Food Research International 145 (2021) 110395

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

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®)

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A R T I C L E I N F O

Keywords:
Mycotoxin
Aflatoxin B1
Fumonisin B1
Bentonite binder
Fumonisin esterase
Fumonisin metabolites
Detoxifiers
Intestinal microbiota
Human intestinal model
Efficacy
In vitro
Toddler SHIME

A B S T R A C T

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. 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 and FB1b concentrations were determined in SHIME samples using a validated ultra-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 AFB1 and FB1 concentrations significantly decreased after the addition of the detoxifiers. Likewise, the concentration of HFB1 significantly increased. 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.

1. Introduction

Food security and safety continue to be one of the world's major challenges as Earth'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 (2019). However, the rapidly augmenting population is

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https://doi.org/10.1016/j.foodres.2021.110395
Received 14 January 2021; Received in revised form 23 April 2021; Accepted 7 May 2021
Available online 12 May 2021
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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 and feed as well as in acute and chronic mycotoxicoses (Bennett & Klich, 2003).

Aflatoxins (AF) and fumonisins (FB) 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 (Zain, 2010). 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 (Matumba, Van Poucke, Ediage, & De Saeger, 2017). Above all, while food security remains a problem (FAO, 2020; FAO, IFAD, & WFP, 2013), food safety is less regulated (FAO, 2004), sporadically leading to fatal mycotoxicoses (Nduti, 2017). In addition, mycotoxin contamination limits trade of agricultural products from sub-Saharan Africa to the rest of the world (Mutegi et al., 2013; Udomkun et al., 2017).

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 (Kemboi, Ochieng, et al., 2020; Kibugu et al., 2019; Mutegi et al., 2013; Sirma et al., 2016; Udomkun et al., 2017; Wangia et al., 2019). Several outbreaks of aflatoxicosis, resulting in a large number of human casualties, have been reported in recent years in Kenya (Probst, Njapau, & Cotty, 2007). 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 (Azziz-baumgartner et al., 2005; CDC, 2004). Aflatoxin B1 is carcinogenic to humans (International Agency for Research on Cancer - IARC Group 1), and is strongly correlated with liver cancer (IARC, 1993, 2002). Co-contamination of cereals with AFB1 and FB1 is of great concern (Mutiga, Hoffmann, Harvey, Milgroom, & Nelson, 2015). Fumonisin B1 is possibly carcinogenic to humans and has been shown to cause oesophageal cancer in laboratory animals (IARC Group 2B) (IARC, 1993). Chronic exposure to AF and FB has also been linked to stunting in children (Gong et al., 2002; IARC, 2015; WHO, 2018; Wild & Gong, 2010). 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 (Kemboi, Antonissen, et al., 2020; Kemboi, Ochieng, et al., 2020). Currently, Kenyans still consume grain and nut products exceeding the national set legal limits of mycotoxins (Kibugu et al., 2019). 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 (Rose et al., 2018). 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 (Luo, Liu, & Li, 2018; Odukoya et al., 2021).

Mycotoxin detoxifiers, such as binders and modifiers, are frequently applied as animal feed additives to prevent acute and chronic mycotoxin toxicity (Dell'Orto, Baldi, & Cheli, 2015). Two specific detoxifiers, a bentonite clay binder and a fumonisin esterase, have been approved by the European Food Safety Authority as feed additives (FEEDAP, 2011; FEEDAP et al., 2017). The AF binder, consisting of bentonite clay, binds AFB1 and other aflatoxins, hence reducing their availability for gastrointestinal absorption. The FB modifier consists of a purified enzyme that is specific for cleaving the FB side chains, resulting in the formation of less toxic FB 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 (EC, 2020). 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 (FEEDAP, 2011; FEEDAP et al., 2017). 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* (Tomasevic-Canovic, Dakovic, Markovic, Radosavljevic-mihajlovic, & Vukicevic, 2000). 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 (Masching, Doupovec, & Schatzmayr, 2017), in chickens after 35 days (BIOMIN, 2014), and in dairy cattle after 7 days (Vetmeduni Wien, 2018). Furthermore, in a 2-weeks and 3-months human trial, performed by Wang et al. (2005) in Texas and Afriyie-Gyawu et al. (2008) 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 (FEEDAP, 2011) 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 (Ouethrani et al., 2013). 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 FB 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 AF and FB 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 FB 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.

2. Materials and methods

2.1. 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*® (see Fig. 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).

2.2. Faecal inoculum

The faeces used to inoculate the SHIME system were obtained from a 2.3 years-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 (David et al., 2014; Goodrich et al., 2016; Lim et al., 2014). 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 μm 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.

2.3. Experimental design and sample collection

The toddler SHIME system consisted of five vessels in series, each in connection through tubes (Bondue, Lebrun, Taminiau, Everaert, LaPointe, Crèvecoeur, et al., 2020; Van Den Abbeele et al., 2010). 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 powder 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 (Van Den Abbeele et al., 2010, 2012). 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 2000 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 Fig. 1. 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.

2.4. Mycotoxin analysis and method validation

Based on a validated multi-mycotoxin UHPLC-MS/MS method for porcine plasma (Lauwers et al., 2019), the analytical protocol to quantify the mycotoxins was adapted and fine-tuned for SHIME medium.

Samples were centrifuged (10 min, 2851*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, 2851*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 UPLC 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. (2011). 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 European Commission (2002). The following parameters were assessed: method linearity, within- and between-day accuracy and precision, limit of detection (LOD) and of limit of quantification (LOQ), specificity, extraction recovery and matrix effect (signal suppression/enhancement). The results for each analyte are summarised in Tables S1 to S3.

