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Enniatin B and beauvericin affect intestinal cell function and hematological processes in Atlantic salmon (*Salmo salar*) after acute exposure

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ABSTRACT

Unintentional use of mold-infested plant-based feed ingredients are sources of mycotoxins in fish feeds. The presence of the emerging mycotoxins ENNB and BEA in Norwegian commercial fish feeds and plant-based feed ingredients has raised concerns regarding the health effects on farmed Atlantic salmon (*Salmon salar*). Atlantic salmon pre-smolts were exposed to non-lethal doses of BEA and ENNB (ctrl, 50 and 500 μg/kg feed for 12 h), after which total RNA sequencing of the intestine and liver was carried out to evaluate gut health and identify possible hepatological changes after acute dietary exposure. ENNB and BEA did not trigger acute toxicity, however ENNB caused the onset of pathways linked to acute intestinal inflammation and BEA exposures caused the onset of hepatic hematological disruption. The prevalence and concentration of ENNB found in today's commercial feed could affect the fish health if consumed over a longer time-period.

1. Introduction

By the early 2010s, a substantial part of the fishmeal and fish oil used in traditional Norwegian salmon feed had been replaced with meal and oil of plant origin, to make the feed more ecologically and economically sustainable (Ytrestøyl et al., 2015). However, plant-based feed ingredients have introduced mycotoxins into marine farmed fish, through the unintentional use of mold-infested cereal grains and pulse crops that are used for production of the meal- and oil feed ingredients. Ingestion of mycotoxins by terrestrial livestock, as well as humans, have resulted in mycotoxicosis and even death (Hussein and Brasel, 2001). Some terrestrial livestock species (e.g., pig) are more sensitive to specific mycotoxins (e.g. deoxynivalenol (DON)) than other livestock species (e.g., ruminants) (Bernhoft et al., 2013a). The use of novel plant-based feed ingredients in marine aquafeeds has also raised concerns regarding the health effects on marine farmed fish species, such as Atlantic salmon (*Salmo salar*), which have not previously been exposed to the terrestrial plant associated mycotoxins (Bernhoft et al., 2013a). Atlantic salmon appears to better tolerate ochratoxin A (OTA) and zearalenone (ZEA), while being rather sensitive to DON exposure (Bernhoft et al., 2018; Moldal et al., 2018; Döll et al., 2010). Several reviews have reported the prevalence of mycotoxins in aquafeed ingredients, aquafeeds and/or transfer to tissues of European farmed fish species (Pietsch, 2020; Tolosa et al., 2021; Bernhoft et al., 2013b, 2017; Nacher-Mestre et al., 2015). As in terrestrial livestock feeds, DON and fumonisins (FBs) were the most prevalent mycotoxins in plant-based aquafeeds used for the two major marine farmed fish species in Europe (Atlantic salmon and gilthead seabream) (Nacher-Mestre et al., 2015).

More recently, the emerging mycotoxins beauvericin (BEA) and enniatin B (ENNB) have been reported in European marine aquafeeds, exhibiting a 100% prevalence in all tested feed samples (Nacher-Mestre et al., 2020; Tolosa et al., 2014). In an *in vitro* study, exposing Atlantic salmon hepatocyte to BEA and ENNB, reduced mitochondrial metabolic activity and altered cellular iron homeostasis were seen, implying that mitochondrial iron retention might have been impaired (Søderstrøm et al., 2022). However, the *in vitro* data on BEA and ENNB toxicity is still limited for fish, and to our knowledge the toxic mode of action and acute effect dose of BEA and ENNB *in vivo* have not been studied in farmed fish species, including Atlantic salmon. Currently, both BEA and ENNB remain non-regulated in the EU feed and food legislation (Lindblad et al., 2013; Bernhoft et al., 2013a; Vaclavikova et al., 2013), as opposed to more well-studied mycotoxins such as DON, FBs, and trichothecenes (e.g., T-2 HT-2) for which guidance values have been established for animal feed including fish feed (Cheli et al., 2014; Pinotti et al., 2016).

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Insufficient toxicity data for establishing tolerable maximum levels of BEA and ENNB (Chain, 2014), highlights the need for more knowledge regarding their potential adverse effects on fish health.

To identify relevant concentrations and prevalence, BEA and ENNB in addition to the mycotoxins aflatoxins (AFs), DON, FBs, T-2 and HT-2, OTA, and ZEA, were analyzed in commercial Norwegian salmon feeds collected from 2015 to 2021 through the National monitoring program for fish feed. Furthermore, a single-dose (one low and one high concentration) gavage feeding trial with Atlantic salmon was carried out to assess the potential acute toxicity of BEA and ENNB using transcriptomic screening. Since the gastrointestinal (GI) tract represents the first barrier after oral exposure, and the liver represents the major detoxification organ after intestinal absorptions, non-targeted intestinal and liver transcriptomics were used to identify dose-responses in the salmon defensome to dietary BEA and ENNB exposure.

2. Materials and methods

2.1. Fish feed samples

Data on mycotoxins in fish feed and feed ingredients were collected through the ongoing National monitoring program of fish feed in Norway, led by the Norwegian Food Safety Authority (NFSA). Commercial fish feeds and feed ingredients were collected annually from authorized Norwegian feed production sites, with sampling randomized with regards to season and location. The samples were collected by inspectors from the NFSA and transported to the Institute of Marine Research (IMR) for data analysis and reporting. In total, 78 plant-based meal and 51 plant-based oil intended for fish feed production, and 245 fish feeds were collected and analyzed for mycotoxins for the years 2015–2021. The plant-based meal ingredients composed of soy protein concentrates (SPC, n = 43), wheat gluten (n = 9), pea protein (n = 5), guar protein (n = 5), faba beans (n = 4), corn gluten (n = 4), sun flour (n = 4), soybean meal (n = 3), and unspecified plant protein meal (n = 1). The plant-based oils composed of rapeseed oil (n = 41), linseed oil (n = 1), camelina oil (n = 1), a blend of rapeseed and linseed oil (n = 1), whereas some of the oils were of unspecified plant origin (n = 7). The data were compiled from the IMR-generated reports Sanden et al. (2017), Sele et al. (2018), Sele et al. (2019), Ørnsrud et al. (2020), Sele et al. (2021).

2.1.1. Analyzes of mycotoxins in feed and feed ingredients

The following mycotoxins were determined; aflatoxin B1, B2, G1, G2, (AFBs), deoxynivalenol (DON), fumonisins (FB1 and FB2), T-2 toxin (T-2), HT-2 toxin (HT-2), ochratoxin A (OTA), zearalenone (ZEA), beauvericin (BEA) and enniatin A, A1, B, B1) (ENNs). BEA and ENNs (A, A1, B, B1) were extracted with ACN/H20, SPE Clean-up, and determined using LC-MS/MS. DON, FBs, HT-2, T-2, and ZEA were also determined using LC-MS/MS, while AFBs and OTA were analyzed by HPLC and fluorescence detection. The limit of quantification (LOQs, in μg/kg) were AFBs <0.1/1.0, DON <10–20, FFBs <10–20, H2 < 10–20, OTA <0.1, T2 < 10, ZEA <10, ENNs <10, BEA <10. Feed and feed ingredient samples analyses were carried out by Eurofins WEJ Contaminants GmbH (Hamburg), using accredited methods (accreditation number D-PL-14602-01-00 and D-PL-14198-01-00). The prevalence (x%) of mycotoxins was calculated by the following equation:

$$\text{Prevalence}(\%) = \frac{\textit{Number of samples over LOQ}}{\textit{Number of samples analysed}} \bullet 100\%$$

The concentrations of ENNB and BEA chosen for the dietary exposure trial with Atlantic salmon were based on results from the monitoring program.

2.2. Fish and husbandry conditions

The feeding trial was carried out at the Institute of Marine Research's (IMR) facility at Matre (North of Bergen, Norway) between the 28th and 30th of January 2020. On-site farmed, freshwater-adapted pre-smolt weighing between 58 and 108 g (average 74 g) (female:male ratio (14:17)) were kept in flow-through tanks (32 tanks, 1 fish in each), in standard freshwater oxygen condition, 10 ± 0.2 °C, with a 12:12 h light/dark cycle regime. The fish was acclimatized for one month prior to the trial before they were placed into separate tanks and fed daily with standard commercial feed pellets through a disk feeder until 24 h prior to the start of the trial.

2.3. Mycotoxin dosing, feed paste preparation, and sampling

The gavage feeding trial was designed as a single-dose exposure experiment testing for non-lethal responses 12 h after the oral administration. The mycotoxins, beauvericin (BEA, cas 26048-05-5) and enniatin B (ENNB, cas 917-13-5), were purchased from AdipoGen® (AdipoGen® Life Sciences, Nordic BioSite, Oslo, Norway). Five different exposure treatments (including control) were administrated, and six biological replicates (n) were used for each treatment test group (Table 1).

