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Replacement of fishmeal with plant protein in the diets of juvenile lumpfish (*Cyclopterus lumpus*, L. 1758): Effects on digestive enzymes and microscopic structure of the digestive tract

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ABSTRACT

This study investigated the effects of plant protein concentrates on the activity of digestive enzymes and microscopic structure of the intestine of juvenile lumpfish. The experiment was carried out using triplicate groups of lumpfish of 7 g average initial body weight, to 40 g average final weight, after 54 days. Four experimental diets were used; a fishmeal (FM) based control diet and three test diets with the plant protein concentrates; soy protein concentrate (SPC) and pea protein concentrate (PPC) (1:1 ratio) replacing FM in proportions of 25% (PP25), 50% (PP50) and 75% (PP75), respectively. Nested ANOVA showed several significant treatment differences in histomorphometry. Overall, fish fed test diets exhibited several changes such as shorter mucosal fold height (MFH) in the anterior intestine (AI), increased number of goblet cells (GCs) and width of lamina propria (WLP) in distal intestine (DI), compared to control. Principal component analysis (PCA) on histological indices showed that all three treatment groups (i.e. PP25, PP50 and PP75) had significantly altered overall intestinal architecture, compared to the control group. The observed enteritis was negatively related with the condition factor (CF). Regarding enzyme activities fewer changes were observed across diets. Fish fed the PP50 diet exhibited an increased activity of leucine aminopeptidase (LAP) in the pyloric caeca and a decrease on chymotrypsin (CHY) in the mid intestine (MI) compared to the control. Nevertheless, the overall changes captured by PCA on enzymes were associated only with condition factor (CF) and not with the diets. Overall, histological evaluation confirmed that lumpfish intestinal morphometry was significantly altered by plant protein ingredients at 25%, 50% and 75% levels, but replacing of FM up to 50% did not affected the growth and the enzyme activities up to 75% inclusion level.

1. Introduction

Lumpfish (*Cyclopterus lumpus*), is currently the second largest farmed species in Norway in terms of numbers, valued for its delousing behavior against the ectoparasite *Lepeophtheirus salmonis* that affects Atlantic salmon (*Salmo salar*) after sea transfer (Powell et al., 2018). In 2019, nearly 42 million lumpfish, were used in the production of Atlantic salmon (Norwegian Directorate Fisheries, 2020). As the demand for juvenile lumpfish grows, husbandry practices, including feeding and nutrition of this species, are rapidly developing in order to both supply the salmon industry and to avoid exploitation of wild populations. To date, very little is known, or published regarding the effect of plant protein ingredients on the growth and development of this species (Willora et al., 2020).

Among the potential fishmeal (FM) substitutes, the main focus has been on soy protein concentrate (SPC) (Colburn et al., 2012; Zhang et al., 2019), which provides a high protein content (650 g kg^{-1}), favorable amino acid profile and lower fiber level (40 g kg^{-1}) (Hardy, 2010; Zhou et al., 2018). A significant amount of research has indicated the suitability of SPC as a protein source in feeds for many farmed fish

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species, and the results have demonstrated that different species have their own unique levels of tolerance towards SPC (Colburn et al., 2012; Escaffre et al., 2007; Kissil et al., 2000; Kokou et al., 2017; Metochis et al., 2016; Storebakken et al., 1998; Wang et al., 2017; Zhang et al., 2019; Zhang et al., 2012). Another potential alternative protein source is pea protein concentrate (PPC) (Nogales-Mérida et al., 2016; Øverland et al., 2009; Penn et al., 2011; Zhang et al., 2012), which has higher protein (350–500 g kg^{-1}), and lower carbohydrate content compared to unprocessed peas (Øverland et al., 2009).

Although, legume meals are also a promising alternative to FM, they are generally high in antinutritional factors (ANFs), and may affect the health and growth performance of animals when ingested, due to their interference with various physiological processes in the intestine (Francis et al., 2001).

Studies conducted to date, have not indicated that SPC promotes any incidence of severe intestine inflammation in salmonids (Escaffre et al., 2007; Green et al., 2013; Krogdahl et al., 2000; Krogdahl et al., 2020). This is probably due to the low amounts of ANFs and the absence of saponins, both of which are considered to be key causal agents of intestinal inflammation (Drew et al., 2007; Krogdahl et al., 2015a; Zhou et al., 2018). However, saponins are still present in PPC (Collins et al., 2013; Storebakken et al., 1998) and incorporation of PPC at high levels in diets of some aquaculture species has been shown to alter the intestinal microscopic architecture (Nogales-Mérida et al., 2016; Penn et al., 2011).

The activity of digestive enzymes in fish may be altered by feed ingredients (Castro et al., 2013), and may also be affected by the general health status of the gastrointestinal tract. Studies on Atlantic salmon diagnosed with soybean meal induced enteritis (SBMIE) show lower brush border membrane (BBM) enzyme activity (LAP) and increased (TRP) activity in the distal intestine (Bakke-McKellep et al., 2000; Chikwati et al., 2013; Kortner et al., 2012; Sahlmann et al., 2013). Feeding Atlantic salmon with PPC also resulted in enzymatic modifications such as those described in salmon fed with SBM diets (Penn et al., 2011). However, the profile of digestive enzymes in lumpfish is unknown, and the activity levels of the most common have not been studied.

A recent study on lumpfish confirmed that 50% of FM could be replaced with a blend of SPC and PPC without compromising growth (Willora et al., 2020). The aim of the present experiment was to investigate the effects on the activity of digestive enzymes and the histomorphology of juvenile lumpfish after feeding them for 54 days with three diets containing a blend of SPC and PPC at 1:1 ratio, compared with a control FM-based diet.

2. Material and methods

2.1. Experimental diets

The experiment was conducted following approval by the institutional Animal Welfare Body in compliance with the guidelines set by the Norwegian Animal Welfare act (LOV-2009-06-19-97) and the European Union directive (EU/2010/63) regarding the use of animals in research. Four experimental diets with nearly 52% crude protein and 21 kJ/g gross energy were manufactured by SPAROS Lda. Olhao, Portugal. A FM-based diet was used as control (CTRL) and three experimental diets were formulated to replace 25% (PP25) 50% (PP50) and 75% (PP75) of FM with a mixture of SPC and PPC (1:1 ratio). The remaining sources of proteins, such as wheat gluten, krill meal, and CPSP 90 were kept constant. The diets were supplemented with L-tryptophan, DL-methionine, L-taurine, and L-histidine to keep these ingredients similar among all diets. Wheat meal was used to balance the starch and carbohydrate content among the diets. Krill oil was used in increasing levels from CTRL to PP75, to increase the content of EPA, DHA, and phospholipids. For details of diet preparation and composition see Willora et al. (2020).

2.2. Fish and sampling

The experiment was carried out using triplicate groups. Farmed lumpfish, average initial body weight 7 g, obtained from Mørkvedbukta AS were randomly allocated into 12 indoor rearing tanks (500 l), with 208 fish per tank, at the research station at the FBA, Nord University, Bodø, Norway (for more details on the experimental conditions, see Willora et al. (2020). Fish were acclimated to laboratory conditions for 2 weeks prior to the experiment, during which time they were fed a commercial diet (Gemma Silk, Skretting, Stavanger, Norway). After 54 days, the fish reached average final weight of about 40 g. At that time, five fish per tank (15 / treatment) were anaesthetized with MS-222 (Tricaine methane sulphonate; Argent Chemical Laboratories, USA; 30 g /l), and sacrificed by a sharp blow to the head. All fish were sampled at least 2–3 h after the last meal. The intestinal tract, including the intestinal content, were removed, together with the adherent tissues and wrapped in aluminum foil; they were immediately frozen in liquid nitrogen and stored at -80 °C until enzymatic analysis. Whole digestive tract from 6 fish per treatment was also sampled for histological analysis. The digestive tracts was cut and separated in anterior and posterior before they were placed in 4% phosphate buffered formalin for 48 h at 4 °C before further processing.

