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# Total replacement of fish meal with plant proteins in diets for Atlantic cod (*Gadus morhua* L.) II — Health aspects

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**Abstract**

Replicate groups of Atlantic cod were fed 5 diets in which fish meal protein was replaced with 25% (25 PP), 50% (50 PP), 75% (75 PP) and 100% (100 PP) of a plant protein mixture containing 50% wheat gluten, 36% soy protein concentrate and 14% bioprocessed soybean meal. After 28 weeks, fish were sacrificed and analysed for growth, feed conversion, nutrient digestibility (see [Hansen, A.-C., Rosenlund, G., Karlsen, Ø., Koppe, W., Hemre, G.-I., Accepted for publication. Total replacement of fish meal with plant proteins in diets for Atlantic cod (Gadus morhua L.) I: Effects on growth and protein retention. Aquaculture]) and health status. Except for small reductions in the mean cell volume and haemoglobin content of red blood corpuscles, health indicators in blood and plasma were mostly unaffected by diet. Although the apparent digestibility coefficient (ADC) of nutrients was only marginally affected by diet [Hansen, A.-C., Rosenlund, G., Karlsen, Ø., Koppe, W., Hemre, G.-I., Accepted for publication. Total replacement of fish meal with plant proteins in diets for Atlantic cod (Gadus morhua L.) I: Effects on growth and protein retention. Aquaculture], faeces moisture content increased non-linearly peaking in fish fed the 100 PP diet where dry matter was only 11% compared to 18% in the control fish. This indicates a progression towards a diarrhoea-like condition in 100 PP fish. Feeding plant proteins did not affect gut relative weight but did affect intestinal morphology. In particular, there was a general increase in cellularity of the lamina propria and a modest goblet cell hypertrophy and hyperplasia which peaked in fish fed the 100 PP diet. More severe enteritis-like conditions were rare and found in only two fish fed the 100 PP diet. The dietary effect on goblet cells may be caused by phytate or fibres rather than the primary enteritis-inducing compounds found in untreated soybean meal. Hindgut segments seemed to be more affected than midgut thus suggesting an up-concentration of the causative agents of intestinal alteration in hindgut regions. Soyasaponins were not degraded during gut transit although the various conjugates were hydrolysed increasing the level of un-conjugated soyasaponins in the faeces. The intestinal level of heat shock protein 70 (HSP70) was unaffected by diet except in one replicate pen of fish fed the 100 PP diet where expression increased fourfold compared to the

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other replicate and other diets. The reason for this remains obscure. In conclusion, the plant ingredients used in the present study appear not to affect cod health to any major degree when used to replace up to 75% of fish meal protein. An increase to 100% may induce enteritis-like conditions in the guts of some fish and also activate HSP70 stress-gene mRNA expression in the intestine.


*Keywords:* Atlantic cod; Plant protein; Digestibility; Feed conversion; Health; Histology; Intestine

## 1. Introduction

In Northern Europe, interest in farming Atlantic cod (*Gadus morhua* L.) has increased steadily over the past decade stimulated by the decline in landings from fisheries and the more predictable supply of juveniles for on-growing (Rosenlund and Skretting, 2006). Compared to more fatty-fish species like Atlantic salmon (*Salmo salar* L.), cod has a relatively low potential to utilize dietary lipid (18%; Rosenlund et al., 2004) and a high requirement for protein (>50%; Rosenlund et al., 2004). Until recently, the protein in cod diets has been based on expensive, high-quality fish meal, high inclusion levels that are neither cost-efficient nor sustainable, and might limit the interest or wishes of growth in this farming sector. Currently, most marine resources are exploited to the highest maximal level (FAO, 2002) and it is therefore essential to evaluate the potential for using more available plant proteins in cod diets.

Substantial effort is being invested in exploring the use of plant proteins as substitutes for fish meal and this presents several challenges. Firstly, the amino acid compositions of many plant proteins differ significantly from that of fish meal and feeding such diets may induce essential amino acid deficiencies that would restrict growth and protein utilisation unless supplemented (Espe et al., 2006; Hansen et al., 2007). Furthermore, most plant protein sources contain antinutritional factors that may affect growth, nutrient utilisation and fish welfare in general (Francis et al., 2001). In salmonids, antinutritional factors can decrease digestion and reduce utilisation of proteins leading to decreased growth rates (Krogdahl et al., 1994; Vielma et al., 2000). In addition, high levels of dietary soybean products may affect intestinal integrity. In salmonids, damage usually affects the distal part of the gut where cells show extensive endocytotic activity and high numbers of intracellular vacuoles. Damage is often characterized by changes in numbers of mucus-producing goblet cells (GCs), intracellular absorptive vacuoles, cellularity of the lamina propria, amount of connective tissue, and degrees of mucosal folding and infiltration of the epithelium or lamina propria by inflammatory cells (van den Ingh et al., 1991; Baeverfjord and Krogdahl, 1996). In extreme cases, massive necrosis is also common, a condition often referred to as soybean-induced enteritis (Baeverfjord and Krogdahl, 1996).

Although the full nature of antinutritional factors has yet to be established, it is acknowledged that most are found in the alcohol-soluble fraction, as addition of an alcohol extract to a standard diet produced intestinal histopathological alterations similar to that of soybean meal (Van den Ingh et al., 1996). Consequently, using soybean concentrates with the alcohol soluble fraction removed will produce growth rates comparable to fish fed a fish meal-based diet, as shown for rainbow trout, *Oncorhynchus mykiss*, (Mambrini et al., 1999). Recently, Knudsen et al. (2007) traced some of the components that cause enteritis in Atlantic salmon to a saponin-containing subfraction of soybean molasses which is a by-product of the aqueous alcohol soy protein concentrate production. In endothermic animals, soyasaponins are degraded by gut microflora (Gestetner et al., 1968; Hu et al., 2004a,b) but this may not occur in Atlantic salmon where they appear to resist degradation during gut transit (Knudsen et al., 2006). As these seem to play a key role in the onset of soybean-induced enteritis in Atlantic salmon, it is of interest to evaluate the fate of soyasaponins during gut passage in Atlantic cod.

