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NUTRITIONAL AND IMMUNOLOGICAL EVALUATION OF SOY-BASED DIETS FOR SUMMER FLOUNDER (*Paralichthys dentatus*) AQUACULTURE

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ABSTRACT

Summer flounder aquaculture has progressed significantly in the last twenty years, and diets have been optimized to provide optimal nutrition, ensuring a high quality final product. Recently, the focus of research has evolved toward finding a replacement for fish meal in diets for many species, as the demand for fish meal worldwide has caused the primary protein product to become increasingly economically volatile. Therefore, a series of experiments were designed to systematically evaluate feeds for summer flounder (*Paralichthys dentatus*) including soy-based products as the primary protein source. The first feed trial involved the comparison of three diets; a fish meal control, a 60% fish meal replacement diet with soybean meal (SBM), and a 60% fish meal replacement diet with soybean meal and soy protein concentrate (1:1 ratio, SBM/SPC), in order to investigate the impact of including soy products in diets for summer flounder on growth and survival following challenge (post-feeding trial) with a bacterial pathogen of summer flounder (*Vibrio harveyi*). The diet with the majority of the FM replaced with SBM/SPC produced significantly greater growth, and survival following bacterial challenge, suggesting that a combination of soybean meal and soy protein concentrate could provide a good alternative to fish meal-based replacement diets by providing equal growth to fish meal diets and enhanced resistance to bacterial challenge. A second trial was designed to systematically evaluate which combination of soybean meal and soy protein concentrate would result in the best growth and survival to bacterial challenge. A diet comprised of 40% fish meal, 48% soy protein concentrate, and 12% soybean meal resulted in significantly better growth and survival following bacterial challenge compared to a fish meal

control diet, confirming the results from the first trial. Additional feed trials were designed to investigate the compounds responsible for both the reduced growth and the increased survival following bacterial challenge, in order to determine which compounds of soybean meal, but absent in soy protein concentrate, may be responsible for the effects on growth and survival to bacterial challenge. In the first feed trial in this series, ,in addition to a fish meal-based control, four additional diets were formulated to contain 60% soy protein concentrate and 40% fish meal as protein sources, with 0.53%, 1.23% or 1.94% soy molasses (w/v) added to the diet. Growth was reduced with soy molasses added to the diet, with the most decreased growth in the group fed the 1.94% soy molasses-supplemented diet, suggesting that antinutritional factors present within soy molasses are responsible for decreased growth observed in summer flounder fed soybean meal-based replacement diets. Therefore, a second feed trial was designed to further investigate the role of compounds present in soybean molasses on growth and survival. Soy molasses was fractionated using *n*-butanol to phase-separate the different compounds within the soy molasses into either a water (primarily enriched with oligosaccharides), interphase (containing a mix of saponins, oligosaccharides, ash and proteins) or butanol (including saponins) phase and 3 diets were prepared by adding each sub-fraction to a soy protein concentrate replacement (60%) diet at a level corresponding to a 12% soybean meal replacement level (best results from trial 2). Growth was significantly lower for all diets with soy molasses fractions added compared to groups fed either the fish meal or soy protein concentrate control diets. Survival following challenge was highest for the groups fed the diets containing either the water phase or butanol phase

of soybean molasses, suggesting that oligosaccharides, alone or in combination with low levels or other antinutritional factors, may be responsible for increased survival to bacterial challenge compared to the fish meal diets. Finally, a feed trial was designed to test the effect of three different levels of oligosaccharide supplementation (0.2%, 0.4% or 0.6% supplementation with stachyose hydrate and raffinose pentahydrate combined at a 3.16:1 ratio (w/w)) to a 60% soy protein concentrate replacement on growth and survival to bacterial challenge. The diet which produced the greatest growth and significantly better survival than the soy protein concentrate-based control diet included an oligosaccharide supplementation level of 0.4% (w/w). These results demonstrate that soy protein concentrate can replace fish meal in summer flounder diets at a high level (60%) with no negative effects on growth. Furthermore, supplementation of the soy protein replacement diet with crystalline oligosaccharides or an oligosaccharide-rich fraction resulting from the phase-separation of soybean meal may decrease susceptibility to disease. These soy-based diets would provide a sustainable, economically viable alternative to fish meal diets for marine finfish aquaculture.

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INTRODUCTION

World capture fisheries have been stable throughout much of the past decade, and it has been estimated that the maximum capture fisheries potential from global waters has been reached (in 2007 as many as 50 percent of stocks were labeled as fully exploited; FAO, 2008). With many US wild groundfish stocks at either maximum catch levels or in decline, the US must increase its aquaculture production, or risk increasing the already $8 billion annual trade deficit in seafood (FAO, 2008). Two of the major challenges to the expansion of marine aquaculture are the cost of feeds and issues with disease (Stentiford et al., 2012).

