



# An *in vitro* model to quantify interspecies differences in kinetics for intestinal microbial bioactivation and detoxification of zearalenone

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## ABSTRACT

Zearalenone (ZEN) is a mycotoxin known for its estrogenic activities. The metabolism of ZEN plays a role in the interspecies differences in sensitivity to ZEN, and is known to occur in the liver and *via* the intestinal microbiota, although the relative contribution of these two pathways remains to be characterized. In the present study a fecal *in vitro* model was optimized and used to quantify the interspecies differences in kinetics of the intestinal microbial metabolism of ZEN in rat, pig and human.  $V_{max}$ ,  $K_m$ , and catalytic efficiencies ( $k_{cat}$ ) were determined, and results obtained reveal that the  $k_{cat}$  values for formation of  $\alpha$ -ZEL and  $\beta$ -ZEL amounted to 0.73 and 0.12 mL/h/kg bw for human microbiota, 2.6 and 1.3 mL/h/kg bw for rat microbiota and 9.4 and 6.3 mL/h/kg bw for pig microbiota showing that overall ZEN metabolism increased in the order human < rat < pig microbiota. Expressed per kg bw the  $k_{cat}$  for ZEN metabolism by the liver surpassed that of the intestinal microbiota in all three species. In conclusion, it is estimated that the activity of the intestinal colon microbiome may be up to 36 % of the activity of the liver, and that it can additionally contribute to the species differences in bioactivation and detoxification and thus the toxicity of ZEN in pigs and rats but not in humans. The results highlight the importance of the development of human specific models for the assessment of the metabolism of ZEN.

## 1. Introduction

Zearalenone (ZEN) is a mycotoxin produced by *Fusarium* species contaminating grains and cereals, particularly wheat and corn. ZEN is known to act as an endocrine disruptor, and exposure to ZEN is known to cause reproductive-toxicity, mediated *via* estrogen receptor agonism [1]. Upon ingestion, ZEN is metabolized to  $\alpha$ -zearalenone ( $\alpha$ -ZEL) and  $\beta$ -zearalenone ( $\beta$ -ZEL). Conversion to  $\alpha$ -ZEL represents bioactivation because  $\alpha$ -ZEL has been shown in different *in vitro* and *in vivo* studies to be on average 60 times more potent as an ER $\alpha$  agonist than ZEN. In contrast,  $\beta$ -ZEL is 5 times less potent than ZEN so that conversion of ZEN to  $\beta$ -ZEL is considered a detoxification [1]. Differences in the reduction of ZEN to  $\alpha$ -ZEL and  $\beta$ -ZEL between species is considered a key factor contributing to differences in the sensitivity to ZEN exposure. Interspecies differences in bioactivation and detoxification of ZEN to  $\alpha$ -ZEL and  $\beta$ -ZEL have been well-described for liver tissue [2]. Pigs, whose liver appears to be more efficient in  $\alpha$ -ZEL production than that of other species are also most sensitive towards ZEN toxicity observed in reproductive organs [3,4]. However, in addition to the liver also the

intestinal microbiota can metabolize ZEN to  $\alpha$ -ZEL and  $\beta$ -ZEL [5], although the relative contribution of conversion by the gut microbiota as well as interspecies differences in bioactivation and detoxification of ZEN by the intestinal microbiota remain to be characterized. Mammalian intestinal microbiota is known to modulate many processes essential to maintain host health, including the biotransformation of xenobiotics [6]. The overall metabolic capacity of the intestinal microbiome has been described to complement the metabolic capacity of the host by encoding for enzymes that the host does not possess itself, with an overall broader substrate specificity [7]. While there is a significant body of literature showing interspecies differences in microbiome composition [8–10] due to lifestyles and diets [11,12], little is known about the potentially resulting differences in functionality. Typical microbial reactions include the reduction of chemicals [11], an important reaction for the bioactivation and/or detoxification of ZEN, so that differences in metabolic capacity of the intestinal microbiota might contribute to the interspecies differences in sensitivity to ZEN exposure.

The aim of the present study was to develop an *in vitro* model system

**Abbreviations:**  $\alpha$ -ZEL,  $\alpha$ -zearalenol;  $\beta$ -ZEL,  $\beta$ -zearalenol; ER, estrogen receptor;  $k_{cat}$ , catalytic efficiency; RP, relative potency; UPLC-PDA, ultra-performance liquid chromatography method with diode array detection; LC-MS/MS, liquid chromatography tandem mass spectrometry; ZEN, zearalenone

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to assess and quantify the contribution of the intestinal microbial metabolism to the bioactivation and detoxification of ZEN. The model to be developed should enable the quantification of kinetics for the conversion of ZEN in incubations with fecal samples from different host species, facilitating the quantitative characterization of species differences in bioactivation and detoxification and thus the contribution of gut microbial metabolism to species differences in sensitivity to ZEN exposure. The development of an *in vitro* model that enables quantification of kinetic parameters for intestinal metabolism is essential for future incorporation of conversion by the intestinal microbiota in alternative testing strategies within the 3R framework for alternatives for animal testing [13].

In the present study an *in vitro* model with fecal slurries was optimized and successfully applied for i) the assessment of interspecies differences in the intestinal microbial metabolism of ZEN in rats, pigs, and humans, and ii) comparison of the relative intestinal and hepatic bioactivation and detoxification of ZEN to  $\alpha$ -ZEL and  $\beta$ -ZEL.

## 2. Materials and methods

### 2.1. Materials

ZEN (CAS registry number 17924-92-4;  $\geq 99.0$  %),  $\alpha$ -ZEL (CAS registry number 36455-72-8;  $> 98$  %),  $\beta$ -ZEL (CAS registry number 71030-11-0;  $> 98$  %) and 17 $\beta$ -estradiol (E2; CAS registry number 50-28-2) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Stock solutions of the test chemicals were prepared in dimethyl sulfoxide (DMSO; CAS registry number 67-68-5) purchased from Merck (Darmstadt, Germany). Cryopreserved UltraPool™ human microsomes (150 mixed gender donors) and pooled human liver S9 (20 mixed gender donors) were obtained from Corning (Woburn, MA, USA).  $\beta$ -Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH; CAS registry number 2646-71-1) was purchased from Carbosynth (Berkshire, UK). Trizma® base (Tris, CAS registry number 77-86-1) and glycerol (CAS registry number 56-81-5) were obtained from Sigma-Aldrich (Steinheim, Germany). Magnesium chloride hexahydrate (MgCl<sub>2</sub>·6H<sub>2</sub>O; CAS registry number 7791-18-6) and formic acid (FA; CAS registry number 64-18-6) were obtained from VWR International (Amsterdam, The Netherlands). Methanol (MeOH, UPLC/MS grade; CAS registry number 67-56-1) and acetonitrile (ACN, UPLC/MS grade; CAS registry number 75-05-8) were purchased from Biosolve (Valkenswaard, The Netherlands). Phosphate-buffered saline (PBS, pH 7.4), Dulbecco's modified Eagle's medium nutrient mixture F12 (DMEM/F12), phenol red-free DMEM/F12, fetal bovine serum (FBS), non-essential amino acids (NEAA), dextran coated charcoal-filtered fetal calf serum (DCC-FBS) and geneticin (G418; CAS registry number 108321-42-2) were obtained from Gibco (Paisley, UK).

