Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
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Plant-based aquafeeds: Carry-over potential of mycotoxins and phytoestrogens from feed to fish and implications for fish health and food safety

Plantebasert fiskefôr: Overføringspotensialet til mykotoksiner og fytoøstrogener fra fôr til fisk og konsekvenser for fiskehelse og mattrygghet

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I like to be like a salmon, fight against the current, overcome the hurdles and reach the destination, no matter how far it is...

Amritha

Abbreviations

15-ADON	15-acetyl-deoxynivalenol
3-ADON	3-acetyl-deoxynivalenol
ADC	Apparent digestibility coefficients
AFL	Aflatoxins
AFB1	Aflatoxin B1
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANF	Anti-nutritional factors
AST	Aspartate transaminase
BEA	Beauvericin
CYP	Cytochrome P450
DAI	Daidzein
DAS	Diacetoxyscirpenol
DEG	Differentially expressed genes
DHA	Docosahexaenoic acid
DON	Deoxynivalenol
DON-3G	Deoxynivalenol-3-glucoside
EA	Ergot alkaloids
EFA	Essential fatty acids
EFSA	European Food Safety Authority
ENNs	Enniatins
EPA	Eicosapentaenoic acid
EU	European Commission
FB1	Fumonisin B1
FCR	Feed conversion ratio
FFA	Free fatty acids
FFDR	Forage fish dependency ratio
FIFO	Fish-In-Fish-Out-ratio
FM	Fish meal

FO	Fish oil
FPH	Fish protein hydrolysate
FUM	Fumonisins
FUX	Fusarenone-X
GEN	Genistein
GLY	Glycitein
HT-2	HT-2 toxin
K	Condition factor
LC	Liquid chromatography
HRMS/MS	High-resolution tandem mass spectrometry
MON	Monoliformin
NIV	Nivalenol
NOAEL	No-observed adverse effect level
OTA	Ochratoxin A
POPs	Persistent organic pollutants
PPC	Pea protein concentrate
PUFA	Poly unsaturated fatty acids
SBM	Solvent-extracted soybean meal
SGR	Specific growth rate
SPC	Soybean protein concentrate
T-2	T-2 toxin
TAG	Triacylglycerol
TG2	Transglutaminase
TG	Triglycerides
TGC	Thermal growth coefficient
Tprot	Total protein
TripleQ-MS/MS	Triple-Quadrupole tandem mass spectrometry
UGT	Uridine-diphosphate-glucuronosyltransferase
WG	Wheat gluten
ZEN	Zearalenone

Summary

The introduction of novel feed ingredients has brought tremendous changes in the field of aquaculture. The shift to non-marine resources was necessary because of rising prices for fish meal and fish oil and their decreasing availability. At the same time, the total global as well as the per-capita fish consumption has increased, which has caused an enormous expansion of the fish farming industry. The introduction of suitable alternatives for the fish-derived feed components was therefore a pre-requisite for the growth of aquaculture. Novel feed ingredients are currently mostly plant-based, including vegetable oils and protein concentrates from different legumes or wheat gluten.

The change in aquafeeds towards "green" ingredients may have negative effects on fish health and product quality, which in turn can lead to economic losses. The main concerns are connected to the presence of plant-borne contaminants including endogenous anti-nutritional factors (ANF). However, new feed processing techniques methods have considerably reduced their occurrence, and sensitive detection methods allow controlling compliance with maximum levels that have been implemented by food and feed safety authorities. Nevertheless, some ANF such as natural toxins, phytoestrogens and allergenic peptides are rather resistant to heat and digestion and have the potential to be carried over into the food chain. The biological activities of these substances in fish is little known and requires more detailed investigation.

This thesis addresses the impact of "green" aquafeeds on fish health and food safety, focussing on mycotoxins and phytoestrogens that are typically present in plant-based ingredients used for fish diets. The plant ingredients used in this study were wheat gluten (WG), soybean protein concentrate (SPC) and pea protein concentrate (PPC), which were fed to zebrafish (*Danio rerio*) and Atlantic salmon (*Salmo salar* L.) with the aim to investigate possible effects on the fish and the transmissibility potentials of target ANF into edible parts in connection with food safety. The project objectives were formulated to address these questions by analysing different aspects of this complex issue.

All experiments performed in the course of the project were based on samples from initially conducted feeding trials in zebrafish and salmon using different custom-made diets produced with definite levels of the three selected plant protein preparations.

Control feed was based on fish meal (FM) as the only source of protein, whereas the test diets contained WG, SPC or PPC at levels of 15% or 30%, replacing the FM. Salmon were exposed to five feed types, i.e. FM, WG15, WG30, SPC15 and SPC30, while zebrafish were additionally exposed to PPC15 and PPC30.

The first analytical project activities were performed with the aim of identifying the carry-over potentials of 25 mycotoxins and phytoestrogens that are frequently occurring in the selected plant protein sources. A quantitative multi-analyte liquid chromatography-high resolution tandem mass spectrometry (LC-HRMS/MS) method for the simultaneous determination of the target substances in feed and fish matrices was developed and validated. Sample preparation was optimised for each matrix by selecting suitable extraction solvents. The analysis of the method performance characteristics showed high specificities for all analytes with sufficient to excellent sensitivities in the different matrices. Linear calibration curves were generated either by using matching stable isotope-labelled derivates or similar-structure homologues as internal standards, or by using matrix-matched external standards. The method precision and accuracy data were in the range suggested in international validation guidelines for quantitative instrumental analysis. Applying the 25-in-1 method to the analysis of feed and fish samples from the feeding trials showed only low levels of the mycotoxin enniatin B in WG-containing diets and of the isoflavones daidzein (DAI), genistein (GEN) and glycitein (GLY) and their respective glucoside forms in SPC- and PPC-based diets, demonstrating the effectiveness of maximum level recommendations and modern feed processing technologies in the Norwegian aquaculture industry. Consequently, carry-over into fish muscle was not observed, confirming that fillets from plant-fed salmon were safe for human consumption.

The second objective in this project was to understand the metabolic fate of major soybean isoflavones in exposed salmon. Products from the hepatic biotransformation of DAI, GEN and GLY in salmon had not been previously identified, but knowledge about possibly harmful metabolites is essential for the assessment of potential risks. Due to the lack of suitable metabolism models for salmon, liver microsomes and primary hepatocytes were prepared in-house for applications in *in vitro* metabolism assays. The salmon liver fractions were characterised with respect to Phase I cytochrome P450 (CYP) and Phase II uridine-diphosphate-glucuronosyltransferase (UGT) enzyme activities using specific probe substrates. Two new methods, one for the simultaneous

measurement of five CYP activities and one for five UGT activities, were developed using LC-Triple-Quadrupole tandem mass spectrometry (TripleQ-MS/MS) for the specific detection of the substrates and their metabolites. The methods were optimised in assays with commercially available human liver microsomes with known enzyme activities and then used for the salmon liver preparations, under consideration that the available probe substrates were specific for human CYP and UGT enzymes. Subsequently, DAI, GEN and GLY were metabolised by using the salmon liver microsomes and primary hepatocytes and metabolites were analysed by LC-HRMS/MS. The isoflavones were effectively eliminated by UGT to metabolites that were preliminarily identified as the 7-O-glucuronides based on the measurement of exact masses, fragmentation patterns, and retention times. In contrast, the production of oxidative metabolites was insignificant. Only small amounts of four mono-hydroxylated DAI metabolites were detectable, when the incubations were upscaled. These findings suggested that bioaccumulation of phytoestrogens in farmed salmon and consumer risks from soybean-containing aquafeeds are unlikely.

The third and fourth project activities were committed to the identification of potential nutrigenomic effects from plant protein-based diets on fish health using transcriptomic analysis of fast muscle, liver and intestine of exposed zebrafish and salmon. Microarray hybridisation analysis was conducted in liver and intestine of salmon fed with FM, WG, and SPC containing diets. Gene expression data were processed and analysed with Nofima's bioinformatics software STARS. In addition, growth performance parameters and apparent digestibilities were recorded, and typical enzyme biomarkers for liver health were analysed in serum. The gene expression profiles in the different salmon tissues showed that several metabolic pathways were the least affected by the SPC15 diet and the most affected by the WG30 diet. The majority of the changes in gene expression patterns occurred in the intestine, in particular in genes related to lipid metabolism and transport, sterol metabolism, immunity and tissue structure and integrity. This study confirmed thus results of previous research on effects of SPC in salmon; however, the observations with regard to WG30 feed implied that the salmon were undergoing nutritional stress and showed symptoms similar to those of gluten sensitivity in humans.

In zebrafish, global transcriptome changes were analysed in fast muscle after exposure to FM, SPC30 or WG30 diets using RNA-seq technology. The analysis was extended to

on-growing salmon by testing selected differentially expressed genes in the zebrafish model using salmon paralogue-specific qPCR assays. Global gene expression changes in the muscle of zebrafish fed with plant diets were moderate, with the highest changes observed in fish fed with the SPC diets, whereas no changes were found for the PPC diets, when compared to the FM-control group. Differentially expressed genes in the SPC and WG feeding groups had important functions in regulating muscle growth, maintaining muscle structure and function, and muscle tissue homeostasis. Most of those genes and their paralogues were similarly affected in salmon fed with the same diets, with some species-specific regulation.

In conclusion, the work presented in this thesis shows that the inclusion of plant-based ingredients into aquafeeds is not of concern for consumers of salmon products regarding potential health risks from the carry-over of mycotoxins and phytoestrogens, but that a high percentage of WG or SPC in the diet can cause adverse effects in fish.

Sammendrag

Introduksjonen av nye ingredienser i fôr har medført store forandringer innen fiskeoppdrett. Overgangen til ikke-marine ressurser har vært nødvendig i forhold til stigende priser og redusert tilgang på fiskemel og fiskeolje. Samtidig har etterspørselen etter fisk økt på både globalt og individuelt nivå, noe som har ført til sterk vekst innen fiskeoppdrett. Innføringen av passende alternativer til fiskebaserte ingredienser i fôret har vært og fortsetter å være grunnleggende for den sterke veksten innen denne sektoren. De fleste nye ingredienser til fiskefôr baserer seg på planter, for eksempel vegetabilske oljer og proteinkonsentrater fra forskjellige erteblomstplanter og hvetegluten.

Overgangen til «grønne» ingredienser i fiskefôr kan ha negative konsekvenser for fiskens helse, og dermed sluttproduktets kvalitet, noe som kan igjen kan føre til økonomiske tap. Hovedbekymringene knytter seg til kontaminanter som forekommer i planter, blant annet endogene ernæringsmotvirkende faktorer (ANF). Likevel har nye fôrprossesseringsteknikker redusert forekomsten av disse, i tillegg til at presise målingsmetoder gjør at nivåene holder seg innenfor begrensningene som er satt av myndighetene. Til tross for dette viser enkelte ANF, blant annet naturlige toksiner, fytoøstrogener og peptider med allergipotensiale, motstandsdyktighet mot varmebehandling og fiskens fordøyelse, og kan muligens bli overført i næringskjeden. De biologiske virkningene til disse stoffene i fisk er lite kjent, og må forskes nøyere på.

Denne avhandlingen omtaler virkningen av «grønt» fiskefôr på fiskens helse og matsikkerhet og fokuserer på mykotoksiner og fytoøstrogener som til vanlig forekommer i plantebaserte fôringredienser for fiskenæring. I denne studien ble det brukt hvetegluten (WG), soyaproteinkonsentrat (SPC) og erteproteinkonsentrat (PPC), som ble fôret til sebrafisk (*Danio rerio*) og laks (*Salmo salar* L.) med målet å undersøke mulige virkninger på fisken og overføringspotensialet av utpekte ANF i henhold til mattrygghet. Prosjektets mål ble utledet for å ta for seg problemstillingen ved å se på de forskjellige aspektene av det sammensatte emnet.

Alle eksperimentene som ble utført i løpet av prosjektet er basert på fôringsforsøk på laks og sebrafisk, som ble utført i begynnelsen av studien, ved hjelp av skreddersydde fôrtyper, med fastsatte nivåer av de tre utvalgte preparatene av planteproteiner. Kontrollfôret var basert på fiskemel (FM) som eneste proteinkilde, imens fôrtypene som

ble testet inneholdt WG, SPC eller PPC i 15% og 30% konsentrasjon, som erstatning for FM. Laksen ble eksponert for fem fôrtyper: FM, WG15, WG30, SPC15 og SPC30, imens sebrafiskene i tillegg ble eksponert for PPC15 og PPC30.

De første analysene i prosjektet ble foretatt med mål om å identifisere overføringspotensialet til 25 ulike mykotoksiner og fytoøstrogener som ofte opptrer i de nevnte planteproteinene. En kvantitativ væskekromatografisk høyoppløsende massespektrometrisk metode (LC-HRMS/MS) som samtidig analyserer og måler de utvalgte analyttene i fiskefôr og fiskeprøver ved å velge ut egnede løsemidler. Metoden viste høy spesifisitet for alle analytter med tilstrekkelig til fremragende følsomhet i forskjellige matriser. Lineære kalibreringskurver ble laget ved å enten benytte stabile isotopmerkede derivater eller homologer med liknende struktur som interne standarder, eller ved å benytte eksterne standarder i sammensvarende matriser. Metodens resultater for presisjon og nøyaktighet var innenfor internasjonalt gitte retningslinjer for kvantitativ instrumentell analyse. Ved å anvende 25-i-én-metoden til å analysere fôr og fiskeprøver fra fôringseksperimentene viste kun lave nivåer av mykotoksinet enniatin B i fôr som inneholder WG, i tillegg til lave nivåer av isoflavonene daidzein (DAI), genistein (GEN) og glycitein (GLY) og deres hendholdsvise glukoside former i SPC- og PPC-baserte fôrsammensetninger. Dette demonstrerer effektiviteten til de satte grenseverdiene samt moderne fôrprosseseringsteknologi i norsk fiskeoppdrett. Det fører til at det ikke ble funnet spor av overført ANF i fiskenes muskelvev, som bekrefter at laks fôret med plantebasert fôr er trygt for mennesker å spise.

Det andre målet med prosjektet var å undersøke og forstå den metabolske nedbrytningsprosessen til soya-isoflavoner i laks. Produkter fra den hepatiske biotransformasjonen til DAI, GEN og GLY i laks hadde ikke blitt identifisert tidligere, men kunnskap om potensielt skadelige metabolitter er avgjørende for å kartlegge mulige risikoer. På grunn av manglende metabolske nedbrytningsmodeller for laks, ble levermikrosomer og primære hepatocytter laget på laboratoriet for å brukes i *in vitro* metabolisme-assayer. Lakseleverfraksjonene ble karakterisert i henhold til fase I cytokrom P450 (CYP) og fase II uridin-difosfat-glukoronosyltransferase (UGT)-enzymaktiviteter ved å bruke spesifikke prøvesubstrater. To nye metoder, hvorav en for samtidig måling av fem CYP-aktiviteter og en for fem UGT-aktiviteter, ble utviklet ved å bruke LC-trippel-kvadrupol-massespektrometri (TripleQ-MS/MS) for å spesifikt kartlegge substratene og deres metabolitter. Metodene ble optimalisert i assayer med

kommersielt tilgjengelige mikrosomer fra menneskelever med kjente enzymaktiviteter, og deretter brukt for lakseleverpreparatene, med forbehold at de tilgjengelige prøvesubstratene var spesifikke for menneskelige CYP og UGT-enzymer. I følge ble DAI, GEN og GLY metabolisert ved å bruke lakselevermikrosomer og primære hepatocytter, og metabolitter analysert med LC-HRMS/MS. Isoflavonene ble effektivt eliminert av UGT til metabolitter som foreløpig ble identifisert som 7-O-glukuronider basert på målinger av eksakte masser, fragmenteringsmønstre og retensjonstider. Til forskjell var produksjonen av oksidative metabolitter ubetydelig. Kun små mengder med fire mono-hydroksilerte DAI-metabolitter var målbare når inkubasjonene ble oppskalert. Disse funnene indikerer at bioakkumulering av fytoøstrogener i oppdrettslaks fra soyaproteiner i fôret er usannsynlig og derfor medfører liten risiko for forbrukere.

Aktivitetene i den tredje og fjerde delen av prosjektet knyttet seg rundt kartlegging av potensielle nutrigenomiske effekter på fiskens helse som følge av et kosthold som baserer seg på planteproteiner, ved hjelp av transkriptomisk analyse av muskler, lever og tarm i eksponert laks og sebrafisk. En microarray hybridiseringsanalyse ble utført i leveren og tarmen til laks fôret med FM, WG og SPC-holdig fôr. Genekspresjonsdataene ble prosessert og analysert ved hjelp av Nofimas bioinformasjonsdatavare STARS. I tillegg ble andre variabler som tilvekst og apparent fordøyelighet, samt typiske enzymatiske biomarkører for leverhelse analysert i serum. Profilene til det genetiske uttrykket i de ulike vevsprøvene av laks, viste at vekst og biosynteseveier ble minst påvirket av fôret som inneholdt SPC15, og mest påvirket av det som inneholdt WG30. Flertallet av forandringene av uttrykkelsen til genmønstre forekom i tarmen, særlig i gener tilknyttet transport av lipoproteiner, sterolmetabolisme, immunitet, samt vevsstruktur og integritet. Studien bekrefter dermed resultatene av tidligere forskning på effektene SPC har i laks. Likevel viser observasjonene i forhold til WG30-fôret at laksen gjennomgikk ernæringsbetinget stress og viste symptomer som liknet glutensensitivitet i mennesker.

I sebrafisk ble forandringer i hele transkriptomet analysert i muskel etter av fiskene var eksponert med FM, SPC30 eller WG30 i fôr ved hjelp av RNA-seq-teknologi. Analysen ble utvidet til laks i vekstfasen ved å teste gener som viste forskjeller i uttrykk i sebrafiskmodellen, ved å bruke paralog-spesifikke qPCR assayer for laks. Forandringer i det helhetlige genetiske uttrykket i musklene til sebrafisk som ble fôret med plantebasert fôr var moderate. De tydeligste forandringene ble funnet i fisk med SPC-

holdig fôr, imens ingen forandringer ble funnet i sammenheng med PPC-holdig fôr, sammenlignet med FM-kontrollgruppen. Forskjellig uttrykte gener hadde viktige funksjoner i regulering av muskelvekst, opprettholdelse av muskelstruktur og funksjon samt homeostase i muskelvevet. De fleste av disse genene og paralogene ble påvirket på liknende måte i laks fôret med samme typer fôr, under forbehold av noe spesiesspesifikk regulering.

Konklusjonen til arbeidet som er presentert i denne avhandlingen er at bruk av plantebaserte ingredienser i fiskefôr ikke utgjør grunn til bekymring for forbrukere av lakseprodukter i forhold til potensielle helserisikoer fra overføringen av mykotoksiner og fytoøstrogener. Likevel kan høye andeler av WG og SPC i fôret ha skadelige virkninger på fisken.

സംഗ്രഹം

മത്സ്യത്തീറ്റയിലെ നൂതന ചേരുവകൾ ആഗോള മത്സ്യകൃഷിയിൽ വരുത്തിയ മാറ്റം വലുതാണ്. മത്സ്യത്തീറ്റയ്ക്കായ് സാധാരണ ഉപയോഗിക്കുന്ന ഭക്ഷ്യയോഗ്യമല്ലാത്ത മത്സ്യം കൊണ്ടുള്ള മീൻപൊടിയുടേയും അതുപോലെ മീനെണ്ണയുടേയും വില വർദ്ധനവും അവയുടെ ലഭ്യതക്കുറവും കാരണം സമുദ്രേതരമല്ലാത്ത ചേരുവകളിലേക്കുള്ള പരിവർത്തനം അനിവാര്യമായി. അതേ സമയം, ആഗോളമായും, വ്യക്തി അടിസ്ഥാനത്തിലുമുള്ള മത്സ്യഉപഭോഗം വർദ്ധിക്കുകയും ചെയ്തു. ഇത് മത്സ്യകൃഷി വ്യവസായ മേഘലയുടെ വിപുലമായ വികാസത്തിന് കാരണമായി. ഇതിനനുസൃതമായി മത്സ്യത്തിൽ നിന്ന് ലഭിക്കുന്ന തീറ്റയുടെ ഘടകങ്ങൾക്ക് അനുയോജ്യമായ ബദലുകൾ വികസിപ്പിക്കുക എന്നത് മത്സ്യകൃഷിയുടെ വളർച്ചയ്ക്ക് ആവശ്യമാണ്. ഇതിൽ പ്രധാനമായുള്ളത്, സസ്യാധിഷ്ഠിത മത്സ്യതീറ്റ ചേരുവകളാണ്. സസ്യത്തിൽ നിന്നും വേർതിരിച്ചെടുക്കുന്ന എണ്ണയും, വിവിധ പയർ വർഗ്ഗങ്ങളിൽ നിന്നോ, ഗോതമ്പിൽ നിന്നോ ഉത്പാദിപ്പിച്ച മാംസ്യവുമാണ് നിലവിൽ നൂതന മത്സ്യത്തീറ്റയിൽ കൂടുതലായും ഉപയോഗിക്കുന്നത്.

സസ്യ ചേരുവകളിലേക്കുള്ള മത്സ്യത്തീറ്റയുടെ മാറ്റം മത്സ്യത്തിന്റെ ആരോഗ്യത്തേയും, അതിന്റെ ഉത്പന്ന ഗുണനിലവാരത്തെയും പ്രതികൂലമായി ബാധിച്ചേക്കാം. ഇത് വരുംകാല സാമ്പത്തിക നഷ്ടത്തിനും കാരണമായേക്കാം. ഇതിൽ ഏറ്റവും ആശങ്കയുളവാക്കുന്നത് എന്തെന്നാൽ സസ്യത്തിൽ നിന്നും മനുഷ്യനിലേക്ക് കടന്നുകയറാൻ സാധ്യതയുയുള്ളതും, സസ്യങ്ങൾക്ക് ആവശ്യവും എന്നാൽ മറ്റുള്ളവയ്ക്ക് ദൂഷ്യവുമായ പോഷക വിരുദ്ധ ഘടകങ്ങൾ (ANF) ആണ്. പക്ഷെ മത്സ്യത്തീറ്റയുണ്ടാക്കുന്നതിലെ നൂതന സാങ്കേതിക വിദ്യകൾ ഇവയുടെ കടന്നുകയറ്റം ഒരു പരിധി വരെ കുറയ്ക്കാൻ സഹായിച്ചു. അതുകൂടാതെ, വളരെ സൂക്ഷ്മമായ പദാർത്ഥങ്ങളെ കണ്ടെത്താൻ തക്കവണ്ണം ഫലപ്രദമായ രീതികളുടെ ഉപയോഗം ഭക്ഷ്യസുരക്ഷാ വകുപ്പ് നടപ്പിലാക്കിയ പരിധി നിയന്ത്രിക്കാൻ സഹായിക്കുന്നു. എന്നിരുന്നാലും, പ്രകൃതിദത്ത വിഷവസ്തുക്കൾ, ഫൈറ്റോഈസ്ട്രജൻ, അലർജിക് പെപ്റ്റൈഡുകൾ എന്നീ ഘടകങ്ങൾ മത്സ്യതീറ്റ ഉത്പാദനത്തിലെ കൂടിയ താപനിലയും, ഉപഭോക്താവിന്റെ ദഹനരസങ്ങളേയും പ്രതിരോധിക്കാൻ ശേഷിയുള്ളവയാണ്. മാത്രമല്ല, അവ പിന്നീട് ഭക്ഷ്യശൃംഘലയിലേക്ക് കടന്നുകയറാനും സാധ്യതയുണ്ട്. മത്സ്യത്തിലെ ഈ പദാർത്ഥങ്ങളുടെ ജൈവിക പ്രവർത്തനങ്ങളെക്കുറിച്ച് വളരെ കുറച്ചു മാത്രമാണ് പഠനങ്ങൾ നടന്നിട്ടുള്ളത്. അതുകൊണ്ട് ഇതേകുറിച്ച് കൂടുതലറിയാൻ ആഴമേറിയ ഗവേഷണങ്ങൾ ആവശ്യമാണ്.

ഈ പ്രബന്ധം മത്സ്യത്തിന്റെ ആരോഗ്യത്തിനും ഭക്ഷ്യസുരക്ഷയ്ക്കും 'സസ്യ'-മത്സ്യത്തീറ്റകളുടെ സ്വാധീനത്തെ അഭിസംബോധന ചെയ്യുന്നു. അതിനായ്, മത്സ്യത്തീറ്റയ്ക്കായ് ഉപയോഗിക്കുന്ന സസ്യധിഷ്ഠിത ചേരുവകളിൽ സാധാരണയായി കാണപ്പെടുന്ന മൈകോടോക്സിൻ, ഫൈറ്റോ ഈസ്ട്രജൻ എന്നിവയിൽ ശ്രദ്ധ കേന്ദ്രീകരിച്ചിരിക്കുന്നു. ഈ പഠനത്തിൽ ഉപയോഗിച്ച

സസ്യഘടകങ്ങൾ ഗോതമ്പ് ഗ്ലൂട്ടൻ (WG), സോയബീൻ പ്രോട്ടീൻ കോൺസൻട്രേറ്റ് (SPC), കടല പ്രോട്ടീൻ കോൺസൻട്രേറ്റ് (PPC), എന്നിവയാണ്. ഇവ നിശ്ചിത അളവിൽ സീബ്രാഫിഷ് (*ഡാനിയോ റീരിയോ*) അല്ലെങ്കിൽ *അറ്റ്ലാന്റിക് സാൽമൺ* (*സാൽമോ സലാർ*) എന്നിവയ്ക്ക് നൽകി, മത്സ്യത്തെ ബാധിക്കുന്ന പ്രത്യാഘാതങ്ങളേയും ഭക്ഷ്യയോഗ്യമായ ഭാഗങ്ങളിലേക്ക് ആഗിരണം ചെയ്യുന്ന ANF-ന്റെ പ്രക്ഷേപണ സാധ്യതയേയും ഭക്ഷ്യസുരക്ഷയുമായി ബന്ധപ്പെടുത്തി വിശകലനം ചെയ്യാനും ലക്ഷ്യമാക്കുന്നു. ഈ സങ്കീർണ്ണമായ പ്രശ്നത്തിന്റെ വിവിധ വശങ്ങൾ വിശകലനം ചെയ്തുകൊണ്ട് പ്രോജക്റ്റിലെ പഠന ലക്ഷ്യങ്ങൾ രൂപീകരിച്ചു.

ഈ പ്രോജക്റ്റിന്റെ ഭാഗമായി നടത്തിയ എല്ലാ പരീക്ഷണങ്ങളിലും ഉപയോഗിച്ചിരിക്കുന്നത്, മൂന്ന് സസ്യമാംസ്യങ്ങൾ തിരഞ്ഞെടുത്ത അളവിൽ ചേർത്ത് വ്യത്യസ്ഥ ഇച്ഛാനുസൃതമായി നിർമ്മിച്ച മത്സ്യത്തീറ്റ നൽകി കൊണ്ട് തുടക്കത്തിൽ സീബ്രഫിഷ്, സാൽമൺ എന്നിവയിൽ നടത്തിയ പരീക്ഷണങ്ങളിൽ നിന്ന് സാമ്പിളുകൾ അടിസ്ഥാനമാക്കിയാണ്. നിയന്ത്രണ തീറ്റയായി (control feed) ഉപയോഗിച്ചിരിക്കുന്നത് മീൻ പൊടിയാണ് (fish meal-FM). അതേസമയം പരീക്ഷണത്തീറ്റകളിൽ (test diet) WG, SPC അല്ലെങ്കിൽ PPC എന്നിവ 15% അല്ലെങ്കിൽ 30% എന്ന അളവിൽ FM-നു പകരം ചേർത്തിരിക്കുന്നു. സാൽമൺ പരീക്ഷണങ്ങളിൽ ഉപയോഗിച്ചത് അഞ്ച് തരം തീറ്റയാണ് FM, WG15, WG30, SPC15, SPC30. എന്നാൽ സീബ്രാഫിഷിൽ PPC15, PPC30 അധികമായി നൽകിയിരിക്കുന്നു.

തിരഞ്ഞെടുത്ത സസ്യമാംസ്യ ശ്രോതസ്സുകളിൽ പതിവായി കാണപ്പെടുന്ന 25 മൈകോട്ടോക്സിനുകളുടേയും ഫൈറ്റോഈസ്ട്രജന്റേയും കൈമാറ്റ പ്രക്രിയയുടെ സാധ്യതകൾ തിരിച്ചറിയുക എന്ന ലക്ഷ്യത്തോടെയാണ് ആദ്യത്തെ വിശകലന പദ്ധതി പരീക്ഷണങ്ങൾ നിർവ്വഹിച്ചത്. ഇതിനായി മത്സ്യത്തീറ്റയിലും മത്സ്യത്തിലും (ഫീഡ്-ഫിഷ്-മാട്രിക്സ്) ഒരേ സമയം മേൽപ്പറഞ്ഞ പദാർത്ഥങ്ങളുടെ അളവ് നിർണ്ണയിക്കുന്നതിനുള്ള ഒരു 'ക്വാന്റിറ്റേറ്റീവ് മൾട്ടി-അനലൈറ്റ് ലിക്വിഡ് ക്രോമാറ്റോഗ്രാഫി-ഹൈറെസല്യൂഷൻ ടാൻഡം മാസ് സ്പെക്ട്രോമെട്രി' (LC-HRMS/MS) രീതി വികസിപ്പിക്കുകയും സാധൂകരിക്കുകയും ചെയ്തു. ANF വേർതിരിച്ചെടുക്കാൻ അനുയോജ്യമായ ലായകങ്ങൾ തിരഞ്ഞെടുക്കുകയും വിവധ മാട്രിക്സിനു അനുയോജ്യമായ സാമ്പിൾ തയ്യാറാക്കൽ രീതികൾ അവലംബിക്കുകയും ചെയ്തു. പിന്നീട് രീതി നിർവ്വഹണത്തിന്റെ വിശേഷ ലക്ഷണങ്ങൾ വിശകലനം ചെയ്യുകയും അതിൽ ഉൾപ്പെടുത്തിയിട്ടുള്ള എല്ലാ അനലൈറ്റ്സിനും വിവിധ മാട്രിക്സുകളിൽ തൃപ്തികരം അല്ലെങ്കിൽ ഉയർന്ന അളവിൽ 'സ്പെസിഫിസിറ്റി'യും, തൃപ്തികരം അല്ലെങ്കിൽ മികച്ചത് എന്ന തോതിൽ 'സെൻസിറ്റിവിറ്റി'യും ലഭിക്കുകയും ചെയ്തു. തുല്യസ്ഥിരതയുള്ള ഐസോട്ടോപ്പ്-ലേബൽ ഡെറിവേറ്റുകൾ ഉപയോഗിച്ചോ അല്ലെങ്കിൽ സമാനഘടനയുള്ള ഹോമോലോഗുകൾ ആന്തരിക മാനദണ്ഡങ്ങളായി ഉപയോഗിച്ചോ, അതുമല്ലെങ്കിൽ മാട്രിക്സുമായി പൊരുത്തപ്പെടുന്ന ബാഹ്യമാനദണ്ഡങ്ങൾ ഉപയോഗിച്ചുമാണ് ലീനിയർ കാലിബ്രേഷൻ ഗ്രാഫ് രൂപീകരിച്ചത്. അതുകൂടാതെ, ഉപയോഗരീതിയുടെ കൃത്യതയുടെ അള

വായ ‘പ്രസിഷൻ’ അഥവാ ‘ആക്കുറസി’ എന്നിവയും കാണ്ടിറ്റേറ്റീവ് ഇൻസ്ട്രുമെന്റൽ വിശകലത്തിനായി അന്താരാഷ്ട്ര മൂല്യനിർണ്ണയ മാർഗ്ഗനിർദ്ദേശപ്രകാരമുള്ള (ISO) തോതിലാണ് കാണപ്പെട്ടത്. ഇങ്ങനെ വികസിപ്പിച്ചെടുത്ത 25-ഇൻ-1 വിശകലന രീതി ഉപയോഗിച്ച് വിവിധ മാട്രിക്സിൽ നടത്തിയ പരീക്ഷണത്തിൽ നിന്നും ഗോതമ്പ് ഉപയോഗിച്ചുണ്ടാക്കിയ മത്സ്യത്തീറ്റയിൽ വളരെ കുറഞ്ഞ തോതിലുള്ള മൈക്രോടോക്സിൻ, ENN B-യും, SPC-, PPC-കലർന്ന മത്സ്യത്തീറ്റയിൽ ഡയാഡ്സിൻ (DAI), ജനിസ്റ്റിൻ (GEN), ഗ്ലൈസിറ്റിൻ (GLY) എന്നിവയും കൂടാതെ ഇവയുടെ ഗ്ലൂകോസൈഡ്സും വളരെ ചെറിയ തോതിൽ കാണപ്പെട്ടു. ഇത് നോർവീജിയൻ മത്സ്യ കൃഷി വ്യവസായത്തിലെ ANF-ന്റെ പരമാവധി അളവ് ശുപാർശകളുടേയും ആധുനിക മത്സ്യതീറ്റ സംസ്കരണ സാങ്കേതിക വിദ്യകളുടെയും ഫലപ്രാപ്തി പ്രകടമാക്കുന്നു. പക്ഷെ, മത്സ്യമാംസത്തിൽ ഈ പദാർത്ഥങ്ങളുടെ സാന്നിദ്ധ്യം കണ്ടെത്താൻ കഴിഞ്ഞില്ല. ആയതുകൊണ്ട്, സസ്യ-മത്സ്യതീറ്റ നൽകിയ സാൽമൺ മനുഷ്യ ഉപയോഗത്തിന സുരക്ഷിതമാണെന്ന് ഉറപ്പുവരുത്തി.

സോയാബീൻ അടങ്ങിയ മത്സ്യതീറ്റ ഭക്ഷിച്ച സാൽമണിൽ പ്രധാന സോയാബീൻ ഐസോഫ്ളാവോണുകളുടെ ഉപാപചയ വിധി മനസ്സിലാക്കുക എന്നതായിരുന്നു ഈ പദ്ധതിയുടെ രണ്ടാമത്തെ ലക്ഷ്യം. സാൽമണിലെ DAI, GEN, GLY എന്നിവയുടെ കരളിലെ ബയോട്രാൻസ്ഫോർമേഷനും അതിലെ ഉത്പന്നങ്ങളെക്കുറിച്ചും (മെറ്റബോലൈറ്റ്സ്) അധികം പഠനങ്ങൾ നടന്നിരുന്നില്ല, പക്ഷെ ഇവയുടെ അപകട സാധ്യതകൾ വിലയിരുത്തുന്നതിന ദോഷകരമായ മെറ്റബൊലൈറ്റ്സിനെ കുറിച്ചുള്ള അറിവ് ആവശ്യമാണ്. ഈ പഠനങ്ങൾക്ക് ആവശ്യമായ സാൽമൺ-കരൾ മാതൃകകളുടെ അഭാവംമൂലം ഇൻവിട്രോ പരീക്ഷണങ്ങൾക്കായുള്ള മൈക്രോസോമുകളും, പ്രൈമറി ഹെപെറ്റോസൈറ്റും പഠന കേന്ദ്രത്തിൽ തന്നെ തയ്യാറാക്കി. ഇവയെ കരളിലെ പ്രധാന ബൈയോട്രാൻസ്ഫോർമേഷൻ രസങ്ങളായ ഫേസ് I സൈറ്റോക്രോമ് പി450 (CYP), ഫേസ് II ഉറിഡിൻ-ഡൈഫോസ്ഫേറ്റ്-ഗ്ലൂകുറോണോസിൽ-ട്രാൻസ്ഫറേസ് (UGT) -ന്റെ പ്രവർത്തനക്ഷമതയ്ക്ക് അനുയോജ്യമായ പ്രോബ് സബ്സ്ട്രേറ്റുകൾ കൊണ്ട് സ്ഥിരീകരിച്ചു. രണ്ട് പുതിയ രീതികൾ; ഒന്ന്, തിരഞ്ഞെടുത്ത അഞ്ച് CYP-ന്റെ പ്രവർത്തനങ്ങൾ ഒരേ സമയം അളക്കുന്നതിനും; മറ്റൊന്ന്, തിരഞ്ഞെടുത്ത അഞ്ച് UGT-യുടെ പ്രവർത്തനങ്ങൾ ഒരേ സമയം അളക്കുന്നതിനായും വികസിപ്പിച്ചെടുത്തു. മേൽപ്പറഞ്ഞ രീതിയിൽ പ്രത്യേക സബ്സ്ട്രേറ്റും അതിന്റെ മെറ്റബോളൈറ്റും അളക്കുന്നതിനായി ‘എൽസി-ട്രിപ്പിൾ-ക്വാഡ്രുപ്പോൾ ടാൻഡം മാസ് സ്പെക്ട്രോമെട്രി’ (TripleQ-MS/MS) എന്ന സാങ്കേതിക വിദ്യ ഉപയോഗപ്പെടുത്തി. ഇങ്ങനെ വികസിപ്പിച്ചെടുത്ത രീതികൾ വിപണിയിൽ ലഭ്യമായ മനുഷ്യ-കരൾ മൈക്രോസോമുകൾ ഉപയോഗിച്ച് ആദ്യവും, പിന്നീട് സാൽമൺ മൈക്രോസോമുകൾ ഉപയോഗിച്ചും, CYP, UGT എന്നിവയുടെ പ്രവർത്തനങ്ങൾ താരതമ്യം ചെയ്തു. പഠനത്തിനായി ഉപയോഗിച്ച പ്രോബ് സബ്സ്ട്രേറ്റുകൾ തിരഞ്ഞെടുത്ത CYP, UGT രസങ്ങൾക്ക് പര്യാപ്തമാണെന്ന അനുമാനത്തിന്റെ അടിസ്ഥാനത്തിലാണ് ഈ പരീക്ഷണങ്ങൾ തുടർന്നത്. പിന്നീട്, DAI, GEN, GLY എന്നീ ഐസോഫ്ളാവോണുകളുടെ ഉപാ

പചയപാത സാൽമൺ കരൾ മൈക്രോസോമുകളും, പ്രൈമറി ഹെപെറ്റോസൈറ്റും ഉപയോഗിച്ച് പഠിക്കുകയും, ഈ പ്രക്രിയയിൽ നിന്നുമുള്ള ഉത്പന്നങ്ങൾ LC-HRMS/MS ഉപയോഗിച്ച് പരിശോധിക്കുകയും അവയെ വിശകലനം ചെയ്യുകയും ചെയ്തു. ഐസോഫ്ളാവോണുകൾ വളരെ ഫലപ്രദമായി UGT-യുടെ സഹായത്താൽ ദോഷകരമല്ലാത്തതും ശരീരത്തിൽ നിന്ന് പുറംതള്ളാൻ കഴിയുന്നതുമായ മെറ്റബോളൈറ്റസ് ഉണ്ടാക്കുകയും, ഇവയെ പിണ്ഡം (എക്സാക്റ്റ് മാസ്), വിഭജനക്രമം (ഫ്രാഗ്മെന്റേഷൻ പാറ്റേൺ), റിട്ടെൻഷൻ ടൈം എന്നീ മാനദണ്ഡങ്ങൾ ഉപയോഗിച്ച് 7-ഒ-ഗ്ലൂകൊറോണൈഡ് ആണെന്ന നിഗമനത്തിലെത്തുകയും ചെയ്തു. ഇതിന വിപരീതമായി ഓക്സിഡേറ്റീവ് ഉത്പന്നങ്ങളുടെ ഉതപാദനം നിസാര അളവിൽ മാത്രമായിരുന്നു. ഇൻക്യുബേഷൻ ലായനിയുടെ സാന്ദ്രത ഉയർത്തി അളന്നപ്പോൾ മാത്രമാണ് നാല് മോണോ-ഹൈഡ്രോക്സിലേറ്റഡ് DAI ഉത്പന്നങ്ങളെ കണ്ടെത്താൻ കഴിഞ്ഞത്. ഈ പഠനങ്ങൾ തെളിയിക്കുന്നത് എന്തെന്നാൽ, ഫൈറ്റോഈസ്ട്രജന്റെ സാൽമണിലുള്ള ജൈവസംഭരണവും അതുപോലെ സോയബീൻ അടങ്ങിയ മത്സ്യത്തീറ്റയിൽ നിന്നും ഉണ്ടാകാനിടയുള്ള അപകട സാധ്യതകൾ തീരെ കുറവാണെന്ന് എന്നതാണ്.

ഈ പ്രോജക്റ്റിന്റെ മൂന്നാമത്തെയും നാലാമത്തെയും ലക്ഷ്യങ്ങൾ കേന്ദ്രീകരിച്ചിരിക്കുന്നത്, സസ്യാധിഷ്ഠിത മത്സ്യതീറ്റ നൽകി വളർത്തിയ സാൽമൺ, സീബ്രഫിഷ് എന്നീ മീനുകളുടെ പേശി (ഫാസ്റ്റ് മസിൽ), കരൾ, കുടൽ എന്നിവയിൽ നിന്നും അവയുടെ ആരോഗ്യത്തെ ബാധിക്കുന്ന ന്യൂട്രിജിനോമിക് മാറ്റങ്ങൾ 'ട്രാൻസ്ക്രിപ്റ്റോമിക്സ്' എന്ന സാങ്കേതിക വിദ്യയുപയോഗിച്ച് തിരിച്ചറിയുന്നതിനു വേണ്ടിയാണ്. ഇതിനായി മാംസ്യ സ്ത്രോതസ്സുകളായ FM, WG, SPC-അടങ്ങിയ മത്സ്യതീറ്റ നൽകിയ സാൽമണിൽ നിന്നും കരൾ, കുടൽ എന്നിവ വേർതിരിച്ച് 'മൈക്രോഅറേ ഹൈബ്രിടൈസേഷൻ' എന്ന സാങ്കേതിക വിദ്യയുപയോഗിച്ച് വിശകലനം നടത്തി. Nofima-യുടെ ബയോഇൻഫൊർമാറ്റിക്സ് പ്ലാറ്റ്ഫോർമായ 'STARS' ഉപയോഗിച്ചാണ് ജീൻ എക്സ്പ്രഷൻ ഡാറ്റ വിശകലനം ചെയ്തത്. അതുകൂടാതെ, സസ്യ-മാംസ്യ സ്ത്രോതസ്സുകൾക്ക് മത്സ്യത്തിൽ ഉണ്ടാക്കിയ വളർച്ചാ സംബന്ധമായ പ്രകടന പരാമീറ്ററുകളും, ദഹനഘടകങ്ങളും (ഡൈജസ്റ്റിബിലിറ്റി) രേഖപ്പെടുത്തുകയും, രക്തത്തിൽ നിന്നും കരളിന്റെ ആരോഗ്യനില അളക്കുന്നതിനായുള്ള ബയോമാർക്കറുകൾ വേർതിരിച്ചെടുത്ത് നിരീക്ഷിക്കുകയും ചെയ്തു. സാൽമണിലെ വിവിധ കോശങ്ങളുടെ രാസവിനിമയ വഴികൾ ജീൻ എക്സ്പ്രഷൻ പൊഫൈലിങ്ങിലൂടെ വിശകലനം ചെയ്തപ്പോൾ മനസ്സിലാക്കാൻ സാധിച്ചത് എന്തെന്നാൽ, ആരോഗ്യ പ്രശ്നങ്ങൾക്ക് കാരണമായ മത്സ്യത്തീറ്റയിൽ മുന്നിൽ നിൽക്കുന്നത് WG30-യും ഏറ്റവും കുറവ് SPC15-നുമാണ്. സാൽമണിലെ പഠന അവയവങ്ങളിൽ, കരളിനെ അപേക്ഷിച്ച് കുടലിനാണ് സസ്യമത്സ്യത്തീറ്റയുടെ ഉപയോഗം ഏറ്റവും പ്രതികൂലമായി ബാധിച്ചത്. വിശദമായ പഠനങ്ങളിൽ നിന്നും മനസ്സിലാക്കാൻ കഴിയുന്നത് എന്തെന്നാൽ, ഈ മത്സ്യങ്ങളിൽ കൊഴിപ്പിന്റെയും, കൊളസ്ട്രോളിന്റെയും ഉപാപചയപാതയും കൈമാറ്റവും, പ്രതിരോധശേഷി, ടിഷ്യുഘടനയും സമഗ്രത

യുമായി ബന്ധപ്പെട്ട ജീനുകളിൽ കൂടുതലായും മാറ്റം സംഭവിച്ചിരിക്കുന്നു. ഈ പഠനത്തിലെ, SPC-മത്സ്യത്തീറ്റയിലെ ഫലങ്ങൾ മുമ്പത്തെ ഗവേഷണ ഫലങ്ങളോട് സാമ്യം പുലർത്തുകയും അതു വീണ്ടും സ്ഥിരീകരിക്കുകയും ചെയ്തു. WG30-മത്സ്യത്തീറ്റയെ സംബന്ധിച്ച നിരീക്ഷണങ്ങൾ സൂചിപ്പിക്കുന്നത്, ഈ തീറ്റ ലഭിച്ച സാൽമണുകൾ പോഷക സമ്മർദ്ദത്തിന വിധേയമാവുകയും, അവയ്ക്ക് മനുഷ്യരിൽ എന്നപോലെ ഗ്ലൂട്ടൻ സെൻസിറ്റിവിറ്റിയ്ക്ക് സമാനമായ ലക്ഷണങ്ങൾ ഉണ്ടാകുന്നുണ്ട് എന്നാണ്. അതേസമയം, സീബ്രാഫിഷിൽ ട്രാൻസ്ക്രിപ്റ്റോമിക് മാറ്റങ്ങൾ അളക്കാൻ 'RNA-seq' എന്ന സാങ്കേതിക വിദ്യയാണ് ഉപയോഗിച്ചത്. ഇതിനായി FM, SPC30, WG30 എന്നീ മത്സ്യതീറ്റ നൽകിയ മീനുകളുടെ ഫാസ്റ്റ് മസിൽ വിശകലനം ചെയ്തു. സീബ്രാ ഫിഷ് മാതൃകയാക്കി അവയിൽ വ്യത്യസ്ഥമായി പ്രകടിപ്പിച്ച ജീനുകളെ സാൽമൺ പാരലോഗ് നിർദ്ദിഷ്ട qPCR പരീക്ഷണങ്ങളിലൂടെ സാൽമണിൽ വിശകലനം ചെയ്തു. സസ്യ-മത്സ്യതീറ്റ നൽകിയ സീബ്രഫിഷിന്റെ പേശിയിലെ ജീൻ എക്പ്രഷൻ പാറ്റേണുകൾ മിതമായ രീതിയിലായിരുന്നു. FM-ആയി താരതമ്യപ്പെടുത്തുമ്പോൾ SPC-മത്സ്യതീറ്റ നൽകിയ മീനിലെ ജീനുകളിൽ കൂടുതൽ മാറ്റം സംഭവിച്ചതായി കണ്ടെത്തി എന്നാൽ, PPC-മത്സ്യ ഗ്രൂപ്പിൽ മാറ്റങ്ങളൊന്നും കണ്ടെത്താനായില്ല. SPC-, WG- ഗ്രൂപ്പുകളിൽ വ്യത്യസ്ഥമായി പ്രകടിപ്പിച്ച ജീനുകൾ പേശികളുടെ വളർച്ച നിയന്ത്രിക്കുന്നതിലും, മാംസപേശികളുടെ സമസ്ഥാപനം കൈവരിക്കുന്നതിലും പ്രധാനപ്പെട്ടവയായിരുന്നു. അവയിൽ ചിലത് സമാന തീറ്റ നൽകിയ സാൽമണിൽ സമാനമായി ബാധിച്ചുവെങ്കിലും, ചില ജീനുകൾ സാൽമണിൽ സീബ്രഫിഷിൽ നിന്നും വ്യത്യസ്ഥമായി കാണപ്പെട്ടു.

ചുരുക്കത്തിൽ, ഈ പ്രബന്ധത്തിൽ അവതരിപ്പിച്ച പരീക്ഷണങ്ങളും അവയുടെ ഫലവും സൂചിപ്പിക്കുന്നത്, മത്സ്യത്തീറ്റയിൽ സസ്യ-മാംസ്യങ്ങളുടെ ഉൾപ്പെടുത്തലുകൾ കൊണ്ട് സാൽമൺ ഉത്പന്നങ്ങളിലേക്കുള്ള മൈകോട്ടോക്സിൻ, ഫൈറ്റോഈസ്ട്രജൻ എന്നിവയുടെ കടന്നുകയറ്റ സാധ്യത ഉപഭോക്താക്കളിൽ ആശങ്കപ്പെടുത്തേണ്ടതില്ലെന്നും, ഇവ ഒരു തരത്തിലും ആരോഗ്യത്തേയും ഭക്ഷ്യസുരക്ഷയേയും ബാധിക്കുന്നില്ലെന്നും കണ്ടെത്തി. പക്ഷെ, ഉയർന്ന അളവിലുള്ള WG അല്ലെങ്കിൽ SPC അടങ്ങിയ മത്സ്യതീറ്റ മത്സ്യത്തിൽ പ്രതികൂല ഫലങ്ങൾ ഉണ്ടാക്കാനിടയുണ്ട്.

List of publications

Paper I

Development and validation of a liquid chromatography high-resolution mass spectrometry method for the simultaneous determination of mycotoxins and phytoestrogens in plant-based fish feed and exposed fish.

Authors: Amritha Johny, Christiane Kruse Fæste, André S. Bogevik, Gerd Marit Berge, Jorge M. O. Fernandes, Lada Ivanova

Published: Toxins 2019, 11:222-243.

https://doi.org/10.3390/toxins11040222

Paper II

Biotransformation of phytoestrogens from soy in enzymatically characterized liver microsomes and primary hepatocytes of Atlantic salmon.

Authors: Amritha Johny, Lada Ivanova, Tone-Kari Knutsdatter Østbye, Christiane Kruse Fæste

Published: Ecotoxicology and Environmental Safety 2020, 197:110611.

https://doi.org/10.1016/j.ecoenv.2020.110611

Paper III

Impact of dietary wheat gluten on feed intake and the intestinal and hepatic transcriptome in Atlantic salmon (*Salmo salar*).

Authors: Amritha Johny, Gerd M. Berge, André S. Bogevik, Aleksei Krasnov, Bente Ruyter, Christiane K. Fæste, Tone-Kari K. Østbye

Submitted to: Scientific Reports

Paper IV

Plant-based diets induce transcriptomic changes in fast muscle of zebrafish and Atlantic salmon.

Authors: Anusha K.S. Dhanasiri, Amritha Johny, Xi Xue, Gerd M. Berge, André S. Bogevik, Matthew L. Rise, Christiane K. Fæste, Jorge M.O. Fernandes

Submitted to: Frontiers in Genetics

1.0 Introduction

1.1 Norwegian aquaculture perspectives of salmon farming

The world population currently is 7.8 billion and is expected to increase 2 billion by 2050. Between 1961 and 2016, the population growth (3.2%) outpaced the food production (1.6%) and also exceeded the total meat production (2.8%) (FAO, 2018). Food fish consumption is increasing constantly at an average rate of 1.5% per year, and the UN estimate that one in five persons depends on fish as the primary source of protein. During the last 70 years, the per capita food fish supply (kg) has constantly increased, even against the background of the rising population (**Figure 1**). This was only possible because the demand has been met more and more by farmed fish.

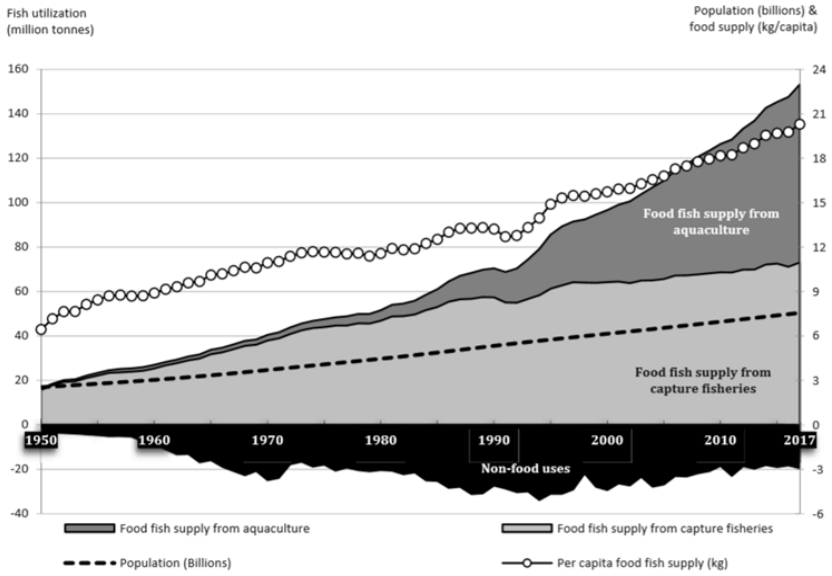


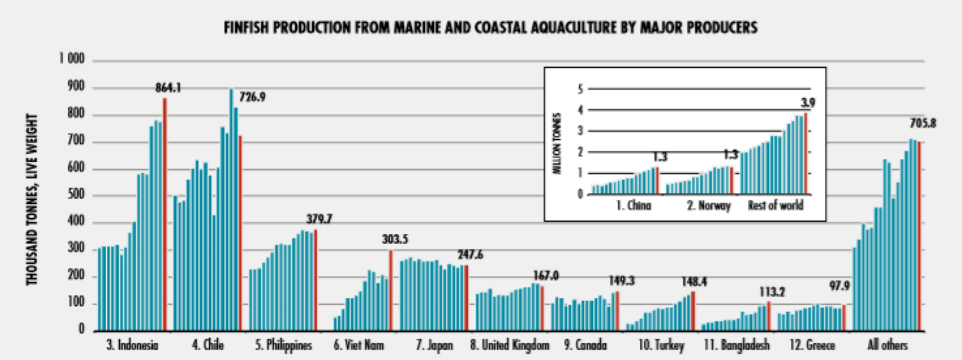
Figure 1. World fish utilisation and supply. [source: FAO, 2019]

This tremendous growth depends directly on the availability of feed resources. Further increase in aquaculture production, however, should, occur in a sustainable manner to avoid harming the environment or threatening biodiversity.

Out of the 171 million tonnes of the total fish production in 2016, 151 million tonnes were used for direct human consumption, whereas 20 million tonnes were used for non-food purpose and mainly, for the production of fish meal (FM) and fish oil (FO). FM and FO are among the main ingredients in feed for farmed fish, especially carnivorous

species like Atlantic salmon (*Salmo salar* L.), as easily digestible and nutritious and moreover, major source of omega-3 fatty acids. Still, their inclusion levels have dropped considerably in recent years (less than 10% in grower diets) due to supply shortages and increased prices and, importantly, due to the introduction of novel protein sources as fish feed ingredients (FAO, 2018; FAO, 2020). FM and FO are mainly replaced by vegetable proteins and vegetable oil, which are sufficient to sustain growth in salmon provided that the feed is fortified with deficient amino acids and macronutrients (NIFES, 2016).

Atlantic salmon is the 9th most produced fish species worldwide and first among the commercially important marine species (**Figure 2**) (FAO, 2018) with its major share



[source: FAO, 2018]

Figure 2. Major finfish-producing countries.

coming from Norway [1.2 million tonnes in 2018, i.e. 94.6% of the total aquaculture production in Norway (Aquaculture, 2020)]. Thus, Norway (2650 km coastline) comes 2nd only to China (14500 km coastline) in the global finfish production from marine and coastal aquaculture.

1.1.1 Salmon - farming in Norway

The first Norwegian legislative for the protection of salmon is dated to the text 'Gulatingsloven' with an origin around 1000 AD: 'The gift from the god (i.e. the migrating salmon) must be allowed to travel from the mountains to the oceans' (Robberstad, 1937). Coincidently, salmon always had an important part in the Norwegian's lives, as a staple food and trading good. The extended Norwegian coastline passing through many coastal islands and fjords is an excellent natural habitat for salmon and thus a preferred place for fishing and, more recently, aquaculture. Years of intensive research and

monitoring with regard to fish growth, production and welfare have helped to turn Norwegian salmon farming into the most important national business area except for the petroleum industry.

According to Norwegian Seafood Council, Norway exported 1.2 million tonnes of farmed

Figure 3. Annual sales of farmed salmon in Norway.

Atlantic salmon with a value of NOK 67.8 billion in 2018, accounting for 68% of the total export value of NOK 99 billion for Norwegian seafood. Since 2012, the salmon production has been about 1.2–1.3 million tonnes per year (**Figure 3**). Although also other species have a certain importance, especially rainbow trout with the sales of NOK 3 billion per year, salmon will continue to be by far the most important farmed fish for many years to come (Nettrapporter/fisken-og-havet 2019).

1.1.2 Salmon - from egg to slaughter-ready fish

Atlantic salmon is an anadromous fish from the Salmonidae family. They spend the first year of life ('fry or alevins') in rivers and streams and then migrate into the sea ('parr') for maturation (**Figure 4**). They are transformed into juvenile fish ('smolt') by a series of physiological changes to adapt from living in freshwater to saltwater (smoltification). The adult salmon later return to their place of birth for spawning, swimming upstream in the rivers and feed on small

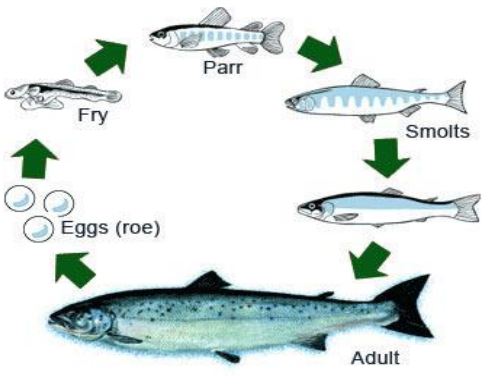


Figure 4. Life cycle of Atlantic salmon.
[source: Atlantic salmon, 2014]

fishes, squids and shrimps.

Salmon farming is consequently organised in two main phases, a freshwater period in land-based hatchery tanks, and a seawater period in ocean pens/cages (**Figure 5**). The parr are kept under optimised conditions with respect to temperature, light, pH, salinity and water flow. They are smoltified by the changing the photoperiod, when they weigh between 40 to 100 g. After the transformation is completed, the smolts are transferred to the seawater pens. Norwegian authorities have established regulations for salmon aquaculture considering fish health and welfare, for example, the fish-to-tank volume and cage parameters in proportion to salmon growth. Feed formulations have been specialised for the different life phases of the salmon considering the growth, weight gain and overall health of the fish. Due to the streamlined rearing conditions, constant availability of energy-rich feed and genetic selection for improved growth, the maturation of farmed salmon is 10 times faster than that of wild salmon. Slaughter-ready fish of about 4 to 6 kg can be harvested from the pens after about two years (Seafood.no).

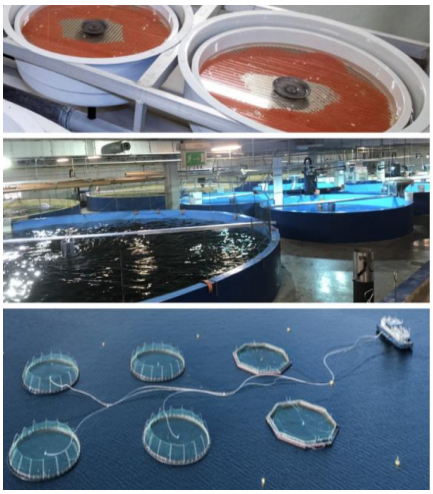


Figure 5. Salmon roe hatchery; rearing tanks; ocean pens. [source: Sinkaberghansen, 2018]

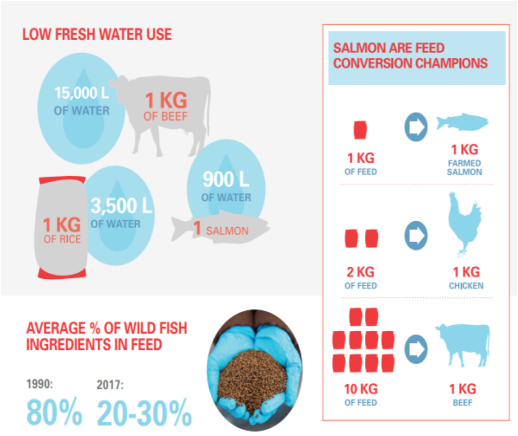


Figure 6. Feed conversion species comparison.
[source: Seafood.org]

Each kilogram salmon requires approximately 1.2 kg fish feed. In its total lifespan, a salmon will consume 6 to 7 kg fish feed, which is very little compared to other animals. For instance, it takes 2 and 3 kg feed to produce 1 kg of chicken and pork, respectively. Strikingly, as much as 10 kg feed are required for 1 kg beef (**Figure 6**) (Seafood.org). The freshwater consumption balance is

equally positive. Fish production is thus much more sustainable than the breeding of land animals when it comes to freshwater usage and feed conversion ratios. Moreover, contaminants such as antibiotics, pollutants, pesticides, and other undesirable factors are monitored in farmed salmon in Norway, and the levels found do not exceed the respective limits laid down in European regulations. Consequently, farmed Atlantic salmon is considered as safe to eat (VKM, 2014).

1.1.3 Challenges to salmon farming in Norway

About 400 million salmon are reared in sea pens along the Norwegian coast at all times (Aquaculture, 2020) at almost 1000 locations (**Table 1**). However, aquaculture is not only an economical success, but also of concern when it comes to the impact of these farms on the environment and wild salmon population.

Table 1. Production data of Atlantic salmon in Norway.

	2015	**2016**	**2017**	**2018**	**2019**
Number of locations (at sea)	990	978	986	1015	966
Slaughter tonnages	1,237,000	1,180,000	1,237,000	1,279,000	1,357,000
Mortality (million fish)	41.3	44.8	45.8	46.2	52.8*
Mortality (%)	14.2	16.2	15.5	14.7	16.2*

*increased death rate due to harmful algal blooms in summer 2019
[source: Sommerset et al., 2020]

Escapees from salmon farms may enter rivers and can interbreed with wild fish, altering the genetic make-up of the native species (Fiske et al., 2006; Zhang et al., 2013), which is a threat to the long-term fitness of wild populations (Naylor et al., 2005; Fiske et al., 2006). Mandatory reporting, extensive monitoring in rivers, identification by genetic assignment and improved pen technologies have helped to reduce escapee numbers, but they are still an ongoing challenge (Karlsson et al., 2016; Diserud et al., 2019). In 2019, almost 290,000 salmon have escaped from Norwegian fish farms (Sommerset et al., 2020). Critical factors are not only the genetic introgression, at least equally important are the spreading of viral and bacterial diseases as well as of parasites (Thorstad et al., 2018; Madhun et al., 2019).

Important virus infecting salmonids and inducing diseases with high lethality rates include the infectious salmon anaemia virus (ISAV) (Madhun et al., 2019), infectious pancreas necrosis (IPN) (Jensen et al., 2019), salmonid gill poxvirus (SGPV) (Garseth et al., 2018), salmonid alphavirus (SAV) (Chang et al., 2017; Madhun et al., 2019), piscine orthoreovirus (PRV) (Dhamotharan et al., 2019) and piscine myocarditis virus (PMCV) (Svendsen et al., 2019). Furthermore, infectious diseases in Atlantic salmon caused by bacterial pathogens such as *Renibacterium salmoninarum, Piscirickettsia salmonis, Vibrio anguillarum*, *Yersinia ruckeri*, *Aeromonas salmonicida* ssp. *Salmonicida* and *Tenacibaculum dicentrarchi* (Lillehaug et al., 2018; Klakegg et al., 2019) can lead to serious losses in aquaculture. Other diseases result from infestation with the amoeba *Paramoeba perurans* (Hellebø et al., 2017) or salmon lice *Lepeophtheirus salmonis* (Overton et al., 2019), or intoxication by toxic metabolites produced by marine algae such as *Pseudochatonella ssp.* and *Chrysochromulina ssp.* (Mardones et al., 2019).

Intensive salmon farming provides favourable conditions for the spreading of bacterial and viral diseases, and parasites because of the huge stocking density in the pens and the transportation of fish between different production sites (Torrissen et al., 2013). In the seawater around pens, increased numbers of harmful biological agents can be measured, leading to increased exposure of wild fish (Egidius et al., 1991; Thorstad and Finstad, 2018).

Different counter measures have been introduced to reduce disease spreading in salmon aquaculture and decrease losses. There are vaccines available for several viral and bacterial diseases (Sommerset et al., 2020). In some regions of Norway, vaccination of smolt against SAV-induced pancreas disease (PD) is mandatory before transfer into the ocean pens (Akvakulturdriftsforskriften, 2019). Other treatments include the application of antibiotics, chemicals, hydrogen peroxide, freshwater bathing, and the introduction of cleaner fish such as *Labridae* ssp. for the delousing of salmon (Skiftesvik et al., 2014). In a risk assessment on the environmental impact of Norwegian Atlantic salmon farming, the five endpoints of the analysis as established by the Norwegian government were spreading of diseases, genetic impact on wild fish, pollution by the discharge of nutrients and organic matters into the surrounding water, influence of the location zoning on pollution, and the sustainability of feed resources (Taranger et al., 2015). The results predicted moderate risks for genetic introgression from escaped salmon and lice infection, whereas minor risks were identified for disease transfer as

well as eutrophication and organic overload beyond the production area. There was, however, no conclusion about sustainable aquafeeds and connected risks.

1.2 The use of zebrafish as a model species

Zebrafish (*Danio rerio* H.) are among the most important vertebrate model organisms due to characteristics that make them suitable for a number of experimental manipulations (Spence et al., 2008). Zebrafish belong to Cyprinidae family and are indigenous to South Asia, especially India, Bangladesh, Nepal, Myanmar and Pakistan. They are asynchronous batch spawners and have a broad habitat preference, mainly in still or slow-moving, slightly alkaline water with relatively high clarity in association with the aquatic vegetation. Zebrafish are omnivorous with the natural diet consisting of worms, insects, larvae, and benthic and planktonic crustaceans. Their life span under laboratory conditions is reported to exceed 5 years. In captivity, they are fed with live feed, artificial diets or a combination of both, but the effects of these diets on fish performance are not very well investigated. Nevertheless, the non-pickiness of zebrafish with respect to feed makes it possible to perform dietary studies with different ingredients including plant proteins.

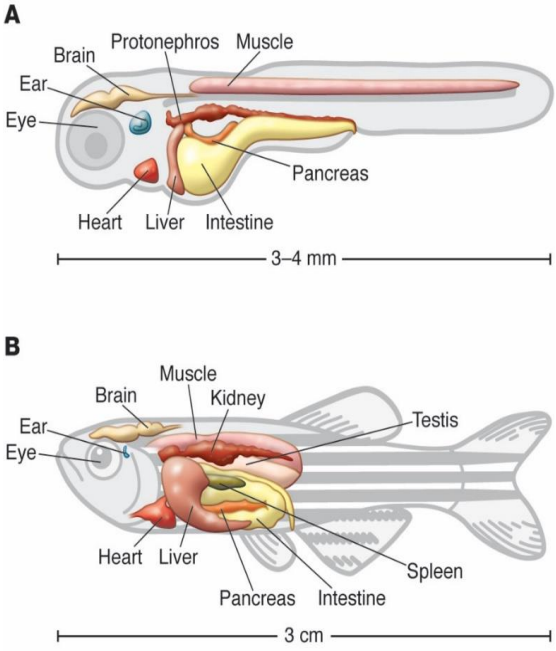


Figure 7. General body composition of a larva (A) 5 days post fertilisation and of a 3-month-old adult (B) zebrafish.

[source: Santoriello et al., 2012; Cassar et al., 2019. Figure re-used with permission from the journal and ACS publications]

Zebrafish offer a number of advantages for biological experiments due to their small size, rapid development (in 90 days from fertilised egg to mature adult) and short generation interval, which are helpful to conduct growth studies in short time. Moreover, their optical transparency during larval stages and the tractability in forward genetic screens are favourable features (Lawrence, 2007). The zebrafish genome has been completely

sequenced and is available in open repositories. A high percentage of homology with human genes contributes to the attractiveness of this animal model (Ulluo et al., 2013). Specific genetic traits can be selected by mating control in breeding programs. Experiments including high numbers of fish to generate powerful data for quantitative evaluations do not afford much space and can be performed cost-effective. The laboratory-rearing conditions for zebrafish are more homogenous and reproducible than farm-rearing conditions.

The rapid development of the embryo into the hatched, free-swimming larvae within 72 hours post fertilisation (hpf) and the subsequent larva maturing allows studying embryogenesis and organogenesis. Liver and pancreas are already fully developed after 76 hpf and the gastrointestinal tract after 96 hpf, and they are comparable to those in a 3-month-old adult fish (**Figure 7**).

In toxicology-related experiments, models using mammals can be very costly, laborious, and often cause ethical concerns. Zebrafish larvae are provided nutrition from their yolk sac during the first 5 days post-fertilisation (dpf), after which they start independent feeding. In this first stage, experimental regulations are limited and zebrafish larvae under 5 dpf have proven their applicability as a *replacement* for *in vivo* studies with mammalian species after validation of the experimental endpoints and can thus help to *reduce* the number of test animals. Furthermore, zebrafish embryos are fertilised externally and are transparent so that the toxic potential of e.g. chemical contaminants can be observed non-invasively, providing a tool for *refinement* and thus supporting the principles of the "3Rs" (Russell and Burch, 1959). Zebrafish larvae are therefore increasingly used in a wide range of developmental and toxicity studies (**Figure 8**) (Cassar et al., 2019).

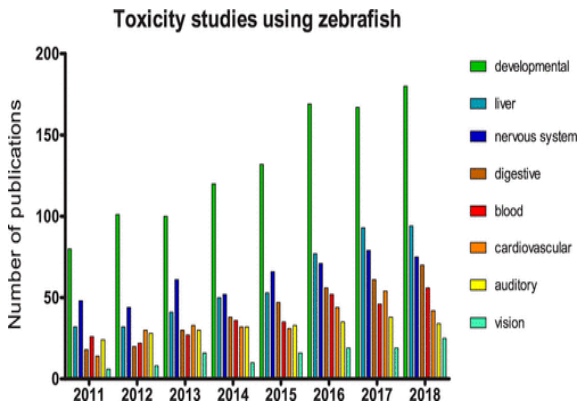


Figure 8. Increasing trend of publications using zebrafish for toxicity studies. [source: Cassar et al., 2019. Figure re-used with permission from the journal and ACS publications]

1.3 Sustainable aquafeeds

1.3.1 Fish feed consumption data and prognosis

Supplying the growing aquaculture with sufficient, safe and nutritious fish feeds is an important task. Feed mainly includes industrially-compounded aquafeeds, farm-made aquafeeds and wild-caught marine organisms of high nutrient value (forage/trash fish, invertebrates, etc.) (FAO, 2006). In 2016, the worldwide total annual production of FM was 4.5 million tonnes and of FO was 0.9 million tonnes, of which, respectively, 69% and 75% were used for aquafeeds (Auchterlonie, 2018; Seafish.org, 2018) (**Figure 9**).

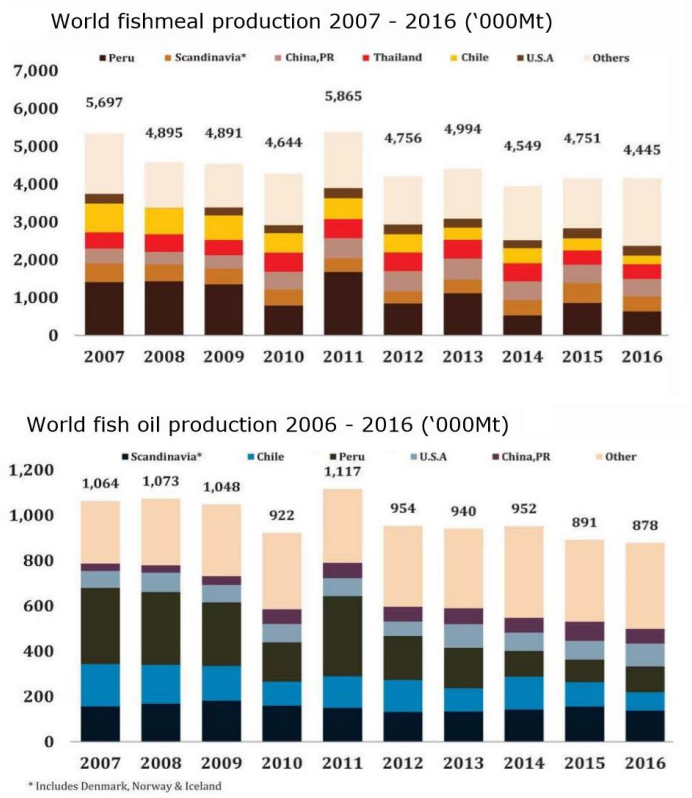


Figure 9. Decrease in global fish meal and fish oil production.
[source: Seafish.org, 2018]

The decrease in the annual FM and FO production in the last decade is in contrast to the overall strong increase of aquafeed production from 12 to 51 million tonnes from 1995 to 2015 (Hasan et al., 2017). In 2006, the total production of compound fish feed in 36 countries was between 20.2 and 22.7 million tonnes with China alone manufacturing 11

to 12 million tonnes. Norway was 4th with an annual production of 940,000 to 960,000 tonnes (Tacon & Meitan, 2008). Remarkably, the global compound aquafeed usage had reached 51.23 million tonnes in 2017, and it is expected to rise to 58.85 million tonnes by 2020, and 73.15 million tonnes by 2025 (Tacon, 2020) (**Figure 10**).

