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Effects of processing full fat soybeans and fish trim for sustainable sablefish *Anoplopoma fimbria* feeds

Running title: Sustainable Sablefish Feed

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ABSTRACT

To quantify the efficacy and cost of different soy protein ingredients, heated (HSB) and underprocessed (SB) full fat soybeans (FFSB), soy protein concentrate (SPC), and trim waste from Pacific whiting (*Merluccius productus*) were incorporated into feeds for the cold water marine sablefish (*Anoplopoma fimbria)*. Feed ingredients for this study were processed using an experimental heated ball mill with demonstrated potential as an affordable process for rural or small scale feed production. The three feed treatments were formulated with equal amounts of soy protein and Pacific whiting process trim. The three feed treatments contained 46% protein and 18% lipid as dry matter. Three 480 L tanks of 10 fish were used to evaluate each of the three feed treatments. Fish were fed to satiation every two days for eight weeks. Weight gain of the treatment groups ranged between 56% and 105% over the course of the study. Fish growth was significantly better among fish receiving the feeds containing HSB and SPC than a feed containing SB. At the conclusion of the study, histomorphologic evaluations of the distal intestines of final fish were conducted. Varying levels of inflammation were measured for all three treatments. Fisher's exact test showed a significant reduction in mononuclear cell infiltration among fish fed heat treated full fat soybeans versus those receiving under-processed full fat soybeans. Fish receiving HSB feed had significantly lower intestinal inflammation than SB fish. Based on our results, a cost savings of $0.253/kg was calculated for the HSB feed over the SPC feed.

Keywords: Sablefish, full fat soybeans, histomorphologic evaluation, cost

INTRODUCTION

Increasing the volume of carnivorous marine finfish in aquaculture is limited by the amount and expense of protein meals from fish and animals for feeds (Hixson, 2014). Advances in nutrition continue to increase the efficiency of feed formulations for marine finfish species; however, industry growth for small scale or rural farmers relies on new ingredient sources and innovations to lower feed costs (Davidson, et al, 2016). Full fat soybeans (FFSB) and fish trim are inexpensive feed ingredients that are not fully utilized in marine fish feeds. The heated ball mill process introduced here is scalable and can process plant and animal materials into shelf stable feed ingredients. The most important features of the heated ball mill design (Figure 1) are the ability to produce a finished shelf stable feed ingredient that is pasteurized, dried, and ground in one unit operation. The design is similar to ore and cement ball mills that are the lowest cost solution for high volume grinding of low value materials. The potential use of automation to control the ball mill can further lower labor costs. Continuous mixing homogenizes feed ingredients, prevents bake-on, and uniformly controls temperature to preserve the nutritional value of ingredients. FFSB on average contain 38% to 40% protein and 18% to 20% oil on a dry matter basis (Yaklich, Vinyard, Camp, & Douglass, 2002). The essential amino acid profile of soy protein is complete with the exception of methionine (Zarkadas, Yu, Voldeng, & Minero-Amador, 1993). Soybean oil is a good source of energy and provides n-6 and n-3 fatty acids in the form of linoleic and linolenic acids. Soybean meal is the predominant soy ingredient for animal feeds worldwide and is a byproduct of the extraction of soybean oil from soybeans. However, when soybean meal is formulated into high energy fish feeds, it is necessary to additionally add plant or animal oils to the formulation to increase caloric content. The use of these oils is often costly to the feed producer. In contrast, the heated ball mill process enables the use of FFSB in high energy feed formulations with little additional oil required and has a cost advantage over soybean meal due to the oil present in the FFSB. Also, the temperature controlled processing of FFSB by the heated ball mill yields a soy protein with similar nutritional value to that of conventional soybean meal.

The main impediment to the use of FFSB in feeds is anti-nutritional factors (ANF), primarily in the form of protease inhibitors and lectins (Dipietro, & Leiner, 1989). Soy protease inhibitors are known to reduce the digestibility of protein in animal feeds. Soy lectins are proteins with specific binding affinity for sugar residues. The lectin-sugar complex interferes with membrane receptors in the gut where it is thought to be responsible for agglutination and mitosis (Van Eys, Offner, & Back, 2004). The soy antigens glycinin and β-conglycinin promote immune response in weaning piglets affecting weight gain and health (Sun, Li, Dong, Qioa, & Ma, 2008). Juvenile carp fed a diet containing 8% β-conglycinin had 15% lower weight gain than a control diet containing no β-conglycinin (Zhang, et al, 2013). Fortunately, soy protease inhibitors and lectins are heat labile and are inactivated with proper heat treatment above 90 ^{o}C. However, excessive heating of FFSB reduces the availability of lysine and cysteine via the Maillard reaction lowering the nutritive value (Caprita, Caprita, Ilia, Cretescu, & Simulescu, 2010). There are other anti-nutritional compounds in soybeans with possible synergies that can affect fish growth and health (Francis, Harinder, Makkar, & Becker, 2001). Some ANF are fish species specific. Further research is needed to understand their effects.