Although feasible, extensive treatment of plant proteins is costly, and will significantly increase feed cost if included into practical diets. Furthermore, this process is not complete, and some antinutrients like phytic acid will remain in the meals (Anderson and Wolf, 1995). Consequently, much research has focused on finding maximum inclusion levels that do not cause harm to the fish, and mixtures of various meals that keep the level of each antinutritional factor low.

Atlantic cod is a promising fish species with regard to new protein sources, as it may tolerate high levels of dietary plant proteins better than salmonids. Recently, Albrektsen et al. (2006) found no negative effect on growth when cod were fed diets supplemented by up to 14% full fat soybean meal (which includes the alcohol soluble fraction) and 28% corn gluten (50% protein replacement). Refstie et al. (2006a,b), found no effect on

growth when extracted soybean meal was used at a level of 25% of the dietary protein but noted that feed intake increased and the apparent digestibility of fat and amino acids decreased. Similarly, when Hansen et al. (2006, 2007) tested various levels of solvent extracted soybean meal (7–30% of protein), corn gluten meal (11–45% of protein), mixtures thereof (19–72% of protein), and mixtures of soybean protein concentrate and wheat gluten (40 and 80% of protein), there were generally few negative effects on cod performance. The exceptions included a small decrease in protein digestibility with higher corn gluten and lower lipid digestibility with higher levels of soybean protein and corn gluten along with a general increase in feed conversion with higher plant protein levels. Hansen et al., 2007 fed cod over 28 weeks with diets in which up to 100% of the fish meal was substituted with plant protein mixtures consisting of wheat gluten, soy protein concentrate and bioprocessed soybean meal in a ratio of roughly 10:7:3. Specific growth rate was reduced in a dose-dependent manner from 0.34 in control fish to 0.29 at 75 PP, before dropping to 0.14 at 100 PP. This was accompanied by a gradual increase in feed conversion ratio (FCR) from 1.08 in controls to 1.49 in the 75 PP group while appetite in the 100 PP group was too low to obtain reliable FCR. Nutrient apparent digestibilities (ADC) were generally unaffected by diet, although a decrease in ADC of starch was observed at 100 PP.

In salmonids, growth and histology are good indicators of the adverse effects of plant nutrients on fish health and welfare. It is worthy of note that no histopathological findings have been reported in studies that have examined the effect of various plant protein sources on cod gastrointestinal tract (Hansen et al., 2006; Refstie et al. 2006b).

Alternative indicators may serve as more-accessible or "early warning" systems for fish performance. In rainbow trout, Martin et al. (2003) found that diets containing 30% of diet as soybean-protein altered the liver protein profile when compared with those fed a diet where 30% total protein was derived from non-soybean sources. Differences included altered levels of several stress proteins (i.e. heat shock proteins, HSPs), suggesting that the diets induced a physiological stress response, presumably related to antinutritional factors (Martin et al., 2003; Vilhelmsson et al., 2004). HSPs are a large family of conserved proteins present in all organisms including fish (Basu et al., 2002). HSP70 is known to assist the folding of nascent polypeptide chains, act as a molecular chaperone, and mediate the repair and degradation of altered or denatured proteins (Basu et al., 2002). It has been widely used as a biomarker of stress (Iwama et al., 1998) and is known to respond to suboptimal diet compositions (Martin et al., 2003; Hemre et al. 2004). Sagstad et al. (2007) found up-regulation of HSP70 expression in hindgut from Atlantic salmon fed diets with full fat soybean meal, and HPS70 was shown to protect the mucosa against toxins and ulcerogenic conditions in mammals (Otaka et al., 2006).

The present paper is the second in a series describing the effect of complete replacement of fish protein by plant protein (Hansen et al., 2007). The first paper described the effect on growth, feed conversion and nutrient digestibility while the present paper focuses on various health parameters, soyasaponin metabolism, intestinal histology and stress gene (HSP70) activation.

## 2. Material and methods

### 2.1. Fish and diets

The fish, diets and experimental design are given in Hansen et al., 2007. In brief, 950 Atlantic cod averaging 1652±6 g (mean±SEM) were distributed into 10 net cages (5×5×5 m) at Austevoll Aquaculture Research Station, Institute of Marine Research, Norway and fed 5 isonitrogenous and isoenergetic extruded diets containing 0 (FM), 25% (25 PP), 50% (50 PP), 75% (75 PP) or 100% (100 PP) of dietary protein as plant protein (Table 1). The latter was a fixed mixture of 50% wheat gluten, 36% soy protein concentrate and 14% bioprocessed soybean meal (Hamlet Protein, Horsens, Denmark). All diets were produced as 9 mm pellets by Skretting ARC (Stavanger, Norway). To avoid nutrient deficiencies, and to comply with the requirements suggested for rainbow trout by NRC (1993), the 100PP diet was further supplemented with methionine and lysine. In addition, the 75 PP and 100 PP diets contained added monosodium phosphate (MSP). Fish oil was added as main lipid source, but in order to adjust for the differences in the levels of the long n-3 fatty acids (EPA and DHA) in response to decreasing levels of fish meal, increasing levels of South American fish oil were added to diets with plant proteins. See Hansen et al. (2007) for further details on diet composition.

### 2.2. Sampling of material

Five fish from the start population and 5 from each cage at termination of trial were anaesthetized in 0.4% benzocaine and killed with a sharp blow to the head. Blood samples were immediately drawn from the caudal

Table 1
Feed ingredients (given as g/kg) and analyzed feed composition (given as g/100 g except for the gross energy given as MJ/kg)