The global salmon production has more than doubled since 2000 and it is expected to grow further (**Figure 11**). The Norwegian salmon production ranked in 2017 first with

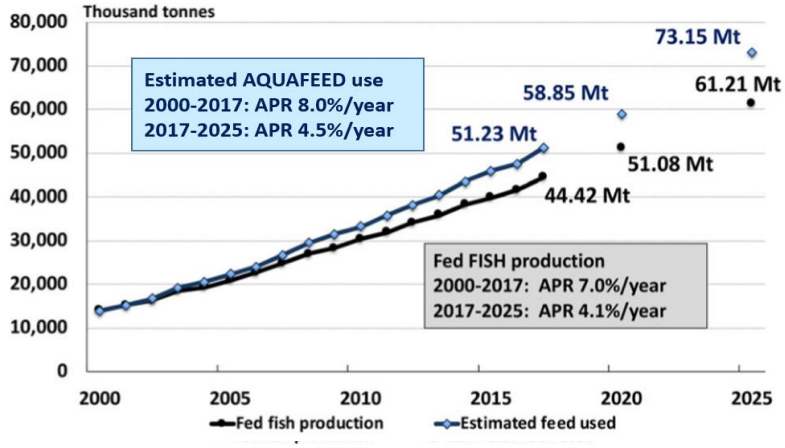


Figure 10. Total estimated commercial aquaculture feed usage by major fed species group: 2000–2017 and estimates for 2020 and 2025 (Tacon, 2020).

about 48% of the worldwide tonnage. Consequently, the demand for salmon feed will continuously increase (Tacon, 2020).

In total 1,252,573 tonnes salmon were produced in Norway in 2016 (**Figure 3**), requiring 1,627,478 tonnes of feed ingredients (Aas et al., 2019). While marine resources made up for 25% of the raw materials, 71% were of plant origin and 4% were micro-ingredients such as vitamin and mineral mixes, phosphorus sources, astaxanthin and crystalline amino acids.

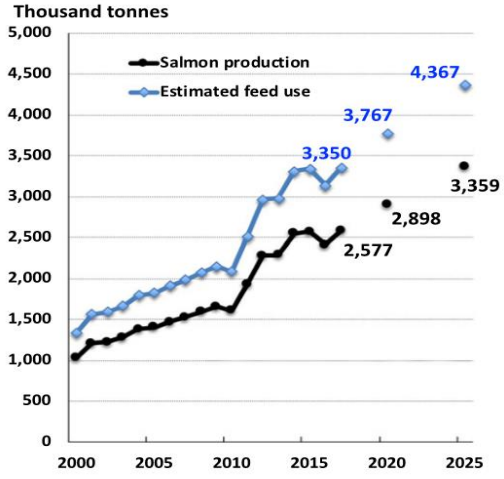


Figure 11. Salmon production and feed usage 2000 to 2017 and projection for 2020 to 2025 (Tacon, 2020).

The use of marine ingredients in salmon feeds is commonly reported in terms of a Fish-In-Fish-Out-ratio (FIFO) (Jackson, 2009). FIFO is a measure for the amount of fish used for the production of one kg farmed salmon. Since the yields and amounts used differ for FM and FO they are considered separately. The forage fish dependency ratio (FFDR) is similar to the FIFO but considers only wild-caught fish as resource, excluding trimmings and offal from slaughtered farmed fish. The FIFO and FFDR for FM and FO for Norwegian salmon have decreased to 0.8 (0.6) and 1.5 (1.1), respectively, between 1990 and 2016 due to a substantial reduction in FM (from 65% to 24%) and FO (from 19% to 11%) inclusion rates (Ytrestøyl et al., 2015) (**Figure 12**).

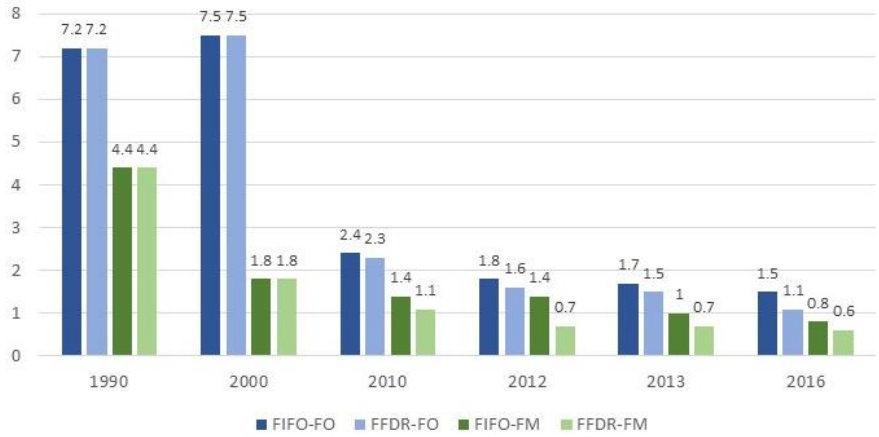


Figure 12. Development of FIFO and FFDR in Norwegian aquafeeds from 1990 to 2016 (numbers from Aas et al., 2019).

This significant change in salmon nutrition led, however, not to a noteworthy drop in the feed conversion ratio (FCR), which is defined as the ratio between feed consumed and salmon produced. The relative stability of the FCR results from the combined effects of genetic optimisation in terms of growth and product quality, as well as improved feed formulations, feed manufacturing methods and on-farm feed management.

The shift to plant-based feed ingredients was a direct consequence of the reduced global availability of FM and FO. Moreover, an increasing market pressure to improve the sustainability of fish farming has encouraged this development (Tacon and Meitan, 2008).

1.3.2 Nutritional requirements of salmon and feed technology

The energy requirement for optimal growth of Atlantic salmon depends not only on the

fish genetic profiles, sizes and water temperature, but also are effected by diet composition, nutrient availability as well as feed formulation and processing (Bendiksen et al., 2003; Krogdahl et al., 2004; Ng et al., 2004). Salmon, like other fish species, do not need specific proteins but require essential amino acids for normal growth. Fast growing fry and juvenile fish need a high protein diet and grower diets contain about 42% to 48% protein (FAO, 2004). This ratio is reduced in on-growing salmon in favour of increased dietary lipids, which improves the efficiency of energy utilisation. Lipids supply not only energy; they contain essential fatty acids (EFA) including long-chain polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Ruyter et al., 2000; Sanden et al., 2011). Carbohydrates do not play a part in the natural diet of salmon; however, starch added to feed at low levels can promote growth after digestibility-improving heat (Hemre and Hansen, 1998). Starches are also added as binders in feed processing to stabilise extruded pellets. Finally, compound aquafeeds have to contain essential fat- and water-soluble vitamins as well as minerals, e.g. phosphorus, magnesium, iron, copper, manganese, zinc, selenium and iodine (Lall, 2008).

The retention of energy and nutrients from feed in salmon is described as retention efficiency by comparing the amount of energy or nutrient incorporated to the amount of energy or nutrient in the feed (Opstvedt et al., 2003; Aas et al., 2019). In 2016, the estimated retention efficiencies of energy, protein, lipid, DHA + EPA and phosphorus in whole salmon was 41.3%, 36.6%, 49.4%, 37.3% and 18.5%, respectively, based on compiled data from Norwegian fish farms. Considering the changes in the FIFO for FM and FO (**Figure 12**), the values were remarkably comparable to those for 2012, with the exception of DHA + EPA, which were reduced by about 60% (Aas et al., 2019).

Feed processing has a significant effect on the nutritional quality and digestible energy of the complete diet (Mundheim et al., 2004; Kraugerud et al., 2011; Morken et al., 2012). For example, it hasbeen shown that soybean meal can cause enteritis in salmon, whereas alcohol-extracted soybean protein concentrate does not affect the intestinal integrity and even enhances growth gain better than FM (Krogdahl et al., 2003). Extensive research on feed processing methodologies and feed compositions has allowed the transition from marine to agricultural resources and the efficient incorporation of extruded plant proteins into aquafeeds. Thus, feed formulation technologies are considered as crucial for successful aquaculture. When salmon farming in Norway first

started in the early 1970, salmon were fed with raw fish (Talbot and Rosenlund, 2002; Tacon and Metian, 2009). Feeding methods developed to using semi-moist and dry pelleted feeds in the 1980s, which was followed by a transition to high-energy extruded pelleted feeds in the 1990s and 2000s (**Figure 13**).

The high content of digestible energy in extruded and coated aquafeeds contributed to

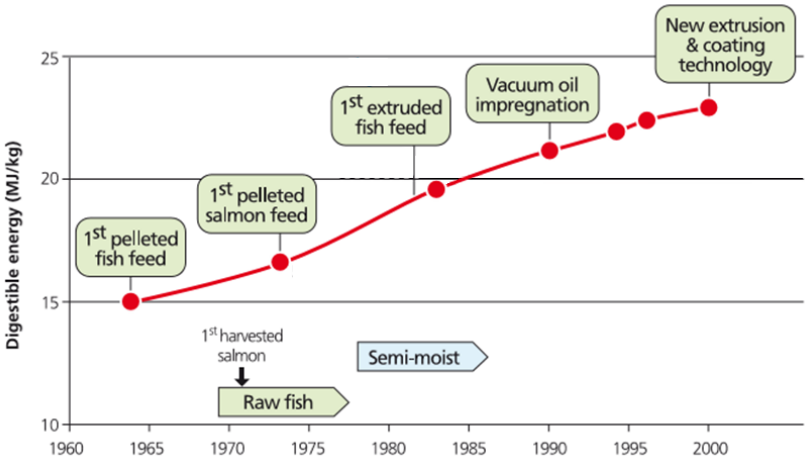


Figure 13. Feed methodologies used in Norwegian salmon farming in comparison to the technology development, and increase in digestible energy (Tacon and Metian, 2009).

the increase of fish growth and feed conversion ratios, resulting in reduced production costs in salmon farming. Mass production and lower retail prices turned salmon into an affordable product for many consumers (Asche et al., 2018) (**Figure 14**).

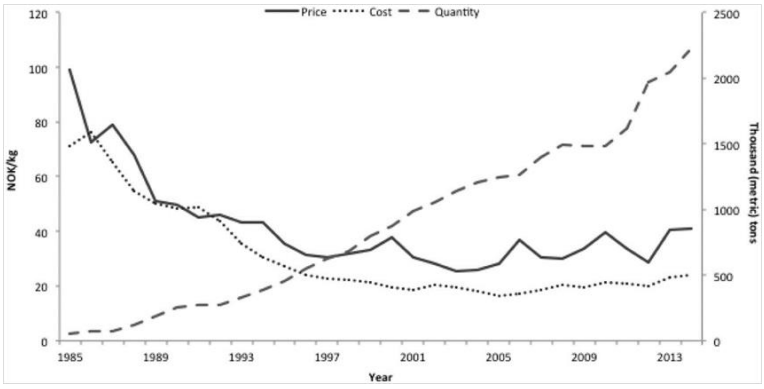


Figure 14. Norwegian salmon price and production costs (in Norwegian kroner (NOK/kg)) and total production (in 1000 tonnes) (Asche et al., 2018).

Since fish in general are the cheapest source of animal protein available, they are an important part of the human diet, contributing with essential amino acids, long-chain omega-3 PUFA including, EPA, DHA, essential minerals (Ca, P, Zn, Fe, Se, I) and vitamins (A, B, D) (VKM, 2014; Hua et al., 2019).

1.3.3 Transition from fish meal to alternative protein sources

The composition of salmon feed has changed significantly since 1990 (**Figure 12**). Plant proteins and plant oils have replaced FM and FO on a grand scale. Extensive research has helped to identify suitable alternatives providing a high protein content, sufficient amino acid profile, high nutrient digestibility, low content of anti-nutrients, fibres and non-soluble carbohydrates, which are at the same time widely available, easy to handle, ship and store, and comparably less expensive (Gatlin et al., 2007; Kraugerud et al., 2011; Ytrestøyl et al., 2015; Salin et al., 2018). Currently, plant materials make up to 70% of the fish feed used in Norwegian salmon aquaculture as compared to only 10% 30 years ago (Aas et al., 2019) (**Figure 15**).

The plant proteins fitting the requirements are mainly produced from legumes such as soybean, pea and lupine as well as from wheat gluten and sunflower seeds. The high

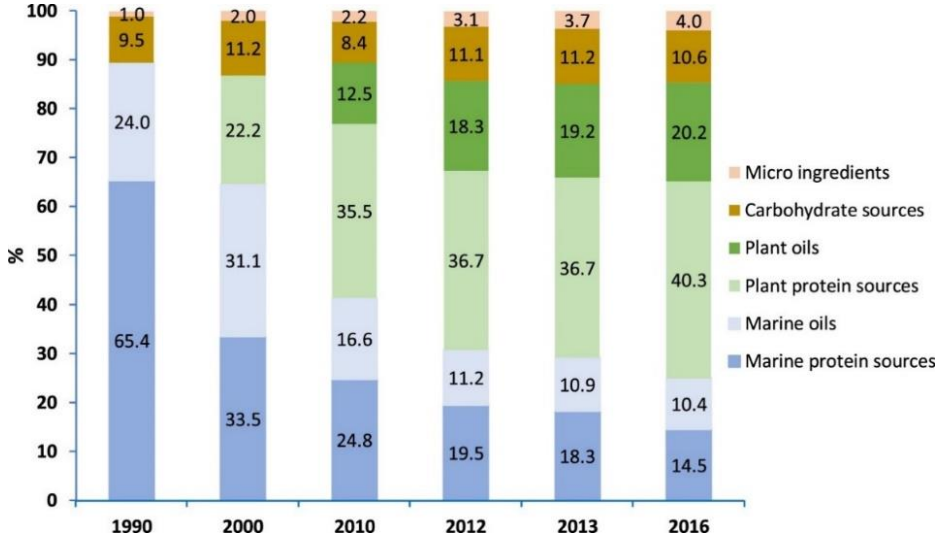


Figure 15. Change (%) of feed ingredient sources in Norwegian salmon feed (Aas et al., 2019).

inclusion ratio of plant ingredients in aquafeeds has caused a reduction in the levels of EPA, DHA and selenium among others in the edible parts of the fish so that less is available for human consumption (Berntssen et al., 2005). However, considering the

average fish intake percapita, these amounts are still sufficient with regard to the levels recommended by the European Food Safety Authority (EFSA) (VKM, 2014).

The complete change in the diet for farmed salmon has led to new challenges regarding fish health and welfare, and product quality (Hardy, 2010). Whereas fish growth and production rates could even be increased (**Figure 14**), the transition from marine to vegetable protein sources for aquafeeds has changed the hazards of exposure to undesired substances for fish and, ultimately, consumers of fish products. Plants produced with high efficiency in commercial agriculture are treated with fertilisers and plant protection products, and are exposed to contaminants in soil, air and water. Favourably, in salmon fed with vegetable oil the levels of waterborne persistent organic pollutants and heavy metals were significantly reduced as compared to FO-fed salmon (Berntssen et al., 2005; VKM, 2014), but very few studies have assessed the transmissibility of natural toxins, bioactive compounds and peptides from plant-based feed to fish to food so far. Finally, the use of plants in aquafeeds concerns also different aspects of sustainability. Plants form a large part of the human consumption and direct use is more energy efficient. Furthermore, cultivation requires vast areas, fertilizers, water, phosphorus, and can lead to the depletion of soil. Thus, aquafeed producers in Norway have become conscious of identifying the origin of the ingredients used, not the least because of rising consumer awareness for sustainability (Alfnes et al., 2018; Abualtaher and Bar, 2020).

1.3.4 Plant-based feed ingredients

Plant ingredients have replaced FM and FO to some extend in aquafeeds (Carter and Hauler, 2000; Kraugerud et al., 2011; Aas et al., 2019). However, not all crops are equally suitable for incorporation into the fish diet. When plant proteins and oils were first tested, some showed an insufficient nutrient and mineral content, which led to deformities of the salmon skeleton, intestinal problems and unfavourable physiological changes (Berntssen et al., 2003; Krogdahl et al., 2003; Helland et al., 2005; Fjelldal et al., 2009; Thomassen, et al., 2012; Bou et al., 2017). Intensive research activities have made it possible to overcome the initial problems by identifying good vegetable resources and optimised vitamin and mineral additives. Mostly used are pulses such as soybean, pea, fava bean and lupine, as well as wheat gluten, maize gluten, barley, tapioca, rapeseed, and cottonseed (Carter and Hauler, 2000; Gatlin et al., 2007; Kraugerud et al., 2011;

Ytrestøyl et al., 2015). The current staple plant source is soybean (Stamer, 2015). The diets can contain up to 50% soybean in differently processed variants (Refstie et al., 2001; El-Saidy et al., 2002). Soybean meal, solvent-extracted soybean meal (SBM), full-fatted SBM, defatted toasted SBM and defatted untoasted SBM, Triple null SBM from a new non-gene-modified cultivar without Kunitz trypsin inhibitor, lectin and the allergen P34/Gly m Bd 30 k, as well as extruded soybean white flakes, soybean protein concentrate (SPC) and soybean protein isolate have been used and examined for their impact on digestibility, fish growth, quality and intestinal health (Refstie et al., 1998; Singh and Muthukumarappan, 2016; Jacobsen et al., 2018; Krogdahl et al., 2020). When SBM products containing anti-nutritional factors (ANF) such as trypsin inhibitors, lectins, phytic acid and saponins are incorporated in salmon feed at a ratio of more than 5 to 10%, they usually cause enteritis in Atlantic salmon (Krogdahl et al., 2015). In contrary, several ANF are reduced in SPC that therefore can be used in higher quantities in aquafeeds without inducing intestinal inflammation (Booman et al., 2018; Krogdahl et al., 2020).

1.3.5 New developments in aquafeeds innovation

Novel sources are explored as ingredients in salmon feed, which do not directly compete with human food chains. The sustainability of aquaculture can be increased by using non-human food constituents with a small environmental footprint, including by-products from fisheries, aquaculture and animal farming, food waste, but increasingly also innovative ingredients processed from microbes, insects, macroalgae, seaweeds and forestry residues (Moroney et al., 2017; Sha et al., 2018; Couture et al., 2019; Wan et al., 2019; Belghit et al., 2019). However, there are still challenges in using unconventional ingredients, such as variable protein contents, unclear nutrient profiles, the feasibility of upscaling the production, establishment of suitable processing technologies, costs and consumer acceptance.

Novel feed ingredients have to be registered in the European Feed Materials Register (EU Feed Chain Task Force, 2019) in accordance with Regulation (EC) No 767/2009 article 24(6) stating that “the person who, for the first time, places on the market a feed material that is not listed in the catalogue shall immediately notify its use to the representatives of the European feed business sectors”. This notification, however, does not mean that the feed ingredient is safe for use. Feed safety is regulated separately with

regard to levels of different contaminants and further rules apply concerning the nutritional value and environmental safety.

1.4 Anti-nutritional factors in plant-based aquafeeds

"Antinutritional factors are defined as the substances, which by themselves, or through their metabolic products arising in living systems, interfere with food utilization and affect the health and production of animals" (Makkar, 1993). These endogenous compounds are produced by plants as part of physiological processes or their defence system. Through carry-over from feed into the edible parts of animals or fish, human consumers can be exposed to ANF and other feedborne contaminants (Shreeve et al. 1979; Hoogenboom et al., 2006; Kan and Meijer, 2007; Leemann et al., 2007). Some ANF can be considered as bioactive compounds with positive health effects, while many have a negative impact depending on dose, frequency of administration, mode of action and species studied (Francis et al., 2001; Gatlin et al., 2007; Mattila et al., 2018) (**Table 2**). For example, in humans, phytic acid, lectins, polyphenols, saponins and phytoestrogens can act beneficially by, respectively, reducing blood glucose and cholesterol levels, decreasing cancer risks, supporting physiological functions, or mitigating hormone imbalances (Popova and Mihaylova, 2019). However, elevated concentrations of ANF in fish feed are undesirable with respect to fish health and their indefinite transmissibility into food products.

ANF have been classified as a) factors affecting protein utilisation and digestion (protease inhibitors, tannins, lectins), b) factors affecting mineral utilisation (phytates, gossypol pigments, oxalates, glucosinolates), c) antivitamins and 4) miscellaneous substances (mycotoxins, mimosine, cyanogens, nitrates, alkaloids, photosensitising agents, saponins and phytoestrogens) (Francis et al., 2001). Moreover, soluble and insoluble fibres with digestive properties, inhibitors of protein, carbohydrate and lipid metabolism, phytosterols interfering with cholesterol and lipids, and oligosaccharides interacting with microbiota are relevant ANF in feed and food (VKM, 2009; Krogdahl et al., 2010).

ANF can be removed from feed by applying specific manufacturing processes including heat treatments, fermentation and extractions (**Table 2**) (Dawood and Koshio, 2019). The application of modern feed technologies, especially extrusion, have not only increased nutrient utilisation from plant resources, but also reduced ANF levels

considerably (**Figure 13**). However, the success rate is substance-dependent and complete removal is not feasible (Khokhar and Apenten, 2003; Bora, 2014).

Table 2. Anti-nutritients in plants typically used in aquafeeds.

ANF	Plant	Effect	Removal
Protein inhibitors	legumes, sunflower seeds, rapeseeds, sesame	reduced gastrointestinal protein digestion	heat
Amylase inhibitors	peas	reduced carbohydrate digestion	heat
Lipase inhibitors	beans	reduced lipid digestion	heat
Lectins	seeds	disruption of intestinal metabolism, morphological changes	heat
Phytic acid	legumes, sesame, cereals, cottonseed	reduced uptake of minerals	mineral supplementation
Fibre	cereals	interference with nutrient absorption and utilisation	dehulling
Tannins	beans, rapeseeds	interference with digestive processes	dehulling
Saponins	legumes	damage to the intestinal mucosa	alcohol extraction
Sterols	legumes	fatty acids and glycogen in liver	alcohol/non-polar extraction
Phytoestrogens	soybean, lupine, cottonseed, linseed	enhanced vitellogenesis, oestrogenic activity	alcohol/non-polar extraction
Mycotoxins	cereals, maize, soybean	toxin-dependent effects	dehulling
Alkaloids	lupine	neurotoxic	aqueous extraction
Gossypol	cottonseed	hepatocyte necrosis, fatty degeneration in the liver	non-polar extraction
Oligosaccharides (e.g. pectins, cellulose, galactans, lignin)	legumes	decrease in nutrient uptake	alcohol/aqueous extraction
Antivitamins	soybean, alfalfa	decreased vitamin efficiency	heat
Glucosinolates	rapeseeds, mustard	impaired iodine uptake, enhanced thyroid activity	heat, aqueous extraction
Allergens	soybean, lupine	enteritis-like changes, (non)specific immune responses	enzymatic digestion

[source: information retrieved from Makkar, 1993; Francis et al., 2001; Gatlin et al. 2007; VKM, 2009; Krogdahl et al. 2010; Mattila et al., 2019.]

Interestingly, many studies have been conducted to identify and quantify individual

compounds, but only a few have analysed co-occurring compounds, investigated combined effects or assessed transmissibility from plant-based feed to fish to food (Nácher-Mestre et al., 2018; Berntssen et al., 2019). Thus, the more than 40-year-old statement from I. E. Liener is still valid, "What has only recently been realized is that although there might not be an immediate violent reaction to a certain food component there might still be a slow cumulative adverse effect resulting in overt disease or less than optimal health. This poses a great challenge, since knowledge of these effects is gained slowly and with difficulty, particularly if the causative principles remain unidentified" (Liener, 1980). Adding the carry-over of ANF from feed to food as an additional factor, further research is much needed. In the current project considering the safety of salmon feed, the focus was thus on ANF that occur regularly in plant ingredients of Norwegian aquafeeds and their health risk potentials for fish and consumers of fish products.

1.4.1 Mycotoxins

1.4.1.1. Important mycotoxins in plant-based aquafeeds

Mycotoxins are secondary metabolites produced by filamentous fungi (Bennet and Klich, 2003). The production of toxins is not essential for cell growth or maintenance of basic cell functions and is potentially a part of the fungi's "chemical warfare", providing some advantage to survive in the environment. Currently, there are more than 20,000 known mycotoxins, and many more supposedly exist (Barac, 2019). About 300 mycotoxins have been studied in detail because of their frequent occurrence in feed- and foodstuffs, and thereof resultant importance for animal and human health (Khaneghah et al., 2019). Agriculture products may become contaminated pre-harvest with mycotoxins produced by fungal genera such as *Fusarium* spp., *Alternaria* spp. and *Claviceps* spp, whereas post-harvest contamination can occur after infection with *Pencillium* spp and *Aspergillus* spp. (Weidenbörner, 2017; Khaneghah et al., 2019).

In moderate climate zones and Nordic countries, *Fusarium* crop infections are the most prevalent fungi contaminations and associated with the considerable occurrence of trichothecenes, zearalenone and enniatins (Champeil et al., 2004; Desjardins and Proctor, 2007; Glenn, 2007; Firakova et al., 2007; VKM, 2013). Trichothecenes are polycyclic sesquiterpenoids containing a double bond between the carbons 9 and 10, a variable number of hydroxyl-, acetoxy- or other functional groups, and a 12, 13-epoxide

ring. They bind to the 60S subunit of eukaryotic ribosomes and inhibit the protein synthesis (Pestka, 2007). The trichothecenes are structurally divided into four main classes (A-D) (Sweeney and Dobson, 1998; McCormick et al., 2011) (**Figure 16 a**). The most important trichothecene mycotoxins belong to the A-type including HT-2 toxin (HT-2), T-2 toxin (T-2) and diacetoxyscirpenol (DAS), and the B-type including deoxynivalenol (DON), its acetylated or glucosidated derivatives 3-acetyl-deoxynivalneol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and deoxynivalenol-3-glucoside (DON-3G), nivalenol (NIV) and fusarenone-X (FUX). In parallel with the trichothecenes' structural diversity, there are great differences in the severity of toxic effects. Whereas e.g. DON, also called "vomitoxin", causes comparable mild acute toxicosis and is more of concern with regard to chronic low-level exposure, T-2 toxin has a considerable acute mortality rate (VKM, 2013). On the molecular level, DON exposure leads to mitochondrial impairment, apoptosis and oxidative stress.

Zearalenone (ZEN) is a macrocyclic b-resorcyclic acid lactone that binds and activates α- and β-oestrogen receptors (Fink-Gremmels and Malekinejad, 2007) (**Figure 16 b**). The mycoestrogen is often found in wheat bran and maize products and can cause

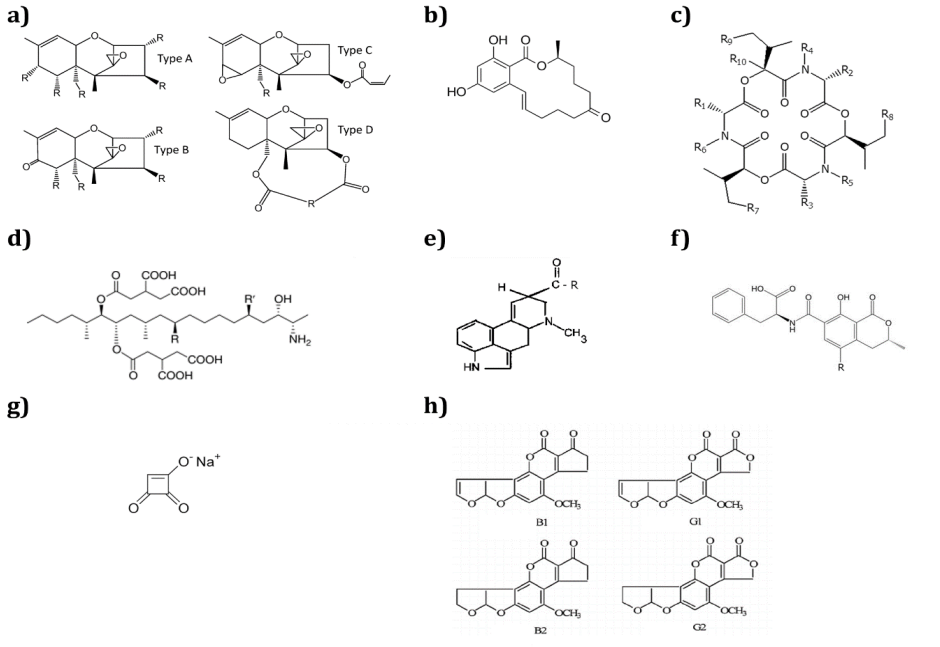


Figure 16. Important mycotoxins; **a)** trichothecenes, **b)** zearalenone, **c)** enniatins, **d)** fumonisins, **e)** ergot alkaloids, **f)** ochratoxins, **g)** moniliformin, and **h)** aflatoxins.

oestrogenic effects after ingestion (VKM, 2013). ZEN is considered as an endocrine disrupter.

Enniatins (ENNs) are ionophoric cyclic depsipeptides (**Figure 16 c**), which are commonly composed of three D-2-hydroxyisovaleric acid (Hiv) residues linked alternately to three N-methyl-L-amino acid residues (N-Me-R). More than 28 homologues have been identified so far, but the most prevalent are ENNB, B1, A, and A1 that are detectable in the majority of grain samples in Norway (Uhlig et al., 2006). The toxic effects of the ENNs are primarily connected to their ability to transport monovalent and divalent cations through biological membranes (Prosperini et al., 2017). Exposure can cause lysosomal disruption, cell cycle arrest, reduced macrophage functions, and altered mitochondrial functions. It can interfere with cell signalling, induce apoptosis or necrosis and nuclear fragmentation. Cases of acute intoxication have not been reported, but chronic exposure is of concern.

Several mycotoxins of considerable global importance in crops are less prevalent in Norway. Fumonisins (FUM), also produced by *Fusarium* ssp., are water-soluble molecules with a long chain-aminopentol backbone esterified with two carbon acids (**Figure 16 d**). More than 28 analogues are known, the most prevalent is fumonisin B1 (FB1). They have structural similarity to the sphingoid bases and can inhibit a key enzyme in the lipid pathway (VKM, 2013). FUM are possibly carcinogenic to humans and animals. Exposure occurs mainly from the intake of contaminated maize and can lead to appetite loss, respiratory problems, hypercholestrolemia, lethargy and immunosuppression. Ergot alkaloids (EA) (**Figure 16 e**) such as ergosine, ergonovine, ergotamine, ergocristin, ergocornine and α-ergocryptine are secondary metabolites of *Claviceps purpurea*. They occur mostly in rye and show moderate acute toxicity. Sublethal exposure causes neurotoxic symptoms such as tremor, rigidity and inhibition of the blood circulation in particular in extremities, which was known in the Middle Ages as Saint Anthony's fire (Florea et al., 2017).

A storage mycotoxin of main concern in Nordic countries is ochratoxin A (OTA), a pentaketidic isocoumarin produced by *Penicillium* or *Aspergillus* ssp. (**Figure 16 f**). The toxin affects several biochemical pathways, e.g. it inhibits peptide elongation in protein synthesis and increases gluconeogenesis and lipid oxidation (VKM, 2013). OTA is considered teratogenic and immunotoxic. The primary clinical effect is nephropathy. Monoliformin (MON), which is also produced by *Fusarium* strains, occurs normally as

the sodium or potassium salt of semisquaric acid (**Figure 16 g**). Concentrations in Norwegian grains are low (VKM, 2013). The primary mode of action is the inhibition of mitochondrial respiration. Toxicological effects include a decrease in protein synthesis, cytotoxicity and chromosome damage, affecting the metabolism and growth rate (Peltonen et al., 2010). Aflatoxins (AFL) are difuranocoumarins that are produced by *Aspergillus* species. They normally do not occur in Norwegian feed commodities because the fungi are not adapted to a colder climate (VKM, 2013; Bernhoft, et al., 2016; Anater et al., 2016). More than 20 aflatoxins have been identified; major analogues are aflatoxin B1, B2, G1 and G2 (**Figure 16 h**). AFL are metabolised in the liver to reactive aflatoxin-8, 9-epoxide that can bind to liver proteins and DNA. Intoxication with AFL can result in acute toxicity or, at lower levels, cause mutagenicity and carcinogenicity (Bbosa et al., 2013).

1.4.1.2. Toxic effects in fish

Crops can be contaminated prior to harvest in the field with DON, NIV, ZEN, HT-2, T-2, ENN, MON, EA and FUM, whereas contamination with OTA and AFL occurs during storage. The sensitivity for mycotoxins after oral uptake with feed or food differs greatly between species. The severity of the toxic effects depends mainly on the level of exposure, but also on age, sex, weight, the presence of other mycotoxins or antinutrients and the nutritional status (Bennett, 1987; Zain, 2011). Clinical effects of acute and chronic exposure with the most important mycotoxins have been performed in farmed animals including pigs and poultry. Moreover, there are a number of dietary studies on farmed finfish. The most studied species are rainbow trout (*Oncorhynchus mykiss*), Nile tilapia (*Oreochromis niloticus*), channel catfish (*Ictalurus punctatus*), Indian major carp (*Labeo rohita*) and common carp (*Cyprinus carpio*), but only a few exposure experiments have been performed in Atlantic salmon (VKM, 2013; Anater et al., 2016).

The observed effects in mycotoxin-exposed fish resemble those found in terrestrial animals (Santos et al., 2010; Matejova et al., 2017a; Gonçalves et al., 2018). AFL can get into aquafeeds through contaminated soybean, maize, cottonseed, peanut or wheat. A level of 200 µg/kg led to cancerous liver damage in tilapia (Selim et al., 2014). AFB1 causes adverse health effects like poor growth, pale gills, hepatic damage and immunosuppression. Early signs of exposure at low levels include oxidative stress and changes in the immune reaction (Pietsch, 2020) (**Figure 17 a**).

ZEN, occurring in maize and soybean, can cause reproductive disorders as shown in rainbow trout (Arukwe et al., 1999; Woźny et al., 2015). The toxin has also been found to be immunotoxic, hepatotoxic, genotoxic, cytotoxic and nephrotoxic (Pietsch, 2020) (**Figure 17 b**).

DON in cereal grain ingredients can cause a reduced feed intake, growth rate and feed efficiency and utilisation as observed in rainbow trout (Hooft et al., 2011; Pietsch, 2020)

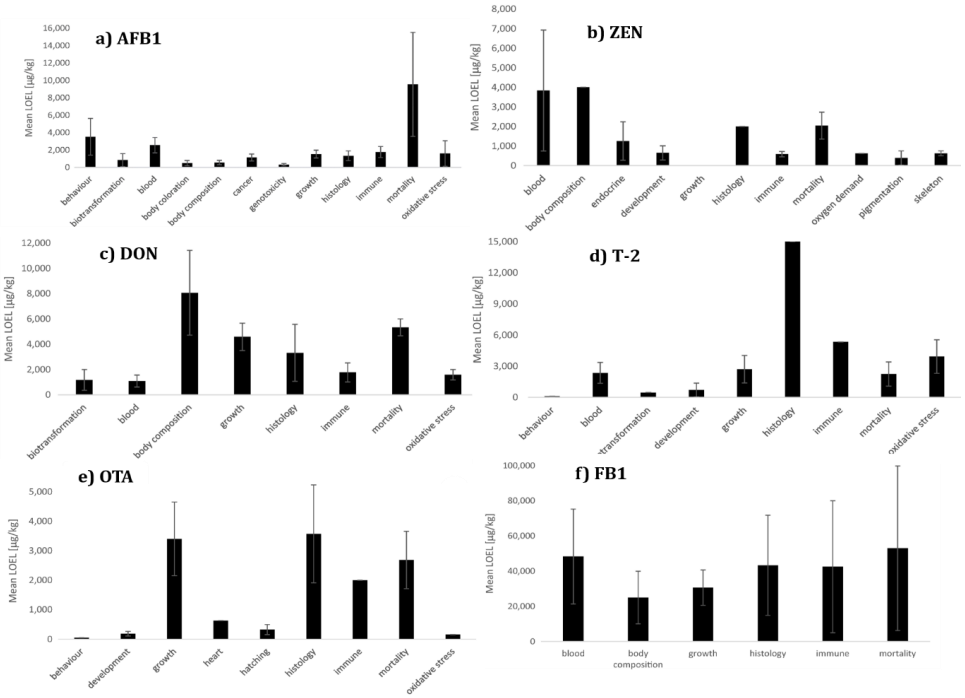


Figure 17. Mean lowest observable effect levels (LOEL) for different endpoints in different fish species for different exposure durations (mean ± SEM) exposed to **a)** AFB1, **b)** ZEN, **c)** DON, **d)** T-2, **e)** OTA, and **f)** FB1 (Pietsch, 2020).

(**Figure 17 c**). Furthermore, the gut epithelium and immune response were impaired in Atlantic salmon (Moldal et al., 2018; Bernhoft et al., 2018). The effects of NIV in fish are still unclear, but the toxic potency is assumed to be similar to that of DON. The exposure of carp to T-2 resulted in changes in the immune response, lipid oxidation in the liver and kidney as well as reduced feed consumption and weight gain (Matejova et al., 2017b; Pietsch, 2020) (**Figure 17 d**). OTA may be introduced in aquafeeds through soybean, maize, cottonseeds or sunflower seeds. Exposure of catfish and seabass at single-digit mg/kg concentrations led to damages to the hepatopancreatic tissue and posterior kidney, and a reduced survival rate (Manning et al., 2003; El Sayed and Khalil, 2009;

Pietsch, 2020) (**Figure 17 e**). In salmon, however, clinical effects were not observed at comparable concentrations (Bernhoft et al., 2018). FB1 impaired the sphingolipid metabolism and thus physiological functions of kidney and liver, resulting in a reduced growth rate and feed efficiency ratio in tilapia (Tuan et al., 2003; Pietsch, 2020) (**Figure 17 f**). It has been shown that the lipophilic ENNs were carried-over from the feed into the fillets of sea bass, sea bream, Atlantic salmon and rainbow trout from aquaculture (Tolosa et al., 2017). MON has been shown to affect cardiac and hepatic tissues, growth and survival in zebrafish (Pietsch, 2020).

Toxicity studies usually investigate individual mycotoxins. Feed- and food-stuffs, however, are normally contaminated with toxin combinations that are produced by one or several fungal species (Lee and Ryu, 2017). Combined effects may be additive synergistic or antagonistic, producing various complex biological effects.

1.4.2 Bioactive compounds

Bioactive compounds are ANF that usually occur in small quantities in feed and food resources and can elicit pharmacological or toxicological effects in humans and animals (Dillard and German, 2000; Guaadaoui et al., 2014; Popova and Mihaylova, 2019). Compounds with bioactivity vary widely in molecule structures and functions (Kris-Etherton et al., 2002). They can be roughly divided into four major groups, i.e. alkaloids including five structurally different classes, glucosides including anthraquinones and cardiac glucosides, polyphenols including flavonoids, tannins and lignans, and terpenes. In plants, these substances have important protective functions or are involved in cell signalling. For example, polyphenolic flavonoids protect against free radicles produced

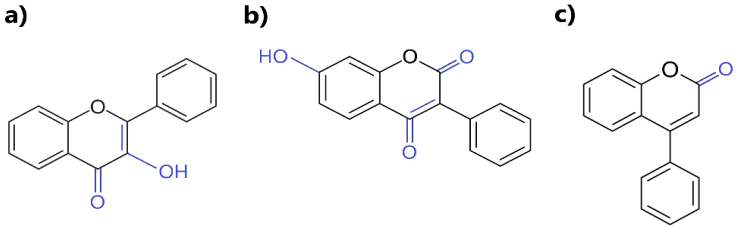


Figure 18. Molecular structure of the backbone of a) (bio)flavonoids, b) isoflavonoids, and c) neoflavonoids. Differences between subclasses are shown in blue. Individual derivates are characterised by the addition of various functional groups (not shown).

during photosynthesis, terpenoids attract pollinators or inhibit competing plants, and alkaloids act as antimicrobial phytoalexins (Bernhoft, 2010). Flavonoids are compounds

with two phenyl rings and one heterocyclic ring occurring in all plants, including plant ingredients used in aquafeeds such as cereals and legumes. Their antioxidant properties have led to widespread interest in potentially health-promoting properties of flavonoid-rich foods under the label of "functional foods" (Holdt and Kraan, 2011). Major structural subclasses of the flavonoids are a) (bio)flavonoids with the subgroups anthocyanidins, anthoxanthins, flavonones, flavanols and flavans, b) isoflavonoids with the subgroups isoflavones, isoflavandiols, coumestans and pterocarpans and c) neoflavonoids with the subgroups neoflavones and neoflavenes (**Figure 18**).

1.4.2.1 Phytoestrogens in plant-based aquafeeds

Legumes, in particular soybean, are much used as protein sources in aquafeeds as a cost-effective and efficient alternative to marine proteins. They are, however, especially rich in isoflavones, compounds with phytoestrogenic activity (Murkies et al., 1998; Fletcher, 2003; Gültekin, 2004). There are four main subclasses of dietary phytoestrogens: isoflavones, prenylflavonoids, coumestans and lignans. They belong structurally to the flavonoids except for the lignans, which are in a different class of the polyphenols. The individual derivates in each category are formed by alkyl group-substitutions in the basic isoflavone or lignin skeleton, respectively (Kurzer and Xu, 1997) (**Figure 19**).

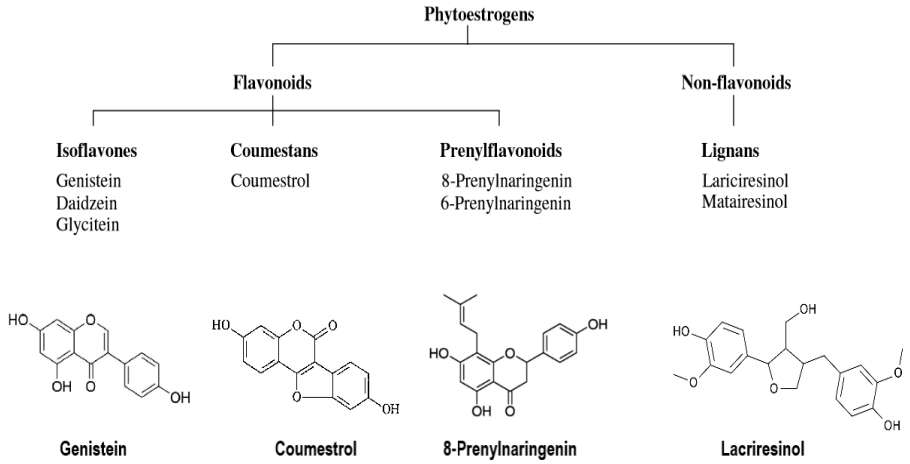


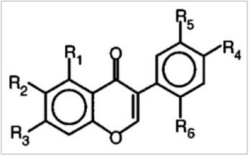
Figure 19. Phytoestrogen subclasses with typical representatives for isoflavones and coumestans (both isoflavonoids), prenylflavonoids (flavonoids) and lignans (non-flavonoids), showing the molecular structures of genistein, coumestrol, 8-prenylnaringenin and lacriresinol, respectively.

Isoflavones are non-steroidal phytochemicals with structural similarity to the sex hormone oestrogen (Song et al., 1999). They possess oestrogen-like biological activity

(Murkies et al., 1998). In soybean, they occur mainly in conjugated form as glucosides or, less frequently, as acetylglucosides and malonylglucosides (Kurzer and Xu, 1997). Important isoflavones in protein preparations of legumes are the glucosides daidzin, genistin and glycitin, of which the respective aglycons are DAI, GEN and GLY (Rietjens et al., 2017) (**Table 3**).

Table 3. Isoflavones occurring in legumes.

Isoflavones

	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**	**R_6**
daidzein	H	H	OH	OH	H	H
daidzin	H	H	O-glucoside	OH	H	H
genistein	OH	H	OH	OH	H	H
genistin	OH	H	O-glucoside	OH	H	H
glycitein	H	OCH_3	OH	OH	H	H
glycitin	H	OCH_3	O-glucoside	OH	H	H
formononetin	H	H	OH	OCH_3	H	H
ononin	H	H	O-glucoside	OCH_3	H	H
biochanin A	OH	H	OH	OCH_3	H	H
sissotrin	OH	H	O-glucoside	OCH_3	H	H
cicerin	OH	H	OH	1,3-dioxol		OCH_3
cicerin-glucoside	OH	H	O-glucoside	1,3-dioxol		OCH_3
homoferreirin	OH	H	OH	OCH_3	H	OCH_3
homoferreirin-glucoside	OH	H	O-glucoside	OCH_3	H	OCH_3

1.4.2.2 Effects of phytoestrogens in fish

The introduction of plant ingredients in aquafeeds, in particular the considerable use of soybean, exposes fish to phytoestrogens that they normally are not confronted with. Studies conducted in different fish species indicate that similar estrogenic effects might occur as have been found in other vertebrates (Setchell and Cassidy 1999; Gudbrandson et al., 2005; VKM, 2009; Zaheer et al., 2017). The soybean isoflavones DAI, GEN and GLY can also act as oestrogen receptor agonists or antagonists in fish (Pastore et al., 2018). GEN at concentrations of up to 10 μM inhibited the hepatic and renal estradiol-17β (E2) metabolism in salmonid fish (Ng et al., 2006). At 1 μM, the three individual compounds and a mix at a typical ratio (1.3:1:0.2) found in soybeans inhibited the oestrogen

metabolism in the liver, but not in the kidney. High levels (> 4 g/kg) of dietary isoflavones reduced significantly the growth of Japanese flounder (*Paralichthys olivaceus*) (Mai et al., 2012), whereas a low intake (40 mg/kg) for eight weeks enhanced the immune ability and hepatic antioxidant abilities of golden pompano (*Trachinotus ovatus*) (Zhou et al., 2015). It has been shown that soybean phytoestrogens induce the vitellogenin production dose-dependently in both male and female carp (*Cyprinius carpio*) in a dose-response manner, indicating disruption of the endocrine system (Turker and Bozcaarmutlu, 2009). The *in vitro* incubation with GEN, DAI, equol, biochanin A or formononetin in rainbow trout hepatocytes showed that all induced the vitellogenin synthesis (Pelissero et al., 1993). Effects of dietary GEN and DAI on sex steroid dynamics has been investigated in a long-term study in goldfish (*Carassius auratus*), demonstrating alterations in the reproductive organs and sex steroidogenesis (Bagheri et al., 2014). Furthermore, GEN concentrations of 10 to 1000 µg/L in the water affected the gonadal development of newly hatched Japanese medaka (*Oryzias latipes*) (Kiparissis et al., 2003). Exposure of male fighting fish (*Betta splendens*) to waterborne phytoestrogens had a significant effect on the behaviour of the fish (Clotfelter and Rodriguez, 2006). In a zebrafish embryo model, GEN and DAI had a disruptive impact (i.e. oestrogenic, apoptotic, thyroid) during embryogenesis and the endotrophic larval period (Sarasquete et al., 2018). When rainbow trout (*Oncorhynchus mykiss*) were fed with an extruded diet, in which more than 50% of the total protein was SPC, the growth rate was significantly reduced (Mambrini et al., 1999). Moreover, isoflavones were detectable in the bile of the fish. There was, however, no detectable negative impact on the reproduction, growth and health of rainbow trout that were exposed to 500 and 1500 mg/kg isoflavones in the diet for 70 days (Pastore et al., 2018).

1.4.3 Bioactive and allergenic peptides in plant-derived feedstuffs

1.4.3.1 Plant peptides in aquafeeds

Proteins are essential constituents of all living organisms. Nutritional proteins are an important source of essential amino acids and energy. They are fragmented after uptake by protease digestion in the gut or by non-thermal processing techniques such as fermentation, aging or ripening (Kamau et al., 2010; Mora et al., 2019). The resulting peptides can be absorbed into the systemic circulation of animals and humans, where they will reach their purpose. Bioactive peptides are specific protein fragments of about 3 to 20 amino acids length, which are inactive within the sequence of the parent protein,

but have various functions after their release by enzymatic hydrolysis (Karami and Akbari-Adergani, 2019). Based on the amino acid composition and sequence structure, they can have different biological effects such as opiate-like, mineral-binding, immunomodulatory, cyto-modulatory, antimicrobial, antioxidative, antithrombotic, hypocholesterolemic, anti-obesity or antihypertensive properties (Sarmadi and Ismail, 2010). Some, bioactive proteins are considered as health-promoting, contributing with beneficial effects to disease prevention or the modulation of physiological systems (Sánchez and Vázquez, 2017) and sometimes marketed as "nutraceuticals". Their content in different food materials has been extensively reviewed but feed ingredients are rarely considered, although cereal grains and legumes are important sources for bioactive proteins (Singh et al., 2014; Maestri et al., 2016; Rizzello et al., 2016).

The activity of some nutritional peptides, however, can also have a negative health impact, so that they are counted among the ANF (**Table 2**). Plants with high nutritional value, especially grains and legumes, which are used in aquafeeds, are also known for their content of major food allergens (Bernhisel-Broadbent et al., 1989). Wheat (gluten), soybean, peanut and lupine are among the 14 major food allergens that have been regulated by the European Union and have to be mandatorily labelled in ingredients lists of food products (EU, 2011). A ten-year (2000 to 2010) survey on allergic reactions to food in Norway showed that IgE-mediated allergies to wheat and several legumes including soybean, peanut, pea, lupine and fenugreek were among the most prevalent, whereas allergy to fish, i.e. salmon and cod, was little developed (Namork et al., 2011). Food allergens have been found to be heat-stable and relatively tolerant to digestion (Sathe et al., 2005). When plant proteins are fragmented by processing methodologies or in the gastrointestinal tract after ingestion, the breakdown into shorter peptides can decrease or release the allergenic properties of, respectively, conformational or sequential IgE-binding, allergy-eliciting epitopes (Sarmadi and Ismael, 2010; Webb et al., 1992; Kaminogawa et al., 1999). The severity of the allergic reaction depends on the specific allergens and the immune system of the affected individual. A weakened immune status, deactivation of peptidases, disrupted intestinal barriers and increased antigen permeability of the gut mucosa are typical symptoms occurring in allergic patients (Majamaa and Isolauri, 1996). The investigation of the transmissibility of allergenic peptides from feed ingredients into food is therefore relevant for consumer health. A substantial carry-over might require labelling in accordance with the food

labelling directives to ensure food safety for allergic/sensitive patients.

1.4.3.2 Effects of bioactive and allergenic plant peptides in fish

The occurrence of peptides in feed ingredients with bioactive properties besides their nutritional value is increasingly investigated in domestic animals and farmed fish (Conceiçao et al., 2012). It has been shown that certain bioactive peptides are beneficial for immunocompetence, control of oxidative stress and disease control of fish in aquaculture. Acidic peptide fractions from fish protein hydrolysate (FPH) had a strong stimulatory effect on the head kidney leucocytes in Atlantic salmon (Gildberg et al., 1996). FPH are frequently applied in animal feed including aquafeeds as they provide a profile of health-promoting bioactive peptides that is different to that in plant peptide hydrolysates (Najafian and Babji, 2012; Halim et al., 2016). The complete exclusion of FPH from fish diets is thus not recommended considering that they can mitigate nutritional stress and promote fish health (Conceiçao et al., 2012). In contrast, potential allergic reactions in fish fed with ingredients that are derived from major allergens such as soybean and wheat have not been identified so far. However, the expression of the heat shock protein 108 was changed in rainbow trout that had been fed with a soybean meal-rich diet for 12 weeks (Martin et al., 2003). Members of this protein family are involved in antigen presentation by association with MHC class I molecules and may thus indicate an immune response. Anyway, with respect to allergen exposure, the safety for consumers of fish products appears to be more relevant, since transmissibility of feedborne peptides has been previously reported. When zebrafish were exposed to a high amount of proteins of the fish parasite *Anisakis simplex*, traces of allergenic anisakid peptides were detectable in the muscle tissue after two weeks of exposure (Fæste et al., 2015). Nevertheless, there are currently no reports of patients with allergy to soybean or wheat that have experienced allergic reactions after the consumption of farmed fish.

1.4.4 Chemical contaminants in plant-based aquafeeds

Aquafeeds may be contaminated with chemical contaminants both from marine and plant ingredients, although with different compound classes (Berntssen et al., 2010). As exogenous compounds, chemical contaminants do not belong to the ANF in the strict sense of the definition. Some substances have, however, comparable active profiles and toxic endpoints as specific ANF, e.g. endocrine disruption (Dang et al., 2011; Søfteland et al., 2014). Their adverse effects can be caused by individual compounds or cocktails.

Fish meal is a major source for heavy metals, i.e. cadmium, arsenic and mercury, and fish oil contains considerable amounts of persistent organic pollutants (POPs) such as dioxin-like (dl-) and non-dioxin-like (non-dl-) polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polybrominated diphenylethers (PBDEs), hexabromocyclodecane (HBCD), poly-aromatic hydrocarbons (PAHs), and organochlorine pesticides (OCPs). The carry-over of these lipophilic contaminants into the edible parts of fish is substantial (Isosaari et al., 2004; Berntssen et al., 2011). In contrast, staple-feed plants including wheat and soybean are produced in industrialised agriculture and exposed to fertilisers and pesticides in addition to environmental contaminants. The transition to plant-derived aquafeeds has thus also resulted in a change of the contaminant content of the finished feed. Vegetable oils still contain POPs, however, the levels are considerably lower than in marine oils (Berntssen et al., 2010). Feeding and survey studies with Atlantic salmon have thus shown significantly reduced levels of POPs in fish fed with plant proteins and oils (Berntssen et al., 2005; VKM, 2014).

Maximum values for POPs in feed ingredients have been established by the European Commission (EU) in 2000, and have been amended several times (EU, 2000). POPs levels in finished feeds containing marine or plant resources are lowered notably through the use of efficient processing techniques so that chemical contaminates are no longer a major health risk for consumers of fish products in Europe. POPs have thus not been included in the scope of this project.

1.4.5 Occurrence of mycotoxins and phytoestrogens in aquafeeds

In Norway, feed regulation is based on EU directives, and additional legislation has been established in the "Forskrift om fôrvarer" (FOR-2002-11-07-1290) and in regulations implemented by the Norwegian Food Safety Authority (Mattilsynet.no). The law shall "ensure that feed is safe and not harmful to human or animal health, or makes food from animals unfit for human consumption. The feed should also not have an adverse effect on the environment" (Lovdata.no). Annual surveys are conducted of feed ingredients and complete feeds for contents of contaminants, for with maximum levels are set by EU directives or Norwegian regulations. These include chemical contaminants, admissible feed additives as well as biological contaminants such as ANF and microbes. Aquafeeds fall under this guidance; however, additional rules and specific survey programmes

have been implemented, determining the contents of contaminants, nutrients, additives and vitamins (Sele et al., 2019).

1.4.5.1 Mycotoxins in fish feed

The world-wide most prevalent and problematic mycotoxins in cereals and other plant-derived feed ingredients are the field mould-produced deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEN), T-2 toxin (T-2), fumonisin B1 (FB1), moniliformin (MON), enniatins (ENNs) and beauvericin (BEA), and the storage mould-produced aflatoxin B1 (AFB1) and ochratoxin A (OTA). Alternaria toxins and ergot alkaloids occur with relatively low frequencies in cereals. In survey programmes, DON, ZEN, T-2, FB1, AFB1 and OTA are regularly monitored, whereas ENNs, BEA and MON have until recently been considered as "emerging toxins" and were rarely included (VKM, 2014).

Table 4. Maximum mycotoxin levels for food and feed in the EU.

Mycotoxin

	Food* **[µg/kg]**	**Feed*** **[µg/kg]**	**Regulation**
DON	200 - 750	900 - 12000	Commission Recommendation of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products, 2013/165/EU Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed
T-2/HT-2	25 - 100	250 - 500	
ZEN	50 - 100	100 - 3000	
FB1	800 - 1000	5000 - 60000	
AFB1	2 - 5	5 - 20	
OTA	2 - 5	50 - 250	

*ranges show limits for different ingredients

Based on risk assessments performed by the European Safety Authority (EFSA), the EU has recommended maximum levels for important mycotoxins in different feed and food commodities (**Table 4**). Moreover, scientific opinions on undesirable compounds in aliments are published by the Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organisation of the United Nations (FAO) and the World Health Organisation (WHO) and national scientific committees and food safety authorities (in Norway: VKM and Mattilsynet, respectively).

The Norwegian guidelines with respect to feeding materials and complete feeds for specific animal species including farmed fish reflect the EU recommendations with the

exception for pigs, for which lower levels of some mycotoxins have been implemented (**Table 5**) (Mattilsynet, 2019). In non-EU countries, other, mainly higher maximum levels might apply for some mycotoxins. ENNs and MON have not been included in recommendations so far.

Table 5. Norwegian recommendations for maximum mycotoxin levels in feed ingredients and composite feeds for different animal species (Mattilsynet, 2019).

Mycotoxin	**Limit [µg/kg]**	**Species**	**Feedstuffs**
DON	8000-12000		cereals, maize
	5000	ruminants, rabbits	complete feeds
	2000	calves, lambs, horses, chicken, dogs, cats, **fish**	complete feeds
	500	pigs	complete feeds
T-2/HT-2	2000		oat bran
	500		cereals
	250	all species incl. **fish**	complete feeds
	50	cats	complete feeds
ZEN	2000-3000		cereals, maize
	1000	cattle, goats, horses, **fish**	complete feeds
	500	sheep, rabbits, dogs, cats	complete feeds
	250	pigs	complete feeds
FUM	60000		maize
	50000	ruminants	complete feeds
	20000	calves, lambs	complete feeds
	10000	chicken, **fish**	complete feeds
	5000	horses, ducks, dogs, cats	complete feeds
	1000	rabbits	complete feeds
	500	pigs	complete feeds
AFB1	20		feed ingredients
	10-20	all species incl. **fish**	complete feeds
	5	piglets, chicken, lambs	complete feeds
OTA	250		cereals
	5000	ruminants	complete feeds
	1000	calves, lambs, horses, rabbits, dogs, cats, **fish**	complete feeds
	50	chicken	complete feeds
	10	pigs	complete feeds

DON is the most prevalent mycotoxin in cereals in Northern Europe (Cheli et al., 2017). In a compilation of survey data from 97 complete fish feeds in Europe, the mean content of DON was about 220 µg/kg (Pietsch, 2020). In the second place was FB1 with about 200 µg/kg, introduced into the feed mainly via maize products (**Figure 20 a**). The

detailed analysis of published data on feed ingredients produced estimated mean mycotoxin levels underlining the importance of DON (**Figure 20 b**).

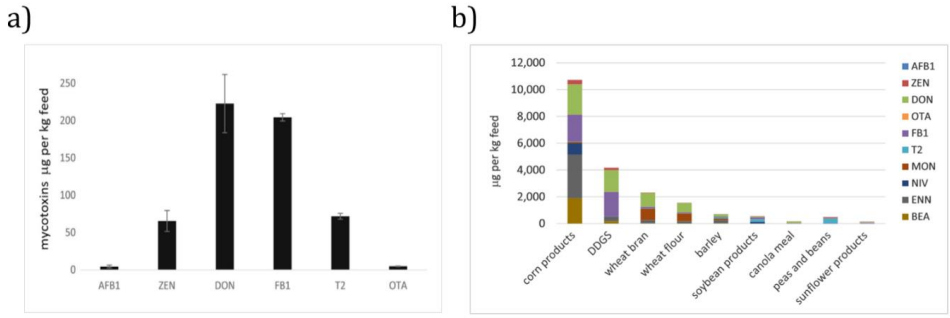


Figure 20 a) Mean contents (± SEM) of major mycotoxins in 97 fish feeds; **b)** Estimated mean mycotoxin contents in fish feed ingredients calculated from 116 published reports on measured levels in food and feed in Europe. DDGS: distillers' grain with solubles. (Pietsch, 2020).

In 2006, VKM concluded in a risk-benefit assessment of seafood in the Norwegian diet that "Little is known about the presence of mycotoxins in fish feed or about the effects of mycotoxins and their potential for accumulating in fish and being transferred to humans" (VKM, 2006). In an update in 2014, mycotoxins were considered as an emerging risk due to the increased use of plant-derived fish feed ingredients, while still stating that there were too little data (VKM, 2014). In an evaluation on the criteria for safe use of plant ingredients in the feeds of aquaculture fish, the lack of sufficient data on mycotoxins in Norwegian aquafeeds was pointed out, but DON, ZEN and FUM were identified as relevant contaminants (VKM, 2009). In the meantime, however, regular surveys have been performed, showing a rather limited occurrence in complete fish feed (Sele et al., 2019).

1.4.5.2 Phytoestrogens in fish feed

Legumes and some seeds are relatively rich in phytoestrogens (Kurzer and Xu, 1997). The most important dietary sources for isoflavones, coumestans and lignans are, respectively, soybeans, clover and alfalfa sprouts, and oilseeds. The glucosidated forms of DAI, GLY and GEN are the major isoflavones in soybean, whereas the glucosides of formononetin and biochanin A, respectively ononin and sissotrin, are present at notable levels in clover. Processing during feed manufacturing has considerable influence of the isoflavone concentrations in the plant materials. Unprocessed soybeans contain about 1.2 to 4.2 mg isoflavones/g dry weight, while soybean flour contains about 1.1 to 1.4

mg/g dry weight (Wang and Murphy, 1994; Fletcher, 2003). Maximum concentrations of DAI and GEN equivalents reached, respectively, 0.7 and 0.9 mg/g in raw soybeans, 1.9 and 2.0 µg/g in chickpeas, 1.0 and 1.8 µg/g in lentils, 1.4 and 70 µg/g in beans, showing that soybean is by far the most important source (Gültekin, 2004). The extrusion of SPC had little effect on the total isoflavone concentration (Mahungu et al., 1999). Coumestans are predominantly produced during germination, for example in sprouting beans and fodder crops (Murkies et al., 1998). They are thus of little relevance for fish feed.

Maximum levels for soybean isoflavones in feed and food have not been established by food authorities. The Italian Health Authority has recommended a limit of 80 mg/day in adults for the sum of all isoflavone isoforms, transferable to 1.14 mg/kg body weight (bw)/day in all age groups in the population (Morandi et al., 2005). The limit was set considering woman taking soybean supplements during menopause, and it thus higher than the estimated average isoflavone intake in East and Southeast Asia (20-50 mg/day). In 2015, EFSA concluded that doses of 35–150 mg/day of isoflavones for 2.5 years do not have adverse effects on oestrogen-responsive tissues (EFSA, 2015). The mean dietary isoflavone exposure in Western countries was estimated until recently to be much lower, i.e. below 1 mg/day in Europe and 3 mg/day in the USA. The introduction of a more global diet and the widespread transition to a vegetarian or vegan lifestyle, however, has resulted in higher isoflavone exposure (Lee et al., 2019a). The use of soybean in Asian diets has a history of safe use, which was extended to Western countries, but modern food preparation styles did not reduce isoflavone levels like traditional cooking (Rizzo and Baroni, 2018). Thus, new processing methods for the removal of isoflavones from modern soybean food products are being considered (Fernandez-Lopez et al., 2016).

Considering the situation for food, the lack of guidance for soybean inclusion levels in fish feed is understandable, particularly as there are tolerance differences between fish species. Furthermore, feed manufacturing has a considerable influence on the isoflavone concentrations in the complete feed. Since many ANF are extractable with alcohol, their levels can be considerably reduced during the preparation of SPC (VKM, 2009). It has, however, been shown in several studies that DAI and GEN from soybean ingredients in aquafeeds can be detected in the blood, liver and muscle tissue of exposed fish (D'Souza et al., 2005; Gu et al., 2006; Kuhnle et al., 2008). Feeding of rainbow trout

with commercial feed containing 3000 mg GEN/kg for 12 months resulted in about 5.4 pmol GEN/mg of fillet. A recent study demonstrated that the isoflavone concentrations in the fillets of trout fed with different soybean-containing commercial aquafeeds were correlated to the levels in the feeds (Merlanti et al., 2018). In soybean-based diets produced for farmed Russian and Siberian sturgeon (*Acipenser gueldenstaedtii* and *Acipenser baerii*), the total isoflavone levels ranged from 1.5 to 50 mg/kg (Rzepkowska et al., 2020). GEN and DAI were most abundant, but also formononetin and biochanin A were detected. Equol, a product of the intestinal biotransformation of DAI, was also present, possibly from added FM. A long-term dietary exposure study with sturgeon larvae receiving soybean-based feed for 800 days resulted in an accumulation in the liver, as well as significant concentrations in blood and in the gonads.

1.5 Impact of plant-based feed on fish health and welfare

1.5.1 Growth, feeding efficiency and digestibility

Initially, plant-based ingredients replacing FM were little processed. They contained a high amount of starch and structural carbohydrates. Their use resulted in reduced nutritional digestibility and growth performance in fish in aquaculture (Krogdahl et al., 2010). In addition, these feeds could impair the immune status, disrupt metabolic pathways and affect the overall health (De Santis et al., 2015). Moreover, the decreased inclusion of FO in favour of vegetable oil limited the availability of long-chain omega 3 PUFA (EPA, 20:5 ω3; DHA, 22:6 ω3), which are crucial for the maintenance of vital biological functions (Ruyter et al., 2000; Sanden et al., 2011; Katan et al., 2019). However, advances in processing technology have reversed this negative development (s. 1.3.2). Alternatively microalagae and genetically engineered oilseed crops make it possible to replace the fish oil fraction to some extent by fulfilling necessary nutritional requirements (Sprague et al., 2017).

The majority of the currently used diets are sufficiently supplemented with essential fatty acids and micronutrients, sustaining the normal growth parameters (final weight, final length, condition factor, weight gain, specific growth rate (SGR), thermal growth coefficient (TGC)) or organ weights. The condition factor (K) describes the ratio of body weight (BW) to fork length (FL) of the fish (K=100×BW/FL) and is thus a measure of regular growth (Fulton, 1902; Moghadam et al., 2007) (**Figure 21**).

Fish growth can be reported by determination of the absolute growth rate (AGR),

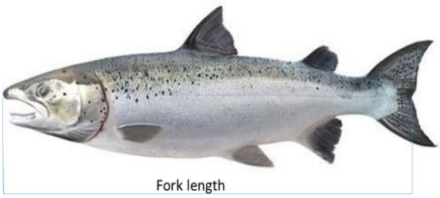


Figure 21. Atlantic salmon (*Salmo salar*). The fork length is the distance measured from the mouth to the middle of the caudal fin or "fork" of the tail.

relative growth rate (RGR), instantaneous growth rate (IGR), SGR and TGC. Since the growth is normally dependent on fish size, meaning that small and large fish have lower $AGR=100\times(BW_2-BW_1)/(t_2-t_1)$ than fish of intermediate sizes, this value gives little information about the daily weight gain (Hopkins, 1992). The $RGR=100\times((BW_2-BW_1)/BW_1)/(t_2-t_1)$ gives the percentage of weight increase, but is only valid for the specific time interval, in which a study is performed, and cannot be extrapolated. IGR is based on AGR by using the natural logarithm of the absolute values. Multiplication by 100 gives the $SGR=100\times(\ln(BW_2)-\ln(BW_1))/(t_2-t_1))$, which describes the percentage of weight increase per day (Lugert et al., 2016). The SGR is mostly used for describing fish growth in aquaculture as it refers to the instantaneous growth, and is applicable to compare fish of different sizes and at different times. However, the underlying assumption of exponential increase of the fish weight is not true over longer periods of time or older fish, so that more refined equations have been developed (Hopkins, 1992). The $TGC=(BW_2^{1/3}-BW_1^{1/3})/(\text{Temp. [°C]}\times\text{days})$, is calculated under the consideration of degree-days in an attempt to improve SGR (Cho, 1992).

Diet composition has a great impact on the FCR in salmon (s. 1.3.1). Three frequently used plant protein sources in aquafeeds are soybean protein concentrate (SPC), pea protein concentrate (PPC) and wheat gluten (WG) (s. 1.3.4.). About 250,000 t SPC, 100,000 t WG and 13,000 t PPC were processed into in total 1,452,000 t salmon feed in Norway in 2012 (Ytrestøyl et al., 2015). In 2016, about 1,628,000 feed was produced, utilising 310,000 t SPC, 146,000 t WG and 22,000 t PPC as protein sources (Aas et al., 2019). SPC is thus the by far most prevalent ingredient in aquafeeds (Stamer, 2015). It has been found to be a valuable replacement for FM, showing comparable feed efficiency (conversion ratio of feed to fish weight, FCR). However, inclusion of 50% SPC or more affected FCR and nutrient retention negatively, potentially due to methionine deficiency

(Mambrini et al., 1999). Supplementation of the feed with limiting amino acids resulted in an improved FCR, although 100% replacement of FM decreased fish growth and feed intake regardless. Many studies have shown the successful partial replacement of FM with soybean protein, mainly SPC, in many fish species like juvenile rainbow trout (Voller et al., 2018), rainbow trout (Kaushik et al., 1995; Mambrini et al., 1999), Atlantic halibut (Berge et al., 1999) and Atlantic salmon (Refstie et al., 2001). In contrast, growth was considerably reduced already with 25% dietary SPC in juvenile Japanese flounder (Deng et al., 2006), which was explained by an imbalance of amino acids and the presence of ANF.

The determination of the digestibility of different nutrients in a diet gives an indication of the ingredients' nutritional values. Furthermore, the energy digestibility is evaluated as a quality parameter. For the calculation of the apparent digestibility coefficients (ADC) of typical feed components such as dry matter, crude protein, crude lipid and energy, an inert marker is included in the complete feed and retrieval ratios are calculated from its recovery in faeces (Engin and Carter, 2002). Thus, ADC (%) can be established for diets as 100 - (100×(% marker in feed marker/% marker in faeces) × (% nutrient or kJ/g energy in faeces/% nutrient or kJ/g energy in feed)). FM is widely used as benchmark ingredient in aquafeeds (Udo et al., 2012). The ADC_I of specific ingredients in a diet with the FM reference can consequently be assessed as $ADC_{Td}+[(ADC_{Td}-ADC_{Ref})\times(a\times N_{Ref}/b\times N_I)]$, considering the test ingredient (I), test diet (T_d), reference diet (Ref), nutrient (%) or energy (kJ/g) in Ref (N_{Ref}), nutrient (%) or energy (kJ/g) in test ingredient and the ratio of reference and I in the diet (a/b) (Rahman et al., 2016; Oeda-Rodrigues et al., 2019). A comparison of ADC for protein and energy for typical protein sources in Atlantic salmon feed shows the advantages of using SPC or PPC compared to the milled pulses, and the high content of WG (**Table 6**). In a study comparing the ADC of pea and soybean-containing diets with FM, PPC in salmon diets in Atlantic salmon it could be shown that up to 50% PPC had no negative impact on the protein, fat, starch and most amino acids digestibility and caused only a slight decrease in the energy ADC (Øverland et al., 2009). In contrast, SBM in the feed led to a decrease of these parameters. Wheat gluten has the highest protein ADC of all commonly used plant-based fish feed ingredients (**Table 6**), which was also shown when diets containing FM, poultry by-products and vegetable proteins were compared in coho salmon and rainbow trout (Sugiura et al., 1998).

WG showed good growth performance parameters in Atlantic salmon (Storebakken et al., 2000). In an experiment with increasing replacement levels of FM by SPC in juvenile pearl gentian grouper (*Epinephelus fuscoguttatus × E. lanceolatus*), SPC 15% achieved higher ADC and FCR than SPC 30% or SPC 45% (Chen et al., 2019). A multitude of studies

Table 6. ADC of typical plant protein sources in feeds for on-growing Atlantic salmon.

Ingredient

	Crude protein (%)	**ADC (%)**	
		Protein	**Energy**
Fish meal (FM) (LT94, Norway)	77.5	95.8	95.2
Corn gluten meal	59.9	88.9	85.6
Wheat gluten (WG)	79.5	98.0	94.5
Canola meal	38.9	76.8	63.6
Soybean meal, dehulled (SBM)	49.8	83.8	70.1
Soybean protein concentrate (SPC)	68.7	93.8	88.8
Pea protein concentrate (PPC)	49.1	90.4	76.2
Lupine meal, white	38.6	88.9	81.2

(source: Lall, unpublished data)

investigating the nutrient values of different plant proteins in a large number of omnivorous and carnivorous fish species have been performed and showed that inclusion at moderate to medium levels did not have a negative impact on fish performance parameters (Daniel, 2018).

1.5.2 Diet-dependent effects on fish intestine and liver

1.5.2.1 Intestine

The fish intestine comes into direct contact with the feed after ingestion and thus is largely affected by the inclusion of certain plant protein into the diet (De Santis et al., 2015; Hansen et al., 2020). The intestinal transcriptome of salmon fed with SBM-containing feed shows mainly changes in the expression of genes related to lipid- and sterol-metabolism, cholesterol biosynthesis, immunity, oxidative stress and disruption of barrier functions. The extent of the observed alterations depends largely on the type of plant protein in the diet. Histological analyses of the mid-intestine in salmon fed with isoenergetic and isonitrogenous diets containing PPC (35%, 50%) or SBM did not reveal significant differences (Øverland et al., 2009). Likewise, the posterior intestine and goblet cells of rainbow trout did not show detectable effects induced by including 10%

of three different wheat protein preparations in a mainly SPC-based (> 40%) diet, whereas the intra-epithelial leukocyte counts and *lamina propia* width were significantly increased (Voller et al., 2018). Morphological changes in the mucosa of the distal intestine of salmonids comparable to the pathological symptoms observed in gluten intolerance in humans were detected in salmon fed with 33% SBM in the diet (Baeverfjord and Krogdahl, 1996). After three weeks, the induced subacute enteritis could be characterised by the widening of the central stroma in the mucosal folds and increase of connective tissue, shortening of heights and fusion of mucosal folds, disappearance of supranuclear vacuolisation of absorptive cells in the intestinal epithelium, and massive infiltration of the lamina propria and submucosa with a mixed population of inflammatory cells (Van den Ingh et al., 1991; Baeverfjord and Krogdahl, 1996). These effects were largely attributed to the presence of ANF in SBM, supported by the observation that they are considerably reduced when using SPC-based diets (s. 1.3.4). A diet with 30% SBM given to salmon for 12 weeks produced a gene expression profile consistent with enteritis in the distal intestine of salmon (De Santis et al., 2015). Interestingly, the majority of salmon fed with diets containing 15% SBM for 18 weeks had changes in the mucosa of the distal intestine, while the inclusion of 35% WG resulted only sporadically in moderate pathological symptoms (Storebakken et al., 2000).

