u een zo representatief mogelijk staal van elke geproduceerde stoelgang in de voorziene staalcontainers. Representatief wil zeggen dat u een beetje van overal (vooraan, midden, en achteraan) uit uw stoelgang collecteert, of indien mogelijk wat mengt alvorens een staal te nemen. Noteer elk tijdstip op **'uw schema'**.

- U kunt de stoelgang opvangen met het voorziene opvangpapier (Faeces Collection Device, Fe-Col) dat u in het toilet hangt. **Plas niet** op dit papier, ander wordt het vochtig en breekt het! Leg er eventueel extra toiletpapier op voor de stevigheid, of gebruik dubbel opvangpapier (in kruisvorm).
- Na het finaliseren van het toiletbezoek, mengt u de stoelgang of neemt u van verschillende plaatsen staaltjes en vult u de voorziene stoelgang container.
- Gebruik steeds een andere container per beurt.
- Vermeld op uw schema welke container gebruikt werd bij welke beurt. Noteer duidelijk een (afgekorte) **code, datum, uur en nummer** op het potje (met een alcoholstift), alsook op uw schema van collectie en eventuele opmerkingen.
- Bewaar uw stoelgang met voorkeur in de diepvries, maar in de frigo mag ook.

MAIS- EN RIJSTVRIJ DIEET

Alle producten die sporen van maïs en rijst bevatten, mogen niet geconsumeerd worden. **Let op!** Men vindt vaak **rijstemeel** of **maïsmeel** terug in verschillende producten. Kijk goed in de **ingrediëntenlijst**! Hier volgt een opsomming van de meest voorkomende maïs en rijst producten:

Ontbijtgranen (Corn flakes, Rice Krispies etc.), gepelde en ongepelde rijst, polenta, orzo, tortilla (maïsmeel), popcorn, chips afkomstig van maïs, maïs, maïsbier, bindingsmiddelen (met maïszetmeel of rijstebloem) in sauzen, pudding, versiering op brood (tijgerbrood), lasagne, paté etc.

Wat er wel mag gegeten worden is:

Alles op basis van andere granen zoals tarwe(bloem) + vis, vlees, yoghurt, vleesvervangers, aardappelen, groenten, fruit, …

Indien per ongeluk toch iets gegeten werd met maïs of rijst, schrijft u dit bij de maaltijd in het voedseldagboek!

VOEDSELDAGBOEK

Gedurende 8 dagen (3 dagen voor tot 4 dagen na, plus de dag van behandeling) noteert u alles wat u consumeert in het voedseldagboek.

Uw schema stoelgang collectie

Code ……

DAG (MA, DI, WO, DO, VR, ZA, ZO)	DATUM STOELGANG COLLECTIE	TIJDSTIP STOELGANG COLLECTIE	GECOLLECTEERD IN CONTAINER NUMMER	OPMERKINGEN

Voedseldagboek

Dag 1

Code ……

Dag (ma, di, wo, do, vr, za, zo) + Datum ……

MAALTIJD *ONTBIJT, LUNCH, DINER, TUSSENDOORTJE, VOCHTINNAME, …*	**TIJDSTIP** *07U15, 12U00, 15U30, …*	**PRODUCTEN + HOEVEELHEID + EENHEID + TOEVOEGINGEN (KRUIDEN, SAUZEN…)** *1 TAS ~150 ML ZWARTE KOFFIE MET KLONTJE SUIKER, 2 BRUINE BOTERHAMMEN MET PINDAKAAS EN RUNDERVLEESWAREN EN MET ROOMBOTER, 200 ML VERS GEPERST FRUITSAP*	**MEDICATIE OF VOEDINGSSUPPLEMENT + OPMERKINGEN** 1 PARACETAMOL VAN 500 MG

6 General discussion and conclusion

6.1 Introduction

Crops are grown and harvested for a variety of purposes, including subsistence and profit [318]. Food and feed crops are crucial nutrient sources for humans and animals, respectively. The most important agricultural commodities that feed the world are maize, rice, and wheat [319]. These cereals are frequently contaminated with multiple mycotoxins, especially in low and middle income countries [37,320]. In African countries, maize is, among others, a key ingredient of the human and animal diet [319]. Maize and maize-based products commonly contain low to high levels of the mycotoxins aflatoxin B1 (AFB1) and fumonisin B1 (FB1). Acute or chronic exposure to these contaminated products results in mycotoxicoses in both humans and animals. For AFB1 intoxication, this entails liver damage and cancer, disrupted reproduction, affected immune function, decreased milk and egg production, and eventually (sudden) death [198,199,284,288]. Consumption of FB1 can possibly result in kidney and liver damage, oesophagus cancer, birth defects, intestinal barrier dysfunction, and death [21,55,66,234,260–262]. Both mycotoxins have also been linked to stunted growth in children by the World Health Organisation (WHO) [21,54–56]. Due to the severity of these possible consequences, intervention strategies to mitigate mycotoxin exposure are highly important. In animal feed, two detoxifiers, an aflatoxin binder and a fumonisin esterase, are commonly used. The efficacy of the possible application of these detoxifiers in human food, using appropriate *in vitro* and *in vivo* models, was evaluated in this doctoral thesis. More specifically, child models instead of adult models were chosen, as children are more susceptible to develop mycotoxin related health effects. Children have a lower detoxification capacity and have a high food intake per kg body weight, due to growth and a higher metabolism. Furthermore, the AFB1 metabolite aflatoxin M1 (AFM1) is excreted in breast milk and therefore poses an additional risk of exposure to mycotoxins in young children [44,49]. The main objectives were described in the Scientific Aims section. These aims were furthermore assessed in research studies and discussed in the chapters of this thesis. Figure 35 summarises the main findings and contributions of this doctoral thesis to the research field.

In **Chapter 1**, the efficacy of the two detoxifiers using an *in vitro* system mimicking the gastrointestinal tract of a human toddler; i.e. simulator of the human intestinal microbial ecosystem (SHIME®), is reported. An UHPLC-MS/MS method was successfully developed and

validated to determine AFB1, FB1, pHFB1a, pHFB1b, and HFB1 concentrations in SHIME samples. This method can be valuable for future research studying the fate of these particular mycotoxins with or without detoxifiers in a SHIME system. Moreover, the environmental conditions in this gut model system can be adapted to mimic other intestinal environments, such as that of adults, elderly, or suboptimal conditions. Furthermore, the effect of these detoxifiers on the microbiota of the large intestine was monitored and evaluated. Short-chain fatty acid (SCFA) concentrations were determined and colonic bacterial communities were analysed. The AFB1 and FB1 concentrations significantly decreased following the addition of the detoxifiers to the system. Complete hydrolysis of FB1 to HFB1 was observed and our study provides strong evidence that this effect was attributed to the enzyme. Furthermore, no significant changes were observed in the bacterial composition and its main metabolites (SCFA), indicating a healthy gut microbiota was maintained. Therefore, the effect of these detoxifiers in reducing human exposure to these mycotoxins seems promising without disrupting the human intestinal microbial environment.

Bentonite clay has already been shown to be effective in reducing aflatoxin exposure in adult humans through the simple consumption of a bentonite suspension in a glass of water [123] and capsule ingestion [121] with the meal. Therefore, **Chapter 2** reports the efficacy of fumonisin esterase using two different administration modes, compared using an *in vivo* pig model. An attractive and popular dosage form for drug and dietary supplement administration in humans consists of capsule ingestion. Capsules can come in various sizes, shapes, and colours. They can also be opened, and the powder from within can be sprinkled on food. Thus, the efficacy of the enzyme was investigated following intraoral (in-feed analogue) and intragastric (capsule analogue) administration. Although a significantly higher Sa/So ratio was shown in the placebo group compared to both administration modes of the esterase (demonstrating the efficacy of the esterase), the efficacy of the enzyme when administered intragastrically was not reflected in the FB1, HFB1, pHFB1a, and pHFB1b levels in serum and faecal samples, while this was observed in the intraoral group. Based on these results, capsule ingestion of the enzyme for human use cannot be recommended; it can be advised to thoroughly mix fumonisin esterase in the food prior to consumption.

Based on the results found in **Chapters 1 and 2**, the efficacy of the detoxifiers seems promising to reduce AFB1 and FB1 exposure in humans when mixed with food prior to

consumption (Figure 35). Therefore, in **Chapter 3**, a cross-over study design was elaborated to evaluate the efficacy of the detoxifiers in a pilot human intervention study in Kenya, followed by a parallel design in South Africa and Belgium. In the latter, only fumonisin esterase was to be evaluated. Due to complications regarding COVID-19 and the legal status or categorisation of fumonisin esterase, none of these intervention studies were performed. Alternatively, a dose titration study was performed to determine the single FB1 dose (1 or 2 µg/kg BW) necessary to quantify FB1 or its metabolites (pHFB1a, pHFB1b, and HFB1) in faeces for future efficacy studies including fumonisin esterase. Based on these results, a dose of 2 µg FB1/kg BW is advised for such efficacy studies. Furthermore, HFB1 was not detected in any samples, possibly making this metabolite a relevant biomarker in human faeces to investigate fumonisin esterase hydrolysis efficacy after a single FB1-dose exposure.

