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ABSTRACT

The impact of the *Fusarium*-derived metabolites beauvericin, enniatin B and B1 (EB) alone or in combination with deoxynivalenol (DON) was investigated in 28–29 days old weaning piglets over a time period of 14 days. The co-application of EB and DON (EB + DON) led to a significant decrease in the weight gain of the animals. Liver enzyme activities in plasma were significantly decreased at day 14 in piglets receiving the EB + DON-containing diet compared to piglets receiving the control diet. All mycotoxin-contaminated diets led to moderate to severe histological lesions in the jejunum, the liver and lymph nodes. Shotgun metagenomics revealed a significant effect of EB-application on the gut microbiota. Our results provide novel insights into the harmful impact of emerging mycotoxins alone or with DON on the performance, gut health and immunological parameters in pigs.

1. Introduction

Cyclic hexadepsipeptides such as enniatins (ENNs) and beauvericin (BEA) are secondary fungal metabolites produced by different fungal genera, but mainly by *Fusarium* species and are considered as emerging mycotoxins (Jestoi, 2008; Urbaniak et al., 2020). Over the past decade, these ionophoric and lipophilic compounds have been the subject of controversy in the literature due to their many detrimental and beneficial effects (Caloni et al., 2020; Gruber-Dorninger et al., 2017; Křížová et al., 2021). On the one hand, they showed cytotoxic, oxidative, proinflammatory and genotoxic effects in numerous cell lines and impaired the gut barrier function of intestinal cells (Albonico et al., 2016; Juan-Garcí et al., 2015; Khoshal et al., 2019; Manyes et al., 2018; Novak et al., 2019; Prosperini et al., 2013; Springler et al., 2016). On the other side, they are also known to possess antibacterial, antifungal and insecticidal properties (Olleik et al., 2019; Urbaniak et al., 2020).

As they occur ubiquitously in feed and food samples, the European Food and Safety Agency (EFSA) published a scientific opinion on their risk as early as 2014, but no regulations or guidelines have been established to date (EFSA, 2014). This is mainly a result of the lack of *in vivo* experiments and long-exposure studies in mammals. The main challenges to perform such *in vivo* trials is the lack of naturally contaminated culture material and the economic feasibility to obtain an affordable amount of those toxins. BEA, enniatin B (ENN B) and enniatin B1 (ENN B1) were detected to 83%, 71% and 69%, respectively, of 1113 feed samples recently analysed (Kovalsky et al., 2016). Another study found a similarly high prevalence of 82% for ENN B and ENN B1, and 67% for BEA in 1141 finished feed samples for swine (Novak et al., 2019). Furthermore, several studies have been published showing different occurrence of those mycotoxins in different geographic regions, different feed commodities and feed destined for different species (Hietaniemi et al., 2016; Juan et al., 2016; Mahnine et al., 2011; Serrano

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et al., 2013). Concentrations of ENNs and BEA vary from low μg/kg to high mg/kg ranges, depending on feed type, climate, country and weather conditions (Medina et al., 2017; Santini et al., 2012; Urbaniak et al., 2020). Furthermore, co-occurrence with more investigated and regulated *Fusarium* mycotoxins, such as deoxynivalenol (DON), was already described (Khoshal et al., 2019; Spanic et al., 2020; Yoshinari et al., 2016). DON is one of the most prevent mycotoxins detected in 55%–95% of analysed feed stuff depending on the region with several described negative effects on animals health, performance and immune system (Holanda and Kim, 2021). Among the known 29 analogues of ENNs, ENN B and B1 seem to occur more often than the others (Novak et al., 2019; Reisinger et al., 2019).

The aim of this study was to investigate the short-term effects of the emerging mycotoxins BEA, ENN B and ENN B1 alone (EB), and together with the regulated mycotoxin DON (EB + DON) on the performance and feed consumption as well as on the intestine and liver of piglets. We chose 28–29 days old piglets for the study, as they are in a critical phase during the first weeks after weaning (Campbell et al., 2013) and thus, are more susceptible to mycotoxins and infectious diseases (Pierron et al., 2016a). Due to the known antibiotic properties of the used emerging mycotoxins, we further investigated their impact on the fecal microbiome by applying a shotgun metagenomics approach. Finally, we analysed the effect of the mycotoxins on several clinical blood parameters, gene expression in liver and jejunum tissue, as well as histology of liver, intestine and lymph nodes.

2. Materials and methods

2.1. Chemical and reagents

Culture material containing 4.67 g/kg beauvericin and 2.50 g/kg enniatins was prepared by Dr. Roman Labudá from the BiMM institute (Bioactive Microbial Metabolites, UFT Tulln, Austria). In brief, 200 g corn kernels were mixed with 200 mL deionized water and soaked for 60 min under room temperature (23 °C ± 1 °C) in polypropylene plastic bags (200 × 300 mm, Roth) before autoclaving at standard conditions (121 °C for 20 min). The autoclaved corn material was then transferred into disposable PD 1200 Microboxes (Nevele, Belgium). A *Fusarium oxysporum* strain RL 108 (personal collection of Dr. Roman Labudá) was used as a production organism. Inoculation was performed with 100 μL conidial suspension (1.0×10^5 conidia/mL) per box, followed by cultivation in darkness at 25 °C for 4 weeks. At the end of cultivation, the cultures were exposed to 75 °C for 2 h (closed boxes) and consequently to 70 °C (open boxes) for the next 4 days till complete dryness. Heat deactivation and drying was performed in a thermostat with forced air circulation (Binder, Germany). The dried cultures were then transferred into a 30 L capacity bag, thoroughly mixed and crushed into smaller pieces. The material was then ground in the mill (Retsch GM 200, Germany), sieved and further homogenized before final sampling. During the milling process, from every portion of freshly ground material (ca. 100 g), several grams were taken as aliquots and all such aliquots were consequently mixed (homogenized) before preparing the final representative sample. The final representative sample (10 g) in duplication was then transferred into 250 mL capacity Erlenmeyer flasks and extracted with 100 mL of solvent mix (ACN:H_2O:AcOH, 70:29:1) by rotary agitating at 160 rpm for 60 min. An aliquot of 100 μL was taken, diluted and filtered for LC-MS/MS analysis.

Culture material with a DON concentration of 15.48 g/kg was obtained by Romer Labs (Tulln, Austria) and produced according to a publication from the year 1994 (Altpeter and Posselt, 1994).

The LC-MS grade eluents used within the analytical method were purchased as followed: acetonitrile (VWR chemicals, USA), methanol (Honeywell Riedel-de-Haën™, Germany), formic acid (Honeywell Riedel-de-Haën™, Germany) and ammonium formate (Merck, Germany).

2.2. Animal and study design

All procedures related to the animal experiment were performed according to Austrian law and following the European Guidelines for the Care and Use of Animals for Research Purpose. The experiment was approved by the office of the Lower Austrian Region Government, Group of Agriculture and Forestry, Department of Agricultural Law (approval code LF1-TVG-57/015–2019) and carried out at the Center of Applied Animal Nutrition (BIOMIN Holding GmbH, Tulln, Austria).

Thirty-two crossbred piglets (Breed Ö-HYB-F1 [Landrace x Large White] x Pietrain, 28–29 days old, average weight 7.32 ± 0.72 kg, mixed sex) were obtained from a local swine producer. Piglets were randomly grouped pairwise in metabolic cages (8 animals/group), had free access to water and were allowed to acclimatize for five days. Feed was provided twice daily in the morning and evening for *ad libitum* intake. Prior to the start of the experiment, the feed was mixed with the respective contaminated culture material and tested for mycotoxin contamination by a multi-toxin LC-MS/MS method (Sulyok et al., 2020).

After an adaptation period of 5 days, piglets received control feed; DON-contaminated feed; enniatin B, B1 and beauvericin (EB)-contaminated diet or an EB + DON-contaminated diet for a period of 14 days (for mycotoxin concentrations see Table 1).

Blood of each piglet was collected after 7 days (D7) and 14 days (D14) on the experimental diets, respectively. Samples were aliquoted and stored at −80 °C until analysis. For blood sampling, about 8 mL full blood was taken with Li-Heparin tubes (S-Monovette 9 mL, Sarstedt, cat. no. 02.1065) and immediately cooled to 4 °C. After transfer to the laboratory, samples were centrifuged at 2000×*g* for 20 min at 4 °C and plasma was aliquoted á 500 μL to labeled 1.5 mL tubes and frozen to −80 °C. Determination of the respective parameters was done within the next 2 months.

Feces samples were taken at the end of the trial from individual piglets during euthanasia (D15/16). About 1 g was transferred into a homemade nucleic acid preservation buffer (NAP) and stored at room temperature (RT) for microbiome analysis.

2.3. Metagenomic analysis

DNA from individual fecal samples was extracted using the QIAamp PowerFecal kit (Qiagen, Crawley, West Sussex, UK) which as shown in a recent study, has minimum effects on the microbial community structure and promising results in terms of the DNA integrity for pig feces samples (Wegl et al., 2021). Briefly, 0.25 mg of digesta/fecal sample was added to the dry bead tube containing 750 μL of bead solution and gently vortexed. C1 solution was added, briefly vortexed, and incubated at 65 °C for 10 min. Samples were shaken in Mo Bio Vortex Adapter Genie2 at maximum speed for 5 min. Samples were centrifuged at 13,000×*g* for 1 min, the supernatant transferred to the 2 mL collection tube and the remainder of the protocol was followed as recommended by the manufacturer. All samples were eluted in 100 μL 10 mM Tris-buffer pH 8.0 after being incubated for 5 min for maximum elution efficiency. The total DNA concentration in each extract were determined fluorometrically using QubitTM dsDNA BR assay kit on a QubitTM 2.0 fluorometer (InvitrogenTM, Unites States) and purity was assessed via 260/280 and

Table 1
Mycotoxin concentration in the respective diet.

Group	Toxin				
	BEA	ENN B + B1	DON	ZEN	FB1
Control	4	161	92	<LOQ	<LOQ
DON	5	186	2524	342	<LOQ
EB	2570	1345	93	<LOQ	<LOQ
EB + DON	3578	1830	2034	263	<LOQ

Concentrations in μg/kg.
<LOQ = below limit of quantification.