The structure of the intestine is strongly related to its function so that the observed morphological changes from SBM-containing diets compromise the operational capability of the intestine. The disruption of the structural and functional integrity of the intestinal epithelium and resulting dysfunctionality significantly contributes to the pathogenesis of intestinal diseases in fish, comparable to the development of inflammatory bowel disease and celiac disease in humans (Marchiando et al., 2010).

The intestinal microbiome has a central role in gut health of the host (Ringø et al., 2016). In endothermic animals as well as in fish, the epithelium-colonising and digesta-associated intestinal microbiota are classified as, respectively, indigenous and transient. Fish have a more fluidic intestinal microbiome than terrestrial vertebrates, which and is highly sensitive to diet changes. It can be affected by feed consistence and the contents of dietary lipids, protein sources, ANF, nutrients, prebiotics, minerals, drugs, and other ingredients. Already the inclusion of 5% or 10% SPC in the diet of salmon altered the intestinal microbiome by increasing the bacterial diversity, introducing bacteria not normally associated with marine fish. This dysbiosis, which is suspected to contribute

to the development of gut pathology, did not occur, when prebiotics such as mannan oligosaccharides (MOS) were added to the diet (Green et al., 2013). The addition of MOS to the feed also proved to boost the intestinal health in trout (Dimitroglou et al., 2009). Since ANF in fish diets can cause intestinal enteritis and disruption of mucosal membranes, the density of fatty acid- and bile acid- transporters in the intestine could be reduced and uptake decreased. This can in turn lead to an increased cholesterol production in the liver and unbalance in the hepatic fatty acid and sterol metabolism (Olsen et al., 2003). However, ANF levels in SPC and WG are lower than in SBM, so that this problem appears to be no longer prominent in modern plant-based aquafeeds.

1.5.2.2 Liver

Replacing FM with plant proteins can alter the liver transcriptome of salmon (Skugor et al., 2011) and other fish species (Geay et al., 2011; Panserat et al., 2009). In general, plant-based diets have a negative impact on lipid digestibility, reduce bile salt levels and can cause hypocholesterolaemia (Kortner et al., 2013). The main changes in liver include altered metabolic functions, especially lipid- and sterol- metabolism (De Santis et al., 2015; Kortner et al., 2013). Changed expression levels of genes related to nutrient metabolism, cell processes and immunity have been observed (Caballero-Solares et al., 2018). The changes may vary according to the sources and combinations of plant proteins and vegetable oils used in a diet, and the connected amount of nutritional stress that is induced in the fish.

Cholesterol, which is produced in the liver, is the main sterol occurring in animal fats and oils. The cholesterol metabolic pathway is strongly affected when FM is replaced by plant proteins and plant oils in aquafeeds. This may be due to antinutritional phytosterols such as stigmasterol in soybean or campesterol in corn oil that can interfere with the cholesterol absorption and metabolism in fish (Krogdahl et al., 2010). Moreover, the fatty acid metabolism in fish is sensitive to dietary plant proteins (Kortner et al., 2012). Changes in the expression levels of relevant enzymes can lead to the accumulation of triacylglycerides in the adipose tissue, muscles, liver and pancreas and consequently to insulin resistance and hyperlipidemia/fatty liver (Ryu et al., 2005). Elevated levels of connected liver enzymes in the blood of plant protein-fed fish are indicative of the inflammation or damage of liver cells. An over-production of cholesterol can also lead to the production of oestradiol, initiating a pathway resulting in the production of vitellogenin in the liver, which is a precursor of egg yolk in

developing oocytes (Carnevali et al., 1998). Thus, diet-depending over-expression of vitellogenin-related genes can have an effect on the reproductive biology of fish.

1.5.3 Major transcriptomic changes associated with plant-based diets

The intestine and liver in fish have been identified as main target organs of diet-induced transcriptome changes. Nutrigenomics, or nutritional genomics, is the analysis of effects on gene expression levels from interactions with food and food ingredients (Tacchi, 2011). Nutrigenomic studies help to understand the effect of dietary ingredients at the molecular level. The interplay between nutrition and changes in the expression of specific genes can be studied by transcriptomic techniques such as microarray and RNA-seq, supports the improvement of aquafeeds functionality and sustainability (Martin and Król, 2017). The identification of biomarkers for health and growth performance of the fish under various experimental conditions is a useful tool in that effort. In this respect, the synthesis of PUFA in fish is of specific interest due to their importance in human nutrition (s. 1.3.2.), and the regulation of the involved lipid pathway enzymes in different tissues of farmed salmon has been investigated in numerous studies (Tacchi, 2011; Tacchi et al., 2012; De Santis et al., 2015).

1.5.3.1 Intestine

In the distal intestine of Atlantic salmon parr fed with a diet containing 45% SPC, a large number of genes were differently regulated as compared to FM-fed fish (Król et al., 2016). Significant changes were among others observed for PUFA and protein metabolism. Several specific genes related to lipid, energy and protein metabolism, mitochondrial activity/kinases, enteritis and transport were upregulated in the mid intestine of salmon fed for about 2 months with feed based on 18% SPC and 15% sunflower meal in comparison to FM-fed control fish (Tacchi et al., 2012). Regarding genes involved in the lipid metabolism, a significantly increased expression was observed for among others PUFA elongase, fatty acid-binding protein (*fabp*), glycolipid transfer protein as well as delta-5 and delta-6 fatty acyl desaturase (*fadsd5/6*). When the molecular responses of the replacement of fish oil by vegetable oil in salmon diets were studied, PUFA biosynthesis genes were found to be upregulated in intestinal tissues (Morais et al., 2012). Genes for fatty acyl elongases (*elovl*) were upregulated in the distal intestine of SBM-fed salmon (De Santis et al., 2015). Genes, *fadsd5/6*, *elovl*, sterol regulatory element binding proteins (*srebp*) and peroxisome proliferator

activated receptor γ (*pparγ*), are important for regulating the lipid and cholesterol metabolism, and themselves controlled by nutritional regulation (Morais et al., 2009; Leaver et al., 2008). The presence of ANF in plant-based fish feed enhances the changes in the lipid metabolism. When salmon were fed diets added 1% soybean saponins, intestinal gene expression profiling revealed an increased expression of a number of key enzymes of the lipid metabolism, including acyl-CoA cholesterol transferase (*acat*), monoacyl glycerol acyltransferase (*mgat*), choline kinase (*chk*), choline phosphate cytidyl transferase (*pcyt1a*), *fabp*, *pparγ* and apolipoproteins (*apo AI, apo AIV, apo B*) (Gu et al., 2014).

Gut inflammation in salmon from SBM- or SPC-induced enteropathy leads to increased expression levels of typical immunity-connected genes (Marjara et al., 2012; Tacchi et al., 2012; De Santis et al., 2015). The activation of T-cell-mediated processes via the up-regulation of cytotoxic T-lymphocyte-associated proteins, interleukins and T-cell receptors and the upregulation of the tumour necrosis factor (*tnf*)-signalling pathway and pro-inflammatory cytokines lead to an inflammatory response (Marjara et al., 2012; Bakke-McKellep et al., 2007a and 2007b). Proteins from these pathways are considered potential markers of the immune response in the salmon.

1.5.3.2 Liver

Changes in the liver transcriptome of fish from plant protein-containing diets are similar to those detected in the intestine (Zheng et al., 2005; Tacchi et al., 2012; Gu et al., 2014; De Santis et al., 2015). The liver is the main anabolic and catabolic organ and responsible for nutrient metabolism, distribution, detoxification and excretion as well as the synthesis of lipids and carbohydrates. Oxidative stress from the environment or the diet, e.g. from the exposure to tocopherol and astaxanthin in plant-based feed, activates response mechanisms in the liver and leads to the production of superoxide dismutase (SOD), catalase, glutathione-S-transferase and glutathione reductase, which can be used to quantify oxidative stress in hepatocytes (Van der Oost, et al., 2003; Olsvik et al., 2011)

Hepatic lipid and transport pathways are particularly affected by SBM- and SPC-containing feeds in Atlantic salmon and several genes have been pointed out as potential biomarkers in a number of studies (Zheng et al., 2005; Leaver et al.; 2008; Morais et al., 2009; Skugor et al., 2011; Gu et al., 2014; De Santis et al., 2015). Differently expressed genes include *fadsd5/6*, *elovl*, *fabp*, high-density-lipoprotein-binding protein and very

long chain acyl-coA synthetase and lipases. Additionally, pancreatic proteases (trypsin and chymotrypsin) and ribosomal proteins were identified as possible biomarkers of dietary effects on the protein metabolism. Impact on the amino acid metabolism could be determined by measuring key genes involved in reactions involving essential amino acids (threonine: glycine C-acetyltransferase; tryptophan: indoleamine 2,3-dioxygenase; methionine: S-adenosylmethionine synthetase). Central genes relevant in energy metabolism pathways belonged to the citric acid cycle (2-oxoglutarate dehydrogenase and succinate dehydrogenase) and the β-oxidation of fatty acids (*acat*). The inclusion of 1% soybean saponins resulted in the down-regulation of the rate-limiting enzyme in bile acid biosynthesis (cytochrome P450 7A1 (*CYP7a1*)), the transcription factor liver X receptor and the bile acid transporter *abcb11* (ATP-binding cassette B11) (Gu et al., 2014).

1.5.4 Epigenetic changes in fish fed on plant proteins

Epigenetics investigates heritable alterations in gene activity and phenotype without manipulating the underlying DNA sequence (Bollati and Baccarelli, 2010). Gene expression is influenced through local modification of the chromatin through epigenetic mechanisms such as DNA methylation and histone modifications, leading to chromatin remodelling (Fymat, 2017) (**Figure 22**). Epigenetic mechanisms are flexible and can change gene functions by influence from exogenous factors, thus altering the gene

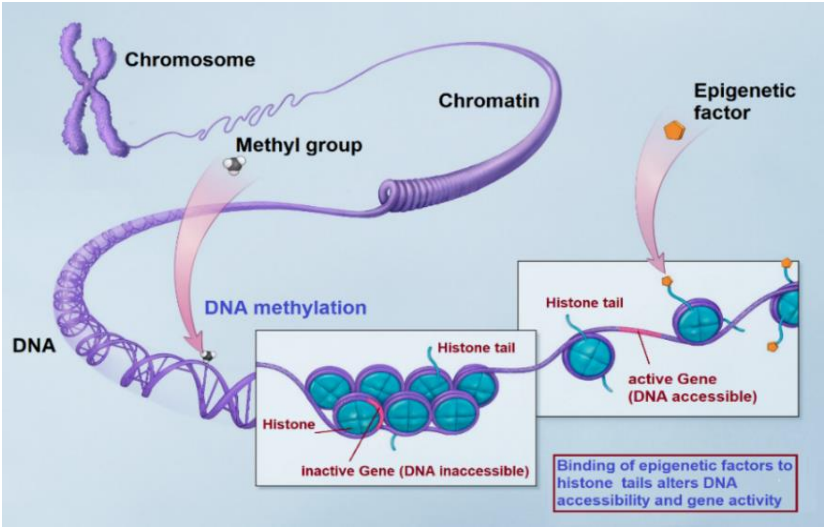


Figure 22. Epigenetic Mechanisms. [source: modified from Fymat, 2017]

expression at transcriptional level and affecting physiological and pathological processes (Choi et al., 2010).

DNA methylation occurs when DNA methyltransferases generate 5-methylcytosines (5mC) in the DNA by transferring methyl groups from *S*-adenosylmethionine (SAM), the universal cellular methyl donor, to the C5 of cytosines (Anderson et al., 2012). SAM is produced intracellularly by one-carbon metabolism from dietary methyl donors including the micronutrients folic acid, vitamin B12, betaine and choline. Effects of the nutritional status and diet compositions on the epigenome are studied by candidate gene detection or, more recently, by genome-wide nutri-epigenomics. In vertebrates including fish, intra- or intergenic demethylation of 5mC is correlated to an increase in transcription rates, and hypermethylation of promotor regions usually results in gene repression or silencing (Strömqvist et al., 2010; Kamstra et al., 2014; Eirin-Lopez and Putnam, 2019). DNA methylation patterns change during development and with age, but gene activity states can also be propagated transgenerationally (Skjærven et al., 2018).

The modification of histones in the nucleosomes affects DNA packaging into chromatin and gene accessibility (Turner, 2009; Gavery and Roberts, 2017). Nucleosomes, the basic DNA packaging units in eukaryotic cells, are octamers consisting of the histones H2A, H2B, H3 and H4 in duplicates around which the DNA strand in wrapped. These core proteins can be modified post-translationally through numerous reactions such as methylation, acetylation or phosphorylation with endogenous molecules, but they are also a target for epigenetic factors, e.g. methyl groups from exogenous sources. Binding to the N-terminal histone tails that are exposed at the nucleosome surface can have an effect on the chromatin structure and dynamics, and consequently gene accessibility (Fymat, 2017).

Since the epigenome is altered by environmental conditions, the identification of epigenetic biomarkers for different stress factors can help in disease assessment and risk prediction (Eirin-Lopez and Putnam, 2019). The phenotypic responses to environmental signals can be temporary or long-lasting. Epigenetic mechanisms have been widely studied in mammals, but are explored more recently also in important aquaculture species (Gavery and Roberts, 2017). In this respect, DNA methylation is the mostly studied epigenetic reaction in fish and shellfish. The impact of environmental exposure on epigenetic traits associated with temperature-induced sex maturation (Navarro-Martin et al., 2011; Shao et al., 2014), smoltification (Moran et al., 2013),

anadromy (Baerwald et al., 2016), growth potential (Campos et al., 2013) are of special commercial interest in aquaculture. Epigenetics are also relevant for sex control (Covelo-Soto et al., 2015), inbreeding (Venney et al., 2016) and adaptation to captivity (Podgorniak et al., 2019).

An important application of epigenetics in the field of aquaculture is the generation of desirable phenotypes by environmental manipulation (**Figure 23**). Epigenetic selection can be used, alone or in combination with genetic selection, to identify individuals with desired traits (Gavery and Roberts, 2017). By altering the environmental conditions for the larvae and breeding adults, within- or between-generation 'epigenetic memories' can be generated.

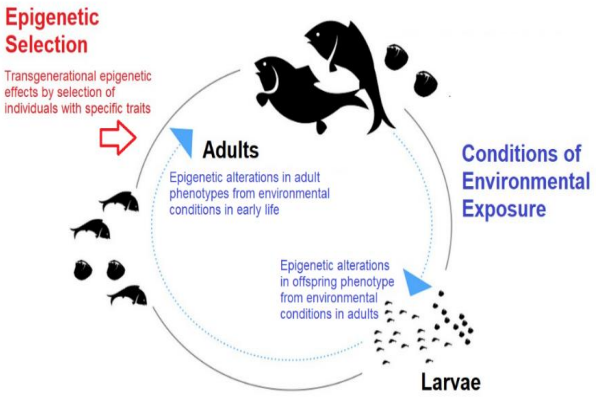


Figure 23. Aquaculture life cycle highlighting how exposure to environmental conditions can lead to transgenerational epigenetic effects.
[source: modified from Gavery and Roberts, 2017]

Environmental stress in marine environments can be caused by the presence of natural toxins, chemical contaminants, metals, parasites and viruses, by changes in water temperature, salinity and oxygen content, or high fish density in aquaculture pens (Eirin-Lopez and Putnam, 2019). Of similar importance are nutritional effects and epigenetic consequences of a multitude of ingredients in fish feeds have been studied. Differentiation between the various epigenetic factors and their respective effects, however, can be difficult due to possible interactions and the complexity of the processes. Mediation of phenotypic responses from exposure to various diets have been studied in European sea bass (Terova et al., 2016) and rainbow trout (Panserat et al., 2017). The studies suggested that specific diet compositions can alter histone modification levels and regulate the expression of histone-associated enzymes. Furthermore, a change in the fatty acid profile in the diet of parental zebrafish was found to affect the DNA methylation at locus-specific sites in the liver of their progeny (Adam et al., 2019). A recent study has revealed that the partial inclusion of plant protein into

the diets can induce genome-wide epigenetic changes in the mid intestine zebrafish (Dhanasiri et al., 2020a). Methylated genes varied among the plant diets and may have specific effects on the intestinal methylome. Many genes related to cell adhesion, neurogenesis, regulation of inflammation and intestinal homeostasis were methylated.

1.5.5 Biomarkers of liver health

Liver health is assessed generally by a number of functional tests measuring certain biomarkers in blood that allow monitoring liver disease or damage. The diagnostic tests help to determine how well the liver is performing its normal functions and measure enzymes that liver cells release in response to damage or disease. Usually evaluated parameters included in the biochemical profiling are albumin and total protein (Tprot), bilirubin, triglycerides (TG), alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP). Hepatic malfunctions can be indicated by decreased albumin and total protein levels, while elevated bilirubin point at a disturbance of the red blood cell metabolism. Triacylglycerol (TAG) levels give information about the status of the fat metabolism. The enzymes ALT, ALP and AST are important in protein and amino acid metabolism and raised concentrations may show pathological reactions.

The liver health biomarkers are also used to access the physiological status of fish (Azeez and Mohammed, 2017). Stress from nutritional or environmental factors can change the expected normal levels of the blood parameters and reveal their impact. In a study on growing Nile tilapia (*Oreochromis niloticus*), the increasing inclusion of FM and SBM in the diet was significantly correlated with a rise of the ALT and AST concentrations (Abdel-Tawwab et al., 2010). The same was observed when FM was replaced by 15 to 75 % SBM in the feed of obscure puffer fish (*Takifugu obscurus*), clearly indicating a form of nutritional stress (Ye et al., 2019). Blood lipid profiling analysing the levels of TAG, free fatty acids (FFA), total cholesterol and high-, low-, and very low-density lipoproteins (HDL, LDL, VLDL), in European sea bass (*Dicentrarchus labrax* L.) fed with diets substituting FM by WG or SBM showed increased cholesterol levels and changes in the fatty acid compositions of TAG (Messina et al., 2013). Moreover, the concentrations of total bile acids, total protein, glucose, sodium and phosphorous were altered as compared to control fish. Changes in the lipid profiles were also observed in Atlantic salmon receiving a diet with a high content of rapeseed oil, leading intestinal problems congruent with a lipid malabsorption syndrome (Hansen et al., 2020).

1.5.6 Biotransformation of xenobiotics in fish

Fish are exposed to xenobiotics in feed and the surrounding water. These substances can enter the body through oral uptake, the gills or skin. They reach the blood circulation either directly after trans-gill or transcutaneous absorption or, after ingestion and passing through stomach and gut, by intestinal absorption and a first liver passage ("first-pass effect"). Vertebrate organisms including fish are equipped with several mechanisms for the detoxification and excretion of xenobiotics, trying to avoid negative health effects and bioaccumulation.

Biotransformation processes occur in several tissues; however, the liver is the major metabolic organ. Accumulation of xenobiotics or their metabolic products occurs, when uptake is faster than elimination from the body (**Figure 24**) (Arnot and Gobas, 2006). The bioaccumulation factor (BAF) of a substance can be calculated from the ratios of the different rate constants: $BAF = C_B/C_{WD} = (k_1+k_D(C_B/C_{WD}))/(k_2+k_E+k_M+k_G)$, with C_B representing the xenobiotic's concentration in the body, and C_{WD} the concentration in water and diet.

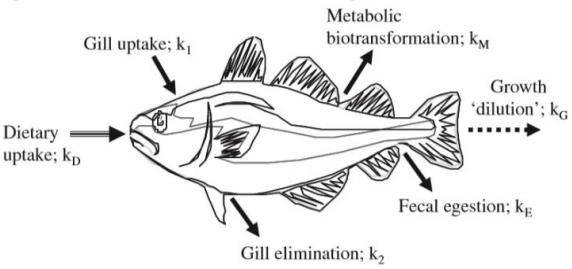


Figure 24. Major uptake and elimination routes for xenobiotics in fish. k: specific rate constants.
[source: Arnot and Gobas, 2006. Figure re-used with permission from the publisher.]

Kinetics has thus the central role in determining the health risk connected to the exposure to xenobiotics (Nichols et al., 2007). The exposure is directly dependent on the oral bioavailability (F (%)) of a substance, which is a multifactorial parameter considering bioaccessibility (F_b), absorption (F_{abs}) and hepatic biotransformation (F_{hep}) together ($F = F_b \times F_{abs} \times F_{hep}$) (Kwan, 1997). Bioaccessibility describes the fraction released from the nutrient matrix in the gut, absorption is the fraction transported across the intestinal epithelium and reaching the hepatic *vena portae*, and biotransformation is the fraction of unmetabolised substance after the first-pass through the liver. After absorption into the organism, a substance is subjected to distribution, metabolism and excretion (ADME). The kinetic performance and metabolic fate are dependent on its molecular properties, resulting in substance-specific ADME parameters. The crucial part of biotransformation in this process is the chemical

modification of the xenobiotic substance to achieve detoxification and facilitate elimination from the organism by converting non-polar, lipophilic molecules to polar, water-soluble metabolites (Schlenk et al., 2008). Usually, the produced metabolites are less toxic than the parent compounds, but in some cases, more reactive metabolites such as epoxides or free radicals are produced that may have a cytotoxic, teratogenic, mutagenic or carcinogenic potential (Buhler et al., 1988).

Biotransformation reactions are divided into two main phases, namely Phase I (functionalisation) and Phase II (conjugative) reactions. Phase I metabolism includes oxidation (aliphatic and aromatic hydroxylation, N-, O-, S-dealkylation, oxidative deamination, N-oxidation), reduction (azo-reduction, nitro-reduction) and hydrolysis (of esters and amides), which introduce or unmask functional groups in the molecule, increasing the hydrophilicity (**Figure 25**). Phase II metabolism forms covalent bonds between a reactive functional group of a substance, or its Phase I metabolite, with an endogenous compound, resulting in glucuronidation, sulphonation, acetylation, methylation or conjugation with amino acids, glutathione or fatty acids. Many biotransformation enzymes are encoded by polymorphic genes and levels can vary considerably between individuals. In addition, physiological and environmental factors such as age, health and diet can have an influence on gene expression and protein levels.

1.5.6.1 Functionalisation reactions (Phase I)

Phase I-metabolising enzymes are usually divided into three groups in accordance with their modes of action (Testa et al., 2012). Oxidoreductases catalyse oxidation-reduction reactions by transferring electrons between molecules. They include oxidases, oxygenases, dehydrogenases and reductases. Hydrolyses are catalysed by enzymes that split molecules by the introduction of water (**Figure 25**).

1) Oxidation

a) Cytochrome P450 (CYP) are haem-containing monoxygenases belonging to an extensive superfamily that is ubiquitous in all eukaryotic organisms (Guengerich, 2010). In vertebrates, they occur at high levels in the liver, but are also present in many other tissues such as intestine, lung, kidney, stomach, brain, heart and skin. Most CYPs enzymes are localised in the membrane of the endoplasmic reticulum (microsomal); however, some specific CYP are integrated in the inner membrane of mitochondria. CYP contribute to 75% of Phase I reactions. They catalyse a multistep cyclic monooxygenase

reaction that requires the CYP enzyme, the electron donors NADPH or NADH as co-factors, CYP P450 reductase, and molecular oxygen (Reed and Backes, 2012). CYP are classified into families according to homologies in the amino acid sequences and named

Figure 25. Phase I and Phase II metabolism reactions. [source: modified from TOX-OER, 2016]

using a specific nomenclature. The CYP families 1 to 4 are mainly involved in the biotransformation of xenobiotics; b) Flavin-containing monooxygenases (FMO) are NADPH- and oxygen-dependent microsomal enzymes that catalyse the oxidation of various nitrogen, sulphur, phosphorous and other nucleophilic heteroatom-containing xenobiotics without the need for additional accessory proteins (Krueger and Williams,

2005). Many substrates can be metabolised by both CYP and FMO, but are often transformed to different metabolites; c) Alcohol dehydrogenases (ADH) are cytosolic (non-microsomal) zinc-containing enzymes that catalyse the oxidation of alcohols to aldehydes and ketones using NAD^+ as cofactor. They are present in many organs including liver, which has the highest content, kidney, lung and gastric mucosa; d) Aldehyde dehydrogenases (ALDH) are $NAD(P)^+$-dependent enzymes that irreversibly catalyse the oxidation of endogenous and exogenous aldehydes to their corresponding carboxylic acids; e) Xanthine and aldehyde oxidases (XO, AOX) are cytosolic molybdenum-containing hydrolases that catalyse the oxidation of xanthine or aldehydes and heterocycles in the presence of oxygen and water; f) Monoamine oxidases (MAO A and B), are flavin-containing enzymes, localised at the outer membrane of mitochondria, that catalyse the oxidation of endogenous or dietary monoamines, diamines and polyamines; g) Peroxidases are haem-containing enzymes that use hydrogen peroxide as electron acceptor and oxygen donor in oxidation reactions without the requirement of NADPH and NADH (Testa et al., 2012).

2) Reduction

Xenobiotics containing metals as well as compounds with multiple bonds such as azo- and nitro-groups, aldehydes, ketones, alkenes, quinones or heteroatom-groups like disulphides, sulphoxides and halides are substrates for reductive reactions (**Figure 25**). These conversions are catalysed by a number of different enzymes: a) Aldo-keto reductases are cytosolic NADPH-dependent oxidoreductases such as aldose reductases, aldehyde reductases, hydroxysteroid dehydrogenases and dihydrodiol dehydrogenases; b) Short-chain dehydrogenases/reductases are NADPH-dependent cytosolic and microsomal enzymes; c) NAD(P)H-quinone reductases are cytosolic flavoproteins that catalyse the two-electron reduction of quinones into hydroquinones utilising NAD(P)H as electron donor; d) Several enzymes catalysing oxidative reactions have at the same time the potential to act as reductases including xanthine oxidoreductase, aldehyde oxidase and mitochondrial amidoxime-reducing component.

3) Hydrolysis

a) Carboxylesterases and cholinesterases catalyse the hydrolysis of ester- or amide-containing substrates into free acids and alcohols or amides; b) Para-oxonases are aryldialkylphosphatases; c) Alkaline phosphorylase (ALP) is a ubiquitous enzyme dephosphorylating substrates; d) Peptidases are catalysing proteolysis; e) Epoxide

hydrolases (EH) are responsible for the hydrolysis of epoxides into dihydrodiols by the addition of water. There are several distinct forms of EH. Microsomal epoxide hydrolase has a broad range of substrates, which includes xenobiotic epoxides (e.g. AFB1), drugs and endogenous lipids (estroxide) (Decker et al., 2009); f) β-glucosidases are responsible for the conversion of glucoside-bound substrates into their free, aglycon forms. The enzymes occur mostly in the liver and in the gut microflora, and they are important in the enterohepatic circulation.

Fish as vertebrate organisms are equipped with the main Phase I enzymes (Schlenk et al., 2008). The complete sequencing of the zebrafish genome has revealed the existence of 94 CYP genes, distributed among 18 genes families, including the xenobiotics-metabolising CYP1 to CYP4 (Goldstone et al., 2010). Orthologous to mammalian enzymes were identified for CYP1, CYP3 and CYP4 enzymes, whereas the diversity among the CYP2 enzymes was great. Across several fish species, more than 137 CYP genes have been identified (Uno et al., 2012; Burkina et al., 2017). Biotransformation activities vary between different species and have a genetic basis, but can also be a result of environmental or dietary influences, or the presence of additive, synergistic or antagonistic effects of substrate mixtures (Celander, 2011). The replacement of FM with a mixture of WG, PPC and maize gluten in feed for Rainbow trouts affected several hepatic metabolism pathways by the up- and down-regulation of important proteins, including electron transfer flavoproteins (Vilhelmson et al., 2004). FMO in fish have a wide substrate spectrum and are involved in the detoxification of environmental pollutants (Schlenk et al., 2008). ADH and ALDH activities have been determined in a number of fish species; however, MAO appears to exist only in one form, and its function is still unclear. The same is true for the contributions of peroxidases and some reductases, but hepatic nitro- and azo-reductase activities were observed in barracuda (*Sphyraena barracuda*) and yellowtail snapper (*Ocyurus chrysurus*). In contrast, carboxylesterases and EH have been detected in several fish species with relevant enzyme activities (Schlenk et al., 2008).

1.5.6.2 Conjugative reactions (Phase II)

Phase II enzymes catalyse reactions that lead to the detoxification of xenobiotics by conjugation with different physiological metabolites or transfer from methyl- and acetyl-coenzyme A (CoA).

1) Glucuronidation is a major pathway, representing about 35% of the Phase II reactions. The transfer of an activated sugar from the co-factor uridine diphosphoglucuronic acid (UDPGA) to the substrate is catalysed by microsomal UDP-glucuronosyltransferases (UGTs) to form more water-soluble metabolites, which are readily excreted through urine or bile. UGTs belong to an enzyme superfamily, expressed by numerous genes and extensively post-translationally modified (Hu et al., 2019). They recognise various functional groups of small lipophilic substrates as sugar-receiving sites, including aliphatic alcohols and phenols (R-OH), carboxylic acids (R-COOH), aliphatic and aromatic amines (R-NH_2 and R-NH-R'), sulphydryl groups (R-SH) as well as nucleophilic carbons.

2) Sulphonation is an important conjugation pathway of substrates that contain phenolic, aliphatic hydroxyl or aliphatic amine groups. It is catalysed by sulphotransferases (SULTs) in the cytosol transferring a sulphate moiety from the co-substrate 3'-phosphoadenosine-5'-phosphosulphate (PAPS) to the acceptor (Jancova et al., 2010). SULTs are regulated by various nuclear receptors that function as sensors of xenobiotics and endogenous molecules such as fatty acids, bile acids and oxysterols.

3) Glutathione conjugation is a major Phase II conjugation reaction. The addition of endogenous tripeptide glutathione (GSH) to electrophilic substrates is catalysed by glutathione S- transferases (GSTs) that can be divided into two distinct groups according to their localisation in the cytosolic or microsomal cell compartment. The soluble cytosolic forms, belonging to several subfamilies, are the ones mainly involved in xenobiotic biotransformation. GST activity is highest in the liver of vertebrates (Jancova et al., 2010).

4) Amino acid conjugation occurs by forming an amide bond between amino acids and a carboxylic or aromatic hydroxylamine group of the substrate, catalysed by aminoacyltransferases with CoA as co-factor.

5) Acetylation of substrates containing amine or hydrazine groups is catalysed by cytosolic N-acetyltransferases (NATs) requiring acetyl-CoA as a co-substrate.

6) Methylation as explained before (s. 1.5.4) transfers a methyl group from SAM to substrates with S- or O- containing functional groups. The enzymes are grouped in accordance to their preferred receptor. In contrast to other biotransformation reactions, S- or O-methylation will decrease the water solubility of a substrate. MT are present in

several cell compartments and different tissues (Jancova et al., 2010). Apart from metabolism function, MT are involved in numerous physiological processes such as the epigenetic modification of the chromatin architecture.

Fish contain all major Phase II enzymes. Glucuronidation is an important pathway for the detoxification of water-borne contaminants (Clarke et al., 1991; Schlenk et al., 2008). Teleost fish have considerably more UGT genes than mammals (George and Taylor, 2002). In zebrafish, 40 genes encoding UGT have been identified, which can be divided into UGT1, UGT2 and UGT5 families (Wang et al., 2014). UGT activities are especially high in liver and intestine. SULTs have been identified in several fish species (Schlenk et al., 2008). Genes for seven different SULTs have been identified in the zebrafish genome. Piscine GST are part of the defence system against oxidative stress. Their expression, especially in the liver, is upregulated by contact with environmental contaminants. Amino acid conjugation in fish has so far been studied for taurine. Detoxification of aromatic substrates by taurine conjugation has been observed winter flounder (*Pseudopleuronectes americanus*) and channel catfish (*Ictalurus punctatus*), and supplementation of feed with taurine is advantageous for some farmed fish (Salze and Davies, 2015). Acetylation occurs extensively in fish in the metabolism of amino group-containing xenobiotics (Schlenk et al., 2008), whereas methylation is an essential part of many endogenous pathways, but also relevant for detoxification.

1.5.6.3 Absorption, distribution, metabolism, excretion (ADME)

Knowledge of a substance's kinetic parameters in humans or animals is of central importance for the assessment of its toxicological potential. The determination of essential ADME parameters such as clearance (CL), plasma half-life ($t_{1/2}$) and volume of distribution (V_d) after intravenous (i.v.) application and maximum plasma concentration (c_{max}), time to c_{max} (t_{max}) and bioavailability (f) after oral (p.o.) application allows describing the concentration-time curve in plasma and thereby exposure to the substance. The kinetic characteristic of a substance are determined in *in vivo* experiments at low doses. However, *in vitro* models applying enzymatically active liver fractions are widely used to reduce the use of experimental animals, in accordance with the principles of the "3Rs", replace – reduce – refine, and are suitable for increasing the substrate throughput (Punt et al., 2018). If a substance is metabolised in hepatic microsomes or primary hepatocytes under the conditions of first-order kinetics, elimination characteristics can be derived, allowing *in vitro*-to-*in vivo* extrapolation

(IVIVE) and the prediction of *in vivo* parameters under consideration of species-specific upscaling parameters such as the microsomal recovery index, hepatocellularity, relative liver weight and hepatic blood flow (Ito and Houston, 2005). Moreover, the empirical observation that anatomical, physiological and biochemical characteristics in mammals are exponentially correlated with the individual bodyweights (BW) has been successfully employed for the allometric scaling of kinetic parameters (Boxenbaum, 1982). These principles and methods have also been employed for fish (Nichols et al., 2006). Since metabolism experiments in fish are not readily feasible, most biotransformation studies have been conducted in *in vitro* systems, allowing for qualitative comparisons among fish species. The determination of kinetic parameters requiring i.v. and p.o. application of a substrate is even more elaborate and only a few studies have been reported. The bioavailability of astaxanthin was approximated in Atlantic salmon by p.o. and intraperitoneal (i.p.) application (Maltby et al., 2003). The pharmacokinetics and bioavailability of oxolinic acid were compared in channel catfish and rainbow trout after p.o. and i.v. application (Kleinow et al., 1994). The kinetic parameters of nalidixic acid were assessed in rainbow trout (Jarboe et al., 1993), of enrofloxacin in juvenile Atlantic salmon (Stoffregen et al., 1997), and of florfenicol in cod (*Gadus morhua*) (Samuelson et al., 2003). Therefore, *in vitro* hepatic metabolism assays are attractive tools to elucidate easily kinetic parameters of xenobiotics in fish (Lee et al., 2019b).

1.5.6.4 In vitro metabolism models

Liver is the main detoxification organ in vertebrates including fish. Liver slices or subcellular fractions prepared from freshly harvested livers can be used in *in vitro* metabolism and kinetic assays under standardised conditions to determine a substrate's metabolites and elimination parameters (**Figure 26**). Slices are directly cut out and transferred into culture buffer. Hepatocytes are prepared by *in situ* digestion with collagenase and washing, whereas S9 fractions, microsomes and cytosol are separated by successive centrifugations of tissue homogenate.

The different models have typical advantages and disadvantages and are selected with regard to the respective research question: I) Liver slices possess intact cell membranes and a complex 3D tissue architecture. They are fully competent with respect to enzyme content and suitable for metabolism studies, but less applicable for kinetic analyses. However, the maintenance of slice cultures is arduous and culture times are limited, the

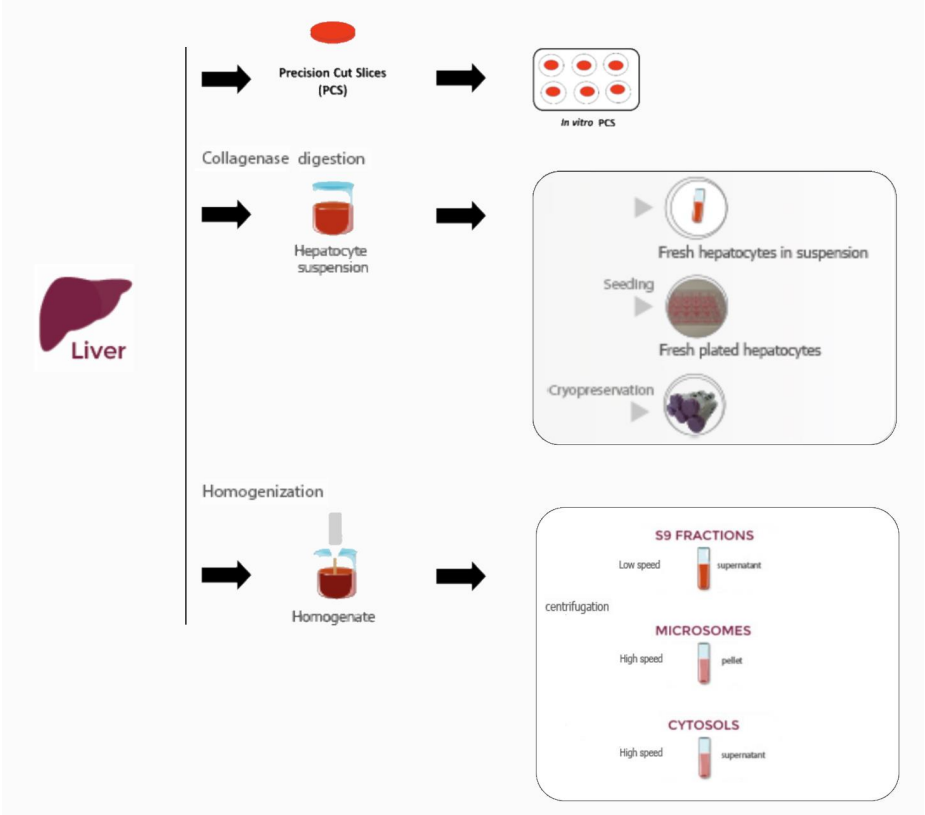


Figure 26. Liver fractions used in *in vitro* biotransformation and kinetics assays. [source: modified based on images available on Liver/ Hepatocytes-fractions, 2020]

free diffusion of compounds through inner layers can be hindered, and inter-assay variability can be considerable (van Delft et al., 2014).

Thus, the use of liver slices is limited to specific applications. II) Hepatocytes are intact cells and comparable to liver tissue in enzyme composition and co-factor content, but miss inter-cell connectivity and tissue architecture. The preparation affords skill to avoid varying cell viabilities. Primary hepatocytes are used directly in suspension for kinetic experiments. Moreover, they can be plated and kept in culture for several days for toxicity and drug interaction studies (Segner, 1998). Hepatocytes can be stored cryopreserved, but should not be used for kinetic experiments after thawing (**Figure 26**). In contrast, permanent cell lines are commercially available and standardised, but are not suitable for metabolism or kinetic studies. III) Subcellular fractions include S9 preparations, microsomes and cytosol, which are easy to produce from liver homogenate. S9 fractions possess both microsomal and cytosolic enzymes as well as co-

factors, but are unsuited for membrane transport assays. Liver microsomes contain the membrane-bound Phase I and Phase II enzymes and require the addition of co-factors to allow functional metabolism reactions. S9 and microsomes can be stored at -80 °C, while maintaining the enzymatic activities. Thus, most *in vitro* metabolism studies are performed using microsomes or S9 fractions (Fitzsimmons et al., 2007). Liver microsomes of a number of mammalian species are commercially available and frequently used in biotransformation experiments. Their versatility makes them an attractive tool also for species, for which material cannot be bought such as fish (Nichols et al., 2006; Smith et al., 2010). Cytosols can be used, when reactions catalysed by soluble hepatic enzymes are of interest. IV) Recombinant enzymes (e.g. specific CYP or UGT) are applied, if information about a substance's metabolic pathway is of interest. They are, however, rarely used for IVIVE due to increased uncertainties in upscaling parameters as compared to the commonly used hepatocytes and microsomes.

Fish are increasingly exposed to water- or feedborne chemicals, pesticides, anti-nutrients and other contaminants (Johny et al., 2020a). Information about their metabolic fate in the fish is relevant for the evaluation of the connected health risks. *In vitro* assays using hepatocytes or liver microsomes provide the opportunity of performing biotransformation studies without the need for live animals, which is in line with ethical considerations.

1.5.6.5 Enzymatic characterisation of liver fractions

Knowledge about the activities of major biotransformation enzymes in hepatocytes or microsomes that are used for metabolism or kinetic experiments allows the quality evaluation of the results and comparison between assays, material batches and species (Johny et al., 2020a). Moreover, combined batches with average enzyme activities can be produced, which is relevant for species with considerable inter-individual differences. Microsomes are usually characterised with regard to total protein content and activities of the most important CYP and UGT. The total CYP content can be determined by measuring the CO difference spectrum at 450 nm (Omura and Sato, 1964). Furthermore, ethoxycoumarin-O-deethylase (ECOD) is used as a marker for the sum of CYP1A1, CYP1A2, CYP2B1, CYP2E1 and CYP2B6 in mammalian microsomes, but has also been determined in microsomes from different fish species (Shen et al., 2016). However, more specific information is obtained by profiling CYP and UGT individually. This is achieved by performing assays with probe substrates that are typical for the

different CYP (Shi et al., 2015) and UGT (Seo et al., 2014). Enzyme activities are described by measuring the concentrations of the specific metabolites produced after definite incubation times with certain amounts of enzyme and different initial substrate concentrations.

1.6 Considerations of consumer safety from the intake of farmed salmon in Norway

Consumers have a right to expect that the food they purchase and consume is safe and of good quality (FAO, 2014). An important aspect in this context is the carry-over of contaminants from animal feed to food, resulting in the exposure of consumers. The connected health risks are dependent on the toxicity of a specific substance, its occurrence in definite food products, and the actual intake by different population groups. A risk assessment considers these elements in a four-step approach: 1) hazard identification, 2) hazard characterisation, 3) exposure assessment and 4) risk characterisation (**Table 7**).

Table 7. Elements of Risk Assessment and their definitions. [source: FAO/WHO, 2006]

Elements of Risk Assessment	
Hazard Identification	Identification of known or potential health effects associated with a particular contaminant.
Hazard Characterisation	The qualitative and/or quantitative evaluation of the nature of the adverse effects associated with a contaminant, which may be present in food. A dose-response assessment should be performed.
Exposure Assessment	The qualitative and/or quantitative evaluation of the degree of intake likely to occur.
Risk Characterisation	Integration of hazard identification, hazard characterization and exposure assessment into an estimation of the adverse effects likely to occur in a given population, including attendant uncertainties.

Risk is a function of the probability of an adverse effect and the magnitude of that effect, consequential to a hazard in food (FAO/WHO, 2006). Risk analysis is a systematic process that investigates the potential adverse health effect consequential to a hazard or condition of a food, develops guidance for mitigating that risk and promotes interactive communication among interested parts.

Three components make up risk analysis: science-based risk assessment, policy-based risk management and interactive risk communication (**Figure 27**). Risk analysis promotes consistency across different scientific disciplines by using the concept of risk

Figure 27. Risk analysis. [source: modified from Milićević et al., 2010]

to establish a priority or level of protection for a food regulatory measure. Risk analysis has thus become the central pillar in the drawing up of food control measures.

In the present study, risks to food safety with regard to the potential carry-over of feed-borne chemical contaminants (s. 1.4.4) and ANF (**Table 2**) from plant-based diets of farmed Atlantic salmon are evaluated, with a special focus on mycotoxins and phytoestrogens. The hazards of the relevant substances are largely identified and adverse effects in humans have been characterised. However, the assessment of exposure to different substances from the consumption of salmon-containing food products is challenging. Both, the occurrence in the edible parts of salmon and the intake of salmon have to be determined before human exposure can be calculated.

FAO has estimated that the mean global consumption of marine and freshwater fish is now above 20 kilos per person, an increase from under 10 kilos in 1960, which was mostly possible due to growth in aquaculture (FAO, 2016). Fish consumption in countries such as Norway, Portugal and South Korea is at more than 50 kg per person

each year. In a survey on the frequency of seafood consumption, more than 50% of the population in ten countries including Norway answered that they eat two or more seafood meals per week (**Figure 28**).

In Norway, national health authorities have recommended the intake of 2 to 3 meals with fish per week for all age groups, in total about 300-450 g fish for adults including 200 g of fatty fish, which is also considered sufficient to meet the body's requirement for vitamin D and essential amino acids (VKM, 2014).

The Norwegian Scientific Committee for Food and Environment (VKM) concluded in their assessment of consumer risk from exposure to chemical contaminants, mercury and veterinary medicinal products including residues of antibiotics in farmed fish that the connected risk was negligible and of no concern considering the recommended tolerable weekly intake (TWI) of the respective substances and the intake of fish products in the Norwegian population (VKM, 2014). With regard to mycotoxins, VKM found that information on transfer from feed into fish was scarce, but that the few available data suggested no risk for the consumers. However, when salmon were exposed to standard feed added with 2 and 6 mg/kg pure DON or 0.8 and 2.4 mg/kg OTA for eight weeks, DON distribution to liver, kidney, plasma, skin, brain and muscle was observed (Bernhoft et al., 2017). The concentrations in liver and muscle accumulated significantly from 3 to 8 weeks of the trial. At study end, the maximal mean DON concentrations in salmon muscle were 8.5 and 18.6 µg/kg for the low and high

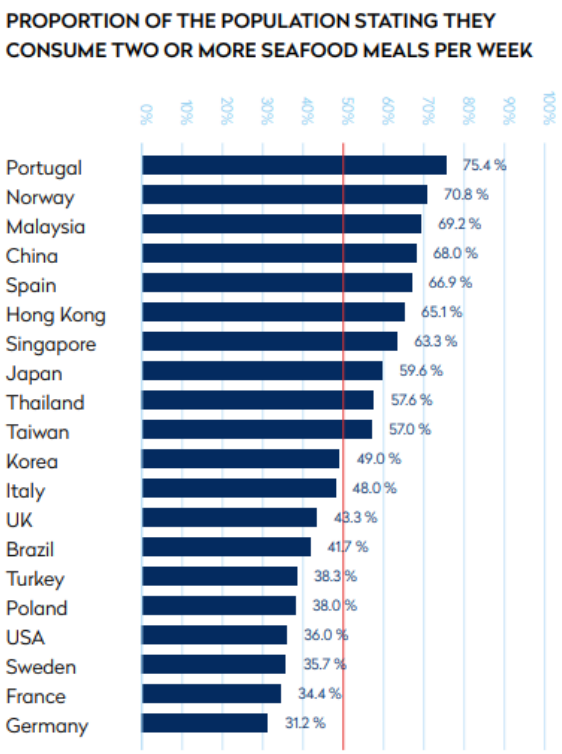


Figure 28. Proportions of the population stating that they consume two or more seafood meals per week in different countries. [source: Norwegian Seafood Council, 2019]

dose, respectively. In contrast, OTA was mainly found in liver and kidney, with concentrations decreasing over time. Nevertheless, using Norwegian food consumption data for the estimation of human exposure from DON in salmon fillet showed that the predicted risk to human health was negligible. VKM identified missing transfer data for contaminants and ANF in new feed ingredients from feed to fish as a data gap and recommended the investigation of their impact on fish health and consumer safety (VKM, 2014).

2.0 Aims and Objectives

The safety assessment of chemical contaminants including natural toxins, agrochemicals, veterinary drugs, and environmental pollutants is a key element of the "farm-to-fork" ("One Health") approach. The project 'SafeFish' extends this principle to the evaluation of fish feed ingredients in a "feed-to-fish-to-fillet" perspective with focus on the possible transfer of mycotoxins, phytoestrogens and peptides from wheat (gluten), soybean and pea.

This PhD project has been conducted as part of the project 'SafeFish' with the aim of assessing the carry-over potential of mycotoxins and phytoestrogens from plant-based ingredients in aquafeeds into fish and implications on fish health and food safety. The PhD thesis addresses the following objectives:

Objectives:

1. Establishment of a validated quantitative liquid chromatography high-resolution tandem mass spectrometry (LC-HRMS/MS) method for the simultaneous analysis of multiple mycotoxins and phytoestrogens in feed and fish matrices, including an optimised preparation of zebrafish and salmon samples and evaluation of the carry-over potential of these anti-nutritional factors from feed to fish [**Paper I**].
2. Elucidation of the biotransformation of major phytoestrogens in salmon using two newly developed *in vitro* metabolism models, i.e. liver microsomes and primary hepatocytes of Atlantic salmon [**Paper II**].
3. Evaluation of the health effects of the partial inclusion of wheat gluten, soybean or pea proteins into fish diets by examining transcriptomic changes in different tissues of zebrafish and salmon [**Paper III** and **Paper IV**].

3.0 Methodological considerations

3.1 Principle outline of the workflow

As an interconnecting part of the SafeFish project, this PhD study has contributed considerably to several of the work packages (**Figure 29**). Effects of the partial inclusion of plant proteins on the growth performance and overall health of zebrafish and salmon

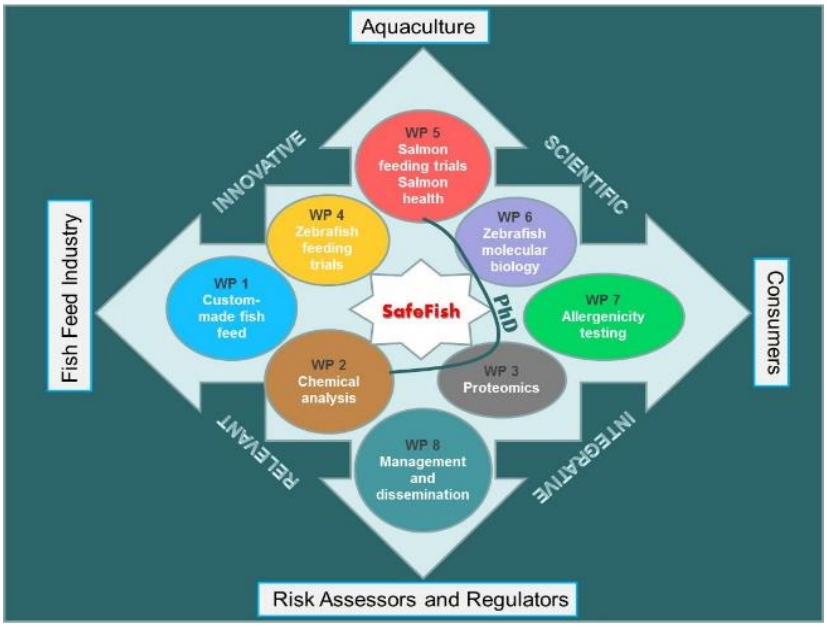


Figure 29. Outline of the Project "SafeFish" showing the different work packages, and the interconnection with the PhD project.

were investigated as given in the PhD project outline (**Figure 30**). Furthermore, concentrations of plant-derived ANF were determined in fish to evaluate potential risks to consumer safety.

The PhD objectives were completed in four phases (**Figure 30**): The study started with feeding trials in salmon and zebrafish with customised diets containing two inclusion levels of either WG, SPC or PPC [**Phase I**]. Advanced chemical analysis by multi-analyte LC-HRMS/MS was applied for the identification of relevant mycotoxins and phytoestrogens in the feeds and in the fish [**Phase II**]. The biotransformation pathways of three major soybean isoflavones were analysed in newly developed and enzymatically-characterised salmon *in vitro* metabolism assays for hazard

characterisation [**Phase III**], and finally, the implications of plant-based diets on fish health were analysed using transcriptomics [**Phase IV**].

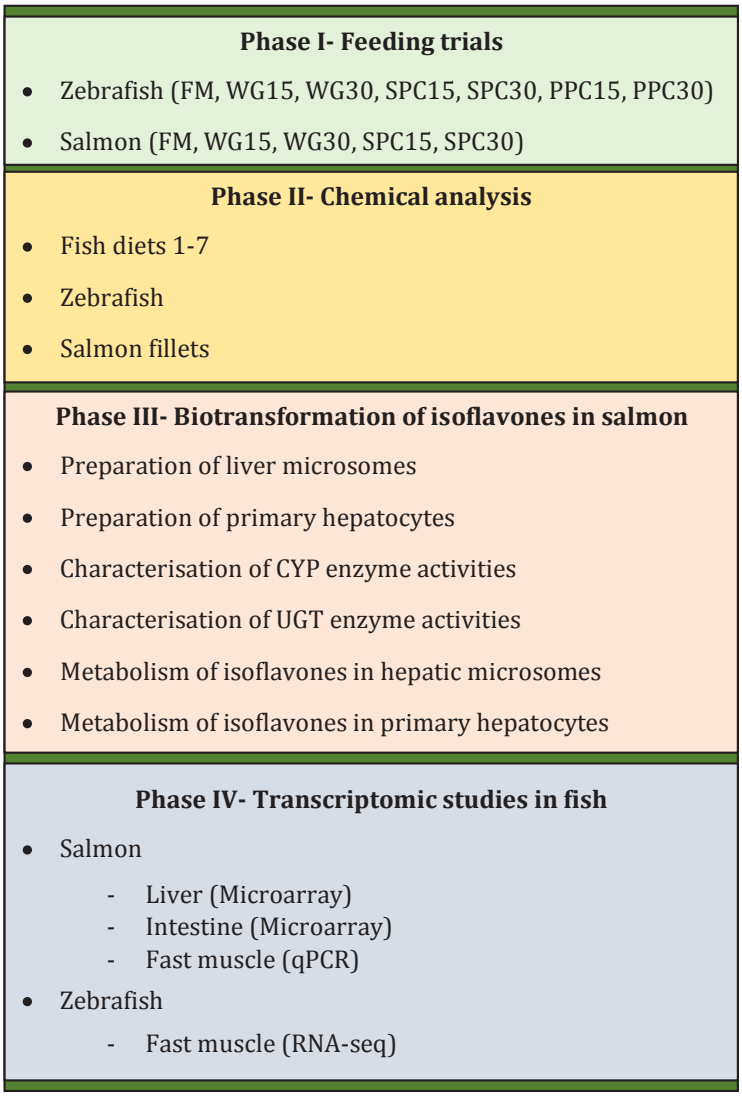


Figure 30. Outline of the PhD project.

3.2 Feeding trials

3.2.1 Ethical considerations

The salmon and zebrafish studies were performed in compliance with the laws regulating experimentation with live animals in Norway. The experimental protocols were approved by the Norwegian Animal Research Authority (Forsøksdyrutvalget/

NARA). The feeding studies were carried out in accordance with the relevant EU guidelines and regulations (EU Directive, 2010). The salmon feeding trial was considered as not requiring a specific license, whereas the zebrafish feeding study required a license (FOTS ID 12581, 27 July 2017) and was approved by the Nord University's (Norway) ethics committee.

3.2.2 Study design

The feeding trials were performed in specialised research centres using custom-made fish feed for zebrafish and on-growing salmon. Mycotoxins and phytoestrogens were measured in feed and the edible parts of the fish by a newly developed and validated multi-analyte LC-HRMS/MS method. Different sample preparation procedures were investigated and optimised for the chosen analytes and matrices. Hepatic metabolism of soybean isoflavones was analysed using in-house prepared and characterised salmon liver models. Nutrigenomic effects of plant protein-containing-diets (WG, SPC) in zebrafish and salmon were identified by measuring global transcriptomic changes in fast muscle using RNA-seq. Furthermore, changes in the intestinal and hepatic physiology in salmon induced by WG or SPC in the diets were determined by direct comparison to control fish receiving FM-containing feed only. Transcriptomic analysis of relevant markers genes was applied for liver samples. At the same time, microarray analysis was used to detect the effects on gut health.

In additional studies performed as part of the SafeFish project, epigenetic changes in the zebrafish intestine from feed obtaining WC, SPC or PPC were investigated by analysing genome-wide methylation patterns. Epigenetic alterations associated with a WG-based diet were also analysed in the livers of Atlantic salmon, and transcriptomic changes were evaluated in spleen of zebrafish fed with WG, SPC or PPC-containing feed. Moreover, the carryover of gluten peptides from feed into fish muscle were measured by LC-HRMS/MS-based proteomic analysis and immunological methods using sera of patients with wheat allergy (**Figure 29**). These experiments were not part of this PhD project, but supported its conclusions. Thus, the combination of the different findings helped to get an extensive understanding of the consequences of plant protein-containing aquafeeds for fish health and consumer safety.

3.2.3 Preparation of customised plant protein-based aquafeeds

The different feed types were custom-made for zebrafish and salmon at Nofima's Feed

Technology Centre (FTC) in Fyllingsdalen, Norway. In total, seven diets were prepared: (1) control feed with fish meal (FM) as the only protein source, and six feeds with partial replacements of FM by, (2) 15% wheat gluten (WG15), (3) 30% wheat gluten (WG30), (4) 15% soybean protein concentrate (SPC15), (5) 30% soybean protein concentrate (SPC30), (6) 15% pea protein concentrate (PPC15), and (7) 30% pea protein concentrate (PPC30) (**Table 8**).

Table 8. Composition of the custom-made salmon and zebrafish feeds.

Ingredients							
	FM	**WG15**	**WG30**	**SPC15**	**SPC30**	**PPC15**	**PPC30**
Fish meal (FM)	63.3	48.3	33.3	48.3	33.3	64.3	49.3
Fish oil (extrusion)	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Fish oil (pellet coating)	16.0	16.0	16.0	16.0	16.0	16.0	16.0
Wheat (as binder)	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Wheat gluten (WG)		15.0	30.0				
Soybean prot. conc. (SPC)				15.0	30.0		
Pea prot. conc. (PPC)						15.0	30.0
Additives	4.7	4.7	4.7	4.7	4.7	4.7	4.7
Sum	100	100	100	100	100	100	100

All diets contained 12% wheat that was required for binding in the extrusion process, and 4% FO. The different diets were produced in pilot scale, dried, and oil-coated with 16% FO. The salmon feed had a pellet size of 3.5 mm, while the zebrafish feed were ground and sieved to a pellet size of 0.6–0.8 mm. The feeds were formulated with the aim to keep the protein-to-energy ratio constant and to supply the dietary needs for protein, but were not formulated to be iso-caloric or iso-nitrogenous. The replacement of FM by alternative raw materials ensured that results from the exposure trials could be considered as a direct effect of the protein source. Balancing of the diets might have masked these correlation. It should be mentioned, however, that the 30%-inclusion of plant proteins used in the present study was higher than the level used in commercial aquafeeds suggesting that the observed results in the present study may represent an overestimation. A considerable number of studies have been performed studying the health effects of plant proteins in fish, but an optimal level of inclusion with regard to fish health is still not totally clear.

The raw materials were purchased from FTC's usual suppliers. The FM used in the control feed was a mix from several fish species, with blue whiting (*Micromesistius*

poutassou) as the major component and minor quantities of byproducts from herring (*Clupea harengus*), whitefish (*Coregonus clupeaformis*) and Atlantic mackerel (*Scomber scombrus*). The ingredients used for the preparation of diets included FM from Norsildmel AS (Bergen, Norway), WG from Tereos Syral (Marckolsheim, France), SPC from Agilia A/S (Videbæk, Denmark) and PPC from AM Nutrition AS (Stavanger, Norway). All diets contained FM to provide sufficient amounts of fatty acids and nutrients to meet the demands of the fish. Additional nutrients were not added into the diets, and they were not balanced for amino acid concentration. The diet compositions and feed preparations are explained in detail in **Paper I**.

3.2.4 Fish husbandry and sampling

Salmon: The feeding trial and sampling of salmon was conducted at the Nofima's Research Station at Sunndalsøra, Norway. The rearing conditions are described in **Paper I**. Briefly, one-year-old post-smolt Atlantic salmon with a mean weight of 223 g were randomly distributed into 15 experimental tanks (1 m^3) filled with seawater (**Figure 31**). The fish were fed in excess to ensure optimal feed intake. Feed rations were based on appetite rather than calculations based on body weight. The amount of feed was adjusted daily, aiming at 20% over-feeding based on the preceding 2 to 3 days' feed intake. The waste feed was collected daily and feed intake was estimated as described in **Paper I**.

Figure 31. Salmon research tanks with automatic feeders and excess water collectors at Nofima, Sunndalsøra, Norway.

The set up for the salmon study included in total 449 fish divided between five dietary groups (FM, WG15, WG30, SPC15, SPC30) in triplicate tanks (n=30 fish per tank except for one FM diet tank with n=29) (**Figure 32**).

The feeding trial was conducted for 9 weeks. Samples were taken at three time points, i.e. at start, after 5 weeks, and at the end of the feeding trial. Samples were collected from 15 fish at the start of the experiment, belonging to the same stock as the fish included in the study. One fish each from the FM and 30% dietary group was sampled

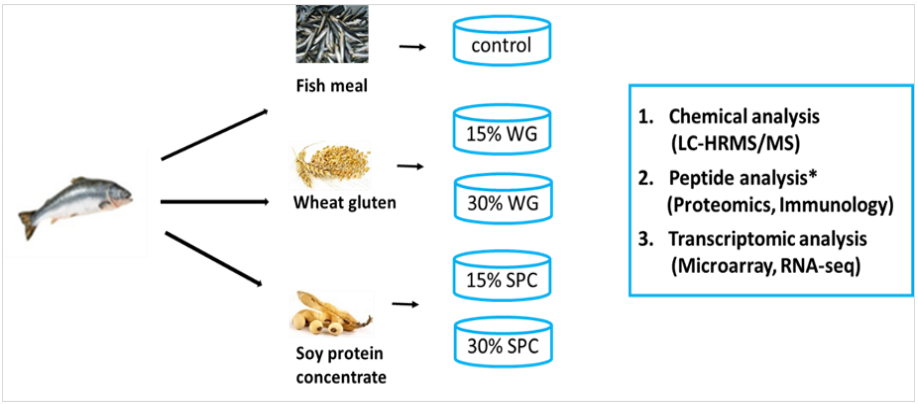


Figure 32. Experimental set-up of the salmon feeding trial.
*Peptide analysis was performed in the SafeFish project, but was not part of the PhD project.

intermediately during the study, and at study end, five fish from each tank (in total = 75 fish) were sampled for the chemical, peptide and microarray analyses, and the remaining fish were weighed in bulk. The fish were dissected, and muscle, intestine, liver and serum were collected and stored at -80 °C until further analysis (**Paper I** and **Paper III**) (**Figure 33**).

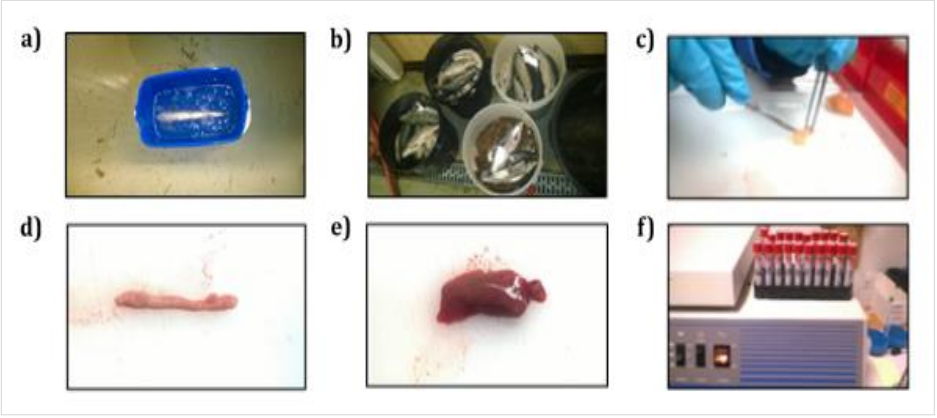


Figure 33. Preparation of salmon samples; a) fish anesthetisation; b) bulk weight assessment for non-filleted fish; c) sampling of muscle; d) mid-intestine, e) liver, and f) blood for the separation of serum and analysis of blood parameters.

Zebrafish: Four-month-old zebrafish with a mean weight of 0.214 g were distributed into 28 3.5 L-tanks in an automated flow-through system at the Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway (**Figure 34**). The experimental conditions are explained in detail in **Paper I** and **Paper IV**.

The feeding study included 336 fish in four replicate groups per each of the seven different diets (FM, WG15, WG30, SPC15, SPC30, PPC15, PPC30) (**Table 8**). Each tank contained six per gender and four spares for the compensation of potential losses, i.e. in

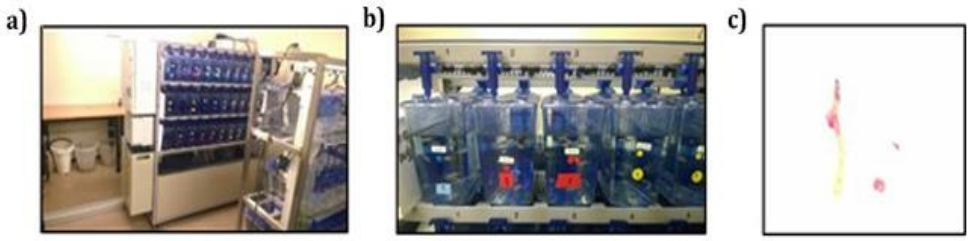


Figure 34. a) Stand-alone rack with flow-through system used in the zebrafish feeding trial; b) Tanks were marked to identify the diet groups; c) Intestine, spleen and liver were sampled.

total 16 fish. They were hand-fed twice daily with a total feed amount equal to 2.5% of their body weight over a period of 46 days. Pea protein concentrate was included in the

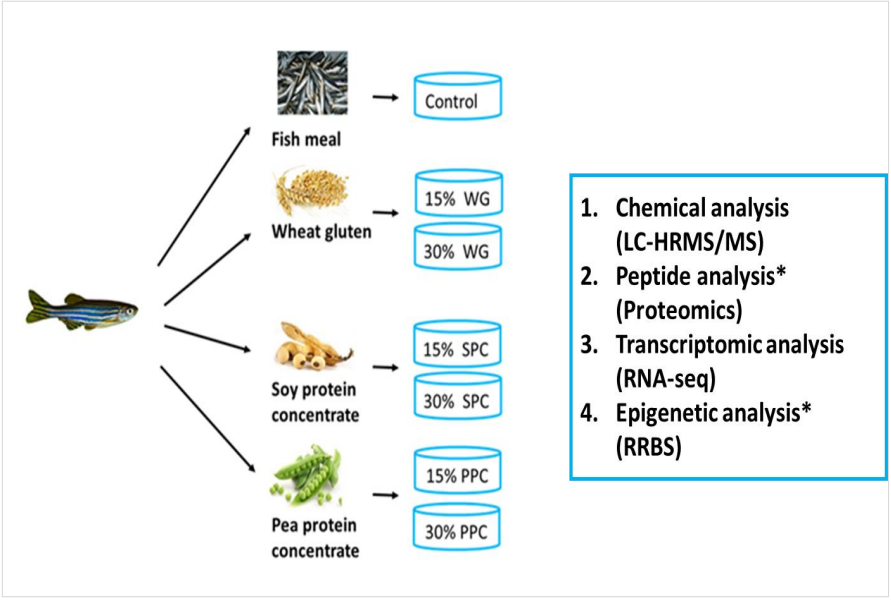


Figure 35. Experimental set-up of the zebrafish feeding trial.
*Peptide analysis and Epigenetic analysis were a part of SafeFish project but not the part of PhD project.

experiment in addition to the diets used for salmon (**Figure 35**). At the end of the study, the fish were not fed for 24 h, separated by gender and euthanised individually by transfer into a tank containing a lethal dose of MS222. The intestine, spleen and liver were carefully dissected under a light microscope (**Figure 34 c**) and immediately frozen

in liquid nitrogen along with the rest of the carcass. All samples were stored at −80°C until further analyses.

3.3 Analytical methods

In the following, a short overview of the main methodologies used for the experiments in the PhD project and described in the Papers I, II, III and IV is given (**Table 9**).

Table 9. Overview of experiments and methodologies included in the PhD project.

Fish	Diets	Tissues analysed	Analyses	Paper No.
Atlantic salmon (one-year-old post-smolt; 223 g at study start)	FM, WG15, WG30, SPC15, SPC30	Muscle	Chemical analysis (LC-HRMS/MS)	I
	FM, WG15, WG30, SPC15, SPC30	Muscle	Transcriptomics (RNA-seq)	IV
	FM, WG15, WG30	Intestine	Transcriptomics (Microarray)	III
	FM, WG15, WG30	Liver	Transcriptomics (Microarray), Liver fat (%)	III
	FM, WG15, WG30	Serum	AST, ALT, FFA, TG, Tprot levels (Enzyme activities)	III
	isoflavones (*in vitro*)	Liver Microsomes/ Primary Hepatocytes	Biotransformation study	II
Zebrafish (four-month-old; 0.214 g at study start)	FM, WG15, WG30, SPC15, SPC30 PPC15, PPC30	Whole body (beheaded)	Chemical analysis (LC-HRMS/MS)	I
	FM, WG15, WG30, SPC15, SPC30 PPC15, PPC30	Muscle	Transcriptomics (RNA-seq)	IV

3.3.1 Chemical analysis

The identification of ANF such as mycotoxins and phytoestrogens in feed ingredients, aquafeeds and fish is important for the assessment of possible health risks from the

consumption of fish fed with plant-derived protein sources (**Paper I**: Johny et al., 2019). Different methods for the individual or simultaneous detection of mycotoxins using liquid chromatography mass spectrometry (LC-MS/MS) in various matrices have been developed, but only few have been applied to fish (Nácher-Mestre et al., 2015; Bernhoft et al., 2017; Gonçalves et al., 2018; Sele et al., 2019). This is even more the case for phytoestrogens, for which just a small number of LC-MS/MS applications for food products have been reported (Kuhnle et al., 2008). Therefore, an LC-HRMS/MS method for the simultaneous determination of naturally-present mycotoxins and phytoestrogens in plant-based aquafeeds and fish matrices was developed and validated in accordance with the relevant ISO standard 17025 (ISO/IEC, 2018) and EU guideline SANTE/12089 (EU, 2016) as first objective of this PhD study. The new method included 19 mycotoxins and 6 isoflavones that are important in the Norwegian context.

3.3.1.1. Preparation of samples for instrumental analytics

Sample preparation is an essential part of every analytical technique and decisive for the assay performance. Sampling strategies for the mitigation of uneven contaminant distribution in different matrices with the aim to achieve homogeneity have been described (Berthiller et al., 2017). Analytes are often extracted by single-step extraction using lipophilic/hydrophilic solvent mixtures, followed by solid-phase extraction (SPE) or immunoaffinity purification (Sun et al., 2015). Matrix effects can be controlled by

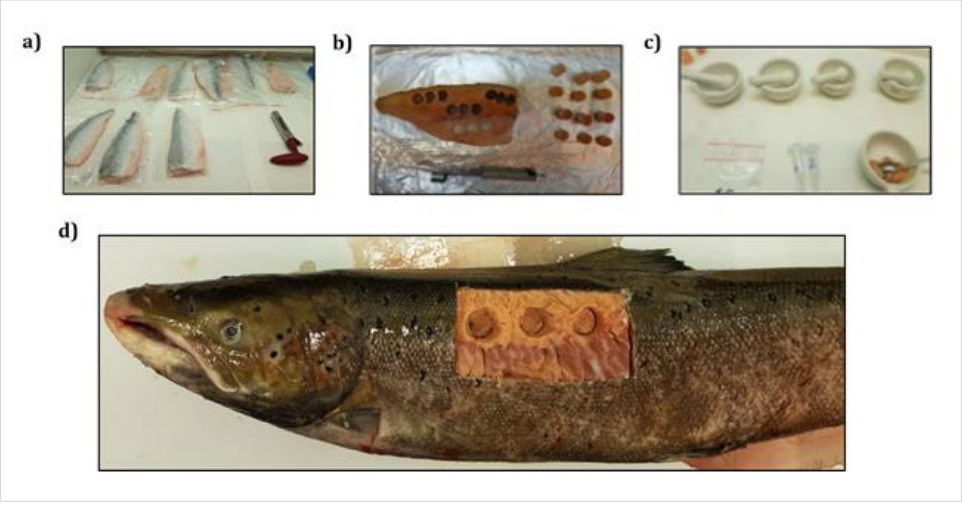


Figure 36. Preparation of salmon samples; a) salmon fillets; b) sampling scheme for homogenous sampling of representative aliquots from a salmon fillet; c) sample homogenisation in liquid nitrogen; d) wild salmon used as a negative control in peptide analysis.

using matrix-matched calibration and isotope-labelled internal standards (ISTD).

Regarding the samples in this project, potential distributional heterogeneity was not an issue in the preparation of zebrafish samples since the whole carcasses of three fish were ground and extracted together. In contrast, the salmon fillets were of considerable size and could not be processed as a whole. Therefore, representative samples were obtained by punching out tissue at different places in the fillet and combining aliquots after grinding (**Figure 36**). The custom-made, extruded diets had already a high degree of homogeneity due to the production process so that targeted analytes were presumably evenly distributed, and samples were taken from a few places in the storage bags and ground together.