| Diet | FM | 25 PP | 50 PP | 75 PP | 100 PP |
|---|---|---|---|---|---|
| *Feed ingredients* | | | | | |
| Fish meal [a] | 69.4 | 54.4 | 36.2 | 18.2 | 0 |
| Wheat gluten [b] | 0 | 8.6 | 17.8 | 27.6 | 36.9 |
| Soy protein concentrate [c] | 0 | 6.1 | 12.8 | 19.7 | 26.4 |
| Soya bean meal [d] | 0 | 2.3 | 4.8 | 7.4 | 10.0 |
| Wheat | 19.0 | 16.2 | 15.0 | 11.7 | 8.2 |
| Fish oil, Nordic | 11.2 | 11.4 | 11.6 | 11.8 | 12.3 |
| Fish oil, South-America | 0 | 0.5 | 1.3 | 2.0 | 2.6 |
| DL-methionine [e] | 0 | 0 | 0 | 0 | 0.028 |
| L-lysine [f] | 0 | 0 | 0 | 0 | 0.573 |
| Monosodium phosphate [g] | 0 | 0 | 0 | 1.1 | 2.7 |
| Constants [h] | 0.48 | 0.48 | 0.48 | 0.48 | 0.48 |
| *Analysed content* | | | | | |
| Crude protein | 50.7 | 52.2 | 53.3 | 53.8 | 53.2 |
| Crude lipid | 18.1 | 16.7 | 16.7 | 16.9 | 16.6 |
| Starch * | 11.2 | 10.2 | 10.1 | 8.2 | 6.8 |
| Dry matter | 91.8 | 91.6 | 93.2 | 92.4 | 92.2 |
| Ash ** | 10.8 | 9.3 | 7.4 | 5.9 | 4.9 |
| Rest[i].*** | 1.0 | 3.3 | 5.7 | 7.6 | 10.7 |
| Gross energy (MJ/kg) [j] | 21.1 | 20.8 | 21.0 | 20.9 | 20.4 |

[a] Norse LT, Vedde Herring Oil Factory, Egersund, Norway.
[b] Gluvtal 21000, Cerestar Scandinavia, Charlottenlund, Danmark.
[c] Soycomil FG, ADM Europort BV, Koog aan de Zaan, The Netherlands.
[d] HP340, Hamlet Protein, Horsens, Denmark.
[e] Degussa, Hanau, Germany; 6: Ajinomoto Eurolysine, Paris, France.
[f] Ajinomoto Eurolysine, Paris, France.
[g] Trouw Nutrition, Boxmeer, The Netherlands.
[h] Constant ingredients; 3.31 g vitamin and mineral premix (proprietary composition, Skretting ARC, Stavanger, Norway), 1 g yttrium premix (100 mg $Y_2O_3$ /kg diet), 0.42 g Betafin (Danisco Animal Nutrition, Marlborough, UK, 0.12 g Lutavit C (BASF, Ludwigshafen, Germany).
[i] Rest = 100% – %protein – %fat – % starch –% ash– %water.
[j] Estimated using the following caloric values: starch 17 MJ/kg, fat 39 MJ/kg and protein 24 MJ/kg.
* $y = 1.47 - 0.04 * x$, $R^2 = 0.93$, $P < 0.01$.
** $y = 10.68 - 0.06 * x$, $R^2 = 0.997$, $P < 0.001$.
*** $y = 0.9 + 0.1 * x$, $R^2 = 0.996$, $P < 0.0001$.

veins using heparinized syringes. Haematocrit (Hct) was determined for each fish immediately after blood sampling. Blood samples were split into two portions. One part was held on ice (4 °C) overnight for analysis of red blood cell count (RBC) and haemoglobin concentration (Hb). The other was centrifuged within 10 min at 2000 ×*g* for 10 min at 4 °C. The resulting plasma was pooled for all fish belonging to the same cage, frozen on dry ice, and stored at −80 °C until analyzed for plasma enzymes.

A lateral incision was made and the intestines dissected out. Sections from midgut (2–3 cm caudal to the last pyloric caeca) and hindgut (middle part of the hindgut) were immediately transferred to McDowell's fixative (McDowell and Trump, 1976) and stored at 6 °C until further processing. For HSP70 analysis, about 1 cm of hindgut tissue from three fish per cage was sampled to obtain RNA for transcription analysis. Samples were taken from the same area of intestine each time and immediately frozen in liquid nitrogen and stored at −80 °C for analysis.

In addition to the five fish collected for blood and organ analyses, another 5 fish from each cage were sampled, anaesthetized, and faeces collected by stripping (Hemre et al., 2003).

### 2.3. Analysis

#### 2.3.1. Blood chemistry

From analyses of Hct, Hb and RBC, we calculated mean cell volume (MCV = (Hct/RBC) × 10), mean cell haemoglobin (MCH = (Hb/RBC) × 10) and mean cell haemoglobin concentration (MCH = (Hb/Hct) × 100). Plasma was analysed for aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) activities on MAXMAT™ PL (MAXMAT S.A., Montepellier, France) using DIALAB kit (Vienna, Australia).

#### 2.3.2. Faeces moisture and intestinal weight

Faeces dry matter was determined gravimetrically after drying at 104 °C for 24 h. For weight measurements, whole intestines were dissected out and the ileal section dislocated from the rectum and the pyloric caeca. The ileal section was then cut open longitudinally and washed with saline solution before being weighed.

#### 2.3.3. Gut histology and ultrastructure

Portions of intestinal segments from 5–10 fish from each dietary group were dissected into smaller pieces for transmission electron microscopy (TEM). These were washed in phosphate buffer (300–320 mOsm, pH 7.4), postfixed for 1.5 h in osmium tetroxide, rewashed in phosphate buffer and stained *en bloc* in 2% aqueous uranyl acetate. After dehydration in a graded series of ethanol concentrations, specimens were embedded in Epon/Araldite via propylene oxide. Semithin (1.0 μm thick) ultramicrotome sections were stained with 2% toluidine blue and examined under a Leica DMLB light microscope at 40× and 100× magnification. Images were acquired by means of Leica DC 300 digital camera and further processed using Olympus Cell a-d ™ software. When required, ultrathin sections (ca. 60 nm thick) were contrasted with uranyl acetate and lead citrate and examined in a Jeol JEM-1230 TEM operated at an accelerating voltage of 80 kV.

