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Monitoring and evaluation of the interaction between deoxynivalenol and gut microbiota in Wistar rats by mass spectrometry-based metabolomics and next-generation sequencing

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ABSTRACT

Published evidence has demonstrated the several toxic characteristics of mycotoxins and their considerable risk to human and animal health. One of the most common uncertainties regards whether if very low concentrations of the mycotoxin deoxynivalenol (DON), easily consumed within the Mediterranean Diet, can cause metabolic alterations; some of them produced by the interaction between DON and gut microbiota. Accordingly, faecal samples were collected from Wistar rats that had consumed the mycotoxin DON at low levels (60 and 120 μg kg^{-1} body weight of DON per day), and were analysed by ultra-high performance liquid chromatography coupled with tandem mass spectrometry detection, in order to monitor the mycotoxin DON and its metabolite de-epoxy deoxynivalenol (DOM-1). The obtained results showed an evolution in DON excretion and the metabolite DOM-1 which has less toxic properties, over the course of the days of the study. To elucidate whether intestinal microbiota had a role in the observed detoxification process, the changes in microbial gut biodiversity were explored through 16s rRNA high throughput sequencing. No main changes were detected but significant increase in Coprococcus genus relative abundance was found. Further studies are needed to confirm if intestinal microbiota composition and function are affected by low mycotoxin concentrations.

1. Introduction

The mycotoxin deoxynivalenol (DON) is a secondary metabolite produced by several Fusarium molds and it is considered the most widespread mycotoxin in food and feed matrices (Payros et al., 2016), especially in cereals and cereal-based products. The consumption of DON may have numerous hazardous effects, ranging from vomiting, diarrhoea, gastroenteritis, growth impairment and immune dysfunction (Pestka, 2007, 2010), to leukopenia, haemorrhage, endotoxemia and shock-like death (Pestka, 2010). The toxicology of DON is dose-dependent and its susceptibility is determined by differences between animal species in terms of DON metabolism, absorption, distribution and secretion. Thus, pigs are the most sensitive animals, while rats have medium sensitivity and ruminants are the least sensitive (Pestka, 2007). There are certain toxicological parameters for examining the degree of toxicity of hazardous compounds, such as the median lethal dose (LD50), the minimum emetic dose (MED) and the no-observed adverse effect level (NOAEL). These parameters also vary between species and the type of administration. In the case of orally administered DON in rats, the estimated NOAEL was found between 150 μg kg^{-1} body weight (b.w.)/day (Pestka, 2007, 2010; European Commission, 1999) and 500 μg kg^{-1} b. w./day (Pestka, 2010; European Commission, 1999), which means that, at this dose, no adverse effects should be observed, but does not mean that intestinal microbiota modifications may not occur. European regulations have set maximum levels for DON in different kind of matrices, especially in cereal and cereal derivatives (Commission Regulation (EC) No. 1126/2007 of 28 September 2007, 2007), varying from 500 μg kg^{-1} to 1750 μg kg^{-1} in adult foodstuffs, to 200 μg kg^{-1} in foods for infants and young children.

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Once DON is consumed, it is mostly absorbed and rapidly distributed to all tissues, with blood, the gastrointestinal tract, the lymphatic system and the immune system being the main targets. Then, it is eventually eliminated, mostly in urine and faeces (Payros et al., 2016), without bioaccumulation, which means that trace concentrations found in food of animal origin are not a public health problem (Pestka, 2007). Of the entire DON structure, the 12,13-epoxy-trichothec-9-ene skeleton can be modified by intestinal bacteria in order to detoxify the mycotoxin. It is known that the 12,13-epoxide group is responsible for the elevated toxicity of the molecule and, specifically, this group is removed by intestinal microbiota for DON detoxification, generating the toxin de-epoxy deoxynivalenol (DOM-1), which is less toxic than its precursor (Payros et al., 2016). This modification of DON into DOM-1 has been observed *in vitro* (Binder et al., 1997; Gratz et al., 2013) and also *in vivo* (Turner et al., 2010) experiments, mostly by anaerobic bacteria from the gut lumen (Maresca, 2013). Nevertheless, not all bacteria can generate DOM-1 through DON detoxification (Karlovsky, 2011). Some researchers suggest that chronic consumption of certain mycotoxins may change the intestinal microbiota in order to try to acquire or increase the detoxification capability (Maresca, 2013; Eriksen et al., 2002). Thus, it is reported that the presence of ochratoxin A and DON can modify animals' intestinal functions (Maresca, 2013) and intestinal microbiota (Guo et al., 2014; Saint-Cyr et al., 2013). In 2009, it was demonstrated for the first time that DON modifies intestinal microflora in pigs (Waché et al., 2009). However, as yet, there has been no identification of the taxonomic categories responsible of these changes in the gut microbiota, which may differ between animal species.

The main objective of the present research is to evaluate gut microbiota to find the bacteria population responsible of the DON metabolism through its administration at NOAEL. Over the course of the present work, the concentration of DON and its derivative DOM-1 in rat faeces were determined by ultra-high performance liquid chromatography coupled with tandem mass spectrometry detection (UHPLC-MS/MS), to monitor and evaluate the possible changes in mycotoxin metabolism. Since the dose of DON administered to rats is low, the presence of this mycotoxin together with their metabolites deoxynivalenol-3-glucoside (DON3G), 3-acetyl-deoxynivalenol (3AcDON) and 15-acetyl-deoxynivalenol (15AcDON) easily found in cereal samples (European Food Safety Authority and EFSA, 2017), was also controlled. These derivatives are easily metabolised to DON by rat gut intestinal microflora (European Food Safety Authority and EFSA, 2017) and their presence might interfere the results of the present research.