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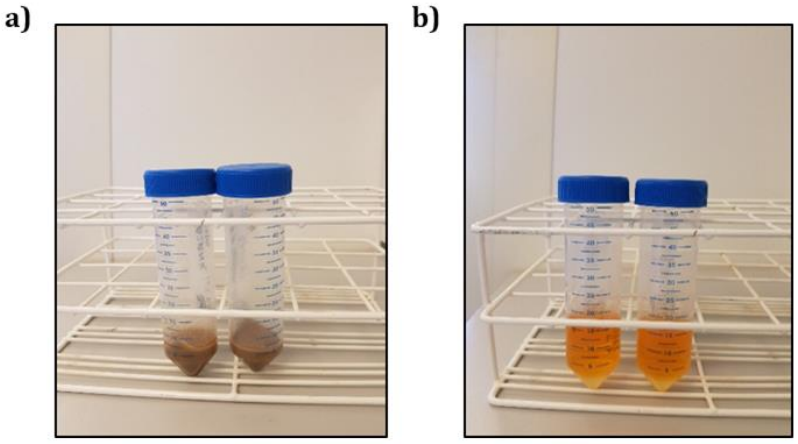

Figure 37. Extraction of fish feed with the validated one step-extraction method; a) fish feed and b) extract of fish feed.

presumably evenly distributed, and samples were taken from a few places in the storage bags and ground together. The mycotoxins and phytoestrogens were extracted from the different matrices using acidic acetonitrile (MeCN)/water (70:30) after testing other

solvent mixtures during and optimisation process (**Figure 37**) (Johny et al., 2019). Homogenised tissue was diluted about ten-fold dilution with the extraction mixture, extracted for 1 hour at room temperature and passed through a submicron filter.

3.3.1.2 Detection by liquid chromatography-mass spectrometry

Liquid chromatography (LC) is a versatile technique for the separation of analytes using molecule properties such as lipophilicity, size and polarity based on partition between specific stationary phases and appropriate mobile phases. The ratio of an analyte's molar concentrations in the stationary phase and the mobile phase is defined as partition coefficient, which is substance-specific and defines the retention time on the chromatographic column. Different analyte mixtures can be separated by using optimised combinations of column fill materials and solvents. After injection of the sample on the column, the analytes are eluted either isocratically by fixed solvent combinations or with a gradient. Depending on the applied separation principle and connected column fill, LC can be classified in, among others, normal phase LC, reversed phase (RP) LC, size-exclusion (SEC) LC and ion-exchange (IEC) LC and hydrophilic interaction (HILIC) LC. RPLC is the most commonly used technique in the analysis of natural toxins, which are separated on non-polar stationary phases by elution with polar mobile phases such as water, methanol, acetonitrile or tetrahydrofuran. In high-performance LC (HPLC), high pressure is applied to achieve chromatographic separation with considerably shortened run times. The retention times of the analytes should be different enough to produce detection signals (chromatographic peaks) that are sufficiently resolved. Resolution (R) is

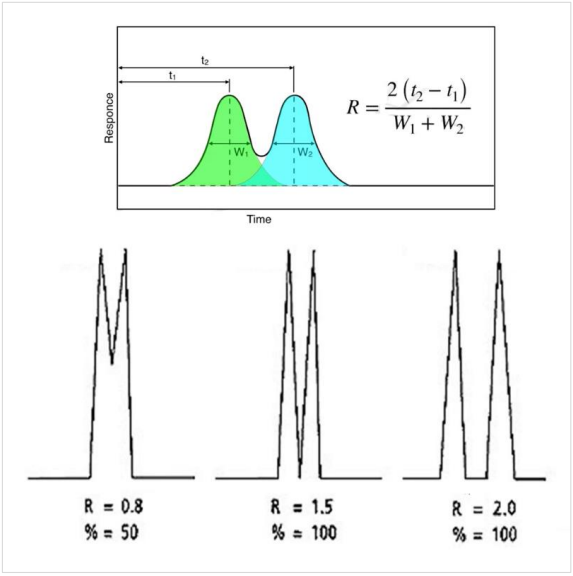

Figure 38. Definition of chromatographic resolution (R) (upper panel), and symmetric analyte peaks separated with R= 0.8, 1.5, or 2.0 (lower panel). Percentage of separation is given. [source: Rich, 2016]

calculated as the difference in retention times (t) of two eluting peaks divided by the average width (W) of the two peaks at baseline (**Figure 38**). It should be at least be 0.8, meaning 50% separation at half of the maximum peak height, whereas a resolution of 1.5 is equivalent to 100% baseline separation.

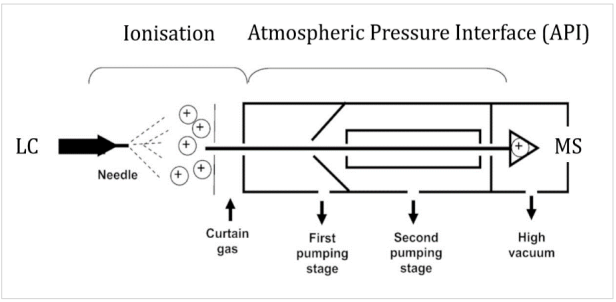

Figure 39. LC-MS interface at atmospheric pressure. [source: modified from Brima et al., 2006]

In the present study, RPLC has been used for the separation of the mycotoxins and isoflavones including their respective metabolites as discussed in the **Paper I** and **Paper II**.

Results of chromatographic separation are made visible by coupling with different detectors. Most commonly used are spectrophotometric (UV-Vis), fluorimetric, electrochemical or mass spectrometric (MS) devices. Mass spectrometry differentiates between ionised analytes according to their mass-to-charge ratio (*m/z*) with high specificity and analytical sensitivity. LC-MS has thus become a frequently used, powerful tool in instrumental analytics. The technique requires the vaporisation and ionisation of the LC eluents, before they enter the MS. The development of interfaces with atmospheric pressure ionisation (API), where ions are generated at ambient atmosphere and sprayed into the low-pressure region of a mass analyser before they are drawn into the high-vacuum part, has greatly increased the applicability of LC-MS (**Figure 39**).

Several types of API sources are available, including the often used Electrospray Ionisation (ESI), Atmospheric Pressure Chemical Ionisation (APCI) and Matrix-Assisted Laser Desorption Ionisation (MALDI) (Bruins, 1991). Of these, ESI has proven its great suitability for the ionisation of a wide range of different analytes such as natural substances, e.g. mycotoxins and isoflavones. It is a soft ionisation technique, because very little residual energy is retained by the analytes and consequently, fragmentation occurs less frequently than with other ionisation methods. HPLC-ESI-MS is often applied in the analysis of small and large molecules of varying polarity in complex matrices. The underlying ionisation mechanism and ion formation consist of the production of charged droplets at the capillary tip, solvent evaporation and the production of gas

phase ions. These processes are determined by the 1) potential difference between capillary and counter electrode, 2) ion formation at the Taylor cone, 3) solvent nebulisation, 4) droplet desolvation, 5) coulombic explosion and 6) release of charged analyte ions (**Figure 40**) (Challamalla et al., 2012). Depending on the direction of the applied voltage, protonated ions $[M+nH]^{n+}$ are produced in positive ESI, and de-protonated ions $[M+nH]^{n-}$ are produced in negative ESI.

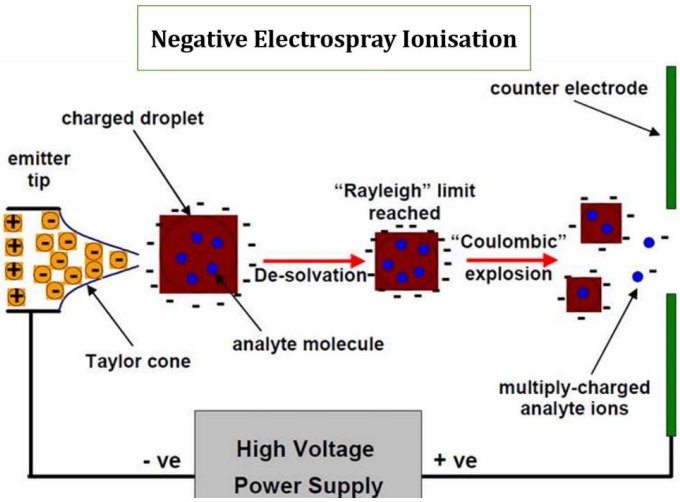

Figure 40. Ionisation mechanism in ESI.
[source: Challamalla et al., 2012]

The choice of the mass spectrometer type is critical for result quality. Commonly used mass analyser are quadrupoles (Q), time-of-flight (TOF) instruments, ion traps (IT), Orbitraps and Fourier transform ion cyclotron resonance (FT) analysers. They differ widely in their functionalities and achieve characteristic parameters for sensitivity, mass resolution, mass accuracy, scan speed and mass range (**Figure 41**). They can be used as stand-alone detectors or combined in tandem mass spectrometry (MS/MS) in typical hybrids such as Q-TOF, Q-IT, TOF-TOF, IT-FT, IT-Orbitrap, Q-Orbitrap and Triple-Q (QqQ). In MS/MS analysis, substrate ions are disintegrated into typical fragments, and the precursor-to-product ion transition is utilised for the specific identification.

MS	Resolution	Scan Speed	Mass Accuracy	Sensitivity
Quadrupole	+	+	+	+
Ion Trap	++	++	+	++
TOF	+ - +++	+++	+++	+++
FT Orbitrap	++++	+ ++	++++	++++

Figure 41. Comparison of MS analysers.
[source: drafted by K.R Jonscher, Uni. of Colorado, Denver, USA]

Quadrupoles are mass filters that let pass only selected *m/z*-values with good reproducibility but limited

resolution and mass accuracy. TOF offers improved resolution, accuracy and a high mass range. It has, however, limitations in the dynamic range and in quantitative applications. Likewise, IT is less suited for quantitative MS but can generate of multiple-stage fragmentation spectra (MS^n) that allow the elucidation of analyte structures. FT offers excellent mass accuracy and resolution power although they are unsuitable for quantification. In contrast, the more recently developed Orbitrap-analysers combine several advantages of other techniques, allowing mass determinations with high accuracy and resolution, quantitative analysis, and as Q-Orbitrap or LT-Orbitrap hybrids, quantitation and the performance of accurate MS^n-experiments.

Orbitrap hybrids, Triple-Q and Q-IT are regularly used in the analysis of natural compounds in different matrices. The MS can be operated in full scan mode, precursor

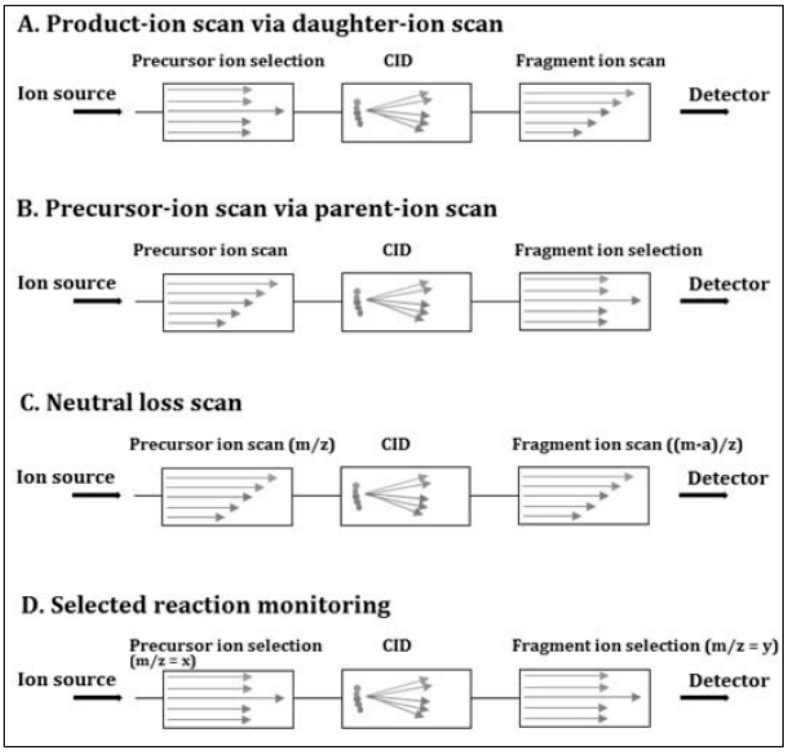

Figure 42. Different scan modes of TripleQ MS. [source: Mika et al., 2019]

ion scan, product ion scan, or neutral loss scan (**Figure 42**). In selected ion monitoring (SIM) and multiple ion monitoring (MIM), the mass filters are adjusted in such a way that only ions with specific *m/z* can pass. Selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) are methods following specific precursor ion-to-

product ion transitions and ideally suited for the unambiguous identification and quantitation of analytes. The hybrid MS combine the favourable characteristics of different mass analysers in a single instrument and have thus increased applicabilities. In the present study, a Q-Exactive instrument (Q-Orbitrap), merging the high selectivity of a quadrupole with the extraordinary sensitivity of an Orbitrap, was used for the development of a quantitative multi-analyte method for mycotoxins and phytoestrogens (**Paper I**).

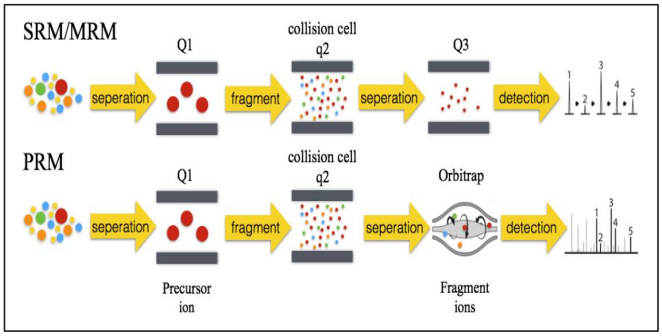

Figure 43. Comparison of selected/multiple reaction monitoring (SRM/MRM) and parallel reaction monitoring (PRM).
[source: Parallel reaction monitoring (PRM) service, 2008-2020. Figure re-used with the permission from the publisher]

Parallel reaction monitoring (PRM) in Orbitrap MS is comparable to SRM/MRM in TripleQ MS and suitable for the absolute quantification of analyte. Unlike SRM, however, which monitors one transition at a time, PRM performs a full scan of all fragments of a precursor ion (**Figure 43**). Specific precursor ions are filtered in the first quadrupole (Q1), fragmented in the collision cell (Q2), and the product ions are separated in the oscillating electric field of the Orbitrap that replaces the third quadrupole (Q3) of a TripleQ MS instruments. All product ions are thus scanned with high resolution and high accuracy, giving PRM not only the ability for targeted quantitative analysis as by SRM/MRM, but adding high result reliability by exact mass determination and the reduction of background interferences. PRM delivers fewer false positive results than SRM/MRM by improving the detection limits, i.e. the sensitivities, in complex matrices and is advantageous in the detection of minor analyte metabolites.

3.3.1.3 Optimisation and validation of analytical methods

International method standardisation organisations such as the International Organization for Standardization (ISO), European Committee for Standardisation (CEN), International Union of Pure and Applied Chemistry (IUPAC) and Association of Official Analytical Collaboration (AOAC) International have established guidelines for the determination of performance characteristics of analytical methods to allow the

comparability of measured results. ISO has issued a guide for method validation of quantitative analysis in chemical testing laboratories (ISO/IEC 17025) that defines the relevant test parameters (**Figure 44**). Furthermore, recommendations for uncertainty limits and acceptance criteria are given. The multi-analyte LC-HRMS/MS method for mycotoxins and phytoestrogens that was developed as part of this PhD project was validated in accordance with these guidelines by considering the following parameters (Johny et al., 2019):

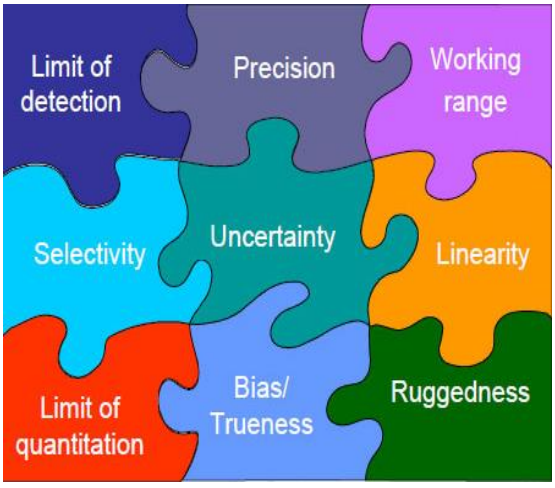

Figure 44. Criteria of analytical method validation. [source: Consultant ISO 17025, 2012]

a) Selectivity, which is defined as the ability to discriminate the target substance in a complex mixture without interferences from other substances or matrices, was determined for all included analytes. They were unambiguously identified by their specific LC retention times and accurate masses in HRMS/MS. The finished method allowed to quantitatively measure 25 substances in parallel in fish and feed matrices.

b) Sensitivity is a measure for the relationship between the concentration of the analyte and the size of the measured signal. It can be determined as the change of the instrumental response divided by the corresponding analyte concentration change, i.e. by the slope of the standard calibration curve. In the present study, six-point calibration curves were calculated by linear regression analysis for all analytes in both solvent (MeCN/water 50:50) and the different matrices. The limit of detection (LOD) and limit of quantitation (LOQ) of an analyte in a matrix are indicators for the method's sensitivity. There are three ways of determining the LOD and LOQ: They can be obtained from 1) the calibration curve, 2) the signal-to-noise ratio, or 3) the standard deviation of a blank matrix sample. The calibration curve is applicable in cases, where an analyte is measurable over a large concentration range, reaching to a multiple of the LOQ. Under these conditions, LOD and LOQ can be estimated from the calibration curve as LOD=3×SD/m and, LOQ=10×SD/m, using the slope (m) and the standard deviation (SD) of the y-intercept of the regression line (Magnussen and Örnemark, 2014). When the

signal-to-noise ratio is used, measured signals from samples with known low analyte concentrations are compared to those of matrix blanks to establish the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio of 3:1 is considered sufficient for LOD, and of 10:1 for LOQ. Finally, the standard deviation of a blank matrix sample (SD_{blank}) is derived from the measured signal intensities of multiple blank samples, and the LOD of the method is estimated as LOD = S_{blank} + $3\times SD_{blank}$. The LOD and LOQ of the 25 analytes included in the newly developed LC-HRMS/MS method were calculated with approach 1 by determining the standard deviations of the intercepts of the respective calibration curves and the corresponding slopes since they showed response linearity over a wide concentration range (Johny et al., 2019). The impact of matrix interference on the individual analyte signals was carefully elucidated by signal suppression and enhancement (SSE) experiments. SSE% is the ratio of the slope of the matrix-assisted standard calibration curve to the calibration curve in MeCN/water (50:50). If SSE% of the individual analytes were above or below 100%, respectively signal enhancement or suppression by the matrix could be assumed and the extent of the interference estimated.

c) Linearity is defined as the concentration interval of the calibration curve, in which the regression coefficient R^2 is ≥ 0.96. The working range extends from the LOQ to the upper limit of the linear concentration interval.

d) Precision is the closeness of agreement between independent test results obtained by a method under stipulated conditions (Thompson et al., 2002). It is thus a general term for the uncertainty of the method by considering the variability between repeated measurements and usually reported as standard deviation. The principal precision measures include repeatability and reproducibility of the test results. Repeatability (intra-day precision) describes the error of a method that is run under repeatability conditions in a single laboratory by the same analyst on the same equipment and within short intervals. It is expressed as standard deviation RSDr of the mean value (x_{mean}) of a least three analyte replicates or the corresponding coefficient of variation %CV ($=100\times RSDr/x_{mean}$). Reproducibility (inter-day precision) is the method error occurring either under internal reproducibility/intermediate precision/ total within-laboratory precision (RSi_R) conditions in a single laboratory on different days and possibly by multiple analysts and on multiple instruments, or under inter-laboratory reproducibility conditions (Horwitz, 1995). It is expressed as RSDR or %CV. Generally,

accepted limits are RSDr < 20% and RSDR < 30%. Precision in terms of RSi_R considering intra-and inter-day variabilities together was determined for all concentration points in the solvent and matrix-assisted standard calibration curves.

e) Accuracy and Trueness of a method are evaluated in terms of bias by using spiked samples or, preferably, (certified) reference materials (CRM). Accuracy is the closeness of agreement between a test result and the accepted reference value, whereas trueness is the closeness of agreement between the average value obtained from a large series of test results and the accepted reference value (Thompson et al., 2002). Bias describes the difference between the expectation of the test results and an accepted reference value. The total bias of a method is composed of the method bias, laboratory bias, run effect and repeatability error. An accepted reference value is an agreed-upon reference for comparison, optimally an appropriate matrix containing a definite, certified amount of the analyte. The accuracy of the 25-in-1 method was assessed by repeatedly performing recovery experiments from matrices spiked with the analyte mixture. Recoveries in the range of 50% to 150% are in general considered as satisfactory.

f) Ruggedness or Robustness of a method describes the impact of minor changes in operational parameters such as temperature, buffer compositions, pH, shaking and centrifugation velocities, instrumentation, etc. on the performance parameters. Robustness testing is useful for the identification of critical points in the workflow and valuable for method optimisation.

3.3.2 *In vitro* metabolism

Liver is the major organ for biotransformation of xenobiotics in vertebrates (s. 1.5.6.4), and *in vitro* incubations with hepatic fractions are well-established for biotransformation studies. In this study, two novel *in vitro* metabolism models for Atlantic salmon have been developed (**Paper II**: Johny et al., 2020a). The determination of important CYP and UGT enzyme activities in the in-house prepared salmon liver microsomes and primary hepatocytes was a prerequisite for their application in biotransformation experiments and comparability to published results in other species (s. 1.5.6.5).

3.3.2.1 Preparation of salmon in vitro biotransformation models

a) Microsomes

Two-year-old Atlantic salmon with a mean body weight of 200 g, kept in tanks with

seawater at the Nofima Research Station, Sunndalsøra, Norway and fed with standard aquafeed, were humanely killed by percussive stunning. The livers were excised, washed with ice-cold physiological saline and stored at -80 °C or in liquid nitrogen. Microsomes were prepared as described in **Paper II**.

Briefly, liver was cut and homogenised in 0.1 M potassium phosphate buffer (pH 7.5)

Figure 45. Potter-Elvehjem homogenisers and Dounce glass tissue grinder (left panel) used for the preparation of salmon liver microsomes (right panel).

with a Potter-Elvehjem homogeniser (**Figure 45**). The homogenate was centrifuged twice for 20 min (4 °C) at 18,000 × g and the resulting supernatant was further centrifuged for 60 min at 100,000 × g using a swing-out rotor in an ultracentrifuge at 4 °C. The precipitated pellet was re-suspended in 0.1 M potassium phosphate buffer (pH 7.5). Pellets from different centrifugation tubes were combined in a Dounce glass tissue grinder and gently homogenised. Aliquots were transferred into cryotubes for storage. All steps were carried out on ice. The total microsomal protein content was determined by Lowry Protein Assay.

b) Primary hepatocytes

Primary hepatocytes were isolated from Atlantic salmon reared at the Norwegian University of Life Sciences, Ås, Norway. Fish (480 g), fed with a commercial diet, were

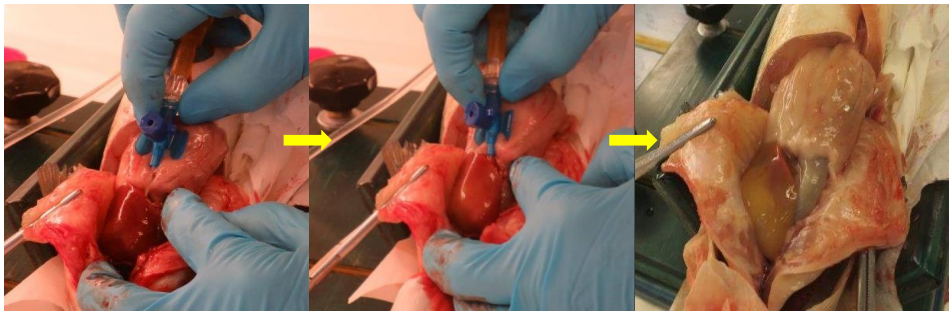

Figure 46. Liver perfusion prior to hepatocyte isolation.

anesthetised using MS-222. The abdominal cavity was opened, the portal vein cannulated and the hepatic artery severed to allow outflow. The liver was perfused *in*

situ via the cannula with a buffer containing EDTA (**Figure 46**), and subsequently with a buffer containing collagenase and HEPES. The liver were excised and the hepatocytes suspended in HEPES buffer. They were filtered, washed and separated by centrifugation. The precipitated hepatocytes were re-suspended in L-15 medium and stored for about 1 h on ice until use. The number of viable cells was determined microscopically by staining with Trypan blue and counting in a Neubauer chamber. After dilution with cold Krebs-Ringer HEPES buffer to reach the intended cell concentration and immediately used in the metabolism assays.

3.3.2.2 Characterisation of selected CYP and UGT enzyme activities

The activities of CYP P450 (s. 1.5.6.1) and UGT enzymes (s. 1.5.6.2) in hepatic fractions can be characterised by using specific probe substrates and their respective metabolism products (s. 1.5.6.5). Enzyme characterisation assays have been mainly established for use in pharmacological studies and the available substrates are optimised for specific human CYP and UGT enzymes (**Table 10**). So far, substances particularly adapted to fish biotransformation enzymes have not been described. In the present project, enzyme characterisation assays were consequently first set up with commercially available human liver microsomes, before they were applied to the in-house prepared salmon liver microsomes and primary hepatocytes. Two new methods, one for the simultaneous measurement of five CYP activities and one for five UGT activities, were developed, using

Table 10. Typical substrates and metabolites of major human CYP and UGT enzymes.

Enzyme	Substrate	Metabolite	Enzyme	Substrate	Metabolite
CYP1A2	PCN	ACP	UGT1A1	E2	E2-17GlcA
CYP2C9	TB	4-OH-TB	UGT1A4	TFP	TFP-GlcA
CYP2D6	DEX	DOR	UGT1A6	NAS	NAS-GlcA
CYP2E1	CH	6-OH-CH	UGT1A9	MA	MA-GlcA
CYP3A4	MDZ	4-OH-MDZ	UGT2B7	NLX	NLX-3GlcA

CYP substrates and respective metabolites: PCN: phenacetin, ACP: acetaminophen; TB: tolbutamide, 4-OH-TB: 4-hydroxytolbutamide; DEX: dextromethorphan, DOR: dextrophan; CH: chlorzoxazone, 6-OH-CH: 6-hydroxychlorzoxazone; MDZ: midazolam, 4-OH-MDZ: 4-hydroxymidazolam;
UGT substrates and respective metabolites: E2: β-estradiol, E2-17GlcA: β-estradiol-17-β-D-glucuronide; TFP: trifluoperazine dihydrochloride, TFP-GlcA: Trifluoperazine-N-β-D-glucuronide; NAS: N-acetylserotonin; NAS-GlcA: N-acetyl serotonin-β-D-glucuronide; MA: mycophenolic acid, MA-GlcA: mycophenolic acid-β-D-glucuronide; NLX: naloxone, NLX-GlcA: naloxone-3-β-D-glucuronide.

LC-TripleQ-MS/MS for the specific detection (by MRM) of the substrates and their metabolites (s. 3.3.1.2). Substrate depletion in assays run under the conditions of first-order kinetics was determined by regression analysis of the measured peak areas versus time curves ($A(t)= A_0 \times e^{-k_{el} \times t}$). Since first-order kinetics means elimination of a constant fraction per time unit, the initial substrate depletion constants (k_{el}) can be used to compare enzyme activities. By considering the amount of microsomal protein in the incubations, assay-independent substrate turnover numbers ($k_{cat}=k_{el}$/[micr. prot.]) can be calculated and used for inter-species comparisons. Additionally, metabolite formation rates of the CYP and UGT probe substrates can be derived from regression analysis of the initial slopes of the different concentration-versus-time curves. The salmon liver microsomes and primary hepatocytes showed the highest activities for CYP3A4- and CYP2D6-like as well as for UGT2B7-, UGT1A9- and UGT1A1-like transformations. Considering that fish genes matching to the human CYP2D are unknown (s. 1.5.6.1), it can be assumed that the reaction was catalysed by other enzymes in the CYP2 family with affinities to the probe substrate.

3.3.2.3 Fragmentation pattern analysis for the elucidation of metabolism pathways

During ionisation in mass spectrometry, molecular ions are fragmented by dissociation of energetically unstable bonds, forming unique substance-specific mass patterns (Demarque et al., 2016). Mass fragment analysis can therefore be used for the identification of substances. Metabolites show usually the same fragmentation pattern of the core molecule structure as the substance they are derived from, which facilitate their identification (Papac and Shahrokh, 2001; Zhu et al., 2013). Moreover, additional information can be gained from typical precursor ion-to-product ion transitions in MS/MS mode (s. 3.3.1.2).

In this PhD study, fragment pattern analysis was applied for the characterisation of soybean isoflavone metabolites that had been produced in *in vitro* assays with salmon liver microsomes or primary hepatocytes. In all species that have been investigated so far, Phase II glucuronidation is the major detoxification pathway of the isoflavones DAI, GEN and GLY. Furthermore, some oxidative metabolites of DAI and GEN, such as dihydrodaidzein (4′,7-dihydroxyisoflavanone) and dihydrogenistein (4′,5,7-trihydroxyisoflavanone) have been identified in human urine (Heinonen et al., 2003). Human liver microsomes transformed DAI to three monohydroxylated and three dihydroxylated metabolites and GEN to four monohydroxylated and two dihydroxylated

metabolites, and similar products were observed in rat microsomes (Kulling et al., 2001). In the salmon liver fractions, isoflavone-7-O-glucuronides were identified as the main metabolites based on the measured exact masses, fragmentation patterns, and retention times. In contrast, the production of oxidative metabolites was insignificant

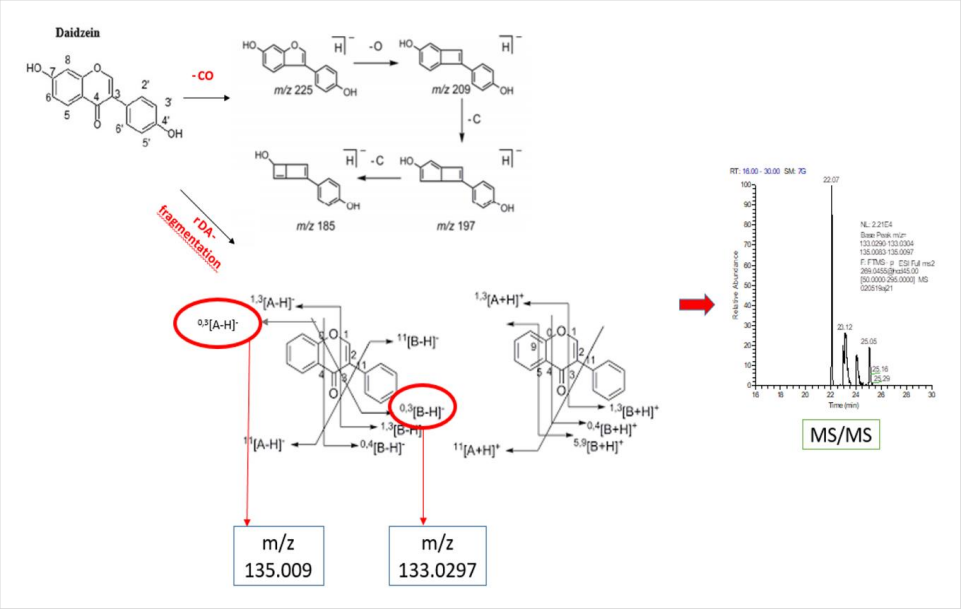

Figure 47. Fragmentation of Daidzein in LC-MS/MS. Characteristic double neutral loss of CO in isoflavones; fragmentation of the molecular structure through retro-Diels Alder rearrangement resulting in typical fragment ions of *m/z* 133.0297 and *m/z* 135.0090; MS/MS fragment ion chromatogram of four DAI-OH metabolites with ion mass *m/z* 269.0455 with typical isoflavone fragments at *m/z* 133.0297 and *m/z* 135.0090.

and only small amounts of mono-hydroxylated DAI were detectable using full-scan LC-MS followed by PRM for specific metabolites (**Figure 47**) (Johny et al., 2020a). DAI-OH was identified by a mass shift of *m/z* 16, whereas a neutral loss of *m/z* 176 was significant for the glucuronide conjugates. Isoflavones show a typical fragmentation pattern resulting from a double neutral loss of –CO (Kuhn et al., 2003). Other diagnostic product ions include two typical product ions resulting from a retro-Diels-Alder (rDA) rearrangement of the isoflavone molecular structure at *m/z* 133.0297 and *m/z* 135.009 (Kang et al., 2006), which supported the identification of Phase I metabolites as mono-hydroxylated DAI-OH metabolites. Metabolite profiling experiments can consist of identification, structural elucidation and quantification steps (Kostiainen et al., 2003),

but this preliminary study was limited to the identification of the newly discovered isoflavone biotransformation products in salmon.

3.3.3 Transcriptomic profiling

Transcriptome referes to the protein-coding part of organisms genome and consists of a set of different RNA species including messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), non-coding RNA (nc-RNA) and microRNA (miRNA) that are present in the cells (Kaur, 2013). Trancriptome analysis gives a global overview of the current gene expression pattern in different tissues, blood, cell cultures, etc. Gene regulation in samples taken under normal (control) and test conditions can be compared and up- and down-regulation due to exposure to specific external factors determined. State-of-the-art techniques for transciptomic profiling are microarray hybridisation and RNA sequencing (RNA-seq) (Anamika et al., 2016).

3.3.3.1 Microarray

Microarray hybridisation is a technique allowing the quantitative assessment of specific mRNA in a particular tissue or cell that is directly correlated with the level of expression of that particular gene (Masotti et al., 2010). After extraction of the mRNA from the sample, complementary DNA (cDNA) strands are produced using labelled nucleotides (**Figure 48**). The labelled cDNA is then hybridised with selected complementary probes on a glass slide or nylon material. The colour intensities in this microarray, which consist of a known set of gene sequences (genome), can be quantified by chemiluminescence or confocal fluorescent scanner, and are directly related to the amount of target mRNA, reflecting the expression level of the individual genes. Thus, microarray hybridisation is a valuable tool for

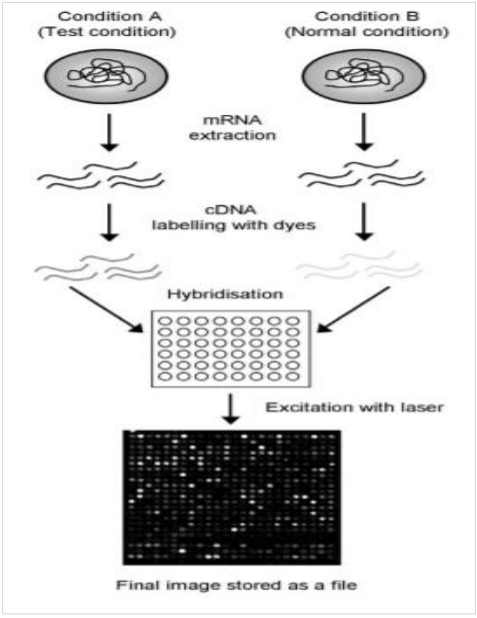

Figure 48. Microarray hybridisation as a tool to study the differential expression of genes.
[source: Babu, 2004. Figure re-used with permission from the author and the publisher]

comparative studies, where effects of different treatments on metabolic processes are compared. Microarray can give important information in nutritional systems biology by identifying nutritional biomarkers. The impact of single nutrients or mixtures can be investigated, and thereby the safety of novel feed ingredients can be evaluated (Masotti et al., 2010).

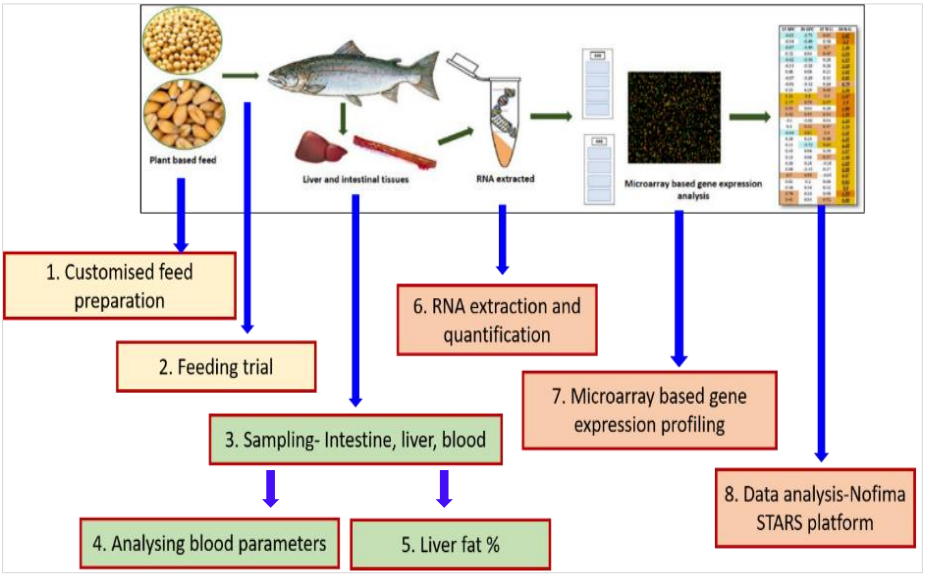

Figure 49. Microarray-based transcriptomic analysis of tissues of salmon fed with plant protein-based diets.

In this study, an oligonucleotide microarray with 4 × 44k 60-mer probes, manufactured by Agilent Technologies (Santa Clara, CA, USA), was used for the analysis of liver and intestine of salmon fed with WG- and SPC-containing diets (**Paper III**: Johny et al., 2020b (submitted)). The sample preparation, microarray hybridisation, washing and scanning were performed according to the manufacturer's instructions using the Low Input Quick Amp Labelling Kit (**Figure 49**). Gene expression data were processed and analysed with Nofima's bioinformatics system STARS (Salmon and Trout Annotated Reference) (Krasnov et al., 2011).

3.3.3.2. RNA-seq

RNA-seq for genome-wide transcriptome characterisation has become possible due to the availability of high-throughput sequencing instruments. Next Generation Sequencing (NGS) enables the sequencing of millions of nucleotide fragments in parallel, making RNA-seq a powerful tool for transcriptome profiling without the necessity for

prior knowledge of genome sequences, which is required in microarray hybridisation analysis (Anamika et al., 2016). With RNA-seq, the transcriptome assembly can be performed reference-guided or *de novo*, depending on the availability of a reference genome. Downstream data processing such as expression profiling, gene ontology and pathway enrichment analyses is helpful in giving a deeper understanding into gene regulation events from e.g. exposure to specific diet components or contaminants.

During this PhD study, global transcriptome changes in fast muscle of zebrafish and salmon fed with plant protein-based diets were analysed using RNA-seq technology (**Paper IV**: Dhanasiri et al., 2020b (submitted)). The samples were prepared for NGS

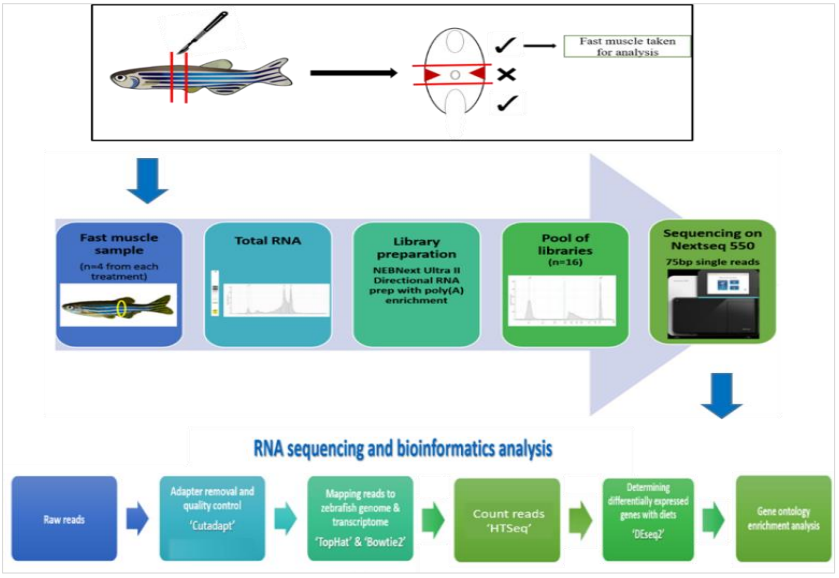

Figure 50. Sample preparation for NGS.

analysis in several extraction steps and the RNA quality was controlled (**Figure 50**). After data pre-processing, transcriptome assembly, data quantitation, statistical analysis and functional annotations, several up- and down-regulated genes were identified. Differentially expressed genes in zebrafish were compared to the results from on-growing Atlantic salmon. The expression of selected Atlantic salmon paralogues of the zebrafish homologues was analysed using paralogue-specific quantitative polymerase chain reaction (qPCR) assays.

4.0 Summary of Papers I to IV

Paper I

Development and validation of a liquid chromatography high-resolution mass spectrometry method for the simultaneous determination of mycotoxins and phytoestrogens in plant-based fish feed and exposed fish

Amritha Johny, Christiane Kruse Fæste, André S. Bogevik, Gerd Marit Berge, Jorge M.O. Fernandes, Lada Ivanova
Toxins 2019, 11:222-243. https://doi.org/10.3390/toxins11040222

New protein sources in fish feed require the assessment of the carry-over potential of contaminants and anti-nutrients from feed ingredients into the fish, and the assessment of possible health risks for consumers. Presently, plant materials including wheat and legumes make up the largest part of aquafeeds, so evaluation of the transfer capabilities of typical toxic metabolites from plant-infesting fungi and of vegetable phytoestrogens into fish products is of great importance. With the aim of facilitating surveillance of relevant mycotoxins and isoflavones, we have developed and validated a multi-analyte LC-HRMS/MS method that can be used to ensure compliance to set maximum levels in feed and fish. The method performance characteristics were determined, showing high specificity for all 25 targeted analytes, which included 19 mycotoxins and three isoflavones and their corresponding aglycons with sufficient to excellent sensitivities and uniform analytical linearity in different matrices. Depending on the availability of matching stable isotope-labelled derivates or similar-structure homologues, calibration curves were generated either by using internal standards or by matrix-matched external standards. Precision and recovery data were in the accepted range, although they varied between the different analytes. This new method was considered as fit-for-purpose and applied for the analysis of customised fish feed containing wheat gluten, soy or pea protein concentrate as well as Atlantic salmon (*Salmo salar*) and zebrafish (*Danio rerio*) fed on diets with these ingredients for a period of upto eight weeks. Only the mycotoxin enniatin B, at a level near the limit of detection, and low levels of isoflavones were detected in the feed, demonstrating the effectiveness of maximum level recommendations and modern feed processing technologies in the Norwegian aquaculture industry. Consequently, carry-over into fish muscle was not observed, confirming that fillets from plant-fed salmon were safe for human consumption.

Paper II

Biotransformation of phytoestrogens from soy in enzymatically characterized liver microsomes and primary hepatocytes of Atlantic salmon

Amritha Johny, Lada Ivanova, Tone-Kari Knutsdatter Østbye, Christiane Kruse Fæste Ecotoxicology and Environmental Safety 2020, 197:110611.
https://doi.org/10.1016/j.ecoenv.2020.110611

Efficient aquaculture is depending on sustainable protein sources. The shortage in marine raw materials has initiated a shift to "green aquafeeds" based on staple ingredients such as soy and wheat. Plant-based diets entail new challenges regarding fish health, product quality and consumer risks due to the possible presence of chemical contaminants, natural toxins and bioactive compounds like phytoestrogens. Daidzein (DAI), genistein (GEN) and glycitein (GLY) are major soy isoflavones with considerable estrogenic activities, potentially interfering with the piscine endocrine system and affecting consumers after carry-over. In this context, information on isoflavone biotransformation in fish is crucial for risk evaluation. We have therefore isolated hepatic fractions of Atlantic salmon (*Salmo salar*), the most important species in Norwegian aquaculture, and used them to study isoflavone elimination and metabolite formation. The salmon liver microsomes and primary hepatocytes were characterized with respect to Phase I cytochrome P450 (CYP) and Phase II uridine-diphosphate-glucuronosyltransferase (UGT) enzyme activities using specific probe substrates, which allowed comparison to results in other species. DAI, GEN and GLY were effectively cleared by UGT. Based on the measurement of exact masses, fragmentation patterns, and retention times in liquid chromatography high-resolution mass spectrometry, we preliminarily identified the 7-O-glucuronides as the main metabolites in salmon, possibly produced by UGT1A1 and UGT1A9-like activities. In contrast, the production of oxidative metabolites by CYP was insignificant. Under optimised assay conditions, only small amounts of mono-hydroxylated DAI were detectable. These findings suggested that bioaccumulation of phytoestrogens in farmed salmon and consumer risks from soy-containing aquafeeds are unlikely.

Paper III

Impact of dietary wheat gluten on feed intake and the intestinal and hepatic transcriptome in Atlantic salmon (*Salmo salar*)

Amritha Johny, Gerd M. Berge, André S. Bogevik, Aleksei Krasnov, Bente Ruyter, Christiane K. Fæste, Tone-Kari K. Østbye
Scientific Reports 2020 (submitted)

Partial or complete replacement of fish meal (FM) with plant proteins in the diets of Atlantic salmon is increasing to meet the demands of the growing aquaculture industry. Wheat gluten (WG) is considered as a valuable protein source in fish diets due to its energy density and relatively low level of anti-nutritional factors (ANF). The main aim of this study was to identify the effects of dietary WG on fish growth, feed efficiency and the hepatic and intestinal transcriptomes of Atlantic salmon. The fish were fed either control diet with FM as the only source of protein or diets, where 15% or 30% of the FM were replaced by WG. The fish had a mean initial weight of 223 g and approximately doubled their weight during the 9-week experiment. Salmon in the WG30 dietary group showed reduced feed intake compared to the WG15 and control groups. The liver fat content and enzyme activities of the liver health markers aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma increased with the inclusion level of WG in the diet. Gene expression analysis showed significant changes in both, intestine and liver of fish fed with WG30. Especially noticeable were changes in the lipid metabolism, in particular in relation to the intestinal lipoprotein transport and sterol metabolism. Moreover, the intestinal transcriptome of WG-fed fish showed shifts in the expression of a large number of genes responsible for immunity and tissue structure and integrity. These observations implied that the fish receiving WG-containing diet were undergoing nutritional stress. Overall, the study provided evidence that a high dietary level of WG has a negative impact on the intestinal and liver health of salmon.

Paper IV

Plant-based diets induce transcriptomic changes in fast muscle of zebrafish and Atlantic salmon

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With the expansion of the aquaculture industry in the last two decades, there has been a large increase in the use of plant ingredients in aquafeeds, which has created new challenges for fish growth, health and welfare. Fish muscle growth is an important trait that is strongly affected by the diet, but our knowledge on the effects of plant protein-based diets on the global gene expression in muscle is still scant. The present study evaluated nutrigenomic effects of the inclusion of proteins from pea, soy and wheat into aquafeeds, compared to a control diet with fish meal as the main protein source using the zebrafish (*Danio rerio*) model by RNA-seq; these results were extended to an important aquaculture species by analyzing selected differentially expressed genes identified in zebrafish on on-growing Atlantic salmon (*Salmo salar*) fed with equivalent plant protein-based diets. The expression of selected Atlantic salmon paralogues of the zebrafish homologues was analyzed using paralogue-specific qPCR assays. Global gene expression changes in the muscle of zebrafish fed with plant-based diets were moderate, with the highest changes observed in the soy diet-fed fish, and no change for the pea diet-fed fish compared to the control diet. Among the differentially expressed genes were *mylpfb*, *hsp90aa1.1*, *col2a1a* and *odc1*, which are important in regulating muscle growth, maintaining muscle structure and function, and muscle tissue homeostasis. Furthermore, those genes and their paralogues were differentially expressed in Atlantic salmon fed with the equivalent percentage of soy or wheat protein containing diets. Some of these genes were similarly regulated in both species while others showed species-specific regulation. The present study expands our understanding on the molecular effects of plant ingredients in fish muscle. Ultimately, the knowledge gained would be of importance for the improved formulation of sustainable plant-based diets for the aquaculture industry.

5.0 General discussion of the key results

Novel feed ingredients will be increasingly tried out and utilised as sources for proteins and oils in aquafeeds, replacing limited marine proteins and fish oil partially or completely. A wide range of materials including different plants, insects and algae are already exploited or under testing. The inclusion of these novel feed constituents is a challenge not only with respect to meeting the dietary needs of the fish, but also regarding the introduction of new chemical contaminants and anti-nutritional factors (ANF) (**Table 2**), which might affect fish health and pose a risk to consumers of fish products.

Currently, plant-based protein sources, especially from wheat and legumes, are still the most used, and therefore, the occurrence of plant-borne substances in fish feed and their carry-over potential are of particular interest. Relevant ANF include different mycotoxins, bioactive compounds and allergenic peptides that can be present in frequently used plant ingredients (Cheli et al., 2013; Goncalves et al., 2020). Maximum levels for some mycotoxins in complete aquafeeds have been set by feed authorities in Norway and the EU (**Tables 4** and **5**), ensuring reduced exposure to these substances in fish. Nevertheless, the Norwegian Scientific Committee for Food Safety (VKM) has pointed out that more knowledge on the transfer potential of several important ANF from plant-based feed into the edible portion of fish is needed (VKM, 2014).

The aim of this PhD project was thus to attempt filling this gap by assessing the use of three common "green" fish feed materials, i.e. wheat gluten (WG), soybean protein concentrate (SPC) and pea protein concentrate (PPC), for effects on fish biology and for risks to consumer health with focus on frequently occurring mycotoxins and phytoestrogens.

Feeding trials run under clearly defined conditions in zebrafish and Atlantic salmon with custom-made diets were the basis for all subsequent experiments (**Figure 35**, **Table 9**). Since the focus of the fish experiments was the potential transfer of ANF from feed to fish and not digestibility or feed utilisation, the diets were balanced with regard to the plant-based ingredients and not to total crude protein and total lipid content (**Table 8**). By keeping the ratio of the plant protein sources constant, comparability between the derived samples was ensured. Selected mycotoxins and soybean isoflavones in feed and

fish were identified and quantified by a newly developed and validated multi-analyte method (**Paper I**).

In contrast to some mycotoxins, there are no recommended maximum levels for isoflavones in fish feed. Since soybean proteins are the most used ingredient for replacing FM and thus the major contributor of isoflavones, understanding their metabolic fate in fish is important for the assessment of possible risks. Therefore, the hepatic biotransformation of daidzein (DAI), genistein (GEN) and glycitein (GLY) was investigated *in vitro* with in-house prepared liver microsomes and primary hepatocytes of Atlantic salmon that first had been enzymatically characterised by specific probe substrates (**Paper II**). Finally, transcriptomic analysis using microarray hybridisation was performed to understand effects of diets containing WG and SPC on the intestine, liver and muscle of salmon (**Paper III** and **Paper IV**). Moreover, transcriptomics by RNA-seq was performed in the muscle of zebrafish fed with WG-, SPC- and PPC-based feeds with the aim of identifying differently expressed genes (**Paper IV**).

Taken together, the findings of the different experiments in this PhD study help to answer two main questions relevant for the aquaculture industry and the consumers of fish products:

- *Are salmon fed with plant-based diets safe to eat?*
- *Have plant-based diets a negative impact on growth performance and health of fish?*

5.1 Are salmon fed with plant-based diets safe to eat?

Human exposure to plant-borne ANF in fillets of farmed salmon is relatively new. Calculating the consumption of salmon from aquaculture in the Norwegian population from national dietary surveys (**Table 11**), the Norwegian National Committee for Food and Environment (VKM) has estimated the intake and exposure to important nutrients such as the long-chain polyunsaturated fatty acids EPA and DHA, vitamin D, iodine and selenium (s. 1.3.2 and 1.3.3) as well as to mercury and major chemical contaminants including dioxins and dl-PCBs (s. 1.4.4) and assessed the connected risks. However, exposure to undesirable substances from new feed sources could not be considered due to a lack of sufficient occurrence data, and the same was true for ANF in currently used ingredients (VKM, 2014).

Table 11. Consumption of fish and farmed salmon in different population groups in Norway (VKM, 2014).

Fish consumption	Number of consumers		All participants (N)	
	n	%	Mean g/day	P95* g/day
2-year-olds (N=1674)				
Fish, in total	1640	98	16	36
Salmon, farmed	1013	61	1	4
Adults (N=1787)				
Fish, in total	1095	61	52	201
Salmon, farmed	323	18	12	83

*P95: 95th percentile; Number of consumers (n) = number of children or adults, who regularly consumed fish and farmed salmon.

The fish consumption data were based on the dietary surveillance studies "Småbarnskost 2007" and "Norkost 3", conducted in 2010 and 2011. Fish consumption in Norway is traditionally relatively high and changes little from year to year. The survey results are therefore considered still reasonably valid. From the 1674 participating 2-year-old children with an average bodyweight (bw) of 12.8 kg, 98% consumed fish regularly and 61% farmed salmon. Considering all participants, 1 g farmed salmon/day was consumed in the mean and 4 g/day by high consumers (95th-percentile). Among the 1787 adults in the study, aged 18 to 70 years and a stipulated average bw of 70 kg, 61% reported fish consume and 18% ate regularly farmed salmon. Based on all participants, 12 g farmed salmon/day was consumed in the mean and 83 g/day by high consumers. Notably, the differences between mean and high fish intake are much bigger in adults than in the 2-year-old children, showing that a lower proportion of the adult population consume farmed salmon, but that the eaten portions were considerably larger.

The assessment of risks to consumer health depends critically on the determination and quantitation of adverse substances of interest in food products. Methods for the specific and sensitive determination of targeted analytes in different matrices have to be available to evaluate the level and frequency of occurrence. As reported, data for important chemical contaminants and heavy metals are available in feed matrices and wild and farmed fish, whereas ANF are much less investigated (s. 1.4.4). Regarding the

ANF studied in this PhD project, mycotoxins have a rather high natural presence in plant sources, but isoflavones are only prevalent in legumes. There are a number of studies on the detection of mycotoxins in feed, poultry, pig and several fish species, but only a few analyses have been conducted in salmon (s. 1.4.1.2), and even less have measured phytoestrogens (s. 1.4.2.1 and 1.4.5.2). In the majority of these studies, feed challenge trials were performed with defined, rather high quantities of the target substances, and only very few have used naturally-contaminated feed or levels that are similar to those in commercially available composite feeds (Pietsch, 2020). In contrast, the present study was based on fish trials conducted with diets that only contained ordinary feed ingredients distributed in Norway. Additional adverse substances were not added during the feed preparation, so that the customisation was restricted to the different contents of, respectively, WG, SPC or PPC (**Table 8**).

Since grain used in Norwegian aquafeeds generally contains only low amounts of mycotoxins (VKM, 2013; Aas et al., 2019), and the maximum ratio of wheat in the experimental diets was 42% in WG30, only marginal levels could be expected to be found in this study. Using the newly developed and validated multi-analyte method for the simultaneous detection of all selected ANF, ENNB was the only mycotoxin measurable at concentrations close to the LODs in WG30 feed (LOD: 41 µg/kg) and interestingly, also in WG30-fed salmon (LOD: 78 µg/kg), indicating a notable carry-over potential (**Paper I**). These findings correlate with the relatively high prevalence of ENNs in Northern Europe (Santini et al., 2012) and with previous reports about the transmissibility of ENNB from feed into poultry and further into eggs (Jestoi et al., 2009) and from feed into the fillets of farmed fish (Tolosa et al., 2017).

ENNB has shown considerable toxic and genotoxic effects *in vivo* after acute and repeated oral administrations in mice (Maranghi et al., 2014) (s. 1.4.1.1). The no-observed adverse effect level (NOAEL) for the most sensitive endpoint was set to 180 µg/kg bw/day. EFSA has estimated that the mean chronic exposure to the sum of ENNs from the consumption of cereals across all age groups using lower bound and upper bound concentrations ranged from 0.42 to 1.82 µg/kg bw/day, and the 95th-percentile exposure ranged from 0.91 to 3.28 µg/kg bw/day (EFSA, 2014). The mean acute exposure estimates of the sum of ENNs across all age groups ranged from 1.01 to 4.67 µg/kg bw/day and the 95th-percentile exposure ranged from 2.20 to 10.1 µg/kg bw/day in adults. With regard to the NOAEL determined in mice, these exposure levels are not

of concern, even for the highest estimates that were derived for 2-year-old children. This is also the case with respect to the ENNB levels detected in the WG30-fed salmon in this PhD study. Considering the occurrence level (about 78 µg ENNB/kg in salmon), the estimated consumption of farmed salmon in the Norwegian population (**Table 11**), and the respective bw of 2-year-old children and adults, exposures could be calculated. Ranging from 0.006 to 0.025 µg ENNB/kg bw/day in children with mean and high consume, and from 0.013 to 0.092 µg ENNB/kg bw/day in mean and high adult consumers, they were far below the NOAEL. Consequently, the risk connected to the intake of ENNB from salmon fed with diets containing high ratios of wheat, which is contaminated with limited ENNs concentrations, was considered as very low.

The new multi-analyte LC-HRMS/MS method, including the optimised sample extraction protocols for different matrices, achieved good performance parameters in terms of linearity, working range, precision and accuracy, allowing the detection of the targeted mycotoxins and isoflavones at low levels in feed and fish. Nevertheless, other mycotoxins frequently occurring in grains such as DON, T-2 or OTA were not detected in the study samples, proving that the use of plant-based fish feed containing mycotoxins below the recommended maximum levels results in negligible health risks for consumers. In other studies using feed that had been spiked with up to 100 µg/kg of different mycotoxins or were naturally contaminated with up to 814 µg DON/kg (Nácher-Mestre et al., 2013 and 2015), showed transfer to exposed fish, but always at levels below the maximum residue limits established by EU (**Table 4**), Generally, risks to consumer health from the carry-over of mycotoxins from plant-based feed to fish appears to be low.

This may, however, change if climate changes lead to higher fungal infestation of feed plants or the establishment of different, thermophilic strains in Northern countries, which could be associated by an increased presence of mycotoxins or occurrence of derivates with high toxic potential such as AFL and FUM (Goncalves et al., 2020). Moreover, the globalisation of trade and import of novel feed resources can also contribute to increased mycotoxin levels in ingredient for aquafeeds. Thus, the developing of methods, which can determine a wide range of substances in different matrices is an important contribution for food safety. In the LC-HRMS/MS method used for the analysis of the samples from the feeding trials AFL and FUM were not incorporated, because they do not occur in Norwegian grains and are surveyed in

imported feed commodities. However, AFL have been intensively studied in fish, demonstrating a considerably high carry-over potential (Pietsch, 2020). Thus, to be equipped for possible future developments, AFL and FUM have been recently included in an updated version of the LC-HRMS/MS method.

Isoflavones in aquafeeds originate from extruded legume meal, in which they occur in relatively high concentrations, mostly in glucosidated form (s. 1.4.2.1). In the present study, the feed analysis by LC-HRMS/MS showed dose-dependent levels of the targeted analytes DAI, GEN, GLY, daidzin, genistin and glycitin in the soybean-containing diets (**Paper I**). The mean concentrations in SPC15 ranged from 21 µg GLY/kg to 786 µg daidzin/kg and in SPC30 from 40 µg GLY/kg to 1356 µg daidzin/kg. Glucosidated forms occurred in higher concentrations than the corresponding aglycons, whereas an increase of the free form had been previously reported in extruded protein preparations (Pandjaitan et al., 2000). In PPC15 and PPC30, 26 and 54 µg GLY/kg were detected, respectively, confirming results from a screening study on fruits and vegetables (Kuhnle et al., 2007). Phytoestrogen levels in food and feed are not regulated so far and the threshold between health benefits and risk from dietary exposure is undecided.

In the present experiment, phytoestrogens were not detected at concentrations above the individual LOQs in the range from 35 µg/kg to 168 µg/kg in SPC15- and SPC30-fed zebrafish or salmon. Considering that the SPC30 feed contained 0.2 mg DAI/kg, it was conclusive that the targeted isoflavones could not be detected in the salmon fillets. In contrast, when gibel carp (*Carassius auratus gibelio*) were exposed to feed with added 40 to 400 mg DAI/kg, the unchanged compound was recovered with 128 and 261 µg/kg in the fish muscle (Li et al., 2016). Muscle samples were not analysed in on-growing farmed Russian and Siberian sturgeon that had received diets with total isoflavone levels in the range of 1.5 to 50 mg/kg from 20 to 800 days post-hatching (Rzepkowska et al., 2020) (s. 1.4.5.2). However, the estimated overall exposure to 1.46 to 1.68 g DAI/fish and 3.56 to 4.09 g GEN/fish during the trial period resulted in levels of 247 µg DAI/kg and 118 µg GEN/kg in blood and 273 µg DAI/kg and 667 µg/kg in liver of Russian sturgeon and 327 µg DAI/kg and 151 µg GEN/kg in blood and 269 µg DAI/kg and 919 µg/kg in liver of Siberian sturgeon that had been starved for 24 h at study end. Since blood flows also through muscle tissue and small amounts of blood are always retained in fresh fishery products, these results are of interest with regard to consumer safety. However, the isoflavone concentrations used in the sturgeon study exceed by far the

levels that are to be expected in Norwegian aquafeeds. Nevertheless, when fillet samples (n=19) from rainbow trout fed with complete soybean meal- and SPC-containing commercial fish diets were analysed in Italy, 5.1 µg DAI/kg, 5.2 µg GEN/kg and 0.62 µg GLY/kg were detected, showing the importance of surveys for food safety assessment (Merlanti et al., 2018).

Information on the uptake of isoflavones in fish is not available, but bioavailabilities differ considerably between warm-blooded vertebrate species (Miura et al., 2016). Preliminary data generated by i.v. and p.o. application of radiolabelled GEN are available for Siberian sturgeon and rainbow trout (Gontier-Latonelle et al., 2007). The measurement of the total radioactivity in the blood of the fish allowed the prediction of relative bioavailabilities of about 19% in the sturgeon and 9.5% in the trout, which is comparable to the value for GEN in women after exposure to soybean isoflavone supplement (Miura et al., 2016).

Major biotransformation pathways of DAI, GEN and GLY in Atlantic salmon were investigated for the first time in this PhD project, using two newly developed and enzymatically-characterised *in vitro* metabolism models (s. 1.5.6.4 and 3.3.2) (**Paper II**). The isoflavones were predominantly metabolised through the formation of glucuronides, which were characterised by their specific retention times, exact masses and fragmentation patterns in LC-HRMS/MS analysis (s. 3.3.2.3) (Heinonen et al., 2003; Zhao et al., 2018). Apart from glucuronidation is sulphonation a major Phase II pathway in some fish species, including Siberian sturgeon (Gontier-Latonnelle et al., 2007). In addition to conjugation reactions, oxidative Phase I metabolism has been reported for DAI, GEN and GLY in humans and the involved CYP enzymes have been identified (Kulling et al., 2001). However, only low levels of four mono-hydroxylated DAI metabolites were found in the incubation mixtures with salmon microsomes and primary hepatocytes (**Paper II**). Equivalent metabolites were not detectable for GEN and GLY.

Both, DAI, GEN and GLY and their respective glucuronide metabolites were not detectable in the fillets of salmon fed with SPC15 or SPC30 diets (**Paper I**), which indicates effective detoxification and elimination processes also *in vivo*, in accordance with what was observed in the *in vitro* biotransformation study (**Paper II**). Salmon, like other species, appear to have the ability to detoxify isoflavones efficiently, so that bioaccumulation in muscle did not occur from the relatively low levels in the feeds in

the present study. It would be interesting to investigate in a follow-up experiment if the salmon metabolism was able to cope with exposure to increased amounts such as used in commercial Italian feeds in the rainbow trout survey (Merlanti et al., 2018). Under the current conditions in Norway, carry-over was not observed.

Together, the results of **Paper I** and **Paper II** allow formulating an answer to the first key question of this PhD project: The potential for transfer of the studied mycotoxins and phytoestrogens from plant-based feed to farmed salmon is low under the tested conditions, rendering fish products safe for consumption (s. 1.1.2). Nevertheless, the metabolites produced could be subjected to further studies such as structure elucidation by nuclear magnetic resonance (NMR) to determine the molecular sites of the chemical modifications. Detailed information on the metabolites will help understand connected effects in biological system and the environment, which is important in risk assessments. In this context, the biotransformation in salmon of different ANF and chemical contaminants originating from novel protein sources in aquafeed will be of increasing importance. The development and characterisation of two mutually confirmative *in vitro* metabolism assays that are generally applicable for the elucidation of xenobiotic detoxification pathways in salmon is thus an added value of this project.

5.2 Have plant-based diets a negative impact on growth performance and health of fish?

Plant-based ingredients, when incorporated into fish feeds, can have a negative impacted on biological functions and the growth performance in fish, mainly due to the presence of ANF (s. 1.5). Consequently, efforts have been made to reduce harmful effects, e.g. by changing processing methods, supplementing essential amino acids, micro- and macro-nutrients, and by introducing genetically-modified plants as feed ingredients. Wheat gluten and protein concentrates of soybean and pea are resources that currently are frequently used in aquafeeds (s. 1.3.4). They may contain different amounts of ANF such as mycotoxins and phytoestrogens. Depending on the fish species, level and duration of exposure and potential of the ANF, effects on biological systems can vary greatly (s. 1.4.1).

The custom-made feeds in this PhD study contained only low concentrations of some of the targeted ANF, which could be expected to not have negative consequences on the health of the exposed zebrafish and salmon (**Paper I**). Substances with oestrogenic

potential such as phyto- or mycoestrogens (e.g. ZEN) can interfere with the oestradiol metabolism by acting as agonists or antagonists for oestrogen receptors (s. 1.4.1.1), functioning as endocrine disruptors by altering hormonal imbalances and thereby affecting fish health (s. 1.4.1.2). However, salmon were shown to have an efficient detoxification mechanism for major soybean isoflavones in this project (**Paper II**). It seemed thus conclusive to assume that observed transcriptomic effects on gene expression patterns in zebrafish and salmon fed with the WG-, SPC- or PPC-containing diets could mainly be attributed to effects of the plant ingredient and not to the presence of ANF (**Paper III** and **Paper IV**).

The most distinct transcriptome changes in salmon intestine were observed with the SPC30 and WG30 diets (**Paper III**), whereby changes in liver were mainly caused by the WG30 diet (**Figure 51**). Generally, the intestine was more affected by the plant-based diets than the liver. In zebrafish muscle, SPC30 cause the greatest transcriptome changes, followed by WG30, while PPC30 diet led not to visible effects (**Paper IV**). In addition, comparable gene up- and down-regulations were observed in salmon muscle. Thus, the different plant-based diets appeared to cause tissue-specific shifts in the expression of genes.

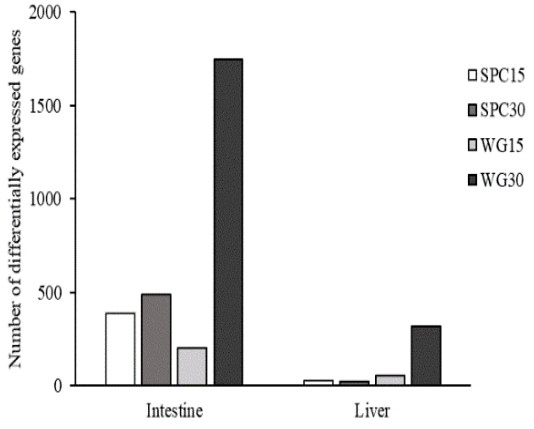

Figure 51. DEG in the intestine and liver of Atlantic salmon fed with SPC- and WG-based diets in comparison to the FM-fed group (n=6). (Johny et al., 2020b, submitted)

In the intestine of salmon fed with WG30, and to a lesser extent fed with SPC30, lipid metabolism- and transport-related genes were upregulated, indicating a compensatory response in the intestine and problems with the transport of lipids from the intestine to the blood circulation (**Paper III**). Similar, but less pronounced changes were observed in the liver. Effects on the lipid metabolism of fish that are exposed to plant-containing diets have been previously reported (Torstensen et al., 2011). Particularly, decreased lipid digestibility, reduced bile salt levels, hypocholesterolaemia have been reported, showing the differentiated reactions to the unfamiliar feed ingredients (s. 1.5.2 and

1.5.3). Other affected metabolic pathways in salmon intestine included xenobiotic-, retinoid-, and protein-metabolism, immunity, cell structure, tissue structure and integrity, tissue neural and cell processes. Dietary effects on the intestine and liver are connected, which became visible from the analysis of gene expression changes in the liver of WG30-fed salmon. Processes linked to lipid and sterol metabolism were largely affected, along with a few genes related to immunity and tissue- and cell-processes (s. 1.5.3). It could therefore be assumed that the intestinal imbalance and metabolic disorder caused by the WG-containing diet lead also to changes in the liver metabolism (**Paper III**). Transcriptome changes in the muscle of SPC-fed zebrafish occurred mainly in genes related to muscle development and function, structure and homeostasis, and these genes and their paralogues were similarly regulated in salmon fed with equivalent diets. Presumably, exposure to the soybean ingredient led to molecular adjustments in the fast muscle to maintain muscle homeostasis following induced imbalances.

Both transcriptomic studies performed in this PhD project confirmed that the inclusion of plant ingredients in fish diets have consequences for the fish physiology. Especially noticeable were the effects of the WG30 diet on the salmon intestine (**Paper III**). Changes in the gene expression patterns were indicative of chronical inflammation, similar to symptoms of gluten sensitivity in humans (Jamnik et al., 2015). The detection of DEG considered as pro-inflammatory and anti-inflammatory markers in humans with gluten sensitivity as well as observed changes in the expression of alpha-macroglobulins and transglutaminase 2 (TG2), were of particular interest. These genes are associated with gluten sensitivity, as they play a role in host defence mechanisms, inflammation and protease inhibition. TG2 can catalyse the deamidation of gliadin peptides and thereby creates epitopes that are recognised by gliadin-specific T-cell in the gut (Molberg et al., 1998). Their activation triggers auto-immune enteropathological responses leading to intestinal lesions and celiac disease (Caputo et al., 2004). Alpha-macroglobulins are inflammation inhibitors. The detected down-regulation of TG2 and up-regulation of alpha-macroglobulins in the intestine of WG30-fed salmon could indicate a physiological effort to reduce the gluten-induced stress.

The histological examination of intestines from WG30-fed zebrafish showed infiltration of eosinophilic granules, giving further evidence of inflammation (Dhanasiri et al., 2020a). Moreover, the intestinal permeability appeared to have increased, which can lead to impaired barrier functions and in turn to changes in the lipid- and sterol-

metabolism (**Paper III**). A leaky gut will allow the entry of excess nutrients, microbes or foreign compounds like large peptides, and will expose the underlying mucosa to antigens from the intestinal lumen. In humans, gluten sensitivity or celiac disease has been shown to be connected with neurological dysfunction in addition to intestinal and extra-intestinal symptoms (Hadjivassiliou et al., 2010). In line with this, a significant increase of DEG in metabolic processes related to cholinergic receptors was observed in intestine and liver of WG30-fed salmon (**Paper III**), and in GO categories related to neurogenesis in zebrafish intestine (Dhanasiri et al., 2020a). With these findings in mind, the effects of WG-based fish diets on neural functions and gut-to-brain signal transmission should be investigated in depth in a follow-up study.

The WG30-related transcriptomic changes in salmon intestine and liver were reflected by a significantly reduced feed intake, specific growth rate (SGR), thermal growth rate (TGR) and final weight (s. 1.5.1) as compared to FM- and SPC-fed fish (**Table 12**) (**Paper III**).

Table 12. Fish weights at study start and end, feed intake, feed conversion ratio (FCR), specific growth rate (SGR) and thermal growth coefficient (TGC) of Atlantic salmon fed with control diet (FM), SPC- or WG-containing diets. Liver fat contents, condition factor (CF) and hepatosomatic index (HSI) of sampled fish.

	FM	**SPC15**	**SPC30**	**WG15**	**WG30**	**p-value˜**
Initial wt (g)	225±2	223±0	223±0	219±4	223±0	0.36
Final wt (g)	548±4^{bc}	608±17^{a}	605±7^{ab}	563±4^{abc}	513±8^{c}	0.02
Feed intake (g)	8251±214^{bc}	9130±393^{a}	8829±45^{ab}	7602±12^{c}	6620±108^{d}	<0.0001
FCR	0.80±0.02	0.80±0.01	0.79±0.02	0.74±0.01	0.76±0.01	0.07
SGR (% d^{-1})	1.53±0.03^{a}	1.64±0.04^{a}	1.62±0.02^{a}	1.54±0.04^{a}	1.36±0.03^{b}	0.001
TGC	3.40±0.06^{b}	3.68±0.10^{a}	3.63±0.05^{ab}	3.41±0.10^{b}	2.97±0.07^{c}	0.001
Liver fat (%)	4.9±0.0^{c}	5.19±0.10^{bc}	6.90±0.41^{ab}	5.6±0.22^{abc}	6.94±0.69^{a}	0.008
CF	1.31±0.02	1.35±0.02	1.35±0.02	1.32±0.02	1.30±0.02	0.12
HSI	1.23±0.03^{b}	1.24±0.03^{b}	1.37±0.04^{b}	1.21±0.02^{b}	1.64±0.09^{a}	0.003

mean ± S.E.M.; n=15, 5 fish per 3 replicate tanks.

$^{a, b, c, d}$ Significant differences ($p<0.05$) between diets are indicated with different letters.

˜p-value: parameters recorded on a group basis were statistically tested by one-way ANOVA and individual measured parameters were tested by a mixed-model.