According to literature, AFB1 (<1–1000 μg/kg) and FB1 (<1–3000 μg/kg) concentrations may be observed in a wide range in African crops (Creppy, 2002; Hove et al., 2016; Maringe et al., 2017; Rodrigues, Handl, & Binder, 2011; Sirma et al., 2016; Ssepuuya et al., 2018). Thus, AFB1 with a concentration of 81.6 μg/kg feed and FB1 with a

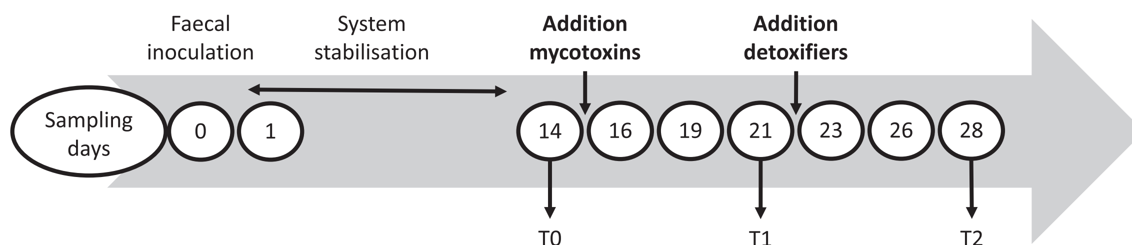

Fig. 1. Timeline presenting the course of the experiment and the sampling days used for determination of mycotoxin concentration, short chain fatty acid concentration and metagenetic analysis. T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 μg/kg food) and fumonisin B1 (FB1) (2000 μg/kg food); T2 = after one week of addition of AFB1 (81.6 μg/kg food) and FB1 (2000 μg/kg food) with bentonite clay – Mycofix® Secure (2.5 g/kg food) and fumonisin esterase – FUM*zyme*® (60 U/kg food).

concentration of 2000 μg/kg feed were employed in the experiment to mimic high contaminations found under African circumstances.

2.5. Short-chain fatty acid analysis

The SCFA analysis was performed following the validated method for SHIME samples by Douny et al. (2019). 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).

2.6. 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 (Illumina, 2013) and the method described by Bondue, Lebrun, Taminiau, Everaert, LaPointe, Hendrick, & et al. (2020). 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 (Bondue, Lebrun, Taminiau, Everaert, LaPointe, Hendrick, & et al., 2020). The obtained operational taxonomic units (OTUs) were used for the inversed Simpson diversity index calculation.

2.7. Statistical analysis

All statistical analyses for mycotoxin concentration, SCFA concentration and metagenetic analysis data were performed using the software package RStudio (R Core Team, 2020). Additionally, statistical analysis regarding the alpha diversity between colon regions, using the inversed Simpson diversity index, was evaluated using AMOVA in Mothur (Schloss et al., 2009).

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. Results and discussion

3.1. Mycotoxin analysis

The SHIME samples collected before mycotoxin inoculation (day 1 and 14, Fig. 1) contained traces of FB1 in all three colon regions of both repetitions, as shown in Fig. 2. 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 (Stanciu et al., 2015), 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 (Creppy, 2002). In 2012, cereal samples collected in Northern Europe had levels for FB1 below the LOD of 100 μg/kg sample (Van Der Fels-Klerx et al., 2012).

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 (Masching et al., 2016). A reduction of the FB1 concentration 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 (Schwartz-Zimmermann et al., 2018). Masching et al. (2016) performed three experiments to determine the FB1 degrading potential of the same esterase. First, within two hours, the enzyme degraded FB1 completely into HFB1 in both jejunum and duodenum segments collected from a pig. Second, a decrease of approximately 46% and of 77% in FB1 was observed in the excreta of turkeys after 7 and 14 days, respectively (Masching et al., 2016). Third, in a pig trial, an average decrease of 72% in FB1 concentration in faeces