2.3. Intestinal histomorphometry

Approximately 0.5 cm in length pieces were cut from the middle of the anterior and posterior third of the formalin fixed intestinal samples, as shown in Fig. 1. All samples were dehydrated in an ethanol gradient (50%, 60%, 70%, 85%, 90% and 96%), using a tissue processor (Shandon Citadel 2000, Thermo Electron Corp., Pittsburg, PA, USA) and embedded in paraffin wax by a paraffin dispenser (Tissue-Tec Wax-Dispenser WD-4, Kunz instruments A/S, Copenhagen, Denmark). Cross sections of 4 μm in thickness (4 sections / sample) were cut on a Shandon Finesse ME microtome (Shandon Lipshaw, Pittsburgh, PA, USA), mounted on glass slides and stained using haematoxylin-eosin (H/E). For the assessment of the goblet cells, Periodic Acid-Schiff and Alcian blue staining was performed on other sections of the same sample. All procedures, including processing, embedding, and staining of the samples, were carried out according to the methods described by Roberts et al. (2012).

Digital images of the sections were captured using a digital camera (Leica MC 170HD, Heersbrugg, Switzerland) connected to a light microscope (Leica DM1000, Wetzlar, Germany) and by using the software Leica Microsystems Framework (LAS V4.12.INK, Heerbrugg, Switzerland). The intestinal histomorphology was evaluated quantitatively. For each section, four histomorphometric indices

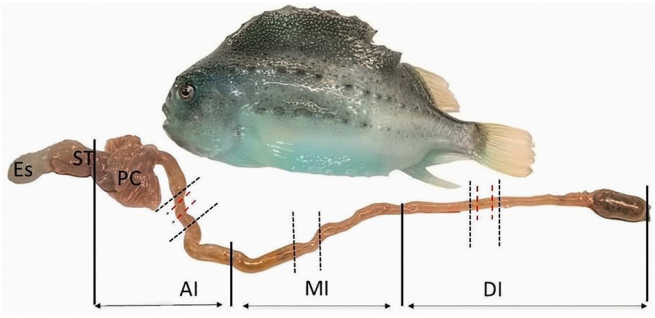

Fig. 1. Photograph of the gastrointestinal tract of juvenile lumpfish (*C. lumpus*) (weight 99,8 g and length 15 cm): Esophagus (Es), stomach (ST), anterior intestine (AI) with pyloric caeca (PC), mid (MI) and distal intestine (DI). Sample sites of AI and DI for gut morphology shown in red and sampling sites of PC, AI, MI and DI for enzyme analysis shown in black. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(measurements) were studied to assess the structure of the intestinal wall and two for the overall complexity and homogenigity of the intestinal mucosa. For the intestinal wall, the indices were: intestinal fold height (MFH), tunica muscularis width (MT), number of goblet cells (GC) and width of lamina propria (WLP). For each histological section, 10 intact folds, which displayed clearly the whole length, were selected to quantify the MFH. The fold height was considered as the length from the bottom to the top of the mucosal fold. For the assessment of the MT index, the distance between the inner and outer layer border was measure in the four locations; top and bottom and left and right sides of the cross section. Before measuring the WLP, all sections were examined for any significant alterations of cell infiltration. The WLP was considered as the width of the connective tissue between the epithelia, and was measured in 5 locations per fold. More details of the measurements are illustrated in Fig. 2B. The number of GC was quantified by counting the cells in the entire mucosal region of 10 randomly selected folds per section and the mean calculated.

For the assessment of the overall mucosal complexity and homogeinity the calculation of fractal dimension (D_B) and lacunarity (L) was performed, according to the method suggested by Sirri et al. (2014), with slight modifications. Briefly, each coloured photograph of the entire cross section was changed to 8 bit b/w and the lumen was selected after applying appropriate thresholding (Fig. 3). The obtained black lumen was used for the calculation of L, while its one-pixel outline was used for the calculation of D_B (Fig. 3). The plugin *FracLac* (Karperien, 2013), was employed for the calculation of the D_B of the outline (box-counting fractal dimension, averaged over the number of scans that were done at different grid positions) and the image lacunarity L (variation in pixel density at different box sizes). The average values per fish were used for the comparisons. For each index, all measurements taken were used to calculate the mean value per fish. All measurements were performed using imageJ 1.52a (Schneider et al., 2012).

Each index was assessed twice by two different researchers and averaged before using it as a representative measurement.

2.4. Digestive enzyme activity and protein assay

Enzyme extracts of pyloric caeca (PC), anterior (AI), mid (MI), and distal intestine (DI) (Fig. 1) were prepared by dilution of 0.1 g tissue samples in milliQ water (1:10 weight: volume) and homogenized using the FastPrep®-24 Classic Instrument (MP Biomedicals, Solon, Ohio, USA) with the following conditions: 6.5 m/s and 20 s. Samples were centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatants stored at -80 °C until analysis. Activities of total alkaline protease (TAP), trypsin (TRP), chymotrypsin activity (CHY), leucine aminopeptidase (LAP) and amylase (AMY), were determined in homogenates of PC, AI, MI and DI as explained below.

2.4.1. Total alkaline protease activity

Alkaline proteinase activity was performed according to the method described by Alarcón et al. (1998). In brief, alkaline protease activity was measured using substrate casein (1%) in 50 mM Tris/HCl buffer (at pH 8.4) with 10 μl extract. The mixture was incubated for 40 min at room temperature (25 °C) and the reaction terminated by adding 0.5 ml of 20% trichloroacetic acid (TCA), followed by cooling the samples for 15 min at -20 °C. After centrifuging at 12,000 g for 10 min, the absorbance of supernatant was measured at 280 nm by spectrophotometer (Shimadzu UV-1800, Shimadzu, Kyoto, Japan). All samples were performed in triplicate, and two controls were run for each sample. Controls were established by adding TCA before enzyme extract. One unit of activity was defined as 1 μg of tyrosine released per min under defined assay conditions (Extinction coefficient = 0.0071 ml μg^{-1} cm^{-1}).

2.4.2. Trypsin activity

Trypsin activity was measured colorimetrically as described by Natalia et al. (2004). Substrates of 50 mM: N-α-benzoyl-DL-arginine-p-nitroanalidine (BApNA) 200 μl with 10 μl of enzyme extract were incubated for 5 min at 37 °C. Absorbance at 405 nm was measured using a multi-scan Ex spectrophotometer (Thermolab Systems, Helsinki, Finland). All measurements were determined in duplicate. Increase in absorbance at 405 nm was measured every 30 s for 5 min. One unit of