The fish were gavage-fed based on the protocol from a previous study (Amlund and Berntssen, 2004). Mycotoxin-supplemented feed pastes where made the day before the trial. BEA and ENNB were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Oslo, Norway) to create two stock solutions of each mycotoxin (stock solution No. 1 = 1 mg/mL, and a stock solution No. 2 = 0.1 mg/mL). Stock solution No. 1 was diluted in the water fraction of the feed paste before being mixed with freshly grinded commercial fish feed in a 2:1 ration to constitute the "high exposure" [500 μg/kg feed], and in the same way stock solution No 2 was used to create the "low exposure" [50 μg/kg feed] feed pastes of BEA and ENNB. The control feed paste was prepared with 0.075% DMSO, equivalent to the concentrations used in the prepared BEA and ENNB feed pastes. The pastes were then pre-loaded into 1 mL syringes with cut off tips and smooth-polished edges and stored inside zip bags at 4 °C until the next day. Prior to administration, the fish were starved for one day to assure empty gastrointestinal tracts. The fish were sedated in 1 g base and 1 g Finquel MS-222 (Tricaine Methanesulfonate) per 10 L tank water before weight and length were recorded. The amount of feed paste administrated was corrected for the weight of the fish and corresponded to 0.85% of their body weight. The 1 mL syringe containing the treatment feed paste was inserted through the mouth and carefully pushed to the posterior end of the stomach, and then slowly retracted meanwhile gently pushing out the feed paste with the plunger. The fish were left to recover in a separate container with tank water, while assuring no regurgitation occurred, before being transferred back to their experimental tanks. One fish escaped from the tank and died during the trial. Exactly 12 h post gavage feeding, the fish were euthanized in 5 g Finquel MS-222 per 10 L tank water. Subsequently, liver- and intestinal tissue samples were dissected, collected, and flash frozen in liquid nitrogen, and stored at −80 °C until further processing for RNA extraction. This *in vivo* gavage feeding trial was approved and carried out in compliance with the current national *animal welfare act - the regulation on animal experimentation* approved by the Norwegian Animal Research Authority and overseen by the Norwegian Food Safety Authority (FOTS ID 21570).

Table 1
Experimental design of exposure treatments with treatment groups, nominal concentration (μg/kg feed) and number of fish in each group.

#	Treatment	Nominal concentration (μg/kg feed)	No. of fish (n)
1	Control	–	6
2	BEA-low	50	5[a]
3	BEA-high	500	6
4	ENNB-low	50	6
5	ENNB-high	500	6

[a] One fish died so n = 5 for this group.

2.4. RNA extraction and sequencing

Total RNA was extracted from the distal intestine and liver tissues sampled using QIAzol® Lysis Reagent and the EZ1® RNA Tissue Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. Frozen liver tissue (50 mg), or intestinal tissue (100 mg), was homogenized in 750 μL QIAzol at 6000 rpm for 3 × 15 s in an Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA was collected and purified using an BioRobot EZ1 Nucleic Acid Purification System (Qiagen) running the program: Total RNA > universal tissue > including DNase. Purified RNA was eluted in 50 μL RNase-free MilliQ H_2O and stored at −80 °C until downstream application. The RNA quantity and purity of all samples (mean ± STD) were measured spectrophotometrically (NanoDrop™ One Microvolume UV–Vis Spectrophotometer, Thermo Fisher Scientific™, Waltham, MA, USA). The A260/A280 and A260/A230 nm ratios for RNA extracted from intestine were 2.09 ± 0.01 and 2.26 ± 0.02, and from liver 2.10 ± 0.24 and 2.16 ± 0.02, respectively. RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) in combination with RNA 6000 Nano LabChips. The RNA integrity numbers (RIN) were 9.02 ± 0.50 for intestine and 9.08 ± 0.24 for liver (indicating non-degraded RNA) for all samples intended for RNA sequencing. Total RNA samples were sent to Novogene Europe, Cambridge, UK, for cDNA library preparation and sequencing using the Illumina NovaSeq 6000 platform for 150 bp paired end reads. Enriched mRNA was prepared using Oligo(dT beads). cDNA libraries were prepared following manufacturer's instructions per the Novogen pipeline (Novogene Europe, Cambridge, UK). A total of 58 libraries were generated, 29 for intestine and 29 for liver comprising all five exposure groups, n = 6 (5 in BEA-low) per experimental treatment. Each library contained an average of 51 ± 8 million reads. Raw reads were submitted to the gene expression omnibus https://www.ncbi.nlm.nih.gov/geo/(GSE213817).

2.5. Differential gene expression

TrimGalore 0.4.2 wrapper tool (https://github.com/FelixKrueger/TrimGalore) was used for removing adaptors and quality trimming, applying the default parameters. Library quality was investigated using fastQC included in the TrimGalore wrapper.

Individual libraries were mapped to the Atlantic salmon genome (RefSeq Assembly ICSASG_v2) using the Hisat2 short read aligner version 2.0.4 (Kim et al., 2015) and the Atlantic salmon NCBI gene annotation file (Salmon_salar, 24/01/2017 GCA_000233375.4_ICSASG_v2_genomic.gff). Transcript levels for the individual libraries were estimated using FeatureCounts (Liao et al., 2014) of the Subread package (http://subread.sourceforge.net/). Read counts were further normalized using Bioconductor R package (version 3.4.4) DESeq2 (version 1.18.1) (Love et al., 2014). Genes of which fewer than 5 samples had gene counts below or equal to 20 reads were excluded from further analysis prior to normalization. DESeq2 was further used for analysis of differentially expressed genes (DEGs) in a pair-wise comparison of each contrast (Ctrl vs BEA-low, Ctrl vs BEA-high, Ctrl vs ENNB-low, Ctrl vs ENNB-high). Venn diagrams were created with EVenn (http://www.ehbio.com/test/venn/#/) (Chen et al., 2021).

2.6. Functional annotation clustering analysis and pathway analysis

Functional annotation clusters enriched by the identified DEGs were generated using the Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis tool (https://david.ncifcrf.gov) (Huang et al., 2009). In short, the salmon NCBI gene ID's of DEG-names were given their human ortholog official gene symbol to generate a gene list. Subsequent analyses were done separately for DEGs with positive and negative fold change (FC) values compared to the control from each exposure group with the following settings: Functional Annotations (KW: disease (DIS), biological process (BP), molecular function (MF)), Gene Ontology (GOTERM: BP, MF). The cutoff criteria for the input data were set to p-adjusted <0.2 (Benjamini and Hochberg correction) and gene count ≥2, and functional annotation clusters were considered significant when Benjamini was <0.1. Pathway analyses were carried out using QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, (www.qiagen.com/ingenuity). The cutoff criteria for the input data were set to p-adjusted <0.2 (Benjamini and Hochberg correction), canonical pathway analysis, disease and biological function analysis and prediction of upstream regulator analysis were conducted. While both canonical pathway analysis and biological function analysis predict affected biological events, the upstream regulator analysis identifies the cascade of upstream transcriptional regulators that can explain the observed gene expression changes. IPA results were filtered using p-adjusted <0.05 (Fisher exact test) and a Z-score >2. The p-value filtering reflects the likelihood of a pathway/function being significantly enriched by the treatments; the Z-score designates the likelihood of a directional association in the dataset. Positive and negative Z-scores imply an increase or decrease in activity/activation of the enriched pathway, respectively.

3. Results

3.1. BEA and ENNB prevalence in Norwegian salmon feed and feed ingredients

Of the feed samples analyzed and above the LOQ, ENNB had the highest prevalence (80%) compared to the other mycotoxins analyzed (i.e., Afs, DON, FB1, FB2, T-2, HT-2, OTA, ZEA) while BEA occurred to a lesser extent (4%), with concentrations up to 250 μg/kg feed for ENNB and 25 μg/kg feed for BEA (Table 2). The highest concentrations were seen for FB1 and FB2, with approximately 30-50-fold higher concentrations than that of ENNB in fish feed (Table 2). In feed ingredients, plant-based protein meals showed a prevalence of 12% for BEA, and 15% of the samples contained ENNB above the LOQ (Table 3), with concentrations up to 2400 μg/kg for BEA, and 530 μg/kg meal for ENNB (Table 3). While the occurrence of ENNB prevailed over BEA, the concentration of BEA exceeded that of ENNB in plant ingredients, though the concentrations of FB1, FB2, and ZEA largely exceeded that of both BEA and ENNB (Table 3). Specifically, none of the plant-based protein meal of SPC contained BEA or ENNB above the LOQ. BEA were detected in guar meal (12–18 μg/kg, n = 3), wheat gluten/meal (11 μg/kg, n = 1)

Table 2
Fish feed. The prevalence (%) and concentrations (mean and standard deviation, μg/kg feed) and range (minimum to maximum concentrations, μg/kg) of mycotoxins in fish feed for the years 2015–2021, with the number of samples analyzed (N).

Mycotoxin	Prevalence (%)[a)]	Mean ± SD (μg/kg)	Range (μg/kg)	N
Aflatoxin B1	<1	< LOQ	0.1	245
Aflatoxin B2	n.d.	< LOQ		244
Aflatoxin G1	n.d.	< LOQ		244
Aflatoxin G2	n.d.	< LOQ		244
Deoxynivalenol	22	37 ± 18	20–99	245
Fumonisin B1	8	205 ± 140	32–620	245
Fumonisin B2	8	104 ± 72	30–280	245
T-2 Toxin	<1	< LOQ	10	245
HT-2 Toxin	<1	< LOQ	12	245
Ochratoxin A	2	1.6 ± 0.7	1.0–2.4	245
Zearalenone	11	74 ± 55	13–290	245
Beauvericin	4	16 ± 5	10–25	200
Enniatin A	0.5	< LOQ	11	200
Enniatin A1	2	12 ± 27	10–16	200
Enniatin B	80	37 ± 35	10–250	200
Enniatin B1	27	18 ± 9	10–54	200

n.d. = non-detected.
[a)] The number of samples above the LOQ of the total number of samples analyzed, in percentage.