2. Results

2.1. Mycotoxin monitoring with UHPLC-MS/MS

2.1.1. UHPLC-MS/MS parameters optimisation

The instrumental optimisation was done for DON and its metabolite DOM-1, but also for DON3G, 3AcDON and 15AcDON, in order to control their possible presence in feed samples. The mobile phase and the chromatographic gradient was selected in accordance with previous research studies in which DON and DON derivatives were separated, except for DOM-1 (Miró-abella et al., 2018). Under these conditions, DOM-1 was injected individually by flow injection analysis (FIA) and its precursor and product ions were selected, with its optimised collision energy. The precursor and product ions for DON, DON3G, 3AcDON and 15AcDON were the same as in the previous research (Miró-abella et al., 2018), ionised in positive mode. DOM-1 also displayed greater abundance in positive mode, with the $[M+H]^+$ ion. Three MRM transitions were selected for every target compound, as detailed in Table 1, so as to enable the correct identification of the mycotoxins in accordance with the recommendations of the EU Directive (SANTE, 2015).

With respect to instrumental optimisation, the detection limits (LOD) and the quantification limits (LOQ) were established by assuming the criteria of a signal-to-noise ratio (S/N) equivalent to 3 for LOD and equivalent to 10 for LOQ. Thus, LODs ranged from $0.01\,\mu g\,L^{-1}$ to $0.25\,\mu g\,L^{-1}$, and LOQs from $0.02\,\mu g\,L^{-1}$ to $0.5\,\mu g\,L^{-1}$. Suitable linearity was obtained (with $r^2 \geq 0.994$) of the linear range which was from LOQ to $200\,\mu g\,L^{-1}$ for DON, 3AcDON and 15AcDON, to $100\,\mu g\,L^{-1}$ for DON3G, and to $40\,\mu g\,L^{-1}$ for DOM-1.

2.1.2. Faecal extraction optimisation

Since only DON and DOM-1 are expected to be quantified in the faecal samples, the method optimisation was developed only for these two mycotoxins. The selection of the solid liquid extraction technique took into account previous studies that extracted mycotoxins from faecal samples (Nagl et al., 2012, 2014; Schwartz-Zimmermann et al., 2014). The selected method was adapted from the literature (Nagl et al., 2012, 2014; Schwartz-Zimmermann et al., 2014), but using a small quantity of faeces, less organic solvents and avoiding a final dilution step if the matrix effects (ME) were sufficiently low to quantify at lower concentration levels.

First of all, faecal samples were analysed as to detect and quantify the possible natural presence of the target mycotoxins, DON and DOM-1, and no presence of these mycotoxins was observed. For the method

Table 1
Parameters of LC-MS/MS for mycotoxin determination in feed and faecal samples.

Start time (min)	Mycotoxin	Retention time (min)	Precursor ion (*m/z*)		Dwell Time (ms)	Product ion (*m/z*) and q/Q relation	CE (eV)
0	DON	2.9	297.1	$[DON + H]^+$	90	248.9 (100)	8
						231.1 (55)	10
						203.1 (60)	8
	DON3G	3.6	297.1	[DON3G – 3G + H]$^+$		248.9 (100)	8
					231.1 (60)	10	
					203.1 (50)	8	
4.4	DOM-1	5.8	281.0	$[DOM\text{-}1 + H]^+$	180	233.2 (100)	8
						109.1 (96)	14
						215.1 (83)	10
7.5	3AcDON	9.8	339.2	$[3AcDON + H]^+$	90	231.0 (100)	8
						203.0 (44)	24
						175.0 (30)	18
	15AcDON	10.1	356.2	$[15AcDON + NH_4]^+$		339.1 (100)	16
					321.0 (38)	12	
					136.9 (35)	4	

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optimisation, faecal samples were spiked and were blended with 1 mL of two different solvents in order to ascertain which is better for extraction: MeOH and MeOH 1% of HCOOH. These samples were sonicated for 15 min, and centrifuged at 15,000 rpm for 10 min. A volume of 700 μL of supernatant which is the maximum quantity of solvent possible to subtract, was evaporated to dryness under nitrogen flow. The extraction was performed twice with the purpose of assessing whether a second extraction was necessary in order to completely extract the mycotoxins. To do so, 1 mL of solvent was added to the wet faecal samples repeating the same procedure as before, but 1 mL of supernatant was evaporated to dryness, instead than 700 μL. Samples were re-dissolved in 1 mL of MeOH/water (20:80, v/v), filtered and analysed by UHPLC-MS/MS.

To obtain quantifiable results, extraction recovery (ER) and the ME were considered. The presence of ME can interfere and cause a systematic error in the determination of the analyte of interest, distorting the signal by enhancing or suppressing it. Thus, the determination of the ME as well as the identification of the %ER, are useful tools for the method validation, which were calculated as follows:

$$\%ER = (C\ before - C\ non\text{-}spiked) / (C\ after - C\ non\text{-}spiked)\ x100, \quad (1)$$

$$\%ME = [(C\ after - C\ non\text{-}spiked) / (C\ calibration\ curve)\ x100] - 100, \quad (2)$$

C indicates concentration, ME = 0 indicates no ME, ME > 0 indicates ion enhancement and ME < 0 indicates ion suppression. The obtained %ER from both types of extraction solvents were similar but slightly higher in the case of MeOH alone (detailed in Table 2) than in the case of MeOH with HCOOH (which were 50% and 70% for DON and DOM-1, respectively). The extraction results obtained from the second extraction were below than 10% and, consequently, one extraction was considered sufficient. Regarding the ME in the tested faecal samples, there was ion suppression due the elevated presence of interferences since the obtained percentage results were −67% for DON and −62% for DOM-1. These results were similar to those obtained from faeces extractions in previous studies (Nagl et al., 2012, 2014). In order to reduce these %ME, samples were diluted 1:1 with MeOH/water (20:80, v/v) before injection into the UHPLC-MS/MS. As a result, MEs were significantly decreased, as detailed in Table 2. Considering these results, faecal sample extractions were finally extracted once with MeOH and re-suspended after supernatant evaporation with 2 mL of MeOH/water (20:80, v/v), since more diluted extracts obtained better results. Obtained method limits for DON and DOM-1 were appropriate for their detection and quantification in faeces and are detailed in Table 2, together with the linearity of the method which was acceptable for both mycotoxins with the r^2 higher than 0.996.