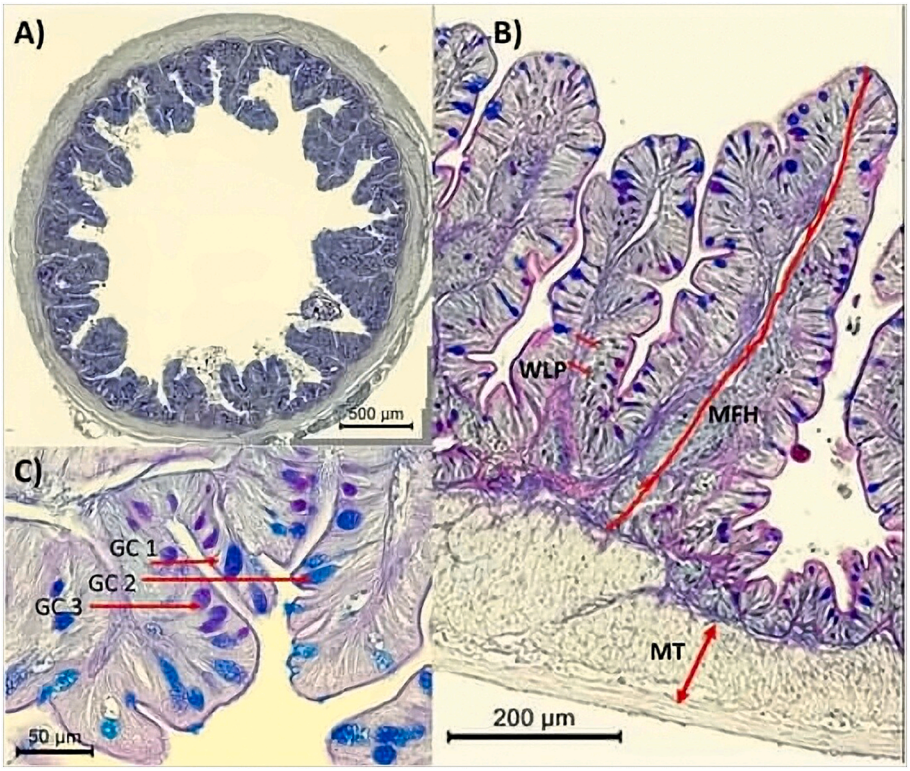

Fig. 2. The histomorphological analysis of juvenile lumpfish intestinal tissue with Periodic Acid-Schiff and Alcian blue stain. A). Cross section of the anterior intestine after scanning under the microscope (X10) B). Vertical and horizontal lines show how the height of the mucosal folds (MFH) and width of muscular thickness (MT) and lamina propria (WLP) were measured (X40) C). Note the three different populations of goblet cells; GC 1 (purple), GC 2 (bright blue) and GC 3 (magenta) stained at pH 2.5 (X40). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

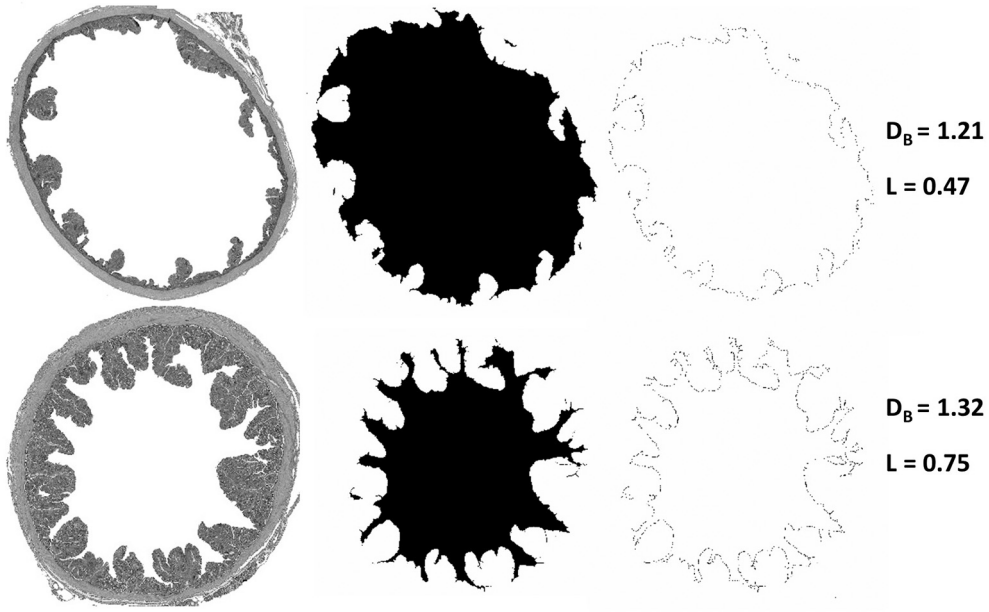

Fig. 3. Transformation of the histological photos for the calculation of D_B and L, using the imageJ software and *FracLac* plugin. Two images of distal intestine from two different fish are used as examples to indicate different measurements.

activity was defined as 1 μM of p-nitroanilide released per min (Molar extinction coefficient = 8800 M/cm).

2.4.3. Chymotrypsin activity

Chymotrypsin activity was measured using 50 mM BApNA (N-α-benzoyl-Larginine-p-nitroanilide hydrochloride, Merck KGaA, Darmstadt, Germany) and 50 mM GApNA (N-glutaryl- *L*-phenylalanine-p-nitroanilide, Merck KGaA, Darmstadt, Germany) as substrates, respectively (Erlanger et al., 1961). Absorbance at 405 nm was measured using a multiscan Ex spectrophotometer (Thermolab Systems, Helsinki, Finland). One unit of activity was defined as 1 μg of p-nitroaniline released per minute.

2.4.4. Leucine aminopeptidase activity

Activity of the brush border membrane enzyme, LAP, was also determined as described by Natalia et al. (2004). Briefly, 0.1 ml of enzyme extract was combined with 1.9 ml of 1.6 mM l-Leucine-p-nitroanilide (LeuNA) containing a substrate of 0.025 M $MgCl_2$ in Phosphate buffer 60 mM (pH 7.0). The increase in absorbance at 405 nm was constantly recorded at 25 °C. One unit of activity was defined as 1 μM of p-nitroanilide released per min (Molar extinction coefficient = 8800 M/cm).

2.4.5. Amylase activity

Amylase activity was determined by measuring the production of reducing sugar from starch using 3, 5-dinitrosalicylic acid (DNS) as described by Miller (1959). The reaction system included 50 μl of enzyme sample and 50 μl of 1.0% starch (Sigma, S2630) solution in 0.1 M citrate-phosphate buffer, (0.1 M, pH 7.5 + NaCl 0.05 M). After 20 min incubation at 37 °C, the reaction was stopped by adding 100 μl of DNS reagent. Blanks contained all the solutions and inactivated (boiled) enzyme sample. One unit of amylase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per minute.

2.4.6. Specific enzyme activities

All enzyme activities were expressed in U per mg of total soluble protein in the samples. Protein cconcentration of soluble protein in extracts was determined according to the Bradford (1976) method, using bovine serum albumin (2 mg / ml) as the standard.

Specific enzyme activities were calculated as follows:

$$\frac{U}{mg\ total\ protein} = \frac{\Delta Abs\ x\ Vtotal}{\varepsilon\ x\ Vsample\ x\ t}\ x\ \frac{ml}{mg\ total\ protein}$$

where *ΔAbs* is the absorbance increase at a determined wavelength; ε is molar extinction coefficient. *Vtotal and Vsample* are the total volume, and volume of extract used in the reaction, *t* is the time of reaction.

2.5. Statistical analysis

All observed variables were checked for normality and homogeneity of variances visually and by using the Shapiro–Wilk and Fligner-Killeen's tests respectively. To account for the hierarchical structure of the study design, nested ANOVA was used for dietary treatment comparison for each observed variable regarding histomorphology where also no missing values existed. Regarding mucosal complexity, because lacunarity was a percentage beta regression was applied to assess the effects of dietary treatments and fractal dimension was normally distributed thus, nested ANOVA was used again (fractal dimension in the anterior part had few missing values which were excluded). When significant effects (p-value<0.05) were present, post hoc pairwise treatment comparison was applied with the Tukey method.