Table 3
Plant proteins. The prevalence (%) and concentrations (mean and standard deviation (μg/kg) and the range: minimum to maximum concentrations, μg/kg) of mycotoxins in plant proteins intended for fish feed production. Data retrieved from the Norwegian monitoring program for fish feed (for the years 2015–2021), and the number of samples analyzed (N).

Mycotoxin	Prevalence (%)[a)]	Mean ± SD (μg/kg)	Range (μg/kg)	N
Aflatoxin B1	10	4.7 ± 5.8	0.7–18	77
Aflatoxin B2	5	0.9 ± 0.6	0.3–1.6	77
Aflatoxin G1	n.d.	< LOQ		77
Aflatoxin G2	n.d.	< LOQ		77
Deoxynivalenol	12	447 ± 406	35–1000	77
Fumonisin B1	6	6825 ± 5165	25–12000	77
Fumonisin B2	6	4084 ± 3350	21–8300	77
T-2 Toxin	1	< LOQ	30	77
HT-2 Toxin	3	10.1 ± 1.1	10–20	77
Ochratoxin A	19	2.0 ± 1.3	0.5–4	77
Zearalenone	10	1107 ± 1573	13–4470	77
Beauvericin	12	416 ± 814	11–2400	67
Enniatin A	1	< LOQ	90	67
Enniatin A1	7	56 ± 49	15–140	67
Enniatin B	15	135 ± 186	11–530	67
Enniatin B1	10	78 ± 63	16–190	67

[a)] The number of samples above the LOQ of the total number of samples analyzed (n.d. = non-detected).

and with the highest concentrations (220–2400 μg/kg, n = 4) in corn meal. ENNB was detected in samples of pea meal (11 μg/kg, n = 1), corn meal (11–130 μg/kg, n = 2), and more frequently in samples of wheat gluten/meal (13–530 μg/kg, n = 7) (Fig. 1). Of the plant-based oil samples analyzed, 10% of the samples contained BEA, and 88% of the samples contained ENNB above the LOQ (Table 4), with concentrations up to 24 μg/kg oil for BEA, and 450 μg/kg oil for ENNB.

3.2. Transcriptional effects of BEA and ENNB

Venn diagrams depicting the individual and overlapping differently expressed genes (DEGs) in the two different tissue types (Fig. 2A–D) resulting from the mycotoxin exposures. Low dose exposure to both BEA and ENNB resulted in a higher number of DEGs compared to the high doses in the intestine (Fig. 2, A, C). While in the liver tissue, the high dose of BEA resulted in 156 more DEGs than in its low dose (Fig. 2, B). BEA caused more transcriptional effects in the liver compared to the intestine (Fig. 2A and B) while ENNB primarily affected the transcription in the intestine, showing far less transcriptional effects in the liver (Fig. 2C and D).

The homeobox (Hox) genes were among the top upregulated DEGs in intestinal tissue exposed to BEA and ENNB (see Supplementary DEG list), of which *hoxa11ab* was represented in all exposure groups, and *hoxa9* and *hoxa7aa* in all but ENNB-low (Fig. 3, A). Several DEGs of cytochrome p450 (CYPs) phase I biotransformation enzymes exhibited increased expression following oral exposure to BEA and ENNB (Fig. 3, B, Supplementary DEG list). BEA-low increased the expression of the *cyp3a27* in the intestine and *cyp1a* in the liver, while ENNB increased the expression of the *cyp2k1* in the intestine (Fig. 3, B).

Table 4
Plant oil. The prevalence (%) and concentrations (mean and standard deviation (μg/kg) and the range: minimum to maximum concentrations, μg/kg) of mycotoxins in plant oil intended for fish feed production, data retrieved from the Norwegian monitoring program for fish feed (for years 2015–2021), and the number of samples analyzed (N).

Mycotoxin	Prevalence (%)[a)]	Mean ± SD (μg/kg)	Range (μg/kg)	N
Beauvericin	10	16 ± 5	10–24	51
Enniatin A	10	20 ± 12	10–38	51
Enniatin A1	29	22 ± 7	11–37	51
Enniatin B	88	114 ± 119	12–450	51
Enniatin B1	65	38 ± 27	10–110	51

[a)] The number of samples above the LOQ of the total number of samples analyzed.

3.3. Common DEGs between annotations

The identified DEGs from each exposure group underwent a UniprotKB Keywords (KW) and Gene ontology (GO) functional enrichment annotation clustering analysis using the DAVID tool. The KW and GO clusters are comprised of the categories termed "disease" (DISs), "biological process" (BPs), and "molecular function" (MFs). In the intestine, the analysis revealed four annotation clusters in the low dose of BEA, and one annotation cluster in the high dose of BEA (Fig. 4). The low dose of ENNB resulted in three annotation clusters in the intestine, while the high dose did not result in any significant annotation clustering (Fig. 4). The top clusters in BEA-low contained downregulated DEGs connected to cell cycle and division, and upregulated DEGs related to developmental proteins (including many homeobox genes) and transcription in both BEA-low and BEA-high (Fig. 4). In the low dose of ENNB, the top cluster based on downregulated DEGs was associated with inflammatory response, followed by kinase activity (most significant) and apoptosis (Fig. 4). The cluster based on upregulated DEGs was related to molecular functions in the extracellular matrix (Fig. 4). ENNB-high did not significantly enrich any clusters.

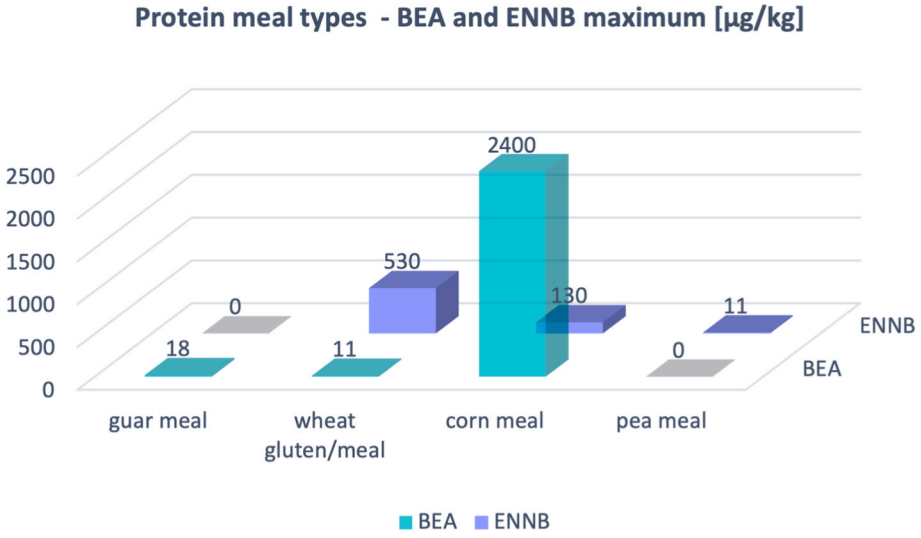

Fig. 1. Plant protein meals. Maximum concentrations (μg/kg) of BEA and ENNB measured in different protein meals: soy protein concentrate (SPC), guar meal, wheat gluten meal, corn meal, and pea meal. None of the SPC contained BEA or ENNB above the LOQ (n = 0 of 43); BEA was detected in guar (12–18 μg/kg, n = 3 of 5), wheat gluten (11 μg/kg, n = 1 of 9), corn (220–2400 μg/kg, n = 4 of 4); ENNB was detected in pea (11 μg/kg, n = 1 of 5), corn (11–130 μg/kg, n = 2 of 4), and wheat gluten (13–530 μg/kg, n = 7 of 9).

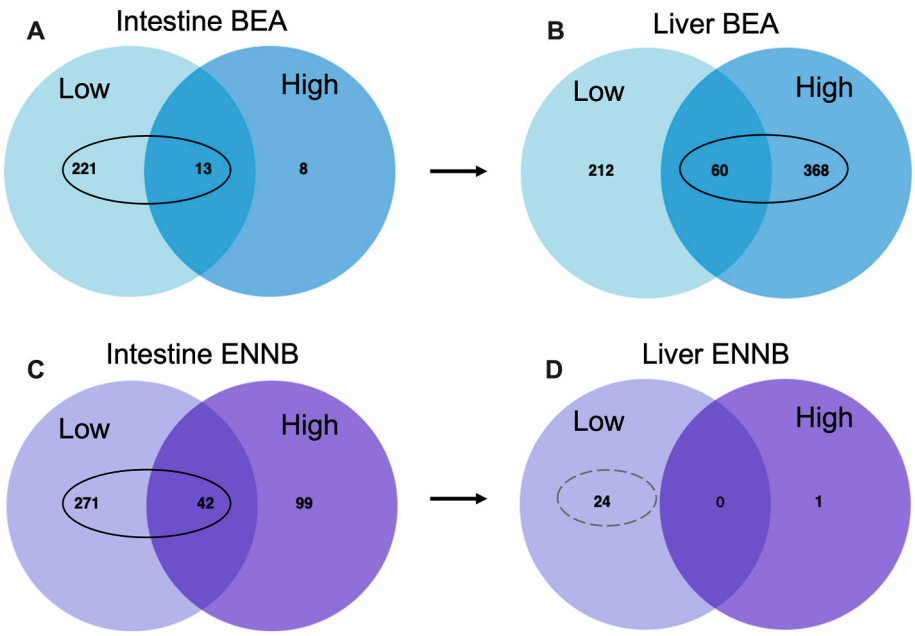

Fig. 2. Venn diagrams depicting the individual and overlapping differentially expressed genes (DEGs) identified in exposure groups of low and high doses of BEA in (A) intestine and (B) liver, as well as low and high doses of ENNB in (C) intestine and (D) liver. n = 6 (BEA-low n = 5), q < 0.2, fold change >1.0.