Fish meal is one of the major components of the diets of popular marine carnivorous fish like flounders, seabasses, and tunas (Murray et al., 2010). The cost of fish meal is highly variable due to the reliance on capture fisheries and the demand for fish meal available for aquafeeds is expected to exceed supply within the next decade (Gatlin et al., 2007). It is now apparent that the aquaculture industry must look to alternative protein sources to keep expanding. Processed soybean (*Glycine max*) produces a high quality source of protein that can replace fish meal as an economically and nutritious alternative (Gatlin et al., 2007; Salze et al., 2010; Riche and Williams, 2011). Crude soybean protein has a balanced amino acid profile, though the presence of several antinutritional factors requires the evaluation of its incorporation into fish diets (Sitja-Bobadilla et al., 2005; Sun et al., 2007; Salah-Azaza et al., 2009). Widespread incorporation of soybean meal or soy protein concentrate into diets for marine aquaculture not only opens up a new market for soy products, it is also one way to

make carnivorous fish culture much more sustainable. Replacing fish meal with soy protein has shown promising results for many species of flatfish, such as Japanese flounder (*Paralichthys olivaceus*) (Kikuchi, 1999; Sun et al., 2007), Egyptian sole (*Solea aegyptiaca*) (Bonaldo et al., 2006), and Atlantic halibut (*Hippoglossus hippoglossus*) (Murray et al., 2010).

Researchers at the University of Rhode Island (URI) previously investigated the use of soybean meal, corn gluten meal, and canola protein concentrate in summer flounder diets either alone or in combination (Enterria et al., 2011). Although flounder growth on the control fish meal diet was superior to that obtained when fish meal was replaced to varying degrees with plant proteins, lowest cost per kg of fish produced was obtained with 40% replacement of fish meal with soybean meal. Further studies at URI investigating the addition of taurine to fish meal – soybean meal diets indicated that soybean meal can replace at least 40% of fish meal in a diet for summer flounder without a significant decrease in growth if taurine is added to the diet (Bengtson et al. in prep.). URI research funded by the United Soybean Board (USB) indicated that a mixture of a low level of soybean meal (~12%) in conjunction with up to 60% soy protein concentrate can replace a majority of the fish meal in diets for summer flounder in a 12-week study. In a subsequent six-month growout trial of juvenile summer flounder funded by USB, no significant differences in growth were seen between a commercial diet and an experimental diet in which 65% of the fish meal was replaced by soy protein concentrate (Bengtson et al. in prep.).

Another key issue facing commercial grow-out of flatfish is disease outbreaks when fish are raised at a high density. Recent outbreaks of vibriosis in summer flounder at both the hatchery and a growout site in the Northeast caused widespread mortality, demonstrating that the disease problems are not limited to the hatchery (George Nardi per. comm.). One of the main disease issues is flounder infectious necrotizing enteritis (FINE), which is caused by the pathogenic bacterium *Vibrio harveyi* (Soffientino et al., 1999). In 2008, an eight-week feeding trial was performed at URI in which summer flounder were fed 0%, 40%, or 70% replacement of fish meal with soybean meal in order to evaluate the upper limit of soybean meal replacement in diets for summer flounder. The fish were then subjected to a seven-day bacterial challenge developed by Soffientino et al. (1999), utilizing a bacterial pathogen of summer flounder, *Vibrio harveyi*. While growth was reduced in groups fed diets with 70% fish meal replaced with soybean meal, survival following bacterial challenge was significantly increased. These results indicate that soybean meal addition improved disease resistance to *V. harveyi*, even though growth was reduced (Lightbourne, 2011). Furthermore, preliminary results from a bacterial challenge following a 6-month feeding trial performed with the 65% soy protein concentrate diet compared to a commercial fish meal diet, indicated that no protection against challenge with *Vibrio harveyi* was provided by soy protein concentrate (Bengtson et al., in prep.).

This apparent health benefit to summer flounder of diets containing soybean meal, but not soy protein concentrate, may be due to immunostimulatory effects of compounds present in soybean meal, as shown in a study with rainbow trout (Rumsey et al., 1994).

Potential immunostimulatory effects have been hypothesized to be attributable to the antinutritional factors (ANFs) found in unprocessed soybean meal, but which have been removed from soy protein concentrate (Grisdale-Helland et al., 2008). These ANFs include protease inhibitors (trypsin inhibitors), oligosaccharides (stachyose, raffinose, etc.), saponins, isoflavones, antigens (glycinin, B-conglycinin, lectins), phytate, and tannins (Refstie et al., 2005; Knudsen et al., 2007; Iwashita et al, 2009). Heat processing of the soybean meal, or alcohol extraction to produce soy protein concentrate, removes many of these factors, and produces a product which may allow for good growth rates without morphological intestinal changes (Francis et al., 2001; Knudsen et al., 2007). The alcohol-soluble fraction in soybean meal contains compounds known to cause morphological changes in the intestines of many species, such as Atlantic salmon (*Salmo salar*) (Knudsen et al., 2007; Yamamoto et al., 2008), rainbow trout (*Oncorhynchus mykiss*) (Rumsey et al., 1994; Yamamoto et al., 2008), and Atlantic cod (*Gadus morhua*) (Olsen et al., 2007). While many researchers have demonstrated that compounds in the alcohol-soluble fraction in soybean meal cause morphological changes in the intestine, some of these compounds may be immunostimulatory and provide a benefit to marine finfish regarding disease resistance. Saponins have been shown to have many antibacterial, antifungal, and antiviral qualities (Francis et al., 2001; Francis et al., 2002; Sparg et al., 2004; Knudsen et al., 2007). Several antigenic compounds present in soybeans have also been hypothesized to cause immunostimulation in fish, such as phenolic compounds or flavonoids (Francis et al., 2001).