In the enzymatic activity dataset several variables did not display normality thus, generalized estimating equation (GEE) was used there. GEE is a marginal modelling approach (Lee and Nelder, 2004; Ziegler, 2011), that can handle correlated, non-normally distributed and heteroscedastic data. The correlation structure was defined as independent. Some of the values were missing at random thus only the complete cases in each variable were included. When significant effects (p-value<0.05) were present, post hoc pairwise treatment comparison was applied by least-square means with the Tukey method.

Furthermore, principal component analysis (PCA) was used separately on the datasets obtained from the histomorphometric indices, the enzymatic activities and mucosal complexity, to assess the overall covariation of their respective variables. Regarding the enzymatic activity and mucosal complexity, as previously, only the complete observations were used for PCA too. In all three cases the scores on the first principal component (PC1) were extracted and used in a nested ANOVA to detect significant mean treatment differences (the PCA scores satisfy the normality and homogeneity of variances assumptions). The PC1s

were also used in a mixed-effects model, as fixed effect along with a random intercept at the tank level, to assess their relationship with condition factor (CF) as response. In both PCAs the variables were scaled and centered. All analysis was performed in the open-source environment R version 3.6.2 (R Core Team, 2020) using the packages "factoextra" (Kassambara, 2020), "lme4" (Bates et al., 2015), "emmeans" (Lenth, 2021), "geepack" (Yan, 2002) and "rstatix" (Kassambara, 2021).

An a priori power analysis, using G* power 3.1.9.2 (Faul et al., 2009), indicated that with the given sample size for the histomorphometry and the enzyme activity, a good power (>0.85) would be obtained for effect size f > 0.8 and f > 0.5 respectively (Cohen, 1988).

3. Results

3.1. Intestinal histomorphometry

The intestine of lumpfish is coiled with several bends, making it nearly twice as long as the body. Macroscopically, no notable differences are observed between the segments of the intestine posterior to the pyloric caeca and anterior to the distal. However, histologically, it could be divided into three, approximately equal regions: the anterior, mid and distal intestine, each accounting for 1/3 of the total length of the intestine (Fig. 1).

Using nested ANOVA, several significant treatment differences were detected in histomorphology (Tables 1, 2). Fish fed the PP25 diet exhibited increased number of GC and WLP in the DI, while MT was decreased in the same location compared to the CTRL. The PP75 group displayed decreased MFH in the AI, along with increased number of GCs and WLP in DI, compared to the CTRL. The number of GC in the DI of the PP75 group was also higher compared to the PP50 group.

Different subpopulations of goblet cells were not quantified. However, PAS staining indicated, two main subpopulations of goblet cells, the bright blue (containing acid mucins) and the magenta (containing neutral mucins) about 50:50, while a few purple goblet cells (containing mixed mucins) appeared scattered among the others (Fig. 2C). No significant differences were noted in the study of fractal dimension and lacunarity (Fig. 4). But the fish fed the plant-based diets, particularly the PP75 diet, tended to exhibit slightly reduced complexity and homogeneity of the intestinal mucosa. (Fig. 4)

3.2. Enzymatic activity

Results of GEE models for each observed variable regarding enzyme activities individually as response are presented in Table S2. Significant pairwise treatment post-hoc comparisons for each observed enzyme activity are presented in Table 3. The PP50 group exhibited significantly higher LAP activity in the PC in the fish of the CTRL group. Similarly, in the DI, the PP25 group had higher LAP activity compared to the CTRL group. Regarding CHY activity in the MI, the PP50 group exhibited significantly lower activity than the CTRL group and PP75 group.

Table 1
Results of nested ANOVA regarding the individual histomorphology variables.

Response variable	Dietary comparisons	Difference	p-value	95% CI
MFH in the AI	PP75 – CTRL	−191.36	0.02	−357.12 / -25.6
GCs in the DI	PP25 – CTRL	32	0.01	7.45 / 56.54
	PP75 – CTRL	47.05	0.0005	22.5 / 71.6
	PP75 – PP50	25.12	0.044	0.57 / 49.66
WLP in the DI	PP25 – CTRL	1.36	0.02	0.24 / 2.49
	PP75 – CTRL	1.66	0.004	0.54 / 2.79
MT in the DI	PP25 – CTRL	−49.04	0.03	−93.04 / -5.04

MFH: mucosal fold height, GCs: goblet cells, WLP: width of laminar propria, MT: muscularis thickness, AI: anterior intestine, DI: distal intestine.

Table 2
Mean values of histological indices measured in the anterior and distal intestine of juvenile lumpfish fed four experimental diets along with significant differences (*P*-value<0.05) from nested ANOVA.

Parameter	CTRL	PP25	PP50	PP75
Condition factor (CF)	0.26 ± 0.02	0.23 ± 0.02	0.24 ± 0.01	0.25 ± 0.02
Mucosal fold height				
Anterior intestine	684.5 ± 33.74 [a]	594.9 ± 21.45 [ab]	493.15 ± 26.34 [ab]	490.22 ± 11.60 [b]
Distal intestine	456.3 ± 9.33	459.5 ± 15.11	476.8 ± 24.54 [b]	397.4 ± 12.92
Muscularis thickness				
Anterior intestine	103.4 ± 5.46	107.5 ± 9.33	79.7 ± 5.82	111.1 ± 4.25
Distal intestine	138.1 ± 2.70 [a]	124.5 ± 5.93 [b]	128.6 ± 5.75 [ab]	101.9 ± 3.29 [ab]
Number of goblet cells				
Anterior intestine	117.9 ± 5.67	131.1 ± 6.44	137.0 ± 3.53	139.9 ± 9.52
Distal intestine	85.2 ± 3.96 [a]	107.2 ± 6.10 [b,c]	132.3 ± 9.51 [a,c]	117.3 ± 3.80 [b]
Width of laminar propria				
Anterior intestine	7.91 ± 0.30	7.72 ± 0.81	7.92 ± 0.38	7.97 ± 0.33
Distal intestine	7.04 ± 0.53[a]	7.98 ± 0.36 [b]	8.28 ± 0.84 [a,b]	8.41 ± 0.29[b]

Values represent mean ± SD ($n = 6$).
CTRL: Control, PP25, PP50 and PP75 are 25% 50% and PP75: 75% of SPC and PPC inclusion respectively.

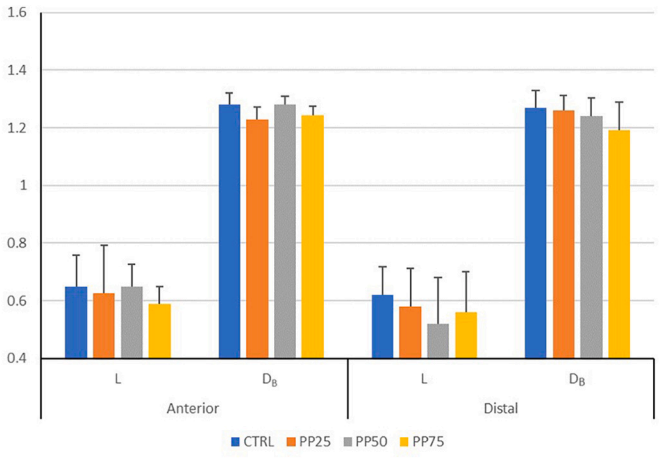

Fig. 4. Measurement of fractal dimension (D_B) and lacunarity (L). Values are presented as mean ± SE.

Table 3
Significant treatment comparisons based on least-square means on GEE models for each observed enzyme activity.

