Different enzyme incorporation strategies in Atlantic salmon diet containing soybean meal: Effects on feed quality, fish performance, nutrient digestibility and distal intestinal morphology.

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Abstract

An enzyme complex containing phytase, protease, xylanase and cellulase produced from *Aspergillus niger* by solid state fermentation was applied to three Atlantic salmon diets. In these diets soy protein concentrate (SPC) was replaced by de-hulled solvent extracted soybean meal (SBM). Three different enzyme application strategies were evaluated: pre-processing SBM with the enzymes, addition of enzymes in the dry mix prior to extrusion and enzyme coating post extrusion. These diets were tested against one with SBM but without enzyme treatment and another one with SPC instead of SBM. All these diets where tested for fish performance, nutrient digestibility, distal intestine morphology and feed pellet physical quality. The feeding trial lasted for 93 days and each diet was fed to triplicate groups of 60 g mean body weight Atlantic salmon. In the end of the trial all the groups at least tripled their initial body weight. The diet with coated enzymes had significantly higher apparent digestibility of dietary Fe ($P < 0.001$) compared to the other diets. The enzyme pre-processed SBM feed had significantly lower apparent protein digestibility ($P < 0.01$) compared to the other diets, the digestibility of some of the dietary amino acids was also significantly lower in this diet. Salmon fed the SBM diet with addition of enzymes in the dry mix prior to extrusion had significantly higher growth and feed intake than the fish fed the SPC diet without enzymes ($P < 0.05$). Hardness was significantly and negatively correlated with feed intake ($P < 0.01$, $r = -0.95$) and growth ($P < 0.01$, $r = -0.95$), which may explain the reduced performance of the fish fed the SPC diet without enzymes. None of the diets caused SBM induced enteritis.

Keywords: Enzyme treatment; soy ingredients; digestibility; Atlantic salmon; physical feed quality

1. Introduction

Vegetable protein sources have replaced large part of the less available fishmeal in Atlantic salmon diets the last decades (Ytrestøyl et al., 2015). However, ingredients of plant origin contain many different anti-nutritional factors (ANFs), which reduce the availability of dietary nutrients and can influence animal health (Francis et al., 2001). Soy protein concentrate (SPC) was the main vegetable protein ingredient used in 2012 in Norwegian aqua-feed production and accounted for 21.3% of the total feed formulation (Ytrestøyl et al., 2015). SPC contains approximately 65% crude protein, 1% fat and 6% ash (Peisker, 2001). The SPC protein content is similar to that of fish meal, which typically ranges between 60-70% (NRC, 1993). Moreover, SPC has a well balanced amino acid profile with methionine being the first limiting essential amino acid compared to fish meal (Gatlin et al., 2007). SPC is more refined than non-GMO de-hulled solvent extracted SBM and is approximately 13.5% more expensive, based on protein cost (internal communication). On the other hand, SBM contains approximately 48-50% crude protein, 1-1.5% fat, 5.5-6% ash and has a higher content of all the water/alcohol soluble ANFs compared to SPC (Peisker, 2001).

Several studies have shown that high dietary inclusion levels of SBM can result in decreased growth performance and feed efficiency in salmonids (Kaushik et al., 1995; Refstie et al., 1998; Refstie et al., 2001; Smith, 1977). Moreover, dietary inclusion of increased levels of SBM has shown to cause enteritis in the distal intestine in salmonids (Baeverfjord and Krogdahl, 1996). This pathology is associated with shortening of intestinal villi, thickening and infiltration of the lamina propria with inflammatory cells and alteration in enterocyte structure (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2000; Rumsey et al., 1994; van den Ingh et al., 1991). Soya saponins, possibly in combination with other unknown components, cause these morphological changes in the distal intestine in salmonids (Knudsen et al., 2007; Knudsen et al., 2008; Sørensen et al., 2011b).

The ANFs are classified into heat-labile and heat-stable (Francis et al., 2001). The heat-labile ANFs, including protease inhibitor and lectins, can be eliminated or reduced by heat treatment (Arndt et al., 1999; Smith, 1977). Heat-stable ANFs in SBM include oligosaccharides, non-starch polysaccharides, saponins, phytate, phytoestrogens and soya antigens (Francis et al., 2001). These can be eliminated or reduced by fractionation, solvent extraction, fermentation or the use of exogenous enzymes (Denstadli et al., 2006; Kaushik et al., 1995; Knudsen et al., 2006; Refstie et al., 2005; Rumsey et al., 1994).

The use of phytase, carbohydrases and protease enzymes in plant based raw material in salmonid diets has been reported before. Phytase is shown to improve the digestibility of dietary phosphorous (Cheng et al., 2004; Dalsgaard et al., 2009; Denstadli et al., 2007; Lanari et al., 1998; Sugiura et al., 2001; Vielma et al., 2004; Wang et al., 2009), other minerals (Cheng et al., 2004; Denstadli et al., 2007; Sugiura et al., 2001; Vielma et al., 2004), protein (Sugiura et al., 2001; Vielma et al., 2004), feed conversion and protein efficiency ratio (Wang et al., 2009). Carter et al. (1994) reported improved growth rate and feed efficiency in Atlantic salmon fed a diet supplemented with trypsin, alkaline protease, acid protease, amyloglucosidase, amylase and cellulase. Moreover, the use of β-glucanase and protease has been shown to improve the apparent digestibility of all nutrients in a diet with a high inclusion rate of SBM (Dalsgaard et al., 2012). Protease and carbohydrases have also been shown to increase the digestibility of non-starch polysaccharides (Dalsgaard et al., 2016). Nevertheless, other studies have not shown any improvement in nutrient digestibility or growth by using carbohydrases or protease in the feed (Carter, 1998; Carter et al., 1992; Denstadli et al., 2011; Ogunkoya et al., 2006; Yigit et al., 2016). The contradicting results of the above sited experiments using enzymes in salmonid diets can be related to the use of different ingredients, type of enzymes, and different ways of adding the enzymes to the feed and the rearing water temperature during the trial period.