The developing marine finfish aquaculture industry requires environmentally sustainable diets that provide both good growth and survival in order to expand in the United States. The work undertaken in this dissertation sought to address that problem by attempting to identify specific components of soybean meal, absent from soy protein concentrate which increase survival to bacterial challenge when incorporated into diets for summer flounder.

CHAPTER 1

INCORPORATION OF SOYBEAN PRODUCTS IN SUMMER FLOUNDER (*Paralichthys dentatus*) FEEDS: EFFECTS ON GROWTH AND SURVIVAL TO BACTERIAL CHALLENGE

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ABSTRACT

As demand for fish meal as a primary protein source in aquaculture feeds has continued to increase, aquaculturists have sought a replacement with similar nutritional profile and more consistent economic value. Soy products have an excellent amino acid profile, consistently lower commodity price as compared to fish meal, and wide availability. However, while soybean meal is much cheaper than fish meal, the inclusion of antinutritional factors within this potential fish meal replacement source limit the potential to include it in diets for summer flounder. Two feed trials were designed to evaluate the replacement of fish meal with soybean meal and soy protein concentrate in diets for summer flounder at different levels. A bacterial challenge utilizing the pathogenic bacterium *Vibrio harveyi* followed each feed trial in order to document the impact on disease susceptibility. In the first trial,

three diets studied were a fish meal-based control (FM), a 60% fish meal replacement with soybean meal (SBM), and a 60% replacement of fish meal with a 1:1 ratio (w/w) of soybean meal and soy protein concentrate (SBM/SPC). At the conclusion of a 12 week feeding trial, fish fed the SBM/SPC diet increased in average weight per fish to 36.40±6.16g, significantly greater than fish fed either the FM control diet (24.40±2.26g) or the group fed the SBM diet (22.54±6.12g) (ANOVA, df=2, F-value=14.5, Pr>F=<0.0001, α=0.05). Survival curves following bacterial challenge were significantly different, with the group fed the control diet having lower survival than the group fed the SBM/SPC diet (Kaplan-Meier Survival Analysis: Log-Rank with All Pairwise Multiple Comparison Procedure, Holm-Sidak method: statistic 6.606, df=2, P value=0.037). The diet with the majority of the FM replaced with SBM/SPC produced significantly greater growth, and survival following bacterial challenge, suggesting that supplementation of a SPC-replacement diet with some SBM may result in similar growth to a fish meal diet but increased survival to bacterial challenge. In the second feed trial 60% of the fish meal was replaced in 6 diets by either soybean meal, soy protein concentrate, or a varying ratio of the two in order to determine the proportion of soybean meal and soy protein concentrate leading to optimal growth and survival. The groups showing the greatest final fish weights were the fish meal control diet (27.58±0.8 g), and the group fed the 60% soy protein concentrate replacement diet (27.30±1.4 g) (ANOVA, df=6, F-value=30.52, Pr>F=<0.0001, α=0.05). The group fed a 12% soybean meal replacement diet had the highest survival to bacterial challenge (45.88±3.7%), which was significantly higher that all other groups except the fish fed 24% soybean meal replacement diet

(36.67±8.3%) (Kaplan-Meier log-rank survival analysis, with all pairwise multiple comparison procedures, Holm-Sidak method, df=6, test statistic= 48.64, $p \leq 0.001$). These results indicated that, although increasing levels of replacement of fish meal with soybean meal resulted in a concomitant decrease in growth compared to the fish meal diet, levels of soybean meal replacement in the diets up to 24% resulted in a significant increase in survival to bacterial challenge. Further research is needed to investigate the specific compounds responsible for both the reduction in growth and the reduction in mortality following bacterial challenge.

INTRODUCTION

As the world population increases, it becomes increasingly important to both international health goals and economics that food resources are increased in an efficient manner that protects both the end consumer and the environment in which that food is raised (FAO, 2012). Wild fish stocks have been repeatedly reported to be at or near maximum sustainable catch levels, and in many cases sustainable yields have been surpassed and populations are in decline (NOAA, 2010). However, there is no indication that the human world population has reached a plateau, and worldwide developing societies are rapidly entering the middle class and increasingly looking for high quality sources of protein to supplement a traditional plant-based diet (Smith et al., 2010; FAO, 2012). These forces acting in concert point to an ever increasing stress on wild fish stocks, with the only answer to global seafood production being an expansion in aquaculture.