### 2.2. Estrogenicity of ZEN, $\alpha$ -ZEL and $\beta$ -ZEL studied in an estrogen receptor-mediated reporter gene (ER $\alpha$ -CALUX) assay

U2OS ER $\alpha$  reporter gene cells, derived from a stably transfected human osteosarcoma cell line expressing ER $\alpha$  were kindly provided by BioDetection Systems (Amsterdam, The Netherlands) [14], cells were cultured in DMEM/F12 supplemented with 10 % FCS, 0.5 % NEAA and 4  $\mu$ g/mL of geneticin. The cells were routinely subcultured every 3–4 days.

For the CALUX assay, cells were seeded in 96-well view plates at a density of  $1 \times 10^5$  cells/mL in 100  $\mu$ l assay medium (phenol red-free DMEM/F-12 supplemented with 5% DCC-FCS and 0.5 % NEAA) and allowed to attach for 48 h with a renewal of assay medium after the first 24 h. 48 h after seeding, the medium was aspirated and replaced with 100  $\mu$ l assay medium containing ZEN (0.1 pM–10 nM),  $\alpha$ -ZEL (0.01–1000 pM), or  $\beta$ -ZEL (0.01 pM–10 nM) added from 200 times concentrated stock solutions in DMSO (0.5 % DMSO maximum final concentration). For each assay, the concentrations were tested in triplicate.

100 pM E2 was used as positive control and 0.5 % DMSO as solvent control. After 24 h, the cells were washed with 100  $\mu$ l 0.5 PBS, and lysed with 30  $\mu$ l low salt buffer (LSB; pH 7.8) containing 10 mM Tris, 2 mM dithiothreitol (DTT), and 2 mM 1, 2-diaminocyclohexanete triacetic acid monohydrate. The plates were placed on ice for 15 min and frozen at  $-80$  °C for at least 2 h. Plates were thawed while being shaken and luciferase activity was measured using a luminometer (Glomax-Multi Detection System, Promega, California) upon adding 100  $\mu$ l of FLASH mix (pH 7.8) containing 20 mM tricine, 1.07 mM (MgCO<sub>3</sub>)<sub>4</sub>Mg(OH)<sub>2</sub>·5H<sub>2</sub>O, 2.67 mM MgSO<sub>4</sub>, 0.1 mM ethylenedinitrilotetraacetic acid disodium salt dihydrate, 2 mM DTT, 0.47 mM D-luciferin and 5 mM adenosine-5-triphosphate.

The luciferase activity was measured in relative light units and used to calculate the fold increases of luminescence relative to the solvent control in MS Excel (2016). The obtained values were normalized to the maximum response of E<sub>2</sub> (100 pM) set at 100 % and the concentration-response curves were fitted in GraphPad Prism 5.04 (GraphPad software, San Diego California, U.S.A.) by using a non-linear regression model (four parameter sigmoidal dose-response) to obtain the half-maximal effect concentrations (EC<sub>50</sub>).

The EC<sub>50</sub> values from other studies using ER activation as endpoint were collected and used to calculate the relative potency factors (EC<sub>50</sub> ZEN/EC<sub>50</sub> metabolite) that could be compared to the relative potency factors obtained in the present study.

### 2.3. Collection of rat, pig and human fecal samples

Fresh fecal samples from Wistar rats (10 male and 15 female) were kindly provided by BASF (Ludwigshafen, Germany). Feces from each individual were obtained by physical stimulation of the abdomen to trigger defecation, weighed and immediately transferred into an anaerobic solution of 10 % (v/v) glycerol in PBS and diluted to a final fecal concentration of 20 % (w/v). Samples were manually homogenized using a sterile glass wand, and tubes flushed with N<sub>2</sub> gas before being frozen to  $-80$  °C. Subsequently, individual samples were mixed and filtered using a woven sterile medical gauze dressing (HeltiQ) under anaerobic conditions (85 % N<sub>2</sub>, 10 % CO<sub>2</sub> and 5% H<sub>2</sub>, in a Bactron EZ anaerobic chamber). Aliquots of the resulting fecal slurry were prepared and stored at  $-80$  °C until further use.

Fecal samples collected from 10 piglets (5 females and 5 males) were kindly provided by Wageningen Livestock Research (Wageningen, The Netherlands) during dissection of untreated control animals of an animal study for which permission (license number 2016.D0136.003) by the Animal Care and Use Committee of Wageningen University & Research (Wageningen, The Netherlands) was obtained. Samples from each individual were treated and stored separately as described. The colorectal part of the intestines containing feces were closed with artery clamps before being removed. Fecal matter from these sections were transferred to 50 mL centrifuge tubes, immediately flushed with N<sub>2</sub> gas and transported in an airtight container into the anaerobic environment of the anaerobic chamber for further processing. Samples were weighed and fecal slurry prepared by diluting the samples with an anaerobic solution of 10 % glycerol in PBS to obtain a final concentration of 20 % (w/v). Samples were manually homogenized with a sterile serological pipette and filtered using a woven sterile medical gauze dressing (HeltiQ). Aliquots of the fecal slurry were prepared and stored at  $-80$  °C until further use. Prior to the experiments a pool of the 10 individuals was prepared.

Human fecal samples were donated by 10 volunteers (7 females and 3 males), aged 24–64 years. Volunteer donors did not consume antibiotics or visit tropical countries for 3 months prior to sample donation and have no history of intestinal diseases. The research protocol for use of these human samples was evaluated by the Medical Ethical Reviewing Committee of Wageningen University (METC-WU) and judged not to require further evaluation within the scope of the Dutch Medical Research Involving Human Subjects Act. Samples from each

individual were treated and stored separately as follows: in specimen tubes, 3–5 g of fecal samples were collected and subsequently transferred into an anaerobic environment within 2 min after donation by the participants. Samples were immediately diluted in an anaerobic solution of 10 % glycerol in PBS to obtain the fecal slurry (20 % w/v). Samples were manually homogenized with a sterile serological pipette and filtered using SpinCon® (Meridian Bioscience Europe) centrifugal filters at 2500 rpm for 5 min. The filtrate was divided into aliquots and stored at -80 °C until further use. Prior to the experiments a pool of fecal samples of 10 individuals was prepared.

