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

Philosophiae Doctor (PhD)
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Intestinal functions and health of Ballan wrasse (*Labrus bergylta*) and lumpfish (*Cyclopterus lumpus*) – Effects of variation in diet composition

Tarmfunksjon og helse hos berggylt (*Labrus bergylta*) og rognkjeks (*Cyclopterus lumpus*) – Effekter av variasjon i fôrets sammensetning

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SUMMARY

To rear cleaner fish together with Atlantic salmon (*Salmo salar*) is a biological strategy to keep sea lice away from the salmon. Ballan (B) wrasse (*Labrus bergylta*) and lumpfish (*Cyclopterus lumpus*) are the two species being farmed in Norway for this purpose. To support a rapid rise of cultivation scale, present knowledge needs strengthening, and in this context, in particular regarding their nutritional biology. Among the many aspects of biology, investigation of digestive physiology and nutrient requirement is necessary to be able to produce feed which supports good growth and high survival. Regarding B. wrasse, a stomach-less fish with short intestine, research conducted until the time of initiation of this PhD work had addressed some aspects of nutrient requirements, the suitability of some plant ingredients as nutrient sources, and the importance of feed consistency. Although some efforts had been made to describe the digestive system of the B. wrasse, essential information was still missing. For lumpfish, a fish with stomach and many pyloric caeca, hardly any knowledge on digestive physiology, nor on nutrient requirement was available. Therefore, the strategy and goal of this PhD work was to strengthen knowledge and understanding on digestive physiology and diet effects on intestinal functions and health in B. wrasse and lumpfish to secure good growth and health. To achieve this, the following strategies and goals were chosen for the present studies of B. wrasse (point 1 and 2) and lumpfish (point 1 and 3):

1. To characterize aspects of intestinal morphology and functions and effects of variation in diet composition for optimization of diet composition in accordance with the biology of the fish.
2. To describe effects of saponins which may occur in plant ingredients and may induce inflammation in the intestine and possible preventive effects of prebiotics.
3. To investigate effects of diet composition on disease resistance.

The work in **Paper I** was carried out to profile key characteristics of intestinal functions and health in wild-caught Ballan wrasse to provide a reference for evaluation of diet effects on intestinal function and health in cultivated fish. To describe functional variation along the intestine, samples were collected from four, consecutive intestinal segments, i.e. IN1, IN2, IN3 and IN4 from the most proximal segment to the most distal segment, respectively. The intestinal structure appeared quite similar in the four different segments. The highest capacity for digestion and absorption was found in IN1. Regarding observed indicators of immune functions, IN4 stood out showing higher gene expression levels of immunoglobulin M (igm), suggesting a more important role of IN4 in the immune responses related to igm. Parasite infection, especially myxozoan parasites like Enteromyxum leei, coincided with infiltration of lymphocytic and eosinophilic granular cells in the submucosa and lamina propria.

The aim of **Paper II**, which comprised a 5-week feeding trial with Ballan wrasse, was to gain better understanding of intestinal functions and health in cultivated Ballan wrasse and to generate knowledge of immune responses in the intestine induced by dietary components. During the trial, the fish were fed either a reference diet, the reference diet supplemented with *i)* soya saponins (0.7%) *ii)* a commercial prebiotic (Aquate™ SG, 0.4%) or *iii)* a combination of soya saponins and the prebiotics. Blood, intestinal tissue, and gut content from IN1 to IN4 were collected. No significant differences in fish growth were observed between the four dietary groups. Saponin supplementation, both alone and in combination with prebiotics, increased weight index of IN2 and IN3 and decreased blood plasma glucose, cholesterol, and total protein. Dry matter of intestinal content and activity of digestive enzymes were not affected by diet. Histomorphological analyses revealed a progressing inflammation with increased infiltration of immune cells particularly into the distal parts of the intestine in fish fed diets with saponins, both alone and in combination with prebiotics. Gene expression profiles obtained by RNA sequencing and quantitative PCR mirrored the histological and biochemical changes induced by the saponin load. The study demonstrated that Ballan wrasse gut health and digestive function may be markedly affected by feed ingredients containing antinutrients.

Paper III presents results from two experiments conducted to deepen the knowledge of lumpfish intestine physiology. Experiment 1 was a 42-day feeding trial in which lumpfish were fed twelve different diets in the following ranges of macronutrients: Protein 43-68%, lipid 4-17%, and carbohydrate 6-17%. Intestinal tissue, gut content and liver were sampled from 6 fish per tank. The results showed that with increasing lipid level and corresponding decrease in protein level, there was a linear decrease in several of the observed biomarkers, including activity of brush border membrane digestive enzymes, expression of genes related to nutrient digestion and transport, ion exchange and immune regulation. Increased intracellular hyper-vacuolization (probably accumulation of lipid) was observed in gut and liver with increasing dietary lipid level. Fewer effects were observed for increased dietary carbohydrate and corresponding decreased protein level. Experiment 2 was a two-week feeding trial for estimation of macronutrient digestibility in which lumpfish were fed three diets, all containing 55% crude protein, with lipid to carbohydrate ratio of the low lipid diet of 7.5%/18.3%, the medium lipid diet of 13.8%/14.6%, and high lipid diet of 18.1%/9.5%. Fecal samples were collected as pooled samples per tank. These results showed that fatty acid digestibility increased as dietary lipid level increased. Of note, starch digestibility decreased greatly as starch level increased, whereas protein digestibility did not change as lipid or starch level varied.

The work in **Paper IV** was conducted to follow up the study in **Paper III**, addressing disease resistance as indicated by the results of a challenge trial with *Aeromonas salmonicida* after a feeding period with diets varying in lipid/carbohydrates composition. Three experimental diets were formulated to have similar content of crude protein and ash but varying in content of lipid/carbohydrates from 6.7 / 18 – 18 / 8.1 %. Lumpfish with average start weight of 1.7 g were fed the experimental diets in triplicate tanks each for a period of 90 days. After termination of the feeding trial and subsequent collection of biological samples, remaining fish were challenged with atypical *A. salmonicida.*

No significant effects of diet were observed for growth performance. Carcass composition showed increasing content of dry matter, lipid, protein, and energy with increasing dietary lipid level. Increasing dietary lipid also increased hepatic dry matter, lipid, and energy levels, while crude protein decreased. Blood plasma nutrient levels and liver function markers showed few significant effects of diet, but dietary lipid level increased plasma cholesterol. Intestinal trypsin activity increased with increasing dietary lipid, whereas activity of other digestive enzymes and digesta bile salt levels were unaffected by diet. Increasing lipid level also increased lipid accumulation in the enterocytes of the proximal and mid intestine. Expression profiling of genes related to digestive and immune function showed few effects of diet. The nutrient transporters *fabp2* and *slc15a1*, as well as the immune genes *mhcII, igm*, and *nfkb* showed increases with dietary lipid levels, whereas the cholesterol transporter *npc1l1* was suppressed. Diet composition did not affect the lumpfish' resistance against *A. salmonicida*. To conclude, the variation in macronutrient composition induced modulations in metabolic, digestive and some immune functions. Modulations seemed however to be within normal ranges and did not produce clear impairments of immune responses to bacterial infection.

Overall, the work of current thesis has increased the knowledge necessary for formulation of optimal diets for cleaner fish production in Norway, comprising, regarding the B. wrasse, general features of the intestine's structure and function as well as inflammatory responses induced by antinutrients in the diet, and regarding lumpfish, knowledge strengthened for key aspect of intestinal structure and functions and effects of diet composition.

SAMMENDRAG (SUMMARY IN NORWEGIAN)

Bruk av rensefisk i oppdrett av Atlantisk laks (*Salmo salar*) er en biologisk strategi for å bekjempe lakselus. Berggylt (*Labrus bergylta*) og rognkjeks (*Cyclopterus lumpus*) er de to artene av rensefisk som det drives aktivt oppdrett på i Norge. Ny kunnskap, særlig innen ernæring, er nødvendig for å øke mulighetene for rensefiskeoppdrett. Blant mange aspekter av rensefiskbiologien, er studier av rensefiskens fordøyelsesfysiologi og ernæringsbehov nødvending, for å kunne tilby rensefisken et fôr som sikrer god vekst og overlevelse i en oppdrettssituasjon. Da dette PhD-arbeidet ble påbegynt fantes det, for berggylt, en mageløs fisk med kort tarm, noe kunnskap om ernæringsbehov, bruk av plantebaserte fôringredienser og viktigheten av pelletkonsistens. Imidlertid manglet mye essensiell kunnskap om berggyltens fordøyelsessystem. For rognkjeks, en fisk med mage og mange blindsekker, fantes det lite eller ingen kunnskap om fordøyelsesfysiologi eller ernæringsbehov. Hovedmålet med dette PhD-arbeidet var derfor å styrke kunnskapsgrunnlaget og forståelsen av hvordan fordøyelsesfysiologi og fôrsammensetning kan påvirke tarmfunksjon og tarmhelse hos berggylt og rognkjeks, for å sikre god vekst og helse. For å oppgå dette målet ble følgende delmål og strategier etablert for studier av berggylt (punkt 1 og 2) og rognkjeks (punkt 1 og 3):

1. Beskrive hvordan variasjoner i fôrsammensetning kan påvirke tarmens struktur og funksjon, for å kunne optimalisere fôrsammensetningen i henhold til fiskens biologi.
2. Beskrive effekter av saponiner, som forekommer i mange plantebaserte fôr og kan forårsake tarmbetennelse, og prebiotika, som kan fungere betennelsesdempende i fisketarmen.
3. Beskrive effekter av endring i fôrsammensetning på mottakelighet for sykdom.

Arbeidet i **Artikkel I** ble gjennomført for å beskrive de viktigste forhold rundt tarmfunksjon og tarmhelse i villfanget berggylt, og dermed etablere et

referansegrunnlag for studier av hvordan variasjoner i fôrsammensetning kan påvirke tarmfunksjon og tarmhelse i oppdrettet berggylt. For å beskrive variasjoner i tarmfunksjon langs tarmkanalen, ble prøver tatt fra fire påfølgende tarmsegmenter fra proksimal til distal del, i.e. IN1, IN2, IN3 og IN4. Tarmstrukturen var relativt lik i de fire segmentene. IN1 viste størst kapasitet for fordøyelse og opptak av næringsstoffer. Blant observasjoner relatert til immunfunksjon, hadde IN4 høyere genuttrykk av immunoglobulin M (*igm*), noe som kan tyde på at IN4-segmentet har en viktig rolle i *igm*-relatert immunfunksjon hos berggylt. Tarminfeksjoner forårsaket av myxozoa-parasitten *Enteromyxum leei* ble observert i sammenheng med økt infiltrasjon av lymfocytter og eosinofile granulocytter i submucosa og lamina propria.

Formålet med studien i **Artikkel II**, som innebar et 5-ukers fôringsforsøk med berggylt, var å øke den generelle forståelsen av tarmfunksjon og tarmhelse hos berggylt i oppdrett, samt å bidra med ny kunnskap om immunresponser i bergylttarm forårsaket av komponenter i fôret. I fôringsforsøket ble fisken tilbudt et referansefôr, samt referansefôret tilsatt *i)* soyasaponin (0,7%), *ii)* et kommersielt prebiotium (Aquate™ SG, 0.4%) eller *iii)* en kombinasjon av soyasaponin og prebiotikumet. Blod, tarmvev og tarminnhold fra IN1 til IN4 ble samlet inn. Ingen signifikante forskjeller i vekst ble observert mellom de fire fôrgruppene. Tilsetning av saponin, både alene og i kombinasjon med prebiotika, økte den relative vekten av tarmavsnittene IN2 og IN3, og førte til reduserte nivåer av glukose, kolesterol og totalprotein i blodplasma. Tørrstoffnivå i tarminnholdet og aktivitet av fordøyelsesenzymer ble ikke påvirket av diett. Histomorfologiske analyser påviste en fremtredende betennelsesreaksjon med økt infiltrasjon av immunceller i de bakre delene av tarmen hos fisk fôret med saponin, både alene og i kombinasjon med prebiotika. Genekspresjonsprofilering med RNA sekvensering og kvantitativ PCR viste effekter av saponinbehandlingen som samsvarte godt med de histomorfologiske og biokjemiske observasjonene. Studien demonstrerte dermed hvordan tarmhelse og fordøyelsesfunksjon hos berggylt kan påvirkes betydelig ved å benytte fôringredienser som inneholder antinæringsstoffer.

Artikkel III presenterer resultater fra to separate studier som ble gjennomført for å styrke kunnskapen om tarmfysiologi hos rognkjeks. Eksperiment 1 var et 42-dagers fôringsforsøk der rognkjeks ble tilbudt tolv forskjellige fôr som varierte i innhold av makronæringsstoffer: Protein 43-68%, fett 4-17%, og karbohydrat 6-17%. Tarmvev, tarminnhold, og levervev ble tatt ut fra 6 fisk per kar. Resultatene viste, ved økende fettnivå og dermed redusert proteinnivå i fôret, en lineær nedgang i nivåer av en rekke biomarkører, inkludert aktivitet av børstesøms-fordøyelsesenzymer og genuttrykk relatert til fordøyelse og næringsstoffopptak, ioneregulering og immunfunksjon. Forøket intracellulær hypervakuolisering (trolig forårsaket av fettakkumulasjon) ble observert i både tarm og lever med økende fettnivå i fôret. Færre effekter ble observert ved å økte fôrets innhold av karbohydrater, og dermed redusere proteinnivået. Eksperiment 2 var et 2-ukers fôringsforsøk som ble gjennomført for å estimere fordøyelighet av makronæringsstoffer hos rognkjeks. Rognkjeks ble tilbudt tre forskjellige fôr som alle inneholdt 55% protein, men som hadde fett til karbohydrat forhold på henholdsvis 7,5%/18,3%, 18,8%/14,6% og 18,1%/9,5% for lavt, medium og høyfett fôr. Faecesprøver ble tatt som samleprøver fra hvert kar. Resultatene viste at fettsyrefordøyeligheten økte med økt tilsetning av fett i fôret. Et hovedfunn fra studien var at stivelsesfordøyeligheten ble kraftig redusert med økende mengde stivelse i fôret. Proteinfordøyeligheten ble ikke påvirket av endringer i fôrets fett- eller karbohydratinnhold.

Arbeidet i **Artikkel IV** ble gjennomført som en oppfølging av resultatene fra studiene i **Artikkel III**. Hovedformålet var å studere sykdomsmottagelighet, basert på en smittestudie med *Aeromonas salmonicida,* etter å ha fôret rognkjeks med ulike dietter som varierte i innhold av fett og karbohydrater. Tre eksperimentelle fôr ble formulert til ha like nivåer av råprotein og aske, men med varierende fett/karbohydrat-ratio fra 6,7 / 18 til 18 / 8,1 %. Rognkjeks med gjennomsnittlig startvekt på 1,7 gram ble fôret på de eksperimentelle diettene i triplikate kar per fôr i 90 dager. Etter avslutning av fôringsforsøket og prøveuttak, ble resten av fisken smittet ble atypisk *A. salmonicida*. Ingen signifikante effekter av fôr ble observert for vekst. Kroppssammensetning viste økte nivåer av tørrstoff, fett, protein, og energi, ved økende fettinnhold i fôret. Økt

fettnivå i fôr ga også økt tørrstoffinnhold, fett, og energinivå i lever, mens nivå av råprotein i lever ble redusert. Næringsstoffnivåer og markører for leverfunksjon i blodplasma viste få effekter av fôr, men økt fettnivå i fôr gav økte kolesterolverdier i blodplasma. Trypsinaktivitet i tarm økte med økende fettnivå i fôret, mens aktivitet av andre fordøyelsesenzymer, og gallesaltnivåer i tarminnhold ikke ble påvirket av endringer i fôrsammensetning. Økt fettnivå i fôret gav også økt grad av fettakkumulasjon i enterocyttene i proksimaltarm og midttarm. Ekspresjonsprofilering av gener relatert til fordøyelse og immunfunksjon viste få effekter av diett. Næringsstofftransportørene *fabp2* og *slc15a1*, samt immungenene *mhcII*, *igm*, og *nfkb* viste økt uttrykksnivå med økende mengde fett i fôret, mens genuttrykket av kolesteroltransportøren *npc1l1* ble redusert. Forsammensetning påvirket ikke dødelighet etter smitte med *A. salmonicida*. Det konkluderes med at variasjoner i fôrets sammensetning av makronæringsstoffer forårsaket endringer i metabolske, fordøyelsesrealterte, og immunrelaterte funksjoner i berggylttarmen. Endringene ble imidlertid vurdert til å være innenfor normal variasjon, og viste ingen klare effekter på immunrespons etter bakteriell smitte.

Overordnet har arbeidet sammenfattet i denne PhD-avhandlingen bidratt med ny kunnskap som vil være nødvending for å kunne formulere optimalt fôr til bruk i rensefiskindustrien i Norge. For berggylt har arbeidet bidratt med ny generell kunnskap om tarmstruktur og tarmfunksjon, samt beskrevet betennelsesreaksjoner i tarm forårsaket av antinæringsstoffer i fôr. For rognkjeks har arbeidet bidratt med ny kunnskap om tarmstruktur, tarmfunksjon og effekter av endringer i fôrsammensetning.

ABBREVIATIONS

IN1 – 4	Intestinal segment 1 – 4
PI, MI and DI	Proximal, mid and distal intestine
IEL	Intraepithelial lymphocyte
BBM	Brush border membrane
TJ	Tight junction
GALT	Gut-associated lymphoid tissues
PRR	Pattern recognition receptors
PAMP	Pathogen-associated molecular pattern
TLR	Toll-like receptor
NLR	NOD-like receptor
IFN	Interferon
IL	Interleukin
TNF	Tumor necrosis factor
CD	Cluster of differentiation
TCR	T cell receptor
OVAT	One variable at a time
LAP	Leucine aminopeptidase
EGC	Eosinophilic granular cell
MOS	Mannan-oligosaccharides

LIST OF PAPERS

Paper I: **Digestive and immune functions in the intestine of wild Ballan wrasse (*Labrus bergylta*).** Weiwen Zhou, Åshild Krogdahl, Øystein Sæle, Elvis Chikwati, Guro Løkka, Trond M. Kortner

Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 260 (2021): 111011. DOI: 10.1016/j.cbpa.2021.111011

Paper II: **Soya saponins and prebiotics alter intestinal functions in Ballan wrasse (*Labrus bergylta*).** Weiwen Zhou, Kai K. Lie, Elvis Chikwati, Katerina Kousoulaki, Ingrid Lein, Øystein Sæle, Åshild Krogdahl, Trond M. Kortner

Accepted for publication in *British Journal of Nutrition*

Paper III: **A screening study of effects of variation in dietary macronutrient composition on gut functions in lumpfish (*Cyclopterus lumpus*).** Weiwen Zhou, Kristin Hamre, Elvis Chikwati, Katerina Kousoulaki, Ingrid Lein, Gerd Marit Berge, Øystein Sæle, Trond M. Kortner, Åshild Krogdahl

Frontiers in Marine Science, 9 (2022):889778. DOI: 10.3389/fmars.2022.889778

Paper IV: **Effects of dietary lipid level on growth, digestive physiology and disease resistance in lumpfish (*Cyclopterus lumpus*).** Gerd Marit Berge*, Weiwen Zhou*, Lill-Heidi Johansen, Elvis Chikwati, Trond M. Kortner, Ingrid Lein, Åshild Krogdahl

Under revision in *Aquaculture*
* Equal contribution

INTRODUCTION

The use of cleaner fish in Norwegian salmon industry

Parasitic infestation, mainly by salmonid specialist *Lepeophtheirus salmonis*, is by many considered as the most severe challenge in Norwegian Atlantic salmon production today. Fish severely infested by sea lice have low survival rates and viability due to the disruption of osmoregulatory and immune functions (Bowers, et al., 2000; Fast, et al., 2006; Mustafa, et al., 2000). Besides being a major welfare issue, lice infestations decrease growth rate and increase mortality, and thus causes economic losses. In addition, sea lice from salmon farms may increase lice infection and mortality of wild stocks and become a major ecological problem (Kristoffersen, et al., 2017). Under these considerations, controlling the number of sea lice is necessary not only for the salmon industry itself, but also for securing responsible aquaculture production for the environment and the society.

Several different treatments and strategies are currently used for reduction of the sea lice challenge in practical production, e.g., chemical treatments through bath treatments or in-feed additives, mechanical treatments, thermal treatments, and biological control (Overton, et al., 2019). In the earlier years of salmon production chemical treatments were used as efficient ways to reduce the number of sea lice, but the long-term use of chemicals has inevitably generated chemical resistance in the lice (Aaen, et al., 2015). Unintended impacts of anti-sea lice chemicals on nontarget crustaceans in the environment have also caused severe concerns (Song, et al., 2016). Non-chemical treatments have therefore become alternative ways to control lice infestations in recent years. Among them, biological control, i.e., co-culturing fish species eating sea lice together with salmon, has become an attractive way with minor negative effects to the salmon. Such fish species are called cleaner fish. In Norway, use of cleaner fish in commercial production has grown rapidly since the early start in 2012 (Overton, et al., 2020). In 2020 the number of fish used reached 51 million (Norwegian Directorate of Fisheries 2021), i.e., a number around 5-fold higher than in 2010. This

increase witness of a success which has created a huge demand for cleaner fish. To fulfil the demand cultivation has become necessary, as catches of wild-caught fish are far from sufficient. Supply is therefore gradually shifting towards use of cultivated fish. This is not only for obtaining more fish of higher quality but also for sustainability reasons of the wild stocks. Cultivated cleaner fish accounted for 64% in 2021 (Calculated number from statistics of Norwegian Directorate of Fisheries: https://www.fiskeridir.no/English/Aquaculture/Statistics/Cleanerfish-Lumpfish-and-Wrasse), which means that still a large of number of cleaner fish are wild caught. At present a challenge for the cleaner fish producers is up-scaling of the production facilities, which takes time and money. A great challenge in this situation is severe lack of basic knowledge of the biology of these species. Although some information has been generated the last decade, we still lack very important information necessary for efficient and healthy production. Many questions need to be answered before the cleaner fish production reaches a well-developed and steady stage.

Figure 1. The appearance of lumpfish (left) and Ballan wrasse (right). Photo modified from Reidun Marie Bjelland /Institute of Marine Research and **Paper I**.

Two fish species are being farmed for use as cleaner fish in Norway, i.e., Ballan wrasse (B. wrasse, *Labrus bergylta*) and lumpfish (*Cyclopterus lumpus*). They are two very different fish species. The B. wrasse belongs to the family of *Labridae*, commonly called wrasses. Fish from this family were the first found to be capable of controlling sea lice

number in salmon, i.e., Goldsinny wrasse (*Ctenolabrus rupestris*), corkwing wrasse (*Symphodus melops*), and rock cook (*Centrolabrus exoletus*) (Bjordal, 1990). The B. wrasse is the largest in size among them and therefore better meets the requirement for delousing in net pens (Skiftesvik, et al., 2014). At present it is the only farmed wrasse species in Norway.

Lumpfish is another fish species which can eat sea lice. Taxonomically it belongs to the family *Cyclopteridae*. It was scientifically demonstrated to be a salmon delouser as late as in 2014 (Imsland, et al., 2014). However, at present, the production of lumpfish outranges other cleaner fish and counted 27 million individuals in 2021. A reason is their better survival and health at low temperatures, which is favourable for their use in Norway, especially the north region of Norway. A recent study showed that after a autumn-winter period, lumpfish had less fin damage and lower mortality than B. wrasse (Geitung, et al., 2020). These observations indicate that use of lumpfish will not only result in a higher delousing efficiency but also reduce other losses in a commercial production.

Many questions regarding biology, which needs answer to secure healthy fish with high capacity for lice cleaning, are still unanswered. Examples are questions regarding general biology, not at least digestive functions, optimal diet composition, welfare and health management, delousing behavior after deployment, genetic background and breeding etc. (Brooker, et al., 2018; Garcia de Leaniz, et al., 2021). Among these issues, the current PhD. work addresses interactions between macronutrient composition of the diet, intestinal functions and health, as well as consequences for disease resistance. Hence the following introductory chapters focus mainly on the aspect of digestive function and nutrition. In the scientific literatures, reports of studies generating such knowledge in B. wrasse, are few (Helland, et al., 2014). It seems that, at larvae stage, B. wrasse need intensively reared copepods, for good growth and adaptation to formulated feed. Regarding the weaning stage, during which formulated feed is introduced, the marine ingredients should be carefully selected. For example, B. wrasse seems to require crustaceans in the diet, such as shrimp and krill, for good growth and

survival, whereas fish meal should be avoided (Kousoulaki, et al., 2015). At later stages, the dependency on crustacean ingredients appears less critical. On the other hand, using plant-based diet seems to be feasible, as the mixture of soy protein concentrate and pea protein can substitute fish meal in diet without compromising growth and feed efficiency (Cavrois-Rogacki, et al., 2022). Present knowledge indicates that the optimal diet for B. wrasse, when based on marine ingredients, contains 65% protein, 12% lipid and 16% carbohydrate, based on the estimate of maximum growth in length (Hamre, et al., 2013). On the other hand, a plant-based diet of 51% protein has been recommended as optimal (Cavrois-Rogacki, et al., 2022). The requirement of micronutrients, i.e. vitamins and minerals has not yet been determined for B. wrasse, but by comparing wild and cultured fish, lower value of vitamins A, K and D were observed in cultured B. wrasse, indicating a need of extra supplement in diet.

Regarding lumpfish, studies related to nutrition are fewer than for B. wrasse. Lumpfish seem to tolerate diets with less marine ingredients and more plant-based ingredients, without impact on growth (Willora, et al., 2020; Willora, et al., 2021). The recent study of Hamre et al. (Hamre, et al., 2022) addressed macronutrient requirement of lumpfish. The work indicates that lumpfish of size 15-50 g should be fed a diet containing 55% protein, 17% lipid, and 6% carbohydrate to reach optimal growth rate. Too low (48%) or too high (68%) protein level led to suboptimal immune responses in isolated head kidney cells.

The anatomy of fish alimentary tracts

As the diversity in anatomy of the digestive tract in fish is tremendous, subdivision of the alimentary tract differs greatly between species (Bjørgen, et al., 2020). To help understanding of the following chapters, a brief introduction is given below.

Gross anatomy

The majority of views of this chapter is referred to the introduction by Egerton and co-workers (Egerton, et al., 2018), and the book chapter by Wilson and Castro (Wilson, Castro, 2010). Fish alimentary tracts can roughly be subdivided into four topographical regions, i.e., head-, fore-, mid- and hindgut (Wilson, Castro, 2010) (Figure 2). The headgut comprises mouth and buccal pharyngeal cavity. Its function is to acquire food and mechanically process it. The foregut comprises the esophagus and stomach where chemical digestion of food begins. However, about 20% of fish species lack a true stomach (Wilson and Castro, 2010). Species that lack stomach include fish in the *Gobiidae* and *Blennidae* families. B. wrasse is also a fish species that has no stomach. Lacking a stomach may be counteracted by other adaptations such as well-developed pharyngeal teeth, pharyngeal pockets, secretory glands in the oesophagus or a muscular gizzard (Egerton, et al., 2018). When presents, the stomach of fish can be classified according to its shape, including straight (I), siphonal (U or J) or cecal (Y) (Wilson, Castro, 2010). Straight stomach is not common, but it is still found in some fish such as mullet, anchovy and menhaden. The U-shaped stomach is more common and is seen in omnivores and carnivores such as seabass (*Dicentrarchus*), salmonids and also in lumpfish. The Y-shaped stomach allows the storage of larger quantities of food and is found in eels (*Anguilliformes*).

The midgut is usually the longest section of the gut. Pyloric ceca, blind-ended ducts that provide additional digestive and absorptive area, are included when present. Due to the long length and the present of pyloric ceca in many species, the midgut generally is where digestion predominantly takes place. Although not always observed, this section often ends with an increase in tube diameter, indicating the beginning of the hindgut. In addition to the change of diameter, a sphincter, valve or a thickening of the circular muscle layer, and/or the change of mucosal folding pattern can also be the indication of the end of the midgut and start of the hindgut (Wilson, Castro, 2010). However, not all fish species have these characteristics, and in these species, it may be difficult to distinguish between midgut and hindgut.

The midgut is sometimes further subdivided into two sections, i.e., the proximal and mid intestine. The proximal intestine is defined as the part of the midgut section where the pyloric caeca are located (Bjørgen, et al., 2020), or, in fish without stomach and pyloric caeca, the section proximal to the first turning of the intestine (Sun, et al., 2019). The mid intestine is the section following the proximal intestine until the beginning of the hindgut. The hindgut is also called the distal intestine.

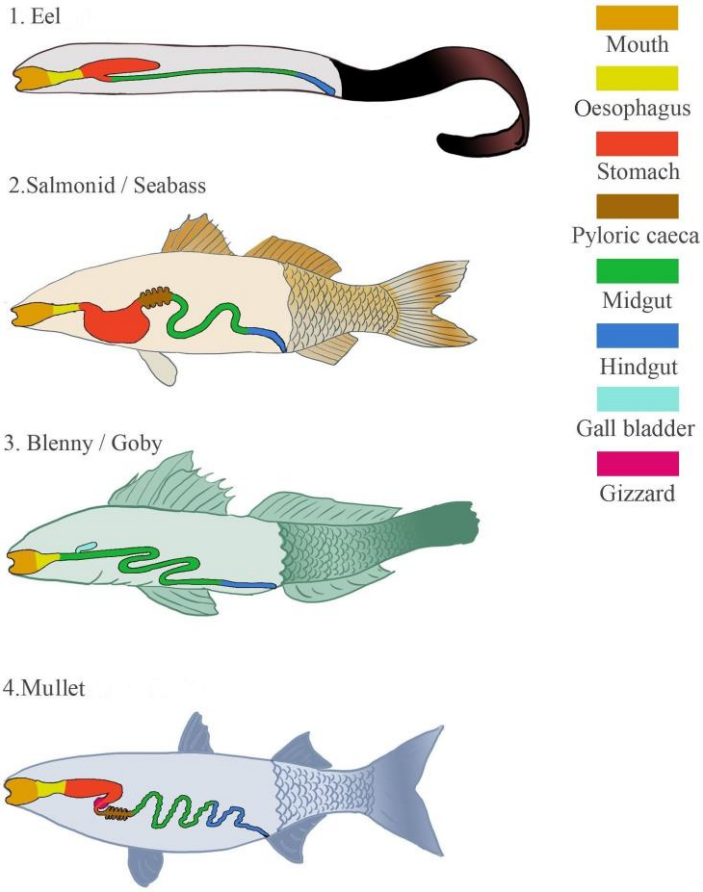


Figure 2. Diagrammatic representation of the different types of digestive systems that can be found in marine fish, including digestive organs that may or may not be present. (Egerton, et al., 2018)

Microanatomy

The gut wall of fish, from inner to outer, consists of mucosa, submucosa, muscularis and serosa (Løkka, Koppang, 2016) all along the gut. However, cell types vary within each section. The mucosa is lined with an epithelial layer. In the esophagus, the epithelium is stratified and becomes columnar towards the posterior region. In the stomach and intestine, the columnar epithelium is the only type of epithelium (Wilson, Castro, 2010). The mucosa rests on the lamina propria which contains connective tissue, blood vessels and scattered leukocytes. The lamina propria can be subdivided into a densely packed layer called stratum compactum, and a looser layer of connective tissue, called stratum granulosum, which may contain eosinophilic granular cells (Bjørgen, et al., 2020; Løkka, Koppang, 2016). Under the lamina propria follows the submucosa which is a deeper layer of loose connective tissue. It may, or may not, be defined as a separate layer, and the distinction between lamina propria and submucosa is not always clear (Løkka, Koppang, 2016; Wilson, Castro, 2010). The muscularis, which supports the submucosa, comprises two layers of muscle fibers. In the esophagus, the inner direction is longitudinal, the outer direction is transversal, whereas in the stomach and intestine, the directions are the opposite. The serosa, a thin coating of connective tissue and a squamous epithelium, defines the outer lining of the gut wall.

A pronounced distinction between each gut section is the cell types of the mucosa. The epithelial cells in stomach and intestine can be classified as columnar and secretory cells. The columnar cells in the stomach are mostly mucous producing. They are tall and have apparent mucous granules in the apical region. The secreted mucus is neutral and Periodic-Acid-Schiff positive, and protects the epithelium from the low pH and enzymatic activity of the gastric juices (Wilson, Castro, 2010). The only secretory cells in fish stomach are called oxynticopeptic cells, with pepsinogen found in zymogen granules in the supranuclear region of the cells (Wilson, Castro, 2010). In intestine, the epithelium consists of a layer of columnar absorptive cells, with goblet cells, lymphocytes and neuroendocrine cells distributed in-between in a scattered manner. The absorptive cells show a well-develop brush border on the apical surface. The brush

border greatly increases the absorptive area, and diverse digestive enzymes are located on it for digestion. After ingestion of feed, absorptive vacuoles are often present in the apical cytoplasm, below the brush border. Goblet cells are the dominant mucous cell type which contain the acidic mucosubstances sialomucin and smaller amounts of sulfomucins (Wilson, Castro, 2010). The immune cells located in the epithelium are commonly named intraepithelial lymphocytes (IELs). The type of IELs in fish might be MHC class II-expressing T cells (Bjørgen, et al., 2020) and IgT positive B cells (Zhang, et al., 2010). Neuroendocrine cells are also present, releasing hormones and other bioactive components into the bloodstream upon stimulation by nutrient, harmful substances and microbes. Their chemoreceptors initiate digestive and immune responses.

Intestinal functions addressed in the present PhD work

The intestine is a multifunctional organ. Digestion and absorption of nutrients represent the fundamental functions. Fish live in water with osmolality which mostly differs from their internal environment, and the intestine is an important osmoregulatory organ, in concert with the gills and the kidney. Moreover, as the gut represents a large surface constantly exposed to the external environment, it must provide defense against harmful components and organisms. Accordingly, it serves as a physical barrier and harbors an extensive immune apparatus. The following chapters give an overview of the most important functions of the gut. In addition, effects of diet composition on the functions of the intestine, relevant for this PhD work, are presented.

Nutrient digestion and absorption

In brief, digestion in the intestine is a process breaking down larger molecules to small absorbable molecules. Figure 3 gives an overview of the main nutrients subject to endogenous digestive and absorptive processes, i.e., lipids, starch, protein, vitamins,

and minerals. The liver and pancreas are involved by secreting digestive enzymes and essential substances for pH adjustment, emulsification and solubilization. As the intestinal structure differs greatly between fish species, the following description of the digestive process in fish is better considered separately for fish with and without stomach, especially regarding protein digestion. The following chapters will therefore cover digestion based on studies in fish with or without stomach, which also correspond to the intestinal structure of lumpfish and B. wrasse.

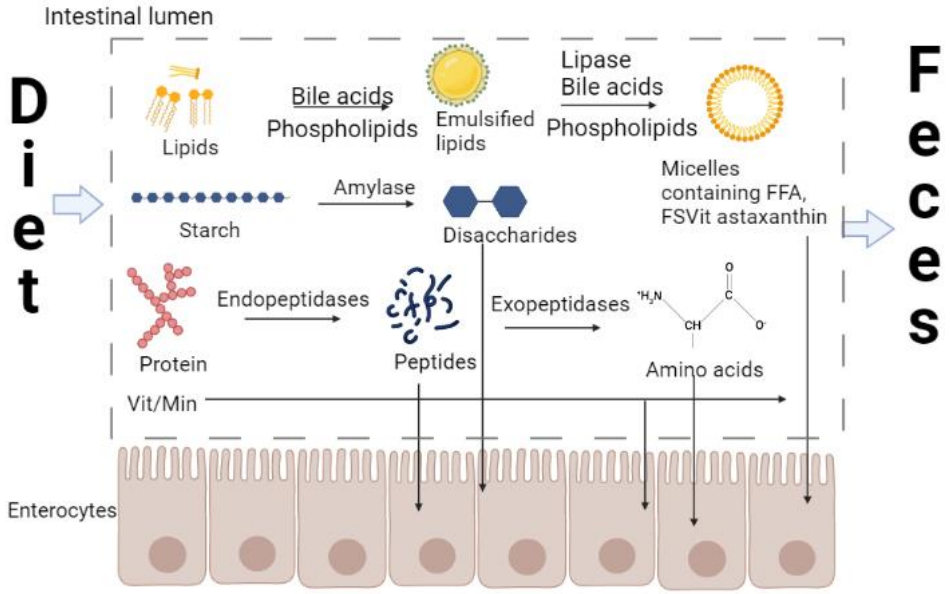


Figure 3. Schematic drawing of the digestive processes in the fish intestine. Digestion at the brush border membrane of enterocytes and absorption are simplified as arrows towards enterocytes. FFA: free fatty acids, FSVit: fat soluble vitamins, Vit/Min: water soluble vitamins and minerals. Modified from (Bakke, et al., 2010) and created with BioRender.com.

A recently published paper (Bjørgen, et al., 2020) presents a comprehensive review of the digestive physiology in Atlantic salmon. In the following it is used as a main source of information for presentation of digestive physiology in fish with stomach and as reference for discussion of our observations regarding B. wrasse and lumpfish.

In gastric fish species, enzymatic digestion starts when food is entering the stomach and is mixed with digestive juice containing acid and pepsin. Pepsin is the dominant digestive enzyme present in the stomach (Bakke, et al., 2010), and therefore, protein may be the only nutrient being digested in this compartment. However, gastric chitinase activity has been documented for some fish species (Bakke, et al., 2010; Danulat, Kausch, 1984), indicative of endogenous gastric chitin digestion. After the digestion in the stomach, the intestinal digestion starts, involving a range of digestive enzymes and essential substances. A major source of digestive enzymes is the exocrine pancreas. Pancreatic enzymes include trypsin, chymotrypsin, elastases, collagenase, carboxy-peptidases, amylase, lipase, phospholipases, cholesterol and wax ester hydrolases, as well as ribo- and deoxyribonucleases (Krogdahl, Sundby, 1999). The digesta is acidic when it enters the intestine and needs pH adjustment to about neutrality to suit the optimum for pancreatic digestive enzymes. Secretion of bicarbonate from the pancreas is regulated to reach this. Another essential mixture of digestive components is found in bile. Bile, secreted from the gallbladder into the intestinal lumen via the bile duct, contains a variety of compounds dominated by bile salts and fats, including fatty acids, cholesterol, and phospholipids (Romano, et al., 2020). Bile components participate in emulsifying function and micelle formation, enhance the hydrolysis of triglycerides by lipases and promote transport of non-polar components from the micelles across the intestinal mucosa. Lipid micelles, containing free fatty acids, mono- and di-triglycerides are products of lipid digestion, while the products of protein and carbohydrate hydrolysis are smaller components in the lumen, e.g., oligo- and di-peptides as well as oligo- and di-saccharides, etc. The products of protein and carbohydrate hydrolysis need further hydrolysis before they are absorbed into the enterocytes. This step takes place mostly in close vicinity to, on or in the brush border membrane (BBM) of the enterocytes. Brush border membrane digestive

enzymes such as aminopeptidases, maltase, sucrases, trehalase, alkaline phosphatases and monoglyceride lipases are involved. After this final digestion, the nutrients in their simplest form are ready for transport across the BBM and through the cell.

The initiation of digestion in stomachless fish is different to that of fish with stomach. Due to the lack of stomach related functions, feed ingested by stomachless fish is not exposed to low pH nor pepsin activity (Horn, et al., 2006), meaning that protein digestion starts when exposed to the pancreatic proteases. The pH is higher in the foregut of stomachless fish than in a functional stomach, but not necessarily alkaline. In some fish species pH is weakly acidic, in others neutral (Manjakasy, et al., 2009) or alkaline (Horn, et al., 2006), which could reflect evolutionary adaptations for optimal digestive enzyme activity. The pattern of digestive enzyme activity along the intestine also shows difference in stomachless fish compared to that in Atlantic salmon, which shows decreased activity towards distal segments of the intestine for most digestive enzymes (Krogdahl, et al., 2015a). In stomachless fish, enzyme activity of the digesta has been observed to be constant along the intestine in some fish species, or to decrease towards the end of the distal intestine in other (Day, et al., 2011; German, 2009).

Nutrient absorption in fish appears to be similar in stomachless fish species and species with stomach. Transporter-mediated absorption across BBM is the dominating route of nutrient absorption from the intestinal lumen to the absorptive cells for small peptides, amino acids and monosaccharides. For instance, the peptide transporters, PepT1 and PepT2, mediate transport of di- or tri- peptides as well as free amino acids (Debnath, Saikia, 2021), and sodium-dependent glucose transporter 1, SGLT1, is involved in glucose transport (Syakuri, et al., 2019). The transporters of amino acids and monosaccharides are usually coupled with ion transporters (Kiela, Ghishan, 2016). When coupled with ions, e.g., Na^+ and H^+, the electrochemical gradient across the brush border membrane can provide driving force for nutrient uptake, so they can be transported from the intestinal lumen into the cytoplasm against their concentration gradient (Thwaites, Anderson, 2007). However, there are still many knowledge gaps regarding nutrient digestion and absorption in fish. Whether monoglycerides formed

during triglyceride hydrolysis in the digesta are absorbed as such, as in monogastric land animals, or if all fatty acids are released as free fatty acids before absorption, is not clear. Fatty acid (Kuz'mina, 2021) and cholesterol transporters (Clifton, et al., 2010) have been identified in fish, but the fate of the lipids after absorption into the enterocyte, i.e. their onward journey to the peripheral tissues, is not described. Until now, a lymphatic system has not been described for fish. In the chicken, which do not have a lymphatic system, lipid pass from the intestine through the liver via the portal vein. However, indications available until now, does not indicate that this is the situation in fish. Regarding micronutrient absorption in fish, knowledge is limited. The fat-soluble vitamins A, D, E, and K and pigment carotenoids such as astaxanthin are thought to be incorporated into the micelles and absorbed when released as they disintegrate when touching the BBM surface. Minerals can, in addition to intestinal absorption, also be absorbed through the gills and skin, and inter-regulatory mechanisms likely exist (Bjørgen et al 2020).

Digestive and absorptive functions of the gut are usually rapidly and closely regulated by changes in diet composition and feed intake. These rapid alterations are achieved by the regulatory actions of the neuroendocrine system, which is considered conserved and similar to that in mammals (Bertucci, et al., 2019). Feeding behaviour and metabolism of absorbed nutrients are regulated by the so-called feeding centre in the brain (Volkoff, 2016). Ingested food is sensed by specific sensors (e.g., SGLT1 for glucose) (Conde-Sieira, Soengas, 2017) regulating production of appropriate hormones. Hormones produced in the brain (e.g., neuropeptide Y and orexins) and peripheral tissues (e.g., ghrelin and cholecystokinin) give the feeding centre signals of food presence and diet composition, and the feeding centre will accordingly adjust food intake, energy expenditure, and homeostasis according to the fluctuation of hormones. The reason why diet composition is a factor influencing hormone production is that to optimize nutrient digestion and utilization in accordance to feed composition and amount ingested, different nutrients are independently sensed by different sensors, and the generated signals may differently influence the hormone production (Bertucci,

et al., 2019), and the released hormones quickly interact between sensors in the intestine, the brain and other regulatory organs in the body.

Osmoregulation

Teleost fish are confronted with great osmotic challenge in both freshwater and seawater. Fish living in freshwater is exposed to hypotonic environment and loose salt continuously across the skin, gills and via the kidney, whilst fish living in seawater must fight dehydration because of the hyperosmolality of the seawater. The intestine plays an important role in keeping optimal ion balance in the fish by regulating ion and water transport according to the demand created by the environment. Marine fish must drink large amounts of water to compensate for the continually water loss to the environment. However, the sea water contains high concentrations of Na^+ and Cl^-, as well as lower concentrations of K^+, Ca^{2+}, Mg^{2+} and SO^{4-} (Whittamore, 2012). Therefore, mechanisms for maintaining ion homeostasis is a key feature of the intestine. Enterocytes absorb Na^+ and Cl^- and K^+ by transporters on the apical side. Simultaneously, enterocytes excrete HCO_3^- which forms precipitates with Ca^{2+} and small amount of Mg^{2+} in the chyme. In this way, osmotic influence of Ca^{2+} and Mg^{2+} is removed, and thus helps maintaining a favourable osmotic gradient for fluid absorption. Transporters involved include Na^+-K^+-Cl^- (NCC) and Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) (Cutler, Cramb, 2008; Esbaugh, Cutler, 2016), and Cl^-/HCO_3^- exchangers (Kurita, et al., 2008).

Water molecules can move across the mucosa paracellularly, i.e., between cells, transcellularly by diffusion, or via water channels, so-called aquaporins (AQPs). Aquaporins increase the osmotic permeability of cell membranes by reducing the activation energy required for dissolution and diffusion of water molecules through the lipid bilayer (Madsen, et al., 2015). Aquaporin 1, 3, 8, and 10 are suggested to be the functional water transporters in teleost (Hu, et al., 2016; Kim, et al., 2010).

Nutrient composition seems to influence intestinal osmoregulation only marginally. Instead, antinutrients originated from plant ingredients may impact intestinal osmoregulation. A common observation in salmon fed diets containing soybean meal is reduced faecal dry matter (Hu, et al., 2016; van den Ingh, et al., 1991), indicating compromised osmoregulatory functions that may eventually result in diarrhea. Increased water content of the chyme may be a result of different factors. On one hand, fluid absorption may be suppressed, as evidenced by down-regulated gene expression of aquaporins and increased blood plasma osmolality (Hu, et al., 2016; Kortner, et al., 2012). Other studies have reported that increased mucosal permeability in salmon fed soybean meal (Knudsen, et al., 2008) likely produced adverse effects on water efflux control mechanisms.

Physical barrier

The physical barrier of the gut consists of a single layer of epithelial cells, connected by the apical junctional complex, composed of tight and adherens junctions and the subjacent desmosomes (Tepass, 2003), and a layer of mucus covering the epithelial surface. Together these layers regulate paracellular and transcellular transport of molecules (Dokladny, et al., 2016), and provide physical and immune defence to fish against invasion of harmful exogenous chemicals and pathogens.

The role of epithelial cells in the physical a barrier is to maintain intestinal luminal milieu. They exert digestion, absorption, and osmoregulation to regulate the movement of nutrients, water, and ion in lumen. The introduction regarding their functions have been mentioned and it will not be expanded in this section.

The mucus is produced by goblet cells located between epithelial cells along the whole intestinal surface. It contains various defence components including mucins, lysozymes, complement components, lectins, antimicrobial peptides and immunoglobulins, which play critical roles in defence against pathogens (Ellis, 2001). Mucins are glycosylated proteins forming the mucus gel matrix. Two types of mucin

genes, annotated as *muc2* and *muc5*, have been widely reported to be present in fish intestine (Peatman, et al., 2015; Sveen, et al., 2017; van der Marel, et al., 2012).

A widely studied structure of the apical junctional complex is tight junction. The tight junction (TJ) is a dynamic and complicated structure at the most apical-lateral space between cellular membranes. They seal the intercellular space and anchor on the epithelial cells which means TJ is under regulation of the epithelial cells. There is no evidence yet indicating that TJ structure in fish differs to that in other animals. As illustrated in Figure 4, TJ in fish comprises claudins, occludins, junctional adhesion molecules (JAMs) and tricellulin. These transmembrane proteins are not solely representing contact between the cell membranes of adjacent enterocytes. They are also directly connected to the intracellular cytoskeleton (actin and microtubules) via zonula occludens (ZOs). Accordingly, tight junctions are affected by both intracellular factors and external luminal substances (Lee, et al., 2018; Shen, 2012). The expressions of TJ genes, and some of the corresponding proteins, have been detected in the intestine of various fish species, including claudin-3, -15, 15a, -25b, -b and -c, occludin, tricellulin, ZO-1 (Ou, et al., 2019; Tipsmark, Madsen, 2012; Tipsmark, et al., 2010; Xu, et al., 2014)

Tight junctions are dynamic structures that may easily be modulated by variation in diet composition via regulation of gene expression. In a study with juvenile tiger puffer (*Takifugu rubripes*), a high-fat diet was found to supress expression of genes coding for TJ proteins (Kong, et al., 2021). The triggering factor may be long chain fatty acids derived from the dietary fat (Suzuki, 2020). Similarly, high-carbohydrate diet was found to decrease the expression of *zo-1*, *occludin*, and *claudin7* (Suzuki, 2020). However, the regulating mechanisms are not clear. An assumption is that the regulation is associated with the glucose transporter, SGLT-1 (Suzuki, 2020). The effects of amino acids on expression of TJ proteins appear complicated. Different amino acids have been reported to exert different effects on the expression of TJ genes. In grass carp, valine deficiency significantly decreased expression of *occludin*, *claudin b*, *claudin c*, *claudin 3*,

and *zo-1*, and induced *claudin 15* expression in intestine (Luo, et al., 2014). In Jian carp,

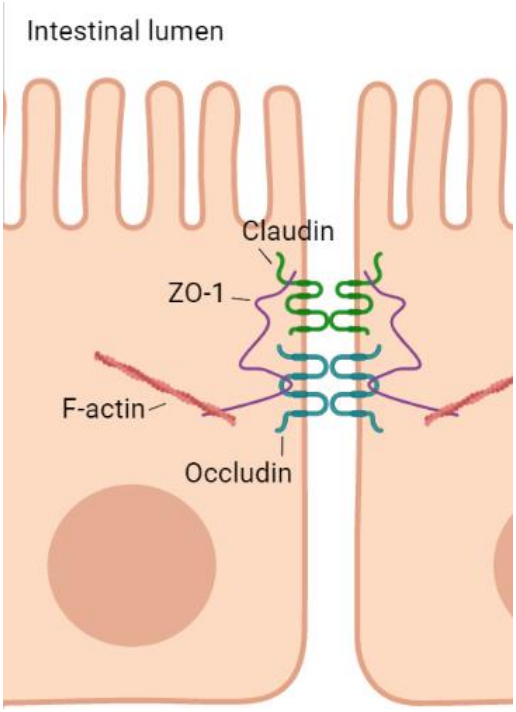


Figure 4. A schematic structure of intestinal epithelial tight junction (TJ) consists of claudin, occludin, ZO-1 and F-actin. Modified from (Lee, et al., 2018; Shen, 2012) and created with BioRender.com.

arginine deficiency increased the expression of *occludin* and *claudin 7* (Wang, et al., 2016), indicating an opposite regulation on *occludin* expression. Some vitamins and minerals, e.g., vitamin A, vitamin D and zinc, are also reported to show effects on expression of TJ proteins (De Santis, et al., 2015; Suzuki, 2020). TJ are dynamic and it appears complicated to elucidate mechanisms how TJ gene expression level affects the physical barrier of intestine. Imbalance of nutrients in diet lead to variation of TJ gene

expression, which might suppress paracellular connection and increase the chance of invasion of alien components, evidencing by increased permeability in intestine of animal fed imbalanced diet (Suzuki, 2020).

Immune function

In addition to the physical barrier, the intestine also harbours a multilayer immune defence system. As other immunogenic organs, the immune system in the fish gut includes both innate and adaptive immune functions (Bjørgen, Koppang, 2022). As in other animals, almost all of the gut immune functions in fish rely on the gut-associated lymphoid tissues (GALT). Microbial pathogens, parasites, and toxins initiate responses in the innate immune system by being recognized by the pattern recognition receptors (PRRs). The PRRs sense conserved molecular structures of a pathogen, known as pathogen-associated molecular patterns (PAMPs), and induce immune responses through multiple signalling pathways. To date, several classes of PRRs, such as Toll-like receptors (TLRs), RIGs-I like receptors (RLRs), NOD-like receptors (NLRs) have been classified in teleosts (reviewed by (Zhu, et al., 2013)). Among them, TLR2, 7, 8 and 9 and NLR3, 5 and X1, have been described to operate in the intestine (Alvarez, et al., 2017; Gao, et al., 2021; Lauriano, et al., 2016; Rawling, et al., 2021). In most cases, resident macrophages are first activated and induce multiple downstream responses, including phagocytosis, cytokine production, and antigen processing and presentation (Forlenza, et al., 2011). Release of cytokines, a family of low molecular weight proteins, is an essential step, initiating cascade reactions which stimulate and recruit leukocytes, e.g., neutrophils, mast cells and lymphocytes. Macrophages, lymphocytes, granulocytes, mast cells, and epithelial cells induced by various pathogens can produce cytokines. Cytokines can be divided into interferons (IFNs), interleukins (ILs), tumor necrosis factors (TNFs), colony stimulating factors, and chemokines (Savan, Sakai, 2006). They regulate the immune responses via different pathways but from the functional aspect, their roles are overlapping. Table 1 categorizes these cytokines by function (modified from (Secombes, 2022)). Briefly described, the functions involve antiviral responses,

initiating inflammatory responses (pro-inflammatory responses), protection against intracellular infection (Type 1 immunity), recruitment of eosinophils as well as basophils and mast cells to inflammation site (Type 2 immunity), protection against pathogens at mucosal surface (Type 3 immunity), inhibition of immune responses (regulatory responses) and migration and recruitment of monocytes, neutrophils, and other effector cells (Secombes, 2022; Zhu, et al., 2013).