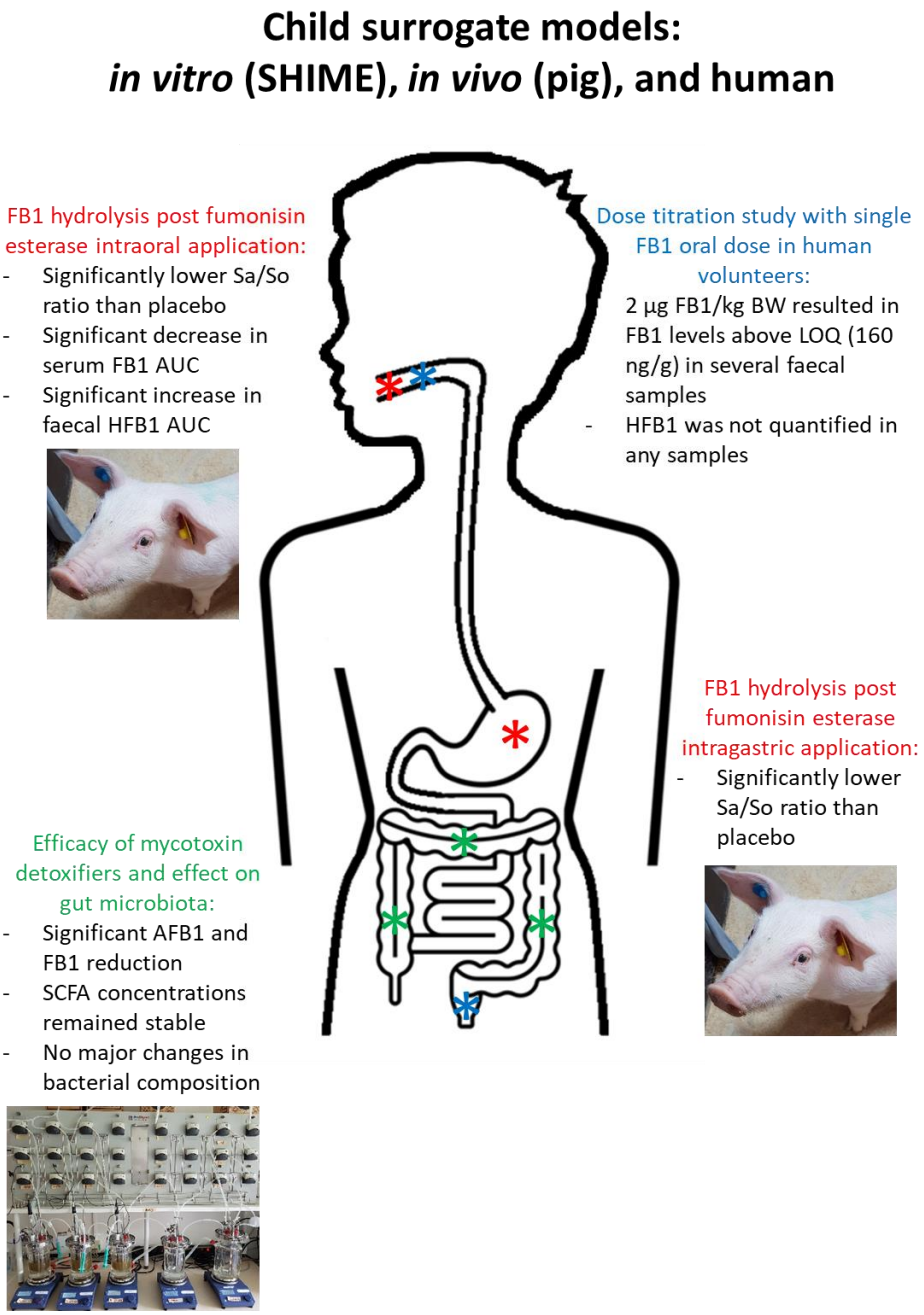


Figure 35. Overview of the objectives and results obtained in this doctoral thesis. The efficacy and effect on gut microbiota of two mycotoxin detoxifiers (aflatoxin binder and fumonisin esterase) were assessed in a human *in vitro* (SHIME) model. The efficacy of two application modes (intraoral and intragastric) of fumonisin esterase was determined and compared in an *in vivo* piglet model. A single oral FB1 dose for quantification in faecal samples was evaluated in humans. The mycotoxins aflatoxin B1 (AFB1) and fumonisin B1 (FB1) were included. Efficacy of the mycotoxin detoxifiers was investigated by analysing AFB1, FB1, sphinganine/sphingosine (Sa/So) ratio, hydrolysed FB1 (HFB1), and partially hydrolysed FB1 (pHFB1a and pHFB1b). The coloured asterisks represent the administration site of the mycotoxins or detoxifiers, or the points of interest investigated in this thesis.

To place the performed research studies in a broader context, a discussion is provided subsequently, where the use of the specific *in vitro* and *in vivo* models employed in this doctoral research are elaborated in more detail. Furthermore, light is shed on the possible legal classifications of fumonisin esterase for human consumption.

6.2 Why employ human *in vitro* models and how applicable is the SHIME as human gut model?

In Europe, the French philosopher, mathematician, and scientist, Descartes (1596-1650), declared animals to be without minds, souls, and consciousness. Therefore, he concluded animals lack the ability to feel pain [321]. As a result, for research purposes, many vivisections on animals were performed throughout Europe without anaesthesia. In 1959, Russell and Burch introduced the principle of the 3Rs, which started out as an ethical response to inhumane animal experiments; to improve animal welfare by minimising cruelty:

- Replacement: the substitution of animals in experiments by alternative methods, strategies, or approaches, e.g. *in vitro* simulation systems (applied in studies reported in **Chapter 1**, i.e. the SHIME).
- Reduction: the minimisation of the number of live animals used in trials to achieve the same objective. Optimisation of the number of animals needed for statistical significance and relevance through power analysis is mandatory (power analysis was performed for the experimental work described in **Chapter 2**).
- Refinement: the minimisation of the incidence and severity of inhumane procedures on animals to minimise pain and distress, as well as the maximisation of animal welfare, e.g. enhancements in animal care, housing, handling, and training (applied in the trial performed in **Chapter 2**).

Throughout the world, this principle has been adopted as ethical framework for improving laboratory animal welfare. It is incorporated in legislation (EU Animal Welfare Directive 2010/63/EU on the protection of animals used for scientific purposes [322]) and is set in the standards of ethical committees for animal experiments; guaranteeing the quality of the trials, preventing suffering, and optimising the use of animals. Nowadays, the 3R principle is not only applied in research from an ethical point of view; the advantages

regarding the opportunity to deliver faster, more reproducible, safer, and more cost-effective results have become evident [321].

Throughout the years, simple and more complex *in vitro* gastrointestinal systems have been developed, widely applied, and constantly improved, striving towards a model mimicking *in vivo* situations as close as possible. To test the efficacy of mycotoxin detoxifiers and their effect on the human gut microbiota, *in vitro* testing was necessary prior to *in vivo* human testing. This has been done in this work using a complex dynamic gastrointestinal tract (GIT) model (**Chapter 1**). Hence, in that chapter, replacement was employed using an *in vitro* gut simulation. The term "*in vitro*" is literally translated from Latin to "in glass". This refers to studies being performed in a test tube rather than in a living organism [323]. It allows research to focus on parts of an organism rather than the whole. However, humans and animals are more complex than what is possible to study in test tubes. Yet, *in vitro* experiments are crucial in research to make scientific progress; they are not a perfect imitation of reality, although they may answer many scientific questions. The mentioned advantages of *in vitro* testing weigh up against live animal or human testing. Furthermore, samples can be obtained in a non-invasive manner.

The choice of the *in vitro* model depends on the study objectives, the advantages and limitations of the experimental systems [324]. The simplest *in vitro* monolayer of an intestinal epithelial cell-line can be sufficient to study cellular processes after exposure to bacteria, food, or drugs. However, when feedback mechanisms need to be studied, this model no longer suffices [325]. To evaluate the efficacy and effect on gut microbiota of two mycotoxin detoxifying animal feed additives, a dynamic *in vitro* simulator of the human intestinal microbial ecosystem (SHIME), is indispensable. A situation closer to reality, where several mycotoxins co-contaminate cereal-based foods, the consideration of the gut dynamic flow, and the effect on the microbiota, provides more information. In the literature, there is not much data available on the effect of the detoxifiers nor of the studied mycotoxins on human gut microbiota. Therefore, the experiment performed in this thesis provided data to start to fill this gap in research.

In vitro gut fermentation models are invaluable in assessing the effect of various substances (dietary ingredients, pathogens, drugs, toxic, or radioactive compounds) on the gastrointestinal environment and microbial composition and vice versa [325]. These models

can vary from static to dynamic systems [326,327]. The former is the simplest and most frequently used model to study gut microbiota within a very limited time frame as an initial screening. It consists of a vessel or test tube, in which specific strains and animal or human microbial communities are added to investigate their metabolism capacity. However, these are far from physiological; the mixing of digestive juices and chymus occurs in a single compartment, and there is accumulation of microbial metabolites, inhibiting further microbial activity [327,328]. The latter is more complex and realistic, with representative environmental conditions; several GIT compartments are connected, and secretion of digestive juices is gradual [327,329]. In the review of O'Farrell et al. [330], several multi-compartmental *in vitro* systems replicating the colonic environment are summarised and compared.

The original multi-compartmental SHIME system was created at Ghent University [331] and has evolved and improved to a technology-driven system [213,332,333]. The validated model [334], used to study microbiota at different digestive levels, has been used in several studies experimenting on anti-, pre-, and probiotics [212,335,336]. Its strength lies in the semi-continuous fermenters, which intermittently add nutrients and remove suspended microbiota, to maintain the inoculated intestinal microbiota [325]. Additional to this study, this system has been used to determine the impact of another mycotoxin, ochratoxin A, on the intestinal microbiota and the occurrence of biotransformation in the gut [208]. The purpose of adopting this system is primarily to study the interactions and influence of additives on the gut microbiota [334]. A variety of additives (e.g. drugs or toxins) can effortlessly be added. Therefore, AFB1 and FB1 were easy to administer to the system. Moreover, the real-life situation of Africa was mimicked by adding the mycotoxins to the food of the system.

A possible critique of this system is that food was provided every 8 h, which creates an unrealistic situation; no individual consistently eats every 8 h. However, this is to ensure stability and reproducibility of the results, and is rather an advantage compared to *in vivo* where standardisation of meal consumption to a similar situation is not possible. This time period for the addition of more nutrients is derived from the validated model to keep a stable and healthy gut microbiota [337]. Furthermore, as single mycotoxin contamination of food is not the norm, but rather an exception, both AFB1 and FB1 and their respective detoxifiers were added together in the SHIME system. Thus, the individual effect of each compound on

the gut microbiota might be disguised. As mentioned previously in this thesis, multi-mycotoxin exposure can result in combined toxic effects, such as additive, synergistic, or antagonistic effects. Additionally, an interference of the adsorbing effect of the binder for AFs due to the presence of FUMs is possible; FUMs may be adsorbed by the binder. Thus, the presence of the binder could possibly decrease the FB1 concentration more than it would with solely the enzyme. However, the HFB1 concentration was also determined. Therefore, it was clear in this study that the enzyme was efficient and hydrolysed FB1. In a study performed by Solfrizzo et al. [338], certain adsorbent materials (1 mg/mL) were tested *in vitro* on their FB1 adsorbing capacity. Bentonite adsorbed only 12% of the toxin in a solution containing 13 µg/mL FB1, whereas cholestyramine and activated carbon each adsorbed 85% and 62%, respectively, in a solution containing 200 µg/mL FB1. In an annual report to the Texas corn producers board of 2012 [339], it is mentioned that activated carbon and bentonites have been used in animal feeding studies, and that they do not effectively reduce fumonisin toxicity. However, there are no references cited for these animal feeding studies. Furthermore, in this report it is mentioned that bentonite was not effective in adsorbing FB1 from aqueous corn meal, nor in water at pH 7, but it was effective in water at pH 4. *In vitro* sodium bentonite (SB) showed strong adsorption of FB1, competing with AFB1, at pH 2 [340,341]. Although, a pH increase resulted in the release of FB1 from the adsorbent. Possibly, the acidic pH protonates FUMs to result in a cationic form that adsorbs to bentonites [339]. In a study performed by Miazzo et al. [342], SB (0.3%) was incorporated in broiler feed during a month containing a combination of 2.5 mg/kg AFB1 and 200 mg/kg FB1. Somehow FB1 interfered with the activity of SB on AFB1 adsorption. The addition of SB to the diet containing AFB1 alone diminished the toxic effect of AFB1 on the relative weights of several organs (liver, kidney, and spleen). However, SB in diets with AFB1 plus FB1 only decreased the weights of the livers. Consequently, it is possible that the binder adsorbed FUMs in the stomach compartment, but they may have been released further in the GIT.