#### 2.4. *In vitro* incubation of ZEN with rat, pig and human fecal samples

To optimize the incubation conditions, linearity of reaction rates over different fecal concentrations and over time was established for rat, pig and human samples. To this end, 20 µM ZEN was incubated under anaerobic conditions with different concentrations of fecal slurry and for different time points. Assuming even distribution of a dose level over the large intestinal content of 0.017, 10.25 and 2.06 L for rats, pigs and humans [15], respectively, an overall concentration of 20 µM would result from dose levels of 44, 261 and 18.8 µg/kg bw, which are above the TDI 0.25 µg/kg bw/day, but in the range of dose levels used in animal studies to detect ZEN mediated (estrogenic) effects compiled in EFSA [4]. To assess linearity over different fecal concentrations, incubation mixes of 100 µL containing 1.4–20 % of fecal slurry (*i.e.* 3.5–50 mg feces/mL) from rats, pigs, and humans in anaerobic PBS (pH 7.4) containing 20 µM ZEN added from a 200x concentrated stock in DMSO (0.5 % DMSO final concentration) were prepared under anaerobic conditions and incubated anaerobically at 37 °C for 5 h. To assess linearity over time, ZEN was incubated with 5% of fecal slurry and sampled every hour for a total of 8 h. To stop the reactions, 100 µl of ice-cold MeOH were added, the samples were vortexed, and kept on ice for 10 min. The samples were centrifuged at 21,500 × g for 15 min at 4 °C, and the supernatant was kept for ultra-performance liquid chromatography photodiode array (UPLC-PDA) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Blank controls were included to assess the stability of ZEN during incubation.

Using the optimized incubation conditions, a range of substrate concentrations of ZEN was incubated to establish reaction kinetics. Incubation mixes of 100 µL were prepared containing 1–250 µM of ZEN added from 200x concentrated stock solutions in DMSO (0.5 % DMSO final concentration), 5% of fecal slurry from rats, pigs, or humans, and PBS (pH 7.4). The samples were incubated anaerobically at 37 °C for 5 h. To stop the reaction, 100 µl of ice-cold MeOH were added, the samples were vortexed, and kept on ice for at least 10 min. The samples were centrifuged at 21,500 × g for 15 min at 4 °C and the supernatant was kept on ice for immediate UPLC-PDA or LC-MS/MS analysis. Three independent experiments for each species were done and data are presented as mean ± standard deviation (SD).

#### 2.5. *In vitro* human hepatic metabolism of ZEN

Human liver microsomal and S9 fraction incubations to characterize the reduction of ZEN by hepatic samples were optimized to establish the linearity over time and protein concentration. To obtain the kinetic parameters, the incubation mixtures with a final volume of 200 µl contained (final concentrations) 2 mM NADPH, 5 mM MgCl<sub>2</sub> and 0.3 mg/mL liver microsomal or S9 fraction proteins in 0.1 M Tris-HCl (pH 7.4). After 1-minute pre-incubation at 37 °C, the reactions were started by the addition of 1–500 µM ZEN (from 100 times concentrated stock solutions in DMSO). The incubations were carried out for 10 min for the microsomes and 5 min for the S9 fractions. To stop the reaction 20 % (v/v) ice-cold ACN was added. Blank incubations were performed without the addition of NADPH. The samples were centrifuged for 5 min at 15,000 × g and the supernatant was kept on ice for immediate UPLC-PDA analysis.

#### 2.6. Kinetic analysis

To derive the kinetic constants for the formation of α-ZEL and β-ZEL by both the microbial and human hepatic metabolism, the amount of metabolites formed expressed per gram of feces or protein, respectively, and per unit of time (rate of formation) were calculated using Microsoft Excel (version 2016) and plotted against the substrate concentrations used. The curve for each metabolite was fitted in GraphPad Prism 5.04 (GraphPad software, San Diego California, U.S.A.) using a standard Michaelis-Menten regression ( $V = V_{max} * [S] / (K_m + [S])$ ) to obtain the *in vitro* kinetic constants,  $V_{max}$  (pmol/min/mg of feces or pmol/min/mg of protein) and  $K_m$  (µM) for the microbial and human hepatic formation of α-ZEL and β-ZEL.

#### 2.7. Comparison of microbial and hepatic metabolism of ZEN

Using the reported average defecation masses of 3 g feces/day for rat [16], 1360 g feces/day for pigs [17] and 128 g feces/day for humans [18], and correcting for average body masses of 0.25 kg for rats [19], 25 kg for pigs [20] and 70 kg for humans [19], the *in vitro*  $V_{max}$  (in pmol/min/mg of feces) was scaled to an *in vivo*  $V_{max}$  (in µmol/h/kg bw).

The hepatic  $V_{max}$  (pmol/min/mg of protein),  $K_m$  (µM) and  $k_{cat}$  (defined as  $V_{max}/K_m$ ) values obtained in this study for human and as reported in literature [3] for rat and pig based on *in vitro* studies with liver microsomes and S9 fractions were used for comparison to the *in vivo*  $V_{max}$  and  $k_{cat}$  values for conversion by the intestinal microbiota. In line with the requirements of the 3Rs (reduction, refinement and replacement) for animal experimentation only the data for hepatic metabolism of human, which were not available in literature, were experimentally determined in this study. The *in vitro*  $V_{max}$  values for conversion by liver samples were scaled to an *in vivo*  $V_{max}$  (in µmol/h/kg bw) using protein yields, liver weight and body mass for each species. Microsomal protein yield used for rat liver was 38 mg/g liver [21], for pig liver it was 32.6 mg/g liver [22] and for human liver it amounted to 32 mg/g liver [23]; S9 protein yield used for rat liver was 87 mg/g liver [21], and it was 127.9 mg/g liver for pig liver [24] and 143 mg/g liver for human liver [25]. Liver weights for rats, pigs and humans were calculated using body masses of 0.25, 25 and 70 kg, respectively, and tissue volumes (% of body weight) of 3.4 [19], 2.9 [20] and 2.6 [19], respectively.

Once the *in vivo*  $k_{cat}$  values were obtained, the percentage of contribution for hepatic (microsomes and S9 fractions) and microbial metabolism were calculated.

#### 2.8. Quantification of α-ZEL and β-ZEL: UPLC-PDA and LC-MS/MS analyses

A UPLC-PDA system (Waters Acquity) was used for the quantification of ZEN, α-ZEL and β-ZEL formed in incubations with rat fecal slurry. The UPLC system was equipped with an Acquity BEH C18 column 1.7 µm, 50 mm x 2.1 mm (Waters) set at 45 °C and a UV detection system recording wavelengths of 190–400 nm. Nanopure water (A) and ACN (B) were used as eluents at a flow rate of 0.6 mL/min with the following gradient profile: 0–25 % B (0–0.5 min), 25–50 % B (0.5–1.2 min), 50–75 % B (1.2–3 min), 75 % B (3–3.5 min), 75–100 % B (3.5–4 min), 100–0 % B (4–4.25 min) and 0 % B (4.25–5 min). Per run, 3.5 µl of sample were injected. ZEN, α-ZEL and β-ZEL were identified using commercially available standards. Chromatograms were presented at 235 nm and chemicals were quantified by comparison of the peak areas at 235 nm to those from standard curves (estimated LOD = 0.02 µM; LOQ = 0.05 µM) prepared using commercially available standards.