The feeds used in marine fish farming are mostly produced by the use of extrusion technology and need to be resistant to mechanical stress during transport, handling and pneumatic feeding. At the same time, the feeds requires a texture and size that facilitate high feed intake and efficient digestion (Aas et al., 2011; Baeverfjord et al., 2006). Too hard pellets may cause digestive disturbances in fish. Overfeeding with hard pellets may result in swelling and rupture of the stomach (Pillay and Kutty, 2005). On the other hand soft pellets or pellets with low water stability may cause oil separation and accumulation of dietary oil in the stomach of fish (Aas et al., 2011; Baeverfjord et al., 2006). Extrusion is a complex process and physical quality, expansion parameters and texture of a feed is affected by the ingredient composition and the extrusion parameters (Aarseth et al., 2006; Glencross et al., 2010; Morken et al., 2012; Refstie et al., 2006; Samuelsen and Oterhals, 2016; Samuelsen et al., 2013; 2014; 2018; Sørensen et al., 2010; Sørensen et al., 2011a; Sørensen et al., 2009).

The objective of the present work is to evaluate the effects on feed quality and salmon performance by the inclusion of an enzyme complex produced by *Aspergillus niger* to diets, containing SBM, in three ways: 1) added prior to extrusion (SBM_E), 2) pre hydrolysing SBM with the enzymes prior to extrusion (SBM_P), 3) adding the enzymes by coating post-extrusion (SBM_C). The different feeds were evaluated against a diet with SBM and without enzymes (SBM_0) and a diet with SPC instead of SBM (SPC_0). Results on fish performance, nutrient digestibility, distal intestine morphology and the physical quality of the feeds are presented.

2. Material and methods

2.1. Experimental diets formulation and production

The SPC_0 diet was formulated based on raw material used in the salmon feed industry in Norway (Ytrestøyl et al., 2015). In the other four diets, SBM was added instead of SPC at

levels providing the same amount of protein deriving from soy in all experimental diets (Table 1). Different enzyme incorporation strategies were applied to three of the SBM diets. The SBM in diet SBM_P was pre-processed with enzymes as described by Jacobsen et al. (2018). Enzymes were added to the dry mix prior to extrusion in diet SBM_E while the enzymes were coated on diet SBM_C prior to the lipid coating of the pellets using the same coater. An equal content of the crude enzyme complex was added to the three feeds (Table 1). In SBM_0 no enzymes were added. The diets where balanced for crude protein, crude lipid and gross energy based on analyses of the feed ingredients (Table 2).

The crude enzyme complex used in this study was produced by *Aspergillus niger* by solid state fermentation. The enzyme activities measured in this enzyme complex at pH 5.5 and 45 °C were: 1253 SPU g^{-1} phytase, 0.196 U g^{-1} protease, 512 XU g^{-1} xylanase and 104 CMCU g^{-1} cellulase as described by Jacobsen et al. (2018).

The experimental diets were produced at Nofima's Feed Technology Centre (Bergen, Norway). The diets were pre-conditioned in an atmospheric double differential conditioner (DDC, Wenger Manufacturing Inc., Sabetha, KS, USA), extruded in a co-rotating twin screw extruder (TX52, Wenger Manufacturing Inc, Sabetha, KS, USA), dried in a dual layer carousel dryer (Model 200.2; Paul Klockner GmbH, Nistertal, Germany) and coated with oil in a Pegasus® vacuum coater (PG-10 VC Lab, Dinnissen BV, Sevenum, Netherlands). The processing parameters are presented in Table 3. The paddle speed in the pre-conditioner, the speed of the extruder screw and the flow rate during all the extrusion experiments were 220 rpm, 400 rpm and 150 kg h^{-1}, respectively. The extrudates were cut at the die surface to equal wet pellet length. The processing temperature was measured at the outlet of the pre-conditioner and in the feed mass upstream the extruder die. The die diameter used was 2.5 mm. The aim was to reach the same temperature at the exit of the conditioner and moisture content at the extruder die for all the feeds. Extruder torque (kW) was recorded during

139 processing, whereas the specific mechanical energy (SME; kJ kg^{-1} wet extrudate) was also
140 calculated.
141
142 2.2. *Feed pellet technical quality*
143 The diameter and length of the pellets were analysed using an electronic sliding calliper and
144 the reported values are the average of 30 measurements per feed.
145 The pellet expansion ratio (%) was calculated as ((pellet diameter-die diameter) × (die
146 diameter)$^{-1}$) × 100.
147 Pellet hardness was measured by a texture analyser (TA-HDi®, Stable Micro Systems LtD,
148 Surrey, UK) using a cylindrical flat-ended aluminium probe (70 mm) as described in
149 Samuelsen and Oterhals (2016). The pellets were treated individually and the reported values
150 were based on the average of 30 analysis.
151 Doris Durability Index (DDI) was measured on oil coated pellets in an DORIS pellet tester
152 (AKVAsmart ASA, Bryne, Norway), by adding a pre-sieved feed sample of 350 g into the
153 inlet of the DORIS durability tester, conveyed by a screw onto a rotating paddle, and re-
154 collected in an accumulation box. The collected sample was then poured on a 2.8 mm screen
155 equipped with a collector, sieved for 30 sec. on an Endecotts Test sieve shaker (Endecotts
156 LTD, London, England). Following sieving, the durability was defined as the percentage of
157 pellets remaining on the 2.8 mm screen. The test was conducted in duplicate samples for each
158 diet.
159 Water stability index (WSI) was analyzed as described by Baeverfjord et al. (2006) with some
160 modifications. Ten g of feed sample was weighted into pre-weighed circular wire netting
161 baskets with a 1.5 mm mesh size and a diameter of 7 cm. The bottom of each basket was flat,
162 and was situated 2 cm above the lower end of the netting tube. Baskets with feed samples
163 were placed in 1000 mL beakers, and 500 mL of tap water was added. Three beakers per diet