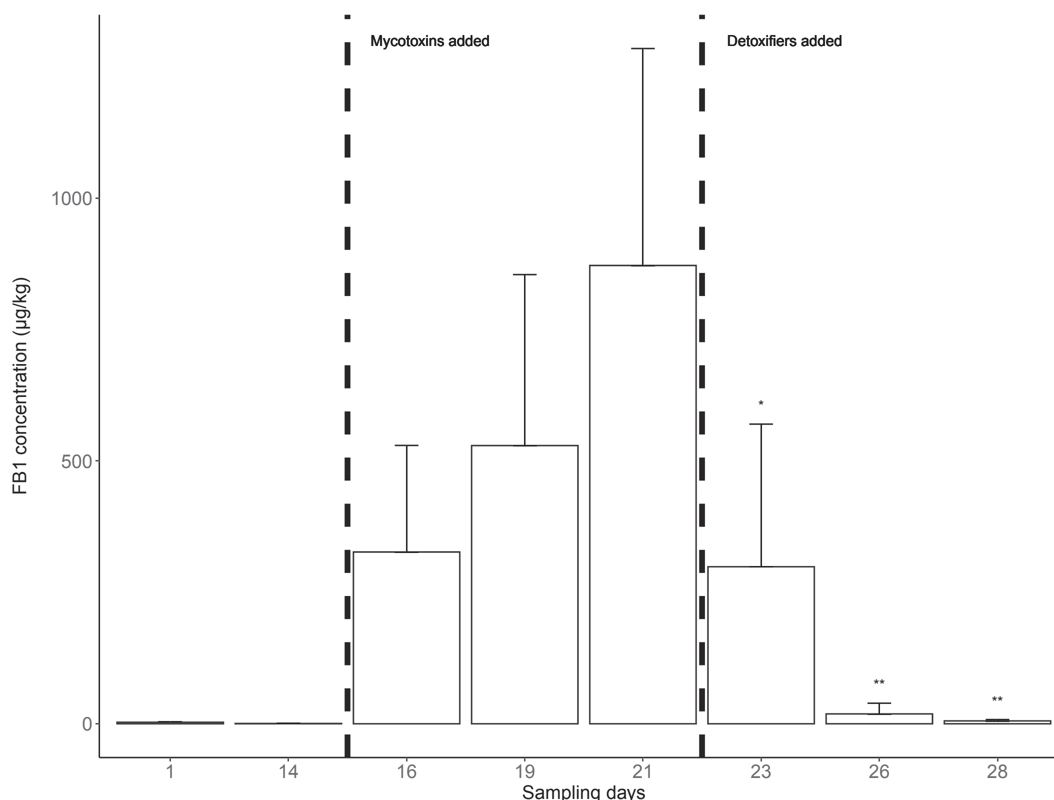

Fig. 2. Mean concentration of fumonisin B1 (FB1) determined in the AC, TC and DC compartment at different sampling days during the SHIME experiment ($n = 2$). Error bars represent the standard deviation. The dashed lines represent the addition of aflatoxin B1 (AFB1) (81.6 μg/kg food) and FB1 (2000 μg/kg 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.

was observed after 14, 28 and 42 days of feeding the piglets fumonisins (2000 μg/kg) and fumonisin esterase (60 U/kg) (Masching et al., 2016). 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. (2016). 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 (Fig. 3). 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.

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 fumonisins in faeces (Schertz et al., 2018; Schwartz-Zimmermann et al., 2018). In this study, pHFB1a and pHFB1b were detected in samples before day 23 (Fig. 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 (Fig. 2) and the concurrent increase of HFB1 concentrations (Fig. 3) 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. (2016) 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. (2012) 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. (2009) 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. (2015) 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 (Fig. 4). 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.

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 (Fig. 4). 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 (EC, 2013b). Bentonite clay has been proven to be safe and effective, and authorised as feed additive in binding AFB1 in animals (ruminants, poultry and pigs) (EC, 2013a; FEEDAP, 2011); a decrease in AFM1 excretion in milk of 55.0 to 68.0% and 17.3 to 21.3% was observed (Maki et al., 2017, 2016), and an *in vitro* study resulted in an AFB1 adsorption capacity from 90.0% to 95.3% (FEEDAP et al., 2017). The

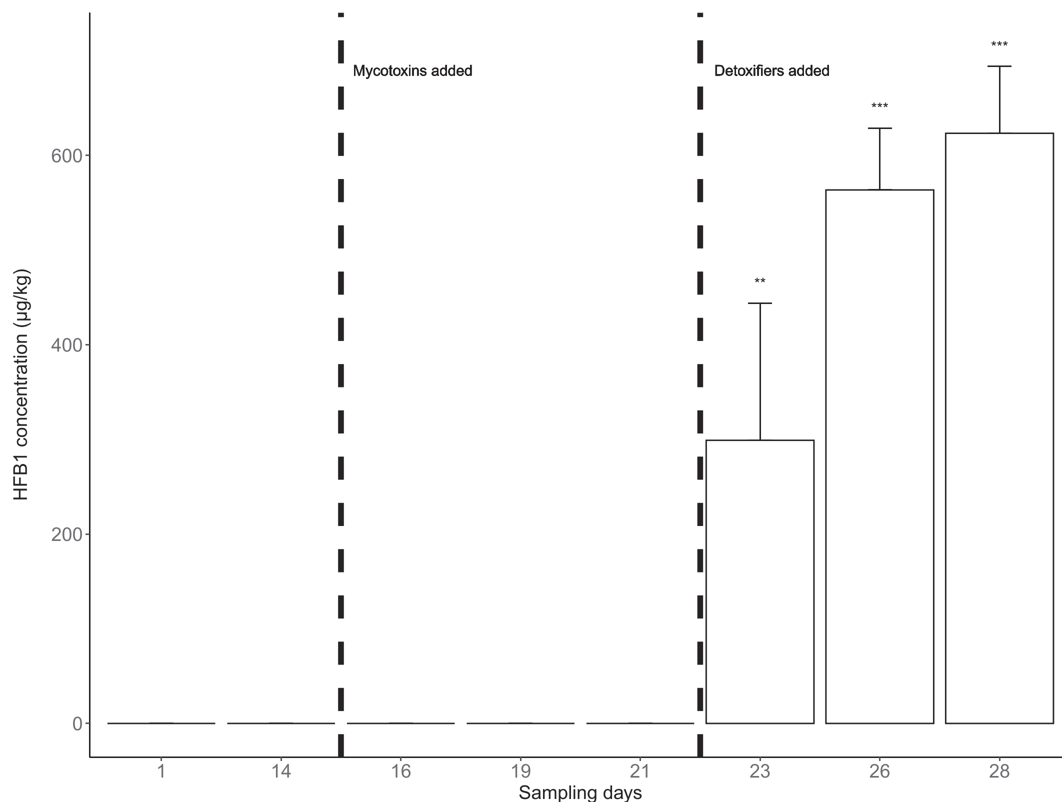

Fig. 3. Mean concentration of hydrolysed fumonisin B1 (HFB1) determined in the AC, TC and DC compartment at different sampling days during the SHIME experiment ($n = 2$). Error bars represent the standard deviation. The dashed lines represent the addition of aflatoxin B1 (81.6 µg/kg food) and fumonisin B1 (2000 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.

effectiveness for detoxification in humans was determined by measuring AFM1 in urine and the AFB1-lysine adduct in serum (Awuor et al., 2017; Pollock et al., 2016; Wang et al., 2005).