Fish trim consisting of heads, back bones, viscera, and skin contains essential amino acids, oils, vitamins, and minerals to support fish growth (Miles, & Chapman, 2006). Co-processing fish trim with full fat soybeans has the potential to produce an economical feed ingredient for finfish culture. Moisture absorbed by FFSB from co-processing with fish trim increases the efficiency of heat inactivation of ANF (Van den Hout, Meerdink, & Van't Riet, 1999). Developing a process that combines fish trim with FFSB would serve small scale feed markets that have access to these raw materials by reducing transportation and ingredient costs. The goals of this project were: 1) Test a new heated ball mill process to produce fish feed ingredients from fish process trimmings and FFSB, 2) Evaluate the growth and health of sablefish (*Anoplopoma fimbria*) fed three feeds containing under-processed FFSB (SB), fully inactivated FFSB (HSB), and soy protein concentrate (SPC).

Materials and Methods

FFSB and Fish Trim

Commodity grade soybeans, grown in the state of Minnesota, were supplied by Cargill (Minneapolis, MN, USA). Average seed count per gram was determined by weighing three samples of 200 soybeans each. Pacific whiting (*Merluccius productus*) trim was collected from the processing line of Ocean Gold Seafood, Inc. (Westport, WA, USA), frozen and held at -28ºC until thawed for use. Trim consisted of heads and viscera. Three one kilogram samples of trim were progressively ground through 1", 1/2", and 1/4" plates in a Hobart Model 4146 meat grinder (Hobart, Troy, OH, USA) and saved for proximate analysis. Proximate analyses of FFSB and the fish trim ingredients are shown in Table 1.

Feed processing

An experimental heated ball mill, previously described (Nicklason, Xu, Johnson, Sommers, & Armbruster, 2016), was used to make a finished feed ingredient consisting of the fish trim and FFSB in a single operation (Figure 1). For the HSB ingredient, the ball mill drum was sealed for 30 minutes at 95°C to cook the starting mixture of dry FFSB and thawed fish trim prior to grinding and drying with forced air. Feed mash processed to make the SB ingredient had no cook step and air circulated continuously during processing. The dry finished feed ingredient passed through a US no.14 sieve. Bone fragments larger than 14 mesh were discarded. Feeds were formulated as listed in Table 2 and were pelleted to 4 mm using a California Pellet Mill (San Francisco, CA, USA).

Fish Feeding

One gram sablefish hatched at the Northwest Fisheries Science Center (NWFSC) Manchester Research Station, Port Orchard, WA were transported to the re-circulating saltwater system at NWFSC Montlake campus in Seattle, WA and raised to approximately 120g on commercial salmon feed (Bio-Fry, Bio-Oregon, Longview, WA, USA). Fish were graded for uniform size, and 10 fish were stocked into each of nine 480 L tanks. Fish were over 100g at the beginning of the experiment. Because of their size, 10 fish were used per tank to account for growth and minimize stress associated with crowding. The three experimental feeds were randomly assigned to three tanks each. Fish were fed to apparent satiation every other day for eight weeks. Fish were bulk weighed by tank every two weeks. Average water temperature, salinity, and pH during the study were 12℃, 30 ppt, and 7.9, respectively. Ammonia concentrations remained below 0.1 mg*L^{-1} during the study. Guidelines for laboratory aquatic animals as established by the National Research council (National Research Council, 2011) were followed for fish care.

Sample collection and analysis

At the conclusion of the trial, fish were fasted for 72 hr., and then five fish from each tank were euthanized with procedures developed by the American Veterinary Medical Association (AVMA, 2007) for tissue sample collection via immersion in a 200 mg L^{-1} solution of tricaine methanesulfonate (Western Chemical Inc., Ferndale, WA, USA) and stunned by a blow to the head. Fish weights and fork lengths were recorded before the livers were excised and weighed. Fultons condition factor (K) was used to assess fish health where K = 100 x weight (grams) x length $(cm)^{-3}$. Hepatosomatic index (HSI) was calculated for each fish using the equation, HSI= liver weight x 100/fish weight. Five whole fish from each tank, including livers, were ground three times through a 1/4" plate in a Hobart meat grinder and thoroughly mixed for whole body proximate analysis as described below.

A 2 cm section of distal intestine, approximately 2 cm from the rectum was excised, cut longitudinally, and placed in a 10% buffered formalin solution. Intestinal samples were sent to Fish Vet Group (Portland, ME, USA) for histomorphologic evaluation. Tissues were prepared by sectioning at 4 µl, and staining with hematoxylin and eosin (H&E).