In response to the increased need for seafood, global aquaculture production is rapidly expanding, and since the mid-1980's aquaculture has maintained an 8.8% annualized rate of growth (NOAA, 2010). In fact, aquaculture production has grown so rapidly in some countries that it is poised to overtake traditional capture fisheries for food fish production. While aquaculture is currently growing faster than any other animal-producing sector, currently most of the growth (89%) is found in Asian countries, especially China (FAO, 2012). With US wild groundfish stocks in decline, the US must increase its aquaculture production, or risk increasing the already $10.4 billion annual trade deficit in seafood (FAO, 2012). Over 86% of seafood consumption in the

US is currently imported, and at least 50% of that is farm-raised (NOAA, 2010; FAO, 2012). Moreover, the US population continues to grow and the per-capita consumption of seafood is expected to increase more than 30% in the next 20 years as farm-raised products become cheaper (FAO, 2012). .

Summer flounder (*Paralichthys dentatus*) are found from Florida through Nova Scotia, with the vast majority located within the Mid-Atlantic Bight (Cape Hatteras, North Carolina through Cape Cod, Massachusetts) (Manderson et al., 2000). Both adult and juveniles inhabit shallow estuarine areas in warmer months, before migrating offshore to the continental shelf in early fall. Summer flounder spawn offshore in late fall to early winter, and larvae are carried inshore on currents, where they enter estuaries to metamorphose and settle to the bottom (Packer, 1999; Able et al., 2011). Both adults and juveniles primarily feed on locally abundant fishes and crustaceans, and are often found in areas of sandy substrates from very shallow (<3m) in the summer to over 150m depths in the winter (Packer, 1999). Currently the stock is not being overfished, though the spawning stock biomass is below the target biomass and the stock was only recently certified as rebuilt in 2010 (Terceiro, 2012).

Summer flounder aquaculture was initially investigated in New England as far back as the 1970's, with a recent resurgence in interest in 1996 (Bengtson, 1999). In 1996 a commercial finfish hatchery (Great Bay Aquaculture) was established in Portsmouth, NH with the goal of supplying a developing summer flounder aquaculture industry with fingerlings. Several issues have plagued the summer flounder industry from taking hold in New England however, and every attempt at commercial

implementation has failed due to disease outbreaks and high production costs (Bengtson per. comm.). High production costs are due, at least in part, to the consistently increasing price for fish meal which is currently the main source of protein in feeds for carnivorous fish (Murray et al., 2010). Fish meal is the protein source of choice for many reasons: high protein content, good amino acid profile, high nutritional digestibility, lack of antinutritional factors, and wide availability (Gatlin et al., 2007). Demand for fish meal for aquafeeds is expected to exceed supply within the next decade, leading to ever increasing feed costs (Gatlin et al., 2007; Olsen and Hasan 2012). The continued growth of sustainable aquaculture production depends upon the development of reliable, ecologically sound protein sources to replace fish meal in aquaculture feeds (Francis et al., 2001; Gatlin et al., 2007).

Many different terrestrial protein and oil sources have been evaluated for potential fish meal replacements in aquaculture diets (Glencross et al., 2007; Olsen and Hasan, 2012). However, the results are highly variable depending on the species cultured, though replacing fish meal with soy protein has shown promising results for many species of flatfish, such as Japanese flounder (*Paralichthys olivaceus*) (Kikuchi, 1999; Sun et al., 2007), Egyptian sole (*Solea aegyptiaca*) (Bonaldo et al., 2006), and Atlantic halibut (*Hippoglossus hippoglossus*) (Murray et al., 2010). The soybean (*Glycine max*), when processed, produces a high quality source of protein (Gatlin et al., 2007) and can replace fish meal as an economically and nutritious alternative. Soybean meal has been found to be a good source of crude protein with a balanced amino acid profile. In some species such as tilapia, soybean meal can replace a majority of the

fish meal in a diet with no adverse impacts to growth or health (El Sayed, 1998; Zhou and Yue, 2010; El Saidy and Saad, 2011). However, in most commonly farmed carnivorous species such as Atlantic salmon (Pratoomyot et al., 2010), coho salmon (Twibell et al., 2012), and Japanese flounder (Ye et al., 2011), soybean meal can only be incorporated into diets at much less than 50% due to the presence of antinutritional factors in soybean meal that cause gastrointestinal enteritis and significant negative health implications and reductions in growth (Sitja-Bobadilla et al., 2005; Sun et al., 2007; Salah Azaza et al., 2009). Soy protein concentrate is a refined soy product in which many of the antinutritional factors have been removed; therefore it can replace the majority or all of the fish meal in diets for carnivorous species (Hardy, 2010; Salze et al., 2010; Colburn et al., 2012).

Researchers at the University of Rhode Island (URI) previously investigated the use of soybean meal, corn gluten meal, and canola protein concentrate in summer flounder diets either alone or in combination (Enterria et al., 2011). Although flounder growth on the control fish meal diet was superior to that obtained when fish meal was replaced to varying degrees with plant proteins, lowest cost per kg of fish produced was obtained with 40% replacement of fish meal with soybean meal. Further studies investigating the addition of taurine and phytase to soybean meal replacement diets indicated that soybean meal can replace at least 40% of fish meal in a diet for summer flounder without a significant decrease in growth if taurine and phytase are added to the diet (Bengtson et al. in prep.). A 12-week feeding study further demonstrated that combinations of soybean meal and soy protein concentrate can replace a majority of

the fish meal in diets for summer flounder. In a subsequent six-month growout trial of juvenile summer flounder, no significant differences in growth were seen between a commercial diet and an experimental diet in which 65% of the fish meal was replaced by soy protein concentrate (Bengtson et al. in prep.).