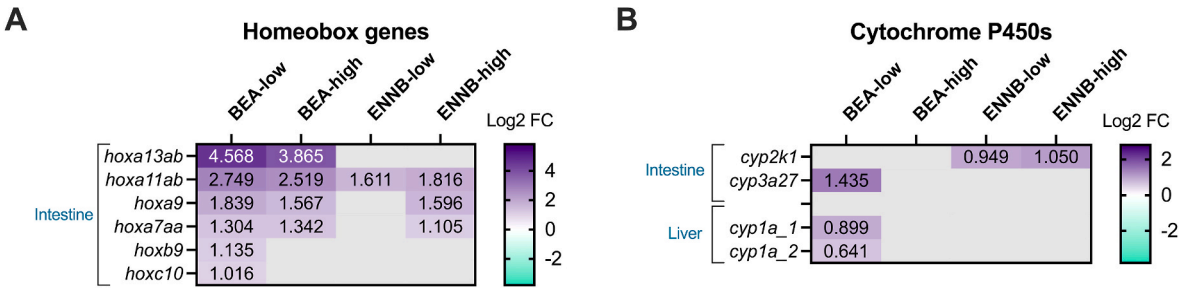

Fig. 3. Heatmaps of differentially expressed genes (DEGs) belonging to (A) homeobox genes, and (B) cytochrome P450 genes resulting from exposure to low and high doses of BEA and ENNB in intestine and liver (n = 6 (BEA-low n = 5), q < 0.2, fold change >1.0).

Only BEA resulted in a sufficient number of regulated DEGs for functional annotation clustering analysis in the liver. Low and high dose oral exposure to BEA resulted in seven annotated clusters (Fig. 5). For BEA-low, the most significant cluster based on DEGs with a negative fold change (FC) was related to hemolytic anemia, followed by actin-binding and detoxification (Fig. 5). In BEA-high, the most significant clusters were associated with the translation machinery (positive FC), and transcription factor activity (negative FC) (Fig. 5).

3.4. Pathway analysis

Ingenuity Pathway Analysis (IPA) of the Atlantic salmon intestine and liver transcriptome were carried out to further elucidate how the DEGs from the low- and high dose of BEA and ENNB translated into biological responses. The analysis of the intestine predicted ENNB-low to downregulate two canonical pathways (e.g., Th2 pathway) and BEA-low downregulated the cell cycle control of the chromosomal replication pathway, while the high doses did not show any effect in this category (Fig. 6, A). In the categories toxicity (Tox), disease and biological function in the intestine, ENNB-low was predicted to downregulate 34 and upregulate 6 pathways, ENNB-high to downregulate 8 and upregulate 2, BEA-low to downregulate 9 and upregulate 2, and BEA-high to downregulate 2 pathways (Fig. 6, B). Notably, both the low and high dose of ENNB upregulated organ inflammation, though the effect in the low dose appeared more pronounced since it also predicted upregulation of immune mediated inflammatory disease, while BEA-low mainly upregulated necrosis (Fig. 6, B). In the category of genes in the immune mediated inflammation/inflammation of organ pathways, ENNB-low exerted most transcriptional effect of the different exposure groups, where haptoglobin (*hp*) was the most upregulated DEG followed by integrin alpha 9 (*itga9*), protein kinase C alpha (*prkca*), while the most downregulated DEG was regenerating protein 1 alpha (*reg1a*) (Fig. 6, C). A similar effect on *prkca* and *reg1a* was observed following exposure to BEA-low (Fig. 6, C).

The liver ENNB-high group was excluded from the IPA since it only had 1 DEG. The analysis of gene expression in the salmon liver tissue predicted that both the low and the high dose exposure to BEA downregulated 4 canonical pathways, while ENNB-low showed no effect on such pathways (Fig. 6, A). In the category toxicity, disease and biological function in the liver, ENNB-low was predicted to downregulate 1 pathway, BEA-low to downregulate 16 and upregulate 5 pathways, while BEA-high downregulated 10 and upregulated 3 pathways (Fig. 7, B). Of note, while both the low and the high dose of BEA were predicted to downregulated hematocrit levels, both doses also upregulated anemia (Fig. 7, B). In addition, BEA-low was predicted to increase inflammatory response, cell death of immune cells, quantity of reticulocytes (immature red blood cells), and cell death of blood cells (Fig. 7, B). Processes related to proliferation of hematopoietic progenitor cells and erythroid precursor generation were among the pathways predicted to be downregulated by BEA-low (Fig. 7, B). In the category of upstream regulators

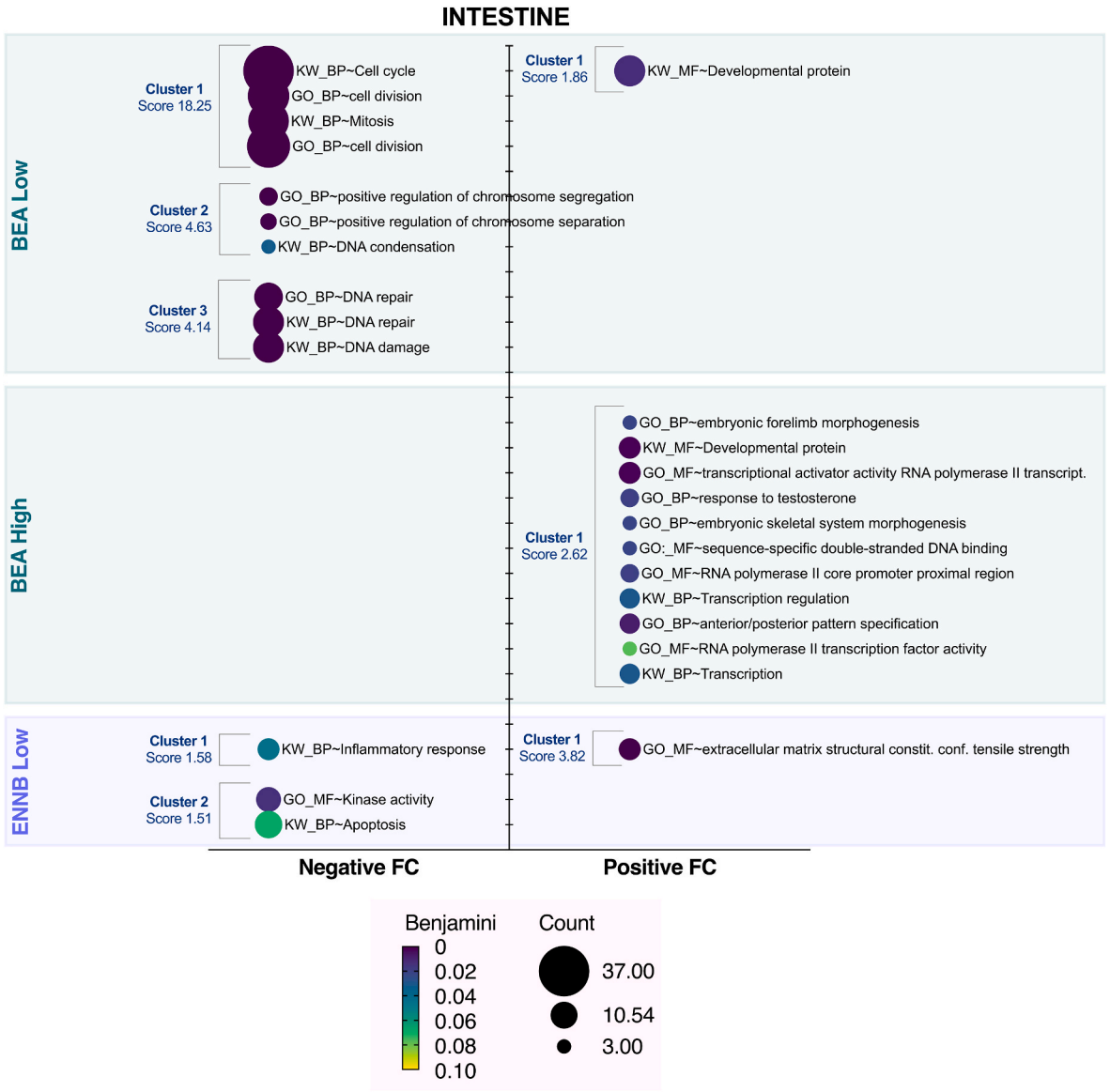

Fig. 4. Functional annotation clustering of differentially expressed gene (DEG) counts in the intestine. KW and GO clustering comprised of the categories disease (DIS), biological process (BP), and molecular function (MF) signified by up- and downregulated DEGs shown on the X-axis for each exposure group (A) BEA-low, (B) BEA-high, (C) ENNB-low, and (D) ENNB-high. The fill colors from yellow to purple denote the Benjamini (significant <0.1), the closer to purple the higher the significance. Bubble sizes depict the count of attributed DEGs in each cluster. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in the liver, the GATA binding protein 1 (*gata1*) transcription factor gene was predicted to be downregulated by both the low and high dose of BEA and by ENNB-low, as were erythropoietin (*epo*) and interferon gamma (*ifng*) (Fig. 7, C). Both the low and the high dose of BEA upregulated the upstream regulators stearoyl-CoA desaturase (*scd*), lysosomal-associated membrane protein 2 (*lamp2*), dexamethasone, and glucocorticoid (in addition to the pharmaceuticals decitabine and budesonide) (Fig. 7, C). More specifically, BEA-low, BEA-high, and ENNB-low affected the genes involved in the hematocrit pathway that was indicated to be decreased (Fig. 7, B). Transcription of the CCAAT Enhancer Binding Protein Alpha (*cebpa*) and cytochrome P450 family 1 subfamily A member 1 (*cyp1a1*) was slightly increased, while and *gata1* and dematin actin binding protein (*dmtn*) were decreased in two and all three exposure groups, respectively (Fig. 7, D). The most downregulated gene was heme oxygenase 1 gene (*hmox1*) in the BEA-low exposure group (Fig. 7, D).