An advantage of this system is that it can simulate a range of species-specific intestinal environments, including that of infants, adults, elderly, specific suboptimal conditions (pathogen infection), as well as that of animals (pig, dog) [333]. For each case, based on the research performed by Minekus et al. (2014) [343], the settings of the system are adjusted to result in the accurate and relevant simulation of the targeted situation; microbial inocula,

residence time per compartment, gastric juice composition, region specific pH, food/feed, feeding timing, and temperature are adjusted.

For each environment, a fermentation profile can be determined per colon region (ascending, transverse, or descending colon). Furthermore, the location of the effect of the added substance on the colonic microbiota can be traced. A disadvantage of this system is that each individual has a specific intestinal microbiota and different people may respond differently to a given treatment. Likewise, faecal microbial composition fluctuates in time due to diet and medication. Therefore, in this study, the SHIME system was inoculated with faeces originating from a child that had not received any medication during the 6 months prior to faeces collection. To obtain a representative human gut microbial composition, pooling different stool samples originating from several individuals seems favourable compared to collecting a sample from one individual [344]. However, pooling causes competition for niches, the microbiota are less stable, and tend not to implant correctly, or at least need more time, needing an extended stabilisation period. Finally, the colonised microbiome resembles that of a single individual [333]. In this system, samples were always taken at a fixed time point prior to a feeding session; important for reproducibility, and bacteria are more active when they receive fresh nutrients, and the SCFA levels are diluted when new SCFA-free nutritional medium and pancreatic juice enter the system.

Colonic microbiota cannot be directly collected from living patients without serious ethical constraints. Therefore, the next best option, is to collect faecal microbiota from an individual presenting the desired intestinal environment to study. However, faecal microbiota significantly differ from *in vivo* gut composition, and therefore dynamic multi-compartment systems were developed [333]. Yet, faecal microbiota can be described as colon microbiota that have undergone community and metabolic changes during passage to the rectum. In a static system, the inoculated faecal microbiota would not adapt nor survive for long, as *in vivo* environmental parameters, such as pH, available nutrients, and microbial dynamics constantly change [333]. Therefore, a suitable adaptation and stabilisation period of two weeks is implemented in the SHIME runs, to allow faecal inoculum to adapt (from *in vivo*) towards a stable *in vitro* microbiota, which is representative for the different colon regions. Upon stabilisation, when treatment is applied, the effects observed are due to the treatment and not due to adaptation of the microbiota to the new environment. Following this stabilisation

period, the SCFA production reaches a steady-state. Practically, a wash-out period can also be implemented in the run to observe if the effect of the treatment is reversible [333]. Noteworthy to mention is that the microbiota obtained *in vitro* are stable and resemble *in vivo* conditions. However, both situations are not identical, a shift, mainly a decrease in butyrate-producing bacteria is present [213].

The food administered to the system in this research, also known as the growth medium, was specifically formulated with ingredients (such as resistant starch) to support the colonic microbiota fermentation. Furthermore, pancreatic juice, bile salts, and sodium bicarbonate are added to simulate the *in vivo* situation of the digestive system [343]. In our experiments, both AFB1 and FB1, and both detoxifiers, were mixed in the mouth compartment, to simulate applicable circumstances. Another possibility, which can be applied in future experiments on the SHIME model, is to separate the mycotoxins from the detoxifiers, and add them together at a later stage of the GIT, e.g. the gastric compartment (as performed in the piglet trial of **Chapter 2**). An advantage of the SHIME system is that part of the orogastrointestinal tract in which the substances are brought together can be selected. Therefore, the study performed in Chapter 2 could also be applied in the SHIME format, as a human *in vitro* model.

A more advanced system, or an extended SHIME system, is the TWINSHIME®. It consists of two SHIME systems in parallel, used to investigate different compounds at the same time [333]. This system could have been applied to our experiment. A parallel placebo-controlled (with calcium carbonate and maltodextrin) run next to a treatment run could have been performed, so both systems would undergo similar disturbances and conditions. Even though in the performed SHIME experiments in this research, no placebos were added, the situation prior to the administration of the detoxifier was considered a control. The SHIME is a simplified environment and only short-term experiments can be conducted.

In a SHIME experiment performed for this research (not reported in the results of this thesis), mould had grown and plugged one of the transit tubes connecting the vessels, resulting in failure of the experiment. This experiment was meant to last four weeks, additional to the 2 weeks of stabilisation period. Therefore, the following two experiments had been restricted in time; two weeks of treatment were implemented (reported in this thesis in Chapter 1). A possibility for a future study would be to compare a candidate detoxifier

to a placebo in a TWINSHIME experimental setup. Another solution would be to replace the tubes during the experiment to prevent plug formation.

A disadvantage of the SHIME system in general, is the lack of gastrointestinal absorption and further metabolism processes that would be present in the body. Therefore, there is no SCFA absorption, nor any possible mycotoxin metabolism by the liver after absorption. However, in this research, we wanted to determine the efficacy of the detoxifiers, which is not hindered by the lack of absorption of the mycotoxins. Furthermore, the SCFAs produced are passed on from the AC, to the TC, and to the DC, which allows quantification of their production by the microbiota. Additionally, this system does not allow culture of the fraction of microorganisms which adhere to the gut mucosa (mucus layer). Therefore, the microbial community is limited to the luminal one, which do not adhere to the gut wall. An alternative system that enables colonisation of mucosal microbiota is available with the M-SHIME technology [327]. Mucin-covered microcosms are added in the colon vessels allowing the culture of both luminal and mucus-associated bacteria, leading to more *in vivo*-like microbial communities.

6.3 How applicable is the piglet as human child model?

Although *in vitro* testing has many advantages, it cannot fully simulate the complexity of living organisms, including gut absorption. Therefore, it cannot completely replace *in vivo* studies. However, performing studies on humans in general require ethical trade-offs between the pros and cons. The most important condition in research when using human participants is that the benefits provided by the generated results must weigh up against the potential harms during research. Performing intervention studies using children is even more delicate than including adults. Especially the ethical issue of consent is an important point of debate when children are included in research [345]. Consent consists of four key aspects; 1) explicit act is necessary (as a verbal or written agreement), 2) being informed about and understanding of the research, 3) participation must be voluntary, and 4) it must be revocable, i.e. participants may withdraw at any time during the research. Due to the ethical difficulties in doing research on children, using a suitable paediatric surrogate animal model is more acceptable and therefore invaluable [346]. Rodents are a frequently used model for humans, and studies using this animal as model dominate literature. However, these animals and their

GIT microbiota are hardly comparable to humans; the development and function of the GIT vary greatly from humans [347]. Therefore, in the pharmaceutical industry, also dogs and non-human primates are used for drug development and testing [274]. Still, even these models are less than ideal; dogs differ substantially from humans in organ dimensions and GIT physiology, and the use of non-human primates is considered unethical. An animal with better anatomical, physiological, and biochemical similarity to humans is thus preferable. The pig has been put forward as a suitable animal model [274,348–350], in which absorption, distribution, metabolism, and excretion (ADME) processes can be studied. To date, several studies have described the many similarities between man and pig both morphologically and physiologically. Pigs have been described to be suitable for human research domains regarding cardiovascular, pulmonary, GIT, renal, immunological, metabolic, embryological, and integumentary systems [274,347,351,352]. In the case of this research, especially the digestive system of the pig which resembles man very closely, regarding GIT anatomy, physiology, biochemistry, and pathology, is of interest [347]. Therefore, strong evidence is provided that the pig is invaluable as animal model for human research. Furthermore, the use of piglets as paediatric model for oral pharmacokinetic (PK) and pharmacodynamic (PD) drug testing seems promising [274,353].

Young animals and infants are more vulnerable compared to adults when it comes to mycotoxin exposure due to metabolic and physiologic immaturity and a relatively higher feed and food intake [48]. Piglets of 3 to 4 weeks of age are comparable to infants of 4 months to 2 years regarding PK parameters [274]. This consists of the vulnerable weaning period when the young are switched from fluid to solid foods or feeds [50]. This period marks a change from a high-fat and low-carbohydrate milk diet to a low-fat and high-carbohydrate diet [155]. Additionally, this dietary shift disrupts the intestinal barrier [50]. This makes piglets prone to post-weaning enteric disorders, and also systemic diseases. Furthermore, a leaky gut leads to increased intestinal permeability [354,355]. In human infants, increased gut permeability is equally described [356]. Therefore, the weaning period marks an important vulnerable period of the GIT to exposure to consumed mycotoxins [47].

Furthermore, age differences in toxicokinetics (TK) of *Fusarium* produced mycotoxins, in particular the modified forms of deoxynivalenol (DON) and zearalenone (ZEN), DON-3-glucoside (DON3G) and zearalenone-14-glucoside (ZEN14G), respectively, have been

demonstrated in piglets following oral exposure [47,353]. A significantly higher absorption of DON3G and ZEN14G fractions was observed in weaned piglets (4 weeks old) compared to older piglets (11 and 8 weeks for DON3G and ZEN14G, respectively), resulting in a higher exposure to these modified mycotoxins. The higher gut permeability in weaned piglets was put forward as causative factor [47,353]. Furthermore, a higher water/fat body ratio and a longer gastrointestinal transit time in weaned piglets were noted to explain other TK differences, such as higher maximum blood concentration and longer elimination half-life, compared to older piglets. These age-related differences could be extrapolated to young human children as piglets have been put forward as suitable human paediatric surrogate model [357]. Therefore, the piglet is an interesting model to use as an alternative for human child testing.

6.4 Fumonisin esterase for human consumption: which legal classification?

Depending on the intended use, the function, and the location of the effect (in food or in the body), a product for human consumption can be defined as a **technological food additive**, a **food supplement**, or a **medicinal product**. Yet, in some cases, the distinction between the legal classifications is subtle and difficult to establish. Fumonisin esterase is one of these cases, where it must be clarified what the function is and where its action takes place. Because of the non-existent legal classification of fumonisin esterase for human consumption, it was impossible to perform intervention studies in humans using this enzyme. Therefore, below, a discussion follows about the possible classification of this specific product and what this could imply.