Response variable	Dietary comparisons	Difference	p-value	95% CI
LAP in the PC	CTRL – PP50	−0.004	0.005	−0.008 / -0.001
LAP in the DI	CTRL – PP25	−0.008	<0.0001	−0.01 / -0.005
CHY in the MI	CTRL – PP50	0.143	0.035	0.007 / 0.28
	PP50 – PP75	−0.36	0.004	−0.6 / -0.08

CTRL: Control, PP25, PP50 and PP75 are 25% 50% and PP75: 75% of SPC and PPC inclusion respectively. LAP: leucine aminopeptidase CHY: chymotrypsin, PC: pyloric caeca, AI: anterior intestine, DI: distal intestine.

3.3. PCA analysis

Regarding PCA, the loadings of each set of variables on their PC1 are shown in S1, S3 and S4. In the PC1 of the histomorphology variables (Fig. 5), the number of goblet cells was positively correlated with lamina propria width in both intestine segments, as the mucosal fold height with tunica muscularis thickness, while those two groups were negatively correlated with each other. This orientation of the variables shows that the first principal component reflects a composite view of enteritis (hereinafter refer to as PC1_Enteritis), that explained 25.7% of the variation observed in all histological parameters. Furthermore, nested ANOVA on PC1_Enteritis showed that all three treatment groups (i.e. PP25, PP50 and PP75) were significantly different from the control group (Fig. S1A). Finally, PC1_Enteritis was also significantly negatively related with CF (Table 4; Fig. 8A).

PCA results for lacunarity and fractal dimension (Fig. 6) showed that PC1 scores (hereinafter refer to as PC1_MComplexity) was reflecting changes in the anterior part and PC2 on the distal part mostly. The PC1_MComplexity explained 40% of the variation observed on its original variables, but did not differ among the dietary treatments and was not significantly associated with CF (Table 4).

In the enzyme activities, all loadings were positively correlated apart from the leucine aminopeptidase activity, which had negative loadings on their PC1 (hereinafter refer to as PC1_Enzymes), that explained 29.3% of the variation observed in all enzyme activities (Fig. 7, S3B). However, no treatment differences were detected with the nested ANOVA and PC1_Enzymes was significantly positively associated with CF (Table 4; Fig. 8B).

Table 4
Mixed-effects model of PC1_Enteritis, PC1_Enzymes and PC1_MComplexity with the condition factor (CF) as response.

Variable name	Coefficient	p-value	95% CI
PC1_Enteritis	−0.008	0.0039	−0.012 / -0.003
PC1_Enzymes	0.006	0.004	0.002 / 0.01
PC1_MComplexity	−0.002	0.67	−0.01 / 0.006

4. Discussion

4.1. Intestinal histomorphometry

Studies on the possible effects on the microscopic anatomy should be included, along with the growth performance and feed utilization, before conclusions are drawn about the potential of any plant ingredients at high incorporation levels (Vatsos, 2021). Among the many histomorphometrical indices used, mucosal fold height and width of lamina propria appear to be the most commonly preferred parameters for studying the effects of different diets, often accompanied by enumeration of goblet cells (Silva et al., 2015). The decreasing mucosal fold height from the anterior towards the rectum in the present experiment, corroborates with studies of most species (Firdaus-Nawi et al., 2013). Anterior intestinal regions with longer folds provide more surface area for efficient absorption of nutrients (Bakke et al., 2010a) and is quantitatively more important for nutrient absorption than the more distal part (Krogdahl et al., 1999). The distal intestine has a more important role in immune functions than the absorption process (Løkka et al., 2013).

Shortening of mucosal folds in distal intestine is often used as an indication of inflammation of the intestinal tract (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003; Urán et al., 2008). This alteration, reduces the total surface area of the intestine and hence the absorption of nutrients, which in turn can affect the growth of fish (Moldal et al., 2014). In the present experiment, none of the dietary treatments caused observable alterations of the mucosal fold height in the DI, which is in agreement with the published literature on meagre (*Argyrosomus regius*) (Ribeiro et al., 2015) and rainbow trout (*Oncorhynchus mykiss*) (Escaffre et al., 2007), fed 50% and 100% of SPC in their diets respectively. In contrast, Kokou et al. (2017), observed moderate alterations in mucosal fold height of the DI in gilthead seabream fed 60% SPC. On the other hand, mucosal fold height in AI was reduced ($p < 0.05$) in the PP75 diet group. This is in line with reports on Atlantic salmon fed 35% of PPC in their diet (Penn et al., 2011). However, it should be noted that not all

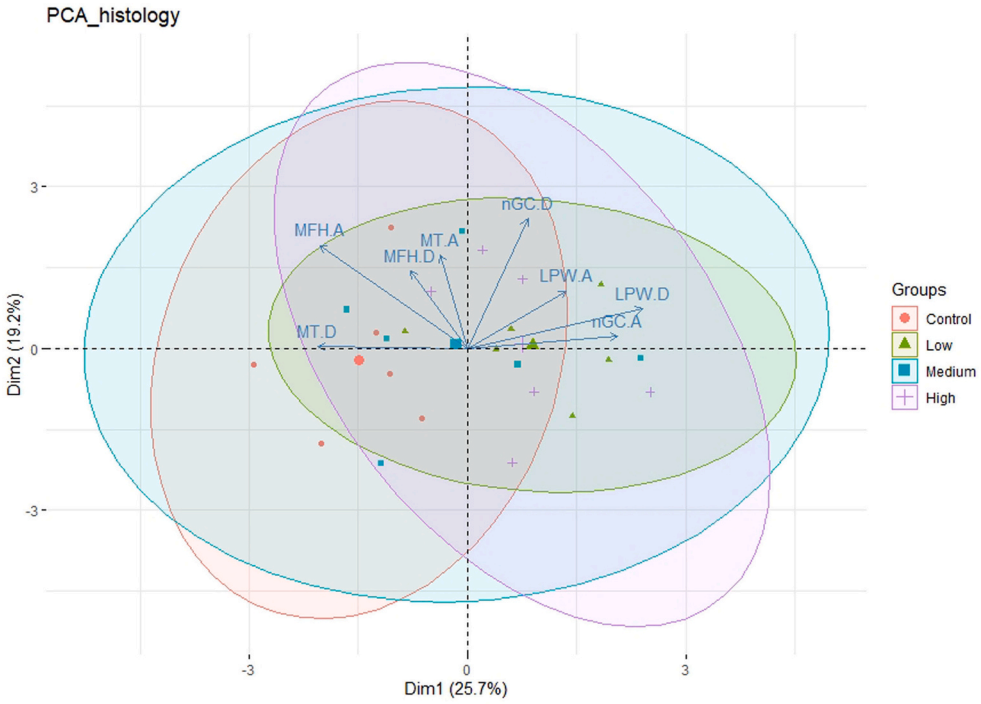

Fig. 5. PCA biplot for histomorphometrical data (PC1 enteritis); intestinal fold height (MFH), tunica muscularis width (MT), number of goblet cells (nGC) and width of lamina propria (WLP) in anterior (A) and distal (D) part; color shows the dietary treatments (CTRL:Control, PP75:High, PP50:Medium, PP25: Low.