260/230 absorbance ratios determined via spectrophotometry (NanoDrop® ND-1000, Thermo Fisher Scientific, United States).

For the size-selected ONT library, 600 ng of genomic DNA was used and quality was controlled using Agilent 4200 TapeStation. The DNA was sheared using Covaris g-Tubes to generate >7–8 kb fragments (Covaris, Inc., Woburn, Ma, USA). After clean-up, DNA was repaired and end-prepared using the NEBNext FFPE DNA Repair kit (New England BioLabs, Ipswich, MA, USA). AMPure XP beads were added to the repaired DNA and incubated on a Hula mixer at RT for 30 min, followed by two washes with 70% EtOH. Beads were then resuspended with 61 μL of nuclease-free (NF) water and incubated on a Hula mixer at RT for 30 min. Then, 61 μL of the eluate was transferred into a clean 1.5 mL Eppendorf tube. The resulting DNA was quantified using the Qubit HS DNA kit. Adapter ligation and clean-up was performed using the Ligation Sequencing Kit SQK-LSK109 (Oxford Nanopore Technologies, Oxford, United Kingdom) with a slightly changed protocol. In brief, ligation buffer, NEBNext Quick T4 DNA ligase, and adapter mix were added to the repaired DNA and incubated at RT for 10 min and then overnight at 4 °C. The ligated sample was purified using 100 μL of AMPure XP beads during a 30 min incubation at RT on the Hula mixer, two bead washing steps using the kit-provided wash buffer and resuspension of the beads in 40 μL of elution buffer at RT for 30 min on the Hula mixer. Then, 40 μL of the eluate was transferred into a clean 1.5 mL tube. The library was then sequenced on a MK1C device (software version 20.03) using R10 flow cell sequencing chemistry, resulting in 11,653,936 1D reads with the quality score >8. Taxonomic profiles of the demultiplexed reads and taxa relative abundance estimated were generated using Kraken2 (Wood et al., 2019) and Bracken (Lu et al., 2017). The R-packages phyloseq (v. 1.30.0) (McMurdie and Holmes, 2013) was used for microbiota data handling and calculating alpha (observed richness and Shannon index) and beta diversity.

2.4. Histological analysis

After euthanasia, samples of jejunum, colon, liver, and lymph nodes were collected from all treatments and fixed in 10% buffered formalin for histopathological evaluation. After fixation, the tissues were dehydrated in a graded series of alcohol, cleared and diaphanized by xylol, and embedded in paraffin for histological sections. Afterwards, 5-μm sections were stained with hematoxylin and eosin or alcian blue for histological analysis. Histological changes were evaluated using a lesion score scale, considering the injury intensity as described by Terciolo et al. (2019). The lesion score was established by considering the degree of severity (severity factor) and the extent of each lesion (according to intensity or observed frequency, scored from 0 to 3). For each lesion, the score of the extent was multiplied by the severity factor.

The morphometric analysis of the number of goblet cells and villi height were adapted from Bracarense et al. (2012). Goblet cells were counted randomly in ten fields per slide at 60X magnification. Villi height was measured randomly on ten villi using a MOTIC Image Plus 2.0 MLw image analysis system (MOTIC Image Plus Motic Instruments, Richmond, Canada).

2.5. Immunoassays and biochemical analysis

A human free IGF-I/IGF-1 immunoassay (Cat. No. DG100B, R&D Systems, Minneapolis, USA), a human FABP2/I-FABP immunoassay (Quantikine® ELISA, Cat. No. DFBP20, R&D Systems, Minneapolis, USA) and a porcine zonulin immunoassay (Cat. No. MBS2607498, MyBioSource, San Diego, USA) were used. The detection limits are indicated as 0.01 ng/mL, 3.63 pg/mL and 0.5 ng/mL, respectively. Determination of the respective protein was done according to the manufacturer's protocol using lithium-heparin plasma taken on D7 and D14. Plasma biochemistry was determined at D7 and D14 at GenoToul-Anexplo platform (Toulouse, France) with a Pentra 400 Clinical Chemistry benchtop analyzer (Horiba, Les Ulis, France).

2.6. Expression of jejunal and liver mRNA by real-time PCR

RT-qPCR assays were performed as previously described (Maruo et al., 2018). Primers obtained from previous studies or designed using PrimerQuest® software were purchased from Sigma (Table 2). Data were analysed with the LinRegPCR 2016.2 program. The expression values of the genes of interest were normalized against three housekeeping genes: hypoxanthine phosphoribosyltransferase 1 (HPRT1), topoisomerase (DNA) II beta (TOP2B) and hydroxymethylbilane synthase (HMBS) and validated with NormFinder software. Gene expression was expressed relatively to the control group.

2.7. Statistical analysis

Performance and feed conversion data were evaluated in the software R-3.5. Boxplots were used to visually inspect the data distribution, variability, and outliers. The model assumptions (normality, homoscedasticity, and independence) were inspected via the residual plots. Mixed Effects Models and Generalized Least Squares with subsequent multiple comparisons were used as indicated to test for differences between groups. The significance level was defined as $p < 0.05$, and $p \geq 0.05$ to <0.10 were considered as tendencies. All histology statistical analyses were performed using GraphPad Prism 9.0.2 software (GraphPad Software Inc., La Jolla, USA). Data were expressed as mean ± SEM (standard error of the mean) for normal distribution or median and interquartile range when data were not normally distributed. They were submitted to statistical analysis, using normality (Shapiro–Wilk) and homogeneity (Bartlett) tests. Significant differences were assessed by one-way ANOVA followed by Tukey's test for parametric data and by Kruskal-Wallis test followed by Dunn's test for non-parametric data. Statistical analysis for all other results were performed by GraphPad Prism 7.05 (GraphPad Software Inc., La Jolla, USA). Values were analysed for normality (Shapiro–Wilk) as well as homogeneity of variance (Levene Statistics). Normally distributed homogeneous data were analysed by one-way ANOVA (Dunnett's multiple comparison test). If normal distribution was violated, the Kruskal-Wallis Test was used. Outliers were identified by Grubbs' test and removed, if applicable.

3. Results

3.1. Growth performance

During the entire study period, the mean body weight gain significantly differed between the groups (Mixed Effects Model, $p = 0.006$). Compared to the control, the weight gain was significantly lower in the EB + DON group (Mixed Effects Model, multiple comparisons, $p = 0.039$) and tended to be lower in the DON group ($p = 0.062$) (Fig. 1A). The DON group differed significantly in the feed consumption per cage during the entire study period if compared to the control group ($p = 0.001$). In the EB-DON group, a slight decrease in feed consumption was determined as well (by trend, $p = 0.076$) (Fig. 1B).

3.2. Plasma parameters

Plasma biochemical parameters revealing liver injury (activities of alkaline phosphatase, ALP; alanine aminotransferase, ALT; aspartate aminotransferase, AST), liver function (concentrations of albumin and total protein), lipid profile, kidney function and metabolism were assessed at D7 and D14 (Table 3).

On D7, a significant increase in albumin concentration was seen in piglets that received either of the EB-containing diets from 445.1 ± 31.4 and 453.4 ± 26.9 μmol/L, respectively compared to 404.6 ± 24.7 μmol/L in the control. Additionally, total protein was higher in the EB group (55.9 ± 3.0 g/L) compared to the control (50.3 ± 3.9 g/L). Plasma calcium was slightly, but significantly higher in piglets that received the EB + DON diet (2.46 ± 0.12 mmol/L) compared to the control (2.30 ±

Table 2
Primer sequences of genes used for qRT-PCR analysis of the jejunum (J) and liver (L).

