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Dietary beauvericin and enniatin B exposure cause different adverse health effects in farmed Atlantic salmon

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ABSTRACT

The extensive use of plant ingredients in novel aquafeeds have introduced mycotoxins to the farming of seafood. The emerging enniatin B (ENNB) and beauvericin (BEA) mycotoxins have been found in the novel aquafeeds and farmed fish. Little is known about the potential toxicity of ENNs and BEA in farmed fish and their feed-to-organ transfer. Atlantic salmon (*Salmo salar*) pre-smolt (75.3 ± 8.10 g) were fed four graded levels of spiked chemical pure ENNB or BEA feeds for three months, in triplicate tanks. Organismal adverse health end-point assessment included intestinal function (protein digestibility), disturbed hematology (red blood cell formation), bone formation (spinal deformity), overall energy use (feed utilization), and lipid oxidative status (vitamin E). Both dietary BEA and ENNB had a low (<~0.01%) transfer to organs (kidney > liver > brain > muscle), with a higher transfer for ENNB compared to BEA. BEA caused a growth reduction combined with a decreased protein digestion and feed conversion rate- ENNB caused a stunted growth, unrelated to feed utilization capacity. In addition, ENNB caused anemia while BEA gave an oxidative stress response. Lower bench-mark dose regression assessment showed that high background levels of ENNB in commercial salmon feed could pose a risk for animal health, but not in the case of BEA.

1. Introduction

Seafood from aquaculture has been identified as a valuable resource to meet the nutritional needs for a growing global population (Costello et al., 2019; Glencross et al., 2020; Naylor et al., 2021). Farmed fish species such as Atlantic salmon (*Salmo salar*) and gilthead sea bream (*Sparus aurata*) have traditionally been fed formulated aquafeeds that were mainly based on protein and fats from feral marine fish species such as blue whiting (*Micromesistius poutassou)*. However, the use of fish oil and fish meal in formulated salmon feeds required the capture of more feral fish (in kg fresh weight) than was produced by farming (Ytrestoyl et al., 2015). This practice makes marine seafood farming less sustainable. Moreover, limited access to feral pelagic fish would not support a growth in farming of marine fish species (Olsen and Hasan, 2012). Consequently, marine feed resources has been replaced with plant based feed ingredients, and during the last few decades formulated Norwegian Atlantic salmon feeds have changed from ~70% marine to ~70% plant-based feed ingredients (Aas et al., 2019). However, the use of novel plant-based feed ingredients in aquafeeds has introduced new contaminants such as mycotoxins, that have not before been associated with the farming of marine fish (Nacher-Mestre et al., 2013). Mycotoxins comprise several groups of diverse chemical structures which are produced by fungi that infect agricultural crops before harvest (field mycotoxins), or post-harvest under certain temperature and humidity conditions (storage mycotoxins) (Bryden, 2012; Magan et al., 2010). Ingestion of mycotoxins by livestock, including fish, can result in several different toxic actions and possibly lead to accumulation in edible parts, raising concern for both animal health and food safety (Glencross et al., 2020; Hussein and Brasel, 2001).

Several reviews have reported on the occurrence of mycotoxins in feed ingredients, aquafeeds, and/or tissues in European farmed fish species (Bernhoft et al., 2013b, 2017; Nacher-Mestre et al., 2013, 2015b; Pietsch, 2020; Pietsch et al., 2013; Tolosa et al., 2021; Wozny et al., 2013). These mycotoxins mainly include well-studied mycotoxins such as deoxynivalenol (DON), fumonisins Bs (FBs), and trichothecenes (e.g., T-2 HT-2), for which guidance values have been established for animal

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feed including fish feed (Cheli et al., 2014; Pinotti et al., 2016). Several studies have investigated the potential adverse effects of these mycotoxins on Atlantic salmon, which appear to tolerate ochratoxin A (OTA) and zearalenone (ZEA), while being rather sensitive to DON exposure (Bernhoft et al., 2018; Döll et al., 2010; Moldal et al., 2018). More recently, the emerging fusarium mycotoxins beauvericin (BEA) and enniatins (ENNs) have been reported in European marine aquafeeds, with enniantin B (ENNB) exhibiting a ~90% prevalence in all tested feed samples (Albero et al., 2022; Nacher-Mestre et al., 2020; Tolosa et al., 2014). Currently, both BEA and ENNB remain non-regulated in legislation for feed or food (Bernhoft et al., 2013a; Krizova et al., 2021; Lindblad et al., 2013; Vaclavikova et al., 2013).

Potential transfer of ENNB and BEA from feed to the fillet of farmed fish could cause seafood to be an extra contributor to mycotoxin exposure to the consumer. Both ENNB and BEA have an extensive intestinal and/or liver biotransformation determining overall feed-to-organ transfer and tissue distribution of parent of the parent compounds (Abd-Allah et al., 1999; Bhateria et al., 2022; Faeste et al., 2011; Ivanova et al., 2011, 2014, 2017, 2019). Still, several ENNs, with ENNB as the most predominant isoform, were observed in the fillet of commercially farmed European sea bass (*Dicentrarchus labrax*) and gilthead sea bream, while BEA was not detected in these fish (Tolosa et al., 2014, 2017, 2021). Similarly, in Norwegian farmed Atlantic salmon fillet, the predominant mycotoxins were ENNs (Tolosa et al., 2020). In an EU surveillance, BEA was observed in a limited number of seafood samples, while ENNs had a high prevalence (EFSA, 2014). However, no apparent feed-to-fillet transfer was observed when gilthead sea bream and Atlantic salmon fillet were fed background levels of ENNB and BEA in plant-based feed (Nacher-Mestre et al., 2020). Extensive metabolization of ENNB or BEA would reduce the potential transfer of the parent compounds to organs.. Several *in vitro* and *in vivo* studies have shown a rapid phase I metabolization of ENNB in farmed terrestrial animals such as broiler chicken (Fraeyman et al., 2016; Ivanova et al., 2014, 2017) and pig (Ivanova et al., 2017). Recently, also BEA has been shown to be *in vitro* and *in vivo* hepatic phase I and phase II metabolized into several metabolites (Yuan et al., 2022). Despite their structural similarity, the metabolism of ENNB and BEA seems to differ in mice, which can affect parent ENNB and BEA organ accumulation/distribution (Rodriguez-Carrasco et al., 2016). To our knowledge, no studies have investigated the potential difference in ENNB and BEA organ distribution/transfer in farmed fish species after spiked dietary exposures (Nacher-Mestre et al., 2020).

One of the main cellular toxic actions of the ionospheric and lipophilic ENNB and BEA (EFSA, 2018) is related to their ability to form channels in the lipid bilayer causing uncontrolled passage of ions (such as Ca^{2+}) over membranes, thereby increasing membrane ion permeability and disturbing the transmembrane potential (Alonso-Garrido et al., 2018, 2020). The *in vitro* disturbance of mitochondrial transmembrane potential is considered to be one of the central toxic modes of action of both ENNB and BEA, leading to the uncoupling of oxidative phosphorylation and caspase-mediated regulated cell death (Alonso--Garrido et al., 2018, 2020; Wang and Xu, 2012). General *in vitro* cellular adverse effect outcomes in different mammalian cell types, include hepatic mitochondrial damage that affects the respiratory chain with following ATP depletion (Kouri et al., 2002, 2005; Tonshin et al., 2010), loss of intestinal cell integrity and gut microbiota (Bertero et al., 2020), differential ENNs and BEA inhibition of osteoclastic bone resorption (Tedjiotsop-Feudjio et al., 2009), eryptosis of red blood cells (Ficheux et al., 2012; Jilani et al., 2011; Qadri et al., 2011), and although debated (Gruber-Dorninger et al., 2017), oxidative stress (Mallebrera et al., 2014; Prosperini et al., 2013; Tedjiotsop-Feudjio et al., 2009), possible leading to depletion of antioxidants such as vitamin E. *In vitro* studies showed that teleost fish derived cell lines are similarly sensitive to BEA as mammalian cell lines (Garcia-Herranz et al., 2019), and both ENNs and BEA exerted a pronounced acute effect in fish RTgill-W1 cell line (Bernal-Algaba et al., 2021). In an recent Atlantic salmon *in vitro* hepatocyte study, BEA and ENNB caused reduced mitochondrial metabolic activity and altered cellular iron homeostasis (Søderstrøm et al., 2022).

Despite the diverse acute toxicity of both ENNB and BEA *in vitro*, most *in vivo* studies indicate no, or only a low, acute toxicity for humans or farmed animals (EFSA, 2014; Faeste et al., 2011; Gruber-Dorninger et al., 2017). Sub-chronic and none acute toxic *in vivo* endpoints include immunotoxicity (EFSA, 2018; Ficheux et al., 2011, 2013), disturbed reproduction (Chiminelli et al., 2022), impaired gut health (Novak et al., 2021; Reisinger et al., 2019; Springler et al., 2016), liver damage as seen from increased plasma alanine aminotransferase (ALAT) (Novak et al., 2021), and reduced feed conversion (Kolawole et al., 2020). Several reviews have summarized the potential *in vivo* chronic toxic consequences of both ENNs and BEA exposures in terrestrial farmed animals (Chiminelli et al., 2022; EFSA, 2014; Gruber-Dorninger et al., 2017; Novak et al., 2021). To our knowledge, no *in vivo* sub-chronic ENNB or BEA exposure studies have been performed on farmed fish species. The present study aimed to establish safe levels of BEA or ENNB in Atlantic salmon aquafeed after sub-chronic (3 months) exposure, using EFSAs Bench-mark Dose Lower (BMDL) model assessments for a dose-response trial. Possible organismal adverse health end-point assessment included intestinal function (protein digestibility), disturbed hematology (hematocrit and red blood cell formation), liver damage (plasma enzymes ASAT), bone formation and mineralization (spinal growth and degree of spinal deformity), overall energy use (feed-intake, growth and following feed conversion), and oxidative stress (vitamin E storage).