*2.3.4. Quantification of soyasaponins in diet and faeces*

Feed and faeces from fish fed the fish meal control diet, 50 PP diet and 100 PP diet were analysed for soyasaponins. The objective was to investigate whether these surface-active components were degraded during gut passage. Extraction, separation and quantification of soyasaponins were performed as described previously (Knudsen et al., 2006). Briefly, separation was achieved by reverse phase high-performance liquid chromatography with diode array detection (HPLC-DAD) using a Hewlett-Packard series 1050 HPLC-DAD system with a 250 mm × 4.6 mm i.d., 5 mm, Supercosil ABZ +Plus, $C_{18}$ reverse phase column (SUPELCO, Bellefonte, Pennsylvania, USA). The mobile phases were 0.05% trifluoroacetic acid in water (solvent A) and 0.05% trifluoroacetic acid in acetonitrile (solvent B). The gradient elution was linear from 25 to 50% B, 0–65 min; linear from 50 to 60% B, 65–70 min; linear from 60–100% B, 70–75 min; isocratic at 100% B, 75–85 min; then linear from 100–25% B, 85–90 min and finally isocratic at 25% B, 90–100 min. The flow rate was 0.5 ml/min, the injection volume was 50 ml and the column temperature was 30°C. Identification of soyasaponins was confirmed by HPLC retention time, UV absorption spectra recorded at 200–350 nm and LC-MS using positive electrospray ionization. The following soyasaponins were detected: Ab, Bb, Bc, Ba-2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (Ba-DDMP), Bb-DDMP and Bc-DDMP (see Knudsen et al. (2006) for molecular structure).

*2.3.5. Hindgut heat shock protein*

*2.3.5.1. RNA extraction.* Total RNA was isolated and extracted from hindgut slices using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Before homogenizing, the tissue (50–70 mg) was cut into pieces and squeezed manually with a disposable plastic stick for 30 s in a 1.5 mL Eppendorf® microcentrifuge tube with Trizol. The tissue was homogenized using a MM301 shaker machine (Retsch, Haan, Germany) at full speed for 6 min. Genomic DNA was eliminated from the samples by DNase treatment according to the manufacturer's instructions (Ambion, Austin, TX, USA), and diluted in 100 µl MilliQ water. The RNA was then stored at −80 °C before further processing.

The quality and quantity of RNA were assessed using the NanoDrop® ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). A 260/280 nm absorbance ratio of 1.8–2.0 indicates a pure RNA sample. To exclude any degradation of RNA, the quality was also evaluated on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), using RNA 6000 Nano LabChip® kit (Agilent Technologies).

*2.3.6. Real-time quantitative RT-PCR*

The PCR primer sequences for hindgut are given in Table 2. The chosen primers for target gene transcript (Atlantic salmon — HSP70 gene), and reference gene transcripts (Atlantic cod — Elongation factor 1α, EF1AA and β-actin genes) have been used previously in real-time RT-PCR analyses of Atlantic cod (Olsvik et al., 2005; Hansen et al., 2006). The RT reaction was run in duplicate on 96-well reaction plates using a Gene Amp PCR 9700 machine from PE Applied Biosystems with the TaqMan Reverse Transcription Reagent containing Multiscribe Reverse Transcriptase (50 U/µl). PCR reactions were incubated at 25 °C for 10 min, reverse transcription at 48 °C for 60 min, and the reverse transcriptase was inactivated at 95 °C for 5 min. The method for cDNA synthesis has been described earlier (Olsvik et al., 2005).

2.5 µl of 10-fold diluted cDNA from each RT reaction was transferred to a new 96-well reaction plate. SYBR® Green PCR Master Mix (0.9 µM) (Applied Biosystems, Foster City, CA, USA) was used to assess the expression of HSP70. The real-time PCR was run on the ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The RT-PCR program included an enzyme step at 95°C (15 min) and 40 cycles of 95 °C (0.15 min) and 60 °C (1.0 min). Efficiency of real-time PCR was monitored using a dilution curve of total RNA with six concentrations from 1000 µl/ng to 31.25 µl/ng. Baseline and threshold for Cycle Threshold calculation were set manually with ABI Prism 7000 SDS software version 1.0 (Applied Biosystems, Foster City, CA, USA), and mean normalized expression was calculated with the Microsoft Excel-based software Q-Gene (Muller et al., 2002). GNorm software (Vandesompele et al., 2002) was used

Table 2
PCR primer sequences and TaqMan MGB probes used for real time qRT-PCR quantification of HSP70, accession numbers and amplicon size

| Gene | Accession number | Forward primer | Reverse primer | Amplicon size (bp) |
|---|---|---|---|---|
| b-actin | CO541508 | CACAGCCGAGCGTGAGATT | ACGAGCTAGAAGCGGTTTGC | 95 |
| EF1AA | CO541820 | CGGTATCCTCAAGCCCAACA | GTCAGAGACTCGTGGTGCATCT | 93 |
| HSP70 | BG933934 | CCCCTGTCCCTGGGTATTG | CACCAGGCTGGTTGTCTGAGT | 121 |

Table 3
Faecal dry matter (%) and gut weight (g) in cod fed diets with increasing levels of plant proteins in the diet

| | FM | 25 PP | 50 PP | 75 PP | 100 PP | Statistics | |
|---|---|---|---|---|---|---|---|
| | | | | | | $P$ | $R^2$ |
| Dry matter | 18.0 | 14.3 | 12.9 | 13.6 | 10.8 | <0.001 | 0.765 |
| Gut weight | 0.83 | 0.74 | 0.80 | 0.75 | 0.86 | ns | |

to evaluate reference gene stability, and EF1AA was found to be most stable. Gene expression data were evaluated statistically using relative expression software tool (REST-384© beta version 2), applying the Pair Wise Fixed Reallocation Randomisation Test© with 2000 randomisations (Pfaffl et al., 2002).

### 2.4. Calculations

Spleen index was calculated as 100 * (spleen weight in grams / live weight of fish in grams) and gut size was calculated as weight of the whole gastrointestinal tract in grams / live weight of the fish in grams.

Mean normalized expression was calculated as $(E_{\text{ref}})^{\text{CT ref, mean}}/(E_{\text{target}})^{\text{CT target, mean}}$ where $E_{\text{ref}}$ is the PCR amplification efficiency of the reference gene, $E_{\text{target}}$ is the PCR amplification efficiency of the target gene, $CT_{\text{ref}}$, mean is the threshold cycle of the PCR amplification for the reference gene and $CT_{\text{target}}$ is the threshold cycle of the PCR amplification for the target gene (Muller et al., 2002).

### 2.5. Statistics

Response parameters were analysed with simple linear regression, where plant protein inclusion level was the dependent factor. All statistical analyses were performed using Statistica™ 7.0 software program (Statsoft, Tulsa, OK, USA, 2005).