Target gene		Primer sequence (5′-3′)	mRNA	Reference
Nitric oxide synthase 2 (NOS2)	F	GAGAGGCAGAGGCTTGAGAC	ENSSSCT00000065180.2	present study
	R	TGGAGGAGCTGATGGAGTAG		
Interferon gamma (IFNG)	F	TGGTAGCTCTGGGAAACTGAATG	ENSSSCT00070017081.1	Gourbeyre et al. (2015)
	R	GGCTTTGCGCTGGATCTG		
Apoptosis inducing factor mitochondria associated 1 (AIFM1)	F	ATCATCTGCTCCAGAAGGA	ENSSSCT00000013838.4	present study
	R	AGTGCCTCCACCAATGA		
Superoxide Dismutase 1 (SOD1)	F	ATCATGGATTCCATGTCCATCAG	ENSSSCG00000021355	present study
	R	GGACCTGCACTGGTACAGCC		
Tumor protein p53 (TP53)	F	AAAAGAAGAAGCCACTGGATGG	ENSSSCT00000019534.4	present study
	R	GTTCACGCCCACGGATCT		
Tumor Necrosis Factor alpha (TNFA)	F	ACTGCACTTCGAGGTTATCGG	ENSSSCT00070048157.1	Gourbeyre et al. (2015)
	R	GGCGACGGGCTTATCTGA		
Toll-like receptor 9 (TLR9)	F	CACGACAGCCGAATAGCAC	ENSSSCT00000012516.5	present study
	R	GGGAACAGGGAGCAGAGC		
Interleukin 1A (IL1A)	F	GCCAATGACACAGAAGAAGA	ENSSSCT00000008863.3	Pierron et al. (2016b)
	R	ATGCACTGGTGGTTGATG		
Nuclear Factor Kappa B (NFkB)	F	CCTCCACAAGGCAGCAAATAG	ENSSSCT00000033438	Maruo et al. (2018)
	R	TCCACACCGCTGTCACAGA		
Mitogen-activated protein kinase 8 (MAPK8)	F	GTGGAATCAAGCACCTTCACTCT	ENSSSCT00000036610.1	present study
	R	GGGCTTTAAGTCCCGATGAATA		
Transforming growth factor beta 1 (TGFB1)	F	GGATACCAACTACTGCTTCAG	ENSSSCT00000036469.3	Gourbeyre et al. (2015)
	R	GGTTCATGAATCCACTTCCA		
Interleukin 10 (IL10)	F	GGCCCAGTGAAGAGTTTCTTTC	ENSSSCT00000017049.5	Pierron et al. (2016b)
	R	CAACAAGTCGCCCATCTGGT		
Interleukin 8 (IL8)	F	GCTCTCTGTGAGGCTGCAGTTC	ENSSSCG00000008953	Cano et al. (2013)
	R	AAGGTGTGGAATGCGTATTTATGC		
Peptide YY (PYY)	F	CTGCGCCACTACCTCAACCT	ENSSSCT00000030078.3	present study
	R	GGGAAGAGCAGTTTGGAGAGAA		
Cholecystokinin (CCK)	F	AAAGCACCTTCTGGCCGAGT	ENSSSCT00000012346.4	present study
	R	GGTCACTTATTCTGTGGCTGGG		
Insulin like growth factor 1 (IGF1)	F	TTCAGTTCGTGTGCGGAGAC	ENSSSCT00000083279.1	present study
	R	CGTACCCTGTGGGCTTGTTG		
Ghrelin (GHRL)	F	AGAAGACAGTGGTGAGGTGGAAG	ENSSSCT00000012660	present study
	R	TGAACCGGATTTCCAGCTTG		
Insulin like growth factor binding protein 1 (IGFBP1)	F	ACCGACATCAAGAAGTGGAAGG	ENSSSCT00000018209.3	present study
	R	CACTTTGTAGAGTTCTCGCTGGC		
Gastric inhibitory polypeptide (GIP)	F	CTCCTGGCAGTGGCGCTA	ENSSSCT00000030856.3	present study
	R	GTGGAATCTGGAGTGACCCTCT		
Leptin (LEP)	F	TTTCACACATGCAGTCTGTCTCC	ENSSSCT00015057529.1	present study
	R	AAGTCCAAACCGGTGACCCT		
Interleukin 1B (IL1B)	F	ATGCTGAAGGCTCTCCACCTC	ENSSSCT00070040964.1	Maruo et al. (2018)
	R	TTGTTGCTATCATCTCCTTGCAC		
Interleukin 6 (IL6)	F	TTCACCTCTCCGGACAAAACTG	ENSSSCT00000023544.3	Gourbeyre et al. (2015)
	R	TCTGCCAGTACCTCCTTGCTGT		
Caspase 3 (CASP3)	F	ATAATAAGAACTTTGATAAAAATACCGGAATG	NM_214131.1	present study
	R	TCCACATCTGTACCAGATCGACAT		
Oxidative stress induced growth inhibitor 1 (OSGIN)	F	CATTGGCAACGGTCCC	ENSSSCT00000066181.2	present study
	R	TATAGCCTGAGAGCAGGTAG		
NADH dehydrogenase subunit 2 (MT-ND2)	F	GCCTCCACTATCAGGATTTATG	ENSSSCT00000019664.4	present study
	R	GGAGTAGGCTAGTCGTATGT		
Mitochondrially encoded cytochrome *c* oxidase III (MT-CO3)	F	GCACTAGGCGTATACTTCAC	ENSSSCT00000019677.1	present study
	R	CCCTGTAGCCACAAAGAAA		
Mitochondrially encoded cytochrome *c* oxidase I (MT-CO1)	F	CTTCCACCATCCTTCCTATTAC	ENSSSCT00000019670.1	present study
	R	GGGCTAAGTTTCCAGCTAAA		
ATP synthase F0 subunit 6 (MT-ATP6)	F	CACCCACCACACAACTATC	ENSSSCT00070061678.1	present study
	R	GTGTTCCTTGTGGTAGAAA		
Cytochrome *b* (MT-CYB)	F	TAGGAGACCCAGACAACTAC	ENSSSCT00000019689.3	present study
	R	GTAGAATAGCGTAGGCGAATAA		
Mitochondrially Encoded NADH Dehydrogenase 1 (MT-ND1)	F	CATACCCACGATTCCGATAC	ENSSSCT00000019660.4	present study
	R	AGGGAGTGAGATGTGTCATA		
RNA, Ribosomal 45S Cluster 2 (MT-RNR2)	F	TCCAGGTCGGTTTCTATCT GGTAGGTCCCATTTCTCTTG	NC_026992.1	present study
	R			
Hypoxanthine phosphoribosyltransferase 1 (HPRT1)	F	CTGACCTGCTGGATTACA	ENSSSCT00065066899.1	present study
	R	CCCGTTGACTGGTCATTA		
Topoisomerase (DNA) II beta (TOP2B)	F	AAGGGCGAGAGGTCAATGAT	ENSSSCT00015080370.1	Park et al. (2015)
	R	ACATCTTCTCGTTCTTGCGC		
Hydroxymethylbilane synthase (HMBS)	F	AGGATGGGCAACTCTACCTG	ENSSSCT00000060506.2	Wang et al. (2018)
	R	GATGGTGGCCTGCATAGTCT		

0.09 mmol/L).

On D14, the liver enzymes ALP, AST and ALT were affected by the mycotoxin-contaminated diets (Table 3). The most remarkable results were observed in the EB + DON group with a decrease in 21–30% for each of the three enzymes compared to the control group.

Potential biomarkers for gut health and growth, such as the intestinal-fatty acid binding protein (i-FABP), the tight junction protein regulator zonulin (ZON) and the insulin-like growth factor 1 (IGF-1) were analysed in heparin plasma samples taken on D7 and D14 (Fig. 2 – 4).

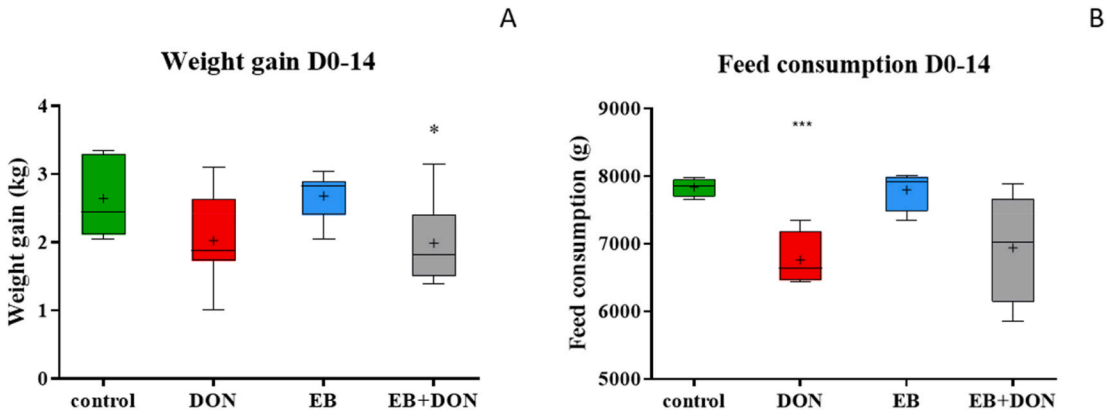

Fig. 1. Boxplot of the body weight gain of piglets (**A**; n = 8) and feed consumption of two piglets per cage (**B**; n = 4) during a time period of D0-14.
The box represents the interquartile range (IQR: 50% of data are found between Q1 to Q3). A line within the box indicates the median. The lines/whiskers outside the box extend by Q1 - 1.5 × IQR (25% of data) and Q3 + 1.5 × IQR (25% of data). The + indicates the mean of the group. Significant differences to the control are indicated with * p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Table 3
Plasma biochemical analysis at D7 (left side) and D14 (right side).

	Animal groups (time and diet)							
	Control D7	DON D7	EB D7	EB + DON D7	Control D14	DON D14	EB D14	EB + DON D14
Alkaline phosphatase (U/L)	313.8 ± 91.5	231.2 ± 51.4	238.6 ± 68.5	236.5 ± 86.7	410.1 ± 91.5	*293.6 ± 51.0**	326.3 ± 94.8	*288.6 ± 95.7**
Alanine aminotransferase (U/L)	32.6 ± 5.3	31.6 ± 4.3	31.4 ± 7.1	31 ± 6.8	57.8 ± 9.4	49.4 ± 8.35	*45.6 ± 5.9**	*45.8 ± 9.4**
Aspartate aminotransferase (U/L)	33.1 ± 8.5	34.8 ± 5.9	31.8 ± 7.6	28.3 ± 5.3	49.3 ± 7.4	46.8 ± 6.0	40.8 ± 6.0	*38.6 ± 5.7**
Albumin (μmol/L)	404.6 ± 24.7	437.5 ± 38.2	*445.1 ± 31.4**	*453.4 ± 26.9**	430 ± 42.7	453.4 ± 18.0	443.2 ± 14.6	461.4 ± 28.2
Total proteins (g/L)	50.3 ± 3.9	52.2 ± 3.3	*55.9 ± 3.0**	52.5 ± 2.3	48.4 ± 3.6	48.9 ± 3.2	48.8 ± 2.3	48.6 ± 2.1
Urea (mmol/L)	0.95 ± 0.21	1.90 ± 1.27	1.91 ± 1.14	1.74 ± 0.68	0.82 ± 0.173	0.86 ± 0.28	0.55 ± 0.12	1.06 ± 0.3
Glucose PAP (mmol/L)	6.05 ± 0.53	5.75 ± 0.39	5.96 ± 0.49	5.66 ± 0.33	6.43 ± 0.46	6.20 ± 0.58	6.45 ± 0.74	5.93 ± 0.48
Cholesterol (mmol/L)	1.81 ± 0.24	1.84 ± 0.34	1.97 ± 0.28	1.96 ± 0.29	2.05 ± 0.27	2.01 ± 0.25	1.92 ± 0.41	1.95 ± 0.27
HDL (mmol/L)	0.97 ± 0.13	0.94 ± 0.14	1.06 ± 0.14	1.05 ± 0.14	1.15 ± 0.11	1.13 ± .0.12	1.08 ± 0.23	1.13 ± 0.17
LDL (mmol/L)	0.65 ± 0.12	0.71 ± 0.20	0.73 ± 0.13	0.71 ± 0.15	0.79 ± 0.14	0.77 ± 0.18	0.74 ± 0.16	0.71 ± 0.10
Triglycerides (mmol/L)	0.47 ± 0.05	0.43 ± 0.08	0.70 ± 0.36	0.49 ± 0.10	0.4 ± 0.10	0.40 ± 0.08	0.35 ± 0.06	0.48 ± 0.14
Creatinine (μmol/L)	117.9 ± 19.2	124.1 ± 18.8	118.7 ± 12.8	110.3 ± 14.7	120.8 ± 16.2	123.4 ± 24.9	113.7 ± 10.1	120.1 ± 16.3
Uric acid (μmol/L)	13.9 ± 7.5	15.0 ± 6.8	15.9 ± 8.8	17.4 ± 6.5	19.4 ± 14.7	18.6 ± 13.6	18.9 ± 10.9	17.5 ± 9.2
Calcium (mmol/L)	2.30 ± 0.09	2.36 ± 0.05	2.39 ± 0.07	*2.46 ± 0.12**	2.64 ± 0.16	2.64 ± 0.11	2.59 ± 0.13	2.55 ± 0.13
Phosphorus (mmol/L)	2.24 ± 0.10	2.22 ± 0.19	2.32 ± 0.15	2.39 ± 0.20	2.77 ± 0.16	2.84 ± 0.17	2.80 ± 0.22	2.85 ± 0.24

Values are means ± SD. Comparison between control group and animals exposed to contaminated diets. n = 8; Significant differences are indicated with * p ≤ 0.05 (marked italics).