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2.1.3. Faecal mycotoxin monitoring

First of all, the feed given to the rats was analysed in order to quantify the possible presence of natural mycotoxins which could contribute to consumption. For that, the presence of DON, DON3G, DOM-1, 3AcDON and 15AcDON was determined. DON was quantified in the tested samples at 3.8 μg kg^{-1}, and the rest of the mycotoxins were not detected. Taking into account the weekly feed controls –each rat consumed daily an average of 24 g of feed– and the daily DON dose administered, the consumption of DON through the feed was below 0.5% of the dose. Thus, the amount of DON consumed through the feed is insignificant and it was discarded.

The target mycotoxins DON and DOM-1, which are those involved in the de-epoxidation process, were monitored and quantified in each faecal sample (35 samples per subject) in order to assess possible concentration variations on all the days of the study. Faecal samples were monitored due to the main excretion of DON metabolites by faeces in rats (Lake et al., 1987).

The quantity of DON and DOM-1 excreted in faeces ranged from 20 μg kg^{-1} to 230 μg kg^{-1} and from 5 μg kg^{-1} to 50 μg kg^{-1}, respectively. These values are very low in comparison to the DON consumed, and it may be due to the excretion of DON sulfonates and DOM sulfonates as major DON metabolites in faeces from rats treated with DON (Schwartz-Zimmermann et al., 2014). From all the faecal concentrations found during the present study, different comparisons were performed between groups using the Student's t-test statistic. As a result, significant differences were found between the control group and the group treated with 60 μg kg^{-1} b.w. of DON per day (P60) and between the control group and the group treated with 120 μg kg^{-1} b.w. of DON per da (P120) in the case of both mycotoxins, DON and DOM-1. At all time points, the concentration of DON found between P60 and P120 was higher than those found in the control faeces. Moreover, comparing both groups of rats that consumed DON at different concentrations (P60 and P120 groups), there were no significant differences in the DON concentration levels present in faeces.

Regarding DOM-1, its concentration is higher in P120 than in P60, in almost all the weeks of study as it is detailed in Fig. 1. The presence of DOM-1 in faecal samples also increased over the seven weeks of study, especially after the fifth week of DON consumption (Fig. 1). The gradual increase in the DOM-1 concentration level was as expected, although the great increase produced in the fifth week surprised the authors. This increase is more prominent in the faeces from rats that con-

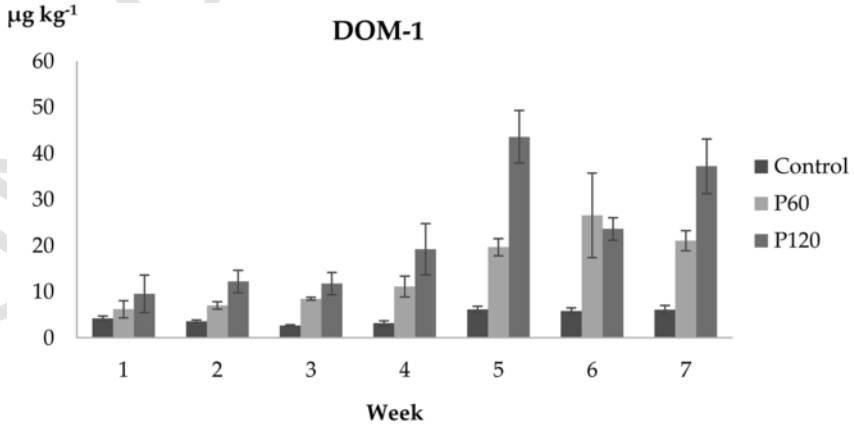

Fig. 1. Concentration of DOM-1 quantified in faecal samples over the 7 weeks of study. Statistic significant differences are shown (one-way ANOVA with Bonferroni post-hoc correction), * $p < 0.05$; ** $p < 0.01$.

sumed 120 μg kg^{-1} per dose, than in faeces from rats that consumed 60 μg kg^{-1} per dose, in which the growth is more progressive. Additional unexpected results were those obtained during the sixth week of the research, where the concentration excreted of DOM-1 decreased instead than increase, as it was expected. Although one should take also into account the great variability of P60 results at sixth week.

2.2. Microbial diversity analysis by high-throughput sequencing

In order to determine and quantify differences between the intestinal microbiota compositions of the three groups in the present research, metagenomic analysis was performed at the end point. The sequencing run produced a total of 6,697,663 paired-end reads that were reduced to 4,117,943 readings after quality filtering. The criteria for quality filtering considered that reads shorter than 50 bp were removed as well as reads with Phred score under 20. These readings were then analysed with QIIME and SPSS. The two phyla Bacteroidetes and Firmicutes dominated the community in all samples (Fig. 2) with varying relative abundance (16.7–45.5% and 52.2–82.2%, respectively), which is consistent with previously published mammalian gut microbiome descriptions (Del Bas et al., 2018; Lamendella et al., 2011).

When the microbial relative abundance between groups was compared, no difference was found at any taxonomic level, except at genus level. The one-way ANOVA test revealed that the relative abundance of the Coprococcus genus was slightly higher in the P60 group compared to the control group (0.24% vs 1.6%, $p = 0.039$) and also higher in the P120 group than in the control group (0.24% vs 1.7%, $p = 0.030$), data not shown. However, when a Kurskall-Wallis test was done with multiple test correction using false descovery rate (q), onlyFig. 3 significant differences were found in Coprococcus genus abundance when treated rats (independently of the dose) and control group were compared (Figure 3b, $q = 0.031$). Those differences were not found when both treated groups were separately compared with control group (Figure 3a, $q = 0.34$).