## 3. Results

Fish were fed the experimental diets for 28 weeks. Growth, FCR and ADC data are given in Hansen et al., 2007). In brief, results showed a small but significant dose-dependent reduction in the specific growth rate (SGR) from 0.34 in control fish to 0.29 at 75 PP, before diving to 0.14 at 100 PP. This was accompanied by a gradual increase in FCR from 1.08 in the control group to 1.49 in the 75 PP group while appetite in the 100 PP group was too low to obtain reliable FCR. The study also showed that nutrient ADC were generally unaffected by diet. However, there was a small reduction in ADC of starch at highest level of plant protein (100 PP).

### 3.1. Faeces moisture and gut weight

There was a good correlation between diet and faecal dry matter decreasing ($Y=16.9–0.06\times$, $R^2=0.77$, $P<0.001$) with increasing level of plant proteins (Table 3). In the FM diet, faecal dry matter was 18%, which dropped to 14.3% in fish fed the 25 PP diet. This level was then relatively stable up to the 75PP diet (13.6%) before falling further to 10.8% in the 100 PP diet.

The relative gut weight was in the range of 0.86 to 0.74. Linear regression analysis did not indicate any effect of diet on relative gut weight ($P=0.704$, $R^2=0.019$) (Table 3).

### 3.2. Saponins

The following soyasaponins were detected in both diets and faecal samples of fish supplemented with soybean products: Ab, Bb, Bc, Ba-DDMP, Bb-DDMP and Bc-DDMP but the levels of Ab and Ba-DDMP were too low for quantitative analysis (Table 4). No soyasaponins were found in the control FM diet. The predominant soyasaponin was Bb contributing 0.236 and 0.474 mg/g diet by dry weight in the 50 PP and 100 PP diets, respectively. This was around 50% of all identified saponins. The second most predominant saponin was Bc being half the level of the Bb level in both diets. The DDMP-conjugates

Table 4
Dietary and faecal content and apparent digestibility coefficient of soysaponins of cod fed control (FM), 50 PP and 100 PP diets

| Diet | FM diet | Faeces | 50 PP diet | Faeces | 100 PP diet | Faeces |
|---|---|---|---|---|---|---|
| *Soy saponins mg/g dry weight* | | | | | | |
| Soyasaponin Ba | n.d.[a] | n.d. | t.a.[b] | t.a. | t.a. | t.a. |
| Soyasaponin Bb | n.d. | n.d. | 0.236 | 1.000 | 0.474 | 2.177 |
| Soyasaponin Bc | n.d. | n.d. | 0.106 | 0.419 | 0.199 | 0.849 |
| Soyasaponin Ba-DDMP | n.d. | n.d. | t.a. | t.a. | t.a. | t.a. |
| Soyasaponin Bb-DDMP | n.d. | n.d | 0.058 | 0.116 | 0.136 | 0.344 |
| Soyasaponin Bc-DDMP | n.d. | n.d. | 0.023 | 0.052 | 0.054 | 0.143 |
| Total saponins | n.d. | n.d. | 0.422 | 1.587 | 0.862 | 3.507 |
| *Soy saponins ADC* | | | | | | |
| Soyasaponin Bb | –[c] | | −17.7 | | −7.4 | |
| Soyasaponin Bc | – | | −10.0 | | 0.2 | |
| Soyasaponin Bb-DDMP | – | | 44.3 | | 40.8 | |
| Soyasaponin Bc-DDMP | – | | 38.0 | | 38.5 | |
| Total saponins | – | | −4.3 | | 5.0 | |

[a]Not detected; [b]trace amounts detected but level was too low for precise quantification, [c]not applicable.

of Bb and Bc made up the remaining 25% of the saponins. Total level of soyasaponins was 0.422 mg/g in the 50 PP diet and 0.862 mg/g in the 100 PP diet.

Compared to dietary content, faecal dry matter levels of soyasaponins increased four-fold to 1.59 mg/g and 3.51 mg/g in fish fed the 50 PP and 100 PP diet, respectively. In both groups, the ADC for Bb and Bc saponins were negative meaning that the content in faeces had increased in relation to the diet. This was most likely due to hydrolysis of the DDMP moiety giving ADC's for Bb-DDMP and Bc-DDMP of around 40% and 38% for these two groups.

### 3.3. Haematology

In general, diets did not appear to affect blood health indicators (Table 5). Hct was in the range 26–28% in the experimental groups, RBC was 1.61–1.86 * $10^{12}$/l while Hb ranged between 4.8 to 5.4 g/100 ml. The only effects observed were small but significant decreases in MCV and MCH with increased plant protein inclusions. Plasma concentrations of ASAT and ALAT were low and did not change with diets. Similarly, the spleen index did not differ between groups (Table 5).

### 3.4. Histology and ultrastructure

Table 6 gives an overview of the number of cod displaying various degrees of morphological changes (as seen by light microscopy). The number 0 indicates essentially normal morphology, 1 indicates moderate/minor effects described in text but evaluated as different from controls, and 2 severe/major effects causing significant cellular damage likely to be pathological.

In control fish midgut, epithelial integrity was good with close approximation of cells and minimal numbers of intraepithelial lymphocytes (IELs), usually near the basal lamina. The microvillous border appeared healthy

Table 6
Number of Atlantic cod displaying various degrees of dietary-induced morphological changes after being fed the diets for 28 weeks

| Diet | Normal (0) | Moderate (1) changes | Severe (2) changes |
|---|---|---|---|
| *Midgut* | | | |
| FM | 7 | 1 | 0 |
| 25 PP | 4 | 1 | 0 |
| 50 PP | 6 | 4 | 0 |
| 75 PP | 3 | 2 | 0 |
| 100 PP | 3 | 3 | 2 |
| *Hindgut* | | | |
| FM | 8 | 0 | 0 |
| 25 PP | 3 | 2 | 0 |
| 50 PP | 6 | 4 | 0 |
| 75 PP | 2 | 3 | 0 |
| 100 PP | 0 | 6 | 2 |

Refer to Results section for description of histological changes. Total number of fish examined in the FM, 25 PP, 50 PP, 75 PP and 100 PP groups were 8, 5, 10, 5 and 8, respectively.