The reduction in the feed intake was in contrast with findings in a previous feeding study, where TGC values in salmon exposed to 35% WG did not significantly differ from those of a FM-fed control group (Storebakken et al., 2000). Moderate pathological symptoms in the intestine were sporadically observed, but were not confirmed in an extensive study with multiple diets including one containing 49% wheat (Aslaksen et al., 2007). In the present study, the decreased feed intake in WG30-fed salmon, accompanied with a significant increase of the hepatosomatic index (HSI) (**Table 12**), demonstrating an increase of the liver weight in comparison to the whole body weight, was in agreement with the observed upregulation of cholecystokinin genes *cck* (**Paper III**). The hormone cholecystokinin regulates food intake, satiation as well as the digestion of fat and protein (Volkoff, 2006). A relatively high level of WG in the diet might therefore have an effect on appetite regulation by signalling satiation.

However, the SGR of zebrafish fed with PPC30, SPC30 or WG30 did not change significantly after 46 days of the feeding trial as compared to the controls fed with a diet containing FM as the only protein source (**Figure 52**) (**Paper IV**). The shorter period of exposure and species differences might contribute to this incongruity, which should be clarified in a futher experiment. Nevertheless, the detected histological changes in the intestines of zebrafish fed with the three plant-based diets showing inflammation-related symptoms supported the perception that plant proteins contents in aquafeeds at levels above 30% can have a negative impact on fish health (Dhanasiri et al., 2020a). Gut inflammation in salmon caused by SBM in the feed had also been previously observed (Van den Ingh et al., 1991; Baeverfjord and Krogdahl, 1996) (s.1.5.2). In the zebrafish, exposure to SPC30 resulted in a considerable number

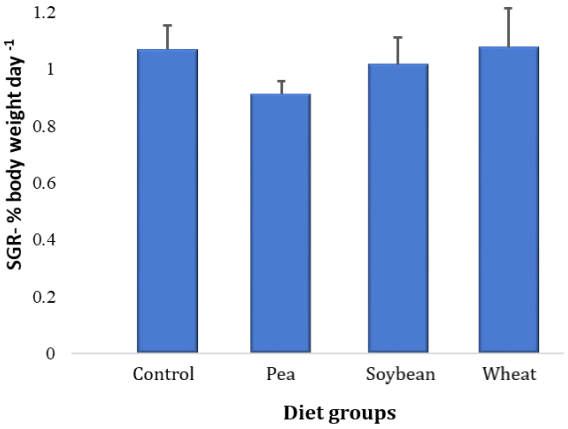

Figure 52. SGR of zebrafish fed for 46 days with PPC30-, SPC30-and WG30-containing diets or the FM control diet. Values are means ± S.E.M (N=32 females) (Dhanasari et al., 2020b, submitted).

of epigenetic changes, followed by WG30, while PPC30 had a considerable weaker effect (**Figure 53**) (Dhanasiri et al., 2020a).

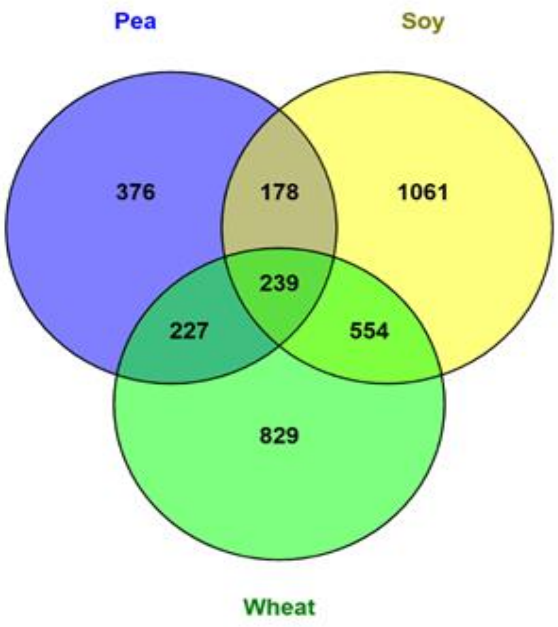

Figure 53. Venn diagram showing total gene numbers with differential methylation in genic and/or intergenic regions of the mid-intestine of zebrafish fed with WG30, SPC30 and PPC30 in comparison to the FM control group (Dhanasari et al., 2020a).

The SPC-receiving salmon showed a slightly improved growth performance and few gene expression changes in the intestine and liver (**Figure 51**) (**Paper III**). SPC is widely used in aquafeeds and considered as an ingredient with a feed conversion efficiency comparable to that of FM (s. 1.5.1). Previous studies have shown that levels of up to 50% SPC can replace FM without negative effects on the growth performance in Atlantic salmon or rainbow trout without the addition of essential amino acids (s. 1.3.4). An imbalance in the amino acid composition (especially with regard to the methionine level) and high contents of carbohydrates and ANF in soybean-derived feed ingredients can affect fish performance and feed digestibility considerably (Krogdahl et al., 2010). As previously observed in salmon (Øverland et al., 2009) and Japanese flounder (Deng et al., 2006; Mai et al., 2012), there was a slight reduction in the ADC for energy with increased inclusion of SPC in the present study (**Table 13**).

The comparison of the diets made it apparent that the ADC for lipids, nitrogen and energy were differently affected by SPC and WG (**Table 13**).

While WG30 slightly reduced ADC_{Lipids}, SPC30 had a noticeable impact on ADC_{Energy}. Good digestibility of WG in both rainbow trout and Atlantic salmon has also been

previously reported (Pfeffer et al., 1992; Sugiura et al., 1998; Storebakken et al., 2000; Apper-Bossard et al., 2013). Furthermore, the plant-based diets had clear effects on the dry matter (DM) content of faeces (**Table 13**). Noticeably, the SPC30 diet reduced DM significantly in comparison to the FM control.

Table 13. Apparent digestibility coefficients (ADC) for lipid, nitrogen and energy in salmon fed with FM, SPC- or WG-containing diets.

	FM	SPC15	SPC30	WG15	WG30	p-value ANOVA
Faecal DM (%)	14.4±1.1^{ab}	12.8±0.1^{b}	11.1±0.12^{c}	16.0±0.4^{a}	15.6±0.1^{a}	0.0003
ADC, Lipids (%)	97.2±0.7	97.6±0.2	97.4±0.3	97.1±0.2	96.8±0.4	0.71
ADC, Nitrogen (%)	86.4±0.7^{c}	86.3±0.5^{c}	85.8±0.5^{c}	89.1±0.1^{b}	91.8±0.3^{a}	<0.0001
ADC, Energy (%)	88.6±0.7^{a}	86.3±0.3^{b}	83.8±0.6^{c}	88.5±0.1^{a}	88.5±0.5^{a}	0.0001

Data are mean ± S.E.M.; n=3 replicate tanks.

$^{a, b, c, d}$ Significant differences ($p<0.05$) between the dietary groups are indicated with different letters.

The high content of indigestible carbohydrates in soybean-based ingredients, which was also determined in the SPC in the present study (data not shown), can be utilised to a lesser extent by carnivorous fish like salmon, causing a reduction in ADC_{Energy}. This lowers the DM amount in the faeces and, at the same time, increases the content of indigestible carbohydrates. Lower digestibility might also prolong the gastrointestinal passage time, which could trigger increased feed intake. Interestingly, the inclusion of 15% or 30% WG in the diets led to a significant increase of $ADC_{nitrogen}$ (**Table 13**), concomitantly with the found reduction of feed intake and growth. The diets in this project were not supplemented to balance amino acid contents and phospholipids, since the focus of the fish experiments was the potential transfer of ANF from feed into fish, and not feed digestibility or utilisation. The influence of the small disparities between the different diets was, however, considered as negligible because the high content of FM even in the SPC30 or WG30 diets was sufficient to provide for all required nutrients, although previous studies in salmonids have shown that the additional supplementation of lysine to WG-containing diets could improve growth (s. 1.5.1). It could also be speculated that differences in nutrient contents had an effect on the feed palatability or caused the increased liver size and fat content in WG30-fed salmon (**Table 12** and **Table 14**) (**Paper III**).

Table 14. Free Fatty Acid (FFA), Total protein (Tprot), Triglycerides (TG), Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) levels in the serum of salmon fed with FM, SPC- or WG-containing diets.

	FM	SPC15	SPC30	WG15	WG30	p-value ANOVA
FFA (mmol/L)	0.41±0.02^{a}	0.31±0.04^{b}	0.27±0.02^{b}	0.29±0.01^{b}	0.31±0.02^{b}	0.001
Tprot (g/L)	42.9 ±1.07^{bc}	44.9±1.17^{b}	44.1±1.31^{bc}	40.7±0.98^{c}	50.3±1.83^{a}	<0.001
TG (mmol/L)	3.75±0.22^{b}	3.54±0.25^{b}	3.60±0.19^{b}	3.48±0.33^{b}	5.15±0.67^{a}	0.011
ALT (U/L)	36.7±2.72^{b}	37.7±5.99^{b}	52.1±11.10^{b}	26.1±2.30^{b}	81.3±17.78^{a}	0.002
AST (U/L)	1082±143^{b}	1393±296^{b}	1911±550^{ab}	763±100^{b}	3116±827^{a}	0.007

Data are mean ± S.E.M. (n=15; 5 fish per 3 replicate tanks).

$^{a, b, c, d}$ Significant differences ($p<0.05$) between the dietary groups are indicated with different letters.

The results from the different experiments in this PhD project that were performed to answer the second key question by studying effects of plant-based diets on fish physiology support findings in published reports showing an imbalance in pathways, mainly related to lipid and cholesterol metabolism and transport, immunity and stress (s. 1.5.2) (**Paper III** and **Paper IV**). Growth performance was only marginally affected and the condition factor (K) was satisfactory for all diet groups in the salmon trial in comparison to the FM-based diet. Apart from confirming the general applicability of SPC and WG in aquafeeds, this project has generated new knowledge about the impact of these plant-based ingredients on gut and liver health as well as of muscle structure in highly exposed fish. These data could be of benefit for the aquaculture industry in the search of improved feed compositions that help to further fish welfare.

6.0 Main conclusions

The aim of this thesis was to evaluate "*Plant-based aquafeeds: Carry-over potential of mycotoxins and phytoestrogens from feed to fish and implications for fish health and food safety*". Specific topics related to both key questions were investigated by analysing samples from feeding trials with custom-made plant-based diets in on-growing salmon and zebrafish with chemical, biological and transcriptomic methods.

Taken together, the results of the different experiments showed that under the chosen conditions, using commercially available Norwegian feed ingredients, carry-over of the anti-nutritional factors (ANF) mycotoxins or phytoestrogens from diets containing wheat gluten (WG), soybean protein concentrate (SPC) or pea protein concentrate (PPC) into the fish was not observed. No risk for consumers of fish products from the targeted ANF was identified. However, the study results also showed that depending on the inclusion level of the plant proteins in the diets, a negative impact on fish health could be determined, especially with regard to enteropathological inflammation.

The different experiments in this project and most relevant results included:

Chemical analysis:

- Development and validation of a 25-in-1 LC-HRMS/MS method that is suitable for the analysis of different mycotoxins and phytoestrogens in feed and fish matrices.
- Mycotoxins at levels above the respective limits of detection (LOD) were not detected in the study diets with the exception of enniatin B (ENNB), which occurred at concentrations close to LOD in WG-containing feed.
- Isoflavones were found in SPC- and PPC-containing fish diets at low levels, but not in salmon fillets or in zebrafish.

Biotransformation analysis:

- Establishment of methods for the simultaneous characterisation of five major CYP P450 and five major UGT enzymes in liver fractions using specific probe substrates and LC-TripleQ-MS/MS analysis.
- Development and enzymatic characterisation of generally applicable salmon *in vivo* biotransformation assays using primary hepatocytes or liver microsomes. Results obtained by both models correlated well. Noticeable enzyme activities

were determined for CYP3A4- and CYP2D6-like as well as for UGT2B7-, UGT1A9- and UGT1A1-like enzymes in the salmon fractions.

- The soybean phytoestrogens DAI, GEN and GLY are in salmon mainly eliminated by glucuronidation (Phase II), possibly as 7-O-glucuronides.
- Four oxidative metabolites (Phase I) of DAI were identified in salmon liver microsomes and primary hepatocytes. Oxidative metabolites were not detected for GEN and GLY.

Growth performance analysis:

- The overall growth performance in salmon and zebrafish fed with plant protein-containing diets was good in comparison to the FM-fed controls. The condition factor K in salmon was satisfactory, showing the general suitability of the used plant-derived feeds.
- The specific growth rates in zebrafish fed with PPC30, SPC30 or WG30 did not significantly change.
- Salmon that were fed with WG-containing diets showed significant, exposure-dependent reductions of feed intake and weight gain. SPC-containing diets did not affect the growth performance.
- The liver fat contents in salmon receiving plant-based diets were increased. The WG30 feed caused a significant increase of the hepatosomatic index (HSI).

Transcriptomic analysis:

- Feeding with plant-based diets induces changes in the gene expression patterns in different organs and tissues of salmon. The highest number of DEG was observed in the intestine of WG30-fed fish, whereas SPC15 had the least effect.
- In intestine, exposure to WG30 mainly affected pathways associated with lipid and sterol metabolism, xenobiotic, retinoid and protease metabolism, immunity, cell stress, tissue structure and integrity and various cell processes.
- In liver, DEG changes induced by the WG30 diet were related to lipid and sterol metabolism, along with some genes related to immunity and tissue- and cell-processes.
- The transcriptome in the muscle of zebrafish showed no detectable changes as a result of exposure to PPC-based diet, whereas SPC- and WG-containing feed caused significant shifts in gene expression patterns. The majority of DEG were

related to muscle growth, structure, function and homeostasis. Specific genes and their paralogues were also similarly expressed in salmon, whereas some of them showed species-specific regulation.

- The observed transcriptomic changes indicated that the fish were trying to maintain homeostasis by regulating compensatory mechanisms to resist effects caused by the plant protein-based diets.
- In salmon receiving increased levels of WG in the feed, symptoms of enteropathological inflammation were observed, comparable to those of gluten sensitivity in humans.

Consumer risk assessment analysis:

- The mycotoxin ENNB was detected in trace concentrations in salmon fillets, showing its carry-over potential. Intake estimations considering the measured occurrence and Norwegian food consumption data led to the assessment that consumer risks were very low.
- The efficient glucuronidation of the analysed major soybean isoflavones in salmon inhibited accumulation in fillets, so that risks to consumer health are considered very low at the occurrence level in feed in this study.

7.0 Future perspectives

The information acquired in this project has contributed to the elucidation of the carry-over potential of selected mycotoxins and phytoestrogens that are commonly found in plant ingredients commercially used in aquaculture. The determination of transfer rates from feed into exposed fish was important to estimate consumer risks and study effects on fish health.

Considering the increasing consumption of farmed fish and associated expansion in aquaculture production and the predicted future development (**Figure 54**), novel feed ingredients, primarily from plant-based resources, will continue to be used as

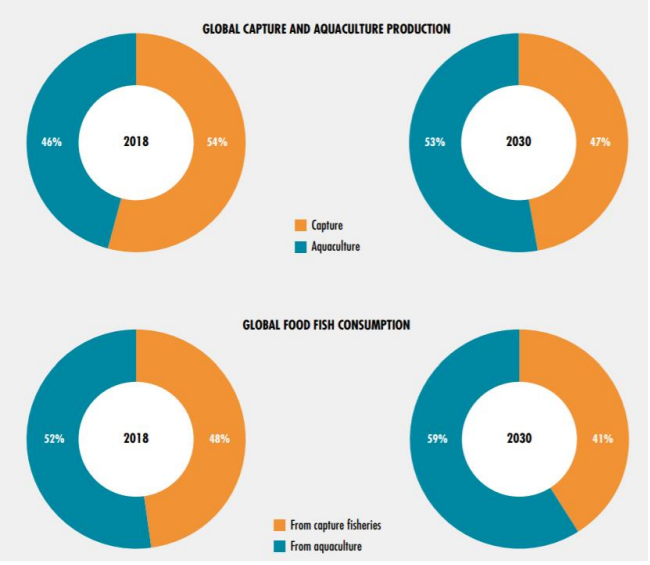

Figure 54. Increasing role of aquaculture in global food fish consumption. (source: FAO, 2020)

replacements for FM. Sustainability in the production of healthy and nourishing fish is essential to ensure food security and safety in accordance with the United Nations' sustainability goals SG2 "Zero Hunger", SG12 "Responsible Consumption and Production" and SG14 "Life below Water" (United Nation, The 17 Goals). The present study has given rise to further research questions that should be answered in follow-up experiments, supporting the production of safe fish and thereby ensuring food safety. Relevant points to be addressed, which were identified in the different activities of this project, are:

- The increasing unpredictability in climatic conditions, including the frequency and extend of extreme weather events and a rise in global temperatures, is a challenge in agriculture, among other things by changing the occurrence of plant-infesting, mycotoxin-producing fungi. Mycotoxins such as AFL and FUM that previously were present mainly in tropical countries are expected to increase in prevalence in moderate climate zones, causing new risks for food safety including fishery products. Analytical methods suitable for the identification and quantification of a wide range of contaminants in complex feed and food matrices are required. In view of the expected developments, work to include AFL and FUM in the LC-HRMS/MS method that has been established in this project, has already started, and further adaptions will be considered.
- Studies focusing on novel feed ingredients and consumer safety are in general limited to the edible portion of fish, mainly the fillet. However, harmful substances might also accumulate in other body parts and enter the food chain through the reuse of fish waste. Therefore, a more detailed analysis of farmed fish, collection of substance occurrence data, proper waste management and tracking of reutilisation are necessary.
- The main elimination route of major soybean isoflavones in salmon is glucuronidation. In addition, oxidative metabolites of DAI were identified in this study. The established *in vitro* biotransformation models should be applied in further experiments for identification of Phase I and Phase II metabolites of different feed-borne ANF and chemical contaminants, helping to understand their metabolism pathways, elimination rates and potential adverse activities of produced metabolites. Moreover, toxicokinetic interaction between commonly co-occurring ANF should be studied together using the *in vitro* models, allowing predictions of the *in vivo* situation and reducing the use of live animals at the same time. With respect to the present project, it would be interesting to investigate if the salmon metabolism was able to cope with exposure to increased amounts of isoflavones as found in commercial fish feeds in different countries.
- Zebrafish is a “model animal” in aquaculture research and were thus included in this project. For the same reason, it would be desirable to establish *in vitro* metabolism assays using zebrafish liver fractions. Since the zebrafish genome has been completely sequenced and is available in data repositories, the

determination of xenobiotic elimination pathways would be facilitated. Interspecies differences in biotransformation are frequently observed, so that the comparison of elimination parameters in zebrafish and salmon is of great interest and useful for the evaluation of zebrafish as a general model for other fish species.

- The gut together with its microbiome is known as the "second brain", which is playing a major role in regulating the physiological responses of an organism to the diet. Furthermore, the impact of gut health on neural functions and well-being has been documented. The effects of feed ingredients on intestinal health and gut-to-brain signal transmission are thus essential for fish welfare and should be included, when the suitability of novel feed ingredients is explored. In this respect, the observations made in the present study that WG can cause enteropathological symptoms in salmon that are similar to those found in humans with gluten sensitivity are remarkable. Moreover, future investigations should include the contribution of the intestinal microbiota in the metabolism and uptake of feed-borne substances.
- Genes involved in neurogenesis and tissue neural metabolism were found to be differentially regulated in zebrafish and salmon intestine receiving WG-containing diets in this project, indicating a neural connection from exposure to gluten peptides. Transcriptomic profiles are useful to trace physiological changes in their initial phase and could be used more intensively in the assessment of novel protein sources for fish feeds.
- Indices for gluten sensitivity in salmon should be further explored by using immunochemical methods. Confirmation, however, could only be achieved by performing feed challenge studies, where fish are given intermittently diets with and without gluten to examine the appearance and disappearance of symptoms. The percentage of gluten in feed that can be tolerated by salmon could thus be determined.
- Methods allowing the detection of fat deposition, tissue- and cell-damages, reduced muscle quality, etc due to unsuitable feed compositions at an early stage in the salmon life-cycle would help to avoid the development of health problems, increase resilience against pathogens and generally improve fish welfare. Techniques like bioelectrical impedance analysis could be explored since they

allow examinations in living fish. Finally, effects of different feed types on fillet quality and shelf life could be analysed, which is relevant with regard to product sustainability and consumer satisfaction.

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9.0 Scientific Papers I to IV

Paper I

toxins

MDPI

Article

Development and Validation of a Liquid Chromatography High-Resolution Mass Spectrometry Method for the Simultaneous Determination of Mycotoxins and Phytoestrogens in Plant-Based Fish Feed and Exposed Fish

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Abstract: New protein sources in fish feed require the assessment of the carry-over potential of contaminants and anti-nutrients from feed ingredients into the fish, and the assessment of possible health risks for consumers. Presently, plant materials including wheat and legumes make up the largest part of aquafeeds, so evaluation of the transfer capabilities of typical toxic metabolites from plant-infesting fungi and of vegetable phytoestrogens into fish products is of great importance. With the aim of facilitating surveillance of relevant mycotoxins and isoflavones, we have developed and validated a multi-analyte LC-HRMS/MS method that can be used to ensure compliance to set maximum levels in feed and fish. The method performance characteristics were determined, showing high specificity for all 25 targeted analytes, which included 19 mycotoxins and three isoflavones and their corresponding aglycons with sufficient to excellent sensitivities and uniform analytical linearity in different matrices. Depending on the availability of matching stable isotope-labelled derivates or similar-structure homologues, calibration curves were generated either by using internal standards or by matrix-matched external standards. Precision and recovery data were in the accepted range, although they varied between the different analytes. This new method was considered as fit-for-purpose and applied for the analysis of customised fish feed containing wheat gluten, soy, or pea protein concentrate as well as salmon and zebrafish fed on diets with these ingredients for a period of up to eight weeks. Only mycotoxin enniatin B, at a level near the limit of detection, and low levels of isoflavones were detected in the feed, demonstrating the effectiveness of maximum level recommendations and modern feed processing technologies in the Norwegian aquaculture industry. Consequently, carry-over into fish muscle was not observed, confirming that fillets from plant-fed salmon were safe for human consumption.

Keywords: Atlantic salmon; zebrafish; liquid chromatography high-resolution mass spectrometry; mycotoxins; phytoestrogens; plant-based feed

Key Contribution: A multi-analyte LC-HRMS/MS method for 25 targeted mycotoxins and phytoestrogens was developed and validated in feed and fish matrices. Mycotoxins above the

respective LOD were not detected in feed and dietary exposed fish, whereas phytoestrogens were found in soy and pea protein-based diets but carry-over into fish was not observed.

1. Introduction

Global fish production reached more than 171 million tonnes by 2016, of which 88% were directly used for human consumption and 12% (20 million tonnes) were used for the production of fishmeal and fish oil in aquaculture [1]. Fish and fishery products are an important source of essential nutrients in the human diet, and demand is growing in line with the increasing world population [2]. Aquaculture is the fastest-growing food industry and the intensification of the production depends on the utilisation of other resources for aquafeeds than fishmeal, for which exploitation is reaching an unsustainable level. Therefore, agricultural crops, mainly legumes, cereal grains and oilseeds, have been introduced in steadily increasing amounts into fish feeds, completely or partially replacing marine protein sources [3].

Plant protein sources mainly include soy, pea, lupine, alfalfa, wheat, corn, rape seeds, sunflower seeds, cotton seeds, sesame seeds, mustard oil cake, and white leadtree leaves [4]. Moreover, proteins from insects, microalgae, krill and single-cell proteins have been explored as replacements for fishmeal, but plant proteins are by far the most used ingredients in feed in aquaculture. The considerable changes in the diet composition of farmed fish include ingredients with physicochemical properties that potentially could lead to challenges regarding fish health and welfare, and product quality [5]. However, new processing technologies for plant protein extraction of undesirable components such as fertilisers, pesticides, persistent organic pollutants and heavy metals have allowed the transition from marine to agricultural sources [6]. The growth performance of plant-fed fish has been found to be adequate in short feeding studies [7], but concern about potential negative health effects from natural toxins and anti-nutritional factors including phytoestrogens remains [4,8]. Some anti-nutritional factors are considerably resistant against heat and digestion and have the potential for carry-over into the food chain. Several studies have shown that bioactive compounds may affect physiological functions in animals and humans including negative effects on intestinal health [9]; however, information for fish is limited [4]. The potential transfer of undesirable substances from new sources of aquafeeds might thus lead to potential health risks for consumers of fish products [10]. The assessment of transmissibility requires analytical methods that can be reliably applied for the detection of relevant natural contaminants in agricultural crops, and the considerable prevalence of mycotoxins and phytoestrogens makes them priority target analytes. However, only a few recent studies have surveyed mycotoxin levels in fish feed or farmed fish [11–16], and phytoestrogens are even less investigated [17,18].

There is a risk of mycotoxicosis in farmed fish due to the presence of mycotoxins in plant feed ingredients, but information on effects in fish is limited [11,19]. Mycotoxins comprise a large variety of secondary metabolites produced by fungi such as *Fusarium* spp., *Aspergillus* spp., *Alternaria* spp. and *Pencillium* spp. that infect agricultural crops both in the field and during storage, depending on their preferred growth conditions [20]. The presence of mycotoxins in practically all feed- and foodstuffs worldwide, although at different levels, is critical for nutritional security and safety, and important for animal and human health and welfare [21]. In moderate climate zones, major mycotoxin classes associated with *Fusarium* crop infections are trichothecenes, zearalenones and enniatins. The most important trichothecenes (polycyclic sesquiterpenoids) are A-type HT-2 toxin (HT-2) and T-2 toxin (T-2) and B-type deoxynivalenol (DON), including the acetylated and glucosidated derivatives 3-acetyl-deoxynivalneol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and deoxynivalenol-3-glucoside (DON-3G), as well as nivalenol (NIV). Furthermore, the mycoestrogen zearalenone (ZEN) shows considerable occurrence and toxicity. The ionophoric enniatins (ENN) B, B1, A, and A1 are detectable in almost all grain samples and considered an emerging threat [22]. In contrast, toxicity caused by ergot alkaloids such as ergosine, ergonovine, ergotamine, ergocristin, ergocornine

and α-ergocryptine in *Claviceps purpurea*-infected cereals has been known as ergotism for centuries. Ergot contamination is a sporadic issue but appears to have increased in recent years. The storage mycotoxin of main concern in Nordic countries is ochratoxin A (OTA), a pentaketidic isocoumarin produced by *Penicillium* or *Aspergillus* sp. In contrast, aflatoxins and fumonisins normally do not occur in Norwegian feed commodities [23]. The European Commission has recommended maximum levels for important mycotoxins in different feed commodities [24]. Fish ingredients and composite fish feed are not specifically mentioned but the guidance levels for DON (5 mg/kg); ZEN (2 mg/kg) and OTA (0.25 mg/kg) also apply to aquaculture. Additionally, an indicative value for the sum of T-2 and HT-2 (250 µg/kg) in compound feed is provided by the EU Commission recommendation [25]. Comparable values have not been established for NIV, enniatins or ergot alkaloids because of the limited occurrence and toxicity data.

Phytoestrogens are plant-derived polyphenolic non-steroidal compounds with structural and functional similarity to animal oestrogens, which can bind to oestrogen receptors and activate oestrogen receptor-dependent pathways in mammals and fish [26]. Thus, they have the potential to disrupt the endocrine system by competing with endogenous hormones. Phytoestrogens can be broadly differentiated into isoflavones, coumestans and lignans, depending on the alkylation pattern in the basic isoflavone molecule structure [27]. Legumes, especially soy, are rich in isoflavones, which occur in plants mainly in glucosidated form, whereas the unconjugated molecules are prevalent after uptake. Important representatives of this substance class are the glucosides daidzin, genistin, glycitin and their respective free counterpart's daidzein, genistein and glycitein [28]. They are also potential substrates for metabolic glucuronidation or sulphatation reactions in the liver and kidneys due to the hydroxyl groups in the molecule and could be excreted as conjugates [29]. Processed soy protein concentrates have an increased aglycon content, which results in improved phytoestrogen absorption from the diet [30]. Exposure of fish to phytoestrogens in feed has been shown to cause reproductive effects and to affect growth and metabolism [31], but the levels in the edible tissue of soy-fed fish and potential human exposure have not been investigated so far.

The assessment of possible health risks from the consumption of fish fed with plant-derived feed requires the development of appropriate analytical methods for the detection of transferred contaminants and bioactive compounds. Mycotoxins are usually analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) with different multi-toxin methods and in various matrices such as bulk cereals, flour, nuts, food products and hay bales [32–40]. Advanced sampling schemes and extraction protocols have been developed, resulting in improved homogeneity and recovery so that method validation can be performed [41]. Sample preparation often includes single-step solvent extraction using acidic acetonitrile/water mixtures, followed by solid-phase extraction (SPE) or immunoaffinity purification [39]. Matrix effects can be controlled by using matrix-matched calibration and isotope-labelled internal standards (ISTD), which are available for trichothecenes but not for enniatins and ergot alkaloids [32,33,36–38,40]. Notably, fewer LC-MS/MS methods have been described for ergot alkaloids than for *Fusarium* toxins, focussing on rye, feed and seeds as typical matrices [34,37]. In contrast, phytoestrogens are mostly measured in physiological samples including human and animal plasma, milk and urine in connection with monitoring of dietary exposure [42,43]. The LC-MS/MS methods developed for the detection of phytoestrogens in soy and food items use methanol-water extraction and reversed-phase (RP) chromatography [44,45].

Earlier studies have measured several mycotoxins in feed ingredients, aquafeeds and fish fillets [11,13,14,16,46] but ergot alkaloids were not among the analytes. In addition, we have found one report of the occurrence of phytoestrogens in foods of animal origin, including a few fish samples [47]. Considering the potential consumer health risk resulting from the extensive introduction of agricultural crops into fish feed and contaminant carry-over, analytical methods for the reliable detection of natural toxins and bioactive compounds are required. The present study was thus intended to fill this gap by developing a multiplexed LC-MS/MS method for the simultaneous quantification of 25 relevant feed-borne mycotoxins and phytoestrogens in feed and fish.

2. Results and Discussion

2.1. Fish Feed with Fixed Contents of Wheat Gluten, Soy Protein or Pea Protein

Finished feed has to comply with national and international legislation regarding maximum contents of certain contaminants including some mycotoxins [24,25]. In the present study, the fish diets were prepared in a fully equipped feed technology research facility based on materials that are commonly used in Norwegian aquaculture. Since the focus of the fish experiments was the potential transfer of natural contaminants from feed into fish, and not digestibility or feed utilisation, the composition was balanced with regard to plant-based ingredients (Table 1). Constant levels of 15% or 30% wheat gluten, soy protein concentrate or pea protein concentrate were achieved by adjusting the amount of fishmeal, which resulted in slight differences in the total crude protein and total lipid contents between the diets (Table 1). By keeping the ratio of plant-derived ingredients constant, comparability of the analytical results for the targeted metabolites was ensured.

Table 1. Composition of customised salmon and zebrafish feed (FM, fish meal; SPC, soy protein concentrate; PPC, pea protein concentrate).

Diet Composition (g/100 g)	FM (Control)	SPC15	SPC30	WG15	WG30	PPC15	PPC30
Salmon							
Fish meal	63.35	48.35	33.35	48.35	33.35	-	-
Wheat	12.0	12.0	12.0	12.0	12.0	-	-
Soy prot. conc.	-	15.0	30.0	-	-	-	-
Wheat gluten	-	-	-	15.0	30.0	-	-
Fish oil	20.0	20.0	20.0	20.0	20.0	-	-
Additives [#]	4.65	4.65	4.65	4.65	4.65	-	-
Total protein	45.2	44.6	44.0	46.7	48.1	-	-
Total lipids	26.5	25.1	23.8	25.4	24.3	-	-
Zebrafish							
Fish meal	79.35	64.35	49.35	64.35	49.35	64.35	49.35
Wheat	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Soy prot. conc.	-	15.0	30.0	-	-	-	-
Wheat gluten	-	-	-	15.0	30.0	-	-
Pea prot. conc.	-	-	-	-	-	15.0	30.0
Fish oil	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Additives [#]	4.65	4.65	4.65	4.65	4.65	4.65	4.65
Total crude protein	56.2	55.6	55.0	57.7	59.1	53.1	49.9
Total lipids	12.0	10.7	9.4	10.9	9.8	11.3	10.7

[#] Additives: Vitamin mix (2%), Mineral mix (0.59%), Monosodiumphosphate-24% P (2%), Yttrium oxide (0.01%), Carophyll Pink-10% (0.05%).

2.2. Exposure of Zebrafish and Salmon to Plant-Derived Aquafeeds

The zebrafish and salmon included in the feeding experiments showed an overall normal growth performance (data not shown). Observable differences in growth rate between diet groups in on-growing salmon in the order of SPC15 > SPC30 > WG15 ≈ FM > WG30 were small but proportional to the feed intake by the same groups. The zebrafish study also included an exposure to PPC15 and PPC30 feed compositions, resulting in a slight growth reduction that had previously also been described for rainbow trout [48]. We considered, however, that the small weight gain differences observed in the present study would not significantly affect the analysis of potentially transmitted contaminants in fish muscle.

2.3. Characteristics of Targeted Analytes in Method

The mycotoxins and phytoestrogens included in the multi-analyte LC-HRMS/MS method had considerable differences in their molecular weights and structures (Table S1). Furthermore, there were

sizeable differences in compound solubilities, e.g., between the hydrophilic DON, DON-3G, 3-ADON, 15-ADON and NIV and the lipophilic enniatins. These differences, as reflected by the logP (Table S1), became obvious in the order of retention on the reversed-phase LC-column (Figure 1). Molecular structure and logP were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Retention times differed with up to 30 min under the optimised chromatographic conditions of the ammonium acetate/MeOH gradient, while peak widths were small demonstrating good signal resolution. MeOH proved to be the best eluent for combining the different analytes in one LC method. Previous studies have shown that MeOH improves peak shape and sensitivity in the analysis of trichothecenes [32,33,35,37–39] and the same solvent has been used for phytoestrogen chromatography [45].

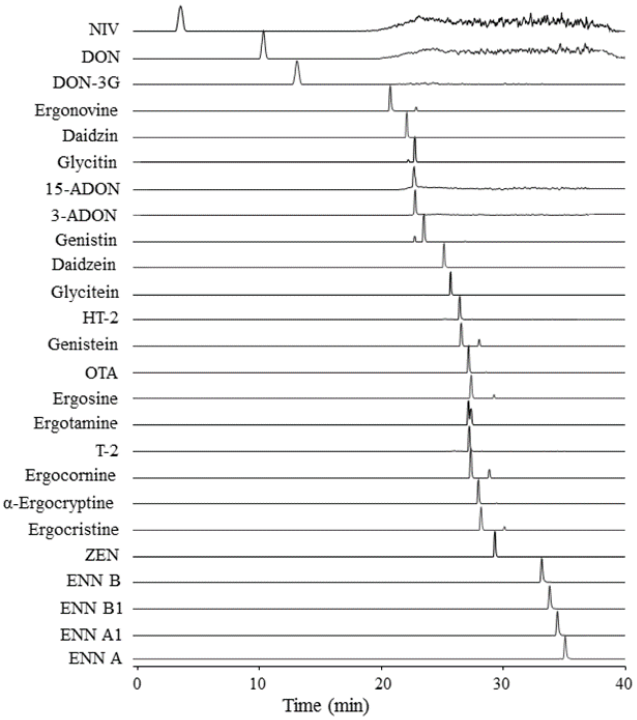

Figure 1. Chromatograms of targeted analysis of 100 µg/L in solvent of the 25 mycotoxins and phytoestrogens included in the multi-analyte LC-HRMS/MS method.

During method development, all compounds were analysed in positive and negative ESI mode for the determination of the highest peak intensities and best target ions, which included proton, ammonium, sodium and acetate adducts (Table 2). The HRMS/MS parameters were adjusted accordingly so that each compound was measured in targeted analysis under optimal conditions.

Table 2. Optimised LC-MS/MS conditions and calibration curve performances (R^2) for target compounds in different matrices.

Compound	Ionisation Mode	Target Ion	RT (min)	Precursor (*m*/*z*)	NCE (ev)	Fish Feed (R^2)	Salmon (R^2)	Zebrafish (R^2)	ISTD
DON	ESI neg	$[M+CH_3COO]^-$	12.3	355.1387	17	0.9996	0.9964	0.9996	^{13}C-DON
3-ADON	ESI neg	$[M+CH_3COO]^-$	23.8	397.1493	15	0.9998	0.9975	0.9999	^{13}C-3-ADON
15-ADON	ESI pos	$[M+Na]^+$	23.7	361.1258	15	0.9999	0.9986	0.9969	^{13}C-15-ADON
DON-3G	ESI neg	$[M+CH_3COO]^-$	15.1	517.1916	17	0.9993	0.9935	0.9851	^{13}C-DON-3G
NIV	ESI neg	$[M+CH_3COO]^-$	5.30	371.1337	17	0.9983	0.9901	0.9972	^{13}C-NIV
T-2	ESI pos	$[M+NH_4]^+$	28.0	484.2541	15	0.9995	0.9978	0.9995	^{13}C-T-2
HT-2	ESI neg	$[M+CH_3COO]^-$	26.4	483.2225	15	0.9998	0.9961	0.9998	^{13}C-HT-2
OTA	ESI neg	$[M-H]^-$	27.3	402.0739	32	0.9992	0.9984	0.9998	^{13}C-OTA
ZEN	ESI neg	$[M-H]^-$	29.5	317.1384	50	0.9999	0.9985	0.9998	^{13}C-ZEN
Ergonovine	ESI pos	$[M+H]^+$	21.9	326.1863	50	0.9996	0.9992	0.9999	MetErg
Ergosine	ESI pos	$[M+H]^+$	27.6	548.2868	27	0.9990	0.9979	0.9999	BromCri
Ergotamine	ESI pos	$[M+H]^+$	28.0	582.2711	32	0.9973	0.9985	0.9999	BromCri
Ergocornine	ESI pos	$[M+H]^+$	28.1	562.3024	25	0.9992	0.9973	0.9998	BromCri
α-Ergocryptine	ESI pos	$[M+H]^+$	28.8	576.3180	25	0.9993	0.9980	0.9999	BromCri
Ergoscristine	ESI pos	$[M+H]^+$	29.0	610.3024	27	0.9984	0.9980	0.9999	BromCri
ENN A	ESI pos	$[M+NH_4]^+$	35.1	699.4903	27	0.9943	0.9981	0.9992	-
ENN A1	ESI pos	$[M+NH_4]^+$	34.4	685.4746	27	0.9984	0.9987	0.9991	-
ENN B	ESI pos	$[M+NH_4]^+$	33.1	657.4433	27	0.9986	0.9952	0.9998	-
ENN B1	ESI pos	$[M+NH_4]^+$	33.8	671.4590	27	0.9993	0.9987	0.9993	-
Daidzein	ESI neg	$[M-H]^-$	26.1	253.0506	75	0.9993	0.9980	0.9982	-
Daidzin	ESI neg	$[M+CH_3COO]^-$	23.1	475.1246	10	0.9997	0.9984	0.9998	-
Genistein	ESI neg	$[M-H]^-$	27.3	269.0455	70	0.9997	0.9986	0.9979	-
Genistin	ESI neg	$[M+CH_3COO]^-$	24.4	491.1195	10	0.9994	0.9974	0.9999	-
Glycitein	ESI neg	$[M-H]^-$	26.4	283.0612	35	0.9998	0.9997	0.9989	-
Glycitin	ESI neg	$[M+CH_3COO]^-$	23.6	505.1351	10	0.9994	0.9979	0.9994	-

2.4. Optimisation of Sample Preparation

Appropriate sampling and sample extraction are prerequisites for the reliability of analytical methods [39–41]. Several studies describing sampling strategies for the mitigation of uneven contaminant distribution in different matrices have been published [34]. Sampling plans should aim at achieving pragmatic fit-for-purpose results, providing homogeneity while limiting sample sizes and numbers. In the present experiment, potential distributional heterogeneity was not an issue in the preparation of zebrafish samples since the whole carcasses of three fish were ground and extracted together. In contrast, the salmon fillets were of considerable size and could not be processed in total. Consequently, we attempted to obtain representative samples by punching out tissue at different places in fillet and combining aliquots after grinding (Figure 2a). Additional tissue punches were gathered for proteomic and immunological analyses that were foreseen for subsequent studies (Figure 2b). The composite diets had already a high degree of homogeneity due to the production process. We assumed therefore that the targeted analytes were evenly distributed in samples taken from a few places in the storage bags and ground together.

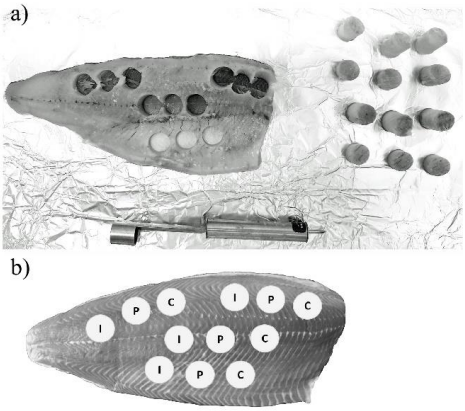

Figure 2. (**a**,**b**) Sampling scheme for homogenous sampling of representative aliquots from a salmon fillet. C: samples used for the chemical analyses in the present study. P and I: samples used for proteomic and immunological analyses in the same project.

Matrix effects impairing analytical method performance can be managed by using clean-up procedures, sample extract dilution, precipitation, filtering, matrix-assisted standard calibration curves and stable-isotope labelled ISTD [34,39]. Clean-up during sample preparation may include passing the extract through immunoaffinity columns or solid-phase extraction (SPE) cartridges, which can be filled with a variety of adsorbents. In the present study, we have not applied clean-up methods during sample preparation to avoid the potential loss of target analytes from surface adhesion. Additionally, the different molecular properties of the 25 compounds would optimally require the use of specific SPE materials. We have therefore attempted to develop a generally applicable sample preparation method by diluting the homogenised material with eight- to tenfold excess of adjusted solvent and using a one-step extraction procedure with subsequent submicron filtering.

Extraction conditions were optimised in a number of preliminary trials by determining recovery rates from spiked matrices with different acidic MeCN/water solvent compositions and, additionally, with a two-step MeCN/water approach [36,40]. However, the two-step extraction produced multiple aqueous and organic layers in the extract, making separation difficult and decreasing analyte recovery. The overall best results for the extraction of the target analytes from feed and fish were achieved with acidic MeCN/water (70:30) (Figure 3), similar to what has been described for other multi-mycotoxin

methods [36,37]. This solvent was also suitable for the phytoestrogens that have been extracted with MeOH/water in previous studies [44,45].

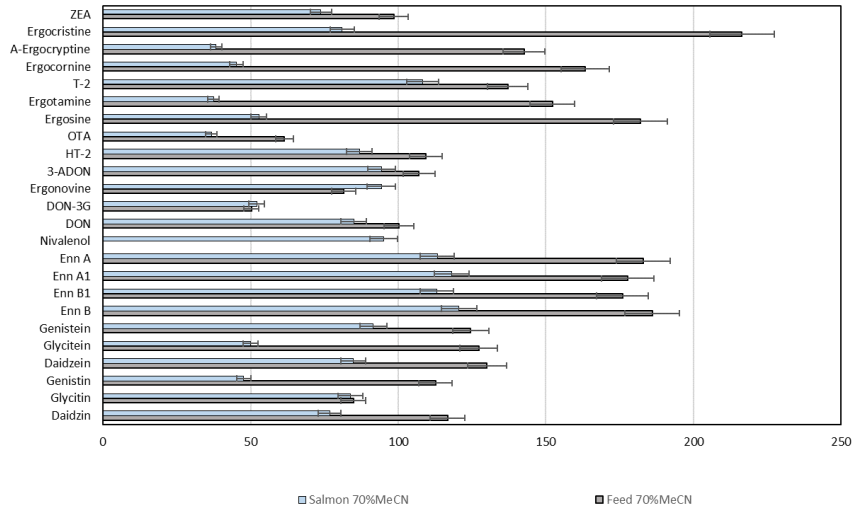

Figure 3. Recovery rates from spiked fish and feed matrices for the mycotoxins and phytoestrogens included in the multi-analyte LC-HRMS/MS method using optimised extraction solvent.

2.5. Performance of the Multi-Analyte LC-HRMS/MS Method

The performance characteristics of the new LC-HRMS/MS method for 25 mycotoxins and phytoestrogens were determined with regard to international standardised guidelines [49,50]. The specificity of the method for the selected analytes was excellent due to the high mass accuracy in full scan mode and targeted fragmentation (dd-MS^2) (Figure 1; Table 2). The total run time was slightly increased in comparison to other multi-mycotoxin methods [32,33,35,37–39,41], leading to good chromatographic separation of the analytes. The high resolution of the analysis allowed us to resolve between isomers such as 3-ADON and 15-ADON, which previously has been sometimes a challenge [41].

The 25 analytes were detected with different sensitivities in fish and feed matrices differed considerably between the 25 analytes. The salmon matrix-assisted standard calibration curves showed high sensitivities for the enniatins, ZEN and the phytoestrogens daidzein and genistein, whereas the curve slopes were less steep for the trichothecenes, OTA, ergot alkaloids and remaining phytoestrogens. Interestingly, this order was not identical for solvent, zebrafish and feed matrices, comparable to results reported for other multi-mycotoxin methods that achieved different analyte sensitivities in matrices such as fruit, yoghurt, soya, hazelnut, pepper, wheat, maize, oat, rice, pasta and bread [33,35–38]. The effect of the signal enhancement or suppression by a specific matrix type can be illustrated by the connected SSE% value. Matrix impact is considered as insignificant for SSE 80-120%, while lower values indicate significant signal decrease and higher values signal increase [32,33,35,37–40]. In the present study, SSE varied from 67% to 115% for control fish feed, 58% to 173% for salmon, and 89% to 181% for zebrafish, with ENN A showing the highest signals in the feed and fish matrices (Table 3). Considering all analytes, the feed matrix generally suppressed signals, whereas the fish matrix caused signal enhancement.

Linearity of the standard calibration curves in different matrices was achieved for all analytes in the range 1.0 to 200 µg/L, with the exception of NIV, OTA, DON-3G and 15-ADON that were linear in the range 5.0 to 200 µg/L. The correlation coefficients (Table 2) were $R^2 > 0.98$ for all calibration curves, irrespectively of whether or not stable-isotope labelled ISTD, similar analogue-ISTD or no ISTD were

included. Considering the eight times or 10 times sample dilution during matrix extraction, the linear ranges corresponded to 8.0 (40)–1600 µg/kg for feed and salmon and 10 (50)–2000 µg/kg for zebrafish.

The limits of detection (LOD) and quantification (LOQ) in solvent, fish feed, salmon and zebrafish matrices are presented for the undiluted commodities (Table 3). The LOD ranged in solvent from 1 µg/L for ENN A1, B, B1 and genistin to 19 µg/L for NIV, in fish feed from 6 µg/kg for 15-ADON to 85 µg/kg for ENN A, in salmon from 21 µg/kg for glycitein to 144 µg/kg for NIV, and in zebrafish from 8.0 µg/kg for ergonovine and α-ergocryptine to 176 µg/kg for DON-3G. The corresponding LOQ were, as per the definition, 3.3 times higher (Table 3). The values were similar to data shown for comparable multi-mycotoxin methods. LOD ranging from 5.4 to 24 µg/kg for DON, 36 to 50 µg/kg for 15-ADON, 2.8 to 50 µg/kg for NIV, 0.2 to 47 µg/kg for ZEN, 1.0 to 18 µg/kg for T-2, and 0.7 to 12 µg/kg were reported in a number of different matrices [32,35–38]. In contrast, two methods that had been specially developed for the analysis of phytoestrogens in food products had established group LODs of, respectively, 250 µg/kg [44] and 15 µg/kg [45].

The precision of our multi-analyte LC-HRMS/MS method was demonstrated on the one hand by good day-to-day congruency of the solvent and matrix-assisted standard calibration curves. The coefficients of variation (% CV) for all data points in six independent experiments were generally less than 20% in solvent and less than 25% in feed, salmon and zebrafish matrices (data not shown), which was well within the guidance criteria [49]. On the other hand, precision was also assessed by intra-day and inter-day analysis of spiked quality control samples. The total within-laboratory precision was in the range of 1% for ZEN and ENN A to 17% for NIV in the feed matrix and 1% for ergonovine to 41% for NIV in the salmon matrix (Table 3). The precision data were comparable to values reported for other multi-mycotoxin methods in a variety of matrices [32,35,37,38,41]. Published precision data for phytoestrogen analysis in food commodities are scarce. When control samples were analysed using standard calibration in solvent, intra-day and inter-day% CV in the range of 1–13% were reached for a number of analytes [45].

Recovery rates in fish feed ranged from 19% to 161% for all mycotoxins and phytoestrogens in the newly developed method, with the exception of DON-3G, NIV, ergosine, ergotamine, ergocornine and α-ergocryptine that were retrieved less efficiently, and ENN A and ENN A1 that showed enhanced recoveries (Table 3). In the salmon matrix, the analytes were recovered with 69–127% except for a reduced performance for NIV and enhancement for genistein. In the zebrafish matrix, recovery rates of 41–98% were reached, except in DON-3G and NIV, which showed reduced values. The recovery rates established in the present study were similar to those determined with comparable methods ranging from 50% to 150% for a number of mycotoxins [32,35–41]. For phytoestrogens, recoveries between 89% and 107% in spiked solvent have been reported [45]. However, in different food matrices the rates were widely varying and in part very low, which is in strong contrast to our new LC-HRMS/MS method, showing remarkably low interference for phytoestrogen analysis in the three matrices considered (Table 3). Spiking experiments are widely used for the determination of recoveries in the validation of analytical methods, although they only can emulate naturally-contaminated samples to a certain extent. Preferably, the accuracy should be verified with a certified reference material, but this is currently not available for all target analytes and selected matrices of the LC-HRMS/MS method.

Table 3. Performance validation parameters for the multi-analyte LC-HRMS/MS method (*n* = number of analysis for each category; a: solvent; b: fish feed; c: salmon; d: zebrafish).

Compound	*n*	LOD				LOQ				SSE (%)	Total within Laboratory Precision (%)		Recovery ± SD (%)		
		(µg/L)	(µg/kg)			(µg/L)	(µg/kg)								
	(a/b/c/d)	(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)	(b/c/d)	(b)	(c)	(b)	(c)	(d)
with ISTD															
DON	4/4/3/3	3	23	67	22	9	78	225	74	77/87/133	4	4	90 ± 7	107 ± 13	92 ± 25
3-ADON	4/4/3/3	4	17	57	9	15	56	189	29	98/116/144	3	3	112 ± 17	96 ± 12	78 ± 20
15-ADON	4/3/3/3	5	6	43	63	16	20	142	210	96/161/141	10	3	133 ± 2	107 ± 25	86 ± 14
DON-3G	4/4/3/3	5	36	115	176	18	121	383	588	85/95/119	11	22	19 ± 9	83 ± 20	48 ± 31
NIV	4/4/3/3	19	59	144	76	63	196	479	252	71/65/115	17	41	57 ± 34	69 ± 33	41 ± 24
T-2	4/4/3/3	4	26	53	26	12	88	176	86	97/136/151	3	3	96 ± 17	99 ± 15	90 ± 19
HT-2	4/4/3/3	2	22	70	15	8	73	235	52	89/129/149	2	3	94 ± 18	96 ± 11	98 ± 15
OTA	4/4/3/3	5	41	44	21	18	138	148	68	105/139/150	6	4	75 ± 13	87 ± 23	83 ± 20
ZEN	4/4/3/3	6	11	43	14	22	38	143	47	90/125/125	1	2	109 ± 5	106 ± 18	96 ± 25
Ergonovine	4/4/3/3	6	23	35	8	19	77	115	26	85/130/106	2	1	84 ± 8	98 ± 13	87 ± 30
Ergosine	4/4/3/3	4	35	52	9	12	117	173	32	79/129/138	7	11	69 ± 27	89 ± 31	72 ± 20
Ergotamine	4/4/3/3	2	59	56	10	8	195	188	35	81/134/155	10	9	64 ± 10	84 ± 26	77 ± 18
Ergocornine	4/4/3/3	3	32	59	16	11	108	196	53	93/129/136	11	11	59 ± 16	90 ± 26	70 ± 14
α-Ergocryptine	4/4/3/3	4	15	38	8	14	50	126	28	67/119/137	7	8	53 ± 7	82 ± 21	70 ± 14
Ergoscristine	4/4/3/3	3	30	51	10	10	100	170	32	70/117/135	8	5	77 ± 22	88 ± 24	54 ± 26
without ISTD															
ENN A	4/4/3/3	4	85	49	32	13	284	165	108	115/173/181	1	8	161 ± 14	117 ± 27	81 ± 17
ENN A1	4/4/3/3	1	45	40	33	5	150	133	111	102/122/148	3	11	147 ± 21	110 ± 29	80 ± 16
ENN B	4/4/3/3	1	41	78	17	4	138	260	57	95/132/152	2	10	117 ± 16	107 ± 30	79 ± 17
ENN B1	4/4/3/3	1	29	40	29	5	96	133	96	102/125/147	3	12	134 ± 9	106 ± 35	79 ± 17
Daidzein	4/4/3/3	13	30	50	48	42	100	168	159	86/120/101	4	13	123 ± 9	122 ± 18	93 ± 15
Daidzin	4/4/3/3	3	19	45	15	12	62	152	51	86/113/140	4	16	93 ± 21	93 ± 6	71 ± 13
Genistein	4/4/3/3	11	20	42	52	37	66	141	172	81/120/104	2	13	114 ± 23	127 ± 28	91 ± 18
Genistin	4/4/3/3	1	29	72	11	5	95	241	35	101/143/149	4	12	101 ± 45	88 ± 1	69 ± 14
Glycitein	4/4/3/3	11	18	21	37	36	58	68	124	80/58/89	2	16	127 ± 3	118 ± 24	96 ± 16
Glycitin	4/4/3/3	4	27	51	26	13	90	170	88	94/123/121	4	23	96 ± 14	113 ± 13	97 ± 22

2.6. Mycotoxins and Phytoestrogens in Fish Feed, Zebrafish and Salmon Tissues

The in-house-validated multi-analyte LC-HRMS/MS method was used for the analysis of the customised fish feed and dietary exposed salmon and zebrafish. The feed analysis did not detect any of the targeted mycotoxins, with the exception of ENN B that was found in concentrations close to LOD in WG30 (data not shown). Norwegian aquafeeds ingredients contain generally only low amount of mycotoxins [13,23]. The highest mean contents were found in wheat (DON: 94 µg/kg; T-2+HT-2: 28 µg/kg) and maize (ZEN: 246 µg/kg), which was in compliance with the recommended maximum levels [24,25,51]. Considering that in the present study, the feed contained a maximum of 42% wheat-derived components (WG30) (Table 1), we did not expect sizable levels in the five diets. In contrast, survey data for finished feeds from Central Europe and Asia contained on average 165 µg DON/kg, 188 µg ZEN/kg and 2 µg OTA/kg [11]. Interestingly, our finding of ENN B in WG30 diets is in line with the relatively high prevalence of enniatins in cereals in Northern Europe. ENNs have shown considerable toxicity in in vitro studies and in mice [52]. Carry-over of ENN B and B1 from poultry feed into eggs has been demonstrated [22], but maximum levels for animal feed have not been established yet.

In view of the low mycotoxin content (<LOQ) in the customised feeds in the present study, we consequently did not detect any of the targeted analytes above the respective LOQ in salmon or zebrafish tissues. There were, however, traces of ENN B in several of the WG30-exposed salmon at concentrations close to the LOD, suggesting the carry-over potential of enniatins. A relatively high occurrence of ENNs, especially ENN B, in fish muscle and livers has been previously reported [22,53] and correlates with our data. Transfer of mycotoxins such as DON, T-2 and OTA from low-level contaminated wheat gluten-containing feed into fish fillets has also been demonstrated [13]. In contrast, when salmon was fed with diets containing 2 and 6 mg DON/kg or 0.8 and 2.4 mg OTA/kg for eight weeks, up to 19 µg DON/kg was measured in the muscle, whereas up to 5 µg OTA/kg was detectable in the fish livers [46]. Human exposure following high consumption of salmon fillets with the highest DON concentrations was estimated to amount to only 2% of the established tolerable daily intake (TDI) [46,54]. Consequently, our results in the present study show that the use of plant-based fish feed containing mycotoxins below the recommended maximum levels results in negligible health risks for consumers.

The phytoestrogen analysis of the diets included in the salmon and zebrafish feeding experiments showed dose-dependent levels of all targeted analytes in the soy protein containing feeds (data not shown). Mean concentrations ranged in SPC15 from 21 µg glycitein/kg to 786 µg daidzin/kg and in SPC30 from 40 µg glycitein/kg to 1356 µg daidzin/kg. Glucosidated forms occurred in higher concentrations than the corresponding aglycons, whereas an increase of the free form had been previously observed in extruded protein preparations [30]. In PPC15 and PPC30, 26 and 54 µg glycitein/kg were detected, respectively, confirming results from a screening study on fruits and vegetables [45]. Phytoestrogen levels in food and feed are not regulated so far, and the health risks or benefits of dietary exposure in humans and animals are still under discussion [28,44]. Still, considerable oestrogenic and thyrogenic activities have been determined in vitro in commercial Spanish fish feeds [18], and further evaluation is required. A survey of the phytoestrogen content in food products of animal origin detected the highest concentrations in soy-containing milk products and farmed salmon contained up to 40 µg/kg [47].

In the present experiment, we did not find phytoestrogen concentrations above LOQ in dietary exposed zebrafish or salmon, not even in the respective SPC30 groups. Information on the uptake of isoflavones in fish is not available, but considerable differences in bioavailabilities and biotransformation are reported for warm-blooded vertebrate species [55]. We have recently studied the metabolism of isoflavones in salmon liver microsomes (article in progress) and characterised the major metabolites. Chromatographic peaks corresponding to the retention times and m/z of these metabolites were, however, absent in the muscle of the dietary exposed fish suggesting an efficient detoxification mechanism and excretion of isoflavones without accumulation in the edible parts of fish. Equol,

an intestinal metabolite of daidzein, has not been studied in our experiment. Previous studies have suggested that isoflavone metabolisation by the intestinal microbiome varies considerably between producers and non-producers of equol [55]. When gibel carp (*Carassius auratus gibelio*) were exposed to 40–400 mg daidzein/kg in feed, the unchanged compound was recovered with 128 and 261 μg/kg in the fish muscle [56]. In contrast, equol was not found in any of the samples suggesting that fish could lack the necessary gut bacteria. Considering that the highest daidzein level in our experiments was with 0.2 mg/kg in SPC30, about 200-fold smaller than the lowest feed concentration in the gibel carp study, and considering the LOQ of the LC-HRMS/MS method in the fish matrix, the non-detectability of the targeted isoflavones in the salmon fillets was conclusive. However, we intend to investigate the metabolic fate of important isoflavones in fish in depth in a follow-up study.

3. Conclusions

The increasing use of vegetable ingredients in aquafeeds has motivated risk evaluations for mycotoxin exposure of farmed fish, which has resulted in the establishment of recommended maximum levels. Furthermore, the potential consequences of the presence of bioactive compounds such as isoflavones in plant-based feed should be monitored. We have therefore developed and validated a 25-in-1 LC-HRMS/MS method that is suitable for the survey of compliance to feed regulations and for the detection of undesirable compounds in fish fillets. The new method has excellent specificity for all analytes, while there are some differences in sensitivity due to the great diversity of molecular structures. The LOD and LOQ in fish feed, zebrafish and salmon matrices are sufficient to ensure that mycotoxin and phytoestrogen levels are below concentrations that might cause negative health effects. The accuracy of the method, described by precision and recovery of the included analytes, is satisfactory, confirming its applicability for screening and surveillance purposes. The applicability range is limited at present, however, due to the exclusion of aflatoxins. They will be added during the planned extension of the multi-analyte method. In zebrafish and salmon exposed to customised feed containing up to 30% wheat gluten, soy or pea protein concentrate, carry-over of mycotoxins or phytoestrogens could not be detected, confirming that fillets from fish fed commercial plant-based diets are safe for consumption.

4. Materials and Methods

4.1. Chemicals

LC-MS grade acetonitrile (MeCN), methanol (MeOH) and water (Optima, LC/MS grade,) were provided by Fisher Scientific (Loughborough, Leics., UK), and ethanol (EtOH) was obtained from VWR International (Lutterworth, Leics., UK). Acetic acid (CH_3COOH) (>99.8%), formic acid (HCOOH) (>98%) and ammonium acetate (CH_3COONH_4) (>98%) were purchased from Merck KGaA (Darmstadt, Germany).

The mycotoxins deoxynivalenol (DON), 3-actetyl-deoxynivalenol (3-ADON), nivalenol (NIV), T-2 toxin (T-2), HT-2 toxin (HT-2), zearalenone (ZEN), deoxynivalenol-3-glucoside (DON-3G), 15-acetyl-deoxynivalenol (15-ADON), ochratoxin A (OTA), ergosine, α-ergocryptine and ergocristine as well as the stable isotope-labelled analogues U-[^{13}C-15]-NIV, U-[^{13}C-15]-DON, U-[^{13}C-21]-DON-3G, U-[^{13}C-17]-3ADON, U-[^{13}C-17]-15ADON, U-[^{13}C-22]-HT-2, U-[^{13}C-24]-T-2, U-[^{13}C-20]-OTA, U-[^{13}C-18]-ZEN were provided by Romer labs (Tulln, Austria) as solutions in MeCN, ranging from 10 to 100 mg/L. Intermediate standard solutions at 10 mg/L were prepared for DON-3G and 15-ADON by dilution of stock solutions with MeCN. The enniatins A, A1, B, and B1 (ENN A, A1, B, B1), ergonovine, ergotamine, ergocornine, methysergide maleate salt (MetErg) and bromocriptine mesylate (BromCri) were provided as solids by Sigma-Aldrich (St. Louis, MO, USA). Stock solutions in MeOH or MeCN were prepared for ergot alkaloids in the range of 100 to 500 mg/L, and for enniatins with 200 mg/L in MeOH. A combined intermediate standard solution with 10 mg/L was prepared for both enniatins and ergot alkaloids by combining appropriate aliquots of

stock standard solutions, evaporating the mixture with a gentle stream of nitrogen and re-dissolving in MeCN/water (50:50). Finally, a combined standard solution containing all mycotoxins (Set A) was prepared by combining aliquots of stock or intermediate standard solutions, evaporating the solvent and re-dissolving in the appropriate volume MeCN/water (50:50) to obtain final concentrations of about 200 µg/L (200.0–200.12 µg/L, depending on the stock solution provided by the manufacturer).

The phytoestrogens daidzin, genistin, glycitin, daidzein, genistein, and glycitein were bought in crystalline form from Sigma-Aldrich (St. Louis, MO, USA), and stock solutions were prepared in MeOH or DMSO (glycitein) ranging from 500 to 1000 mg/L. Individual intermediate standard solutions at a concentration of 5 mg/L were prepared by dilution with MeOH. A combined standard solution (Set B; 200 µg/L) containing all phytoestrogens was prepared by further dilution in MeCN/water (50:50). The finished Set A and Set B solutions were stable at −20 °C for several months and used for the preparation of standard calibration curves.

Additionally, a 25-in-1 multi-analyte mixture was prepared and used in spiking experiments. All analytes were combined with regard to the concentrations of their respective stocks or intermediate standard solutions so that a final concentration of 25 µg/L per analyte was reached after spiking into feed, zebrafish and salmon samples. The multi-analyte mixture was evaporated and re-dissolved in MeCN/water (50:50). It was stable at −20 °C for about a month.

A combined internal standard (ISTD) solution for 15 mycotoxins, containing stable isotope-labelled analogues and the ergot homologues MetErg and BromCri, was prepared in MeCN/water (50:50) to reach final concentrations of 251 µg/L U-[^{13}C-18]-ZEN, 500 µg/L U-[^{13}C-22]-HT-2, 443 µg/L U-[^{13}C-22]-T-2, 506 µg/L U-[^{13}C-15]-DON, 502 µg/L U-[^{13}C-17]-3ADON, 500 µg/L U-[^{13}C-17]-15ADON, 500 µg/L U-[^{13}C-20]-OTA, 530 µg/L U-[^{13}C-15]-NIV, 530 µg/L U-[^{13}C-21]-DON-3G, 624 µg/L BromCri and 500 µg/L MetErg. The different concentrations were chosen with regard to the respective measurement sensitivities in the developed multi-analyte LC-HRMS/MS method. The ISTD solution was stored at −20 °C, adjusted to room temperature (RT) and mixed thoroughly prior to use. It was added in a ratio of 1:5 to the study samples.

4.2. Preparation of Fish Diets

Diets with definite amounts of wheat gluten, soy protein concentrate or pea protein concentrate were produced at Nofima Feed Technology Centre, Fyllingsdalen, Norway. The diets were based on fishmeal (FM) as main protein source, which was replaced by 15% or 30% plant proteins. All diets contained 12% wheat that was required for binding in the extrusion process, in addition to minor inclusion of wheat as carrier for some of the additives used (Table 1). In total seven diets were produced: (1) control feed (FM), (2) 15% soy protein concentrate (SPC15), (3) 30% soy protein concentrate (SPC30), (4) 15% wheat gluten (WG15), (5) 30% wheat gluten (WG30), (6) 15% pea protein concentrate (PPC15), and (7) 30% pea protein concentrate (PPC30). The ingredients used for the preparation of diets included FM Norsildmel AS (Bergen, Norway), SPC from Agilia A/S (Videbæk, Denmark), PPC from AM Nutrition AS (Stavanger, Norway) and WG from Tereos Syral (Marckolsheim, France). All diets had an inclusion of 4% fish oil at extrusion. The feed were produced on a pilot scale twin-screw, co-rotating Wenger TX 52 extruder (Wenger, Sabetha, KS., USA) with a die of 2.5 mm diameter. After extrusion, the diets were dried for 40–70 min in a carousel dryer (Paul Klöckner, Verfahrenstechnik GmbH, Hachenburg, Germany) at 65 °C to a water content of 7–8%. The salmon diets 1 to 5 were, in addition, oil-coated with 16% fish oil after extrusion by vacuum-coating (Dinnissen, Sevenum, Netherlands) to meet the standard dietary inclusion of oil for the fish size studied. The salmon feed had a pellet size of 3.5 mm, while the zebrafish feed were ground and sieved to a pellet size of 0.6–0.8 mm.

4.3. Feeding Studies in Zebrafish and On-Growing Salmon

4.3.1. Zebrafish

Four-month-old zebrafish (*Danio rerio*) (AB strain) with a mean weight of 0.214 g were distributed into 28 tanks ($n = 16$) and were maintained in a flow-through system with 20 % water exchange per hour (ZebTEC Stand-Alone Toxicology Rack, Techniplast, London, UK) under daily-monitored standard husbandry conditions, including a stable temperature of 28 ± 0.5 °C, pH 7.5, water conductivity of 1500 μS/cm and photoperiod of 12 h light:12 h dark at the Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway. The feeding study included 336 fish that were distributed into the system's 3.5-litre tanks according to the seven experimental diets. Four replicate groups per diet, each consisting of 12 fish (six per gender) in one tank (and an additional four fish to compensate for potential losses during the study period), were hand-fed twice daily with a total feed amount equal to 2.5% of their body weight over a period of 46 days. The feeding behaviour and health and welfare of the fish were regularly controlled. At the end of the study, the fish were not fed for 24 h prior to sampling. They were separated by gender and euthanised individually by transfer into a tank containing a lethal dose of 200 mg/L tricaine methanesulfonate (MS222) (Sigma-Aldrich, St. Louis, MO, USA), buffered with an equal amount of sodium bicarbonate. The liver, spleen and intestines were carefully dissected under a light microscope and immediately frozen in liquid nitrogen along with the rest of the carcass. All samples were stored at −80 °C for further analyses.

The zebrafish feeding study was conducted in compliance with the guidelines provided by the Norwegian Animal Research Authority (FOTS ID 12581, 27 July 2017) and approved by the Nord University (Norway) ethics committee.

4.3.2. Salmon

One-year-old post-smolt Atlantic salmon (*Salmo salar*; salmo breed strain) with a mean weight of 223 g were randomly distributed into 15 experimental tanks (1 m^3; $n = 32$) filled with seawater at the Nofima Research Station, Sunndalsøra, Norway. The oil-coated diets 1–5 were given to randomised triplicate tanks by automatic disc feeders. Excess feed was collected once daily for calculation of feed intake. The water temperature was maintained at an average of 10.6 (±0.6) °C. The oxygen level at the tank outlets was higher than 90% at study start and about 80% at the study's end. The water flow in each tank was set to 20 L/min.

The feeding was conducted for nine weeks. At the start of the experiment, 15 fish were sampled, and the muscle, liver and intestine were collected. After five weeks, muscle was sampled from one fish from each tank of the FM, SPC30 and WG30 groups. At the termination of the study, five fish from each tank were collected and weighed. The sampled fish were anaesthetised with 60–80 mg/L MS222, transferred and euthanised with a double dose (120–160 mg/L) MS222. Blood was drawn from the caudal vein using 2.5-mL vacutainers (VACUETTE® 2.5 mL Z serum separator clot activator; Greiner Bio-One, Kremsmünster, Austria) and centrifuged at 2500× *g* for 15 min at 4 °C (Allegra 6R Centrifuge, Beckman, Indianapolis, IN, USA), and sera were stored at −20 °C. The livers and intestines of the fish were removed, and tissue samples were frozen with liquid nitrogen and stored at −80 °C. Fillets were stored at −20 °C. The remaining fish in each tank were weighed in bulk, and their mean weight was calculated, including the sampled fish.

The salmon feeding study was performed in compliance with the national regulations for the use of animals in experiments [57]. The experiment was classified as not requiring a specific license [58] as none of the planned experimental treatments were expected to cause any distress or discomfort for the fish.

4.4. Extraction of Fish Feed, Zebrafish and Salmon Samples

4.4.1. Fish Feed

Fish feed pellets were homogenised with a grinding mill (Retsch, Haan, Germany), and 2.5 g were weighed into 50-mL polypropylene tubes. After the addition of 20 mL extraction solvent, the samples were vortexed for 1 min, extracted on a horizontal shaker (Edmund Bühler, Tübingen, Germany) with 200 min^{-1} at room temperature (RT) for 30 min, and centrifuged with 2000× *g* for 10 min at 4 °C (Beckman Coulter, Brea, CT, USA). The supernatants were transferred into fresh 50-mL tubes and let to settle overnight (ON) at 4 °C. Subsequently, 0.5 mL of the supernatants were centrifuged for 1 min at 20,000× *g* through 0.22 µm nylon filters (Costar Spin-X; Corning, Inc., Corning, NY, USA) and 40 µL of the filtrates were transferred into LCMS vials. Finally, 10 µL ISTD solution were added to each vial. Samples were store refrigerated until analysis by LC-HRMS/MS.

The composition of the extraction solvent was optimised during method development in spiking experiments. Multi-analyte mixture (50 µL) was added to 2.5 g ground feed, which was then kept under a laminar hood for 30 min, allowing the solvent to evaporate. Extractions were performed either in one step with 20 mL acidic (0.1% formic acid (FA)) MeCN/water mixtures of different compositions (50:50; 60:40; 70:30; or 80:20) or in two steps with acidic MeCN/water (I: 80:20; II: 20:80). Based on the best recovery rates for mycotoxins and phytoestrogens, MeCN/water (70:30; 0.1% FA) was selected for all further experiments.

4.4.2. Zebrafish

Three frozen, gutted zebrafish, for each replicate and diet, were thawed and, after separation of the heads, ground to a fine powder with pestle and mortar in liquid nitrogen. The powdered tissue (0.1 g) was weighed and extracted with 1 mL extraction solvent (MeCN/water 70:30; 0.1% FA). The mixture was homogenised by ultra-sonication (Branson, Danbury, CT, USA) for 10 min at 30 °C, centrifuged at 4000× *g* for 10 min at 4 °C (Thermo Scientific, Waltham, MA, USA), and the supernatant was transferred into fresh 5-mL tubes. An aliquot (0.5 mL) was filtered as described before, and 40 µL of the filtrates were transferred into LCMS vials, mixed with 10 µL of the ISTD solution, and analysed by LC-HRMS/MS.

The recoveries of mycotoxins and phytoestrogens from the zebrafish matrix was investigated during method development by different acidic MeCN/water extraction solvents in spiking experiments with multi-analyte mixture.

4.4.3. Salmon

The salmon fillets were half-thawed. Tissue pieces of equal size were sampled from four different areas using a steel puncher (0.5 cm in diameter) (Figure 2). The tissue samples were ground with a pestle and mortar, combined, and 2.5 g were transferred into a 50-mL tube, extracted with 20 mL extraction solvent (MeCN/water 70:30; 0.1% FA) and thoroughly homogenised for 40 s by ultra-turrax (Janke & Kunkel, IKA-Werke, Staufen, Germany). To avoid cross-contamination, the ultra-turrax was washed with water for 20 s between samples from the same fish tank and with water and MeOH for 40 s between samples from different tanks. The samples were vortexed for 30 s and extracted using a horizontal shaker (Edmund Bühler) with 200 min^{-1} at RT for 1 h. Subsequently, they were centrifuged with 2000× *g* for 10 min at 4 °C (Beckman Coulter), and the supernatants were transferred into fresh 50-mL tubes and let to settle overnight at 4 °C. Subsequently, 0.5-mL aliquots were filtered as described before, and 40 µL of the filtrates were transferred into LCMS vials, mixed with 10 µL of the ISTD solution, and analysed by LC-HRMS/MS. The recovery of mycotoxins and phytoestrogens from the salmon matrix was investigated as described for zebrafish.

