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In vitro digestibility based on fish crude enzyme extract for prediction of feed quality in growth trials

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Abstract: Biochemical structure of protein (reactive SH content, content ratio of SH/S—S and concentration of D-Asp as % of total (D+L)-Asp) indicating digestibility of dietary protein was changed under different processing conditions. Based on fish crude enzyme extract, *in vitro* digestibility of different fish materials processed under different conditions correlated positively with reactive SH content and content ratio of SH/S—S and negatively with D-Asp concentration. *In vitro* digestion of different experimental feeds, based on Atlantic salmon crude enzyme extracts, was studied in association with growth trials in order to investigate its value as a criterion for industrial strategy in predicting feed quality. Crude enzymes were extracted from the pyloric caeca before feeding. Significant differences in *in vitro* digestibility between the experimental feeds were observed whereby there would be differences in feed conversion efficiency within 3 months of feeding. There were associations between the *in vitro* digestibility and other parameters for dietary quality, such as mink digestibility and the biochemical structure parameters of the dietary protein due to different processing conditions. Crude enzyme extracts from rainbow trout and European seabass were also used for *in vitro* digestibility study of different experimental feeds by standardising trypsin activity to that of Atlantic salmon crude enzyme extract. The results indicated that different fish species have different digestion ability to the same feed types, and the effective time for feed utilisation and growth is dependent on fish sensitivity and the extent of difference in digestibility between the feeds consumed as observed in the Atlantic salmon trials. For the species investigated, sensitivity ranking of the enzymes to feed quality under the condition studied was Atlantic salmon > rainbow trout > European seabass. The results indicated that *in vitro* digestibility study of experimental feeds using pyloric caecal crude enzyme extract from a specific species at an age of interest could be a practical, quick and reliable method for testing feed quality in growth trials. By standardising the crude enzyme extract with regards to trypsin activity, the *in vitro* digestibility values could be comparable not only within the same species but also between different species.
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Keywords: fish meal; fish feed; *in vitro* digestibility; trypsin; chymotrypsin; sulphydryl group; disulphide bond; D/L-aspartic acid; Atlantic salmon; European seabass; rainbow trout; mink

INTRODUCTION

In vitro digestion is being used to predict the quality of experimental feeds. Many *in vitro* methods have been developed and tested for measuring digestibility of different dietary proteins.[1–3] So far the methods have not been fully validated for industrial use, as the enzymes used for digestion were not suitable and/or optimised.[4–7] The enzymes from animal intestine, such as intestinal fluid from pig[8] and caecal enzymes from salmonids,[3,9] were reported to be appropriate for use in the *in vitro* digestibility study of dietary proteins. To optimise the use of animal intestinal enzymes for *in vitro* digestibility study, the *in vitro* digestion process should be standardised with regards to proteolytic activity of the animal crude enzyme extract. Trypsin is a primary important factor affecting feed conversion efficiency (FCE) and specific growth rate (SGR).[10–12] Studies on trypsin isozymes in the pyloric caeca of

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salmonids, especially Atlantic salmon (*Salmo salar* L), have illustrated the special role of trypsin as a key enzyme in the digestion process.[13] Besides, enzyme extracts from pyloric caeca of Atlantic salmon with different trypsin phenotypes showed different digestion ability *in vitro* for the same dietary proteins.[14] Therefore trypsin/trypsin-like activity is suggested as reference for standardising the enzymatic activity in the crude extracts employed *in vitro*.

To evaluate the method, dietary quality was studied using *in vitro* digestibility by Atlantic salmon crude enzyme extracts standardised with regards to trypsin activity, in association with *in vivo* trials for FCE, SGR and protease activities of trypsin and chymotrypsin after feeding. In comparison with other parameters for dietary quality, the parameters showing biochemical structure (contents of SH group/S—S bond[15,16]) and aspartic acid racemisation (D-Asp as % of total (D+L)-Asp[17,18]), as affected by different processing conditions, were determined. Mink digestibility was performed as it has been used for estimating dietary protein quality for salmonids. In order to compare different fish species, *in vitro* digestibility of the experimental diets by crude enzyme extracts from rainbow trout (*Oncorhynchus mykiss* Walbaum) and European seabass (*Dicentrarchus labrax* L) was also determined.

EXPERIMENTAL

Fish materials, fishmeals and experimental fish feeds

Twenty-seven different fish materials (laboratory-scale preparations of fish meal) were provided by the Norwegian Herring Oil and Meal Industry Research Institute (Fyllingsdalen, Norway). The first set of 13 fish materials was treated at different temperatures (70, 95 and 120 °C) for times varying from 5 to 120 min. The second set of 13 fish materials was treated at a fixed temperature of 95 °C for different times (10–120 min) with pH reduced from 7 to 4 by formic acid, or oxygen pressure reduced from atmospheric pressure to <0.8%, or moisture content reduced from 80% to 30 or 15% by freeze-drying. A control fish material was produced under normal conditions of atmospheric pressure, unheated, pH 7 and 80% moisture. Details on production of these fish materials were described by Luzzana *et al.* [18]

Four different fish meals, FM1, FM2, FM3 and FM4, were used as the main dietary protein sources for production of experimental feeds. The fish meals FM1 and FM2 were produced in a pilot plant (Norwegian Herring Oil and Meal Industry Research Institute, Fyllingsdalen, Norway) under low- and high-temperature exposure respectively as described by Opstvedt *et al.* [19] The fish meals FM3 and FM4 were commercially produced by Norsildmel AS (Bergen, Norway) and certified as low-temperature fish meal Norse LT94® and a lower-quality standard NorseaMink® respectively. Three different extrusion conditions (ET1, gentle/low; ET2, medium; ET3, tough/high temperature) were used for producing three different feed qualities from each fish meal type.[19] Altogether, 12 experimental feeds were produced. The feeds FM1ET1, FM1ET2, FM1ET3, FM2ET1, FM2ET2 and FM2ET3 were produced by the Norwegian Herring Oil and Meal Industry Research Institute in a pilot plant and were used in Experiment 1 as described below. The feeds FM3ET1, FM3ET2, FM3ET3, FM4ET1, FM4ET2 and FM4ET3 were produced in a commercial plant by ASA srl-Agridea (San Martino BA, Verona, Italy) and were used in Experiment 2 as described below. The compositions of the experimental feeds are shown in Table 1.