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 (Wang et al., 2008). 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.

3.2. 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 (Bernalier-Donadille, 2010). 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 (Demigne, Remesy, & Morand, 1999). 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 (Fig. 5), nor in the GIT (Fig. 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. (Cinquin, Le Blay, Fliss, & Lacroix, 2006) noticed a molar ratio of 75/19/6 in infant faeces and Cummings (1981) 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) (Bondue, Lebrun, Taminiau, Everaert, LaPointe, Crèvecoeur, et al., 2020; Bondue, Lebrun, Taminiau, Everaert, LaPointe, Hendrick, & et al., 2020).

Similar to the study of Bondue, Lebrun, Taminiau, Everaert, LaPointe, Hendrick, & et al. (2020), the SCFA concentration profile in the AC was evidently different from TC and DC (Fig. 5), while it was nearly identical in TC and DC. This corresponds to what is commonly seen in the microbial profile, discussed later on in Section 3.3.2. 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 (Fig. 5). 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* (Venegas et al., 2019), 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

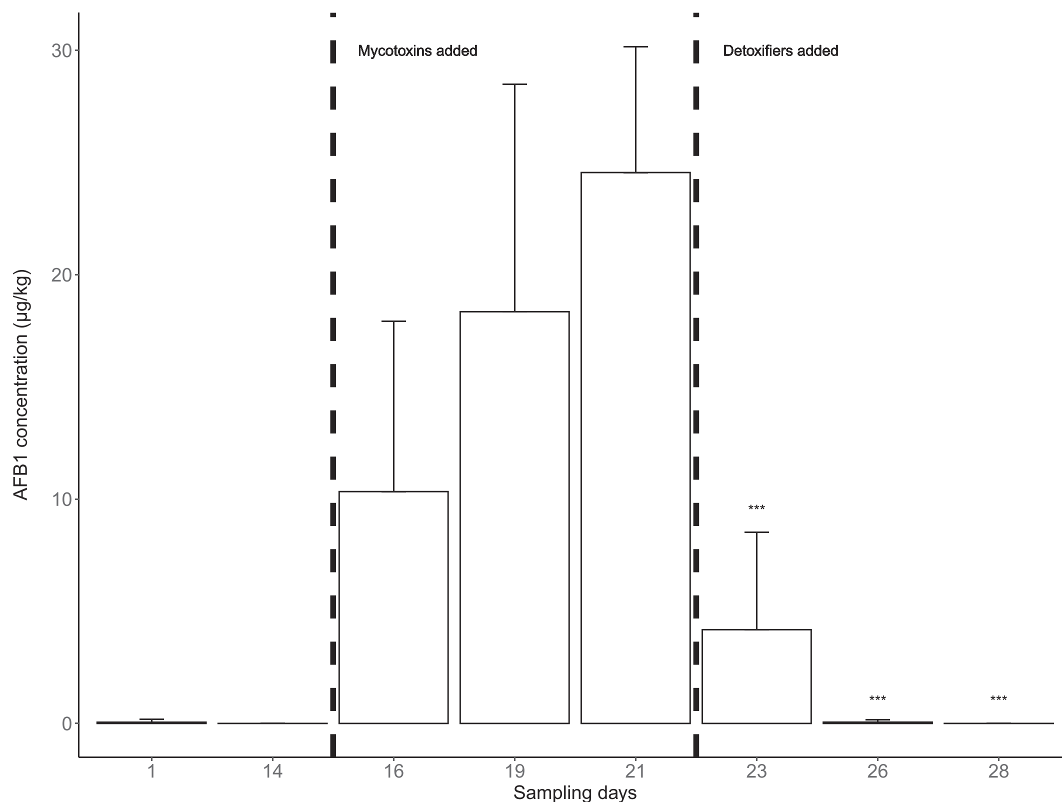

Fig. 4. Mean concentration of aflatoxin B1 (AFB1) determined in the AC, TC and DC compartment at the different sampling days during the SHIME experiment (n = 2). Error bars represent the standard deviation. The dashed lines represent the addition of AFB1 (81.6 µg/kg food) and fumonisin B1 (2000 µg/kg 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.

slight decrease from T0 to T1 was due to natural fluctuations in time or method related variability. In the study of Zhou et al. (2018), the oral exposure of rats to 5, 25 and 75 µg AFB1/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 (Bernalier-Donadille, 2010; Louis, Hold, & Flint, 2014). 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 (Hernández, Canfora, Jocken, & Blaak, 2019).

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 (Cinquin et al., 2006; Reichardt et al., 2014; Van Den Abbeele et al., 2010).

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 (Bondue, Lebrun, Taminiau, Everaert, LaPointe, Hendrick, & et al., 2020).

As for propionate, butyrate is produced by a restricted number of bacterial groups. Butyrate can be produced using 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 (Louis et al., 2014). Therefore, the production of butyrate occurs in the distal part of intestinal microbiota, where acetate is high. The main butyrate producers are *Faecalibacterium prausnitzii* (*Clostridium* cluster IV), *Anaerostipes*, *Eubacterium* and *Roseburia* spp. (*Clostridium* cluster XIVa) (Rivière, Selak, Lantin, Leroy, & De Vuyst, 2016). 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 repetitions, indicating anaerobic conditions were maintained in the system (Bondue, Lebrun, Taminiau, Everaert, LaPointe, Hendrick, & et al., 2020; Onrust et al., 2015).

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.