Table 1 Grouping of teleost cytokines by function

Function	**Cytokines**
Antiviral responses	Type I IFN: group 1 (IFNa,d,e,h), group 2 (IFNb,c), group 3 (IFNf)
Pro-inflammatory responses	IL-1β, TNFα, IL-6, IL-11, M17
Type 1 immunity	IFN-γ, IFN-γ-rel, IL-2, IL-15, IL-15L, IL-12 family, IL-18
Type 2 immunity	IL-4/13A, IL-4/13B, IL-20L
Type 3 immunity	IL-17A/F, IL-17B, IL-17C, IL-17D, IL-17N, IL-21, IL-22, IL-26
Regulatory responses	nIL-1F, IL-10, IL-35, TGF-β1
Leukocyte recruitment to infected sites	CC chemokines, CXC chemokines and their receptors

* Modified from Secombes (2022)

The categorization of the cytokines does not indicate that they are independent of each other. On the contrary, most cytokines participate in various signal transduction mechanisms, so elucidating all functions for one cytokine is complicated and not appropriate. IL-1β, TNFα, IL-8 and IFNγ are pro-inflammatory cytokines commonly observed to be induced during inflammatory reactions, e.g., in the intestines of fish (Couto, et al., 2014; Kortner, et al., 2012; Krogdahl, et al., 2015b; Wang, et al., 2020). Aside from cytokines, antimicrobial peptides, complement components, and lectins also play important roles in fish innate immunity (Zhu, et al., 2013). For example,

complement components and lectins have been found to respond to parasite infection in fish intestine (Martin, et al., 2016).

The adaptive immune system is activated when lymphocytes interact with antigens presented by antigen presenting cells (APCs) of the innate immune system. B and T lymphocytes (B and T cells) together with antibodies primarily mediate adaptive immune responses in fish. B cells and the antibody-secreting function is conserved throughout evolution in all vertebrates (Parra, et al., 2013). In addition, B cells play roles in innate immunity, e.g., phagocytic activity and cytokine production, which are also conserved (Parra, et al., 2015). Immunoglobulins (Igs) are antibodies secreted by B cells. The isotypes identified in fish include IgM, IgD and IgT/IgZ. Among them, IgM and IgT/IgZ dominate the humoral adaptive immunity in the intestine. Particularly, IgT/IgZ has been demonstrated to have a specific role in intestinal mucosal immunity. In rainbow trout (*Oncorhynchus mykiss*), a B-cell lineage was found to only express IgT (Zhang, et al., 2010). The same study also showed that the IgT concentration was greatly induced by a parasite infection in the intestine, whereas the concentration of IgM was unaffected. Regarding the role of IgD in the intestine, it remains unclear. It has been detected in the intestine, but its gene expression was not clearly induced by a bacterial infection (Xu, et al., 2019).

T cells act mainly as coordinators of other immune cells and as effector cells, killing infected or tagged cells. T cells are characterized by the presence of a specific receptor, the T cell receptor (TCR) through which they recognize antigens. The cluster of differentiation (CD) 3 forms a complex with TCR and generates an activation signal intracellularly (Blanchard, et al., 2002). In teleosts, three chains have been identified (CD3-γδ, -ε and -ζ) (Laing, Hansen, 2011). T cells can be subdivided into T cytotoxic (Tc) or T helper (Th) cells, depending on the type of CD expressed on their cellular membrane. Tc cells express CD8 and are able to kill infected cells. Th cells express CD4 and produce cytokines to regulate the action of other immune cells, mainly B cells. The number of gut-associated intraepithelial T cells seems comparable to the number of T cells from other secondary lymphoid organs, an indication of the importance of the

intestine in T cell responses (Tafalla, et al., 2016). Expression of the genes coding for CD4α, TCRγ, CD8α, CD8β, CD3γδ, etc., are widely used as biomarkers to indicate immune responses of T cells in fish intestine (Blanchard, et al., 2002; Liu, et al., 2015; Tafalla, et al., 2016; Wang, et al., 2020).

Intestinal immune regulation involves a large number of immune components and shows responses to variation in diet composition, in particular to deficiencies and excesses. It is well known from the study of the immune apparatus in land animals (Calder, 2013) that suboptimal supply of macronutrients, as well as of most essential micronutrients affect intestinal immunity by altering gene expression of inflammatory cytokines introduced above, e.g., genes coding for TNFα, IL-1β and IL-8, activities of immune relevant enzymes, e.g., lysozyme, and acid phosphatase, and contents of complement C3. Similar alterations were also observed in fish. In grass carp intestine, dietary tryptophan inclusion level showed a quadratic relationship with immune-relevant biomarkers, i.e., expression of IL-10 and TGF-β increased firstly till 3.1-4.0% and then decreased, while expression of TNF- α, IL-8 and TOR showing an opposite trend (Wen, et al., 2014). Varying vitamin A level in diet also showed quadratic relationship with immune biomarkers in grass carp intestine (Zhang, et al., 2017). Lysozyme activity, contents of component 3 and 4, gene expression of anti-inflammatory cytokines (*il-10*, *il-11*, *tgf-1b*) and antibacterial peptides (*β-defensin-1, hepcidin, leap-2a* and *leap-2b*) were increased with increased vitamin A level, reached a peak between 0.42-0.62 mg/kg, and decreased thereafter, while gene expression of pro-inflammatory cytokines (*tnf-α, ifn-γ2, il-1b, il-6, il-8, il-15* and *il-17d*) showed opposite trends. In largemouth bass intestine, increasing starch level in diet from 5% to 15% increased expression of *tnf-a*, *il-1b*, *il-10*, *il-8* and *tgf-1b* (Zhou, et al., 2021).

Antinutrients, prebiotics and their effects in the fish intestine

Antinutrients are a group of very different endogenous components in feed ingredients, mostly of plant origin, which may affect normal physiological processes in an animal

(Krogdahl, Bakke, 2015). Increasing the proportion of plant-based ingredients in fish diets thus increases exposure to antinutrients, which may severely affect intestinal health. In the present context, the compounds mentioned in the following are of particular relevance. Many soybean-based ingredients contain saponins, steroid or triterpenoid glycosides which are heat-stable and amphipathic. Saponins have been shown to impair intercellular junctions and increase membrane permeability in the intestine (Gu, et al., 2018; Knudsen, et al., 2008). The increase in permeability may lead to increased influx into the tissue of antigens and other alien components, resulting in a progressing inflammatory response which triggers several immune functions, such as production of cytokines (Kortner, et al., 2012; Krogdahl, et al., 2015b) and migration of lymphocytes into the tissue (Chikwati, et al., 2012). Lectins, another antinutrient present in soybean-based ingredients, have also been shown to affect intestinal health, as they may bind to cellular receptors and other cell components with carbohydrate-rich side chains on the epithelium of intestine (Hendriks, et al., 1990). When present in the diet at a level of 3.5%, morphological changes in the distal intestine of Atlantic salmon have been observed, including cellular infiltration in the submucosa and lamina propria and alterations in the villous integrity (Buttle, et al., 2001).

The term prebiotic refers to a group of non-digestible feed ingredients which may beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. They may also act by direct effects on immune functions, and thus improve host health (Gibson, Roberfroid, 1995). Due to their expected health-promoting properties, prebiotics are now extensively used in fish diets. In aquaculture, β-glucans are one type of commonly used prebiotics. In fish intestine they seem to be recognized by pattern recognition receptors (PRRs) like Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and complement receptor type 3 (CR3) (Reviewed by Ching et al (2021). The induced immune responses comprise, as examples, production of proinflammatory cytokines like IL-1β and TNF and increase of intraepithelial lymphocytes (Ching, et al., 2021). Despite numerous experiments demonstrating positive effects of prebiotics on immune regulation (Guerreiro, et al., 2018), the basic mechanisms for their actions are not well-established. In some cases,

however, studies of effects of prebiotics have not shown the expected health promoting effects (Wang, et al., 2020). Therefore, when new species are selected for cultivation, such as lumpfish and B. wrasse, for which fundamental knowledge of every aspect is lacking, effects of prebiotics must be carefully evaluated before use.

The digestive tract of Ballan wrasse and lumpfish

B. wrasse and lumpfish are two fish species with a digestive tract of very different structure. The digestive tract of the B. wrasse is studied to a greater extent than the lumpfish. Therefore, the knowledge and understanding of the function of the digestive tract of the wrasse is more solid than that for the lumpfish. In the following sections, present knowledge for the two species is reviewed in separate chapters.

Ballan wrasse

B. wrasse is a special fish species regarding its intestinal structure and food preference. It is carnivorous and naturally preys on hard-shelled crustaceans, e.g., molluscs, decapods and isopods (Dipper, et al., 1977). It is a stomachless fish with a short intestine, which differs from most of carnivorous fish species. The intestine is usually 2/3 of the body length (Fig 5 A). It can be divided into four segments of similar length (Fig 5 B). A bulb is observed in the most proximal part of the intestine (IN1). The following segments (IN2 and IN3) lie in the abdomen in one loop before the most distal part of the intestine (IN4).

The work of Lie et al (Lie, et al., 2018) shows that the absence of a stomach is not only anatomically but also functionally as genes related to stomach functions are absent. Genes related to functions of the intestine, however, are the same as found in other species. The proximal intestine shows high expression of genes relevant for digestion and absorption, less so for immune genes. In the distal intestine genes relevant to immune functions are more prominent. These results were confirmed in the work of Bilal et al (Bilal, et al., 2019) showing that the distal intestine in B. wrasse expresses

genes coding for immunoglobulin M, immunoglobulin T and T cell receptor alpha. Also the PhD work by Le (Le, 2019), published in three scientific papers (Le, et al., 2019a; Le, et al., 2021; Le, et al., 2019b), describes important features of the intestine in B. wrasse. This work indicates that, for B. wrasse, moisture level in the diet affects localization of the digestive processes. Increasing moisture content moved protein and carbohydrate digestion and absorption distally but did not affect total apparent digestibility of these nutrients (Le, et al., 2019b). Moreover, receptors for cholecystokinin (CCK), a hormone which, in other animals, modulates digestive mechanisms by inhibiting gastric emptying, were identified (Le, et al., 2019a). A follow-up *in vitro* study showed that the receptors were functioning, and modified motility patterns of intestine. The *in vitro* studies also indicated how protein, lipid and cellulose may influence intestinal motility patters (Le, et al., 2021). Protein and lipid reduced the frequency of both non-propulsive and propulsive contractions and prolonged the residence of the chyme in the proximal segment, supposedly to secure optimal utilization of the nutrients, which also influences genes regulating intestinal evacuation rate. Cellulose, on the other hand, induced propulsive contractions which rapidly moved this indigestible material towards the distal segments.

The fact that the alimentary tract of the B. wrasse lacks a stomach, and correspondingly, lack of adidic protein digestion, may demand species-specific characteristics of the diets to be used in cultivation. A recent study documented, and confirmed earlier indications, that pellet consistency greatly affected intestinal functions, health and production of B. wrasse (Kousoulaki, et al., 2021). In this study agglomerated, cold-extruded and extruded feed were compared. The feed did not influence the growth of fish. However, mortality was increased in the fish group fed the extruded diet. The same regarded level of triglycerides and cholesterol in the blood. In the intestine higher specific activity of leucine aminopeptidase and maltase were observed. Moreover, B. wrasse fed diets with plant ingredient as protein sources showed increased number of intestinal mucus cells, while growth, survival and feed efficiency were not affected (Cavrois-Rogacki, et al., 2022). This indicates that some components coming from plant ingredients, such as antinutrients, may interact and alter mucus production in the intestine.

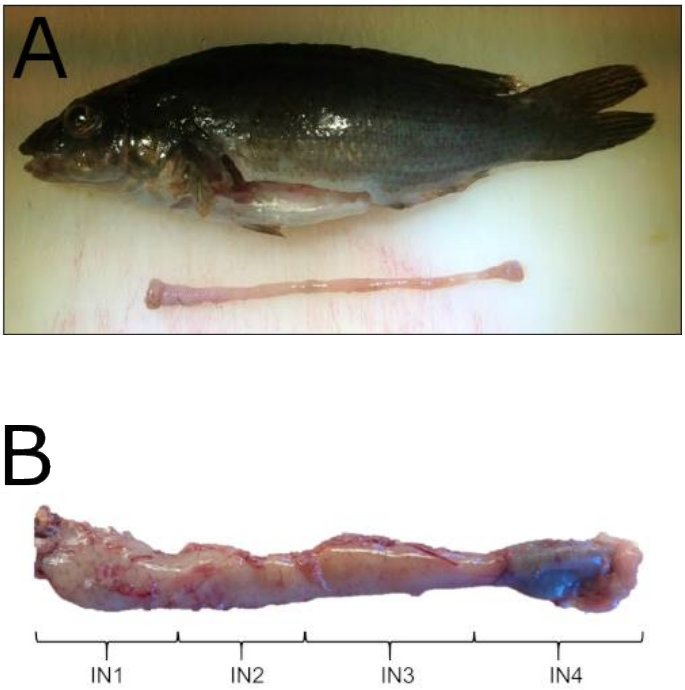


Figure 5. Ballan wrasse and its intestine (A) and the sectioning of intestine (B). Images modified from Kousoulaki, et al. (Kousoulaki, et al., 2014) and **Paper I**.

Lumpfish

Lumpfish has an alimentary tract very different from B. wrasse, in particular as it comprises an intact stomach. Moreover, just distal to the pyloric sphincter, a number of pyloric ceca are attached (Fig 6). The intestine is almost twice as long as the body. The intestinal tract terminates with a bulb-like structure which is connected to the anus. The intestine of the lumpfish is quite similar to that of the intestine of Atlantic cod (Fig 6c). Zhukova and Stroganov (Zhukova, Stroganov, 2022) described the anatomical structure of the alimentary tract in lumpfish, with focus on esophagus and the stomach.

The oesophagus is relatively short with a thick muscular wall whose thickness may increase more than two folds during contraction. The stomach is distinguishably divided into cardiac, fundus, and pyloric parts. A high density of gastric glands are located in the fundus part and less in the cardiac part. They are absent in the pyloric part. The location of these cells indicates that the highest digestive activity occurs in the fundus part. The intestinal length is estimated to be 1.2 times of the standard body length, and the histological structure found the same throughout its entire length.

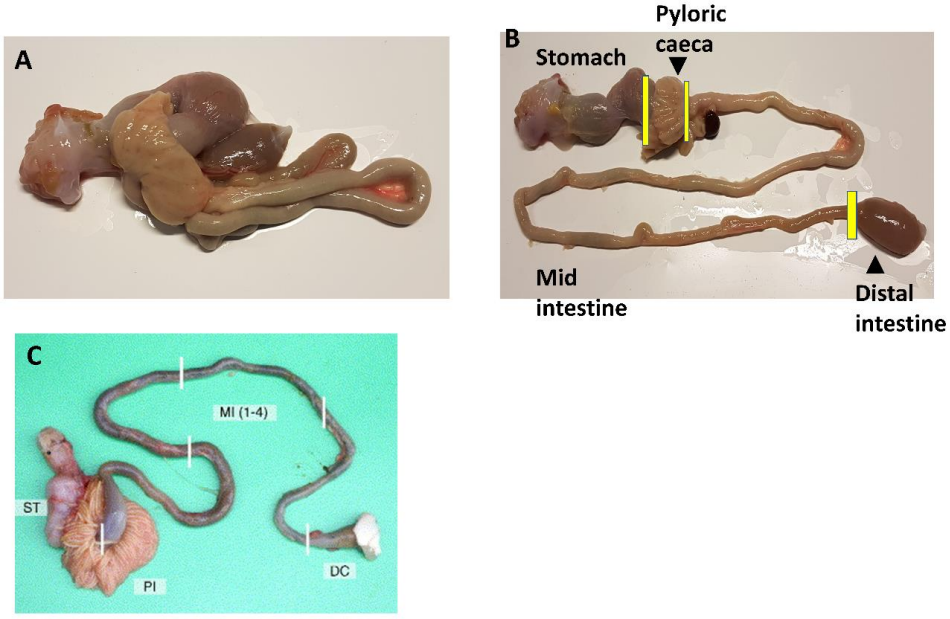


Figure 6. Lumpfish gastrointestinal tract in situ (A), expanded (B) and Atlantic cod gastrointestine (C). Localization of stomach, pyloric caeca, mid intestine and distal intestine is given for lumpfish; and localization of stomach (ST), pyloric intestine with caeca (PI), mid intestine (MI, sectioned into four parts), and distal chamber (DC) is given for Atlantic cod. Figure A and B are derived from Paper III. Figure C is derived from Restie et al (Refstie, et al., 2006).

In this context, intestinal characteristics of Atlantic cod might be relevant for comparison to lumpfish as they, macroscopically, look quite similar. However, at least one physiological characteristic differs clearly, i.e., the lumpfish expresses a much lower amylase activity in the intestinal chyme (2U/mg dry matter) than the Atlantic cod (56U/mg dry matter) (values from **Paper III** and (Refstie, et al., 2006)). At present, published studies of the function of the intestine of the lumpfish are few. Marthinsen (2018) investigated ontogenetic development and function of the digestive system in lumpfish larvae revealing close similarities with other teleosts. The study was conducted employing three diets: dry feed, live copepods or live *Artemia*. *Artemia* gave the highest growth rate and earliest maturation of many histological characteristics of the gastrointestinal tract, whereas the other two diets resulted in slower development than *Artemia*. A very recent study (Willora, et al., 2022) showed that exchanging a diet with 75% of fishmeal for a diet in which the fish meal was replaced by soy and pea protein concentrate, did not affect specific activity of alkaline protease, chymotrypsin, trypsin, leucine aminopeptidase and amylase. However, fish fed the plant-based diet showed structural changes in the intestine, including decreased mucosal fold height, increased number of goblet cells, and increased thickness of the muscularis layer. These results indicate that inclusion of plant ingredients modulate intestinal function, but this may take place without alteration of digestive capacity.

Summary of the study progresses in B. wrasse and lumpfish

Present knowledge of the structure of the digestive tract of the two cleaner fish species, for which cultivation recently started, is fairly well described, whereas knowledge of functions of the digestive organs has many gaps, in particular for lumpfish. Regarding B. wrasse, studies have identified mRNA profiles of segments from proximal to distal in B. wrasse, which provide an overview of functions exerted by each segment. Motility patterns of the intestine to nutrients have also been elucidated. Besides this, intestinal

responses to diet moisture have been studied in B. wrasse. For lumpfish, anatomical characteristics of the alimentary tract has been documented, with focus on stomach and oesophagus. A study also demonstrated the effects of plant ingredients in diet on intestinal function. However, other information relevant to the intestine is rare, especially the effects of diet composition. Compared to fish species which are well studied regarding intestinal functions, e.g., Atlantic salmon, considerable basic knowledge needs to be generated, including but not limited to effects of varying diet composition on gut function and health, immune responses to antinutrients and general morphological features of intestine, etc., before a similar level of understanding is reached. Strengthening of the understanding of digestive functions and factors which may compromise them, is necessary for formulation of diets which satisfy the nutrient requirement of fish. Meeting the requirement levels for energy, macro- and micronutrients is a prerequisite for securing good growth, health and welfare of all animals. However, also knowledge on nutrient requirements for cleaner fish has severe knowledge gaps. At the time of the start of this PhD. project, the commercially available feeds for B. wrasse and lumpfish were formulated mainly based on estimates of minimum requirement of nutrients for other fish species. As the physiology of B. wrasse and lumpfish gut has important differences, it is likely that these two species have different nutrient requirements. Studying effects of diet composition on intestinal function and health generates knowledge of another aspect, apart from growth, and facilitate the understanding of their nutrient requirements.

AIMS OF THE WORK

The overall goal of this PhD work was to strengthen the knowledge and understanding of diet effects on intestinal functions and health in both Ballan wrasse and lumpfish as basis for formulation of diets with optimal composition for production of healthy cleaner fish. To achieve this, the following subgoals were formulated:

1. To characterize and compare intestinal morphology and functions of wild-caught and cultivated Ballan wrasse for better understanding of their digestive physiology and implication for nutrient utilization (**Paper I & II**).

2. To understand mechanisms underlying diet-induced inflammation in the intestine of Ballan wrasse by use of soybean saponins and prebiotics (**Paper II**).

3. To profile general characteristics regarding intestinal function and health in lumpfish (**Paper III & IV**).

4. To understand the effects of diet composition on intestinal function and health disease resistance in lumpfish (**Paper III & IV**).

HYPOTHESES

The following hypotheses were formulated based on the information presented in the introductory chapter. These hypotheses were the basis for the aims of the current thesis work:

- Intestinal morphology and functions of wild-caught and cultivated Ballan wrasse slightly differ from each other.

- Inclusion of soya saponins in the diet will lead to inflammatory reactions in B. wrasse, and the inclusion of prebiotics cam improve intestinal health and reduce the inflammatory reactions caused by soya saponins.

- The characteristics regarding intestinal functions in lumpfish are highly similar to those of other carnivorous fish species with stomach.

- Variation in diet composition results in alteration of intestinal functions in lumpfish.

SUMMARY OF PAPERS

Paper I: Digestive function and intestinal immune apparatus in wild Ballan wrasse (*Labrus bergylta*)

The work in **Paper I** was carried out to profile key characteristics of intestinal functions and health in wild-caught Ballan wrasse to provide a reference for evaluation of diet effects on intestinal function and health in cultivated fish. To study variation in physiological characteristics along the intestine, samples were collected from four intestinal segments, named IN1, IN2, IN3 and IN4 for the most proximal segment to the most distal segment, respectively. The intestinal structure appeared quite similar in the four segments with regards to mucosal fold height and degree of fold branching, lamina propria and submucosal width and cellular composition and thickness of the muscle layers. The highest digestion and absorption activity were identified in IN1. The gene expression of *igm* were higher in IN4, suggesting an important role of the posterior part of the intestine in the immune responses related to *igm*. However, other immune related genes, *lyz*, *il1b*, *il6*, *cd40* as well as the cell proliferation marker *pcna*, showed no difference between the proximal and the distal part of the intestine. infiltration of lymphocytic and eosinophilic granular cells in the submucosa and lamina propria was observed in all segments, seemingly following presence of parasites, especially myxozans such as *Enteromyxum leei*. Blood plasma nutrient concentration were lower in wild Ballan wrasse compared to cultivated fish except for glucose, which was higher in wild wrasse, suggesting that the wild fish were in an unfed or starving status.

Paper II: Soya saponins and prebiotics alter intestinal functions in Ballan wrasse (*Labrus bergylta*)

The aim of the work published in this paper, which comprised a 5-week feeding trial with the cleaner fish Ballan wrasse (*Labrus bergylta*), was to gain better understanding of the basic biology of intestinal functions and health in B. wrasse, and how plant

components might affect the functions and thereby strengthen our knowledge basis for developing suitable diets for this species. During the trial, effects in the fish of four diets were evaluated: a reference diet or the reference diet supplemented with *i)* antinutrient soya saponins (0.7%) *ii)* a commercial prebiotic (Aquate™ SG, 0.4%) or *iii)* a combination of soya saponin and the prebiotic. Blood, intestinal tissues, and gut content from four consecutive intestinal segments (IN1 – IN4) were collected.

No significant differences in fish growth were observed between fish fed the four diets. Saponin supplementation, both alone and in combination with prebiotics, increased weight index of IN2 and IN3 and decreased blood plasma glucose, cholesterol, and total protein. Dry matter of intestinal content and activity of digestive enzymes were not affected by diet. Histomorphological analyses revealed a progressing inflammation with increased infiltration by immune cells particularly into the distal parts of the intestine in fish fed diets with saponins, both alone and in combination with prebiotics. Gene expression profiles obtained by RNA sequencing and quantitative PCR mirrored the histological and biochemical changes induced by the saponin load. The study demonstrated that Ballan wrasse gut health and digestive function may be markedly affected by feed ingredients containing antinutrients.

Paper III: A screening study on effects varying dietary macronutrient composition on gut functions in lumpfish (*Cyclopterus lumpus*)

This paper presents results from two experiments conducted to deepen our knowledge on lumpfish intestine physiology. Experiment 1 was a 42-day feeding trial in which lumpfish were fed twelve different diets in the following ranges of macronutrients: Protein 43-68%, lipid 4-17%, and carbohydrate 6-17%. Intestinal tissue, gut content and liver were sampled from 6 fish per tank. The results showed that with increasing lipid level and corresponding decrease in protein level, there was a linear decrease in several of the observed biomarkers, including activity of brush border membrane digestive enzymes, expression of genes related to nutrient digestion and transport, ion

exchange and immune regulation. Increased intracellular hyper-vacuolization (probably accumulation of lipid) was observed in gut and liver with increasing dietary lipid level. Fewer effects were observed for increased dietary carbohydrate and corresponding decreased protein level.

Experiment 2 was a two-week feeding trial for estimation of macronutrient digestibility in which lumpfish were fed three diets, all containing 55% crude protein, with lipid to carbohydrate ratio of the low lipid diet of 7.5%/18.3%, the medium lipid diet of 13.8%/14.6%, and high lipid diet of 18.1%/9.5%. Fecal samples were collected as pooled samples per tank. These results showed that fatty acid digestibility increased as dietary lipid level increased. On the other hand, starch digestibility decreased greatly as starch level increased, whereas protein digestibility did not change as lipid or starch level varied. Taken together, the results of the studies presented in this paper indicated that increasing lipid level in the diet with corresponding decrease in protein level affects digestion, absorption, as well as immune functions in the lumpfish intestine. Variation in dietary carbohydrate to protein level showed less effects, possibly due to low starch digestibility which makes the variation in available carbohydrates much less than the variation in the analysed level of dietary carbohydrates.

Paper IV: Effects of dietary lipid level on growth, digestive physiology and disease resistance in lumpfish (*Cyclopterus lumpus*)

The work in **Paper IV** was conducted to follow up the study in **Paper III** and investigated effects of feeding lumpfish diets varying in lipid/carbohydrate ratio on the outcome of infection with *Aeromonas salmonicida*. Three experimental diets were formulated to have similar content of crude protein and ash but varying in content of lipid/carbohydrates from 6.7/18.0 – 18.0/8.1 %. Lumpfish with average start weight of 1.7 g were fed the experimental diets in triplicate tanks for a period of 90 days. After termination of the feeding trial and subsequent collection of biological samples, remaining fish were challenged with atypical *A. salmonicida.*

No significant effects of diet were observed for growth. However, carcass composition showed increasing content of dry matter, lipid, protein and energy with increasing dietary lipid level. Increasing dietary lipid also increased hepatic dry matter, lipid, and energy level, while crude protein decreased. Blood plasma nutrient levels and liver function biomarkers showed a few significant effects of diet, such as increasing effect of increased dietary lipid level on plasma cholesterol. Intestinal trypsin activity increased with increasing dietary lipid, whereas activity of other digestive enzymes and digesta bile salt levels were unaffected by diet. Increasing lipid level also increased lipid accumulation in the proximal and mid intestine. Expression profiling of genes related to digestive and immune function showed few effects of diet, but the nutrient transporters *fabp2* and *slc15a1*, as well as the immune genes *mhcII, igm*, and *nfkb* increased with dietary lipid levels, whereas the cholesterol transporter *npc1l1* was suppressed. Diet composition did not affect the lumpfish' resistance against *A. salmonicida*. To conclude, the variation in macronutrient composition induced modulations in metabolic, digestive and some immune functions. Modulations seemed however to be within normal ranges and did not produce affect immune responses to bacterial infection.

METHODOLOGICAL CONSIDERATIONS

As available information relevant for intestinal functions in B. wrasse and lumpfish at start of this PhD work was limited, comparison to biomarkers that showed responses in other species was the strategy of the PhD work. Intestinal function and possible effects of diet were evaluated by using biomarkers at different levels of biological organization, including digestive enzyme activity, gene expression profiling, blood plasma biochemistry, and histomorphological analyses. Among them, enzymatic assays and gene expression profiling are further discussed this chapter.

Using mixture design to investigate intestinal functions

The study presented in **Paper III,** aimed to find optimal balance between macronutrients in lumpfish diets. In most studies of optimal macronutrient level in fish diets conducted until now, the 'one-variable-at-a-time' (OVAT) design has been used. However, the close relationship between the content in the diet, the fact that they all provide energy, but at different concentration, and the difference between them regarding functions in the body, makes it very difficult to vary only one nutrient at a time without affecting the effects in the body of the other two. Adjusting the level e.g. of protein, given e.g., as percent or weight per kilo of the diet, will change the energy concentration of the diet, and inevitably lead to changes in value and function of other components (Ruohonen, Kettunen, 2004). To overcome such dependencies the use of non-nutritive fillers has been common (Ruohonen, Kettunen, 2004), but use of fillers may induce other unintended effects, not at least regarding digestive functions. To reach good estimates of optimal balance between protein, lipid, and carbohydrate in diets for an animal species, by using the OVAT design, many experiments must be conducted, and the results need to be integrated in meta-studies, which all in all becomes time consuming and resource demanding.

An alternative experimental design, the mixture design, has recently been put to use in aquaculture to find optimal macronutrient balance in fish diets and was used in the study presented in **Paper III**. The method was first developed to find the optimal balance in mixtures of more than two ingredients in which interaction between the ingredients is of great importance for the characteristics of the mixture (Scheffé, 1958). It allows the interacting components to be changed simultaneously, continuously, and systematically and focuses on responses of variables which depend on the relative proportion of the ingredients. The mixture design is the most used methodology in many industrial areas, e.g. in concrete (Jiao, et al., 2018), chemical (Akalin, et al., 2010), pharmaceutical (Mahdhi, et al., 2010), and food industry (Nikzade, et al., 2012; Toker, et al., 2013).

Four studies have been found in the scientific literature, addressing optimal macronutrient balance in fish diets, all from the same research group, i.e., a group involved also in the present work (Hamre, Mangor-Jensen, 2006; Hamre, et al., 2013; Hamre, et al., 2003; Zhang, et al., 2012). The experience gained shows that the mixture model supplies useful information and may save time, resources, and fish, in the search for improvement of fish diets. However, to find the optimal balance between three components by using the mixture design, implies evaluation of effects of many diets varying in composition of the components over wide ranges. Accordingly, such experiments are demanding regarding number of treatments, tanks, fish, labour, as well as cost. Compromises must therefore be made regarding and levels observed for each component and number of replicates, resulting in less accurate results than wished for. However, the mixture design can supply better information on optimal nutrient balance than the traditional experimental designs and save time and money.

In the present Thesis work the experiments presented in **Paper III** and **IV** can illustrate some of the challenges experienced in the nutrition area which may be related to differences in experimental design and/or variation between experiments in factors which may influence the results. The work in **Paper IV**, conducted based on the OVAT design, was a follow-up of the work in **Paper III**, conducted according to the mixture

design. The cause of the follow-up was questions which arose regarding digestibility, in particular regarding carbohydrates, an important variable of importance for interpretation of the results. However, faeces had not been collected from the first study, necessary for analysis of nutrient digestibility. A digestibility experiment with a limited number of treatments was therefore conducted to get indications whether starch digestibility might be affected by starch level in the diet. Carbohydrate digestibility has been found to vary greatly between fish species (Krogdahl, et al., 2005). For this discussion, it suffices to mention the results for expression of the *igm* gene in the tissue of the distal intestine. In the follow-up study presented based on OVAT, the *igm* expression increased with lipid level from 6.7% to 13.7% and seemed to plateau or decrease at higher level up to 18%. In the work employing the mixture design, the results for igm were quite different. For a constant protein level of 53%, the picture seemed very different, decreasing with increasing lipid level between 6.7 and 13.7%, and increasing thereafter.

The cause of this apparent difference in results may be lower accuracy of the estimates of *igm* expression in the experiment with the mixture design, i.e., that the indicated differences between the diets were far from significant. Other possible causes may be related to the experimental conditions which were different, as they were performed at different places, with differences in facilities and water quality, with fish from different sources, and with new batches of diets. The physiological meaning of the differences in results for *igm* is also difficult to suggest. Further studies are clearly needed to be able to conclude regarding the magnitude and direction of effects on *igm*-expression in the studies, and to understand the consequences of the effects.

Enzymatic assays

Enzyme activity assays are conventional methods to obtain information on digestive functions. The methods established in our group comprise activity of leucine aminopeptidase, maltase, trypsin, α-amylase, and lipase, which are indicators of protein,

starch, and lipid hydrolysis. In biochemical contexts, enzyme activity is often expressed as product formed (or substrate converted) per unit of pure enzyme per time unit (Bisswanger, 2014), termed specific activity. The term is used also in nutritional context, but with a different definition i.e. as product formed per milligram of total protein in a tissue homogenate or extract. The difference in definition of a term used by two closely related research fields may cause misunderstandings and should be kept in mind when comparing biochemical and nutritional results. In most nutritional studies the enzyme preparations are homogenates of a tissue or organ, or an extract of gut content or faeces. However, these enzyme sources are very heterogeneous, not at least regarding content of other than enzyme protein. This fact must be taken into consideration when interpreting effects on specific activity. For example, a decrease in specific activity may be due to an increase in other proteins than the one of interest. Expressing the activity as enzyme capacity, i.e., enzyme activity per organ, normalized to fish weight, often gives a better picture of effects on digestive functions.

Gene expression profiling

Gene expression profiling has become a widely applied analytical approach for functional studies. For intestinal functions which are difficult to determine at protein, metabolite or structural level, determining them at transcriptional level is a good alternative. Quantitative real time PCR (qPCR) and RNA sequencing (RNAseq) were the two methods used for investigating gene expression in the current PhD work. The fundamental difference between these two methods of gene expression profiling is that RNAseq generates an untargeted, global transcriptome dataset, whereas qPCR is used for targeted expression profiling of specific biomarker genes of interest. RNAseq is therefore often applied in studies of more explorative nature where there is little prior knowledge on how gene expression may respond. On the other hand, qPCR can be a favourable and less time- and resource demanding approach in studies where good biomarker genes of different functional categories are known. For B. wrasse, global RNAseq was firstly applied as a holistic approach to increase the general knowledge of B. wrasse gut functions at molecular level, and to monitor responses to soya saponins

and prebiotic inclusion (**Paper II**). For the wild fish study of **Paper I**, a targeted qPCR approach was chosen. Genes belonging to different functional categories were selected based on results from the RNAseq study in **Paper I**, as well as previous experience from related studies in salmon and other fish species. The studies in lumpfish also applied qPCR for expression profiling. Genes of interests were selected to cover important gut functions such as digestion, osmoregulation, barrier function and immune regulation. By the time of selecting genes, there was very little sequence data, and no reference genome, available for lumpfish. Primers for qPCR assays were designed based on a de novo genome, at that time unpublished, and in the process of being assembled and annotated. This raised the problem that some of primers were incorrectly designed. When **Paper III** was drafted, a high-quality annotated reference genome for lumpfish was uploaded to NCBI, and we got a chance to better validate the designed primers by comparing the primers to the reference sequence database of RNA. Eventually, primers initially designed to amplify aquaporin 1 (*aqp1*) and interleukin 8 (*il8*) were found to be amplifying tight junction protein 1 (*tjp1*) and chemokine (C-X-C motif) ligand 19 (*cxcl19*), whereas primers for t cell receptor (*tcr*) and cluster of differentiation 40 (*cd40*) got no matches to the reference sequence database. The other genes of interest were also blasted against the reference genome and were confirmed to be correct.

DISCUSSION OF MAIN RESULTS

Intestinal morphology and functions of Ballan wrasse

Digestive function of B. wrasse

The current work characterised intestinal functions in both wild (**Paper I**) and cultured B. wrasse (**Paper II**). By comparing biomarkers of digestive processes in the proximal intestine, where most of the nutrients are absorbed, wild B. wrasse seemed to have the highest digestive capacity. This is based on the fact that, in the wild fish relative weight of the intestine was approximate 0.8%, LAP capacity was about 65U/kg fish, and maltase capacity was 50U/kg fish, while in cultured fish, the relative weight of intestine was 0.4%, LAP capacity was 25 U/kg fish and maltase capacity was 5 U/kg. The reason for these differences can be several. The results indicate that fish weight was not an important contributor to the differences. As shown in figure 7, values of the three biomarkers are generally higher in wild fish than in the cultured fish, independent of weight, e.g., at about 100 g weight. Therefore, even though the captured wild fish had higher average weight, higher digestive capacity in wild fish should not be ascribed to higher average weight. Among other possible explanations for this difference are dietary composition and frequency of nutrient supply. The natural diet of the B. wrasse mostly comprises prey such as echinoderms, gastropods, and decapods many of which contain tough chitinous exoskeletons, and meals come irregularly and at very different sizes (Figueiredo, et al., 2005). Cultured B. wrasse eat feed with composition which over long time has a stable composition and is served in rather constant portions and at regular interval. This may be less demanding and be the cause of lower capacity of digestive enzymes. It is well known from other animals that production and secretory capacity for proteolytic enzymes in the pancreas increases, e.g., when ingredients containing protease inhibitors are included in the diet, and the weight of the proximal intestines may increase as well (Ge, Morgan, 1993). This is also the case in Atlantic salmon (Olli et al., 1994). Stable supply of a diet with constant content of highly digestible nutrients, may require less digestive capacity than what is the situation for

wild wrasse which eat irregularly and with great variation in meal size and content of material with low digestibility, a situation which may require higher digestive capacity. Protein in formulated diets is in general heat treated by extrusion and thereby denatured, and therefore easier to digest, particularly in a species without stomach. Native proteins, as in natural prey fish, needs denaturation before digestive processes can take place. In a stomachless fish, lack of initial acid denaturation of the proteins, may be a challenge and increase time of digestion, and demand higher digestive capacity.

On the other hand, the histological structure appeared largely similar in wild and cultured B. wrasse. However, the minor differences observed may be important for the function of the short intestine of B. wrasse. Firstly, thicker fibrous submucosa and lamina propria in the wild B. wrasse is likely an adaptation to the natural diet containing tough chitinous exoskeletons from the ingested prey (Figueiredo, et al., 2005). Secondly, the submucosa and lamina propria were wider in the wild fish. The explanation for this observation cannot be found in the present results. In Atlantic salmon, widening of the submucosa, and lamina propria are often associated with inflammation (Krogdahl, et al., 2003). However, as other typical signs of inflammation were not observed, such as infiltration of leukocytes, the difference in width of the lamina propria is less likely to indicate inflammation in the tissue. Lastly, supranuclear vacuoles were not detected in the enterocytes in any of the investigated intestinal regions. In salmonids, supranuclear vacuoles are normally found in the distal intestine, while in cyprinids, they are located in the second segment of the mid intestine (Ng, et al., 2005; Rombout, et al., 1985; Wallace, et al., 2005). The supranuclear vacuoles are known to diminish upon starvation and intestinal inflammation (Baeverfjord, Krogdahl, 1996; Uran, et al., 2008). However, these may not be the reasons in B. wrasse as the absence was also observed in cultured fish fed reference diet in **Paper II**, which was supposed to be healthy and well-fed. Therefore, this observation could be a species-specific characteristic of B. wrasse.

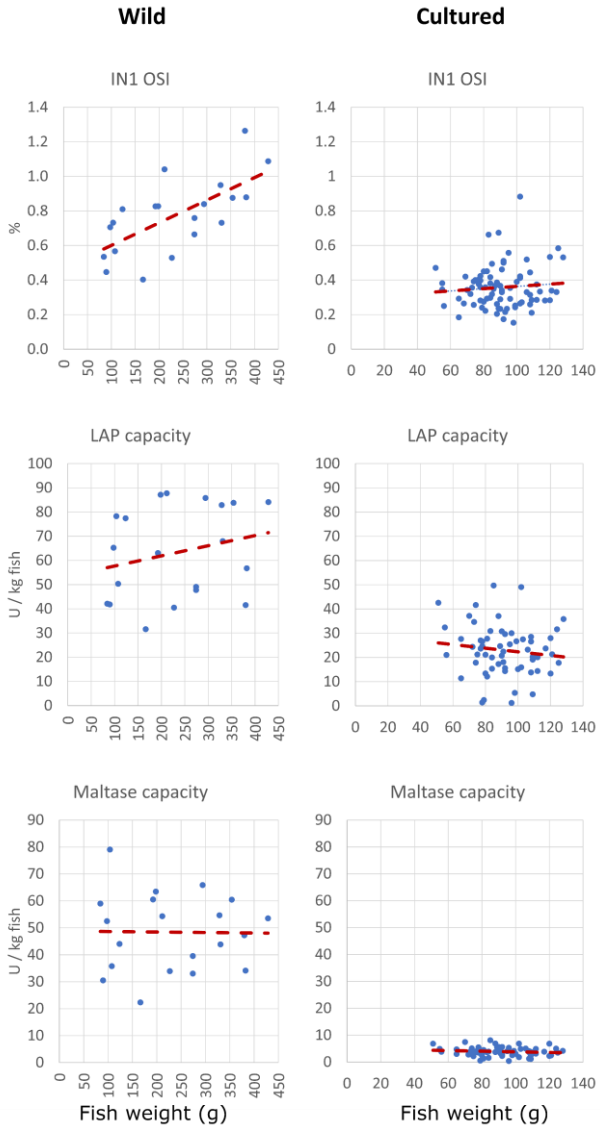


Figure 7 Comparison of relative organ weight and capacity of leucine aminopeptidase (LAP) and maltase in the proximal part of the intestine (IN1) as function of fish weight between wild and cultured B. wrasse, based on results of **Paper I** and **Paper II**. A linear trend line (red dashed line) is provided to illustrate the change of mean values.

Immune function of the B. wrasse intestine

Although the intestinal structures were observed to be largely similar in the sections along the intestine of B. wrasse, some other biomarkers showed difference between intestinal sections, indicating that there may be spatial difference in immune function from proximal to distal sections of the intestine. For instance, a higher gene expression of *igm* was detected in IN4 than in the other sections (**Paper I)**, and more marked inflammatory changes regarding infiltration of leukocytes were observed in IN3 and IN4 after exposure of the fish to diets containing soya saponins (**Paper II).** This corresponds to the results of a transcriptomic study in B. wrasse (Lie, et al., 2018), in which the highest expression of immune-relevant genes was detected in the most distal segment. Moreover, also a study of Bilal et al (Bilal, et al., 2019) indicated that the distal segment of intestine play an important role in immune regulation in B. wrasse, as expression level of genes coding for two immunoglobulins, IgM and IgT, and a T cell receptor, TCRα, tended to be higher in the distal compared to the three more proximal intestinal segments. Considered together these results suggest that the distal section of intestine is the most active immunogenic part of the intestine in B. wrasse. Similar conclusions have been reached for other teleosts as well (Bjørgen, et al., 2020; Calduch-Giner, et al., 2016; Løkka, et al., 2014), suggesting that this characteristic is common for fish.

Although the distal sections of the intestine seem to be more immunologically active than the more proximal, our work revealed that some immunological activities may be more active in the proximal sections. Infiltration of eosinophilic granular cells (EGCs) was observed in the proximal compartments of the intestine, less in the more distally located, in both wild B. wrasse (**Paper I**) and cultivated B. wrasse (**Paper II**). An increase in number of EGCs in the intestine is often accompanied by inflammatory reactions (Reite, Evensen, 2006). However, no signs of inflammation were observed in the tissues in the experimental B. wrasse. The cause of the higher number EGC in the proximal intestinal sections, is difficult to suggest. One possible explanation is that a component in the diet stimulated migration of EGCs into the tissue, and that this factor

was digested or otherwise eliminated along the intestine and therefore, caused no change in more distal part of the intestine. On the other hand, the adaptive immunity showed more important role in the intestine of B. wrasse, especially to resist the challenge of soya saponins, as the transcriptomic results in **Paper II** showed that induced responses were mostly related to lymphocytes. The further discussion is given in the next chapter.

Soya saponins and prebiotics altered intestinal functions in Ballan wrasse

In the experiment presented in **Paper II**, soya saponins was used as a tool with the aim to modulate intestinal digestive and immune functions, based on experience from other fish species (Couto, et al., 2014; Krogdahl, et al., 2015b; Yamamoto, et al., 2012). The results indicated that soya saponins is a useful tool for this purpose also in the B. wrasse. The responses to soya saponins included negative effects on cholesterol uptake and fatty acid transport, as well as stimulation of intraepithelial lymphocyte infiltration. These responses were quite similar to those observed in some other fish species such as sea bream, Atlantic salmon and rainbow trout (Couto, et al., 2014; Krogdahl, et al., 2015b; Yamamoto, et al., 2012), indicating that mechanisms affected by the presence of soya saponin in the diet modulating immune and digestive functions are similar in B. wrasse and some other fish species. As mentioned in previous chapter, lymphocyte-mediated immunity seemed to play a dominant role of attenuating the challenge of soya saponins in intestine of B. wrasse, but the reactions were very complex and of a wide range, and to clarify the mechanisms based on the current results is difficult. The involvement of diverse T cells may be a strategy for fighting the consequences of soya saponins in fish, as this has been commonly observed in Atlantic salmon (Marjara, et al., 2012; Romarheim, et al., 2013; Sahlmann, et al., 2013), which may be triggered by the increase in mucosal permeability by saponin, allowing entrance of alien compounds (Knudsen, et al., 2008). T cells may also be the key immune cells in B. wrasse as results

in **Paper II** showed that various pathways of T helper cells signaling were induced. As for B cells, its involvement in the responses to soya saponins was demonstrated, as IgM level was elevated in mid and distal intestine in Atlantic salmon (Krogdahl, et al., 2000). In rice field eel (*Monopterus albus*), on the contrary, adding soya saponin to diet decreased the IgM level in the intestine (Hu, et al., 2021). The different responses of IgM level in intestine implied B-cell mediated responses may be of species difference. However, the function of B cells in the responses to soya saponins is rarely documented. The question whether B cells directly interact with soya saponins, as phagocytosis was also identified in B cells (Øverland, et al., 2010), or the immunoglobulins interact with saponins directly cannot be answered with the information available at present. The results in **Paper II** showed an induced pathway of "B cell receptor signaling", with no significant change of *igm* expression. The role of B cells in responses to soya saponins in the intestine of B. wrasse therefore needs further study.

Alterations induced by prebiotics in B. wrasse in **Paper II** were also observed. They indicate that the addition of prebiotics to the feed induced various responses in the intestine of B. wrasse, as the number of genes showing differential expression was the highest. Categorized by canonical pathway analysis, these genes were either involved in lipogenesis, fatty acid oxidation and signaling of T helper cells which were suppressed, or other immune-relevant signaling which were induced. However, these responses did not affect digestive enzyme activity and histological results, suggesting that these responses did not have great influence on the gut health in the B. wrasse. The prebiotics used in the present study was a mixture of yeast (*Saccharomyces cerevisiae*) extract which contains functional ingredients such as mannan-oligosaccharides (MOS), and dried algae. Its effects in fish would therefore be expected to be comparable to effects of MOS. As summarized by Torrecillas et al. (Torrecillas, et al., 2014), supplementing a diet with MOS can induce increase in lysozyme activity, mucus secretion and cytokine production, and enhance lymphocyte responses, including both T- and B-cell responses (Torrecillas, et al., 2015). The intestine of B. wrasse, however, did not show such effects of the supplementation with the prebiotic. Moreover, the prebiotic did not counteract the inflammation induced by soya saponins in B. wrasse,

but rather stimulated the inflammatory responses in the intestine as shown in **Paper II**. The explanation for the absence of the expected effects may be species differences in the gut microbiota and physiology. If the microbiota in B. wrasse differs in composition from that of other fish species, the effects of the prebiotics may also differ. Species differences in responses in the microbiota upon exposure to prebiotics have been observed earlier. In gilthead sea bream (*Sparus aurata*), adding MOS to the diet did not change richness and diversity of intestinal microbiota, while in in turbot, *Scophthalmus maximus* (Bai, et al., 2017), soybean-meal-induced changes on microbiota abundance were counteracted, however, with no counteracting effects on soybean-meal-induced inflammation. The intestinal microbiota may also have been involved in the saponin-induced inflammatory response in our study in B. wrasse. However, during the presence of the prebiotics, which may have altered the composition of microbiota, the effects of soya saponins was enhanced rather than diminished. Profiling the intestinal microbiota in B. wrasse should therefore be done for better understanding of their potential role in soya saponin induced inflammation.

All in all, the present work showed that the intestine of B. wrasse seemed to respond to soybean saponins with similar inflammatory responses as other investigated fish species, including inhibition of fatty acid transport and intraepithelial lymphocyte infiltration. During the process, lymphocytes, both T and B cells, seemed to play important roles in the immune regulation. Eosinophilic granular cells may also be involved in diet-induced inflammation, but the modulating factors are unknown. To understand possible species differences in responses to the prebiotic, the role of the microbiota in these aspects should be investigated.