Table 1. Composition of experimental feeds

	Atlantic salmon experiments				*Rainbow trout and European seabass experiments*			
Feed composition	*FM1ET*	*FM2ET*	*FM3ET*	*FM4ET*	*FM1ET*	*FM2ET*	*FM3ET*	*FM4ET*
Composition ($g\,kg^{-1}$)								
Herring meal	651	651	656	656	716	716	721	721
Fish oil	208	205	200	200	130	125	120	120
Wheat flour	126	129	129	129	139	144	144	144
Choline chloride (70%)	1	1	1	1	1	1	1	1
Vitamin C[a]	1	1	1	1	1	1	1	1
Vitamin/mineral premix[a]	3	3	3	3	3	3	3	3
Analytical values (%)								
Moisture	5.4	4.7	6.5	7.2	5.3	5.4	8.2	8.2
Crude protein	50.7	51.2	38.9	39.3	55.6	55.4	53.7	53.7
Crude fat	25.5	25.7	27.6	27.4	18.1	18.6	18.5	18.5
Ash	10.2	9.8	7.9	8.5	11.2	10.9	8.1	8.1
Nitrogen-free extract[b]	8.2	8.6	19.1	17.6	9.8	9.7	11.5	11.5

[a] Providing per kg of feed: 250 mg vitamin C (Rovimix Saty C 35%), 3000 IU vitamin A acetate, 1600 IU vitamin D_3, 160 IU α-tocopheryl acetate, 12 mg vitamin K_3, 12 mg thiamine-HCl, 24 mg riboflavin, 12 mg pyridoxine-HCl, 120 mg niacin, 6 mg folate, 0.024 mg vitamin B_{12}, 0.6 mg biotin, 48 mg Ca-pantothenate, 3 mg Cu as $CuSO_4 \cdot 5H_2O$, 2.4 mg I as $Ca(IO_3)_2$, 24 mg Fe as $FeSO_4 \cdot 7H_2O$, 21 mg Mn as MnO_2, 30 mg Zn as ZnO and 0.1 mg Se as Na_2SeO_3.

[b] Calculated by difference.

Determination of reactive sulphydryl (SH) group of cysteine

Formation of disulphide (S—S) bonds of cystine from pairs of cysteine in proteins of the fish materials, fish meals and fish feeds due to the different processing conditions was studied. The content of reactive sulphydryl (SH) group of cysteine in the dietary protein was determined by a newly developed monobromobimane method modified from Cotgreave and Moldèus.[20] Approximately 20 g of fish product was extracted with petroleum spirit (BP 40–60 °C) for 2 h using Soxhlet extraction apparatus. The extracted material was air-dried and ground to a fine powder using a mortar and pestle. The defatted and ground fish sample of 15 mg was homogenised on ice for 2 min with 4740 µl of degassed 0.2 M Tris-HCl buffer (pH 7.4) containing 0.2 M EDTA. The solution was added to 60 µl of 0.092 M monobromobimane (thiolyte) reagent and 1.2 ml of 10% SDS and mixed on a wheel (a test tube rotator) in the dark for 1 h at 37 °C. Protein in 0.3 ml of the solution was precipitated with 80% acetone on ice for 30 min. The precipitate was washed twice with 80% acetone and dissolved (30 min on the wheel) in 1 ml of the Tris/EDTA buffer containing 2% SDS and 40 mM NaCl (and also 10 µg of proteinase K if the material was fish feed). A 1 ml aliquot of the reaction mixture was diluted with 3 ml of Tris/EDTA buffer before measuring fluorescence at excitation and emission wavelengths of 394 and 475 nm respectively. β-Lactoglobulin of known SH content (2 mol mol^{-1} dimer) was used as a standard protein range of 0–0.044 µmol SH (in 0.5% sulphosalicylic acid). An *N*-ethylmaleimide (NEM)-treated fish sample was used as a negative control (background values) by adding to 15 mg of the defatted and ground fish sample 4140 µl of the Tris/EDTA buffer and 600 µl of NEM (25 mg ml^{-1} in the same buffer) before the addition of monobromobimane and following the above procedure. Total content of SH groups was determined using Ellman's method[21] as described by Opstvedt *et al*,[16] after reduction with sodium borohydride ($NaBH_4$),[22] and reduced glutathione as reference standard, with the following modifications: NEM-treated samples were used as negative controls (background values) together with reagent blanks (background values plus reagent values were never higher than 10% of the sample values.). The content of the SH group of cysteine forming the S—S bond of cystine (referred to as S—S content) was calculated by the difference between the total SH group and the reactive SH group.

Determination of D-aspartic acid content

Racemisation of L-Asp to the corresponding D-enantiomer in the fish materials, fish meals and fish feeds due to the different processing conditions was measured as described by Luzzana *et al.* [18] Briefly, samples were hydrolysed in 6 M HCl at 100 °C for 6 h to reduce the degree of hydrolysis-induced racemisation.[23] D-Asp content was determined by RP-HPLC with fluorescence detection after a pre-column automatic derivatisation with *o*-phthaldialdehyde together with *N*-isobutyryl-D-cysteine.[24] The results of D-Asp (%) are expressed in relation to total (D + L)-Asp, calculated as $100 \times$ D/(D + L),[25] and referred to as % D/(D + L).

Determination of enzymatic activities of trypsin and chymotrypsin

Trypsin/trypsin-like and chymotrypsin/chymotrypsin-like activities of pyloric caecal crude enzyme extract were determined using the method of Rungruangsak-Torrissen and Sundby[26] modified by studying the initial reaction as described by Sunde *et al.* [12] Briefly, in a reaction mixture containing 1000 µl of trypsin substrate (1.25 mM benzoyl-L-arginine-*p*-nitroanilide dissolved in 5% dimethylformamide and made up to solution with 0.2 M Tris-HCl buffer, pH 8.4) and 10 µl of crude enzyme extract, trypsin activity was determined by measuring the rate of *p*-nitroaniline production during the first 30–60 s of reaction at an optimal temperature of 50 °C[13] at the absorbance of 410 nm. Chymotrypsin activity was similarly determined in a reaction mixture containing 1000 µl of substrate (0.1 mM *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide dissolved in 5% dimethylformamide and made up to solution with 0.2 M Tris-HCl buffer, pH 8.4) and 10 µl of crude enzyme extract by measuring the rate of *p*-nitroaniline production during the first 30–60 s of reaction at an optimal temperature of 40 °C[13] at the absorbance of 410 nm.

Protein concentration in the pyloric caecal crude enzyme extract was determined by Lowry *et al*'s method[27] using the Bio-Rad DC (detergent-compatible) protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The enzyme activity was expressed as µmol *p*-nitroaniline produced h^{-1} mg^{-1} protein.