Technological food additive

In animals, the safety and efficacy of fumonisin esterase in all species has been established by categorising this product as a EFSA-evaluated and EU-approved "technological feed additive" in animal nutrition [300,358–361]. It is designated to the functional group "substances for the reduction of the contamination of feed by mycotoxins", with intended use to reduce the contamination of feed by fumonisins in complete feeds (for pigs and poultry) and in fermenting maize-based silages (for all animal species) [300,358–361]. In this case, it is clearly defined what the intended use and function is, and where the action of the product is

meant to take place. A similar explicit purpose must be determined for the use of this enzyme in human foods or for human consumption.

When extrapolating this product from animal to human consumption, it could be assumed it would be classified as a food additive. The authorisation procedure, namely the rules on **food additives**; definitions, conditions of use, labelling, and procedures, are described in Regulation (EC) No 1333/2008 [362]. In this regulation, a food additive is defined as "any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food, whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods." In summary, food additives are defined as substances that are not normally consumed as food themselves but are intentionally added to food for a technological purpose. Their use must be safe, supported by a technological need, must not mislead the consumer, and must present benefit to the consumer. The EU legislation describes 26 technological purposes or functional classes (summed up in Annex I of EC No 1333/2008 [362]).

At a first glance, fumonisin esterase meets the aforementioned criteria; it is not a "food" itself and is added to food with the intention of reducing exposure to fumonisins. However, it must be clarified where the enzyme performs its detoxifying effect. Based on the regulation, it can be deduced that for food additives the effect of the enzyme would have to be completed in the food, prior to consumption, as a food additive "or its by-products become directly or indirectly a component of such foods." However, this is not specified and could depend on one's perspective. This is in contrast with the assumption that the enzyme needs to come in contact with saliva to become activated and perform its effect orally.

When taking a closer look at the criterium of "a technological need", a peculiarity is that unlike in the regulation on feed additives, there is no mention of the functional group "substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action" in the technological category [363]. Therefore, this could be a reason to reject this enzyme to be classified as a food additive. The one functional group listed in the food additives that could be applied to fumonisin esterase is "preservatives" in the broad definition. These

are “substances which prolong the shelf-life of foods by protecting them against deterioration caused by micro-organisms and/or which protect against growth of pathogenic micro-organisms.” Yet mycotoxins are not ‘micro-organisms’, but they are toxins produced by fungal micro-organisms. Furthermore, it can be debated if fumonisin esterase prolongs shelf-life.

Interestingly, there are many more described functional groups for feed additives than the 26 for food additives. Feed additives that have a specific effect on the feed or animals directly are divided into 5 categories (technological additives, sensory additives, nutritional additives, zootechnical additives, and coccidiostats and histomonostats), which are further subclassified functional/additive groups. In contrast, the list of food additives is much more restricted, and there is no mention of having a possible effect on humans directly. There is a need for expanding the list of functional groups, or at least the inclusion of a specific category concerning reducing mycotoxins in foods for human consumption.

Moreover, in the regulation on food additives, it is mentioned that “food enzymes should not fall within the scope of that regulation.” Therefore, food enzymes is a particular approach worth investigating as fumonisin esterase is an enzyme that could be added to food. Before 2008, food enzymes, other than those used as food additives, were not regulated at EU level, or regulated as processing aids under the legislation of Member States. **Processing aids** can be defined as “substances not consumed as food itself but used intentionally in the processing of (raw) foods (with technological purpose during treatment or processing), which only remain as (technically unavoidable) residues (or its’ derivatives) in the final food, and which do not have a technological effect in the final product (and do not present any health risk)”. If the purpose of fumonisin esterase was to treat food during processing and no longer have an effect by the time food is ready for consumption, it could be classified as a processing aid. However, many enzymes are sensitive to temperature and pH changes, and therefore susceptible to denaturation during food processing. According to EFSA and the manufacturer, this enzyme, which is a protein, will be degraded in the gastrointestinal tract of animals [185]. Therefore, the risk of denaturation during food processing, except for the washing procedure, before complete detoxification, is legitimate. The laws regarding **food enzymes** are listed in Regulation No 1332/2008 [364]. This regulation covers enzymes that are added to food to perform a technological function in the manufacture, processing, preparation, treatment, packaging, transport, or storage of such food, including enzymes used as processing aids. This

scope does not cover enzymes intended for nutritional (vitamins and minerals) or digestive purposes. Furthermore, enzymes used exclusively in the production of food additives (such as amylolytic enzymes to modify starch) fall under the regulation EC No 1333/2008 on food additives. However, these food enzymes are used as such in food, they are covered by Regulation No 1332/2008. A food enzyme will be included in the official EU list of approved food enzymes if it does not pose any health concerns to the consumer (EFSA evaluates the safety); if there is a technological need for its use, and if its use does not mislead consumers. So far, classification of fumonisin esterase as a food enzyme seems promising. A food enzyme is legally defined as "a product obtained from plants, animals, or micro-organisms, or products thereof, including a product obtained by a fermentation process using micro-organisms:

a) containing one or more enzymes capable of catalysing a specific biochemical reaction; and
b) added to food for a technological purpose at any stage of the manufacturing, processing, preparation, treatment, packaging, transport, or storage of foods".

The esterase in FUM*zyme*® was initially identified and isolated from a soil bacterium, *Sphingopyxis* sp. MTA144 [182,184,185]. Therefore, fumonisin esterase complies to the definition of "a product obtained from micro-organisms". Yet, the genes encoding the enzyme were then used to transform the yeast *Komagataella pastoris* (previously known as *Pichia pastoris*) into a fumonisin esterase secreting recombinant strain (*K. pastoris* DSM 26643) [184,185]. Yeasts are single-celled micro-organisms and members of the fungus kingdom. However, currently, there is no list of EU authorised food enzymes, but a list of applications considered for inclusion is available [365]. Evaluations for market authorisation are ongoing. In this preliminary register, a trypsin produced by *Fusarium venenatum* and a phospholipase C produced by *Pichia pastoris* are listed. This same yeast, albeit from a different strain, was genetically transformed into a fumonisin esterase secreting recombinant strain [184,185]. The registration of fumonisin esterase as food enzyme seems conceivable if, again, it is used to treat the food prior to ingestion. Presently, esterases are used in the food and alcoholic beverage industry as food (microbial) enzymes with the following purposes: enhancement of flavour and fragrance in fruit juice, de-esterification of dietary fibre, and production of flavour esters [366].

Food supplement

Laws on **food supplements** are outlined in the Directive 2002/46/EC [367] and defined as "foodstuffs the purpose of which are to supplement the normal diet and which are concentrated sources of nutrients (i.e. vitamins, minerals) or other substances with a nutritional or physiological effect (i.e. amino acids, essential fatty acids, fibre, various plant, and herbal extracts), alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills, and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities."

One could argue that fumonisin esterase is indeed a supplement to the normal diet, designated to be consumed in small quantities, and could be packaged in a dose form mentioned in the directive. However, the substance itself does not have any nutritional or physiological effects. Yet, the consequence of adding this product to food could result in a difference in physiological effect, namely preventing symptoms of fumonisin intoxication. The product would have to comply with certain criteria listed in the directive, but which would be difficult to establish, such as 'the percentage of the reference intake values'. There are no known reference intake values for fumonisin esterase. In summary, supplements are meant to correct nutritional deficiencies, maintain adequate intake of certain nutrients, or support physiological functions. Furthermore, it is specified that food supplements are not medicinal products, and thus they cannot exert pharmacological, immunological or metabolic action, and their use is not intended to treat or prevent disease in humans or modify physiological functions.

Novel food

Personal communication with the Health, Innovation, and Research Institute - Clinical Trials Unit (HIRUZ-CTU) (19/05/2021) confirmed the complexity of the matter. The institute informed that enzymes cannot be classified as a food supplement under the **novel food legislation**, as they need to first meet the criteria of a food supplement (be safe for consumption and have a technological function). Further on, the R&D Department of the Federal Agency for Medicines and Health Products (FAMHP) (22/09/2021) informed that provided the action of fumonisin esterase takes place post-consumption, it could be possible

to register it under the novel food legislation. This relatively new Regulation (EU) 2015/2283 [368] defines novel food as "food that was not used for human consumption to a significant degree within the Union before 15 May 1997, irrespective of the dates of accession of Member States to the Union, and that falls under at least one of the (ten) following categories". The category of interest for fumonisin esterase is "food consisting of, isolated from, or produced from micro-organisms, fungi or algae." This legislation seems to be the best chance for fumonisin esterase to be added to food, if it is accepted as safe for consumers, the foodstuff properly labelled (as not to mislead the consumers), and not nutritionally disadvantageous for the consumer. If a new technological need concerning "mycotoxin reduction" in food is added to the list, the enzyme could be classified under Regulation (EC) No 1333/2008 for technological food additives instead of under the novel food legislation.

Medicinal product

Another possibility, which would be relevant in case the enzyme is still active in the orogastrointestinal tract, is that it could be seen as a **medicinal product** for human use. A medicinal product is defined by the European Medicines Agency (EMA) as "a substance (of human, animal, vegetable, or chemical origin) or combination of substances that is intended to treat, prevent or diagnose a disease, or to restore, correct or modify physiological functions by exerting a pharmacological, immunological or metabolic action." The legal framework for the authorisation, manufacturing, and distribution of human medicines in the EU is described in Directive 2001/83/EC [369]. Toxins and micro-organisms are specified as substances of animal and vegetable origin. Therefore, as the esterase was initially identified and isolated from a soil bacterium, fumonisin esterase could be considered a medicinal product. However, the process of drug development to marketing is very expensive, with several clinical phases and procedures, and can take up to 15 years [370].

6.5 Future perspectives

To tackle the mycotoxin problem and in particular AFB1 and FB1 in sub-Saharan Africa, there is a need for more data collection and public access to its reporting. Especially for sub-Saharan Africa and for FB1, little occurrence data is available in the literature. For example, in the study performed by Gruber-Dorninger et al. [371], only 208 samples were collected in sub-Saharan Africa and analysed, compared to the 1,000 to 21,000 samples collected in all other parts of the world. Yet, 72.6% of the samples originating from sub-Saharan Africa were positive for fumonisins. The first step in the direction of mycotoxin mitigation is to **identify the extent of the mycotoxin problem**.

The importance of the health effects associated with mycotoxins in both humans and animals is being more and more acknowledged; there is a worldwide increase in several country regulations implemented for maximum allowed mycotoxin contamination levels in foods/feeds or guidance values thereof [191]. However, in African countries legislative regulations are generally limited to aflatoxins and few other mycotoxins in certain foods, while in some countries legislations are entirely lacking [191,372]. This is not entirely surprising, as the establishment of mycotoxin regulations requires sufficient occurrence data, as well as certain high standard facilities. This includes, among others, availability to toxicological data, exposure data, analytical facilities and methods, and experienced personnel for operation [191]. In South Africa, high exposure to fumonisins through maize consumption has been shown in several studies during many years [134,191,373,374]. Yet, the country has only recently incorporated the set limits of the Codex Alimentarius into its own legislations [112]. The second step is to **set national mycotoxin limits and implement them**.