were then incubated in a water bath (Julabo SW22, JULABO GmbH, Seelbach, Germany) at 9 °C and 145 shakings per min for 24 hours. The baskets were then gently removed from the beakers, dried with paper tissues and weighed, then placed into a heating cabinet at 105 °C for 18 h. The baskets were weighed again after drying to determine the residual dietary dry matter in each basket.

2.3. *Experimental fish, rearing facilities and conditions*

The feeding experiment was carried out using 1125 Atlantic salmon (*Salmo salar*) post-smolts of the Bolaks (BO 4-15) SalmoBreed (Bergen, Norway) strain at Nofima's indoor tank facilities at Sunndalsøra (Norway). Groups of 75 fish with a mean body weight of 60 g were distributed into 15 experimental tanks (0.5 m^3) supplied with 20 L min^{-1} seawater. The sea water used was pumped from 40 meter depth, filtered and UV-treated. The mean seawater temperature during the trial was 10.9 °C. Prior to the start of the experiment, the fish had been reared under continuous light in similar tanks and fed a commercial feed (Nutra Olympic, 3.0 mm pellets, Skretting AS, Stavanger, Norway). Triplicate groups of fish were fed one of the five experimental feeds continuously at 120% of the *ad labium* daily feeding level using automatic disk feeders. Feed waste was collected from the effluent water in wire mesh boxes, and weighed daily to estimate feed intake. Uneaten pellets were analyzed for dry matter content. Feed intake was calculated on a dry matter basis for each tank by taking into account the uneaten pellets and the percentage recovery of dry matter from the diet in the system (Helland et al., 1996), and was used to calculate feed intake per kg gain (FCR). The fish were reared with continuous light and the feeding trial lasted for 93 days from June to September 2016.

2.4. *Sampling*

189 Prior to the start of the experiment, 36 fish were sampled for initial tissue chemical
190 composition and histology analysis. In triplicate pooled samples of 15 fish we analysed the
191 vertebra mineral composition, whole body chemical composition, whereas gut histology was
192 studied in 6 fish from the initial trial fish population. At the end of the experiment, all the fish
193 in the experimental tanks (five each time) were killed by an overdose of the anaesthetic
194 tricaine methanesulfonate (MS-222: 0.05-0.08 g L^{-1}), individually weighed and measured for
195 fork length. Faeces were stripped from all fish except those used for tissue sampling. The
196 pooled faecal material collected from each tank was frozen and freeze-dried prior to chemical
197 analyses. Five fish per tank were sampled for whole body and vertebra chemical composition,
198 for the measurement of organ somatic indices and study of the distal intestine (DI)
199 histomorphology. Only fish with digesta throughout the intestinal tract were sampled to
200 ensure intestinal exposure to the diets. The fish were dissected and the gastrointestinal tract
201 removed and the liver weighed for the calculation of the hepatosomatic index (HSI).
202 Associated adipose tissue was removed from the gastrointestinal (GI) tract and then mid (MI)
203 and DI where separated and weighed after gently removing their faecal content. DI samples
204 were immediately fixed in 4% phosphate buffered formalin for histological evaluation. The
205 same vertebral section from each sample fish, situated between the rear part of the dorsal fin
206 and the anterior part of the anal fin, was dissected. The neural and haemal arches where cut
207 off close to the attachment of the backbone. The vertebra segments were manually cleaned for
208 soft tissue and blood by scrubbing with a stiff brush. The cleaned vertebral segments of the
209 five fish per tank where pooled, homogenized, freeze-dried and analyzed.
210
211 2.5. *Chemical analyses*
212 Moisture and ash were determined gravimetrically after drying at 105 °C for 16 h (ISO 6496-
213 1999) and incinerating at 500 °C for 12 h (ISO 5984-2002), respectively. Crude protein was

214 determined by the Kjeldahl method (N × 6.25) (ISO 5983-1997) and amino acids by HPLC
215 following acid hydrolysis (ISO 13903:2005). The lipid content was quantified by HCl
216 hydrolysis (COMMISSION REGULATION (EC) No 152). The mineral content in feed and
217 whole body was analyzed by ICP-MS (ISO 17294-4). The bone mineral content was assessed
218 by a spectrometric method (ISO 6491-1998). Total starch and degree of starch gelatinisation
219 were measured utilising a modification of the glucoamylase methodology described by
220 Chiang and Johnson (1977) and Samuelsen and Oterhals (2016). Yttrium was determined by
221 ICP-AES (ISO 11885-1996).
222
223 2.6. *Histology analysis*
224 Formalin fixed distal intestinal tissue samples were processed according to standard histology
225 procedures. Slides were stained using a mixture of haematoxylin and eosin (H&E) and Alcian
226 blue at pH 2.5. The slides were evaluated blindly using a Nikon Eclipse E200 light
227 microscope (Nikon Instruments Europe B.V., Netherlands). A semi-quantitative scoring
228 system was used as adapted by Uran et al. (2008), where mucosal folds, goblet cells, lamina
229 propria, supranuclear vacuoles, eosinophilic granulocytes and sub-epithelial mucosa are
230 evaluated. A score of 1 represents normal morphology, a score of 2-3 was attributed to mild
231 morphological changes compatible with signs of inflammation, while a score of 4-5 was given
232 to progressively more marked morphological symptoms of severe enteritis.
233
234 2.7. *Calculations*
235 Condition factor (CF) was calculated as $\mathrm{CF} = 100 \times \mathrm{W_i} \times (\mathrm{L_i^3})^{-1}$, where $\mathrm{W_i}$ is the individual
236 weight and $\mathrm{L_i}$ is the individual fork length. Specific growth rate (SGR) was calculated as
237 following: $SGR = 100 \times [(lnW_1) - (lnW_0)] \times D^{-1}$, where $\mathrm{W_0}$ and $\mathrm{W_1}$ is initial and final
238 weight, respectively, and D is the number of feeding days. Feed conversion ratio (FCR) was