Slides were scanned digitally at 40x magnification on a Leica SC2 scanner and tissues were examined blindly by a single pathologist using ImageScope software (Leica Biosystems, Richmond, IL, USA). Cellular characteristics were quantitatively and semi-quantitatively assessed as described in Table 3. Semi-quantitative scoring was recorded as “1”-minimal to “5”-severe change. Advanced change was defined as a score of 3 to 5.

2.5 Proximate analysis

Moisture and ash were determined according to standard methods (AOAC, 1980). Protein was measured by determining nitrogen concentration by Dumas combustion methodology with a LECO FP-2000 nitrogen analyzer (LECO Corporation, St. Joseph, MI, USA), and multiplying the result by 6.25. Fat content was determined by Soxhlet extraction using methylene chloride as a solvent.

Urease testing

All feeds were ground with a small food mill to pass a 40 mesh screen. Urease activity in the ground samples were measured using AOCS Official Method Ba 9-58. Triplicate measurements were made for each.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed to detect significant differences in fish growth, feed performance, body indices, intestinal measurements, and whole body proximate composition attributable to experimental feed using R version 3.4.3 (The R foundation for Statistical Computing, Vienna, Austria). When significant differences were detected by ANOVA, the Tukey HSD test was subsequently employed to assess the significance of differences between treatment means. Differences in the occurrence of an intestinal pathology attributable to a feed were statistically evaluated using Fisher's exact test. Differences were deemed significant when $P < 0.05$.

RESULTS

Juvenile sablefish fed HSB and SPC feeds had extremely similar weight gain and feed conversion ratios (FCR) exceeding the performance of SB fish over the course of the trial (Figure 2). The weight gain, thermal growth coefficient, and feed intake were significantly different for HSB and SPC versus SB (Table 4). Whole body proximate analysis of final fish (Table 5) showed moisture content of SB fish was significantly higher than HSB fish ($P = 0.01$) and SPC fish ($P = 0.005$). Conversely, whole body lipid content of SB fish was significantly lower than HSB fish ($P = 0.006$) and SPC fish ($P = 0.004$). Significant differences were also detected for hepatosomatic index and condition factor between SB fish and the HSB and SPC fish ($P < 0.005$).

Histological scoring is shown in Table 6. There were varying levels of inflammation in the distal intestine for all three treatments. SB fish displayed increased inflammatory changes characterized by a relative increase in mononuclear cell infiltration vs. HSB fish. The occurrence of advanced (score of 3 to 5) mononuclear cell infiltration was significantly higher among SB fish than HSB fish. HSB fish intestines were similar to those of SPC fish. Figure 3 shows a representative distal intestine of a SB fish and a HSB fish. Numerically, lamina propria width was greatest among SB fish and showed greater variation between fish in this group as defined by the standard deviation, but was not statistically different than HSB and SPC fish.

The ΔpH mean of urease activity of the SPC and HSB feeds was 0.0 and 0.0 (n=3). The ΔpH mean of urease activity of the SB feed was 0.44 at 30 minutes, 0.69 at 60 minutes, and 2.1 at 20 hrs (n=3).

DISCUSSION

The inactivation of urease in FFSB by heating is highly correlated to inactivation of serine protease inhibitors and other anti-nutritional factors (Caprita, Caprita, Gheorghe, Cretescu, & Simulescu, 2010). Lectin in soybeans interferes with digestion by binding to and agglutinating intestinal membranes. Inactivation of lectins also correlates highly with urease inactivation (Fasina, Classen, Garlich, Swaisgood, & Clare, 2003). For carnivorous fish feeds, a urease test resulting in a pH increase (ΔpH) of less than 0.04 after 30 minutes is targeted for inactivation of ANF. A ΔpH of 0.05 to 0.20 after 30 minutes will have variable impacts on feed performance depending on the species. A urease test with a ΔpH above 0.20 limits the amount of soy that can be used in feed for fish and many other animal species (Van Eys, Offner, & Back, 2004). In the present study, FFSB and fish trim were co-processed with the heated ball mill. The first 30 minutes of heating of HSB feed mash occurred in a sealed mill. As the mixture was heated, free water released from the fish trim was absorbed by the FFSB. This aided in the heat transfer and inactivation of ANF. Samples of FFSB taken during HSB feed processing had moisture contents in the range of 39% to 54% after 30 minutes of heating. The mill was then unsealed and air circulation was turned on to dry the product. The ΔpH for the HSB batches was 0.0 indicating inactivation of the major ANF lectins and protease inhibitors. The FFSB/fish trim batches for the SB feed had the same initial moistures as those for the HSB feed, but were processed differently. The differences were the mill was not sealed during the first 30 minutes of heating and air circulation was turned on. The process time was shorter and the amount of time the FFSB was above 90ºC was less due to evaporative cooling. This resulted in a product with active urease ΔpH of 0.44 after 30 minutes. At 20 hours the ΔpH was 2.1, which is the stoichiometric endpoint for the conversion of 3.0% urea to ammonia. This is of particular concern for sablefish which are suspected of having an extended gut retention time and would be especially susceptible to the ongoing activity of under-processed FFSB.