Since *gata1* was represented both on the upstream regulator category (Fig. 7, C), as well as in the hematocrit pathway (Fig. 7, D), the GATA binding protein 1 (GATA1) pathway was also signaled as significantly affected (Fig. 8, A). ENNB-low had the least effect and primarily downregulated predicted genes (Fig. 8, A). Notably, despite that ENNB-low only resulted in 24 DEGs in the liver (Fig. 2, D) five of these were represented in the GATA1 pathway. BEA-low had most effect and downregulated 15 and upregulated 5 genes, finally BEA-high affected 6 genes 3 were downregulated and 3 were upregulated (Fig. 8, A). Evident from the Ferris wheel plot (Fig. 8, B) showing how BEA-low affected genes downstream of GATA1, the mitochondrial 5-aminolevulinate synthase (*alas2*) was also predicted to be downregulated (Fig. 8, B).

4. Discussion

Evaluation of the prevalence and concentrations of mycotoxins in plant-based feed ingredients and Norwegian fish feeds identified ENNB as one of the most prevalent mycotoxins, and BEA to a lesser extent. To assess whether ENNB and BEA pose a concern to fish health, an acute

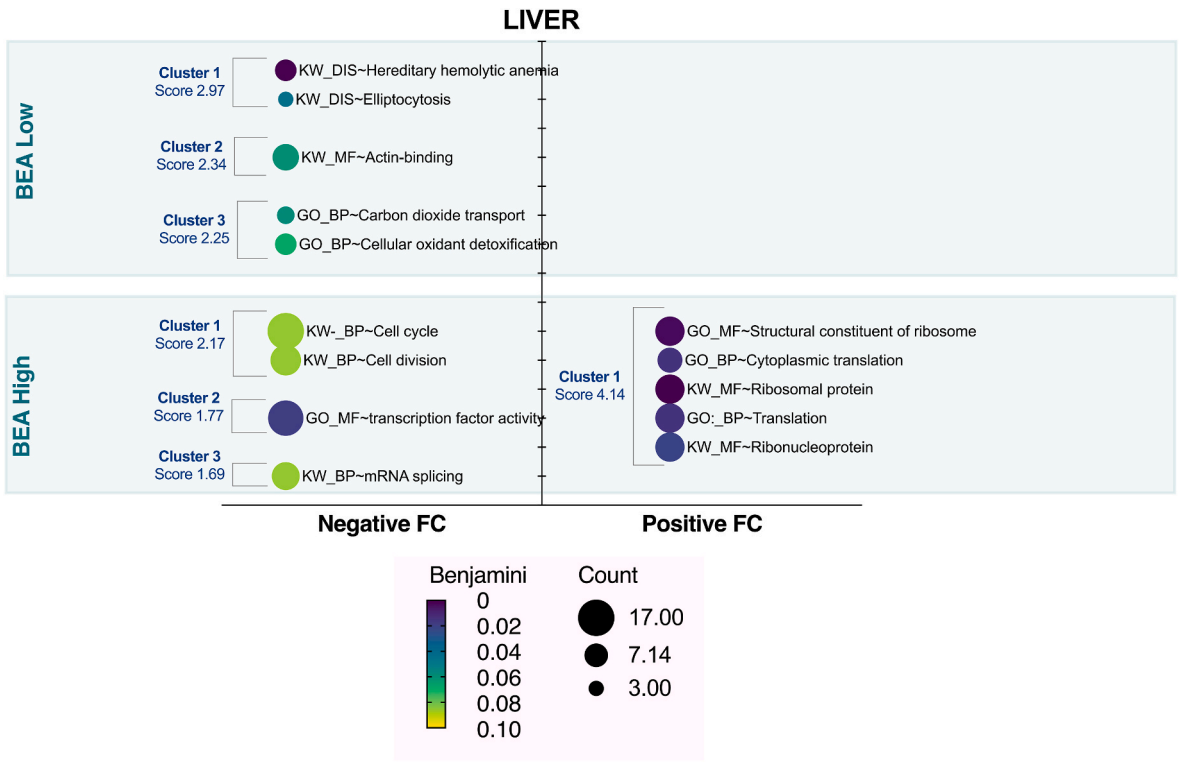

Fig. 5. Functional annotation clustering of differentially expressed gene (DEG) counts in the liver. Clustering comprised of CCs, BPs, and MFs signified by up- and downregulated DEGs shown on the X-axis for each exposure group (A) BEA-low, and (B) BEA-high. The fill colors from yellow to purple denote the Benjamini (significant <0.1), the closer to purple the higher the significance. Bubble sizes depict the count of attributed DEGs in each cluster. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

gavage feeding trail on Atlantic salmon was performed. To fully understand the complex xenobiotic defensome and stressor responses occurring in teleost (Eide et al., 2021), a non-targeted toxicological evaluation of the intestines and livers following short-term dietary exposure to BEA and ENNB were performed to map the initial transcriptomic response.

4.1. BEA and ENNB prevalence in Norwegian salmon feed (2015–2021)

Analysis of salmon feed and feed ingredients showed higher prevalence and concentrations of ENNB in comparison to other emerging mycotoxins such as other ENNs and BEA. Although the concentration of ENNB was lower than FBs and ZEA in some samples, the prevalence of ENNB in the surveyed salmon feeds was substantially higher than the more routinely monitored mycotoxins, i.e., AFB_1, FBs, HT-2, T-2, OTA, ZEA, and DON for which recommended or maximum levels in feed and commodities have been established by the European Commission (Cheli et al., 2014; Pinotti et al., 2016; EC, 2016). Analyses of plant-based feed ingredients indicated that rapeseed oil and wheat- and corn gluten were major sources of ENNB, and that corn gluten was the primary source of BEA. In a previous survey on Sea bream (*Sparus aurata*) and Seabass (*Dicentrarchus labrax*) aquafeeds from 2014, ENNB and BEA had a 100% prevalence with mean occurrence levels of 0.89 and 1.4 μg/kg (0.1–3.2 μg/kg and 0.1–6.6 μg/kg, min max, n = 20), respectively (Tolosa et al., 2014). In a more recent study on Atlantic salmon and Sea bream plant-based feeds all twenty surveyed feeds had detectable levels of BEA and ENNB, with the highest concentrations being 80.4 and 32.8 μg kg, respectively (Nacher-Mestre et al., 2020). In agreement with these surveys, ENNB showed a high prevalence in Norwegian fish feeds and feed ingredients surveyed in 2015–2020, and the concentrations of ENNB in fish feed were consistently higher than BEA (250 and 25 μg/kg, respectively). In plant-based feed ingredients, however, BEA was occasionally measured with extreme concentrations in corn meal.

4.2. Difference in BEA and ENNB intestinal-hepatic transcriptomic responses

In the current feeding trial with Atlantic salmon pre-smolt, the low environmentally relevant doses (50 μg/kg feed) of both BEA and ENNB caused a higher number of DEGs in the intestine than the non-lethal high doses (500 μg/kg feed). That fewer DEGs were observed in the high dose of BEA and ENNB could indicate that the toxic reactions are overcoming the adaptive transcriptional stress responses in the intestine. Similarly in an *in vitro* study, Jurkat cells exposed to three concentrations (1.5, 3, and 5 μM) of ENNB exhibited the highest number of DEGs in the mid-concentration (Alonso-Garrido et al., 2018). The authors suggested two hypotheses as to why the highest number of DEGs was observed in the mid-concentration, either due to time-dependent transcriptomic damage or that the toxicity of the highest concentration of ENNB was blocking it from entering the cells. However, while exposing the same cell line to BEA the number of DEGs increased with increasing concentration (Escrivá et al., 2018). The transcriptional effects following exposure to ENNB mainly occurred in the intestine while limited effects were observed in the liver. In comparison, BEA affected the liver transcriptome to a larger degree than the intestinal. Previous *in vivo* and *in vitro* studies on rodents indicated enterohepatic recycling with increased tissue levels of BEA, while the metabolism of ENNB occurred both in the intestine and liver in rodents (Rodríguez-Carrasco et al., 2016; Fæste et al., 2011; Ivanova et al., 2011). In the present *in vivo* study, ENNB increased the expression of *cyp2k1* in the intestine, while BEA increased the expression of *cyp3a27* in the intestine and *cyp1a* in the liver. This is in line with a previous salmon hepatocyte *in vitro* study, were ENNB affected the transcription of *cyp2k1*, while BEA affected *cyp3a27* and *cyp1a* transcription (Søderstrøm et al., 2022) (Supplementary DEG list). Phase I CYP enzymes have been reported to be important for biotransformation of ENNB in human microsomes (Fæste et al., 2011). In fish, cyp2k1 is primarily expressed in the digestive tract where it has been