3.3. Metagenetic analysis

3.3.1. 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 inversed Simpson diversity index were observed on genus level in AC, TC, DC nor in the total GIT (Figs. S4-7) after administration of mycotoxins and detoxifiers to the SHIME system. This is in contrast to the study of Mateos et al. (2018), where six piglets were exposed to fumonisins (10.2 mg FB1, 2.5 mg FB2 and 1.5 mg FB3/kg feed), and after 15 days of exposure the inversed Simpson index significantly decreased compared to the control. For AFB1, Yang et al. (2020) 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 (Fig. 6). 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

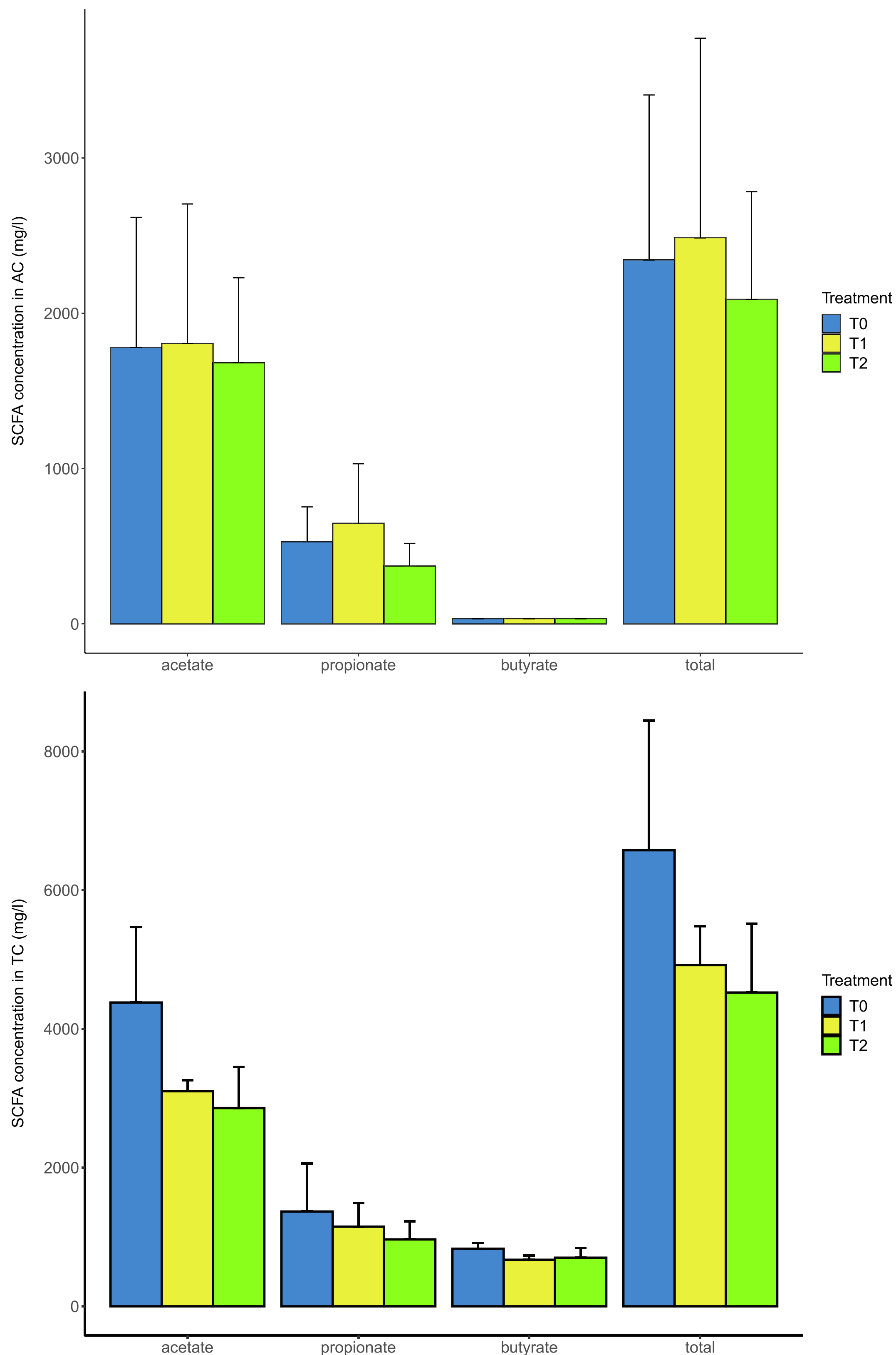

Fig. 5. Mean concentrations of short-chain fatty acids (SCFAs) in the a) ascending (AC), b) transverse (TC) and c) descending colon (DC) ($n = 2$). Error bars represent the standard deviation. Total is the sum of acetate, propionate, butyrate, isobutyrate and isovalerate. T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 μg/kg food) and fumonisin B1 (FB1) (2000 μg/kg food); T2 = after one week of addition of AFB1 (81.6 μg/kg food) and FB1 (2000 μg/kg food), together with bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food).