In vitro digestibility

In vitro digestibilities of different fish materials and fish meals were investigated using an enzyme extract from the pyloric caeca of Atlantic salmon of about 1 kg weight. For the experimental feeds, *in vitro* digestibilities were determined using crude enzyme extracts from the fish in the experiments described later. The crude enzyme extract was prepared and the *in vitro* digestibility study was performed for each fish sample using the method modified from Bassompierre *et al*[14] as described below.

Crude enzymes were extracted from the pyloric caeca. After removing the fat by dissection, the pyloric caeca were homogenised (1:3 w/v) in 50 mM Tris buffer (pH 8.0) containing 200 mM NaCl at 4 °C. The homogenate was centrifuged at 15 000 × *g* for 60 min at 4 °C and the supernatant was dialysed against 10 mM phosphate buffer (pH 7.8) overnight at 4 °C using a Pierce Slide-A-Lyzer® dialysis cassette (Pierce Chemical Co, USA). The dialysed crude enzyme extract was obtained by centrifuging at 15 000 × *g* for 60 min at 4 °C and kept frozen at

−80 °C until final use. Trypsin activity of each dialysed crude enzyme extract was determined using the method described earlier.

In vitro digestibility was performed in approximately 20 mg of sample. To the known amount of ground sample, 40 ml of 10 mM phosphate buffer (pH 8.2) and 0.2 ml of 0.5% chloramphenicol (in 96% ethanol) were added and mixed thoroughly. The mixture was incubated overnight in a shaking incubator at a controlled temperature of 15 °C. The samples were run in triplicate. Before performing digestion, 0.5 ml of each mixture was sampled as a control, immediately heated at 100 °C for 5 min to inactivate the enzymes, and rapidly frozen at −80 °C for later determination of control reactive amino group using the trinitrobenzene sulphonic acid (TNBS) assay[1,14] as described below. *In vitro* digestion was started by adding 0.5 ml of the dialysed crude enzyme extract. The digestion process was performed in a shaking incubator at a controlled temperature of 15 °C to complete digestion. After 24 h of digestion, at least 0.3 ml of each digested mixture was sampled, immediately heated at 100 °C for 5 min and rapidly frozen at −80 °C for later determination of liberated reactive amino group of the peptides produced using the TNBS method[1,14] as described below.

A solution of 0.2 ml of either the undigested control (0 h) or the digested mixture (24 h) with 2 ml of 10 mM phosphate buffer (pH 8.2) was mixed thoroughly with 1 ml of 0.1% TNBS in 10 mM phosphate buffer (pH 8.2) and incubated at 60 °C for 1 h in the dark. The reaction was stopped by adding 1 ml of 1 M HCl and cooling to room temperature. The absorbance was measured at 420 nm and the concentration of the reactive amino group was calculated using DL-alanine as a standard.

The *in vitro* digestibility was expressed as µmol DL-alanine equivalent liberated reactive amino group of cleaved peptides per 100 µg sample.

Mink digestibility

The study of true protein digestibility of fish feeds in mink (*Mustela vison*; standard 2 kg mature male fed the experimental feeds for 7 days) was performed at the Norwegian Herring Oil and Meal Industry Research Institute using the method modified from Skrede[28] as described by Opstvedt *et al.*[19] Digestibility in mink was determined by total faecal collection. Nitrogen (N) content was analysed in freeze-dried samples of feeds and faeces according to ISO 5983,[29] and protein calculated as 6.25 × N. Corrected 'true' digestibility was calculated using standard values for endogenous nitrogen excretion.[28]

In vitro digestibility in comparison with growth study in Atlantic salmon trials

Experiment 1

The experiment was carried out at Matre Aquaculture Research Station, Norway. Atlantic salmon of about 150 g weight were raised in 3 m^3 circular tanks (110 fish per tank) supplied with seawater of salinity 30–33 (on the Practical Salinity Scale 1978) at an average temperature of 10.8 ± 0.2 °C for 3 months. The fish were fed in triplicate the six experimental feeds FM1ET1, FM1ET2, FM1ET3, FM2ET1, FM2ET2 and FM2ET3 (18 rearing units) at a daily feeding rate of 1% body weight for 6 h by automatic feeder from 08:30 to 14:30. Daily consumption rate of each tank was recorded by collecting surplus feed. Feed conversion efficiency (FCE) was calculated as the ratio of weight gain to the amount of feed consumed during the whole experimental period. Fish weights were measured individually at the start and every 30 days until the end of the experiment at day 90. Specific growth rate (SGR) of the fish in each tank was calculated according to Houde and Schekter[30] as

$$\mathrm{SGR}(\%\ \mathrm{day}^{-1}) = 100(e^{g} - 1)$$

where $g = (\ln W_t - \ln W_0)/(t - t_0)$, W_t is the weight at day t and W_0 is the weight at day t_0.

In order to study whether *in vitro* digestibility could be used as a reliable indicator of feed quality to predict feed utilisation and growth of the fish, enzyme extracts from Atlantic salmon were obtained at the start before feeding the experimental diets (day 0) and at the end of the growth trial after 3 months of feeding (day 90). Pyloric caecal samples were collected from each tank, from three fish at the start and 25 fish at the end of the experiment. The 54 (3 × 18) samples at the start were pooled, extracted for crude enzymes and used for the *in vitro* digestibility study (as described earlier) of the six experimental feeds. For the 25 samples at the termination, each sample was divided into two parts. The first part was used for determinations of trypsin and chymotrypsin activities for studying development of the enzyme activities of individual salmon after feeding. The pyloric caeca were extracted in 1 mM HCl (1:5 w/v) at 4 °C. After centrifuging at 15 000 × *g* for 60 min at 4 °C, the supernatant was kept frozen at −80 °C until final use. Trypsin and chymotrypsin activities were determined using the method of Rungruangsak-Torrissen and Sundby[26] modified by studying the initial reaction according to Sunde *et al*[12] as described earlier. The second parts of the 25 samples were pooled for each tank, extracted and used for the *in vitro* digestibility study (as described earlier) of each respective experimental feed used for each tank. Trypsin in all pooled enzyme extracts for the *in vitro* digestibility study was adjusted by dilution with 10 mM phosphate buffer (pH 7.8) to the same activity before use. To make it practical, standardised trypsin activity in the crude enzyme extracts was chosen from the pooled extract with lowest enzyme activity, and the extracts were not diluted to the same activity as for the fish material study as it was not necessary for comparison.