Weight gain, thermal growth coefficient, and feed intake were significantly greater for HSB and SPC fish versus SB fish. Mean feed consumption of SB fish averaged 45% lower than the other treatments. Feed formulations of the SB and HSB feeds were nearly identical as was the processing of the FFSB with fish trim ingredient used in the feeds. This suggests feed palatability is likely not a factor driving feed intake. A review of ANF in plant derived feed ingredients found that compounds such as lectins, phytic acids, protease inhibitors, and allergens can influence feed intake in fish (Francis, Harinder, Makkar, & Becker, 2001). Work with Atlantic salmon (*Salmo salar*) and soy ingredients showed differences in growth, feed intake, and FCR between fish fed diets with standard soybean meal (SBM) and soybean meal processed to remove oligosaccharides, trypsin inhibitors, and lectins (RO-SBM; Refstie, Storebakken, & Roemb, 1998). For all these parameters, fish fed the RO-SBM feed outperformed fish fed the SBM feed. The authors found the increase in FCR among fish fed the SBM feed was correlated with lower protein, fat, and energy digestibility. Of particular interest to the current study, a palatability study followed the growth study where fish were fed a combination of the fishmeal control feed and SBM or RO-SBM feeds marked with different inert rare-earth markers. The authors demonstrated that palatability was not a major factor driving the previously observed lower feed consumption of SBM feed vs the RO-SBM feed for Atlantic salmon. In a separate study Nile tilapia (*Oreochromis nilotica*) were fed six diets for 70 days with increasing levels of unheated FFSB that retained proportional levels of trypsin inhibitor (Wee & Shu, 1989). Significant differences in specific growth rate became apparent after 4 weeks. Additionally, feed efficiency, digestibility and feed intake trends decreased with the increasing levels of unheated FFSB indicating a reduction in fish health from under processed FFSB.

In the present study, FCR of the SB feed was higher, but not significantly different than that of the HSB and SPC feeds and similar to those observed previously for this species (Reid, Forster, Cross, Pace, Balfry, & Dumas, 2017; Johnson et al, 2016). In other studies with Atlantic salmon, soybean diets caused alterations in the distal intestine irrespective of heat treatment (Van den Ingh, Olli, & Krogdahl, 1996; Refstie, Sahlstrom, Brathen, Baeverfjord, & Krogedal, 2005). Heat stable alcohol soluble soybean oligosaccharides and saponins were suspected to be likely causes. In addition, distal intestine morphology was measured for larger Atlantic salmon in net pens fed diets containing 30% FFSB meal, 50% herring meal, or 26% soy protein concentrate for four months (Van den Ingh, Krogdahl, Olli, Hendriks, & Koninkx, 1991). Soy protein concentrate fish showed no abnormalities and intestinal health was similar to that of herring meal fish whereas FFSB fish had marked changes in the epithelium and microvilli of the distal intestine. Profine VF, the sole soy protein source in the SPC feed in the current study, is processed to remove oligosaccharides and other antagonistic compounds and would be expected to perform better than HSB feed; however, the HSB and SPC feeds performed similarly. Possible explanations include the higher level of wheat flour in the SPC feed countered any performance gain in SPC, or the heated FFSB in the HSB feed performed as well as the SPC. In a study with coho salmon (*Oncorhynchus kisutch*) heated defatted soy flour was found to have similar protein digestibilty to that of Profine VF (Arndt, Hardy, Sugiura, & Dong, 1999).

There have been numerous feeding studies investigating the potential of raw or processed FFSB ingredients for a variety of fish species. Similar to the previously cited salmon studies, under processed FFSB with residual ANF negatively impacted fish growth or fish health in virtually every study. In the current study, decreases in HSI, condition factor, and whole body lipid content in SB fish were correlated with increased inflammation in the distal intestine of sablefish. This included more intense infiltration of the tissue with mononuclear cells and a trend towards increased lamina propria width that may be associated with space occupying inflammatory changes such as mononuclear cell infiltration or edema fluid. In a study with Asian seabass (*Lates calcarifer*), fish fed a feed containing raw FFSB had significantly lower growth and higher intestinal pathologies than fish that received a feed with steamed FFSB (Boonyaratpalin, Sutaneiranat, & Tunpibal, 1998). Fish receiving the raw FFSB feed had a decreased number of absorptive vacuoles in the mucosal epithelium, microvilli were not present, and mononuclear cell infiltration of the lamina propria was observed. Atlantic halibut (*Hippoglossus hippoglossus*) were less affected when fed graded level of toasted FFSB up to 36% fishmeal nitrogen substitution. The authors observed no significant differences in growth, feed efficiency, or intestinal morphology, but HSI significantly decreased with FFSB addition (Grisdale- Helland, Hellend, Baeverford, & Berge, 2002). Heat treated full fat soybean meal (FFSM) was added to rainbow trout diets in place of LT fish meal, wheat, and fish oil up to 25% with no effect on fish growth or survival (Morris, Gallimore, Handley, Hide, Haughton, & Black, 2005). The FFSM was prepared by pressure cooking cracked and dehulled FFSB followed by drying and grinding. There was no observed increase in intestinal enteritis among study fish, but again, HSI decreased with FFSM addition suggesting a reduction in fish health.