Setting mycotoxin limits in legislation is one thing, implementing them is another. Sub-Saharan Africa has a large subsistence farming population [37,40,43]. Where food is commercialised, safety has been assessed according to the national regulations. However, where people consume home-grown maize, these food products are not subjected to any inspection nor analysis prior to consumption. For example, in the region of the Eastern Cape Province of South Africa, the difference in fumonisin contamination in maize flour is believed to be due to the commercial versus traditional milling, where in the latter, reduction of the high fumonisin contamination in home-grown maize is insufficient [112]. Therefore, many

people remain vulnerable to exposure with high mycotoxin levels. **Identify**, **reach** and **protect people at risk** are key factors to be addressed.

To **identify** people at risk, reliable and validated biomarkers for exposure and effect for AFB1 and FB1 in easy-to-collect human biological samples can be advantageous. Further research on these biomarkers is needed to reach a consensus on which ones are sensitive and trustworthy in determining acute and chronic mycotoxin exposure and effect. Urine and faeces seem like the most straightforward and practical samples to collect without the intervention of medically trained personnel.

The seemingly most promising human biomarkers for fumonisin exposure and effect are those related to the disruption of *de novo* sphingolipid biosynthesis. Elevated levels of Sa or Sa/So ratio have been extensively investigated and observed in a variety of animal species (horses, pigs, rodents, trouts, monkeys, rabbits, ducks, and chickens) following FB1 exposure [143,260]. This biomarker is commonly regarded as a validated biomarker for fumonisin exposure [143]. However, an increase in the Sa levels or in the Sa/So ratio in blood or urine have been difficult to correlate with fumonisin exposure in humans. These sphingoid bases are naturally present in blood and urine of healthy individuals, and their normal ranges vary widely and change over time [143–145]. Furthermore, the change in serum ratio occurs in a FB1 dose-dependent manner; the lower exposures to fumonisins in practice compared to the higher experimentally-induced exposures, can make the detection of changes in Sa or Sa/So ratio more challenging in live situations. Previous studies analysing human-derived samples have determined FB1 and Sa/So in urine [39,131,268,375] and Sa/So in plasma [375]. Although a difference in the urinary Sa/So ratio between the FB1 exposed groups and the intervention-applied groups was observed, this difference was not always statistically significant. The question remains if this ratio is sensitive enough to act as a biomarker at natural or toxicological relevant FB1 contamination levels [375].

As unaltered FB1 has been detected in urine of animals [143], it might be more sensible to determine this molecule itself in human urine and blood as biomarker. However, as only up to 1% of the *per os* administered dose is recovered in the urine [61], FB1 exposure must be high or chronic to be detected in this matrix, and sensitive analytical methods are necessary. Yet, in a previous study with human subjects, FB1 and its metabolites were analysed in urine, and urine was put forward as a suitable matrix to determine biomarkers of exposure in

humans [69,128,134]. However, a low urinary excretion of FB1 and a high inter-individual variation, might make faeces a more reliable matrix. Low FB1 oral bioavailability is a common feature across many species (ranging from <1% [376] to 5% [61]), and in pigs, up to 96% of FB1 is excreted in faeces. A similar situation is expected in humans. Therefore, FB1 and its metabolites (pHFB1a, pHFB1b, and HFB1) may be suitable faecal biomarkers. Yet, Shephard et al. [143] suggested that the collection and use of faeces in large-scale studies would be unpractical. Most likely the established cultural practice of 'peeing in a cup' is more acceptable than collecting faeces. Moreover, the volume of the produced samples, and the nature of the samples make this matrix less attractive to both the researchers and the public. Animal and human hair has also been successfully applied for the determination of FB1 [143]; this could potentially be a useful matrix to assess FB1 exposure [377]. However, the discovery and confirmation of FB1 exposure would always come some time after the event occurred, and it would therefore be too late to intervene. Furthermore, the advancement in microsampling techniques, which are less invasive, such as dried blood spots (DBS) or Volumetric Absorptive Microsampling (VAMS), which require blood drops obtained through a simple finger prick, have been put forward as practical multi-mycotoxin exposure monitoring techniques in resource-limited areas [308]. The VAMS have the advantage of absorbing a fixed amount of blood, while the DBS technique suffers from varying haematocrit (hct) levels that can influence the spread of blood on filter paper. Therefore, the use of VAMS overcomes this hct issue and is a preferred method over DBS. Specifically, the possibility of storage of these devices at room temperature makes them attractive; in remote areas, time will elapse between sampling and analysis. These sampling methods can also easily be applied to monitoring of the most vulnerable of the population, namely children [378].

More research has been performed on the biomarkers of AFB1 exposure and there is a better consensus compared to fumonisins. For recent human exposure to aflatoxin through ingestion, urinary biomarkers are useful. Urinary AFM1 and AFB1-N7-guanine (both for recent exposure), and serum AFB1-albumin or the dominant AF-bound amino acid in albumin, AFB1-lysine (for chronic exposure), are regarded as good biomarkers due to their strong correlation with aflatoxin intake [129,130,132–134]. Contrarily, urinary AFB1 and its metabolites AFP1 and AFQ1 have not demonstrated a good correlation with intake [133]. Urinary AFB1 can show the event of an exposure, but cannot tell anything about the quantity of ingested toxin.

Especially urinary AFM1 is a well-established and validated biomarker of aflatoxin exposure [126–132]. In a study performed by Ayelign et al. [379], morning urine was collected from 200 children and screened for AFB1, AFB2, AFG1, AFG2, and AFM1. All the toxin variants were detected except AFB1. Aflatoxin M1 was the most frequently detected, namely in 7% of all collected samples. Noteworthy is that the regions for the sampling had been selected based on relatively high total aflatoxin levels (exact levels not reported) in foods and stunting prevalence in children. This indicates that urine is an accessible and practical fluid even for the most vulnerable, namely children. Mykkänen et al. [126] noted higher excreted levels of AFQ1 and AFM1 in faeces compared to urine. Faeces remains an important route of AFB1 metabolites excretion. Therefore, faeces is useful as predictive marker for AFB1 exposure and worthy of further research, however the same practical issues apply as have been mentioned for fumonisins.

To **reach** out to vulnerable populations at risk, investment in and maintenance of strong sustainable collaborations are needed. An example is the overarching project of this PhD thesis, MycoSafe-South [380], the "European–African partnership for safe and efficient use of mycotoxin-mitigation strategies in sub-Saharan Africa". A specific goal of this project is "to improve sustainability of the acquired results by organising education programmes and awareness campaigns that will facilitate best practices, transfer the acquired knowledge , and help stakeholders to understand mycotoxin-associated health risks." Important for successful and effective collaboration is to involve local stakeholders of the food supply chain, such as producers, farmer organisations, processors, importers, exporters, retailers, consumers, community groups, public health organisations, and all levels of government. The most vulnerable populations are those in remote areas where survival is based on subsistence farming, mostly in villages. Cooperation between local (health or agricultural) authorities and local organisations to reach out and involve village leaders or spokespersons is essential to address and reach village or district residents. Locally-lead organisations are best to assess the situation in their communities and respond accordingly. There is a higher inclination to participation and acceptance of the residents if information is brought by familiar and trusting sources, and in the locally-spoken language. In a study performed by Afriyie-Gyawu et al. [121], collaboration with the District Health Director and health personnel was established to enter the community and bring the researchers in touch with the leaders and residents of the

aflatoxin-affected communities. “Networks and inter-continental partnerships for mycotoxin research, education, and service to society”, such as established by MYTOX-SOUTH [381], must be preserved to continue to improve food safety and security through mitigation of mycotoxins at local and global level. A Farmer’s lab gathering in Soweto (Johannesburg, South Africa) was organised by a local innovation platform iZindaba Zokudla [382] (Zulu language for ‘Conversations about Food’) together with the University of Johannesburg during the conduct of this project. Many stakeholders gathered together, including subsistence farmers, making it the perfect opportunity to exchange information and reach people at risk of mycotoxicosis. Collaboration with similar local action projects and working groups must be maintained and further expanded to reach the intended population.

To **protect** populations at risk of mycotoxin exposure, awareness campaigns with knowledge dissemination, education, and trainings must be organised, preferably on location, near the most likely exposed people. An important aspect of the work is generating and disseminating information and developing education strategies to help reduce contamination risks worldwide. Many individuals remain unaware of the mycotoxins present on their harvested crops. In a first step, it is important to provide information regarding the health threats these mycotoxins pose on both animals and humans, and their origin. In a next step, active prevention measures must be elucidated and taught. Both pre- and post-harvest mycotoxin mitigation techniques can be communicated and illustrated. On farmers’ level, practical, and culturally accepted methods are important to, at the very least, reduce mycotoxin contamination.

On a more national level, the government would have to provide support or subsidies to encourage different stakeholders to apply intervention techniques to safeguard the food chain from farm to fork. Preferably, the authorities would provide safe and effective mycotoxin detoxifiers to mycotoxin-affected regions, as soon as these are available.

Mycotoxin detoxifiers are globally used in the animal industry. Specifically, the two EU-approved detoxifiers employed in this thesis, an aflatoxin bentonite binder and a fumonisin esterase enzyme, are often applied in the feed of several animal species and are commercially available. Thus, the safety and efficacy of these detoxifiers in animals has been scientifically demonstrated. The fact that these detoxifiers are practical and easy-to-apply, would make their adoption in humans at risk very attractive. From the above-mentioned (“Under which

legal classification can fumonisin esterase for human consumption fall?"), it is clear that it is not straightforward to determine into which category the enzyme belongs. Yet, the classification of "food enzyme" seems to be the most applicable and the preferable legal route to follow. Furthermore, authorisation of bentonite (E558) as food additive ended in 2013 [137], due to the high intake of aluminium present in bentonite [138]. However, according to the FDA, bentonite is considered as a "Generally Recognized as Safe" (GRAS) substance [139].

The downside of these detoxifiers is that they come with a monetary price. However, pre- and post-harvest techniques require time and effort. Possibly the cost of the detoxifiers might outweigh the cost spent on more practicable mitigation strategies. Furthermore, the government could assist in the regions at risk of mycotoxicosis. In Kenya, a study performed by Awuor et al. [123] tested the efficacy, acceptability, and palatability of a similar bentonite aflatoxin binder. Nearly all of the surveyed participants would be willing to consume this clay and give it to their children if it could protect them from aflatoxicosis. However, for both detoxifiers, one must be wary of the long-term effects. Continuous health monitoring and surveillance would be necessary. Bentonite has an aspecific action, but an affinity for the adsorption of aflatoxins has been demonstrated [383]. On the other hand, the enzyme was specifically designed to cleave the ester bonds within fumonisins [361]. Yet, perhaps other endogenous biochemical substances with similar chemical structure could be targeted, such as sphingolipid bases? Further research is mandatory on the safety of both detoxifiers in humans, especially on the long run.