3. Discussion

As has already been hypothesised (Maresca, 2013; Eriksen et al., 2002), the observed rise of the faecal presence of DOM-1 must presumably be caused by gut microbiota increasing detoxification capability. Probably, intestinal microbiota from rats increased the detoxification ability during the treatment. These results can be compared with a study of faecal samples from pigs (Eriksen et al., 2002) in which the de-epoxidation ability was found only in animals fed with contaminated feed from the second week of exposure onwards. Additionally, the intestinal microbiota composition was different between animals that had increased the ability to de-epoxidate mycotoxins in comparison to animals that were not exposed to mycotoxins.

Regarding the microbial diversity analysis, the gut abundance of Coprococcus has changed due the treatment with the mycotoxin DON.

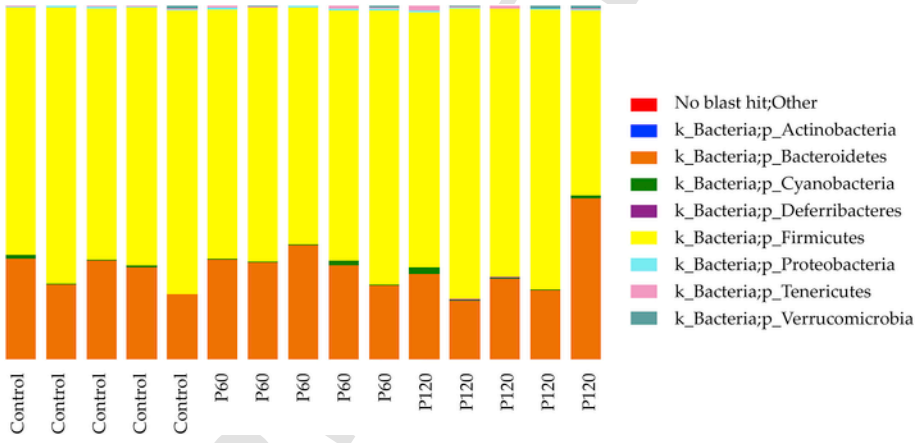

Fig. 2. Phylum-level taxonomic distribution. Bars represent the relative abundance (percentage) of each phylum detected per sample.

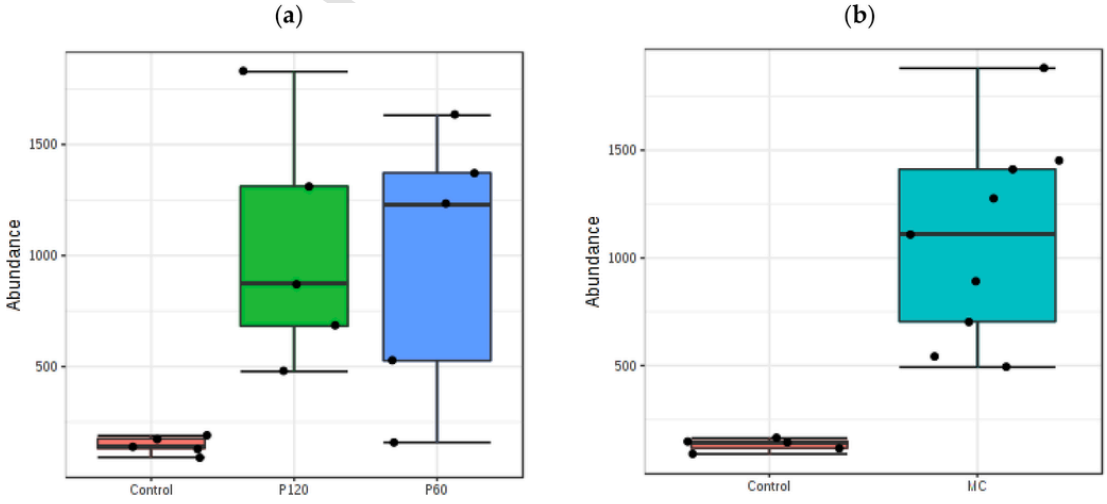

Fig. 3. Graphics from Kurskall-Wallis tests comparing the abundance of the Coprococcus genus from the tested groups (applying false discovery rate correction). Abundance refers to the number of readings for the Coprococcus genus. (a) Comparison between the group control, P60 and P120; (b) Comparison between the treated and the non-treated rats; P60 + P120 refers to a whole group of treated rats with DON (P60 together with P120), *$q < 0.05$.

However, Coprococcus belongs to a group of anaerobic cocci that are known to produce butyrate, which is an essential metabolite in the human colon. Butyrate is the preferred energy source for the colon epithelial cells. It contributes to the maintenance of the gut barrier functions, and has immunomodulatory and anti-inflammatory properties (Rivière et al., 2016). Evidence indicating that disruption of the intestinal epithelial barrier due to DON exposure is well established (Robert et al., 2017). Further research is needed to know if the Coprococcus genus can play a role in the detoxification of DON, which may explain the increased amount of DOM-1 in faeces, described in the UHPLC-MS/MS results section.

Nevertheless, the results reported here do not agree with already published *in vivo* studies. For example, Saint-Cyr and colleagues (Saint-Cyr et al., 2013) used quantitative PCR to determine an increment of Bacteroides and Prevotella genera, as well as a decrease in Escherichia coli in stool samples from rats after the administration of 100 $\mu g\,kg^{-1}$ b. w. for four weeks. These findings were not corroborated, probably because the microbiota of these rats was of human origin and transplanted. Another study by Waché and colleagues (Waché et al., 2009) reported that DON had a moderate effect on cultivable bacteria and on capillary electrophoresis single strand conformation polymorphism patterns corresponding to the Eubacteria genus in the pig intestine. They found that aerobic mesophilic bacteria increased while anaerobic sulphite-reducing bacteria remained unchanged. These outcomes were not replicated in our tests nor in a later study conducted also on a pig model (Piotrowska et al., 2014), in which, moreover, no change in microbiota composition in response to DON administration was found. However, our study is the first to apply a metagenomic approach sequencing regions of 16s RNA gene, providing a general overview of microbiota composition, and all the previous publications mentioned only looked for specific bacteria. Therefore a variation in the Coprococcus genus may have gone unnoticed.