calculated as: $FCR = F \times G^{-1}$, where F is total feed consumption in dry matter and G is the total fish weight gain. Thermal growth coefficient (TGC) was calculated as: $TGC = \left(W_1^{1/3} - W_0^{1/3}\right) \times (\sum D^{\circ})^{-1}$, where $\sum D^{\circ}$ is the thermal sum (feeding days × average temperature, °C). Crude and digestible protein retention (PRC and PRD) was calculated as: $PRC\%\ and\ PRD\% = (Protein\ gain)/p \times 100$, where P is the protein fed in PRC% and digested protein in PRD%. The hepatosomatic index (HSI) and organosomatic indices (OSI) of the MI and DI were calculated as the percentages of the weight of the samples in relation to the whole body weight of fish, calculated as: HSI and $OSI = (mT \times bw^{-1}) \times 100$, where mT is the weight of tissue and bw final body weight. The fish dress out percentage (D%) was calculated as: $D\% = (Fw \times bw^{-1}) \times 100$, where Fw is the gutted fish weight and bw is the body weight prior gutting. The apparent digestibility coefficient (ADC) of the nutrients and minerals was calculated as following: $ADC(\%) = 1 - \left[100 \times \left((D_i \times F_i^{-1}) \times (F_n \times D_n^{-1})\right)\right]$, where Di and Fi is the inert marker concentration in the diet and faeces, and Fn and Dn is the concentration of nutrient in diet and faeces, respectively.

2.8. *Statistics*

The results were statistically analysed by one-way analysis of variance. The significant difference between means ($P < 0.05$) were determined using Tukey`s multiple range test. Correlation coefficients on physical quality characteristics and growth were carried out using the Pearson correlation coefficient procedure. Statistical analyses were performed with STATISTICA (v.12.0) from Statsoft (Tulsa, OK, USA).

3. Results

3.1. *Feed processing*

Due to the high moisture content in the pre-processed soybean meal, the moisture content in the SBM_P feed mixture was 18.9% prior to extrusion. The moisture content in the other feed mixtures was 8.5, 8.3, 8.4 and 8.5 for SPC_0, SBM_0, SBM_E and SBM_C, respectively. Therefore only steam was added to the SBM_P mix in the conditioner to increase the temperature and moisture content while both steam and water was added in the other diets. Due to lower conditioner temperature and no steam addition in the extruder (Table 3) the die temperature only reached 114 °C during processing of the SBM_P feed, whereas the temperature reached 120-122 °C at the die in the other diets.

3.2. *Pellet technical quality*

The experimental feeds were significantly different in terms of expansion ratio, with the SPC_0 diet having significantly highest and SBM_P significantly lowest expansion ratio (Table 4). The pellet hardness was significantly higher for the SPC_0 diet compare to the other diets (Table 4). The durability of all the experimental feeds was high with very small variation, still with some significant differences, with SBM_C showing the highest durability (Table 4). The water stability index was significantly different between the diets with SPC_0 the highest and SBM_P the lowest water stability index (Table 4). The pellet length was not significant between the feeds (Table 4).

3.3. *Fish performance*

The fish more than tripled their initial weight with minor incidences of mortality, non-significantly different among the treatments. The main fish performance results are presented in Table 5. The final weight, growth and TGC were significantly higher in fish fed the SBM_E diet compared to the SPC_0 diet. The total feed intake of fish fed the SBM_E and SBM_C diets was significantly higher than that of fish fed the SPC_0 diet. The FCR, SGR,

PRC%, PRD%, CF, D%, HSI, OSI MI and OSI DI did not show significant differences between fish feed the different feeds.

3.4. *Apparent digestibility coefficient of dietary macro and micro nutrients*

The chemical analysis of the feeds shows that there were only minor differences in the chemical composition between the diets (Table 2). The SPC_0 diet had a slightly higher starch content (158 g kg^{-1}) compared to the SBM diets with or without enzymes (114-105 g kg^{-1}). The apparent digestibility of protein was significantly lower in the SBM_P diet compared to the SBM_E and SBM_C diets. Apparent energy digestibility was significantly higher in SBM_C compared to SBM_P diet. No significant differences were found in the apparent fat and ash digestibility coefficient of the experimental diets (Table 6).

The apparent digestibility of some of the amino acids in SBM_P was significantly lower compared to the SBM_E and SBM_C diets (Table 6).

The apparent phosphorus digestibility was not affected significantly by the use of enzymes. The digestibility of iron was significantly highest for SBM_C and significantly lowest for SBM_0 with negative values. The apparent zinc digestibility of the SBM_0, SBM_E and SBM_C was significantly higher than that of SPC_0 (Table 6).

3.5. *Whole body macro and micro nutrient composition*

No statistical significant differences were found in the whole body chemical composition and bone mineralisation of the fish fed the different feeds (results not shown).