Omnivorous fish such as Nile tilapia (*Oreochromis niloticus*) are less affected by under processed FFSB. To encourage the creation of simple feeds prepared on-farm for tilapia, the nutritive value of boiled FFSB was investigated (Wee, & Shu, 1989). The authors observed boiling to improve both digestibility and retention of FFSB protein. Fish grew well on a diet of 58% boiled FFSB with residual 0.6 mg trypsin inhibitor/g diet. The 58% boiled FFSB treatment group also had the highest body lipid content. In a different study, replacing soybean meal protein with raw soybeans in tilapia diets at levels of 15%, 25%, and 35% affected the weight gain, feed intake, and protein retention (Martins, Pezzato, Guimaraes, Padovani, & Mazini, 2017). HSI and whole body lipid content were lowest in the 35% group in addition to fish having a significantly higher plasma neutrophil count. In another omnivorous species, mirror carp (*Cyprinus carpo*) were fed diets containing 62% fishmeal, a commercial trout diet, or 50% heated FFSB and 31% fishmeal (Abel, Becker, Meske, & Friedrich, 1984). Five FFSB feeds were included in the study, which differed in how FFSB was processed (i.e. the use of mild or intense, dry or wet heat, and a raw FFSB treatment). Three of the heated FFSB feeds outperformed the commercial diet, but not the high fishmeal diet. The authors speculated further improvements in growth could be achieved by supplementing the FFSB feeds with synthetic amino acids.

In all the previous studies, fish exhibited reduced growth or compromised health when fed under processed FFSB. Tilapia and carp were the most tolerant of active FFSB. Salmon species were sensitive to several ANF, including oligosaccharides and saponins. In this study, juvenile sablefish had reduced growth and feed consumption when fed a feed containing FFSB with active protease inhibitors and lectins. In contrast, there was no significant difference in the performance of heat inactivated FFSB and soy protein concentrate feeds indicating juvenile sablefish may be tolerant to oligosaccharides and saponins. Results indicate that properly processed FFSB performs as well as more expensive soy protein concentrates for this marine species.

The heated ball mill process has the potential to lower capital and operating costs for small or remote feed producers. The ingredients that varied between the three formulations and their prices are listed below and were used to compute differences in formulation costs.

FFSB = \$0.42 kg^{-1} SPC = \$2.09 kg^{-1}

Wheat = \$0.33 kg^{-1} Fish oil = \$1.76 kg^{-1} Poultry fat = \$0.88 kg^{-1}

Based on the formulations in Table 2, the ingredient cost for HSB is \$0.253 kg^{-1} lower than SPC formula. The fat price used for this estimate is for poultry fat, instead of the fish oil used in the feed formulations. Also, the whiting trim ingredient provides 5.4% fish oil, which is an additional cost savings of these feeds, and would contribute approximately 1.1% of essential long chain n-3 high unsaturated fatty acids to the dry diet.

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Data Availability Statement. Data generated in this study will be made available upon request.

Conflict of Interest. There are no commercial or personal conflicts of interest with authors and associated members of this study.

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Table 1. Nutrient composition of full fat soybeans, Pacific whiting trim, and seed count.

	Full fat soybeans (FFSB)	Pacific whiting trim
Chemical analysis (g/100g, dry weight basis)		
Protein	38.6	58.4
Fat	18.8	23.4
Ash	4.8	17.8
Moisture	11.0	78.2
Seed count/gram	7.1	

Table 2. Formulas and chemical composition of experimental diets.

[a] Profine VF, Central Soya Company, Inc., Fort Wayne, IN.

	Active Soy Feed (SB)	Inactive Soy Feed (HSB)	Soy Protein Conc. Feed (SPC)
Ingredient (g/100g)			
Soy protein concentrate [a]	18.6	16.5	25.6
Active FFSB	19.5	-	-
Inactive FFSB	-	18.4	-
Corn protein concentrate [b]	9.7	10.3	10.0
Wheat flour	14.3	16.8	23.8
Pacific whiting trim	22.0	23.2	22.1
Menhaden oil [c]	9.6	8.5	12.2
Vitamin pre-mix [d]	1.0	1.0	1.0
Mineral pre-mix [e]	0.1	0.1	0.1
Stabilized vitamin C	0.5	0.5	0.5
Choline	0.5	0.5	0.5
Dicalcium phosphate	2.0	2.0	2.0
L-Lysine	0.2	0.2	0.2
L-Methionine	0.15	0.15	0.15
Taurine	1.0	1.0	1.0
Lignin sulfonate	1.0	1.0	1.0
Antioxidant [f]	0.03	0.03	0.03
Chemical composition (g/100g, dry weight basis)			
Protein	45.8	46.0	46.3
Lipid	18.3	18.5	18.4
Ash	7.5	7.7	7.4

[b] Empyreal 75, Cargill Inc., Blair, NE.