The willingness of people to use mycotoxin detoxifiers in food, in low and middle income countries, must also be addressed. There are not many studies available in literature that have investigated this issue. In the study performed by Awuor et al. [123] in Kenya, the acceptability of calcium montmorillonite clay use to reduce AFB1 dietary exposure was assessed. The conclusion was that most participants (n = 50) had no concerns about the clay and would be willing to take ACCS100 (98%) and give it to their children (98%). However, this assessment was solely based on a questionnaire at the end of the study. The percentage greatly decreased when asked if participants would be willing to take the clay for at least 2 weeks (40%) or as long as recommended (38%). Although, most participants (96%) had heard of AFs prior to the study, and most (91%) worried about becoming sick as a result of exposure to AFs. Therefore, awareness of the adverse health risks associated with the consumption of mycotoxin

contaminated food might not be enough to convince people to apply mycotoxin detoxifiers. In a study performed by Mutua et al. [384], in Kenya, the availability and use of mycotoxin binders in selected urban and peri-urban areas of the Nairobi (Kasarani sub-county) and Kisumu counties in smallholder dairy systems were evaluated. This study suggests four points to increase acceptability of mycotoxin binders (in feed). First, and most important, is still to promote awareness about the health risks of AFs, and on the existence of mycotoxin binders. While all the millers were aware of mycotoxins binders only half of the 17 agrovet dealers were aware of them and fewer than 10% sold them. Noteworthy is that 30% of the dealers indicated that the customers do not ask for binders. Second, is to address the affordability; selling mycotoxin binders in smaller quantities (1 kg vs. 25 kg) may encourage more farmers to purchase the product. Third, farmers must be made aware that the use of binders is not a stand-alone strategy; good production, handling, and manufacturing practices are the primary mycotoxin control strategies, and control is required along the food chain at several points. Fourth, it is important to have information on the efficacy of binders in order to regulate them appropriately. Lack of regulation and information on the efficacy exposes the farmers to products that are not effective. Perhaps if the government would create incentives, or aid in promoting the positive effects of the detoxifiers and making these detoxifiers available in the regions at risk, it would help increase the willingness to use detoxifiers. Additionally, some farmers used motorcycle operators to purchase and deliver feeds to their farms [384], this could also be a way of distributing binders to remote locations.

Other questions that must be raised and addressed in future research, is the administration route for the detoxifiers in humans, the effective dose, and their (long-term) effect on nutritional status in humans. Current available research indicates inconsistent results regarding the effect of bentonite to adsorb minerals and vitamins. Monitoring mineral, vitamin, and haematological parameters are important for long-term human application of the detoxifiers. The efficacy of bentonite was demonstrated by ingestion with a glass of water. However, further research on the most effective application strategy of the enzyme for human consumption, if brought on the market, is mandatory. A possibility is to detoxify food before ingestion, during food processing, or adding and mixing it in soaked food. Furthermore, the nutritional status of food must be monitored for eventual changes. The challenge of adding the enzyme to the food is the homogenous mixing to hydrolyse all fumonisins present. To

determine the most effective detoxifier dose, practical administration must be considered. The dose could be based on body weight of the consumer, or on meal weight; which is currently set for feed. A fixed amount would be easier for human administration, instead of weighing each meal (dry or wet) and calculating the necessary amount to be added.

6.6 Conclusion

This doctoral research contributed to the knowledge of mycotoxin mitigation strategies in humans through the application of detoxifiers. A method was developed and validated to analyse AFB1, FB1, pHFB1a, pHFB1b, and HFB1 in samples obtained from the SHIME system. The SHIME system proved to be a useful *in vitro* model to determine the efficacy of detoxifiers in the gastrointestinal tract of young children, as well as to monitor the effect on microbiota. This research provided evidence for the efficacy of both detoxifiers for human consumption, without any negative effects on the gut microbiota. Furthermore, the piglet was used as child model to investigate the most effective administration method (in-food or in-capsule) of fumonisin esterase. Mixing the enzyme in food, prior to ingestion, seems to be the most promising application method, based on the results from the piglet study. For future research, a pilot human intervention study was planned and drafted in this thesis. Finally, under controlled experimental conditions, a single oral FB1 dose of 2 µg/kg BW was observed to be necessary to quantify FB1 and its metabolites in human faeces, which can then be used as biomarkers to demonstrate the efficacy of fumonisin esterase. Furthermore, analysing HFB1 in human faeces seems promising to determine the efficacy of fumonisin esterase in future human intervention studies. Yet, prior to the execution of human intervention studies, more research on the safety of consumption of the detoxifiers is needed. Furthermore, these detoxifiers need to be legally regulated.

Summary

In sub-Saharan Africa, crop contamination with mycotoxigenic fungi remains an important problem. Some of their secondary metabolites are classified as mycotoxins that can cause severe adverse health effects in both animals and humans after exposure. In Africa, the largest part of the population practices subsistence farming. Hereby, the produce is not subjected to any form of monitoring, and thus mycotoxin contamination poses a real threat to food safety and security. In particular, the mycotoxins aflatoxin B1 (AFB1) and fumonisin B1 (FB1), which regularly contaminate corn and peanuts and their derived products, are of great concern. Furthermore, maize is a staple diet in many African countries, increasing the risk and exposure to these specific mycotoxins.

The **general introduction** of this doctoral thesis introduces mycotoxins, giving some background information, including their history, occurrence, and toxicity in humans, with a particular focus on children. Furthermore, the introduction highlights several applied pre- and post-harvest mycotoxin mitigation techniques, and a section on mycotoxin detoxifiers, adsorbers (e.g. clay binders) and biotransformers (e.g. enzymes), is added. In a last section, possible interactions between mycotoxins and human intestinal microbiota are considered.

The **general objective** of this thesis was to investigate the efficacy of two mycotoxin detoxifiers in humans, with focus on children. Two effective European Food Safety Approved (EFSA)-evaluated and EU-approved mycotoxin detoxifier feed additives, an aflatoxin bentonite clay binder and a fumonisin esterase enzyme, are currently used to prevent mycotoxicoses in animals. For this project, these two detoxifiers were identified as potential candidates to decrease mycotoxin exposure in humans. Furthermore, their effect on gut microbiota was studied, as well as an alternative method of application for fumonisin esterase was tested. Therefore, a suitable and validated *in vitro* human gastrointestinal (GIT) model and an *in vivo* animal model for the human were employed. In the former model, the efficacy of the detoxifiers in a human GIT environment was determined, as well as their effect on the gut microbiota was monitored. In the latter animal model, the efficacy of fumonisin esterase in two application methods, intraoral and intragastric, were compared in piglets. Lastly, human intervention studies were planned in Kenya, South Africa, and Belgium to test the detoxifiers directly in humans. Unfortunately, due to COVID-19 and legal complications regarding the undetermined status of fumonisin esterase for human consumption, these intervention studies were not able to be performed. Whereas in Kenya and South Africa the food is

naturally contaminated with AFB1 and FB1, in Belgium, the food consumed during the intervention study was to be experimentally contaminated with FB1. Therefore, a single FB1 dose determination study was performed including four volunteers.

The efficacy of the two detoxifiers was to be tested in the intended target population. However, before applying these detoxifiers directly to humans, *in vitro* and *in vivo* models were used to safely and ethically test these detoxifiers in a simulated human environment. In **Chapter 1**, an *in vitro* child gut model, the Simulator of Human Intestinal Microbial Ecosystem (SHIME®), was employed to determine the efficacy of the two aforementioned mycotoxin detoxifiers, as well as their effect on the gut microbiota. The system was inoculated with faeces obtained from a 2.3 year-old male child of African descent living in Merelbeke, Belgium. After two weeks of system stabilisation, the mycotoxins AFB1 and FB1 were repeatedly added to the system through the food during the third week at levels comparable to high contamination in African food, according to the literature. During the fourth week, both mycotoxins and detoxifiers were added together. An ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analytical method specifically for SHIME matrix was developed and validated to determine AFB1, FB1, and FB1 hydrolysed metabolites (partially hydrolysed FB1a (pHFB1a), pHFB1b, and completely hydrolysed FB1 (HFB1)) concentrations. Furthermore, the effect of the detoxifiers on colonic microbiota was investigated. The short chain fatty acid concentrations were analysed with a validated gas chromatography-mass spectrometry method. The colonic bacterial communities were analysed using metabarcoding. The two detoxifiers demonstrated their effectiveness; both mycotoxins were nearly completely removed from the system after five days. Furthermore, the composition of the healthy gut microbiota remained stable throughout the experiment. The results obtained in this *in vitro* human GIT model indicated that the detoxifiers are effective in reducing AFB1 and FB1 concentrations in a human child intestinal environment, without compromising the intestinal microbiota.

In the first chapter, the efficacy of an aflatoxin binder and a fumonisin esterase in the human GIT was demonstrated. Next, the optimal method of oral administration of these detoxifiers to humans was evaluated. In a previous study in the literature, it was already determined that a similar aflatoxin binder was effective in humans when mixed in the food or ingested with water straight into the stomach. The focus of **Chapter 2** was therefore on the

administration method of fumonisin esterase. An attractive and standard dosage form is through capsule ingestion. Hence, the efficacy of fumonisin esterase through two modes of application was compared in piglets, which are a suitable biomedical large animal model for humans. The enzyme was administered intraorally (in-feed analogue) or intragastrically (capsule analogue), in combination with FB1. Besides the two treatment groups, a placebo group was also included. Using a validated UHPLC-MS/MS method, suitable biomarkers for exposure, i.e. FB1, pHFB1a, pHFB1b, and HFB1 were analysed in serum and faeces. Based on these results, major toxicokinetic parameters were calculated; maximum observed concentration (C_{max}), time where maximum concentration was observed (T_{max}), and area under the concentration-time curve from time zero to time of last quantifiable concentration ($AUC_{0\rightarrow t}$). In serum, a significant and relevant decrease of 60% was observed in the FB1 AUC following intraoral administration of the enzyme compared to the placebo group. Additionally, in faeces, a significant increase of 119% in the HFB1 AUC was observed in the intraoral treatment group compared to the placebo group. Furthermore, the serum sphinganine/sphingosine (Sa/So) ratio, a validated biomarker of effect, was determined using UHPLC-MS/MS. The prevention of the increase in the Sa/So ratio by both intragastric and intraoral administrations of the fumonisin esterase enzyme when compared to the placebo group demonstrated the efficacy of the enzyme. However, the efficacy of the enzyme when administered intragastrically was not reflected by the other analysed biomarkers of exposure in serum and faecal samples, while this was observed in the intraoral group, as mentioned above. Although fumonisin esterase provided in a capsule could be an attractive strategy against fumonisin intoxication in humans, based on the results of this study, it cannot be recommended. Thorough mixing of fumonisin esterase in food prior to consumption seems to be the most effective approach.