ZEN, α-ZEL and β-ZEL formed in pig and human incubations were quantified by LC-MS/MS. The analysis was performed on a Nexera XR LC-20AD SR UPLC system coupled to a triple quadrupole LCMS 8040

mass spectrometer (Shimadzu Benelux, 's Hertogenbosch, The Netherlands) with electrospray ionization (ESI) interface. The UPLC system was equipped with a Kinetex® C18 column 1.7  $\mu\text{m}$ , 50 mm x 2.1 mm (Phenomenex) and set at 40 °C with a flow rate of 0.3 mL/min. The mobile phases consisted of nanopure water containing 0.1 % (v/v) formic acid (A) and ACN containing 0.1 % (v/v) formic acid (B). The total run time was 14 min with the following gradient profile: 0–40 % B (0–1.3 min), 40–50 % B (1.3–5.7 min), 50–100 % B (5.7–6 min), 100 % B kept for 2 min and 100–0% B (8–8.1 min) for equilibration. Per run, 1  $\mu\text{l}$  of sample was injected. MS/MS analysis was operated in the positive ion mode and the MRM mode with a spray voltage of 4.5 KV. The transitions monitored were ( $m/z$ ) 319.2  $\rightarrow$  301.2, 319.2  $\rightarrow$  283.1, 319.2  $\rightarrow$  187.1 for ZEN; ( $m/z$ ) 321.2  $\rightarrow$  303.1, 321.2  $\rightarrow$  285.1, 321.2  $\rightarrow$  131.0 for  $\alpha$ -ZEL; and 321.2  $\rightarrow$  303.2, 321.2  $\rightarrow$  285.15, 321.2  $\rightarrow$  267.0 for  $\beta$ -ZEL. The Postrun Analysis function from the LabSolutions software (Shimadzu, Kyoto, Japan) was used to obtain the peak area of the total ion chromatogram (TIC) for each compound. For quantification of the compounds, the areas were compared to standard curves made using commercially available standards (estimated LOD = 0.01  $\mu\text{M}$ ; LOQ = 0.04  $\mu\text{M}$ ).

### 3. Results

#### 3.1. Estrogenicity of ZEN, $\alpha$ -ZEL and $\beta$ -ZEL

The concentration-response curves (Supplementary Fig. B.1) for the induction of ER $\alpha$ -mediated gene expression were used to derive EC<sub>50</sub> values of 1.6 nM, 0.03 nM and 41.6 nM for ZEN,  $\alpha$ -ZEL and  $\beta$ -ZEL, respectively. EC<sub>50</sub> values, from this study and other relevant literature, are listed in Table 1 together with the calculated relative potencies (RP) of the metabolites compared to ZEN. The relative potencies (RP) of the metabolites compared to ZEN were calculated and are included in Table 1. Substantial variation in RPs between the studies is observed, with, in spite of this,  $\alpha$ -ZEL always showing higher potency than ZEN being 12- to 200-fold more potent, while the RP for  $\beta$ -ZEL was always lower amounting to 0.002- to 0.6 times the RP of ZEN. In all cases, the potency ranking of the compounds is  $\alpha$ -ZEL > ZEN >  $\beta$ -ZEL, with  $\alpha$ -ZEL being on average 72.3 times more potent and  $\beta$ -ZEL on average 5.5 times less potent than ZEN. Therefore, the formation of  $\alpha$ -ZEL from ZEN is considered a bioactivation while the formation of  $\beta$ -ZEL a detoxification.

**Table 1**

EC<sub>50</sub> values and relative potencies for ER $\alpha$  agonist activity of ZEN,  $\alpha$ -ZEL and  $\beta$ -ZEL as derived from the ER $\alpha$  -CALUX assay in the present study or taken from literature.

	EC <sub>50</sub> (nM)			Relative potency <sup>h</sup>	
	ZEN	$\alpha$ -ZEL	$\beta$ -ZEL	$\alpha$ -ZEL	$\beta$ -ZEL
Activation of reporter gene (ER $\alpha$ -CALUX) <sup>a</sup>	1.58	0.03	41.59	55	0.04
Activation of reporter gene (ER $\alpha$ -CALUX) <sup>b</sup>	0.49	0.01	2.50	51	0.20
Activation of reporter gene (MMV-Luc) <sup>c</sup>	1.60	0.02	3.90	73	0.41
MCF7 cell proliferation <sup>d</sup>	3.81	0.06	8.49	64	0.45
MCF7 cell proliferation <sup>e</sup>	0.31	$1.4 \times 10^{-3}$	5.20	221	0.06
MCF7 cell proliferation <sup>f</sup>	1.64	0.05	20.01	33	0.08
Ishikawa cell proliferation <sup>g</sup>	0.06	0.01	25	8.79	0.002
Average ( $\pm$ SD)	1.3 ( $\pm$ 1.2)	0.03 ( $\pm$ 0.02)	15.2 ( $\pm$ 14.4)	72.3 ( $\pm$ 69.0)	0.18 ( $\pm$ 0.18)

<sup>a</sup> This study.

<sup>b</sup> Ehrlich et al. [61].

<sup>c</sup> Frizzell et al. [62].

<sup>d</sup> Molina-Molina et al. [66].

<sup>e</sup> Minervini et al. [65].

<sup>f</sup> Malekinejad et al. [64].

<sup>g</sup> Le Guevel and Pakdel [63].

<sup>h</sup> EC<sub>50</sub> ZEN/EC<sub>50</sub> metabolite.

#### 3.2. Conversion of ZEN in *in vitro* incubations with rat, pig and human fecal slurries

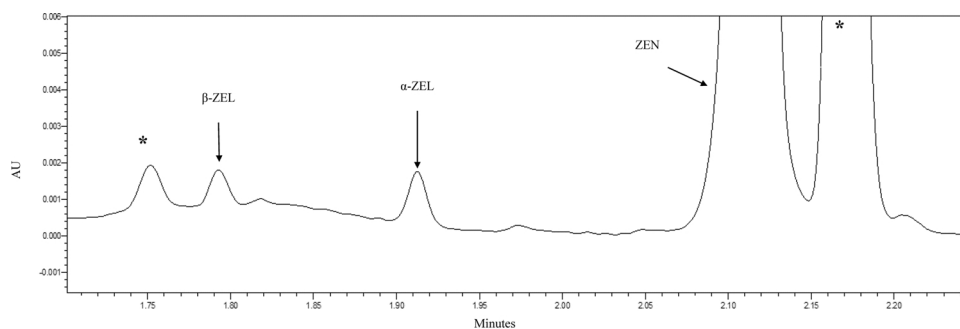
To study its intestinal microbial metabolism, ZEN was incubated anaerobically with rat, pig and human fecal slurries. This resulted in the formation of two metabolites identified as  $\alpha$ -ZEL and  $\beta$ -ZEL (Fig. 1). Under the conditions applied no other metabolites were formed at a detectable level. Incubation conditions were optimized for subsequent study of reaction kinetics defining the range where metabolite formation was linear with respect to time and fecal slurry concentration. Based on the experimental results obtained (Supplementary Fig. A.1), optimal conditions for subsequent kinetic experiments were defined as 5-h incubation time using 5% fecal slurry (equal to 12.5 mg feces/mL).