Intestinal morphology and functions of lumpfish

Digestive function of lumpfish

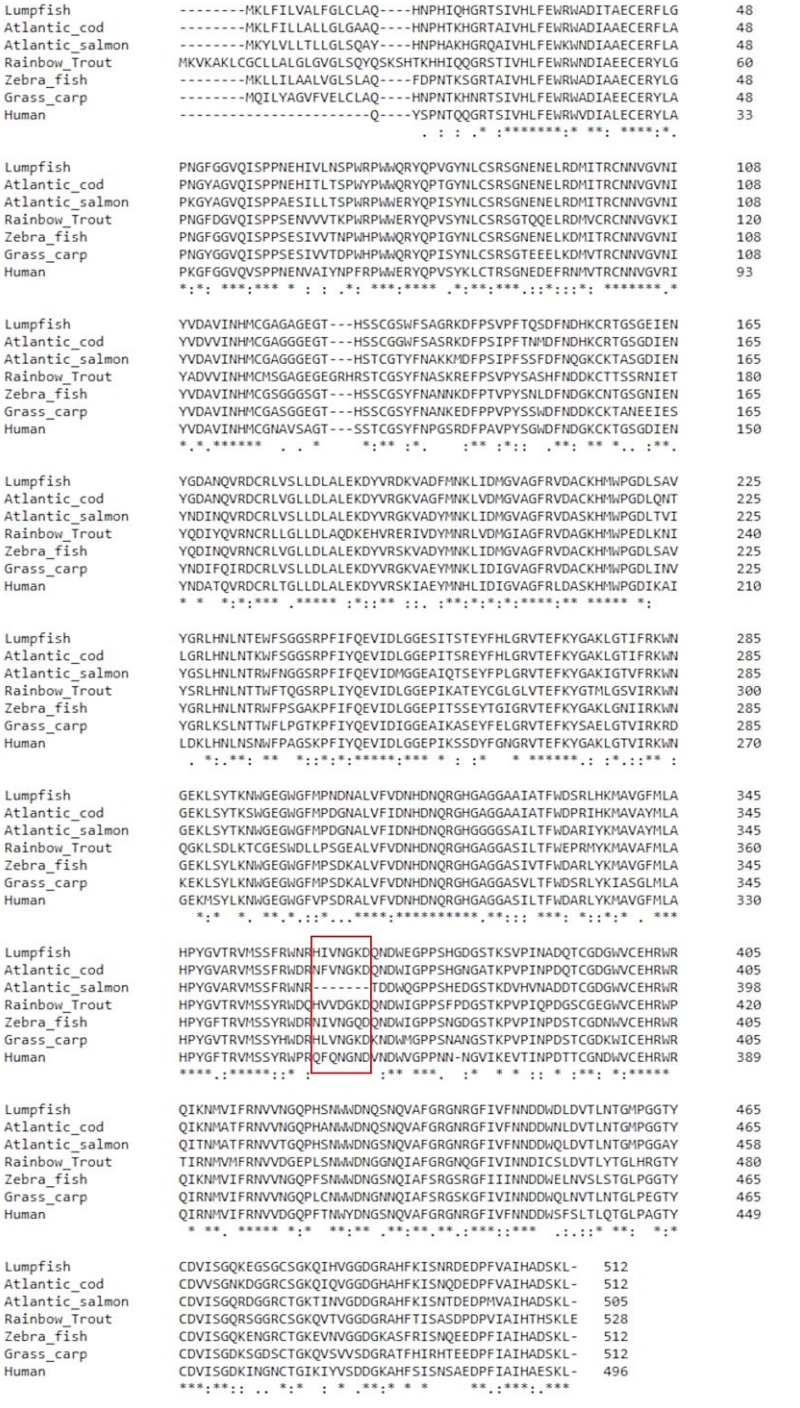
The work presented in **Paper III** greatly improves our knowledge regarding digestive characteristics of the lumpfish intestine. The intestinal functions of lumpfish showed

partial similarities to other fish species. Compared to Atlantic salmon and Atlantic cod (Kraugerud, et al., 2007; Refstie, et al., 2006), the weight of PI in lumpfish comprised a lower proportion of the weight of the intestine. On the other hand, the MI was larger in the lumpfish than in the salmon, but similar to that in cod. The DI in the lumpfish comprised a smaller part of the intestine in both lumpfish and salmon. The relative weight of intestinal sections may reflect digestive capacity. In lumpfish, the PI and MI showed similar values of LAP and maltase capacity (U/kg fish), in the range 40 to 50% of the total capacity. In the salmon, 70 – 80% of the total LAP and maltase capacity was in the PI (Bakke-McKellep, et al., 2008). However, in the cod, in which relative weight of PI and MI were similar to lumpfish, 80 – 90% of total LAP and maltase capacity was observed in the PI (Hansen, et al., 2008). The digestive capacity correlated closely with tissue weight, as also observed for Atlantic salmon. Based on weight and digestive enzyme capacity, we can conclude that both the PI and MI are major sites for digestion. The DI also shows digestive capacity but much lower than the more proximal sections. Digestibilities in lumpfish were also determined in **Paper III**. The protein digestibility was about 83%. This is a rather low digestibility coefficient, in particular as the protein source was mainly fish meal which can be assumed to have high protein value. Although not directly comparable, protein digestibility coefficients of about 90 - 95% (Bendiksen, et al., 2003; Sajjadi, Carter, 2004) and 85 - 90% (Grisdale-Helland, et al., 2008; Hemre, et al., 2003) have previously been reported for salmon and cod, respectively. The lipid digestibility was about 95% in lumpfish and was similar to those in salmon and cod, which was about 95-99% (Bendiksen, et al., 2003; Carter, et al., 2003) and 88 – 96% (Hansen, et al., 2008; Karlsen, et al., 2017), respectively. Similar to observations in Atlantic salmon (Krogdahl, et al., 2005), the starch digestibility was low, indicating that starch utilization is also poor in lumpfish. The reason why starch digestibility was low could be a result of low amylase secretion or specific activity. As determined in **Paper III**, amylase activity was only 2U/g dry matter, which is comparable to that observed in Atlantic salmon but much lower than cod and rainbow trout (Froystad, et al., 2006). In salmon, the amylase amino acid sequence was found to lack 7 amino acids which was suggested to play important roles of the interaction with starch molecules (Froystad, et

al., 2006). The same may be the situation for lumpfish. Therefore, to better understand the low amylase activity, an alignment of alpha-amylase amino acid sequences from lumpfish, Atlantic cod, Atlantic salmon, rainbow trout, zebra fish, grass carp and human was conducted. As displayed in figure 8, the amino acid sequence did not differ much among the different species, except for the earlier observed deletion of the 7 amino acids in the Atlantic salmon (Froystad, et al., 2006). The results suggest that the cause of the low amylase activity in lumpfish may not be related to the absence of the 7 amino acids in the sequence, but to some other unidentified characteristic. The practical consequence of the low amylase capacity in lumpfish, as well as in Atlantic salmon, is that starch should be included at low level in the feed to avoid intestinal challenges, waste of nutrients, and low feed utilization.

The general histological appearance of the lumpfish intestine, as presented in **Paper III**, was mostly similar to that of Atlantic salmon (Bjørgen, et al., 2020). However, two characteristics which differed can be mentioned herein. Firstly, a structure located adjacent to the pyloric caeca, which was fused with and shared muscular and serosal wall with the caeca, was observed. The same structure has also identified in Atlantic cod (Peruzzi, et al., 2013). As lumpfish and cod have similar alimentary tracts, this may be an evolutionary adaptation to densely arranged pyloric caeca. In contrast to salmon in which pyloric caeca attach to a long region of the intestine, pyloric caeca in lumpfish and cod attach to a very short region of the intestine, close to the stomach.

Figure 8 (See next page). Alignment of α-amylase amino acid sequences from lumpfish, Atlantic cod, Atlantic salmon, rainbow trout, zebra fish, grass carp and human. Identical residues in all sequences are indicated by (∗) under the column, conserved substitutions are indicated by (:), and semi-conserved substitutions are indicated by (.). Deletions are indicated by dashes. The red rectangular indicates the region where the 7 amino acid deletion was identified by Froystad et al. (Froystad, et al., 2006) in Atlantic salmon.


Lumpfish --------MKLFILVALFGLCLAQ----HNPHIQHGRTSIVHLFEWRWADITAECERFLG 48
Atlantic_cod --------MKLFILLALLGLGAAQ----HNPHTKHGRTAIVHLFEWRWADIAAECERFLA 48
Atlantic_salmon --------MKYLVLLTLLGLSQAY----HNPHAKHGRQAIVHLFEWKWNDIAAECERFLA 48
Rainbow_Trout MKVKAKLCGCLLALGLGVGLSQYQSKSHTKHHIQQGRSTIVHLFEWRWNDIAAECERYLG 60
Zebra_fish --------MKLLILAALVGLSLAQ----FDPNTKSGRTAIVHLFEWRWADIAAECERYLG 48
Grass_carp --------MQILYAGVFVELCLAQ----HNPNTKHNRTSIVHLFEWRWADIAAECERYLA 48
Human ----------Q----YSPNTQQGRTSIVHLFEWRWVDIALECERYLA 33
Lumpfish PNGFGGVQISPPNEHIVLNSPWRPWWQRYQPVGYNLCSRSGNENELRDMITRCNNVGVNI 108
Atlantic_cod PNGYAGVQISPPNEHITLTSPWYPWWQRYQPTGYNLCSRSGNENELRDMITRCNNVGVNI 108
Atlantic_salmon PKGYAGVQISPPAESILLTSPWRPWWERYQPISYNLCSRSGNENELRDMITRCNNVGVNI 108
Rainbow_Trout PNGFDGVQISPPSENVVVTKPWRPWWERYQPVSYNLCSRSGTQQELRDMVCRCNNVGVKI 120
Zebra_fish PNGFGGVQISPPSESIVVTNPWHPWWQRYQPIGYNLCSRSGNENELKDMITRCNNVGVNI 108
Grass_carp PNGYGGVQISPPSESIVVTDPWHPWWQRYQPISYNLCSRSGTEEELKDMVTRCNNVGVNI 108
Human PKGFGGVQVSPPNENVAIYNPFRPWWERYQPVSYKLCTRSGNEDEFRNMVTRCNNVGVRI 93
Lumpfish YVDAVINHMCGAGAGEGT---HSSCGSWFSAGRKDFPSVPFTQSDFNDHKCRTGSGEIEN 165
Atlantic_cod YVDVVINHMCGAGGGEGT---HSSCGGWFSASRKDFPSIPFTNMDFNDHKCRTGSGDIEN 165
Atlantic_salmon YVDAVINHMCGAGGGEGT---HSTCGTYFNAKKMDFPSIPFSSFDFNQGKCKTASGDIEN 165
Rainbow_Trout YADVVINHMCMSGAGEGEGRHRSTCGSYFNASKREFPSVPYSASHFNDDKCTTSSRNIET 180
Zebra_fish YVDAVINHMCGSGGGSGT---HSSCGSYFNANNKDFPTVPYSNLDFNDGKCNTGSGNIEN 165
Grass_carp YVDAVINHMCGASGGEGT---HSSCGSYFNANKEDFPPVPYSSWDFNDDKCKTANEEIES 165
Human YVDAVINHMCGNAVSAGT---SSTCGSYFNPGSRDFPAVPYSGWDFNDGKCKTGSGDIEN 150
Lumpfish YGDANQVRDCRLVSLLDLALEKDYVRDKVADFMNKLIDMGVAGFRVDACKHMWPGDLSAV 225
Atlantic_cod YGDANQVRDCRLVGLLDLALEKDYVRGKVAGFMNKLVDMGVAGFRVDACKHMWPGDLQNT 225
Atlantic_salmon YNDINQVRDCRLVSLLDLALEKDYVRGKVADYMNKLIDMGVAGFRVDASKHMWPGDLTVI 225
Rainbow_Trout YQDIYQVRNCRLLGLLDLAQDKEHVRERIVDYMNRLVDMGIAGFRVDAGKHMWPEDLKNI 240
Zebra_fish YQDINQVRNCRLVGLLDLALEKDYVRSKVADYMNKLIDMGVAGFRVDACKHMWPGDLSAV 225
Grass_carp YNDIFQIRDCRLVSLLDLALEKDYVRGKVAEYMNKLIDIGVAGFRVDACKHMWPGDLINV 225
Human YNDATQVRDCRLTGLLDLALEKDYVRSKIAEYMNHLIDIGVAGFRLDASKHMWPGDIKAI 210
Lumpfish YGRLHNLNTEWFSGGSRPFIFQEVIDLGGESITSTEYFHLGRVTEFKYGAKLGTIFRKWN 285
Atlantic_cod LGRLHNLNTKWFSGGSRPFIYQEVIDLGGEPITSREYFHLGRVTEFKYGAKLGTIFRKWN 285
Atlantic_salmon YGSLHNLNTRWFNGGSRPFIFQEVIDMGGEAIQTSEYFPLGRVTEFKYGAKIGTVFRKWN 285
Rainbow_Trout YSRLHNLNTTWFTQGSRPLIYQEVIDLGGEPIKATEYCGLGLVTEFKYGTMLGSVIRKWN 300
Zebra_fish YGRLHNLNTRWFPSGAKPFIFQEVIDLGGEPITSSEYTGIGRVTEFKYGAKLGNIIRKWN 285
Grass_carp YGRLKSLNTTWFLPGTKPFIYQEVIDIGGEAIKASEYFELGRVTEFKYSAELGTVIRKRD 285
Human LDKLHNLNSNWFPAGSKPFIYQEVIDLGGEPIKSSDYFGNGRVTEFKYGAKLGTVIRKWN 270
Lumpfish GEKLSYTKNWGEGWGFMPNDNALVFVDNHDNQRGHGAGGAAIATFWDSRLHKMAVGFMLA 345
Atlantic_cod GEKLSYTKSWGEGWGFMPDGNALVFIDNHDNQRGHGAGGAAIATFWDPRIHKMAVAYMLA 345
Atlantic_salmon GEKLSYTKNWGEGWGFMPDGNALVFIDNHDNQRGHGGGGSAILTFWDARIYKMAVAYMLA 345
Rainbow_Trout QGKLSDLKTCGESWDLLPSGEALVFVDNHDNQRGHGAGGASILTFWEPRMYKMAVAFMLA 360
Zebra_fish GEKLSYLKNWGEGWGFMPSDKALVFVDNHDNQRGHGAGGASIVTFWDARLYKMAVGFMLA 345
Grass_carp KEKLSYLKNWGEGWGFMPSDKALVFVDNHDNQRGHGAGGASVLTFWDSRLYKIASGLMLA 345
Human GEKMSYLKNWGEGWGFVPSDRALVFVDNHDNQRGHGAGGASILTFWDARLYKMAVGFMLA 330
Lumpfish HPYGVTRVMSSFRWNRHIVNGKDQNDWEGPPSHGDGSTKSVPINADQTCGDGWVCEHRWR 405
Atlantic_cod HPYGVARVMSSFRWDRNFVNGKDQNDWIGPPSHGNGATKPVPINPDQTCGDGWVCEHRWR 405
Atlantic_salmon HPYGVARVMSSFRWNR--------TDDWQGPPSHEDGSTKDVHVNADDTCGDGWVCEHRWR 398
Rainbow_Trout HPYGVTRVMSSYRWDQHVVDGKDQNDWIGPPSFPDGSTKPVPIQPDGSCGEGWVCEHRWP 420
Zebra_fish HPYGFTRVMSSYRWDRNIVNGQDQNDWIGPPSNGDGSTKPVPINPDSTCGDNWVCEHRWR 405
Grass_carp HPYGVTRVMSSYHWDRHLVNGKDKNDWMGPPSNANGSTKPVPINPDSTCGDKWICEHRWR 405
Human HPYGFTRVMSSYRWPRQFQNGNDVNDWVGPPNN-NGVIKEVTINPDTTCGNDWVCEHRWR 389
Lumpfish QIKNMVIFRNVVNGQPHSNWWDNQSNQVAFGRGNRGFIVFNNDDWDLDVTLNTGMPGGTY 465
Atlantic_cod QIKNMATFRNVVNGQPHANWWDNQSNQVAFGRGNRGFIVFNNDDWNLDVTLNTGMPGGTY 465
Atlantic_salmon QITNMATFRNVVTGQPHSNWWDNGSNQVAFGRGNRGFIVFNNDDWQLDVTLNTGMPGGAY 458
Rainbow_Trout TIRNMVMFRNVVDGEPLSNWWDNGGNQIAFGRGNQGFIVINNDICSLDVTLYTGLHRGTY 480
Zebra_fish QIKNMVIFRNVVNGQPFSNWWDNGSNQIAFSRGSRGFIIINNDDWELNVSLSTGLPGGTY 465
Grass_carp QIRNMVIFRNVVNGQPLCNWWDNGNNQIAFSRGSKGFIVINNDDWQLNVTLNTGLPEGTY 465
Human QIRNMVIFRNVVDGQPFTNWYDNGSNQVAFGRGNRGFIVFNNDDWSFSLTLQTGLPAGTY 449
Lumpfish CDVISGQKEGSGCSGKQIHVGGDGRAHFKISNRDEDPFVAIHADSKL- 512
Atlantic_cod CDVVSGNKDGGRCSGKQIQVGGDGHAHFKISNQDEDPFVAIHADSKL- 512
Atlantic_salmon CDVISGQRDGGRCTGKTINVGDDGRAHFKISNTDEDPMVAIHADSKL- 505
Rainbow_Trout CDVISGQRSGGRCSGKQVTVGGDGRAHFTISASDPDPVIAIHTHSKLE 528
Zebra_fish CDVISGQKENGRCTGKEVNVGGDGKASFRISNQEEDPFIAIHADSKL- 512
Grass_carp CDVISGDKSGDSCTGKQVSVVSDGRATFHIRHTEEDPFIAIHADSKL- 512
Human CDVISGDKINGNCTGIKIYVSDDGKAHFSISNSAEDPFIAIHAESKL- 496

Secondly, in contrast to the Atlantic salmon, at least in the fed state, the enterocytes in distal intestine of fed lumpfish showed little or no vacuolization (Baeverfjord, Krogdahl, 1996; Knudsen, et al., 2007). As the loss of distal intestine enterocyte vacuolization is a key feature associated with distal intestinal inflammation in salmon (Agboola, et al., 2022; Krogdahl, et al., 2003; Marjara, et al., 2012), the lack of vacuolization in the distal intestine, indicates that reduced vacuolization might not be a valid biomarker of inflammation for lumpfish.

Immune function of the lumpfish intestine

In **Paper III**, although genes showing differential expression upon variation in diet composition were observed mostly in MI, this may be natural adaptations to changes in diet without further consequences for intestinal immune function, as there were no changes of other immune-relevant biomarkers, especially regarding infiltration of leukocytes. This may indicate that the intestinal immune regulation of lumpfish is relatively resilient to variations in macronutrient composition of the diet. As evidence, in Paper **IV**, varying lipid/carbohydrate composition affected expression of a few immune-relevant genes, without other impacts on immune-relevant biomarkers, neither on the survival rate after challenge with atypical *A. salmonicida*. However, excluding the possibility that macronutrient variation did not impact intestinal health may not be suitable at this stage because possible changes in intestine after bacterial challenge was not investigated in **Paper IV**. To gain better understanding of whether variation in macronutrient composition has effects on intestinal health, future studies with other pathogen challenges are required. In addition, when comparing gene expression between different intestinal segments, they did not show spatial difference. Genes showing differential expression was not associated with specific immune responses neither. These may also be attributed to the lack of pathogen challenge, which may trigger different pattern of immune responses.

Effects of diet composition on intestinal function of lumpfish

The work in **Paper III** and **Paper IV** provided basic information for understanding alterations of intestinal function induced by differences in diet composition. The most apparent change was triggered by variation in dietary lipid level. The results demonstrated that increasing dietary lipid content induced expression the fatty acid transport gene *fabp2* and resulted in intracellular accumulation of lipids in the enterocytes as indicated by an increase in enterocyte vacuolization, similar to observations in other fish species (Kjaer, et al., 2009; Kowalska, et al., 2011; Penn, et al., 2011). The increase in vacuolization may a result of excessive dietary lipid supply, or deficiency of substances essential for onward lipid transport and metabolism. Since expression of genes coding for a fatty acid transporter and lipid digestibility showed positive correlation with dietary lipid level, it is likely that the deficiency of essential substances was the cause. Deficiency of essential fatty acids have been observed to cause lipid accumulation in the enterocytes of Atlantic salmon (Bou, et al., 2017; Olsen, et al., 1999; Olsen, et al., 2003). Also, insufficient choline supply is a possible explanation for the increase in vacuolization, as recently documented in Atlantic salmon (Hansen, et al., 2020). Both essential fatty acids and choline are needed for aggregation of the components constituting the lipoproteins which are vehicles for lipid export from the intestinal epithelium to the other organs and tissues of the fish body (Krogdahl, et al., 2020; Liland, et al., 2018). Whether deficient supply of essential fatty acids, choline, or some other essential nutrient involved in lipid transport and metabolism were causing the histological alterations observed in the present study, cannot be concluded upon until further studies have been conducted.

As discussed in the previous chapter, lumpfish showed low capacity for starch digestion. This fact prevents the use of starch as energy source for lumpfish, above a certain dietary level. On the other hand, it may prevent lumpfish from the impact of high carbohydrate supply. Excessive dietary starch has been reported to result in hyperglycemia, glycogenic hepatopathy and impaired growth of carnivorous fish (Li, et al., 2022; Zhang, et al., 2020). However, results from the same study as presented in **Paper III**, published elsewhere by Hamre et al. (Hamre, et al., 2022), revealed that

lumpfish fed high starch diets did not show such negative effects. Although increased enzymatic activity and expression of a gene coding for disaccharidase were observed with increasing starch content of the diet (**Paper III**), most likely induced by the small but significant increase in production of disaccharides from the starch (5.6, 6.6 and 8.1 g/100g diet in E2 of **Paper III**), such small amount of digested starch seemed not to be harmful to the lumpfish. The present results suggest that a starch level lower than 6% is suitable for lumpfish.

Regarding effects of dietary protein level on gut function, due to the limitations of the mixture design, it is difficult to draw conclusions in light of the interactions between the three macronutrients. It can, however, be stated that low protein level in the diet was associated with low values of the biomarkers showing significant responses in the intestine, and vice versa (Table 5 in **Paper III**). A high-protein diet seemed to be important for intestinal functions in lumpfish. However, too high protein levels in the diet might compromise immune responses in other organs, such as the head kidney (Hamre, et al., 2022).

MAIN CONCLUSIONS

Regarding B. wrasse

- The proximal segment of the intestine showed highest digestive capacity, while the distal segment seemed to be the most important segment for immune functions.

- Soybean saponins in the diet altered immune and digestive functions and induced inflammation similar to the effects observed in Atlantic salmon.

- A mixture of prebiotics modulated gene expression, but did not prevent the saponin-induced inflammation.

Regarding lumpfish

- High digestive capacity was found in both proximal and mid segment of the intestine.

- Increasing lipid level in the diet did not affect intestinal immune function but caused hyper-vacuolization of the enterocytes in the pyloric caeca.

- Capacity for starch digestion is low and a dietary level above 6% will increase faecal nutrient waste and reduce feed utilization.

FUTURE PERSPECTIVES

The current thesis work has strengthened our knowledge regarding intestinal functions and health of Ballan wrasse and lumpfish, and how variation in diet composition may affect gut function, growth and susceptibility towards certain production disorders and infectious disease. However, many questions remain to be answered in order to produce cleaner fish feed which supports good growth and high survival. Among the questions arising in the present context, which most urgently need to be answered are the following:

- What are the micronutrient requirements of these species for optimal growth and disease resistance?

- Are the nutrient requirements different between development stages in lumpfish?

- What are the nutritive vales of available alternative nutrient sources for cleaner fish production?

- How does intestinal immune apparatus response to nutritional variation in lumpfish?

- Will antinutrients induce inflammation in lumpfish as they do in B. wrasse?

- What roles does the intestinal microbiome play for the health and function of the intestine and how are they affected by variation in diet composition and supplementation with prebiotics?

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PAPER I-IV

PAPER I

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Digestive and immune functions in the intestine of wild Ballan wrasse (*Labrus bergylta*)

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ABSTRACT

This study was carried out to profile key characteristics of intestinal functions and health in wild-caught Ballan wrasse. To describe functional variation along the intestine, samples were collected from four intestinal segments, named from the proximal to the distal segment: IN1, IN2, IN3 and IN4. The sections showed quite similar structure, i.e. regarding mucosal fold height and branching, lamina propria and submucosal width and cellular composition and thickness of the muscle layers. Leucine aminopeptidase and maltase capacity decreased from IN1 to IN4, suggesting a predominant role of IN1 in digestion. Gene expression levels of vitamin C transporter (*slc23a1*) and fatty acid transporters (*cd36* and *fabp2*) were higher in IN1 than in IN4, indicating a more important role of the proximal intestine regarding transport of vitamins and fatty acids. Higher expression of the gene coding for IgM heavy chain constant region (*ighm)* was found in IN4 than in IN1, suggesting an important immune function of the distal intestine. Other immune related genes *il1b*, *il6*, *cd40,* showed similar expression in the proximal and the distal part of the intestine. Parasite infection, especially the myxozoan parasite *Enteromyxum leei*, coincided with infiltration of lymphocytic and eosinophilic granular cells in the submucosa and lamina propria. The present study established reference information necessary for interpretation of results of studies of intestinal functions and health in cultured Ballan wrasse.

1. Introduction

Ballan wrasse belongs to the family of *Labridae*, commonly called the wrasses. The wrasses inhabit the coastal waters of the North-eastern Atlantic Ocean, from Morocco to Norway, and are primarily associated with rocky reefs and kelp beds (Whitehead et al., 1984). Their nature as protogynous hermaphrodites fish species, i.e. are born as females and can change to male, (Dipper et al., 1977), gives them a complex hierarchy and a highly skewed sex ratio (Leclercq et al., 2014b). The alimentary tract of Ballan wrasse is short, lacks a stomach, and genes coding for stomach functions are absent from the genome (Lie et al., 2018). Earlier studies indicate that feed passes rapidly through the digestive tract, and that the reaction rates of digestives processes are high (Horn and Messer, 1992). Ballan wrasse are omnivorous, tending towards carnivorous. Their natural diet consists of hard-shelled crustaceans, e.g., molluscs, decapods and isopods (Dipper et al., 1977). Thick projecting lips and short blunt pharyngeal teeth are seemingly adaptions to the diet (Dipper et al., 1977) and make them able to feed from lice attached to other fish. These characteristics make Ballan wrasse suitable as lice cleaner fish in salmon production, and they are used by the industry at increasing numbers to combat the severe consequences of lice infection.

In Europe and Canada, *Lepeophtheirus salmonis* is the most common sea louse infecting salmonids (Aaen et al., 2015). At present, it represents a major challenge for production of Atlantic salmon (*Salmo salar*) in Norway. Atlantic salmon infected by L. *salmonis* suffers from osmoregulatory failure due to skin lesions which may develop into serious wounds (Grimnes and Jakobsen, 1996), resulting in decreased growth rates and high mortality. Earlier, the most common method for delousing of Atlantic salmon was medicinal treatments, but frequent use has inevitably led to the sea lice developing resistance towards the medicine (Aaen et al., 2015). The use of cleaner fish to remove sea lice from cultured Atlantic salmon was first based on wild caught Ballan wrasse. However, as demand increases, the wild stock is suffering, and

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the threat of transferring pathogens from wild Ballan wrasse populations to the cultured salmon is of concern. To achieve independence of wild caught fish, the salmon industry is making great efforts to farm Ballan wrasse. Major challenges inhibiting large-scale production of the fish include complicated brood stock management, long production period, high mortality rate at larval stage and unclear factors causing deformities in juveniles (Brooker et al., 2018). Some of these challenges are most likely related to the lack of knowledge on the digestive physiology of wild Ballan wrasse, i.e. knowledge which may be necessary for optimization of the diet composition and physical quality.

Studies on cultured Ballan wrasse have shown that the proximal intestinal segment represents the major segment regarding digestive and absorptive capacity (Krogdahl et al., 2014; Le et al., 2019), while the distal segment seems to harbour most important immune functions. A study of Bilal et al. (Bilal et al., 2019) revealed that the hindgut of Ballan wrasse expressed high transcriptional level of secreted-form IgM, which may provide strong immune activity. These results are fully in line with those of a transcriptomic study by Lie et al. (Lie et al., 2018). However, as there is hardly any information regarding intestinal functions in free-living, wild Ballan wrasse, it is difficult to fully understand these characteristics in the cultured fish. Therefore, the current study was conducted to establish knowledge of the intestinal morphology and mucosal functions of wild-caught Ballan wrasse focusing on characteristics studied earlier in cultured fish and which are considered as most important for understanding of the gut functions of these fish. Detailed histological examinations were performed to describe morphological characteristics of the gut. Activity of maltase and leucine aminopeptidase (LAP) were measured as representatives of digestive enzymes located in the brush border of the mucosa, as frequently used in studies of Atlantic salmon (Krogdahl et al., 2003; Krogdahl et al., 2014). Gene expression levels in the gut tissue were measured to get information on other gut functions, including nutrient absorption, cholesterol synthesis, mucin secretion and immune regulation. Blood plasma variables were measured to evaluate nutrient status and liver function of the fish.

2. Materials and methods

2.1. Fish and sampling

Wild Ballan wrasse were obtained from local fishermen fishing in the waters west of Bergen, Norway, during the summer of 2018. The fish were caught with traps using cooked shrimp as bait. Thereafter, the fish were handled in accordance with laws regulating the experimentation with live animals in Norway. The fish were transported alive in large plastic bags with oxygenation. Before sampling, fish were firstly anaesthetized and euthanized immediately by cervical dislocation. Blood samples were collected with heparinized vacutainers from the caudal vein for plasma preparation prior to tissue sampling. The abdominal cavity was opened and the whole intestine removed from the abdominal cavity. The intestine was cut in four segments as shown in Fig. 1, as defined previously (Lie et al., 2018), and were named as intestinal segments 1–4, respectively (IN1, IN2, IN3 and IN4). The amount of intestinal content was highly variable and in general low. As the fish had been caught with cooked shrimp as bait, intestinal chyme samples were considered inappropriate for the present study and not collected.

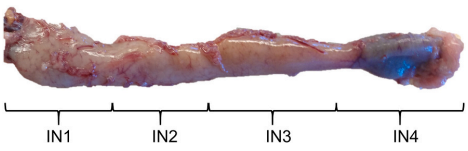

Fig. 1. The intestine of Ballan wrasse showing how it was separated into four segments. IN1 to IN4 are short for intestinal segment 1 to 4.

The sections were rinsed in saline and tissue samples were collected from each segment for histology, RNA extraction and brush border membrane enzyme activity assessment. The samples collected for histological evaluation were fixed in 4% phosphate-buffered formaldehyde solution for 24 h, and subsequently stored in 70% ethanol until further processing. Samples for RNA extraction were fixed in RNAlater (Ambion, Carlsbad, CA) at 4 °C for 24 h, and stored at −20 °C. The remaining tissue of each segment was collected, snap frozen in liquid N_2 and stored at −80 °C for brush border digestive enzyme activity assessment.

2.2. Histomorphology

Tissue processing, sectioning, and staining with haematoxylin and eosin (H&E) followed the methodology described in our previous work (Le et al., 2019). Intestinal tissues were oriented for longitudinal sections. Evaluation of the histology sections used the Zeiss AxioScope A1, (Carl Zeiss AS, Oslo, Norway) light microscope mounted with an IDS UI3260CP-C-HQ 2.3 MP camera (IDS Imaging Development Systems GmbH, Obersulm, Germany) for image capture using the MicroVisioneer whole-slide scanning software (MicroVisioneer, Freising, Germany). Histomorphological examination primarily focused on key descriptive morphological features of the intestine that included height of mucosal folds, degree of mucosal fold branching, cellular composition and structure of the epithelial barrier, width and cellular composition of the lamina propria and submucosa compartments, and appearance of the muscle layer. Pathological changes in the intestinal sections, such as degeneration, inflammation, and/or presence of infectious or parasitic organisms were noted and scored on a scale from 0 to 4 that corresponded to a grading of normal and healthy, or mild, moderate, marked, or severe changes.

2.3. Brush border membrane enzyme activity

The intestinal tissue samples were thawed on ice and homogenized (1:20 *w/v*) in ice-cold 2 mM Tris/50 mM mannitol, pH 7.1, containing phenyl-methyl-sulphonyl fluoride (P-7626, Sigma, Norway) as serine protease inhibitor. The homogenates were sonicated, aliquoted and stored at −80 °C until analysis. LAP activity was measured employing the Sigma procedure no. 251 also used by Krogdahl et al. (Krogdahl et al., 2003), using l-leucyl-b-naphthylamide as the substrate. To measure maltase activity, the method described by Dahlqvist (Dahlqvist, 1968) was applied, using maltose as substrate.

2.4. Quantitative Real Time PCR (qPCR)

Total RNA was extracted from IN1 and IN4 samples (~20 mg) of all fish using Trizol reagent and PureLink™ RNA Mini Kit (Thermo Fisher Scientific). RNA was purified by an on-column DNase kit (PureLink™ DNase Set, Thermo Fisher Scientific) according to the manufacturer's protocol. Total RNA purity and concentration were measured using the Epoch Microplate Spectrophotometer (BioTeK Instruments, Winooski, USA). The RNA integrity was verified by the 2100 Bioanalyzer (Agilent Technologies) in combination with RNA Nano Chip (Agilent Technologies, Santa Clara, CA, USA). First-strand complementary DNA (cDNA) was synthesized from 1.0 μg total RNA in a 20-μl reaction that contained 4 μl mastermix from the kit SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific). Negative controls were performed in parallel by omitting RNA or enzyme.

The qPCR primers were designed using the Primer-BLAST tool of NCBI. Primer information are shown in Table 1. The selected genes were related to digestion (*ctra*), nutrient absorption (*cd36*, *fabp2, slc23a1* and *aqp8*), cholesterol synthesis (*cyp51a1* and *sqle*), immunity (*lyz*, *cd40*, *il1b*, *il6* and *ighm*), cell proliferation (*pcna*), cell remodelling (*mmp13*) and mucin-localization (*fcgbp*). All primer pairs were first used in gradient reactions to determine optimal annealing temperatures. To

Table 1
Primer pairs and related information for real-time PCR assays.

Gene symbol	5′ -3′ primer sequence Forward	Reverse	Amplicon size (bp)	Annealing temperature (°C)	Efficiency	Gene bank accession no.
gapdh2	TATTTGTGTCCGTGTGCCCC	GCCTCCGTCCACTGATGAAT	129	62	1.99	XM_020633887.1
ctra	AGCGTCCCAGTAGAGAAGGT	ACACTGGAGCTGAATCTGGC	110	60	2.1	XM_020657256
cd36	ACGGAGGGATAAAACGCACA	TATGCTGTGGTTCCAGGCTC	181	62	2.01	XM_020649455.1
fabp2	TACAGCCTTGCGGATGGAAC	ATCCTCTTAGCCTCCACACCT	173	60	1.95	XM_020643842.1
slc23a1	CCCACTGAACACCTCACACA	AGACCAATCAGCAGCTCCAC	93	60	1.83	XM_020655303
aqp8	TTGGCTCCTTTCCTTGTGGG	CCGAGAATGAGCCTGAGCAA	197	60	1.95	XM_020642545.1
cyp51a1	AAGGACTGCTGTTCCGATGG	CCTCTCCACAAAACCACCGA	113	60	1.79	XM_020648620
sqle	ACGAGAGATCAGCGACCAAC	CAGGTTCTGGAGCCACTGTT	117	62	1.94	XM_020635029
lyz	CTTGGGACAGCGAGGAACAC	TCCATCGCCCATGTTGTAGG	140	62	1.96	XM_020660641
cd40	AGCAGTAAACCCGACTGAGG	GCTTTGGTCGTCCTCGTTCT	85	60	1.99	XM_020651338.1
il1b	AAGGACGGTGATGAGGCAAC	GAAACCGAACCATGTCGCTG	94	57	1.87	XM_020651384.1
il6	GATCCTTGGTGCGACCGAT	GAAGGGCAGCTTTCTGGGAG	117	57	1.95	XM_020631726.1
ighm	ATCTCTTGTGGAACAGGGCAC	CCTTGAAGTCAGCAAAACGCT	101	55	1.89	XM_020660315.1
pcna	GCCAACAACACACAAAGGCT	TCGTCTTTCTGCGTCACTCC	106	62	1.88	XM_020647462.1
mmp13	TCTCGACGCCGCTTATGAAA	CACGCACGGGTTTATAGCCA	95	60	1.9	XM_020631204.1
fcgbp	CAACTCTCCCTGTCTCTCCAG	GCTTCACAGAGGCAATTCTCC	126	62	2.04	XM_020655516.2

gapdh2, glyceraldehyde-3-phosphate dehydrogenase 2; *ctra*, chymotrypsin A; *cd36*, cluster of differentiation 36; *fabp2*, fatty acid-binding protein 2 isoform; *slc23a1*, solute carrier family 23 member 1; *aqp8*, aquaporin 8; *cyp51a1*, cytochrome P450 family 51 subfamily A member 1; *sqle*, squalene epoxidase; *lyz*, lysozyme; *cd40*, cluster of differentiation 40; *il1b*, interleukin 1 beta; *il6*, interleukin 6; *ighm*, immunoglobulin M heavy chain constant region; *pcna*, proliferating cell nuclear antigen; *mmp13*, matrix metalloproteinase 13; *fcgbp*, Fc fragment of IgG binding protein.

confirm amplification specificity, the PCR products from each assay were subjected to melting curve analysis and visual inspection by agarose gel electrophoresis. PCR efficiency for each gene assay was determined using 2-fold serial dilutions of randomly pooled complementary DNA.

Expression of target genes was analyzed using the LightCycler 96 (Roche Diagnostics, Basel, Switzerland) with a 10-μl DNA amplification reaction. Each 10-μl DNA amplification reaction contained 2 μl PCR grade water, 2 μl of 1:10 diluted cDNA template, 5 μl LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) and 0.5 μl (10 μM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no-template control. The three-step qPCR run included an enzyme activation step at 95 °C (5 min), 40 cycles at 95 °C (10 s), annealing temperature (10 s), and 72 °C (15 s) and a melting curve step. The mean normalized expression of the target genes was calculated from raw Cq values (Muller et al., 2002). Genes of glyceraldehyde-3-phosphate dehydrogenase 2 (*gapdh2*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (*ywhae*) and DNA Topoisomerase II Alpha (*top2a*) were selected as reference gene candidates. Ultimately *gapdh2* was selected as the reference gene in terms of its stability among different fish (Kortner et al., 2011).

2.5. Plasma biomarkers

Plasma was analyzed at the Central Laboratory of the Norwegian University of Life Sciences, Faculty of Veterinary Medicine, Oslo. The concentration of free fatty acids, bile salts, glucose, cholesterol, total protein, sodium and the activity of aspartate transaminase and alanine transaminase were measured automatically following standard procedures.

2.6. Statistical analysis

Data were processed using R (version 3.5.2, 2018) in the integrated development environment Rstudio (version 1.1.463, 2018). Data quality diagnosis was conducted in terms of the discussion by Kozak and Piepho (Kozak and Piepho, 2018). Data of digestive enzyme activity and gene expression were tested for normality of distribution of residuals using normal quantile-quantile plot as well as the Shapiro-Wilk test. The homogeneity of variance was examined using standardized residuals-versus-fitted value plot followed by the Bartlett's test. Data that violated either normality of distribution of residuals or homogeneity of variance were transformed using Box-Cox transformation. Data of digestive enzyme activity were tested for difference using one-way analysis of variance (one-way ANOVA, or Welch's ANOVA for data violating homogeneity of variance), followed by Tukey's Honest Significant Difference test (or Games-Howell post-hoc test for data violating homogeneity of variance). Student's *t*-test was applied to test difference in gene expression data. Student'st-test was also used for comparison of the present results from the wild fish with our previous results from cultured fish. The significance level was set to 0.05 ($\alpha = 0.05$) for all hypothesis tests in this study.

3. Results

Most of the sampled fish investigated in the present study had empty shrimp shells in their stomach, indicating that they were not in a clear starving situation, but the time since the last meal might have been several hours.

3.1. Plasma biomarkers

Results regarding plasma samples, collected to get a general impression of the nutritional status and health of the liver, are shown in Table 3.

3.2. Histomorphology

As shown in Fig. 2a, the intestinal wall tissue layers observed in the wild Ballan wrasse consisted of the inner folded mucosa (Fig. 2a, black and yellow arrowhead) followed by a submucosal layer (Fig. 2a yellow arrow), a muscle layer (Fig. 2a black and blue arrow) and a thin serosa demarcating the intestinal wall. The mucosa comprised an epithelial monolayer (Fig. 2a, black arrowhead) and a supportive layer of connective tissue known as the lamina propria (Fig. 2a, yellow arrowhead), while no muscularis mucosa, normally present in mammals, was observed. The epithelium lining the intestinal lumen consisted of columnar enterocytes with a brush-border membrane, as well as mucous or goblet cells, and a high number of intraepithelial cells. The intraepithelial cells included lymphocytes, eosinophilic granular cells (EGCs; Fig. 2b, blue arrow) and occasional rodlet cells (Fig. 2b, black arrow). Supranuclear vacuoles were not detected in the enterocytes in any of the investigated intestinal regions. The lamina propria was observed as a fibrous compartment sparsely filled with blood vessels and cells

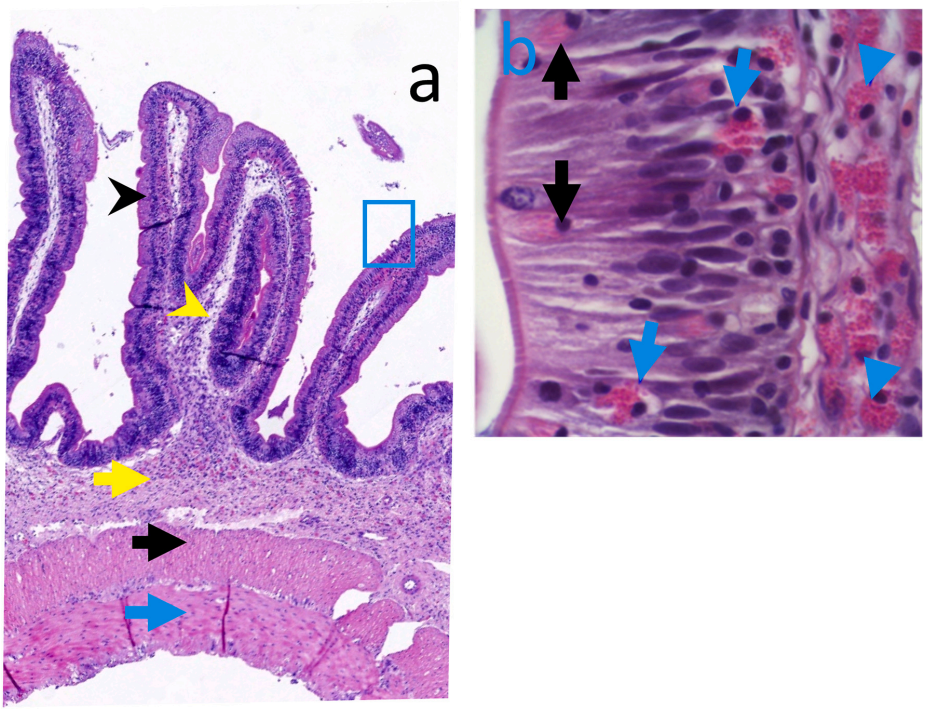

Fig. 2. The microscopic appearance of the intestinal wall from IN2 of an individual Ballan wrasse. (a) The overall structures showing epithelia monolayer (black arrow), lamina propria (yellow arrowhead) and submucosa (yellow arrow), inner circular muscle layer (black arrow) and an outer longitudinal muscle layer (blue arrow). (b) Enlarged section of mucosal fold. Eosinophilic granular cells were observed in epithelium (blue arrow) and in lamina propria (blue arrowhead) as well as rodlet cells in epithelium (black arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

including EGCs (Fig. 2b blue arrowhead). The lamina propria was about 3–5 cell layers thick. Following the mucosa, the submucosal layer was recognized to be a thick compartment comprising fibrous mesenchymal tissue that was similarly sparsely populated by cells as the lamina propria. The muscle layer consisted of an inner compartment of circularly arranged smooth muscle fibers and an outer compartment of longitudinally arranged smooth muscle fibers.

The intestinal structure appeared quite similar in the four different segments with regards to mucosal fold height and degree of fold branching, lamina propria and submucosal width and cellular composition and thickness of the muscle layers (Fig. 3). Both simple, slender mucosal folds and thicker folds with branches where the submucosa extended up into the folds were observed in all intestinal regions. The majority of the 20 fish displayed varying degrees of infiltration of leucocytes and EGCs in the lamina propria and submucosa that may indicate an inflammatory reaction (Fig. 4). Such changes were more visible

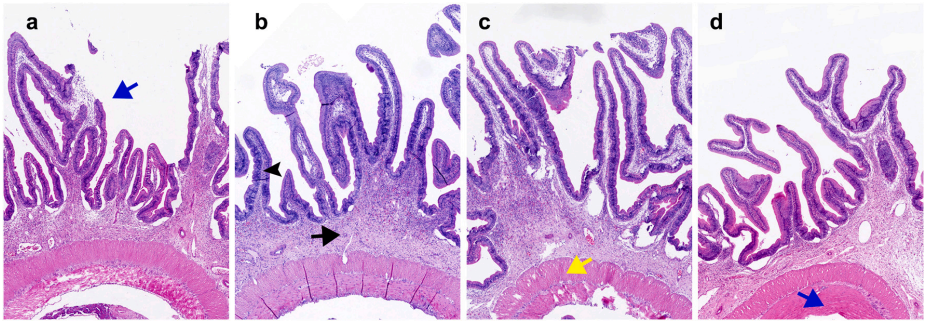

Fig. 3. Comparison of the microscopic appearance of the intestinal wall from different regions of an individual Ballan wrasse showing the structures of mucosal folds (blue arrow in a), lamina propria (black arrowhead) and submucosa (black arrow), as well as the muscle layer comprising of an inner circular layer (yellow arrow) and an outer longitudinal layer (blue arrow in d). Images a, b, c, d represent the regions IN1, IN2, IN3, IN4, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

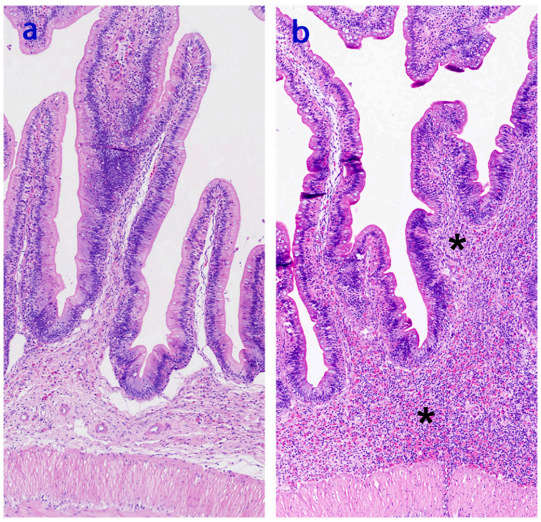

Fig. 4. Representative images of the mucosa and submucosa of the IN1 region in wild Ballan wrasse intestine illustrating (a) normal and healthy lamina propria and submucosa compartments with fibrous tissue and sparse numbers of cells alongside (b) a strong inflammatory reaction characterised by a marked increase in the cellularity of the lamina propria and submucosa (asterisks) due to an infiltration by a mixed population of cells chiefly, lymphocytes and eosinophilic granular cells (EGCs).

in the two proximal intestinal segments, less in IN3, whereas IN4 was largely normal and healthy in appearance (Fig. 5).

Nine out of the 20 observed fish had parasitic organisms in the intestine. Parasites were observed in all intestinal segments, but with higher prevalence in the proximal and mid segments compared to the most distal part (Table 2).

Various parasite species were identified and are shown in Fig. 6. Typical intraepithelial trophozoite stages of a myxozoan parasite were observed in the IN1 region. Surface-attached parasitic organisms, possibly a different developmental stage of the myxozoan *Enteromyxum leei,* were detected in multiple fish in all intestinal regions. Cestode parasites were observed in IN3 and IN4 regions in 3 fish.

3.3. Brush border digestive enzyme activity

Total capacity as well as specific activity of leucine aminopeptidase (LAP) and maltase decreased gradually from IN1 to IN4 (Fig. 7). The capacity decreased more pronounced than the specific activity indicating a change in the ratio of enzyme to total protein in the tissue along the intestine.

3.4. Gene expression

The expression levels (Fig. 8) of membrane fatty acid transporter (*cd36*), intracellular fatty acid binding protein (*fabp2*) and vitamin C transporter (*slc23a1*) were significantly higher in IN1 than in IN4, while the expression of chymotrypsin A (*ctra*) and cholesterol synthesis genes (*cyp51a1* and *sqle*) did not differ significantly between intestinal segments. On the other hand, the expression of the water channel gene *aqp8* was substantially higher in IN4 than in IN1 with an approximately 1200-fold difference. Also expression of immunoglobulin M (*ighm*) and mucin-forming-related gene, *fcgbp*, were significantly higher in IN4 than IN1, while the other measured barrier-related genes, including *cd40*, *lyz*, *il1b* and *il6*, showed no difference between IN1 and IN4 (Fig. 8). The same was the result for *pcna* which indicates proliferation rate of the enterocytes. The expression of the tissue remodelling collagenase 3 (*mmp13*) showed no difference between IN1 and IN4.

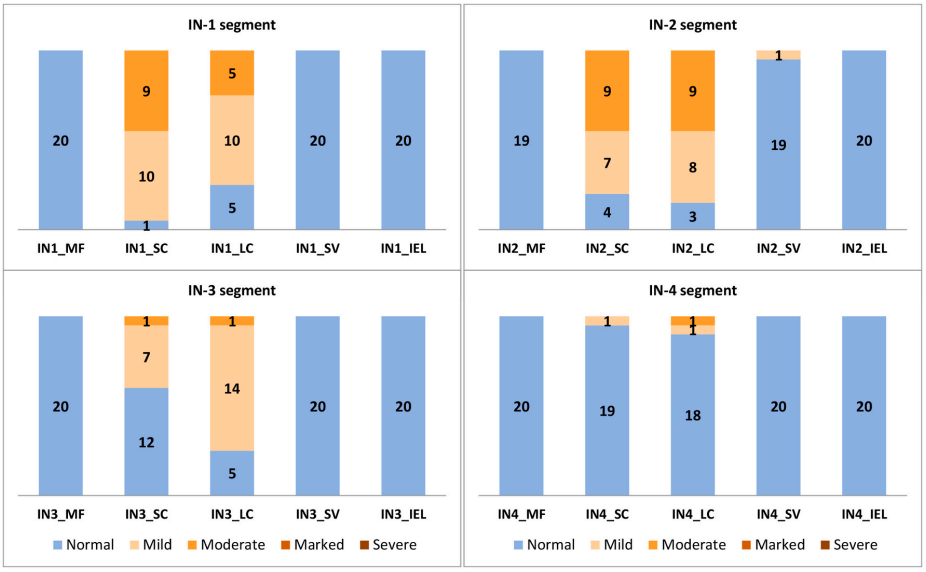

Fig. 5. Number of evaluated Ballan wrasse fish (N = 20) that were observed with and graded for degree of change in selected morphological features including mucosal fold height (MF), increased cellularity of the submucosa (SC) and lamina propria (LC) in inflamed fish, supranuclear vacuolization in enterocytes (SV), and increase of cells in the intraepithelial compartment by lymphocytes and/or degranulated eosinophilic granular cells (IEL).

Table 2
Blood plasma variables of the wild Ballan wrasse.

	FFAs (mmol/L)	TBAs (μmol/L)	Glu (mmol/L)	Chol (mmol/L)	Tprot (g/L)	TGs (mmol/L)	Sodium (mmol/L)	ALT activity (U/L)	AST activity (U/L)
Mean	3.1	8	6.2	4.4	32	4.8	177	33	125
SD	0.9	8	3.0	0.6	3	1.8	29	79	5
n	19	19	19	19	19	19	20	19	19

Abbreviations: FFAs: free fatty acids; TBAs, total bile acids; Glu, glucose; Chol, cholesterol; Tprot, total protein; TGs, triglycerides; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Table 3
The prevalence of parasitic infection in different intestinal segments

Segment	Prevalence (%)	n
IN1	25	20
IN2	26	19
IN3	25	20
IN4	10	20

4. Discussion

The aim of this study was to gain information on structure and function of the intestine of wild Ballan wrasse, as a basis for interpretation of corresponding results from cultured Ballan wrasse studied in a previous experiment in our laboratory from which the main part of results have been published earlier (Le et al., 2019). In the discussion below the results from the present and previous study are compared. Some results from the previous study, which have not been published earlier, as indicated in the figure captions, are also included in the discussion. To facilitate comparison of the present results with those of the experiment of Le et al. (Le et al., 2019), *t*-tests were performed. The results are illustrated in Fig. 9–11.

4.1. Blood plasma variables

The plasma nutrient levels observed for the wild Ballan wrasse in the present study, except for glucose, were about half the values observed earlier in cultured fish (Fig. 9), and the statistical comparison showed significant difference for all these biomarkers. Lower level of lipids and cholesterol in the wild fish suggests that the fish in the present study were in a postabsorptive state when they were caught, whereas the cultured fish all were in the fed state. Effects of fasting and starvation have been studied in several species, with the same result, decreased plasma nutrient levels, e.g. in Atlantic salmon (Krogdahl et al., 1999), European bass (*Dicentrarchus labrax*) (Perez-Jimenez et al., 2007) and tinfoil barb (*Barbonymus schwanenfeldii*) (Eslamloo et al., 2017). Although responses to starvation in Ballan wrasse have not been described in the scientific literature, a general strategy for fish in unfed state is to reduce their metabolic rate to save energy for vital metabolism (Gingerich et al., 2010). For instance, during starvation European bass liver lipogenesis was observed to be down-regulated (Perez-Jimenez et al., 2007) whereas the use of amino acids for glucose production was upregulated. It can be assumed that Ballan wrasse responds similarly.

Plasma glucose in the present wild fish was three times higher than in the cultured Ballan wrasse (Le et al., 2019). This very high level in the wild fish could be a result of stresses during capture and transportation. Hemre et al. summarised that physical handling of fish, in general, may result in increased plasma cortisol levels and secondarily elevated plasma glucose concentration (Hemre et al., 2002). Leclercq et al. demonstrated that 30-min-air exposure resulted in 4-fold increase of plasma cortisol level and significantly increased plasma glucose level by 45% in cultured Ballan wrasse (Leclercq et al., 2014a). In the present study, stresses were inevitably introduced to the fish during capture and transportation, which may explain the elevated plasma glucose levels.