3.6. *Histology analysis of distal intestine*

No statistically significant histomorphological differences were found in the distal intestine of the fish fed the different feeds and there were no signs of soybean meal induced enteritis (Table 7).

3.7. *Physical feed quality and effect on fish performance*

Feed pellet hardness was negatively correlated to TGC, growth and feed intake. Hardness did also have a positive correlation trend to starch content and gelatinized starch. Starch content and gelatinized starch content were negatively correlated with feed intake. Starch content and gelatinized starch did also have a negative correlation trend to growth and TGC. Starch content showed a positive correlation trend to WSI. Feed intake was positive correlated to TGC and growth (Table 8).

4. Discussion

4.1. *Feed process and technical quality*

The observed higher SME for the SBM_P was probably an effect of the reduced viscosity compared to the other processed feeds, as a reduced temperature in the extruder barrel will increase the viscosity in the melt during processing (Mercier and Feillet, 1975). The measured lower expansion ratio of the SMB_P diet (Table 4) may be related to the lower die temperature and increased viscosity (higher SME) compared to the other diets. Previous studies have shown an inverse relationship between feed expansion ratio and the physical quality parameters of hardness and durability (Aarseth et al., 2006; Hansen and Storebakken, 2007; Morken et al., 2012; Sørensen et al., 2010; Sørensen et al., 2011a; Sørensen et al., 2009). Contrary, our results show that the SPC_0 diet had both the highest expansion and pellet breaking force compared to the others tested feeds. The feeds were balanced for total soy protein level, thus the SPC_0 diet contained more wheat and thus more starch, compared

to the SBM diets, which contained more non-starch carbohydrates. Except for the starch levels, there were only minor differences in the chemical composition between the diets (Table 2). Starch is primarily used as a digestible binder and this study demonstrated a positive correlation trend between pellet hardness and dietary starch content and gelatinized starch which is in accordance to Wood (1987). The positive correlation trend between starch and WSI can be explained by the higher binding capacity of diet with higher starch content.

4.2. *Fish growth parameters and nutrient digestibility*

Previous results (Jacobsen et al., 2018; Romarheim et al., 2005) have shown that heat treatment of soybean meal can improve the protein and amino acid digestibility which is related to the reduced protease inhibitors. Trypsin inhibitors are affected by the temperature range used (Johnson et al., 1980). Morken et al., 2011; 2012 documented increased protein and amino acid digestibility by increasing the extruder temperature in the range of 110-150 °C probably caused by thermal denaturation. The achieved lower temperature in the extruder for SBM_P in our study may explain the resulting lower apparent protein and amino acid digestibility of this diet.

The negative correlation between hardness and feed intake and growth parameters in our study indicates that the physical quality of the feed may be of importance for fish performance. Harder pellets may be more difficult to hydrate and dissolve, which would slow down the gastric evacuation time, resulting in reduced feed intake, as reported previously (Aas et al., 2011; Venou et al., 2009), which could lead to poorer growth despite the high apparent digestibility of the dietary nutrients.

The difference in the starch concentration between the diets in this study did not have an influence on the apparent dietary fat or energy, which is not in line with Aksnes, 1995 and Hemre et al., 1995. However the starch variation in this study (105-158 g kg^{-1} DM) is much

lower than in the previous published studies (0-602 g kg^{-1}). The starch content was positively associated to pellet hardness (Table 8) and the negative correlation between starch content and feed intake may therefore be a result of increased hardness. Previous studies have demonstrated that starch levels above 10% result in reduced feed utilization and this is compensated by increased feed intake to maintain growth (Hemre et al., 2002).

The feed processing parameters of the SBM_0, SBM_E and SBM_C feeds were the same (Table 3), excluding any impact of the process on pellet hardness. This indicates that the enzymes added to the mix prior to feed processing may have influenced physical feed quality and consequently feed intake and fish growth. Previous result with β-galactosidase in bread baking, α-amylase in maltodextrin systems and lipoxygenase in a glucose calcium-alginate gel have shown that these enzymes are more heat stable in systems with reduced moisture content than in aqueous solutions (Liou, 1982; Samborska et al., 2005; Zhang et al., 2017). This may indicate that the enzymes have been active in the feed process and/or in the 20 minutes drying process after extrusion altering physical feed quality by making it softer.

4.3. *Digestibility*

Dietary phosphorus apparent digestibility was not significantly affected by enzymatic pre-processing of SBM even though 84% of the IP6 could be hydrolysed by this process as reported by Jacobsen et al. (2018). This indicates that the proportion of IP6 phosphorous in the diet is low and the improved bioavailability of the phosphorus is masked by the already high available phosphorous in the diet. Accordingly, there were no significant differences in the salmon whole body and vertebra phosphorous levels of our study. The improved apparent dietary Fe and energy digestibility by coating the pellets with enzymes found in our study is in line with previous results (Vielma et al., 2004; Wang et al., 2009), which were however from experiments conducted at higher water temperatures (14-18 °C). Denstadli et al. (2007)