Using the conditions thus established for linear conversion with respect to incubation time and fecal concentration, the ZEN concentration-dependent conversion of ZEN to  $\alpha$ -ZEL and  $\beta$ -ZEL was quantified (Fig. 2). The formation of  $\alpha$ -ZEL and  $\beta$ -ZEL followed Michaelis-Menten behavior (Fig. 2) and allowed determination of the kinetic constants  $V_{\text{max}}$  and  $K_m$ . The *in vitro* kinetic constants and catalytic efficiencies ( $k_{\text{cat}}$ ; calculated as  $V_{\text{max}}/K_m$ ) for the formation of  $\alpha$ -ZEL and  $\beta$ -ZEL by fecal samples of the three species are presented in Table 2. In all three species,  $\alpha$ -ZEL was formed at a higher rate than  $\beta$ -ZEL at all substrate concentrations tested. The  $k_{\text{cat}}$  values for formation of  $\alpha$ -ZEL in incubations with fecal slurries from rats and pigs appeared to be comparable, with the  $k_{\text{cat}}$  for the pooled fecal samples from rats, being 1.23 times higher than that of the pooled fecal samples from pigs. Among the three species, the highest  $k_{\text{cat}}$  for the formation of  $\alpha$ -ZEL was observed with the pooled human fecal samples, mainly due to a higher  $V_{\text{max}}$ . When comparing human with rat and pig fecal samples, the  $k_{\text{cat}}$  for the formation of  $\alpha$ -ZEL by human fecal samples was 1.8–2.0 times higher. The  $k_{\text{cat}}$  for formation of  $\beta$ -ZEL for rat and pig fecal samples was also comparable, with a 1.1 times higher value for pig samples. Human fecal samples showed for  $\beta$ -ZEL formation a  $k_{\text{cat}}$  that was 1.6–2.0 fold lower than that for the other two species, suggesting a less efficient detoxification.

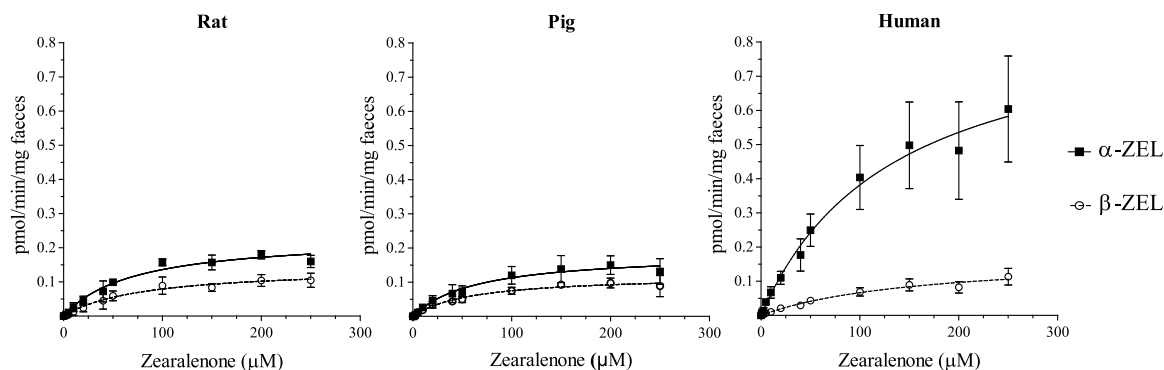
As shown in Table 2, the resulting ratio between  $k_{\text{cat},\alpha\text{ZEL}}$  and  $k_{\text{cat},\beta\text{ZEL}}$  is highest for human (6:1) indicating human to be the species with the highest relative level of bioactivation of ZEN to  $\alpha$ -ZEL with relatively low detoxification to  $\alpha$ -ZEL by the fecal samples.

In a next step the *in vitro*  $V_{\text{max}}$  and  $k_{\text{cat}}$  values obtained for the formation of  $\alpha$ -ZEL and  $\beta$ -ZEL were scaled to *in vivo*  $V_{\text{max}}$  and  $k_{\text{cat}}$  values expressed per kg bw using the defecation volume per day and the respective body masses as scaling factors (Table 2). After this conversion,





**Fig. 1.** Chromatogram of the incubation of 20  $\mu\text{M}$  ZEN with fecal slurries from rats for 5 h at 37  $^{\circ}\text{C}$  under anaerobic conditions. Peaks marked with an \* represent peaks also present in blank incubations without ZEN and originating from the fecal slurry.



**Fig. 2.** ZEN concentration-dependent formation of  $\alpha$ -ZEL and  $\beta$ -ZEL in incubations with pooled fecal samples of rat, pig and human. Each data point represents the mean  $\pm$  SD of three independent experiments.

**Table 2**

Kinetic parameters for the intestinal microbial formation of  $\alpha$ -ZEL and  $\beta$ -ZEL from ZEN scaled to the *in vivo* situation for rat, pig and human.

	Metabolite	$V_{\text{max}}$ , in vitro <sup>a</sup>	$K_m$ <sup>b</sup>	$k_{\text{cat}}$ , in vitro <sup>c</sup>	Scaled $V_{\text{max}}$ , in vivo <sup>d</sup>	$k_{\text{cat}}$ , in vivo <sup>e</sup>	Ratio $k_{\text{cat},\alpha\text{-ZEL}}/k_{\text{cat},\beta\text{-ZEL}}$
Rat	$\alpha$ -ZEL	0.23	66	3.5	0.17	2.60	2.0
	$\beta$ -ZEL	0.14	80	1.8	0.10	1.30	
Pig	$\alpha$ -ZEL	0.19	65	2.9	0.61	9.40	1.5
	$\beta$ -ZEL	0.12	61	2.0	0.38	6.30	
Human	$\alpha$ -ZEL	0.90	135	6.6	0.10	0.73	6.0
	$\beta$ -ZEL	0.18	163	1.1	0.02	0.12	

<sup>a</sup> pmol/min/mg of feces.

<sup>b</sup>  $\mu\text{M}$ .

<sup>c</sup> ( $10^{-3}$ )  $\mu\text{l}/\text{min}/\text{mg}$  feces.

<sup>d</sup> Expressed as  $\mu\text{mol}/\text{h}/\text{kg}$  bw and calculated from  $[(V_{\text{max}}, \text{in vitro}) \times (\text{defecation volume in mg}) \times (60 \text{ min. h})] / (10^6 \mu\text{mol}/\text{pmol}) / (\text{kg bw})$ .