Additionally, some *in vitro* studies have demonstrated the ability of certain bacterial species to promote DON metabolism, by binding or detoxification (Gratz et al., 2013; Kollarczik et al., 1994; Zou et al., 2012; Young et al., 2007), but none of the genus where these species belong to were found to be significantly increased in our treated groups. An explanation for this may be that these species do not react to mycotoxin exposure *in vivo* or perhaps they respond by increasing gene expression of proteins related to DON detoxification rather than by increasing their cellular abundance.

Overall, technical issues coupled with the dose and the extent and duration of treatment, as well as the animal model, could explain the discordances between the results of the *in vitro* studies published and our *in vivo* study.

4. Conclusions

Mycotoxins are fungal secondary metabolites, potentially hazardous to human and animal health following direct consumption through contaminated food or feed. The impact of mycotoxin consumption in microbial gut composition has already been demonstrated and our results support the hypothesis that microbiota composition slightly changes in response to mycotoxin consumption. In this regard, several *in vitro* and *in vivo* studies have identified a modulation of certain groups of intestinal bacteria due to mycotoxin exposure but, to date, this is the first metagenomic study that assesses the response of gut microbial composition to DON administration. An increase in DOM-1 was found in faecal samples due to mycotoxin consumption and a correlation of this rise with a significant increase in the relative abundance of the Coprococcus genus.

Long-term exposure to mycotoxins may produce significant changes in microbiota composition and their metabolic activity, and these issues require further experimentation to elucidate the mechanism of action in order to promote them and find a new way of preventing or treating the effects of mycotoxin. Thus, further *in vivo* and *in vitro* studies are needed to shed some light on the response of microbiota to mycotoxins. According to present knowledge, the identification of specific bacterial genus or species that have detoxification capability opens the possibility of their use as feed additives (Vinderola and Ritieni, 2014). For example, *Eubacterium* strains reduce the epoxy group from mycotoxins and their effect has already been reported in chicken models (Awad et al., 2006). Thus, the administration of this species as feed additives in high-probably exposed populations could prevent, palliate or even restore the chronic damage caused by DON and other mycotoxins.

However, metagenomic analysis is restricted to the identification of microbial diversity, while the molecular functionality of this community remains ignored. As most biological mechanisms involve more than one type of biomolecule, further studies should combine multiple omic strategies (i.e. metagenomics and metaproteomics) to achieve a comprehensive, structured and interactive overview of the mycotoxin-microbiota interplay.

5. Materials and methods

5.1. Experimental design

Male Wistar rats (7-weeks-old, 200–240 g b. w.) were purchased from Charles River Laboratories (Barcelona, Spain). Water and radiated pelleted feed from Teklad Global Diets (Madison, WI, USA), free of fungi that can generate mycotoxins, were provided ad libitum. The eighteen rats were housed individually in polycarbonate cages under controlled conditions in terms of temperature (22 ± 2 °C), humidity (50–60% relative humidity) and a light/dark cycle (12h). The animals were acclimatised for five days and were distributed fairly into three groups taking into account their b. w. and their contents in fat, lean tissue, free water and total water, obtained through Nuclear Magnetic Resonance (NMR) measurements. The study groups (N = 6) were depending on the mycotoxin administration: P60 group was treated with 60 $\mu g\,kg^{-1}$ b.w. per day of DON and P120 group was treated with 120 $\mu g\,kg^{-1}$ b.w. of DON per day, using in all cases 600 μL of diluted condensed milk, five days per week for eight weeks. Following the same administration protocol, 600 μL of diluted condensed milk were used as the vehicle for the control group. The individual dose of toxin was revised weekly according to their b.w. Faecal samples were individually collected five days per week for seven weeks and stored at −80 °C until analysis. Water, feed and b. w. controls were performed weekly. At the end of the eighth week of study, the rats were anaesthetised using pentobarbital sodium and then decapitated. The kidneys, liver, brain, muscle, the caecum and the heart were removed and weighed as a control parameter, and caecum was frozen with liquid nitrogen and stored at −80 °C until metagenomic analysis. The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all of the procedures.

5.2. Standard mycotoxin solutions

Methanol (MeOH, for LC-MS grade) and ethanol (EtOH) were purchased from Panreac (Barcelona, Spain), and formic acid (HCOOH) ~98% was acquired from Fluka (St. Louis, MO, USA). Acetone, 10 M ammonium formate (NH_4HCOO) aqueous solution and pentobarbital sodium salt were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure-grade water was obtained by a Milli-Q water purification system (Millipore, Darmstadt, Germany).

The target mycotoxins were DON and DOM-1, both obtained from Bioser (Barcelona, Spain), and DON3G, 3AcDON and 15AcDON were purchased from Romer Labs (Union, MO, USA). DON, 3AcDON and 15AcDON were obtained in powder form and DOM-1 and DON3G were

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purchased in acetonitrile (ACN) solution at 50 mg L^{-1} and at 50.9 mg L^{-1}, respectively. DON was dissolved at $1.8{\cdot}10^{3}$ mg L^{-1} and 3AcDON and 15AcDON were dissolved at 104 mg L^{-1}, all in water/EtOH (80:20, v/v) and stored at 4 °C during the experiment. A mixed stock solution of all mycotoxins was prepared at different concentrations depending on their response in (ESI)MS/MS, in order to obtain similar response values. Thus, DON, DON3G, 3AcDON and 15AcDON were at 0.5 mg L^{-1} and DOM-1 was at 0.1 mg L^{-1}, in water/MeOH (80:20, v/v), and this mix solution was stored at −20 °C for six months.