In **Chapter 3**, three pilot human intervention study designs, including the administration of the binder for AFB1 and the enzyme for FB1, were developed for Kenya, South Africa, and Belgium. Furthermore, their respective dossiers for ethical application, informed consent forms, and information leaflets were drafted. Based on the results obtained in the second chapter, both detoxifiers were to be mixed in every ready-to-consume meal. Due to COVID-19, and its complementary restrictions for close human contact needed for blood sampling, and the non-existence of a legal status of the enzyme for human consumption, these studies

could not be performed. For the execution of future intervention studies with fumonisin esterase, legal status and categorisation of fumonisin esterase must be investigated and determined. Furthermore, the safety of the enzyme in humans should be investigated before the implementation of an efficacy study. Whereas in Kenya and South Africa the food is naturally contaminated with AFB1 and FB1, in Belgium, the food consumed during the intervention study was to be experimentally contaminated with FB1. Therefore, a single FB1 dose determination study was performed including four volunteers. A single FB1 dose of 1 or 2 µg/kg BW was administered, corresponding to the legal tolerable daily intake (TDI) and twice the TDI, respectively. The participants were on a controlled diet (no consumption of maize and rice containing products) during eight consecutive days. All faecal samples were collected. These samples were analysed for FB1 and its metabolites, pHFB1a, pHFB1b, and HFB1, using a validated UHPLC-MS/MS method. A single FB1 dose of 1 µg/kg BW resulted in one faecal sample with levels above the limit of quantification (LOQ, 160 ng/g) per participant. A single dose of 2 µg FB1/kg BW resulted in FB1 levels above the LOQ in several faecal samples. Therefore, a FB1 dose of 2 µg/kg BW is advised for future experimentally-controlled fumonisin esterase efficacy studies when no naturally contaminated food is available. Hydrolysed FB1 could not be detected at concentrations higher than the LOQ in any of the samples, providing evidence of this metabolite to be a relevant biomarker in human faeces to determine fumonisin esterase efficacy following the administration of a single FB1 dose. Further research would have to prove if this is also the case in real life, with chronic FB1-contaminated food consumption.

In the **general discussion**, the reason for adopting *in vitro* techniques was highlighted. The advantages and disadvantages were pointed out. Furthermore, the benefits and shortcomings of the adoption of the SHIME as human gut model was discussed in more detail. Challenges that were encountered while using this system and its assets specifically to achieve the research objectives of this doctoral thesis were specified. In a second point, the existing legal classifications regarding foodstuffs, and more specifically the possible categories under which fumonisin esterase could be allocated were discussed in-depth. Depending on the intended use, the function, and location of the effect (in food or in the body), fumonisin esterase could fit into several categories. Yet, the most important issue that arose was that, contrary to animal feed legislation, for human food there is no specific technological need for

mycotoxin reduction mentioned in the regulations. However, this problem can be circumvented by the novel food legislation. The specific category for "food consisting of, isolated from, or produced from microorganisms, fungi or algae" is possibly applicable for the enzyme. Furthermore, some future research opportunities are presented to help fill gaps in research.

In **conclusion**, this doctoral thesis provides strong evidence of the efficacy of an aflatoxin binder and a fumonisin esterase in humans, based on an *in vitro* gut model and *in vivo* study with piglets. In addition, the administration of fumonisin esterase in ready-to-consume meals seems to be more effective than ingestion through a capsule. Furthermore, the completely hydrolysed FB1 metabolite, HFB1, is put forward as a suitable biomarker to determine fumonisin esterase efficacy in humans following the administration of a single FB1 dose. For future human intervention studies under controlled experimental conditions, where no FB1-contaminated food is available, it is advised to provide a FB1 dose of 2 µg/kg BW to individuals to obtain quantifiable concentrations of FB1 and its metabolites in faecal samples. Furthermore, the legal classification of fumonisin esterase must be established before the conduct of such efficacy studies. Ultimately, future studies are advised to investigate the safety, especially for long-term application, of the detoxifiers in humans.

Samenvatting

In sub-Sahara Afrika blijft besmetting van gewassen met mycotoxigene schimmels een groot probleem. Hun secondaire metabolieten, mycotoxinen, kunnen ernstige gezondheidsproblemen veroorzaken na opname door zowel dier als mens. In Afrika doet het grootste deel van de bevolking aan zelfvoorzienende landbouw. Hierdoor ontlopen hun geteelde producten iedere vorm van controle, en is er een reële kans op mycotoxinebesmetting, wat voedselveiligheid en -zekerheid in gevaar brengt. Vooral de mycotoxinen aflatoxine B1 (AFB1) en fumonisine B1 (FB1), die regelmatig maïs en pindanoten en hun afgeleide producten besmetten, baren grote zorgen. Bovendien is maïs een basisvoedsel in vele Afrikaanse landen, waardoor het risico van en de blootstelling aan deze specifieke mycotoxinen verhoogd is.

De **algemene inleiding** van dit proefschrift introduceert mycotoxinen, waarbij achtergrond informatie gegeven wordt, waaronder hun geschiedenis, voorkomen en toxiciteit bij mensen, met bijzondere aandacht voor kinderen. Verder belicht de inleiding verschillende voor- en naoogststrategieën die kunnen toegepast worden als mycotoxine mitigatietechnieken, en is er een sectie over mycotoxine detoxifiers, adsorbeerders (zoals kleibinders) en biotransformeerders (zoals enzymen) toegevoegd. In een laatste sectie worden de mogelijke interacties tussen mycotoxinen en darmmicrobiota aangehaald.

De **algemene doelstelling** van dit proefschrift beoogt de werkzaamheid van twee mycotoxine detoxifiers bij mensen te onderzoeken, met bijzondere aandacht voor kinderen. Twee werkzame, door de Europese Autoriteit voor Voedselveiligheid (EFSA) beoordeelde en nadien door de Europese Commissie goedgekeurde voederadditieven, een aflatoxine bentoniet kleibinder en een fumonisine-esterase enzym, worden momenteel bij dieren gebruikt om mycotoxicose te voorkomen. Voor dit onderzoeksproject werden deze twee detoxifiers geïdentificeerd als potentiële kandidaten om de gevolgen van mycotoxine blootstelling in de mens te verminderen. Verder werd hun effect op de darmmicrobiota bestudeerd en werd een alternatieve toedieningsmethode voor fumonisine-esterase getest. Hiervoor werd een geschikt en gevalideerd *in vitro* spijsverteringsstelsel (SVS) model en een *in vivo* diermodel voor de mens gebruikt. In het eerste model werd de werkzaamheid van de detoxifiers in een humaan SVS omgeving nagegaan, evenals hun effect op de darmmicrobiota. In het tweede model werd de werkzaamheid van twee toedieningswegen van fumonisine-

esterase, intra-oraal en intra-gastrisch, vergeleken in biggen. Ten slotte waren er humane interventiestudies gepland in Kenia, Zuid-Afrika en België om de detoxifiers rechtstreeks bij de mens te testen. Helaas konden deze interventiestudies niet worden uitgevoerd, omwille van COVID-19 en door juridische complicaties met betrekking tot de onbestaande status van fumonisine-esterase voor humane consumptie. Terwijl het voedsel in Kenia en Zuid-Afrika van nature gecontamineerd is met AFB1 en FB1, zou in België het voedsel dat geconsumeerd zou worden tijdens een interventiestudie, experimenteel gecontamineerd moeten worden met FB1. Daarom werd een FB1-dosisbepalingsstudie uitgevoerd met vier vrijwilligers.

De werkzaamheid van de twee detoxifiers diende getest te worden in de beoogde doelgroep. Vooraleer deze detoxifiers rechtstreeks aan mensen toe te dienen, werden *in vitro* en *in vivo* modellen gebruikt om ze veilig en ethisch te testen in een gesimuleerde menselijke omgeving. **Hoofdstuk 1** behandelt het gebruik van een *in vitro* darmmodel voor kinderen, de Simulator of Human Intestinal Microbial Ecosystem (SHIME®), in het onderzoek naar de werkzaamheid van de twee detoxifiers, en hun effect op de darmmicrobiota. Het systeem werd geïnoculeerd met feces verkregen van een 2,3 jaar oud mannelijk kind van Afrikaanse afkomst dat in Merelbeke, België, woont. Na twee weken systeemstabilisatie, werden de mycotoxinen AFB1 en FB1 gedurende één week toegediend via het voedsel, aan niveaus vergelijkbaar met hoge besmetting in Afrikaans voedsel, volgens data gevonden in de literatuur. De daaropvolgende week werden zowel de mycotoxinen als de detoxifiers samen toegediend. Een ultrahoge performante vloeistofchromatografie tandem massaspectrometrie (UHPLC-MS/MS) analytische methode werd specifiek voor de SHIME-matrix ontwikkeld en gevalideerd om AFB1, FB1 en FB1 gehydrolyseerde metabolieten concentraties te bepalen (gedeeltelijk gehydrolyseerd FB1a (pHFB1a), pHFB1b en volledig gehydrolyseerd FB1 (HFB1)). Verder werd het effect van de detoxifiers op de darmmicrobiota onderzocht. De concentraties korte keten vetzuren werden geanalyseerd met een gevalideerde gaschromatografie massaspectrometrie methode. De bacteriële gemeenschappen in het colon werden geanalyseerd met behulp van metabarcodering. De twee detoxifiers hebben hun werkzaamheid aangetoond; na vijf dagen waren beide mycotoxinen bijna volledig uit het systeem verwijderd. Bovendien bleef de samenstelling van de gezonde darmmicrobiota gedurende het hele experiment stabiel. De resultaten verkregen van dit *in vitro* humane SVS-model geven aan dat deze detoxifiers effectief zijn in het verlagen van AFB1- en FB1-

concentraties in de darmomgeving van mensen, zonder de darmmicrobiota in gevaar te brengen.