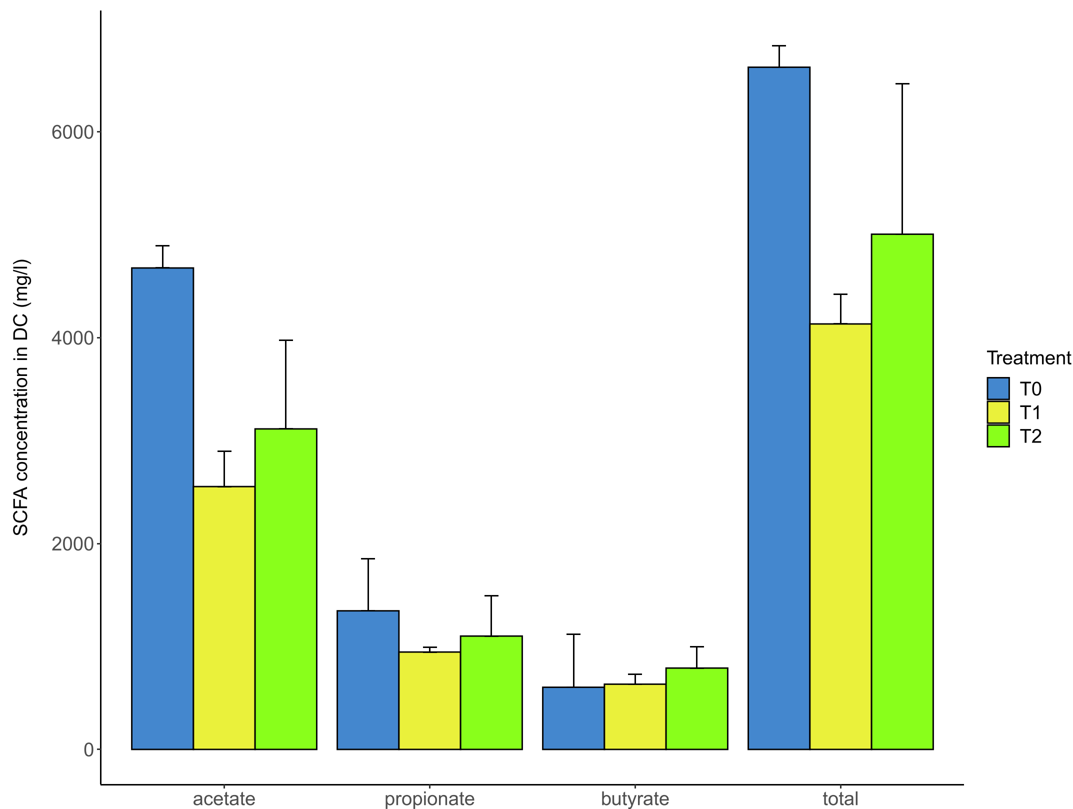

Fig. 5. (*continued*).

diversity (Bondue, Lebrun, Taminiau, Everaert, LaPointe, Crèvecoeur, et al., 2020; Van Den Abbeele et al., 2010). AC was dominated by the genus *Veillonella*, whereas TC and DC are mainly composed of *Lachnoclostridium*, *Bacteroides* and unidentified genera from the Lachnospiraceae family.

3.3.2. Beta diversity

Across all the phyla (Fig. 7), families (Fig. 8) and genera (Figs. 9 and 10), 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. (2018), where Firmicutes (82%) were most abundant, followed by Bacteroidetes (14%), Proteobacteria (1.8%), Spirochaetes (1.5%) and Actinobacteria (0.7%). Similarly, Grosu et al. (2019) found Firmicutes (58.3%), Bacteroidetes (34.5%), Proteobacteria (1.2%) and 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. (2019), 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 Fig. 7.

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, Lebrun, Taminiau, Everaert, LaPointe, Hendrick, & et al. (2020). Similar to the families found in piglet faeces by Mateos et al. (2018), *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. (2018), a decrease in proportion of *Actinobacteria*, *Proteobacteria, Lachnospiraceae* and *Veillonellaceae* was