5.3. Faecal mycotoxin monitoring

5.3.1. Mycotoxin faecal sample extraction and method validation

Aliquots of 100 mg of freeze-dried and homogenised faecal samples were blended with 1 mL MeOH and sonicated for 15 min. Samples were centrifuged at 15,000 rpm for 10 min, and 700 μL aliquots of supernatant were evaporated to dryness under nitrogen air and re-suspended in 2 mL of MeOH/water (20:80, v/v). Before analysis by UHPLC-(ESI)MS/MS, the extracts were filtered with a 0.45 μm nylon filter (Phenomenex, Torrance, CA, USA) and stored at 4 °C. For the method optimisation, faecal samples without the presence of mycotoxins, or the least possible presence, were used to obtain the ER and ME values. To do so, 100 mg of homogenised freeze-dried faecal samples were spiked with the mycotoxins at two different concentrations: DON was at 50 μg kg^{-1} and DOM-1 was at 10 μg kg^{-1}. Samples were spiked with 200 μL of mycotoxin solution in acetone in order to distribute it homogeneously throughout the matrix, and left overnight. Then, samples were treated as detailed above. The method validation was done for the two faecal target mycotoxins DON and DOM-1. It was performed by studying the method detection limits (MDL) and method quantification limits (MQL), linear range, accuracy, repeatability and reproducibility. To do so, 100 mg of faecal sample was analysed following the extraction method described above. MDLs and MQLs were estimated by the criteria of a signal-to-noise ratio (S/N) equivalent to 3 and 10, respectively. The repeatability (n = 5, intra-day) and the reproducibility (n = 5, inter-day) of the method were below 4% and 12%, respectively, expressed as the relative standard deviation (%RSD). These results were obtained by spiking the samples at a concentration near to the MQL and at a concentration ten times higher than the MQL, for each compound. Thus, repeatability and reproducibility were acceptable in accordance with current guidelines (SANTE, 2015).

5.3.2. UHPLC-MS/MS analysis

DON and DON derivatives were analysed chromatographically on an Agilent 1290 Infinity LC Series UHPLC system (Agilent Technologies, Waldbronn, Germany), and separated using a Cortecs UHPLC C_{18} column (100 mm × 2.1 mm, 1.6 μm) from Waters (Wexford, Ireland). The column was kept at 40 °C and mycotoxins were eluted at a flow rate of 0.45 mL min^{-1} using a binary mobile phase constituted of water (eluent A) and MeOH (eluent B), both with 5 mM of NH_4HCOO and 0.1% of HCOOH. The gradient elution was the same as the previous research (Miró-abella et al., 2018), the injection volume was 10 μL and the autosampler was kept at 4 °C.

Then, the LC eluent was directed into a 6495 iFunnel Triple Quadrupole MS/MS with an electrospray ionization (ESI) interface from Agilent Technologies. Source parameters were taken from previous research (Miró-abella et al., 2018) for DON, DON3G, 3AcDON and 15AcDON, and they were also applied for DOM-1 as follows: capillary voltage of 3500 V, desolvation gas flow of 18 L min^{-1}, desolvation gas temperature of 160 °C, nebuliser pressure of 35 psi, nozzle voltage of 500 V, fragmentor voltage of 380 V, cell acceleration voltage of 5 V, sheath gas flow of 11 L min^{-1} and sheath gas temperature of 350 °C. The high and low pressure funnel parameters were 150 and 60 V, respectively. All compounds were acquired by Multiple Reaction Monitoring (MRM) mode in positive polarity, in which three characteristic MRM transitions were monitored for each mycotoxin, in accordance with the European Commission guidelines (SANTE, 2015). Suitable collision energies were tested for each transition, and different time segments were optimised in order to improve analyte sensitivity. All these parameters mentioned above are detailed in Table 1.

5.4. 16s rRNA-metagenomics

5.4.1. DNA extraction

To obtain DNA from caecum samples, the QIAmp DNA Stool Kit (Qiagen, Venlo, the Netherlands) was used replacing the 70 °C lysis incubation recommended by the protocol, by a 95 °C lysis. DNA purity and integrity were assessed using spectrophotometry (NanoDrop, Thermo Fisher Scientific, Massachusetts, USA).

5.4.2. Partial 16S rRNA gene amplification and purification

Sequences from the V3 and V4 regions of 16S rRNA gene were amplified from the extracted faecal DNA through two primer pairs: 341F-532R (5′-CCTACGGGRSGCAGCAG-3'; 5′-ATTACCGCGGCTGCT-3′) for the V3 region, 515F-806R (5′-GTGCCAGCMGCCGCGGTAA-3'; 5′-GGACTACHVGGGTWTCTAAT-3′), for the V4 region. These primers comprise, at their 5′ end, one of the two adaptor sequences used in the Ion Torrent sequencing library preparation protocol linking a unique Tag barcode of 10 bases to identify different samples. To perform the V3 region amplification, 1 μL of extracted DNA (50 ng mL^{-1}), 7.5 μL of water, 12.5 μL of AmpliTaq Gold 360 (Applied Biosystems, California, USA), 2 μL of each primer Forward (5 μM) and 2 μL of the primer Reverse (5 μM) were mixed in this order. Meanwhile, to perform the V4 region amplification, 1 μL of extracted DNA (50 ng mL^{-1}), 8.5 μL of water, 12.5 μL of AmpliTaq Gold 360, 1.5 μL of each primer Forward (5 μM) and 1.5 μL of the primer Reverse (5 μM) were mixed, also in this order. Different Polymerase Chain Reaction (PCR) cycle parameters were used for the V3 and V4 regions. For the V3 region, the parameters were 5 min at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 1.3 min at 72 °C, followed by 10 min at 72 °C. For the V4 region, the parameters were 3 min at 94 °C, 30 cycles of 30 s at 94 °C, 45 s at 57 °C and 1 min at 72 °C, followed by 2 min at 72 °C. Reactions were carried out by using a Verity Thermocycler (Applied Biosystems, Waltham, MA, USA). In order to confirm the PCR products, a 2% agarose gel was used and the specific bands were excised and purified with the Nucleospin Gel and the PCR clean-up kit (Macherey-Nagel, Berlin, Germany). The concentration of the PCR amplicons was analysed by electrophoresis on a Bioanalyser (Agilent Technologies). Equimolar pools of each fragment and sample were combined to obtain a multiplexed pool.