Experiment 2

The six experimental feeds FM3ET1, FM3ET2,

FM3ET3, FM4ET1, FM4ET2 and FM4ET3 were used. The experiment was carried out in duplicate (12 rearing units) at Matre Aquaculture Research Station, Norway. Atlantic salmon of about 2 kg weight were raised in $5 \times 5 \times 5\,m^3$ rectangular sea cages (150 fish per cage) at an average temperature of $13.4 \pm 0.1\,°C$ for 3 months. The fish in each sea cage were fed manually to satiation for about 30 min each day and the amount of feed consumed was recorded. Fish weights were individually measured at the start (day 0) and every 30 days until the end of the experiment (day 90). FCE and SGR values were calculated as in Experiment 1.

Pyloric caecal samples were collected from each sea cage, from three fish at the start and 15 fish at the end of the experiment. The 36 (3×12) samples at the start were pooled, extracted and used for the *in vitro* digestibility study (as described earlier) of the six experimental feeds. For the 15 samples at the termination, each sample was divided into two parts. The first part was extracted and used for determinations of trypsin and chymotrypsin activities for studying development of the enzyme activities of individual salmon after feeding as described above. The second parts of the 15 samples were pooled for each tank, extracted and used for the *in vitro* digestibility study (as described earlier) of each respective experimental feed used for each tank. Trypsin in all pooled enzyme extracts for the *in vitro* digestibility study was adjusted by dilution with 10 mM phosphate buffer (pH 7.8) to the same activity as in Experiment 1 before use.

In vitro digestibility by enzyme extracts from different fish species

A new batch of 12 fish feeds was used. The feeds were produced under the same conditions as described for the experimental feeds for the Atlantic salmon experiments using the same fish meals FM1, FM2, FM3 and FM4. Compositions of the feeds are shown in Table 1. The contents of the SH group and S—S bond were determined in these feeds as described earlier.

In order to compare enzymes from different fish species, the *in vitro* digestibility of different experimental feeds was investigated using crude enzyme extracts from the pyloric caeca of rainbow trout (250–300 g) and European seabass (350–400 g). The sample of each species was pooled from five fish. The crude enzyme extracts were prepared from fish that had never been fed the experimental feeds before. The *in vitro* digestibility study was performed for each experimental feed as described earlier. In order to compare with the enzyme extracts from Atlantic salmon experiments at day 0, the enzyme extracts from different fish species were adjusted to have the same trypsin activity as in the salmon enzyme extract experiments before being used for the *in vitro* digestibility study.

Statistical analyses

The values are given as mean ± standard error of mean (SEM) throughout. Data were analysed using Statistica 5.1 software (StatSoft, OK, USA) and Microsoft Excel (Microsoft Corp, Redmond, WA, USA). Linear regression and analysis of variance at 95% significance level were performed. The *t*-test was used to compare values between groups.

RESULTS

Associations between *in vitro* digestibility by Atlantic salmon enzyme extract, SH content and D-Asp concentration of fish materials

The dialysed crude enzyme extract from 1 kg Atlantic salmon had a trypsin specific activity value of 272 µmol *p*-nitroaniline produced $h^{-1}\,mg^{-1}$ protein and was used for *in vitro* digestion of the fish materials. *In vitro* digestibility of the 27 fish materials studied clearly showed a significant linear correlation ($n = 27$, $R^2 = 0.68$, $P < 0.0001$) with reactive SH content without being affected by total SH content (Fig 1). This resulted in a significant correlation ($n = 27$, $R^2 = 0.78$, $P < 0.0001$) between *in vitro* digestibility and content ratio of SH/S—S (Fig 2). The *in vitro* digestibility method under the conditions studied seemed to be less sensitive if the difference in the SH group between the fish materials was less than 0.65 mmol per 100 g

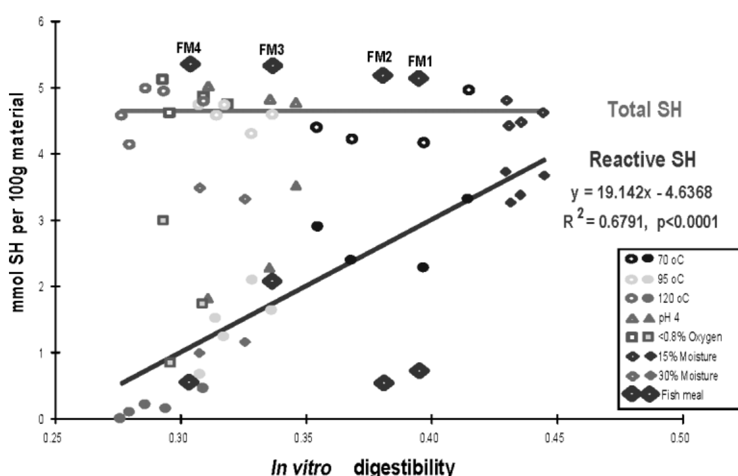

Figure 1. Effect of reactive SH content in 27 fish materials produced under different processing conditions on *in vitro* digestibility (µmol DL-alanine equivalent liberated reactive amino group of peptides produced per 100 µg fish material) by Atlantic salmon pyloric caecal crude enzyme extract having trypsin specific activity of 272 µmol *p*-nitroaniline produced $h^{-1}\,mg^{-1}$ protein. The values of the fish meals FM1, FM2, FM3 and FM4 are also illustrated (not included in the regression). The range of standard error of mean (SEM) for SH content was 0.013–0.028 mmol SH per 100 g sample, and for *in vitro* digestibility 0.004–0.010 µmol DL-alanine equivalent liberated reactive amino group of peptides produced per 100 µg sample.

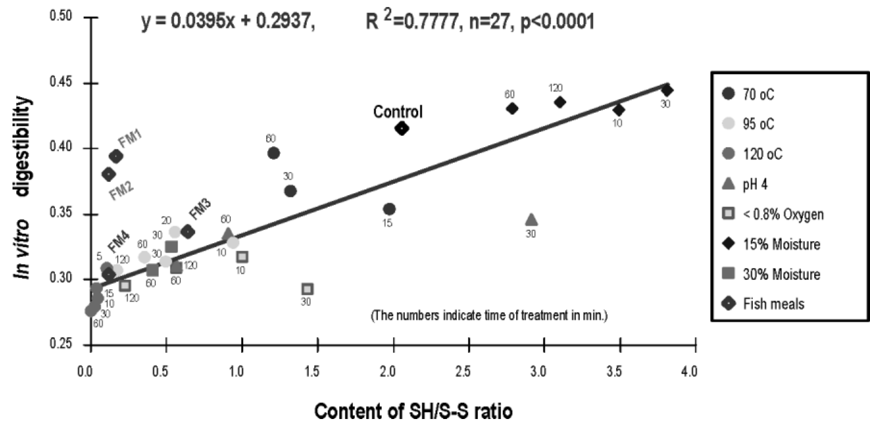

Figure 2. Relationship between *in vitro* digestibility (μmol DL-alanine equivalent liberated reactive amino group of peptides produced per 100 μg fish material) by Atlantic salmon pyloric caecal crude enzyme extract (having trypsin specific activity of 272 μmol *p*-nitroaniline produced $h^{-1}mg^{-1}$ protein) and SH/S—S content ratio of 27 different fish materials produced under different processing conditions. The values of the fish meals FM1, FM2, FM3 and FM4 are also illustrated (not included in the regression). The range of standard error of mean (SEM) for SH/S—S content ratio was 0.007–0.011, and for *in vitro* digestibility 0.004–0.010 μmol DL-alanine equivalent liberated reactive amino group of peptides produced per 100 μg sample.