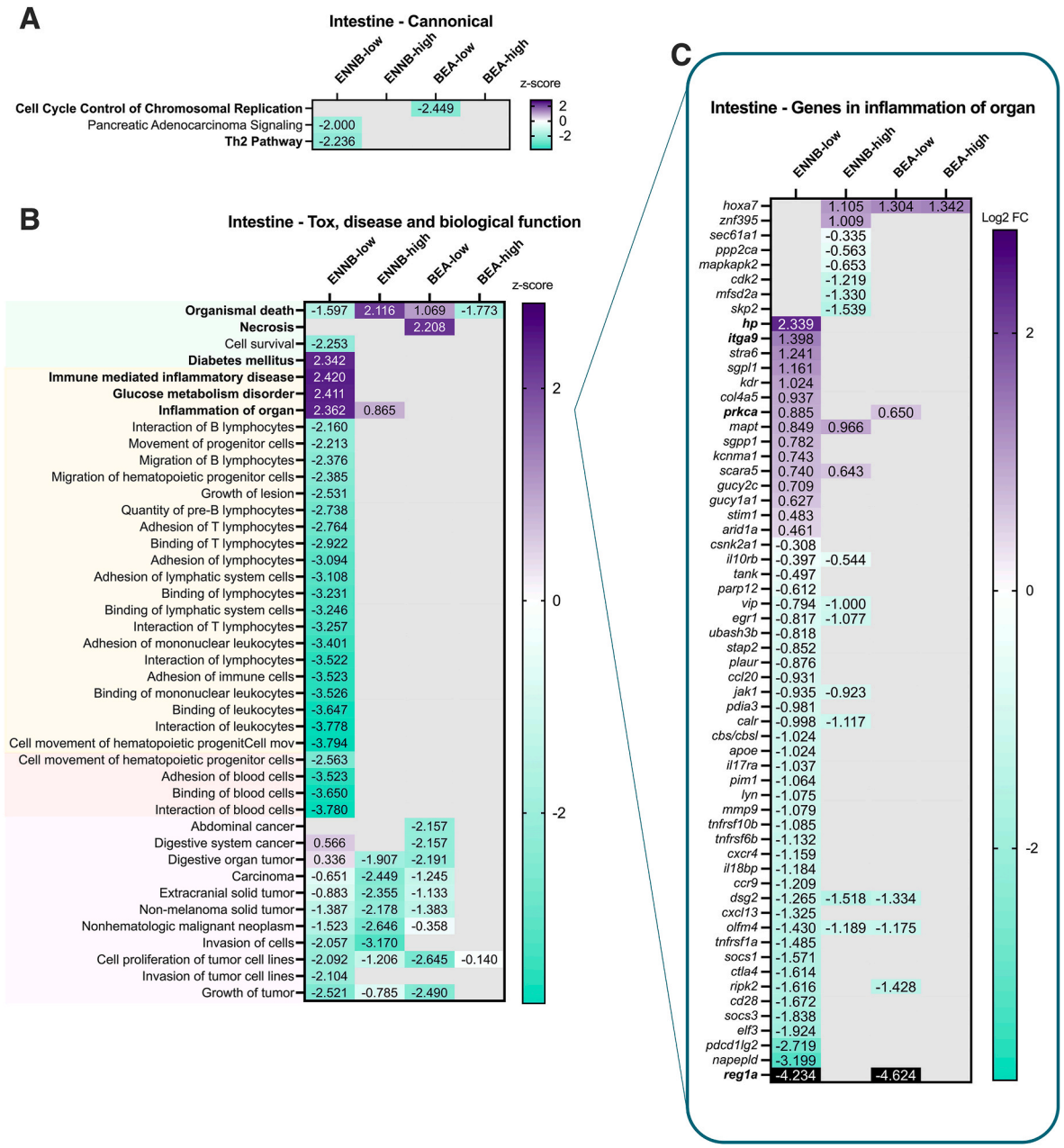

Fig. 6. IPA Pathway analysis of gene regulation patterns in the intestine in response to BEA and ENNB exposure. Heatmaps depicting (A) canonical pathways, (B) toxicity, disease and biological functions in intestine, and (C) genes in organ inflammation. Predicted upregulated pathways/genes indicated by dark purple, and downregulated genes indicated by turquoise. p-adjusted <0.2 and all pathways were subjected to z-score filtering >2, genes depicted as log2 fold change. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

shown to be involved in the metabolism (Schlenk et al., 2008) and bioactivation of aflatoxin B1 (AFB1) (Yang et al., 2000; Wang-Buhler et al., 2005). Thus, it could be hypothesized that *cyp2k1* prevents ENNB from reaching the liver which could explain the difference in transcriptomic response between BEA and ENNB in the present study. However, further toxicokinetic research is needed to establish the fate of ENNB and BEA in Atlantic salmon following dietary exposure.

4.3. Effects of dietary exposure to ENNB and BEA in the intestine

4.3.1. Intestinal responses following ENNB exposure

In this study, transcriptional analysis of intestinal tissues from fish exposed to ENNB-low indicated an increase in inflammation-related processes (immune-mediated inflammation, glucose metabolism disorder, and organ inflammation). Haptoglobin (*hp*), an acute phase protein, was the most upregulated DEG in the immune mediated inflammation pathway in the intestine by ENNB. Tissue inflammation is often associated with tissue damage and the release of red blood cells that have ruptured (de la Rubia Ortí et al., 2021; Jeney, 2018). The main function of haptoglobin is to bind and remove free hemoglobin from damaged red blood cells to prevent heme iron (Fe^{2+}) causing oxidative damage such as lipid peroxidation (Alayash, 2011), or to reduce availability of free heme iron that could be utilized by invading pathogens (Díaz et al., 2021). Haptoglobin has previously been identified to be the most sensitive marker of acute inflammation in Wistar Han rats (Giffen et al., 2003). The observed upregulation of haptoglobin by ENNB-low suggested damage of intestinal cell and possibly red blood cells. Tissue injuries resulting in immunogenic cell death (e.g., necrosis, ferroptosis,

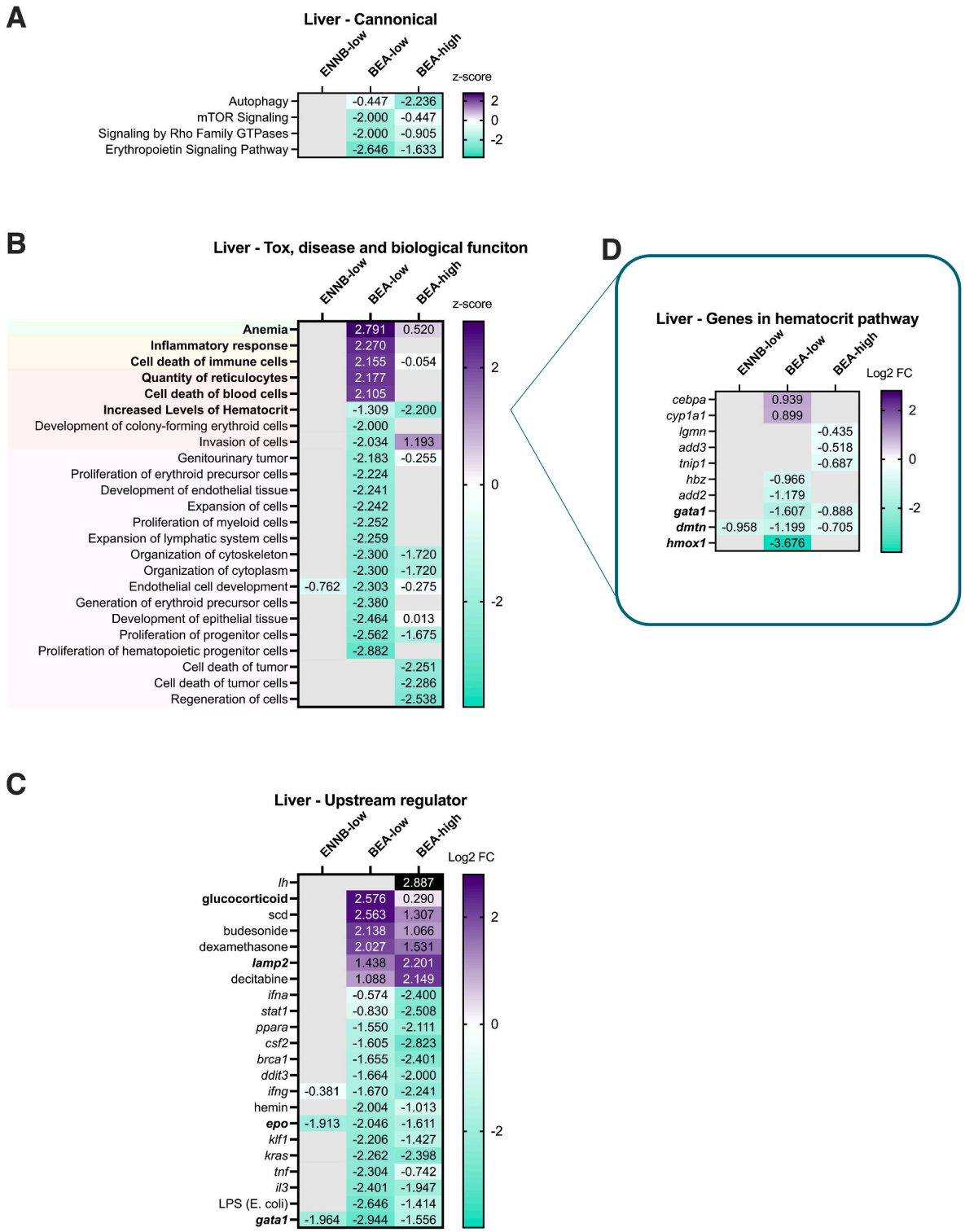

Fig. 7. IPA Pathway analysis of gene regulation patterns in the liver in response to BEA and ENNB exposure. Heatmaps depicting (A) canonical pathways, (B) toxicity, disease and biological functions, (C) upstream regulators, and (D) genes in hematocrit pathway in liver tissues. Predicted upregulated pathways/genes indicated by dark purple, and downregulated genes indicated by turquoise. p-adjusted <0.2 and all pathways were subjected to z-score filtering >2, genes depicted as log2 fold change. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