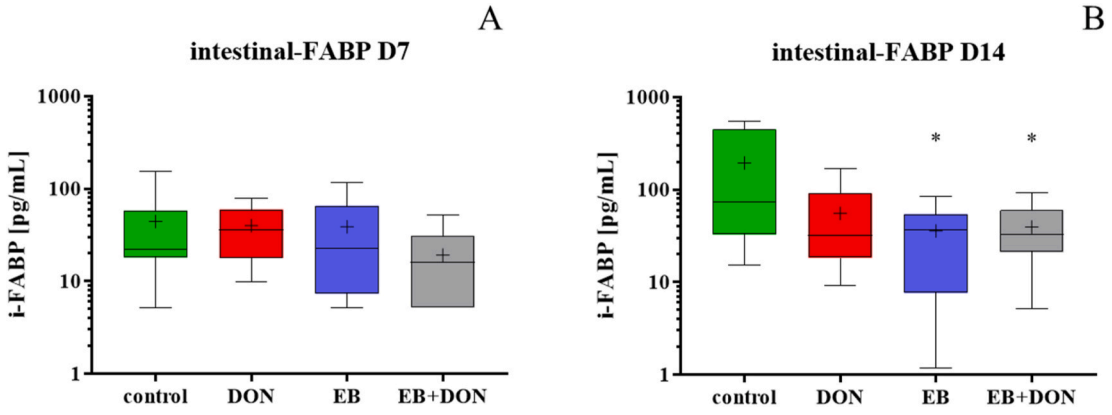

Fig. 2. Intestinal-fatty acid binding protein (pg/mL) measured in heparin plasma samples from D7 (**A**) and D14 (**B**); n = 8; Significant differences are indicated with * p ≤ 0.05.

As shown in Fig. 2, the i-FABP concentration was significantly decreased in the EB group (p = 0.028) and the EB + DON group (p = 0.032) on D14 compared to the control. This protein was also reduced in the DON-fed group, but not significantly (p = 0.059). IGF-1 protein, which might be a parameter for growth reduction, was not significantly affected by the different diets on any sampling day. However, p-value between control and EB + DON group was 0.092 on D14, which could indicate a declining trend. Likewise, a decrease in IGF-1 mean values in

the DON group on both days is evident, from 50.1 ng/mL (control) to 43.1 ng/mL (D7) and from 83.1 ng/mL (control) to 68.9 ng/mL, respectively (Fig. 3). ZON, described as permeability marker in chronical bowel diseases, did not vary between the groups, although a decreasing trend was seen in the DON group ($p = 0.099$) on D14 (Fig. 4).

3.3. mRNA expression in jejunum and liver tissue

The mRNA expression of genes related to immune response (NOS2, IFNG, AIFM1, SOD1, TP53, TNFA, TLR9, IL1A, NFKB1, MAPK, TGFB1, IL10, IL8) as well as to satiety and growth (PYY, CCK, IGF1, GHRL, IGFBP1, GIP, LEP) was analysed in the jejunum. The results are presented in Fig. 5. Expression profile of most genes showed a slight, but not significant upregulation in the DON group or the EB + DON group.

In the liver tissue, the expression of genes coding for inflammatory mediators (IL1B, AIFM1, TP53, TNFA, SOD1, TGFB1, IL6, CASP3, OSGIN1) and of mitochondrial genes (MT-ND2, MT-CO3, MT-CO1, MT-ATP6, MT-CYB, MT-ND1, MT-RNR2) was affected but to a lesser extent (Fig. 6). Oxidative Stress Induced Growth Inhibitor 1 (OSGIN1) tended to be up-regulated in the two groups receiving DON.

3.4. Histology

In jejunum, a significant increase ($p = 0.007$) in the lesion score was detected in animals fed the multi-toxin contaminated diet (EB + DON) compared to the control (Fig. 7A). Control animals showed well-delineated villi lined by columnar enterocytes (Fig. 7B), while changes such as lymphatic vessel dilation and interstitial edema with mild intensity were also observed in these animals. Animals fed the DON-contaminated diet showed villi atrophy, as well as flattening and cytoplasmic vacuolation of enterocytes (Fig. 7C). In animals that received the EB diet, cytoplasmic vacuolation of enterocytes was the most frequently observed change (Fig. 7D). Animals receiving the EB + DON-contaminated diet showed mainly villi atrophy and vacuolation of enterocytes (Fig. 7E). A significant decrease in the number of goblet cells in the jejunum was observed in DON-fed animals ($p = 0.029$, Fig. 8A). Furthermore, villi height was decreased in the DON- ($p = 0.008$) and EB + DON-fed ($p = 0.022$) groups (Fig. 8B).

Colon samples were analysed, however, no significant difference was observed in the lesion score between treatments (Fig. 9A). The control group showed a normal histological structure (Fig. 9B). The main histological findings in pigs fed the mycotoxin-contaminated diets were edema of the lamina propria, flattening and cytoplasmic vacuolation of apical enterocytes as well as focal areas of enterocytes necrosis (Fig. 9C–E).

In the liver, animals fed EB and EB + DON diets showed a significant increase ($p = 0.043$ and $p = 0.011$, respectively) in histological changes when compared to the control (Fig. 10A). Control animals showed well-organized hepatocytes forming trabeculae, while mild cytoplasmic vacuolation of hepatocytes was frequently detected in this group (Fig. 10B). Animals receiving the contaminated diets showed mainly moderate to severe vacuolation of hepatocytes and megalocytosis (Fig. 10C–F). Apoptosis of hepatocytes and focal necrosis were also detected in DON and EB + DON groups (Fig. 10C + E).

A significant increase in histological changes ($p = 0.022$) was detected in lymph nodes of animals exposed to DON in comparison to control. Lymph nodes of control animals presented a histological aspect within normal limits. In pigs fed the DON or EB + DON contaminated diets, the main findings were lymphoid depletion and apoptosis, while in the EB group lymphoid hyperplasia was the most frequent change (Fig. 11).

3.5. Metagenomic data of the fecal microbiome

Mycotoxin applications substantially impacted the diversity and structure of fecal microbiota in weaned piglets. Shotgun metagenomics of 32 collected fecal samples using the MK1C platform generated over 11 million 1D reads with the quality score >8 with a mean number of about 300 k reads per sample. Taxonomic profiling of the fecal samples in terms of the most abundant microbial phyla and species is shown in Fig. 12. In total, 1945 different microbial species were observed, of which Firmicutes, followed by Ascomycota and Proteobacteria, were the most dominant members of the piglet's fecal microbiota. Interestingly, the phyla Actinobacteria was more dominant in the EB group which goes along with a reduction in Ascomycota (Fig. 12A). In regards to the most abundant species, *Aureobasidium namibiae* completely disappeared in the EB and EB + DON groups, while the *Mycobacterium branderi* is found in a remarkable amount in the EB group, but not in the others (Fig. 12B).

Permutational Multivariate Analysis of Variance (PERMANOVA) statistical analysis calculated on Bray–Curtis dissimilarity matrices from Kraken2 and Bracken data showed that the difference in the treatment groups of the piglets explained 14% of the variation in fecal microbiota profiles of the piglets in the current study ($R2 = 0.13351$, $p = 0.005$), showing a significant impact of the applied mycotoxins on the structure and community membership of the piglet's fecal microbiota. Furthermore, pairwise post-hoc comparisons (https://rdrr.io/github/GuillemSalazar/-EcolUtils/man/adonis.pair.html) showed that among different groups, fecal microbiota of the piglets in the EB group showed the most significant difference in the profiles of the relative abundance of taxa compared to the animals in the control group (control vs EB, $R2 = 0.10$, FDR-corrected $p = 0.005$), followed by DON (control vs DON, $R2 = 0.072$, FDR-corrected $p = 0.02$) and EB + DON (control vs EBD, $R2 = 0.068$, FDR-corrected $p = 0.02$). In line with the observation from the beta diversity analysis, a significant effect of mycotoxins contamination

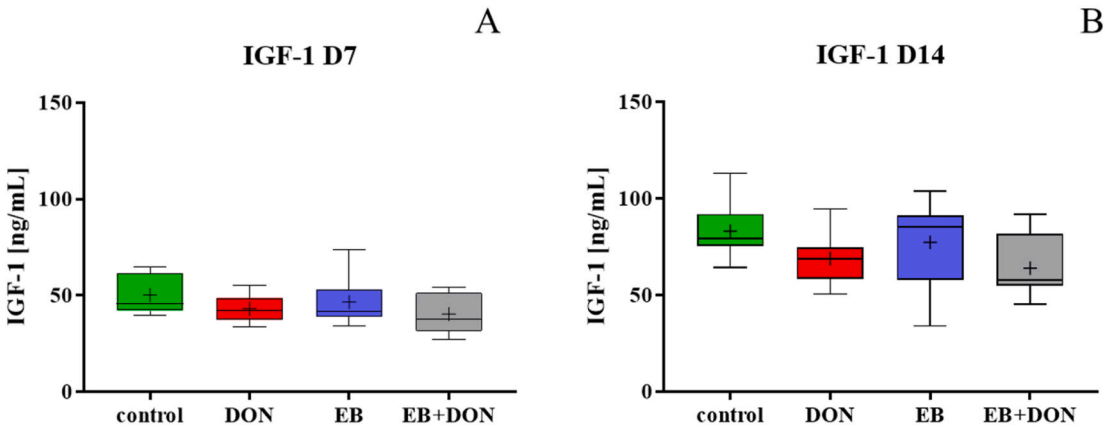

Fig. 3. IGF-1 concentration (ng/mL) measured in heparin plasma samples from D7 (**A**) and D14 (**B**); n = 8.