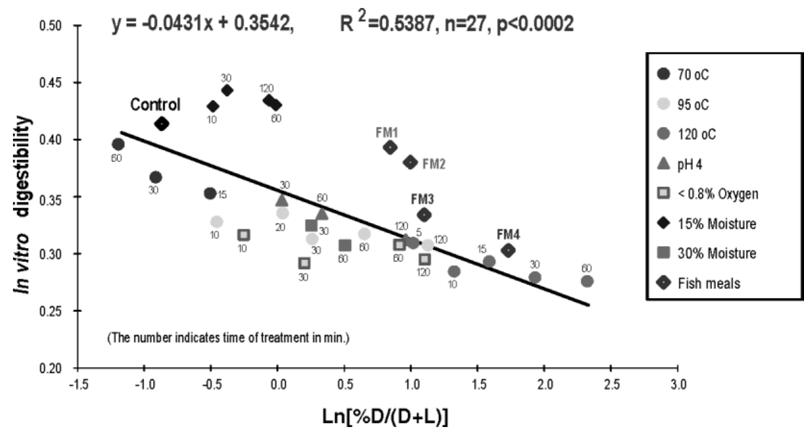

Figure 3. Relationship between *in vitro* digestibility (μmol DL-alanine equivalent liberated reactive amino group of peptides produced per 100 μg fish material) by Atlantic salmon pyloric caecal crude enzyme extract (having trypsin specific activity of 272 μmol *p*-nitroaniline produced $h^{-1}mg^{-1}$ protein) and D-Asp content (as ln[% D/(D + L)]) of 27 different fish materials produced under different processing conditions. The values of the fish meals FM1, FM2, FM3 and FM4 are also illustrated (not included in the regression). A single value of D-Asp content was obtained for each sample. The range of standard error of mean (SEM) for *in vitro* digestibility was 0.004–0.010 μmol DL-alanine equivalent liberated reactive amino group of peptides produced per 100 μg sample.

sample. *In vitro* digestibility inversely correlated ($n = 27$, $R^2 = 0.54$, $P < 0.0002$) with D-Asp concentration (Fig 3). The values of fish meal samples (FM1, FM2, FM3 and FM4) shown in Figs 1–3 are not included in the regressions.

Significant linear correlation ($n = 27$, $R^2 = 0.70$, $P < 0.0001$) was observed between changes in SH content of cysteine due to S—S formation of cystine and in Asp racemisation following different processing conditions (Fig 4). The values of the experimental fish meals and fish feeds are also shown but are not included in the regression.

Effects of different experimental feeds on *in vitro* digestibility in comparison with feed conversion efficiency (FCE) and specific growth rate (SGR) in Atlantic salmon experiments

Fish growth

The experimental feed FM2ET1 had to be studied in duplicate instead of triplicate owing to a problem of

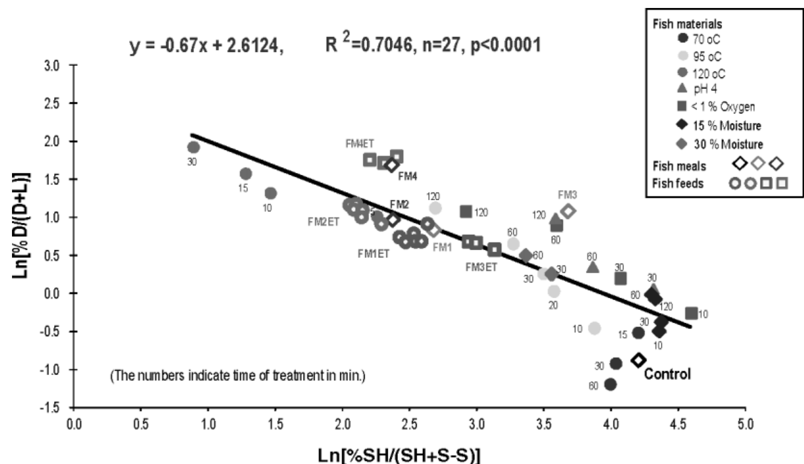

Figure 4. Relationship between SH and D-Asp contents of 27 different fish materials produced under different processing conditions. The values of four experimental fish meals, 12 experimental fish feeds in Atlantic salmon experiments and six experimental fish feeds (produced from fish meals FM1 and FM2) for *in vitro* digestibility study of rainbow trout and European seabass enzymes are also illustrated (not included in the regression). A single value of D-Asp content was obtained for each sample. The range of standard error of mean (SEM) for SH content was 0.53–1.27% SH/(SH + S—S).

water supply in one of the three tanks during the trial. An effect of feed quality was observed in the fish experiments within 3 months of the experimental period. Two-way ANOVA of the main factors fish meal (FM) types and extrusion (ET) conditions indicated that FM types, but not ET conditions, had significant effects ($P < 0.0001$) on both FCE and SGR values after 90 days of feeding. Therefore the results were grouped according to the fish meal types (FM1ET ($n = 9$), FM2ET ($n = 8$), FM3ET ($n = 6$) and FM4ET ($n = 6$)) and are shown in Table 2.

The SGR values increased during the second month of feeding in Experiment 1, while they decreased in Experiment 2, possibly owing to restricted feeding (Table 2). The fish showed a steady growth rate during the last 2 months, as stable SGR values were observed during days 30–90 in both experiments (Table 2). Significant differences in FCE of Atlantic salmon fed different experimental diets were observed between the feeds FM3ET and FM4ET in Experiment 2 ($P < 0.05$) but not between the feeds FM1ET and FM2ET in Experiment 1 (Table 2). There were no significant differences in SGR among fish fed different experimental feeds in either Experiment 1 or Experiment 2 (Table 2).