5.4.3. Sequenced-based microbiome analysis and statistics

The library pool was diluted to a DNA concentration of 60 pM prior to clonal amplification. The Ion 520 & Ion 530 Kit-Chef (Life Technologies, Carlsbad, California, USA) was employed for template preparation and sequencing according to the manufacturer's instructions. Prepared samples were loaded on to a 530 chip and sequenced using the Ion S5 system of the Ion Torrent Platform (Life Technologies). Once sequencing was achieved, Ion Torrent Suit software removed low quality and polyclonal sequences and those readings were then analysed using QIIME (v1.9.1), selecting only sequences with 150–200 bp and omitting homopolymers greater than 6 (Caporaso et al., 2011; Edgar, 2010; Altschul et al., 1990; McDonald et al., 2012; Lozupone and Knight, 2005). QIIME was used to summarise the relative abundance of microbial clades at different taxonomic levels, generating an OTU (operational taxonomic unit) table for each taxonomy level. Afterwards, SPSS (IBM Corp. Released, 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp) was used with data contained in each OTU table to determine significant differences between phylum,

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classes, orders, families and genus between the groups of interest. To perform statistical analysis, relative abundance of microbial clades lower than 0.01% were ignored. A one-way ANOVA test with a Bonferroni correction for post hoc analysis was performed between all the groups for each taxonomy level.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.08.006.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Awad, W.A., Bohm, J., Razzazi-Fazeli, E., Ghareeb, K., Zentek, J., 2006. Effect of addition of a probiotic microorganism to broiler Diets contaminated with deoxynivalenol on performance and histological alterations of intestinal villi of broiler chickens. Poultry Sci. 85, 974–979. https://doi.org/10.1093/ps/85.6.974.

Binder, J., Horvath, E.M., Schatzmayr, G., Ellend, N., Danner, H., Krska, R., Braun, R., 1997. Screening for deoxynivalenol-detoxifying anaerobic rumen microorganisms. Cereal Res. Commun. 25, 343–346.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., Mcdonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2011. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303.

Commission Regulation (EC) No 1126/2007 of 28 September 2007, 2007. Amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards Fusarium toxins in maize and maize products. OJEU, L255/14-17.

Del Bas, J.M., Guirro, M., Boqué, N., Cereto, A., Ras, R., Crescenti, A., Caimari, A., Canela, N., Arola, L., 2018. Alterations in gut microbiota associated with a cafeteria diet and the physiological consequences in the host. Int. J. Obes. https://doi.org/10.1038/ijo.2017.284.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461. https://doi.org/10.1093/bioinformatics/btq461.

Eriksen, G.S., Pettersson, H., Johnsen, K., Lindberg, J.E., 2002. Transformation of trichothecenes in ileal digesta and faeces from pigs. Arch Tierernahr 56, 263–274.

European Commission, 1999. Opinion on Fusarium toxins, Part 1: deoxynivalenol (DON). Scientific Committee on Food 1–9.

European Food Safety Authority, EFSA, 2017. Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. EFSA J 15, 4718. https://doi.org/10.2903/j.efsa.2017.4718.

Gratz, S.W., Duncan, G., Richardson, A.J., 2013. The human fecal microbiota metabolizes deoxynivalenol and deoxynivalenol-3-glucoside and may be responsible for urinary deepoxy-deoxynivalenol. Appl. Environ. Microbiol. 79, 1821–1825. https://doi.org/10.1128/AEM.02987-12.

Guo, M., Huang, K., Chen, S., Qi, X., He, X., Cheng, W.H., Luo, Y., Xia, K., Xu, W., 2014. Combination of metagenomics and culture-based methods to study the interaction between ochratoxin a and gut microbiota. Toxicol. Sci. 141, 314–323. https://doi.org/10.1093/toxsci/kfu128.

Karlovsky, P., 2011. Biological detoxification of the mycotoxin deoxynivalenol and its use in genetically engineered crops and feed additives. Appl. Microbiol. Biotechnol. 91, 491–504. https://doi.org/10.1007/s00253-011-3401-5.

Kollarczik, B., Gareis, M., Hanelt, M., 1994. In vitro transformation of the Fusarium mycotoxins deoxynivalenol and zearalenone by the normal gut microflora of pigs. Nat. Toxins 2, 105–110. https://doi.org/10.1002/nt.2620020303.

Lake, B.G., Phillips, J.C., Walters, D.G., Bayley, D.L., Cook, M.W., Thomas, L.V., Gilbert, J., Startin, J.R., Baldwin, N.C.P., Bycroft, B.W., Dewick, P.M., 1987. Studies on the metabolism of deoxynivalenol in the rat. Food Chem. Toxicol. 25, 589–592 https://doi.org/10.1016/0278-6915(87)90019-6.

Lamendella, R., Santo Domingo, J.W., Ghosh, S., Martinson, J., Oerther, D.B., 2011. Comparative fecal metagenomics unveils unique functional capacity of the swine gut. BMC Microbiol. 11, 103. https://doi.org/10.1186/1471-2180-11-103.