4.5. Preparation of Matrix-Assisted Standard Calibration Curves

Calibration curves in solvent were prepared by evaporating 200 µL Set A solution with nitrogen and re-dissolving with 200 µL Set B, resulting in a standard solution with 200 µg/L for all 25 analytes included in this study. The standard solution was serially diluted with MeCN/water (50:50) to produce calibrants with 200, 100, 50, 10, 5 and 1 µg/L. For the preparation of the matrix-assisted standard calibration curves, 40 µL aliquots of the calibrants were transferred into LCMS vials and 10 µL ISTD solution was added. They were evaporated with nitrogen at 40 °C and re-dissolved in the same volume of blank matrix extract that had been prepared either from control feed or from zebrafish or salmon in the respective FM-control groups by pooling equal volumes of replicates. The calibration standards were transferred into LCMS vials and analysed by LC-HRMS/MS.

4.6. Development of the Multi-Analyte Liquid Chromatography High-Resolution Mass Spectrometry (LC-HRMS/MS) Method

Multi-analyte analysis was performed on a Q-Exactive™ Hybrid Quadrupole-Orbitrap HRMS/MS equipped with a heated electrospray ion source (HESI-II) and coupled to a Vanquish UHPLC system (Thermo Scientific). The instrument setup was similar to that described in a previous study [36]; however, there were several modifications and different analytes were included. The HESI-II interface was operated at 300 °C, alternatively in positive and negative mode during one run. The parameters were adjusted as follows: spray voltage 3.2 and 2.5 kV (positive and negative mode, respectively), capillary temperature 280 °C, sheath gas flow rate 35 L/min, auxiliary gas flow rate 10 L/min, and S-lens RF level 55.

The Q-Exactive HRMS/MS was operated in full scan (FS) mode with the inclusion of targeted fragmentation (data-dependent MS/MS: dd-MS^2). For full scans, the mass ranges were set to *m*/*z* 90–900 and 200–900 in negative and positive mode, respectively. FS data were acquired at a mass resolution of 70,000 full width half-maximum (FWHM) at *m*/*z* 200, while mass resolution was set to 17,500 FWHM at *m*/*z* 200 during dd-MS2. The automated gain control (AGC) target was set to 5×10^5 ions for a maximum injection time (IT) of 250 ms in the FS mode, whereas for dd-MS^2 mode the AGC target was 1×10^5 and the IT was 100 ms. The inclusion list for the targeted analysis contained the *m*/*z*, retention times (RT), and normalised collision energies (NCE) (Table 2). NCE values were determined by direct infusion of standard solutions in the mobile phase (MeCN/water (50:50), containing 5 mM ammonium acetate and 0.1% acetic acid) by using a syringe pump at a flow rate of 5 µL/min. The quadrupole mass filter was operated with an isolation window of *m*/*z* 3. External mass calibration of the Q-Exactive HRMS/MS was performed every three days over the mass range *m*/*z* 90–2000, in accordance with the manufacturer's instructions. The identification of the 25 mycotoxins and phytoestrogens included in the multi-analyte method was supported by the determination of specific retention times, fragmentation patterns and accurate masses, which were obtained using a mass accuracy window of ±5 ppm with respect to the theoretical accurate masses (Table S1). Chromatographic separation was achieved at 30 °C on a 150 × 2.1 mm Kinetex reversed-phase F5 column (2.6 µm, 100Å; Phenomenex, Torrance, CA, USA) with a 0.5 µm × 0.004" ID, HPLC KrudKatcher Ultra Column In-Line filter. The flow rate of the mobile phase was 0.25 mL/min, and the injection volume was 1 µL. Eluent A was water and eluent B was MeOH (both containing 5 mM ammonium acetate and 0.1% acetic acid). Since the solubility of ammonium acetate in MeOH is limited, it was first dissolved in 25 mL water before MeOH was added. The total run time was 43 min, and gradient elution was employed starting at 3% B for 1 min, linearly increasing to 15% B in 15 min, to 79% B in 10 min, and finally, to 100% B in 13 min. After washing the column for 2 min with 100% B, the mobile phase was returned to the initial conditions and the column was eluted isocratically for 2.5 min. The column was regularly washed with 70% methanol to prevent cross-contamination. Calibration standards and samples were analysed in randomised order and intercepted with blank solvent samples to minimise analytical bias from sample positions and to reduce sample-to-sample carry-over.

4.7. Validation of the Multi-Analyte LC-HRMS/MS Method

The method was validated with regard to the guidelines established by the International Organization for Standardization [49,50]. The analytical selectivity was determined by the combination of LC retention time and high-resolution mass detection including dd-MS^2 product ion qualifying of the different analytes. Measured peak areas were used for quantification. Sensitivity for the different analytes was expressed, by the slope of the respective six-point standard calibration curves (mean of three to four independent experiments) that were calculated by linear regression analysis in both solvent (MeCN 50:50) and the different matrices. The linear range was defined as the concentration interval, in which the regression coefficient R^2 was ≥ 0.96. Although internal standard calibrations were used for 15 of the analytes for the compensation of matrix interferences, potential suppression and enhancement (SSE%) of signals from the co-eluting matrix were estimated for all analytes as the ratio of the slope of the matrix-assisted standard calibration curve to the calibration curve in MeCN/water (50:50). If SSE values were above or below 100%, signal enhancement or suppression by the matrix could be assumed.

Considering the negligible noise in the extracted high-resolution mass chromatograms, the limits of detection (LOD) and limits of quantification (LOQ) of the 25 analytes were calculated based on the standard deviation of the y-intercept of the respective calibration curves and their corresponding slopes (m) as $LOD = 3 \times \frac{SD}{m}$, $LOQ = 10 \times \frac{SD}{m}$ [59]. The accuracy of the method was assessed by determining recovery by spiking experiments and precision in terms of total within laboratory precision (RSi_R) considering intra- and interday variabilities together [60]. Furthermore, coefficients of variation (% CV) were determined for all concentration points in the solvent and matrix-assisted standard calibration curves. Recovery rates were calculated for all analytes as the mean of three to four experiments at a spiking level of 25 µg/L. In a few cases, where the matrix-assisted standard curves in feed or fish matrices did not pass through the origin but showed a positive signal on the ordinate due to background noise, this was corrected by virtually moving the curve with parallel shift on the abscissa. The corresponding concentration difference was added to the spike concentration used in the recovery experiments according to $Recovery_{(spike\ corrected)}$ = (measured concentration − blank)/(spiked concentration + concentration difference to origin).

Measured results for fish feed and fish study samples were converted from concentrations (µg/L) into content in the respective matrix (µg/kg) by using the factors 0.1 for zebrafish and 0.125 for salmon and feed.

4.8. Data Analysis

The Q-Exactive was calibrated using Xcalibur software, version 2.2 (Thermo Scientific). The molecular formulas and exact masses of the target analytes were calculated using the built-in Qualbrowser of the Xcalibur 2.2 software, which was also applied for signal quantification. Microsoft Excel (Version 2016, Microsoft Corporation, Redmond, WA, USA) was used for basic statistics (e.g., calculation of mean, minimum and maximum values, regression and relative standard deviation).

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6651/11/4/222/s1, Table S1: Molecular characteristics of target analytes.

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Supplementary Materials: Development and Validation of a Liquid Chromatography High-Resolution Mass Spectrometry Method for the Simultaneous Determination of Mycotoxins and Phytoestrogens in Plant-Based Fish Feed and Exposed Fish

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Table S1. Molecular characteristics of target analytes.

Compound	Structure	Formula	MW (g/mol)	LogP
DON		$C_{15}H_{20}O_6$	296.319	–0.7
3-ADON		$C_{17}H_{22}O_7$	338.356	–0.1
15-ADON		$C_{17}H_{22}O_7$	338.356	–0.7
DON-3G		$C_{21}H_{30}O_{11}$	458.46	–2.3
NIV		$C_{15}H_{20}O_7$	312.318	–1.7
T-2		$C_{24}H_{34}O_9$	466.527	0.9
HT-2		$C_{22}H_{32}O_8$	424.490	0.4
OTA		$C_{20}H_{18}ClNO_6$	403.815	4.7
ZEN		$C_{18}H_{22}O_5$	318.369	3.6
Ergonovine		$C_{19}H_{23}N_3O_2$	325.412	1.8
Ergosine		$C_{30}H_{37}N_5O_5$	547.656	1.8

Ergotamine		$C_{33}H_{35}N_5O_5$	581.673	2.0
Ergocornine		$C_{31}H_{39}N_5O_5$	561.683	2.4
α-Ergocryptine		$C_{32}H_{41}N_5O_5$	575.71	2.7
Ergoscristine		$C_{35}H_{39}N_5O_5$	609.727	3.0
ENN A		$C_{36}H_{63}N_3O_9$	681.912	7.6
ENN A1		$C_{35}H_{61}N_3O_9$	667.885	7.3
ENN B		$C_{33}H_{57}N_3O_9$	639.831	6.5
ENN B1		$C_{34}H_{59}N_3O_9$	653.858	6.9
Daidzein		$C_{15}H_{10}O_4$	254.241	2.5
Daidzin		$C_{21}H_{20}O_9$	416.382	0.7
Genistein		$C_{15}H_{10}O_5$	270.240	2.7
Genistin		$C_{21}H_{20}O_{10}$	432.381	0.9
Glycitein		$C_{16}H_{12}O_5$	284.267	2.4
Glycitin		$C_{22}H_{22}O_{10}$	446.408	0.6

Paper II

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Biotransformation of phytoestrogens from soy in enzymatically characterized liver microsomes and primary hepatocytes of Atlantic salmon

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ABSTRACT

Efficient aquaculture is depending on sustainable protein sources. The shortage in marine raw materials has initiated a shift to "green aquafeeds" based on staple ingredients such as soy and wheat. Plant-based diets entail new challenges regarding fish health, product quality and consumer risks due to the possible presence of chemical contaminants, natural toxins and bioactive compounds like phytoestrogens. Daidzein (DAI), genistein (GEN) and glycitein (GLY) are major soy isoflavones with considerable estrogenic activities, potentially interfering with the piscine endocrine system and affecting consumers after carry-over. In this context, information on isoflavone biotransformation in fish is crucial for risk evaluation. We have therefore isolated hepatic fractions of Atlantic salmon (*Salmo salar*), the most important species in Norwegian aquaculture, and used them to study isoflavone elimination and metabolite formation. The salmon liver microsomes and primary hepatocytes were characterized with respect to phase I cytochrome P450 (CYP) and phase II uridine-diphosphate-glucuronosyltransferase (UGT) enzyme activities using specific probe substrates, which allowed comparison to results in other species. DAI, GEN and GLY were effectively cleared by UGT. Based on the measurement of exact masses, fragmentation patterns, and retention times in liquid chromatography high-resolution mass spectrometry, we preliminarily identified the 7-O-glucuronides as the main metabolites in salmon, possibly produced by UGT1A1 and UGT1A9-like activities. In contrast, the production of oxidative metabolites by CYP was insignificant. Under optimized assay conditions, only small amounts of mono-hydroxylated DAI were detectable. These findings suggested that bioaccumulation of phytoestrogens in farmed salmon and consumer risks from soy-containing aquafeeds are unlikely.

1. Introduction

Fish in aquaculture are dietary exposed to a range of substances, which usually are not present in their natural habitat. Since marine reserves are limited, sustainable plant-based protein sources for aquafeeds are explored (Espe et al., 2006; Gatlin et al., 2007), and legumes, cereal grains and oilseeds are most commonly used (Kraugerud et al., 2011; Ytrestøyl et al., 2015). The complete diet change has led to new challenges regarding fish health and product quality as well as consumer risks due to a potential carry-over from feed to fish. Anti-nutritional substances present in plant-based feed include phytoestrogens such as the isoflavones daidzein (DAI), genistein (GEN) and glycitein (GLY). They occur in relatively high concentrations in extruded legume meal, especially soy, mostly in glucosidated form as daidzin, genistin and glycitin that are cleaved after ingestion by β-glucosidase (Mahungu et al., 1999). Isoflavones were observed in fish bile and shown to inhibit hepatic and renal estradiol-17β metabolism in salmonid fish, acting as endocrine disruptors (Mambrini et al., 1999; Ng

Abbreviations: Cytochrome P450, CYP; UDP-glucuronosyltransferase, UGT; Human liver microsomes, HM; Fish liver microsomes, FM; Fish hepatocytes, FHep; Daidzein, DAI; Genistein, GEN; Glycitein, GLY; β-estradiol, E2; β-estradiol 17- β-D-glucuronide, E2-17GlcA; N-Acetylserotonin, NAS; N-Acetyl Serotonin β-D-Glucuronide, NAS-GlcA; Naloxone, NLX; Naloxone-3ß-D-glucuronide, NLX-3GlcA; Mycophenolic acid, MA; Mycophenolic acid-β-D-glucuronide, MA-GlcA; Trifluoperazine dihydrochloride, TFP; Trifluoperazine N-ß-D-glucuronide, TFP-GlcA; Phenacetin, PCN; Acetaminophen, ACP; Tolbutamide, TB; 4-Hydroxytolbutamide, 4-OH-TB; Chlorzoxazone, CH; 6-Hydroxychlorzoxazone, 6-OH-CH; Midazolam, MDZ; 4-Hydroxymidazolam, 4-OH-MDZ; Dextromethorphan, DEX; Dextrophan, DOR; Triple-quadrupole mass spectrometry, TQMS; High-resolution mass spectrometry, HRMS; mass-to-charge ratio, *m/z*; tandem mass spectrometry, MS^2

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et al., 2006). The characterization of their detoxification pathways in the piscine metabolic system is thus relevant for the assessment of possible health risks for fish and the consumers of fish products.

Biotransformation is essential for all living organisms, both for providing physiologically important components and the detoxification of harmful xenobiotics (Parkinson and Ogilvie, 2007; Timbrell and Marrs, 2009). In vertebrates, the liver is the main metabolism organ. The hepatocytes contain oxidative (phase I) and conjugative enzymes (phase II) (Sevior et al., 2012). Since the determination of biotransformation pathways and kinetic parameters is of central importance for the assessment of the pharmacological or toxicological potential of, respectively, drugs or food contaminants, test systems and procedures for their elucidation have been established (Shu et al., 2008). Apart from *in vivo* studies, *in vitro* models applying enzymatically active liver fractions are widely used. Hepatic microsomes and primary hepatocytes assays run under definite conditions allow the determination of a substrate's typical metabolites and the prediction of elimination characteristics (Ito and Houston, 2005). The *in vitro* models help reduce the use of experimental animals, in trait with the principles of "3 R", replace – reduce – refine, and are suitable for increasing the substrate throughput (Wetmore, 2015; Punt et al., 2018).

Liver microsomes of several mammalian species are commercially available and suited for the application in biotransformation experiments (Di et al., 2003; Jia and Liu, 2007). They are characterized with regard to total protein content and activities of the most important cytochromes P450 (CYP) and uridine-diphosphate-glucuronosyltransferases (UGT), enabling comparison between lots and standardization. The total CYP content can be determined by measuring the carbon monoxide (CO) difference spectrum at 450 nm (Omura and Sato, 1964). Furthermore, ethoxycoumarin-O-deethylase (ECOD) is used as a marker for the sum of CYP1A1, CYP1A2, CYP2B1, CYP2E1, and CYP2B6 in mammalian microsomes (Shen et al., 2016). However, more specific information is obtained by characterizing CYP and UGT enzyme activities individually. This is achieved by performing assays with probe substrates that are typical for the different CYP (Li et al., 2007; Dinger et al., 2014; Shi et al., 2015) and UGT (Walsky et al., 2012; Joo et al., 2014; Seo et al., 2014). Enzyme activities are described by measuring the concentrations of the specific metabolites produced after definite incubation times with certain amounts of enzyme and different initial substrate concentrations (Kuś et al., 2015; Achour et al., 2017). Knowledge about enzymatic capacities allows the evaluation of metabolization results obtained with a specific microsomal lot. Moreover, combined batches with average enzyme activities can be produced, which is relevant for species with considerable inter-individual differences (Snawder and Lipscomb, 2000; Mondal et al., 2008).

The applicability of liver microsomes to generate relevant biotransformation data makes them an attractive tool also for species, for which material cannot be bought such as fish and birds (Smith et al., 2010; Ivanova et al., 2014). Information on hepatic metabolites is not only interesting for pharmaceutical research but increasingly applied for risk assessments on environmental or dietary exposure of humans and animals to chemical contaminants and natural toxins. The comparability of results obtained in such experiments will be increased, if the metabolism enzyme activities have been determined first.

It was therefore our aim in the present study to establish methods for the simultaneous measurement of CYP and UGT enzyme activities in liver fractions and apply them to the characterization of hepatic microsomes and primary hepatocytes from Atlantic salmon (*Salmo salar*), which were subsequently used for the *in vitro* metabolism of phytoestrogens from soy in newly developed biotransformation assays.

2. Materials and methods

2.1. Chemicals

Optima LC-MS grade acetonitrile (MeCN), methanol (MeOH) and water were provided by Fisher Scientific (Waltham, MA, USA). Dimethyl sulfoxide (DMSO, pro analysis quality), ethanol (EtOH) from VWR Chemicals (Lutterworth, UK) and formic acid (HCOOH) were purchased from Merck (Darmstadt, Germany).

Enzyme activities in hepatic fractions were characterized by using specific probe substrates and their respective metabolism products. Phenacetin (PCN), acetaminophen (ACP), tolbutamide (TB), 4-hydroxytolbutamide (4-OH-TB), dextromethorphan (DEX), dextrophan (DOR), chlorzoxazone (CH), 6-hydroxychlorzoxazone (6-OH-CH), midazolam (MDZ) and 4-hydroxymidazolam (4-OH-MDZ) were purchased from Sigma-Aldrich (Millipore-Sigma; St. Louis, MO, USA), as well as β-estradiol (E2), β-estradiol 17-(β-D-glucuronide) sodium salt (E2-GlcA), N-acetyl serotonin (NAS), naloxone (NLX), naloxone-3β-D-glucuronide solution (NLX-GlcA), mycophenolic acid (MA), mycophenolic acid-β-D-glucuronide (MA-GlcA), trifluoperazine dihydrochloride (TFP) and trifluoperazine N-β-D-glucuronide (TFP-GlcA). N-acetyl Serotonin β-D-glucuronide (NAS-GlcA) was supplied by Santa Cruz Biotechnology (Dallas, TX, USA). Substrate stock solutions were prepared in MeOH except for MA-GlcA and NLX-GlcA that were solved in MeCN and MeOH:H_2O (9:1 v/v), respectively.

The isoflavones daidzein (DAI), genistein (GEN) and glycitein (GLY) and their respective glucosides daidzin, genistin and glycitin were bought in crystalline form from Sigma-Aldrich, and stock solutions were prepared in MeOH or DMSO (GLY) containing 0.5 or 1 mg/L compound.

Buffer and substances used for the *in vitro* metabolism experiments including β-nicotinamide adenine dinucleotide phosphate sodium salt ($NADP^+$), β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), D-glucose-6-phosphate sodium salt, D-glucose-6-phosphate dehydrogenase from baker's yeast (*Saccharomyces cerevisiae*), Uridine 5′-diphosphoglucuronic acid tri-sodium salt (UDPGA), Uridine 5′-diphospho-N-acetylglucosamine sodium salt (UDPAG), $MgSO_4$, KH_2PO_4, $CaCl_2$ and HEPES buffer were purchased from Sigma-Aldrich. UDP-glucuronosyltransferase (UGT) Reaction Solution B containing 250 mM Tris-HCl, 40 mM magnesium chloride and 0.125 mg/mL alamethicin in water was supplied by BD Biosciences (Woburn, MA, USA). NaCl, KCl and Na_2HPO_4 were bought from Merck (Darmstadt, Germany) and $MgCl_2 \times 6H_2O$ was from Honeywell Fluka (Bucharest, Romania).

For the isolation of salmon hepatocytes, NaCl, KCl, HEPES, EDTA disodium salt, $CaCl_2$, fetal bovine serum and sodium bicarbonate were purchased from Merck (Darmstadt, Germany), Collagenase Type 1 from Worthington (NJ, USA), and L-15 medium, L-15 GlutaMAX and Antibiotic-Antimycotic from ThermoFisher (MA, USA).

2.2. Simultaneous characterization of five major CYP enzyme activities

The probe substrates PCN, TB, DEX, CH and MDZ were used to measure activities of, respectively, CYP 1A2, CYP 2C9, CYP 2D6, CYP 2E1 and CYP 3A4 in hepatic preparations. Initially, assay conditions were determined by incubating the substrates separately with different start concentrations (1–10 μM) and by measuring the production of the respective main oxidative metabolites ACP, 4-OH-TB, DOR, 6-OH-CH and 4-OH-MDZ. The final method used a combined substrate solution containing 715 μM PCN, 710 μM TB, 715 μM DEX, 1444 μM CH and 2824 μM MDZ in MeOH, which after dilution in the assay resulted in start concentrations of 2.5 μM (PCN, TB and DEX), 5 μM (CH) and 10 μM (MDZ).

The experiments to establish the CYP characterization method were performed with pooled human liver microsomes (HM) (20 mg/mL, 150-Donor pool; Lot IHE) from Bioreclamation IVT (Westbury, NY, USA) in a previously described assay format (Fæste et al., 2011). Substrates were incubated at 37 °C in a shaking water bath (OLS 200; Grant, Cambridge, UK) with a reaction mixture containing NADPH generating system (0.91 mM NADPH, 0.83 mM NADP, 19.4 mM glucose 6-phosphate, 1 U/mL glucose-6-phosphate dehydrogenase, 9 mM

$MgCl_2 \times 6H_2O$) and 2 mg microsomal protein in 1 mL incubation buffer (45 mM HEPES pH 7.4). The reaction was started after preincubation for 3 min by addition of the substrate mixture (3.5 μL). Aliquots of the reaction mixture were drawn after 0, 5, 10, 15, 30 and 60 min and mixed with equal volumes of ice cold 100% MeCN. Samples were kept on ice until centrifugation (Eppendorf, Hamburg, Germany) at 20,000 × *g* for 10 min to precipitate the proteins. Supernatants were placed on 0.22 μm Costar Spin-X filters (Corning Inc., Corning, NY, USA), centrifuged at 15,000 × *g* for 1 min (Eppendorf), and the filtrates were transferred into 300 μL fixed-insert HPLC vials (Thermo Fisher Scientific, Waltham, MA, USA) for analysis on the same day. Reaction samples without the NADPH-regeneration system served as negative controls, and reaction samples without substrate or without microsomes were used as vehicle controls for background subtraction. All incubations were performed at least two times and in duplicate.

2.3. Simultaneous characterization of five major UGT enzyme activities

The probe substrates E2, TFP, NAS, MA and NLX were used to evaluate the glucuronidation capacities of, respectively, UGT1A1, UGT1A4, UGT1A6, UGT1A9 and UGT2B7 in hepatic preparations. Assay conditions were optimized in substituted HM by incubating the substrates individually with different start concentrations (0.5–10 μM), while measuring the production rates of the respective glucuronides E2-GlcA, TFP-GlcA, NAS-GlcA, MA-GlcA and NLX-GlcA. The final method used a combined substrate solution containing 250 μM E2, 83 μM TFP, 250 μM NAS, 83 μM MA and 167 μM NLX in 100% MeOH, which after dilution in the assay resulted in start concentrations of 1.5 μM (E2, NAS), 0.5 μM for TFP, 0.2 μM for MA and 1 μM for NLX.

The glucuronidation experiments were performed as described before (Fæste et al., 2018). Substrates were added to 0.5 mL 7.4 mM UDPGA, 0.3 mM UDPAG and Reaction Solution B (1:5) containing 2 mg/mL microsomal protein after pre-incubation for 2 min at 37 °C in a shaking water bath.

Aliquots of the reaction mixture were drawn after 0, 5, 10, 15, 30 and 60 min and mixed with equal volumes of ice cold 100% MeCN. They were precipitated by centrifugation, filtered (Costar Spin-X) and transferred into HPLC vials as described above. Likewise, the same control samples were included. All incubations were performed at least two times and in duplicate.

2.4. Preparation of liver microsomes from Atlantic salmon

Two-year-old Atlantic salmon with a mean body weight of 200 g, kept in tanks with seawater at the Nofima Research Station, Sunndalsøra, Norway and fed with standard aquafeed, were humanely killed by percussive stunning. The livers were extracted, washed with ice-cold physiological saline and stored at −80 °C until further use. Microsomes were prepared by differential centrifugation as described before (Ivanova et al., 2014). Liver was cut and homogenized in 100 mL 0.1 M potassium phosphate buffer (pH 7.5) with a Potter-Elvehjem homogenizer (Sigma-Aldrich). The homogenate was centrifuged twice for 20 min (4 °C) at 18,000 × *g* (Beckman Instruments, Palo Alto, CA, USA), and the resulting supernatant was further centrifuged for 60 min at 100,000 × *g* using a swing-out rotor SW41Ti in an ultracentrifuge (Beckman) at 4 °C. The precipitated pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.5). All steps were carried out on ice. Total microsomal protein was determined by Lowry Protein Assay (BioRad Laboratories, Hercules, CA, USA), and the salmon liver microsomes (FM) were stored at −80 °C.

The CYP and UGT enzyme activities in the salmon liver microsomes were determined by incubation with the specific probe substrates in the same assay formats as described above, with exception of the temperature, which was set to 20 °C after exploratory runs at 17–22 °C. All incubations were performed at least two times and in duplicate.

2.5. Preparation of primary hepatocytes from Atlantic salmon

Primary hepatocytes of Atlantic salmon with a mean body weight of 480 g were isolated as previously described by a procedure approved by the Norwegian Animal Research Authority (NARA) (Kjær et al., 2016). The fish were kept at the Norwegian University of Life Sciences, Ås, Norway and fed with a commercial diet. After anesthetization with Metacain (MS-222, Norsk Medisinaldepot, Norway), the abdominal cavity was opened, the portal vein cannulated and the hepatic artery severed to allow outflow. The liver was perfused *in situ* via the cannula with a buffer containing EDTA (22 mM EDTA, 143 mM NaCl, 7 mM KCl, 10 mM HEPES pH 7.4) at a flow rate of 4 mL/min and thereafter with a buffer containing collagenase (0.1% collagenase, 1 mM $CaCl_2$, 143 mM NaCl, 7 mM KCl, 10 mM HEPES pH 7.4). The cells were filtered through a 100 μm nylon filter, washed three times in L-15 medium and centrifuged for 2 min at 50*g*. The supernatants were discarded and the precipitated hepatocytes re-suspended in L-15 GlutaMAX containing 10% fetal bovine serum, 4.5 mM sodium bicarbonate and 5 mM HEPES. They were stored for about 1 h on ice until use. The number of viable cells was determined microscopically by staining with Trypan blue (0.4%; Sigma-Aldrich) and counting in a Neubauer chamber. The primary salmon hepatocytes were diluted with cold Krebs-Ringer HEPES buffer (KRB pH 7.4; 145 mM NaCl, 5.4 mM KCl, 0.33 mM Na_2HPO_4, 0.8 mM $MgSO_4$, 0.34 mM KH_2PO_4, 2 mM $CaCl_2$, 20 mM HEPES) to 2.5×10^6 living cells/mL and immediately used for *in vitro* biotransformation assays (Moldes-Anaya et al., 2009).

The CYP and UGT enzyme activities in the primary salmon hepatocytes were determined by incubation with the specific probe substrates in an assay format previously described for primary rat hepatocytes (Moldes-Anaya et al., 2009), with exception of the temperature, which was set to 13 °C. All incubations were performed at least two times and in duplicate.

2.6. Metabolism of the isoflavones daidzein, genistein and glycitein in salmon liver microsomes

Phase I incubations of the three phytoestrogenic isoflavones DAI, GEN and GLY were performed individually in the assay format used for CYP activity characterization as described above (Fæste et al., 2011). After pre-incubation of 1 mL reaction mixture containing 2 mg/mL salmon liver microsomes at 20 °C for 3 min under shaking, 0.5 μM, 1 μM, or 5 μM of the respective substrate were added. Aliquots were drawn after 0, 5, 10, 15, 30 and 60 min and prepared for analysis. In a subsequent experiment, the DAI reaction mixture was concentrated to increase the possibility of identifying oxidative metabolites. Ethyl acetate (600 μL) was added to 400 μL of 60 min-incubation aliquots, vortexed for 15 s and centrifuged at 2000 × *g* for 5 min. The ethyl acetate fraction was transferred into a new tube and the procedure was repeated twice. All ethyl acetate fractions were combined, evaporated to the dryness using a gentle stream of nitrogen at 40 °C and reconstituted in 70 μL MeCN/water (50:50). After filtration (Costar Spin-X), the concentrated sample was analyzed using LC-HRMS method.

Phase II incubations of the three isoflavones were performed in accordance with the method used for UGT enzyme activities characterization as described above (Fæste et al., 2018). DAI, GEN (both with 0.5 μM, 1 μM or 5 μM) or GLY (1 μM, 5 μM or 10 μM) were added to 0.5 mL reaction mixture containing 2 mg/mL salmon liver microsomes after 2 min pre-incubation at 20 °C under shaking. Aliquots were taken after 0, 5, 10, 15, 30 and 60 min and prepared for analysis. Control samples were included in both methods. All experiments were performed at least twice and in duplicate.

2.7. Metabolism of the isoflavones daidzein, genistein and glycitein in primary salmon hepatocytes

The isoflavones (2 μM) were incubated individually in 2.5 mL

hepatocyte suspension containing 1.3 × 10^6 cells/mL in KRB under shaking in accordance to a previously described method (Moldes-Anaya et al., 2009), but at 13 °C. Aliquots were drawn after 0, 2.5, 5, 7.5, 10, 15, 30 and 60 min and precipitated with the same volume of ice-cold MeCN. Controls consisting of samples without hepatocytes, or containing deactivated hepatocytes were included. Samples were kept on ice until centrifugation (Eppendorf) for 10 min with 20,000 × *g* at 4 °C. Supernatants were filtered (Costar Spin-X) and transferred to HPLC vials for analysis as described above. All incubations were performed at least two times and in duplicate.

2.8. Preparation of calibration standards for probe substrates

Stock solutions of the main oxidative metabolites ACP, 4-OH-TB, DOR, 6-OH-CH and 4-OH-MDZ of the respective CYP probe substrates were combined for the preparation of a mixed solution containing 2 mg/L of each compound in MeCN/water (50:50). The mixture was used to produce calibration standards in solvent at 7.5 μg/L, 15 μg/L, 75 μg/L, 150 μg/L and 300 μg/L for quantitative analysis by TQMS.

Likewise, stock solutions of the main metabolites E2-GlcA, TFP-GlcA, NAS-GlcA, MA-GlcA and NLX-GlcA of the respective UGT probe substrates were combined in a mixed solution containing 2 mg/L of each compound, which was used to prepare calibration standards at 500 μg/L, 250 μg/L, 125 μg/L, 25 μg/L and 12.5 μg/L in in MeCN/water (50:50) immediately before use.

Additionally, matrix-assisted calibration standards were prepared both for phase I and phase II experiments by using filtrate from the preparation of control incubations containing only the liver microsomes. Aliquots were transferred into HPLC vials, evaporated and re-suspended by adding the mixed calibration standards at different concentrations.

2.9. Ultra-high pressure liquid chromatography (UHPLC) triple-quadrupole mass spectrometry (TQMS) for the quantitation of CYP and UGT probe substrates and metabolites

Samples from the CYP and UGT activities characterization experiments were analyzed by using an Agilent 1290 Infinity Binary UHPLC System with vacuum degasser and thermostatted column department (30 °C) interfaced by electrospray ionisation (ESI) with an Agilent 6470 TQMS (Agilent Technologies, Santa Clara, CA, USA). The instrument parameters, including the precursor ion-to-product ion transition, mass-to-charge values (*m/z*), fragmentor voltage (FV), collision energy (CE), cell accelerator voltage (CAV) for the multiple reaction monitoring (MRM) of all analytes (Table 1), were developed by infusing the compounds (1 μg/mL in MeOH) isocratically with a flow of 0.4 mL/min (MeCN:water 50:50, both containing 0.1% formic acid) and applying the instrument's MassHunter™ Optimizer software (version B 08.00; Agilent). Several transitions were monitored for each compound, and the most suitable transition was used for the quantitative analysis. Furthermore, the ion source parameters were optimized for both positive and negative ionisation in order to minimize in-source fragmentation and achieve the greatest possible intensity for all compounds included in the present study (Supplementary Table 1).

Quantitation was performed in dynamic multiple-reaction monitoring (dMRM) mode with the Q1 and Q3 resolutions set to unit. Rapid switching between positive and negative ion mode (ESI pos/neg) in the same analytical run was facilitated by defining fixed retention time windows for the individual probe substrates.

Chromatographic separation was achieved using a 150 × 2.1 mm i.d. Kinetex F5 2.6 μm UHPLC column with a 0.5 μm × 0.004 in i.d. KrudKatcher Ultra Column in-line filter (Phenomenex, Torrance, CA, USA). Phase I compounds probe substrates and their metabolites (injection volume 1 μL) were eluted using a water (A)/MeCN (B) (containing 0.1% formic acid) binary gradient at a flow rate of 0.25 mL/min, starting at 18% B for 1 min and linearly increasing to 55% B at 13 min and to 95% at 14 min. After washing for 3 min with 95% B, the column was re-equilibrated for 4 min with 18% B. The total run time was 21 min. Phase II probe substrates and their metabolites were eluted under the same conditions, but with the binary gradient starting at 2% B for 1.5 min, linearly increasing to 39% B at 4 min, to 55% B at 10.5 min, and to 95% B at 11 min. After washing the column for 2 min with 95% B, the mobile phase was returned to the initial conditions and the column was eluted isocratically for 2 min. The total run time was 15 min.

The different CYP and UGT probe substrates were semi-quantified based on measured peak areas, whereas their respective main metabolites were quantified using matrix-assisted calibration curves. Data acquired by TQMS were analyzed with MassHunter™.

2.10. UHPLC high-resolution mass spectrometry (HRMS) for the characterization of isoflavones and metabolites

The isoflavones analysis was performed on a Q-Exactive™ Hybrid Quadrupole-Orbitrap HRMS/MS equipped with a heated electrospray ion source (HESI-II) and coupled to a Vanquish UHPLC system (Thermo Fisher Scientific). The instrument was operated with a set up similar to that described in a previous study (Johny et al., 2019). The HESI-II interface was operated at 300 °C, alternating between positive and negative mode in a run, and the parameters were adjusted to 3.2 (ESI pos) and 2.5 kV (ESI neg) spray voltage, 280 °C capillary temperature, 35 L/min sheath gas flow rate, 10 L/min auxiliary gas flow rate, and S-lens RF level 55. The HRMS was run in full scan (FS) mode with the mass ranges set to *m/z* 90–900 and 200–900 in negative and positive mode, respectively, and a mass resolution of 70,000 full width half-maximum (FWHM) at *m/z* 200. The automated gain control (AGC) target was adjusted to 5 × 10^5 ions for a maximum injection time (IT) of 250 ms, and the quadrupole mass filter was operated with an isolation window of *m/z* 3. External mass calibration of the HRMS over the mass range *m/z* 90–2000 was performed every third day in accordance with the manufacturer's instructions. Isoflavones were identified by their specific retention times, fragmentation patterns and accurate masses, which were determined with a mass accuracy window of ± 5 ppm with respect to the theoretical accurate masses (Table 2).

The compounds (1 μL injection volume) were separated chromatographically at 30 °C on a 150 × 2.1 mm Kinetex reversed-phase F5 column (2.6 μm, 100 Å; Phenomenex) with a 0.5 μm × 0.004 in. ID HPLC KrudKatcher Ultra Column in-line filter using a flow rate of 0.25 mL/min and a binary water (A)/MeOH (B) gradient (both phases containing 5 mM ammonium acetate and 0.1% acetic acid). Elution started with 3% B for 1 min, linearly increasing to 15% B at 16 min, to 79% B at 26 min, and finally to 100% B at 39 min. The column was washed for 2 min with 100% B before it was re-equilibrated to the initial conditions for 2 min. The total run time was 43 min. DAI, GEN and GLY and their glucuronides were semi-quantified based on the measured peak areas. The Q-Exactive was calibrated using Xcalibur, version 2.2 (Thermo Scientific). The molecular formulas and exact masses of the target analytes were calculated using the software's built-in QualBrowser.

In addition to full MS/dd-MS^2, parallel reaction monitoring (PRM) scan mode was performed for the identification and characterization of the potential phase I biotransformation products of DAI. PRM fragmentation mass spectra were recorded in negative ionisation mode at a mass resolving power of 35,000 FWHM with a quadrupole isolation window of 3 *m/z* for precursor ions. Other HRMS parameters for PRM were set to: default charge 1, inclusion on, maximum IT 120 ms, AGC target 5.0 × 10^5, and NCE/stepped 35. The accurate masses for DAI and its oxidative metabolites were *m/z* 253.0506 and *m/z* 269.0455, respectively.

Table 1
dMRM parameters for the TQMS analysis of CYP and UGT probe substrates and their main metabolites.

Enzyme	Compound	Transition[a] [m/z]	CE[b] [eV]	FV[c] [V]	CAV[d] [V]	Polarity
CYP (phase I)						
CYP1A2	Phenacetin (PCN)	179.1 → 110.0	22	80	4	ESI pos
	Acetaminophen (ACP)	152.1 → 110.0	14	70	4	
CYP2C9	Tolbutamide (TB)	271.1 → 91.0	35	65	4	ESI pos
	4-Hydroxytolbutamide (4-OH-TB)	287.1 → 74.0	10	80	4	
CYP2D6	Dextromethorphan (DEX)	272.0 → 215.0	26	130	4	ESI pos
	Dextrophan (DOR)	258.2 → 157.0	43	145	4	
CYP2E1	Chlorzoxazone (CH)	168.0 → 132.0	22	100	4	ESI neg
	6-Hydroxychlorzoxazone (6-OH-CH)	184.0 → 120.0	18	90	4	
CYP3A4	Midazolam (MDZ)	326.1 → 291.0	30	140	4	ESI pos
	4-Hydroxymidazolam (4-OH-MDZ)	342.1 → 297.0	34	130	4	
UGT (phase II)						
UGT1A1	β-Estradiol (E2)	255.2 → 159.0	18	110	4	ESI neg
	β-Estradiol 17-β-D-glucuronide (E2-GlcA)	447.2 → 271.1	31	160	4	
UGT1A4	Trifluoperazine dihydrochloride (TFP)	408.2 → 70.1	55	70	4	ESI pos
	Trifluoperazine N-ß-D-glucuronide (TFP-GlcA)	584.2 → 408.2	26	145	4	
UGT1A6	N-Acetylserotonin (NAS)	219.1 → 160.0	18	70	4	ESI pos
	N-Acetyl Serotonin β-D-Glucuronide (NAS-GlcA)	395.2 → 160.0	34	55	4	
UGT1A9	Mycophenolic acid (MA)	319.1 → 275.1	15	115	4	ESI neg
	Mycophenolic acid-β-D-glucuronide (MA-GlcA)	495.1 → 319.1	19	110	4	
UGT2B7	Naloxone (NLX)	328.2 → 310.1	18	70	4	ESI pos
	Naloxone-3ß-D-glucuronide (NLX-GlcA)	504.2 → 310.1	34	155	4	

[a] Precursor ion-to-product ion transition.
[b] Collision energy.
[c] Fragmentor voltage.
[d] Collision cell accelerator voltage.

Table 2
HRMS parameters for the analysis of soy isoflavones and their main metabolites.

Compound	Ionisation mode	Target ion	RT[a] [min]	Ion mass [m/z]
DAI	ESI neg	$[M-H]^-$	26.2	253.0506
DAI (O)	ESI neg	$[M-H]^-$	22.0/23.1/24.0/25.0	269.0455
DAI-GlcA	ESI pos	$[M+H]^+$	21.1	431.0973
GEN	ESI neg	$[M-H]^-$	27.3	269.0455
GEN-GlcA	ESI pos	$[M+H]^+$	22.8	447.0922
GLY	ESI neg	$[M-H]^-$	26.4	283.0612
GLY-GlcA	ESI pos	$[M+H]^+$	22.4	461.1078

[a] Retention time.

2.11. Determination of substrate turnover numbers and metabolite formation rates

Substrate depletion by CYP and UGT enzyme activities in HM and FM was approximated by regression analysis of the measured peak areas versus time curves ($A(t) = b + a*e^{-k_{el}*t}$). The initial substrate depletion constants (k_{el}) were used to calculate the substrate turnover numbers (k_{cat}) considering the amount of microsomal protein in the incubations. Potential protein binding of the substrates in the assay was not considered.

Metabolite formation rates of CYP and UGT probe substrates in HM, FM and FHep assays were determined by regression analysis from the initial slope of the concentration versus time curves.

3. Results

3.1. Analysis of CYP and UGT probe substrates and metabolites by dMRM

TQMS methods for the parallel analysis of the CYP probe substrates and their metabolites and of the UGT probe substrates and their metabolites were developed by determining specific mass ion transitions, polarities as well as instrument and ion source parameters (Table 1; Supplementary Table 1). Calibration curves measured for the metabolites showed in part considerable compound-dependent slope differences between calibrants dissolved in solvent or incubation assay buffer, demonstrating the effect of matrix interferences (Supplementary Fig. 1). Consequently, all analyses were performed with matrix-assisted standards. The measured metabolite concentrations were used in the determination of metabolite formation rates, while depletion constants were derived from the peak area versus time curves of the probe substrates.

3.2. Method for the simultaneous characterization of CYP enzyme activities

Specific probe substrates for the human phase I enzymes CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 were incubated with HM individually at different concentrations and optimal start conditions were determined by calculating substrate depletion constants and considering metabolite formation (data not shown). A mixed solution containing all probe substrates at their optimized concentrations was incubated with HM and performances regarding depletion and metabolite formation were compared to the results of the individual assays (Fig. 1). There were no significant differences in the curve slopes for the elimination of PCN, TB, DEX, CH and MDZ or the production of ACP, 4-OH-TB, DOR, 6-OH-CH and 4-OH-MDZ, showing that the chosen concentrations were appropriate and that the use of a substrate mixture for the characterization of CYP enzyme activities is functional. The CYP enzyme activities in the HM were further described by calculating the turnover numbers and metabolite formation rates (Supplementary Table 2). Comparison showed that CYP3A4-catalyzed MDZ-elimination and 4-OH-MDZ formation was the occurred most rapidly, whereas the enzyme reactions catalyzed by CYP1A2, CYP2C9 and CYP2D6 were much slower.

3.3. Method for the simultaneous characterization of UGT enzyme activities

Specific probe substrates for the human phase II enzymes UGT1A1, UGT1A4, UGT1A6, UGT1A9 and UGT2B7 were incubated in substituted HM with specific probe substrates individually for the optimization of the start conditions. Depletion of the substrates E2, TFP, NAS, MA and NLX and formation of the respective metabolites E2-GlcA, TFP-GlcA, NAS-GlcA, MA-GlcA and NLX-GlcA was compared in the finished assay

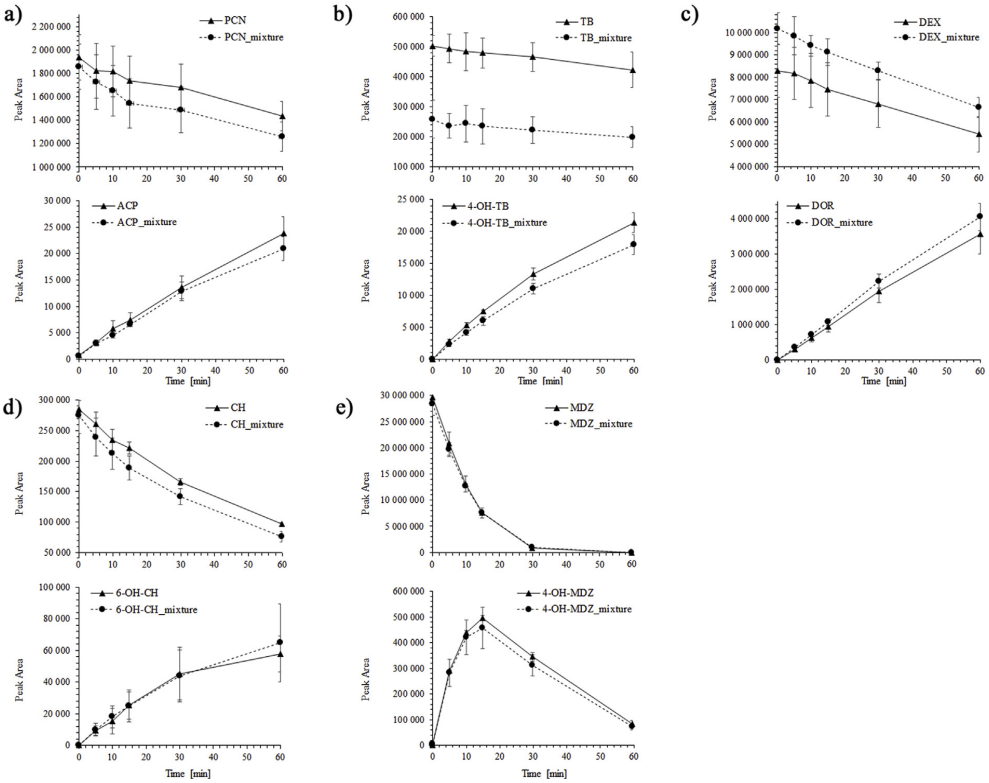

Fig. 1. Characterization of CYP enzyme activities in HM using specific probe substrates individually and in a mixture **a)** CYP1A2: elimination of PCN (2.5 μM) (upper panel) and production of ACP (lower panel); **b)** CYP2C9: elimination of TB (2.5 μM) (upper panel) and production of 4-OH-TB (lower panel); **c)** CYP2D6: elimination of DEX (2.5 μM) (upper panel) and production of DOR (lower panel); **d)** CYP2E1: elimination of CH (5 μM) (upper panel) and production of 6-OH-CH (lower panel); **e)** CYP3A4: elimination of MDZ (10 μM) (upper panel) and production of 4-OH-MDZ (lower panel). Means of two independent experiments performed in duplicates.

separately and by using a substrate mixture, showing no significant differences (Fig. 2). The UGT enzyme activities in HM were calculated (Supplementary Table 2), showing that MA elimination and MA-GlcA formation by UGT1A9 was rapid, while UGT1A4-catalyzed TFP elimination was much slower.

3.4. Production yield of microsome and primary hepatocyte isolation

Considering the total protein content in the prepared microsomes (43.5 mg/mL) and the amount of liver used, the yield was determined as 98 mg microsomal protein/g liver, corresponding to about 50% of the estimated total protein content in homogenized rainbow trout liver (Han et al., 2008). The yield (total cell number) of the hepatocyte isolation was established as 6.3×10^7 cells/g liver, which was about 12% of the approximated hepatocellularity (5.1×10^8 cells/g liver) in rainbow trout (Fay et al., 2014). The viability of the Atlantic salmon hepatocytes was higher than 97% in all isolations performed. The relative liver weight of on-growing Atlantic salmon was determined as 12.2 g liver/kg fish using data of 15 control fish included in a dietary exposure study (Johny et al., 2019).

3.5. CYP and UGT enzyme activities in salmon liver microsomes and primary hepatocytes

Incubation of the salmon microsomes with the mixture of CYP probe substrates showed the formation of DOR and 4-OH-MDZ, and to a much lesser extent 6-OH-CH (Fig. 3a), indicating enzyme activities related to CYP2D6, CYP3A4 and some CYP2E1 (Supplementary Table 2), which were considerably lower than in HM. Performed under identical assay conditions, FM demonstrated about 50% of the metabolite formation capacity by CYP3A4 and CYP2D6 in HM. Using the mixture of UGT probe substrates, production of NLX-GlcA, NAS-GlcA, MA-GlcA and E2-GlcA was detected (Fig. 3b), suggesting UGT2B7, UGT1A6, UGT1A9, and UGT1A1-related enzyme activities (Supplementary Table 2). The FM reached 80% of the metabolite formation rate by UGT2B7 in HM.

The experiments were repeated in primary salmon hepatocytes, revealing similar results. The production of the DOR, 4-OH-MDZ and 6-OH-CH was confirmed, demonstrating CYP2D6, CYP3A4 and CYP2E1-connected enzyme activities (Fig. 3c). In addition the formation of ACP was observed, indicating some CYP1A2-associated activity (Supplementary Table 2). The substrate depletion and metabolite formation rates were not directly comparable to those in HM and FM, since the FHep assays were run under different conditions. Still, the formation rates of DOR and 4-OH-MDZ were the highest as observed in FM. Regarding the UGT enzyme activities, the production of NLX-GlcA, NAS-GlcA, MA-GlcA, and E2-GlcA were confirmed, demonstrating UGT2B7, UGT1A6, UGT1A9, and UGT1A1-related enzyme activities (Fig. 3d; Supplementary Table 2). The formation of MA-GlcA and NLX-GlcA, catalyzed by, respectively, UGT1A9 and UGT2B7-like activities, were the most rapid reactions, adequate to what was observed in the microsomes.

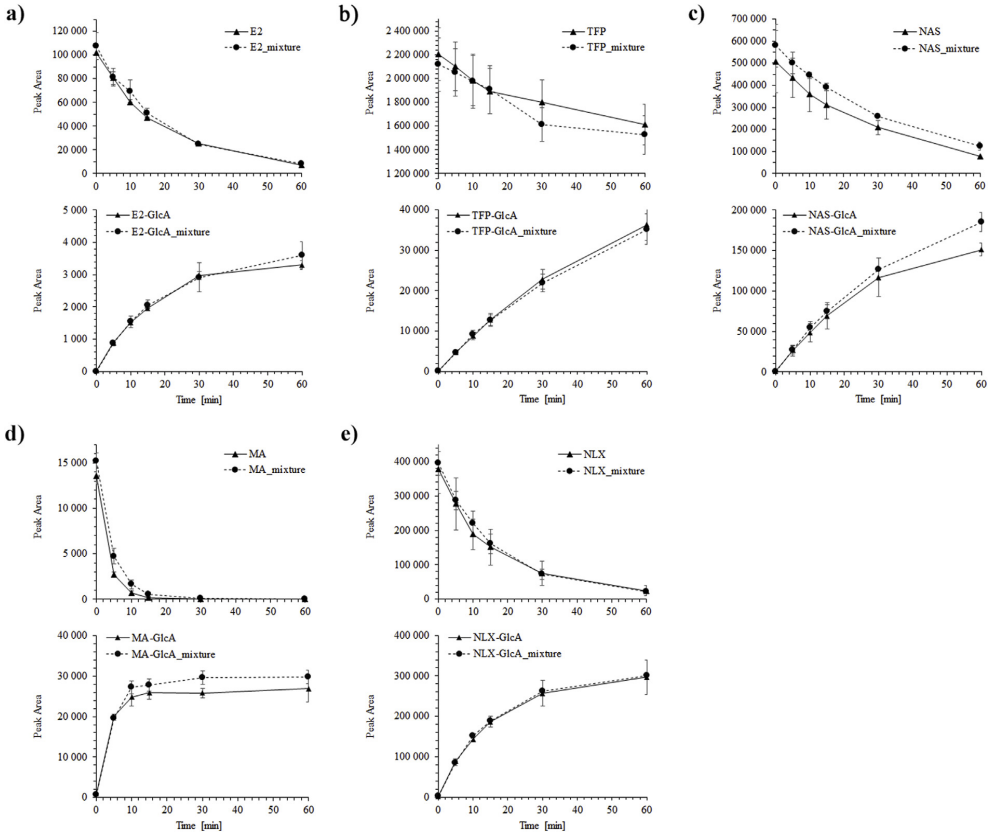

Fig. 2. Characterization of UGT enzyme activities in HM using specific probe substrates individually and in a mixture. **a)** UGT1A1: elimination of E2 (1.5 μM) (upper panel) and production of E2-GlcA (lower panel); **b)** UGT1A4: elimination of TFP (0.5 μM) (upper panel) and production of TFP-GlcA (lower panel); **c)** UGT1A6: elimination of NAS (1.5 μM) (upper panel) and production of NAS-GlcA (lower panel); **d)** UGT1A9: elimination of MA (0.2 μM) (upper panel) and production of MA-GlcA (lower panel); **e)** UGT2B7: elimination of NLX (1.5 μM) (upper panel) and production of NLX-GlcA (lower panel). Means of two independent experiments performed in duplicates.

3.6. Biotransformation of isoflavones in salmon liver microsomes

The soy phytoestrogens DAI, GEN and GLY and their respective glucosidated precursors daidzin, genistin and glycitin were incubated with different start concentrations in the optimized salmon liver microsome assay under phase I conditions and phase II conditions. The glucosidated isoflavones precursors were inactive in all assays (data not shown), probably because glucosidase was lacking in the hepatic preparations. They were therefore excluded from further experiments. Elimination of the aglycones and formation of potential oxidized (MW +16) or glucuronidated (MW+176) metabolites was measured by HRMS (Table 2; Fig. 4a and b). The quantitative determination of metabolites was not possible due to the lack of available reference materials. Glucuronidation was the major elimination reaction pathway for the three studied isoflavones (Figs. 4b and 5). The production of DAI-GlcA, GEN-GlcA and GLY-GlcA started immediately after incubation start and reached a plateau after 15 min incubation. GLY elimination was fast. The substrate was no longer detectable after 10 min when the same 1 μM start concentration as for DAI and GEN was used. A ten-time increase saturated the system and some remaining GLY was measurable after 60 min incubation.

In comparison, the formation of oxidative DAI, GEN and GLY metabolites appeared to be insignificant in FM. A decrease of the initial assay concentration during incubation was not observable (Supplementary Fig. 2). However, the base peak chromatogram at *m/z* 269.0455, corresponding to the mass ion of mono-oxidized DAI, showed small peaks that were not present in the matrix control (data not shown). The incubation aliquot at 60 min was therefore concentrated, and re-analysis allowed the determination of four DAI (O) metabolites with distinct retention times (Fig. 4b). In contrast, small signals in the respective base peak chromatograms of potential mono-oxidized GEN and GLY could not be distinguished from matrix background noise.

3.7. Biotransformation of isoflavones in primary salmon hepatocytes

Metabolization of DAI, GEN and GLY in the optimized primary salmon hepatocyte assay confirmed the liver microsome experiments. The respective glucuronides were the major products, whereas oxidative metabolites could be detected only for DAI after 60 min incubation (Fig. 4c). Four different DAI (O) metabolites were distinguishable, apparently with the same intensity profile as measured for the microsomes. The formation of the DAI, GEN and GLY glucuronides with 5 μM as start concentration in the assay were comparable (Supplementary

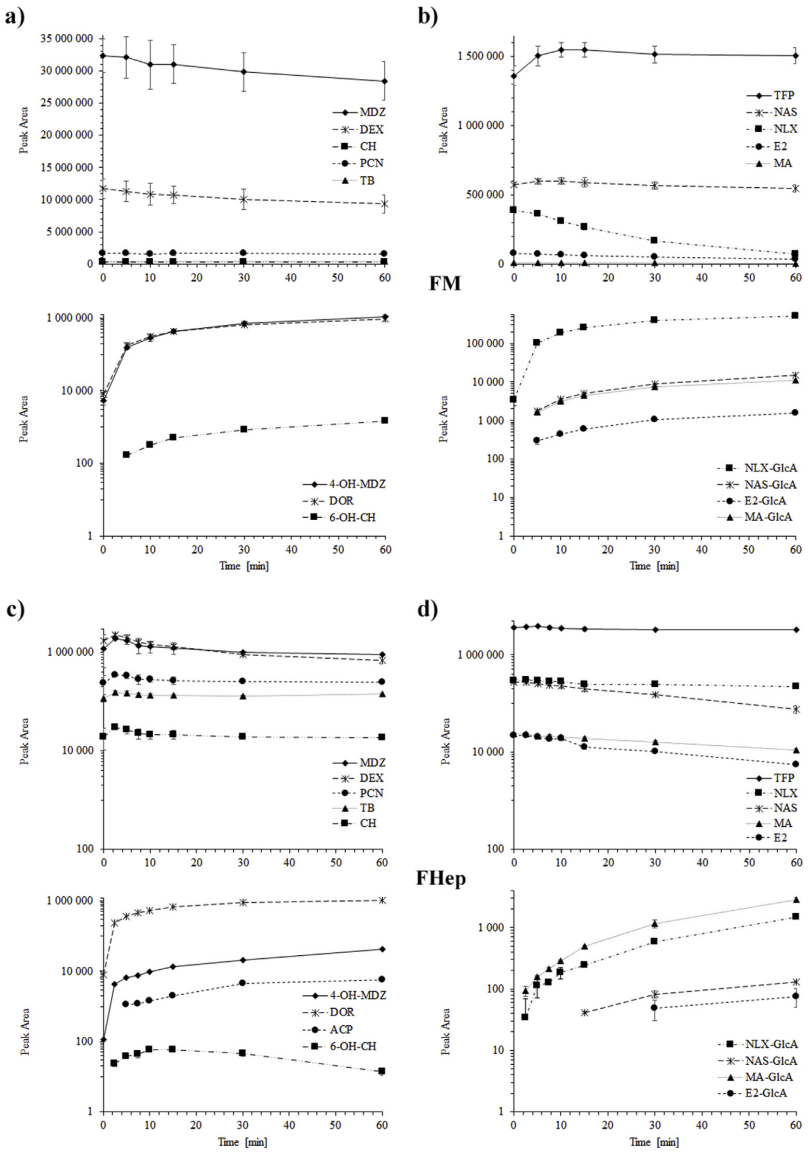

Fig. 3. Characterization of CYP and UGT enzyme activities in FM and FHep using specific probe substrate mixtures. **a)** CYP- and **b)** UGT-dependent substrate elimination (upper panels) and metabolite production (lower panels) in FM (initial concentrations as in Figs. 1 and 2); **c)** CYP- and **d)** UGT-dependent substrate elimination (upper panels) and metabolite production (lower panels) in FHep (initial concentrations 2 μM). Means of two independent experiments performed in duplicates.

Fig. 3).

3.8. Characterization of oxidative DAI-metabolites

The MS^2-spectra of DAI, GEN and GLY showed typical fragment peak ions (Supplementary Fig. 4), which could be used for metabolite identification through comparison (Supplementary Fig. 5). In the absence of suitable reference material, the positive identification of the four potential DAI (O) metabolites (M1 to M4) was thus achieved by HRMS analysis with mass filter on *m/z* 269.0455 (Supplementary Fig. 6a) with particular focus on two prominent fragments at *m/z* 133.0297 and *m/z* 135.0090 in the MS^2-spectrum (Supplementary Fig. 6b). The fragmentation patterns and major fragment ions of DAI and the suggested mono-oxidized M1 to M4 matched consistently, confirming the identity of the metabolites.

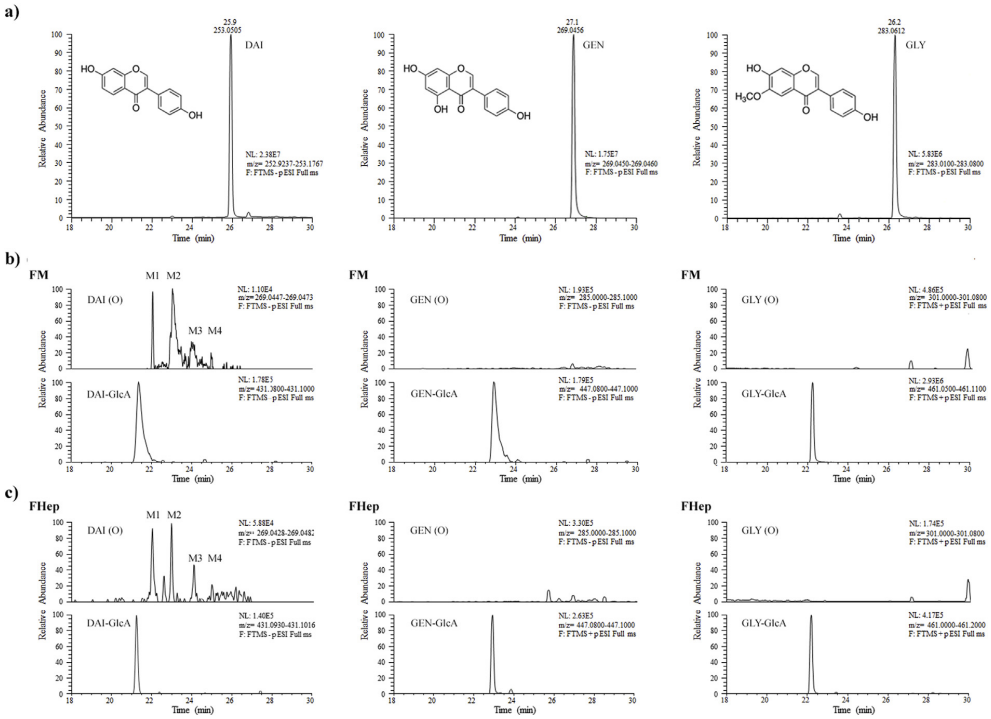

Fig. 4. HRMS analysis of DAI, GEN and GLY and their metabolites detected in FM and FHep. **a)** molecular structures, base peak chromatograms showing RT and ion masses (*m/z*) (upper panels); **b)** base peak chromatograms of mono-oxidized metabolites and glucuronides produced in FM (60 min) (intermediate panels); **c)** base peak chromatograms of mono-oxidized metabolites and glucuronides produced in FHep (60 min) (lower panels).

4. Discussion

The shift to new protein sources for aquafeeds brings new challenges for fish health and consumer safety. Plant ingredients may contain a variety of bioactive compounds, natural toxins and chemical contaminants from agricultural practices to which fish are not exposed in their natural environment. Depending on how the physiological processes in the fish can handle unfamiliar feed constituents, toxic effects might occur. Furthermore, carry-over into edible parts could be relevant for the assessment of consumer risks. In this context, information on the biotransformation of xenobiotics in farmed fish is important. Liver microsomes or primary hepatocytes are frequently used for the characterization of elimination pathways as an integrated part in toxicity risk assessment (Coecke et al., 2013); however, these *in vitro* models are not commonly applied for fish.

As part of good practice-considerations for *in vitro* metabolism models (Jia and Liu, 2007), methods for the specification of CYP and UGT activities in different liver fractions such as hepatic microsomes, hepatocytes, S9 fractions or organ slices are important. Probe substrates, mostly for human enzymes, are used individually or in mixtures for the simultaneous determination of activities. We identified PCN, TB, DEX, CH, and MDZ for, respectively, CYP1A2, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 as a consensus set from several references (Walsky and Obach, 2004; Kuś et al., 2015; Schelstraete et al., 2019), whereas E2, TFP, NAS, MA and NLX were considered as suitable for assessing UGT1, UGT1A4, UGT1A6, UGT1A9 and UGT2B7 (Walsky et al., 2012; Joo et al., 2014; Achour et al., 2017). However, other substrates and the inclusion of additional CYP and UGT have been

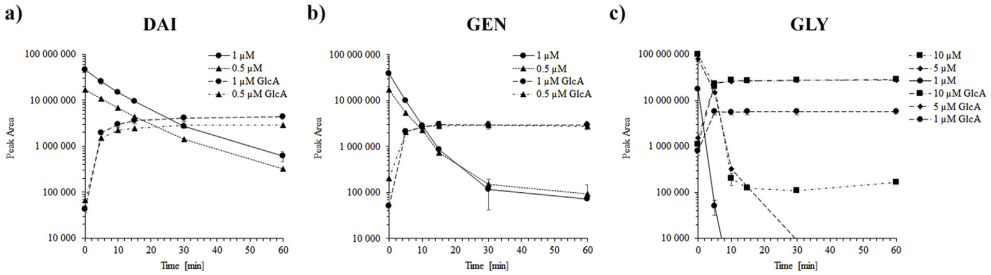

Fig. 5. Phase II metabolization of isoflavones in FM. **a)** DAI, **b)** GEN, and **c)** GLY elimination and glucuronide production with two start concentrations. Means of three independent experiments performed in duplicates.

described.

The state-of-the-art for simultaneous screening of enzyme activities involves incubation with probe substrate mixtures and quantification of the formed metabolites by liquid chromatography mass spectrometry. In our study, we used UHPLC-TQMS for the sensitive and specific determination of substrates and metabolism products. Method performance was comparable to similar assays (Joo et al., 2014; Kuś et al., 2015). The analysis was based on matrix-assisted calibration because of analyte-dependent signal suppression or enhancement from non-specific protein binding (Walsky and Obach, 2004; Lee et al., 2015).

Functionality testing of our CYP and UGT profiling methods with pooled human microsomes showed enzyme activities in the order CYP3A4 > CYP2E1 > CYP2D6 > CYP2C9 > CYP1A2 and UGT1A9 > UGT2B7 > UGT1A1 > UGT1A6 > UGT1A4, resembling observations made with identical probe substrates (Walsky and Obach, 2004; Schelstraete et al., 2019; Walsky et al., 2012; Achour et al., 2017). However, varying assay conditions or enzyme polymorphism can contribute to differences in metabolite formation efficiencies.

Inter-individual variances in the expression and composition of biotransformation enzymes due to genetic, environmental and life-style influences are of considerable size in humans, and thus preparations of pooled hepatic microsomes are preferred for *in vitro* experiments. In contrast, this is less pronounced in experimental and domestic animals including farmed fish that are often inbred and kept under standardized conditions. Inter-species differences are, however, relevant, as demonstrated for major CYP in humans and several animal species (Fink-Gremmels, 2008). Interestingly, CYP2E and CYP3A activities were similar in all tested species but varied widely for other CYP. Congruently, glucuronidation activities in seven mammalian species depended on UGT families and the substrate's molecular sizes (Boutin et al., 1984).

The results from the characterization experiments with the new salmon liver microsomes and primary hepatocytes were comparable. We saw the highest activities for CYP3A4-and CYP2D6-as well as for UGT2B7-, UGT1A9-and UGT1A1-like transformations, adequate to the incubations with human microsomes, but metabolite formation by the other enzymes included in the test was insignificant or undetectable. Information on CYP and UGT in several fish species are available through reviews (Clarke et al., 1991; Uno et al., 2012) and from the assembly of the zebrafish (*Danio rerio*) genome (Goldstone et al., 2010; Wang et al., 2014). Fish have 18 CYP gene families including many genes in CYP5 to CYP51 that are direct orthologues of mammalian CYP and involved in endogenous functions, and more diverse genes in CYP1 to CYP4 that are mainly involved in xenobiotics metabolism. Interestingly, CYP3A was present and CYP2C absent in all examined fish species, confirming our findings for Atlantic salmon. Induction of CYP1A expression has been shown *in vivo* after exposure to a xenoestrogen (Meucci and Arukwe, 2006), whereas we detected only a weak activity in the primary hepatocytes from untreated salmon. The identification of fish-human orthologues is most difficult for CYP2 due to little sequence homologies (Goldstone et al., 2010). Matching fish genes to the human CYP2D and CYP2E are unknown, so that the considerable formation of DOR and CH in our salmon assay was probably catalyzed by other CYP2 with affinities to the respective probe substrates.

The situation is equally complex for UGT. Although glucuronidation is known as an important detoxification pathway for environmental toxicants in fish, information on piscine UGT is limited (Wang et al., 2014). Teleost fish have considerably more UGT genes than mammals. In zebrafish, 40 genes encoding UGT have been identified, which can be divided into UGT1, UGT2 and UGT5 families. Prediction of substrate specificities is difficult since orthologues to mammalian UGT have not been identified on the protein level. Therefore, activities were mapped using typical probe substrates (Wang et al., 2014), showing sizable metabolite formation by UGT1A1 and UGT1A9, comparable to our result with salmon microsomes and primary hepatocytes. UGT2B7-related glucuronidation of NLX, which was remarkably high in our salmon assays, is not described for other fish, so that the identity of the catalyzing UGT is unclear.

After the enzymatic profiling we applied the salmon liver fractions to study the biotransformation of three major soy isoflavones. Total isoflavones contents in different soy varieties fluctuate from year to year in a range from 1.2 to 3.3 mg/g, whereof DAI, GEN and GLY, together with the respective glucosides, account for in average 26%, 32% and 4.6% (Wang and Murphy, 1994). Their estrogenic activities result from structural similarities with endogenous estradiol and binding affinity to the estrogen receptor. Biotransformation of food-borne phytoestrogens has been investigated in humans *in vivo* after dietary exposure and *in vitro* with liver and intestinal microsomes, hepatocytes and enterocytes, using pure standards (Heinonen et al., 2003; Atherton et al., 2006). The main detoxification process is glucuronide formation. Both, oxidative pathways (Kulling et al., 2002) and glucuronidation patterns (Tang et al., 2009) of DAI, GEN and GLY have been determined. Metabolism enzymes involved are CYP1A2 and potentially CYP2E1 (Atherton et al., 2006), while UGT1A1 and UGT1A9 are mainly responsible for the glucuronidation, in addition to the less effective UGT1A10 and UGT1A8 (Pritchett et al., 2008; Tang et al., 2009). GLY is much faster conjugated than DAI or GEN.

These findings in humans fitted well with the results of the *in vitro* salmon experiments in the present study. The salmon liver microsomes and hepatocytes contained the relevant CYP and UGT according to the enzyme characterization performed. We observed the formation of glucuronides, and GLY was more rapidly glucuronidated than DAI and GEN. However, oxidative reactions appeared to be less effective, since only weak signals of four DAI-monohydroxylated metabolites were detectable. By assay upscaling and concentration we will investigate further whether phase I metabolites of GEN and GLY are also formed.

The isoflavones metabolites were characterized by LC-HRMS, using retention times, exact masses and fragmentation patterns by ESI-MS^2 for provisional identification (Heinonen et al., 2003; Zhao et al., 2018). Without NMR analysis, however, the exact position of biotransformation was not determinable. DAI and GEN are oxidized at C6, C8, C3′ and C2 (Kulling et al., 2002), whereas GLY, adequate to 6-methoxy-DAI, is oxidized at C8, C3' and C2 (Rüfer et al., 2007). Di- and trihydroxylated metabolites are known and *in vivo*, DAI can be converted into the isoflavan equol. Comparing RT and peak intensities of the salmon DAI (O) with published human data generated under similar LC conditions, the two strongest signals might represent 8-OH-DAI and 6-OH-DAI.

Glucuronidation of isoflavones occurs preferably at C7, while the reaction at C4′ is secondary (Pritchett et al., 2008). Regarding the similarities in RT and peak intensities between the human data and our salmon experiments, we identified the observed major glucuronidation products preliminarily as DAI-7-O-GlcA, GEN-7-O-GlcA and GLY-7-O-GlcA. *In vivo* phase II conjugation of GEN has been previously described for rainbow trout (*Oncorhynchus mykiss*) and Siberian surgeon (*Acipenser baeri*) (Gontier-Latonnelle et al., 2007). Equivalent to our results, 7-O-GlcA was the main metabolite in trout, which is in the same salmonid family, whereas GEN-4′-O-sulfatation was preferred in sturgeon.

In conclusion, information on biotransformation processes in fish helps evaluating risks from aquafeeds. Bioactive phytoestrogens in soy-rich diets can have negative health impact (Ng et al., 2006), and derivatives of oxidized isoflavones are potential endocrine disruptors or carcinogens. The finding that in salmon detoxification of DAI, GEN and GLY occurs mainly by conjugation to inactive glucuronides suggested thus a certain tolerance level towards dietary soy.

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CRediT authorship contribution statement

Amritha Johny: Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Lada Ivanova:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Supervision. **Tone-Kari Knutsdatter Østbye:** Resources, Writing - review & editing. **Christiane Kruse Fæste:** Conceptualization, Methodology, Formal analysis, Writing - review & editing, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.110611.

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Supplementary information

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Title:

Biotransformation of phytoestrogens from soy in enzymatically characterized liver microsomes and primary hepatocytes of Atlantic salmon

Journal title:

Ecotoxicology and Environmental Safety

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Supplementary Table 1

Ion source parameters for TQMS with positive/negative switching.

	Phase I CYP substrates	**Phase II UGT substrates**
Drying gas temperature [°C]	300	300
Drying gas flow [L/min]	8	10
Nebulizer [psi]	35	30
Sheath gas heater [°C]	350	375
Sheath gas flow [L/min]	11	12
Capillary [V]	2500 (ESI+/-)	4000 (ESI+/-)
Nozzle voltage [V]	0 (ESI+/-)	500/1000 (ESI+/-)

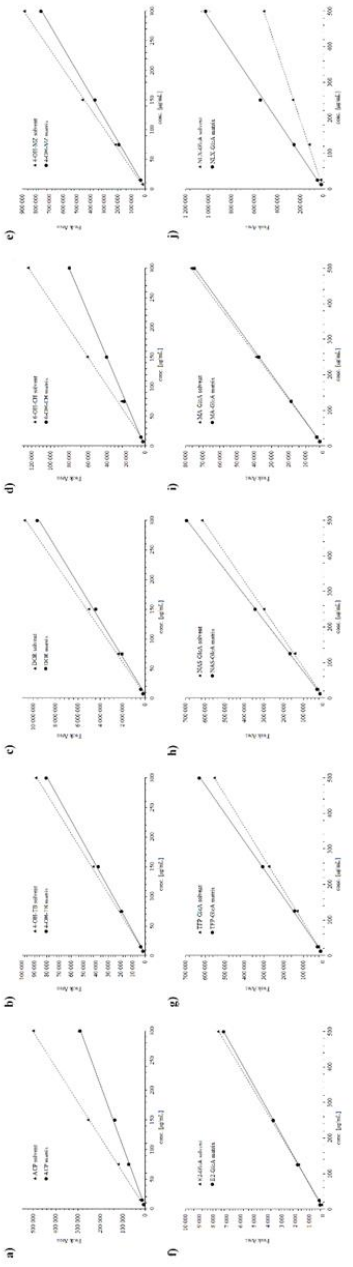
Supplementary Table 2

Initial turnover numbers and metabolite formation rates of CYP and UGT enzymes with specific probe substrates[#] in different hepatic preparations.