4.2. Histomorphological characteristics

The current assessment of the morphological appearance of the

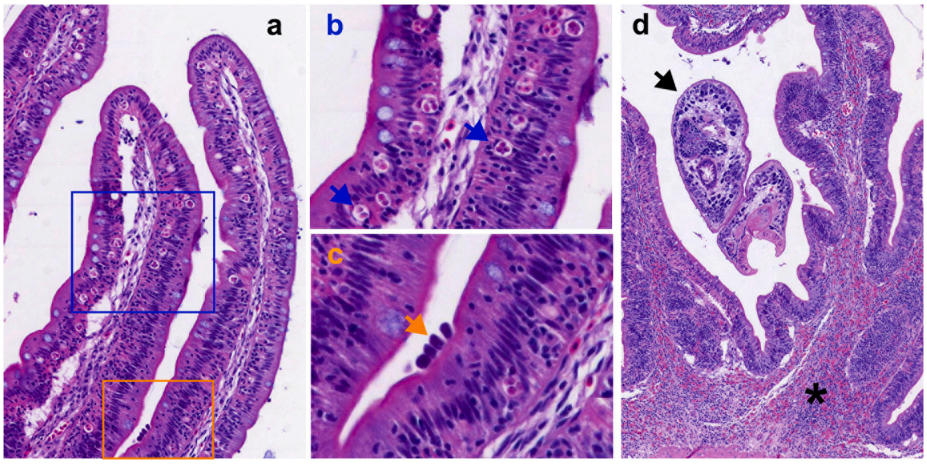

Fig. 6. Different intestinal parasites observed in the histological assessment of tissue from wild Ballan wrasse. (a) Myxozoan parasitic stages (possibly of *Enteromyxum leei*) observed in the intraepithelial space (blue rectangle) and possibly on the mucosal surface (orange rectangle). (b) and (c) Higher magnifications of the myxozoan parasite stages showing trophozoites in the intraepithelial space (blue arrows) and on the epithelial surfaces (orange arrow). (d) A cestode (tapeworm) parasite in the intestinal lumen (black arrow) alongside a strong inflammatory reaction (asterisk) in the submucosa and lamina propria in the IN4 intestinal region. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

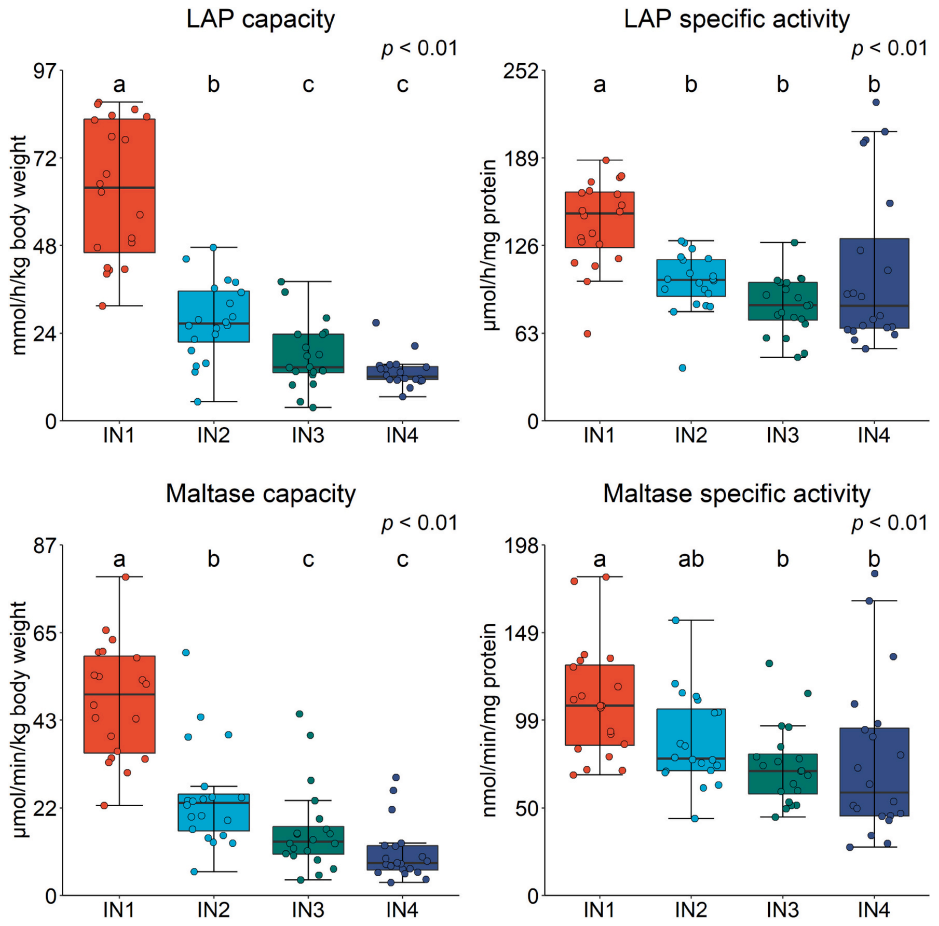

Fig. 7. Box-whisker plots illustrating LAP capacity, LAP specific activity, maltase capacity and maltase specific activity in different intestinal segments, with coloured points illustrating the distribution of individual values. Lowercase letters on top of each graphs indicates significant differences between intestinal segments.

intestine in wild Ballan wrasse revealed a largely similar intestinal architecture across all regions of the fish's short intestinal tract. The observations are in agreement with the observations made in cultured Ballan wrasse (Le et al., 2019). The similarity between the intestinal sections indicates that the whole intestinal tract is potentially available to carry out most of the vital functions depending on the physiological demand. It may also allow this stomach-less fish to use the whole intestinal tract as a food reservoir or 'stomach' in situations of high feed availability.

The thick fibrous submucosa and relatively thick lamina propria are possibly structural adaptations that add mechanical strength to the intestinal wall to allow the fish to cope with its natural diet mostly comprised of tough chitinous exoskeletons from prey that often includes echinoderms, gastropods and decapods (Figueiredo et al., 2005). The degree of mucosal fold branching in both wild and cultured Ballan wrasse is lower than expected for fish with such a short intestinal tract as only primary branching, i.e. a mucosal fold branched into two, and no higher branching was observed.

Supranuclear vacuoles in the enterocytes have not been detected in the intestine of the Ballan wrasse, neither in the wild in the present study nor in cultured fish ((Le et al., 2019). In salmonids, supranuclear vacuoles are normally found in the distal intestine (van den Ingh et al., 1991), while in cyprinids, they are located in the second segment of the mid intestine (Ng et al., 2005; Rombout et al., 1985; Wallace et al., 2005). In Atlantic salmon, the supranuclear vacuoles are known to diminish upon starvation and intestinal inflammation (Baeverfjord and Krogdahl, 1996), probably due to either reduced need for absorption capacity during starvation, or as a direct consequence of tissue damage and loss of function during an ongoing inflammatory reaction. Absence of enterocyte vacuoles in the Ballan wrasse could therefore reflect that the fish were in an unfed state. However, as supranuclear vacuoles are absent also in well fed cultured Ballan wrasse ((Le et al., 2019), this morphological feature might be lacking in the intestine of the Ballan wrasse.

The inflammatory morphological changes observed were likely caused by the parasitic organisms. The mucosal surface-attached parasite, probably *Enteromyxum leei*, a species of the myxozoan parasites (Cuadrado et al., 2007), are known to affect wrasses and considered quite significant in Mediterranean aquaculture (Katharios et al., 2011). In addition, a common feature observed in the current fish as well as in cultured Ballan wrasse (Le et al., 2019) was the strong presence of the eosinophilic granular cells (EGC). In teleost fish, the EGCs are

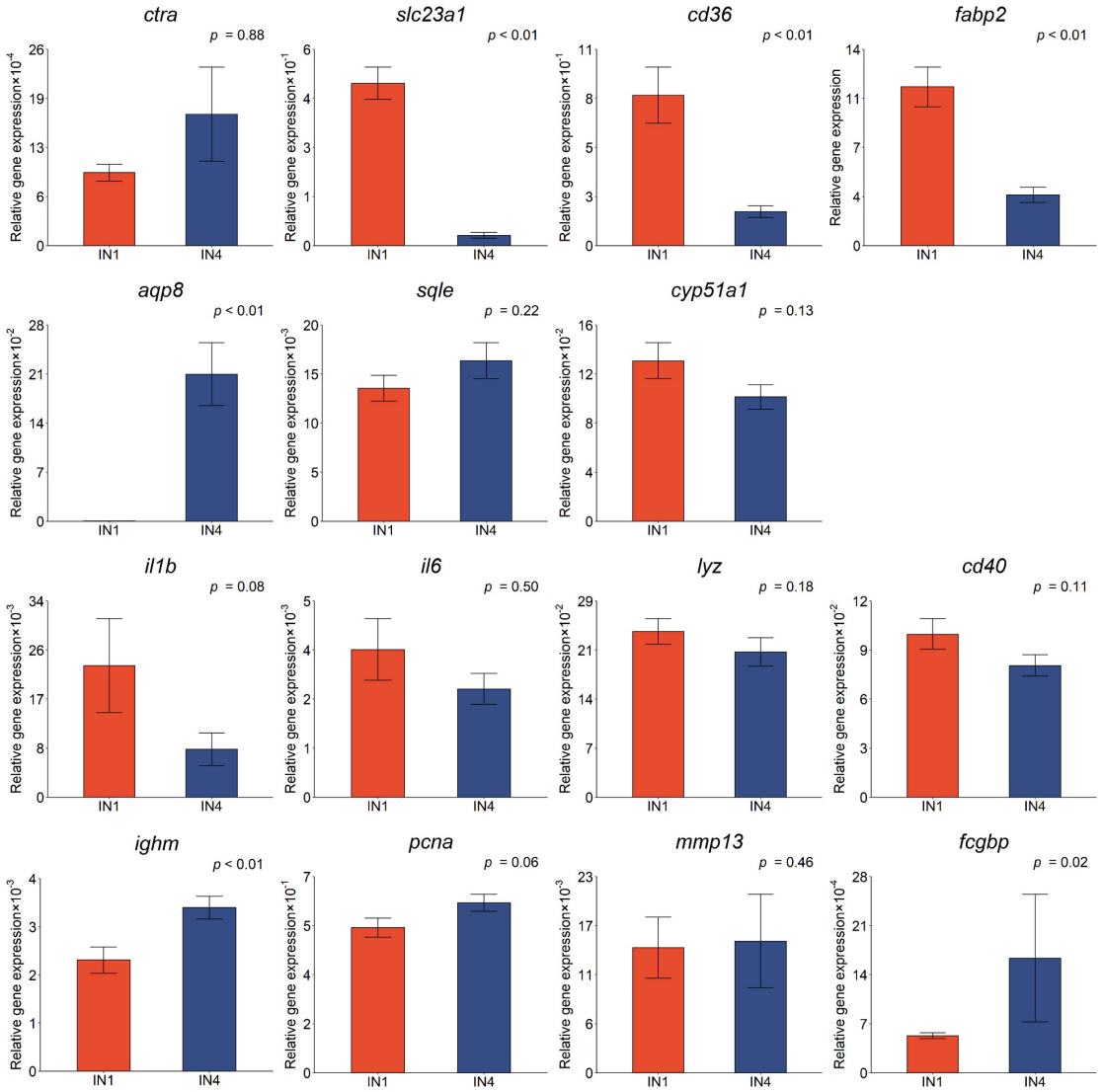

Fig. 8. Gene expression in IN1 and IN4 of: chymotrypsin A (*ctra*), vitamin C transporter (*slc23a1*), cluster of differentiation 36 (*cd36*), fatty acid binding protein (*fabp2*), aquaporin 8 (*aqp8*), squalene epoxidase (*sqle*) and lanosterol 14α-demethylase (*cyp51a1*), interleukin 1 beta (*il1b*), interleukin 6 (*il6*), lysozyme (*lyz*), cluster of differentiation 40 (*cd40*), immunoglobulin M heavy chain constant region (*ighm*), proliferating cell nuclear antigen (*pcna*), matrix metallopeptidase 13 (*mmp13*), IgGFc-binding protein (*fcgbp*). Values are means with standard errors as error bars. *P* values derived from statistical analysis are given.

granulated and are participants of the innate immune system. They have been proposed to be mast cell analogues (Hellberg et al., 2013; Reite and Evensen, 2006). In assessments of both wild and cultured Ballan wrasse, EGC number were markedly elevated not only in the submucosa and lamina propria, but EGCs had also migrated into the intraepithelial space. Consequently, the intraepithelial space of the Ballan wrasse showed a marked infiltration with EGCs, lymphocytes and occasionally, rodlet cells, which are thought to function to combat parasitic infections (Reite, 2005). Similarly, infections by a range of parasites in the gut were also observed in wild Ballan wrasse from British south-west coast (McMurtrie et al., 2019). Here, parasite infections did not cause any pathological changes, but a high number of eosinophils were observed, which is in line with the histological results of the present study.

4.3. Brush border digestive enzymes

The high specific activity and capacity of LAP and maltase observed in the IN1, gradually decreasing towards the distal end, strongly indicate that IN1 plays the most prominent role in digestion and absorption of macronutrients. These results correspond to the transcriptomic results of Lie et al. (2018) showing that digestion-related genes were expressed more abundantly in the proximal segment in cultured Ballan wrasse.

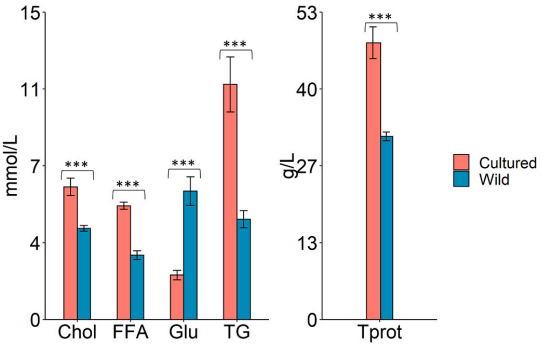

Fig. 9. Comparison of blood plasma variables between wild and cultured Ballan wrasse. Values are means ($N = 20$ for wild fish and N = 12 for cultured fish) with standard errors as error bars. "***" indicates p value is less than 0.01. Results of cultured fish are presented in Le et al. (2019). Abbreviations: Chol, cholesterol; FFAs: free fatty acids; Glu, glucose; TGs, triglycerides; Tprot, total protein.

Fig. 10 illustrates the present results in comparison to unpublished results from our earlier work on cultured Ballan wrasse (Le et al., 2019). Also, the cultured fish showed decreasing enzyme capacity along the intestine, but the absolute values were much higher in the wild than the cultured fish. This difference may partly be due to differences in developmental stage of the fish. Many studies have demonstrated that specific activity of digestive enzymes increases as fish grows (Faulk et al., 2007; Kim et al., 2001; Oozeki and Bailey, 1995; Sahlmann et al., 2015; Tramati et al., 2005). The average weights were 232 g for the current wild Ballan wrasse and 87 g for the cultured Ballan wrasse, indicating that wild fish were at a more advanced developmental stage and may therefore have higher LAP and maltase capacity. However, as Ballan wrasse can reach a size of 4 kg, it is unlikely that the difference in developmental stage, reflected in a difference in weight of 150 g, explains the great differences in enzyme characteristics between the wild and the cultured fish. Another possible explanation might be differences in nutrient sources and protein level in the diet, knowing that digestive capacity in most animals is very flexible and easily adapts to diet composition (Cahu and Infante, 1995; Nicholson et al., 1974).

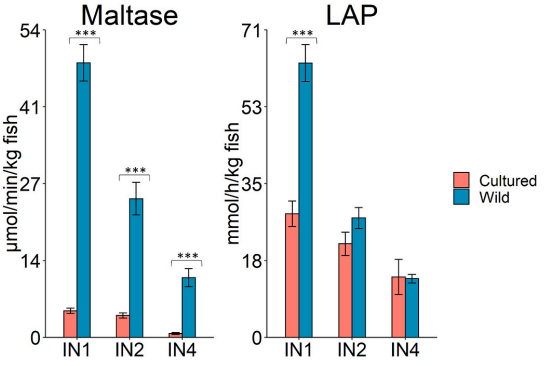

Fig. 10. Comparison of maltase and LAP capacity between wild and cultured Ballan wrasse. Values are means (N = 20 for wild fish and $N = 12$ for cultured fish) with standard errors as error bars. Results of cultured fish are from unpublished data of Le et al. (2019).

4.4. Gene regulation in the intestinal mucosa

4.4.1. Nutrient absorption

The results in the present study showing higher expression of *cd36* and *fabp2* in IN1 than in IN4 are supporting the argument above that IN1 is the major site for macronutrient absorption. These observations are in line with the previous results from cultured Ballan wrasse, which showed that the IN1 segment accounted for 50% of dietary lipid absorption (Le et al., 2019). The comparison of expression levels of these genes in IN4 of wild and cultured fish revealed substantially lower expression of *fabp2* in the wild fish than in the cultured, and *cd36* showed a same trend (Fig. 11), a result which may be related to lower feed intake in the wild fish.

The higher expression level of *slc23a1* in IN1 than in IN4 indicates that IN1 is predominant site also for vitamin C absorption. The major absorption site of vitamin C seems to differ between animals. In guinea pig and common carp (Dabrowski, 1990; Hornig et al., 1973), the proximal intestine was predominant, but in rat and humans, the ileum show the highest absorption (Hornig et al., 1973; Malo and Wilson, 2000). No clear difference between wild and cultured fish was observed in IN4 regarding expression of this gene.

The substantially higher expression levels of *aqp8* in IN4 than in IN1 is in agreement of that observed in cultured fish in which the expression level of *aqp8* increased gradually from the proximal intestine towards the distal intestine (Le et al., 2019). The aqp8 protein is involved in various functions such as water and ammonia transport (Saparov et al., 2007) and H_2O_2 efflux from mitochondria (Danielli et al., 2019), but which function drives its higher expression in IN4 cannot be identified based on its gene expression. In Atlantic salmon, the intestinal *aqp8b* expression is highly induced during sea water acclimation, indicating that osmoregulation could be the main influence of *aqp8* expression (Tipsmark et al., 2010). Therefore, it could also be the explanation that high *aqp8* expression in IN4 is reflecting that this site is important for adjustment of water absorption. Expression of *aqp8* in IN4 in wild compared to the cultured fish showed similar values (Fig. 11).

4.4.2. Intestinal cholesterol metabolism

The observation of similar gene expression level of *cyp51a1* and *sqle* in IN1 and IN4 is likely an indication of similar cholesterol synthesis level, as transcription levels of these genes show good correspondence with total cholesterol load and synthesis rate (Kortner et al., 2014). In mammals, intestinal cholesterol synthesis accounts for 15% ~ 35% of total body cholesterol synthesis, but different intestinal regions have different synthesis rate, and is highest in the proximal small intestine and decreases towards the distal regions (van der Wulp et al., 2013). This is contrary to the current observation in wild Ballan wrasse, a contrast which may be related to the postabsorptive status of the observed wild wrasse. Cholesterol synthesis has been observed to decline during fasting in humans (Browning and Horton, 2010), rat (Caimari et al., 2010) and chicken (Desert et al., 2008). In accordance with this consideration the expression of the *cyp51a1* gene, was much lower in the wild than the cultured, well fed Ballan wrasse (Fig. 11), whereas *sqle*, showed no such difference.

4.4.3. Immune response in the intestine

The higher expression level of *ighm* in IN4 than in IN1 is in agreement with the observation in the study on cultured Ballan wrasse by Bilal et al. (2019). As an important component in humoral immunity, *ighm* is usually highly expressed in systemic immune organs such as head kidney and spleen (Piazzon et al., 2016; Press and Evensen, 1999). However, the study by Bilal et al. showed that in both wild and cultured Ballan wrasse, *ighm* expression in intestine was even higher than that in head kidney and spleen (Bilal et al., 2019). Experiments have shown up-regulation of IgM expression, both regarding mRNA and protein, when the fish were exposed to various factors, including dietary soybean molasses (Krogdahl et al., 2000), infection (Estensoro et al., 2012) and

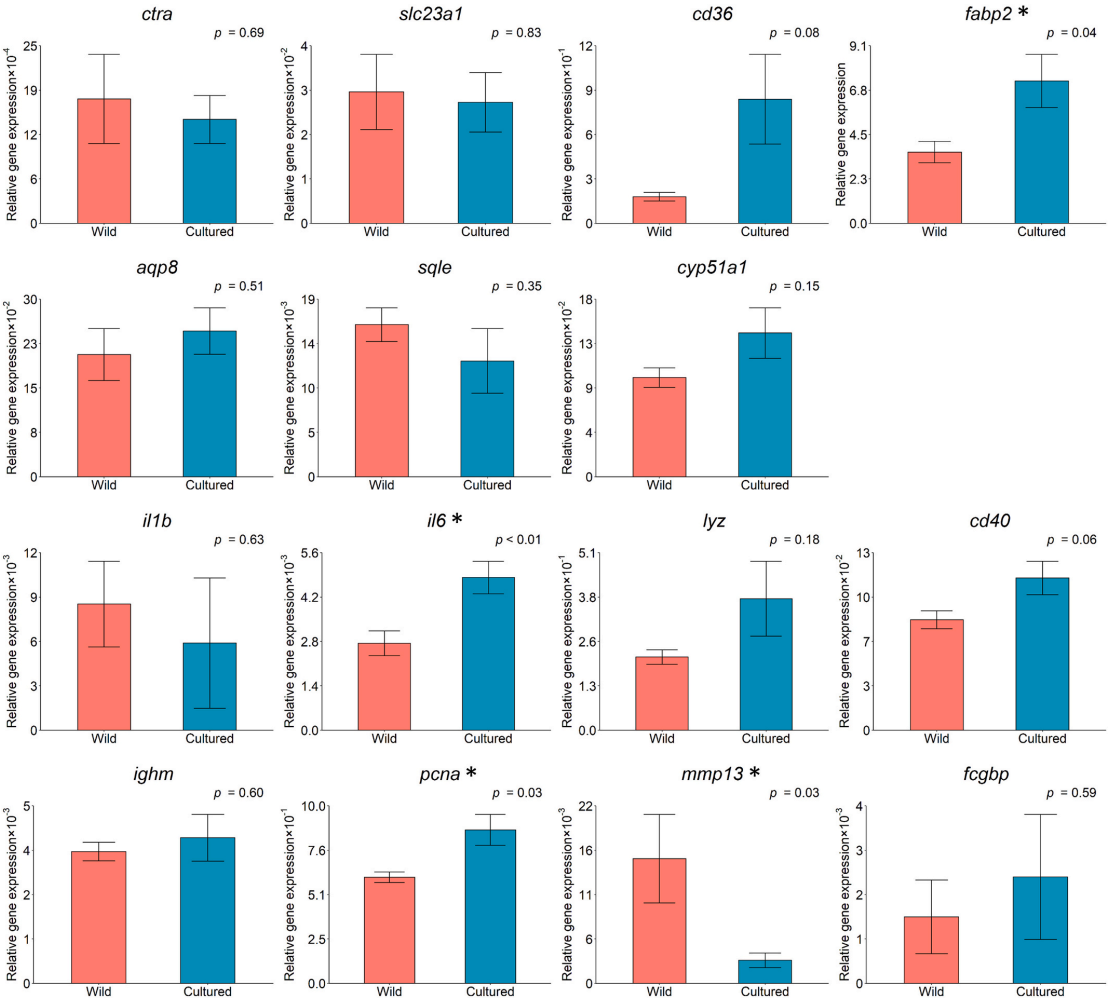

Fig. 11. Comparison of gene expression level in IN4 between wild (present results) and cultured (unpublished data of Le et al. (2019)) Ballan wrasse for genes showing significant (*) or no indications of differences.

environmental stresses (Huang et al., 2011), demonstrating that *ighm* serves as an immune factor that can respond to a variety of stimuli. Expressing *ighm* at high level may give IN4 more protection towards antigens from the anal opening. In the present comparison, regarding expression of *ighm* in IN4 in wild and cultured fish, similar values were observed.

In a previous study conducted with cultured Ballan wrasse, most immune-related genes were expressed at higher levels in the distal intestinal segment than in the proximal (Lie et al., 2018). However, in the present study, important intestinal immune genes such as *lyz*, *il6*, *il1b*, *cd40* showed no difference in expression levels between IN1 and IN4. One possible explanation for this discrepancy could be related to the parasite infections, which showed higher prevalence in IN1 than in IN4 in the current study. As all the above-mentioned genes have been reported to respond to parasite infection in fish (Alvarez-Pellitero, 2008; Dezfuli et al., 2012), the higher infection prevalence observed in IN1 might have elevated mRNA abundance of these genes in IN1 and thereby levelled out potential differences in basal expression levels between IN1 and IN4. However, *ighm* expression remained higher in IN4, indicating that an IgM response might not be involved in the intestinal responses to parasite infection in Ballan wrasse. This is in agreement with studies in other fish species (Piazzon et al., 2016; Zhang et al., 2010) showing that IgT level but not IgM was elevated in the intestine during parasite infection.

Comparison between the present results and the results of our previous study on cultured Ballan wrasse, shows lower values in the wild fish for two inflammation and tissue damage related genes, *il6* and *pcna* (Kelman, 1997; Tanaka et al., 2014), while the expression of *mmp13*, which is also related to inflammation (Xu et al., 2018), was higher (Fig. 11). On the other hand, several of the observed immune genes involved in various immune responses did not differ between wild and cultured fish. Present knowledge on gut immune functions in fish in general, and on Ballan wrasse in particular, is not sufficient to conclude whether these wild fish had more healthy intestines than the cultured fish in our studies. However, the parasites found in the wild fish intestines indicate that the observed differences between the wild and the

cultured fish might be related to the parasite challenge in the wild fish.

5. Conclusions

The present study revealed characteristics of morphology and digestive and immunological functions along the digestive tract in wild Ballan wrasse. Based on these observations it appears that the whole intestinal tract is potentially available to carry out most of the vital functions depending on the physiological demand of the fish. Regarding digestive and absorptive activities, IN1 seems as the predominant site. Intestinal immune function was also profiled in wild Ballan wrasse, but in terms of parasite infection in the gut, which may have modulated gene expression profile, a general picture could not be elucidated. Eosinophilic granular cells and rodlet cells seemed to be the most active cells involved in the parasite response. IgM seemed to play an important role in intestinal immunity, especially in the distal segment, which was also observed in cultured Ballan wrasse. However, it may not be involved in the immune response to the parasite infection.

In comparison to the cultured Ballan wrasse, the wild Ballan wrasse had lower level of plasma nutrients including protein, cholesterol, fatty acids and triglyceride, which indicated a postabsorptive status of the wild fish. The higher LAP and maltase capacity suggest that wild Ballan wrasse have higher digestive capacity to amino acids and maltose, which may be a result of differences in diet composition. Status of the intestinal immune apparatus did not show apparent differences between wild and cultured Ballan wrasse.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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PAPER II

Soya saponins and prebiotics alter intestinal functions in Ballan wrasse (*Labrus bergylta*)

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Abstract

A 5-week feeding trial was conducted in the cleaner fish Ballan wrasse (*Labrus bergylta*) for a better understanding of the basic biology of the intestinal functions and health in this stomach less species. During the trial, Ballan wrasse was fed either a reference diet, the reference diet supplemented with *i)* a commercial prebiotic (Aquate™ SG, 0.4%) expected to have beneficial effects, *ii)* soya saponins (0.7%) expected to induce inflammation, or *iii)* a combination of the prebiotics and the soya saponins to find a remedy for gut inflammation. Blood, intestinal tissue, and gut content from four consecutive intestinal segments (IN1 – IN4) were collected. No significant differences in fish growth were observed between the four dietary groups. Saponin supplementation, both alone and in combination with prebiotics, increased weight index of IN2 and IN3 and decreased blood plasma glucose, cholesterol, and total protein. Dry matter of intestinal content and activity of digestive enzymes were not affected by diet. Histomorphological analyses revealed a progressing inflammation with increased infiltration by immune cells particularly into the distal parts of the intestine in fish fed diets with saponins, both alone and in combination with prebiotics. Gene expression profiles obtained by RNA sequencing and quantitative PCR mirrored the histological and biochemical changes induced by the saponin load. The study demonstrated that Ballan wrasse gut health and digestive function may be markedly affected by feed ingredients containing antinutrients.

Key words: Cleaner fish, Ballan wrasse, Gut health, Antinutrients, Prebiotics

1. Introduction

The Ballan wrasse (*Labrus bergylta*) has, during the recent years, become a domesticated fish species due to its ability to feed on sea lice living on the surface of Atlantic salmon (*Salmo salar*)[1,2,3]. Sea lice infestation has become the most important challenge for Atlantic salmon production in Norway and neighboring countries [1,4] as a result of increased lice resistance against delousing chemicals [5]. Ballan wrasse is considered as an effective therapeutic and preventive biological control agent against salmon lice [6,7]. Although wild caught fish are still the main source of Ballan wrasse used in salmon production, farmed Ballan wrasse is increasingly being used. However, the challenges in this production are many and several knowledge gaps must be filled. The present study seeks to find answers to questions regarding which nutrients sources can secure or compromise gut health in Ballan wrasse. The observation of severe symptoms of gut inflammation in an earlier survey of gut health in cultivated Ballan wrasse [8] demonstrated the urgency for better understanding of the general structure and physiology of the digestive tract of this species, and relationships between diet composition and digestive and immune functions in the intestine. The present study aimed to strengthen knowledge on such aspects of the short and agastric digestive tract of the Ballan wrasse[9,10].

We chose soya saponins and a commercial prebiotic as stimulus to trigger immune responses in gut. In studies with Atlantic salmon, soybean meal was documented to induce gut inflammation [11,12,13] due to its content of saponins. Saponins seem to impair intercellular junctions and increase membrane permeability when they present in intestine [14]. The increase in permeability, and the resulting increase in influx into the tissue of antigens and other alien components, results in triggering of several immune functions, such as production of cytokines [15,16] and migration of lymphocytes into the tissue [17].

Supplementing fish with prebiotics has been reported to enhance immune status of intestine, improve gut structure, and disease resistance with possible direct or indirect modulatory effects on gut immune responses [18]. Therefore, using prebiotics is a useful way to explore mechanisms of gut functions and health in fish. The prebiotic used in the current study is a commercial product (Aquate, Alltech) composed of contains a cell wall extract from baker's yeast (*Saccharomyces cerevisiae*) and dried algae. It is claimed by the producer that it maintains gastrointestinal integrity and stability, improves digestibility and intestinal health. (see https://www.knowde.com/stores/alltech/products/aquate/).

The inclusion of saponins and prebiotic in diet in the present study was to use them, either alone or in combination, as tools for the study of mechanisms underlying gut function and health in Ballan wrasse. We hypothesized that soya saponins in diet impair intestinal immunity, while the prebiotic may trigger immune reactions in the intestine and counteract with saponins when they present in diet together.

2. Materials and methods

2.1 Experimental animals, diets, and sampling

The experimental procedure of the present study comprised a five week feeding trial with four diets, i.e. a reference diet (Ref), and three experimental diets made by supplementation with a prebiotic (Pre) expected to improve gut health and function at a level suggested by the producer, soya saponins (Sap) at a level found in soybean meal and found to induce inflammation in Atlantic salmon [15,19], or a mixture of the two (P+S) which might indicate whether gut inflammation can be prevented by functional ingredients. Together observations made on fish fed these four diets was expected to fulfil the aims of this study, i.e., to be able to improve diet composition and thereby health and function of the fish by strengthening knowledge of the general structure and physiology of the digestive tract of Ballan wrasse, and relationships between diet composition and digestive and immune functions in the intestine.

The feeding trial followed the Norwegian animal welfare act guidelines, in accordance with the Animal Welfare Act of 20th December 1974, amended 19th June 2009. The facility at Sunndalsøra, Nofima (division 60) got a permission granted by the Norwegian Food Safety Authority, (FOTS ID 8060) to run the experiment. The decision was made on the basis of Regulation 18. June 2015 on the use of animals in experiments, §§ 6, 7, 9, 10 and 11. The 3R principles were used as a foundation for the design of the experiment, i.e.to "Reduce, Refine, Replace" which means to minimize the use of experimental animals to a minimum, to keep the animals in such a way that the burden on the animals is as small as possible, and when possible, animal experiments are replaced by alternative methods. As effects of antinutrients and prebiotics on digestive functions and gut health, indicated by biochemical, histological, and molecular responses, were the main goals of the present work, and effects of growth was not in focus, two tanks per treatment was considered sufficient for generation of information on tank effects. The variables addressed have shown negligible tank effects from our previous work with antinutrient in diets for Atlantic salmon. Moreover, five weeks feeding was considered sufficient, as development of effects of saponins in Atlantic salmon takes only about two weeks to reach full inflammation pictures [20,21].

The fish used for the trial had been reared for one year in a commercial farm (MOWI, Bergen, Norway) before transfer to the Nofima AS land-based research facility in Sunndalsøra, Norway. Upon arrival, the fish were distributed randomly to 10 cylindrical flat-bottomed black tanks with a volume of 350 l, equipped with separate light sources and artificial kelp. The photoperiod was 24 hours per day. A flow through system was used in the tank, with seawater pumped from the Sunndal fjord and filtered by drumfilters of approximately 20 µm. The water salinity 32 ppt and water temperature in tanks during the experiment was 14.5-17 °C. All fish were individually tagged (passive integrated transponder (PIT), weighed and length measured at the same time as they were tagged. Because of the

sensitivity of Ballan wrasse to the change of feed and tank environment, fish were kept on the same diet before the start of the experiment. After two-week rearing, the feed uptake remained low, so fish were reared for another two weeks in the experimental tanks. During these four weeks the fish were fed the diet later referred to as the reference diet. At the start of the experiment, fish weight was 73.5g and the densities were adjusted to 16 kg/m^3 per tank (80 fish).

One reference diet (Ref) containing high quality marine raw materials was made (Table 1). The diet choice of ingredients and formulation was based on experience from previous Ballan wrasse feeding trials [22,23]. It contained 61% protein, 16% lipid, 7% carbohydrate and 16% ash (of dry matter). Three experimental diets were made by supplementing the Ref diet with either a commercial prebiotic (Aquate™ SG, 0.4%, a yeast product containing *Saccharomyces cerevisiae* extract and algae, included at a level recommended by the producer Alltech) (Pre), soyasaponin (0.7%) (Sap), or the combination of prebiotics and soyasaponin (P+S). Feed was produced by extrusion (2 mm pellet size) at Nofima's Aquafeed Technology Center in Bergen, Norway. Feed was distributed randomly to duplicate groups of fish per diet for 24 hours with 10 min feeding intervals, using automatic belt feeders for the duration of the experiment. Each belt feeder (one per tank) was loaded with feed equivalent to 80 g feed/kg fish per day. Before loading to each belt automat, the 80 g of feed was soaked in 10 ml of water for 4-5 minutes to soften the feed. Feed intake was not recorded.

The feeding trial ran for five weeks. At termination of the feeding trial, fish were randomly sampled from the tanks and fully anesthetized and euthanized with 110 mg/l tricaine methane sulfonate (MS-222, Argent Chemical Laboratories). From 6 fish per tank, blood was sampled by venipuncture of the caudal vein. Blood was collected in vacutainers containing lithium heparin and stored on ice until centrifugation. Plasma was separated and immediately frozen in liquid nitrogen and stored at -80°C until analysis. After blood withdrawal, fish were killed

by a blow to the head before organ sampling. Thereafter, fish were opened, and the viscera was removed from the abdominal cavity for dissection. The intestine was cleaned of mesenteric fat and divided into four segments (IN1-4) as previously described [9]. Each segment was opened longitudinally, and the gut content was collected, snap frozen in liquid nitrogen and stored at -80°C before further processing. From the same intestinal segments, tissue samples were taken for RNA extraction (submerged in RNAlater solution, incubated at 4 °C for 24 h and stored at -20 °C) and histomorphological evaluation (fixed in 4% phosphate-buffered formaldehyde solution for 24 h and transferred to 70% ethanol for storage).

2.2 Blood plasma variables

Blood plasma was analysed for cholesterol, free fatty acids, total triglycerides, total protein and glucose at the Central Laboratory of the Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo, according to standard, medical procedures. The instrument used was a Siemens Atellica CH fra Siemens Healthineers. The procedures are available in supplementary files S1-S5.

2.3 Histology

Formalin fixed tissue samples were processed using standard histological techniques and stained with haematoxylin and eosin (H&E). Examination was conducted blindly and in randomized order. The following histological characteristics associated with inflammatory reactions in the intestinal mucosa were evaluated: length and fusion of mucosal folds, cellular infiltration, width of the lamina propria and submucosa, enterocyte vacuolization, nucleus position within the enterocytes and the relative number of goblet cells. The morphological assessment was guided by our experience with salmonid intestinal histopathology [24] as well

as an examination of the histomorphology of the intestine in wild ballan wrasse that we describe in Zhou et al [25].

2.4 Trypsin activity and total bile salts in the digesta

Trypsin activity and total bile salt levels were measured in pooled freeze-dried gastrointestinal contents from the four separate intestine segments (IN1-4). Trypsin activity was determined colorimetrically as described by Kakade and co-workers [26] using the substrate benzoyl arginine p-nitroanilide (Sigma no. B-4875, Sigma Chemical Co., St. Louis, MO) and a curve derived from standardized bovine trypsin solution.

Bile salt levels were determined using the enzyme cycling amplification/Thio – NAD method (Inverness Medical, Cheshire, UK) in the ADVIA®1650 Chemistry System (Siemens Healthcare Diagnostics Inc.) at the Central Laboratory of the Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo.

2.5 Brush border membrane enzyme activity

The activity of the brush border membrane enzymes leucine aminopeptidase (LAP) and maltase was measured in tissue homogenates from IN1, IN2 and IN4 as described previously [27]. Due to an analytical error, some data from IN3 were lost and are therefore not presented. Activities were calculated as mmol substrate hydrolysed per unit time in the whole tissue per kg body weight (enzymatic capacity). Protein was analysed using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany).

2.6 Gene expression

RNA sequencing

Gene expression profiling was conducted using samples from IN4, based on our previous finding that the distal intestine harbors the most active immune functions [28], in line with what is the case also in Atlantic salmon which shows distinct structural changes in this section upon feeding with diets containing saponins [15]. Gene expression analyses were performed using n=3 fish per tank (n=6 fish per diet) fed the three experimental diets and the reference diet. Total RNA was extracted using a BioRobot® EZ1 and RNA Tissue Mini Kit (Qiagen, Hilden, Germany) including a DNase treatment step according to the manufacturer's instructions. RNA quantity and quality were assessed using a NanoDrop ND-1000 UV–vis Spectrophotometer (NanoDrop Technologies, Wilmington, USA). All samples had 260/230 and 260/280 ratios above 2.0 and 2.2 respectively, indicating high RNA purity. Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, USA) were used for assessment of RNA integrity for the individual samples. The average RNA integrity number (RIN) of all samples was 7.8 ± 0.5. RNA sequencing was performed by the Norwegian Sequencing Centre (www.sequencing.uio.no). Sequencing libraries were prepared with an automated NeoPrep platform (Illumina) using 90 ng total RNA input to the TruSeq Stranded mRNA Library Prep Kit (Illumina) and standard Illumina adaptors included in the kit. The libraries were sequenced using HiSeq4000 according to manufacturer instructions, generating paired end libraries with an average library size of 14.2 ± 1.4 million reads Prior to mapping, remaining sequencing adaptors were removed using Cutadapt [29]. Subsequent trimming and filtering were conducted using Sickle (https://github.com/najoshi/sickle) with a 40 bp minimum remaining sequence length, Sanger quality of 20, and no 5' end trimming. The individual libraries were mapped to the Ballan wrasse genome (European Nucleotide Archive accession number: PRJEB13687, http://www.ebi.ac.uk/ena/data/view/PRJEB13687), using the short read aligner TopHat2 [30], and transcript abundances was estimated using the FeatureCounts [31]. Differentially expression of genes (DEGs)were analyzed using the

DEseq2 package [32], taking advantage of the 2x2 factorial design and enabeling analysis of combining effects between saponin and prebiotic. Features (genes) with row sums less than 50 were excluded prior to analysis of the results for differently expressed genes. RNAseq can be accessed through the Gene Expression Omnibus (GEO accession number GSE152475). An adjusted p value (p-adjust) of < 0.1 (Benjamini and Hochberg correction) was applied for further downstream analysis. Pathway and functional analysis of DEGs was conducted using the Ingenuity Pathway Analysis (IPA) software (IPA, Qiagen, Redwood City, CA, USA). Canonical pathway analysis, disease and biological function analysis and prediction of upstream regulators were conducted on the dataset. While both canonical pathway analysis and biological function analysis predict affected biological events, the upstream regulator analysis identifies the cascade of upstream transcriptional regulators that can explain the observed gene expression changes. IPA results were filtered using $p < 0.05$ (Fisher exact test) and a Z-score > 2 or higher depending on the need for reducing the dataset. While the p value filtering reflects the likelihood of a pathway/function being significantly affected by the treatment, the Z-score describes the likelihood of a directional association in our dataset. Positive and negative Z-scores implies an increase or decrease in activity/activation of the enriched pathway respectively.

Quantitative real-time PCR

In addition to RNA sequencing, quantitative real-time PCR (qPCR) was used as an independent gene expression profiling method to examine genes of interest in detail. Assays were carried out according to MIQE standards [33] on separate RNA aliquots from all samples used for RNA sequencing. Complementary DNA synthesis, qPCR assays, primer design, optimization and validation was carried out as described previously [34]. For reference gene selection, we searched for stably expressed genes in the RNA sequencing data set by

employing the following filter strategy: base mean >200, log2fold <0.01 and >0.01. Three putative reference genes (*ywhae*, *gapdh2*, *top2a*) were selected from the list, and *gapdh2* was ultimately used as the only reference gene according to its stability in different diet groups. Target gene expression was normalized according to Muller et al. [35]. Details of qPCR assays are given in Table 2. In total, we profiled 19 genes based on either significant differential expression as obtained with RNAseq data analysis, or by selecting gene candidates for assessment of gut functions and health.

2.7 Calculations

Thermal-unit growth coefficient (TGC) was calculated as: $TGC = 1000*(FBW^{1/3} - IBW^{1/3}) \times (\Sigma D°)^{-1}$, where IBW and FBW are the initial and final body weights (tank means) and ΣD° is the thermal sum (feeding days x average temperature in °C).

The specific growth rate (SGR) was calculated using the tank means for initial body weight (IBW) and final body weight (FBW) as follows: SGR = [(ln FBW – ln IBW) /number of days] x 100.

Organosomatic indices (OSI) were calculated as percentages of the weight of the organ in relation to body weight.

2.8 Statistical analyses

Data were processed using R (version 3.5.2, 2018) in the integrated development environment Rstudio (version 1.1.463, 2018). Tank effect was firstly examined by point plot followed by comparison of mixed effect models. As tank effect in this study were not significant for any of the biomarkers, individual fish was used as the statistical unit. Data were then diagnosed for normal distribution of residuals and homogeneity of variance

246 according to Kozak and Piepho [36]. Data that violated either normality of distribution of
247 residuals or homogeneity of variance were subject to Box-Cox transformation.
248 Two-way ANOVA was used for statistical evaluation with diet and gut sections as class
249 variables. When the interaction of factors was significant, one-way ANOVA followed by
250 Tukey's test was applied to investigate effects of diets in individual gut segments. Kruskal–
251 Wallis test was applied for those data that could not meet normal distribution of residuals,
252 followed by Dunn's multiple comparison test if significant. Differences were considered
253 significant at $p < 0.05$.
254 Differences in histological scores for the various evaluated morphological characteristics
255 were analyzed using ordinal logistic regression. When score differences were only 2 levels,
256 statistical significance was assessed using the Fisher exact test. Post hoc analysis for
257 significant test results was conducted using the Chisq.post.hoc test (Fifer package in R).
258 Differences were considered significant at $p < 0.05$.
259

260 3. Results
261 In accordance with the goals of the present work presentation of the results are organized in
262 two subchapters. The first reports results regarding the general physiology of the intestine of
263 the Ballan wrasse, and the second effects of the prebiotic and the saponins, alone and in
264 combination.
265

266 3.1 General characteristics of the intestine
267 The histological observations showed that both branched and unbranched mucosal folds
268 appeared in the intestine of Ballan wrasse, and the branching occurred in all regions of the
269 intestinal tract. The branching was mostly simple bifurcation (Figure 1), and they were most
270 extensive in the IN1 and IN4. The submucosa and lamina propria were wide, containing

fibrous tissue and showed varying degrees of cellularity (Figure 2a). The most common cell type in these compartments were the eosinophilic granular cells (EGCs). A stratum compactum delimiting the submucosa from the muscular layers, observed in some other species, was absent in the Ballan wrasse. The Ballan wrasse intestinal mucosa showed a high degree of intraepithelial lymphocytes. The enterocytes seemed to lack extensive supranuclear vacuolization (Figure 3) normally found in the distal intestine of Atlantic salmon [13].

Table 3 shows the two-way ANOVA results of variables including OSI, LAP and maltase capacity, dry matter, trypsin activity and bile salt level in digesta. The OSIs, given as percent of body weight, of the intestinal segments were 0.37±0.02, 0.35±0.02, 0.23±0.01, and 0.13±0.01 for IN1, IN2, IN3 and IN4, respectively (mean±S.E.M, unit: % of body weight), showing a decreasing trend towards the distal segments.

Trypsin activity, analyzed in the intestinal chyme, LAP and maltase activity in homogenates of the mucosa of the measured segments demonstrated digestive capacity throughout the intestinal tract, decreasing trend towards the distal part of the intestine (Figure 4 and Figure 5). Bile salts were also detected in digesta collected from IN1 to IN4 with the highest level in IN2 (Figure 6).

3.2 Effects of saponins and prebiotics

Fish growth

Final weight and length, condition factor, thermal growth coefficient, and specific growth rate are presented in Table 4. There were no significant differences in fish growth performance between the treatments. Regarding the mortality, except for 2.7% fish died due to an incidence with the oxygen supply in one tank, there was no fish died during the feeding trial.

296 Blood plasma biochemistry

297 Results of the blood plasma variables are presented in Table 5. Adding the prebiotic alone to
298 the diet (Pre) reduced plasma glucose in fish compared to that of the Ref diet. Fish fed the
299 Sap and the P+S diets showed similar decreasing trends. The Pre diet did not affect plasma
300 cholesterol level whereas Sap and P+S diets reduced cholesterol level. Plasma total protein
301 level was unaffected by Pre, showed a decreasing trend for the Sap diet, and a significant
302 decrease in fish fed the P+S diets. Regarding plasma triglyceride level, none of the
303 supplemented diets caused significant changes.
304

305 Histomorphology of the intestine

306 Adding the prebiotic alone (Pre) to the Ref diet did not alter the cellularity of the lamina
307 propria (Figure 7) nor the intraepithelial lymphocyte infiltration (Figure 8), but significantly
308 increased intraepithelial eosinophilic granular cells in IN3 (Figure 9). Adding soya saponins
309 alone (Sap) caused moderate to marked changes regarding intraepithelial lymphocyte
310 infiltration in IN3 and IN4 (Figure 8), indicating a progressive inflammatory process.
311 However, cellularity of the lamina propria (Figure 7) and the number of eosinophilic granular
312 cells were not affected (Figure 9). Combining the prebiotic and the saponin (P+S) did not
313 eliminate the saponin effects but rather increased immune cell infiltration in lamina propria in
314 IN4 (Figure 7), and intraepithelial lymphocyte infiltration in IN3 and IN4 (Figure 8).
315 In some individuals marked infiltration of eosinophilic granular cells and increased rodlet cell
316 numbers in the mucosal epithelium were observed in IN1 and IN2. However, the changes did
317 not show relationship to dietary treatments.
318

Activity of digestive enzymes in the brush border membrane

The results for maltase capacity in the brush border along the intestine, observed for the sections IN1, 2 and 4, are presented in Figure 4. No significant diet effects were observed in IN1 for any of the diets. In IN2 Pre did not affect maltase activity, whereas significant stimulating effects of Sap and P+S were observed. The results for IN4 showed a trend towards stimulating effects of Pre but showed no effect of Sap or P+S. Regarding LAP capacity (Figure 5), no significant changes in any of the intestinal segments were observed. However, the results showed a trend towards increased capacity for fish fed Sap and P+S, in parallel to the result observed for maltase.

Bile salt concentration, dry matter, and trypsin activity in intestinal content

The Pre diet, compared to the Ref diet, elevated chyme bile salt levels significantly in IN2 and showed the same trend for IN1 and IN3 (Figure 6). Neither the Sap nor the P+S diet caused significant effects on bile salt concentration in any of the intestinal segments. This indicates that the effect of the prebiotic was eliminated when combined with the saponins.

For dry matter and trypsin activity in the intestinal content, no diet effects were observed (Table 3). The trypsin activities averaged 181, 110, 110 and 131U/mg dry matter whereas digesta dry matter were 16, 16, 17, and 16% in IN1 to IN4, respectively.

Gene expression in IN4

RNA sequencing

As illustrated in Figure 10, a total number of 4544 genes showed effect of diet on their expression, as compared to the expression in fish fed the Ref diet. Among the treatments, fish fed the Pre diet demonstrated effect on the greatest number of genes, 2817 of which 2128

were unique for this treatment. Fish fed the Sap diet showed the lowest number: 1055 of which 308 were unique, and those fed the P+S diet showed effects on an intermediate number of genes:1975, of which 1078 were unique for this treatment. Among the DEGs, 273 were common for all the three supplementations, 133 for Sap and Pre, 341 for Sap and P+S, 283 for Pre and P+S. The complete list of DEGs is shown in supplemental table S6.

The 10 most upregulated genes (log2 fold change >2) were for fish fed the Pre diet relatively diverse in their biological function such as: *histone h2a* in gene regulation, *mtrf1l* in translation termination, *l1td1* in stem cell function, *mks1* in cell surface structure maintenance, *jam3* in regulation of tight junctions, *igkv4-1* in antigen binding, *ly75* in antigen processing, *itih3* in handling of oxidative stress, *me2* in mitochondrial energy metabolism, and *hkdc* in glucose metabolism. For the Sap treatment, in contrast, most of the top upregulated genes were involved in lymphocyte function and signaling as a response to infestation, such as *neurl3*, involved in protein degradation, the pro-inflammatory chemokines *ccl11* and *ccl4*, *pnp* in purine metabolism, *mov10b* in RNA binding, and the pro-inflammatory *il8r1*. A similar picture was observed for P+S for which *neurl3, ccl11, ccl4* were among the top abundant genes.

The top 10 most down-regulated genes for fish fed the Pre diet belonged to diverse functional categories, such as *amdhd* in histidine metabolism, *blm* involved in DNA damage response, and *ccer2* in microtubule bundling. Several of the most suppressed genes in Pre-fed fish fed Pre diet pointed towards cell development, differentiation, and turnover, including *anxa4*, *ccnb*, *daam2* and *cdh2*. A more uniform picture of genes responsible for nutrient digestion, transport and metabolism was evident among the most down-regulated genes in fish fed Sap and the P+S combination. In particular, reduced transcript levels of genes involved in fatty acid uptake and metabolism (*cd36*, *fabp2*, *acsl5*, *nr1d2*, *acsl5*, *asah2*), lipoprotein synthesis (*apoeb*, *dgat2*), and sterol metabolism (*abcg8*, *cyp26a1*) were observed for fish fed Sap or

P+S. Also, amino acid (*slc1a4*), peptide (*slc15a1*/*pept1*), starch (*mgam*) and vitamin A (*rbp2*) transporters were reduced by the Sap or the P+S.

Functional analyses of DEGs conducted by IPA software analyses revealed that several processes were affected by the individual dietary supplements (Pre and Sap) and that there was a significant interaction between prebiotics and saponin (Supplementary figures S7 – S9). Following hierarchical clustering analysis of "Canonical pathways" (Z-score < 2)," Disease and function" (Z-score < 2,5) and "Upstream regulators" (Z-score < 6), the Sap group and the P+S combination group of DEGs clustered together in all three analysis (Supplementary figure S10) suggesting that the expression fingerprint of the P+S was more similar to the Sap fed fish than to the Pre fed fish.