Table 5
Haematological values and plasma ASAT and ALAT activity in cod fed diets with increasing levels of plant protein in December (initial sampling), and in June (28 weeks), given as mean ($n$=2 cages)

| Clinical parameters | Initial | End | | | | | Statistics | |
|---|---|---|---|---|---|---|---|---|
| | | FM | 25 PP | 50 PP | 75 PP | 100 PP | $P$ | $R^2$ |
| Hct[a] (%) | 30 | 28 | 28 | 27 | 26 | 27 | ns | |
| RBC[b] ($10^{12}$/l) | 1.70 | 1.65 | 1.73 | 1.61 | 1.74 | 1.86 | ns | |
| Hb[c] (g/100 ml) | 4.5 | 5.4 | 5.3 | 4.8 | 5.1 | 5.1 | ns | |
| MCV[d] ($10^{-15}$/l) | 174 | 174 | 165 | 156 | 148 | 143 | <0.01 | 0.836 |
| MCH[e] ($10^{-6}$ g) | 27 | 33 | 31 | 29 | 29 | 28 | <0.05 | 0.516 |
| MCHC[f] (g/100 ml) | 15 | 19 | 19 | 18 | 20 | 19 | ns | |
| ASAT[g] (U/l) | 30 | 21 | 21 | 38 | 32 | 17 | ns | |
| ALAT[h] (U/l) | 16 | 16 | 10 | 13 | 19 | 12 | ns | |
| Spleen index[i] | | 0.16 | 0.15 | 0.15 | 0.14 | 0.16 | ns | |

Linear regression is given were $y$=level of parameter measured and $x$=the level of plant protein in diet.
[a] Haematocrit.
[b] Red blood cell count.
[c] Haemoglobin concentration.
[d] Mean cell volume.
[e] Mean cell haemoglobin.
[f] Mean cell haemoglobin concentration.
[g] Aspartate aminotransferase.
[h] Alanine aminotransferase.
[i] Spleen index=(spleen weight g/live weight g)*100.

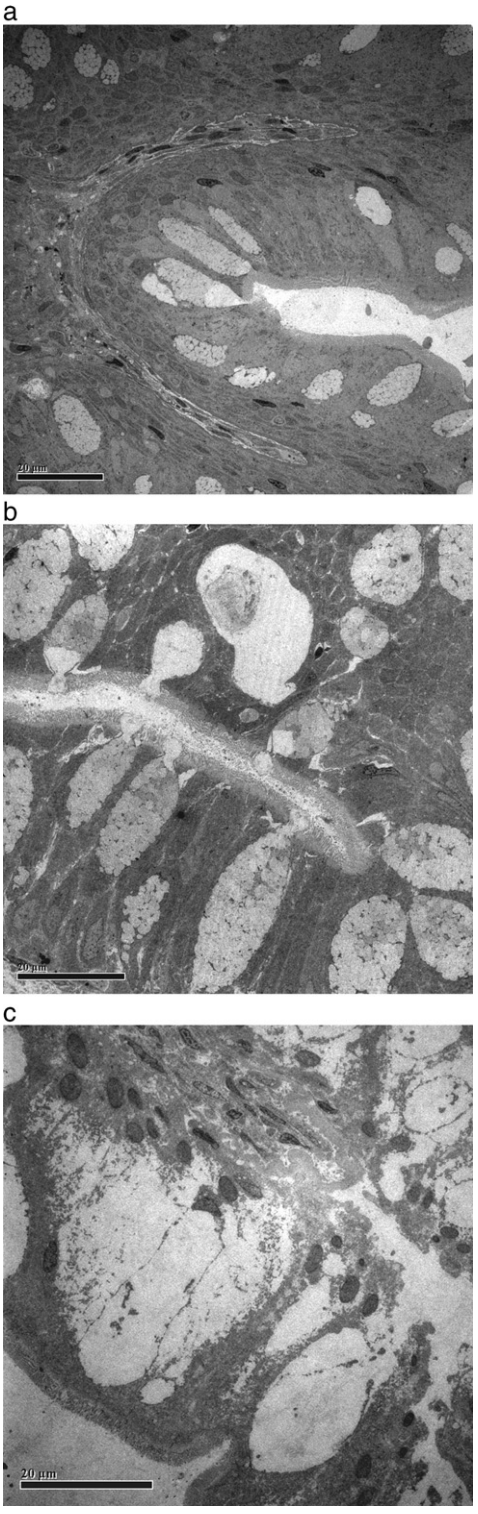


Fig. 1. TEM images of midgut of Atlantic cod fed the experimental diets. a) Fish fed the control diet (FM). Normal enterocytes. Note the high density and a normal looking lamina propria. $X=1500$; b) fish fed with the 100 PP diet. Note increased number and size of goblet cells. $X=1500$. c) Fish fed with the 100 PP diet. Necrotic tissue as found in two fish. $X=2000$.

with normal intercellular junctional complexes (Fig. 1a). Occasionally, absorptive cells showed lipid vacuoles in the supranuclear region and sometimes these were found in the intercellular spaces. Samples from one fish had moderate hypertrophy and hyperplasia of goblet cells (GC) (Table 6). With increased dietary content of plant proteins, there was an increased frequency of fish displaying cellular alterations in midgut. The number of fish displaying such alterations increased from ca 1 in every 5 fish in the 25 PP group to around 2 in every 5 fish in the 50 PP and 75 PP groups. In all cases, the major effect was a notable and apparently gradual increase in GC hypertrophy and hyperplasia. This was accompanied by, what appeared to be, a lower density material in secretory vacuoles, and many appeared empty. There were also increased incidences of IELs and increased cellularity of the lamina propria. In all these diets, cellular damages were relatively rare. The level of alterations appeared to peak in the 100 PP diet as did the number of fish displaying such changes (only 3 of 8 fish were similar to the control, Fig. 1b). However, in this group there were also 2 fish with extensive cellular damages in the midgut. At the most extreme, we observed almost complete loss of epithelial integrity with swollen cells, lost microvilli and swollen disintegrating nuclei (Fig. 1c). Coupled with IELs at all levels in the epithelium, this suggests either widespread necrosis or anoikis (apoptosis with secondary necrosis). GC contents in this group had very low density.