Since the summer flounder industry started in 1995, disease outbreaks have been a constant concern, and commercial aquaculture companies have shown particular interest in any opportunities to manage disease outbreaks. One of the main disease issues is flounder infectious necrotizing enteritis (FINE), caused by the pathogenic bacterium *Vibrio harveyi* (Soffientino et al., 1999). The effect of feeding summer flounder for 8 weeks with soybean meal replacement diets (0%, 40% or 70%) on disease susceptibility was recently evaluated using a bacterial challenge. Interestingly, this study demonstrated that soybean meal replacement may improve resistance to intraperitoneal injection of *V. harveyi*, even though growth was significantly reduced (Bengtson et al. in prep.). Results from another bacterial challenge performed following a 6-month feeding trial in which a 65% soy protein concentrate diet was compared to a commercial fish meal diet indicated that no protection against challenge with *V. harveyi* was provided by the inclusion of soy protein concentrate in the diet (Bengtson et al. in prep.). This apparent health benefit of diets containing soybean meal, but not soy protein concentrate, may be due to immunostimulatory effects of compounds present in soybean meal, as described in a study with rainbow trout (Rumsey et al., 1994). Although partial replacement of fish meal with soybean meal in marine finfish diets may result in decreased growth, they may also provide several

economical benefits to farmers that need to be considered, including lower cost and protection against disease outbreaks.

Reduction of disease and sustainable feed production are two of the main obstacles facing aquaculture expansion. This research sought to determine the relative impact of soybean meal and soy protein concentrate replacement diets on growth, survival to disease challenge, and non-specific immune responses in summer flounder.

MATERIALS AND METHODS

Diet Preparation: All diets were prepared at the Food Science and Nutrition Research Center, University of Rhode Island. Diets were formulated to be isocaloric, isonitrogenous, and contain 50% total crude protein (CP). All soybean-based diets were supplemented with taurine at 1% and diets containing soybean meal had phytase added at 0.2-0.3%. All diets were analyzed for proximate composition: crude protein (CP), crude lipid (CL), moisture, fiber, and ash, using the AOAC (1995) methods. Soy products (soybean meal and soy protein concentrate) were donated by ADM (Decatur, IL). Fish meal (International Protein Corp.: anchovy, 69.6% protein, 9% oil) was donated by the USDA-ARS Fish Technology Center (Bozeman, Montana), and fish oil was donated by Omega Protein (Houston, TX). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

Trial 1:

Diet formulation: Three diets were formulated as follows (Table 1): Control (FM): all protein from fish meal; soybean meal (SBM): partial replacement of 60% fish meal with soybean meal; and soybean meal/soy protein concentrate (SBM/SPC): partial replacement of 60% fish meal with a 50:50 mix of soybean meal and soy protein concentrate.

Fish, rearing conditions, and sampling protocol: The first feed trial was carried out at the Marine Biological Laboratory (MBL, Woods Hole, MA; IACUC approval number 10-55), on a temperature and lighting (12L:12D) controlled recirculating system. Two-hundred juvenile summer flounder (*Paralichthys dentatus*) (length 7.1 ± 0.1cm, weight 3.6 ± 0.1g; mean ± SD throughout text, unless otherwise noted) were obtained from GreatBay Aquaculture (Portsmouth, NH) and were transported to the MBL prior to the start of the feed trials. All fish were fed a commercial diet (Skretting Gemma Diamond 0.8 mm, Stavanger, Norway) twice daily by hand during the 2 week acclimation period. The fish were held in a recirculating system at the MBL with controlled filtration and temperature (17.6 ± 0.1ºC) throughout the feeding trial. Twenty fish were randomly distributed to each of nine 75 L aquaria, comprising triplicate aquaria for each of the three dietary treatments. Fish were fed twice daily at the rate of 5% wet body weight per day (recalculated every 2 weeks), for a period of 12 weeks. All uneaten feed was siphoned every day. Individual fish wet weight and total length were determined every two weeks and feed levels adjusted accordingly. Weight gain (WG), feed conversion ratio (FCR), specific growth rate (SGR), condition factor (K), and survival were calculated according to standard formulas (Hopkins, 1992).

Bacterial challenge: Following the conclusion of the feeding trial, all fish were transferred the Blount Aquaculture Research Laboratory at URI (URI-BARL; IACUC approval number AN10-10-008). Fish from all the tanks for each individual treatment were pooled, and randomly redistributed into 75L aquaria as follows: each dietary treatment (three total) comprised four tanks, of which two tanks had 10 fish each (challenged, for bacterial injection), and two tanks had seven fish each (control, filtered artificial seawater (FASW) injected). The fish were fed twice daily with their respective diet (5% body weight) and left for one week to acclimate to the new tanks.