Selected IPA terms related to immune function are presented in Figure 11. Fish fed Pre showed significant induction of several immune pathways, including IL-8 signaling, reactive oxygen species (ROS) production and T-cell function, that pointed towards upstream immune regulators such as Tumor necrosis factor (TNF), lipopolysaccharide, NFkB and Toll-like receptor 7 (TLR7). For fish fed Sap, a large number of immune pathways were significantly induced, including B- and T-cell development, and signaling, and apoptosis. Correspondingly, a number of upstream regulators related to these pathways also showed significant induction after Sap treatment, including interferon gamma (IFNG), TNF, TLR7, immunoglobulins and T-cell receptor (TCR). Interestingly, the P+S combination seemed to potentiate the effect of Sap alone, showing strong induction of most of the same immune pathways related to B-cell, T-cell, and macrophage function. A range of inflammatory and immunological molecules were predicted to be upstream regulators affecting the transcriptome of the P+S fed fish, with IFNG being the predicted molecule with the highest z score.

In addition to the effects observed on the immune function terms, many IPA terms related to lipid metabolism were significantly affected by diet (Figure 12). In particular, cholesterol biosynthesis was not affected by Pre, but strongly induced by Sap, whereas the combination of P+S seemed to alleviate the effect of saponins on cholesterol synthesis. Triglyceride biosynthesis and fatty acid alpha oxidation were negatively affected by Pre and by the combination P+S. Several upstream regulators involved in lipid and sterol metabolism were also affected by the Sap and P+S treatments, such as progesterone (PGR), estrogen (ESR1) and aryl hydrocarbon (AHR) receptors, and the Carbohydrate-responsive element-binding protein encoded by MLXIPL.

qPCR

Results from the qPCR analyses are illustrated in the figures 13A and B, sorted in two panels according to their roles in the gut. Panel A includes genes related to digestive processes, i.e. protein digestion (*ctra*), nutrient absorption (*slc23a1*, *cd36*, *fabp2* and *aqp8*) and sterol metabolism (*sqle* and *cyp51a1*), and panel B includes genes related to immune regulation (*il1b*, *il6*, *nlrc3*, *lyz*, *cd40*, *igm*, *cd38* and *mrc1*), cell proliferation (*pcna*), oxidative stress (*cat*), cell remodeling (*mmp13*) and mucin formation (*fcgbp*).

Regarding the genes related to the digestive processes (13A), fish fed the Pre diet showed expression not significantly different from fish fed the Ref diet, although the results seemed to indicate a trend towards upregulation of the *ctra* gene coding for the principal precursor of the pancreatic proteases. In fish fed the Sap and P+S diets upregulation of was indicated for *slc23a1*, the vitamin C transporter, downregulation of one of the fatty acids transporters, *cd36*. Expression of *fabp2*, another fatty acid transporter, was downregulated only by the P+S diet. Fish fed the Sap diet exhibited a trend towards increased expression of genes involved in sterol metabolism (*sqle* and *cyp51a1*). The expression level of *mrc1,* a receptor mediating the

endocytosis of glycoproteins by macrophages, was downregulated in fish fed all the three supplemented diets compared to the Ref diet.

Regarding the immune related genes subject of qPCR analyses, most of them, including the mucin-forming-related gene, *fcgbp*, did not show significant effects of any of the supplemented diets. A trend towards elevation of expression level of *cd38,* an enzyme synthesizes cyclic ADP-ribose and NADP, as well as *fcgbp* was observed in fish fed the Pre diet. Whereas, fish fed the P+S diet showed lower expression of *cat*, the catalase.

4. Discussion

4.1 General characteristics of intestinal tract of the Ballan wrasse

The relative weights (OSI) of the intestinal sections of the fish, showing values decreasing along the intestine from 0.37 to 0.13%, in sum comprising 1.1% of body weight, were about half the values observed in our previous studies [8,37]. The reason for this may be the difference of fish weight, which was three times as high in the former studies as in the present, about 50-60g. On the other hand, trypsin activity and bile salt concentration of the chyme were at the same level. The same was true for the total capacity of brush border LAP and maltase. A difference between the present results and the previous were the capacities of the sections along the intestine [8]. The explanation for this difference was most likely differences in the positioning of the division between IN1 and IN2 of the intestine, which in the two first studies were more proximal than in the present [8,37]. The reason for this change, was difficulties in defining the end of the first section, a bulb-like structure. In the latter study, the intestine was divided in four sections of similar length.

The results regarding the brush border enzyme capacity were quite similar in the present and the previous study [8,37,38], i.e., the enzyme capacity in IN1 and IN2 was about 10 and 110 U/kg fish for maltase and LAP in the present study, about 10 and 50 U/kg fish in the previous

443 ones. Also, trypsin activity and bile salt concentration in the intestinal content were similar.
444 In the previous study observations were not available for IN1. In IN2 the values in the present
445 study were about 70 U and 40 mg/g dry matter, respectively, and in the previous study, the
446 values were about 110 U and 40 mg/g dry matter.
447 The results for the distal most section indicated lower capacity than for the other sections, in
448 the present as well as in the earlier studies[8,37,38]. The results confirm that IN1 – 3 have
449 similar absorptive capacity, and that reabsorption of enzymes and bile salts mostly take as the
450 chyme passes through IN4. Efficient absorption of nutrients in IN1 – 3 seems to be secured
451 by retrograde movements while the feed present in the intestine [39].
452 At histological level, Ballan wrasse possesses wide submucosa and lamina propria.
453 Thickening of submucosa and lamina propria usually is associated with unhealthy status in
454 fish intestine [24,40,41,42,43,44]. Since wide submucosa and lamina propria commonly exist in
455 Ballan wrasse, it may not be appropriated to involve them as a sign of unhealthy status of
456 intestine. Instead, increase of cellularity that comprised of leukocytes [45], would be the more
457 appropriate marker of unhealthy intestine in Ballan wrasse, which has also been observed in
458 our previous study in wild Ballan wrasse [25].
459
460 4.2 Effects of prebiotics
461 The present study clearly indicated that supplementation with the prebiotics produced local
462 effects in the gut on a molecular level, including modulation of innate and adaptive
463 immunity. These responses were, however, not manifested in any clear histomorphological
464 alternations in the gut. Previous studies indicate that prebiotics may strengthen disease
465 resistance (summarized by [18]). Potentially the influenced genes are involved in pathogen and
466 disease resistance and therefore, changes were not triggered without infestations, in spite that
467 in other fish species [46,47], enhanced immunity was observed before pathogen challenge.

The prebiotics used in the present study is a product of yeast (*Saccharomyces cerevisiae*) extract which contains functional ingredients such as mannan-oligosaccharides (MOS). Its effects in fish would therefore be expected to be comparable to effects of MOS. In general, prebiotics appear to activate the innate immune system, directly or by enhancing the growth of commensal microbiota [48] but the effects seem to differ among different fish species. For instance, lysozyme expression was not altered in the present study, in line with observations in channel catfish (*Ictalurus punctatus*) [49] and European sea bass (*Dicentrarchus labrax*) [50] fed MOS supplemented diets. These results, however, differ from effects observed in rainbow trout (*Oncorhynchus mykiss*) [51] and red drum (*Sciaenops ocellatus*) [52] in which dietary MOS supplementation caused increase lysozyme activity. Adaptive immunity can also be influenced by prebiotics. For instance, lymphocyte responses, including both T- and B-cell responses, were enhanced in European sea bass (*Dicentrarchus labrax*) fed concentrated MOS supplemented diet [53]. The lymphocyte responses in Ballan wrasse, however, was not widely influenced by the supplemented prebiotics.

4.3 Effects of soya saponins

The lowered plasma level of cholesterol and the trend towards lowered concentration of bile salts in the content of IN4 observed in fish fed Sap and P+S supports results from studies conducted with other fish species [15,54,55]. In the current study, saponin supplementation seemed to increase cholesterol synthesis in enterocytes, as indicated by the upregulation of *cyp51a1* and an elevating trend of *sqle* in fish fed Sap. These results agree with observations in Atlantic salmon showing increased synthesis of cholesterol and bile salts in the liver by feeding with diets containing soybean meal [56]. The intestine play important roles in cholesterol homeostasis through absorption and excretion. In addition, the enterocytes have the capacity to synthesize cholesterol. Enhancing cholesterol synthesis in enterocyte is likely

493 a manner to counteract the lowering effect of saponins on plasma cholesterol level also in
494 Ballan wrasse. As a demonstration of this assumption, the experiment in mouse showed
495 upregulating the expression of *Srebp2* gene, a regulator of sterol and fatty acid synthesis, in
496 intestine increased plasma cholesterol by inducing expression of genes related to cholesterol
497 synthesis, including *sqle* and *cyp51a1* [57].
498 The reduced expression in IN4 of genes involved in fatty acid uptake and metabolism, such
499 as *cd36* and *fabp2* in the Sap and P+S treatments and other related genes verified by IPA in
500 Sap treatment, is in line with results from studies in Atlantic salmon [15,16] and gilthead sea
501 bream [54], suggesting that saponin inclusion also influences fatty acid transport in the distal
502 most compartment, directly or indirectly. However, blood plasma levels of free fatty acids
503 and triglycerides were not significantly affected, indicating that the reduced expression of
504 *cd36* and *fabp2* did not reduce availability of fatty acids to the peripheral tissues of the fish.
505 The main reason for lack of effect on plasma lipid levels may be the fact that most lipids are
506 absorbed in the more proximal compartments [38]. Similarly, in Atlantic salmon, it has been
507 observed that dietary inclusion of soya saponin may reduce expression of *fabp2* and reduce
508 apparent digestibility of lipid in the distal intestinal segment, but, as in the present study,
509 plasma levels of free fatty acids and triglycerides remained unchanged [15].
510 It is well established that soya saponins induce intestinal inflammation in fish, and the most
511 typical signs are mobilization of the immune apparatus, loss of digestive functions and major
512 histological alterations [13,15,58]. The present results indicate that similar processes take place
513 in the distal intestine of the Ballan wrasse. In Atlantic salmon the affected genes are in
514 particular related to T and B cell regulation in [21]. In the present study, all the top 10 genes up
515 regulated by Sap, are involved in immune functions such as lymphocyte functions and
516 signaling, and pathways related to immunity like "T_h2 pathway" and "CD28 signaling in T

helper cells" were also induced. We therefore conclude that soya saponins induce similar responses in Ballan wrasse as in Atlantic salmon.

The histological changes observed in the present Ballan wrasse study indicated intraepithelial infiltration of lymphocytes. The type of lymphocyte infiltrated was most likely T cells as in the intestine of higher vertebrates, intraepithelial lymphocytes were identified as diverse T cells [59]. The involvement of T lymphocytes is suggested to be a strategy for fighting the consequences of soya saponins as this has been commonly observed in Atlantic salmon [20,21,60], which may be triggered by the increase mucosal permeability by saponin, allowing entrance of alien compounds [61]. The present results indicate that the increased exposure to feed and microbial components from the intestine induced a wide range of very complex reactions with pro- and anti-inflammatory changes occurring simultaneously in the distal intestine. For instance, the downregulation of gene *mrc1* may be a reflection of anti-inflammation because it is considered a marker of macrophage in intestine [62], and is found to be reduced also in gilthead seabream displaying anti-inflammatory status [63]. The inhibition of IL-8 signaling pathway was likely an indication of anti-inflammation processes since IL-8 participates in the proinflammatory signaling cascade [64]. The presence of anti-inflammatory changes may be a reaction for protection the fish from negative effects of the induced inflammatory responses.

4.4 Combination effects of saponin and prebiotics

The fact that the gene expression profile for P+S clustered with that of Sap, indicates that the modulatory effects of prebiotics, were overridden by the challenging effects of the Sap. The responses to P+S differed from the responses to Pre in particular regarding intraepithelial lymphocyte infiltration, however, were more similar to responses to Sap with enhanced lymphocyte responses. According to gene expression, we can assume the type of

lymphocytes particularly involved in the P+S treatment. For instance, T helper cell 17 (T_h17) was particularly activated. It differentiates from CD4+ T cells together with regulatory T cells (T_{reg}). It is demonstrated to participate in pathogen clearance and the balance of T_h17 and T_{reg} influence autoimmunity [65,66] and these may also be affected in the Ballan wrasse. Besides, the type of B cells seems not to be IgM expressing, considering that *igm* expression showed no significant effect, but rather a decreasing trend. Instead, it might be IgT- or IgD-expressing B lymphocytes which, in earlier studies, have been found to be expressed in IN4 in Ballan wrasse [67].

However, present information regarding combination effects of prebiotics and soya saponins in fish intestine are not enough to understand the mechanisms underlying the combined effects of the two substances. Considering the properties of the prebiotics it is highly likely that effects on gut microbiota were important factors. However, one study with Nile tilapia has indicated that prebiotics and saponins do not necessarily alter the composition of gut microbiota [68].

Altogether the results of the present study indicate that use of prebiotics should be done with care. Interaction with antinutrient effects, such as effects of saponins, might eliminate their potential beneficial effects and possibly potentiate challenging effects of the antinutrients.

5. Conclusions

In the present study, the Ballan wrasse responded to dietary soya saponins in a similar way as Atlantic salmon. Observed responses included decreased cholesterol levels in blood, and clear alterations in the histomorphology and gene expression of the distal intestine, indicative of a progressing inflammation with increased intraepithelial lymphocyte infiltration. The study also suggested that dietary supplementation of prebiotics had modulatory, potentially

beneficial, effects on gut health and function. However, interaction with plant antinutrients such as saponins may prevent these effects.

Competing interests

No competing interest exists for the scientists involved in this work

Author's contributions

The authors' contributions were as follows:

WZ's contribution in this work regarded expertise in gene expression analyses and data processing and drafting and completing of the manuscript.

KKL analyzed the transcriptomic data, joined the data processing and writing of the manuscript.

EC performed histological analysis

KK participated in planning of the experimental design, and feed formulation, was in charge of feed production, participated in discussion of results and manuscript development.

IL was project leader, participated in planning of the experimental design, and feed formulation, participated in discussion of results and manuscript development.

ØS was involved in molecular analysis and evaluation, in discussion of results and manuscript development.

ÅK was the responsible scientist at NMBU for the study, designed the study, overviewed the analyses, joined the data processing and writing of the manuscript.

TMK planned and overviewed the analyses, joined the data processing and writing of the manuscript.

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Data availability statement

RNA sequencing data can be accessed through the Gene Expression Omnibus (GEO accession number GSE152475).

Supplementary materials

The supplementary files include the detection methods of blood plasma variables S1 – S5, tables S6-S9 and figure S10. S1-S5, detection methods for cholesterol, fatty acids, total protein, glucose and triglycerides, respectively. Table S6. The complete list of differentially expressed genes (DEGs). Table S7. IPA analysis of DEGs of Pre group. Table S8. IPA analysis of DEGs of Sap group. Table S9. IPA analysis of DEGs of P+S group. Figure S10. Comparative Ingenuity Pathway analysis.

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819 Tables
820
821 Table 1. Basic feed formulation (%)

	Ref
[1]Shrimp meal	28.50
[1]Cod meal	24.83
[2]Krill hydrolysate	6.44
[3]Reference fishmeal stick water	12.00
[1]Squid meal	11.00
[4]Fish oil	3.50
[5]Krill oil	2.50
[6]Wheat	7.00
[7]Vitamin mix	2.65
[7] Mineral mix	5.00
[8] Soya lecithin	2.00
[9] Additives	2.08
[10] Yttrium oxide	0.05
Water	-7.55
Sum	100.00

822 [1] Provided by Seagarden AS, Norway. [2] Antarctic krill (*Euphausia superba*) provided by RIMFROST AS,
823 Norway. [3] Provided by Pelagia AS, Norway. [4] Provided by Norsildmel AS, Norway. [5] Provided by Aker Biomarine
824 Antarctic AS, Norway. [6] Provided by Norgesmøllene, Norway. [7] Supplied by Vilomix, Norway. [8] Provided by
825 Denofa, Norway. [9] Cholesterol from Grudlita, Lithuania; Choline chloride, Carophyll Pink (10%), Stay-C and
826 Methionine from Vilomix, Norway; Lysine from Agrocorn, Denmark; and Taurine from VWR, Norway. [10]
827 Provided by Metal Rare Earth Limited, China. 0.4 %Aquate (provided by Alltech, Norway) and/or 0.7% soya
828 saponins (purity ≥80%, provided by Shanxi Pioneer Biotech Co., Ltd., Xian, China) were added to treatment diets
829 (Pre, Sap and P+S).
830

Table 2 Primers and related information for qPCR assays

Gene symbol	Gene function	5' - 3' primer sequence		Amplicon size (bp)	Annealing temperature (°C)	Efficiency	Gene bank accession no.
		Forward	Reverse				
gapdh2	Synthesizes pyruvate from D-glyceraldehyde 3-phosphate	TATTTGTGTCCGTGTGCCCC	GCCTCCGTCCACTGATGAAT	129	62	1.99	XM_020633887.1
ctra	Protein digestion	AGCGTCCCAGTAGAGAAGGT	ACACTGGAGCTGAATCTGGC	110	60	2.00	XM_020657256
slc23a1	Vitamin C transport	CCCACTGAACACCTCACACA	AGACCAATCAGCAGCTCCAC	93	60	1.83	XM_020655303
cd36	Lipid transport	ACGGAGGGATAAAACGCACA	TATGCTGTGGTTCCAGGCTC	181	62	2.00	XM_020649455.1
fabp2	Lipid transport	TACAGCCTTGCGGATGGAAC	ATCCTCTTAGCCTCCACACCT	173	60	1.95	XM_020643842.1
aqp8	Water channel	TTGGCTCCTTTCCTTGTGGG	CCGAGAATGAGCCTGAGCAA	197	60	1.95	XM_020642545.1
sqle	Cholesterol biosynthesis	ACGAGAGATCAGCGACCAAC	CAGGTTCTGGAGCCACTGTT	117	62	1.94	XM_020635029
cyp51a1	Cholesterol biosynthesis	AAGGACTGCTGTTCCGATGG	CCTCTCCACAAAACCACCGA	113	60	1.79	XM_020648620

il1b	Pro-inflammatory cytokine	AAGGACGGTGATGAGGCAAC	GAAACCGAACCATGTCGCTG	94	57	1.87	XM_020651384.1
il6	Pro-inflammatory cytokine	GATCCTTGGTGCGACCGAT	GAAGGGCAGCTTTCTGGGAG	117	57	1.95	XM_020631726.1
nlrc3	Negative regulator of innate immune responses	GGACTTGTTCCTCCGCTTCTCC	CTGATTGGTCTGTGAGCCACTTC	88	62	1.98	XM_020659897.1
lyz	Antibacteria	CTTGGGACAGCGAGGAACAC	TCCATCGCCCATGTTGTAGG	140	62	1.96	XM_020660641
cd40	Activates antigen presenting cells	AGCAGTAAACCCGACTGAGG	GCTTTGGTCGTCCTCGTTCT	85	60	1.99	XM_020651338.1
igm	Humoral immunity	ATCTCTTGTGGAACAGGGCAC	CCTTGAAGTCAGCAAAACGCT	101	55	1.89	XM_020660315.1
cd38	Synthesis and hydrolysis of cyclic ADP-ribose and NADP	CCTGCACAGATGACGACCAA	TTGCACATGTTTGGCACGAG	81	62	2.00	XM_020633573
mrc1	Mediates endocytosis of glycoproteins by macrophages	TGTCCGGTTACATCCAAAGGA	TTATTACCCCCAGAACCTGTGA	185	57	2.00	XM_020635202.1
pcna	Cell proliferation	GCCAACAACACACAAAGGCT	TCGTCTTTCTGCGTCACTCC	106	62	1.88	XM_020647462.1
cat	Catalysis of hydrogen peroxide	TGTGAGCCGTTACAACAGTGC	TTGGATGAAGAGCTGGGCTCC	142	60	1.62	XM_020660768.1

mmp13	Tissue remodelling	TCTCGACGCCGCTTATGAAA	CACGCACGGGTTTATAGCCA	95	60	1.90	XM_020631204.1
fcgbp	Mucin forming	CAACTCTCCCTGTCTCTCCAG	GCTTCACAGAGGCAATTCTCC	126	62	2.00	XM_020655516.2

831

Table 3. *P* values of the results based on Two-way ANOVA

	P value			
Parameters	Model	Diet	Segment	Diet×Segment
OSI (% of body weight)	< 0.01	0.049	< 0.01	0.32
Maltase capacity (μmol/min/kg fish)	< 0.01	0.08	< 0.01	< 0.01
LAP capacity (mmol/h/kg fish)	< 0.01	0.04	0.06	0.046
Dry matter (%)	0.48	0.09	0.64	0.22
Trypsin activity (U/g DM)	0.75	0.69	0.77	0.94
Bile salt level (mg/g DM)	< 0.01	0.09	0.04	0.03

OSI, organosomatic index; LAP leucine aminopeptidase; DM, dry matter.

860 Table 4. Initial and final weight, length, condition factor (CF), thermal growth coefficient
861 (TGC) and specific growth rate (SGR)

	Ref	Sap	Pre	P+S	*P* value	Pooled SEM
Initial weight (g)	72.4	73.8	70.7	72.9	0.19	0.83
Final weight (g)	89.7	92.5	87.5	93.9	0.56	1.67
Initial length (mm)	162.1	162.3	160.6	161.6	0.38	0.38
Final length (mm)	170.4	171.8	169.4	173.1	0.69	0.88
CF (g cm^{-3})	1.79	1.81	1.78	1.79	0.69	0.008
TGC ($g^{1/3}$(°C day)$^{-1}$)	0.29	0.28	0.26	0.32	0.78	0.013
SGR (% day^{-1})	0.32	0.31	0.29	0.35	0.79	0.014

862

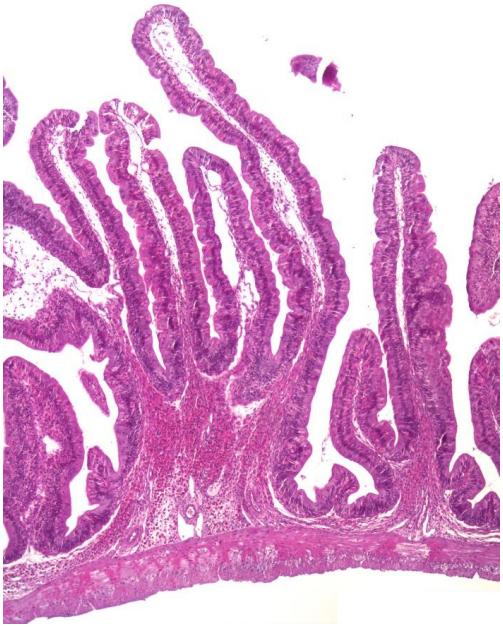
Table 5 Blood plasma variables[1]

	Ref	Sap	Pre	P+S	*P* value	Pooled SEM
Glu (mmol L^{-1})	2.1^{a}	1.5^{ab}	1.4^{b}	1.5^{ab}	0.01	0.5
FFA (mmol L^{-1})	5.5	5.7	5.7	5.9	0.15	0.4
TG (mmol L^{-1})	11.3^{ab}	11.2^{b}	15.8^{a}	12.2^{ab}	0.01	3.8
Chol (mmol L^{-1})	6.4^{a}	3.7^{b}	6.3^{a}	4.1^{b}	< 0.01	1.2
Tprot (g L^{-1})	48^{a}	40^{ab}	48^{a}	38^{b}	< 0.01	8

863 [1]Abbreviations: FFA, free fatty acid; Glu, glucose; Chol, cholesterol; Tprot, total protein; TG,
864 triglycerides. Values with different superscript letters within the same row denotes significant
865 difference ($P < 0.05$).

866

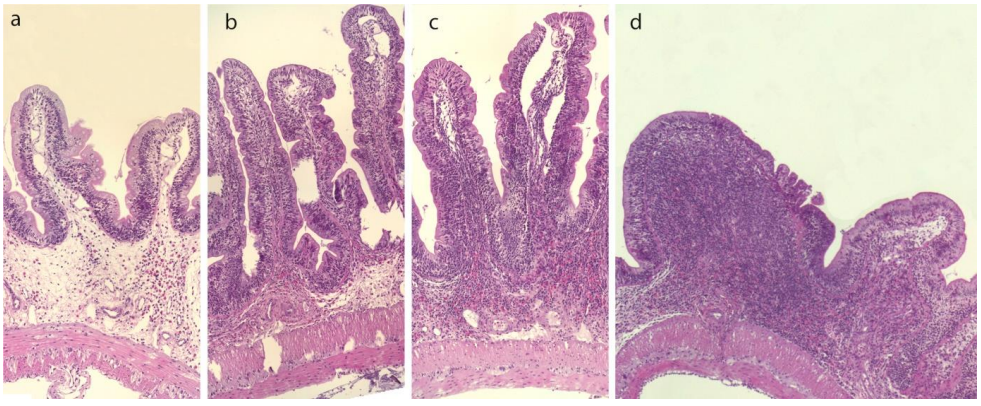
867 Figures



868

869 **Figure 1.** A representative image showing the bifurcation of intestinal mucosal fold.

870



871

872 **Figure 2**. Representative images of the mucosa of Ballan wrasse intestine that were scored as
873 normal, mild, moderate, and marked (images a-d, respectively) for infiltration of lamina
874 propria by inflammatory cells. Image d shows a notable increase in width of the lamina
875 propria.

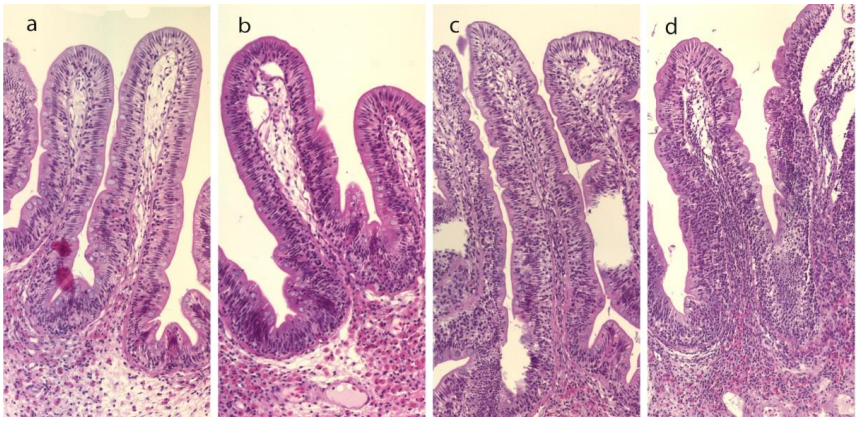


Figure 3. Representative images of the mucosal epithelium of the Ballan wrasse intestine that were scored as normal, mild, moderate, or marked for intraepithelial lymphocyte infiltration (images a-d, respectively).

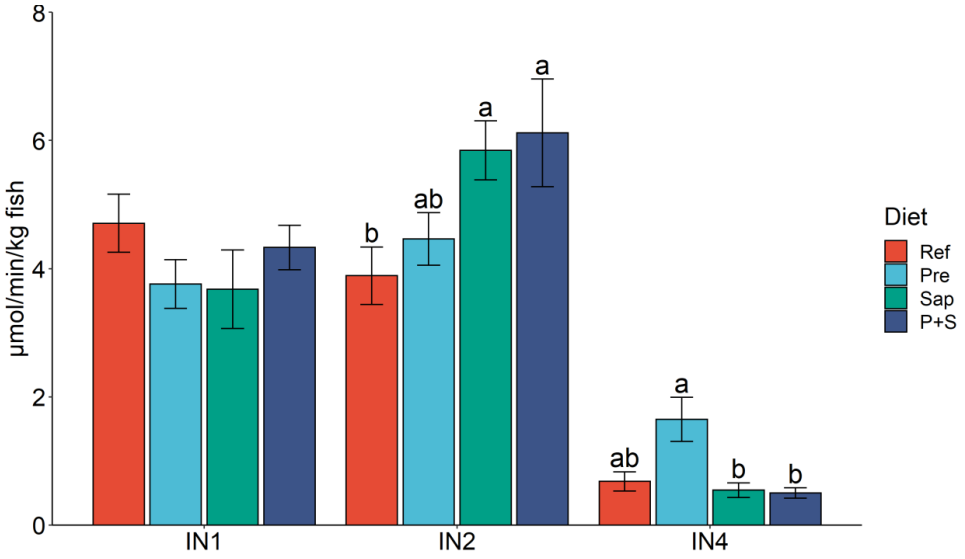


Figure 4. Maltase capacities in IN1, IN2 and IN4. Values are means with standard errors as error bars. Mean values with different letters are significantly different. *P* values derived from one-way ANOVA for diet effect are 0.30, less than 0.01 and less than 0.01 in IN1, IN2

and IN4, respectively. IN1, IN2 and IN4, intestinal regions 1, 2 and 4 in a proximo-distal axis.

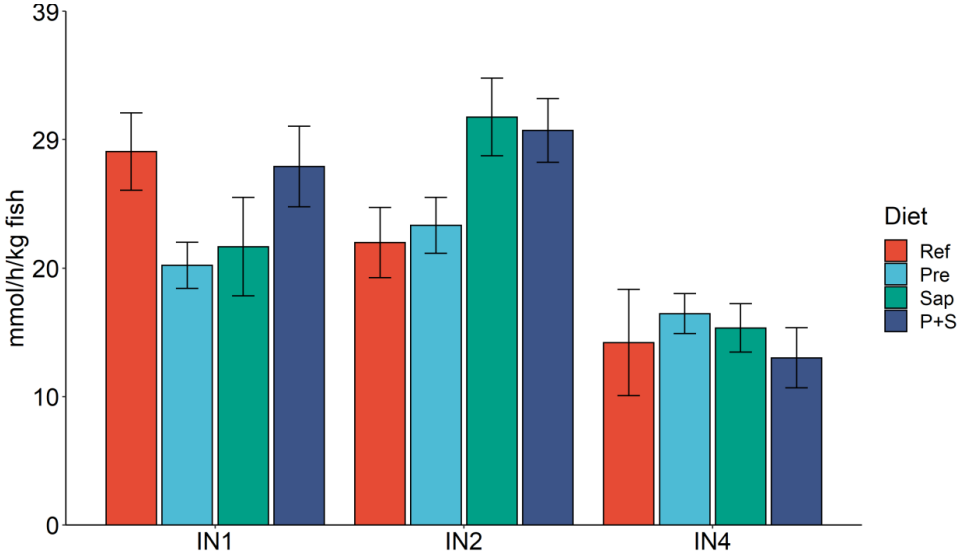
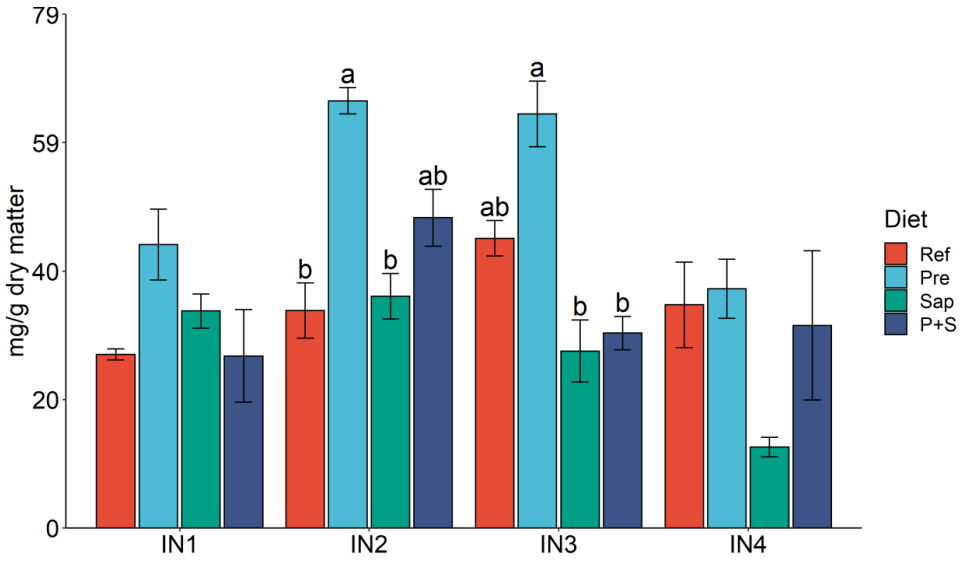


Figure 5. LAP capacities in IN1, IN2 and IN4. Values are means with standard errors as error bars. Mean values with different letters are significantly different. *P* values derived from one-way ANOVA for diet effect are 0.11, 0.22 and 0.39 in IN1, IN2 and IN4, respectively. IN1, IN2 and IN4, intestinal regions 1, 2 and 4 in a proximo-distal axis.



893

894 **Figure 6.** Digesta bile salt concentration in IN1 to IN4. Values are means with standard

895 errors as error bars. Mean values with different letters are significantly different. *P* values

896 derived from one-way ANOVA for diet effect are 0.16, 0.01, less than 0.01 and 0.20 from

897 IN1 to IN4, respectively. IN1-4, intestinal regions 1-4 in a proximo-distal axis.

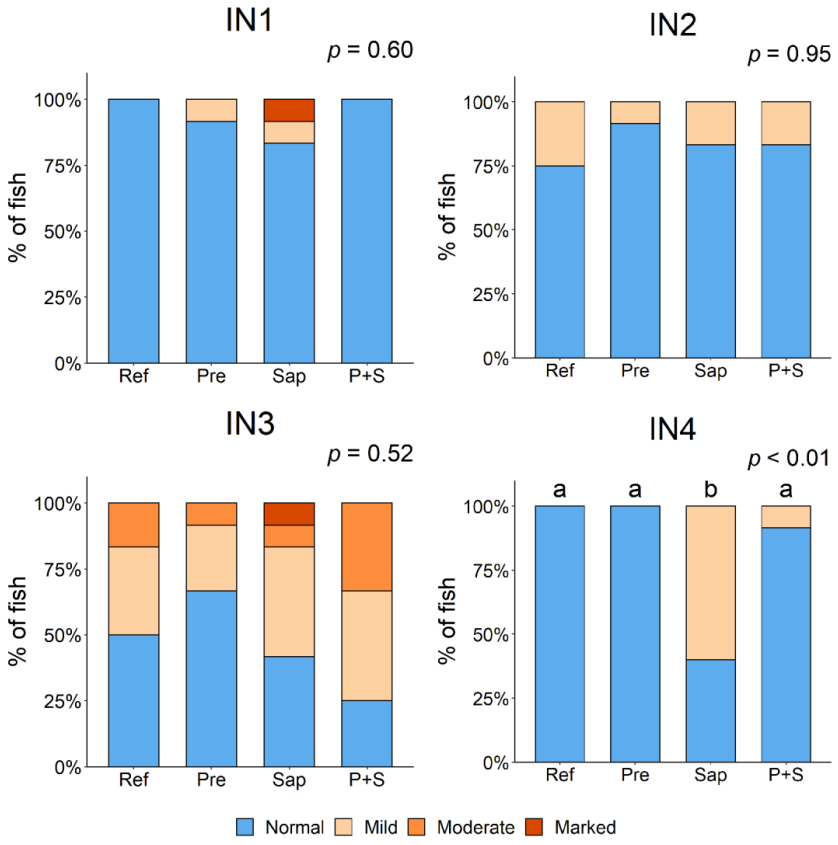


Figure 7. Results of statistical analysis of diet effects on the cellularity of the lamina propria in the different regions of the Ballan wrasse intestine. LP, lamina propria. IN1-4, intestinal regions 1-4 in a proximo-distal axis.

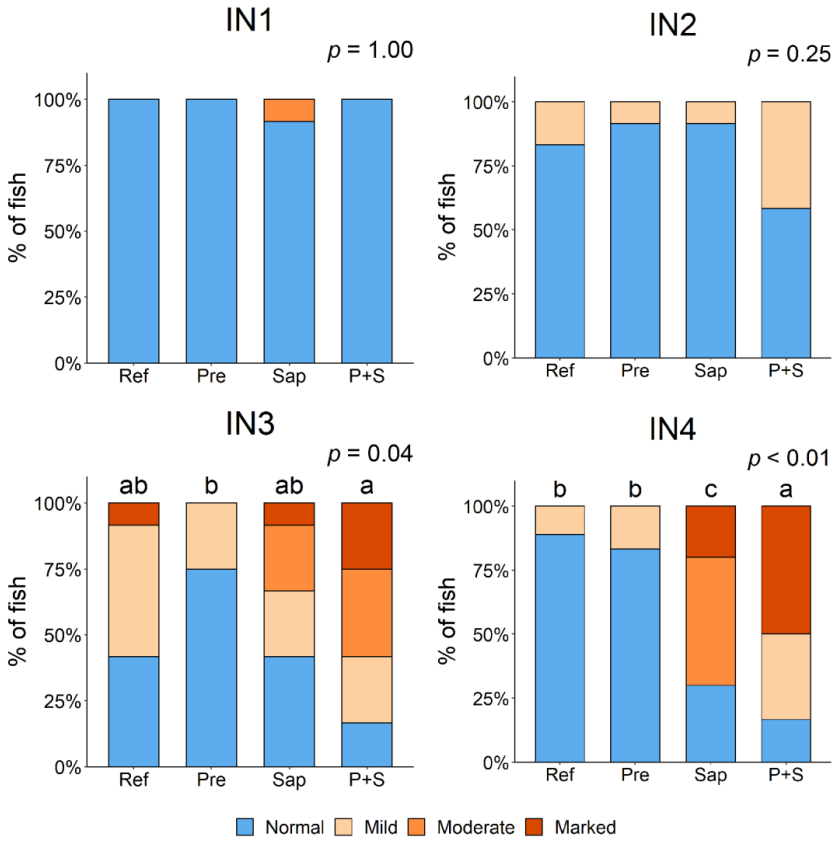
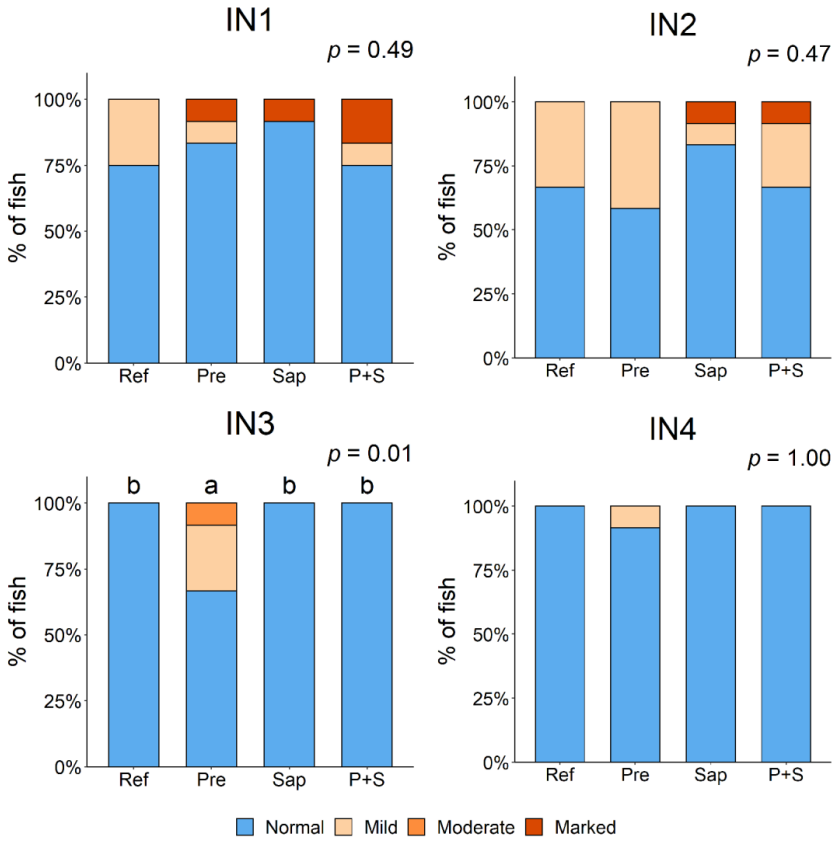


Figure 8. Results of statistical analysis of diet effects on the intraepithelial lymphocyte infiltration in the different regions of the Ballan wrasse intestine. IEL, intraepithelial lymphocyte. IN1-4, intestinal regions 1-4 in a proximo-distal axis.

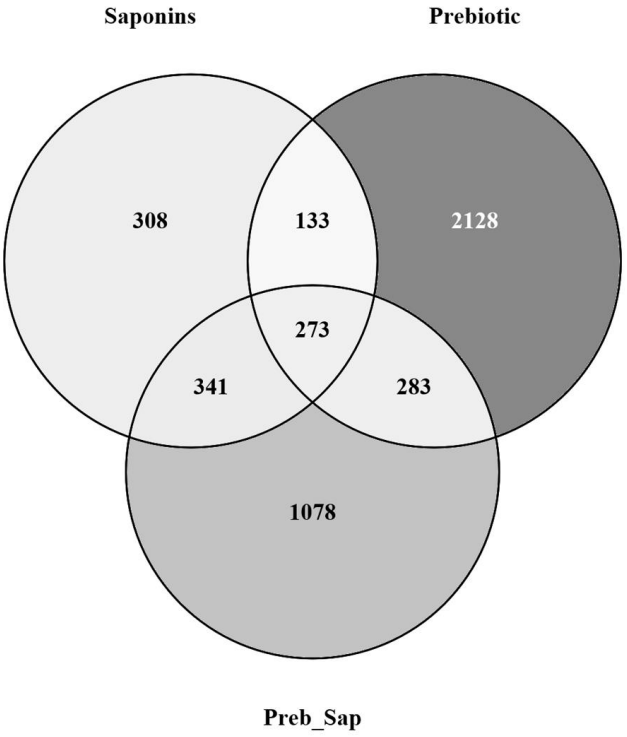


906

907 **Figure 9.** Results of statistical analysis of diet effects on the numbers of intraepithelial rodlet

908 cells and eosinophilic granular cells (EGCs) in the mucosal epithelium in the different

909 regions of the Ballan wrasse intestine. IN1-4, intestinal regions 1-4 in a proximo-distal axis.



910

911 **Figure 10.** Venn diagram showing the number of differentially expressed genes affected by
912 either saponin, prebiotic or their combination. Among them, 308, 2128 and 1078 genes were
913 exclusively affected by saponin, prebiotic and combination treatments, respectively; 133
914 genes were influenced by both saponin and prebiotic treatments; 341 genes were influenced
915 by both saponin and combination treatments; 283 genes were influenced by both prebiotic
916 and combination treatments; 273 genes were influenced by all treatments. Venn diagram was
917 created by using venny 2.1.0.

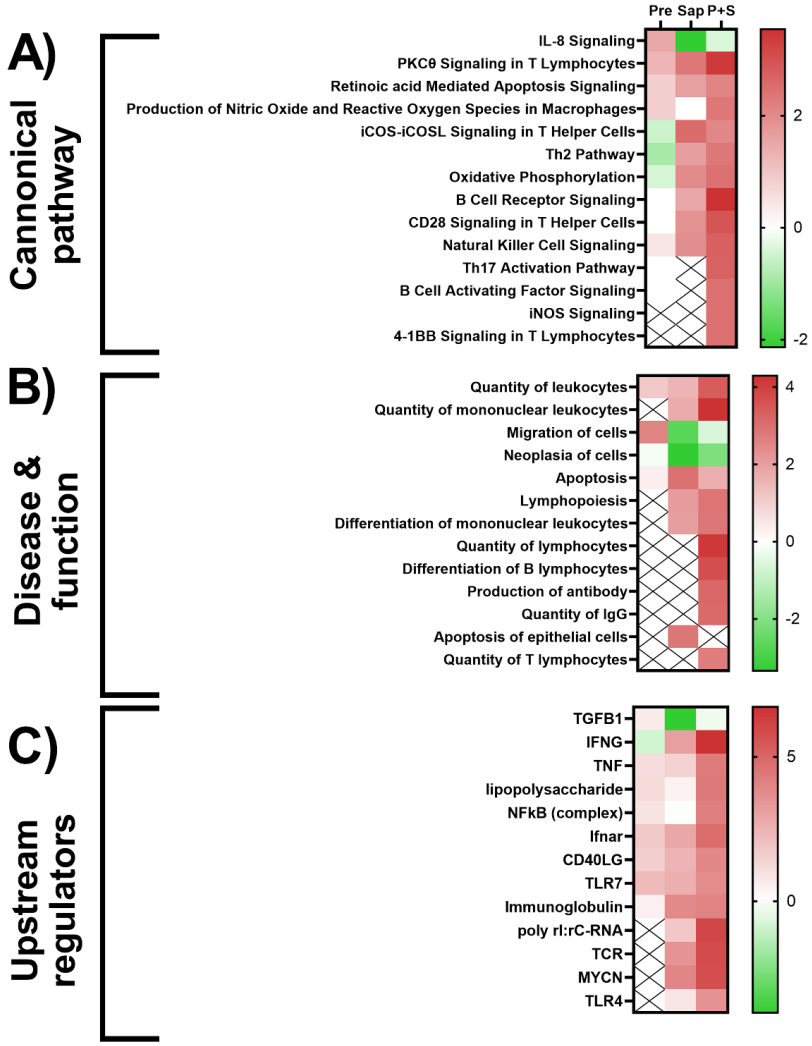
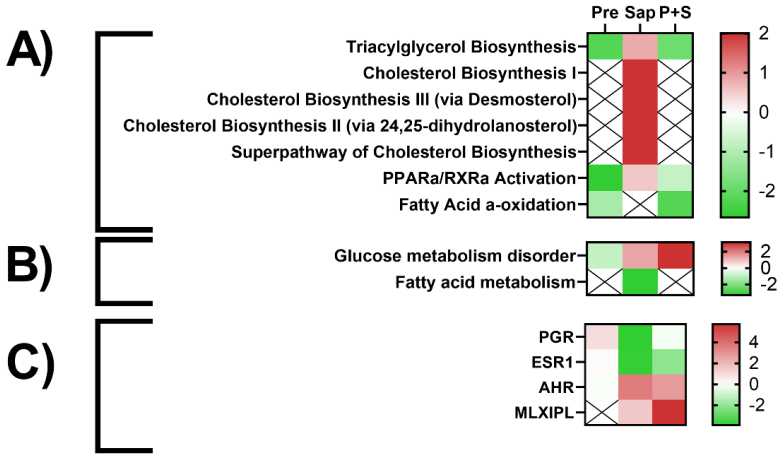


Figure 11. Comparative Ingenuity Pathway analysis showing A) significant canonical pathways (Z-score > 2) B) Disease and function (Z-score > 2.5) and C) predicted upstream regulators (Z-score > 4) related to immune function in Ballan wrasse (*Labrus bergylta*) as a result of adding either saponins, prebiotics or saponin + prebiotic in the feed. All analysis were filtered using $p < 0.05$ in addition to Z-score filtering. Non-significant terms are marked as "X".



925

926 **Figure 12.** Comparative Ingenuity Pathway analysis showing A) significant canonical
927 pathways (Z-score > 2) B) Disease and function (Z-score > 2.5) and C) predicted upstream
928 regulators (Z-score > 4) related to lipid metabolism in Ballan wrasse (*Labrus bergylta*) as a
929 result of adding either saponins, prebiotics or saponin + prebiotic in the feed. All analysis
930 were filtered using $p < 0.05$ in addition to Z-score filtering. Non-significant terms are marked
931 as "X".

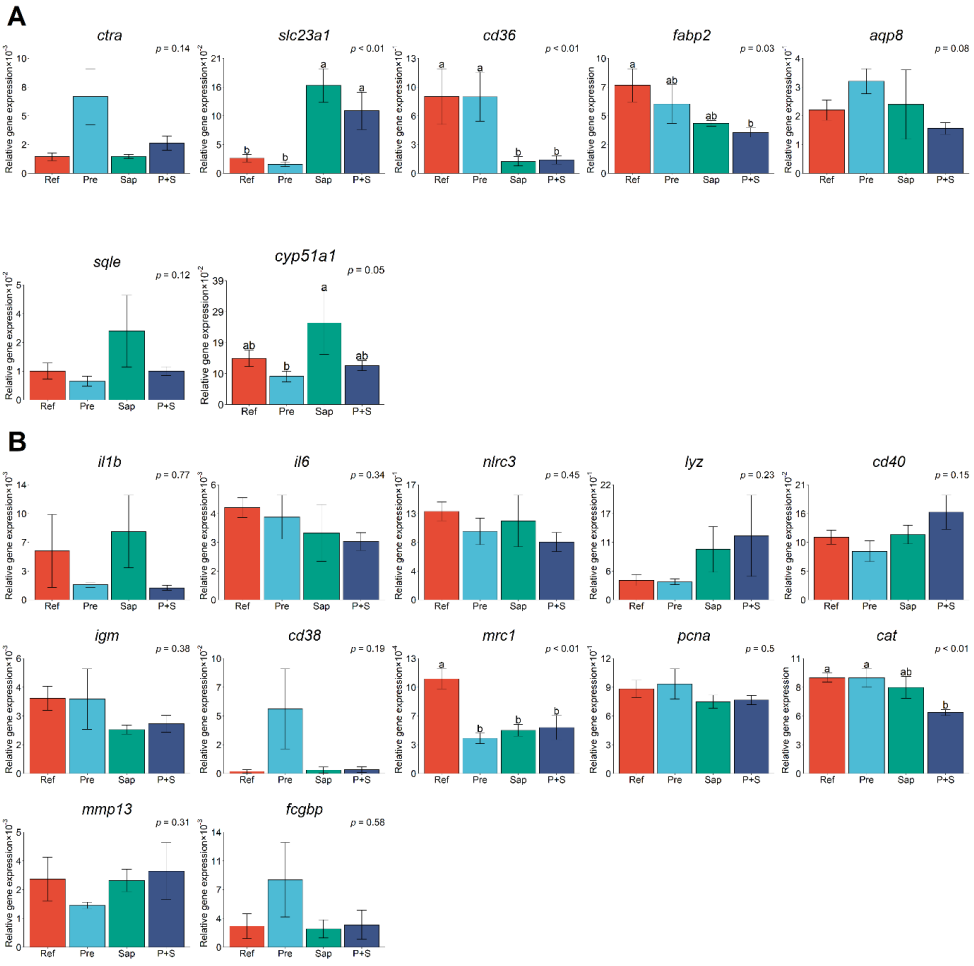


Figure 13. Distal intestine gene expression of A: chymotrypsin A (*ctra*), vitamin C transporter (*slc23a1*), cluster of differentiation 36 (*cd36*), fatty acid binding protein (*fabp2*), aquaporin 8 (*aqp8*), squalene epoxidase (*sqle*) and lanosterol 14α-demethylase (*cpy51a1*). B: interleukin 1 beta (*il1b*), interleukin 6 (*il6*), NOD-like receptor family CARD domain containing 3 (*nlrc3*), lysozyme (*lyz*), cluster of differentiation 40 (*cd40*), immunoglobulin M (*igm*), cluster of differentiation 38 (*cd38*), mannose receptor C-type 1 (*mrc1*), proliferating cell nuclear antigen (*pcna*), catalase (*cat*), matrix metallopeptidase 13 (*mmp13*), IgGFc-binding protein (*fcgbp*). Values are means with standard errors as error bars. Mean values

941 with different letters are significantly different. *P* values derived from statistical analysis are

942 given.

PAPER III

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ORIGINAL RESEARCH
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A Screening Study on Effects Varying Dietary Macronutrient Composition on Gut Functions in Lumpfish *(Cyclopterus lumpus)*

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Cultivation of lumpfish (Cyclopterus lumpus) as lice cleaner fish for salmon is now expanding. For successful cultivation of a new species, understanding the basic biology of digestive functions is vital to facilitate and optimize diet formulation. This paper presents results from two experiments conducted to deepen our knowledge on lumpfish intestine physiology. Experiment 1 was a 42-day feeding trial in which lumpfish were fed twelve different diets in the following ranges of macronutrients: Protein 43-68%, lipid 4-17%, and carbohydrate 6-17%. Intestinal tissue, gut content and liver were sampled from 6 fish per tank. The results showed that with increasing lipid level and corresponding decrease in protein level, there was a linear decrease in several of the observed biomarkers, including activity of brush border membrane digestive enzymes, expression of genes related to nutrient digestion and transport, ion exchange, immune regulation, and cell remodeling. Increased intracellular accumulation of lipid (steatosis) was observed in gut and liver with increasing dietary lipid level. Fewer effects were observed for increased dietary carbohydrate and corresponding decreased protein level. Experiment 2 was a two-week feeding trial for estimation of macronutrient digestibility in which lumpfish were fed three diets, all containing 55% crude protein, with lipid to carbohydrate ratio of the low lipid diet of 7.5%/18.3%, the medium lipid diet of 13.8%/14.6%, and high lipid diet of 18.1%/9.5%. Fecal samples were collected as pooled samples per tank. These results showed that fatty acid digestibility's increased as dietary lipid level increased. Of note, starch digestibility decreased greatly as starch level increased, whereas protein digestibility did not change as lipid or starch level varied. Taken together, the present studies indicated that increasing lipid level in the diet with corresponding decrease in protein level affects digestion, absorption, and immune responses in the lumpfish intestine. Variation in dietary carbohydrate to protein level showed less effects, possibly due to low starch digestibility which makes the variation in available carbohydrates much less than the variation in the analysed level of dietary carbohydrates.