386 did not find any mineral digestibility improvement by coating phytase onto the pellets which
387 were fed to fish reared at 8 °C. In the aforementioned experiment, the researchers did not use
388 the same enzyme complex and had different feed formulations compared to our study.
389 Previous experiments have shown that the whole body mineral concentration is
390 homeostatically controlled (Satoh et al., 1987; Shearer, 1984), i.e. if the whole body mineral
391 levels are lower than normal it is an indication of a subclinical mineral deficiency. The
392 present experiment lasting for 93 days did not show any differences in whole body and
393 vertebra mineral composition between the five diets even though some diets showed
394 improved apparent mineral digestibility.
395
396 4.4. *Histology*
397 The relative little impact and non significant difference between the SPC_0 diet and SBM_0
398 diet on the distal intestine structure in this experiment when using approximately 20% of de-
399 hulled solvent extracted SBM, is not in accordance to previous results (Król et al., 2016).
400 Previous experiments conducted with fish meal based diets where soybean meal replaced part
401 of the fish meal (Baeverfjord and Krogdahl, 1996; Chikwati et al., 2012; Knudsen et al., 2007;
402 Knudsen et al., 2008; Krogdahl et al., 2003; Król et al., 2016; Sørensen et al., 2011b; Uran et
403 al., 2008; van den Ingh et al., 1991) or the use of soya saponin concentrates (Chikwati et al.,
404 2012; Knudsen et al., 2007; Knudsen et al., 2008; Krogdahl et al., 2015) have shown soybean
405 meal induced enteritis in fish. The high inclusion rates of concentrated soya saponins used in
406 previous trials corresponds to 24-140% SBM in the diet when the saponin content is 7.1 g kg^{-1}
407 as analysed by Knudsen et al. (2008). The saponin content in the SBM in this trial is 5.65 g
408 kg^{-1} DM (Jacobsen et al., 2018) which corresponds to 1.12 g kg^{-1} soya saponins in the diet.
409 Knudsen et al. (2008) analysed the saponin content in SBM and in a soya saponin concentrate,
410 and found that the DDMP-conjugated group B and group A saponins where not present in the

concentrate. That study demonstrated that soya saponin concentrate in combination with lupin kernel meal did not have the same impact as the soybean meal on the intestinal morphology changes and it was concluded that the DDMP-conjugated B group saponins and group A saponins may have enhanced the intestinal morphology changes. The DDMP-conjugated saponin and A group saponin levels in the SBM used in this study (Jacobsen et al., 2018) are lower than in the SBM used in Knudsen et al. (2008). This fact may therefore explain why we did not see the common highly inflamed distal intestine in the fish of our study as reported previously and indicates the importance of detailed knowledge of the processing parameters and chemical composition of raw materials to be used in aquatic diets.

5. Conclusions

The present experiment showed that the physical quality of the feed pellet has an influence on fish performance and that this should be taken into consideration when designing fish feeding and digestibility trials with different feeds. To the author`s knowledge similar effects of pellet hardness on fish performance have not been reported previously. The use of soybean meal did not induce the common soybean meal induced enteritis in the distal intestine in this experiment. The mechanism behind this effect is not clear and further research is needed to clarify which of the saponin groups in the soybean meal induce enteritis in the distal intestine in Atlantic salmon. The addition of enzymes prior to extrusion may have an effect on the physical quality of the feed. The study demonstrates that addition of enzymes post extrusion can improve Fe and energy digestibility. Pre-processing SBM with the used enzymes and adding it to an extruded feed in this study demonstrated reduced fish performance.

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651

652 Table 1. Formulation of the experimental diets in dry matter (g kg^{-1}).

Diet code	SPC_0	SBM_0	SBM_P	SBM_E	SBM_C
Soy protein concentrate[1]	143.0				
Soy bean meal[2]		198.0		198.0	198.0
Pre-processed Soy bean meal[2]			198.0		
Enzyme[3]			0.6	0.6	0.6
Fishmeal[4]	204.0	204.0	204.0	204.0	204.0
Krill hydrolysate (wet)[5]	13.6	13.6	13.6	13.6	13.6
Corn gluten[1]	26.0	26.1	26.1	26.1	26.1
Wheat gluten[6]	180.0	191.0	191.0	191.0	191.0
Wheat[7]	151.0	85.0	85.0	85.0	85.0
Fish oil[4]	108.6	108.6	108.6	108.6	108.6
Linseed oil[8]	50.1	51.0	50.7	50.7	50.7
Rapeseed oil[9]	44.0	43.0	42.7	42.7	42.7
Choline chloride[10]	4.9	4.9	4.9	4.9	4.9
Cholesterol[11]	4.9	4.9	4.9	4.9	4.9
Soya lecithin[12]	4.9	4.9	4.9	4.9	4.9
vitamin mix[10]	29.7	29.7	29.7	29.7	29.7
mineral mix[10]	6.0	6.0	6.0	6.0	6.0
NaH_2PO_4[10]	25.0	25.0	25.0	25.0	25.0
Lys (99%, 19,41% Cl)[13]	1.7	1.7	1.7	1.7	1.7
Methionine 99%[13]	2.0	2.0	2.0	2.0	2.0
Carophyll Pink (10%)[14]	0.5	0.5	0.5	0.5	0.5
Yttrium oxide[15]	0.1	0.1	0.1	0.1	0.1
Sum	1000	1000	1000	1000	1000

653 [1]Provided by Agrokorn, Denmark.
654 [2]Provided by DSM, Switzerland.
655 [3]Enzymes from Aspergillus niger processed under solid state fermentation, the enzyme activity: 1253
656 SPU g^{-1} phytase, 0.196 U g^{-1} protease, 512 XU g^{-1} xylanase and 104 CMCU g^{-1} cellulase as described by
657 Jacobsen et al. (2018).
658 [4]Norse-LT, provided by Norsildmel AS, Norway.
659 [5]Antarctic krill *(Euphausia superba),* Olympic AS, Ålesund, Norway.
660 [6]Amytex 100 vital, Provided by Tereos Syral, France.
661 [7]Provided by Norgesmøllene, Norway.
662 [8]Provided by Holtermann, Norway.
663 [9]Provided by Emmelev, Demark.
664 [10]Provided by Vilomix, Norway.
665 [11]Provided by Grudlita, Lithuania.
666 [12]Provided by Denofa, Norway.
667 [13]Provided by Evonik Industries AG, Essen, Germany.
668 [14]Provided by G.O. Johnsen AS, Oslo, Norway.
669 [15]Provided by Metal rare earth Limited, Shenzhen, China.
670