<sup>e</sup> ml/h/kg bw.

the *in vivo*  $k_{\text{cat}}$  of pigs for the formation of  $\alpha$ -ZEL appeared to be almost 3.6 and 12.8 times higher than that for rats and humans, respectively, revealing that on a per kg basis pigs represent the species with the highest potential of the three investigated species for the formation of  $\alpha$ -ZEL. For  $\beta$ -ZEL, the *in vivo*  $k_{\text{cat}}$  values for pigs were 4.9 and 52.4 times higher than those in rats and humans, respectively. In spite of humans showing the highest *in vitro*  $k_{\text{cat}}$  for  $\alpha$ -ZEL and  $\beta$ -ZEL, they appeared to have the lowest *in vivo*  $k_{\text{cat}}$  due to a relatively lower defecation volume per kg bw.

### 3.3. *In vitro* liver conversion of ZEN by microsomal and S9 fractions

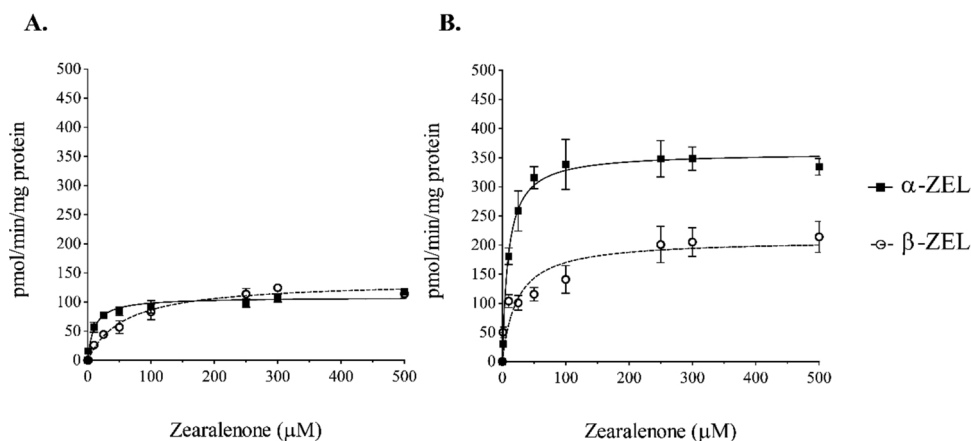
To enable comparison of intestinal microbial conversion to

conversion by the liver, the *in vitro* kinetic data for hepatic metabolism of ZEN in humans were determined in this study from *in vitro* incubations with liver microsomes and S9 fractions, where the formation of  $\alpha$ -ZEL and  $\beta$ -ZEL followed Michaelis-Menten behavior (Fig. 3).  $V_{\text{max}}$ ,  $K_m$  and  $k_{\text{cat}}$  values derived from these data are presented in Table 3, together with the kinetic constants  $V_{\text{max}}$ ,  $K_m$  and  $k_{\text{cat}}$  obtained from literature for conversion of ZEN by liver microsomes and S9 from rats and pigs [3]. Also these  $V_{\text{max}}$  and  $k_{\text{cat}}$  values were scaled to the *in vivo* situation and the values thus obtained are also presented in Table 3. The catalytic efficiencies for liver metabolism of ZEN in humans and pigs showed a preference for the formation of  $\alpha$ -ZEL over  $\beta$ -ZEL, while in rats  $\beta$ -ZEL was formed more efficiently than  $\alpha$ -ZEL. Overall human liver catalyzed the conversion of ZEN to  $\alpha$ -ZEL 7 and 75 times more efficiently (based on microsomes and S9 liver fractions, respectively) than liver from pigs. The lowest  $k_{\text{cat}}$  for the formation of  $\alpha$ -ZEL were observed for rat microsomes and S9 fractions with 8 and 899 times lower  $k_{\text{cat}}$  values than obtained for human liver microsomes and S9 fractions, respectively. The highest  $k_{\text{cat}}$  for the formation of  $\beta$ -ZEL was observed for rat microsomes and human S9 fractions, pig liver samples showing the lowest preference for  $\beta$ -ZEL formation.

### 3.4. Comparison of intestinal microbial and hepatic metabolism of ZEN

The *in vivo*  $k_{\text{cat}}$  values derived for the metabolism of ZEN to  $\alpha$ -ZEL and  $\beta$ -ZEL from the *in vitro* liver and fecal incubations allow the comparison of the activities of the liver and colonic microbiome, revealing marked species differences (Fig. 4). When comparing these values, the *in vivo*  $V_{\text{max}}$  and  $k_{\text{cat}}$  appear to be higher for liver (microsomes and S9 fractions) than for the intestinal microbiota for all three species. While for pigs and rats the  $K_m$  for metabolism of ZEN by the intestinal microbiota was lower than the  $K_m$  for liver, for humans the  $K_m$  for the intestinal microbiota was higher than that for liver.

For the three species, the relative activity of the colonic microbiome compared to the liver for the formation of  $\alpha$ -ZEL ranged from 0.1 % (in



**Fig. 3.** Substrate (*i.e.* ZEN) concentration-dependent formation of  $\alpha$ -ZEL and  $\beta$ -ZEL in incubations with pooled human liver microsomes (A) and pooled human S9 fractions (B). Each data point represents the mean  $\pm$  SD of three independent experiments.

**Table 3**

Kinetic parameter for the formation of  $\alpha$ -ZEL and  $\beta$ -ZEL *via in vitro* rat, pig and human hepatic metabolism of ZEN.

	$V_{max}$ , in vitro (pmol/min/mg of protein)	$K_m$ ( $\mu$ M)	$k_{cat}$ , in vitro ( $\mu$ l/min/mg protein)	Scaled $V_{max}$ , in vivo ( $\mu$ mol/h/kg bw) <sup>a</sup>	$k_{cat}$ , in vivo (ml/h/kg bw)	Ratio $k_{cat}$ , $\alpha$ -ZEL/ $k_{cat}$ , $\beta$ -ZEL
<b>Microsomes</b>						
Rat <sup>b</sup>	$\alpha$ -ZEL 80	89	0.9	6.2	70	0.02
	$\beta$ -ZEL 495	10	49.5	38.4	3837	
Pig <sup>b</sup>	$\alpha$ -ZEL 796	566	1.4	45.8	81	4.70
	$\beta$ -ZEL 512	1696	0.3	29.4	17	
Human <sup>c</sup>	$\alpha$ -ZEL 108	10	10.7	5.4	536	4.60
	$\beta$ -ZEL 136.5	58	2.4	6.8	118	
<b>S9 fractions</b>						
Rat <sup>b</sup>	$\alpha$ -ZEL 32	592	0.05	5.7	10	0.016
	$\beta$ -ZEL 72	21	3.40	12.8	609	
Pig <sup>b</sup>	$\alpha$ -ZEL 480	936	0.50	108.3	116	3.10
	$\beta$ -ZEL 208	1243	0.17	47.0	38	
Human <sup>c</sup>	$\alpha$ -ZEL 358.7	9	38.7	80.02	8623	4.30
	$\beta$ -ZEL 209.3	23	9.02	46.7	2014	

<sup>a</sup> Calculated from  $[(V_{max}, in vitro) \times (mg \text{ microsomes or S9/g liver}) \times (60 \text{ min. h})] / (10^6 \mu\text{mol}/\text{pmol}) / \text{kg bw}$ .