2. Material and methods

2.1. Feeding trial

The trial was conducted according to the guidelines of the Norwegian Regulation on Animal Experimentation and EC Directive 86/609/EEC. The National Food Safety Authorities approved the protocol (identification number: ID 26017). The trial started the August 17th of August 2021 and ended the 25th of December 2021 and was conducted at Matre Research Station (Institute of Marine Research, Norway). Individually passive integrated transponder (PIT) tagged pre-smolt Atlantic salmon (*Salmo salar* L.) (Stead and Laird, 2002) of both sexes (Aquagen strain) were distributed among 21 conical fiberglass tanks (500 L; 1 m Ø x 0.9 m) with 39 fish per tank. Initial weight and length (fork-tail) were respectively 75.3 ± 8.10 g and 18.5 ± 6.12 cm (mean ± standard deviation; n = 819). Pre-smolt salmon was chosen as juvenile fish have a higher relative feed intake (1–2% of body weight (BW) per day) than adult fish (<0.8% of BW/day), and a poor health status prior to smoltification (pre-smolt) is considered as the one of the main courses for increased mortality, reduced growth, increased bone deformity observed in adult salmon reared in sea cages. See Supplementary Fig. 1 for a schematic representation for the experimental set-up and time-line of the feeding trial.

During a three-week acclimatization period to the holding facilities, all fish were fed a marine based control diet, that was designed to have no background levels of mycotoxins which normally are associated with plant ingredients (non-detectable ENNB, BEA, DON, FBs, OTA, ZEA levels with a detection limit of <0.01 mg kg^{-1}). The marine control diet (produced by Cargill Aqua Nutrition) was composed of fish meal (70% North Atlantic), plant binders (starch sources) (12%), fish oil (15.5%, Northern hemisphere). Vitamin and mineral premix (2.4%) were supplemented as estimated to cover requirements according to NRC (2011). Yttrium oxide (0.02%) was added to the feed as an inert marker to assess nutrient digestibility as a measure for intestinal functionality. Thereafter triplicate tanks received one of the seven experimental diets: either control or ENNB- or BEA-spiked feeds at three different (low, medium, or high) concentrations (for description of chosen levels, see below). The BEA diets were fed for ~3 months (76 days) while ENNB diets were fed for ~2.5 months (69 days) due to a limit access to ENNB enriched feed

compared to BEA feed. Fish were fed by automatic feeders in two meals per day. Feed intake per tank was measured by collecting feed waste ~30 min after each meal, to assess daily feed intake (~1.2% body weight (BW) day^{-1}). The fish were reared in sea water (30 g L^{-1}, 10.2 °C) using 24 h light. The O_2 saturation of the outlet water was always above 80%. Mortality was recorded on a daily basis.

The ENNB or BEA spiked diets were prepared by dissolving these lipophilic mycotoxins directly into the feed oil and further vacuum-coated on the basal pellet (3 mm diameter, lacking 6% oil) at a level of 6% fish oil inclusion. The mycotoxin BEA (cas 26048-05-5) and ENNB (cas 917-13-5) were purchased from AdipoGen® (AdipoGen® Life Sciences, Nordic BioSite, Oslo, Norway). Control diets were vacuum-coated with 6% of ENNB or BEA-free feed oil. Samples were taken from each feed batch and analysed for supplemented levels and were below limit of quantification (LOQ) 0.01 (control) and 0.3, 5.2, 83 mg ENNB kg^{-1} feed wet weight (ww) for ENNB diet, and 0.3, 4.8, 46 mg BEA kg^{-1} feed ww for BEA diet, respectively. The levels in the experimental levels were chosen as to be able to perform a EFSA bench-mark (BMD) dose regression assessment (EFSA, 2017)(see section Statistics). This includes a very high dose (high levels, ~40–80 mg kg^{-1}) as a "positive toxic control" and part of the upper-plateau of the dose-response BMD assessment (EFSA, 2017). The medium concentrations are near the theoretically maximum contamination (~5 mg kg^{-1}) scenarios using contaminated plant feed ingredients in fish feeds (Pietsch, 2020). The lower exposure concentration is near the higher range of levels observed (~0.3 mg kg^{-1}) in Norwegian commercial salmon feeds (IMR surveillance data from 2015 to 2021, n = 200 commercial samples, (IMR, 2015). Pellets were stored at −18 °C until fed to the fish. Feed samples were taken at the end of the trial, and after 3 months of storage, and analysis showed no significant ENNB or BEA degradation during the frozen (−18 °C) storage.

At the middle and end of the trial of the trial, six fish per tank were randomly sampled (n = 18 per dietary group). Sampled fish were sacrificed in a bath of tricaine methanesulfonate (Tricaine Pharmaq; ~40 mg L^{-1}). The fish blood samples were taken from the caudal vein quickly following the initial anesthetization, using a heparinized VACUETTE® blood collection tube with 21G x 1' needle. Whole blood was divided into two aliquots, one of which was used for immediate on-site analyses of haematocrit and preparation of plasma. The other aliquot (~2 mL) was kept on ice for erythrocyte count and haemoglobin determination, which were performed within two days after sampling. For plasma samples, whole blood was centrifuged at 3500g for 10 min, and the plasma was snap-frozen in liquid nitrogen and stored at −80 °C until further analysis. Body weight and length of each fish were recorded, and liver was weighed and divided into two parts, one for biochemical analyses (MDA, vit E) and the other for parent BEA or ENNB levels. Brain, muscle, and kidney samples were taken and pooled for all fish per tank to obtain sufficient material for parent ENNB or BEA analyses. Similarly, pooled feces samples were taken from all sampled fish per tank to obtain sufficient material for protein and yttrium marker analysis. All samples were immediately frozen in liquid or solid nitrogen followed by storage at −80 °C until biochemical analyses.

2.2. Tissue levels, of ENNB and BEA and tissue retention

Tissues were homogenized, and aliquots (0.5 g) were combined with internal standard (20 ng T2-toxin, CAS 21259-20-1) and 5 mL of acetonitrile:water:formic acid, 75:24:1. Samples were shaken for 1 h at 2500 rpm with a benchtop, multi-tube vortex, then centrifuged at 3000 rcf for 3 min. The supernatant was transferred to a new tube and frozen at −20 °C for at least 2 h. Finally, the top layer (organic phase) was filtered through 0.2 μm regenerated cellulose filters into 1.5 mL analysis vials. Quantitative analysis for parent BEA or ENNB were performed with an Agilent G6460C triple quadrupole mass spectrometer paired with an Agilent 1290 Infinity II liquid chromatography system, using Agilent Jetsteam electrospray ionization in positive mode. The injection volume was 2 μL; the column was Agilent Zorbax RRHD StableBond C18, 2.1 × 150 mm, 1.8 μm; and column heater was set at 45 °C. Methanol (A) and water (B), each with 2 mmol ammonium acetate were the mobile phases. With a constant flow rate of 0.4 mL/min, the gradient started at 5% A; increased from 0.2 min to 10 min up to 98% A; held until 11 min; returned to 5% A by 11.3 min; and held until end at 14 min. The gas temperature was 300 °C with flow at 5 L min^{-1}. Sheath gas temperature was 250 °C with a flow at 11 L min^{-1}. Nozzle voltage was 500 V, and capillary voltage was 3500 V. The following transitions (collision energy, eV) were monitored: 784.4 → 134.1 (70) and 784.4 → 244.1 (30) for BEA; 640.4 → 196.1 (22) and 640.4 → 86.2 (70) for ENNB; and 489.2 → 327.0 (22) and 489.2 → 387.1 (18) for T2-toxin internal standard. Targets were quantified using Agilent Masshunter software against the relative response of the internal standard in matrix-matched calibration curves. Limits of detection ranged between 0.09 and 0.24 ng g^{-1} ww for each compound in the different tissue types.

Tissue retention (%) of parent dietary BEA and ENNB was calculated in kidney, liver, brain, and muscle in pooled fish per tank as following

$$\text{Tissue BEA or ENNB retention } (\%) = \left(\frac{\text{Total tissue weight gain (g ww)} * \text{concentration of BEA or ENNB (ng g}^{-1}\text{)}}{\text{total feed eaten (g ww)} * \text{concentration of BEA of ENNB (ng g}^{-1}\text{)}} \right) * 100$$

2.3. Growth rate and feed conversion

Specific growth rate (SGR), specific length increase rate (SLR) feed intake (FI) and feed conversion rate (FC) were calculated with the following equations. SGR and SLR were assessed for individual pit-marked fish and tank growth, while daily feed intake and feed conversion rate were based on tank level.

$$\text{Specific growth rate (SGR) tank or individual} = \left(\frac{\ln(\text{Final body weight (g)}) - \ln(\text{Mean initial body weight(g)})}{\text{days of feeding experiment}} \right) * 100$$

$$\text{Specific lenght increase rate (SLR) tank or individual} = \left(\frac{(\text{Final body length (mm)}) - (\text{Initial body lenght (mm)})}{\text{days of feeding experiment}} \right) * 100$$

$$\text{Daily feed intake} * \text{fish}^{-1}(\text{FI}) = \frac{\text{Recorded feed intake } * \text{tank}^{-1} * \text{day}^{-1}(\text{g})}{\text{Number of fish } * \text{tank}^{-1}}$$

$$\text{Feed conversion rate}(\text{FC}) = \left(\frac{\text{Total feed intake} * \text{fish}^{-1}(\text{g})}{\text{Body weight gain (g)}}\right)$$

$$\text{Condition factor}(\text{C}-\text{factor}) = \left(\frac{\text{Weight (g)}}{\text{Length}^3\ (\text{mm})}\right)$$