671 Table 2. Chemical composition of the diets in g kg^{-1} DM.

Diet code	SPC_0	SBM_0	SBM_P	SBM_E	SBM_C
Crude protein	465	465	465	464	463
Crude Fat	242	244	245	242	246
Ash	76	82	81	82	82
Starch	158	114	114	112	105
Gelatinized starch	145	102	108	105	102
Energy (Mj/kg)	24.0	23.9	24.0	23.8	24.0
Minerals					
P	13.9	13.9	13.9	13.9	13.2
Fe	0.3	0.3	0.3	0.3	0.3
Ca	10.1	9.9	9.5	10.4	9.9
Mg	2.4	2.5	2.4	2.6	2.5
Zn	0.2	0.3	0.3	0.3	0.3
Essential Amino acids					
Lysine	23	22	23	22	22
Threonine	16	16	16	16	15
Methionine	11	11	11	11	11
Valine	20	20	20	20	20
Isoleucine	19	19	18	19	19
Leucine	35	34	34	34	34
Phenylalanine	21	21	21	21	21
Histidine	10	10	10	10	10
Arginine	24	23	24	23	23
Non-essential Amino acids					
Aspartic acid	32	32	32	32	32
Serine	21	21	21	21	21
Glutamic acid	106	107	106	107	105
Proline	36	35	35	35	34
Glycine	21	22	21	22	22
Alanine	20	20	19	20	20
Tyrosine	13	13	12	14	13

672

673

674 Table 3. Feed Processing parameters during extrusion of the experimental diets.

Diets[1]	SPC_0	SBM_0	SBM_P	SBM_E	SBM_C
Conditioning					
Temperature (°C)	91	85.5	83.5	85	85
Water (kg h^{-1})	21.6	18.9	0	18.6	18.6
Steam (kg h^{-1})	11	11	12	11	11
Moisture (%)	24.0	21.8	24.0	22.2	22.0
Extrusion					
Steam (kg h^{-1})	5	5	0	5	5
Temp. behind die (°C)	120	121	114	122	122
pressure, Section 7 (bar)	13	14	14	14	14
Moisture at die (%)	26.1	24.0	24.0	24.4	24.2
SME[2] (Wh kg^{-1} wet)	30.4	31.2	37.2	32.8	32.3

675 [1] Diets formulated as in Table 1.

676 [2]Specific mechanical energy.

677

678 Table 4. Technical quality of experimental diets.

	SPC_0	SBM_0	SBM_P	SBM_E	SBM_C	s.e.m.*	P-value**
Expansion ratio (%)	45.9^{c}	35.9^{b}	21.9^{a}	34.4^{b}	36.0^{b}	1.1	<0.001
Length	3.7	3.6	3.5	3.6	3.6	0.1	ns***
Hardness (N)	29.7^{c}	24.3^{ab}	25.6^{b}	21.3^{a}	22.8^{ab}	0.8	<0.001
Doris durability index (%)	99.7^{a}	99.7^{a}	99.6^{a}	99.7^{a}	99.9^{b}	0.0	<0.01
Water stability (%)	82.6^{d}	75.9^{c}	69.2^{a}	75.8^{c}	72.2^{b}	0.6	<0.001

679 *Pooled standard error of means.

680 **Values not sharing common superscript letters are significantly different (P<0.05) determined by

681 ANOVA followed by Tukey post hoc test.

682 *** Non-significant.

683

684 Table 5. Growth, feeding performance and biometrics of Atlantic salmon fed the five different diets.

685 Values are mean (n=3 tanks per treatment).

	SPC_0	SBM_0	SBM_P	SBM_E	SBM_C	s.e.m.*	P-value**
Start fish	75	75	75	75	75		
Mortality (%)	0	0	0.9	1.8	0	0.4	ns
Initial weight (g)	59.7	60.6	59.7	60.5	60.4	0.3	ns***
Final weight (g)	212.0^{a}	229.2^{ab}	222.6^{ab}	237.6^{b}	227.7^{ab}	4.1	<0.05
Growth (g)	152.3^{a}	168.6^{ab}	162.9^{ab}	177.1^{b}	167.3^{ab}	4.2	<0.05
Total feed intake (kg DM)	8.15^{a}	9.13^{ab}	8.90^{ab}	9.52^{b}	9.16^{b}	0.21	<0.05
FCR (DM)	0.71	0.72	0.74	0.73	0.73	0.01	ns
TGC (*1000)	2.03^{a}	2.16^{ab}	2.12^{ab}	2.24^{b}	2.15^{ab}	0.04	<0.05
SGR	1.36	1.43	1.41	1.47	1.43	0.02	<0.1
PRC (%)	55.7	54.7	54.0	54.2	54.4	0.8	ns
PRD (%)	61.1	60.0	59.7	59.1	59.4	0.8	ns
CF	1.24	1.26	1.23	1.26	1.23	0.01	ns
D%	89.6	90.1	89.7	89.9	90.1	0.4	ns
HIS	1.14	1.14	1.12	1.17	1.10	0.03	ns
OSI Mi	0.17	0.17	0.17	0.17	0.16	0.01	ns
OSI DI	0.47	0.47	0.48	0.45	0.45	0.01	ns

686 *Pooled standard error of means.

687 ** Values not sharing common superscript letters are significantly different (P<0.05) as determined

688 by ANOVA followed by Tukey post hoc test.

689 ***Non-significant.