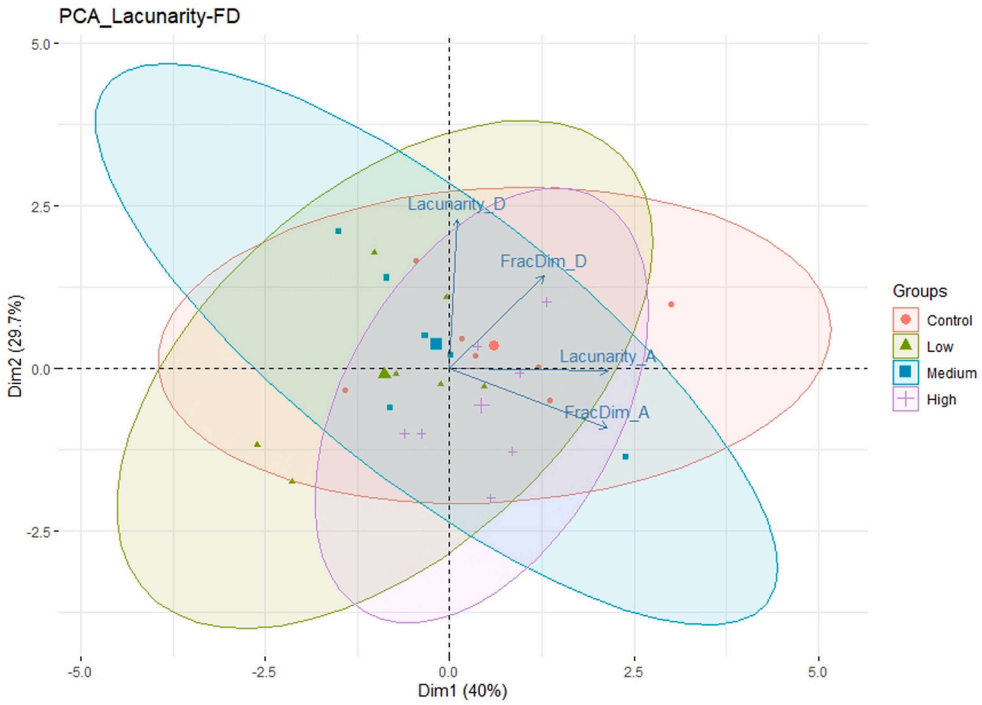

Fig. 6. PCA biplot for fractal dimension (D_B) and lacunarity (L); color shows the dietary treatments (CTRL:Control, PP75:High, PP50:Medium, PP25: Low.

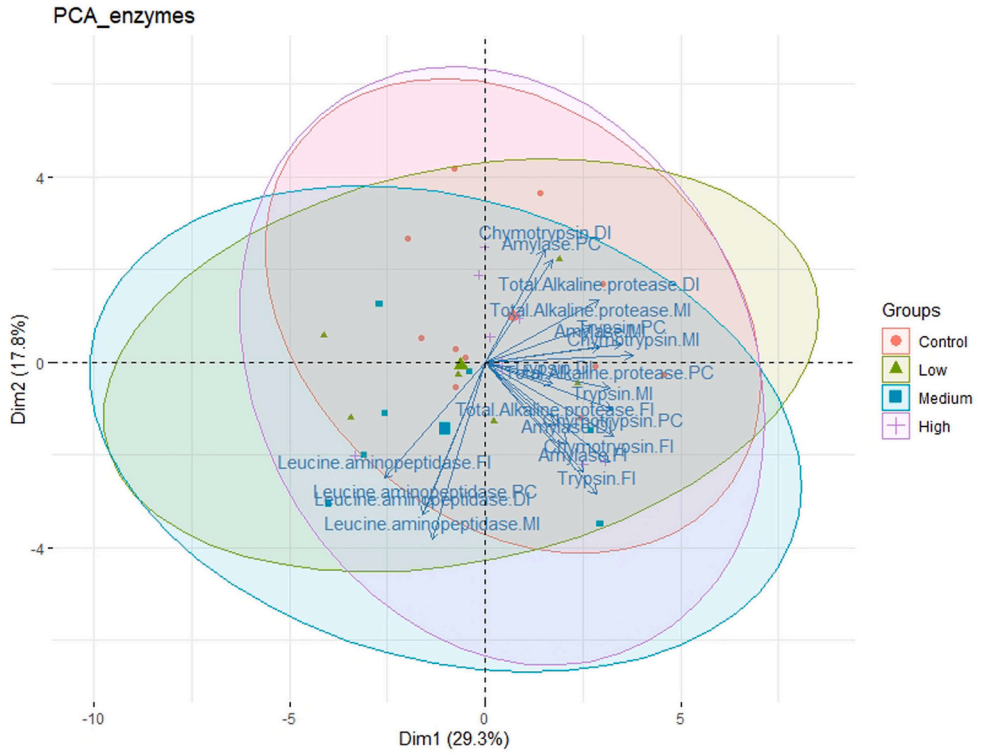

Fig. 7. PCA biplot for enzyme activities; color shows the dietary treatments (CTRL:Control, PP75:High, PP50:Medium, PP25: Low.

species fed with high dietary levels of plant proteins respond in the same manner. For example, sharp-snout sea bream (*Diplodus puntazzo*), an omnivorous fish, fed a diet with 48% PPC showed the longest mucosal folds in the DI (1164 μm) compared to a FM based diet (810 μm) (Nogales-Mérida et al., 2016). Thus, it appears that dietary preference should be taken into consideration when evaluating the effects of any dietary ingredients.

The present study, demonstrated a significant increase in the thickness of lamina propria in DI, in fish fed the PP75 and PP25 diets, compared to the fish fed the control diet. This indicates that lumpfish cannot tolerate such a high incorporation level of these plant protein concentrates in the feed. A widening of lamina propria is mainly caused

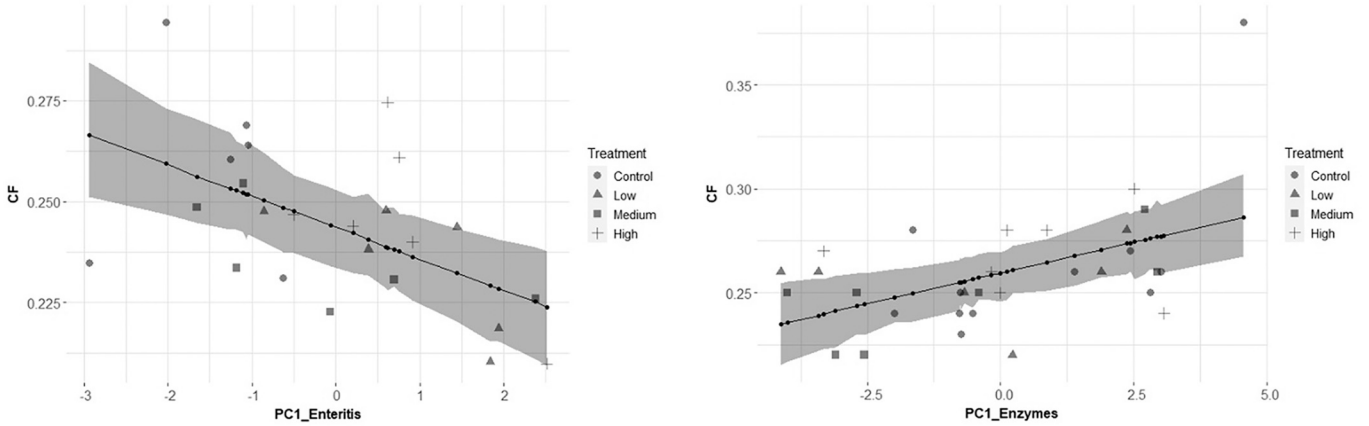

Fig. 8. A). Mixed-effects model of PC1_Enteritis and condition factor B). Mixed-effects model of PC1_Enzymes and condition factor.

by tissue proliferation and/or infiltration of the inflammatory cells (Baeverfjord and Krogdahl, 1996; Van den Ingh et al., 1991) and thus it is a sign of intestinal inflammation. Widening of lamina propria is usually not observed in fish fed SPC incorporated diets (Escaffre et al., 2007; Kokou et al., 2017; Ribeiro et al., 2015). Nevertheless, a recent study showed widening of lamina propria in zebrafish (*Danio rerio*) fed 30% of SPC, associated with gut inflammation in the MI (Dhanasiri et al., 2020). Inclusion of PPC showed a similar picture in salmon fed a diet containing 30% PPC (Penn et al., 2011), and sharp-snout sea bream fed a diet containing 48% PPC (Nogales-Mérida et al., 2016). Therefore, the level of this tissue alteration, induced by these plant ingredients, appears to be species-specific.