After 90 days of feeding, the SGR values of Atlantic salmon showed significant linear correlations positively with mink digestibility ($n = 18$, $R^2 = 0.74$ and $n = 12$, $R^2 = 0.69$, $P < 0.05$) and negatively with D-Asp concentration ($n = 18$, $R^2 = 0.72$ and $n = 12$, $R^2 = 0.69$, $P < 0.05$) of the experimental feeds in both Experiment 1 and Experiment 2, while positive correlation with SH content was potential ($n = 18$, $R^2 = 0.62$, and $n = 12$, $R^2 = 0.58$, $P < 0.082$) (data not shown). Significant linear correlation between SGR and FCE values ($n = 12$, $R^2 = 0.66$, $P < 0.05$), on the other hand, was observed only in Experiment 2 (data not shown), where differences in FCE values were detected between the feeds produced from the fish meals FM3 and FM4 (Table 2). No significant correlations ($P > 0.05$) were observed in either Experiment 1 or Experiment 2 between SGR and *in vitro* digestibility by the enzyme extracts from either day 0 or day 90. Although the FCE values were significantly different ($P < 0.05$) only in Experiment 2, they showed an association with *in vitro* digestibility within 3 months of feeding in both experiments (see Table 2).

Comparison between different parameters for dietary quality

The trypsin activity value from the dialysed crude enzyme extract with the lowest activity was 454 μmol *p*-nitroaniline produced $h^{-1} ml^{-1}$ and this value was chosen for standardising the crude enzyme extracts for *in vitro* digestion study of the experimental fish feeds. Two-way ANOVA of the main factors FM types and ET conditions indicated that FM types, but not ET conditions, had significant effects ($P < 0.01$) on *in vitro* digestibility by the enzyme extracts from either day 0 or day 90. Therefore the results were also grouped according to the fish meal types (FM1ET ($n = 9$), FM2ET ($n = 8$), FM3ET ($n = 6$) and FM4ET ($n = 6$)).

A significant difference in *in vitro* digestibility ($P < 0.02$) by the crude enzymes of unadapted fish (at day 0) was observed in Experiment 2, where differences in FCE between the diets FM3ET and FM4ET were detected within 90 days of feeding (Table 2). On the other hand, *in vitro* digestibility by the enzyme extracts of adapted fish (at day 90) showed significant differences ($P < 0.04$) between the diets FM1ET and FM2ET in Experiment 1 (Table 2), suggesting a potential of differences in FCE between these groups within the next 3 months if the experiment had been prolonged. The 150 g Atlantic salmon

Table 2. Relationship between different quality parameters of experimental feeds expressed as content of SH group of cysteine and S—S bond of cystine, % D/(D+L)-aspartic acid, mink digestibility and *in vitro* digestibility (μmol DL-alanine equivalent liberated reactive amino group of peptides produced per 100 μg feed), together with feed conversion efficiency (FCE), specific growth rate (SGR) and protease activity ratio of trypsin to chymotrypsin (T/C) in growth trials. The values with an asterisk and with different letters are significantly different ($P < 0.05$)

	Experiment 1		*Experiment 2*	
Parameter	*FM1ET*	*FM2ET*	*FM3ET*	*FM4ET*
SH/S—S content ratio	0.147 ± 0.011a	0.096 ± 0.007b	0.258 ± 0.020c	0.112 ± 0.007a
% SH/(SH + S—S)	12.81 ± 0.81a	8.74 ± 0.56b	20.45 ± 1.27c	10.06 ± 0.53b
% D/(D + L)-aspartic acid	2.20 ± 0.15a	2.77 ± 0.18a*	1.93 ± 0.07a*	5.83 ± 0.12c*
Mink digestibility (%)	91.41 ± 0.35a	89.13 ± 0.48b	91.17 ± 0.12a	87.07 ± 0.17c
In vitro digestibility at day 0	0.352 ± 0.026	0.299 ± 0.015	0.162 ± 0.007*	0.116 ± 0.004*
In vitro digestibility at day 90	0.235 ± 0.018*	0.176 ± 0.019*	0.122 ± 0.009	0.106 ± 0.008
FCE	1.47 ± 0.01	1.44 ± 0.02	0.93 ± 0.03*	0.84 ± 0.04*
SGR (% day^{-1})				
Day 0–30	0.84 ± 0.04	0.85 ± 0.03	0.68 ± 0.03	0.56 ± 0.04
Day 30–60	1.17 ± 0.03	1.08 ± 0.04	0.34 ± 0.01	0.29 ± 0.04
Day 60–90	1.10 ± 0.01	1.07 ± 0.01	0.35 ± 0.02	0.34 ± 0.03
Day 0–90	1.05 ± 0.01	1.01 ± 0.01	0.48 ± 0.02	0.42 ± 0.01
T/C ratio at day 90	0.327 ± 0.018	0.377 ± 0.033	0.693 ± 0.068*	1.063 ± 0.067*

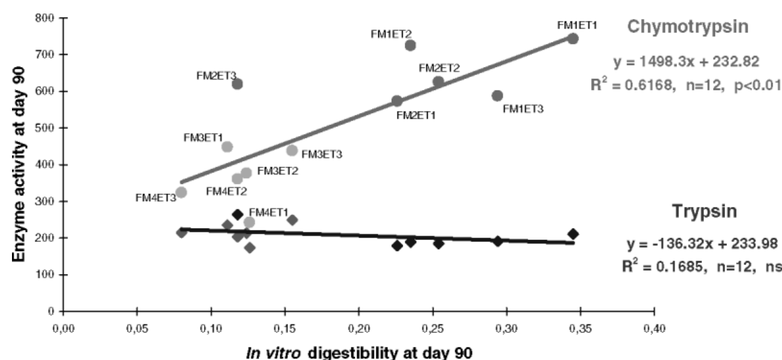

Figure 5. Relationship between *in vitro* digestibility (μmol DL-alanine equivalent liberated reactive amino group of peptides produced per 100 μg feed) and trypsin and chymotrypsin specific activities (μmol *p*-nitroaniline produced $h^{-1} mg^{-1}$ protein) after feeding (at day 90) of Atlantic salmon, during adaptation/steady growth phase, fed different experimental feeds in both Experiment 1 and Experiment 2. The same trypsin activity of 454 μmol *p*-nitroaniline produced $h^{-1} ml^{-1}$ was used for *in vitro* assay. Each *in vitro* assay was run in triplicate and determinations of trypsin and chymotrypsin activities were performed on individuals. The range of standard error of mean (SEM) for *in vitro* digestibility was 0.006–0.019 μmol DL-alanine equivalent liberated reactive amino group of peptides produced per 100 μg sample, and for enzyme specific activities 7–26 and 17–49 μmol *p*-nitroaniline produced $h^{-1} mg^{-1}$ protein for trypsin and chymotrypsin respectively. ns, Not significant.

in Experiment 1 showed higher FCE, SGR and *in vitro* digestibility at day 0 than the 2 kg fish in Experiment 2 (Table 2). A significant reduction ($P < 0.04$) in *in vitro* digestibility was observed at day 90 compared with the values at day 0 in Atlantic salmon in Experiment 1 (Table 2).