The dietary influence on hindgut was, in many respects, similar to that observed in the midgut. Slightly more fish appeared to be affected by the diets accompanied by a higher degree of change, especially in GC hyperplasia and hypertrophy (Table 6). For example, on the 100PP diet, the number of fish unaffected by diet decreased from 3 (of 8) in the midgut to 0 in the hindgut. However, the number of fish displaying massive intestinal damage was similar (2 of 8) to that observed in the midgut. Damage included loss of epithelial integrity with swollen cells, loss of microvilli and swollen disintegrating nuclei and invasion of IELs at all levels in the epithelium. There were also cases of expanded intercellular clefts, and TEM images showed loss of nuclear organisation with focal clumping of chromatin around what looked like central nucleolar remnants.

### 3.5. Heat shock protein

Real-time PCR amplification efficiency for HSP70, β-actin and EF1AA was 106%, 93% and 97%, respectively, indicating that the PCR assays had reliable

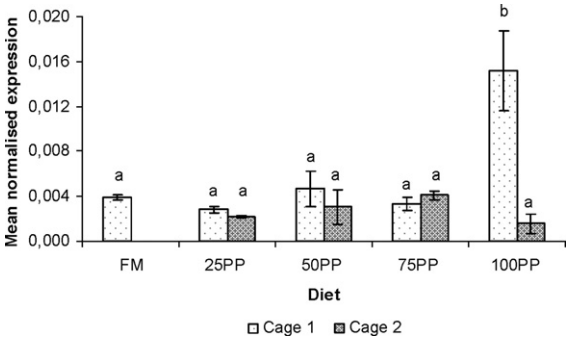


Fig. 2. Mean normalized expression of HSP70 in hindgut from cod fed diets 0–100 PP. Different letters show expressions that are significantly different from each other.

reaction efficiency. Gnorm showed EF1AA to be the most stable gene and was used to normalize the data in Qgene (Muller et al., 2002). RNA from one of the replicates given FM control diets degraded under extraction (results not presented). There were no significant differences in expression of HSP70 between the duplicate cages for fish fed the 25 PP, 50 PP and 75 PP diets or the single cage of fish fed the FM diet (Fig. 2). Fish from one of the cages given 100 PP showed significant up-regulation of HSP70 compared to the other cage given the same diet. This replicate of 100 PP was also significantly up-regulated compared to control fish (regulation factor of 2.953, $P<0.001$), and to all other diet groups.

## 4. Discussion

Plant ingredients in cod diets did not appear to have a generally adverse affect on fish health. Leakage of the organ specific enzymes ASAT and ALAT, commonly used as indicators of organ damage or dysfunction (Racicot et al., 1975), were low and comparable to the controls regardless of dietary group. This agrees with previous reports from Atlantic salmon fed 12–17% full-fat soybean meal (Hemre et al., 2005; Sanden et al., 2006). Likewise, blood haematology was essentially normal and values were within previously reported levels for cod (Rosenlund et al., 2004). One of the few effects observed, was a significant reduction of MCV as the content of plant proteins increased. This has also been observed in some groups of salmon fed soybean products (Hemre et al., 2005). As this observation appeared to coincide with increased spleen size (Hemre et al., 2005), it was suggested that some of the plant ingredients may cause early release of immature erythrocytes. This did not appear to be the case in the present study where spleen size was similar regardless of diet. There was, however, a reduced MCH and an apparent (though not significant) increase in RBC. Although this finding was not investigated further, it could be related to other causes such as iron deficiency or liver abnormalities. A close relationship between dietary iron availability and haemoglobin has been reported in Atlantic salmon (Vangen and Hemre, 2003). Furthermore, antinutrients like phytic acid, found in soybean meal, are known to chelate di- and trivalent ions (Francis et al., 2001) and may have reduced iron uptake from the gut. No measurement of the iron status was however performed in the present study.

In the current study, fish showed relatively small but significant decreases in SGR with increased plant protein inclusion up to 75 PP. This seemed to coincide with decreases in feed conversion ratio. At 100 PP, growth rate decreased over 50% accompanied by severe loss of appetite. These effects appeared not to be related to the ADC of nutrients which was only marginally affected by diet (Hansen et al., 2007). The effects may have been caused in part by nutritional imbalances, particularly shortage in methionine.

Present results do not exclude the possibility that some of the effects on growth and feed conversion are the result of direct effects of dietary plant ingredients on the gastrointestinal tract. A particular case is the tendency of increased faecal water content with increased content of plant proteins. This indicates progression of a diarrhoea-like condition which is commonly observed in salmonids (Refstie et al., 2000, 2004) and cod (Refstie et al., 2006a) fed plant proteins. The effect is mostly attributed to the osmotic influence of soybean α-galactosides or non-starch polysaccharides (see Refstie et al., 1999, 2005 and references therein) which would also have increased in the present study (Table 1, indicated through an increase in the "remains" fraction from 1% in control to over 10% in the 100 PP diet). It was also interesting to note that such observations are generally accompanied by reduced nutrient digestibility of lipid and sometimes also energy and protein. As this did not appear to be the case in the present study (Hansen et al., 2007), it may be that nutrient digestibility is not directly linked to increased faecal moisture.

A frequent observation made on salmonids fed high levels of dietary soybean products is the enteritis-like condition observed in intestinal enterocytes. Such damage is usually related to distal parts of the gastrointestinal tract and characterized by goblet cell hypertrophy and hyperplasia, decreases in the number of absorptive vacuoles, increased cellularity of the lamina propria, increased amount of connective tissue, decreased mucosal foldings and infiltration of inflammatory cells in lamina propria or epithelium (van den Ingh et al., 1991,

1996; Baeverfjord and Krogdahl, 1996). Many of these earlier studies used large amounts of untreated soybean products now known to cause such problems. In order to remedy such effects, feed manufacturers now tend to move towards cleaner and more purified products. In the present study, the main ingredients were bioprocessed soybean meal, soybean concentrate and wheat gluten. Wheat gluten and soybean concentrate, the latter being highly purified and low in antinutritional factors, are good protein sources for fish and are reported to give comparable performance to fish meal at high inclusion levels in both Atlantic salmon (Storebakken et al., 1998) and cod (Hansen et al., 2007). The same would apply to bioprocessed soybean meal that has higher digestibility than ordinary soybean meal due to significantly reduced levels of trypsin inhibitors and indigestible oligosaccharides (www.hampletprotein.com). The processing also involves phytase treatment. These products would however still contain some antinutrients. The concentrate will for example have significant amounts of phytin as phytase treatment improves protein utilisation (Storebakken et al., 1998). The same applies to wheat gluten which contains some fibres and anti-nutrients, such as protease inhibitors and phytin (Francis et al., 2001).