Keywords: cleaner fish, lumpfish, gut functions, macronutrient requirement, digestibility

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1 INTRODUCTION

Cultivation of lumpfish (*Cyclopterus lumpus*) expanded rapidly during the past decade, i.e. approximate 34 million individual fish for delousing of Atlantic salmon were produced in Norway in 2020 (statistics by Norwegian Directorate of Fisheries, 2021), and in Scotland the number of lumpfish produced was 6.6 million (Munro, 2020). For successful cultivation of a new species detailed knowledge of several physiological aspects is necessary, particularly regarding digestive physiology and nutrient requirement. Powell et al. stated that one third of lumpfish die of starvation within a few weeks in salmon cages (Powell et al., 2018), indicating supplementary feed is necessary for lumpfish after deployment. This may be associated with its feeding preference. Lumpfish is an opportunistic, omnivorous feeder that not only feed on sea lice after deployment (Powell et al., 2018). Naturally, lumpfish seem to predominantly prey on crustacean, followed by molluscs and small fish (Ingolfsson and Kristjansson, 2002; Imsland et al., 2015). A diet of high protein content seems to be optimal in terms of its natural preference. Although plant ingredient tolerance has been tested for lumpfish (Willora et al., 2020; Willora et al., 2021), studies investigating nutrient requirement of lumpfish are rare. No relevant scientific information is available currently for the feed producers. Present commercial diets seem to be produced based on knowledge from other fish species as well as experience generated from inhouse, unpublished trials and commercial production of lumpfish.

Present knowledge of digestive physiology in lumpfish is very limited. Zhukova and Stroganov (Zhukova and Stroganov, 2021) described the anatomical structure of the alimentary tract in lumpfish, with focus on esophagus and the stomach. The oesophagus is reported to be relatively short with a thick muscular wall whose thickness may increase more than two folds during contraction. The stomach was observed with well-developed gastric glands and distinguishably divided into cardiac, fundus, and pyloric parts. Intestinal length was estimated to be 1.2 times of the standard body length, and the histological structure found the same throughout its entire length. Some studies on lumpfish employed histopathological evaluation of intestine as an indicator of inflammatory responses after changes in diet composition or feeding strategy (Imsland et al., 2018; Imsland et al., 2019a; Imsland et al., 2019b). The observed responses, however, were mild or none.

The gut is primarily an organ for digestion and absorption of nutrients. For fish, it also serves as an important organ for osmoregulation (Grosell, 2006; Gregorio and Fuentes, 2018). As the intestinal epithelial surface is constantly exposed to the elements of the external environment, it therefore must also provide defence, which herein refers to the physical barrier and immune response, against alien components and organisms (Zhu et al., 2013; Garcia-Hernandez et al., 2017). Studies in other fish species have clearly demonstrated how diet composition can influence nearly all aspects of the intestine, i.e., digestion and absorption (Krogdahl et al., 1999; Silva et al., 2010; Gu et al., 2014), osmoregulation (Taylor and Grosell, 2006), physical barrier (Hu et al., 2016) and immune regulation (Krogdahl et al., 2015). The present study was therefore conducted to strengthen knowledge of effects of variation in macronutrient diet composition on gut function and thereby to gain information necessary for successful cultivation of lumpfish.

The results presented herein are based on investigation of samples collected in two feeding experiments. The main part of the results stems from a feeding experiment (E1) conducted to estimate optimal balance between protein, lipid, and carbohydrates in diets for lumpfish of 15-50 g, based on observations of growth and feed conversion ratio, physiological characteristics of the intestine, mucosal and systemic immune responses. Results regarding growth performance, with more than three times increase in weight, body composition, welfare scores, and systemic immune responses are published in the paper of Hamre et al. (Hamre et al., 2022) with the following conclusion: The best growth and welfare were obtained in fish fed a diet with 55% protein, 17% lipid and 6% carbohydrates. However, to meet the wish from farmers of reducing growth, diets for lumpfish weighing from 10-50 g body weight should contain approximately 55% protein, minimum 10% lipid and maximum 10% carbohydrate. The work presented in the former paper also stated that the latter composition would be suitable also for lumpfish of 1.7-10g.

The present study comprises results regarding characteristics of the intestinal tract based on samples from E1. However, as the E1 experiment did not give sufficient fecal sample material for estimation of nutrient digestibility, an additional experiment (E2) was conducted addressing specifically macronutrient digestibility, the results of which are also included in the present paper. In addition to the macronutrient digestibility analyses, the lumpfish intestine was characterized in detail by histomorphological evaluations, digestive enzyme activity and bile acid assays, as well as by expression profiling of genes involved in nutrient digestion and transport, ion exchange, immune regulation, and cell remodeling. Liver structure and function was also evaluated to provide information on nutrient turnover and integrated metabolism.

2 MATERIALS AND METHODS

The feeding trials were conducted in accordance with Norwegian laws and regulations concerning experiments with live animals. Experiments were overseen by the Norwegian Food Safety Authority.

2.1 Experimental Fish and Feed

2.1.1 Fish and Feed for Experiment 1

This experiment evaluated effects of diet composition on digestive functions in lumpfish with an initial body weight of 15 g growing to 50 g. The experiment was conducted based on a three-component mixture design (Cornell, 2011), set up with Design Expert ver. 8.0.4. (Stat-Ease Inc. MN, USA). As it is a special type of response surface methodology for experimental design, for which replicates are not necessary (Hamre et al., 2003; Hamre and Mangor-Jensen, 2006; Hamre et al., 2013), we set one tank for most diets. In total, twelve different diets were

formulated and fed to fish in one tank for each one of 11 diets. The 12th diet was fed to fish in 3 tanks to obtain a measure of tank variation. The experimental diets were produced at the Aquafeed Technology Centre of Nofima in Bergen, Norway, in the same production series, using a Wenger TX-52 co-rotating twin-screw extruder with 150 kg/h capacity. The dietary oil was added in the different feed mixes prior to extrusion. The settings of the extruder were "normal"; i.e., the production can be upscaled to a feed factory (extruder settings considered: screw configuration (D), die opening (1.5 mm), knife speed (2671-3108 rpm), feed rate (110-150 kg/h), at the DDC the amount of added steam was 10-12 kg/h, and water 0.16-0.18 kg/min, and in the extruder there was added 0.24-0.45 kg/min water and no steam. The ingoing temperature of the feed mass in the extruder was 79-84°C and the outgoing 103-118°C. The produced pellets were air-dried in a carousel dryer (Model 200.2, Paul Klöckner GmbH, Nisteral, Germany) at 85°C for 10-12 min to a final moisture level between 6.36 and 8.26%. The size of the dried pellets was approx.: 1:5 – 1:7 × approx.: 2:3mm with bulk density between 443 and 582 g/L. Diet recipes and proximate composition are shown in **Table 1**, and **Figure 1** illustrates the dietary design of experiment E1. The following ranges of macronutrients were covered: Protein 43-68%, lipid 4-17%, and carbohydrate 6-17%. For most observed biomarkers, samples from all treatments were investigated. For some, due to resource restrictions, it was possible to investigate only selected macronutrient combinations.

Details of the experimental conditions are published in (Hamre et al., 2022). In brief, the fish were produced by a commercial hatchery and transported to Nofima's research facility at Sunndalsøra, Norway. Fourteen 150 L conical tanks were used, each with 90 fish. Dead fish were removed daily, counted, and weighed. Feed was distributed continuously (15 sec feeding every 5 minutes) using small belt automatic feeders above each tank. The fish were fed to satiation, and the feed rations increased from 30 to 68 g/tank in tanks according to appetite. The fish were fed 1-1.6mm pellets until 20 g size and 1.6-2.3mm until the end of the experimental period. Due to the small pellet sizes, feed intake could not be recorded by our system. The temperature was set to a mean of 9.8°C (min 8.9°C, max 10.6°C). The water flow was set to 4 l/min and oxygen was adjusted to 80-100% by adding oxygen to the water holding tank when needed. Temperature was recorded daily, and oxygen was measured and adjusted 2-3 times per week. As the trial was run in a flow-through system, ammonia nitrogen and nitrate nitrogen were not recorded.

TABLE 1 | Diet formulation and proximate composition for experiment 1 (E1).

Diet no.	1	2	3	4	5	6	7	8	9	10	11	12
Basic mix:												
Fish meal[1]	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5
Krill hydrolysate[2]	3	3	3	3	3	3	3	3	3	3	3	3
Vitamin mix[3]	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435
Krill oil[4]	1	1	1	1	1	1	1	1	1	1	1	1
Mineral mix[5]	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84
Lys[6]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Choline chloride[7]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Cholesterol[8]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Aquate[9]	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Biomoss[9]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Taurine[10]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Carop. Pink[11]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Yttrium oxide[12]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Varying ingredients:												
Cod muscle meal[13]	30.5	26.1	23.3	28.4	24	21.2	23.4	19.1	16.3	20	15.6	12.9
Fish oil[1]	0.05	7.9	12.85	0.1	7.95	12.9	0.35	8.15	13	0.5	8.25	13.1
Wheat gluten[14]	30.5	26.1	23.33	28.38	23.98	21.2	23.43	19.08	16.3	19.95	15.58	12.85
Wheat meal[15]	1	1.5	1.8	5.1	5.6	5.9	14.5	15.05	15.4	21.15	21.7	22.05
NaH_2PO_4[16]	4.2	4.65	4.95	4.3	4.75	5.05	4.55	4.9	5.25	4.7	5.15	5.4
Analysed composition %												
Crude protein	68	62	58	65	58	55	59	52	48	54	48	45
Total lipid	4	11.8	16.4	4.1	11.9	16.9	4.2	12	16.5	4.5	12.3	17.3
Carbohydrates	5.9	6	5.8	8.3	8.2	8.1	13.7	13.6	13.7	16.9	17.5	17.7
Ash	11.6	11.2	11.2	11.2	11.4	11.4	11.3	11.1	11.2	11.2	11.1	10.9
Choline	0.503	–	0.473	–	0.483	–	–	–	–	–	–	0.446
Dry matter	92	93	93	92	92	92	92	92	92	91	92	93

Values are in %. [1]Norsildmel, Egersund, Norway; [2]Approx. 60% dry matter, Olympic, Herøy, Norway; [3]Provides in the final feed 3000 IU vit D3, 410 mg/kg vit E, 20 mg/kg vit K3, 700 mg/kg vit C, 20 mg/kg B1, 30 mg/kg vit B2, 25 mg/kg B6, 0.05 mg/kg vit 12, 60 mg/kg pantothenic acid, 10 mg/kg folic acid, 200 mg/kg niacin, and 1 mg/kg biotin; [4]Aker Biomarine, Oslo, Norway; [5]Provides in the final diet 50 mg/kg Mn, 750 mg/kg Mg, 150 mg/kg Fe, 120 mg/kg Zn, 10 mg/kg Cu, 800 mg/kg Na, 0.2 mg/kg Se and 0.2 mg/kg Co; [6]L-lysine, delivered by Vilomix, Hønefoss, Norway; [7]Choline chloride, delivered by Vilomix, Hønefoss, Norway; [8]Cholesterol, Carbogen Amics B.V., Veenendaal, The Netherlands; [9]Aquate and Biomos, Alltech Norway AS, Førde. Norway; [10]Taurine, VWR, Oslo, Norway; [11]Taurine, VWR, Oslo, Norway; [12]Yttrium oxide, Y2O3 (99.9%), delivered by Vilomix, Hønefoss, Norway; [13]Seagarden, Karmøy, Norway; [14]Wheat gluten, Amytex 100, Tereos Syral, Aalst, Belgium; [15]Wheat meal, Norgesmøllene AS, Bergen, Norway; [16]MSP (26% P), Mono sodiumphosphate, delivered by Vilomix, Hønefoss, Norway.

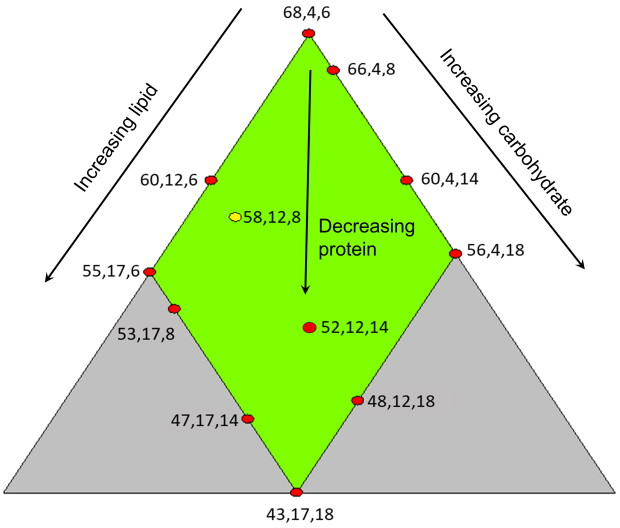

FIGURE 1 | Illustration of feed design. Each feed is indicated with a red dot and numbers beside the dot presenting macronutrient composition in the following sequence, from left to right, level of crude protein, crude lipid and total carbohydrates. The yellow dot represents diet was fed to fish in three tanks.

2.1.2 Fish and Feed for Experiment 2

An additional experiment, E2, was conducted to gain knowledge on macronutrient digestibility. Starch digestibility was of particular interest as great variations have been observed between other species (Krogdahl et al., 2004). Feed formulations are shown in **Table 2**. The diets were formulated to contain 55% crude protein with low (Diet 1), medium (Diet 2) and high (Diet 3) levels of lipid, and with carbohydrate level of contrary variation to lipid level. The same ingredients were used for all three diets, and the different lipid and carbohydrate levels were balanced by differing the dietary inclusion levels of lipid and wheat meal, respectively. The diets were produced at Aquafeed Technology Centre of Nofima in Bergen, Norway. Each of the three diets were fed to fish in triplicate tanks for a period of 14 days.

Lumpfish with an average body weight of around 100 g were distributed to 9 tanks, 100 individuals per tank, providing a biomass of around 10 kg in each tank. The tanks were cylindrical, with flat bottom and black inner surface, and an efficient water volume of 350 L. All tanks were equipped with automatic belt feeders for distribution of feed, and a separate light source mounted on each tank. Inlet water was run through a 10 μm filter and UV treated. Water flow was set at 12 L/min and oxygen saturation was kept within 80-100%. Average water temperature was 11.6°C (10.9-12.4°C).

2.2 Sampling

2.2.1 Sampling for E1

The trial was terminated after 42 days. At termination of the experiment, 6 fish from each tank were sampled at random, weighed, and intestinal tissues collected for analyses. The intestine was cleaned of mesenteric fat and divided into three segments, i.e., pyloric intestine including the pyloric caeca (PI), mid intestine (MI) and distal intestine (DI) (See **Supplementary Figure S1**). Each segment was opened longitudinally. Limited by fish size, gut content was only collected from MI, where the amount was enough for analysis. The collected gut content of MI was snap frozen in liquid nitrogen and stored at -80°C before further processing and analysis of activity of digestive enzymes and concentration of bile salts. Samples of tissue from all sections, located in the middle of the sections were collected for RNA extraction (submerged in RNAlater solution, incubated at 4°C for 24 h and stored at -20°C) and histomorphological evaluation (fixed in 4% phosphate-buffered formaldehyde solution for 24 h and transferred to 70% ethanol for storage). The remaining tissue was collected for brush border digestive enzyme assessment, snap frozen in liquid nitrogen and stored at -80°C.

2.2.2 Sampling for E2

The trial was terminated after 14 days, when fecal samples were collected as pooled samples per tank. Because of the very short length of DI of lumpfish, it cannot be stripped for feces like salmonids, therefore fish were euthanized by an overdose of anesthetic (Tricaine mesylate, MS-222) and the total content of the DI was collected by dissecting the intestine.

2.3 Analytical Procedures

2.3.1 Activity of Digestive Enzymes and Bile Salt Concentration

The intestinal tissue samples were thawed and homogenized (1:20 w/v) in ice-cold 2 mM Tris/50 mM mannitol, pH 7.1, containing

TABLE 2 | Diet formulation and proximate composition for experiment 2 (E2), g/kg.

	1	2	3
Ingredients:			
Fish meal[1]	366.2	362.9	387.6
Wheat gluten[2]	183.1	181.5	193.8
Wheat meal[3]	188	125	40
Fish oil[4]	5	73	120.9
Codfish powder[5]	100	100	100
Krill hydrolysate[6]	20	20	20
Krill meal[7]	40	40	40
Krill oil[8]	10	10	10
Vitamin mix[9]	30	30	30
MSP[10]	24	24	24
Mineral mix[11]	8.4	8.4	8.4
Biomos[12]	4	4	4
Cholesterol[13]	5	5	5
Choline cloride[14]	5	5	5
Lysine[15]	6	6	6
Taurine[16]	2	2	2
Stay-C[17]	2.2	2.2	2.2
Carphyll pink[18]	1	1	1
Yttrium oxide[19]	0.1	0.1	0.1
Analysed content, %:			
Dry Matter	89.4	92	93.2
Lipid	7.5	13.8	18.1
Carbohydrates	18.3	14.6	9.5
Starch	16	11.7	6.6
Protein	55.3	55.3	57.3
Ash	8.2	8.3	8.4
Choline	0.521	0.516	0.54
Energy, MJ/kg	19.9	20.9	22.2

[1] Fishmeal, Norse-LT, Vedda AS, Langevåg, Norway,; [2] Wheat gluten, Amytex 100, Tereos Syral, Aalst, Belgium [3] Wheat meal, Norgesmøllene AS, Bergen, Norway; [4] Fish Oil, NorSalmOil, Pelagia, Egersund, Norway; [5] Codfish powder, Seagarden AS, Avaldsnes, Norway; [6] Krill hydrolysat, Rimfrost AS, Ålesund, Norway; [7] Krill meal, Rimfrost AS, Ålesund, Norway; [8] Krill oil, Aker BioMarine, Lysaker, Norway; [9] Vitamin premix, Nofima vitamin premix, Vilomix, Hønefoss, Norway [10] MSP (26% P), Mono sodiumphosphate, delivered by Vilomix, Hønefoss, Norway [11] Mineral premix, Nofima mineral premix, Vilomix, Hønefoss, Norway [12] Biomos, Alltech Norway AS, Førde. Norway; [13] Cholesterol, Carbogen Amics B.V., Veenendaal, The Netherlands [14] Choline chloride, delivered by Vilomix, Hønefoss, Norway [15] L-lysine, delivered by Vilomix, Hønefoss, Norway; [16] Taurine, VWR, Oslo, Norway;[17] Stay-C 35%, delivered by Vilomix, Hønefoss, Norway; [18] Carophyll pink (10% astaxanthin), delivered by Vilomix, Hønefoss, Norway [19] Yttrium oxide, Y_2O_3 (99.9%), delivered by Vilomix, Hønefoss, Norway.

phenyl-methyl-sulphonyl fluoride (P-7626, Sigma, Norway) as serine protease inhibitor. The homogenates were sonicated, aliquoted and stored at -80°C until analysis.

Leucine aminopeptidase (LAP) and maltase were the brush border digestive enzymes assessed. The activity of LAP was measured employing the Sigma procedure no. 251 (Krogdahl et al., 2003). L-leucyl-b-naphthylamide is used as the substrate and is reacted with diluted homogenates at 37 °C for 1 h. The reaction is terminated by HCl. A subsequent color reaction is done by adding sodium nitrite, ammonium sulfate and *N*-(1-Naphthyl)ethylenediamine dihydrochloride in order at room temperature. A standard curve is made for calculation by measuring serial diluted 2-Naphthylamine. The color absorbance is read at 580nm. To measure maltase activity, the method described by Dahlqvist (Dahlqvist, 1970) was applied. Maltose is used as substrate and is reacted with diluted homogenates at 37 °C for 1 h. The reaction is terminated and colorized by TGO solution (mixture of Trizma buffer, detergent, o-dianisidine and peroxidase). A standard curve is made for calculation by measuring serial glucose dilution. The color absorbance is read at 480nm.

Activity of trypsin, amylase and lipase, and total bile salt level were measured in pooled freeze-dried content from MI. Trypsin activity was determined colorimetrically (Kakade et al., 1973). Benzoylarginine p-nitroanilide (Sigma no. B-4875, Sigma Chemical Co., St. Louis, MO) is used as substrate and is reacted to freeze-dried content solution at 37 °C for 10 min. The reaction is terminated by 30% acetic acid. The color absorbance is read at 410nm. Absorbance variance between sample and blank is defined as enzyme unit instead of international enzyme unit. Lipase activity was determined as described in (Brockman, 1981). 4-Nitrophenyl myristate (Sigma 70124, Sigma Chemical Co., St. Louis, MO) is used as substrate and the reaction temperature is 37 °C. The absorbance is read at 405nm every 30 sec for 7 times. The enzyme unit is defined as absorbance variance per minute. Amylase was determined as described by (Froystad et al., 2006), using a Randox amylase assay kit (AY3805, Randox Laboratories Ltd., Crumlin, UK). The kit employs Ethylidene PNPG7 method (Kruse-Jarres et al., 1989) for α-amylase measurement. Ethylidene-blocked p-nitrophenyl-maltoheptaoside is the substrate in the enzyme assay reagent and reaction temperature is 25°C. Absorbance is read at 405nm after 20, 30 and 40 min of reaction and the enzyme unit is defined as absorbance variance per minute. Bile salt level was determined using the enzyme cycling amplification/Thio – NAD method (Inverness Medical, Cheshire, UK) in the ADVIA®1650 Chemistry System (Siemens Healthcare Diagnostics Inc.) at the Central Laboratory of the Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo. The assay measures total 3αOH of cholic acid, whereas the reported results indicate the corresponding level of bile salt as taurocholate.

2.3.2 Quantitative Real-Time PCR

Total RNA was extracted from tissue samples of PI, MI, DI, and liver. (~20 mg) of all fish using Trizol reagent (PureLink™ RNA Mini Kit, Thermo Fisher Scientific). RNA was purified by an on-column DNase kit (PureLink™ DNase Set, Thermo Fisher Scientific) according to the manufacturer's protocol. RNA purity and concentration were measured using the Epoch Microplate Spectrophotometer (BioTeK Instruments, Winooski, USA). The RNA integrity was verified by the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) in combination with RNA Nano Chip (Agilent Technologies, Santa Clara, CA, USA). First-strand complementary DNA (cDNA) was synthesized using 1.0 μg RNA from two fish of the same tank, namely 0.5 μg RNA of each two fish were combined as a unit of RNA sample for cDNA synthesis. The reaction volume was 20 μl, including 4 μl mastermix of from the kit SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific). Negative controls were performed in parallel by omitting RNA or enzyme.

Lumpfish mRNA sequences were derived from NCBI database. The selected genes include elongation factor 1-alpha (*ef1a*), beta-actin (*bactin*), hypoxanthine-guanine phosphoribosyltransferase

1 (*hprt1*), tubulin beta chain (*tubb*), sucrase-isomaltase (*si*), solute carrier family 27 member 4 (*slc27a4*), solute carrier family 15 member 1 (*slc15a1*), niemann-pick C1-like 1 (*npc1l1*), solute carrier family 12 member 1 (*slc12a1*), tight junction protein 1a (*tjp1a1*), occludin (*occludin*), cyclooxygenase-2 (*cox2*), immunogobulin M (*igm*), inhibitor of nuclear factor kappa B kinase subunit beta (*ikbkb*), complement component 5 (*c5*), chemokine (C-X-C motif) ligand 19 (*cxcl19*), tumor necrosis factor alpha (*tnfa*), nuclear factor kappa-light-chain-enhancer of activated B cells (*nfkb*), transcription factor p65 (*rela*), major histocompatibility complex II (*MHCII*), matrix metallopeptidase 13 (*mmp13*) and proliferating cell nuclear antigen (*pcna*). The selected genes cover the functions of disaccharide digestion (*si*), nutrient transport *(slc27a4*, *slc15a1*, *npc1l1*), ion-exchange (*slc12a1*), tight junction forming (*tjp1a1*, *occludin*), immune regulation (*cox2*, *igm*, *ikbkb*, *c5*, *cxcl19*, *tnfa*, *nfkb*, *rela*, *MHCII*, *mmp13*) and cell proliferation (*pcna*). Reference gene candidates include *ef1a*, *tubb*, *hprt1* and *bactin*.

Primer information are shown in **Supplementary Table S2**. The qPCR primers were designed using the Primer-BLAST tool of NCBI. All primer pairs were first used in gradient reactions to determine optimal annealing temperatures. To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and visual inspection of the PCR products by agarose gel electrophoresis. PCR efficiency for each gene assay was determined using 2-fold serial dilutions of randomly pooled complementary DNA.

Expression of target genes were analyzed using the LightCycler 96 (Roche Diagnostics, Basel, Switzerland) with a 10-µl DNA amplification reaction. Each 10-µl DNA amplification reaction contained 2 µl PCR grade water, 2 µl of 1:10 diluted cDNA template, 5 µl LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) and 0.5 µl (10 µM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no-template control. The three-step qPCR run included an enzyme activation step at 95°C (5 min), 40 cycles at 95°C (10 s), annealing temperature (10 s), and 72°C (15 s) and a melting curve step. The mean normalized expression of the target genes was calculated from raw Cq values (Muller et al., 2002). Reference genes selected was done in terms of its stability among different fish (Kortner et al., 2011). The chosen reference genes are: *ef1a*, *tubb* and *hprt1* for PI and liver, *ef1a*, *bactin* and *hprt1* for MI and *tubb*, *bactin* and *hprt1* for DI.

2.3.3 Histological Evaluation

The samples fixed for histological evaluation from all intestinal segments collected, i.e., PI, MI, and DI, were processed using standard histological methods and stained with hematoxylin and eosin (H&E). The histological sections were estimated to describe the general structure as well as assessing for any morphological changes in the intestinal mucosa such as inflammation. Since the relative weights (OSI) of intestinal sections and liver seemed to vary more specifically to dietary lipid content, histological examination was conducted for the six sampled fish fed the following four diets spanning from low to high lipid content: i.e., 6, 21, 28, and 38 g lipid/100g protein (Diet 1, 5, 3 and 12, respectively). The evaluated morphological characteristics included changes in cellularity of submucosa and lamina propria, enterocyte supranuclear vacuolization and numbers of intra-epithelial lymphocytes. The degree of changes was graded as normal, mild, moderate, marked and severe, and was scored from 0 to 4, respectively.

2.3.4 Macronutrients and Yttrium in Feed and Feces

Samples of the feed and feces were analyzed at the Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, Ås, Norway. Dry matter was determined by oven drying at 105°C, 16–18 h, to constant weight. Nitrogen was determined using the Kjeldahl method (Kjeltech Auto Analyser, Tecator, Höganäs, Sweden) (crude protein: N×6.25), fatty acid composition by FAME analysis (O'Fallon et al., 2007) in a Trace GC Ultra with auto injector (Thermo Scientific), and starch was measured as glucose after hydrolysis by α-amylase and amyloglucosidase, followed by glucose determination by the 'Glut-DH method'. Yttrium was analyzed by dissolution of ashed samples with hydrochloric acid and nitric acid by heating, then dissolved in 5% nitric acid. Yttrium was then detected with an ICP-AEF, Optima 3000 V (Perkin Elmer, USA).

2.4 Calculations and Statistics

Organosomatic indices of the intestinal sections = tissue weight/body weight × 100.

Apparent digestibility coefficient (referred to as digestibility in the text) =

$100 - 100 \times (\%$ nutrient in feces/% nutrient in diet) $\times (\%\ Y_2O_3$ in diet/% Y_2O_3 in feces)

Models describing the effects of macronutrient composition on the different responses were calculated using the software Design Expert ver. 8.0.4. (Stat-Ease Inc. MN, USA). Different models were fitted to the data and the recommended model with the best fit was chosen. When no model had a significant fit to the response data, only mean and standard deviation of the total dataset are given. Models and coefficients were considered significant at $p < 0.05$. Models where $0.05 < p < 0.1$ were considered a trend and presented.

Differences in histological scores for the various evaluated morphological characteristics were analyzed using ordinal logistic regression. When score differences were only 2 levels, statistical significance was assessed using the Fisher exact test. *Post hoc* analysis for significant test results was conducted using the chi-square test (chisq.post.hoc function of Fifer package in R). Differences were considered significant at $p < 0.05$.

3 RESULTS

3.1 Digestive Enzyme Activities in Gut Tissues and Digesta, and Nutrient Digestibility

As the present study is among the very first studies investigating the physiology of the intestinal tract of lumpfish, some summary statements regarding the general characteristics are given: The average OSI of the three intestinal sections, PI, MI, and DI, was 2.1, 1.6, and 0.6% of body weight and they comprised 49,

38, and 13% of the intestinal weight, respectively. Of total LAP capacity, i.e., units per kg fish, 34, 50, and 16% were observed in PI, MI, and DI, respectively, and of maltase capacity, 48, 40, and 12%. Digestive enzyme activities in chyme, results only available from MI, were as follows: trypsin activity averaged 25 U/mg DM, amylase 2 U/mg DM, and lipase 0.27 U/mg DM. Bile salt concentration in chyme from MI was 49 mg/g DM. Average digestibility, as measured in collected feces, was for protein: 83%, sum of fatty acids: 95%, and starch: 64%.

3.2 Effects of Macronutrient Composition on Intestinal and Liver Functions

In the following content we have chosen to present the results and comments as follows: For results showing linear relationship to the macronutrient levels, effects of decreasing protein level are presented first, in light of possible effects of protein deficiency at low dietary levels. Thereafter effects of increasing level of lipid are presented and finally effects of increasing level of carbohydrates. For results showing more complicated relationships, the comments are presented as found most suitable for the outcome of the data evaluation. Linear relationships were found for most of the observed variables, but for those explicitly mentioned in the text showing no relationship with diet macronutrient composition or following a model other than a linear one. To check model parameters, one can refer to **Tables 3**, **4**, and **Supplementary Tables S3**, **S4** listing the coefficients derived from the model equation.

3.2.1 Organosomatic Indices

Variation of diet composition significantly affected the intestinal weight of PI and MI (**Figure 2** and **Table 3**). In PI, OSI increased with decreasing protein level, increased with increasing dietary lipid level, and had no response with carbohydrate variation. In MI, OSI increased with decreasing protein level, increased with increasing dietary lipid level, and increased with increasing carbohydrate level.

3.2.2 Brush Border Digestive Enzyme Activities

Specific activity of both LAP and maltase was highest in MI, lowest in the PI, and intermediate in DI. Also, the value for LAP capacity, i.e., per kg of fish, was highest in the MI, whereas for maltase, the highest value was observed for PI. The DI showed the lowest capacity values for both LAP and maltase.

In PI, specific activity of LAP and maltase showed significant relationships with diet macronutrient composition ($p = 0.032$ and 0.034, **Table 3** and **Figure 3**). The specific LAP and maltase activities (U/mg protein) decreased with decreasing protein level, decreased with increasing dietary lipid level and, to lesser extent, increased with increasing carbohydrate level. On the other hand, the enzyme capacity (U/kg fish) of LAP and maltase ($p = 0.090$ and $p > 0.10$, **Table 3** and **Figure 3**) showed no significant correlations with diet macronutrient composition in this section.

In MI, both specific activity and capacity of LAP and maltase showed clear relationships with diet composition ($p = 0.005$, 0.016, 0.001 and 0.002, **Table 3** and **Figure 3**), i.e., a decrease with decreasing protein level, a decrease with increasing dietary lipid level, and an increase with increasing carbohydrate level.

In DI, there were no significant effects for either specific activity or capacity and the analysis by software cannot illustrate a clear trend neither ($p = 0.080$, 0.1, 0.060 and 0.070, **Table 3**).

3.2.3 Enzyme Activities and Bile Salt Level in MI Digesta

Lipase activity in digesta from MI showed a significant, linear, relationship with dietary protein level, decreasing with decreasing protein level ($p = 0.020$, **Table 3** and **Figure 3**). Lipase activity decreased with increasing lipid level as well as with increasing carbohydrate level. The trypsin and amylase activities in digesta from MI did not show significant relationships ($p > 0.10$, **Table 3**) with diet macronutrient composition. Bile salt concentration showed a tendency towards a similar response pattern as for the lipase ($p = 0.077$, **Table 3** and **Figure 3**), i.e., decrease with decreasing protein, increase with increasing lipid and carbohydrate level.

3.2.4 Gene Expression in Intestinal Tissue

In PI, *si* and *slc15a1*, coding for a disaccharidase and a peptide transporter, respectively, were the two genes showing significant responses to diet macronutrient composition ($p = 0.039$ and 0.050, respectively, **Table 4** and **Figure 4**). Their relationships

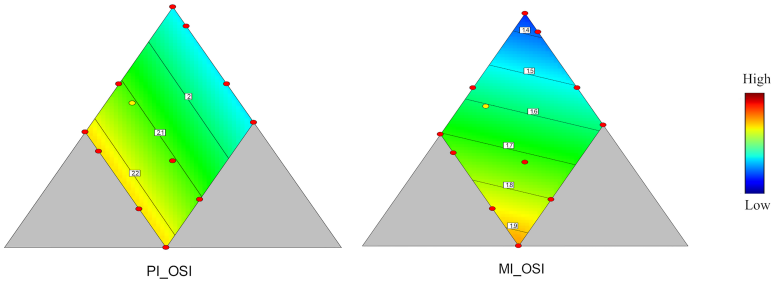

FIGURE 2 | Organosomatic indices of PI and MI. Color scale from blue to red indicates value changes from low to high. The yellow dot represents diet was fed to three tanks. Macronutrient composition of each dot can refer to **Figure 1**.

TABLE 3 | Result summary: Organ indices and digestive enzymes.

Response	Minimum	Maximum	Mean	Std. Dev.	Ratio	Model	P model
PI_OSI	1.76	2.37	2.09	0.16	1.3	Linear	**0.004**
MI_OSI	1.32	2.04	1.64	0.20	1.5	Linear	**0.002**
DI_OSI	0.41	0.69	0.57	0.07	1.7	Mean	
PI_LAPprot	35	79	52	13	2.2	Linear	**0.032**
PI_LAPkg	31	65	44	9	2.1	Linear	0.090
PI_MALTprot	8.7	17	11	3	2.0	Linear	**0.034**
PI_MALTkg	5.8	14	9.3	1.9	2.4	Mean	
MI_LAPprot	88	188	131	35	2.1	Linear	**0.005**
MI_LAPkg	51	80	66	10	1.5	Linear	**0.016**
MI_MALTprot	9.9	27	16	5	2.7	Linear	**0.001**
MI_MALTkg	5.8	11	7.8	1.51	1.9	Linear	**0.002**
DI_LAPprot	61	182	115	37	3.0	Linear	0.080
DI_LAPkg	11	32	21	6	2.8	Linear	0.1
DI_MALTprot	7.1	21	12	4	2.9	Linear	0.060
DI_MALTkg	1.2	3.6	2.3	0.7	2.9	Linear	0.070
MI_Lipase	0.067	0.42	0.27	0.11	6.3	Linear	**0.020**
MI_Bile salt conc.	32	68	49	10	2.1	Linear	0.077
MI_Amylase	0.5	6	2.0	1.5	11	Mean	
MI_Trypsin	13	36	25	7	2.7	Mean	

Data series minimum and maximum values, means and standard deviations, the ratio between max and min, the model that fitted the data best and probability that the data distribution is random (different from mean). The names of responses comprise intestinal sections and biomarkers tested. PI, proximal intestine. MI, mid intestine. DI, distal intestine. OSI, organosomatic index (%). LAPprot, LAP specific activity (U/mg protein). LAPkg, LAP capacity (U/kg fish). MALTprot, maltase specific activity (U/mg protein). MALTkg, maltase capacity (U/kg fish). Lipase, lipase activity (U/mg dry matter). Bile salt conc, bile salt concentration (mg/g dry matter). Amylase, amylase activity (U/mg dry matter). Trypsin, trypsin activity (U/mg dry matter). Bold value indicates significance (p model < 0.05).

with diet composition were similar, i.e., decreasing in value with decreasing protein content, decreasing with increasing lipid level and increasing, although only slightly, with increasing carbohydrate. Expression of *rela*, *igm* and *cxcl19*, all involved in immune functions, tended to vary with diet composition ($p = 0.080$, 0.073 and 0.094, respectively, **Table 4** and **Figure 4**). The trend observed for expression of *rela* was a decrease with decreasing protein level, decrease with increasing lipid level and increase with carbohydrate level, while *igm* expression decreased with decreasing protein level, decreased with increasing lipid level, but increased slightly with increasing carbohydrate level. The trend of *cxcl19* expression fitted a quadratic model, where the minimum was at medium protein, high lipid, and low carbohydrate level, and the maximum was at medium protein and lipid level, and high carbohydrate.

In MI, more genes responded significantly to variation in diet composition. As in PI, significant response was observed for *si* and *slc15a*, and the pattern of responses was quite similar, with a decrease with decreasing dietary protein, a decrease with increasing lipid and an increase with dietary carbohydrate level ($p = 0.026$ and 0.001, **Table 4** and **Figure 5**). Also, *slc12a1*, the gene involved in ion exchange expression decreased with decreasing protein level, decreased with increasing lipid ($p = 0.031$, **Table 4** and **Figure 5**) and increased with increasing carbohydrate level. Regarding genes involved in immune functions, linear effects of diet composition were seen for *igm and mmp13*, and quadratic responses were seen for *rela* and *cxcl19* (for p values see **Table 4** and **Figure 5**). For the former four, expression decreased with decreasing protein level, most pronounced for *mmp13*, decreased with increasing lipid level as well as with increasing carbohydrate level.

For *rela*, which fitted a quadratic model, the maximum expression was found near the center of the design, i.e., at medium levels of all the three macronutrients. Two low values were seen, i.e., at medium protein, high lipid, and low carbohydrates, and at low protein, high lipid, and high carbohydrates. The highest value for *cxcl19* was observed at the highest protein level, at which both lipid and carbohydrates were low. Lowest value was found at medium protein and lipid level and high carbohydrate level. For some genes showing fewer clear relationships with diet composition, trends ($0.05 < p < 0.10$) were indicated, i.e., for *npc1l1*, *cox2*, and *pcna* (for p values see **Table 4**). The relationship of expression of *npc1l1*, the cholesterol transporter, showed decreased level with decreasing protein level, decreased level with increasing lipid level and increased level with increasing carbohydrate level. The expression *cox2* and the cell proliferating related gene *pcna* fitted quadratic models. The maximum expression of *cox2* was at high protein, low lipid, and low carbohydrate level, with a lower peak at low protein level, high lipid, and high carbohydrate level. The minimum was shown at medium protein level, medium lipid, and high carbohydrate level. Maximum expression of *pcna* was at medium lipid and protein level and low carbohydrate level.

In DI, only expression of the tight junction related gene *occludin* and the immune related gene *igm* were significantly influenced by diet composition ($p = 0.003$ and 0.004 respectively, **Table 4**). Expression of *igm* was best explained by a quadratic model (**Figure 6**), with two maxima, one at medium protein, high lipid, and low carbohydrate level, and the other at medium protein level, low lipid, and high carbohydrate level. The minimum was observed at low protein, high lipid, and high carbohydrate level. The *occludin* expression, following a linear

TABLE 4 | Result summary: Expression of selected genes in liver and intestine.

Response	Minimum	Maximum	Mean	Std. Dev.	Ratio	Model	P model
L_*ikbkb*	0.020	0.037	0.027	0.005	1.8	Mean	
L_*rela*	0.176	0.386	0.271	0.065	2.2	Mean	
L_*igm*	0.0022	0.0043	0.0032	0.0007	2.0	Mean	
L_*cox2*	0.0025	0.0042	0.0033	0.0006	1.7	Mean	
L_*tjp1a1*	0.034	0.052	0.045	0.005	1.5	Mean	
L_*pcna*	0.210	0.412	0.313	0.055	2.0	Quadratic	**0.020**
L_*c5*	0.645	1.171	0.845	0.144	1.8	Linear	**0.025**
L_*mmp13*	0.021	0.048	0.034	0.008	2.3	Mean	
L_*MHCII*	0.025	0.052	0.038	0.009	2.1	Mean	
L_*cxcl19*	0.002	0.019	0.006	0.005	9.5	Special Cubic	0.179
L_*tnfa*	0.0005	0.0018	0.0012	0.0004	3.2	Mean	
L_*nfkb*	0.139	0.237	0.172	0.030	1.7	Mean	
L_*npc1l1*	0.041	0.089	0.068	0.016	2.2	Mean	
PI_*ikbkb*	0.060	0.085	0.073	0.009	1.4	Mean	
PI_*rela*	0.271	0.478	0.370	0.066	1.8	Linear	0.080
PI_*igm*	0.0009	0.0028	0.0016	0.0005	3.2	Linear	0.073
PI_*cox2*	0.0039	0.0097	0.0065	0.0016	2.5	Mean	
PI_*tjp1a1*	0.054	0.096	0.068	0.010	1.8	Mean	
PI_*pcna*	0.313	0.386	0.351	0.021	1.2	Mean	
PI_*c5*	0.007	0.017	0.010	0.002	2.4	Mean	
PI_*mmp13*	0.013	0.033	0.025	0.005	2.5	Mean	
PI_*MHCII*	0.256	0.638	0.376	0.097	2.5	Mean	
PI_*slc27a4*	0.160	0.303	0.216	0.034	1.9	Mean	
PI_*slc12a1*	0.205	0.335	0.253	0.040	1.6	Mean	
PI_*occludin*	0.020	0.032	0.025	0.004	1.6	Mean	
PI_*slc15a1*	0.144	0.243	0.185	0.031	1.7	Linear	**0.050**
PI_*cxcl19*	0.0024	0.0207	0.0082	0.0046	8.7	Quadratic	0.094
PI_*tnfa*	0.0023	0.0069	0.0042	0.0012	3.0	Mean	
PI_*nfkb*	0.181	0.276	0.224	0.028	1.5	Mean	
PI_*npc1l1*	0.147	0.280	0.205	0.039	1.9	Mean	
PI_*si*	0.275	0.431	0.343	0.052	1.6	Linear	**0.039**
MI_*ikbkb*	0.046	0.060	0.051	0.005	1.3	Mean	
MI_*rela*	0.208	0.279	0.245	0.020	1.3	Quadratic	**0.005**
MI_*igm*	0.00067	0.00162	0.00104	0.00031	2.4	Linear	**0.010**
MI_*cox2*	0.0038	0.0070	0.0048	0.0008	1.9	Quadratic	0.066
MI_*tjp1a1*	0.034	0.045	0.039	0.003	1.3	Mean	
MI_*pcna*	0.266	0.353	0.310	0.022	1.3	Quadratic	0.062
MI_*c5*	0.011	0.017	0.014	0.002	1.5	Mean	
MI_*mmp13*	0.009	0.020	0.013	0.004	2.2	Linear	**0.004**
MI_*MHCII*	0.059	0.124	0.080	0.017	2.1	Mean	
MI_*slc27a4*	0.134	0.189	0.160	0.017	1.4	Mean	
MI_*slc12a1*	0.057	0.094	0.078	0.012	1.7	Linear	**0.031**
MI_*occludin*	0.016	0.021	0.018	0.001	1.3	Mean	
MI_*slc15a1*	0.076	0.133	0.099	0.018	1.8	Linear	**0.001**
MI_*cxcl19*	0.0021	0.0063	0.0041	0.0015	3.0	Quadratic	**0.045**
MI_*tnfa*	0.0009	0.0018	0.0014	0.0003	2.1	Mean	
MI_*nfkb*	0.103	0.154	0.124	0.013	1.5	Special Cubic	0.181
MI_*npc1l1*	0.121	0.208	0.164	0.021	1.7	Linear	0.098
MI_*si*	0.205	0.325	0.267	0.034	1.6	Linear	**0.026**
DI_*ikbkb*	0.061	0.093	0.076	0.009	1.5	Mean	
DI_*rela*	0.196	0.287	0.251	0.028	1.5	Mean	
DI_*igm*	0.0014	0.0030	0.0020	0.0005	2.1	Quadratic	**0.003**
DI_*cox2*	0.0039	0.0065	0.0050	0.0006	1.7	Mean	
DI_*tjp1a1*	0.033	0.049	0.038	0.003	1.5	Mean	
DI_*pcna*	0.283	0.352	0.312	0.022	1.2	Mean	
DI_*c5*	0.0035	0.0080	0.0054	0.0012	2.3	Mean	
DI_*mmp13*	0.013	0.026	0.019	0.004	2.0	Special Cubic	0.119
DI_*MCHII*	0.066	0.153	0.102	0.030	2.3	Mean	
DI_*slc27a4*	0.059	0.102	0.077	0.014	1.7	Mean	
DI_*slc12a1*	0.106	0.188	0.137	0.026	1.8	Mean	
DI_*occludin*	0.013	0.021	0.016	0.003	1.6	Linear	**0.004**
DI_*slc15a1*	0.039	0.076	0.056	0.013	2.0	Mean	
DI_*cxcl19*	0.0031	0.0072	0.0045	0.0013	2.3	Quadratic	0.104

(Continued)

TABLE 4 | Continued

Response	Minimum	Maximum	Mean	Std. Dev.	Ratio	Model	P model
DI_*tnfa*	0.0014	0.0027	0.0020	0.0005	2.0	Mean	
DI_*nfkb*	0.095	0.149	0.120	0.015	1.6	Mean	
DI_*npc1l1*	0.084	0.130	0.100	0.016	1.5	Mean	
DI_*si*	0.295	0.474	0.393	0.053	1.6	Mean	

The names of responses comprise intestinal sections and genes measured. L, liver. PI, proximal intestine. MI, mid intestine. DI, distal intestine. ikbkb, inhibitor of nuclear factor kappa B kinase subunit beta. rela, transcription factor p65. igm, immunogobulin M. cox2, cyclooxygenase-2. tjp1a1, tight junction protein 1a. pcna, proliferating cell nuclear antigen. c5, complement component 5. mmp13, matrix metallopeptidase 13. MHCII, major histocompatibility complex II. cxcl19, chemokine (C-X-C motif) ligand 19. tnfa, tumor necrosis factor alpha. nfkb, nuclear factor kappa-light-chain-enhancer of activated B cells. npc1l, niemann-pick C1-like 1. slc27a4, solute carrier family 27 member 4. slc12a1, solute carrier family 12 member 1. occludin, Occludin protein. slc15a1, solute carrier family 15 member 1. si, sucrase-isomaltase. Bold value indicates significance (p model < 0.05). Data series minimum and maximum values, means and standard deviations, the ratio between max and min, the model that fitted the data best and probability that the data distribution is random (different from mean).

model, showed highest values at high protein a low lipid level, with minor, but positive dependency on carbohydrate level.

3.2.5 Gene Expression in the Liver

Among the genes observed in the liver, only *c5* and *pcna*, coding for proteins participating in immune regulation and cell proliferation, respectively, showed significant responses to the dietary variation ($p = 0.020$ and 0.025, respectively, **Table 4**). The expression of *c5* was affected only to a minor degree by protein level, decreased with increasing lipid, and increased with increasing carbohydrate level (**Figure 7**). The *pcna* expression data fitted a quadratic model (**Figure 7**) with two maxima, one at medium protein, high lipid, and low carbohydrate level, the other at low protein, high lipid, and high carbohydrate level. One minimum was apparent, i.e., at high protein, low lipid and medium carbohydrate and high protein level.

3.2.6 Histology of the Alimentary Tract and Liver

The general histological appearance of the gut mucosa is presented in **Figures 8A–D**. The stomach structure was quite similar to that observed in other fish species with simple columnar epithelium, gastric glands, and a thick smooth muscle layer with two layers, the inner circular and the outer longitudinal (**Figure 8A**). The structure of the pyloric caeca (**Figure 8B**) also appeared similar to what has been observed in other fish species. The caeca were surrounded by pancreatic tissue and were of thin wall with simple unbranched mucosal folds. Lamina propria and submucosa were thin. Fused caeca, due to branching of pyloric caeca were a unique finding for the lumpfish with shared muscular and serosal wall (**Figure 8B**, blue arrow). The mid intestine showed structures comparable to those observed in Atlantic salmon with short mucosal folds and short branching, thin

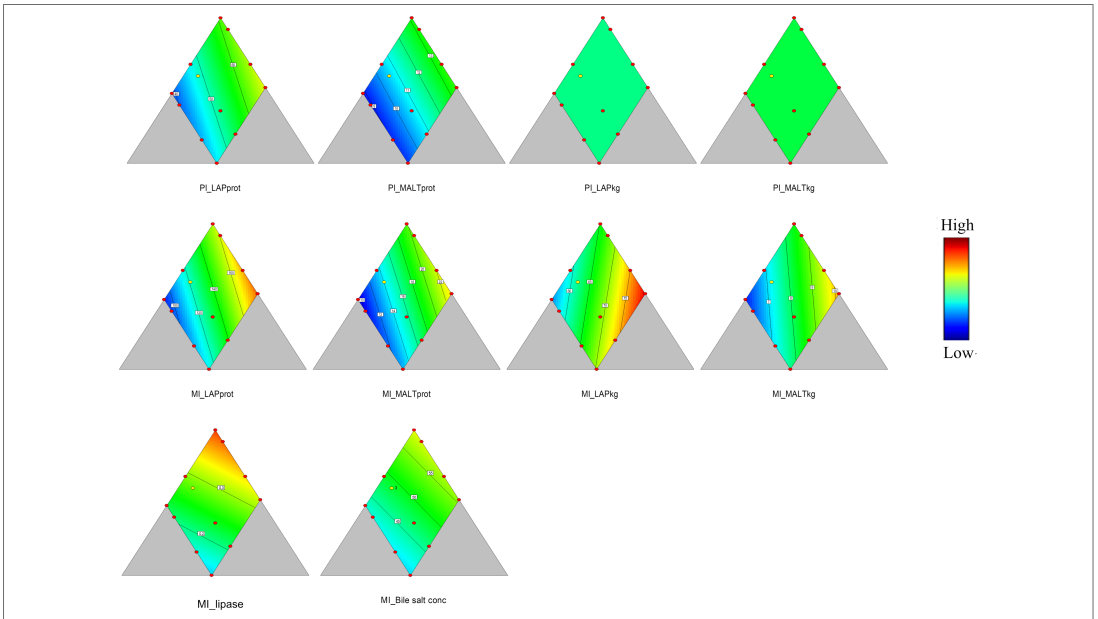

FIGURE 3 | Leucine aminopeptidase (LAP) and maltase specific activity and capacity in proximal intestine (PI) and mid intestine (MI), and lipase activity (units/mg dry matter) and bile salt concentration (mg/g dry matter) in digesta from MI. LAPprot and MALTprot, LAP and maltase specific activity (units/mg protein). LAPkg and MALT kg, LAP and maltase capacity (units/kg fish). Lipase, lipase activity (units/mg dry matter). Bile salt conc, bile salt concentration (mg/g dry matter). Color scale from blue to red indicates value changes from low to high. The yellow dot represents diet was fed to three tanks. Macronutrient composition of each dot can refer to **Figure 1**.