690

691 Table 6. Apparent digestibility coefficient (%) of macronutrients, amino acids and minerals. Values

692 are mean (n=3 tanks per treatment).

	SPC_0	SBM_0	SBM_P	SBM_E	SBM_C	s.e.m.*	P-value**
Crude Protein	91.2^{ab}	91.1^{ab}	90.4^{a}	91.6^{b}	91.7^{b}	0.2	<0.01
Fat	97.4	97.3	97.6	97.7	97.6	0.1	ns***
Ash	14.1	18.2	14.8	18.0	22.7	2.0	<0.1
Energy MJ/kg	84.5^{ab}	84.5^{ab}	84.1^{a}	85.6^{ab}	86.0^{b}	0.4	<0.05
Essential Amino acids							
Lysine	92.8^{ab}	92.5^{ab}	91.8^{a}	93.0^{b}	92.9^{b}	0.2	<0.05
Threonine	90.4	90.3	89.8	90.9	90.6	0.3	ns
Methionine	94.2	94.3	94.4	95.0	94.7	0.3	ns
Valine	93.4	93.3	92.9	93.8	93.7	0.2	ns
Isoleucine	94.4^{ab}	94.2^{ab}	93.5^{a}	94.7^{b}	94.6^{b}	0.2	<0.05
Leucine	94.9	94.6	94.2	94.9	95.0	0.2	<0.1
Phenylalanine	94.6	94.3	93.8	94.6	94.7	0.2	<0.1
Histidine	91.7^{ab}	92.0^{ab}	91.3^{a}	92.5^{b}	92.3^{ab}	0.2	<0.05
Arginine	95.7^{b}	95.5^{ab}	94.8^{a}	95.7^{b}	95.5^{ab}	0.2	<0.05
Non-essential Amino acids							
Aspartic acid	81.5^{a}	83.1^{ab}	82.2^{ab}	84.0^{b}	84.5^{b}	0.5	<0.01
Serine	92.8	92.6	92.1	93.2	92.9	0.2	<0.1
Glutamic acid	96.8	96.6	96.2	96.9	96.8	0.1	<0.1
Proline	95.7	95.5	95.2	95.8	95.5	0.2	ns
Glycine	89.0	89.5	88.7	90.1	90.3	0.4	<0.1
Alanine	92.7	92.7	92.0	93.1	93.2	0.3	<0.1
Tyrosine	93.7^{b}	93.2^{ab}	92.2^{a}	94.0^{b}	93.9^{b}	0.3	<0.01
Minerals							
P	51.5	52.0	53.0	53.8	52.8	0.9	ns
Fe	12.7^{b}	-6.4^{a}	11.2^{b}	17.0^{b}	23.7^{c}	1.3	<0.001
Zn	22.0^{a}	28.9^{b}	27.5^{ab}	29.2^{b}	32.9^{b}	1.4	<0.01

693 *Pooled standard error of means.

694 **Different superscript letters denote significant differences (P<0.05) and tendencies (0.10 > P >

695 0.05) determined by ANOVA followed by Tukey post hoc test.

696 ***Non-significant.

697

698 Table 7. Histological evaluation of distal intestine[a].

	SPC_0	SBM_0	SBM_P	SBM_E	SBM_C	s.e.m.*	P-value**
Mucosal folds	1.28	1.56	1.61	1.22	1.56	0.24	ns***
Supra nuclear vacuoles	2.33	1.89	1.94	2.06	2.22	0.35	ns
Goblet cells	2.67	2.28	2.33	2.33	3.00	0.24	ns
Eosinophilic granulocytes	2.11	2.56	2.28	2.61	2.72	0.23	ns
Lamina propria	1.33	1.56	1.17	1.44	1.78	0.21	ns
Sub-epithelial mucosa	2.28	2.28	2.06	2.11	2.28	0.25	ns
Mean score	2.00	2.02	1.90	1.96	2.26	0.21	ns

699 [a]Intestinal cuts were scored according to Uran et al. (2008). A score of 1 represent normal

700 morphology, a score of 2-3 was given to mild morphological changes while a score of 4-5 represent

701 sever enteritis. Reported data are mean values (n=3 tanks / n= 6 fish per tank).

702 *Pooled standard error of means.

703 **Values not sharing common superscript letters are significantly different (P<0.05) determined by

704 ANOVA followed by Tukey post hoc test.

705 ***Non-Significant.

706

Table 8. Correlation coefficients of significant physical feed quality and fish performance variables.

	Growth	TGC	FI	Exp	Hard	DDI	WSI	Starch	Gel
Growth	1.00*								
TGC[1]	0.99*	1.00*							
FI[2]	0.99*	0.99*	1.00*						
Exp[3]	-0.36	-0.40	-0.46	1.00*					
Hard[4]	-0.95*	-0.95*	-0.95*	0.20	1.00*				
DDI[5]	0.29	0.28	0.35	0.22	-0.56	1.00*			
WSI[6]	-0.42	-0.45	-0.56	0.91*	0.38	-0.17	1.00*		
Starch[7]	-0.81[T]	-0.82[T]	-0.90*	0.66	0.81[T]	-0.40	0.83[T]	1.00*	
Gel[8]	-0.83[T]	-0.83[T]	-0.91*	0.63	0.81[T]	-0.31	0.77	0.99*	1.00*

[1]TGC, temperature growth coefficient

[2]FI, feed intake

[3]EXP, expansion ratio

[4]Hard, hardness

[5]DDI, doris durability index

[6]WSI, water stability index

[7]Starch, starch content

[8]Gel, gelatinized starch

*Denotes significant correlation coefficients ($P < 0.05$).

[T]Denotes correlation coefficients tendencies ($0.10 > P > 0.05$).