One would not expect to find significant effects of these diets on cod gastrointestinal tract. This is supported by previous studies where moderate inclusions of purified soybean products did not cause major alterations in intestinal morphology (Hansen et al., 2006; Refstie et al., 2006b). The present study appears to support this finding but some effects seemed to be induced by the diets. In most cases, these alterations were minor and involved mostly goblet cells. Although the incidences, and, to some extent, the severity tended to increase from 25 PP up to 75 PP, the condition would still be considered mild. Even at 100 PP, most fish were only mildly affected, except for two cod where elements of a classical enteritis-like condition described in salmonids appeared to occur. This might be taken as an indication of a suboptimal diet. The observation also coincided with the drop in appetite and growth.

In salmonids, soybean-induced gut damages are usually related to distal parts of the gastrointestinal tract affecting cell types with extensive endocytotic activity and high levels of intracellular vacuoles. In salmonids, the proximal part of the gut, where most nutrients are being absorbed, contains different cell types distinguishable from distal cells in not having endocytotic activity and intracellular vacuoles. The only significant effect of soybean diets in this region is goblet cell hypertrophy and hyperplasia (van den Ingh et al., 1991). Cod does not have the same differentiation of cell types (Odense and Bishop, 1966), and most of the intestine contains cells that do not appear to be actively endocytotic. This altered structure may have implications for regional sensitivity to dietary antinutrients. However, we did observe some attenuation of responsiveness to the diet in passing from proximal to distal parts of the gut. This raises the possibility that some of the effects observed in proximal parts of both cod and salmonids are related to an up-concentration of antinutrients in the undigested digesta eventually reaching toxic levels. Thus, analysing this part of the gastrointestinal tract for antinutrient concentration could help establish critical upper levels of these compounds.

What compounds cause the effects seen in the present study is still a matter of debate. The agents causing soybean-induced enteritis in salmonids are known to be alcohol soluble, with possible candidates being saponins (Knudsen et al., 2007) and oligosaccharides (van den Ingh et al., 1996). Earlier studies have shown that soyasaponins are degraded by gut microflora in endothermic animals (Gestetner et al., 1968; Hu et al. 2004a,b). Interestingly, soyasaponins were recently found to resist degradation during gut passage in Atlantic salmon, except for the conversion of DDMP-conjugated soyasaponins to their non-conjugated counterparts (Knudsen et al., 2006). Similar findings were observed in the present study where the DDMP-moiety was partially hydrolysed but the overall apparent digestibility of soyasaponins was close to zero. We conclude that soyasaponins also retain their amphiphilic properties during gut passage in Atlantic cod. The lack of degradation caused the soyasaponins to be strongly concentrated in the digesta as feed ingredients were absorbed and suggests they are strong candidates as enteritis-causing agents. As recent studies has provided evidence that soyasaponins do induce enteritis in salmon (Knudsen et al., 2007), maximum advisory levels in the feed should be established.

As the incidences of enteritis-like conditions were relatively few in the present study, it is also possible that goblet cell changes are not directly related to this condition. All plant ingredients used in the present trial contain significant amounts of fibres and phytin. This is also highlighted in Table 1 where the amount of dietary "rest" compounds that would have been mainly fibre increased from 1% in the fish meal diet to 10% in the 100 PP diet. In mammals, these compounds tend to increase intestinal size in general, and goblet cell volume and numbers in particular (Lundin et al., 1993). Furthermore, additions of phytase and xylanase tend to restore these variables to control values (Wu et al., 2004). Although we did not observe any effect of diet on intestinal weight, previous studies have observed increases in gut weight in cod fed plant protein sources (Refstie et al., 2006a).

Another approach to study suboptimal diets and their effect on the animal is to evaluate various stress-gene activations. HSPs protect and maintain cell integrity and are expressed when the animal is subjected to various kinds of stress, including hyperthermia, transient anorexia and nutritional changes, i.e. antinutritional factors (Tsukimi and Okabe, 2001; David et al., 2002; Martin et al. 2003). Plant proteins do not seem to induce HSP activation in the liver (Hansen et al. 2006) and it is still largely unknown if these factors influence the expression of HSP in the intestine of fish. However, up-regulation of HSPs was found in hindgut in salmon fed with soybean (Sagstad et al., 2007). In this context, we found that HSP70 gene expression was not significantly different among dietary treatments and this suggests that expression is not associated with plant proteins in the diet. However, taking into account the ADC and histological findings presented here, a key decrease in starch digestibility was found when plant protein was increased from 75 PP to 100 PP (Hansen et al., 2007). This could be due to significant up-regulation of HSP70 in one of the cages given 100 PP (Fig. 1). Why the other cage was unaffected remains obscure. Therefore, we conclude that the expression of HSP70 in the intestine was modulated by dietary treatment, the results depending on the HSP, histological/ultrastructural part of intestine and ADC. However, the actual consequences of such changes on the overall protection of the intestine of fish are not known at present. Further investigations are warranted to examine the influences of dietary plant protein inclusion and the general stress associated with heat shock proteins.

In conclusion, increasing the mixture of plant proteins (wheat gluten, soy concentrate, bioprocessed soybean meal; 50, 36, 14 w/w, respectively) up to 75% of diet protein produced only marginal effects on fish growth, health and apparent nutrient digestibility. The latter was largely unaffected except for a small increase in protein digestibility and a reduction in carbohydrate utilisation. There was also a tendency towards increased faeces moisture. Intestinal morphology was mildly affected in about half of the fish examined, the most prominent effect being increased size and number of intestinal goblet cells. There was also a tendency for midgut regions to be less affected than hindgut. Increasing the plant protein content to 100% tended to induce alterations that may cause concern. Faeces moisture content increased which indicates diarrhoea. Within this group, there were also some fish displaying severe intestinal necrotic damages in all gut segments, and heat shock 70 mRNA expression increased. Finally, it has been shown that soyasaponins found in diets were not degraded and accumulated in the faeces.

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