2.4. Protein digestibility

Feed and fecal samples were homogenized and freeze-dried (until successive weighing was unchanged) and analysed for total protein and yttrium. Crude total protein, including both structural and soluble protein, was determined by nitrogen combustion of 0.5 g of freeze-dried material with a Dumas and Liebig nitrogen analyzer (PE 2410, USA). Nitrogen was detected by thermal conduction and crude protein was calculated as Nx6.25 (Crooke and Simpson, 1971). Casein (C-8654, Sigma, Dorset, UK) was used as reference material. The yttrium oxide concentrations in freeze-dried feed and feces were analysed according to (Ottera et al., 2003). Briefly, yttrium oxide was quantified by inductively coupled plasma mass spectrometry after wet digestion in a microwave oven (Ottera et al., 2003). Apparent digestibility (AD) of total proteins was calculated using the formula described by (Maynard and Loosli, 1969).

$$\text{Apparent digestibility (AD)} = 100 - \left(\frac{\text{yttrium concentration diet } \left(\frac{\text{mg}}{\text{kg}}\right) * \text{protein content feaces } \left(\frac{\text{mg}}{\text{kg}}\right)}{\text{yttrium concentration feaces } \left(\frac{\text{mg}}{\text{kg}}\right) * \text{protein content diet } \left(\frac{\text{mg}}{\text{kg}}\right)}\right)$$

2.5. Bone deformity and vertebrae Ca and P content

Bone deformity was assessed by x-ray according to (Witten et al., 2009). Whole fish were radiographed with a Direct Radiology System (Canon CXDI-410C Wireless, CANON INC., Kawasaki, Japan) using a portable x-ray unit (Portable X-ray Unit Hiray Plus, Model Porta 100 HF, JOB Corporation, Yokohama, Japan) at 85 cm distance with 40 kV and 4 mAs. The degree of deformity was defined as the percentage of fish per diet with one or more identified deformed vertebrae.

For vertebrae Ca and P content, three vertebrae from the same anatomical region were isolated from three fish of each experimental replicate (9 fish per treatment group). After removing attached soft tissues, the vertebrae were dehydrated in a drying hoven at 70 °C over night. Dried samples were weighted and digested in nitric acid at 60 °C for 5 h. The calcium and phosphorous mineral content were quantified using Microwave Plasma-Atomic Emission Spectrometry (MP-AES; Agilent) according to Tarasco et al. (2022).

2.6. Hematology and plasma biochemistry

Hematocrit (Hct) was determined immediately from individual sampled blood using Vitex Pari microhematocrit capillary tubes (Vitrex Medical A/S, Denmark) and a microhaematocrit centrifuge (Hematophagy, Heraeus-Christ GmbH, Germany). The number of red blood cells (RBC) and amount of hemoglobin (Hb) in full blood were measured in a Cell Dyn 400 Hematological Analyzer (Sequoia-Turner) according to the manufacturer's instructions, using Para 12 Extend control blood (Streck, MedMark Ref:218777) for calibration. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated from Hct, RBC and Hb as described in Sandnes et al. (1988). Plasma concentrations, albumin, total protein (tot prot), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), bilirubin, creatinine and were measured on a PL multipurpose diagnostic analyzer (Maxmat S.A., Montpellier, France) using DIALAB diagnostic kits (Vienna, Austria).

2.7. Liver oxidative stress

In order to assess liver oxidative stress the fat soluble antioxidant vitamin E was analysed in liver samples pooled from six fish per tank. Individual samples were analysed for the lipid peroxidative product malondialdehyde (MDA). Vitamin E was analysed as α-, β-, γ- and δ-tocopherol isomers and α-, β-, γ- and δ-tocotrienol by high performance liquid chromatography (HPLC) according to the method described by Hamre et al. (2010). In short, the homogenized liver samples were saponified (20 min at 100 °C) using ethanol, potassium hydroxide, pyrogallol, ascorbic acid and EDTA, before the samples were extracted three times with hexane. The solvent was subsequently evaporated under nitrogen and the samples were diluted with a standard volume of hexane before injection into the HPLC and detection by fluorescence detector. The method for determination of MDA is described in Hamre et al. (2022). Extraction was performed as in Hamre et al. (2001). Briefly, homogenized fish tissue was weighed and extracted with chloroform:methanol (2:1, containing 0.005% BHT) and saturated EDTA. The methanol: water phase supernatant was transferred to a new tube and TBA reagent was added. This system was heated at 100 °C for 30 min. After cooling, an aliquot was placed in an autosampler vial and subjected to HPLC separation with UV detection at 532 nm. Quantification was obtained by comparison with an external MDA standard, prepared from 1,1,3,3- tetraethoxypropane (TEP).

2.8. Statistics and benchmark dose modelling

To account for the variance among experimental tanks within a dietary treatment, as well as variance among fish within an experimental tank, multiple comparison nested ANOVA, followed by Tukey's HSD post hoc test were used. Significant differences among group were set at $p < 0.05$. All statistics were performed using the program Graphpad Prism 9 (Dotmatics Inc.). Benchmark dose (BMD) analysis was conducted on the responses of the graded dietary exposures according to the EFSA's benchmark dose technical guidance (EFSA, 2017). Nested (response per diet nested in tank) individual data were fitted on two model families (exponential and Hill), using the EFSA BMD platform (Proast, version 66.5 https://shiny-efsa.openanalytics.eu/app/bmd). Selection of models (significantly better model fit) was based on the

Table 1
Kidney, liver, muscle, and brain BEA and ENNB concentration (triplicate per diet, mean ± SD, pooled sample of six fish per tank, N = 3, μg kg^{-1}) for Atlantic salmon (*Salmo salar*) fed control (no detectable mycotoxins) or three graded levels of beauvericin (BEA-$_{low,\ medium,\ high}$: 0.3, 4.8, 46 mg kg^{-1} feed) or enniatin B (ENNB-$_{low,\ medium,\ high}$: 0.3, 5.2, 83 mg kg^{-1}) for 76 and 69 days, respectively.

Feed(mg kg^{-1})	Control	BEA_{low}	BEA_{medium}	BEA_{high}
	<0.01	0.3	4.8	46
kidney	nd	nd	0.169 ± 0.217	6.00 ± 1.93
liver	nd	nd	nd	4.70 ± 6.41
muscle	nd	nd	nd	0.118 ± 0.113[b]
brain	nd	nd	nd	nd
Feed(mg kg^{-1})	**control**	**$ENNB_{low}$**	**$ENNB_{medium}$**	**$ENNB_{high}$**
	<0.01	0.3	5.2	83
kidney	nd	nd	0.485 ± 0.841	24.0 ± 11.5
liver	nd	nd	0.668 ± 0.954	10.6 ± 7.65
muscle	nd	nd	nd	4.87 ± 2.3
brain	nd	nd	nd	2.82 ± 1.19

nd = not detected, limit of detection (LOD) for ENNB or BEA, respectively in liver (0.19 and 0.23 μg kg^{-1}), kidney (0.09 and 0.20 μg kg^{-1}), brain (0.24 and 0.10 μg kg^{-1}), muscle (0.10 and 0.16 μg kg^{-1}).

Akaike information criterion (AIC). A default value of 2 units difference between AICs is considered as the critical value by the EFSA (EFSA, 2017). BMD models were accepted when the AIC of the model was lower than the AIC of the null model (no dose response) −2 (AIC < AICnull-2), and the model with lowest AIC (AICmin) was lower than the AIC of the full model +2 (AICmin < AICfull+2) (EFSA, 2017). Model averaging was performed for continuous data as available in the current version of Proast. The 90% lower and upper confidence intervals for the BMD (BMDL and BMDU, respectively) were estimated including bootstrap with standard 200 Bootstraps. The BMDL is defined as the dose not expected to give an adverse effect. A default benchmark response (BMR) of 5% change was used as starting point for model fitting of apparent adverse effects (EFSA, 2017) such as reduced growth.

3. Results

3.1. Tissue ENNB and BEA concentration and tissue retention

Kidney was the organ with highest BEA or ENNBBEA levels after feeding high or medium ENNB or BEA feed levels (kidney levels were 6.0 vs 24.0 and 0.17 vs 0.0.49 μg kg^{-1} ww, respectively) (Table 1). For liver tissue, fish fed high BEA or ENNB spiked feed had detectable levels of parent BEA and ENNB (4.7 and 10.6 μg kg^{-1}ww, respectively). However, at medium BEA or ENNB feed exposure level, only detectable levels were found in the liver of fish fed ENNB and not BEA fed fish (0.67 and < 0.23 μg kg^{-1} ww) (Table 1). Both BEA and ENNB were detected in muscle tissue of high BEA and ENNB exposed fish, with a 40-fold higher level for ENNB fed fish compared to BEA fed fish (4.87 vs 0.118 μg kg^{-1} ww, respectively) (Table 1). At medium exposure levels no parent BEA or ENNB were observed in muscle tissue (Table 1). None of the BEA spiked feeds exposure groups (high, medium, or low) gave detectable BEA levels in brain (Table 1), while fish fed only the high spiked ENNB feed had detectable levels of parent ENNB in the brain (2.82 μg kg^{-1} ww), and brain was the organ with lowest ENNB levels (Table 1).

The retention of dietary ENNB or BEA in was relatively low in all tissues (<0.01%). The retention of parent ENNB or BEA was highest in muscle tissue with a significantly higher retention for ENNB compared to BEA exposed fish (0.01% versus 0.0003%, respectively) (Fig. 1), reflecting the higher muscle ENNB levels compared to BEA levels (Table 1). Similar for kidney, the retention was higher for ENNB compared to BEA fed fish (0.0005 versus 0.0002%, respectively), with a lower kidney retention compared to muscle (Fig. 1). The ENNB and BEA liver retention was lowest (~0.00025%) with no differences in retention between BEA and ENNB (Fig. 1).