[c] Omega Protein Inc. Houston, TX.

[d] ARS 702; contributed, per kg diet; vitamin A 9650 IU; vitamin D 6600 IU; vitamin E 132 IU; vitamin K3 1.1 gm: thiamin mononitrate 9.1 mg; riboflavin 9.6 mg; pyridoxine hydrochloride 13.7 mg; pantothenate DL-calcium 46.5 mg; cyancobalamin 0.03 mg; nicotinic acid 21.8 mg; biotin 0.34 mg; folic acid 2.5 mg; inositol 600 mg.

[e] ARS 860, contributed in mg/kg of diet; manganese 13; iodine 5; copper 9; zinc 40.

[f] Tenox™ 4 Antioxidant, Eastman Chemical Company, Kingsport, TN.

Table 3. Histomorphological evaluation rubric for distal sablefish intestines. Prepared by David Marancik for Fish Vet Group.

Parameter	Score*				
	1	2	3	4	5
Supranuclear vacuoles	Occupy entire area of enterocytes	Medium-sized in less than half of the enterocytes	Small-sized in many enterocytes	Scattered and small in few enterocytes	No vacuoles observed
Goblet cells Infiltration of eosinophilic granulocytes Mononuclear cell infiltration	Few observed, scattered	Increased number, but sparsely distributed	Increased number, and widely spread	Densely grouped	Highly abundant and tightly packed
Mucosal length (µm) Lamina propria width (µm) Submucosal width (µm)	Performed via digital measurements. Average of four areas per tissue.				

*Semi-quantitative scoring was ranked as “1”-minimal to “5”-severe change. For quantitative scoring, relatively longer mucosal length and shorter lamina propria and submucosal width were interpreted to represent healthier tissue.

Table 4. Growth performance of juvenile sablefish fed plant based feeds containing different soy proteins. Values are mean ± SD, *n=3*. Lowercase superscript letters within a row indicate significant difference between treatment means (P<0.05).

	SB Feed	HSB Feed	SPC Feed	P-value
Survival (%)	97 ± 6	100 ± 0	87 ± 23	0.506
Initial weight (g)	120 ± 0	119 ± 1	119 ± 1	0.167
Final weight (g)	187 ± 23 ^{a}	243 ± 3 ^{b}	245 ± 5 ^{b}	0.004
Weight gain (%)	56 ± 19 ^{a}	105 ± 4 ^{b}	105 ± 6 ^{b}	0.003
TGC	1.16 ± 0.36 ^{a}	1.97 ± 0.05 ^{b}	1.98 ± 0.08 ^{b}	0.005
FI (g fish^{-1})	91 ± 23 ^{a}	166 ± 2 ^{b}	168 ± 6 ^{b}	0.001
FCR	1.43 ± 0.24	1.34 ± 0.02	1.35 ± 0.04	0.701
PRE (%)	22.4 ± 3.7	23.9 ± 0.6	23.9 ± 1.6	0.678

Note. TGC: thermal growth coefficient, FI: feed Intake, FCR: feed conversion ratio, PRE: protein retention efficiency.

Table 5. Body indices and nutrient composition of juvenile sablefish fed plant based feeds containing different soy proteins. Values are mean ± SD of tank composite samples of 5 fish each, *n=3*. Lowercase superscript letters within a row indicate significant difference between treatment means (P<0.05).

	Initial fish*	SB Feed	HSB Feed	SPC Feed	P-value
Body indices					
Length (mm)	233 ± 1	279 ± 9	293 ± 3	290 ± 3	0.059
CF (g/cm^3 x 100)	0.83 ± 0.00	0.92 ± 0.02 ^{a}	1.01 ± 0.02 ^{b}	1.00 ± 0.02 ^{b}	0.002
HSI	-	1.7 ± 0.2 ^{a}	2.2 ± 0.1 ^{b}	2.3 ± 0.1 ^{b}	0.001
Whole body chemical analysis (g/100g wet weight)					

Protein	14.5 ± 0.2	14.4 ± 0.0	14.6 ± 0.2	14.7 ± 0.3	0.480
Lipid	11.3 ± 0.3	13.6 ± 0.4 [a]	16.6 ± 0.2 [b]	16.9 ± 1.2 [b]	0.003
Ash	2.4 ± 0.0	2.3 ± 0.1	2.2 ± 0.2	2.2 ± 0.0	0.265
Moisture	72.2 ± 0.4	70.0 ± 0.4 [b]	67.4 ± 0.3 [a]	67.0 ± 1.1 [a]	0.004

*Initial fish values are mean ± SD of 3 composite samples of 5 fish each.