The differences in % SH group (4.07%) and % D-Asp content (0.57%) between the feeds in Experiment 1 were less than the differences in % SH group (10.39%) and % D-Asp content (3.90%) between the feeds in Experiment 2 (see Table 2). The results of differences in the *in vitro* digestibility and growth trials were in agreement with the extent of differences in the SH group and D-Asp contents between the experimental feeds and were dependent on fish size. Within each experiment, the higher the SH group and the lower the D-Asp contents the dietary proteins have in the experimental feeds, the higher are the mink digestibility, *in vitro* digestibility, FCE and SGR (Table 2).

Interestingly, induction of trypsin activity was similar while induction of chymotrypsin activity was different after 90 days of feeding of the different diets. This caused the *in vitro* digestibility at day 90 not to be correlated with trypsin activity, while it correlated significantly with chymotrypsin activity ($n = 12$, $R^2 = 0.62$, $P < 0.01$), regardless of fish size (Fig 5). An importance of protease activity ratio of trypsin to chymotrypsin (T/C ratio) for feed utilisation and growth in animals was observed. There were differences in the enzyme induction by feeding, and differences in the T/C ratio values were observed only between the FM3ET and FM4ET diets in Experiment 2 where a difference in FCE was detected (Table 2). At zero value of *in vitro* digestibility ($x = 0$) a very similar specific enzyme activity (234 and 233 μmol *p*-nitroaniline produced $h^{-1} mg^{-1}$ protein) of trypsin and chymotrypsin (T/C ratio = 1) was observed, probably indicating trypsin activating chymotrypsin with the same specific activity at normal biological condition when no digestion process occurred (Fig 5).

Comparison of *in vitro* digestibility of experimental feeds by enzyme extracts from different fish species

The % SH/(SH + S—S) content and SH/S—S ratio of the feeds FM1ET (12.37 ± 0.21% and 0.141 ± 0.003 respectively) and FM2ET (8.16 ± 0.18% and 0.089 ± 0.002 respectively) produced for the experiments on rainbow trout and European seabass were similar to the values of the feeds for Atlantic salmon in Experiment 1 (see Table 2 for salmon). On the other hand, the% SH content and SH/S—S ratio of the feeds FM3ET (11.17 ± 2.94% and 0.128 ± 0.036 respectively) and FM4ET (4.24 ± 0.33% and 0.044 ± 0.004 respectively) were lower than the values of the feeds for Atlantic salmon in Experiment 2 (see Table 2 for salmon). The differences between the feeds FM3ET and FM4ET for rainbow trout and European seabass (6.93% and 0.084 unit respectively) were less than between the Atlantic salmon feeds (10.39% and 0.15 unit respectively) (see Table 2 for salmon).

In vitro digestibility of the experimental feeds by the crude enzyme extracts from rainbow trout and European seabass in comparison with the crude enzyme extracts from Atlantic salmon in Experiment 1 and Experiment 2 are shown in Fig 6. Significant differences in feed quality were due to fish meal types (Fig 6(A)) but not due to extrusion conditions (Fig 6(B)). Rainbow trout and European seabass enzymes could differentiate the feeds FM1ET and FM2ET from the feeds FM3ET and FM4ET ($P < 0.05$) similarly to Atlantic salmon, but they could not differentiate between the feeds FM3ET and FM4ET as Atlantic salmon could (Fig 6(A)). This may be due to the fact that differences between the feeds FM3ET and FM4ET for rainbow trout and European seabass as described by the other chemical methods used (SH and D-Asp determinations) were less than those measured for Atlantic salmon feeds as explained earlier. *In vitro* digestibility of the feed group FM3ET, FM4ET was significantly lower ($P < 0.05$) than that of the feed group FM1ET, FM2ET by the enzyme extracts from every fish species studied (Figs 6(A) and

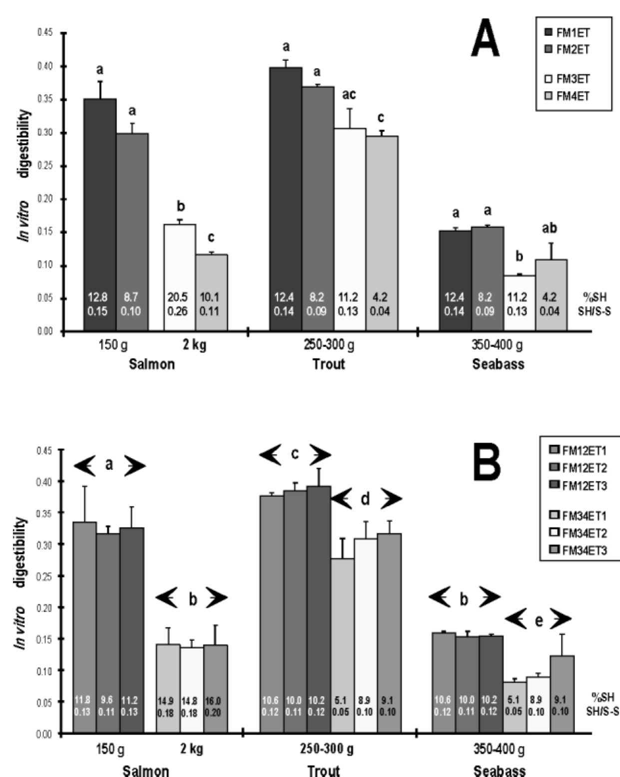

Figure 6. Association of SH content and SH/S—S content ratio of different experimental feeds with *in vitro* digestibility (μmol DL-alanine equivalent liberated reactive amino group of peptides produced per 100 μg feed) by crude enzyme extracts from different fish species that had never been fed experimental feeds before. The same trypsin activity (454 μmol *p*-nitroaniline produced h^{-1} ml^{-1}) in the dialysed enzyme extracts was used for all species. (A) *In vitro* digestibility grouped by fish meal types. Within species the bars with different letters are significantly different ($P < 0.05$). (B) *In vitro* digestibility grouped by extrusion conditions. The bars with different letters are significantly different ($P < 0.05$).