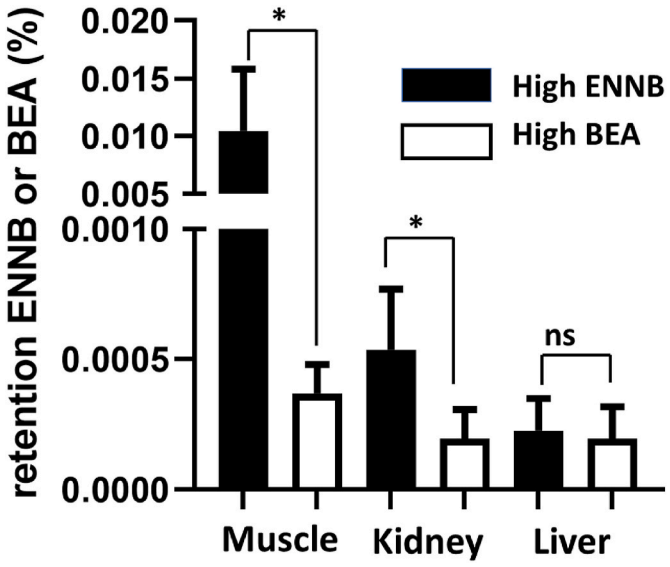

Fig. 1. Retention (%) of consumed enniatin B (ENNB) and beauvericin (BEA) in muscle, kidney, or liver (triplicate tanks per diet, mean ± standard deviation, pooled sample of six fish per tank, N = 3) for Atlantic salmon (*Salmo salar*) fed high levels of high ENNB or (83 or 46 mg kg^{-1}, black or open column, respectively) for 69 or 76 days, respectively. Organs with a significant difference in relative retention between ENNB or BEA fed fish are indicated with an asterisk (*) (nested one-way ANOVA, Tukey's *t*-test, p-value). Ns = not significant.

3.2. Growth, length, feed conversion, feed intake

Table 1 in supplementary data gives individual specific length increase rate (SLR), specific growth rate (SGR), weight, length, c-factor as well as tank-based feed intake and feed conversion rate in fish fed graded levels of BEA or ENNB for 32 days (mid sampling) or 76 and 69 days (end sampling), respectively. Most significant differences ($p < 0.05$, ANOVA Tukey's-t test) for BEA and ENNB fed fish compared with the control groups were observed at the end of the chronic exposure period (Supplementary Table 1). Results will hence be presented from the middle to end period of the trial for SLR and SGR, and the end of the trial for weight, length, or c-factor. Specific length increase rate was significantly reduced in fish fed the medium and high ENNB diets (5.2 and 83 mg kg^{-1}) compared to the control groups (no detectable mycotoxins), while BEA exposure had no significant effect on SLR (Fig. 2A). Specific growth rate was significantly affected by both BEA and ENNB exposures (Fig. 2B). ENNB had a stronger adverse effect on SGR than BEA, as fish fed medium and high ENNB had a reduced SGR compared to control, while BEA reduced SGR only at the high exposure level (46 mg kg^{-1}). As BEA reduces growth in weight but not length, BEA reduces the condition factor with a significantly reduced c-factor in fish fed high levels, compared to the control groups. ENNB is reducing both weight and length increase, and ENNB exposures up to 83 mg kg^{-1}, did not affect condition factor (Fig. 2C).

Fish fed BEA had an increased feed conversion rate (FCR) with significantly increased FCR levels in fish fed medium and high BEA levels, compared to control fish (Fig. 3A). ENNB exposure did not cause any change in feed conversion rate (Fig. 3A). The fish fed high BEA levels had an increased feed intake (FI), compared to control fish (Fig. 3B). For ENNB fed fish, there were no significant differences in FI (Fig. 3B).

3.3. Digestibility

Fish fed the highest level of BEA (46 mg kg^{-1}) had a significantly lower protein digestion compared to control, while none of the ENNB exposure levels significantly affected protein digestibility (Fig. 4).

3.4. Vertebra deformity

No significant differences were seen in spinal deformity as expressed as percentage fish with one or more deformed vertebrae (Fig. 5). The vertebrae Ca and P levels as well as Ca/P ratio was not significantly affected by BEA or ENNB exposure (data not shown).

3.5. Hematology and plasma chemistry

Fish fed BEA had no significant changes in Hct, RBC, Hgb. However, the mean corpuscular volume (MCV) was significantly decreased in fish fed all three BEA levels (Table 2). Fish fed ENNB at all three exposure levels, had a significantly reduction in Hct and Hgb compared to control, whereas RBC was only significantly reduced at the medium -and high ENNB exposure levels (Table 2). Mean corpuscular haemoglobin (MCH) increased in the second highest and highest ENNB exposure levels compared with control and mean corpuscular haemoglobin concentration (MCHC) was significantly increased in the high ENNB exposure group compared with control.

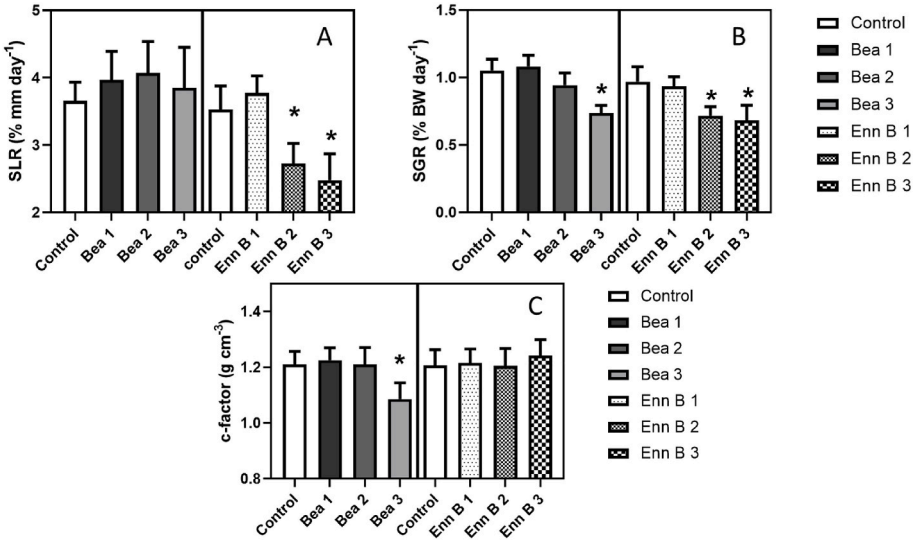

Fig. 2. (A–C). Individual specific length increase rate (SLR% mm day^{-1} A), specific growth rate (SGR,% BW day^{-1}, B), and condition factor (c-factor, g cm-^{3}, C) (triplicate tanks per diet, mean ± standard deviation, 16 fish per tank, n = 48) for pit-tagged Atlantic salmon (*Salmo salar*) fed control (no detectable mycotoxins) or three graded levels of beauvericin (BEA1-3, 0.3, 4.8, 46 mg kg^{-1} feed) or enniatin B (ENNB 1–3, 0.3, 5.2, 83 mg kg^{-1}) for 76 and 69 days, respectively. Values are given from middle to end sampling (44 or 37 days for BEA or ENNB, respectively). Columns (mean ± SD) with an asterisk (*) are significantly different from the control group (nested one-way ANOVA, Tukey's *t*-test, p-value).

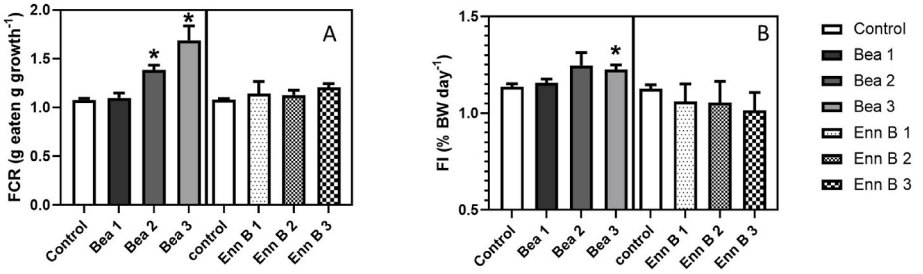

Fig. 3. Feed conversion rate (FCR, g feed eaten/g weight gain, A) and feed intake (FI, % BW day^{-1}, B) (triplicate tanks per diet, mean ± standard deviation, pooled sample of six fish per tank, N = 3) for Atlantic salmon (*Salmo salar*) fed control (no detectable mycotoxins) or three graded levels of beauvericin (BEA1-3, 0.3, 4.8, 46 mg kg^{-1} feed) or enniatin B (ENNB 1–3, 0.3, 5.2, 83 mg kg^{-1}) for 76 and 69 days, respectively. Values are given from middle to end sampling (44 or 37 days for BEA or ENNB, respectively). Columns (mean ± SD) with an asterisk (*) are significantly different from the control group (nested one-way ANOVA, Tukey's *t*-test, p-value).