Lozupone, C., Knight, R. UniFrac, 2005. A new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71, 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005.

Maresca, M., 2013. From the gut to the brain: journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol. Toxins 5, 784–820. https://doi.org/10.3390/toxins5040784.

McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., Desantis, T.Z., Probst, A., Andersen, G.L., Knight, R., Hugenholtz, P., 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 6, 610–618. https://doi.org/10.1038/ismej.2011.139.

Miró-Abella, E., Herrero, P., Canela, N., Arola, L., Ras, R., Fontanals, N., Borrull, F., 2018. Determination of trichothecenes in cereal matrices using subcritical water extraction followed by solid-phase extraction and liquid chromatography-tandem mass spectrometry. Food Anal. Methods. 11, 1113–1121. https://doi.org/10.1007/s12161-017-1089-6.

Nagl, V., Schwartz, H., Krska, R., Moll, W.D., Knasmüller, S., Ritzmann, M., Adam, G., Berthiller, F., 2012. Metabolism of the masked mycotoxin deoxynivalenol-3-glucoside in rats. Toxicol. Lett. 213, 367–373. https://doi.org/10.1016/j.toxlet.2012.07.024.

Nagl, V., Woechtl, B., Schwartz-Zimmermann, H.E., Hennig-Pauka, I., Moll, W.D., Adam, G., Berthiller, F., 2014. Metabolism of the masked mycotoxin deoxynivalenol-3-glucoside in pigs. Toxicol. Lett. 229, 190–197. https://doi.org/10.1016/j.toxlet.2014.06.032.

Payros, D., Alassane-Kpembi, I., Pierron, A., Loiseau, N., Pinton, P., Oswald, I.P., 2016. Toxicology of deoxynivalenol and its acetylated and modified forms. Arch. Toxicol. 90, 2931–2957. https://doi.org/10.1007/s00204-016-1826-4.

Pestka, J.J. Deoxynivalenol, 2007. Toxicity, mechanisms and animal health risks. Anim. Feed Sci. Technol. 137, 283–298. https://doi.org/10.1016/j.anifeedsci.2007.06.006.

Pestka, J.J. Deoxynivalenol, 2010. Mechanisms of action, human exposure, and toxicological relevance. Arch. Toxicol. 84, 663–679. https://doi.org/10.1007/s00204-010-0579-8.

Piotrowska, M., Ślizewska, K., Nowak, A., Zielonka, , Zakowska, Z., Gajecka, M., Gajecki, M., 2014. The effect of experimental Fusarium mycotoxicosis on microbiota diversity in porcine ascending colon contents. Toxins 6, 2064–2081. https://doi.org/10.3390/toxins6072064.

Rivière, A., Selak, M., Lantin, D., Leroy, F., De Vuyst, L., 2016. Bifidobacteria and butyrate-producing colon bacteria: importance and strategies for their stimulation in the human gut. Front. Microbiol. 7, 979. https://doi.org/10.3389/fmicb.2016.00979.

Robert, H., Payros, D., Pinton, P., Théodorou, V., Mercier-Bonin, M., Oswald, I.P., 2017. Impact of mycotoxins on the intestine: are mucus and microbiota new targets?. J. Toxicol. Environ. Health B Crit. Rev. 20, 249–275. https://doi.org/10.1080/10937404.2017.1326071.

Saint-Cyr, M.J., Perrin-Guyomard, A., Houée, P., Rolland, J.G., Laurentie, M., 2013. Evaluation of an oral subchronic exposure of deoxynivalenol on the composition of human gut microbiota in a model of human microbiota-associated rats. PLoS One 8, 1–9. https://doi.org/10.1371/journal.pone.0080578.

SANTE, 2015. European Commission Document No SANTE/11945/2015. Guidance Document on Analytical Quality Control and Method Validation Procedures for Pesticides Residues Analysis in Food and Feed. 2015.

Schwartz-Zimmermann, H.E., Hametner, C., Nagl, V., Slavik, V., Moll, W.D., Berthiller, F., 2014. Deoxynivalenol (DON) sulfonates as major DON metabolites in rats: from identification to biomarker method development, validation and application. Anal. Bioanal. Chem. 406, 7911–7924. https://doi.org/10.1007/s00216-014-8252-3.

Turner, P.C., Hopton, R.P., Lecluse, Y., White, K.L.M., Fisher, J., Lebailly, P., 2010. Determinants of urinary deoxynivalenol and de-epoxy deoxynivalenol in male farmers from normandy, France. J. Agric. Food Chem. 58, 5206–5212. https://doi.org/10.1021/jf100892v.

Vinderola, G., Ritieni, A., 2014. Role of probiotics against mycotoxins and their deleterious effects. J. Food Res. 4, 10. https://doi.org/10.5539/jfr.v4n1p10.

Waché, Y.J., Valat, C., Postollec, G., Bougeard, S., Burel, C., Oswald, I.P., Fravalo, P., 2009. Impact of deoxynivalenol on the intestinal microflora of pigs. Int. J. Mol. Sci. 10, 1–17. https://doi.org/10.3390/ijms10010001.

Young, J.C., Zhou, T., Yu, H., Zhu, H., Gong, J., 2007. Degradation of trichothecene mycotoxins by chicken intestinal microbes. Food Chem. Toxicol. 45, 136–143. https://doi.org/10.1016/j.fct.2006.07.028.

Zou, Z.Y., He, Z.F., Li, H.J., Han, P.F., Meng, X., Zhang, Y., Zhou, F., Ouyang, K.P., Chen, X.Y., Tang, J., 2012. In vitro removal of deoxynivalenol and T-2 toxin by lactic acid bacteria. Food Sci. Biotechnol 21, 1677–1683. https://doi.org/10.1007/s10068-012-0223-x.

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