In het eerste hoofdstuk werd de werkzaamheid van een aflatoxinebinder en een fumonisine-esterase in het humaan SVS aangetoond. Vervolgens werd de optimale methode voor orale toediening van deze detoxifiers aan mensen geëvalueerd. In een eerdere studie was al aangetoond dat een vergelijkbare aflatoxinebinder effectief was bij mensen wanneer het in het voedsel gemengd werd of rechtstreeks met water in de maag werd ingenomen. In **Hoofdstuk 2** lag de focus daarom enkel op de toedieningsmethode van fumonisine-esterase. Een aantrekkelijke en standaard vorm van supplement toediening is via capsules. Daarom werd de werkzaamheid van fumonisine-esterase via twee toedieningswijzen vergeleken *in vivo* bij biggen, als geschikt biomedisch groot diermodel voor de mens. Het enzym werd intra-oraal (in voedsel analoog) of intra-gastrisch (capsule-analoog) toegediend in combinatie met FB1. Naast de twee behandelingsgroepen was er ook een placebogroep. Met een gevalideerde UHPLC-MS/MS methode werden geschikte biomerkers voor blootstelling geanalyseerd in serum en feces, namelijk FB1, pHFB1a, pHFB1b en HFB1. Op basis van deze resultaten werden belangrijke toxicokinetische parameters berekend; de maximaal waargenomen concentratie (C_{max}), de tijd waarop maximale concentratie werd waargenomen (T_{max}), en oppervlakte onder de concentratie-tijdscurve van tijdstip nul tot het tijdstip van de laatste meetbare concentratie ($AUC_{0\rightarrow t}$). In serum werd een significante en relevante afname van 60% waargenomen in de FB1 AUC na intra-orale toediening van het enzym in vergelijking met de placebogroep. Bovendien werd in de feces een significante toename van 119% van de HFB1 AUC waargenomen in de intra-orale behandelingsgroep tegenover de placebogroep. Verder werd de serum sfinganine/sfingosine (Sa/So) ratio, een gevalideerde biomerker voor effect, bepaald met UHPLC-MS/MS. De verhindering in toename van de Sa/So ratio door zowel intra-gastrische als intra-orale toediening van het fumonisine-esterase in vergelijking met de placebogroep, toonde de efficaciteit van het enzym aan. De werkzaamheid van het enzym bij intra-gastrische toediening werd echter niet weerspiegeld in de overige geanalyseerde biomerkers voor blootstelling in de serum en fecale stalen, terwijl dit wel werd waargenomen in de intra-orale groep, zoals eerder vermeld. Hoewel toediening van fumonisine-esterase in een capsule een aantrekkelijke strategie zou kunnen zijn tegen fumonisine-intoxicatie bij mensen, kan het, op basis van de resultaten die in dit onderzoek zijn gevonden, niet worden

aanbevolen. Grondig mengen van fumonisine-esterase in voedsel voorafgaand aan de opname lijkt de meest effectieve aanpak.

In **Hoofdstuk 3** zijn drie pilootstudies voor humane interventiestudies uitgeschreven, waarbij de AFB1-binder en het fumonisine-esterase zouden toegediend worden, in Kenia, Zuid-Afrika en België. De respectievelijke dossiers voor ethische goedkeuring, formulieren voor geïnformeerde toestemming en informatiefolders werden opgesteld. Op basis van de resultaten gevonden in het tweede hoofdstuk, moeten beide detoxifiers in kant-en-klare maaltijden worden gemengd. Vanwege COVID-19 en de daarbij horende beperkingen bij nauw menselijk contact (bv. voor bloedafname), en het ontbreken van een wettelijke status van het enzym voor humane consumptie, konden deze onderzoeken niet worden uitgevoerd. Voor de uitvoering van toekomstige interventiestudies met fumonisine-esterase moet de juridische status en classificatie van fumonisine-esterase worden onderzocht en bepaald. De veiligheid van het enzym bij mensen dient onderzocht te worden vooraleer een efficaciteitsstudie kan worden uitgevoerd. Terwijl in Kenia en Zuid-Afrika het voedsel van nature gecontamineerd is met AFB1 en FB1, zou het voedsel, dat geconsumeerd zou worden tijdens de interventiestudie in België, experimenteel gecontamineerd moeten worden met FB1. Daarom werd een FB1-dosisbepalingsstudie uitgevoerd met vier vrijwilligers. Een enkele FB1-dosis van 1 of 2 µg/kg LG werd toegediend, overeenkomend met respectievelijk de wettelijk toelaatbare dagelijkse inname (TDI) en het dubbele van de TDI. De deelnemers volgden gedurende acht achtereenvolgende dagen een gecontroleerd dieet (geen maïs- en rijsthoudende producten). Alle fecale stalen werden verzameld. Deze stalen werden geanalyseerd op FB1 en diens metabolieten, pHFB1a, pHFB1b en HFB1, met een gevalideerde UHPLC-MS/MS methode. Een enkele FB1-dosis van 1 µg/kg LG resulteerde in één staal per deelnemer met niveaus boven de bepaalbaarheidsgrens (LOQ, 160 ng/g). Een enkele dosis van 2 µg FB1/kg LG resulteerde in FB1-niveaus boven de LOQ in verschillende fecale stalen. Daarom wordt een FB1-dosis van 2 µg/kg LG geadviseerd voor toekomstige experimenteel gecontroleerde efficaciteitsstudies met fumonisine-esterase wanneer er geen natuurlijk gecontamineerd voedsel beschikbaar is. Gehydrolyseerd FB1 werd in geen van de stalen gekwantificeerd, wat kan wijzen op het feit dat deze metaboliet een relevante biomerker in humane feces zou kunnen zijn om de werkzaamheid van fumonisine-esterase te bepalen na toediening van een enkele FB1-dosis.

Nader onderzoek zou moeten uitwijzen of dit ook het geval is bij chronische FB1-gecontamineerd voedsel opname.

In de **algemene discussie** belicht het eerste deel de reden voor het toepassen van *in vitro* technieken. Er wordt gewezen op de voor- en nadelen. Verder worden de voordelen en tekortkomingen van het gebruik van de SHIME als humaan model voor het SVS in detail besproken. Uitdagingen die we ondervonden bij het gebruik van dit systeem en de troeven, specifiek voor de onderzoeksdoelstellingen van dit proefschrift, worden besproken. In een tweede deel worden de bestaande wettelijke classificaties met betrekking tot levensmiddelen, en meer specifiek de mogelijke categorieën waaronder fumonisine-esterase zou kunnen worden ingedeeld, uitgebreid besproken. Afhankelijk van het beoogde gebruik, de functie en de plaats van het effect (in het voedsel of in het lichaam), kan fumonisine-esterase onder verschillende categorieën vallen. Het belangrijkste probleem dat zich echter voordoet, is dat er, in tegenstelling tot de diervoederwetgeving, voor humane voeding geen specifieke technologische noodzaak is voor mycotoxine reductie. Dit probleem kan echter worden omzeild door de nieuwe voedselwetgeving. Mogelijk is voor het enzym de specifieke categorie "voedsel bestaande uit, geïsoleerd uit of geproduceerd uit micro-organismen, schimmels of algen" van toepassing. Bovendien worden enkele toekomstige onderzoeksmogelijkheden gepresenteerd om hiaten in het onderzoek te helpen opvullen.

Als **conclusie** levert dit proefschrift sterk bewijs voor de werkzaamheid van een aflatoxinebinder en een fumonisine-esterase toepassing bij de mens, gebaseerd op een *in vitro* model voor het spijsverteringsstelsel en een *in vivo* biggenstudie. Daarnaast geeft dit onderzoek aan dat het toedienen van fumonisine-esterase in kant-en-klare maaltijden effectiever is dan inname via een capsule. Bovendien wordt de volledig gehydrolyseerd FB1 metaboliet, HFB1, naar voren gebracht als een geschikte biomerker om de werkzaamheid van fumonisine-esterase bij mensen te bepalen na toediening van een enkele FB1-dosis. Voor toekomstige humane interventiestudies, onder gecontroleerde experimentele omstandigheden waar geen FB1-besmet voedsel beschikbaar is, wordt geadviseerd om individuen een FB1-dosis van 2 µg/kg LG te verstrekken om kwantificeerbare concentraties van FB1 en zijn metabolieten in fecale stalen te verkrijgen. Bovendien moet de wettelijke classificatie van fumonisine-esterase worden vastgesteld voordat dergelijke

efficaciteitsstudies worden uitgevoerd. Voor toekomstige studies wordt geadviseerd om de veiligheid, vooral op lange termijn, van de toepassing van deze detoxifiers bij de mens te onderzoeken.

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Curriculum vitae

Kaat Neckermann was born in Wetteren on the 30th of May, 1993. After obtaining the European Baccalaureate at the European School of Mol (scientific orientation), she started the study Veterinary Medicine at Ghent University. In 2017, she obtained her Master in Veterinary Medicine (main subject research) with honors.

In November 2017, she started working as a veterinary inspector at the Federal Agency for the Safety of the Food Chain in the primary sector of the living animals. In September 2018, she started an international joint-PhD project (MycoSafe-South) at the Laboratory of Pharmacology and Toxicology of the Faculty of Veterinary Medicine at Ghent University, as well as at the department of Food Sciences of the Faculty of Veterinary Medicine at the University of Liège. This project aimed at reducing aflatoxin B1 and fumonisin B1 exposure in both animals and humans through mitigation strategies, including the use of two detoxifiers. Kaat Neckermann's part focussed on the human aspect.

Kaat Neckermann is author of two scientific publications, presented at several national and international conferences and supervised students of the Faculty of Veterinary Medicine during their master thesis. In 2022, she completed the training programmes of the Doctoral Schools of Life Sciences and Medicine at both the University of Ghent and Liège.

Kaat Neckermann werd geboren te Wetteren op 30 mei 1993. Na het behalen van het Europees baccalaureaat (wetenschappelijke richting), begon zij in 2011 aan de studies diergeneeskunde aan de UGent. In 2017 behaalde zij met onderscheiding een Master in de Diergeneeskunde (afstudeerrichting onderzoek).

In november 2017 ging zij aan de slag als Inspecteur Dierenarts bij het Federaal Agentschap voor de Veiligheid van de Voedselketen in de primaire sector van de levende dieren. In september 2018 begon zij aan een internationaal joint-PhD project (MycoSafe-South) tussen het Laboratorium voor Farmacologie en Toxicologie van de Faculteit Diergeneeskunde van de Universiteit Gent en de vakgroep Voedingswetenschappen van de Faculteit Diergeneeskunde van de Universiteit van Liège. Dit project had als doel de blootstelling aan aflatoxine B1 en fumonisine B1 in dier en mens te verminderen, inclusief het gebruik van twee detoxifiers. Kaat Neckermann haar deel was gericht op het humane aspect.

Kaat Neckermann is auteur van twee wetenschappelijke publicaties, presenteerde op nationale en internationale congressen en begeleidde studenten van de faculteit Diergeneeskunde bij het schrijven van hun masterthesis. In 2022 voltooide zij de opleiding van de Doctoral Schools of Life Sciences and Medicine van zowel UGent als van ULiège.

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Oral presentations

Neckermann, K.; Antonissen, G.; Uhlig, S.; Gathumbi, J.; Delcenserie, V.; Croubels, S. Safety and efficacy of mycotoxin binders/modifiers *in vitro* (SHIME), *in vivo* (piglets) and human intervention study in Kenya. MycoSafe-South kick-off meeting; Cape Town, South Africa, 2018

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