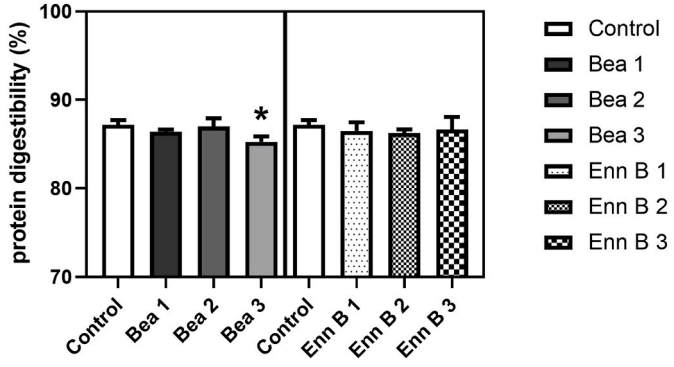

Fig. 4. Protein digestibility (%) (triplicate tanks per diet, mean ± standard deviation, pooled sample of six fish per tank, N = 3) for Atlantic salmon (*Salmo salar*) fed control (no detectable mycotoxins) or three graded levels of beauvericin (BEA1-3, 0.3, 4.8, 46 mg kg^{-1} feed) or enniatin B (EnnB1-3, 0.3, 5.2, 83 mg kg^{-1}) for 76 and 69 days, respectively. Values are given from middle to end sampling (44 or 37 days for BEA or ENNB, respectively). Columns (mean ± SD) with an asterisk (*) are significantly different from the control group (nested one-way ANOVA, Tukey's *t*-test, p-value).

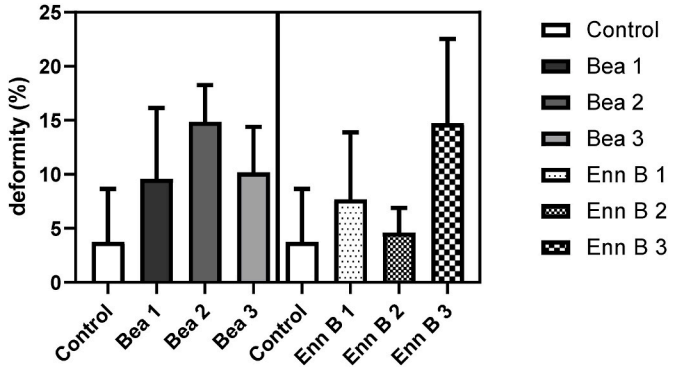

Fig. 5. Degree of spinal deformity as % of fish per diet with one or more deformed vertebrae (triplicate tanks per diet, mean ± standard deviation, 16 fish per tank, n = 48) for Atlantic salmon (*Salmo salar*) fed control (no detectable mycotoxins) or three graded levels of beauvericin (BEA1-3, 0.3, 4.8, 46 mg kg^{-1} feed) or enniatin B (ENNB1-3, 0.3, 5.2, 83 mg kg^{-1}) for 76 and 69 days, respectively. Values are given from middle to end sampling (44 or 37 days for BEA or ENNB, respectively).

BEA did not significantly affect the levels of plasma liver enzymes ASAT and ALAT. In contrast, the highest level of ENNB caused an increase in ASAT and ALAT, compared with control fish (Table 3). The levels of creatinine, urea, or total protein were not significantly affected by either dietary ENNB or BEA exposures.

3.6. Vitamin E, D and C, and MDA

Fish fed all levels of BEA had significantly reduced hepatic vitamin E alfa-tocopherol concentrations (Fig. 6A), and significantly increased delta-tocopherol and alfa-tocotrienol concentrations (Fig. 6B and D, respectively), compared to control. No significant differences were observed in gamma-tocopherol concentrations in BEA exposed fish (Fig. 6 C), compared to control. The tocopherol isoform, beta-tocopherol

Table 2
Hematocrit (Hct, %), red blood cell count (RBC, 10^{12} l^{-1}), haemoglobin (Hgb, g 100 ml^{-1}) mean corpuscular volume (MCV, × 10^{-15} 1), mean corpuscular haemoglobin (MCH, 10^{-6} g), and mean corpuscular haemoglobin concentration (MCHC, g 100 ml^{-1}) in full blood (triplicate tanks per diet, mean ± standard deviation, six fish per tank, n = 18) for Atlantic salmon (*Salmo salar*) fed control (no detectable mycotoxins) or three graded levels of beauvericin (BEA1-3, 0.3, 4.8, 46 mg kg^{-1} feed) or enniatin B (ENNB 1–3, 0.3, 5.2, 83 mg kg^{-1}) for 76 and 69 days, respectively (BEA1-3 and ENNB 1–3 = BEA- or ENNB-low, medium, or high). Values in rows with the same superscripts (or lack of superscripts) are not significantly different (nested one-way ANOVA, Tukey's *t*-test, p-value).

	Control	BEA 1	BEA 2	BEA 3	p =
Hct	44.8 ± 2.47	46.5 ± 2.77	45.5 ± 2.50	47.7 ± 3.92	0.0623
RBC	1.24 ± 0.13	1.29 ± 0.099	1.31 ± 0.079	1.34 ± 0.18	0.2211
Hgb	8.56 ± 1.22	9.75 ± 1.09	9.72 ± 1.08	9.72 ± 0.81	0.0591
MCV	417.9 ± 39.9^{a}	361.4 ± 31.9^{b}	349.3 ± 31.0^{b}	352.6 ± 56.3^{b}	*0.0115*
MCH	72.8 ± 9.72	75.1 ± 4.52	72.7 ± 6.15	73.3 ± 8.43	0.9146
MCHC	17.3 ± 1.22	20.9 ± 2.24	21.1 ± 2.58	20.6 ± 2.50	0.0503
	Control	ENNB 1	ENNB 2	ENNB 3	p =
Hct	45.0 ± 2.12^{a}	40.5 ± 3.01^{ab}	40.7 ± 2.49^{b}	36.4 ± 2.77^{c}	<0.0001
RBC	1.21 ± 0.13^{a}	1.10 ± 0.15^{ab}	0.87 ± 0.083^{b}	0.78 ± 0.049^{b}	0.0002
Hgb	8.41 ± 1.22^{a}	7.49 ± 0.73^{ab}	7.55 ± 0.68^{b}	7.48 ± 0.53^{b}	<0.0001
MCV	411.3 ± 39.9	455.2 ± 63.2	463.7 ± 37.2	463.3 ± 33.7	0.1309
MCH	72.8 ± 9.72^{a}	81.3 ± 8.92^{ab}	86.9 ± 4.82^{b}	95.8 ± 6.67^{b}	0.0015
MCHC	17.37 ± 1.22^{a}	18.28 ± 1.58^{a}	18.48 ± 1.57^{ab}	20.82 ± 1.80^{b}	0.0036

Table 3
Alkaline phosphatases (ALP, $U\mu l^{-1}$), alanine aminotransferase (ALAT, $U\mu l^{-1}$), aspartate aminotransferase (ASAT, $U\mu l^{-1}$), creatinine (mg L^{-1}), urea (nmol L^{-1}), total protein (g L^{-1}), in plasma (triplicate tanks per diet, mean ± standard deviation, six fish per tank, n = 18) for Atlantic salmon (*Salmo salar*) fed control (no detectable mycotoxins) or three graded levels of beauvericin (BEA1-3, 0.3, 4.8, 46 mg kg^{-1} feed) or enniatin B (ENNB 1–3, 0.3, 5.2, 83 mg kg^{-1}) for 76 and 69 days, respectively (BEA1-3 and ENNB 1–3 is BEA- or ENNB-low, medium, or high). Values in rows with the same superscripts are not significantly different (nested one-way ANOVA, Tukey's *t*-test, p-value).

	Control	BEA 1	BEA 2	BEA 3	p =
ALP	213.3 ± 69.6	194.5 ± 44.2	197.8 ± 49.1	177.5 ± 75.7	0.5454
ALAT	23.2 ± 3.35	21.5 ± 3.89	20.8 ± 4.63	21.5 ± 6.12	0.2735
ASAT	625.2 ± 136.7	567 ± 192.2	527.4 ± 151	555.5 ± 190.9	0.4566
Creatinine	60.6 ± 33.1	70.2 ± 42.8	60.0 ± 33.6	86.4 ± 51.0	0.2437
Urea	0.50 ± 0.17	0.44 ± 0.15	0.41 ± 0.16	0.37 ± 0.12	0.1068
Total protein	36.3 ± 4.25	34.7 ± 3.51	34.8 ± 3.10	35.5 ± 2.87	0.2437
	Control	ENNB 1	ENNB 2	ENNB 3	p =
ALP	219.3 ± 48.2	202.3 ± 64.4	256.4 ± 39.0	243.9 ± 52.7	0.2378
ALAT	22.1 ± 2.08^{a}	22.3 ± 4.74^{a}	25.8 ± 5.26^{ab}	30.6 ± 4.26^{b}	*0.0021*
ASAT	621.9 ± 132.8^{a}	632.3 ± 134.9^{a}	691.5 ± 164.0^{ab}	851.2 ± 106.0^{b}	*0.0464*
Creatinine	62.3 ± 27.1	69.1 ± 32.3	80.0 ± 37.4	89.7 ± 52.9	0.3037
Urea	0.47 ± 0.19	0.30 ± 0.18	0.36 ± 0.19	0.41 ± 0.16	0.1614
Total protein	35.5 ± 4.75	33.7 ± 2.43	33.4 ± 3.12	34.8 ± 4.77	0.7989

was not quantifiable (LOQ <0.08 mg kg^{-1} ww) in the liver of any of the experimental groups, as well as the delta-, and gamma-trienols isoforms (LOQ <0.04 and < 0.08, respectively mg kg^{-1} ww). As opposed to dietary BEA exposures, ENNB had no significant effects on the liver tocopherol and tocotrienols levels at any dietary exposure level (Fig. 6A–D), compared to control. As opposed to tocopherols and tocotrieonols, none of the dietary BEA or ENNB exposures affected vitamin C or vitamin D3 levels, compared to control (data not shown). The levels of malondialhyde (MDA), a marker of lipid peroxidation, were not significantly increased compared to control on all dietary BEA or ENNB, exposure groups, although there was a dose-response correlation in fish fed elevated graded levels of BEA (Fig. 6E).