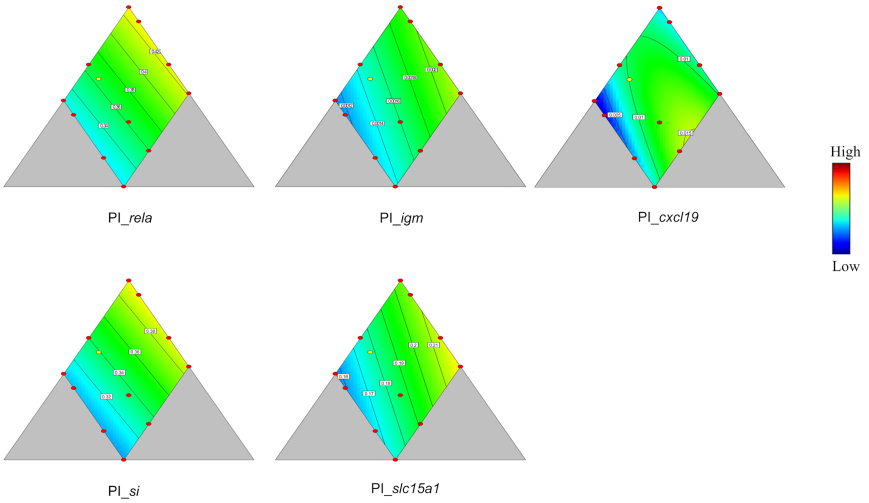

FIGURE 4 | Genes showing significant responses ($p < 0.05$) or trend ($0.05 < p < 0.1$) in proximal intestine (PI). *rela,* nuclear factor NF-kappa-B p65 subunit; *igm,* immunoglobulin M; *cxcl19,* chemokine ligand 19; *si*, sucrase-isomaltase; *slc15a1*, peptide transporter 1. Color scale from blue to red indicates value changes from low to high. The yellow dot represents diet was fed to three tanks. Macronutrient composition of each dot can refer to **Figure 1**.

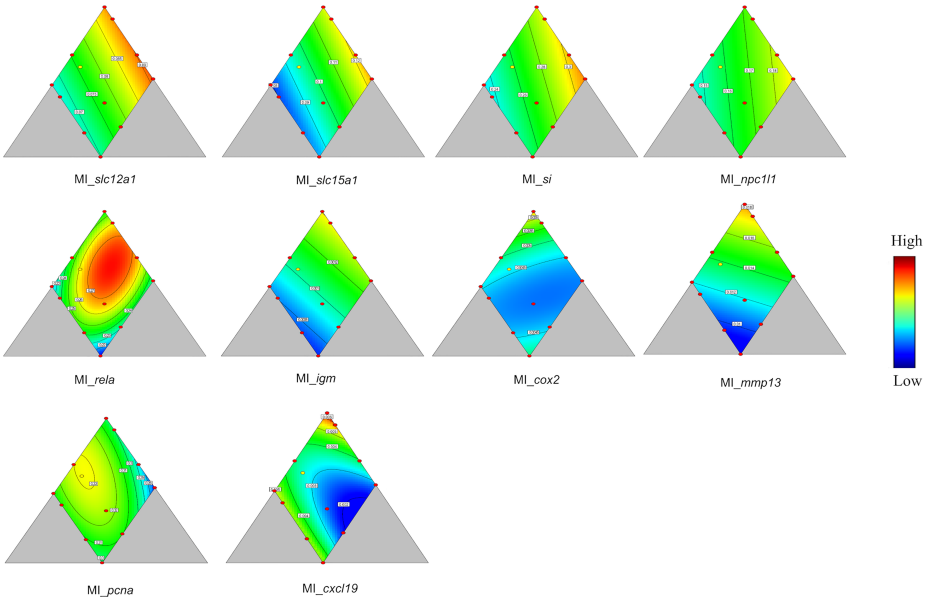

FIGURE 5 | Genes showing significant responses ($p < 0.05$) or trend ($0.05 < p < 0.1$) in mid intestine (MI). *slc12a1,* Na-K-Cl cotransporter; *slc15a1,* peptide transporter 1; *si*, sucrase-isomaltase; *npc1l1*, Niemann-pick C1-like 1; *rela*, nuclear factor NF-kappa-B p65 subunit; *igm*, immunoglobulin M; *cox2*, cyclooxygenase 2; *mmp13*, matrix metallopeptidase 13; *pcna*, proliferating cell nuclear antigen; *cxcl19*, chemokin ligand 19. Color scale from blue to red indicates value changes from low to high. The yellow dot represents diet was fed to three tanks. Macronutrient composition of each dot can refer to **Figure 1**.

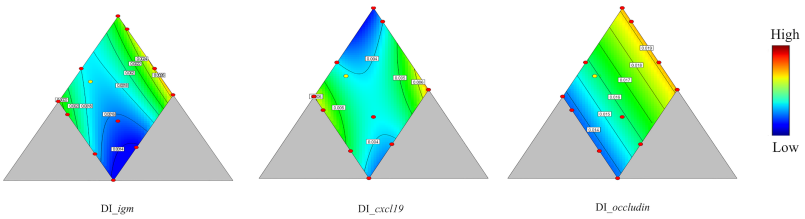

FIGURE 6 | Genes showing significant responses ($p < 0.05$) or trend ($0.05 < p < 0.1$) in distal intestine (DI). *igm*, immunoglobulin M; *cxcl19*, chemokine ligand 19; *occludin*, an integral membrane protein at the tight junctions. Color scale from blue to red indicates value changes from low to high. The yellow dot represents diet was fed to three tanks. Macronutrient composition of each dot can refer to **Figure 1**.

lamina propria and submucosa with sparse cellularity, but thick muscle layer (**Figure 8C**). The structure of the distal intestine (**Figure 8D**) appeared similar to that in Atlantic salmon with simple (Baeverfjord and Krogdahl, 1996; Knudsen et al., 2007), and complex (branched) mucosal folds which were taller than in mid intestine, and lamina propria and submucosa with fibrous tissue and little cellular composition. The enterocytes showed little or no vacuolization, a feature which may distinguish the lumpfish from Atlantic salmon which, in the fed state, show high vacuolation of the distal intestinal enterocytes.

Figures 8E–H show histological features of the cells of the PI and liver in fish fed diets with high protein and low lipid and low protein high lipid levels. Hyper-vacuolization, a symptom of excessive lipid accumulation, so-called steatosis, was observed in both PI and liver. The steatosis level was scored and analyzed for PI, MI, and liver (**Figure 9**), clearly showing increasing steatosis with increasing lipid level.

3.2.7 Digestibility

The results of the additional digestibility experiment, E2, are illustrated in **Figure 10**, showing effects of dietary lipid level on starch, protein, and fatty acid digestibility. Protein digestibility did not change as lipid or starch level varied. Fatty acid digestibility increased as dietary lipid level increased. For saturated fatty acids this effect was not significant, but the results showed the same trend as for other fatty acids ($p = 0.06$). The digestibility of ω-6 fatty acids was relatively low, between 60% and 68%. Noticeably, starch digestibility decreased greatly as starch level increased, from 84.3% at 6.6% inclusion to 50% at 16% inclusion. This means a mean partial digestibility of the starch increment from 6.6% to 16.0% of about 27% and increase in available starch level from 55-80 g/kg diet.

4 DISCUSSION

Lumpfish belongs to the family *Cyclopteridae* in which only lumpfish is commercially produced. It means gut characteristics of evolutionarily relevant species of lumpfish is lacking. In this context, comparing to other fish species of similar feeding habit is a better way to understand our results in lumpfish. Despite of shorter intestinal length to body length compared to lumpfish, Atlantic salmon is also a carnivorous fish with stomach, with available well-defined gut characteristic. Therefore, Atlantic salmon is chosen as a major species for comparison in the following discussion.

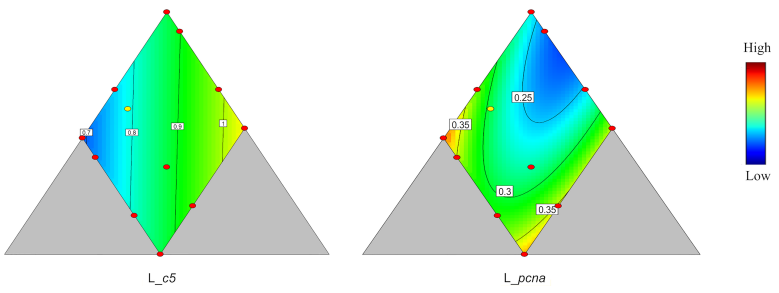

FIGURE 7 | Genes showing significant response ($p < 0.05$) responses in liver. *c5*, complement component 5; *pcna*, proliferating cell nuclear antigen. Color scale from blue to red indicates value changes from low to high. The yellow dot represents diet was fed to three tanks. Macronutrient composition of each dot can refer to **Figure 1**.

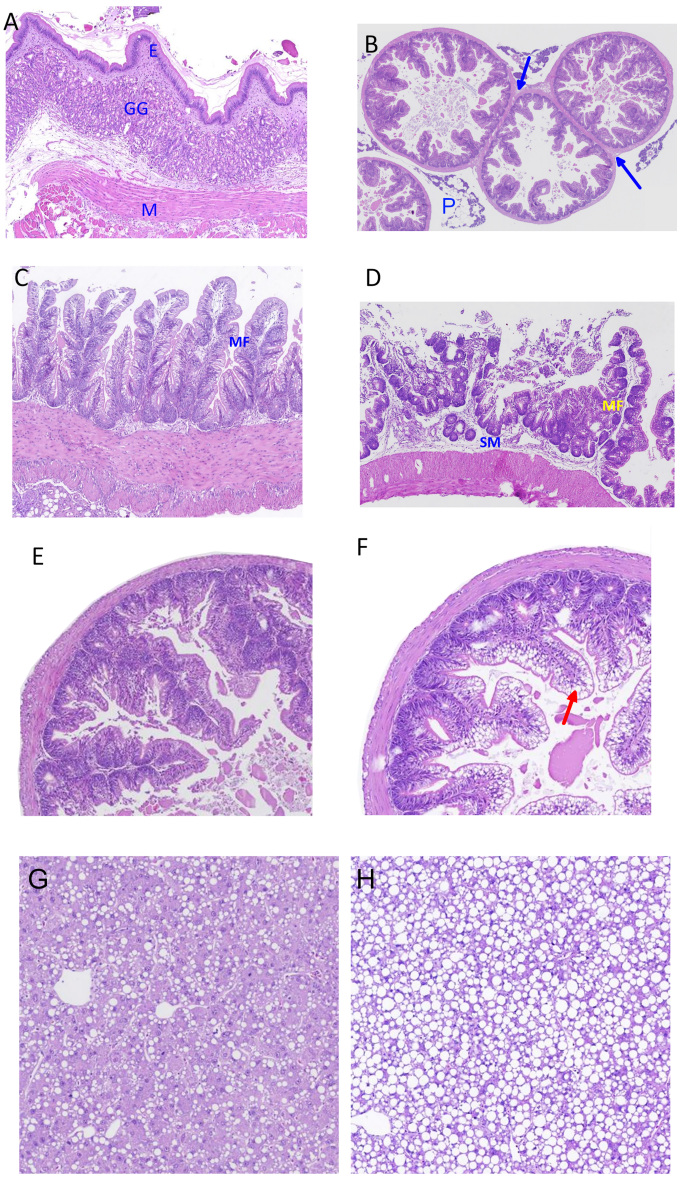

FIGURE 8 | Images of histological characteristics of the mucosa in the stomach **(A)**, pyloric intestine **(B)**, mid intestine **(C)** and distal intestine **(D)** of lumpfish, as indicated by H&E staining, and images of pyloric caeca with normal appearance **(E)** and with clear signs of hypervacuolation/steatosis **(F)**, and images of liver with normal appearance **(G)** and with clear signs of hypervacuolation/steatosis **(H)**. Image A: E, simple columnar epithelium; GG, gastric glands; M, smooth muscle layer with inner circular and outer longitudinal indicated. Image B: blue arrows indicate the unique feature of fused caeca, the share of muscular and serosal wall, and indicates a point of branching in pyloric caeca; P, pancreatic tissue. Image C: MF, short mucosal folds with short branching and with thin lamina propria and submucosa with sparse cellularity, and a thick muscle layer are apparent. Image D: MF, simple and branched mucosal folds (MF) with enterocytes of no vacuolation; SM, submucosa with and little cellularity. Image F, red arrow indicates vacuolation in enterocytes which is considered a sign of steatosis

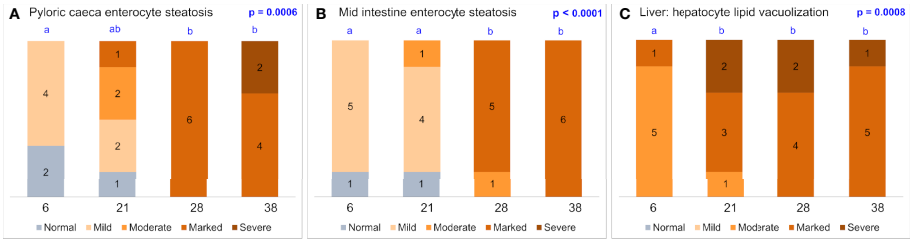

FIGURE 9 | Results of histological scoring of vacuolation in the enterocytes of the pyloric ceaca **(A)**, mid intestine **(B)** and hepatocytes **(C)**. The numbers of the x-axis indicate level of lipid in the diets, i.e. (Protein (P)/Lipid (L)/Carbohydrates (C): Diet 1: 68/4.3/5.9%; Diet 5: 58/11.9/8.2%; Diet 3: 58/16.4/5.8% and Diet 12: 45/17.3/17.7%, respectively. The x-values 6, 21, 28, and 38 represent the ratio lipid to 100g protein in the diets. Stack bars with different letters above indicate significant difference.

4.1 General Features of Lumpfish Intestine

The present study greatly improves general knowledge regarding characteristics of the lumpfish intestine and makes comparison with other fish species possible. As studies of similar characteristics of the intestinal tract of Atlantic salmon, employing the same methods and assays as in the present work is available, the present lumpfish results are compared to similarities and differences relative to Atlantic salmon. The weight of the pyloric intestine of lumpfish comprised a much lower proportion of the weight of the intestinal tract than observed in salmon,

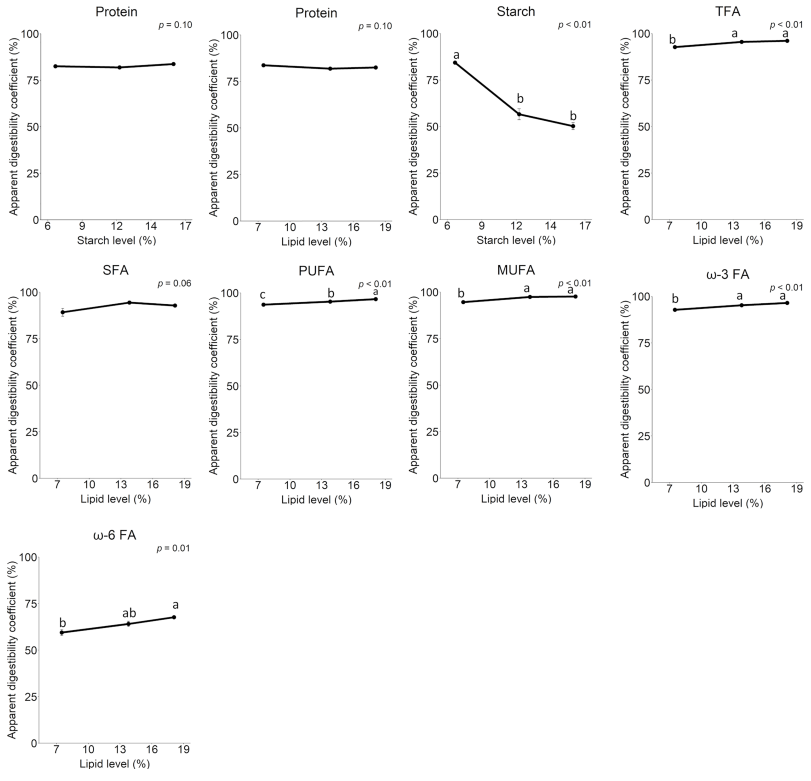

FIGURE 10 | Apparent digestibility coefficients of protein, starch, and total fatty acids. Starch digestibility is related to dietary starch level, total fatty acid digestibility is related to dietary lipid level, and protein digestibility is related to both starch and lipid level. TFAs, total fatty acids; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; ω-3 FA, omega-3 fatty acids; ω-6 FA, omega-6 fatty acids.

TABLE 5 | Indication of position in the triangle of minimum and maximum for the biomarkers which showed apparent relationship with macronutrient content of the diets*.

		Minimum									Maximum								
		Protein			Lipid			Carbo			Protein			Lipid			Carbo		
		L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
PI OSI	*Organ indices*		x	x	x						x	x				x			
MI OSI				x	x			x			x					x			x
PI LAPprot	*Digestive functions*		x				x	x				x		x					x
PI MALTprot	*in PI*	x	x				x					x	x	x					
PI *si*		x	x				x					x	x	x					
PI *slc15a1*			x				x	x				x		x					x
PI *rela*	*Immune functions*	x	x				x					x	x	x					
PI *igm*	*in PI*		x				x	x				x		x					x
PI *cxcl19*			x				x	x			x				x				x
MI LAPprot	*Digestive functions in MI*		x				x	x				x		x					x
MI LAPkg			x				x	x				x		x					x
MI MALTprot			x				x	x				x		x					x
MI MALTkg			x				x	x				x		x					x
MI *si*			x				x	x				x		x					x
MI *slc15a1*			x				x	x				x		x					x
MI lipase		x					x			x			x	x			x		
MI bile salt		x					x			x			x	x			x		
MI *slc12a1*	*Osmoregul. in MI*		x				x	x				x		x					x
MI *rela*	*Immune functions in MI*		x				x	x				x			x			x	
MI *rela*		x					x			x									
MI *igm*		x					x			x			x	x			x		
MI *mmp13*		x					x			x			x	x			x		
MI *cxcl19*			x			x				x			x	x			x		
MI *cox2*			x			x			x				x	x			x		
MI *cox2*											x					x			x
MI *pcna*	*Cell division in MI*		x		x					x		x			x		x		
DI *igm*	*Immune functions in DI*	x					x			x			x	x					x
DI *igm*												x				x	x		
DI *cxcl19*				x	x			x				x		x					x
DI *cxcl19*		x					x			x		x				x	x		
DI *occludin*	*Tight junction in DI*	x	x				x					x	x	x					
L *pcna*	*Cell division and immune function*			x	x				x			x				x	x		
L *pcna*	*in liver*										x					x			x
L *c5*			x				x	x				x		x					x

*The indictors are categorized as low (L), medium (M) and high (H) as interpreted from the respective graphs shown in ***Figures 3–7***. Background colors represent low (blue), medium (green) and high (red) for each nutrient.

about 50% compared to about 75% in salmon (Kraugerud et al., 2007). On the other hand, the MI was much larger in lumpfish, about 40% compared to about 5% in salmon, whereas the DI comprised a small part of the intestine of both species, about 10 and 20%, respectively. In the salmon, the enzyme capacities of these intestinal sections reflect the weigh differences, with 70 – 80% of the total capacity (U/kg fish) in the PI (Bakke-McKellep et al., 2008). In the lumpfish, however, of total LAP and maltase capacity, the PI and MI showed similar values, in the range 40 to 50%. The activity (U/mg digesta dry matter) of the pancreatic enzymes, trypsin, amylase, and lipase, measured in content from MI, showed values very different from values observed in Atlantic salmon, in particular for trypsin and lipase. In lumpfish the trypsin activity was 1/6 and lipase 1/10, of values often observed in salmon (Chikwati et al., 2013). Amylase showed values quite similar to those observed in salmon (Froystad et al., 2006). The species differences observed for these pancreatic enzymes may be related to differences in diet composition between the experiments. However, the high activities of trypsin and lipase in the salmon, may also be a result of the breeding program steady improving growth rate and capacity for feed intake and utilization. The similarity of the observed amylase activities in lumpfish and salmon is remarkable, as salmon amylase is known to be rather ineffective. The salmon amylase has at least two amino acid sequence modifications greatly reducing its specific activity, compared, for example, to the amylase of rainbow trout (*Oncorhynchus mykiss*) which shows values 10 times higher than observed for the Atlantic salmon and herein for lumpfish (Froystad et al., 2006). Our results suggest that similar limitations as in the Atlantic salmon may be present also in the amylase of the lumpfish. Further evidence supporting this is that the starch digestibility decreased to around 50% when dietary starch level increased to 16%, indicating the starch was not efficiently digested and absorbed at levels above 6%.

The decreasing digestibility of starch with increasing dietary inclusion level implicates that the variation in available

carbohydrates of the diets was much less than the variation in analyzed level of carbohydrates, in both the E1 and E2 experiment. Variation in analyzed carbohydrates in E1 was from 6 to 18%, whereas available carbohydrate, based on estimates from E2, varied from about 5.5 to 8.1% (almost 30%). This means that the quantitative aspects of effects of analyzed and available carbohydrate levels on the relationships between diet composition and the observed biomarkers might differ substantially. For protein and lipid, the analyzed and available levels differed much less. The impact of variation in these nutrients on the various observed biomarkers would therefore be expected to be quite similar whether based on chemically analyzed values or as levels of digestible nutrients.

Although the amylase activity was not affected by dietary starch, it seemed that the disaccharidase activities were induced at the transcriptional level (*si* expression) as well as on the enzymatic level (maltase specific activity and capacity) in MI, shown as slight positive relationship with carbohydrate level. Similar relationships have been observed also in Atlantic salmon and rainbow trout (Krogdahl et al., 2004). The mechanisms underlying such effects cannot, however, be found based on the available results in the present study. These effects may be related to the increased amount of digested starch (5.6, 6.6 and 8.1 g/100g diet in E2) when dietary starch increased as the digested starch provided substrates, i.e., maltose, which may have induced disaccharidase expression and activity. Besides this, bacteria inhabiting the intestine of fish and the metabolites produced by these, may influence host intestinal metabolism and homeostasis, i.e. macronutrient and cholesterol metabolism (Le Roy et al., 2019), as well as immune regulations (Zhang et al., 2017). Therefore, the undigested starch remaining in the lumen might influence intestinal metabolism and immune regulations in lumpfish by altering intestinal microbiota composition. This suggestion is in line the results recently reported from experiments with the pompano (*Trachinotus ovatus)* (Zhao et al., 2020) and largemouth bass (*Micropterus salmoides)* (Zhang et al., 2020). Gut microbiota were not investigated in the present study but deserves attention in further studies. All in all, it can be concluded that the diet for lumpfish should not contain high level of dietary starch, as this will not be utilized by the fish, and instead increase environmental pollution, and may also harm production and health of the lumpfish.

4.2 Lipid Accumulation in the Lumpfish Intestine

The diet effects observed for OSI of PI and MI in E1, with a positive, linear relationship with dietary lipid level, indicate increased lipid accumulation in the intestinal tissue, and were confirmed by the histologically apparent elevation of the number of vacuoles in enterocytes of PI and MI. These changes may result from either excessive dietary lipid content, or deficiency of substances essential for lipid transport and metabolism. Enterocyte lipid accumulation has been observed in studies of Atlantic cod (*Gadus morhua*) (Kjaer et al., 2009), pikeperch (*Sander lucioperca*) (Kowalska et al., 2011) and Atlantic salmon (Penn, 2011) in which lipid accumulation was observed as an increase in the presence of lipid droplet in enterocytes of fish fed high-fat diets. The cause of the lipid accumulation in these fish may be deficient supply of essential nutrients necessary for efficient transport of lipid through the enterocytes. Deficiency of essential fatty acids have been observed to cause lipid accumulation in the enterocytes of Atlantic salmon (Olsen et al., 1999; Olsen et al., 2003; Bou et al., 2017).Choline is another nutrient recently shown to be essential for Atlantic salmon and signs of deficiency comprise similar symptoms (Hansen et al., 2020). Whether deficient supply of essential fatty acids, choline, or some other essential nutrient involved in lipid transport and metabolism are causing the histological alterations observed in the present study, cannot be concluded upon due to lack of knowledge. Great efforts are needed to define nutrient requirement of lumpfish, necessary to secure good health and welfare.

Among the genes investigated in the present work, two are involved in lipid transport, i.e., *slc27a4*, coding for the principal long chain fatty acid transporter (Krammer et al., 2011; Mitchell et al., 2011), and *npc1l1*, a cholesterol transporter and key actor in absorption of cholesterol by enterocytes and hepatocytes (Davis et al., 2004; Betters and Yu, 2010). The independence of expression of *slc27a4* in PI and MI of the lumpfish on dietary lipid and choline level, is in line with results from Atlantic salmon (Hansen et al., 2020). The trend of decreasing expression of *npc1l1* with increasing lipid level and decreasing choline level indicates that this might be a compensatory mechanism related to the increasing dietary cholesterol. This is in line with the results from salmon fed a choline deficient diet (Kortner et al., 2014; Krogdahl et al., 2020).

4.3 Macronutrient Composition Affected Immune Responses in Lumpfish Intestine

The results observed for the genes, *mmp13*, *rela*, *igm* and *cxcl19*, all involved in regulation of immune responses, suggest that the immune functions located in MI are more responsive to macronutrient variation than those located in PI and DI. The decrease in expression of *mmp13* and *igm*, with increasing lipid level indicate responses in the activity of B cells (*igm*), and lymphocyte recruitment (*mmp13*) (Xu et al., 2018). These responses may be related to the increasing level of ω3-PUFAs with increasing dietary lipid level, i.e., increased levels of fish oil. Earlier studies have shown that ω3-PUFAs, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), may inhibit expression of immune relevant genes (Teitelbaum and Walker, 2001; Calder, 2008). Also results of *in vitro* studies have shown suppression of B cells by EPA and DHA (Wang et al., 2011; Yang et al., 2016).

The results which showed effects of carbohydrate level on the intestinal immune regulation, indicated by altered expression of *rela*, *cox2*, *mmp13*, *cxcl19*, and *pcna* in MI, must be interpreted with caution, as the digestibility, and therefore the availability of the starch, decreased greatly with increasing dietary inclusion level. This fact makes interpretation of the results of the four genes *rela*, *cox2*, *cxcl19*, and *pcna* particularly challenging as they showed quadratic responses and with different maxima and minima. Further studies are needed before the results regarding

effects of variation in carbohydrate level can be discussed and concluded upon.

Although certain effects of diet composition on immune related genes were observed, the histological observations did not show important symptoms of inflammation. The diet effects on expression of immune genes can therefore merely be reflections of normal adjustments to variation in diet composition. It is also possible, however, that they indicate altered resistance to pathogens such as bacteria or virus, for the better or worse. Challenge tests should therefore be conducted to clarify such possible effects.

4.4 Indication of Optimum Balance Between the Dietary Macronutrients

To get an overview of the present results, they are summarized in **Table 5** with indication of the nutrient level (low, medium or high) which gives minimum and maximum for the variables which showed a statistically significant relationship with diet composition and visually apparent relationship with dietary level of macronutrients in **Figures 3–10**. The MI seemed to show the clearest picture, whereas the pictures for DI and the liver were less clear. Based on the results for MI, the maximum results for the biomarkers of digestive functions were observed for diets with medium to high protein level. For the indicators of protein digestion (LAP and *slc15a1*), the highest value was observed at medium protein, low lipid, and high carbohydrate level, for carbohydrate digestion (MALT and *si*) the highest results were found for medium protein, high carbohydrate, and low lipid level, whereas for lipid digestion (lipase) the highest results were observed for diets with high protein, low lipid, and low carbohydrate level. For the biomarkers of immune functions in MI, high values were found at low lipid and carbohydrate levels for most of the biomarkers. The minimum results were found at low to medium level of protein and high level of lipid and carbohydrates. As the activity of lipase (Krogdahl and Sell, 1989) and maltase, as well as expression of a number of immune related genes showed the lowest values at low protein level, the results indicate that the diets with low protein level were protein deficient. It is well known that protein deficiency severely affects the immune functions and disease resistance in animals including humans (Carrillo et al., 2014). It should be kept in mind that the gut mucosa is among the most dynamic tissues in an animal body and has a great capacity to adapt to diet composition (Olli et al., 1994). Therefore, the finding that level of LAP was low at low protein level may reflect low production of peptidases at low dietary protein level rather than protein deficiency, in accordance with the need for efficient protein digestion. The effects of carbohydrate level were less clear regarding indication of minimum and maximum of the biomarkers. This may be related to the fact that the range of available carbohydrates among the diets was rather small, resulting in less clear relationships. Based on the present results, it is not possible to conclude firmly regarding optimal macronutrient balance in lumpfish diets.

As mentioned in the introduction, our previously published results from E1, indicated that peak performance of lumpfish weighing between 15 and 50g, based on growth, body composition, welfare score and immune responses in isolated head kidney cells, would be obtained with a diet containing 55% protein, minimum 17% lipid and maximum 6% carbohydrate (Hamre et al., 2022). The present results, showing very low starch digestibility, suggest that dietary starch level should be limited to about 6%. The intestinal immune related biomarkers showed a linear relationship with the dietary protein/lipid ratio at low carbohydrate, with the highest response at maximum protein level. Since it is not certain if strong immune responses in unchallenged tissues is positive or negative for the fish, a recommendation on the optimal macronutrient composition cannot be given based on the present results regarding immune function. However, according to Hamre et al. (2022), head kidney cells from fish fed the diet with the maximum level of protein, showed a suboptimal immune response when challenged with lipopolysaccharide (LPS). Fish fed the diet with the highest growth (55, 17, 6) had a normal immune response. In line with this, the present results do not suggest alteration in the conclusion presented in our previous paper, i.e., that for peak performance a diet with high lipid, minimum carbohydrate and medium protein is optimal for lumpfish of 10-50g.

5 CONCLUSIONS

The current study improved knowledge regarding responses to dietary macronutrient composition in lumpfish intestine *via* a three-component mixture design. The intestinal functions were broadly impacted by increasing lipid level in the diet with corresponding decrease in protein level, including digestion of polypeptides, maltose and lipid, absorption of fatty acids and peptides, and immune regulations that involve *rela*, *igm* and *cxcl19* and *mmp13*. Elevated lipid digestibility by dietary lipid level was also observed. Although alteration by dietary carbohydrate to protein level was observed on multiple parameters, such as the expression of *igm* and *mmp13*, we should be careful considering the influence of carbohydrate variation because of low starch digestibility when dietary starch level was above 6%. In all, a diet of 55% protein, 17% lipid and 6% carbohydrate is recommended for lumpfish.

Among the many questions to which answers are urgently needed for the feed industry to be able to produce nutritionally well-balanced feeds for lumpfish, the present study answers only a few. Further information is urgently needed and should be addressed in future research. Examples of questions which need answers regard optimal inclusion of available macronutrients, vitamin and minerals, for growth, function as lice cleaners and, not at least, immune functions and disease resistance.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Norwegian Food Safety Authority.

AUTHOR CONTRIBUTIONS

WZ analyzed samples and data processing, and drafting and completing of the manuscript. ÅK and TK overviewed the analyses, joined the data processing and writing of the manuscript. KK, KH, ØS, IL and GB participated in planning of experimental design and feed formulation. KK was in charge of feed production. GB was in charge of the feeding trial. EC performed histological analysis and related statistics. KH conducted statistics related to the mixture design. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.889778/full#supplementary-material

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PAPER IV

Effects of dietary lipid level on growth, digestive physiology and disease resistance in lumpfish (*Cyclopterus lumpus*)

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Abstract

Lumpfish (*Cyclopterus lumpus*) aquaculture has expanded greatly in recent years due to demands for sea lice cleanerfish from the salmon industry. There are knowledge gaps in lumpfish digestive physiology, nutrient requirement and implications of nutrition for health and disease susceptibility. The present study, conducted to follow up our recent screening trials for estimation of optimal balance of protein, lipid and carbohydrate in diets for lumpfish, involved challenging the fish with *Aeromonas salmonicida* after a feeding period with diets varying in lipid composition. Three experimental diets were formulated to have similar content of crude protein and ash but varying in content of lipid/carbohydrates from 6.7 / 18 – 18 / 8.1 %. Lumpfish with average body weight at start of 1.7 ± 0.03 g were fed the experimental diets in triplicate tanks each (110 fish per tank, in total 990 fish in 9 tanks) for a period of 90 days. After termination of the feeding trial and subsequent collection of biological samples, remaining fish were challenged with atypical *A. salmonicida.* No significant effects of diet were observed for growth performance. Carcass composition showed increasing content of lipid, protein, and energy with increasing dietary lipid level. Increasing dietary lipid also increased hepatic dry matter, lipid and energy levels, while crude protein decreased. Blood plasma nutrient levels and biomarkers of liver function showed few significant effects of diet, but dietary lipid level increased plasma cholesterol. Intestinal trypsin activity increased with increasing dietary lipid, whereas activity of other digestive enzymes and digesta bile salt levels were unaffected by diet. Increasing lipid level also increased lipid accumulation in the proximal and mid intestine. Expression profiling of genes related to digestive and immune function showed few effects of diet, but the nutrient transporters *fabp2* and *slc15a1*, as well as the immune genes *MHCII, igm*, and *nfkb* showed increases with dietary lipid levels, whereas the cholesterol transporter *npc1l1* was suppressed. Diet composition did not affect the lumpfish' resistance against *A. salmonicida*. To conclude, the variation in macronutrient composition induced modulations in metabolic, digestive and some immune functions. Modulations seemed however to be within normal ranges and did not produce clear compromises in immune responses to bacterial infection.

Keywords: Lumpfish; lipid requirement; growth; intestinal functions; disease resistance

Introduction

The infestation by salmon louse (*Lepeophtheirus salmonis*) is a great challenge in Atlantic salmon production (Forseth, et al., 2017; Kristoffersen, et al., 2017). Lumpfish (*Cyclopterus lumpus*) has become an important species for co-culture with Atlantic salmon because of its capacity to remove sea lice, offering a biological alternative to chemicals and mechanical solutions (Stien, et al., 2020). Initially, wild caught lumpfish were used. However, wild stocks are limited and may carry pathogens causing diseases, e.g., amoebic gill disease (Haugland, et al., 2017), in the lumpfish as well as in the salmon they are meant to protect. Cultivated lumpfish have therefore become the most commonly used, and at present, lumpfish production is the third largest fish production in Norway with an annual production number of about 30 million individual fish (Stien, et al., 2020). However, for successful cultivation of a new species, a solid knowledge basis regarding key physiological and health related aspects, and not in the least, nutrient supply required for normal function and disease resistance must be built. Regarding lumpfish, knowledge on nutrient requirement has substantial holes, and knowledge from other fish species cannot be expected to be relevant, at least not for a species with very different body composition than most fish for which nutrient requirements have been described so far (Council, 2011; Willora, et al., 2021). It is highly likely that high mortality and prevalence of health issues such as cataract in lumpfish co-cultured with salmon (Stien, et al., 2020; Garcia de Leaniz, et al., 2021; Willora, et al., 2021), at least partly, are due to deficient and/or an imbalanced supply of essential nutrients, as shown for other fish species for vitamin A (Fernandez, et al., 2015), vitamin D (Miao, et al., 2015), vitamin E (Lim, et al., 2009; Lim, et al., 2010) and vitamin C (Delavari, et al., 2021). We recently published findings from the first study to estimate optimal balance between macronutrients in diets for lumpfish (Hamre, et al., 2022). Obtained results indicated that, based on observation of effects on growth rate and immune function biomarkers, the diet for lumpfish weighing 10-50 g should contain approximately 55% protein, minimum 10% lipid, and maximum 10% carbohydrate. In a follow-up study, we proposed that, due to a very low capacity in lumpfish for starch digestion, optimal carbohydrate level should be below 10% (Zhou, et al., 2022).

Mortality is high in lumpfish production and use, often due to infection with atypical *Aeromonas salmonicida* (Ronneseth, et al., 2017; Kverme, et al., 2022). The close relationship between nutrition, immune function, and disease resistance, is well documented for humans and for most other production animals than fish (Maggini, et al., 2018). As nutrient requirement for optimal growth and optimal disease resistance is not necessarily the same (Wen, et al., 2009; Wen, et al., 2010), knowledge on both is necessary for formulation of optimal diets. Moreover, too high supply of certain nutrients may also be detrimental and should be investigated (Guo, et al., 2017; Guo, et al., 2018). Accordingly, disease resistance should also be a criterion for estimation of nutrient

requirement over a wide range of dietary inclusion levels, but no such information is available in the scientific literature for lumpfish. Therefore, the aim of the present work was to generate knowledge addressing effects of lipid level in the diet on disease resistance in lumpfish by challenging the fish with *A. salmonicida* after a feeding period with diets varying in lipid level. The hypothesis was that high lipid level affects disease resistance, since high lipid level in diet was found to cause low expression of immune-relevant genes in our previous study (Zhou, et al., 2022).

Materials and methods

Diets

Three experimental diets were produced at Nofima's Aquafeed Technology Center in Bergen, Norway. The diets (Table 1) were formulated to have similar content of digestible protein and carbohydrate, and to vary only in level of digestible lipid. The varying level of lipid, 6.7, 13.7 and 18%, were chosen based on results regarding nutrient digestibilities from our previous studies on lumpfish (Hamre, et al., 2022; Zhou, et al., 2022). The particle sizes were adjusted accordingly to suit the fish size. During the challenge test a commercial diet (Lumpfish Grower, BioMar, 2.0 mm) was used.

Experimental design

The trial was conducted according to the Norwegian experimental animal regulations and was approved by The Norwegian Food Safety Authority (FOTS ID 22076). The trial was comprised of two parts, a feeding period carried out at Nofima's research facility at Sunndalsøra and a challenge test conducted at the Aquaculture Research Station in Tromsø. At the start of the experiment, fish average initial weight was 1.7 ± 0.03 g. The fish were distributed to 150-liter tanks, 110 fish per tank, and triplicate tanks per diet. The tanks were flat bottom with black walls, equipped with a flow-through system with seawater filtered to 10 µm and UV treated. Temperature and salinity were measured daily, while oxygen was measured twice a week. Temperature and salinity were measured in tank water while oxygen was measured in water from the outlet. The mean temperature in the tanks was 11.6°C (max 12,4 and min 10,9°C) and the mean salinity was 31.7 ppt (max 32.6 and min. 30.7 ppt). The oxygen varied from 79-96% but was adjusted daily with the aim to keep the saturation between 80 and 100%. Each tank was equipped with a LED light dimmed to 4 % and fish were kept at 24 h light regime. The tanks were equipped with automatic belt feeders. Feed was distributed continuously, according to appetite. Feeding level was set according to growth tables and adjusted

visually to have some overfeeding to ensure access to feed for all individual fish. Any dead fish were removed daily from the tanks, counted, and weighed. Total duration of the feeding trial was 90 days.

Data recording and sampling

All fish were weighed individually at start of the trial. Thirty fish were scored for welfare characteristics. These 30 fish were thereafter euthanized by an overdose of anesthetic (tricaine mesylate, Finquel vet®(MS-222)) and were frozen for whole body analyses. The fish in each tank were weighed in bulk after 4 and 8 weeks.

At termination of the feeding trial individual weight and length (both with and without caudal fin) were measured for all fish. Six fish per tank were randomly selected, anaesthetized and euthanized with 110 mg/l MS-222, for sample collection. Blood was collected in vacutainers containing lithium heparin from the caudal vein and stored on ice until centrifugation for plasma. Plasma was separated and immediately frozen in liquid nitrogen and stored at -80°C until analysis. After blood withdrawal, fish were opened, the organ package was carefully removed from the abdominal cavity, and the organs separated. Liver and gonads (if present) were weighed. The intestine was cleaned free of mesenteric fat and divided into four segments, namely pyloric ceca (PC), mid intestine 1 (MI1), mid intestine 2 (MI2) and distal intestine (DI). Each segment was opened longitudinally, and the gut content was collected and snap frozen in liquid nitrogen and stored at -80°C before further processing. The cleaned gut segments were weighed before tissue samples were taken for RNA extraction (kept in RNAlater solution, incubated at 4 °C for 24 h and stored at -20 °C), and for histological analysis (fixed in 4% phosphate-buffered formaldehyde solution for 24 h and transferred to 70% ethanol for storage). For each intestinal segment, the weight was recorded. Remaining tissue was put back with the carcass of the fish. Pooled samples per tank, of carcass and of livers, were frozen for later analyses.

Challenge test with atypical A. salmonicida

The remaining fish were transported by truck to the Aquaculture Research Station in Tromsø where a challenge test with atypical *A. salmonicida* was performed. Before the transport to Tromsø, all fish were individually tagged with electronic tags ((Unique 125 kHz, Sokymat tags, RFID solution) and sorted into two identical groups. Each group consisted of 340 fish with a mean body weight of 57 grams. Each of the two groups consisted of a total of 113-114 fish from each of three diets, and the fish were collected from each of three parallel tanks per diet. Upon arrival in Tromsø the two fish groups were transferred to two 1800 l tanks and held under continuous light at 8°C, i.e. the same temperature as in the transport truck at arrival. Acclimatization to 14°C was done over a 7-days

period. The fish started to eat immediately, and only one fish died during the first days after arrival. The fish were fed the same diet (Lumpfish Grower, BioMar, 2.0 mm) at maintenance level during the experimental period. The feeding was stopped for 24 hours before start of the challenge test.

Bacteria cultivation:

Atypical *A. salmonicida* newly passed and re-isolated from lumpfish was used in a bath infection experiment. The isolate originated from a diagnosed outbreak of atypical furunculosis, the disease caused by *A. salmonicida* infection, in lumpfish at a commercial production facility and was received from the Veterinary Institute in Harstad, Norway (2013-70-F-524.2/ A-layer type VI). The bacterial culture which was frozen with glycerol was thawed and transferred to blood agar (Blood agar base, Oxoid, with 5% human blood concentrate and 2% NaCl). The plates were incubated at 18°C for 4 days until bacterial growth was visible. A single colony was transferred to 20 ml Brain Heart Infusion (BHI) medium (Oxoid) as pre-culture and incubated with gentle shaking for 24h at 18°C. OD520nm was measured and 10 ml from the pre-culture per flask was used to inoculate the 200 ml main cultures. After 24 hours growth the OD520 nm was measured before harvesting the bacterial cultures. Titer (cfu/ml) was determined by titration on blood agar. The agar plates were incubated at 18°C for 4 days before counting the colonies.

Infection procedure:

The bath infection experiment was performed at 14°C. The fish density was approximately 57 kg/m^3 during the infection procedure. The infection dose was 10^7 cfu/ml, based on experience from the established challenge model with lumpfish (previously present as conference poster (Johansen, et al., 2017)). The water was oxygenated and monitored during this procedure which lasted for 2 hours. Mortality was recorded daily for 49 days. The experiment was terminated when no dead or moribund fish had been recorded for three days and all remaining fish were euthanised by an overdose of the anaesthetic metacaine (0.6 ml/l, Finquel vet®). Bacterial streaks from the head kidneys of the first moribund fish in each tank were used to confirm successful systemic infection by atypical *A. salmonicida*, the most likely cause of the morbidities and mortalities observed. This was done for 24 fish from both tanks. The plates were incubated at 18°C until colonies were visible.

195 Chemical analyses

196 Feed samples were analyzed for dry matter (105°C, until constant weight), ash (550°C until constant
197 weight), lipid after HCl hydrolysis (Soxtec HT6, Tecator, Höganäs, Sweden) and nitrogen (AOAC
198 2001.11; Kjeltec 8400 Analyzer Unit, Tecator, Höganäs, Sweden), content of carbohydrates was
199 calculated by difference. Carcass and liver samples were analysed for dry matter, ash, lipid, crude
200 protein, methods as described above, and energy (Parr 63000 Bomb calorimeter).
201

202 Blood plasma variables

203 Blood plasma was analyzed for cholesterol, monoglycerides, total triglycerides, glucose, activities of
204 aspartate transaminase (AST) and alanine aminotransferase (ALT) at the Central Laboratory of the
205 Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, according to standard,
206 medical procedures.
207

208 Activity of digestive enzymes and bile salt concentration

209 The intestinal tissue samples were thawed and homogenized (1:20 w/v) in ice-cold 2 mM Tris/50 mM
210 mannitol, pH 7.1, containing phenyl-methyl-sulphonyl fluoride (P-7626, Sigma, Norway) as serine
211 protease inhibitor. Tissue samples from MI1 and MI2 were homogenized together to represent the
212 whole mid intestine (MI). The homogenates were sonicated, aliquoted and stored at -80 °C until
213 analysis.

214 Leucine aminopeptidase (LAP) and maltase were the brush border digestive enzymes assessed. LAP
215 activity was measured by employing the Sigma procedure no. 251, which was also used by Krogdahl
216 et al. (Krogdahl, et al., 2003), using l-leucyl-b-naphthylamide as the substrate. To measure maltase
217 activity, the method described by Dahlqvist (Dahlquist, 1995) was applied, using maltose as
218 substrate.

219 Trypsin activity and total bile salt level were measured in pooled freeze-dried intestinal contents
220 from MI1, MI2 and DI. Trypsin activity was determined colorimetrically as described (Kakade, et al.,
221 1973), using the substrate benzoylarginine p-nitroanilide (Sigma no. B-4875, Sigma Chemical Co., St.
222 Louis, MO) and a curve derived from standardized bovine trypsin solution. Lipase activity was
223 determined as described (Brockman, 1981), using 4-Nitrophenyl myristate (Sigma 70124, Sigma
224 Chemical Co., St. Louis, MO) as substrate with sodium taurocholate hydrate as buffer (Sigma 86339.
225 Lot 0001428479).

226 Bile salt level was determined using the enzyme cycling amplification/Thio – NAD method (Inverness
227 Medical, Cheshire, UK) in the ADVIA®1650 Chemistry System (Siemens Healthcare Diagnostics Inc.) at

the Central Laboratory of the Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås. The assay measure total 3αOH of cholic acid, whereas the reported results indicate the corresponding level of bile salt as taurocholate.

Quantitative real-time PCR

Total RNA was extracted from tissue samples of PC and DI (~20 mg) of all fish using Trizol reagent (PureLink™ RNA Mini Kit, Thermo Fisher Scientific). RNA was purified by an on-column DNase kit (PureLink™ DNase Set, Thermo Fisher Scientific) according to the manufacturer's protocol. RNA purity and concentration were measured using the Epoch Microplate Spectrophotometer (BioTeK Instruments, Winooski, USA). The RNA integrity was verified by the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) in combination with RNA Nano Chip (Agilent Technologies, Santa Clara, CA, USA). First-strand complementary DNA (cDNA) was synthesized using 1.0 µg RNA from two fish of the same tank, namely 0.5 µg RNA of each two fish were combined as a unit of RNA sample for cDNA synthesis. The reaction volume was 20 µl, including 4 µl mastermix of the kit SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific). Negative controls were performed in parallel by omitting RNA or enzyme.

Primer information are shown in supplementary table S1. Lumpfish mRNA sequences were derived from NCBI database. The selected genes cover the functions of disaccharide digestion (*si*), nutrient transport *(slc27a4*, *slc15a1*, *npc1l1*), ion-exchange (*slc12a1*), tight junction forming (*tjp1*, *occludin*), immune regulation (*cox2*, *igm*, *ikbkb*, *c5*, *cxcl19*, *tnfa*, *nfkb*, *rela*, *il1b*, *MHCII*, *mmp13*) and cell proliferation (*pcna*). The qPCR primers were previously designed and used in our earlier published study.

Expression of target genes were analyzed using the LightCycler 96 (Roche Diagnostics, Basel, Switzerland) with a 10-µl DNA amplification reaction. Each 10-µl DNA amplification reaction contained 2 µl PCR grade water, 2 µl of 1:10 diluted cDNA template, 5 µl LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) and 0.5 µl (10 µM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no-template control. The three-step qPCR run included an enzyme activation step at 95°C (5 min), 40 cycles at 95°C (10 s), annealing temperature (10 s), and 72°C (15 s) and a melting curve step. The mean normalized expression of the target genes was calculated from raw Cq values (Muller, et al., 2002). Reference genes selected was done in terms of its stability among different fish (Kortner, et al., 2011). The chosen reference genes are hypoxanthine phosphoribosyltransferase 1(*hprt1*), actin beta (*bactin*) and elongation factor 1 alpha (*ef1a*) for PC, and elongation factor 1 alpha (*ef1a*), actin beta (*bactin*) and tubulin beta chain (*tubb*) for DI.

Histological examination

The samples fixed for histological evaluation from all intestinal segments were processed using standard histological methods and stained with haematoxylin and eosin (H&E). The histological sections were evaluated to describe the general structure as well as observing for histological alterations associated with inflammatory reaction in the intestinal mucosa. The evaluated morphological characteristics included cellularity of the submucosa and lamina propria, enterocyte supranuclear vacuolization, and intra-epithelial lymphocytes. The degree of changes was graded as normal, mild, moderate, marked, or severe. The morphological assessment was guided by our experience with salmonid intestinal histopathology (Penn, et al., 2011) as well as an examination of the histomorphology of the intestine in lumpfish (Zhou, et al., 2022).

Calculations and statistical methods

Specific growth rate (%BW d^{-1}); SGR = $(\ln W_2 - \ln W_1) * (t_2 - t_1)^{-1} * 100$

Thermal growth coefficient; TGC= $(W_2^{1/3} - W_1^{1/3})/((t_2 - t_1) \times T)^{-1} * 1000$

where W_1 and W_2 are body weights (g) at time (days) t_1 and t_2, respectively, and T is average water temperature over the test period.

Mortality per tank (%): total number of dead fish/initial number of fish *100.

Hepatosomatic index (HIS, %): liver weight/whole body weight * 100.

Organosomatic indices of the intestinal sections (%) = tissue weight/whole body weight * 100.

Results recorded on group basis (mean per tank) were statistically tested by a one-way ANOVA, assessing the effect of diet, and using Duncan's multiple range test to rank the dietary treatments. For individual measured parameters (weight and length) from the final sampling, a mixed model was used, with diet as a fixed variable and tank as a random variable, using tank within diet as error term for testing.

Gut-related results and blood plasma results: Data were processed using R (version 3.5.2, 2018) in the integrated development environment Rstudio (version 1.1.463, 2018). A mixed model was used for testing, with diet as a fixed variable and tank as a random variable, followed by pairwise multiple comparison of estimated marginal means as post hoc analysis. When the singular fit was found, one-way ANOVA followed by Tukey's test as post hoc analysis was applied. Differences were considered significant at $p < 0.05$.

Differences in histological scores for the various evaluated morphological characteristics were analyzed using ordinal logistic regression. When score differences were only 2 levels, statistical significance was assessed using the Fisher exact test. Post hoc analysis for significant test results was

conducted using the Chisq.post.hoc test (Fifer package in R). Differences were considered significant at $p < 0.05$.

Results from the challenge test were evaluated employing the Chi-Squared test.

Results

Growth performance

Results on growth and mortality are shown in Table 2. The lumpfish grew from an average weight of 1.7 g to final weights around 60 g. There was no significant difference in final weight or body length of the fish. Specific growth rates (SGR) averaged 3.9, and thermal growth coefficients 2.8. No significant diet effects were observed. The total body length, with caudal fin included, tended to be lower ($p=0.10$) in fish fed diet L. Mortality during the feeding period was highest (26%) in fish groups fed the diet H, and lowest (20%) in groups fed the diet L. However, the variation within diet was high, and no significant differences were revealed.

Body composition

Significant differences were found in composition of both carcass (Table 3) and liver (Table 4). The carcass showed increasing content of dry matter, lipid, crude protein and energy with increasing content of lipid in the diet. In the liver an increase with increasing lipid level in the diet was seen in dry matter, lipid and energy, while crude protein decreased.

Blood plasma variables

Results for blood plasma biomarkers are illustrated in Fig 1. Cholesterol level was significantly elevated as lipid level increased from 6.7% to 14% but did not change with further increase in lipid level, i.e., from 14 to 18%. Monoglyceride, triglyceride, glucose and activities of AST and ALT did not show significant difference between diet groups. However, triglyceride and activities of AST and ALT tended to show responses to lipid level. Triglyceride level increased as lipid level increased from 6.7% to 14%, and its level slightly decreased when lipid level increased from 14% to 18%. Activities of AST and ALT shared a same trend, i.e., the activity decreased as lipid level increased from 6.7% to 14%, and it remained similar when lipid level increased from 14% to 18%.

Brush border digestive enzyme activities

Results of LAP and maltase specific activity and capacity are shown in Table 5. LAP specific activity was significantly affected by dietary lipid level in MI and DI. In MI, the activity decreased as lipid level increased from 6.7% to 18%. In DI, on the contrary, the activity increased as dietary lipid level

increased from 6.7% to 18%. Although the activity was not significantly influenced in PC, it showed a decreasing trend as lipid level increased from 6.7% to 18%.