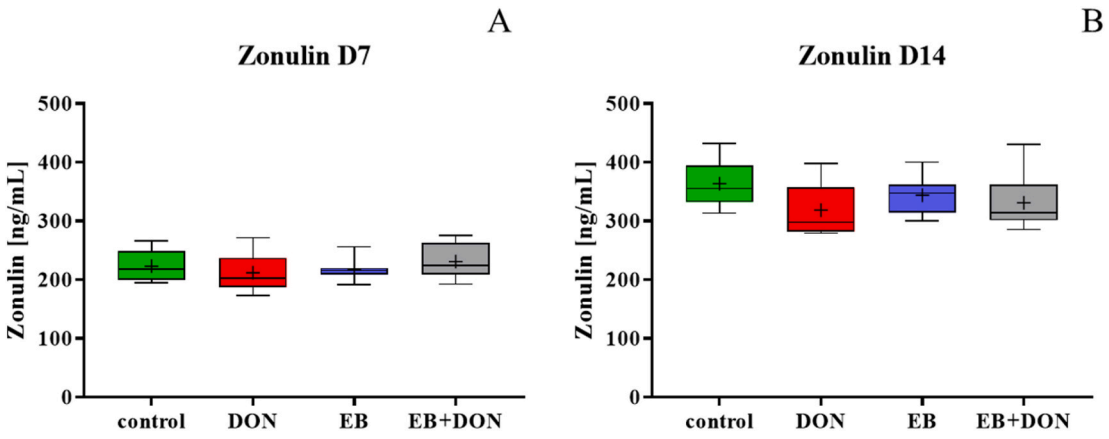

Fig. 4. Zonulin concentration (ng/mL) measured in heparin plasma samples from D7 (**A**) and D14 (**B**); n = = 8.

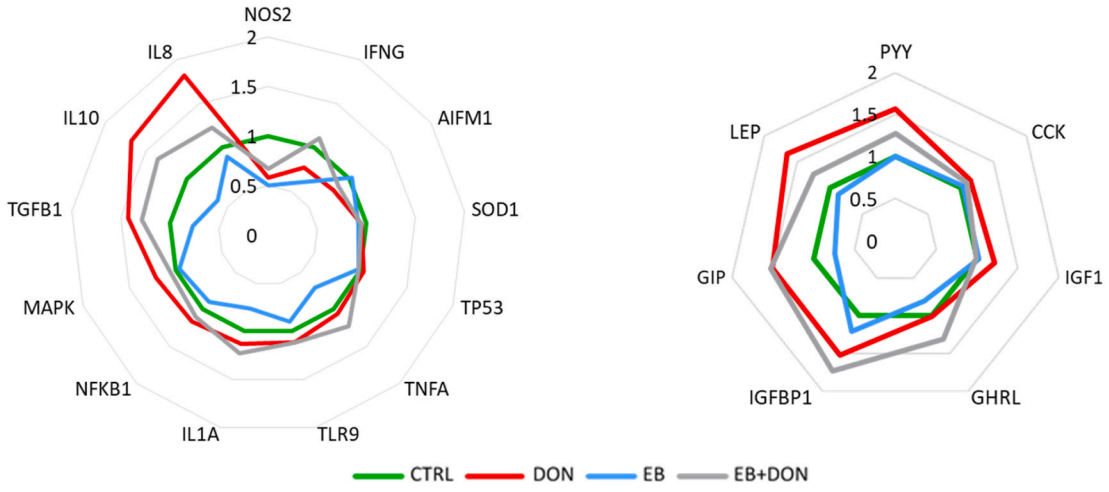

Fig. 5. mRNA expression of selected genes in the jejunum of animals fed a control or contaminated diet. Mean values of gene expression levels for the control group are set to 1.

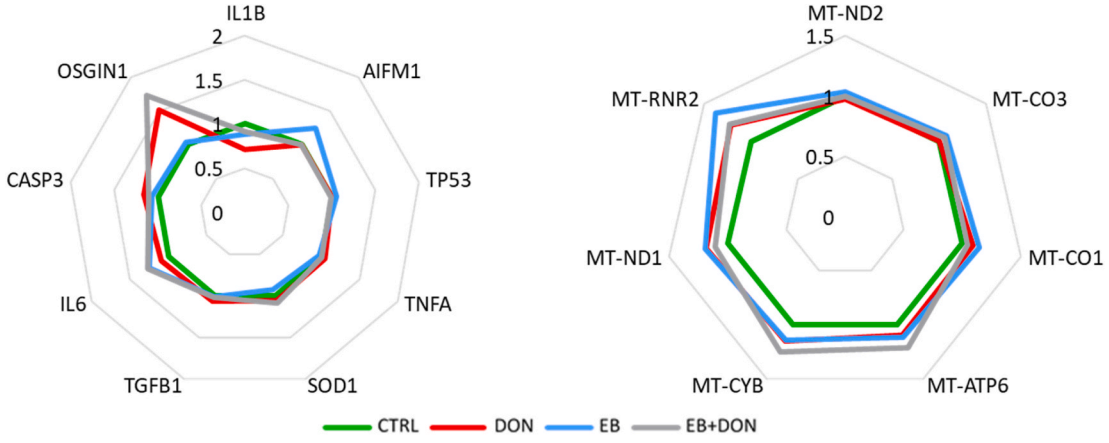

Fig. 6. mRNA expression of selected genes in the liver of animals fed a control or contaminated diet. Mean values of gene expression levels for the control group are set to 1.

on the alpha diversity indices was observed, with the EB + DON resulted in a significantly lower microbial diversity (measured by Shannon index, p = 0.004 and 0.088, respectively) and richness (measured by Observed index, p = 0.035 for EB) in the fecal microbiota of the piglets (Fig. 13).

4. Discussion

In this study, we investigated, for the first time the effects of a diet contaminated with the emerging mycotoxins enniatin B, enniatin B1 and beauvericin (EB) on piglets. As enniatins (ENNs) and BEA frequently co-occur with the regulated *Fusarium* mycotoxin DON (Kovalsky et al.,

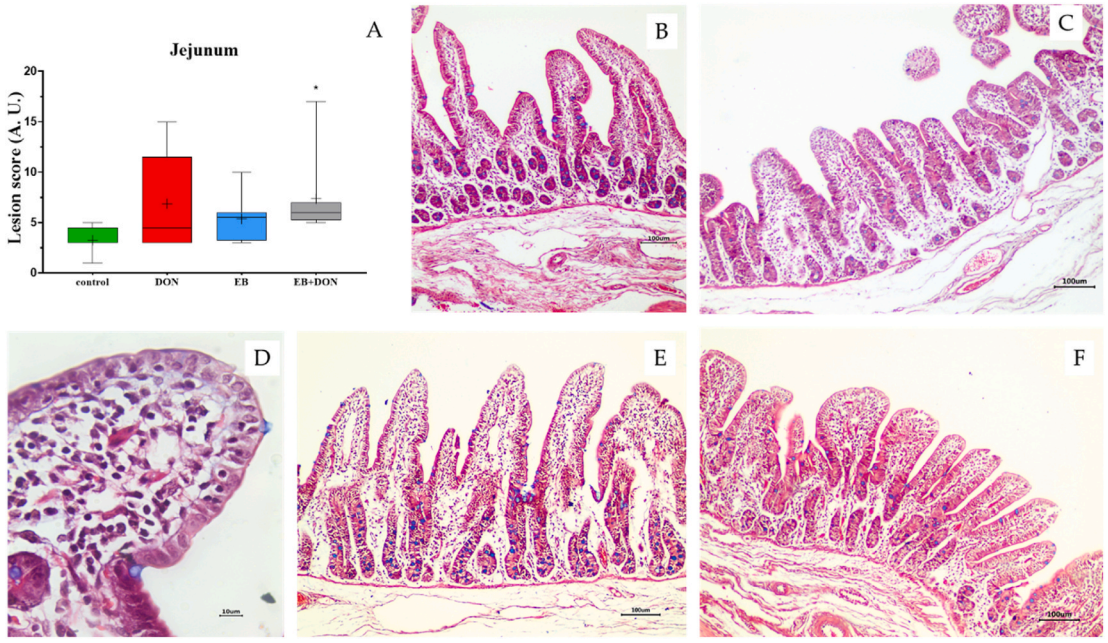

Fig. 7. Lesion score of jejunum tissue (**A**, n = 8) and histological images of experimental groups. Control (**B**) showing a normal aspect of villi; DON (**C**) with villi atrophy and edema of lamina propria; EB (**D**) showing enterocyte flattening; EB + DON showing edema of lamina propria (**E**) and villi atrophy (**F**). Alcian blue staining. Scale bar 100 μm (B, C, E, F), 10 μm (**D**). Significant differences are indicated with * $p \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

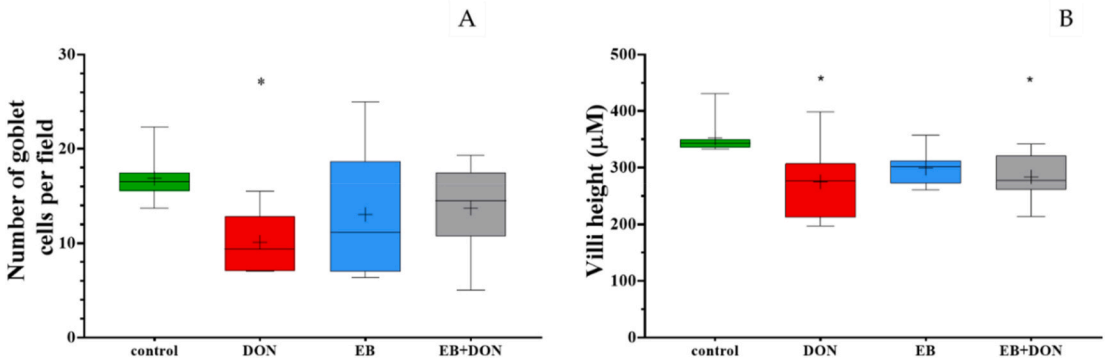

Fig. 8. Number of goblet cells per field (**A**) and villi height (**B**) in the jejunum (n = 8). Significant differences are indicated with * $p \leq 0.05$.