<sup>b</sup> Malekinejad et al. (2006).

<sup>c</sup> This study.

**Table 4**

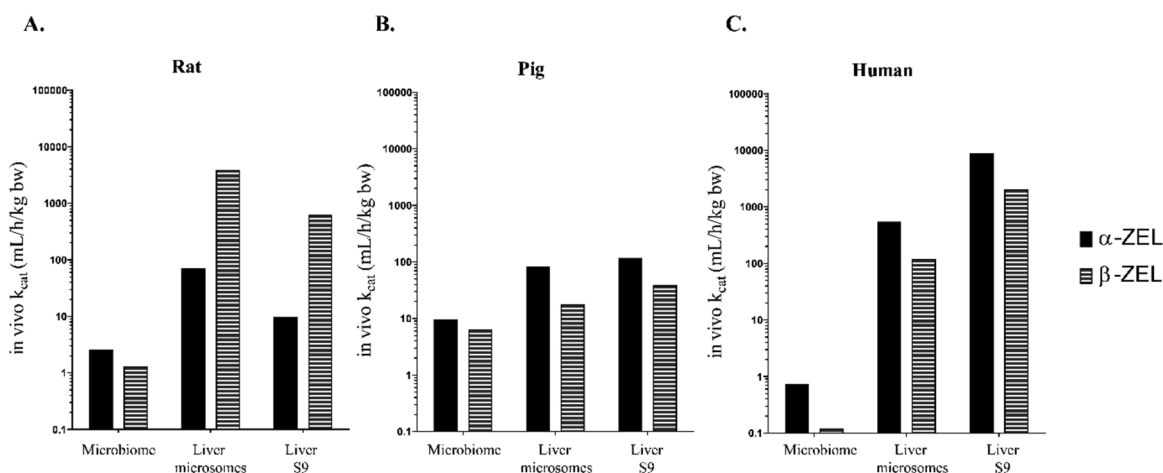
Activity (%) of the intestinal colonic microbiome metabolism to the overall conversion of ZEN to  $\alpha$ -ZEL and  $\beta$ -ZEL compared to the hepatic metabolism based on *in vivo*  $k_{cat}$  values. The liver microsomes or S9 fraction activity represent 100 % of the activity.

	% activity of the microbiome vs liver microsomes		% activity of the microbiome vs liver S9 fractions	
	$\alpha$ -ZEL	$\beta$ -ZEL	$\alpha$ -ZEL	$\beta$ -ZEL
Rat	4	$\leq 0.1$	27	$\leq 0.2$
Pig	12	36	8	17
Human	$\leq 0.1$	$\leq 0.1$	$\leq 0.1$	$\leq 0.1$

human) to 8–12 % (in pigs) to 4–27 % (in rats), while for  $\beta$ -ZEL the values ranged from 0.1 % (in human) to 0.1–0.2% (in rats) and 17–36 % (in pigs) (Table 4). In pigs, the relative activity of the intestinal colonic microbiota was the highest for both  $\alpha$ -ZEL and  $\beta$ -ZEL formation, followed by rats where the activity of the colonic microbiota to form  $\alpha$ -ZEL was a quarter of the activity of liver S9. This comparison suggests that overall the  $k_{cat}$  of the liver is higher than of the microbiota for the reduction of ZEN.

#### 4. Discussion

The aim of the present study was to develop an *in vitro* model system to predict and quantify intestinal microbial metabolism, and to apply this model system to study interspecies differences in the intestinal



**Fig. 4.** Calculated *in vivo*  $k_{cat}$  from microbial and hepatic (microsomes and S9 fractions) formation of  $\alpha$ -ZEL and  $\beta$ -ZEL from ZEN in (A) rats, (B) pigs and (C) humans.

microbial metabolism of ZEN. The level of reduction of ZEN to  $\alpha$ -ZEL has been linked to interspecies differences in sensitivity to the estrogenic effects of ZEN [3,26] given that  $\alpha$ -ZEL has a significantly higher estrogenic potency than ZEN. While kinetics are considered to play a major role in interspecies differences [4], it should be noted that also other factors, such as differences in the estrogen receptors themselves [27,28] and differences in estrogen receptor activation sites [29] can be involved in the interspecies differences in sensitivity to ZEN exposure. The results from the ER $\alpha$ -CALUX assay used in this study showed that  $\alpha$ -ZEL was 55 times more estrogenic than ZEN, while  $\beta$ -ZEL was 25 times less estrogenic than ZEN, an observation in line with other studies comparing the relative potencies of ZEN,  $\alpha$ -ZEL and  $\beta$ -ZEL for ER $\alpha$ -mediated responses although the size of the relative differences varied with the study (Table 1). Prior studies have also reported that ZEN and its metabolites can bind to and activate ER $\beta$ , but it is not clear if there is a receptor subtype specific preference [30,31]. Despite the variation in the size of the differences, all studies corroborate that reduction of ZEN to  $\alpha$ -ZEL is a bioactivation pathway, while the reduction to  $\beta$ -ZEL can be considered a detoxification [32].

In the *in vitro* model for intestinal microbial conversion of ZEN developed in this study, the intestinal microbiota were derived from fecal slurries prepared from fecal samples from rat, pigs and humans. Intestinal microbial metabolism has been studied previously by the use of synthetic communities or isolated microbial strains [33], but one of the main advantages of using fecal samples for metabolic studies is that they allow for studying interspecies differences, as well as inter-individual differences. Another advantage of the use of fecal samples is that these can be sampled non-invasively, as opposed to collection of microbial communities from other parts of the intestinal tract, usually involving individuals with compromised health status [34–36]. When using fecal samples to characterize intestinal microbial metabolism, it is of importance to note that there are differences in bacterial numbers and compositions along the intestinal tract, which might lead to region-specific differences. However, the colon, harboring 70 % of the total bacteria present in the gut, is the main site for bacterial fermentation [37]. The bacterial communities in colon and feces have been reported to be highly comparable [38–40]. Furthermore, proofs of principle that show that anaerobic *in vitro* incubations using fecal communities can be used to predict intestinal microbial metabolic activities have been reported [41], supporting the use of anaerobic fecal samples as a representative population of intestinal microbes. Therefore, the use of anaerobic fecal samples for the study of intestinal microbial metabolism represents a first tier approach to estimate its contribution to the total metabolism in the host. An additional advantage of using fecal samples as inoculum is the high yield of material obtained, which, together with the short incubation times and volumes, allows for a high-throughput application required in *in vitro* testing [42]. The effect of freezing, storing and thawing on the metabolic activity of the fecal samples for conversion of ZEN was tested and shown not to affect the metabolism (data not shown).