6(B)). Rainbow trout enzymes showed a higher *in vitro* digestion ability (Figs 6(A) and 6(B)), suggesting a possibility of higher feed utilisation than the other species studied under the same conditions at around 15 °C (temperature used for *in vitro* digestibility study). Considering all the enzyme extracts used having the same standardised trypsin activity, enzyme sensitivity to feed quality under the conditions studied could be ranked according to fish species as Atlantic salmon > rainbow trout > European seabass.

DISCUSSION

Quick and reliable analytical methods to test the quality of the feed produced are undoubtedly a major need for the aquafeed industry. The experiments described in the present paper suggest that in order to optimise the *in vitro* digestibility method, the enzymes for digestion should come from the pyloric caecal (intestinal) crude extract of a specific species at an age of interest, and digestion ability should be based on standardisation of enzymatic activity of trypsin for comparison.

In order to recommend the *in vitro* digestion method for testing feed quality, Atlantic salmon growth experiments were set up using different feeds of known different protein structures in terms of reactive SH content, SH/S—S ratio and D-Asp concentration due to different processing conditions. The content ratio SH/S—S may be suitable for use in comparison between different protein sources, while in the same protein source only the reactive SH content could be used. The results indicated that the FCE value was more sensitive than the SGR value (Table 2). SGR is the terminal indicator of protein quality after a number of changes in biochemical and physiological parameters, such as trypsin activity, tissue free amino acids and hormone insulin secretion.[26,31] Differences in the *in vitro* digestibility of diets using crude enzyme extracts before feeding (at day 0) were observed only when differences in FCE would be detected within the next 3 months of feeding (Table 2). This indicates that the differences in *in vitro* digestibility between the diets could predict potential differences in future growth of the animal studied. The effective time for feed utilisation and growth should be dependent on the extent of difference in digestibility between the feeds consumed (Table 2). Increases in S—S bond formation[16] and Asp racemisation[17,18,25] reduced the nutritional quality of fish proteins. *In vitro* digestibility correlated with these parameters (Figs 1–3) and could be used to detect an extent of difference between the diets (Table 2) as actually detected by the animal itself.

The nature of protein structure affected digestion ability of the fish through trypsin expression.[32,33] Luminal secretion of trypsin due to feeding resulted in high secretion of chymotrypsin,[13] which differed depending on dietary protein quality (Fig 5). This makes the activity ratio of trypsin to chymotrypsin (T/C) suitable as an indicator for digestion ability of the animal[12,13,34] and dietary quality (Table 2), similar to the *in vitro* digestibility by crude enzyme extract (Table 2). Trypsin is the key protease when growth potential is considered,[12,13,34] while chymotrypsin seems to play a major role during starvation when there is no growth potential,[34] and this could affect the T/C ratio at different phases. During the growing phase the T/C value correlates with trypsin activity, and these values correlate with SGR.[12] Fish with potentially higher growth efficiency will have a higher T/C value during growth,[12] while the value will be lower during the adaptation/steady growth phase owing to a higher level of chymotrypsin activity as shown in the current experiments. An inverse association between lower T/C ratio and higher *in vitro* digestibility, regardless of fish size (Table 2), should be a common phenomenon of the non-growing/steady growth phase, as SGRs were stable during the last 2 months in both Experiment 1 and Experiment 2. It is interesting to note that during the steady growth phase, trypsin activity was also stable, while chymotrypsin activity varied. This has made trypsin the suitable enzyme for standardisation, as the *in vitro* digestion ability will depend on the variation in chymotrypsin activity in the crude enzyme extract. If

chymotrypsin had been used for standardisation, differences in the *in vitro* digestibility would be opposite, as the trypsin activity would become lower in the enzyme extract from fish fed higher digestible feed. Chymotrypsin activity is not suitable as a reference for standardisation if the crude enzyme extracts are from fish at the steady growth phase.

Study of *in vitro* digestibility using enzyme extracts from different fish species and based on the same trypsin activity indicated that rainbow trout enzymes did differentiate the experimental feeds similarly to Atlantic salmon enzymes, while the enzymes from European seabass did not (Fig 6). Atlantic salmon enzymes seemed to be the most sensitive to different feed quality compared with the enzymes from the other species studied. A higher *in vitro* digestibility by the rainbow trout enzymes indicated that this species could probably utilise the feeds better than Atlantic salmon and European seabass under the conditions studied (Fig 6). Based on Atlantic salmon experiments, the differences in *in vitro* digestibility between the two feed groups FM1ET, FM2ET and FM3ET, FM4ET by rainbow trout and European seabass enzymes (Fig 6) suggested that there would be a possibility for these two species to differentiate the two feed groups within 3 months of feeding.

Although trypsin activity in each enzyme extract used was the same, the *in vitro* digestibility values were different among fish species and fish ages (Fig 6). Therefore it is important to use enzyme extract from a specific species and at the age of interest to test the quality of the experimental feeds. If significant differences are observed in any of the parameters studied (mink digestibility, D-Asp content, SH content and *in vitro* digestibility by animal crude enzyme extract), significant differences in FCE and SGR could be expected. However, the time of growth difference will depend on the extent of quality difference between diets and fish sensitivity. Although there are differences in biochemical structure of the dietary proteins, there is a certain range of the difference for each fish species to differentiate the quality of the feeds by affecting feed utilisation and growth as shown in the current study. This seemed to make the *in vitro* digestibility less sensitive than the other methods studied, but it could predict an actual time effect on growth difference between the diets consumed, as *in vitro* digestibility associated with FCE values within 3 months of feeding, which is a common experimental period for growth study. The method might be able to be optimised by increasing the standardised trypsin activity by concentrating the crude enzyme extracts to ensure complete *in vitro* digestion, as well as increasing the concentration of phosphate buffer to ensure buffering capacity and using the actual rearing temperature of each species for *in vitro* digestibility study.

In vitro digestibility by animal crude enzyme extract seems to be a reliable indicator for the actual difference in feed quality as detected by the animal itself. *In vitro* digestibility based on trypsin activity of animal crude enzyme extract from a specific species and at the age of interest could be a practical, quick and reliable method for testing protein nutritional quality in diets. The method could be used before (without)/during growth trials depending on the extent of the differences in feed quality studied. By standardising the trypsin activity of the crude enzyme extracts, the *in vitro* digestibility values could be comparable not only within the same species but also between different species. The method should be applicable not only to fish but probably also to other animal species, and supports an ethical point of view in helping to reduce the number of experimental animals used.

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