or excessive apoptosis), causes release of endogenous damage-associated molecular patterns (DAMPs) (i.e., cellular debris) that can activate the innate immune system and triggers acute inflammation to promote damage repair (Anders and Schaefer, 2014; Shi et al., 2021; Jaeschke, 2006). Meanwhile, pathogen-associated molecular pattern molecule (PAMP) (i.e., a foreign antigen), such as beta-glucan in the fungal cell wall, directly activates the receptors of the innate immune system (e.g., Toll-like receptors (TLRs)) (Medzhitov, 2008; Rauta et al., 2012). It might therefore also be possible that mycotoxins, such as ENNB and BEA, are recognized by TLRs of the innate immune system similar to fungal PAMP (Gruda et al., 2018; Perincherry et al., 2019), and directly trigger the receptors of the innate immune system

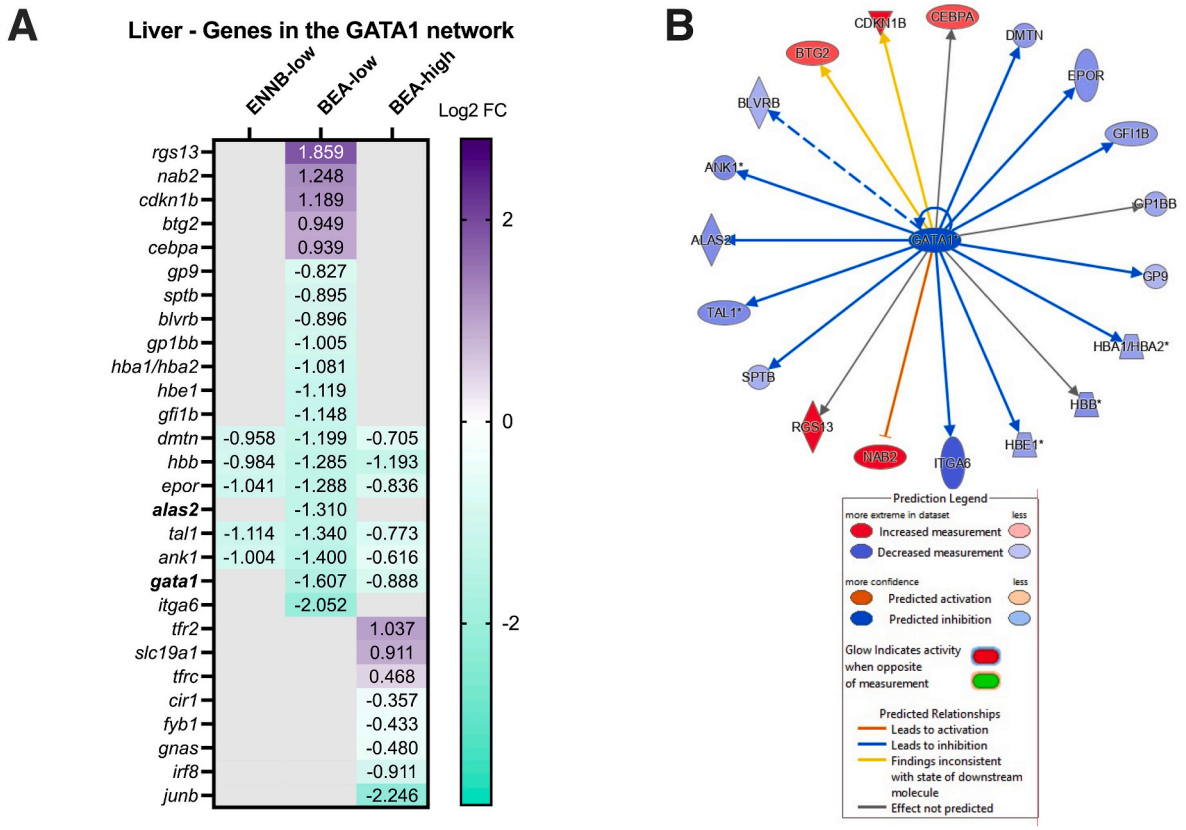

Fig. 8. IPA Pathway analysis of gene regulation patterns and Ferris wheel graph of GATA1 downstream target genes in response to ENNB-low, BEA-low, and BEA-high exposure. (A) Heatmap and (B) Ferris wheel showing predicted upregulated genes indicated by red, and downregulated genes indicated by blue. p-adjusted <0.2, and all pathways were subjected to z-score filtering >2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Medzhitov, 2008; Rauta et al., 2012). Unlike mammalian red blood cells, in teleost these cells are nucleated and contain organelles such as mitochondria and lysosomes (Glomski et al., 1992), and teleost red blood cells partake in the immune response against infections since they can release cytokines, modulate leucocyte activity, and are able to phagocytose (Passantino et al., 2002, 2004, 2007). Recently, BEA was reported to exhibit immunostimulatory effects via the toll-like receptor 4 (TLR4) signaling pathway on murine dendritic cells (Yang et al., 2022). In a study with RAW 267.4 murine macrophages, ENNB was found to induce an inflammatory response that was proposed to occur through lysosomal damage releasing DAMPs that secondarily triggered the cellular inflammasome (Gammelsrud et al., 2012). ENNB was previously shown to impair mitochondria and lysosomes *in vitro* in a study with Atlantic salmon primary liver cells (Søderstrøm et al., 2022). However, in the current study, it remains to be elucidated whether ENNB secondarily triggers the cellular PRRs by causing DAMP release due to oxidative stress-induced cell damage, or if ENNB itself is recognized by PRRs and directly triggers the innate immune response.

ENNs ability to cause oxidative stress in intestinal tissue was previously suggested to impair the intestinal barrier function in Wistar rats (Cimbalo et al., 2021). Through their ionophoric activities, ENNs and BEA have been proposed to cause decreased tight junction resistance, and hence intestinal barrier impairment, by inducing the activity of protein kinase C alpha which is initiated by increased intracellular calcium ion levels (Tai et al., 1996; Bertero et al., 2018). Thus, the increase in *prkca* (protein kinase C alpha) expression in the intestine could indicate that ENNB has the potential to impair processes that affect the intestinal barrier if prolonged dietary exposure occurred. Homeobox genes (Hox genes) were among the most upregulated DEGs in the intestinal tissue responding to both mycotoxins. Hox genes have been shown to contribute to wound healing (Chuong, 2003), and adult cell renewal in mammals (Kachgal et al., 2012). Thus, the upregulation of Hox genes in the intestine may suggest that ENNB damaged the intestinal cells causing an upregulation of Hox genes to mitigate the damage.

While chronic inflammation could be harmful for tissues, acute inflammation is a rapid and short-term adaptive response characterized by initial recruitment of neutrophils to the injured site from the surrounding blood vessels, to resolve the damaging insult and reestablish homeostasis (Medzhitov, 2008; Germolec et al., 2018; Kolaczkowska and Kubes, 2013). Overall, the upregulation of responses related to inflammation indicate an early innate immune response to ENNB since immune functions related to the delayed adaptive immune system and the production of antibodies i.e., lymphocytes and the Th2 pathway, were downregulated by ENNB. The current study was a short-term trial (12 h) and activation of the adaptive immune response would have been expected to occur later (days, weeks) (Terrazas et al., 1998), if activated at all. Acute inflammation is a normal biological response that is rapidly triggered after an insult or injury, intended to quickly resolve and repair damaged cells and tissues to restore homeostasis (Medzhitov, 2008; Germolec et al., 2018; Kolaczkowska and Kubes, 2013). In this study, the upregulation of the function "inflammation of organ" (intestine) by the ENNB-low was stronger than by ENNB-high. While future research is needed to fully clarify this, it could be hypothesized that the acute inflammatory response is both time- and dose-dependent, where a higher dose would trigger a stronger initiation of the inflammatory response that would start to dab off as the response reaches the resolving phase and the damage is repaired. Thus, the transcriptomic data suggested that the cell damaging activity of ENNB most likely triggered the acute inflammatory response, which potentially could lead to immunological dysfunction of the intestinal barrier in Atlantic salmon if dietary

exposure would become prolonged.