3.7. BMDL modelling

For individual whole body parameters such as specific growth rate (SGR), specific length rate (SLR), condition factor (c-factor), feed conversion rate (FCR), Feed intake (FI) and hematology red blood cell count (RBC) and plasma (ASAT), a lower bound benchmark dose (BMDL) could be assessed as the dose-response model was significantly better than the response model that predicts no dose-response (null model) (AIC < AICnull-2), and the best fitted dose-response model (lowest AIC) was better than the full response model (AICmin < AICfull+2) (Table 4). The parameters SGR, SLR, c-factor, RBC, and ASAT had a low ratio (<10) between upper bound BM (BMDU) and BMDL. For the FCR and FI parameters in BEA exposed fish, the ratio between significant BMDL and BMDU was too high (>10) for set assessment (Table 4).

4. Discussion

4.1. Feed to organ transfer

ENNB was quantified in the brain of fish exposed at the highest dietary ENNB level. The lipophilic and ionospheric chemical properties of ENNB (EFSA, 2014) causes it to cross the blood brain barrier. Similarly, intraperitoneally or intracerebroventricularly BEA and ENNB exposed mice showed brain BEA and ENN accumulation (Rodriguez-Carrasco et al., 2016; Taevernier et al., 2016). Highest organ levels for both ENNB and BEA exposed fish, were found in kidney, followed by liver, muscle, and brain. Although parent ENNB and BEA was found in several organs in high BEA and ENNB exposed fish, the overall accumulation potential of dietary ENNB or BEA was relatively low as seen from a low ENNB and BEA retention in all organs. The dietary retention of ENNB and BEA in kidney, liver, and muscle was at the level ~0.0005% for kidney, ~0.00025% for liver, and ~0.0003–0.01% in muscle. The relative low feed-to-organ transfer of parent BEA and ENNB in the present study with spiked feeds, agrees with the reported absence of detectable BEA or ENNB levels in sea bream and Atlantic salmon fillets when fed low natural background BEA and ENNB levels (Nacher-Mestre et al., 2015a). The low potential for feed-to-tissue transfers for parental dietary BEA and ENNB, reflects ENNB and BEA toxico-kinetics with either a low oral uptake, high metabolization, and/or high excretion. Earlier *in vitro* ENNB toxicokinetic studies with intestinal CaCO-2 cells, showed a high bioavailability (~80%) (Meca et al., 2012), and *in vivo* pig trials also reported a high bioavailability (~90%) for ENNB (Devreese et al., 2014). Parent BEA and ENNB does not seemed to be excreted by urine, as no BEA and ENNB found in urine of intraperitoneally BEA and ENNB exposed mice (Rodriguez-Carrasco et al., 2016). In the present study, both BEA and ENNB was found in the kidney tissue at the two highest exposure level. However, as no urine was sampled in fish, the kidney BEA and ENNB levels might only reflect circulating parent BEA and ENNB. Biotransformation of ENNB *in vitro* and *in vivo*, showed an extensive combined intestinal and liver biotransformation with the formation of several metabolites as the product of hydroxylation, carbonylation, carboxylation and oxidative demethylation reactions (Bhateria et al., 2022; Faeste et al., 2011; Ivanova et al., 2011, 2014,

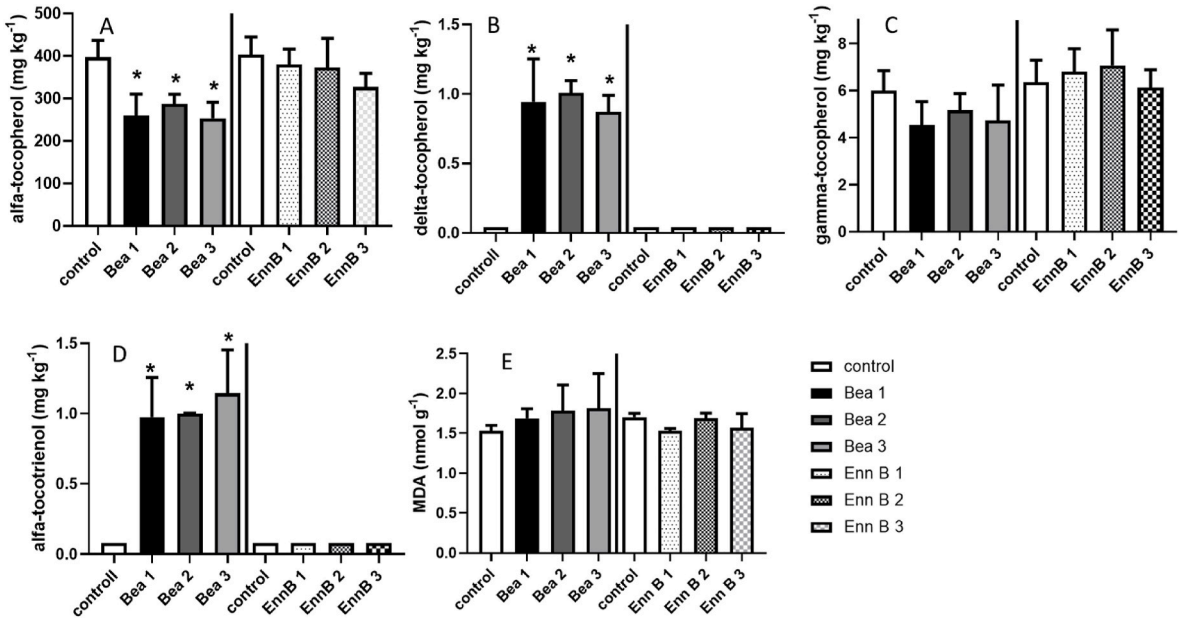

Fig. 6. (A–E). Levels (mg kg^{-1} ww) of alfa-tocopherol (A), delta-tocopherol (B), gamma-tocopherol (C), alfa tocotrienol (D), and malondialdehyde (MDA) (E) (nmol g^{-1} DW)(triplicate tanks per diet, mean ± standard deviation, pooled samples per tank, n = 3) in the liver of Atlantic salmon (*Salmo salar*) fed control (no detectable mycotoxins) or three graded levels of beauvericin (BEA1-3, 0.3, 4.8, 46 mg kg^{-1} feed) or enniatin B (ENNB1-3, 0.3, 5.2, 83 mg kg^{-1}) for 76 and 69 days, respectively (BEA1-3 and ENNB 1–3 is BEA- or ENNB-low, medium, or high). Columns (mean ± SD) with an asterisk (*) are significantly different from the control group (nested one-way ANOVA, Tukey's *t*-test, p-value <0.05).

Table 4
Lower Benchmark doses (BMDL) (μg kg^{-1}), Upper benchmark dose (BMDU) (μg kg-1), and ratio BMDU/BMDL for relevant responses (for abbreviations see main text) in Atlantic salmon (*Salmo salar*) fed control (no detectable mycotoxins) or three graded levels of beauvericin (BEA1-3,300, 4800, 46000 μg kg^{-1} feed) or enniatin B (ENNB1-3, 300, 5200, 83000 μg kg^{-1}) for 76 and 69 days, respectively. Ns = no significant BMD assessment, na = not assessed.

	BEA			ENNB		
	BMDL	BMDU	ratio	BMDL	BMDU	ratio
SGR	4560	25300	5.6	248	1050	4.2
SLR	ns	ns	na	99	900	9.1
c-factor	7520	38500	5.1	ns	ns	na
FCR	6.8	2100	307	ns	ns	na
FI	0.0014	42600	$30*10^6$	ns	ns	ns
RBC	ns	ns	na	216	123	1.8
ASAT	ns	ns	na	312	134	2.3

2017, 2019). Recent intra-species *in vitro* and *in vivo* studies, also showed that BEA is metabolized into several metabolites after oxygenation, demethylation, and phase II conjugation (Yuan et al., 2022). A high metabolization followed by a rapid elimination of the metabolites from the systemic circulation, might hence explain the low potential for feed-to-tissue transfer of parent BEA and ENNB in the present study. No metabolite assessment was performed in the present study.

Despite the low feed-to-organ transfer of parental dietary ENNB or BEA, there was a significantly higher transfer of ENNB compared to BEA and the relative retention of ENNB in kidney and muscle was significantly higher than that of BEA. The largest difference in ENNB or BEA transfer was seen for muscle tissue. The fillet levels in the highest BEA fed fish were a 40-fold lower than fish fed highest ENNB spiked feed, even though the highest BEA and ENNB feed levels only differed 2-fold. For both ENNB and BEA an extensive liver metabolization has been reported (Bhateria et al., 2022; Faeste et al., 2011; Ivanova et al., 2011, 2014, 2017, 2019; Yuan et al., 2022). However, in a comparative study with ENNB and BEA intraperitoneally exposed mice, no metabolites for BEA were observed as opposed to ENNB, indicating a differential metabolism of ENNB and BEA in mammals, which can affect parent ENNB and BEA organ accumulation/distribution (Rodriguez-Carrasco et al., 2016). This further highlights the need for comparative ENNB and BEA *in vivo* metabolization studies in species such as Atlantic salmon to assess feed-to-fillet transfer. A difference in transfer for ENNB compared to BEA, might explain the differences seen in analysed farmed sea bream, with detectable levels of ENNB but not BEA (Tolosa et al., 2014, 2017, 2021). Similarly, seafood samples surveilled on the European market had few cases of detectable levels of BEA in contrast to ENNB (EFSA, 2014). The relatively low fillet ENNB and BEA retention, likely due to a high liver/intestine metabolization, is unlikely to cause a high challenge for food safety. In EU surveillance studies, the food category "fish and seafood" seems to be a minor contributor to ENNs and BEA exposure for the general population, especially compared to grain-based food products (EFSA, 2014).