Bacteria used for the challenge was a previously isolated DN01 strain of *Vibrio harveyi* from an outbreak of FINE in summer flounder (Soffientino et al., 1999) and further characterized by Gauger et al. (2006). A sterile loop was used to streak bacteria from the glycerol stock stored at -80˚C onto a Luria-Bertani agar plate with 20 g/L NaCl. The bacteria were incubated at room temperature overnight until colonies were observed. Single colonies were attained with a sterile loop, mixed in 20 ml LB20 broth, placed on a shaker table, and incubated with agitation at 28˚C for 24 hrs. In order to activate the bacteria by passage, non-experimental juvenile summer flounder were anesthetized with tricaine methane sulphonate (MS-222; 100 mg/L for 5 min) and 100 µl of a twice serial-diluted bacterial solution were injected into the peritoneal cavity. Fish were monitored for signs of disease, a necropsy procedure was performed on morbid fish, and ascites fluid was streaked onto LB20 plates. Examination of the morphology of the cultured colonies confirmed the presence of *V. harveyi*. *Vibrio*

harveyi isolated from the ascites fluid of passage fish was selected for the bacterial challenge. Single colonies were attained with a sterile loop and mixed in 20 ml of LB20 broth. LB broth solutions were placed in a shaker and incubated with agitation for 24 hrs at 28°C. In order to determine bacterial concentration, 100 µl of the bacterial broth was placed into the well of a 96-well plate in duplicate. Serial dilutions were made by pipetting aliquots of 10 µl of broth solution out of the previous well down the plate and adding 100 µl of filtered artificial seawater (FASW). The plate was immediately inserted into a spectrophotometer and absorbance (optical density) at 600 nm was measured. Bacterial concentration in colony forming units (CFU) ml^{-1} was determined based on a previously established relationship between optical density at 600 nm and colony forming units (Gauger et al., 2006).

The optimum bacterial cell concentration to use in the challenge (LD_{50}, bacterial dose lethal to 50% of the fish) was determined prior to the challenge by the Karber method (Barros et al., 2002). Fish were placed in 75L tanks and injected intraperitoneally (i.p.) with 100 µl containing $9.14x10^7$ CFU *Vibrio harveyi*, or 100 µl FASW. Mortality was monitored and recorded twice daily for seven days following injection. Post-challenge, bacteria were isolated from the peritoneal fluid of moribund fish, and confirmed to be *V. harveyi* by amplification and sequencing of a portion of the 16S rDNA gene using polymerase chain reaction (Gauger et al., 2006). At the conclusion of the challenge (day 7) all remaining fish (survivors) were anesthetized with tricaine methane sulphonate (MS-222; 100 mg/L for 5 min). Blood was drawn from the caudal vein of each fish (less than 0.5 ml per fish) using a 27-gauge needle attached to a 1ml syringe

rinsed with 0.2 mM EDTA and was placed in a 1.5-ml eppendorf tube with 2 drops 0.2mM EDTA as an anticoagulant. Following blood drawing, all of the fish were euthanized with an overdose of MS-222 (250 mg/L for 10 min). Samples of blood from fish within each tank were pooled.

Hematocrit determination: Hematocrit was immediately measured by filling a hematocrit microcapillary tube two-thirds full with whole blood from each individual fish in duplicate, and then centrifuged for 5 min at 12000g. Percentage of packed cell volume (PCV) (hematocrit) was determined using the hematocrit reader (Ibraham et al., 2010).

Respiratory burst activity: The remaining blood was stored on ice, and transported to the Center for Biotechnology and Life Sciences, at the URI-Kingston campus for further analysis. Respiratory burst activity (RBA) was measured using a modified protocol based on Brubacher and Bols (2001) and Cathcart et al. (1983), which measures fluorescence of dichlorofluorescin (DCF) when converted from 2',7'-dihydrodichlorofluorescin-di-acetate (H_2DCFDA) through radical oxygen species. H_2DCFDA (Invitrogen, USA) was converted to DCF by incubating H_2DCFDA (0.5 ml of 1 mM in ethanol) with 2.0 ml 0.01N NaOH for 30 min at room temperature in the dark. The hydrolysate was then neutralized with 10 ml of 25-mM sodium phosphate buffer, diluted by 100x, and stored on ice. Whole blood (100 µl) was placed into the wells of flat-bottom 96-well microtiter plates in triplicate. Each plate was then incubated for 1 hr at 16ºC to allow cells to adhere to the sides of the microplate. The

supernatant was removed and the wells were rinsed 3x with phosphate-buffered saline (PBS). The activated substrate DCF (50 µl) was then added, as well as 50 µl of the blood cell activator phorbolmyristate acetate (PMA; Sigma, St. Louis, MO, USA, final concentration of 1 µg/ml). Fluorescence was measured immediately using a spectrofluorometer (excitation at 480 nm and emission at 530 nm) and then every 5 min after for 75 min, at which point the values began to plateau. Values are represented as relative fluorescence units, after the value of the blank has been removed.