The contraction and relaxation of tunica muscularis facilitates transport of the intestinal content towards the anus (Shahrooz et al., 2018). Thinner muscularis layer was observed in fish fed the highest incorporation of plant protein concentrates compared to the control diet in the DI. Similarly, thinner muscularis in DI has been reported in Northern Snakehead (*Channa argus*) and Japanese seabass (*Lateolabrax japonicus*) fed diets containing 75% and 50% SBM respectively. However, reports are conflicting and seem to depend on species, feeding habits, and protein source. For example, sharp-snout sea bream fed diets containing 48% PPC showed thicker tunica muscularis in AI compared to the FM diet (Nogales-Mérida et al., 2016), and sea bream fed a diet containing a 90% mixture of PPC and rice protein concentrate (1:1.3 proportion) did not show any significant change in thickness of the tunica muscularis (Baeza-Ariño et al., 2016).

The mucus covering the intestinal mucosa has many functions, such as protection against mechanical or chemical damage, enhancing digestion and absorption of nutrients, buffering intestinal fluids, and assisting in the movement of intestinal content towards the rectum (Lommel and Alfons, 2003). The mucus is produced by goblet cells located in the intestinal epithelium, and their number and size can be affected by many factors, including the diet (Fronte et al., 2019; Torrecillas et al., 2013). Significantly increased number of goblet cells in DI in fish fed the PP75 diet is in agreement with other studies, which contained a 90% blend of PPC and rice protein (Baeza-Ariño et al., 2016), or 48% PPC (Nogales-Mérida et al., 2016). Thus, the increased number of goblet cells concurrent with higher mucin production, indicates increased lubrication and protection of the mucosa, as a response to fish fed high inclusion level of plant proteins, as previously reported in several farmed fish (Baeverfjord and Krogdahl, 1996; Baeza-Ariño et al., 2016; Nogales-Mérida et al., 2016). The application of Alcian blue-PAS staining showed that the majority of acidic (bright blue) and neutral (bright magenta) goblet cells were scattered within the epithelium in both regions of the intestine. A few purple (a color between blue and magenta) goblet cells were also observed, which probably reflected different ages or stages of cell development and differentiation. Similar differentiation stages were reported for the goblet cells of zebra mbuna (*Maylandia zebra*) after being treated with alcian blue at pH 2.5 followed by PAS (Leknes, 2010).

In general, the number of goblet cells increase from the anterior to posterior segments in the intestine as well as in the rectum (Machado et al., 2013). In the present study however, the number of goblet cells in AI was slightly higher compared to DI, which might be a species-specific feature of lumpfish. For example, two species from the family Gobidae showed that the distribution of goblet cells in the intestine varied between species; for instance, the Giurine goby (*Rhinogobius giurinu*) has more goblet cells in the AI (641 $\pm$ 107) than the posterior intestine (379 $\pm$ 101), and the number of goblet cells decreased towards the rectum. In contrast, the Hairychin goby (*Sagamia geneionema*) showed nearly a 2-fold increase in goblet cells in the DI compared to the AI (Hur et al., 2016). Another explanation is that prolonged feeding with a diet that increased mucus production might have led to a gradual depletion of the goblet cells. This has been noted in many cases of chronic enteritis, where the initially increased number of mucous cells decreased dramatically after a certain period of time (Dharmani et al., 2009).

The negative effects of dietary SBM on intestinal morphology have been associated with the content of ANFs (Couto et al., 2015; Krogdahl et al., 2015a). Fish with omnivorous feeding habits, may have higher tolerance to plant ingredients compared to carnivorous habits (Silva et al., 2010). However, some omnivorous species, like carp (*Cyprinus carpio*) do exhibit an inflammatory response in the DI, similar to soybean meal induced enteritis in salmonids (Urán et al., 2008), when fed 20% SBM. The relatively high tolerance to plant protein concentrate observed in the present experiment may be attributed to the omnivorous feeding behavior of lumpfish (Davenport, 1985; Ingólfsson and Kristjánsson, 2002; Machado et al., 2013).

In the present study, in addition to the histomorphometric indices commonly used in fish nutrition studies, two additional indices were assessed. The calculation of fractal dimension is used to evaluate the complexity of a shape, in this case a line, while lacunarity also known as 'gappiness' is a complementary index of heterogeneity, that shows how similar the different regions or parts of a shape are (Dong, 2000). Both indices have been used in the study of various phenomena and their usefulness in human histopathology has been known for many years. The plugin used here for the calculation of the two indices is also known to be very efficient, particularly for the evaluation of histological images (Andjelkovic et al., 2008). In fish histomorphometry, and particularly in nutritional studies, these two indices have not been used that much. Some examples include the study of gills of seabass (*Dicentrarchus labrax*) exposed to different toxic substances (Manera et al., 2016) and the study of the effects of mussel meal on the intestine of sole (*Solea solea*) (Sirri et al., 2014). In the current study, fractal dimension and lacunarity were used to assess the effect of the experimental diets on the overall

trophic condition of the intestinal mucosa. Previous published studies have already demonstrated that the proliferation rate of enterocytes appears to exhibit a positive correlation with fractal dimension measurement (Sirri et al., 2014) and therefore, increased rate, as for instance, occurs in various intestinal cancers, results in increased fractal dimension (Liu et al., 2007). In the present study, although no statistically significant effects were noted, a slight reduction in both the complexity and the heterogeneity of the mucosa was observed, particularly in the fish fed the diet the PP75. This result, in connection with the reduction of the height of the villi, could partially explain the reduced growth that was observed in this group too (Willora et al., 2020). However, as their effects were not significant, mainly other factors contributed in the reduced growth performance.

Previous research on lumpfish intestinal health associated to diet is limited. For example, Imsland et al. (2019), reported the effect of feeding frequencies on gut health. The overall histological evaluation confirmed that lumpfish are sensitive to the inclusion of plant ingredients in their diet. Reduction in mucosal fold height, increased number of goblet cells and the slight reduction in both the complexity and the heterogeneity of the mucosa were associated with the highest inclusion level of plant protein concentrate. It should be noted though, that the duration of the experiment may have been too short to manifest more notable differences in the histomorphology of the intestine between groups (Torrecillas et al., 2017), and therefore, long-term studies are recommended to establish a more solid conclusion of the effect of plant proteins on intestinal health of juvenile lumpfish.

4.2. Enzyme activity

The present experiment measured the activity of TAP, TRP, CHY, LAP and AMY throughout the digestive tract and in the PC of juvenile lumpfish. Individuals in current study did not show any signs of a specific enzymatic zonation, suggesting that the same enzymes are released and absorbed over the length of the intestinal tract.

Leucine aminopeptidase and trypsin activities were found to be significantly higher in MI. In general, digestive enzymes synthesized in the fish exocrine pancreas are secreted into the anterior section of the digestive tract (Bakke et al., 2010a, 2010b). This section, therefore, seems to be a main site for nutrient digestion and absorption than more posterior sections. In common carp, the mid intestinal segment is the major intestinal area for enzymatic hydrolysis of protein and starch, as well as for the absorption of amino acids (Dabrowski, 1986a).