4.2. Growth and protein digestibility

Both dietary ENNB and BEA caused a reduced specific growth rate (SGR), with a more pronounced effect of ENNB exposed fish than BEA fed fish. ENNB gave reduced SGR at medium and high exposure level while BEA caused reduced SGR only at the high exposure level. Exposure to ENNB did not affect feed intake, protein digestibility, or feed conversion, thus indicating that ENNB-mediated reduced growth is not due to a disturbed intestinal nutrient uptake, nutrient conversion, or energy expenditure. The reduced growth seems to be mostly related to a reduced length (skeleton) growth, rather than impaired energy use. In contrast to the ENNB findings in the present *in vivo* study, earlier, *in vitro* hepatocyte Atlantic salmon studies showed both ENNB and BEA caused mitochondrial disruption with altered energy expenditure (Soderstrom et al., 2022). Mitochondrial disruption as one of the main ENNB and BEA cellular adverse effects (Bertero et al., 2020; Gruber-Dorninger et al., 2017; Prosperini et al., 2017; Shiwali and Kumar, 2022), and *in vitro* adverse effect outcomes in different mammalian cell types include hepatic mitochondrial damage affecting the respiratory chain and causing ATP depletion (Kouri et al., 2002, 2005; Tonshin et al., 2010). However, ENNs have a lower *in vitro* potential than BEA in causing mitochondrial impairment (Tonshin et al., 2010). In the present study, a low *in vitro*

potential for ENNB-induced mitochondrial disfunction, did not seem to cause an *in vivo* disturbed overall energy expenditure, as feed conversion was not significantly affected.

In contrast to dietary ENNB, BEA caused a growth reduction with increased feed conversion rate and reduced feed conversion, indicating that consumed feed is less well converted into weight in BEA exposed fish. Dietary BEA exposure caused a decrease in total protein digestibility in fish fed the highest BEA levels, while ENNB exposure did not affect protein digestion. Several *in vitro* intestinal model studies of ENNs and BEA effects have been performed, and have been reviewed by Bertero et al. (2020). They indicate that both BEA and ENNs affect cell lines in an intestinal environment (Bertero et al., 2020). Few *in vivo* studies have assessed ENNB's and BEA's potential adverse effects on the intestinal tissue or intestinal microbial environment. Weaning piglets fed ENN spiked feed alone or in combination with DON, showed a decrease in the diversity of the gut microbiome, altered histological changes in the intestine and other organs, and reduced growth (Novak et al., 2021). In the present trial, no apparent decreased intestinal function or feed utilization was seen after ENNB exposure, while BEA did reduce digestibility leading to reduced feed conversion and consequently weight growth. The adverse effects of BEA on intestinal digestible function seems to contribute to the reduced growth and feed conversion, possibly in combination with overall impaired mitochondrial energy formation. BEA exposed fish had a significant increase in feed intake, likely as a compensation of reduced feed utilization. Although both ENNB and BEA are known to cause *in vitro* toxicity in intestinal cells, no comparative *in vivo* studies exist on intestinal impairment of ENNB or BEA. The *in vivo* differential effect between BEA and ENNB on intestinal function despite an apparent similar *in vitro* intestinal effect, might be related to differential intestinal metabolization and/or enterohepatic cycling of the two mycotoxins. Previous studies on rodents indicated enterohepatic recycling for BEA and not for ENNs as parent BEA was found in the colon, while ENNB not (Rodriguez-Carrasco et al., 2016). Differential *in vivo* enterohepatic kinetics of BEA and ENNs are likely to cause different effects of these two otherwise structural similar mycotoxins on the intestinal function as seen in the present trial.

4.3. Vertebrae deformity and stunted growth

Few studies have focused on the effects of BEA and ENNB on bone formation, however, both BEA and ENN inhibited osteoclast bone resorption and induced osteoclast apoptosis *in vitro* (Forsdahl et al., 2008). No studies were performed on the effect of ENN B or BEA on human osteoblast, however other mycotoxins such as citrinin are known to induce apoptosis in human osteoblasts (Huang et al., 2009). However, in chickens fed diets contaminated with fusarium it was found that the presence of fusarochromanone was the causing factor of avian tibial dyschondroplasia and the characteristic bone deformities presented by affected animal (Krogh et al., 1989). In fish, osteoclasts have little role in the growth of vertebrae, however are important in the mineral reabsorption in the development of haemal and neural vertebral arches, which are also involved in the bone growth (Witten and Huysseune, 2009). In the present study, neither ENNB nor BEA seemed to be able to affect bone formation as assessed from x-ray evaluation. However, despite no significant differences detected by x-ray in spinal deformity and no altered vertebrae Ca and P levels among the dietary exposure groups, fish fed dietary ENNB showed a significant stunted growth as seen from the reduced length growth (SLR). The reduced length growth did not affect condition factor, indicating that the observed reduced specific growth rate (SGR) in dietary ENNB fish is most likely to a reduced skeleton growth rather than an impaired feed utilization capacity. In contrast, the reduced SGR in dietary BEA exposed fish was not accompanied by reduced length growth, and in the dietary BEA groups feed utilization was impaired. The reduced length growth in dietary ENNB exposed fish in the present trial, could be due to ENNB induced effects on osteoclast thus impairing bone formation. ENNB and BEA have different effects on mammalian osteoclast. The survival of mammalian osteoclasts is affected by ENN but not by BEA (Tedjiotsop-Feudjio et al., 2009). No information is available on the effects of ENN or BEA on osteoblasts. Similarly to the differential effects of ENN and BEA on human osteoclasts, in the present Atlantic salmon study ENN could have affected osteoclast and or osteoblast causing hampered skeleton length growth.

4.4. Hematology

The present study showed a difference in chronic ENNB or BEA induced hematology toxicity, with ENNB being more hemotoxic than BEA. ENNB exposures caused reduced hematocrit (HCT), red blood cells (RBC) count, and hemoglobin (Hgb). The ENNB mediated reduced red blood cell counts in the present trial, can have origins in destruction in the circulatory system and/or to insufficient new production (Ficheux et al., 2012). Earlier, *in vitro* studies with human RBCs, showed that ENNB caused increased cytosolic Ca^{2+} activity, eliciting hemolysis (Jilani et al., 2011). However, as the mean corpuscular volume (MCV) of the red blood cells in ENNB exposed fish in the present study did not change, the reduced RBC number was likely not due to hemolysis, but rather a reduction in new RBC formation (myelotoxicity). The increased mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) in ENNB exposed fish, indicates an increased Hgb in red blood cell, possibly compensating for reduced red blood cell oxygen transport. In addition to hematological toxicity, ENNB fed fish also showed liver toxicity as seen from increased plasma ASAT values which indicated liver injury with release of liver enzymes ASAT to blood circulation.

In contrast to ENNB exposed fish, BEA did not significantly affect hematocrit (HCT), red blood cells (RBC) count, and hemoglobin (Hgb). However, BEA significantly reduced the RBC mean corpuscular volume (MCV). Early human RBC *in vitro* trials showed that the ionophoric properties of BEA causes stimulation of Ca^{2+} entry causing cell shrinkage potentially leading to regulated death or eryptosis (Qadri et al., 2011). The BEA mediated reduced RBC volume in the present study, could represent BEA-induced RBC shrinkage, however, reduced MCV was not accompanied by reduced RBC numbers or blood Hgb levels.

4.5. Vitamin E

BEA, but not ENNB, caused significant effects on the levels of different liver vitamin E (vit E) isoforms. As opposed to the other responses (SGR, SLR etc.), the effects on vit E isoforms were not dose-responsive. A similar effect of BEA on the different isoforms was seen at all exposure levels compared to control fish. The BEA induced response on vit E was different among the different tocopherol and tocotrienol isoforms. Strongest effects were seen for liver alfa-tocopherol (α-TOH), with a similar ~ 25% reduction in all BEA exposure groups. Delta tocopherol (δ-TOH) and alfa tocotrienol (α-TOT) had a significant increase rather than decrease, and gamma-tocopherol (γ-TOH) was not significantly affected. The tocopherols and tocotrienols are fat soluble antioxidants that protect polyunsaturated lipids against oxidation, and the biological activity of α-TOH by far exceeds that of the other vit E isoforms (Hamre, 2011). This is because α-TOH is preferentially retained in the liver compared to the other tocopherols, probably due to the high affinity of the tocopherol transfer protein (TTP) for α-TOH. TTP has a lower affinity for the other vit E isoforms which are in greater extent excreted in the bile after intestinal uptake (Hamre, 2011). The reduction of α-TOH could indicate a use of fat-soluble antioxidants to protect against PUFA oxidative stress. However, if BEA induces oxidative stress, the reduction in α-TOH should be dose dependent. There was also no clear effect of BEA on MDA, an important product of lipid oxidation. This indicates that oxidative stress is not an important effect of BEA

contamination at these levels. It can be speculated that the effects on tocopherols are associated with changes in the function of TPP, since α-TOH retention is reduced while that of other tocopherols are slightly increased. The body level of vitamin E depends on the dietary supplementation and α-TOH was significantly reduced. Therefore, oxidation due to vitamin E deficiency caused by BEA could happen if dietary vitamin E is low.

Whereas BEA caused reduced liver α-TOH, ENNB did not affect liver α-TOH indicating a different potential for BEA and ENNB to cause *in vivo* lipid oxidative stress. Several *in vitro* mammalian studies have shown that both ENNB and BEA cause the formation of reactive oxygen species (ROS) generation lipid peroxidative stress (LPO) which seem to occur downstream the ENNB-induced cytotoxic events by the mitochondria (Caloni et al., 2020; Mallebrera et al., 2018; Prosperini et al., 2017). However, diverse cellular and molecular assays indicated that oxidative stress does not contribute to ENN- and BEA-induced cytotoxicity (Dornetshuber et al., 2009).