Specific activity of maltase showed significant diet effect in MI and DI but not in PC. In MI, the activity decreased when lipid level increased from 6.7% to 18%. In DI, the activity increased when lipid level increased from 6.7% to 13.7% but did not change with further increase in lipid level.

For capacity of LAP and maltase, there was no significant diet effect for any of the intestinal sections.

Trypsin and lipase activities and bile salt level in intestinal content

The results regarding trypsin and lipase activity are shown in Table 6. In MI1 and MI2 trypsin activity showed no significant treatment effects, however the mean value showed increasing trend as lipid level increased from 6.7% to 13.7%. In DI, trypsin activity was the lowest in diet of 6.7% and was increased to the highest as lipid level increased to 13.7% and 18%. Lipase activity did not show significant diet effect in any of the segments. The same trend observed regarding chyme bile salt level, however, a decreasing trend was observed in DI when lipid level increased from 6.7% to 18%.

Gene expression in PC and DI

Table 7 shows the gene expressions measured in PC and DI. In PC, the intracellular fatty acid transporter *fabp2* and the antigen-presenting peptide *MHCII* were the gene transcripts significantly affected by diet, and the response patterns were similar (Fig 2). The expression of *fabp2* significantly increased when lipid level increased from 13.7% to 18% as the mean values were the lowest in L and M group and the highest in H group. The expression of *MHCII* significantly increased when lipid level increased from 6.7% to 18%.

In DI, the cholesterol transporter *npc1l1*, the peptide transporter *slc15a1*, immunglobulin *igm*, and the immune signalling *nfkb* were influenced by diet composition (Fig 3). The expression of *igm* and *nfkb* increased as lipid level increased from 6.7% to 13.7%, and slightly decreased as lipid level reached 18%. The expression of *slc15a1* was increased with increasing lipid level. The expression of *npc1l1*, on the contrary, was decreased with increasing lipid level.

Histopathology

The pyloric caeca showed mild to severe lipid accumulation (steatosis) in the enterocytes (see Fig 4 a and b for representative images). Fig 4c illustrates the differences in enterocyte vacuolization between the groups. Fish from the L group (6.7% lipid) showed 66% prevalence of mild to moderate vacuolization. Fish from the two other diet groups were all scored with mild to severe steatosis, and the 18% lipid group showed the strongest responses. These apparent differences were statistically

verified as indicated by different letters in Fig 4c. None of the observed indicators of inflammation showed significant diet effects (Fig 4d).

Mild to severe steatosis in the enterocytes was also observed in the mid intestine (see Fig 5 a and b for representative images). The pattern for the MI was similar to that for the PC with the least changes observed in L (6.7% lipid) and the most changes for H (18% lipid) (Fig 5c). As for PI, no significant changes in morphological features of mucosal inflammation were observed in the MI (Fig 5d).

The distal intestinal tissue was observed with largely normal and healthy morphology as observed in Fig 6.

Challenge trial

Both external and internal symptoms of the infection are typical for fish suffering from infection with *Aeromonas salmonicida*. However, the evaluation of external signs of disease revealed only a few symptoms on a few of the fish. A few fish had small wounds and erosions around the cartilage peaks on the lateral sides. Likewise, no visible gross changes were observed in the internal organs. As total tank mortality at the end of the experiment did not differ significantly between replicate tanks ($p > 0.05$), the data were pooled. Mortality was 45% for diet L (6.7% lipid), 54% for diet M (13.7% lipid) and 49% for diet H (18% lipid) (Fig 7). The statistical evaluation showed no significant diet effects ($p > 0.05$). However, there was a tendency to lower mortality in the fish group fed the low-fat diet (diet L).

Discussion

The observations made and issues raised in our study which deserve attention in this discussion are the following: a) The nutritional value of the diet indicated by analyses of proximate composition differed importantly from the value indicated by the observed level of digestible nutrients; b) Diet composition did not affect growth performance but changed body composition; c) Increasing dietary lipid level/decreasing carbohydrate level increased chyme trypsin activity along the intestine, but did not affect lipase activity, nor capacity of LAP and maltase in the brush border of MI and DI; d) In MI diet composition affected expression of two of the 21 observed genes, i.e. one involved in lipid transport, the other involved in antigen presentation, whereas in DI four of the genes were significantly affected, i.e. two involved in nutrient transport, and two in immune functions; e) Increasing lipid level/decreasing carbohydrate level increased lipid vacuolization of PC and MI; f) Increasing dietary lipid level/decreasing carbohydrate level increased plasma cholesterol and

decreased plasma AST and ALT activities; g) Diet composition did not affect the lumpfish' resistance against *A. salmonicida.*

Considerations regarding diet composition, growth performance, and body and liver lipid content

The intention to formulate the diets with constant protein level and inversely varying content of lipid and carbohydrates was reached according to the chemical analyses which was made. However, animals sense and respond to the balance in supply of absorbable nutrients, which can be expressed as the balance between digestible protein (DP) and energy (DE). Information on effects of dietary inclusion level on digestibility of macronutrients, generated in a study conducted in parallel to the present study showed great inverse effects for starch, only minor effects for protein and lipid (Zhou, et al., 2022). The implication of this difference is that the diets varied more in protein value than indicated by the proximate composition. Table 8 shows estimated values for digestible macronutrients in the diets as well as estimated DP/DE ratio, which decreased from 32 to 27 g/MJ with increasing lipid level in the diet. In our recent study conducted to find optimal balance between macronutrient in lumpfish diet, the conclusion was that the best result was obtained with 55% protein, minimum 10% lipid and maximum 10% carbohydrate, as measured by proximate analyses (Hamre, et al., 2022). A diet with these nutrient levels would contain, employing the same digestibility values as above, 29 g DP/MJ DE. If lipid level is increased to 18% and carbohydrate reduced to 6%, the ratio would become about 26 g/MJ DE. This indicates that the range of the DP/DE ratio in the present study was within the range suggested to support good performance. The lack of effects of diet composition on growth in the present study is in line with these considerations. The diet effects on body composition, with increase in dry matter, lipid as well as protein with increasing lipid level in the diet, are difficult to explain. In most situations when animals, including humans, are putting on fat, dry matter is increasing whereas protein level decreases (Sarett, et al., 1966). The same was true for lumpfish in an experiment evaluating diets with the same nutrient content but differing nutrient sources, in which also growth performance differed (Sarett, et al., 1966). However, lumpfish has a body composition deviating greatly from that of most other cultivated animals (Willora, et al., 2020; Ageeva, et al., 2021), i.e., with much higher water and hence, much lower protein content. Differences induced by variation in nutrient content of the diet without growth effects, may result in a different picture, than if the variation is due to differences in nutrient sources and growth rate is affected. Further investigations into the variation in body composition of the lumpfish, as well as causes and implications of such variations, is needed for better understanding of the lumpfish physiology.

The observation that liver lipid content increased with increasing dietary lipid level, is in agreement with the results of our previous screening study aiming to find optimal protein, lipid, and carbohydrate level of lumpfish diets (Hamre, et al., 2022). However, in our previous study, with diets containing lipid in the same range as the present, the liver lipid content was lower, ranging from 10 to 25%, compared to the variation in the present study between 30 to 36%. A recent screening of 5200 lumpfish in four Faroese Atlantic salmon farming sites (Eliasen, et al., 2020) showed values between 1.2% to 24.7%. Lumpfish is clearly a fish species which use the liver as a major lipid storage tissue. The observed liver lipid levels in the present study, were well below lipid levels observed in other species with the same characteristic, such as the cod, which may show levels above 80% (Kjaer, et al., 2009; Weil, et al., 2013).

Effects on activity of digestive enzymes

The general understanding of regulation of capacity of digestive enzymes in the intestine is that an increase in content of protein, lipid, or carbohydrate, will induce an increase in activity in the chyme and brush border of the corresponding digestive enzymes (Krogdahl, et al., 2011). However, the present results seem to indicate the opposite regarding trypsin activity in the chyme along the intestine, as the activity increased with decreasing protein level, significantly so for DI with the same trend for MI1 and MI2. However, as digestibility of the macronutrients differs greatly, the composition of the chyme dry matter changes continuously as it passes along the intestine. Information given in Table 8 intends to explain this development in numbers. Even though protein digestibility was high compared to starch digestibility, the proportion of protein in dry matter of the chyme increased along the intestine and was completely dominating towards the end. The explanation for the increasing trypsin activity with increasing lipid content in the diet, may therefore be, that in the chyme, the protein level increased, eliciting signals from the gut to the pancreas for more trypsin. Another consequence of high level of dietary protein in the chyme was, supposedly, decreased inactivation and digestion of the trypsin and other active proteases. As long as there is dietary protein in the gut, autolysis is slow, due to higher affinity of the protases for feed protein than endogenous functional proteins.

Similar considerations can be made regarding chyme lipase activity, which was not affected significantly by diet composition. The high digestibility observed, may indicate that most of the lipid was removed from the chyme in the proximal compartments of the intestine, and that very little lipid entered the more distal compartments. Hence, activity of lipase in the more distal intestinal segments was less affected by lipid level in the diet. Results of studies in other fish species may indicate that under other circumstances, e.g., with other lipid sources, the picture may differ (Infante, Cahu, 1999; Gomez-Requeni, et al., 2013; Li, et al., 2016; Chang, et al., 2018). The high lipid

digestibility observed in the present trial, show that lipase activity was not a limiting factor for efficient lipid digestion.

The decreasing effect of increasing dietary lipid level on specific activity of LAP, i.e. activity per weight of tissue protein, in the brush border in MI and the opposite effect in DI, are difficult to explain. However, total capacity was not significantly affected, indicating that the effects on specific activity was due to alterations in protein level in the tissue rather than enzyme activity. The same consideration is valid for the maltase activity and capacity.

Effects on nutrient transport

Among the genes involved in lipid transport, which were observed in the present study, only *fabp2* in PC showed significant diet effect, increasing with increasing dietary lipid level, as expected. The *fabp2* is highly expressed in the teleost intestine and shows a decreasing expression along the proximal to distal axis. The gene product is involved in transport of long chain fatty acids, saturated as well as unsaturated, across the mucosa (Storch, McDermott, 2009), forwards free fatty acids to different metabolic pathways, and modulate enzyme activity involved in lipid metabolism (Ordovas, 2001). The importance of the FABP2 protein is indicated by its abundance in the enterocyte, which, according to studies in humans, may exceed 3% of the enterocyte's cytoplasmic mass. It is also linked to mitochondrion β-oxidation and cholesterol uptake (Montoudis, et al., 2008).

The signs of lipid accumulation (steatosis) in PC and MI which increased in severity with increasing lipid level in the diet, indicate that the capacity for lipid transport in the mucosa was insufficient for effective transfer from the gut to the systemic circulation. Similar results were seen in our previous digestibility study with similar diets (Ref. submitted manuscript). In Atlantic salmon (Bou, et al., 2017) and Arctic char (Olsen, et al., 2000) steatosis has been associated with deficient supply of long chain ω-3 fatty acids. However, as supply of EPA and DHA was high in the present study, also other possible limiting factors should be considered. As lipid transport in the enterocytes is dependent on supply of choline, choline supply may have been insufficient for efficient transport in the present study. However, choline requirement of lumpfish has not been investigated. According to the diet formulation, choline level in the diets were about 5000 mg/kg, i.e. well above estimates of choline requirement in Atlantic salmon for efficient lipid transport across the intestine (Hansen, et al., 2020b; Hansen, et al., 2020a). Whether or not delayed lipid transport, causing signs of steatosis, is harmful for the fish cannot be concluded upon at present. Choline requirement of lumpfish should however be estimated in future studies, in which also effect on other functions of choline, such as donation of methyl groups in epigenetic processes, which are key elements in activation of immune function (Zeisel, 2017) should be observed.

Effects on cholesterol absorption and metabolism

The diet effect on expression of *npc1l1*, coding for a key transporter for intestinal cholesterol uptake, i.e. reduction with increasing lipid level, is in agreement with results in Atlantic salmon (Kortner, et al., 2014) and mammals (den Bosch, et al., 2008; Alvaro, et al., 2010). This effect was likely a result of high content of n-3 polyunsaturated fatty acids (Alvaro, et al., 2010; Yang, et al., 2018), as fish oil was the ingredient varied for adjusting lipid level. Another explanation would be dietary cholesterol uptake correspondingly increased with the increased uptake of dietary fat, resulting in increased cholesterol levels in the blood. The increased blood cholesterol level thereafter suppressed expression of *npc1l1*, as a manner of reducing continuous increase of cholesterol level (Davis, et al., 2004; Davies, et al., 2005). However, our results differ from the experiment in lumpfish studying macronutrient requirement where plasma cholesterol level and *npc1l1* expression was not influenced (Hamre, et al., 2022; Zhou, et al., 2022). The difference could be caused by different experimental design. Protein, lipid, and carbohydrates were simultaneously varied in the study by Hamre et al. This may also influence other factors regulating cholesterol homeostasis and resulted in unchanged plasma cholesterol and *npc1l1* expression.

Effects on plasma biomarkers

Among the observed plasma biomarkers, all related to nutrient metabolism, cholesterol was the only showing significant effect of diet composition. Similar relationship with dietary lipid content has been observed also in other fish species (Yun, et al., 2011; Kortner, et al., 2014; Yan, et al., 2015). Although the ANOVA did not show significant alteration in triglyceride level, the correlation with cholesterol level was high (Pearson correlation analyses: $p<0.0001$; $r^2=0.78$). This result is as expected as increased lipid absorption increase plasma concentration of lipoproteins in which cholesterol and triglyceride are main components.

The about 50% drop in activity of AST and ALT also deserves attention. These aminotransferases are found in high concentrations in liver tissue, at low concentration in other tissues (Giannini, et al., 2005). Increased activity in plasma may therefore, depending on the magnitude of increase, be a symptom of altered liver function, or disease and malfunction of the liver. However, in humans, alterations in the range >5 times normal range, is considered to indicate moderately challenged liver. If lumpfish, in this regard, respond similarly, the observed decrease in AST and ALT with increasing dietary lipid level/decreasing protein level, indicates a physiologically normal response to changes in diet composition, and not necessarily an improvement in liver function. Excess supply of protein, as

was the case for fish fed the low lipid diet, represents a metabolic burden on the liver related to deamination of amino acids and production of NH_3. The relatively high value for these fish, may be reflecting such alterations in the liver metabolism.

Effects on immune functions and disease resistance

No information on expression of genes involved in immune functions in lumpfish, induced by bath exposure to *A. salmonicida* has been found. However, as rainbow trout infected with this bacterium have shown effects on expression of a wide range of inflammatory, antimicrobial peptide and complement genes similar functions would be expected also in lumpfish, and that the pathogen should be suitable for the present investigation (Castro, et al., 2015). The low number of immune related genes showing effect of diet in the present study, i.e. two out of 11, indicates that immune function was not severely affected by the variation in macronutrient composition. Increasing lipid level induced pyloric caeca expression of *MHCII,* involved in antigen presentation. In the distal intestine induction was observed for *igm*, induced by initial exposure of antigens, and *nfkb,* a molecule characterized as a "rapid-acting" primary transcription factor. These responses indicate modulation by diet composition of processes in adaptive immune functions. However, as no structural or cellular alterations were observed, the response in these two genes was most likely not indicating important immunological effects. The absence of lymphocyte infiltration in intestinal tissues supports this consideration and are in line with results of our previous screening study (submitted mns.). The results of the challenge test with *A. salmonicida*, in which cumulative mortality did not differ significantly between treatments, supports this suggestion. It also indicates that the variation in nutrient composition of the diets did not compromise other important immune functions (Estensoro, et al., 2012; Torrecillas, et al., 2017).

Conclusions

The current study demonstrated effects of variation in dietary lipid level on fatty acid transport, cholesterol transport and metabolism, which macroscopically resulted in intracellular lipid accumulation and elevated level of blood cholesterol. Body and liver composition were also affected by dietary lipid variation, but growth performance was not affected. With variation in dietary lipid as in the present study (6.7% - 18%), we expected to observe immune-relevant changes. However, there was no significant change indicating that fish health was impacted during the normal feeding period, or after challenge by *A. salmonicida*. Lumpfish immune apparatus therefore seem tolerate high lipid diets, but too high lipid levels may result in metabolic disorders.

Data Availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Supplementary materials

The supplementary files including table S1. S1 listed primer information of genes involved in the present study.

Author contributions

WZ analyzed samples and processed the data, drafted and completed the manuscript. ÅK and TK joined the planning of the experiment, overviewed the analyses, and joined the data processing. IL and GB were in charge of the experimental design and feed formulations, and as well as of the feeding trial. EC performed histological analysis and related statistics. LJ was in charge of the challenge trial. All authors contributed to the article and approved the submitted version.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Tables and figures

Table 1. Diet levels of feed ingredients and estimated chemical composition

	L	M	H
Ingredients (g/kg):			
Fish meal[1]	366.2	362.9	387.6
Wheat gluten[2]	183.1	181.5	193.8
Wheat meal[3]	188.0	125.0	40.0
Fish oil[4]	5.0	73.0	120.9
Codfish powder[5]	100.0	100.0	100.0
Krill hydrolysate[6]	20.0	20.0	20.0
Krill meal[7]	40.0	40.0	40.0
Krill oil[8]	10.0	10.0	10.0
Vitamin premix[9]	30.0	30.0	30.0
Organic mineral premix [10]	8.4	8.4	8.4
Mono sodium phosphate[11]	24.0	24.0	24.0
Biomos[12]	4.0	4.0	4.0
Cholesterol[13]	5.0	5.0	5.0
Choline chloride[14]	5.0	5.0	5.0
L-lysine[15]	6.0	6.0	6.0
Taurine[16]	2.0	2.0	2.0
Stay-C[17]	2.2	2.2	2.2
Carphyll pink (10% AX)[18]	1.0	1.0	1.0
Yttrium oxide[19]	0.1	0.1	0.1
Chemical composition:			
Analyzed values, %			
Dry matter	90.5	92	91.4
Protein	54.1	52.7	53.3
Lipid	6.7	13.7	18
Ash	11.2	11.4	11.8
Starch	16	11.7	6.6

L, low lipid diet (6.7% lipid); M, medium lipid diet (11.7% lipid); H, high lipid diet (18% lipid).

[1] Fishmeal, Norse-LT, Vedde AS, Langevåg, Norway.

[2] Wheat gluten, Amytex 100, Tereos Syral, Aalst, Belgium.

[3] Wheat meal, Norgesmøllene AS, Bergen, Norway.

[4] Fish Oil, NorSalmOil, Pelagia, Egersund, Norway.

[5] Codfish powder, Seagarden AS, Avaldsnes, Norway.

[6] Krill 23ydrolysate, Rimfrost AS, Ålesund, Norway.

[7] Krill meal, Rimfrost AS, Ålesund, Norway.

[8] Krill oil, Aker BioMarine, Lysaker, Norway.

[9] Vitamin premix (per kg diet), 0.5% Nofima vitamin premix, Vilomix, Hønefoss, Norway. Vitamin A, 3000 IU; vitamin D3, 3800 IU; vitamin E, 300 mg; vitamin K3, 30 mg; vitamin B1, 30 mg; vitamin B2, 45 mg; vitamin B6, 38 mg; vitamin B12, 0.08 mg; niacin, 300 mg; Ca-D-pantothenate, 90mg; biotin, 1.5 mg; folic acid, 15 mg; vitamin C, 300 mg.

[10] Mineral premix (per kg diet), 0.5 % Nofima mineral premix, Vilomix, Hønefoss, Norway. Fe,60mg; Mn, 30mg; Zn, 130mg; Cu, 6mg; I, 5mg; Co, 0.05mg; Se, 0.3mg.

[11] MSP (26% P), Mono sodiumphosphate, delivered by Vilomix, Hønefoss, Norway.

[12] Biomos, Alltech Norway AS, Førde, Norway.

[13] Cholesterol, Carbogen Amics B.V., Veenendaal, The Netherlands.

[14] Choline chloride, delivered by Vilomix, Hønefoss, Norway.

[15] L-lysine, delivered by Vilomix, Hønefoss, Norway.

[16] Taurine, VWR, Oslo, Norway.

[17] Stay-C 35%, delivered by Vilomix, Hønefoss, Norway.

[18] Carophyll pink (10% astaxanthin), delivered by Vilomix, Hønefoss, Norway.

[19] Yttrium oxide. Y_2O_3 (99.9%), delivered by Vilomix, Hønefoss, Norway.

Table 2. Fish weights (g). length (mm). growth rates and mortality (% of wet basis, mean ± s.e.m., n = 3)

	L	M	H	p-value
Weight, day 0	1.73 ± 0.01	1.73 ± 0.02	1.71 ± 0.01	0.57
Weight, day 90	61 ± 4.2	62 ± 0.9	59 ± 2.6	0.76
Length1, day 90	90 ± 1.3	92 ± 0.4	90 ± 1.5	0.45
Length2, day 90	98 ± 1.3	104 ± 0.9	101 ± 2.2	0.10
SGR. % d^{-1}	3.9 ± 0.09	3.9 ± 0.01	3.9 ± 0.07	0.94
TGC	2.8 ± 0.10	2.9 ± 0.02	2.8 ± 0.07	0.87
Mortality. %	20 ± 2.2	22 ± 4.5	26 ± 2.0	0.4

789 L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%); Length 1, body length
790 without caudal fin; Length 2, total length with caudal fin included.

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Table 3. Chemical content in carcass ((% of wet basis, mean ± s.e.m., n = 3)

	L	M	H	p-value
Dry matter. %	11.3 ± 0.03^{c}	12.2 ± 0.1^{b}	12.9 ± 0.2^{a}	<.0001
Ash. %	1.7 ± 0.03	1.7 ± 0.0	1.7 ± 0.0	0.42
Lipid. %	1.6 ± 0.00^{c}	2.4 ± 0.1^{b}	3.1 ± 0.1^{a}	<.0001
Crude protein. %	7.8 ± 0.03^{b}	8.0 ± 0.1^{ab}	8.1 ± 0.1^{a}	0.04
Energy. MJ kg^{-1}	2.4 ± 0.01^{c}	2.7 ± 0.1^{b}	3.0 ± 0.1^{a}	0.006

792 L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%).

793 Values with different superscript letters within the same row denotes significant difference ($p <$
794 0.05), n = 3.

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800

Table 4. Chemical content in liver (mean ± s.e.m., n = 3)

	L	M	H	p-value
Dry matter. %	44.4 ± 0.3^{c}	46.4 ± 0.2^{b}	49.6 ± 0.1^{a}	<.0001
Ash. %	2.2 ± 0.2	2.3 ± 0.03	2.3 ± 0.1	0.85
Lipid. %	29.7 ± 0.3^{c}	32.7 ± 0.1^{b}	36.4 ± 0.2^{a}	<.0001
Crude protein. %	9.9 ± 0.1^{a}	9.8 ± 0.1^{a}	9.2 ± 0.1^{b}	0.01
Energy. MJ kg^{-1}	14.6 ± 0.1^{c}	15.7 ± 0.04^{b}	17.0 ± 0.1^{a}	<.0001

L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%).

Values with different superscript letters within the same row denotes significant difference ($p < 0.05$).

Table 5 Brush border digestive enzyme capacity (EC) and specific activity (SA) in PC, MI, and DI (mean ± s.e.m., n = 3)

		Diet			
	Tissue	L	M	H	p value
Maltase SA (nmol/min/mg protein)	PC	4.6 ± 0.6	3.8 ± 0.6	7.0 ± 1.1	0.15
	MI	38 ± 3.3a	27 ± 2.7b	22 ± 1.5b	0.03
	DI	44 ± 3.5b	55 ± 2.6a	59 ± 3.3a	< 0.01
Maltase EC (µmol/min/kg fish)	PC	2.1 ± 0.3	2 ± 0.4	4.4 ± 0.7	0.08
	MI	1.9 ± 0.1	1.9 ± 0.1	1.8 ± 0.1	0.97
	DI	4.8 ± 0.3	5.2 ± 0.2	5.2 ± 0.3	0.78
LAP SA (µmol/h/mg protein)	PC	100 ± 5	92 ± 4.8	82 ± 5.4	0.31
	MI	268 ± 24^{a}	209 ± 14^{ab}	171 ± 12^{b}	0.05
	DI	518 ± 43^{b}	545 ± 30^{ab}	613 ± 28^{a}	0.04
LAP EC (mmol/h/kg fish)	PC	46 ± 1.7	48 ± 2.7	50 ± 2.7	0.52
	MI	13.1± 0.7	15.2 ± 0.8	14.5 ± 0.7	0.13
	DI	56 ± 2.5	51 ± 2.8	54 ± 2.4	0.78

L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%).
Values with different superscript letters within the same row denotes significant difference ($p < 0.05$).

Table 6 Activity of trypsin and lipase and bile salt level in MI1. MI2 and DI (mean ± s.e.m., n = 3)

		Diet			
	Section	L	M	H	p value
Trypsin activity (U/mg dry matter)	MI1	7.6 ± 1.5	16.1 ± 0.9	16.5 ± 4.2	0.09
	MI2	5 ± 1.8	13.9 ± 3.4	16.2 ± 3.7	0.09
	DI	6.5 ± 1.4[b]	21.9 ± 1.6[a]	25.3 ± 2.1[a]	< 0.01
Lipase activity (U/mg dry matter)	MI1	1.7 ± 0	1.7 ± 0.3	1.6 ± 0.1	0.85
	MI2	1.6 ± 0.2	1.5 ± 0.2	1.4 ± 0.3	0.78
	DI	0.4 ± 0	0.4 ± 0.1	0.6 ± 0.1	NA*
Bile salt level (mg/g dry matter)	MI1	64.4 ± 11.7	72.5 ± 8	73.9 ± 3.9	0.71
	MI2	52.8 ± 7.9	50.4 ± 5.2	46.1 ± 3.2	0.72
	DI	6.8 ± 0.9	4.1 ± 1.3	2.6 ± 0.5	0.06

* The gut content of L group from DI was merely enough for analysis in one tank. The significant test was therefore not applicable. L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%). Values with different superscript letters within the same row denotes significant difference ($p < 0.05$).

Table 7 Relative gene expression in PC and DI (mean ± s.e.m., n = 3)

Gene category	Gene symbol	PC				DI			
		L	M	H	p value	L	M	H	p value
Nutrient transport	***slc27a4***	$1.8 \pm 0.1 \times 10^{-1}$	$1.8 \pm 0.2 \times 10^{-1}$	$1.8 \pm 0.1 \times 10^{-1}$	0.88	$4.0 \pm 0.1 \times 10^{-2}$	$4.1 \pm 0.2 \times 10^{-2}$	$4.0 \pm 0.09 \times 10^{-2}$	0.81
	cd36	$2.5 \pm 0.2 \times 10^{-1}$	$2.7 \pm 0.1 \times 10^{-1}$	$2.5 \pm 0.2 \times 10^{-1}$	0.66	$0.5 \pm 0.1 \times 10^{-3}$	$1.5 \pm 0.7 \times 10^{-3}$	$0.6 \pm 0.1 \times 10^{-3}$	0.59
	fabp2	2.7 ± 0.1 **b**	2.9 ± 0.1 **b**	3.6 ± 0.2 **a**	< 0.01	$5 \pm 0.6 \times 10^{-2}$	$6.1 \pm 1 \times 10^{-2}$	$6.5 \pm 1 \times 10^{-2}$	0.54
	cav1	$4.1 \pm 0.3 \times 10^{-2}$	$4.4 \pm 0.2 \times 10^{-2}$	$4.2 \pm 0.2 \times 10^{-2}$	0.72	$2.4 \pm 0.1 \times 10^{-2}$	$2.7 \pm 0.2 \times 10^{-2}$	$2.7 \pm 0.07 \times 10^{-2}$	0.08
	npc1l1	$2.4 \pm 0.1 \times 10^{-1}$	$2.2 \pm 0.2 \times 10^{-1}$	$2.1 \pm 0.1 \times 10^{-1}$	0.27	$6.9 \pm 0.4 \times 10^{-2}$**a**	$5.2 \pm 0.4 \times 10^{-2}$**b**	$4.9 \pm 0.4 \times 10^{-2}$**b**	0.03
	slc15a1	$1.7 \pm 0.1 \times 10^{-1}$	$1.6 \pm 0.07 \times 10^{-1}$	$1.6 \pm 0.09 \times 10^{-1}$	0.29	$3.2 \pm 0.1 \times 10$-^{2}**b**	$4 \pm 0.2 \times 10^{-2}$**a**	$4.5 \pm 0.3 \times 10^{-2}$**a**	< 0.01
Disaccharide digestion	***si***	$4.3 \pm 0.2 \times 10^{-1}$	$4.0 \pm 0.1 \times 10^{-1}$	$3.9 \pm 0.1 \times 10^{-1}$	0.3	$2.9 \pm 0.08 \times 10^{-1}$	$3.1 \pm 0.1 \times 10^{-1}$	$2.9 \pm 0.1 \times 10^{-1}$	0.43
Ion exchange	***slc12a1***	$3.0 \pm 0.2 \times 10^{-1}$	$3.0 \pm 0.3 \times 10^{-1}$	$2.6 \pm 0.2 \times 10^{-1}$	0.34	$6.7 \pm 0.5 \times 10^{-2}$	$6.3 \pm 0.5 \times 10^{-2}$	$5.7 \pm 0.4 \times 10^{-2}$	0.33
Tight junction proteins	***occludin***	$1.7 \pm 0.09 \times 10^{-2}$	$1.8 \pm 0.08 \times 10^{-2}$	$1.9 \pm 0.09 \times 10^{-2}$	0.42	$7.9 \pm 0.1 \times 10^{-3}$	$8.1 \pm 0.3 \times 10^{-3}$	$7.3 \pm 0.2 \times 10^{-3}$	0.08
	tjp1	$7.5 \pm 0.4 \times 10^{-2}$	$7.9 \pm 0.5 \times 10^{-2}$	$7.4 \pm 0.4 \times 10^{-2}$	0.64	$2.1 \pm 0.1 \times 10^{-2}$	$2 \pm 0.08 \times 10^{-2}$	$2.0 \pm 0.07 \times 10^{-2}$	0.58
Immune regulation	***cox2***	$5.4 \pm 0.3 \times 10^{-3}$	$5.2 \pm 0.3 \times 10^{-3}$	$5.6 \pm 0.4 \times 10^{-3}$	0.76	$2.2 \pm 0.1 \times 10^{-3}$	$2.4 \pm 0.2 \times 10^{-3}$	$2.4 \pm 0.1 \times 10^{-3}$	0.47
	igm	$1.1 \pm 0.2 \times 10^{-3}$	$0.9 \pm 0.1 \times 10^{-3}$	$0.9 \pm 0.07 \times 10^{-3}$	0.53	$3.3 \pm 0.3 \times 10^{-4}$**b**	$4.9 \pm 0.5 \times 10^{-4}$**a**	$4.3 \pm 0.3 \times 10^{-4}$**ab**	0.03
	rela	$4.0 \pm 0.08 \times 10^{-1}$	$3.9 \pm 0.1 \times 10^{-1}$	$3.8 \pm 0.1 \times 10^{-1}$	0.5	$1.3 \pm 0.04 \times 10^{-1}$	$1.3 \pm 0.04 \times 10^{-1}$	$1.3 \pm 0.04 \times 10^{-1}$	0.69
	ikbkb	$8.1 \pm 0.5 \times 10^{-2}$	$6.9 \pm 0.2 \times 10^{-2}$	$7.3 \pm 0.2 \times 10^{-2}$	0.14	$4.2 \pm 0.1 \times 10^{-2}$	$4.3 \pm 0.2 \times 10^{-2}$	$3.9 \pm 0.2 \times 10^{-2}$	0.26
	nfkb	$1.7 \pm 0.03 \times 10^{-1}$	$1.6 \pm 0.03 \times 10^{-1}$	$1.6 \pm 0.06 \times 10^{-1}$	0.4	$5.2 \pm 0.09 \times 10^{-2}$**b**	$5.8 \pm 0.2 \times 10^{-2}$**a**	$5.5 \pm 0.2 \times 10^{-2}$**ab**	0.02
	c5	$1.3 \pm 0.2 \times 10^{-2}$	$1.1 \pm 0.1 \times 10^{-2}$	$1.3 \pm 0.2 \times 10^{-2}$	0.69	$2.6 \pm 0.4 \times 10^{-3}$	$2.3 \pm 0.2 \times 10^{-3}$	$2.2 \pm 0.3 \times 10^{-3}$	0.78
	cxcl19	$2.8 \pm 0.3 \times 10^{-3}$	$2.6 \pm 0.3 \times 10^{-3}$	$2.3 \pm 0.1 \times 10^{-3}$	0.58	$7.8 \pm 1.2 \times 10^{-4}$	$7.7 \pm 1.5 \times 10^{-4}$	$9.9 \pm 1.9 \times 10^{-4}$	0.65
	tnfa	$4.8 \pm 0.2 \times 10^{-3}$	$4.5 \pm 0.3 \times 10^{-3}$	$3.7 \pm 0.3 \times 10^{-3}$	0.17	$8.0 \pm 0.7 \times 10^{-4}$	$1.3 \pm 0.06 \times 10^{-4}$	$1.1 \pm 0.1 \times 10^{-4}$	0.09
	MHCII	$2.6 \pm 0.1 \times 10^{-1}$**b**	$3 \pm 0.2 \times 10^{-1}$**ab**	$3.5 \pm 0.2 \times 10^{-1}$**a**	0.02	$5.2 \pm 0.3 \times 10^{-2}$	$6.9 \pm 0.4 \times 10^{-2}$	$6.4 \pm 0.3 \times 10^{-2}$	0.07
	mmp13	$3.8 \pm 0.3 \times 10^{-2}$	$3.4 \pm 0.3 \times 10^{-2}$	$3.6 \pm 0.2 \times 10^{-2}$	0.81	$6.7 \pm 0.4 \times 10^{-3}$	$7.7 \pm 0.7 \times 10^{-3}$	$8.7 \pm 1 \times 10^{-3}$	0.49
Cell proliferation	***pcna***	$2.9 \pm 0.06 \times 10^{-1}$	$3.1 \pm 0.07 \times 10^{-1}$	$3.1 \pm 0.08 \times 10^{-1}$	0.14	$1.1 \pm 0.02 \times 10^{-1}$	$1.2 \pm 0.03 \times 10^{-1}$	$1.2 \pm 0.04 \times 10^{-1}$	0.16

L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%). Values with different superscript letters in bold within the same row denotes significant difference ($p < 0.05$).

Table 8. Estimates for nutrient digestibility, content of digestible nutrient in the diets, and level of macronutrients in the faeces[1]

Diet	L	M	H
Digestibility, %[1]			
Protein	84	82	83
Lipid	93	96	96
Starch	50	57	84
Dry matter	59	64	68
Level of digestible nutrient[1]*:*			
Protein (DP), %	45	43	44
Lipid, %	6	11	15
Carbohydrates, %	7	8	9
Digestible energy (DE), MJ/kg	15	17	18
DP/DE, g/MJ	31	26	24
Proportion of macronutrients in faeces DM, % of sum			
Crude protein	52	63	85
Carbohydrates	46	34	9
Lipid	2.4	3.3	5.6

[1]Estimated employing results from a previous experiment with diets based on the same formulation (Zhou, et al., 2022). L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%).

Figures

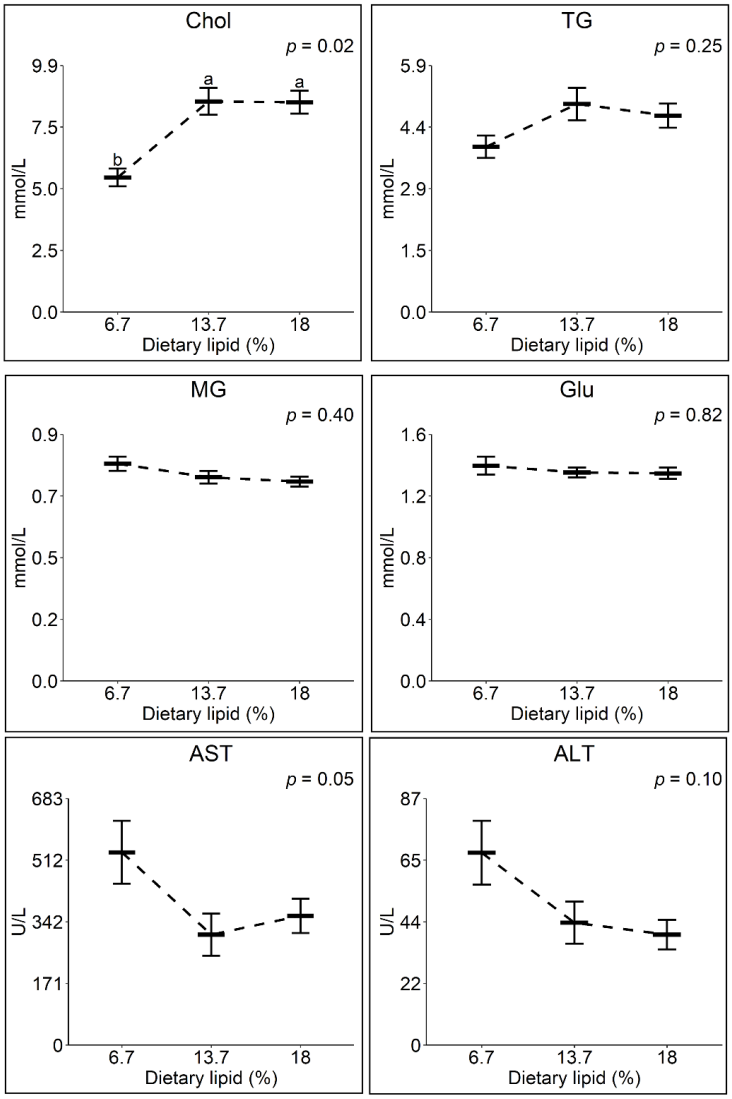

Fig 1. Effects of dietary lipid variation (%) on blood plasma variables. Mean values are denoted as horizontal bars. Error bars indicates standard error. Dashed lines connecting means between groups are illustrated for understanding changing trends, without any statistical meanings.

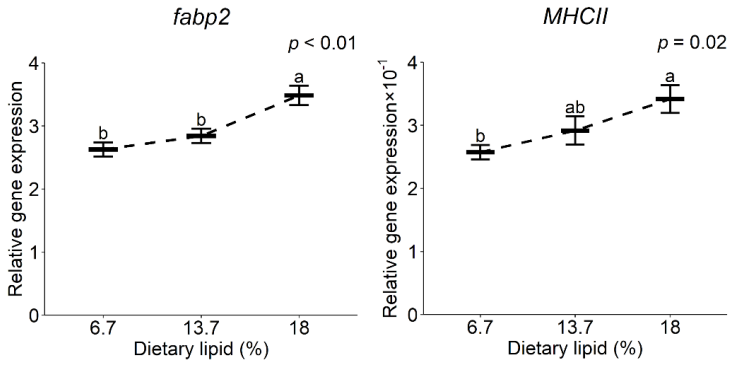

Fig 2. Genes showing significant responses in PC. *fabp2.* fatty acid binding protein 2; *MHCII*. major histocompatibility complex class II. Mean values are denoted as horizontal bars. Error bars indicates standard error. Dashed lines connecting means between groups are illustrated for understanding changing trends, without any statistical meanings.

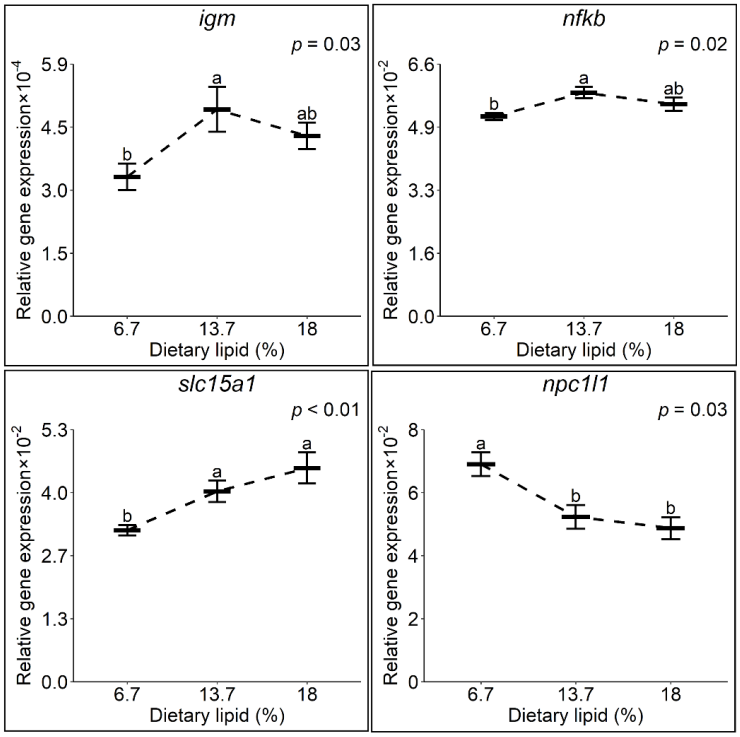

Fig 3. Genes showing significant responses in DI. *igm:* immunoglobulin M; *nfkb*: nuclear factor kappa-light-chain-enhancer of activated B cells; *slc15a1*: peptide transporter 1; *npc1l1*: Niemann-Pick C1-Like 1. Mean values are denoted as horizontal bars. Error bars indicates standard error. Dashed lines connecting means between groups are illustrated for understanding changing trends, without any statistical meanings.

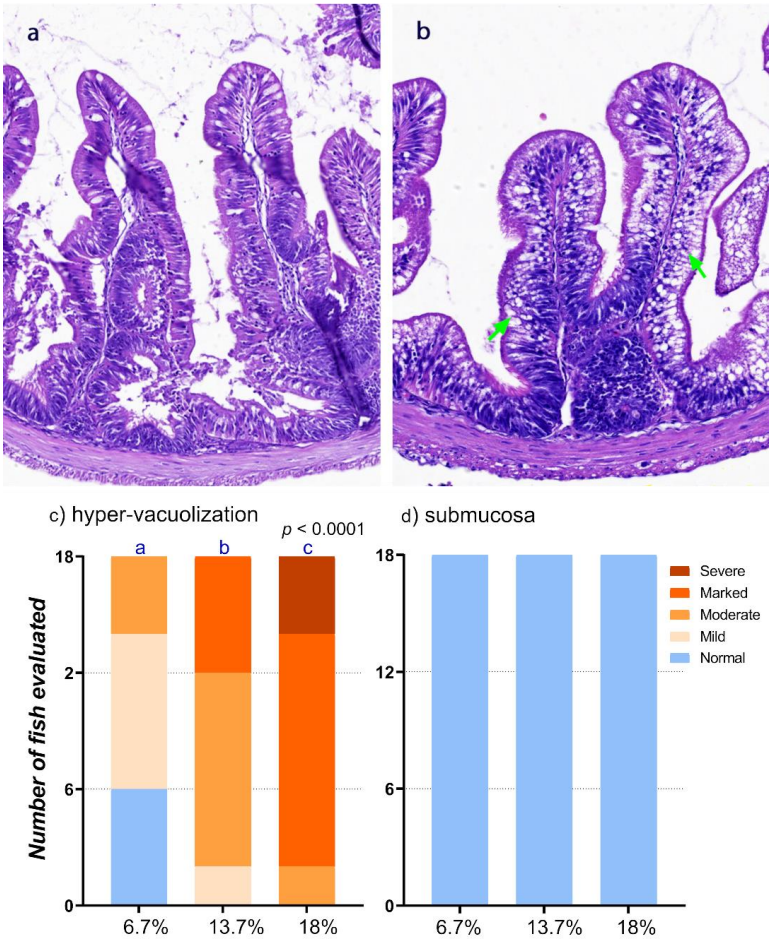

Fig 4. Representative images of the pyloric caeca sections that were scored as a) normal and healthy and b) showing marked lipid accumulation (steatosis) appearing as vacuoles (green arrow) above the enterocyte nucleus; and the number of pyloric caeca tissue sections that were scored for c) enterocyte steatosis or hyper-vacuolization and d) increases in the width and inflammatory cell infiltration in the submucosa. The horizontal axis denotes dietary lipid in diet. P-values represent outcomes of an ordinal logistic regression for differences in the distribution of the histological scores between the diet groups. Columns not sharing a letter label are statistically different from the group 0 (reference group in the ordinal logistic regression analysis conducted).

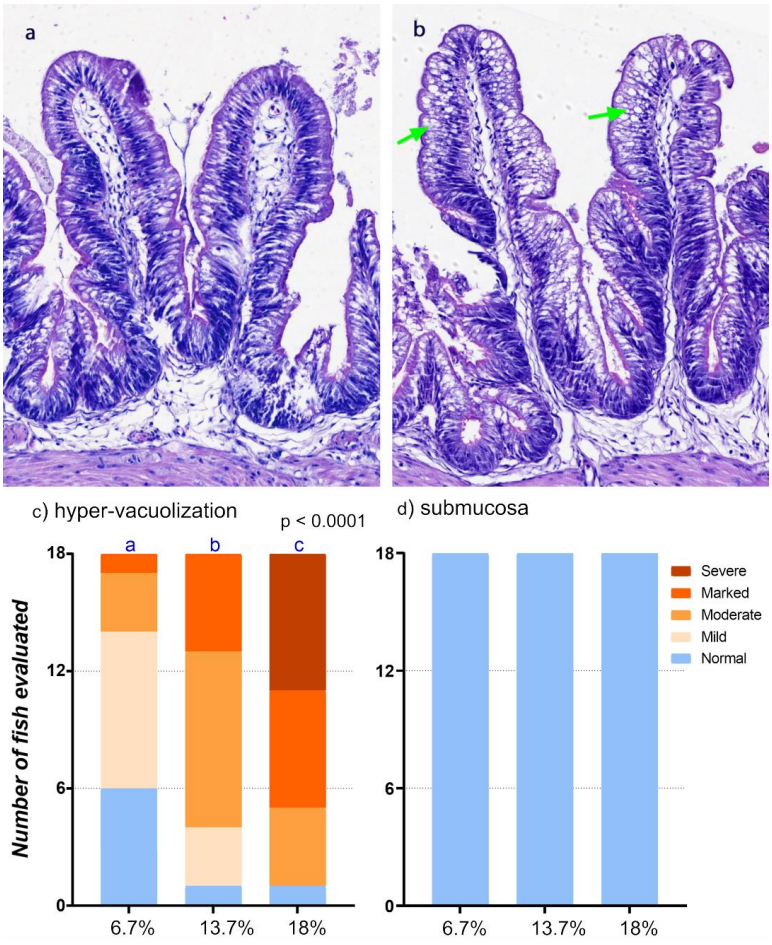

Fig 5. The upper pictures show representative images of sections from the mid intestine scored as a) normal and healthy; b) with marked lipid accumulation (steatosis) indicated as enlargement of vacuoles (green arrow) above the nucleus in the enterocytes. The lower left graph (c) shows number of mid intestinal tissue sections scored as normal, mild, moderate, marked, or severe regarding enterocyte steatosis. The right graph (d) shows number of mid intestinal tissue sections scored as normal, mild, moderate, marked, or severe regarding increases in the width and inflammatory cell infiltration in the submucosa. Columns not sharing a letter label are statistically different.

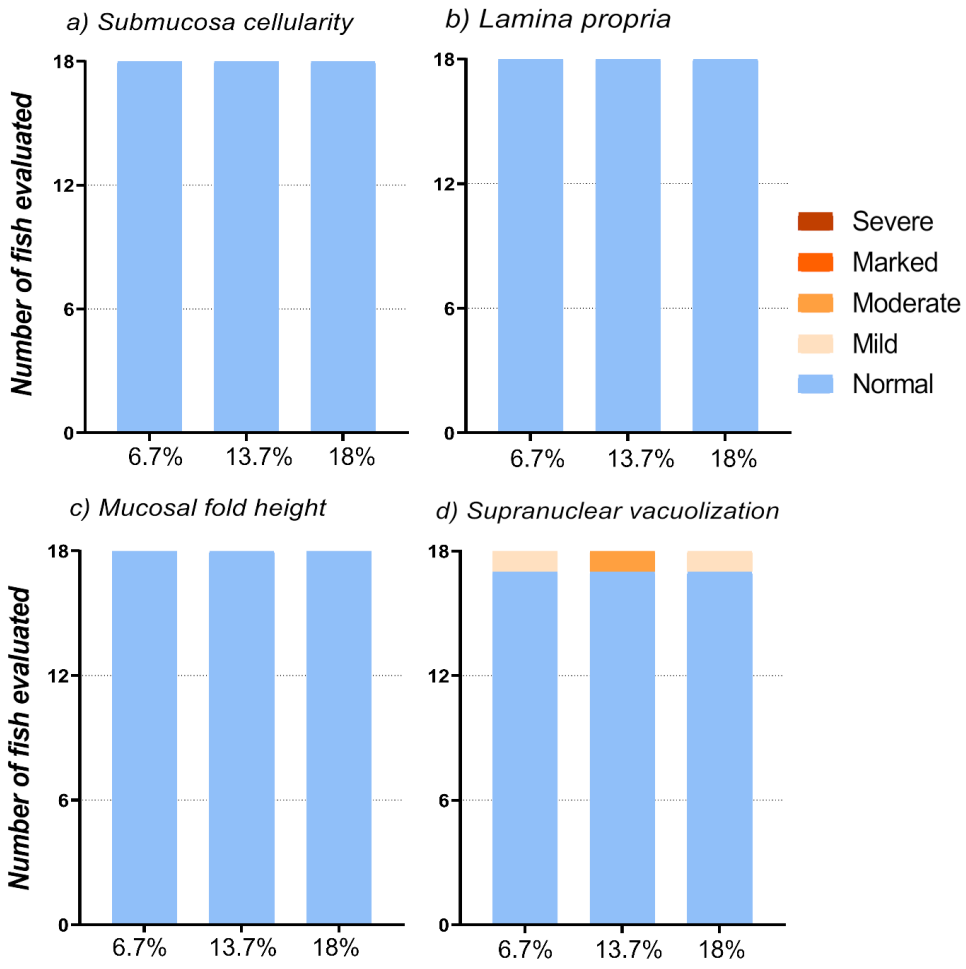

Fig 6. Number of distal intestine tissue sections from the gut health assessment for (a) increase in width and inflammatory cell infiltration of the submucosa. (b) increase in width and inflammatory cell infiltration of the lamina propria. c) change of the mucosal fold height and d) loss in enterocyte supranuclear vacuolization.

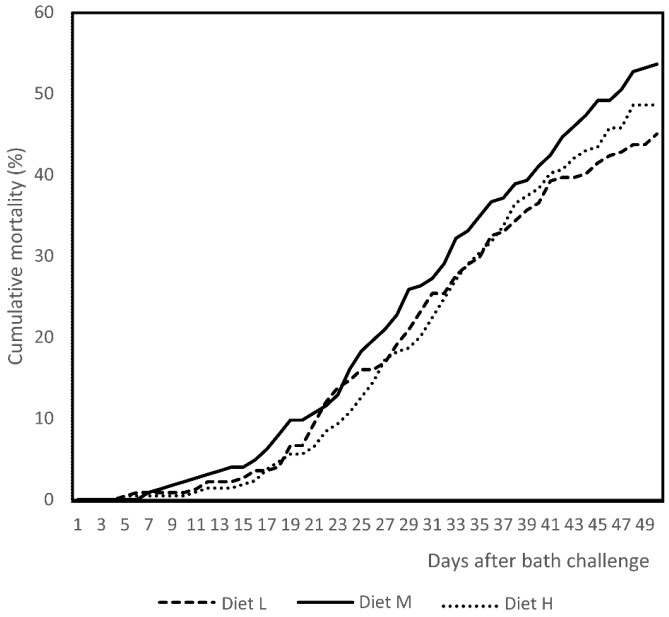

Fig 7. Cumulative mortality (%) in lumpfish fed diet L, M and H after bath challenge with atypical *Aeromonas salmonicida.* L, low lipid diet (6.7%); M, medium lipid diet (11.7%); H, high lipid diet (18%). Data are average from two parallel challenge tanks.

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