2016; Lindblad et al., 2013), we included a combinatory group to determine possible combined effects. We used a BEA concentration of 2570 μg/kg and 3578 μg/kg, as well as a joint ENN B and ENN B1 concentration of 1345 μg/kg and 1830 μg/kg in the EB and EB + DON group, respectively. In the field, BEA is found in rather low concentrations (~100 μg/kg), however, also high contamination up to 26300 μg/kg were already determined in specific feed stuff and countries. Regarding ENN B and B1, the usual contamination is higher with detected maximum levels of 81100 μg/kg and 795000 μg/kg, respectively (Fraeyman et al., 2017). However, concentrations are difficult to compare as many variables have to be considered such as feed stuff, region, analytical method, sampling as well as weather and storage conditions.

As a first indicator, we determined weight gain and feed consumption of the animals. In the 14 experimental days, the weight gain of 2.64 ± 0.56 kg in the control group was significantly decreased to only 1.99 ± 0.60 kg in the EB + DON group, but remained constant in the EB group with 2.68 ± 0.34 kg. As with 2.03 ± 0.66 kg the weight gain in the DON group was also low (by trend, Fig. 1A), the observed negative effect of EB + DON derived due to an enhanced intake of DON through the gut barrier, triggered by the ionophoric properties of the emerging mycotoxins (Tonshin et al., 2010). Also *in vitro* experiments showed synergistic effects of ENNs with DON on the gut integrity in intestinal porcine epithelial cells (Springler et al., 2016). The direct reason might be the reduced feed consumption in both DON groups. Animals which received the DON-contaminated diet ate 6763 ± 404 g of feed on average; EB + DON-fed animals only 6943 ± 832 g (Fig. 1B). A significant difference was detected only for the DON group compared to the control (7839 ± 135 g). A lower consumption of DON-contaminated feed has been described by several other scientists (FAO/WHO, 2001; Prelusky, 1997; Rotter et al., 1996; Trenholm et al., 1994) and is believed to be a consequence of the impact of DON on satiety hormones, such as peptide YY (PYY) and cholecystokinin (CCK) (Flannery et al., 2012; Knutsen et al., 2017). However, only a slight, but not statistically significant up-regulation of the expression of genes coding for satiety and growth hormones was observed (Fig. 6), which could be a matter of sampling time point (Taylor et al., 2019). One of the most remarkable effects of DON is the anorexia and also emesis that are elicited by the direct action on the central nervous system and by the indirect action of enteroendocrine cells that secretes several gut hormones (Terciolo et al.,

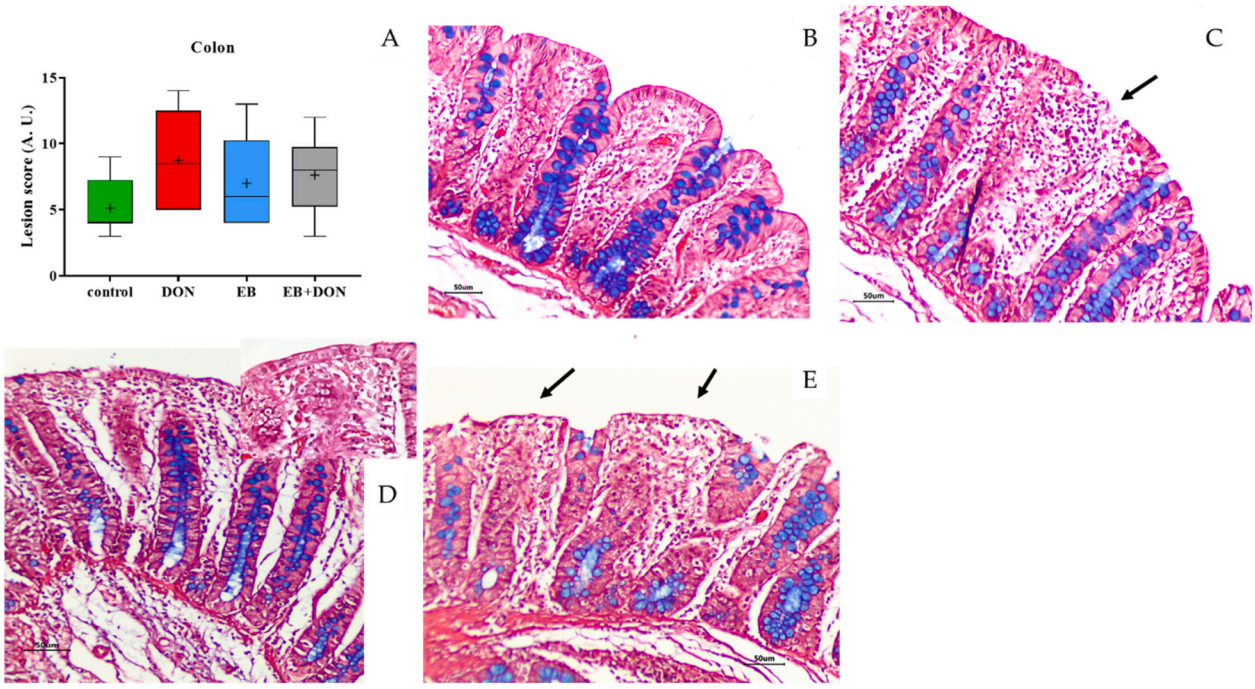

Fig. 9. Lesion score of colon tissue (**A**). Control: Normal aspect of colon (**B**). DON: Edema of the lamina propria, focal necrosis of enterocytes (arrow) (**C**). EB: Edema of lamina propria, flattening of enterocytes. Insert: Flattening of enterocytes (**D**). EB + DON: Multifocal flattening of enterocytes and necrosis (arrows) (**E**). Alcian blue staining. Scale bar 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

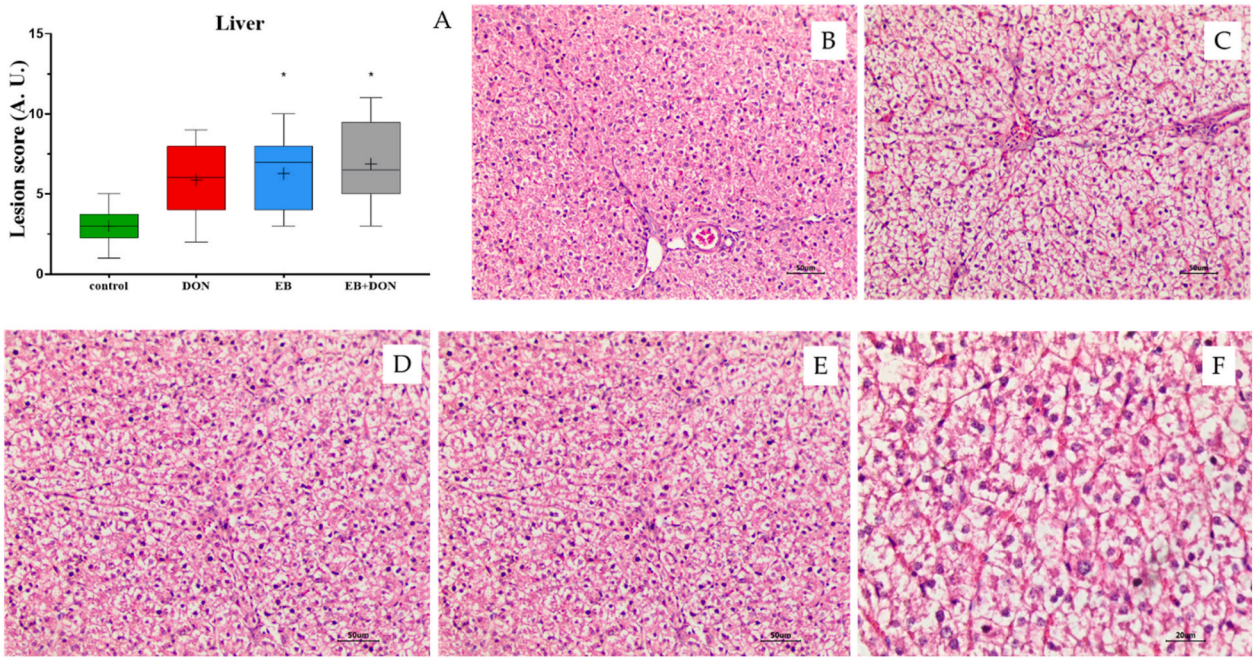

Fig. 10. Lesion score of liver tissue (**A**) and histological images of control (**B**) showing a normal aspect of liver; DON (**C**) showing diffuse and severe vacuolar degeneration of hepatocytes; EB (**D**) showing moderate vacuolar degeneration of hepatocytes; EB + DON showing moderate vacuolar degeneration of hepatocytes (**E**) and megalocytosis (**F**). Hematoxylin and Eosin (H&E) staining. Scale bar 100 μm (**B-E**), 20 μm (**F**). Significant differences are indicated with * $p \leq 0.05$.

2018).