Plasma protein: Blood samples were centrifuged at 3500 rpm for 30 min, and the plasma was removed and transferred to separate eppendorf tubes by pipette. The plasma samples were stored at 4°C for 24 hrs until further analysis. Plasma protein was measured following the Coomassie (Bradford) Protein Assay (Bradford, 1976) using bovine serum albumin as the standard.

Plasma lysozyme content: Plasma lysozyme content (per mg of protein) was measured following the standard hen egg-white lysozyme (HEWL) turbidimetric assay (Litwack, 1955). Briefly, 50 µl plasma or lysozyme (HEWL) standard dilution (2.0-0.625 mg/ml) was added to individual wells of a 96-well microtiter plate in triplicate. A solution of reconstituted lyophilized *Micrococcus lysodeikticus* (150 µl of 0.75 mg/ml) was added to each well and the absorbance was read at 450 nm immediately. The absorbance was read again after 5 minutes. The trendline equation (R^2>0.980) from plotting the lysozyme standard curve was used to determine plasma lysozyme

content, considering that each decrease in absorbance of 0.001/min is 1 international unit (IU) lysozyme.

Plasma bactericidal activity: Plasma bactericidal activity was determined following a modified procedure of El-Boshy et al. (2010). Fresh plasma (40 µl) or Hank's Balanced Salt Solution (HBSS; positive control) were added in triplicate to wells of 96-well microtiter plate and incubated for 2.5 h with 20 µl aliquots of an overnight culture of *V. harveyi*. Following incubation, 20 µl MTT (2.5 mg/ml; Sigma) was added, and the plate was incubated at room temperature for 10 min to allow the formation of formazan. The plates were then centrifuged for 10 min at 3200 rpm, the supernatant discarded, and the interphase was dissolved in 150 µl of DMSO. The absorbance of the dissolved formazan was read at 580 nm, and the bactericidal activity was calculated as the decrease in the number of viable *V. harveyi* cells by subtracting the absorbance of samples from that of controls and reported as absorbance units.

Trial 2:

Seven diets were formulated as shown in Table 2. The FM (control) diet contained no soy protein, with all protein from fish meal. All other diets contained 40% fish meal and the rest (60%) of the protein was from various combinations of soybean meal or soy protein concentrate, as follows: 60% SBM: 60% soybean meal and 0% soy protein concentrate; 48% SBM: 48% soybean meal and 12% soy protein concentrate; 36% SBM: 36% soybean meal and 24% soy protein concentrate; 24% SBM: 24% soybean

meal and 36% soy protein concentrate; 12% SBM: 12% soybean meal and 48% soy protein concentrate; 0% SBM: 0% soybean meal and 60% soy protein concentrate.

Fish, rearing conditions, and sampling protocol: The second feeding trial was carried out at the URI-BARL, within a temperature controlled (18ºC target temperature), flow-through system, with 12L:12D lighting throughout. Juvenile summer flounder (*Paralichthys dentatus*) (700; length 7.9 ± 0.1cm, weight 5.2 ± 0.3g) were obtained from GreatBay Aquaculture (Portsmouth, NH), and were transported to the URI-BARL prior to the start of the feeding trial. All fish were fed a commercial diet (Skretting Gemma Diamond 1.2 mm, Stavanger, Norway) twice daily by hand during the two-week acclimation period. Twenty fish were randomly distributed to each of 35 75 L aquaria, comprising five replicate aquaria for each of the seven dietary treatments. Fish were fed twice daily to satiation, for a period of 9 weeks, and all uneaten feed was siphoned once daily. Individual fish wet weight and total length were determined initially and at the conclusion of the feeding trial, with total tank biomass calculated every two weeks. Final weight, final length, FCR, SGR, K, and survival were calculated following the conclusion of the trial (Hopkins et al., 1992).

Bacterial challenge: Following the conclusion of the feeding trial, a bacterial challenge was administered through i.p. injection as described above. Each dietary treatment comprised five tanks; of which the fish from two tanks were each injected with 100 µl of FASW, and the fish from the other three tanks were each injected with 100 µl of *V. harveyi* ($4.0x10^7$cells).

Statistics: All data other than the bacterial challenge survival data were analyzed using the General Linear Model procedure using SAS computer software (SAS 9.2, Cary, NC, USA). Mean results were subjected to one-way analysis of variance (ANOVA), and significant results were further analyzed using Tukey's post test. If results were not normally distributed, Kruskal-Wallis Test Nonparametric ANOVA was used. Bacterial challenge survival data were analyzed using the Kaplan-Meier survival analysis, log-rank, with all pairwise multiple comparison procedures (Holm-Sidak method) using SigmaStat (Systat Software, San Jose, CA, USA). The significance level of 0.05 was chosen, and all data are presented as mean ± SD.