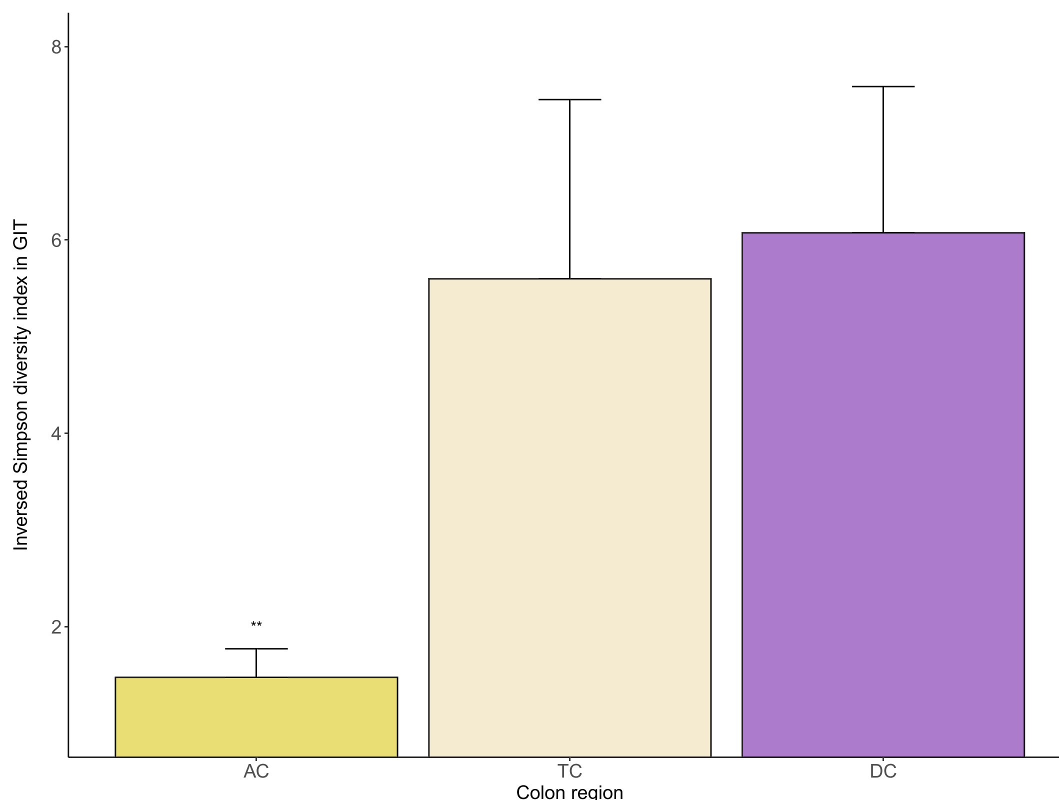

Fig. 6. Inversed Simpson diversity index means per colon region in the gastrointestinal tract (GIT) on genus level (mean ± SD). The colon regions stand for ascending colon (AC), transverse colon (TC) and descending colon (DC). T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 μg/kg food) and fumonisin B1 (FB1) (2000 μg/kg food); T2 = after one week of addition of AFB1 (81.6 μg/kg food) and FB1 (2000 μg/kg food), together with bentonite clay (2.5 g/kg food) and fumonisin esterase (60 U/kg food). The asterisks represent the significant statistical difference in AC ($p < 0.01$) compared to TC and DC.

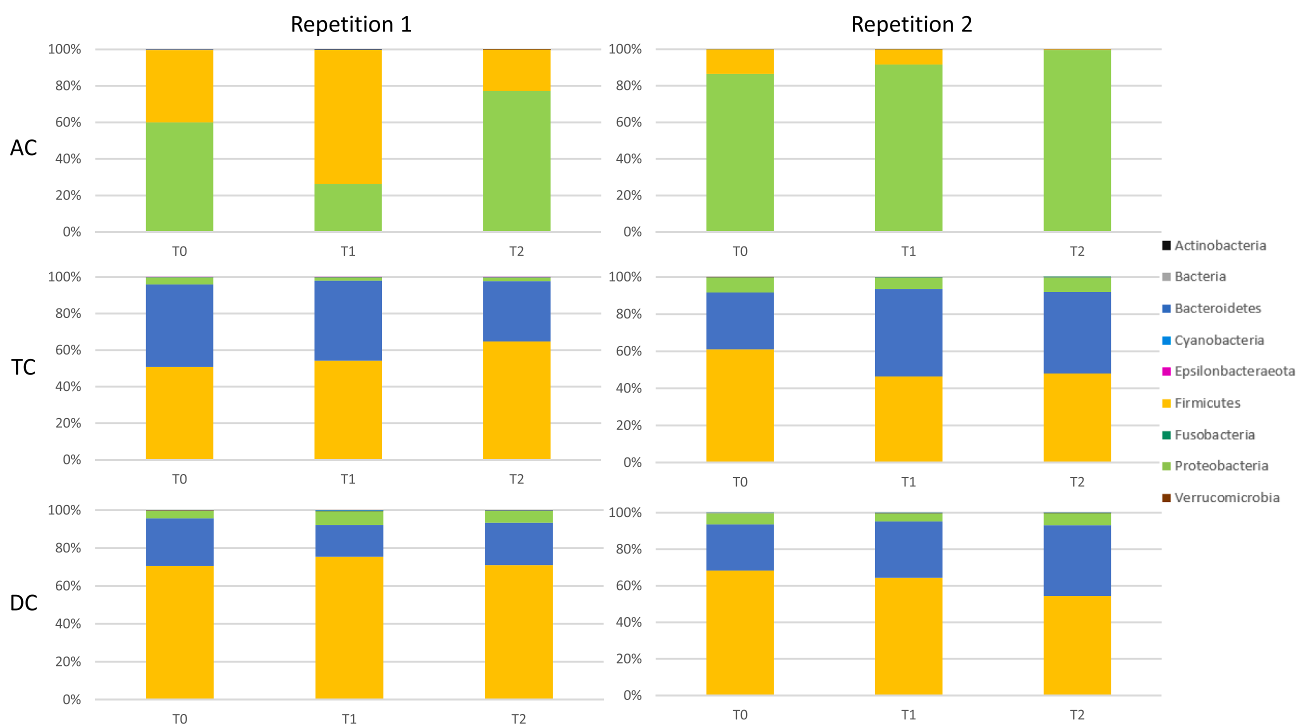

Fig. 7. Microbial phyla present in the SHIME samples at T0, T1 and T2, in the AC, TC and DC of both repetitions. T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 μg/kg food) and fumonisin B1 (FB1) (2000 μg/kg food); T2 = after one week of addition of AFB1 (81.6 μg/kg food) and FB1 (2000 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.