4.6. Safe levels in feed

In general, both ENNs and BEA are cytotoxic at low micromolar concentrations in mammalian *in vitro* models (EFSA, 2014; Gruber-Dorninger et al., 2017). Acute *in vivo* mammalian trials did not show acute toxicity, which has been related to a high metabolization and/or following elimination (Faeste et al., 2011). For several farmed terrestrial animals, no acute toxicity is reported, and broiler chicken also seem to tolerate prolonged exposure to ENNs (EFSA, 2014). In contrast, adverse effects such as alteration of microbial community composition, increased organ lesions, and impaired growth performance were seen in weaning piglets fed ENNs for two weeks (Novak et al., 2021). In the present study, no acute toxicity was observed, however, sub-lethal dose-response adverse effects occurred after sub-chronic (10% life cycle) exposure at high dietary ENNB and BEA.

In establishing safe dietary limits, not only the threshold levels of toxicity are of importance but also the dietary levels that give no toxic effect. The introduction of the lower bound bench mark dose (BMDL) allows establishing of a safe limit based on a common assessment of different dose-response models fits that are associated with a specific change in response (the bench mark response; BMR) (EFSA, 2017). The lower bound bench mark dose (BMDL) gives the lower 90% variance of the dose-response model fit, which is defined as the dose that is not giving an adverse effect and is hence an alternative to the use of non-observed effect level (NOEL), which is assessed as statistical differences between exposure groups (EFSA, 2017). In the present study for ENNB dose-response exposure, a significant BMDL regression assessment could be set for SGR, SLR, RBC, and ALAT parameters. All these parameters had a low (<10) ratio between upper and lower bound BMD (BMDU:BMDL), indicating a low variation within the BMD assessment. Based on BMDL, safe levels for ENNB in the present trial could be set on 99–312 μg kg^{-1} feed. For BEA dose-response exposure, a significant BMDL regression assessment could be set for SGR, C-factor, FCR, and FI. However, only for SGR and C-factor a low (<10) ratio between BMDU and BMDL was seen. For the feed conversion and feed intake, the ratio between BMDU and BMDL was high (>300), indicating a large variation and uncertainty for use of BMDL. Based on BMDL with a low variation, safe levels of BEA in the present experimental trial could be set at 4560–7520 μg kg^{-1} feed. In risk assessment, extrapolation from sub-chronic experimental trials to whole-cycle production of food producing animals includes the use of uncertainty factors (UF) (EFSA, 2012). To account for inter-species variability in toxicodynamic (biological effect) studies, a UF of 2.5 is suggested (EFSA, 2012). For extrapolation from sub-chronic to chronic study duration in rodents, EFSA recommends the use of an UF of 2 (EFSA, 2012). For fish studies, no specific UF have been established, but if an interspecies variability UF of 2.5 is used and a sub-chronic to chronic extrapolation of 2 is added the total UF would be 5. When applying the UFs to the significant BMDLs, for ENNB a safe limit of 20–50 μg kg^{-1} based on reduced SLR, SGR and RBC (Table 4). For BEA a safe limit can be set on 912–1504 μg kg^{-1} based on c-factor and SGR.

Highest levels observed in Norwegian commercial salmon feeds currently on the market were 25 μg kg^{-1} for BEA and 250 μg kg^{-1} for ENNB, with a lower prevalence for BEA (<5%) compared to ENNB (near 80%) (IMR surveillance data from 2015 to 2021, n = 200 commercial samples, (IMR, 2015–2021) (Table 4). Moreover, in a recent risk assessment for mycotoxin contamination in fish feeds in Europe levels of BEA and ENNs were calculated in fish feeds based on a wide-scope assessment of inclusion percentages of plant-based feed ingredients in fish feed and the reported mycotoxin contamination levels of the plant-based fish feed ingredients (Pietsch, 2020). The calculated weighted mean levels (MW± SEM) were 105.3 ± 21.9 μg kg^{-1} for BEA and 210.4 ± 36.7 μg kg^{-1} for ENNs. However, when batches of high contaminated wheat are used, calculated "worst case scenario" levels were 420.2 ± 28.2 μg kg^{-1} for BEA and 14937.0 ± 720.5 μg kg^{-1} for ENNs (Pietsch, 2020). When comparing proposed safe ENNB feed levels (20–50 μg kg^{-1}) with highest levels found in commercial Norwegian salmon feeds (250 μg kg^{-1}), or theoretically future feed levels (210.4 μg kg^{-1}), it can be concluded that ENNB in commercial plant-based salmon feed could form a risk for salmon health. In contrast, based on the assessed safe levels for BEA in salmon feeds (912–1504 μg kg^{-1}) and the levels found in commercial salmon feeds or theoretically calculated levels (25 or 105 μg kg^{-1}, respectively), it is unlikely that BEA levels in salmon feeds would be a risk for Atlantic salmon health (Table 5).

4.7. In conclusion

For ENNB, exposure to high contaminated commercial salmon feed currently surveyed on the Norwegian market (250 μg kg^{-1}) during an entire aquaculture Atlantic salmon production cycle, could potentially

Table 5
Overview of Lower Benchmark doses (BMDL) (μg kg^{-1}), in Atlantic salmon (*Salmo salar*) fed control (no detectable mycotoxins) or three graded levels of beauvericin or enniatin B, implementation of uncertainty factors (UF) for extrapolation of sub-chronic experimental results to interspecies variation (UF = 2) and chronic exposure (UF = 2.5) in a life-cycle farmed production of Atlantic salmon, safe levels for commercial feeds based on BMDL multiplate with UF, maximum levels observed in commercial Norwegian produced salmon feed (μg/kg), and finally the risk potential for high ENNB and BEA levels of ENNB in commercial exceeding the estimated safe levels (yes or no).

	Statistics		Chronic& species variation	Safe levels		Salmon feeds		Potential risk ?	
	BMDL		Uncertainty factor (UF)	BMDL x risk factor		max levels found			
	BEA	ENNB	2 (UF)*2.5 (UF)	BEA	ENNB	BEA	ENNB	BEA	ENNB
	μg/kg	μg/kg	X	μg/kgx	μg/kgx	μg/kg	μg/kg		
Specific growth rate (SGR)	4560	248	5	912	50	25	250	No	yes
Specific length rate (SLR)	n	99	5	–	20	25	250		yes
Condition-factor (C-factor)	7520	n	5	1504	–	25	250	No	
Red blood cell count (RBC)	n	216	5	–	43	25	250		yes
Liver damage (ASAT)	n	n	5	–	–	25	250		

impair weight and length growth as well as giving anemia, as BMDL safe levels are set on 20–50 μg kg^{-1}. In contrast, for BEA it seems unlikely that levels will give health effect based on the BMDL safe levels of 912–1504 μg kg^{-1} set for growth and feed conversion and observed highest levels in Norwegian commercial salmon feeds of 25 μg kg^{-1}. In addition, when calculated levels are used based on the contamination level of plant-feed ingredients, "worst case scenarios" for BEA levels in fish feeds (420.2 ± 28.2 μg kg^{-1}) are still under the set safe limits of 912–1504 μg kg^{-1}. In contrast, for ENN calculated worst case scenario levels (14937.0 ± 720.5) by far exceed the safe limits of 20–50 μg kg^{-1} (Table 4).

Fish fed BEA spiked feed had significantly reduced levels of the antioxidant vitamin E and red blood cells size, without causing anemia. These parameters, however, were not dose-dependent affected and hence no BMDL could be assessed. The significant effects were seen at the lowest exposure level of 300 μg kg^{-1}, which is not in the range of highest BEA levels observed in commercial salmon feeds (25 μg kg^{-1}). Furthermore, the BEA induced reduction in vit E did not seem to lead to oxidative damage of the susceptible polyunsaturated fatty acids which are rich in salmonids. In addition to reduced vit E, BEA also caused increased feed intake and reduced feed conversion. However, the BMD assessment had a large variation, giving a large uncertainty in BMDL assessment. However, fish fed 300 μg kg^{-1} had no significant (ANOVA) effect on feed intake of feed conversion, indicating that BEA levels in salmon feed are unlikely to cause effects on feed conversion or feed intake.

In the present study, only the most dominant ENNs isoform in aquafeed was assessed (ENNB). However, ENNs have different isoforms which are also detected in aquafeeds (e.g. ENN-A1 and ENNB1), and *in vitro* studies showed that the mixture of ENNs (7% A, 20% A1, 19% B, 54% B1) was more mitochondrial cytotoxic than ENNB alone (Tonshin et al., 2010). Furthermore the fusarium mycotoxins BEA, ENNs and DON often co-occur (Novak et al., 2021), and the present study only investigated separate ENNB and BEA exposure to assert the relative accumulation and toxic potential of these two related mycotoxins in farmed fish. Future studies are needed to assess the potential differential toxicity of the ENNs isoforms also in relation with other well-known fusarium mycotoxins such as DON.

CRediT authorship contribution statement

M.H.G. Berntssen: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **P.G. Fjeldal:** Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing. **P.J. Gavaia:** Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing. **V. Laizé:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **K. Hamre:** Conceptualization, Writing – review & editing, Supervision. **C.E. Donald:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **J.V. Jakobsen:** Conceptualization, Methodology, Resources, Writing – review & editing. **Å. Omdal:** Methodology, Validation, Formal analysis. **S. Søderstrøm:** Investigation, Methodology, Writing – review & editing. **K.K. Lie:** Conceptualization, Methodology, Validation, 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

Data will be made available on request.

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Appendix A. Supplementary data

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