Enzyme	Substrate	-k_{cat}[*] [min^{-1} mg protein^{-1}]		Metabolite	Formation rate [ng mL^{-1} min^{-1}]		
		HM	FM		HM	FM	FHep[¤]
CYP1A2	PCN (2.5 µM)	0.006	0.0002	ACP	0.342	n.d.	0.045
CYP2C9	TB (2.5 µM)	0.003	n.d.	4-OH-TB	1.117	n.d.	n.d.
CYP2D6	DEX (2.5 µM)	0.004	0.0025	DOR	2.103	0.899	1.848
CYP2E1	CH (5.0 µM)	0.011	0.0002	6-OH-CH	7.267	0.081	0.039
CYP3A4	MDZ (10 µM)	0.067	0.0010	4-OH-MDZ	19.373	9.204	0.450
UGT1A1	E2 (1.5 µM)	0.043	0.0140	E2-GlcA	5.098	2.008	0.064
UGT1A4	TFP (0.5 µM)	0.009	n.d.	TFP-GlcA	0.508	n.d.	n.d.
UGT1A6	NAS (1.5 µM)	0.026	0.0020	NAS-GlcA	3.802	0.165	0.004
UGT1A9	MA (0.2 µM)	0.219	0.0090	MA-GlcA	15.950	1.628	0.280
UGT2B7	NLX (1.0 µM)	0.049	0.0290	NLX-GlcA	10.234	7.961	0.142

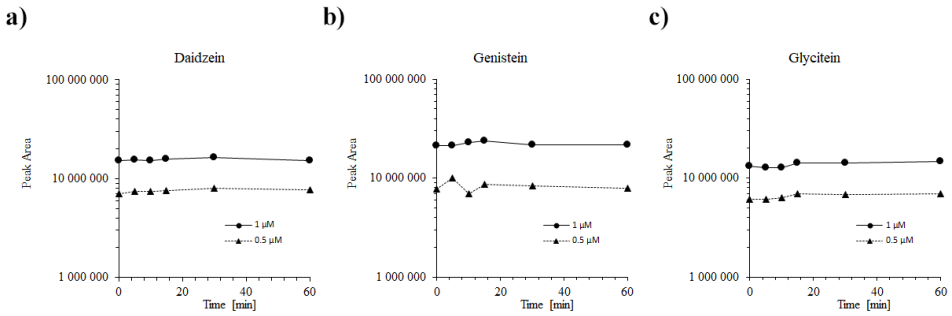
[*]turnover number
[#]substrate mixture
[¤]initial assay concentration of probe substrates was 2 µM for FHep
n.d.: not detected

Supplementary Figure 1



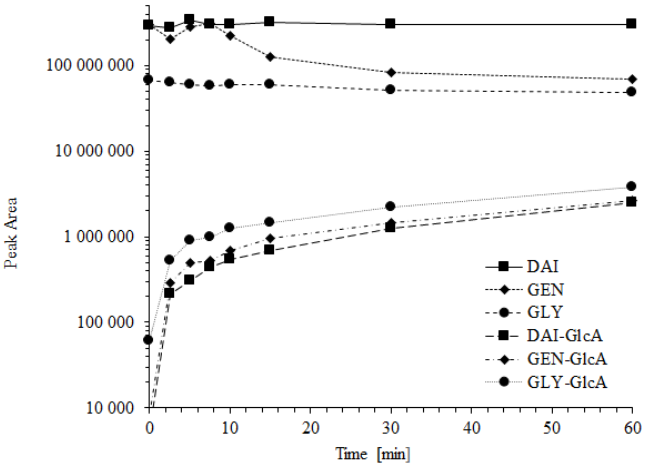
Calibration curves (in solvent or matrix-assisted) in TQMS of metabolites produced from specific probe substrates by CYP and UGT enzyme activities. **a)** ACP; **b)** 4-OH-TB; **c)** DOR; **d)** 6-OH-CB; **e)** 4-OH-MDZ; **f)** E2-GlcA; **g)** TFP-GlcA; **h)** NAS-GlcA; **i)** MA-GlcA; and **j)** NLX-GlcA.

Supplementary Figure 2



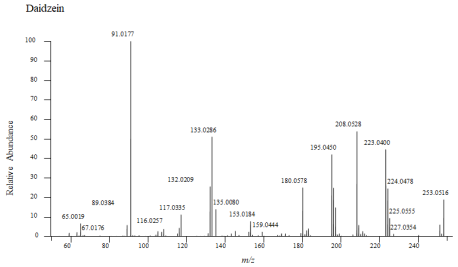
Phase I metabolization of **a)** DAI, **b)** GEN, and **c)** GLY in FM. Means of three independent experiments performed in duplicates.

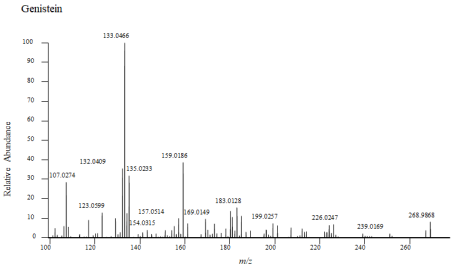
Supplementary Figure 3

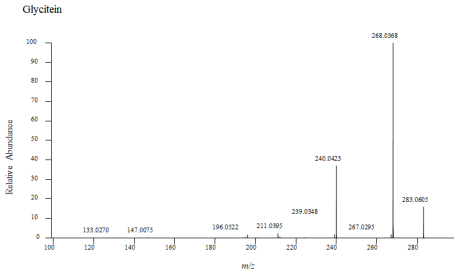


Elimination and glucuronides production of isoflavones (5 µM) in FHep.

Supplementary Figure 4

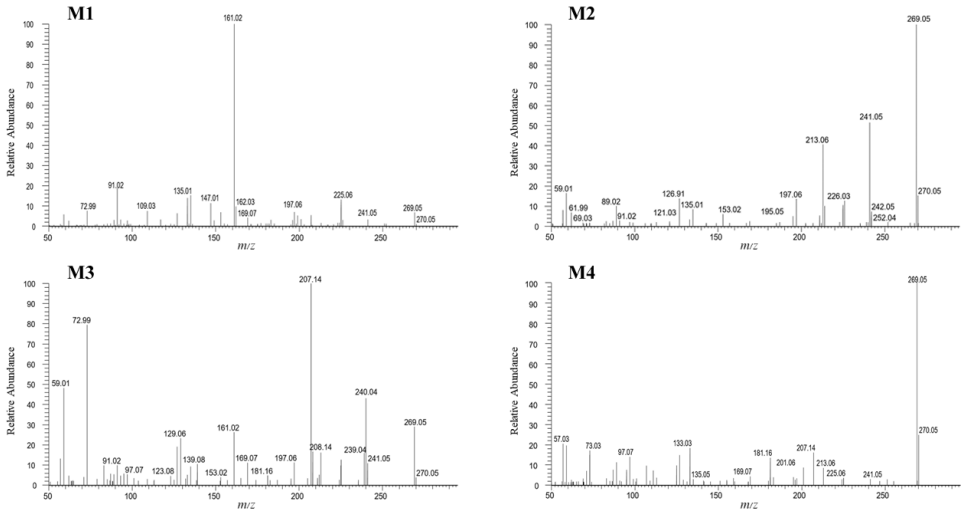






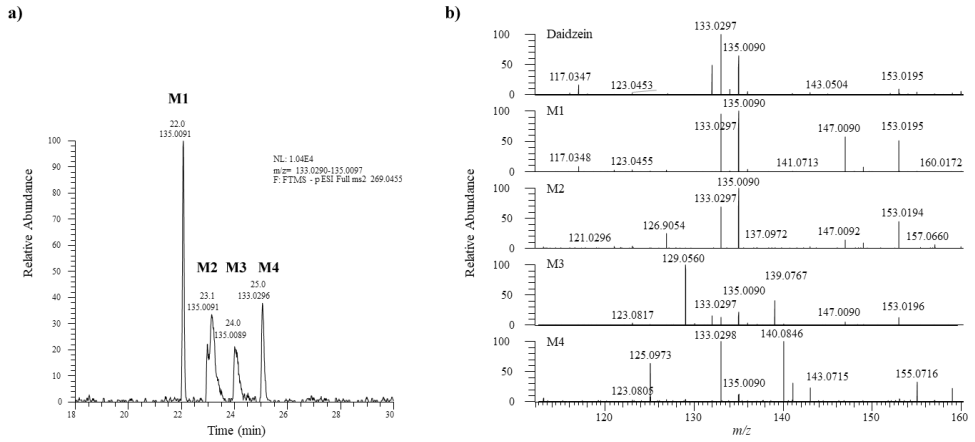
MS^2-spectra of DAI, GEN and GLY.

Supplementary Figure 5



MS^2-spectra of four mono-oxidized DAI metabolites.

Supplementary Figure 6



Identification of mono-oxidized DAI-metabolites. **a)** Fragment ion chromatogram at *m/z* 133.0297 and *m/z* 135.0090 of four DAI (O) metabolites with ion mass *m/z* 269.0455 **b)** MS^2-spectra of DAI and four DAI (O) metabolites showing identical fragmentation patterns.

Paper III

Impact of dietary wheat gluten on feed intake and the intestinal and hepatic transcriptome in Atlantic salmon (*Salmo salar*)

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Declaration of interest: none

Abstract

Partial or complete replacement of fishmeal (FM) with plant proteins in the diets of Atlantic salmon is increasing to meet the demands of the growing aquaculture industry. Wheat gluten (WG) is considered as a valuable protein source in fish diets due to its energy density and relatively low level of anti-nutritional factors (ANFs). The main aim of this study was to identify the effects of dietary WG on fish growth, feed efficiency and the hepatic and intestinal transcriptomes of Atlantic salmon. The fish were fed either control diet with FM as the only source of protein or diets, where 15% or 30% of the FM were replaced by WG. The fish had a mean initial weight of 223 g and approximately doubled their weight during the 9-week experiment. Salmon in the WG30 dietary group showed reduced feed intake compared to the WG15 and control groups. The liver fat content and activities of the liver health markers aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma increased with the inclusion level of WG in the diet. Gene expression analysis showed significant changes in both, intestine and liver of fish fed with WG30. Especially noticeable were changes in the lipid metabolism, in particular in relation to the intestinal lipoprotein transport and sterol metabolism. Moreover, the intestinal transcriptome of WG-fed fish showed shifts in the expression of a large number of genes responsible for immunity and tissue structure and integrity. These observations implied that the fish fed receiving WG-containing diet were undergoing nutritional stress. Overall, the study provided evidence that high dietary level of WG in the diet have a negative impact on the intestinal and liver health of salmon.

Introduction

The world population is currently 7.8 billion and expected to increase with 2 billion by 2050. Between 1961 and 2016, the population growth (3.2%) outpaced the food production (1.6%) and also exceeded the total meat production (2.8%) [1]. However, the fish consumption is increasing constantly at an average rate of 1.5% per year, and the UN estimate that one in five persons depends on fish as the primary source of protein. This tremendous growth depends directly on the availability of feed resources. Atlantic salmon (*Salmo salar*) is a commercially important species [1], with the major share produced in Norway, even increasing in export volume by 4% from 2019 to 2020 [2]. Fish consumption is expected to increase in the coming years to meet the demands of the growing population. Fish is an important part of the human diet, contributing with essential amino acids, omega-3 long chain polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid, essential minerals (Ca, P, Zn, Fe, Se, I) and vitamins (A, B, D) [3]. Growth in aquaculture is necessarily accompanied by an increase in feed production, and consequently by a need for alternative feed ingredients. The inclusion rates of fishmeal (FM) and fish oil (FO) in the diets of Atlantic salmon have decreased from 90% in 1990 to 14.5% in 2016 [4, 5]. The shift to plant-based feed ingredients is a direct consequence of the reduced global availability of FM and FO. Moreover, a rising market pressure to improve the sustainability of fish farming has encouraged this development [6]. This caused the marine protein dependency ratio to decrease from 3.8 kg (1990) for 1 kg of salmon to 0.7 (2013), along with a parallel decrease in the use of marine oils [4].

Novel protein sources, e.g. from insects, are commercially available, but plant-based materials continue to be the prevalent replacement for marine ingredients. Mostly used are soybean protein concentrate (SPC), wheat and wheat gluten (WG), along with corn, faba beans, sunflower meal, pea protein concentrate and other vegetable proteins [5]. The optimal growth of Atlantic salmon depends not only on the fish genetic profiles, rearing conditions and diet

composition but also on the feed formulation and processing [7]. Feed processing has a significant impact on the nutritional quality and digestible energy of the complete diet [8]. It has been shown that soybean meal can cause enteritis in salmon, whereas alcohol-extracted SPC appears not to affect the growth and intestinal integrity, and even enhances weight gain better than FM [9]. However, some studies have indicated that also protein concentrates from soybean and pea can trigger moderate changes in the intestine of salmon, while comparable effects were not reported for WG [10]. WG is a good pellet binder in extruded diets. It is highly digestible [11] and can replace up to 35% of FM in salmonid diets without significant negative effects [12]. However, higher levels can only be incorporated after adequate supplementation with limiting amino acids, especially lysine.

Inclusion of plant ingredients pose a potential threat to fish due to the presence of various undesirable substances including anti-nutritional factors (ANFs) such as phytoestrogens, mycotoxins and plant peptides as well as chemical contaminants, which can interfere with nutrient digestibility, absorption and utilization, and negatively affect growth and health [13]. Undesirable substances may be present in feed at low levels and thus remain unnoticed due to lack of analysis, methods with insufficient detection limits or the non-availability of reference standards, yet their presence alone or in combination may have negative implications. Dietary exposure to harmful contaminants can be detected by transcriptomic analysis showing affected pathways. WG contains comparably less ANF than protein concentrates from legumes [11]. However, major protein fractions in WG, i.e. the gliadins and glutenins [14], are associated to a range of intestinal health effects and disorders in humans [15]. So far, gluten sensitivity has not been reported in fish.

The present study was designed to identify the impact of WG on growth, feed efficiency, metabolism and intestinal health in Atlantic salmon. WG was administered at two inclusion levels (15% or 30%) in the diet and compared to a diet containing only FM as the protein source.

Blood parameters and liver fat were analysed to identify potential health effects. Gene expression profiling by microarray analysis was performed to study the response in two metabolic important tissues, intestine and liver.

Results

Growth performance.

Growth performance was significantly affected by the WG diets (Table 1). Increasing inclusion of WG in the diet resulted in reduced feed intake, with the WG30-fed salmon showing significantly lower feed intake than the FM group. The WG30 group had the lowest final weight but was not significantly different from the other groups. There was no difference in growth rates (specific growth rate (SGR) and thermal growth coefficient (TGC)) between the WG15 and FM groups, whereas the WG30 group had lower SGR and TGC than the other groups. There was no measurable effect of the different diets on the condition factor (CF), ranging from 1.30 to 1.35, indicating the overall good condition of the salmon in the feeding trial. Feed conversion ratios (FCR) also did not differ between groups (Table 2). In contrast, there was a clear effect of the diets on the apparent digestibility coefficients (ADC) for nitrogen, which were higher in both WG groups than in the FM group. The ADC for energy and lipids were not different between the groups (Table 2). No mortality was observed during the feeding trial.

Blood serum analysis.

The diets had a significant effect on several of the measured serum parameters (free fatty acids (FFA), total protein (Tprot), triglyceride (TG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) (Table 3). The FFA levels were lower in both WG groups as compared to the FM-fed control group. However, the WG30 group showed higher levels of Tprot, and the

liver enzyme (ALT) than both the WG15 and FM group. Moreover, there was a strong trend towards higher AST levels in the WG30 group.

Hepatosomatic index and liver fat content.

The fat contents in the salmon livers were within the normal range of 4.9 to 6.9% (Table 1). There was, however, a significant dietary effect with increasing WG in the diets leading to higher liver fat percentages. The hepatosomatic index (HSI) in the WG30 group was significantly higher than in the other groups, ranging from 1.21 in the FM to 1.64 in the WG30 group.

Gene expression profiling.

The numbers of genes, which fulfilled the criteria for differential expression (> 1.754-fold, $p < 0.05$) in the WG-fed groups, when compared to the FM group, were 204 and 1748 in intestine and 55 and 318 in liver for, respectively, the WG15 and WG30 group (Fig. 1). Thus, the group showing the highest number of differentially expressed genes (DEG) and the largest fold change in gene expression was in the salmon fed with 30% of WG. Of the two tissues studied, intestine was more affected by WG inclusion in the diet than the liver. The major pathways that have been altered in the intestinal transcriptome included metabolic processes related to lipid metabolism and transport, sterol metabolism, immunity, tissue structure and integrity and cell stress (Table 4. a. 1, b. 1; Fig. 2. a-e). In salmon liver, processes linked to lipid metabolism and sterol metabolism were largely affected, along with a few genes related to immunity and cell-processes (Table 4. a. 2, b. 2; Fig. 3. a-d). Gene expression was generally more up-regulated in the WG30 than in the WG15 group, both in intensity and number of DEG in intestine and liver.

Intestinal gene expression

The partial inclusion of WG in the diets induced significant changes in the expression of genes associated with lipid metabolism (Fig. 2a). In general, genes related to a) lipoprotein assembly (*choline-phosphate cytidyltransferase* (*pcyt1B*), *sodium-dependent lysophosphatidylcholine symporter 1-B-like genes* (*msfd2a*), *choline kinase* (*chkb*), *CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase-like gene* (*pgs1*), *N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D-like* (*nape-pld*), *phosphoethanolamine methyltransferase* (*pemt*), *apolipoproteins* (*apo*), *perilipins* (*plin*), *microsomal triglyceride transfer protein* (*mtp*)), b) fatty acid synthesis (*fatty acid desaturase* (*fads5*), *elongation of very long chain fatty acids protein* (*elovl4*, *elov5*, *elov6*), *long-chain-fatty-acid-CoA ligase* (*acsbg1*), *sterol-C5-desaturase* (*sc5d*)), *diacylglycerol O-acyltransferase 2* (*dgat2*), *phospholipase* (*ddhd2*)), and c) fatty acid transport (*fatty acid binding protein* (*fabp7*)), along with the associated transcription cofactors (*sterol regulatory element-binding protein 1* (*srebp1*) *and peroxisome proliferator-activated receptor gamma* (*pparγ*)) were the most affected in the salmon intestine.

Inclusion of WG in the diets altered genes involved in choline-based phospholipid synthesis including *pcyt1B* and *msfd2a*, involved in lipoprotein assembly, and *chkb*, which catalyses the first reaction in choline pathway for phosphatidylcholine biosynthesis. Several genes of the same pathway were down-regulated in the WG30 group, including *nape-pld* and *pgs1*. Furthermore, *pemt* was upregulated in the WG30, but not in WG15 group.

The *apo* genes (*apoA-IV, apoEB, apoB-100, apoC1, apoCII, apoE*), which play a key role in transporting lipids from intestine and liver to peripheral tissues, were up-regulated in the WG30 and to a lesser extent in the WG15 group. The lipid droplet-associated protein, *plin2* showed increased expression with both WG diets in comparison to the FM diet. Likewise, up-regulation of the *mtp gene*, involved in the transport of glyceride, cholesterol ester and phospholipids, was observed. Several other genes involved in fatty acid and TG synthesis such as*srebp1, fabp7, fads5, elovl4, elov5, elov6, acsbg1, sc5d, dgat2* and *ddhd2* were also differentially expressed,

with the highest expression found in the WG30 group. A number of genes involved in vesicle formation and transport including *ADP-ribosylation factors* (*arf*) were significantly up-regulated with the WG-containing diets, with the highest increase in the WG30 group.

Sterol or cholesterol metabolism-related genes (Fig. 2b) including *fatty acid hydroxylase* (*faxdc2*), *sodium/bile acid co-transporter-like genes* (*slc*), *neutral cholesterol ester hydrolase* (*nceh1*), *dehydro cholesterol reductase* (*dhcr7*) and bile acid receptors were upregulated in the WG30 dietary group. Furthermore, *cholesterol 7-alpha-monooxygenase-like* (*cp7a1*), which is involved in the conversion of cholesterol into 7-α-hydroxycholestrol, was upregulated in both WG groups. Some genes from the same group were down-regulated including *bile salt export pump* (*abcb11a*). *Scavenger receptor class B member* (*scarb1*), which is a receptor for different phospholipid ligands, and *cholecystokinin* (*cck*), were also upregulated in both WG groups as compared to the FM group.

Expression of immunity-related genes (Fig. 2c) indicated a hypo-immune status of fish fed a high level of WG. Various classes of pro-inflammatory and anti-inflammatory genes responsible for antigen presentation, inflammation and overall immune response were differentially regulated in the intestine. *MHC class I and II histocompatibility antigens* (h2-q10), *NF-kappa-B inhibitor zeta-like* (*nfkbiz*), *interleukins* (*il17, il22, il10*), *TNF receptor-associated factor* (*traf2*), *T-cell specific surface glycoprotein* (*cd28*), *cytotoxic T-lymphocyte-protein 4-like gene* (*ctla4*) along with several others were downregulated in the WG30 group, whereas *annexins* (*anxa2, anxb11*), *nuclear factor interleukin-3-regulated protein* (*nfil3*), *complement C1q-like protein 2* (*ciql2*) *and TNF-like domains* (*tnf*), *nattectin precursor* (*natte*) and *toll like receptors* (*tlr5*) were upregulated. *Macrophage stimulating receptor* (*mst1r*) was up-regulated in both WG groups.

Dietary content of WG affected the expression of several genes related to the maintenance of tissue structure and integrity (Fig. 2d). Differential regulation of genes for mucosal proteins (*mucin-2, mucin 5b*) along with strong down-regulation of genes for extracellular matrix components (*fibronectins, collagen alpha- 3-(IV)* (*col4a3*), *neuropilin, catenin, semaphorin 4E, transcription cofactor HES-6 like* (*hes6*) *and F-box genes* (*fbxo30*), and up-regulation of genes involved in various cellular processes (*occludin, Rho GTPase activating proteins, myosin* and *cyclin-dependent kinase inhibitor 1B* (*cdkn1b*)) was observed in the WG30 group. A suite of proteases and protease inhibitors were up-regulated in the intestine of WG30-fed salmon (*alpha-1-microglobulins* (*a1m*), *trypsin inhibitor ClTI-1-like* (*citi1*), *serine proteases* (*prss33*), *meprin A* (*mep1a*) and *cathepsin S* (*ctss*)), whereas *calpain* (*capn1*) and *alpha-2-macroglobulin-like* genes (*a2ml*) were down-regulated.

Compared to the FM group, the WG groups showed increased expression of several stress-related genes (Fig. 2e) (*growth arrest and DNA damage inducible, beta* (*gadd45b*), *junC* and *DNA damage inducible protein* (*ddi3*)).

Hepatic gene expression

Genes involved in lipid metabolism, sterol metabolism and immunity were significantly up-regulated in the livers of WG30-fed salmon. Among the up-regulated genes associated with lipid- and sterol-metabolism (Fig. 3a, b) were *fatty acid synthase* (*fasn*), *acetoacetyl-CoA synthetase* (*aacs*), *phosphatidyl serine synthase* (*ptdss1*), *beta-1,3-N-acetylglucosaminyltransferase* (*b3gnt5*), *retinol dehydrogenase* (*rdh10, rdh11*), *hydroxymethylglutaryl-CoA synthase, cytoplasmic* (*hmgcs1*), *isopentenyl-diphosphate delta-isomerase-1* (*idi1*), *farnesyl diphosphate synthase* (*fdps*), *7-dehydrocholesterol reductase* (*dhcr7*) as well as several genes functionally related to DEG of the intestine (*mfsd2a, fads5, fads6, elov6, fabp, sc5d and ddhd2*). The few down-regulated genes in liver included *lipase*

maturation factor 2 (*lmf2*), *nape-pld*, *monoglyceride lipase* (*mgl*) and *carnitine palmitoyltransferase 1* (*cpt1*). The upregulated immunity-related genes (Fig. 3c) in the WG30 group (*serum amyloid A5* (*saa5*), *angiogenin-1 precursor* (*ang1*), *phospholipase A2-inhibitor* (*pla2-inhibitor*), *saxitoxin and tetrodotoxin-binding protein 2-like* (*psbp*), *perforin-1-like* (*prf1*), *arginase-1* (*arg1*), *alpha-2-macroglobulin* (*a2m*)) are involved in inflammation. Several genes connected to cell cycle including *cell division cycle-associated protein 3* (*cdca3*), *cyclinB2 (ccnb2*), and *G2/mitotic-specific cyclin B1-like* (*ccnb1-like*) were down-regulated in the WG30 group (Fig. 3d).

Discussion

The present study reports the effects of the dietary inclusion of the plant ingredient WG on the growth and nutrient metabolism in Atlantic salmon. In agreement with previous studies, there were no major effects on the digestibility and growth rate of fish fed with diet at moderate inclusion (15%) of WG [12, 16, 17]. There was, however, a significant reduction of the feed intake in the WG30 group, resulting in significantly lower growth rate and a tendency towards a decreasing final weight with the increased inclusion of WG. The lower feed intake is in agreement with the observed upregulation of cholecystokinin genes *cck* in both WG groups, most pronounced in the WG30 group. Cholecystokinin is a hormone involved in the regulation of food intake and satiation and regulates the digestion of fat and protein [18]. The differential expression of these genes as well as the growth hormone secretagogue receptors (*ghsr-a*: *motilin receptor* and *ghsr-1: ghrelin*) indicate an effect of gluten in the diets on appetite regulation by signalling satiation that will reduce feed intake in the salmon. The increased expression of *cck* might be caused by a gluten-induced metabolic disorder in the intestine. The lower feed intake in the WG groups might thus be explained by an imbalance in the intestine caused by low tolerance for gluten in salmon, which has not been reported previously. This is in contradiction to previous studies showing no signs of intestinal pathology in salmon fed with up to 35% crude WG protein [12] or with wheat meal [19]. A comprehensive transcriptomic analysis of the effects of the dietary inclusion of WG in salmon has, however, not been performed so far.

Our findings with regard to a supposed gluten sensitivity in salmon were further supported by the increased expression of genes involved in lipid metabolism and transport in fish receiving the WG-containing diets, indicating a compensatory response in the intestine and problems with the transport of lipids from the intestine to the blood circulation. Similar responses were observed in the liver, however to a lower extent, comparable to observations in previous studies

[20, 21]. Changes in lipid metabolism and transport, steroid biosynthesis and protein synthesis by dietary inclusion of plant proteins in fish have been reported in a number of studies [21, 22, 23].

In several vertebrate species, reduced tolerance to gluten can cause intestinal inflammation and malabsorption syndromes [24]. In our study, high dietary levels of WG resulted in similar symptoms of intestinal lipid malabsorption as previously described for choline deficiency in Atlantic salmon [21]. Fish meal is the main source of choline in salmon diets. Since the customised diet in the present study had a relatively high level of FM varying from 33.4 to 63.4%, it was expected to cover the requirement for phospholipids and choline. We therefore reckon that the observed up-regulation of several genes involved in the choline pathway (*chka, chkb, pcyt1b, pmt2, mfsd2a, pgs1*) is not a result of choline deficiency but probably a response to an imbalance in the synthesis of lipoproteins that are involved in intestinal lipid transport. Similar changes were found in the liver transcriptome of WG30-fed fish, showing altered expression of genes for phosphatidyl choline biosynthesis (*mfsd2a* and *ptdss1*). Thus, it can be assumed that the intestinal imbalance and metabolic disorder caused by the WG-containing diet lead also to changes in the liver metabolism.

The imbalance in the intestine induced by the WG diets is further evidenced by an up-regulation in the bile acid and fatty acid metabolism, especially in the WG30 dietary group. The cholesterol biosynthetic pathway was affected in both liver and intestine of WG30-fed fish, indicating hypocholesterolaemia and an upregulated capacity for cholesterol synthesis. The observed effects are similar to those reported in previous studies on the use of plant proteins in salmon diets, where decreased lipid digestibility, reduced bile salt levels and hypocholesterolaemia were observed [25]. The detected up-regulation of the squalene and lanosterol biosynthetic pathway, producing precursors of cholesterol biosynthesis, in the WG30 group could be a compensatory mechanism for low cholesterol body pools or an imbalance in the metabolism.

The up-regulation of markers for cholesterol biosynthesis (*dhcr7, srepb1, pparγ, hmgcs*) in the WG30 group indicated increased production of cholesterol. This might be a consequence of a reduced dietary level of cholesterol due to the exchange of marine proteins by plant proteins in the diet. Alternatively, impaired cholesterol and bile acid reabsorption may result from intestinal inflammation as observed in a study on soybean meal in salmon diets [22]. An imbalance in the cholesterol and bile acid absorption might also be the cause for the detected strong down-regulation of intestinal ABC transporters (*abcb11*) in the WG30 group. *Abcb11* mediates the efflux of cholesterol and bile acids from liver into bile [22, 26], and its mRNA expression is positively regulated by bile acid receptors (*fxr*) [27]. Although the bile acid receptor level was unaffected in the liver of the WG-fed salmon, it was up-regulated in the intestine along with bile acid co-transporters.

Dietary effects on intestine and liver are connected, which we could confirm with our findings regarding WG-containing salmon. Apart from transcriptome changes we also detected that typical makers for liver damage were elevated in the WG30 dietary group. The increase was significant for ALT and close to significant for AST. The liver fat levels were within the normal range, but were notably increased by WG dietary inclusion. The HSI of fish in the WG30 group was higher than that of the other groups. This may be caused by increased fat retention or liver fatty acid synthesis, which was observed in salmon fed with mainly plant protein- and plant oil-containing diet [28]. The assumed connection between dietary WG and an increased fatty acid synthesis in our study is supported by the detected up-regulation of the transcription factor *srebp1* and the lipid metabolism genes *fadsd5, fadsd6, fasn, elovl6, fabp, sc5d, b3gnt5, ddhd2* and *aacs. Acetoacetyl-coA synthetase* (*aacs*) catalyses the first reaction in fatty acid metabolism and plays a major role in the lipid synthesis of triacylglycerols, phospholipids and cholesterol esters [29]. These responses to the WG diets, in combination with the down-regulation of lipid-hydrolysing lipases (*lmf2, nape-pld, mgl*) and *cpt1*, which is involved in the beta-oxidation of

fatty acids, might be a compensatory response to the malabsorption of lipids in the intestine [30].

The present study also showed a high up-regulation of lipid transport proteins, apolipoproteins and perilipins, supporting an imbalance in the intestine caused by gluten sensitivity-like reactions. Deficiency in the bile transport, as discussed previously, can cause lipid droplet accumulation and triggers the increase of transport-related genes [22]. Apolipoproteins are proteins, which bind to lipids and form lipoproteins and thereby act as transport vehicles [31]. These proteins play an important role in the transport of cholesterol, triglycerides, phospholipids and fat-soluble vitamins between the intestine, liver and peripheral tissues. We observed the up-regulation of several apolipoproteins in the intestine of both WG-fed groups as compared to the FM group, indicating an accumulation of lipids in the intestine and reduced transport of lipids to the circulation. A similar effect has been reported in a previous study on salmon fed with diets containing considerable levels of plant protein and oils [32]. The increased expression of *plin2,* involved in the coating of lipid droplets, in the intestine of both WG dietary groups further supports this possible link. Our findings are, however, in contrast with a salmon study on choline deficiency, where reduced intracellular lipid levels were reflected by the suppression of *plin2* expression [21]. However, WG-containing diets seem to primarily affect the absorption and transport of lipids in the intestine.

The detected changes in several genes connected to pathways important for maintaining the intestinal balance that were induced by the WG-containing diets, especially at the high inclusion level, gave additional evidence for the proposed connection. The regulation of the genes such as *pept1*, *occludin*, *Rho GTPase activating proteins*, *myosin*, *cck*, *ghsr*, *cdkn1b*, *fibronectin*, *collagen*, *mucin*, *hes* and *calpain* indicated a direct effect of WG on the salmon intestine. Up-regulation of *pept1*, involved in peptide transport across the enterocyte membrane [33], might be a compensatory response to alterations in the intestinal membrane and increased peptide

absorption. Expression of *pept1* has been shown to be affected by dietary plant protein sources in sea bream [34]. *Occludin* expression was up-regulated in the WG30 group and may indicate WG-induced reorganisation or strengthening of cell junctions. A similar response was observed in a study on salmon fed with pea proteins combined with soybean saponins [35]. The *cdkn* inhibitors, linked to cell cycle progression, were induced by inclusion of WG in the diet in our study, and might be a compensatory mechanism to reduce the rapid proliferation of cells in the intestine, whereas the downregulation of *fibronectin, collagen* and *mucin* genes in WG30-fed salmon might further indicate an altered intestinal integrity. The downregulation of genes belonging to extracellular matrix (*mucins* and *collagen*) and proteases (*hes* and *calpain*) involved in intestinal development and homeostasis in the WG30 group point also at a negative effect on intestinal integrity, as observed for soy bean enteritis in salmon [36].

The present study showed that WG altered the expression of many immune genes, which has been reported in studies on chronically-inflamed intestine in salmon fed with plant diets [20, 35, 37]. We observed down-regulation of the *CD28 antigen, cytotoxic T-lymphocyte-associated protein 4, IL-17, IL-22*, T-*cell receptor-signalling pathway* and *TNF-like domains, NF-kB* and *macrophage stimulating receptor* genes, along with an increase in anti-inflammatory markers in the WG30 group. These findings support the assumption that salmon have a low tolerance to gluten and that exposure can lead to an imbalance in the intestine with increasing inclusion of WG in the diet. Interestingly, genes related to cell apoptosis (cell death activator genes) were seen strongly up-regulated in both WG dietary groups, and the most in the WG30 group. The up-regulated *annexin A1 and annexin A2 (anxa2, anxb11), cannabinoid 2 receptors and alpha-1-macroglobulins* (*a1m*) are inflammation inhibitors [20, 35]. Previous studies on humans with gluten sensitivity also showed increased plasma concentrations of these biomarkers during increased gluten intake [38]. Moreover, the up-regulation of *serum amyloid A protein (SAA5), phospholipase A2 inhibitor-like genes (differentially regulated trout protein 1), arginase 1,*

angionenin 1 precursor, pstbps and a2m supports an inflammatory effect of the WG30 diet in salmon.

Conclusion

The partial inclusion of WG in salmon diets resulted in reduced feed intake. The transcriptomic changes in the intestinal and liver metabolism indicated an imbalance in the intestinal integrity and function, with the intestine being more affected than the liver. There was a dose-dependent effect of dietary WG inclusion. Genes related to lipid and sterol metabolism, immunity and intestinal integrity were the most affected, suggesting that the fish tried to maintain homeostasis and to compensate for the negative effects of the high dietary WG level.

Materials and Methods

Preparation of customised salmon diets.

The fish diets were prepared at Nofima Feed Technology Centre, Fyllingsdalen, Norway by incorporating WG as the plant protein source, replacing 15% or 30% of the fish meal (FM). The feed materials were purchased from the commercial companies Norsildmel AS (FM) (Bergen, Norway) and Tereos Syral (WG) (Marckolsheim, France). The plant ingredients used were of good quality and contained only low levels of undesirable substances such as mycotoxins, often below the limit of detection [39]. Therefore, we assumed that any change in the gene expression in different salmon tissues was not caused by these contaminants, but was a direct consequence of the presence of WG in the diet. The manufacturing process of the experimental diets for salmon has been reported previously in detail [39]. Diet formulations and compositions are given in Table 5. The fish diets were formulated in such a way that contents of total proteins, dry matter, lipids and energy were approximately the same for all diet groups. The pellet size was adjusted to the size of the on-growing salmon. All non-oil ingredients were mixed, extruded, dried and coated with oil. Yttrium oxide was included at 0.01% as an indigestible inert marker to quantify apparent nutrient digestibility. The diets were not balanced for amino acids and the other nutrients.

Fish and feeding trial.

The experimental set-up, feeding trial and sampling have been previously explained in detail [39]. Briefly, one-year-old post-smolt Atlantic salmon (*Salmo salar*) with a mean weight of 223 g were used in a nine-week feeding trial at the Nofima's Research Station, Sunndalsøra, Norway. The experimental groups consisted of a control group receiving diet with FM as the only protein source, and two groups fed with WG-containing diets with, respectively, 15% or 30% replacement of FM. Fish were randomly distributed to the tanks (1 m^3; n = 30 fish per

tank, except one tank of the FM group, which had 29 fish) supplied with seawater, using three replicate tanks for each of the diet groups. Bulk weight of the fish per tank was recorded at the start of the experiment. Excess feed was collected daily from the tanks for the calculation of feed intake [40]. Fish health, feed intake and overall welfare were regularly monitored. The water temperature was maintained at an average of 10.6 (±0.6) ˚C. The oxygen level at the tank outlets was higher than 90% at study start and about 80% at study end. The water flow in each tank was set to 20 L/min.

At the end of the trial, the fish were anaesthetised with a standard dose of tricaine methanesulfonate MS222 (Sigma-Aldrich, St. Louis, MO, USA), transferred to a smaller tank and euthanised with a lethal dose of 200 mg/L of the same chemical. Individual weights, liver weights and lengths were recorded for the sampled fish (n=5 fish per tank, i.e. in total 15 for each of the diets) and the rest weighed in bulk. Tissues and blood samples were taken. The sampled fish were opened and the mid-intestine was dissected out and digesta removed. The intestines were rinsed clean with phosphate-buffered saline (PBS) pH 7.4 and snap-frozen in liquid nitrogen. Livers were removed, weighed and cut into small pieces of 1 cm before snap-freezing in liquid nitrogen. The liver and intestine samples were stored at –80 °C until analysis.

Fecal samples were collected, pooled per tank and stored at -20 °C prior to analysis for calculating apparent digestibility coefficients (ADC). The ADC of lipid, nitrogen and energy in the experimental diets were determined by using the equation ADC (%) = $100 - 100 \times (Y_d \times N_f) / (Y_f \times N_d)$, where d is for diet, f for faeces, Y for yttrium content, and N for nutrient content. The growth rates were calculated according to the equation for the specific growth rate SGR (%) = $(\ln W_1 - \ln W_0)/d) \times 100$, where W_1 is the final weight, W_0 the initial weight, and d are the days in experiment, and for the thermal growth coefficient TGC = $1000 \times (W_1^{(1/3)} - W_0^{(1/3)}) / ddg$, where ddg represents degree-days, i.e. the product of the water temperature (°C) and the number of days at this temperature in the experiment. Furthermore, the feed conversion

ratio (FCR = feed consumed/biomass increase), condition factor (CF = (fish weight (g)/fish fork length (cm) × 100), and hepatosomatic index (HSI = 100 × (liver weight/total fish weight) were calculated.

Measurements of blood parameters.

Blood samples were centrifuged for serum preparations, and FFA, Tprot, TG, ALT and AST were analysed according to standard procedures at the Central Laboratory of the Norwegian University of Life Science (NMBU), Oslo, Norway.

Chemical analysis of liver fat.

Total lipids were extracted as previously described [41]. Briefly, 0.4 g liver samples of five fish in a tank were pooled, homogenised in chloroform/methanol (2:1, v/v), filtered, washed in isotonic saline and quantified gravimetrically.

RNA extraction.

Total RNA was extracted from the mid-intestine and liver samples (~10 mg) using a Biomek 4000 Automated Workstation (Beckman Coulter, Indianapolis, US), applying the Agencourt® RNAdvance tissue kit according to the manufacturer's instructions: The tissues (10 mg) were transferred into 1.2 mL collection microtubes (QIAGEN, Venio, Netherlands) with 3 mm magnetic beads, 400 µL lysis buffer and 1 mg proteinase K. The samples were homogenised in a tissue lyser (180 sec, 1800 rpm; FastPrep-96, Beckman Coulter), centrifuged (1min, 1600 rpm, Avanti™ J-301, Beckman Coulter) and placed in a heated cabinet (Termaks, Bergen, Norway) for 25 min or more until the tissue samples were completely lysed. Subsequently, the samples were processed in the Biomek 4000 workstation. RNA concentration and quality were determined using a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Bremen, Germany) and a 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA), applying

the Agilent RNA 6000 Nano Kit in accordance to the respective instruments protocols. An RNA integrity number (RIN) value above 7.5 was considered as satisfactory. The RNA samples were stored at -80 °C until use.

Microarrays

Nofima's genome-wide Atlantic salmon oligonucleotide microarray with 4 × 44k 60-mer probes, manufactured by Agilent Technologies (Santa Clara, CA, USA), was used [42]. All reagents and equipment were purchased from the same source. Two fish from each replicate tank, in total six fish per dietary group for both intestine and liver, were selected for the microarray analysis. Amplification and labelling of the total RNA (200 ng / reaction) with cyanine 3- cytidine triphosphate (CTP) was performed with the Low-Input Quick-Amp Labeling Kit. cRNA was quantified using the NanoDrop 8000 Spectrophotometer. The Gene Expression Hybridization Kit was used for fragmentation of the labelled RNA. The samples were immediately loaded onto the microarray for hybridisation for 17 hours in a hybridisation oven at 65 °C with a rotation speed of 10 rpm. The arrays were then washed for 1 min at room temperature with Gene Expression Wash Buffer I and for 1 min with Gene Expression Wash Buffer II, the latter was pre-warmed and kept at 37 °C until use. The microarrays were scanned with a SureScan Microarray Scanner from the same manufacturer.

Gene expression data were processed and analysed with Nofima's bioinformatics system STARS (Salmon and Trout Annotated Reference Sequences) [42]. Global normalisation was performed by equalising the mean intensities of all microarrays. The normalised values for the individual features were then divided by the mean value of all samples, thus determining the specific expression ratios (ER). Since the expression changes in the WG-containing diets were correlated, just the scale being greater for the WG30 diet, the results were combined for further

analysis. Finally, log_2-ER were calculated and normalised by locally-weighted non-linear regression (Lowess).

Data analysis.

The growth rates and other parameters (body weights, feed intake, FCR and ADC) of salmon fed with different diets were recorded for each dietary group (considering the means of three replicate tanks, n=3) and statistically tested by one-way ANOVA to assess the effects of the diets. For comparison of the individual measured parameters from the final sampling (HSI and CF), a nested mixed model was used with diet as a fixed variable and tank as a random variable, and by using tank within diet as error term for testing. Statistical differences between the dietary groups with respect to the blood parameters and liver fat contents were evaluated by one-way ANOVA followed by Duncan's multiple range test. In microarray analysis, DEGs were selected by difference from control (>1.75-fold and $p < 0.05$). Enrichment of GO terms and KEGG pathways was assessed with Yates' corrected chi-square test.

Ethical statement

The study was performed in compliance with the laws regulating experimentation with live animals in Norway and the experimental protocol was approved by the Norwegian Animal Research Authority (Forsøksdyrutvalget). The methods were carried out in accordance with the relevant guidelines and regulations by EU [43]. The experiment was considered as not requiring a specific license as the fish received uncontaminated feed, were not subjected to experimental treatments, and euthanised by an ethically-approved method.

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Author Contributions

A.J., T-K.K.Ø., G.M.B., A.B. and C.K.F. designed the study; A.J., G.M.B., A.B. and T-K.K.Ø contributed towards methodology; A.K. and G.M.B. performed bioinformatics; A.J., A.K., G.M.B., B.R. and T-K.K.Ø. analysed and interpreted the data; T-K.K.Ø. and C.K.F. acquired resources; A.J. wrote the original draft; T-K.K.Ø, B.R. and C.K.F. supervised A.J.; G.M.B. and C.K.F. was responsible for the project administration; C.K.F. acquired funding for the study. All authors were involved in drafting the article or revising the content, and all authors approved the final version to be published.

Additional Information

The authors declare no conflict of interest.

Figure legends

Figure 1. Differentially-expressed genes (DEG) in the intestine and liver of salmon fed with WG-containing diets (15% or 30%) in comparison to the FM control group (n=6).

Figure 2. Major differentially-expressed genes (DEG) in the intestine of salmon fed with WG containing diets; genes are related to a) Lipid metabolism and transport, b) Sterol metabolism, c) Immunity, d) Tissue structure and integrity, e) Cell stress. Data are folds to the FM-fed control group, DEG (>1.754-fold, $p<0.05$) are indicated with underlined italics.

Figure 3. Major differentially-expressed genes (DEG) in the liver of salmon fed with WG containing diets; genes are related to a) Lipid metabolism, b) Sterol metabolism, c) Immunity, d) Cell-processes. Data are folds to the FM-fed control group, DEG (>1.754-fold, $p<0.05$) are indicated with underlined italics.

Figures

Figure 1.

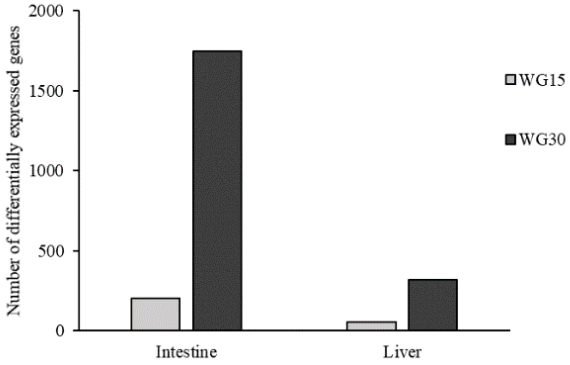

Figure 2.

a) Lipid metabolism and transport

WG15	WG30	
1.05	*1.57*	Delta-6 fatty acyl desaturase
-0.01	*0.81*	Sterol-C5-desaturase
2.24	*3.95*	Stearoyl-CoA desaturase b
0.83	*1.86*	Fatty acid desaturase 1
1.07	*2.45*	Elongation of very long chain fatty acids protein 5
1.21	*1.79*	Polyunsaturated fatty acid elongase 12
0.63	*1.29*	ELOVL family member 6
0.38	*1.04*	Long-chain-fatty-acid—CoA ligase
0.23	*0.90*	Globoside alpha-1,3-N-acetylgalactosaminyltransferase 1
0.71	*1.28*	Lipid phosphate phosphohydrolase 2
2.18	*3.51*	ADP-ribosylation factor 4-like
2.36	*3.37*	ADP-ribosylation factor 5
0.54	*1.19*	3-hydroxyacyl-CoA dehydratase
0.81	*0.96*	Choline kinase beta
0.85	*1.10*	Choline-phosphate cytidylyltransferase B-like
-0.16	*-0.84*	Phospholipase C
0.56	*1.10*	Phospholipase DDHD2-like
0.28	*0.98*	Phospholipase B-like 2
1.20	*2.02*	Phospholipase A2 group XIIA secretory
-0.49	*-1.02*	Phospholipase D1
0.37	*1.14*	Fat storage-inducing transmembrane protein 2-like
0.37	*0.81*	Non-specific lipid-transfer protein
-0.60	*-1.05*	Diacylglycerol O-acyltransferase
-0.32	*-1.06*	Sphingosine 1-phosphate receptor 1
-1.17	*-1.63*	Low-density lipoprotein receptor-related protein 1
-0.40	*-0.94*	Glycolipid transfer protein
0.66	*1.00*	3-hydroxyacyl-CoA dehydratase 2
-0.73	*-1.29*	Ceramide synthase 5
-1.38	*-1.86*	Low density lipoprotein receptor-related protein 1Bb
0.80	*1.40*	Beta-carotene dioxygenase 1
0.89	*1.21*	Fibrous sheath CABYR-binding protein-like
0.66	*1.00*	Epidermal retinol dehydrogenase 2
-0.37	*-1.16*	CDP-diacylglycerol—glycerol-3-phosphate 3-phosphatidyltransferase-like
0.83	*1.42*	Phosphate cytidylyltransferase 1, choline, alpha b
0.31	*0.80*	Lysophosphatidic acid receptor 6
0.67	*1.50*	Diacylglycerol O-acyltransferase 2
0.62	*1.24*	Acyl-CoA synthetase long-chain family member 4a
-0.38	*-0.82*	Sphingosine-1-phosphate lyase 1
-0.90	*-1.02*	Sphingosine kinase 2
-1.05	*-1.56*	Apolipoprotein A-IV-like
0.73	*1.58*	Apolipoprotein E
1.22	*1.49*	Apolipoprotein A-I-like
1.45	*3.27*	Apolipoprotein B-100
2.12	*3.15*	Apolipoprotein Eb [Apo-Eb]
2.43	*4.26*	Apolipoprotein C-I
0.47	*0.98*	Apolipoprotein CII
3.52	*4.77*	Apolipoprotein A-IV
1.41	*1.99*	Perilipin
0.49	*0.90*	Microsomal triglyceride transfer protein large subunit
1.00	*1.79*	Group XIIA secretory phospholipase A2-like
0.66	*0.96*	Lysocardiolipin acyltransferase 1
-0.64	*-1.05*	Acyl-CoA synthetase long-chain family member 1a
-0.20	*-0.96*	ABC transporter G family member 20-like
1.26	*1.75*	Sodium-dependent lysophosphatidylcholine symporter 1-B-like
0.66	1.39	Fatty acid binding protein 10a
1.25	*1.84*	Oligopeptide transporter, solute carrier family 15 member 1
0.95	*1.86*	Solute carrier family 22 member 4-like
-1.04	*-1.86*	N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D-like
0.63	*1.41*	Phosphoethanolamine methyltransferase
0.34	*0.95*	Sterol regulatory element-binding protein 1
0.12	*0.80*	Peroxisome proliferator-activated receptor gamma coactivator 1 beta - Ident
1.04	0.60	Cholinergic receptor, muscarinic 5a
0.31	*0.98*	Retinol dehydrogenase 11
-0.71	*-1.30*	Aryl hydrocarbon receptor nuclear translocator-like

b) Sterol metabolism

WG15	WG30	
0.62	*1.30*	Fatty acid hydroxylase domain-containing protein 2
0.68	*1.10*	7-dehydrocholesterol reductase
0.69	*1.11*	Sodium/bile acid cotransporter-like
1.29	*2.13*	Scavenger receptor class B member 1
0.77	*0.88*	Cytochrome P450 7A1
0.47	*1.03*	Neutral cholesterol ester hydrolase 1-like
0.41	*0.82*	Bile acid receptor-like
-0.09	*-0.88*	Bile salt export pump

c) Immunity

WG15	WG30	
-0.27	*-0.88*	MHC class I antigen
-0.78	*-0.86*	MHC class II antigen
-0.50	*-0.89*	HLA class II histocompatibility antigen gamma chain-like
-0.54	*-0.85*	Phosphatidylinositol 4,5-bisphosphate 3-kinase
-0.38	*-1.58*	Complement C1q-like protein 2
-0.40	*-1.07*	Lipopolysaccharide-induced tumor necrosis factor-alpha factor homolog
-0.78	*-1.28*	Putative interferon-alpha/beta receptor alpha chain
-0.96	*-0.91*	Interleukin-17
-0.34	*-0.99*	Interleukin-22
-0.37	*-1.01*	Interleukin 10
-1.20	*-2.02*	Cytokine receptor-like factor 1b
-0.34	*-0.89*	Macrophage colony-stimulating factor 1 receptor
-0.56	*-1.08*	T-cell receptor beta chain
-1.09	*-1.81*	Modified T cell receptor alpha
-0.62	*-1.32*	CD28 T-cell-specific surface glycoprotein
-0.31	*-1.01*	Cytotoxic T-lymphocyte protein 4
-0.89	*-1.18*	T-cell-specific surface glycoprotein CD28-like
0.73	*1.47*	T-cell activation inhibitor
-0.55	*-1.26*	Tumor necrosis factor
-0.40	*-1.57*	Mitogen-activated protein kinase-activated protein kinase 3
-0.62	*-0.96*	NF-kappa-B inhibitor zeta-like
-0.90	*-1.59*	TRAF-interacting protein
0.68	*0.93*	Jun C
1.64	*2.82*	C1q and TNF-like domains
0.97	*1.58*	Macrophage stimulating 1 receptor
0.38	*0.98*	Nuclear factor interleukin-3-regulated protein
0.74	*1.09*	Interferon-induced protein with tetratricopeptide repeats 2-like
0.18	*1.04*	Annexin A2a
0.18	*0.98*	Annexin A11b
0.92	*0.80*	Nattectin precursor
0.29	*0.84*	Toll-like leucine-rich receptor 5
1.79	*3.48*	Perforin-1-like

d) Tissue structure and integrity

WG15	WG30	
0.89	*1.64*	Neural-cadherin-like
1.12	*1.68*	Pannexin-2
-0.80	*-1.28*	Catenin
-0.64	*-0.88*	Semaphorin-4E-like
-0.45	*-0.90*	Neuropilin 2a
0.45	*1.01*	Occludin
2.31	*2.47*	Troponin C, skeletal muscle
0.38	*1.19*	fast skeletal myosin heavy chain
1.43	*0.84*	Myosin, heavy chain b
0.35	*1.02*	Rho-related GTP-binding protein
0.63	*1.14*	Serine/threonine-protein kinase
1.51	*5.98*	Signal sequence receptor, gamma
-0.35	*-0.90*	Alpha-2-macroglobulin-like
-1.09	*-1.50*	Mucin-2-like
-0.73	*-0.93*	Dipeptidase 1
-0.15	*-0.86*	Fibronectin
-0.27	*-1.03*	Collagen alpha-3
0.44	*1.03*	Mucin-5B-like
0.57	*0.84*	Mucin-2-like
0.78	*0.85*	Cannabinoid receptor 2-like
0.97	*1.58*	Macrophage stimulating 1 receptor
-0.39	*-0.81*	Insulin-like growth factor I
-0.42	*-1.37*	Transcription cofactor HES-6-like
0.42	*0.96*	Growth hormone secretagogue receptor a
-0.21	*-0.97*	F-box only protein 30
-0.78	*-1.11*	Growth hormone secretagogue receptor 1
-0.22	*-1.07*	Transforming growth factor beta receptor type 3-like
1.04	0.60	Cholinergic receptor, muscarinic 5a
1.02	*1.71*	f-box
0.69	*0.81*	Estrogen receptor 1
1.27	*2.18*	C-type natriuretic peptide
0.41	*0.90*	Steroid hormone receptor ERR1-like
0.53	*0.89*	Annexin A2a
0.64	*1.25*	Catalase
0.39	*1.35*	F-box/LRR-repeat protein 20
1.22	*1.95*	Cell death activator CIDE-3
1.38	*2.29*	Alpha-1-microglobulin
0.11	*0.83*	Trypsin inhibitor CITI-1-like
0.20	*0.81*	Aminopeptidase N
0.45	*1.06*	Cathepsin S-like
0.34	*1.26*	Cathepsin D
0.39	*0.81*	CNDP dipeptidase 2
0.14	*0.97*	Carboxypeptidase O
0.42	*1.25*	Serine protease 33-like
0.19	*1.03*	Meprin A subunit alpha-like
0.60	*0.93*	Trypsin-2
0.63	*1.09*	Disintegrin and metalloproteinase domain-containin
0.39	*0.87*	Phosphatidylethanolamine binding protein
-0.43	*-0.92*	Calpain 9
-0.35	*-0.90*	Alpha-2-macroglobulin-like

e) Cell stress

WG15	WG30	
0.32	*0.92*	**DNA damage-inducible transcript 3 protein**
0.50	*0.91*	**AP1-1**
0.35	*1.06*	**Growth arrest and DNA-damage-inducible, beta b**
0.66	*0.98*	**Jun C**
-0.46	*-0.79*	**Mitogen-activated protein kinase kinase kinase 5**

Figure 3.

a) Lipid metabolism

WG15	WG30	
0.45	*1.34*	Delta-6 fatty acyl desaturase
0.50	*2.47*	Fatty acid synthase
0.97	*1.90*	ELOVL family member 6
0.28	*1.86*	Acetoacetyl-CoA synthetase
0.54	*1.59*	Phosphatidylserine decarboxylase
0.04	*1.33*	Fatty acid-binding protein
0.40	*1.31*	Acyl-CoA-binding protein-like
0.46	*1.31*	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5
0.85	*1.18*	Proline-rich extensin-like protein EPR1
0.39	*1.07*	Acetyl-CoA acetyltransferase
0.57	*1.06*	Sterol-C5-desaturase
-0.28	*1.05*	Fatty acid desaturase 1
0.27	*0.88*	Phospholipase DDHD2-like
-0.05	*0.87*	Protein tyrosine phosphatase-like
0.08	*0.82*	Long-chain-fatty-acid--CoA ligase ACSBG1
0.32	*0.80*	Lipid phosphate phosphohydrolase 1-like
0.19	*1.42*	Sodium-dependent lysophosphatidylcholine symporter 1-B-like
0.71	*0.83*	Phosphatidylserine synthase 1
-0.03	*-0.94*	Monoglyceride lipase
-0.05	*-0.83*	Lipase maturation factor 2
-0.06	*-1.11*	Carnitine palmitoyltransferase 1B (Muscle)

b) Sterol metabolism

WG15	WG30	
0.61	*1.61*	Hydroxymethylglutaryl-CoA synthase, cytoplasmic
0.38	*1.50*	Isopentenyl-diphosphate Delta-isomerase 1
0.70	*1.36*	Farnesyl diphosphate synthase
0.45	*1.14*	7-dehydrocholesterol reductase
0.28	*1.11*	Squalene synthase
0.26	*1.05*	Lanosterol 14-alpha demethylase
0.33	*0.81*	Mevalonate kinase
0.26	*0.79*	Methylsterol monooxygenase 1
0.59	*1.56*	Retinol dehydrogenase 11
0.57	*1.17*	Retinol dehydrogenase 10

c) Immunity

WG15	WG30	
0.08	*1.95*	Serum amyloid A5
0.52	*0.86*	Differentially regulated trout protein 1
0.62	*1.26*	Angiogenin-1 precursor / RNase ZF3
0.54	*1.12*	Saxitoxin and tetrodotoxin-binding protein 2-like
0.63	*0.90*	Perforin-1-like
0.49	*0.88*	Arginase-1
0.18	*1.00*	Alpha-2-macroglobulin-like

d) Cell-processes

WG15	WG30	
-0.41	*-0.87*	G2/mitotic-specific cyclin-B1-like
-0.40	*-0.89*	Kinesin-like protein
-0.63	*-0.95*	Cyclin B2
-0.40	*-0.96*	Protein regulator of cytokinesis 1
-0.55	*-1.00*	Cell division control protein 2 homolog
-0.15	*-0.83*	Cell division cycle-associated protein 3
-0.48	*-1.01*	Cell division cycle protein 20 homolog
-0.67	*-0.80*	NUF2, NDC80 kinetochore complex component
-0.29	*-0.82*	Ubiquitin-conjugating enzyme E2 C

Tables

Table 1. Body weights (bw) at study start and end, feed intake, feed conversion ratios (FCR), specific growth rates (SGR) and thermal growth coefficients (TGC) of Atlantic salmon (mean ± S.E.M.; n=3 replicate tanks) fed with control diet (FM) or WG-containing diets (WG15, WG30). Liver fat contents, condition factors (CF) and hepatosomatic index (HSI) of sampled fish, mean ± S.E.M.; n=15, 5 fish per 3 replicate tanks. [a, b, c, d] Significant differences ($p<0.05$) between diets are indicated with different letters.

	FM	**WG15**	**WG30**	**p-value**
Initial wt (g)	225 ± 2	219 ± 4	223 ± 0	0.31
Final wt (g)	548 ± 34	563 ± 4	513 ± 8	0.28
Feed intake (g)	8251 ± 214[a]	7602 ± 12[b]	6620 ± 108[c]	0.0005
FCR	0.80 ± 0.02	0.74 ± 0.01	0.76 ± 0.01	0.08
SGR (% d^{-1})	1.53 ± 0.03[a]	1.54 ± 0.04[a]	1.36 ± 0.03[b]	0.02
TGC	3.40 ± 0.06[a]	3.41 ± 0.10[a]	2.97 ± 0.07[b]	0.01
Liver fat (%)	4.9 ± 0.0[b]	5.6 ± 0.2[ab]	6.9 ± 0.7[a]	0.04
CF	1.31 ± 0.02	1.32 ± 0.02	1.30 ± 0.02	0.83
HSI	1.23 ± 0.03[b]	1.21 ± 0.02 [b]	1.64 ± 0.09 [a]	0.01

Table 2. Apparent digestibility coefficients (ADC) for lipid, nitrogen and energy in salmon fed with FM or WG-containing diets. Data are mean ± S.E.M.; n=3 replicate tanks. Significant differences ($p<0.05$) between the dietary groups are indicated with different letters.

Digestibility	FM	WG15	WG30	p-value
Lipid	97.2 ± 0.7	97.1 ± 0.2	96.8 ± 0.4	0.86
Nitrogen	86.4 ± 0.7 ^{c}	89.1 ± 0.1 ^{b}	91.8 ± 0.3 ^{a}	0.0005
Energy	88.6 ± 0.7	88.5 ± 0.1	88.5 ± 0.5	0.97

Table 3. Free Fatty Acid (FFA), Total protein (Tprot), Triglycerides (TG), Alanine aminotransferase (AT) and Aspartate aminotransferase (AST) levels in the serum of salmon fed with FM or WG-containing diets. Data are mean ± S.E.M. (n=15; 5 fish per 3 replicate tanks). Significant differences ($p<0.05$) between the dietary groups are indicated with different letters.

	FM	**WG15**	**WG30**	**p-value**
FFA (mmol/L)	0.41 ± 0.02^{a}	0.29 ± 0.01^{b}	0.31 ± 0.02^{b}	0.01
Tprot (g/L)	42.9 ± 1.1^{b}	40.7 ± 1.0^{b}	50.3 ± 1.8^{a}	0.02
TG (mmol/L)	3.75 ± 0.22	3.48 ± 0.33	5.15 ± 0.67	0.31
ALT (U/L)	36.7 ± 2.7^{b}	26.1 ± 2.3^{b}	81.3 ± 17.8^{a}	0.04
AST (U/L)	1082 ± 143	763 ± 100	3116 ± 827	0.09

Table 4. Functional GO categories and KEGG pathways of genes that were differentially expressed in tissues of salmon fed with the WG-containing diets in comparison to the FM controls (n=6; 2 fish per 3 replicate tanks); a) GO categories, 1) mid-intestine and 2) liver; b) KEGG pathways, 1) mid-intestine and 2) liver. Features represent the number of DEG that were enriched of the genes included in the microarray platform. The significance (p-value) of the enrichment was assessed by the Yates' corrected chi-square values.

a) 1.

Mid intestine					
GO categories	**Features**	**p-value**	**GO categories**	**Features**	**p-value**
Antioxidant activity	13/57	<0.001	Lipid transport	25/207	<0.001
Autophagy	23/324	0.020	Lipoxygenase pathway	5/38	0.032
Cadherin binding	76/1263	0.002	Liver development	31/481	0.027
Cholesterol biosynthetic process	16/109	<0.001	Long-chain fatty-acyl-CoA biosynthetic process	11/63	<0.001
Cholesterol efflux	18/52	<0.001	MHC class II protein complex	5/24	0.001
Cholesterol homeostasis	34/250	<0.001	MHC class II protein complex binding	8/54	0.001
Cholesterol metabolic process	26/230	<0.001	Muscle organ development	25/287	0.048
Defence response	19/223	0.004	Myosin filament	15/174	0.011
Defence response to virus	39/535	0.001	Phospholipase A2 activity	11/55	<0.001
Fatty acid biosynthetic process	12/131	0.015	Phospholipid metabolic process	17/158	<0.001
Glycolipid biosynthetic process	8/51	<0.001	Retinoid metabolic process	22/196	<0.001
Immune response	59/971	0.006	Steroid binding	10/95	0.009
Innate immune response	74/1196	0.001	Sterol metabolic process	6/54	0.043
Interferon-gamma-mediated signalling pathway	35/241	<0.001	Transforming growth factor beta receptor binding	10/116	0.046
Iron ion binding	29/403	0.006	Triglyceride catabolic process	19/63	<0.001
Keratinization	16/199	0.018	Triglyceride metabolic process	10/77	0.001
Linoleic acid metabolic process	12/82	<0.001	Ubiquitin protein ligase activity	45/657	0.001

Lipid catabolic process	32/235	<0.001	Ubiquitin-dependent protein catabolic process	51/865	0.022
Lipid homeostasis	18/159	<0.001	Very-low-density lipoprotein particle	10/52	<0.001
Lipid metabolic process	36/572	0.023	Wound healing	25/361	0.021
Lipid particle	26/300	<0.001	Xenobiotic metabolic process	21/228	<0.001

a) 2.

Liver		
GO categories	**Features**	**p-value**
Cholesterol biosynthetic process	12/109	<0.001
Fatty acid biosynthetic process	6/131	<0.001
Iron ion binding	8/403	0.010
Lipid metabolic process	10/572	0.011
Liver development	8/481	0.040
Peptidase inhibitor activity	7/153	<0.001
Receptor-mediated endocytosis	7/394	0.039
Sterol biosynthetic process	5/35	<0.001
Triglyceride metabolic process	5/77	<0.001
Xenobiotic metabolic process	6/228	0.004

b) 1.

Mid-intestine		
KEGG pathways	**Features**	**p-value**
alpha-Linolenic acid metabolism	13/32	<0.001
Arachidonic acid metabolism	10/90	0.005
Biosynthesis of unsaturated fatty acids	8/40	<0.001
Ether lipid metabolism	12/64	<0.001
Fc epsilon RI signalling pathway	15/180	0.015
Focal adhesion	46/716	0.006
Glycerophospholipid metabolism	20/196	<0.001
GnRH signalling pathway	21/288	0.020
Linoleic acid metabolism	10/38	<0.001
mTOR signalling pathway	12/148	0.043
PPAR signalling pathway	19/164	<0.001
Retinol metabolism	18/98	<0.001
Steroid hormone biosynthesis	7/70	0.049
Vascular smooth muscle contraction	29/418	0.011

b) 2.

Liver		
KEGG pathways	**Features**	**p-value**
Arginine and proline metabolism	6/196	0.001
Cell cycle	6/273	0.016
Drug metabolism - other enzymes	5/78	<0.001
p53 signaling pathway	6/150	<0.001
PPAR signaling pathway	6/164	<0.001

Table 5. Formulation and chemical contents of the custom-made salmon diets used in this study. The control diet contained only fish meal (FM) as protein source, which was partly replaced by wheat gluten (WG) at 15% and 30% inclusion levels.

[#]Additives include Vitamin mix (2%), Mineral mix (0.59%), Monosodiumphosphate-24% P (2%), Yttrium oxide (0.01%) and Carophyll® Pink-10% (0.05%).

	FM	**WG15**	**WG30**
Diet composition (g/100 g)			
FM	63.4	48.4	33.4
Wheat	12.0	12.0	12.0
WG	-	15.0	30.0
Fish oil	20.0	20.0	20.0
Additives[#]	4.7	4.7	4.7
Chemical content (% in diet)			
Dry matter	93.6	93.9	94.1
Protein	45.2	46.7	48.1
Lipid	26.5	25.4	24.3
Ash	13.5	11.0	8.6
Energy (MJ/kg)	21.3	21.2	21.1
Yttrium	0.007	0.007	0.007

Paper IV

Plant-based diets induce transcriptomic changes in fast muscle of zebrafish and Atlantic salmon

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Keywords

Plant-based proteins; fast muscle; pea protein concentrate; soy protein concentrate; wheat gluten; gene expression

Abstract

With the expansion of the aquaculture industry in the last two decades, there has been a large increase in the use of plant ingredients in aquafeeds, which has created new challenges in fish growth, health and welfare. Fish muscle growth is an important trait that is strongly affected by diet, but our knowledge on the effect of plant protein-based diets on global gene expression in muscle is still scant. The present study evaluated nutrigenomic effects of the inclusion of proteins from pea, soy and wheat into aquafeeds, compared to a control diet with fishmeal as the main protein source using the zebrafish model by RNA-seq; these results were extended to an important aquaculture species by analyzing selected differentially expressed genes identified in the zebrafish model on on-growing Atlantic salmon fed with equivalent plant protein-based diets. Expression of selected Atlantic salmon paralogues of the zebrafish homologues was analyzed using paralogue-specific qPCR assays. Global gene expression changes in muscle of zebrafish fed with plant-based diets were moderate, with the highest changes observed in the soy diet-fed fish, and no change for the pea diet-fed fish compared to the control diet. Among the differentially expressed genes were *mylpfb*, *hsp90aa1.1*, *col2a1a* and *odc1*, which are important in regulating muscle growth, maintaining muscle structure and function, and muscle tissue homeostasis. Furthermore, those genes and their paralogues were differentially expressed in Atlantic salmon fed with the equivalent percentage of soy or wheat protein containing diets. Some of these genes were similarly regulated in both species while others showed species-specific regulation. The present study expands our understanding on the molecular effects of plant ingredients in fish muscle. Ultimately, the knowledge gained would be of importance for the improved formulation of sustainable plant-based diets for the aquaculture industry.

Introduction

Exploration of alternative feed ingredients for fishmeal and fish oil has become a necessity for sustainable growth of the aquaculture sector (Carter and Hauler, 2000; Gatlin et al., 2007; Hardy, 2010). The industry has seen a steady increase in utilization of plant-based ingredients, mainly legumes, cereal grains and oil seeds in the fish feeds throughout the past years. For instance, the Norwegian salmon industry reported a large increase in use of plant ingredients, from 10% in 1990 to 60.5% in 2016 (Ytrestøyl et al., 2015; Aas et al., 2019). This huge transition to plant-based ingredients in the diet of farmed fish has led to new challenges in fish growth, health and welfare, and product quality (Hardy, 2010). Some of these challenges have been successfully addressed through advanced feed processing technologies and extensive research on the influence of dietary incorporation of plant ingredients on health, growth and nutrient utilization (Drew et al., 2007; Gatlin et al., 2007), but many challenges remain.

Plant-based proteins in fish diets can affect growth as a consequence of their nutritional and non-nutritional characteristics (Gatlin et al., 2007). Plant proteins also have some nutritional limitations such as the presence of high amounts of carbohydrates and imbalance in amino acid profiles compared with fishmeal. Generally, they are limited in some essential amino acids such as lysine and/or methionine, and low in available phosphorus and cationic minerals that mainly exist in bound form with phytic acid (Gatlin et al., 2007). With the inclusion of plant ingredients in the diets, fish could be exposed to various phytochemicals including anti-nutritional factors (ANFs) and phytoestrogens. ANFs (e.g. fibers, enzyme inhibitors, lectins, saponins etc.) can interfere with nutrient digestibility, absorption and utilization, ultimately negatively affecting growth and health (Francis et al., 2001; Krogdahl et al., 2010). Phytoestrogens are compounds with estrogenic activity in animals, which can have an impact on the processes regulated by estrogens (Rietjens et al., 2013). Phytoestrogens have been reported to mostly exert a positive effect on fish growth (Chakraborty et al., 2014).

Among the widely used plant-based protein sources in aquafeeds, soy protein concentrate (SPC) produced from acid alcohol extraction has become the preferred choice compared to soy bean meal due to the absence of the ANF saponins as well as low contents of trypsin inhibitors, storage globulins with antigenic properties and oligosaccharides (Drew et al., 2007; Zhou et al., 2018). Nevertheless, it has been shown that diets with SPC caused intestinal enteritis in salmonids

(Escaffre et al., 2007; Penn et al., 2011). Furthermore, inclusion of SPC in diets affects growth, depending on the percentage of replacement and fish species, e.g. partial replacement (50%) in diet of Atlantic salmon, *Salmo salar* (Storebakken et al., 2000a) and total replacement (100%) in diet of rainbow trout, *Oncorhynchus mykiss* (Kaushik et al., 1995) of fishmeal with SPC caused no changes in the growth of respective fish species. In contrast, a decrease in growth was observed in Japanese flounder, *Paralichthys olivaceus* (Deng et al., 2006), and gilthead sea bream, *Sparus aurata* (Kokou et al., 2015), with 25% of fishmeal replacement and 40% of inclusion with SPC in the diets, respectively.

The inclusion of pea protein concentrate (PPC) in aquafeeds also showed its potential as an alternative to fishmeal in farmed fish (Thiessen et al., 2003; Øverland et al., 2009; Zhang et al., 2012). Production of PPC by applying dehulling and air classification processes has enhanced the protein content, reduced the starch content and lowered certain ANF such as tannins compared to pea meal (Drew et al., 2007). However, it can still contain protease inhibitors, phytic acid, α-galactosidases and saponins in considerable amounts (Drew et al., 2007). PPC caused intestinal inflammation in the Atlantic salmon, similarly to soy-induced enteritis, and reduction in growth when included at a level of 35% in the diet (Penn et al., 2011). However, another study has reported that even inclusion levels of 50% of the dietary protein did not show any unfavorable effect on growth in rainbow trout (Zhang et al., 2012).