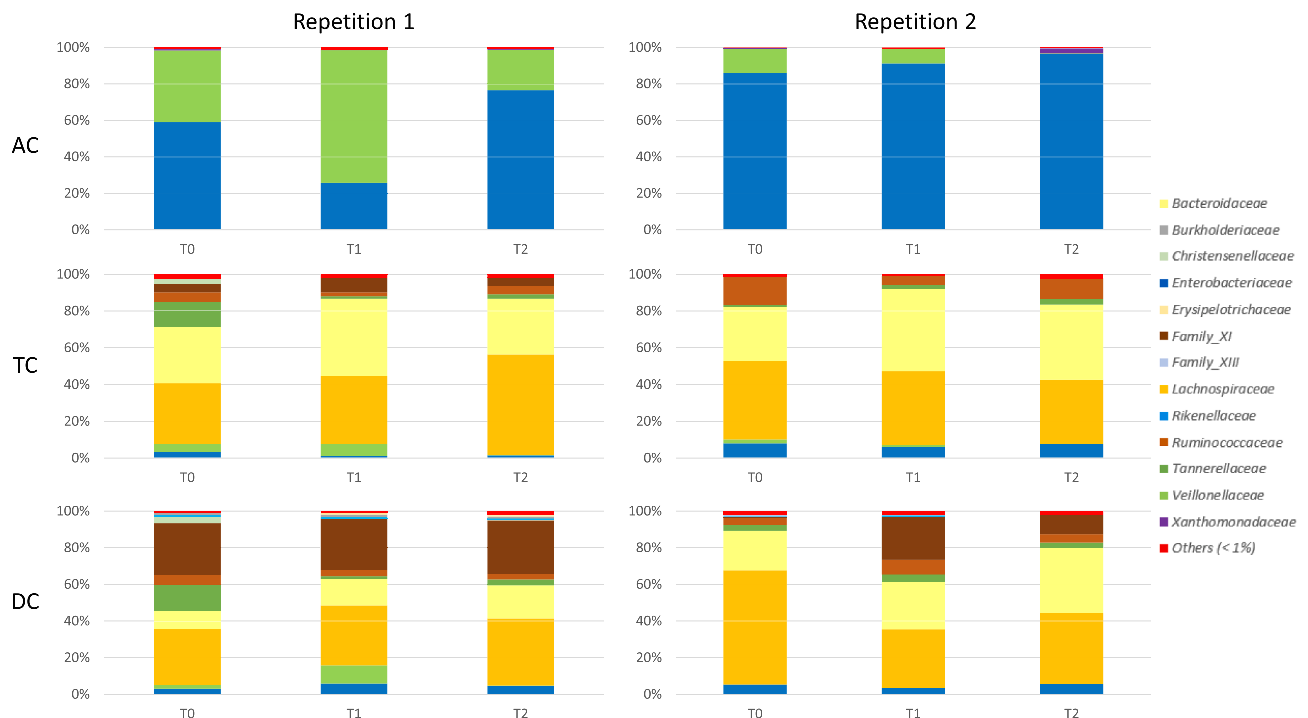

Fig. 8. Microbial families present in the SHIME samples at T0, T1 and T2, in the AC, TC and DC of both repetitions. T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 μg/kg food) and fumonisin B1 (FB1) (2000 μg/kg food); T2 = after one week of addition of AFB1 (81.6 μg/kg food) and FB1 (2000 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.

Fig. 9. 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) (2000 μg/kg food); T2 = after one week of addition of AFB1 (81.6 μg/kg food) and FB1 (2000 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.

observed in piglets after an exposure period to a combination of fumonisin B1, B2 and B3 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. (2018), 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. (2017), 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 (Jiang et al., 2020). Furthermore,

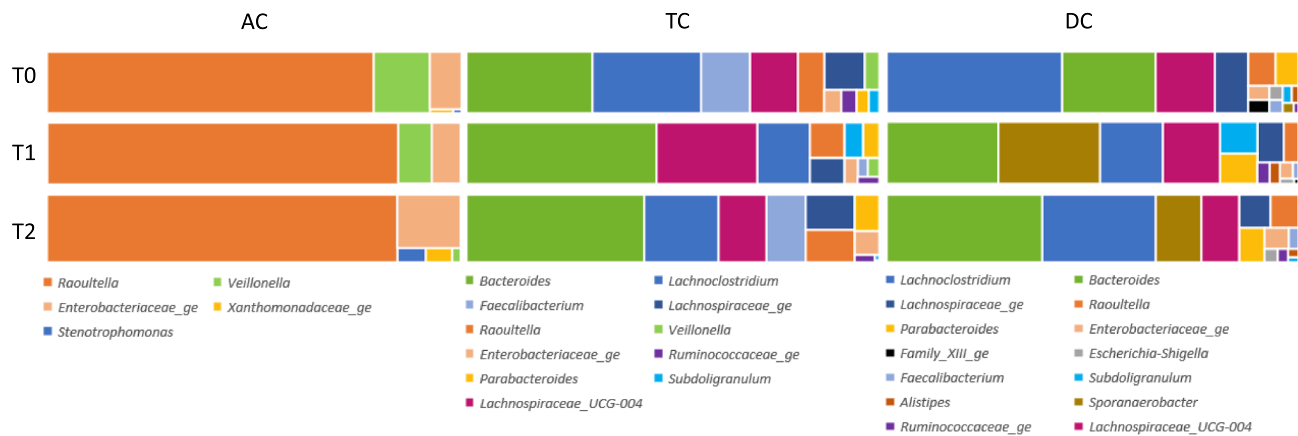

Fig. 10. Tree map of microbial genera present (≥1%) in the second repetition in %, at T0, T1 and T2, in the AC, TC and DC. T0 = after two weeks of stabilisation and before addition of mycotoxins; T1 = after one week of addition of aflatoxin B1 (AFB1) (81.6 μg/kg food) and fumonisin B1 (FB1) (2000 μg/kg food); T2 = after one week of addition of AFB1 (81.6 μg/kg food) and FB1 (2000 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.

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.

4. 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 FB; 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

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 detoxifiers Mycofix® Secure and FUM*zyme*® were provided by BIOMIN Research Center, Tulln, Austria.

CRediT authorship contribution statement

Kaat Neckermann: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Gregor Claus:** Formal analysis, Writing - review & editing. **Siegrid De Baere:** Conceptualization, Validation. **Gunther Antonissen:** Conceptualization, Funding acquisition, Project administration. **Sarah Lebrun:** Conceptualization, Methodology, Investigation. **Céline Gemmi:** Investigation. **Bernard Taminiau:** Formal analysis. **Caroline Douny:** Formal analysis, Writing - review & editing. **Marie-Louise Scippo:** Writing - review & editing, Funding acquisition, Project administration. **Dian Schatzmayr:** Conceptualization, Resources. **James Gathumbi:** Funding acquisition, Project administration. **Silvio Uhlig:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition, Project administration. **Siska Croubels:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition, Project administration. **Véronique Delcenserie:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

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

Acknowledgements

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.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110395.

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