In the present study, the microbiota obtained from fecal samples enabled characterization and also quantification of the kinetic interspecies differences in the metabolism of ZEN. Fecal samples have previously been used successfully in anaerobic incubations with pharmaceuticals and foodborne chemicals to assess their susceptibility to microbial metabolism [43–46]. These studies, however, assessed the presence or absence of metabolism in only a qualitative or semi-quantitative manner, which does not allow for the definition of *in vitro* kinetic constants of intestinal microbial metabolism. Optimizing the incubation conditions as done in the present study for linearity in time and with the amount of fecal slurry, allows definition of kinetic constants. Definition of these kinetic constants would be an essential requirement for integration of intestinal microbial metabolism in so-called physiologically based kinetic (PBK) models used in *in vitro-in silico* based testing strategies developed to replace *in vivo* testing [47,48]. To our knowledge, the results of the present study provide the

first proof-of-principle to use *in vitro* fecal incubations to quantify kinetic constants for intestinal microbial metabolism, and to use these *in vitro*  $k_{cat}$  values obtained for conversion to *in vivo*  $k_{cat}$  for interspecies comparisons.

In the current study, it was shown that ZEN was reduced to both  $\alpha$ -ZEL and  $\beta$ -ZEL (Fig. 1) under anaerobic conditions, which corroborates qualitative results from previous studies reporting incubations of ZEN with human fecal material [5].  $\alpha$ -ZEL and  $\beta$ -ZEL appeared to be the major metabolites in the incubations (Fig. 1), which indicates that the results from the *in vitro* fecal incubations are in line with the *in vivo* observation that  $\alpha$ -ZEL and  $\beta$ -ZEL are the major metabolites of ZEN observed in different species such as rats, pigs, chickens and humans [49–54]. Conversion of ZEN to  $\alpha$ -ZEL and  $\beta$ -ZEL represents a chemical reduction and is in line with the notion of Spanogiannopoulos et al. [55] who suggested that anaerobic respiration of the intestinal microbiota could be facilitated by the use of the broad range of xenobiotics available as terminal electron acceptors, as in anaerobic environments no oxygen is available to fulfil this function.

Our results show species differences in the *in vitro* formation of  $\alpha$ -ZEL and  $\beta$ -ZEL by the intestinal microbiota under anaerobic conditions, supporting the idea that the differences in the intestinal microbial composition may affect the metabolic activity [11,56]. Comparison of the estimated *in vivo*  $k_{cat}$  (Table 2) of the different species showed that the microbiome of pigs was overall most efficient in metabolizing ZEN, followed by that of rats and humans. Although the relatively high sensitivity of pigs to adverse effects of ZEN has been linked to the higher formation of  $\alpha$ -ZEL in the liver [3,4], the results from microbial metabolism now obtained indicate that metabolism by the gut microbiota may contribute to this interspecies difference in sensitivity where the highest  $\alpha$ -ZEL formation was observed in pigs (Table 4). Additionally, the preference for the detoxification of ZEN together with the lower intestinal microbial metabolism observed for rats compared to pigs is in line with the lower sensitivity to ZEN reported for this species [4].

The *in vivo*  $k_{cat}$  for the formation of  $\alpha$ -ZEL by the human intestinal microbiota is lower than that for pigs and rats, but so is the detoxification to  $\beta$ -ZEL. The human intestinal microbiota strongly favors the formation of  $\alpha$ -ZEL over  $\beta$ -ZEL, with ratios of formation for  $\alpha$ -ZEL: $\beta$ -ZEL around 6:1 in humans, an observation in line with what was observed for the hepatic metabolism ratio for  $\alpha$ -ZEL: $\beta$ -ZEL of 4:1. In addition to having a high preference for the formation of  $\alpha$ -ZEL, as shown also by Bravin et al. [57], and in contrast to what was observed for the intestinal microbial metabolism, expressed per kg bw human hepatic metabolism appeared to show the highest *in vivo*  $k_{cat}$  of the three species. The establishment of safe dose levels of exposure to ZEN for humans has been based on pigs as model species due to similarities in physiological and anatomical characteristics, as well as their efficient formation of  $\alpha$ -ZEL [4,58], however, the higher preference for the bioactivation of ZEN shown in the present study together with the low preference for detoxification to  $\beta$ -ZEL, 4–6 times lower than  $\alpha$ -ZEL formation, indicate a need for human-specific models to study metabolism and sensitivity to ZEN exposure. There is an urgent need for more human-relevant models in toxicology, as has also been demonstrated by the other researchers highlighting the differences in kinetics as an important contributor to species differences [59]. A better understanding of human ADME can further aid the development of human biomonitoring strategies to assess exposure to mycotoxins. This is particularly useful for mycotoxins such as ZEN, for which the occurrence of conjugated forms have been reported, but are difficult to quantify due to the lack of commercially available analytical standards [60].

The comparison of the relative catalytic efficiency of microbial and the hepatic metabolism of ZEN showed that humans compared to pigs and rats, have the lowest microbial activity with less than 0.1 % of  $\alpha$ -ZEL and  $\beta$ -ZEL formed by the microbiota. Although the use of fecal samples as a source of gut microbiota may represent a first tier

approach to estimate and quantify intestinal microbial activity it likely adequately reflects the relative interspecies differences in the intestinal microbial metabolism of ZEN.

Overall, the developed *in vitro* model was able to capture interspecies differences in the formation  $\alpha$ -ZEL and  $\beta$ -ZEL by intestinal microbiota, which can also be applied to study interindividual differences in the conversion of other chemicals known to be converted by the intestinal microbiota. The model system enables quantification of kinetic data that can be used to integrate intestinal microbial metabolism in so-called PBK models for quantitative *in vitro* to *in vivo* extrapolations (QIVIVE).

It is concluded that the intestinal colonic microbial activity may be up to 36 % of the activity of the liver and that it can additionally contribute to the species differences in bioactivation and detoxification and thus the toxicity of ZEN in pigs and rats but not in humans. The results highlight the importance of the development of human specific models for the assessment of the metabolism of ZEN.

#### CRedit authorship contribution statement

**Diana M. Mendez-Catala:** Conceptualization, Methodology, Writing - original draft. **Albertus Spenkeliink:** Methodology, Investigation. **Ivonne M.C.M. Rietjens:** Conceptualization, Writing - review & editing. **Karsten Beekmann:** Conceptualization, Methodology, Writing - review & editing.

#### Declaration of Competing Interest

The authors state no conflict of interests.

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