Table 6. Histomorphological measurements of sablefish distal intestines. Length and width results are mean ± SD of individual fish samples, *n=8*. Number of fish showing an advanced pathology are out of 8 fish per treatment. Lowercase superscript letters within a row indicate significant difference between treatment means (P<0.05).

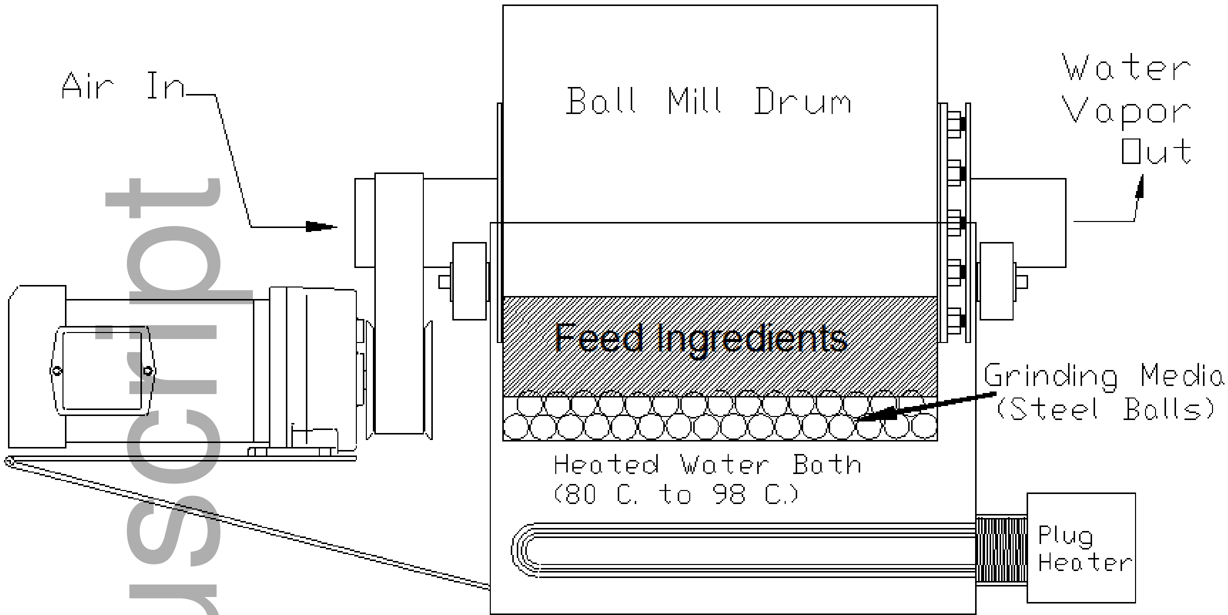
	Feed SB	Feed HSB	Feed SPC	P-value
Parameter				
Mucosal length (um)	982 ± 131	877 ± 117	894 ± 206	0.368
Lamina propria width (um)	9.1 ± 5.3	6.6 ± 2.0	7.0 ± 1.8	0.312
Submucosal width (um)	23.0 ± 3.1	23.8 ± 3.2	22.0 ± 3.3	0.544
Fish with an advanced pathology (score of 3 to 5)				
Supranuclear vacuoles density	4	3	2	0.866
Goblet cell density	4	4	7	0.261
Eosinophillic granulocytes infiltration	6	6	7	1.000
Mononuclear cell infiltration	5 [b]	0 [a]	2 [ab]	0.031

Figure Legends

Figure 1. Heated ball mill designed for simultaneous grinding, mixing, and drying of fish feed ingredients. Full-fat soybeans and Pacific whiting trim ingredients used in the present study were processed with the heated ball mill.

Figure 2. Growth of juvenile sablefish fed feeds containing different soy proteins during the eight week study. Mean weight of fish is shown by treatment; SPC feed (- - ● - -), HSB feed (—♦—), SB feed (– –▲– –), *n=3*. At 8 weeks, the mean weight of SB fish was significantly less than mean weights of SPC and HSB fish (P=0.004).