Wheat gluten (WG) has become an attractive alternative protein source for aquafeeds in several farmed species (Apper-Bossard et al., 2013), since it has a comparatively high crude protein content than fishmeal. Moreover, WG is used as pellet binder in feed manufacturing. It is high in sulfur amino acids and glutamate but generally low in lysine compared to the other commonly used plant protein sources (Storebakken et al., 2000b; Apper-Bossard et al., 2013). In a study on rainbow trout, it was found that WG was able to replace FM completely or to a large proportion without any unfavorable effect on growth, when lysine was supplemented in the diet (Pfeffer et al., 1992; Davies et al., 1997). Furthermore, moderate FM replacement with WG did not show any effect on growth in Atlantic salmon (35%) (Storebakken et al., 2000b) and Nile tilapia, *Oreochromis niloticus* L. (15%) (Schneider et al., 2004).

Muscle growth is an important trait affected by the nutritional status and diet (Valente et al., 2013). Some previous studies have examined the transcriptomic changes linked to muscle growth and dietary manipulation in fish (Alami-Durante et al., 2010; Bower and Johnston, 2010; Ulloa

et al., 2013; Ulloa et al., 2015; Valente et al., 2016). However, knowledge on the effect of plant protein-based diets on global gene expression in muscle is still scant. In the present study, we have evaluated nutrigenomic effects of the inclusion of plant proteins SPC, PPC and WG into aquafeeds, compared to a control diet with fishmeal as the main protein source, using the zebrafish as a model. Global transcriptome changes were analyzed in fast muscle using RNA-seq technology. The analysis was extended to a commercially important species by testing selected genes from the zebrafish model on on-growing Atlantic salmon fed with custom-made feeds containing the same plant ingredients. Considering the salmonid-specific genome duplication event (Lien et al., 2016), expression of selected paralogues of respective selected genes was analyzed using paralogue-specific qPCR assays.

Materials and Methods

Feeding experiments

Feeds: Custom-made diets for zebrafish and Atlantic salmon were produced by extrusion at the Nofima Feed Technology Centre, Fyllingsdalen, Norway as described by Johny et al. (2019). The control diet included 79.4% and 63.4 % fishmeal as protein source for, respectively, zebrafish and salmon (dietary composition is presented in Supplementary Table 1). Both control diets had 12% wheat as pellet-binding component, and 4.6% of additives including monosodium phosphate-24% P, vitamins and mineral mix. Considering the high fish oil requirement of salmon, 20% fish oil was added into the salmon diets as compared to 4% in the zebrafish diets. Plant protein-based diets were prepared by replacing 30% fishmeal in the control diet with PPC, SPC or WG as protein source. The total protein content in all diets was 50 to 59 g/100 g feed for zebrafish and 44 to 48 g/100g feed for salmon, while the lipid content ranged from 9 to 11% and from 24 to 26% for, respectively, zebrafish and salmon. The plant-based diets were not supplemented with amino acids.

Zebrafish feeding trials and sampling: Feeding trials were conducted using four-month-old zebrafish (AB strain, mean weight 0.214 g) for 46 days at Nord University, Bodø, Norway. Sixteen fish (1:1 sex ratio) were distributed into each of four randomly assigned replicate tanks (3.5 L volume) per dietary group in a flow-through system with 20% water exchange per h (ZebTEC stand-alone toxicology rack; Tecniplast, Buguggiate, Italy). Standard husbandry

conditions were maintained with a stable temperature of 28 ± SD 0.5 °C, pH 7.5, water conductivity of 1500 µS/cm and photoperiod of 12 h light/12 h dark. Feeding was performed twice a day with a total daily feed amount equal to 2.5% (w/w) of the body weight. Feeding behavior and health were regularly observed. The fish were fasted for 24 h prior to sampling. During the sampling, the fish in each tank were separated by gender and weighed. After euthanasia with a lethal dose of 200 mg/L tricaine methanesulfonate (MS222) (Sigma-Aldrich, St. Louis, USA), buffered with an equal amount of sodium bicarbonate, the fish were immediately frozen in liquid nitrogen and stored at -80 °C. Pectoral fast muscle was later dissected (after removing the skin from the frozen specimens) for RNA-seq analysis. Six female fish were randomly selected, including at least one fish from each of the four tanks per treatment. Fish from the same gender was selected to avoid sex effects that might interfere with the interpretation of gene expression changes induced by plant-based diets.

Statistical analysis of differences in specific growth rate among the dietary groups was performed with one-way ANOVA using R (https://www.r-project.org/). Assumptions for statistical analysis, normality of the distribution and homogeneity of variances were assessed, respectively, using the Shapiro-Wilk test and Levene's test. Pairwise comparisons were analyzed using Tukey's honest significance test, and p-value after adjustment for the multiple comparisons < 0.05 was considered as statistically significant.

Salmon feeding trials and sampling: Feeding trials were conducted using one-year-old post-smolt Atlantic salmon (SalmoBreed, Lønningdal, Norway) with a starting mean weight of 223 g for 63 days at Nofima Research Station, Sunndalsøra, Norway as detailed by Johny et al. (2019). Each feeding group was allocated to three randomly distributed tanks (1 m^3 and n=15) and fish were fed to satiation with automatic disc feeders. They were reared at an average temperature of 10.6 ± SD 0.6 °C and a seawater flow of 20 L/min. The oxygen levels of the tank outlets were above 80%. At the end of the feeding trials, the fish were anaesthetized with 60–80 mg/L MS222, weighed and then euthanized with a double dose (120–160 mg/L) of MS222. Pectoral fast muscle was carefully dissected after removing the skin, frozen in liquid nitrogen and stored at -80 °C until RNA isolation for qPCR analysis.

Specific growth rates among the dietary groups were analyzed using one-way ANOVA, followed by Duncan's multiple range test using SAS software (as detailed in the related manuscript under

preparation). All the pertinent assumptions, normality of the distribution and homogeneity of variances were checked before performing ANOVA.

RNA Sequencing

Total RNA was extracted from zebrafish fast muscle samples using QIAzol lysis reagent (Qiagen, Hilden, Germany), following the manufacturer's protocol. Tissues were homogenized twice at 5000 x g for 15 s with zirconium oxide beads (1.4 mm; Precellys, Montigny-le-Bretonneux, France) using MagNALyser (Roche, San Francisco, USA). RNA integrity was checked using an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, USA), and RNA quantity was determined with a Qubit fluorometer (Invitrogen, ThermoFisher Scientific, Waltham, USA). Libraries for RNA-seq were prepared using the NEBNext ultra II directional RNA library preparation kit with a poly(A) mRNA magnetic isolation module (NEB #E7490) in accordance to the manufacturer's protocol (New England BioLabs Inc., Ipswich, UK). Briefly, after poly(A) enrichment from 1 µg total RNA, mRNA was fragmented to about 100–200 nt lengths and used for 1st and 2nd strand cDNA synthesis. After A-tailing, end repair and adaptor legation, the second strand was selectively removed using uracil-specific excision reagent (USER® II Enzyme; New England BioLabs Inc.). The resulting directional RNA-seq libraries were amplified with 8 PCR cycles and later purified using AMPure XP beads (Beckman Coulter, Inc., Brea, USA). Quality and quantity of the RNA-seq libraries were assessed using the Agilent 2200 TapeStation. Sequencing was performed on the Illumina NextSeq platform (Illumina, San Diego, USA) at Nord University using a single-end 75 bp high-throughput sequencing kit with 4% Phix control DNA (Illumina) as internal control.

RNA-seq data analysis

Raw sequencing data were processed for quality and adapter trimming using Cutadapt (Martin, 2011) with -q 25, 20, quality-base = 33, trim-n -m 20 parameters, followed by a further quality check with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Quality trimmed reads were mapped to the zebrafish genome and transcriptome downloaded from Ensembl (http://www.ensembl.org release 91) with TopHat2, version 2.1.0 (Kim et al., 2013). Indexing of the genome prior to mapping was done with Bowtie 2 (Langmead and Salzberg,

2012) and Tophat2. HTSeq (Anders et al., 2014) was used to compute gene expression values. Differentially expressed genes in fish fed with plant protein-based aquafeeds in comparison to their control diet counterparts were determined using DESeq2 (Anders and Huber, 2010) with the criteria adjusted p-value (q) with the Benjamini-Hochberg procedure ≤ 0.05 and absolute fold-change ≥ 1.5.

qPCR analysis

We used paralogue-specific qPCR assays to determine the expression of Atlantic salmon putative orthologues of several differentially expressed genes in zebrafish fed with soy or wheat diets as compared to the fishmeal control diet (Table 1). The Atlantic salmon had been fed with customized diets containing equal percentages of the same plant proteins as the zebrafish. Total RNA was extracted from Atlantic salmon fast muscle (n=9) using the above protocol, treated with DNase I (RNase-Free DNase Set from Qiagen) to digest any residual genomic DNA, and subjected to column-purification using RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. RNA integrity was confirmed by 1 % (w/v) agarose gel electrophoresis, and purity was assessed using a NanoDrop UV spectrophotometry (NanoDrop, Wilmington, USA). First-strand cDNA templates for qPCR were synthesized in 20 µL reactions from 1 µg of DNaseI-treated, column-purified total RNA using random primers (250 ng; Invitrogen/Life Technologies), dNTPs (0.5 mM final concentration; Invitrogen/ Life Technologies), and M-MLV reverse transcriptase (200 U; Invitrogen/Life Technologies) with the manufacturer's first strand buffer (1× final concentration) and DTT (10 mM final concentration) at 37 °C for 50 min.

Putative paralogous genes in Atlantic salmon corresponding to selected zebrafish genes were identified by BLASTn searches against the non-redundant nucleotide and expressed sequence tags (EST) databases at NCBI. Genomic location and pairwise alignment details of paralogues are presented in Supplementary Table 2. All identified paralogues were aligned using ClustalW (https://www.ebi.ac.uk/Tools/msa/clustalw2/), and paralogue-specific primers were designed to target regions where there were at least 2 bases different between paralogues to ensure specificity. Alignment of the paralogues with primers highlighted are presented in the Supplementary

Document 1. Primers were designed either with primer-BLAST (NCBI) or manually and analyzed with Net Primer (http://www.premierbiosoft.com/netprimer/) (Table 2).

Primer quality was assessed to ensure amplification of a single product without primer dimers by melt curve analysis using cDNA prepared with a pool of RNA with equal contribution from all the samples used in the qPCR study. The size of the amplicons corresponding to each primer pair was verified by 2 % (w/v) agarose gel electrophoresis using 1 kb plus ladder (Invitrogen/Life Technologies). Amplification efficiencies (Pfaffl, 2001) of primer pairs were calculated using a 5-point 1:3 dilution series starting with the pooled cDNA representing 10 ng of input total RNA. qPCR was performed in triplicate using Power SYBR Green I dye chemistry in 384-well format on a ViiA 7 real time PCR system (Applied Biosystems, Foster City, CA) for the normalizer and target genes. Each 13 µL reaction mixture contained 1x Power SYBR Green PCR Master Mix (Applied Biosystems), 50 nM of both the forward and reverse primers, and 4 µL diluted cDNA (corresponding to 5 ng of input total RNA). The thermocycling profile was as follows: 1 cycle at 50 °C for 2 min and 1 cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The fluorescence signal data were collected after each 60 °C step. Minus reverse transcriptase and no-template reactions were used as negative controls to confirm that there were no genomic and reagent contaminations, respectively.

Reference genes for normalization were selected after analyzing six candidate reference genes, including *actb* (*β-actin*), two paralogues of *elongation factor 1 alpha* [*ef1a1* (Olsvik et al., 2005) and *ef1a2* (Xue et al., 2015)], *pabpc1* (*polyadenylate-binding protein 1*), *rpl32* (*60S ribosomal protein L32*) (Xue et al., 2015), and *eif3d* (*eukaryotic translation initiation factor 3 subunit D*) (Caballero-Solares et al., 2017). Evaluation of normalizer genes was carried out using all the replicate samples from each of the control and plant protein diet-fed groups using *geNorm* (Vandesompele et al., 2002). ***actb*** and ***ef1a2*** were selected as normalizer genes as they were found to be the most stable of the tested six reference genes (i.e. lowest M-value, a gene stability measure). The relative quantity (RQ) of each transcript was determined using a qBase relative quantification framework (Hellemans et al., 2007; Xue et al., 2019), with normalization to the expression levels of *actb* and *ef1a2*. The sample with the lowest normalized expression level was used as calibrator (i.e. assigned RQ 1.0) when determining the RQ of each gene.

Statistical analysis was performed using R (https://www.r-project.org/). Changes in gene expression between the control and each of the plant-based diets were determined by either parametric unpaired t-test or the nonparametric Wilcoxon rank sum test with continuity correction, based on the fulfillment of all pertinent assumptions for statistical analysis. Normality of the distribution was analyzed using the Shapiro-Wilk test, and homogeneity of variances was assessed by F-test. Gene expression changes were considered statistically significant at $p \leq 0.05$.

Results

Plant-based diets induced moderate transcriptomic changes in zebrafish fast muscle

We used RNA-seq to determine the global transcriptomic changes in the fast muscle of zebrafish in response to plant protein-based diets that included 30% PPC (pea diet), SPC (soy diet) or WG (wheat diet), as well as a control diet containing fishmeal as the main protein source (Supplementary Table 1). The number of raw sequences ranged from 18 to 35 million per library. We obtained 18 to 31 and 16 to 30 million trimmed and mapped reads, respectively, per library (Figure 1). Each library had less than 7% of multiple mapping.

The analysis of global transcriptomic changes in the fast muscle of fish fed with the plant-based diets in comparison to the control group revealed 137 and 29 significantly differentially expressed genes (DEGs, fold change ≥1.5, Benjamini-Hochberg adjusted p-value < 0.05) in soy and wheat diet groups, respectively, with a higher proportion of downregulated genes (Figures 2A and 2B). However, the pea diet group did not show any significant changes compared to the control group within the applied statistical criteria.

Feeding soy and wheat diets resulted in the regulation of genes involved in growth, structure and function of fast muscle in zebrafish

GO enrichment analysis of DEGs in the soy or wheat diet groups failed to show any enriched biological processes, most likely due to the relatively low number of DEGs. However, functional annotation revealed that several DEGs are involved in regulating fast muscle growth and its structure and function in zebrafish. The list of annotated DEGs in the soy and wheat diet groups compared to the control group and associated GO terms is presented in Supplementary Tables 3

and 4, respectively. An important gene for fast and slow skeletal muscle development, *myosin light chain, phosphorylatable fast skeletal muscle b* (*mylpfb,* 2.7-fold), and a component of the myosin complex, *myosin heavy polypeptide 1.1* (*myhz1.1,* 2.7-fold), were downregulated in zebrafish fed with the soy diet, but not with the wheat diet. Whereas, *popeye domain-containing* (*popdc3*), needed for skeletal muscle development, was upregulated in the same group of fish. Another gene involved in muscle development, *heat shock protein hsp90-alpha1* (*hsp90aa1.1*, 3.9-fold), was downregulated in zebrafish fed with the wheat diet, but its expression did not significantly change in the zebrafish fed with the soy diet. Two of the upregulated genes in zebrafish fed with the soy diet were *activating molecule in beclin-1-regulated autophagy* (*ambra1a*, 2.1-fold), a positive regulator of autophagy during skeletal muscle development, and *ryanodine receptor 1a* (*ryr1a*, 2.2-fold), an essential component of all skeletal muscle fiber calcium-release channels. On the other hand, a gene coding for a vital component of the skeletal muscle extracellular matrix, *collagen type II, alpha 1* (*col2a1a,* 2.6-fold), was downregulated in the same group of fish. Zebrafish fed with soy diet also showed downregulation of *ornithine decarboxylase* (*odc1*, 2.6-fold), which is important for polyamine biosythetic processes and myoblast proliferation. *betacellulin* (*btc,* 2.6-fold), a member of the epidermal growth factor family that mediates diverse processes including proliferation and differentiation, was upregulated in fast muscle of wheat diet-fed zebrafish.

Furthermore, several genes involved in metabolic processes including proteolysis (i.e. *dipeptidyl-peptidase 6b, dpp6b,* (3.3-fold increase)), cholesterol biosynthetic process (i.e. apolipoproteins, *apoa1a, apoa1b* and *apoa1bb.1* (2.7-, 2.8-, 2.9-fold decrease respectively)) and glucose metabolism (i.e. *glyceraldehyde-3-phosphate dehydrogenase, gapdh* (1.6-fold decrease) were differentially regulated in soy diet-fed zebrafish.

Among the genes differentially regulated in both soy and wheat diets-fed fish were *PDZ and LIM domain 1 (pdlim1*, downregulation), involving in actin cytoskeleton organization; serotransferrin (*tfa,* downregulation), important for ion transport as well as response to bacterium; *mutS homolog 3* (*msh3*, upregulation), vital for DNA repair; and *activating transcription factor 3* (*atf3*, downregulation), needed for regulation of transcription and fish immune responses (Feng and Rise, 2011).

A considerable number of *crystallin* genes (*crystallin, gamma* and *crystallin, beta*), mainly known to be important for visual perception, were downregulated in the soy diet fed group. Even though chaperone-like activity was described for mammalian *crystallin, gamma* (Andley et al., 1996), to our knowledge, the importance of this gene for fast muscle growth and homeostasis in mammals or fish has not been reported.

Differential expression of Atlantic salmon homologues related to zebrafish genes that are differentially expressed by feeding with plant-based diets

The expression of selected genes that were differentially regulated with the diet in zebrafish fast muscle was subsequently examined in the fast muscle of Atlantic salmon fed with equivalent diets. Considering the salmonid genome duplication event, we used paralogue-specific qPCR assays to quantify the transcript levels of Atlantic salmon homologues of the selected zebrafish genes (Table 1).

Expression profiles of the selected paralogous genes in fast muscle of Atlantic salmon are presented in Figures 3 and 4. The paralogues *mylpfba* and *mylpfbb* were upregulated in the soy diet group (Figure 3A1) whereas the zebrafish putative orthologue was downregulated in those fed with the same plant-based diet. There was no significant regulation of *mylpfb* observed for wheat diet fed zebrafish or Atlantic salmon. Both the *hsp90aa1.1a* and *hsp90aa1.1b* paralogues (Figure 3B2) were upregulated (2.5- and 2.2-fold respectively) in wheat diet-fed Atlantic salmon, contrary to the downregulation observed in wheat diet-fed zebrafish. All three *ambra1a* paralogues were not significantly regulated in soy diet-fed Atlantic salmon (Figure 3C1) even though the putative orthologue was upregulated in the zebrafish counterparts. They were also not significantly changed in Atlantic salmon fed with the wheat diet (Figure 3C2). Similarly to the findings in zebrafish, *col2a1aa* and *col2a1ab* were downregulated (1.7-fold each) in Atlantic salmon fed with the soy diet (Figure 4A1,). The expression of *btca* and *ryr1aa* paralogues did not change significantly in soy or wheat diet-fed Atlantic salmon (Figures 4B1 and 4B2). Both *odc1* paralogues were downregulated (1.2-fold each) in soy diet-fed Atlantic salmon (Figure 4C1), comparable to their zebrafish counterparts. Further, *odc1* paralogues were also downregulated in Atlantic salmon fed with wheat diet (Figure 4C2) while they were not significantly changed in the zebrafish fed with the similar plant-based diet.

We also analyzed the expression of genes involved in insulin-like growth factor (IGF) signaling in the fast muscle of Atlantic salmon fed with soy and wheat diets. The transcript expression of *insulin-like growth factor I* (*igfI*) did not show any significant changes with respect to the diet (Figures 5A and 5B). In contrast, *insulin-like growth factor II* (*igfII*, 1.5- and 1.9-fold respectively for soy and wheat diet groups) and *insulin-like growth factor binding protein 1 paralog A2* (*ifbp-1a2*, 2.1- and 31.8-fold respectively for soy and wheat diet groups) were upregulated with both soy and wheat diet groups (Figures 5A and 5B).

Growth performances of the zebrafish and Atlantic salmon during feeding study

The specific growth rate of zebrafish fed with diets including 30% PPC, SPC or WG did not significantly change after 46 days of the feeding trial as compared to their counterparts fed with a diet containing fishmeal as the main protein source (Figure 6). Moreover, inclusion of 30% SPC also did not show any significant changes in the specific growth rate of Atlantic salmon compared to counterparts fed with a diet containing fishmeal as the main protein source; on the other hand, inclusion of 30% WG had a significant reduction in specific growth rate in salmon compared to the control group (growth data are included in the related manuscript under preparation).

Discussion

The present study reports the nutrigenomic effects of fish diets containing partial inclusion of plant-based proteins, e.g. pea, soy and wheat, in the fast muscle of zebrafish. Global gene expression changes in the muscle tissues of zebrafish fed with plant-based diet were moderate yet included several genes important for the regulation of muscle growth, maintenance, function and homeostasis.

The specific growth rate of zebrafish was not significantly affected by any of the plant protein-based diets used in the present study after a 46-day feeding trial. Inclusion of plant-based proteins in aquafeeds can affect fish growth, depending on the percentage of replacement and species. For instance, 50% replacement of fishmeal with SPC did not affect the growth rate in Atlantic salmon (Storebakken et al., 2000a), whereas 25% replacement of fishmeal with SPC significantly

reduced the growth rate of Japanese flounder (Deng et al., 2006). The total replacement of marine protein sources with plant-based proteins from 35 to 98 days post-fertilization reportedly decreased the specific growth rate of zebrafish (Ulloa, Peña et al. 2013). The plant-based diets used in our study (Johny et al., 2019) contained still a considerable amount of fishmeal (49.4%) in addition to the 30% plant protein, resulting in a crude protein content of 50 g pea, 55 g soy and 59 g wheat per 100 g feed, which was similar to the control diet with 56 g crude protein /100 g feed. The relatively high ratio of fishmeal in the plant-based diets has probably helped the zebrafish to maintain a similar growth rate as their control counterparts fed with 79.4% fishmeal. Moreover, we found that Atlantic salmon fed with plant-based diets containing 33.4% fishmeal in addition to 30% SPC also did not show any significant changes in the specific growth rate; however, salmon fed with the diet containing 30% WG had a growth rate decreased by 11.1% compared to the controls that were fed with 63.4% fishmeal.

The analysis of DEGs in the muscle of zebrafish fed with the plant protein-containing diets showed that the DEG numbers varied with the plant ingredient. They were highest in fish fed with the soy diet followed by the fish fed with the wheat diet. Surprisingly, the pea diet did not induce any significant gene expression changes as compared to the fishmeal control group. Among the DEGs changed in the soy and wheat diet-fed groups, there were genes associated with muscle growth, function, metabolism and homeostasis, some of which were also differentially expressed in Atlantic salmon fed with the same plant-based protein diets, while others showed species specific regulation.

All teleosts share three rounds of whole-genome duplication (WGD), 1R, 2R and 3R where the last was a teleost-specific WGD (Glasauer and Neuhauss, 2014; Lien et al., 2016). Additionally, a fourth (4R) WGD occurred in the common ancestor of salmonids (Glasauer and Neuhauss, 2014). WGDs are important for evolutionary adaptations and innovations; complexity and diversifications. The duplicated genes after WGDs can undergo different fates including: nonfunctionalization (deleterious mutations occur in one of the duplicates leading to loss of expression); subfunctionalization (mutations in paralogous genes leading to preservation of both duplicates); neofunctionalization (one of the genes acquires a novel function) (Glasauer and Neuhauss, 2014). Differential expression patterns of Atlantic salmon homologues (putative orthologues and paralogues) related to zebrafish genes regulated by dietary inclusion of plant-based diets observed in our study detailed in the following sections, provide possible evidence

for gene duplication and divergence. However, functional studies are needed to clarify the fate of the Atlantic salmon paralogous genes to determine whether they were subfunctionalized or neofunctionalized.

Skeletal muscle is a highly adaptable tissue, which changes in size and cell composition in response to environmental and physiological conditions. Its homeostasis can be challenged by both short and long term nutritional changes (Matsakas and Patel, 2009). Skeletal muscle fibers consist of myofibrils that are made up of sarcomeres containing organized arrays of actin thin filaments and myosin thick filaments along with accessory proteins (Johnston et al., 2011). Several muscle subtypes are described based on the expression of numerous isoforms of myosin light and heavy chains. Myosin heavy chain is mainly responsible for the functional and phenotypic diversity of muscles (Pette and Staron, 2000). The regulatory light chain of myosin in fast skeletal muscle is encoded by the *mylpf* gene in mammals (Wang et al., 2007), and orthologue, *mylpfb*, exists in zebrafish. In mouse, *mylpf* knockdown resulted in the complete lack of skeletal muscle during development, indicating the importance of *mylpf* for the growth of both fast and slow skeletal muscle (Wang et al., 2007). *mylpfb* was downregulated in soy diet-fed zebrafish and its homologues, *mylpfba and mylpfbb* were upregulated in soy diet-fed Atlantic salmon, with no significant changes in the growth of the respective species, suggesting a species-specific regulation of *mylpfb* gene.

Several studies in zebrafish and Atlantic salmon demonstrated that heat-shock proteins 90α (Hsp90α), especially Hsp90α1, play an important role in myosin folding, myofibril assembly and myosin thick filament organization (Du et al., 2008; Garcia de la serrana and Johnston, 2013; He et al., 2015). Loss of Hsp90α1 function resulted in increased myosin protein degradation (Du et al., 2008) and disruption of all sarcomeric structures, including both thick and thin filaments in skeletal muscles in zebrafish (Codina et al., 2010). We observed a downregulation of *hsp90aa1.1* in zebrafish fed with the wheat diet. On the other hand, both the *hsp90aa1.1a* and *hsp90aa1.1b* paralogues were upregulated in the same group of salmon. This species-specific regulation of *hsp90aa1* needs further studies.

Autophagy is an evolutionarily conserved catabolic process important for protein and organelle clearance, as well as for maintenance of muscle mass and myofiber integrity (Masiero et al., 2009). It is also vital for cell survival under stressful conditions and protein degradation in

skeletal muscle during starvation (Masiero et al., 2009). *ambra1* is a positive regulator of the beclin-1-dependent autophagy and encoded by two paralogous genes in zebrafish: *ambra1a* and *ambra1b*. Studies with *ambra1a* and *ambra1b* in zebrafish morphants showed that silencing these genes leads to a severe myopathy with structural and functional defects of skeletal muscles (Skobo et al., 2014). In the present study, upregulation of *ambra1a* was observed in soy diet-fed zebrafish; in contrast, changes were not observed for its homologues in salmon fed with the same plant-based diet, indicating that regulation of this gene may be species-specific.

In addition to genes coding for fast muscle components or directly involved in muscle growth and function, some genes indirectly involved in muscle functions, such as *col2a1a*, were downregulated in zebrafish and salmon fed with the soy diet. The skeletal muscle extracellular matrix (ECM) is vital for muscle fiber force transmission, maintenance and repair (Gillies and Lieber, 2011). Collagen is a major structural protein in muscle ECM even though it is a minor constituent (1–10%) of the muscle mass dry weight. *collagen type II* (*col2a1*) is expressed during skeletal muscle development in mammals (Gillies and Lieber, 2011) and zebrafish embryogenesis (Dale and Topczewski, 2011). Therefore, the downregulation of *col2a1a* caused by the soy diet in adult zebrafish and Atlantic salmon warrants further investigation. Moreover, both zebrafish and Atlantic salmon fed with the soy diet showed downregulation of *odc1*, a gene coding for ornithine decarboxylase, which is important for polyamine biosynthetic processes. Studies in mouse myoblasts and human skeletal muscle cell lines showed that *odc1* gene promotes myoblast proliferation (Lee et al., 2011). The downregulation of *odc1* in both zebrafish and salmon fed with the soy diet could be linked to high polyamine levels present in SPC, since ornithine decarboxylase levels are modulated by the available polyamine concentration (Pegg, 2006). This could have ultimately delayed myoblast proliferation and affected myogenesis.

Muscle growth is regulated by growth hormones acting directly through sarcolemmal receptors and indirectly *via* the insulin-like growth factor (IGF) pathway. The IGF system comprises IGF-I, IGF-II, several receptors and six binding proteins (IGFBPs) (Johnston et al., 2011). IGFs stimulate muscle growth in fish by promoting the proliferation of myogenic cells, protein synthesis and hypertrophy, as well as inhibiting protein degradation and muscle atrophy (Fuentes et al., 2013). The availability of IGFs is regulated by IGFBPs, and generally IGFBP-1 and -2 inhibit the growth-promoting functions of IGFs in both mammals and fish (Fuentes et al., 2013). Zebrafish selected for large body size showed higher *igf1* and lower *igfbp1a* mRNA levels

compared to those selected for small body size in a study investigating, responses to changes in the nutritional input (Amaral and Johnston, 2012). Interestingly, a study in Atlantic salmon showed that switching to fast growth involves the local upregulation of *igfI*, *igfbp-5.2* and *igfbp-4* accompanied by downregulation of *igfbp-2.1* and *igfII* in the skeletal muscle (Bower et al., 2008). We did not observe differential expression of genes from the GH-IGF signaling system in zebrafish fed with any of the three-plant protein-based diets as compared to the fishmeal diet, which is in agreement with the lack of growth rate differences between the feeding groups. On the other hand, *igfII* and *igfbp-1a2* were upregulated in Atlantic salmon fed with the soy and wheat diets with normal and reduced growth respectively, compared to the control group. Further studies are needed to explain the involvement of specific components of the GH-IGF system in regulating the growth changes observed in Atlantic salmon fed with different plant-based diets.

Conclusions

The partial inclusion of proteins from soy and wheat into zebrafish feed resulted in the regulation of, respectively, 137 and 29 genes in fast muscle. In contrast, the inclusion of pea protein concentrate did not induce changes in the gene expression as compared to a control group receiving a fishmeal-based diet. Among the differentially expressed genes, several were important for muscle growth, structure, function and homeostasis. Some of these genes and their paralogues were similarly regulated in Atlantic salmon fed with equivalent diets while others showed species- specific regulation. Our results revealed that molecular adjustments occurred in the fast muscle of zebrafish and Atlantic salmon, probably to maintain muscle homeostasis following imbalances caused by dietary plant ingredients. Ultimately, this knowledge may be applied for the improved formulation of sustainable plant-based diets for the aquaculture industry.

Data availability statement

Sequence data were deposited in the NCBI Sequence Read Archive (SRA) with specific reference number (PRJNA577226, SUB6971208).

Ethics statement

All experiments involving zebrafish were conducted in agreement with the guidelines provided by the Norwegian Animal Research Authority (FOTS ID 12581, 27 July 2017) and approved by the Nord University (Norway) ethics committee.

Author contributions

AD, JF and CF Conceived and designed the study. CF, JF and MR contributed reagents and materials for the experiments. AD, AJ and XX performed laboratory work. AD and GB conducted, respectively, zebrafish and Atlantic salmon feeding trials. AB prepared the feeds. AD, JF and XX analyzed the data. AD, JF, XX, MR and CF wrote the manuscript. All authors read and approved the manuscript.

Disclosure statement

The authors reported no potential conflicts of interest.

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Figure Legends

Figure 1. RNA-seq library characterization of individual replicates from zebrafish in control (C1-C4), pea (PPC1-PPC6), soy (SPC1-SPC6) and wheat (WG1-WG4) diet groups. Raw read counts were trimmed for quality and the adaptors were removed. The number of reads that could be aligned to the zebrafish genome and transcriptome (Ensembl release 91) is presented.

Figure 2. Heat maps generated by complete linkage method with Euclidean distance measure **(A1, B1)** and pie charts **(A2, B2)** showing significantly differentially expressed genes (absolute fold change ≥1.5, $q < 0.05$) in the fast muscle of zebrafish fed with the soy **(A)** and wheat **(B)** diets in comparison to controls fed with a diet containing fishmeal as the main protein source. Gradient scale indicates Z-scores of DEGs where red signifies most induced expression and green represents most reduced expression.

Figure 3. Expression profiles of *mylpfb*, *hsp90aa1.1* and *ambra1a* paralogues in the fast muscle of Atlantic salmon fed with the soy **(A1, B1 and C1)** and wheat **(A2, B2 and C2)** diets compared to controls fed with a diet containing fishmeal as the main protein source. Values are means ± s.e.m (N=7-9). Asterisks above the bars indicate significant changes ($P < 0.05$) in transcript levels.

Figure 4. Expression profiles of *col2a1a*, *btc*, *ryr1a* and *odc1* paralogues in the fast muscle of Atlantic salmon fed with the soy **(A1, B1 and C1)** and wheat **(A2, B2 and C2)** diets compared to controls fed with a diet containing fishmeal as the main protein source. Values are means ± s.e.m (N=7-9). Asterisks above the bars indicate significant changes ($P < 0.05$) in transcript levels.

Figure 5. Expression profiles of IGF-signaling genes in the fast muscle of Atlantic salmon fed with the soy **(A)** and wheat **(B)** diets compared to controls fed with a diet containing fishmeal as the main protein source. Values are means ± s.e.m (N=9). Asterisks above the bars indicate genes, whose transcript levels changed significantly ($P < 0.05$) with the plant-based diet.

768 **Figure 6.** Specific growth rates of zebrafish fed for 46 days with diets containing 30% plant
769 proteins from pea, soy and wheat or with a fishmeal-based control diet. Values are means ± s.e.m
770 (N=32 females).

771

772 **Figure 1.**

773

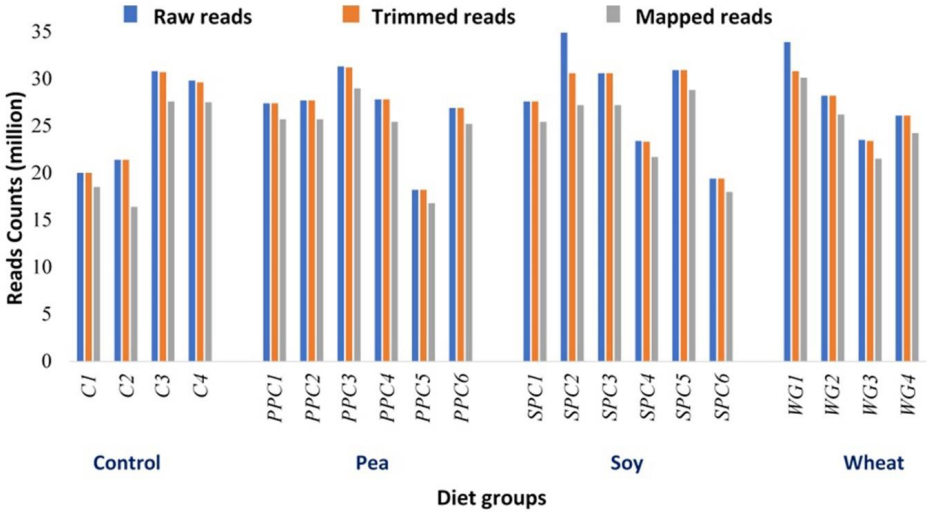

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Figure 2

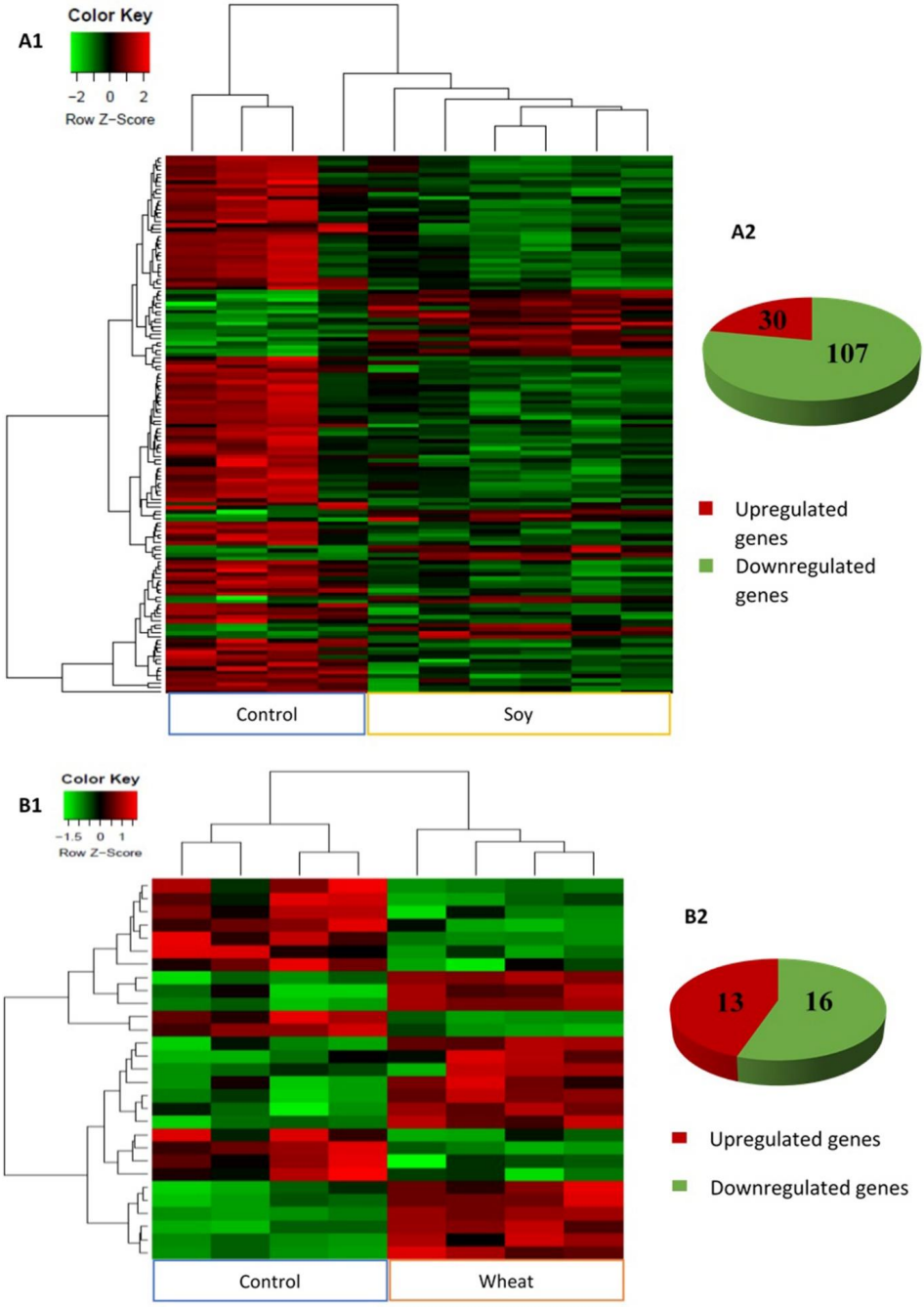
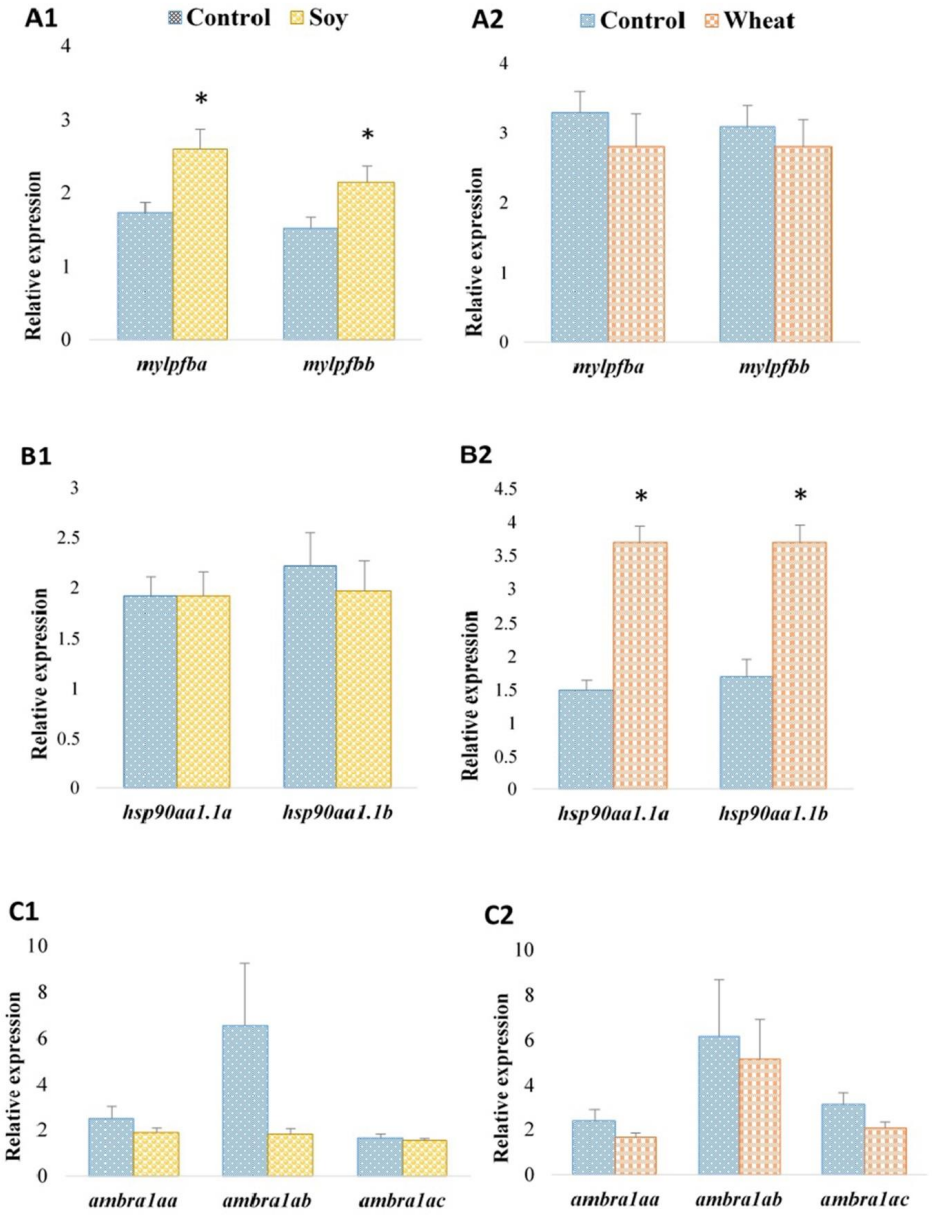

A1
Color Key
−2 0 2
Row Z−Score
Control
Soy
A2
30
107
Upregulated genes
Downregulated genes
B1
Color Key
−1.5 0 1
Row Z−Score
Control
Wheat
B2
13
16
Upregulated genes
Downregulated genes

Figure 3


A1
Control
Soy
Relative expression
mylpfba
mylpfbb
A2
Control
Wheat
Relative expression
mylpfba
mylpfbb
B1
Relative expression
hsp90aa1.1a
hsp90aa1.1b
B2
Relative expression
hsp90aa1.1a
hsp90aa1.1b
C1
Relative expression
ambra1aa
ambra1ab
ambra1ac
C2
Relative expression
ambra1aa
ambra1ab
ambra1ac

781 **Figure 4**

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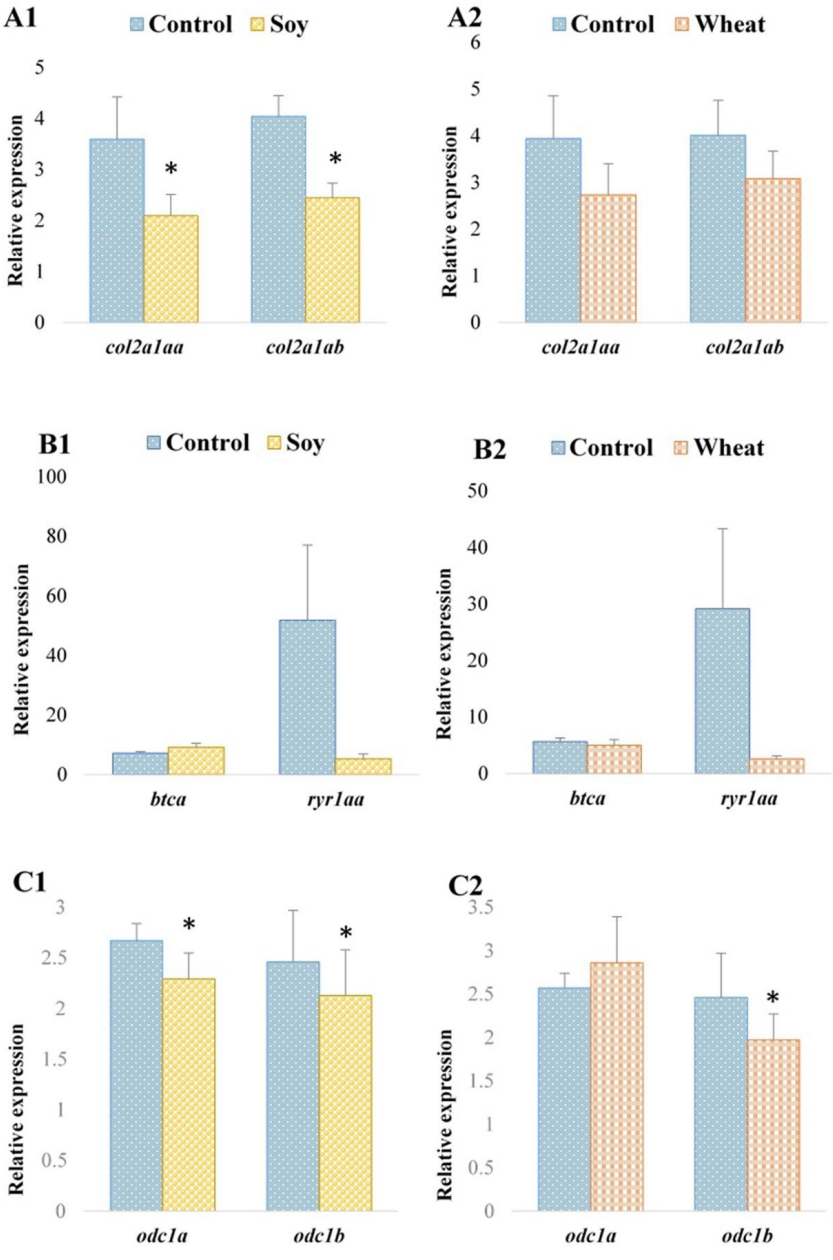

Figure 5

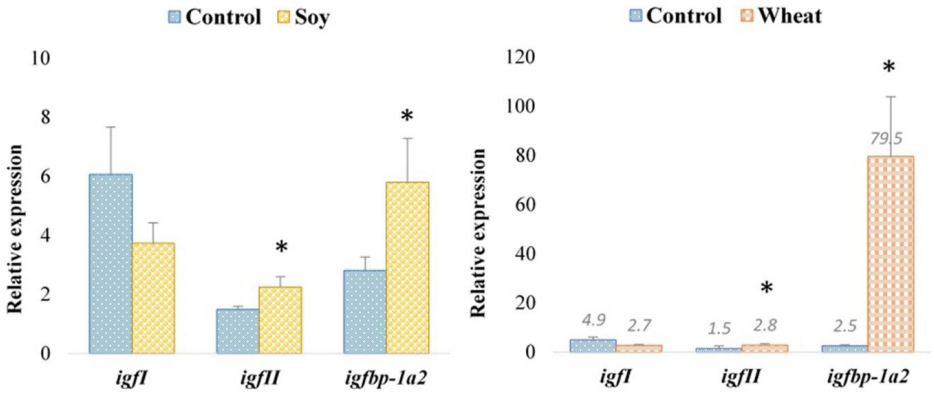

Figure 6

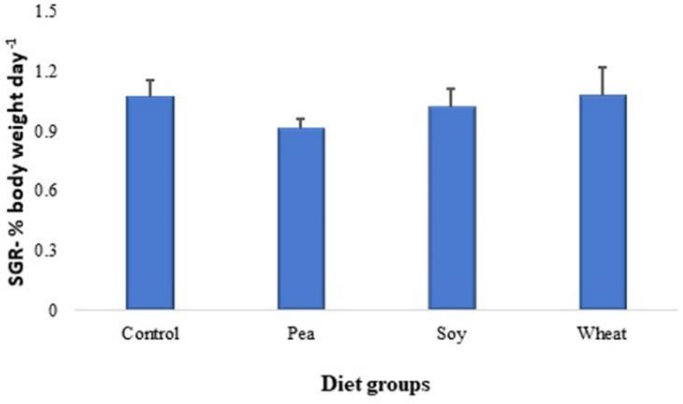

Table 1. Atlantic salmon homologues related to zebrafish genes that are differentially expressed by feeding with plant-based diets.

	Zebrafish		Atlantic salmon	
Gene name (symbol)	NCBI accession number	Differential expression	Paralogues*	NCBI accession number
myosin light chain, phosphorylatable fast skeletal muscle b (*mylpfb*)	NM_001004668.1	Soy group 2.7-fold decrease	***mylpfba***	**XM_014203362.1**
			mylpfbb	**NM_001123716.1**
			mylpfbc	XM_014161797.1
heat shock protein hsp90-alpha1 (*hsp90aa1.1*)	NM_131328.1	Wheat group 3.9-fold decrease	***hsp90aa1.1a***	**XM_014205881.1**
			hsp90aa1.1b	**XM_014144832.1**
activating molecule in beclin-1-regulated autophagy (*ambra1a*)	NM_001281992.1	Soy group 2.1-fold increase	***ambra1aa***	**XM_014175106.1**
			ambra1ab	**XM_014126406.1**
			ambra1ac	**XM_014147648.1**
			ambra1ad	XM_014123983.1
collagen type II, alpha 1 (*col2a1a*)	NM_131292.1	Soy group 2.6-fold decrease	***col2a1aa***	**XM_014134054.1**
			col2a1ab	**XM_014168236.1**
			col2a1ac	XM_014135018.1
			col2a1ad	XM_014145553.1
betacellulin (*btc*)	NM_001044764.3	Wheat group 2.6-fold increase	***btca***	**XM_014129351.1**
			btcb	XM_014138426.1
ryanodine receptor 1a (skeletal) (*ryr1a*)	XM_021479405.1	Soy group 2.2-fold increase	***ryr1aa***	**XM_014196267.1**
			ryr1ab	XM_014129250.1
ornithine decarboxylase (*odc1*)	NM_131801.2	Soy group 2.6-fold decrease	***odc1a***	**XM_014211026.1**
			odc1b	**XM_014192087.1**

* Genomic location and pairwise alignment details of paralogues are presented in Supplementary Table 2.

Paralogues used for qPCR analysis are shown in bold letters.

Paralogues, *btcb* (XM_014129351.1) and *ryr1ab* (XM_014129250.1) were not used for qPCR analysis, since the primers tested failed to show amplification.

Paralogues *mylpfbc* (XM_014161797.1), *ambra1ad* (XM_014123983.1), *col2a1ac* (XM_014135018.1) and *col2a1ad* (XM_014145553.1) had very low expression levels in all dietary groups and therefore were not included in the differential expression analysis.

804 **Table 2.** Primers employed for paralogue-specific qPCR study in Atlantic salmon fast muscle.

Gene	Accession number	Sequence 5′–3′ §	Efficiency (%)	Size (bp)
mylpfba	XM_014203362.1	F-CTGTTCTCTCATACCTTCCTTCTCTT R- CACCCGCTCTCACACAAATATC	110.5	113
mylpfbb	NM_001123716.1	F-CTATTCTCTTTATGTGTACTCGTGTGC R- GCCCGCTCTCACACAAATGT	81.3	96
hsp90aa1.1a	XM_014205881.1	F-GAAGTGTATAGAGCTTTTCACAGAACTC R- CAGGGAAACCATTTCATCACC	87.7	178
hsp90aa1.1b	XM_014144832.1	F-AAAGTGTATGGATCTTTTCATCGAG R- TCAGGGAGACCATTTCGTCAG	93.3	179
ambra1aa	XM_014175106.1	F- CGTAGCAGTGGGAAAAGCTAAC R- GCCATTGCATGGTGAAATCCA	86.1	78
ambra1ab	XM_014126406.1	F- GGCAACATTGTCATCAGCTCC R-CAGAACCATGTTTGTAACTAACTAGCTA	103.5	103
ambra1ac	XM_014147648.1	F- AACAACAGCAGCAGCAGAGATAG R- AGCATCGTCACTAGCATCAGTC	107.7	136
col2a1aa	XM_014134054.1	F- CATGGTGCCTCAGCCCTATC R- CATTGGCTGCCTCTTTCTCC	86.6	125
col2a1ab	XM_014168236.1	F- CAGCGGTCTGGGTCTGGC R- CAGTTGGCGGACGGAGG	100.0	112
btc	XM_014129351.1	F-CAGGGTATCCTACTAAACCGAGGA R- TAGGGCCAAAGCTGTGGCTAT	105.1	168
ryr1a	XM_014196267.1	F- CTGCTGAACCTGCTCCTGCT R- TTTTCAGTGTCTGCCTTCTCGG	97.4	76
odc1a	XM_014211026.1	F-CTTGTCCAATTCTTTTGTGACAATCT R- CAGGAAGACAAAATCAGGAGCA	90.5	78
odc1b	XM_014192087.1	F-CCAGTTTTCTTTTGTGACCAATCTA R- TCATCCGACATGGACATCTCA	87.9	143
*igf1**	NM_001123623.1	F-CCTGTTCGCTAAATCTCACTTC R-TACAGCACATCGCACTCTTGA	91.5	227
*igfII**	NM_001123647.1	F-GAAAACACAAGAATGAAGGTCAA R-CCACCAGCTCTCCTCCACATA	124.1	127

*igfbp-1a2**	NM_001279137	F-CTAAACCCCCAAACCCAGAT R-GTTGTGGCCAGGAAGGTAGA	134.8	104
ef1a2¤	BG933853	F- GCACAGTAACACCGAAACGA R- ATGCCTCCGCACTTGTAGAT	88.0	132
actb¤	BG933897	F- CCAAAGCCAACAGGGAGAAG R- AGGGACAACACTGCCTGGAT	99.5	91

§ Alignment of the paralogues with primers highlighted are presented in the Supplementary document 1

* Genes were tested in addition to the selected genes that were differentially regulated in the zebrafish feeding experiment: *igfI-insulin-like growth factor I, igfII-insulin-like growth factor II, igfbp-1a2-insulin-like growth factor binding protein 1 paralog A2*

¤ Genes selected as reference genes for qPCR analysis

Note: Supplementary document 1 contains multiple sequence alignments of putative paralogues (*my1pfb, hsp90aa1.1, ambra1a, col2a1a, btc, ryr1a, odc1*) from Atlantic salmon and highlighted primers. This document is not presented in the thesis along with the article due to its large size (60 pages) and neither the online link is currently available. The sequence information can be provided upon request.

Supplementary Tables 1-4

Plant-based diets induce transcriptomic changes in fast muscle of zebrafish and Atlantic salmon

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Supplementary Table 1. Composition of the zebrafish and Atlantic salmon diets

Diet composition (g/ 100g)	**Control**	**PPC**	**SPC**	**WG**
Zebrafish				
Fish meal	79.35	49.35	49.35	49.35
Wheat	12.0	12.0	12.0	12.0
PPC.	-	**30.0**	-	-
SPC	-	-	**30.0**	-
WG	-	-	-	**30.0**
Fish oil	4.0	4.0	4.0	4.0
Additives *	4.65	4.65	4.65	4.65
Total crude protein	56.2	49.9	55.0	59.1
Total lipids	12.0	10.7	9.4	9.8
Atlantic Salmon				
Fishmeal	63.35	-	33.35	33.35
Wheat	12.0	-	12.0	12.0
SPC	-	-	**30.0**	-
WG	-	-	-	**30.0**
Fish oil	20.0	-	20.0	20.0
Additives *	4.65	-	4.65	4.65
Total protein	45.2		44.0	48.1
Total lipids	26.5		23.8	24.3

* Vitamin mix (2%), Mineral mix (0.59%), Monosodium phosphate-24% P (2%), Yttrium oxide (0.01%), Carophyll Pink-10% (0.05%)

PPC- pea protein concentrate, SPC- soy protein concentrate and WG- wheat gluten

Supplementary Table 2. Genomic location and pairwise alignment details of paralogues used in this study.

Paralogues	**Genomic location**		**Pairwise alignment details***			
	Chromosome	Location	Pairwise alignment	Length (bp) of aligned region	% identity over aligned region	Associated E-value of aligned region
mylpfba	ssa06	24880606-24885367	*mylpfba : mylpfbb*	805	97	0.0
mylpfbb	ssa03	75692468-75696877	*mylpfba* : *mylpfbc*	468	71	1e-50
mylpfbc	ssa20	37559259-37568216	*mylpfbb* : *mylpfbc*	468	70	3e-48
hsp90aa1.1a	ssa06	72144452..72149825	*hsp90aa1.1a* : *hsp90aa1.1b*	2744	92	0.0
hsp90aa1.1b	ssa15	46699126..46705169				
ambra1aa	ssa26	9393199..9499944	*ambra1aa* : *ambra1ab*	4750	93	0.0
ambra1ab	ssa11	9352168..9464323	*ambra1aa* : *ambra1ac*	1850	76	0.0
ambra1ac	ssa16	1802087..1853353	*ambra1aa* : *ambra1ad*	1847	77	0.0
ambra1ad	ssa10	63803157..63851652	*ambra1ab* : *ambra1ac*	1851	75	0.0
			ambra1ab : *ambra1ad*	1842	75	0.0
			ambra1ac : *ambra1ad*	4728	88	0.0
col2a1aa	ssa12	84293497-84386605	*col2a1aa* : *col2a1ab*	2082	93	0.0
col2a1ab	ssa22	53429386-53512213	*col2a1aa* : *col2a1ac*	1533	84	0.0
col2a1ac	ssa13	21505732-21531640	*col2a1aa* : *col2a1ad*	1980	82	0.0
col2a1ad	ssa15	62188998-62218204	*col2a1ab* : *col2a1ac*	4169	83	0.0
			col2a1ab : *col2a1ad*	4706	82	0.0
			col2a1ac : *col2a1ad*	4473	93	0.0
btca	ssa01	117982644-17989859	*btca* : *btcb*	794	85	0.0
btcb	ssa13	89439773-89448007				
ryr1aa	ssa04	35701429-35771978	*ryr1aa* : *ryr1ab*	10715	95	0.0

ryr1ab	ssa11	77701604-77778088				
odc1a	ssa09	27129401-27134433	*odc1a* : *odc1b*	2636	86	0.0
odc1b	ssa01	29219775-29225435				

*Pairwise alignment details were manually obtained on 14.06.2020 from BLAST alignment of respective sequences in NCBI's nt database

Supplementary Table 3. List of selected differentially expressed genes in zebrafish muscle fed with the soy diet compared to their counterparts fed with fishmeal.

Gene symbol	Gene name	Selected GO terms*	Fold change	q-value
Upregulated				
klhl30	*kelch-like family member 30*	Protein ubiquitination	5.9	9.8E-16
dpp6b	*dipeptidyl-peptidase 6b*	Proteolysis	3.3	0.0014
gnsb	*Glucosamine(N-acetyl)-6-sulfatase*	Glycosaminoglycan metabolic process	3.1	0.0105
lmo7b	*LIM domain 7b*	Regulation of signaling	2.8	0.0013
loxl2b	*lysyl oxidase-like 2*	Oxidation-reduction process	2.7	0.0451
evpla	*envoplakin a*	Epidermis development	2.5	0.0470
caly	*calcyon neuron-specific vesicular protein*	Dopamine receptor signaling pathway	2.4	0.0498
dhrs11b	*dehydrogenase/reductase (SDR family) member 11b,*	Oxidoreductase activity [MF]	2.3	0.0331
si:ch211171h4.3 ¤	*serine/threonine-protein kinase SBK1* #	Activation of MAPK activity	2.3	0.0203
limch1a	*LIM and calponin homology domains 1a*	Actomyosin structure organization	2.2	0.0277
ryr1a	*ryanodine receptor 1a*	Calcium ion transmembrane transport	2.2	0.0022
dhx32a	*DEAH (Asp-Glu-Ala-His) box polypeptide 32a*	mRNA splicing, via spliceosome	2.2	0.0274
coro6	*coronin 6*	Actin cytoskeleton organization	2.1	0.0357
slc22a16	*solute carrier family 22 member 16*	Transmembrane transport	2.1	0.0244
msh3	*mutS homolog 3(E. coli)*	Mismatch repair	2.1	0.0200
ambra1a	*activating molecule in beclin-1-regulated autophagy*	Skeletal muscle fiber development	2.1	0.0045
si:ch211242b18.1¤	*Myomegalin* #	Microtubule organizing center [CC]	2	0.0075
mpp7a	*MAGUK p55 subfamily member 7*	Adherens junction [CC]	1.9	0.0160
crot	*carnitine O-octanoyltransferase*	Fatty acid metabolic process	1.8	0.0327

si:ch211196f2.3 ¤	*mucin-3A* #	Integral component of membrane CC	1.8	0.0470
popdc3	*popeye domain-containing 3*	Skeletal muscle tissue development	1.7	0.0084
Downregulated				
rsph3	*radial spoke 3 homolog*	No evidence	4.0	0.0013
crygm2d17	*crystallin, gamma M2d17*	Visual perception	3.4	0.0033
hspa1b	*heat shock protein family A (Hsp70) member 1B*	Chaperone cofactor-dependent protein refolding	3.4	0.00005
crybb1	*crystallin, beta B1 (Fragment)*	Visual perception	3.3	0.0011
fetub	*fetuin B*	Negative regulation of endopeptidase activity	3.2	0.0064
opn1sw2	*opsin-1, short-wave-sensitive 2*	G-protein coupled receptor signaling pathway	3.2	0.0033
crygm2d1	*crystallin, gamma M2d1*	Visual perception	3.1	0.0045
crygm2d13	*crystallin, gamma M2d13*	Visual perception	3.1	0.0045
cyt1	*type I cytokeratin, enveloping layer*	Structural molecule activity [MF]	3.1	0.0016
desi1a	*desumoylating isopeptidase 1a*	Protein modification by small protein removal	3.1	0.0064
ifitm1	*interferon induced transmembrane protein 1*	Response to biotic stimulus	3.1	0.0001
opn1lw2	*red-sensitive opsin-2*	G-protein coupled receptor signaling pathway	3.1	0.0014
prelid3b	*PRELI domain containing 3B(PRELID3B))*	Phospholipid transport	3.1	0.0007
crygm2d12	*crystallin, gamma M2d12*	Visual perception	3.0	0.0218
crygm2d21	*crystallin, gamma M2d21*	Visual perception	3.0	0.0110
crygm2d7	*crystallin gamma EM2-7*	Visual perception	3.0	0.0219
fgfbp2a	*fibroblast growth factor-binding protein 2a*	Growth factor binding [MF]	3.0	0.0200
slc12a10.3	*solute carrier family 12 member 10, tandem duplicate 3*	Ion transport	3.0	0.0050
apobb.1	*apolipoprotein Bb, tandem duplicate 1*	Cholesterol biosynthetic process	2.9	0.0084
cryba1b	*crystallin, beta A1b*	Visual perception	2.9	0.0042
crybgx	*crystallin beta gamma X*	Visual perception	2.9	0.0071
crygm2d15	*crystallin, gamma M2d15*	Visual perception	2.9	0.0151

hbae3	*hemoglobin alpha embryonic-3*	Oxygen transport	2.9	0.0045
rho	*rhodopsin*	G-protein coupled receptor signaling pathway	2.9	0.0151
apoa1b	*apolipoprotein A-Ib*	Cholesterol biosynthetic process	2.8	0.0302
atf3	*activating transcription factor 3*	Regulation of transcription by RNA polymerase II	2.8	0.0223
capn3a	*calpain 3a*	Negative regulation of apoptotic process	2.8	0.0116
ccl34b.4	*chemokine (C-C motif) ligand 34b, duplicate 4*	Inflammatory response	2.8	0.0219
crybb1l1	*crystallin, beta B1,-like 1*	Visual perception	2.8	0.0116
crygm2d14	*crystallin, gamma M2d14*	Visual perception	2.8	0.0162
crygm2d20	*crystallin, gamma M2d20*	Visual perception	2.8	0.0327
crygm2d8	*crystallin, gamma M2d8*	Visual perception	2.8	0.0084
crygn2	*crystallin, gamma N2*	Visual perception	2.8	0.0151
gngt2b	*guanine nucleotide-binding protein subunit gamma*	G-protein coupled receptor signaling pathway	2.8	0.0327
ngs	*notochord granular surface*	Notochord morphogenesis	2.8	0.0151
rspo3	*R-spondin 3*	Wnt signaling pathway	2.8	0.0188
serpina1	*serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1*	Negative regulation of endopeptidase activity	2.8	0.0212
slc32a1	*solute carrier family 32 (GABA vesicular transporter), member 1*	Amino acid transmembrane transport	2.8	0.0226
apoa1a	*apolipoprotein A-1a*	Cholesterol biosynthetic process	2.7	0.0365
arr3a	*arrestin 3, retinal (X-arrestin), like*	Signal transduction	2.7	0.0033
chia.2	*chitinase, acidic.2*	Chitin metabolic process	2.7	0.0378
gnb3b	*guanine nucleotide binding protein (G protein), beta polypeptide 3b*	Signal transduction	2.7	0.0033
myhz1.1	*myosin, heavy polypeptide 1.1, skeletal muscle*	Somite specification	2.7	0.0488
mylpfb	*myosin light chain, phosphorylatable, fast skeletal muscle b*	Calcium ion binding [MF]	2.7	0.0212

opn1mw1	*green-sensitive opsin-1*	G-protein coupled receptor signaling pathway	2.7	0.0219
syt5b	*synaptotagmin Vb*	Regulation of calcium ion-dependent exocytosis	2.7	0.0178
tfa	*serotransferrin*	Ion transport	2.7	0.0161
col2a1a	*collagen type II, alpha 1*	Extracellular matrix structural constituent [CC]	2.6	0.0244
crybb1l2	*crystallin, beta B1,-like 2*	Visual perception	2.6	0.0045
crygm2d2	*crystallin, gamma M2d2*	Visual perception	2.6	0.0295
crygm2d3	*crystallin, gamma M2d3*	Visual perception	2.6	0.0031
crygmxl2	*crystallin, gamma MX,-like 2*	Visual perception	2.6	0.0346
krtt1c19e	*keratin type 1 c19e*	No evidence	2.6	0.0219
odc1	*odc1 protein*	Ornithine decarboxylase activity	2.6	0.0425
sncb	*beta-synuclein*	Dopaminergic neuron differentiation	2.6	0.0470
ucp1	*uncoupling protein 1*	Cellular response to estrogen stimulus	2.6	0.0197
arrdc3b	*arrestin domain-containing 3b*	Protein transport	2.5	0.0255
col9a2	*procollagen, type IX, alpha 2*	Extracellular matrix organization	2.5	0.0327
cryba1l1	*beta A1-2-crystallin*	Visual perception	2.5	0.0354
cryba2b	*betaA2-2-crystallin*	Visual perception	2.5	0.0212
opn1sw1	*opsin SWS-1*	G-protein coupled receptor signaling pathway	2.5	0.0197
pck1	*phosphoenolpyruvate carboxykinase 1 (soluble)*	Polyamine biosynthetic process	2.5	0.0256
rbp4l	*retinol binding protein 4, like*	Transport	2.5	0.0002
crygm2d18	*crystallin, gamma M2d18*	Visual perception	2.4	0.0480
cyt1l	*type I cytokeratin, enveloping layer,-like*	No evidence	2.4	0.0478
gnat2	*guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2*	Signal transduction	2.4	0.0488
col9a3	*collagen, type IX, alpha 3*	Extracellular matrix organization	2.3	0.0224
fosl2	*fos-like antigen 2*	Regulation of transcription, DNA-templated	2.3	0.0425

gbp1	*guanylate-binding protein 1*	Cell-cell signaling	2.3	0.0367
nupr1	*nuclear protein 1*	Cellular response to estrogen stimulus	2.2	0.0480
pdlim1	*PDZ and LIM domain 1*	Actin cytoskeleton organization	2.1	0.0084
si:dkey183i3.5 ¤	*thread keratin alpha #*	Intermediate filament [CC]	2.1	0.0022
arrdc2	*arrestin domain-containing 2*	Protein transport	2.0	0.0256
mrpl20	*mitochondrial ribosomal protein L20*	Translation	2.0	0.0425
mrps10	*mitochondrial ribosomal protein S10*	Translation	2.0	0.0100
si:ch211217a12.1 ¤	*alanine aminotransferase 2-like #*	Catalytic activity	2.0	0.0002
atp1b1a	*sodium/potassium-transporting ATPase subunit beta*	Ion transport	1.9	0.0045
ciapin1	*cytokine induced apoptosis inhibitor 1*	Apoptotic process	1.9	0.0335
rmdn1	*regulator of microtubule dynamics 1*	No evidence	1.9	0.0061
timm17a	*translocase of inner mitochondrial membrane 17A*	Intracellular protein transport,	1.9	0.0431
cox10	cytochrome c oxidase assembly factor heme A	Heme biosynthetic process	1.8	0.0084
alas1	*5-aminolevulinate synthase (Fragment)*	Heme biosynthetic process	1.7	0.0045
fkbp3	*peptidylprolyl isomerase*	Protein folding	1.7	0.0313
mrpl42	*mitochondrial ribosomal protein L42*	Mitochondrial large ribosomal subunit [CC]	1.7	0.0019
pmpcb	*peptidase (mitochondrial-processing) beta*	Proteolysis	1.7	0.0461
sumo2b	*small ubiquitin-related modifier 2*	Protein sumoylation	1.7	0.0137
tmem258	*transmembrane protein 258*	Protein N-linked glycosylation	1.7	0.0378
wbp1	*WW domain-binding protein 1*	No evidence	1.7	0.0496
gapdh	*glyceraldehyde-3-phosphate dehydrogenase*	Glucose metabolic process	1.6	0.0498
mpc1	*mitochondrial pyruvate carrier*	Mitochondrial pyruvate transport	1.6	0.0084
mrpl47	*mitochondrial ribosomal protein L47*	Mitochondrial translation	1.6	0.0238
ndufb4	*NADH:ubiquinone oxidoreductase subunit B4*	NADH dehydrogenase (ubiquinone) activity [MF]	1.6	0.0123
ppifb	*peptidyl-prolyl cis-trans isomerase*	Protein folding	1.6	0.0470

psmd3	*proteasome (Prosome, macropain) 26S subunit, non-ATPase, 3*	Regulation of protein catabolic process	1.6	0.0327
rbp4	*retinol binding protein 4(plasma)*	Transport	1.6	0.0488
tmem11	*transmembrane protein 11, mitochondrial*	Mitochondrion organization	1.6	0.0498
reep5	*receptor expression-enhancing protein*	Integral component of membrane [CC]	1.5	0.0349
si:dkey44g23.5 ¤	*MAPK regulated corepressor interacting protein 2* #	No evidence	1.5	0.0346

* Selected GO biological process terms presented here unless specified as [CC] for cellular component or [MF] for molecular function where there is no biological process GO terms related to the gene

¤ Official symbol provided by zebrafish nomenclature committee

Preferred name indicated in NCBI

Supplementary Table 4. List of selected differentially expressed genes in zebrafish muscle fed with the wheat diet compared to their counterparts fed with fishmeal

Gene symbol	Gene name	Selected GO terms*	Fold change	q-value
Upregulated				
mhc1uba	*major histocompatibility complex class I UBA*	Antigen processing and presentation	18	4.01E-30
si:ch211-242b18.1 ¤	*Myomegalin #*	Microtubule organizing center [CC]	8.6	0.0001
si:ch211-278j3.3 ¤	*RING-type E3 ubiquitin transferase #*	Protein ubiquitination	7	0.0158
ttll2	*tubulin tyrosine ligase-like family*	Cellular protein modification process	3.2	0.0353
raver2	*ribonucleoprotein*	mRNA splicing, via spliceosome	2.7	0.0008
btc	*betacellulin*	Epidermal growth factor receptor signaling pathway	2.6	0.0084
msh3	*mutS homolog 3 (E. coli)*	Mismatch repair	2.2	0.0222
Downregulated				
rsph3	*radial spoke 3*	No evidence	11	0.016
elmo2	*engulfment and cell motility 2*	Cytoskeleton organization	10.5	0.001
tfa	*serotransferrin*	Ion transport	8.7	0.005
atf3	*activating transcription factor 3*	Regulation of transcription by RNA polymerase II	8.6	0.026
fabp10a	*fatty acid-binding protein 10-A*	Transport	7.8	0.022
zbtb16a	*zinc finger and BTB domain containing 16a*	Protein ubiquitination	6.2	0.008
angel1	*angel homolog 1*	3'-5'-exoribonuclease activity [MF]	6.2	0.013
pdlim1	*PDZ and LIM domain 1*	Actin cytoskeleton organization	4.3	0.016
hsp90aa1.1	*heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 1*	Skeletal myofibril assembly	3.9	0.013
aimp1b	*aminoacyl tRNA synthetase complex interacting multifunctional protein 1b*	RNA binding [MF]	3.5	0.013

hspa1b	*heat shock protein family A (Hsp70) member 1B*	Chaperone cofactor-dependent protein refolding	7	0.012

* Selected GO biological process terms presented here unless specified as CC for cellular component or MF for molecular function where there is no biological process GO terms related to the gene

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Errata list

PhD candidate: Amritha Johny
Thesis: Plant-based aquafeeds: Carry-over potential of mycotoxins and phytoestrogens from feed to fish and implications for fish health and food safety
Date: 29/10/2020

Page	Line	Original text	Corrected text
10	Figure 10	2075-2025	2017-2025
13	Figure 14 legend	Norwegian crown	Norwegian kroner
25	Line 7	Isoflavone , isoflavone (repeated twice)	isoflavone
36	Line 19	13000 PPC	13000 t PPC
47	Line 20	$k_2+k_B+k_M+k_G$	$k_2+k_E+k_M+k_G$
72	Line 1	(Johny et al., 2019),	2019).
110	Line 26	life animals	live animals

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