4.3.2. Intestinal responses following BEA exposure

Both the IPA (canonical) and the DAVID analyses indicated pathways involved in cell cycle arrest and DNA repair were affected in intestinal cells exposed to BEA-low. BEA-high on the other hand, appeared to impact the cells' transcriptional machinery, as seen from the absence of affected genetic pathways other than organismal death. The effects on intestinal cell cycle arrest and DNA repair at low BEA exposure, but absence of these biological process pathway responses at high BEA exposure, suggest that the intestinal cells temporarily stopped the cell cycle after BEA exposure to mitigate the inflicted stress and to allow cellular damage to be repaired (Pietenpol and Stewart, 2002). Similar disruption of cell cycle progression has previously been demonstrated in CHO–K1 cells (Mallebrera et al., 2016) and in SH-SY5Y neuronal cells (Agahi et al., 2021). The current transcriptomic analyses suggest that cell cycle control pathways were affected by BEA to prevent the development of necrosis in the salmon intestine. Like ENNB, BEA upregulated hox genes in the intestine which could mitigate the cell damage inflicted in the intestine.

4.4. Effects of dietary exposure to BEA in the liver

In the liver, representing the major detoxification organ, effects on gene transcription were primarily observed following BEA exposure. The functional analysis showed BEA to primarily cause hematological effects in the liver, such as decreased levels of hematocrit and anemia. Although kidney and spleen are the major erythropoietic organs, liver is the primary location for maintaining iron homeostasis and erythrocyte recycling involving Kupffer cells (Theurl et al., 2016). Gata1, one of the main upstream regulators in the production of red blood cell (erythropoiesis), was predicted downregulated in the present dataset. Improper expression of Gata1 has been reported to result in anemia (Gutiérrez et al., 2020). In addition, both doses of BEA downregulated the canonical erythropoietin (EPO) signaling pathway. EPO is a hormone-acing cytokine that can facilitates activation of Gata1 (Zhao et al., 2006), thereby linking the transcriptomics to dysregulation of maturation and production of red blood cell. Heme oxygenase 1 (*hmox1*), a rate-limiting enzyme in heme degradation and iron recycling (Bach, 2002), was the most downregulated gene (in terms of fold change) by BEA-low in the liver and related to the predicted reduction in hematocrit levels. Interestingly, mice lacking functional Hmox1 expression also developed anemia (Poss and Tonegawa, 1997). Alas2, the first rate limiting enzyme for heme biosynthesis acting downstream of Gata1 (Tanimura et al., 2016) was downregulated by BEA in the present study. Transcription of *alas2*, which is specific for blood cells (Muckenthaler et al., 2017), is normally inhibited when intracellular iron levels are low (Poli et al., 2021). This suggests exposure to BEA caused a reduction in bioavailable cellular iron in the salmon liver. BEA was shown to affect iron homeostasis in salmon primary hepatocytes *in vitro* (Søderstrøm et al., 2022), thus correlating to the transcriptional changes in the liver in the present *in vivo* trial, where BEA resulted in hematological effects (e.g., reduction of hematocrit levels and anemia). Although very few genes were affected by ENNB in the liver, like BEA, *dmtn*, *hbb*, *epor*, *tal1*, and *ank1* were downregulated, while *gata1* and *epo* were predicted as upstream regulators in ENNB-exposed livers. The *dmtn* gene was also denoted in the hematocrit pathway, and this gene encodes a membrane stabilizing protein important for the structure of erythrocytes (Wang et al., 2022). The function "*quantity of reticulocytes*" was predicted upregulated by IPA following exposure to BEA in the liver, and both IPA and DAVID suggested BEA to trigger regenerative hemolytic anemia, since increased levels of reticulocytes is characteristic of hemolytic anemia (Barcellini and Fattizzo, 2015; Grimes and Fry, 2015). While Olleik et al. (2019) reported BEA to exhibit a weak hemolytic activity towards human erythrocytes, Qadri et al. (2011) instead reported BEA to trigger eryptosis by scrambling the membranes of red blood cells. Ionomycin, a well-known calcium (Ca^{2+}) ionophore with high resemblance to the ionophoric BEA and ENNB, induced eryptosis and engulfment of injured and deformed human red blood cells by macrophages (Bigdelou and Farnoud, 2020). Eryptosis is a controlled removal of defective red blood cells to avoid hemolysis, though excessive eryptosis may however cause anemia (Lang et al., 2008; Lang and Lang, 2015).

Like the intestine, the liver transcriptome in BEA-exposed fish indicated promotion of cell death of immune cells and upregulation of inflammatory responses. Since oxidative stress can trigger cell death, it is interesting that the lysosomal-associated membrane protein 2 (*lamp2*) was predicted as an upstream regulator in the liver following BEA exposure. Lamp2 has been described to mediate removal of Zn^{2+}-induced reactive oxygen species in A549 cells (Qin et al., 2017), which suggests that BEA exposure caused oxidative stress in the salmon liver. This is in line with a previous *in vitro* study where BEA generated oxidative stress in primary salmon hepatocytes, impaired lysosomal and mitochondrial function, altered iron homeostasis, and increased Gpx activity in a concentration-dependent manner (Søderstrøm et al., 2022). Further, the inflammatory response triggered by BEA could be linked to the above-mentioned hematological effects and possible hemolytic activity. Previous studies have shown that increased production of pro-inflammatory cytokines (e.g., Tnfα, Il-1β, and Il-6) can lead to anemia by exerting a hampering effect on Epo production in mammals (Morceau et al., 2009). In the present study, Epo signaling was predicted by IPA to be downregulated in all exposure groups. Increased inflammatory signaling was also indicated by the predicted effect on the upstream regulators related to glucocorticoid, since glucocorticoids have been found to be anti-inflammatory and immune suppressive in various tissues e.g. mice intestine (Noti et al., 2010) and in airway epithelial cells and macrophages (Miyata et al., 2015). Since hemoglobin and red blood cell formation appeared affected by BEA in liver, it is noteworthy that the Hox genes were upregulated in the intestinal tissue by both mycotoxins. Hox genes in blood cells are key regulators during hematopoiesis (blood and plasma production), and dysregulation of their expression has been linked to various hematological abnormalities such as Hoxa9 (Sio et al., 2013; Morgan et al., 2005), that was upregulated by BEA-low and ENNB-high in intestinal tissue in the current study. Overall, the transcriptomics indicated BEA to primarily affect heme and blood related components negatively, in addition to hamper different transcriptional units and processes indicating oxidative stress, which was supported by earlier salmon hepatocyte *in vitro* studies showing an altered intercellular iron homeostasis and upregulated antioxidant defense (Søderstrøm et al., 2022). Whether the inflammatory response BEA triggered in the liver was due to oxidative stress-induced cell damage (including potential damage to red blood cells), or whether BEA itself directly triggers the innate immune response needs further research to elucidate.

4.5. Potential implications of exposure to BEA and ENNB for Atlantic salmon

Using earlier published data, Pietsch (2020) theoretically estimated maximum mycotoxin levels in farmed fish based on different contamination scenarios of European feed ingredients with mean predicted concentrations of 41.5 μg/kg for BEA (predicted maximum 2692 μg/kg) and 107 μg/kg for total ENNs (predicted maximum 68472 μg/kg). The low dose in the current study (50 μg/kg feed) was lower than the maximum measured concentration of ENNB in Norwegian commercial salmon feeds (250 μg/kg feed). Interestingly, the low dose in this study resulted in the onset of genes related to acute inflammation in intestinal tissue and possible impairment of intestinal barrier integrity. BEA-low (50 μg/kg feed) was comparable to the highest observed concentration (80 μg/kg) in salmon feed (Nácher-Mestre et al., 2020), and also indicated negative effects on intestinal barrier integrity and caused alterations in gene expression of the hematocrit and anemia pathways in the liver. Considering the measured concentrations of ENNB and BEA in

Norwegian salmon feed, and the concentrations of BEA and ENNs in the predicted scenarios by Pietsch (2020), the present study shows that the concentrations occasionally found in commercial fish feed could potentially constitute a problem for fish health if frequent re-exposure occurs. Thus, whether the toxic responses following repeated exposure to ENNB, and BEA can be mitigated and resolved needs further research, preferably by performing a long-term feeding trial using Atlantic salmon to test for chronic effects.

5. Conclusions

In accordance with other studies, ENNB was found to be one of the most prevalent mycotoxin in Norwegian fish feed, and the feed ingredients rapeseed oil and wheat gluten were major sources of ENNB, while BEA was primarily found in corn meal. Both BEA and ENNB elicited effects on transcription in the intestine, where the low doses caused the onset of adaptive transcriptional mechanisms, whereas the responses to high doses resulted in fewer DEGs indicating toxic reactions overcoming adaptive stress responses. BEA exerted greater transcriptional effects in the liver contra the intestine. The present acute dietary *in vivo* Atlantic salmon study showed that ENNB triggered an acute inflammatory response in the intestine, while BEA negatively affected heme biosynthesis and blood homeostasis in the liver.

CRediT authorship contribution statement

Sofie Søderstrøm: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing, Visualization. **Liv Søfteland:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition. **Veronika Sele:** Conceptualization, Formal analysis, Resources, Writing – review & editing, Visualization. **Anne-Katrine Lundebye:** Conceptualization, Writing – review & editing. **Marc HG. Berntssen:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition. **Kai K. Lie:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

https://www.ncbi.nlm.nih.gov/geo/(GSE213817)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2022.113557.

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