In general, due to the inter-individual variability and the limited number of animals in this trial, the number of genes whose expression was significantly regulated by the ingestion of a mycotoxin-contaminated diet was limited. However, the tendency of some genes to be upregulated was consistent with previously described effects of DON. Increased production of cytokines upon DON exposure has already been demonstrated in human cells (Maresca et al., 2008), in porcine intestinal cells (Cano et al., 2013), in jejunal explants from pigs (Pierron et al., 2016b) as well as *in vivo* in pigs and mice (Azcona-Olivera et al., 1995; Bracarense et al., 2012, 2020; Pierron et al., 2018). In the present study, a 2-weeks-week exposure to ~2 ppm DON showed a tendency to elicit a pro-inflammatory response. As zearalenone (ZEN) concentration with 342 and 263 μg/kg, respectively, in both DON groups was quite

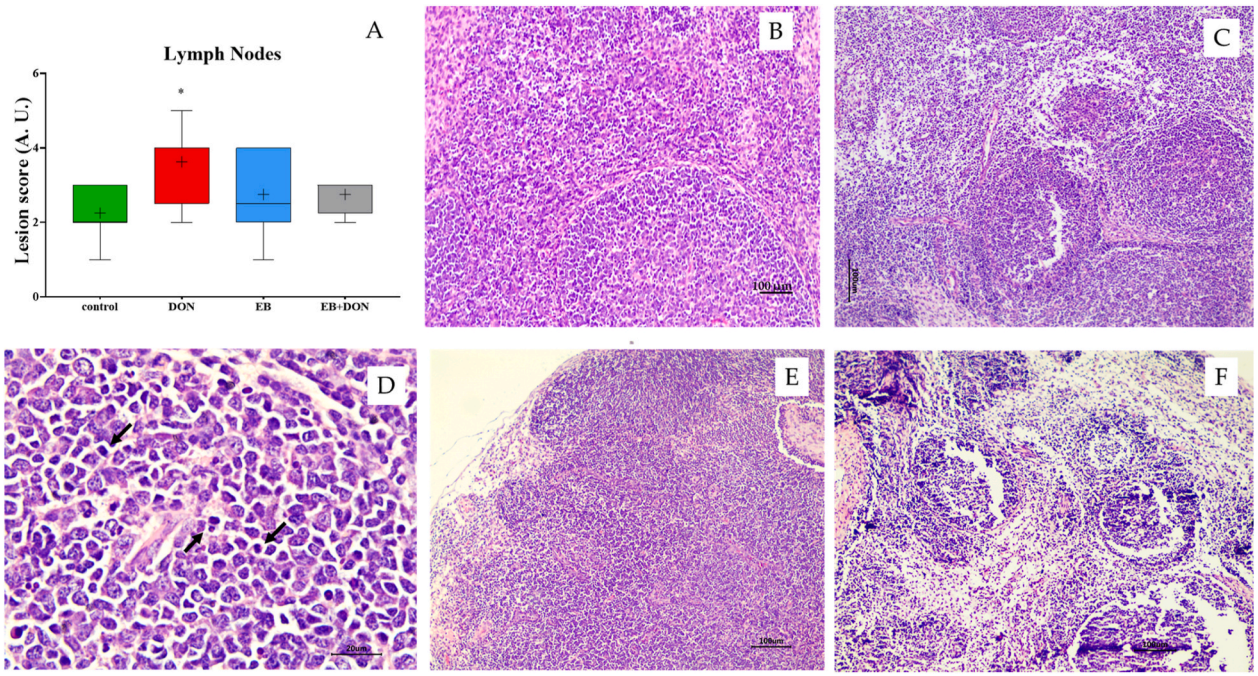

Fig. 11. Lesion score of lymph nodes tissue (**A**) and histological images of control (**B**) showing a normal aspect of lymph node; DON showing lymphoid depletion (**C**) and lymphocyte apoptosis (**D**); EB (**E**) showing lymphoid hyperplasia; EB + DON (**F**) showing severe lymphoid depletion. Hematoxylin and Eosin (H&E) staining. Scale bar 100 μm (**B, C, E** and **F**), 20 μm (**D**). Significant differences are indicated with * $p \leq 0.05$.

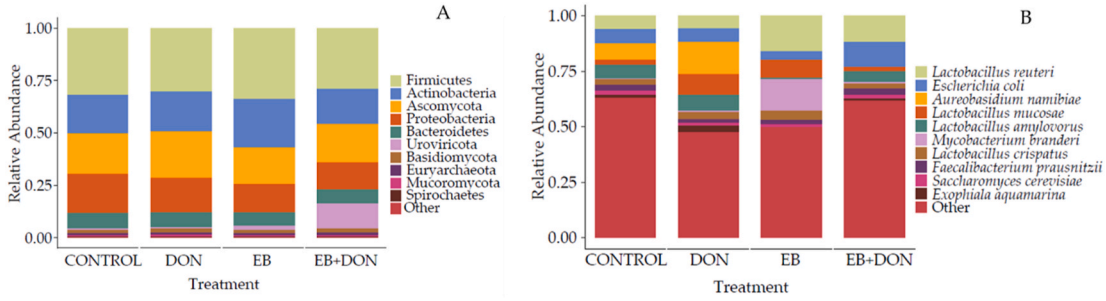

Fig. 12. Relative abundance of 10 most abundant microbial phyla (**A**) and microbial species (**B**) found in the fecal microbiome of pigs fed with the different diets.

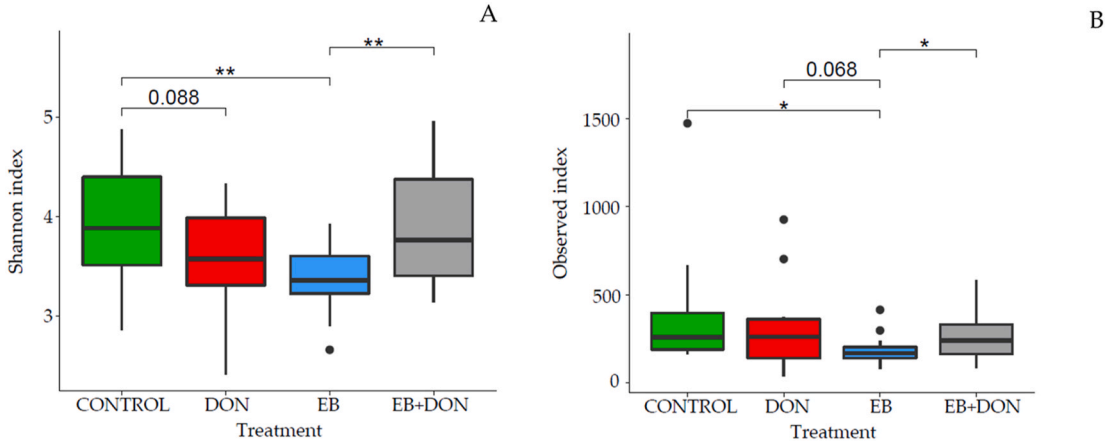

Fig. 13. Alpha diversity analysis of the taxa in pig fecal microbiome (**A**: Shannon index, **B**: observed index); n = = 8; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

high, an impact of ZEN on inflammation mediators could be possible (Pistol et al., 2015). However, clinical effects on estrogenic parameters such as an increased, swollen vulva in female pigs were not detected (Gajecki, 2002). Since ZEN is not known to elicit any effect on our analysed parameters, we assume that our findings are mainly caused by EB and/or DON. Most interestingly, co-exposure to EB seemed to reduce

the inflammatory potential of DON, and led to a down-regulation of cytokines (Fig. 6), possibly due to their ionophoric properties disrupting several functions of cells (EFSA, 2014). Antagonistic cytotoxic effects of DON and EB have already been described in IPEC-1 cells (Khoshal et al., 2019). Our results, both on feed consumption and growth and on mRNA expression, corroborate the indirect action of hormones on the anorexic mechanism of DON and, again, the association with EB partially antagonized this effect. Recent studies have highlighted the involvement of mitochondrial genes in the acute toxicity of DON (Wang et al., 2021), BEA and ENNs (Alonso-Garrido et al., 2018; Escrivá et al., 2018, 2019) in cultured cells. Our experiment indicates that the expression of these genes was also slightly exacerbated upon *in vivo* chronic exposure to the toxins (Fig. 6).

Besides tissue samples from different organs, we also took plasma samples at the middle and at the end of the trial. Increased concentrations of albumin and total proteins were observed in pigs exposed to EB at D7 (Table 3). Co-exposure to EB and DON showed a similar effect on albumin but not on total proteins (Table 3). Albumin has a turnover of about 25 days and there is a relative consensus in the literature that hyperalbuminemia, an increase in the albumin concentration in serum is mainly due to dehydration (Busher, 1990; Levitt and Levitt, 2016). Increased serum albumin levels in our trial could be due to the ionophoric effect of ENNs and BEA on cell membranes leading to a disturbance of osmosis. The lack of increase in total protein concentration in the EB + DON group could be related to the well-characterized inhibitory effect of DON on protein synthesis after its binding to the ribosome (Payros et al., 2016). The increase in albumin level in the EB + DON group accompanied an increase in circulating calcium (Table 3). The lack of an increase in calcium in the EB group could be due to the fact that BEA increases cytoplasmic calcium concentration, thereby reducing the amount of circulating calcium (Chen et al., 2006). These effects seem to be temporary since no differences between the groups were seen at D14.

At D14, the ALP concentration was decreased in DON-receiving groups (Table 3), although usually an increase in liver enzyme activity is associated with liver damage (Giannini et al., 2005). An explanation could be that enzymes increase rapidly after the first days of exposure and on D14, the activity has already dropped to a lower level. A positive correlation between food consumption and serum ALP was reported previously (Amacher, 2002). So, the decreased feed consumption may have led to a reduction in serum ALP in this study. Another reason could be a zinc deficiency elicited by an inadequate absorption and which correlates with a low ALP (Cho et al., 2007). ALP activity is a good marker of intestinal function. Indeed, it was shown to be higher in rat intestinal mucosa than in liver (Ramaiah, 2007). ALP participates in the metabolism of vitamin B6 which is a co-factor for both ALT and AST (Ono et al., 1995). In fact, both groups exhibiting ALP reduction (DON, EB-DON) also presented reductions in ALT and AST activity (Table 3). (EB-DON). Although an increase in liver enzyme activity is associated with liver injury, also reductions on ALT and AST activities have been seen in cases of chronic liver diseases usually associated with other pre-existent condition such as kidney disease (Ono et al., 1995).