In salmon, nutrient absorption is known to take place in the PC and the AI and, to a lesser extent, the posterior intestine (Krogdahl et al., 2015b). In case of common carp, the mid-intestine is relatively more capable of absorbing nutrients than its anterior counterpart (Dabrowski, 1986b). However, among all tissue analyzed in the present study, specific enzyme activities found in PC were lower compared to the other intestinal segments. In contrast to our findings, Gai et al. (2012), reported generally higher aminopeptidase, amylase and lipase activity in the PC compared to AI in rainbow trout fed a fishmeal diet and/or rice protein concentrate at 20% and 53%. Furthermore, elevated digestive enzyme activities (TRP, AMY, and CHY) in the pyloric caeca was observed in white snook (*Centropomus viridis*) fed 15% SPC in their diet (Arriaga-Hernández et al., 2021). Lumpfish have relatively short PC, the number of which are 36–79, compared to 31–147 in rainbow trout (Bergot et al., 1981). According to Marthinsen (2018), PC appear 34 days post hatch and consist of goblet cells and enteroendocrine-like cells in the mucosa of lumpfish. However, detailed studies of normal intestinal morphology in lumpfish is still lacking.

Production of digestive enzymes is affected by a number of factors in fish, such as feeding habits, food preferences and diet formulations (Castro et al., 2013; Pavasovic et al., 2007). Protein digestion in intestine happens mainly by the action of alkaline proteases such as trypsin, chymotrypsin, and cytosolic peptidases (Zambonino Infante and Cahu, 2001). Our nested ANOVA results showed that a few enzyme activities had a significance difference across diets, while the effect of diet on the overall changes observed in the enzyme activities was limited, since PC1_Enzymes was not significantly different across the dietary treatments. In addition, PC1_Enzymes had low loadings across all enzymes, indicating that the changes observed were of equal scale.

In this study aminopeptidase activity was not affected by diet, in contrast to observations of aminopeptidase in the Senegal sole (*Solea senegalensis*) fed 28% PPC in their diet, which lowered the activity compared to those fed both SBM and SPC (Rodiles et al., 2012). Similar effects were also reported for tilapia fed 100% SBM in their diets which reduced the aminopeptidase levels (Pervin et al., 2020).

Among the digestive proteinases, trypsin activates other enzymes initially generated as inactive zymogens, such as chymotrypsin or carbopeptidases, thus, it is referred to as a key enzyme in protein digestion (Ágeirsson et al., 1989). Intestinal proteases tended to be lower in the posterior (distal) intestine than in more anterior regions (Krogdahl et al., 2015a). Activity of trypsin is readily affected by plant protein ingredients in different ways. Previous studies reported a reduced trypsin activity in intestinal AI and MI of salmon fed SBM (Olli et al., 1994). Similarly, significantly lower trypsin levels in the DI found for the Senegal sole fed 28% PPC was suggested to be related to the presence of a noticeable amount of protease inhibitors in the PPC (Rodiles et al., 2012). On the other hand, Lilleeng et al. (2007), found increased trypsin-like activity in the distal intestinal wall of salmon fed 30% SBM diet. The authors proposed that the immune cells in the inflamed DI tissue could be a source of trypsin activity. Moreover, elevated trypsin activity in the digesta from distal intestinal region has been often reported for fish suffering from SBMIE (Chikwati et al., 2013; Krogdahl et al., 2003; Lilleeng et al., 2007), and similar kinds of inflammation detected in salmon fed 35% PPC (Penn et al., 2011). In contrast to the results reported in previous studies, the present work did not find any diet related trypsin changes.

Leucine aminopeptidase split the dipeptides and oligopeptides into free amino acids that can be absorbed into the enterocyte (Chikwati et al., 2013; Tibaldi et al., 2006). In the present study, leucine aminopeptidase activity was relatively uniform among experimental groups (Table S5). This is in line with results reported by Fraisse et al. (1981), who showed that leucine aminopeptidase was uniformly present along the gut of carp. Replacing 10–35% FM with SBM in diets for Atlantic salmon has gradually reduced leucine aminopeptidase activity in the DI (Chikwati et al., 2013; Krogdahl et al., 2003; Lilleeng et al., 2007; Marjara et al., 2012). The reduced BBM enzyme activities of maltase and leucine aminopeptidase in the distal intestinal mucosa with increasing dietary SBM levels was seen in salmon fed PPC (Øverland et al., 2009; Penn et al., 2011). The decreased leucine aminopeptidase activity in those studies mirrored the tissue dysfunctions resulting in SBMIE (Chikwati et al., 2013; Krogdahl et al., 2003).

Lumpfish are believed to be omnivorous, thus their carbohydrate utilization expected to be better than carnivorous fish. Based on nested ANOVA, reduced AMY activity found in PC in fish fed the PP75 diet, which is in agreement with the study on juvenile Chinese sucker (*Myxocyprinus Asiaticus*) fed 60% of SBM (Yu et al., 2013).

Condition factors are used for comparing the ‘condition’, ‘fitness’, or ‘well-being’ of fish, based on the assumption that heavier fish of a given length are in better condition (Froese, 2006). In our study, the control diet showed overall better growth performance, but not at a significant level. Here, we also employed PCA to assess the overall health status of the intestine, as well as the enzyme activities and how they were related to CF individually. This approach has been used before in histological assessment in mice and to our knowledge not yet in fish and not in relation to growth (McNulty et al., 2011). Indeed, PC1_Enteritis and PC1_Enzymes were associated with growth performance, as it has been recommended by Vatsos (2021). The regression of the PC1_Enteritis with CF resulted in a negative coefficient (i.e. -0.008; Table 4), which showed that fish with higher PC1_Enteritis scores were associated with lower weight. Having increased values on PC1_Enteritis meant having

lower mucosal fold height and muscular thickness and higher number of goblet cells and lamina propria width which is representative of intestinal inflammation. The causal pathways that a feed can induce lower CF are multiple. In this study, it was seen that histological alterations were related both with CF and dietary treatments revealing their intermediary position to that relationship. Thus, the non-significance of the CF differences across diets, was most likely due to small sample size and/or limited experimental period as previously mentioned. For the enzyme activities that was not the case. Although, they have an effect on CF, which noted their important role on growth, they were equal across diets, showing that these diets could not have affected CF by limiting enzyme activity.

5. Conclusion

In this study PCA was used to study the overall effect of the diets on the architecture of intestine and the enzyme activities and subsequently, to associate any observed changes with the growth of the individual fish. Examination of individual indices can provide some specific information, but a broader evaluation can give more informative assessment, particularly in relation to the growth performance. Many other factors, such as variations in amino acid profile, and digestibility (not investigated in this study) could also exert an adverse impact on growth. Based on our assessment, diet had significant effects on individual variables in both histology (excluding mucosal complexity) and enzymes, but it was significantly associated with the main common changes observed in the former only. Overall, histological evaluation confirmed that lumpfish intestinal morphometry was significantly altered by plant protein ingredients at 25%, 50% and 75% levels, but replacing of FM up to 50% did not affect the growth and the enzyme activities up to 75% inclusion level.

CRediT authorship contribution statement

Florence Perera Willora: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Ioannis N. Vatsos:** Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Panagiotis Mallioris:** Methodology, Software, Formal analysis, Investigation, Visualization, Writing – review & editing. **Francesco Bordignon:** Investigation. **Sven Keizer:** Investigation. **Silvia Martınez-Llorens:** Investigation, Writing – review & editing. **Mette Sørensen:** Conceptualization, Methodology, Validation, Resources, Supervision, Project administration, Writing – review & editing. **Ørjan Hagen:** Conceptualization, Methodology, Validation, Resources, Supervision, Project administration, Writing – review & editing.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

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