Figure 3. Histological slides of distal intestines from juvenile sablefish fed (a) an untreated full fat soybean feed, SB, and (b) a heat treated full fat soybean feed, HSB, for 8 weeks. SB fish displayed increased mononuclear cell infiltration within the lamina propria vs. HSB fish. Histomorphologic characteristics of HSB fish intestines were similar to those of fish fed a soy protein concentrate feed throughout the study. Histomorphological results are summarized in Table 6. GC-goblet cells, EG-eosinophilic granulocytes, MC- mononuclear cells.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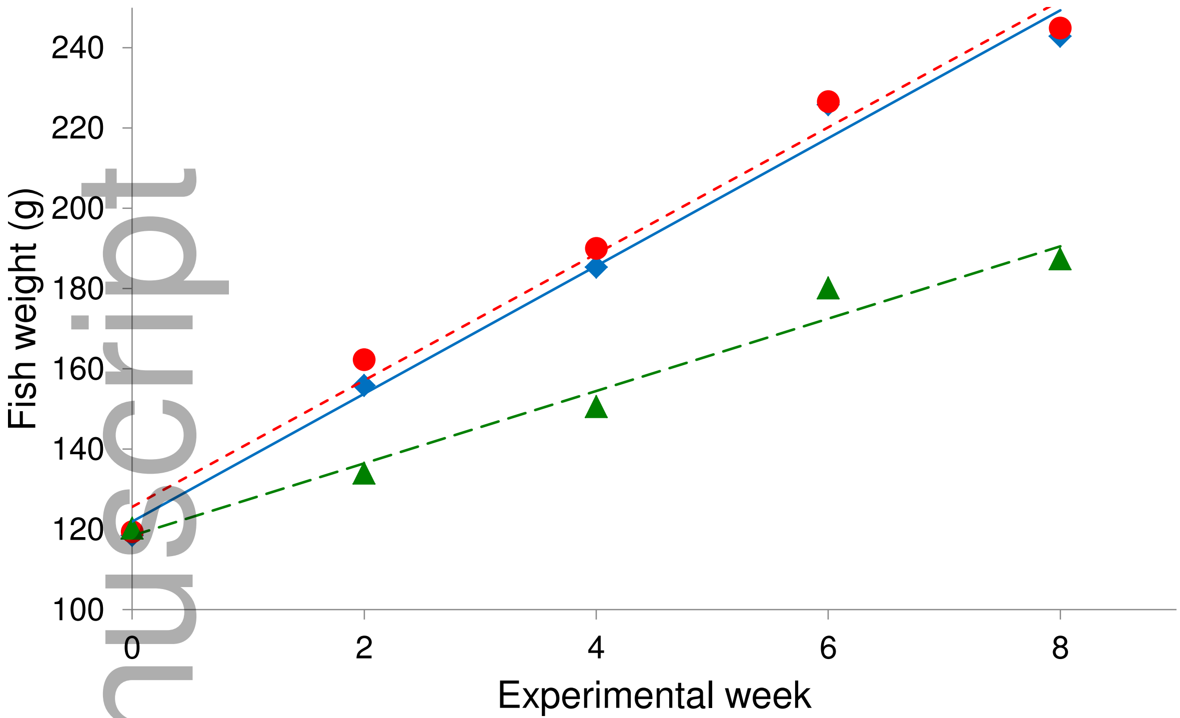
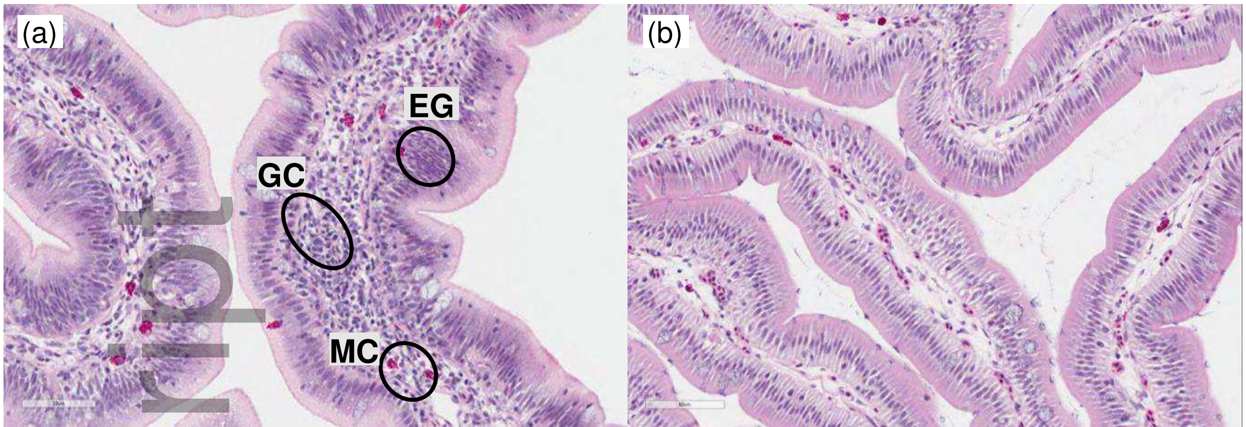
Ball Mill Drum
Water Vapor Out
Feed Ingredients
Grinding Media (Steel Balls)
Heated Water Bath (80 C. to 98 C.)
Plug Heater


Fish weight (g)
240
220
200
180
160
140
120
100
0
2
4
6
8
Experimental week


(a)
EG
GC
MC
(b)