We additionally analysed the effects of mycotoxin-contaminated diets on proteins in porcine plasma associated with growth or gut barrier functionality, i.e. i-FABP, IGF-1 and ZON, in the porcine plasma. IGF-1 is known to correlate with growth performance and a reduction of this biomarker has been already described upon DON exposure (Pestka, 2010; Voss, 2010). However, in our study, no significant impact of mycotoxin-contaminated diets on IGF-1 in serum was seen (Fig. 4), although trends were determined in the DON group on both sampling days as well as in the EB + DON group on D14. Additionally, the IGF-1 mRNA expression in jejunum was slightly increased (Fig. 5), which is in accordance with a previous study in mice, in which a higher gene expression in the liver was observed, but circulating IGF-1 was decreased (Amuzie and Pestka, 2009).

ZON, a widely-discussed biomarker for mucosal barrier integrity (Fasano, 2020), has been described in humans suffering from inflammatory bowel disease (Caviglia et al., 2018). In DON-fed piglets a significant increase in ZON was associated with the damaging effect of this mycotoxin on the gut barrier (S. Wang et al., 2018). In contrast to our expectations, we observed a slightly lower ZON level in the DON group (318.7 ng/mL) compared to the control group (363.6 ng/mL). This has also been shown in another study in patients with chronic kidney diseases and the authors of this study hypothesized that low ZON concentration is caused by impaired defensive mechanisms due to a suppressed immune system (Lukaszyk et al., 2018).

As another biomarker for intestinal permeability, we analysed i-FABP, which is considered to be released into the blood stream from damaged mucosal tissue (Funaoka et al., 2010). Again, we detected a significant decrease in i-FABP in both EB-containing diets on D14, instead of the expected increase (Fig. 2). However, Lau et al. also reported an unexpected decrease in i-FABP plasma levels with a parallel increased gene expression in the jejunum (Lau et al., 2016). They hypothesized that increased i-FABP levels could be a result of an increased production by enterocytes rather than tissue damage. Furthermore, EB inhibit protein biosynthesis (Olleik et al., 2019) and thereby, might lead to a reduced i-FABP plasma concentration.

As blood parameters alone can be difficult to interpret due to limitations in methods, dependence on sampling time point and choosing the right parameter (Celi et al., 2019), we additionally investigated the extent of histological lesions in selected organs. As the intestine and the liver are the main sites of drug metabolism (Chhabra, 1979), we focused on jejunum, colon, liver and additionally on lymph nodes. DON-containing diets led to significant changes in the jejunum seen as villi atrophy, flattening and cytoplasmic vacuolation of enterocytes, reduced number of goblet cells and shorter villi (Fig. 8A + B). This is in accordance with several other studies (Bracarense et al., 2012; Gerez et al., 2015; Pinton and Oswald, 2014; Przybylska-Gornowicz et al., 2018). The most frequent change observed in EB-fed animals was vacuolation of enterocytes. This change may be associated with the ionophoric properties of ENNs and BEA resulting in an imbalance of cell membrane permeability. Enniatins have the ability to incorporate into cell membranes forming cation selective pores with high affinity to Na+ (Kouri et al., 2003). Increases in cytosolic Na+ is followed by intracellular water rise and cell swelling (Myers et al., 2012). No significant effects were seen in the histological part of colon tissue between the different groups. Most likely, the parent fungal compounds were already metabolized into less-toxic substances as known for DON and its metabolite deepoxy-DON (DOM-1) (Bracarense et al., 2020). In general, the effects of EB were more significant on liver tissue than in the intestine (Figs. 7–10), which might be due to rapid absorption known for ENN B1, and its fast metabolism in the liver (Devreese et al., 2014). Piglets receiving the EB-diets showed moderate to severe vacuolation of hepatocytes and megalocytosis (Fig. 10). This could be due to a possible bioaccumulation of lipophilic ENNs and BEA in fat-rich tissue as seen in mice (Rodríguez-Carrasco et al., 2016). In addition, the administration of 50, 100 or 200 mg/kg b.w. of BEA or ENN B by oral gavage in mice induced mild toxic effects in the liver (Maranghi et al., 2018). However, those doses are very high and perhaps mice may be more tolerant to these mycotoxins than pigs, but it is hard to make this conclusion confirm due to the lack of *in vivo* studies with emerging mycotoxins. In total, apoptosis and necrosis were detected only in animals fed the DON-diets. An increase in the lesion score of piglets fed with DON-diets was reported previously (Bracarense et al., 2020; Mikami et al., 2010; Pierron et al., 2018), but apoptosis was not present in the liver of rats despite the increased lesion score in this organ (Bracarense et al., 2017). In lymph nodes, a significant increase in the histological score was detected in DON-exposed animals showing lymphoid depletion and apoptosis (Fig. 11). This is in accordance with several studies performed by our group, which found that DON increased the lesion score in the lymph nodes in both, piglets and rats (Bracarense et al., 2017, 2020; Gerez et al., 2015; Mikami et al., 2010; Pierron et al., 2016a). In this

study, lymphoid hyperplasia was frequent in the EB group, but the lesion score did not differ from the control group (Fig. 11).

The antibiotic activity of ENNs and BEA against mostly gram-positive bacteria and fungi due to their ionophoric properties has already been studied (Olleik et al., 2019; Sy-Cordero et al., 2012), and could explain differences in fecal microbiome between groups in our study. The fecal microbiome of animals receiving the EB diet showed a lower diversity compared to the control group, but also compared to the EB + DON group. Therefore, it is tempting to speculate that DON antagonized the antibiotic effect of EB. Furthermore, a change in the microbial pattern was seen in all mycotoxin-containing groups (Fig. 12). This life stage of pigs, only two weeks after weaning, is crucial as the microbial flora is not fully developed which can lead to higher susceptibility to diarrhea, weight loss and mortality (Rhouma et al., 2017). The importance of the intestinal gut microbiome has been studied more extensively in the last decade due to development of the "omics" methodologies. Therefore, we already know the strong correlation between a high diverse microbiota and appropriate functions such as enhanced feed efficiency, production of volatile fatty acids, resistance against pathogenic bacteria and improved immune system (Ghanbari et al., 2019; Guevarra et al., 2019; Kraler et al., 2016; Stokes, 2017; Yang et al., 2017). One study showed that DON, applied in a concentration of 2.8 mg/kg, changed the microflora balance and modified the richness index in 9-week-old pigs, but did not influence the diversity (Waché et al., 2009). In our study, DON alone also led to a slight decrease in the diversity of taxa, but not significantly ($p = 0.088$) and in the EB + DON group no effect was found (Fig. 13). A higher translocation of a pathogenic *Escherichia coli* strain was seen *in vitro* using intestinal porcine cells (IPEC-1), when treated with DON (Pinton et al., 2009). The relative abundance of *E. coli* in the DON group was not increased (Fig. 12). However, in animals that received DON + EB, an increase in *E. coli* abundance was seen. Another remarkable alteration in EB-fed piglets was the increase in *Actinobacteria* and *Mycobacterium branderi* with a parallel decrease in *Ascomycota* and the species *Aureobasidium namibiae*. An alteration of the mycobiome (the fungal part of the microbiome) has been associated with several diseases in humans (Chin et al., 2020). There is not much literature about the species *M. branderi*, belonging to the phylum *Actinobacteria*, except that it is a potential pathogen first isolated from human respiratory tract (Koukila-Kahkola et al., 1995). Also, the abundant *Lactobacillus* species L. *amylovorus* known to exhibit positive probiotic activities in weaning piglets (Konstantinov et al., 2006) was reduced only in the EB-fed group, but not in the EB + DON group. On the other hand, the relative abundance of *L. reuteri* was increased in the EB group and also, to a lesser extent, in the EB + DON group compared to the control and the DON group. *L. reuteri* is a lactic acid bacterium and proven to reduce *Cryptosporidium parvum*-induced diarrhea in piglets (Casas and Dobrogosz, 2000). This is in contrast to a recent publication (Reddy et al., 2018), in which *Lactobacillus* was significantly more abundant in piglets fed a DON-diet (7.38 mg/kg) compared to the control. However, in this study 8-week-old piglets were used for a 4-week trial with a quite high DON contamination, which is in contrast to our approach.

5. Conclusion

All together it could be seen that a contaminated diet including EB led to a decrease in the diversity of the gut microbiome as well as to a shift in certain fungal, viral and bacterial genera in weaning piglets. Interestingly, these effects were not so evident when DON was additionally included in the diet, which indicates a combinatory effect. The EB + DON diet significantly decreased the weight gain within the short period of 14 days, which was not seen in the EB or DON groups. Furthermore, all mycotoxins-receiving animals showed moderate to severe histological changes in the intestine, the liver and the lymph nodes to different extents, although plasma biomarkers were not as clearly affected. Our study shows that the emerging mycotoxins alone, but also with the frequently co-occurring metabolite DON, impact the growth performance, lead to organ lesions and to an alteration of microbial community composition for which later consequences have to be in the focus of further studies. Regarding the high prevalence and co-occurrence of EB with regulated mycotoxins in different feed commodities, more *in vivo* results must be generated also in other animal species to provide sufficient data for a reliable risk assessment.

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CRediT authorship contribution statement

Barbara Novak: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – original draft, preparation, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Amanda Lopes Hasuda:** Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – original draft, preparation, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Mahdi Ghanbari:** Formal analysis, Data curation. **Viviane Mayumi Maruo:** Formal analysis, Data curation. **Ana Paula F.R.L. Bracarense:** Data curation, Writing – review & editing. **Manon Neves:** Methodology. **Caroline Emsenhuber:** Methodology. **Silvia Wein:** Writing – review & editing. **Isabelle P. Oswald:** Writing – review & editing, Supervision, Funding acquisition. **Philippe Pinton:** Conceptualization, Formal analysis, Data curation, Writing – review & editing, Funding acquisition. **Dian Schatzmayr:** Conceptualization, Supervision.

Declaration of competing interest

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

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