RESULTS

Trial 1:

Growth and feed conversion: All diets were formulated to be isonitrogenous and isocaloric (Table 1). Initial fish weights and lengths were not significantly different. The increase in both length and weight of fish during the feeding trial was significantly higher in the group fed the SBM/SPC diet than in fish fed FM or SBM (ANOVA, df=2, F-value=14.5, Pr>F=<0.0001, α=0.05; Fig. 1). The SBM/SPC-fed group had a significantly higher SGR compared to the other two experimental groups (ANOVA, df=2, F-value=29.43, Pr>F=0.001, α=0.05; Table 3). Food conversion rate (FCR) for the SBM/SPC-fed group was significantly lower than that of the SBM-fed group, but not the FM-fed control group (ANOVA, df=2, F-value=7.77, Pr>F=0.021, α=0.05; Table 3). Condition factor (K) was only different between the FM control and

SBM-fed groups (ANOVA, df=2, F-value=10.38, Pr>F=0.011, α=0.05; Table 3). There were no differences in survival among groups (ANOVA, df=2, F-value=4.20, Pr>F=0.0723, α=0.05, Table 3). Mortality was low throughout the entire feeding trial (11.7% throughout the entire 12 weeks), and most of the mortality was due to fish jumping out of tanks during the first days of the trial, before nets were placed on top of the tanks.

Survival and Immune Parameters after Bacterial Challenge: All of the fish injected FASW as a control survived the 7-day challenge period. Within the groups of fish that were experimentally injected with *V. harveyi*, the group fed the FM control had significantly lower survival than the group fed the SBM/SPC diet (Kaplan-Meier Survival Analysis: Log-Rank, df=2, p=0.037, p≤0.05; Holm-Sidak Pairwise Multiple Comparison; Table 4). Hematocrit was approximately 29% for all groups when injected with FASW, which for all groups was significantly higher than the same treatment group injected with bacteria (Two-Way ANOVA, df=1, F-value=6.93, Pr>F=0.0389, α=0.05; Table 4). No significant differences among groups of fish fed the different diets were observed for the hematocrit of fish challenged with bacteria (ANOVA, df=5, F-value=1.41, Pr>F=0.3419, α=0.05). For the control groups injected FASW, plasma protein was highest for the SBM group (Table 4), and, for the groups injected with bacteria, plasma protein was highest for the SPC group, though the results were not significantly different among treatments (FASW or bacteria injected) due to high variability (ANOVA, df=5, F-value=0.98, Pr>F=0.5002, α=0.05; Table 4).

Respiratory burst activity (RBA) was measured using adherent blood cells. At no point throughout the 75-min period in which fluorescence was measured was a statistically significant difference observed among the experimental groups. Samples from all diet groups showed higher RBA values after injection with bacteria as compared to FASW (Table 4). Of those injected with bacteria, the group fed the FM control diet also resulted in higher RBA after 75 minutes (91.5 ± 14.7 RFU) as compared to the two experimental diets (78.3 ± 7.4 RFU for SBM, and 78.3 ± 5.7 RFU for SBM/SPC) however, this difference was not significant (ANOVA, df=5, F-value=0.77, Pr>F=0.6017, α=0.05). The group fed the SBM diet showed the lowest level of lysozyme activity in plasma for the groups challenged with FASW (Table 4). Lysozyme in plasma from fish in the group fed the FM control diet was slightly higher than the group fed the SBM/SPC diet. There were no differences between the groups injected with bacteria, however lysozyme levels in plasma of fish from the SBM/SPC-fed group injected with bacteria was lower compared to all other groups (Table 4). Regardless of diet following either FASW or bacteria injection, fish in the group fed the FM control diet showed higher bactericidal activity (lower levels of formazan indicate less bacteria in the sample). However due to high variability in the two groups fed the experimental diets, there were no statistically significant differences in plasma bactericidal activity between any of the groups (ANOVA, df=5, F-value= 0.60, Pr>F=0.7016, α=0.05).

Trial 2:

Growth and feed conversion: The seven experimental diets were all relatively uniform in chemical and nutritional composition (Table 2). Mortality was low throughout the entire 9-week feeding period (95-100% percent survival across all tanks, not shown), and there were no significant differences among treatments (ANOVA, df=6, F-value=0.96, Pr>f=0.4732, α=0.05). Initial fish weights and lengths were not significantly different (ANOVA, df=6, F-value=1.41, Pr>F=0.2462, α=0.05, not shown). There was a trend for lower growth with increasing inclusion of soybean meal in the diet. The presence of soybean meal in the SPC replacement diets led to significant reductions in the length and weight of the fish (Fig. 2). Final fish length and weight was significantly greater in the groups fed the FM control and the 0% SBM replacement diets, compared to all other diets (ANOVA, df=6, F-value=45.24, Pr>F=<0.0001, α=0.05 for length; df=6, F-value=30.52, Pr>F=<0.0001, α=0.05 for weight, Fig. 2). Fish fed the 12% SBM replacement diet were also significantly longer than all other fish fed with higher (24 – 60%) percentages of SBM and significantly heavier than fish fed 48 and 60% SBM (Fig. 2).

The groups with no soybean meal in the diets had significantly higher SGR than all other groups (ANOVA, df=6, F-value=31.74, Pr>F=<0.0001, α=0.05) (Table 5). Accordingly, FCR increased with increasing amount of soybean meal in the diet, with the groups of fish fed 60% and 48% SBM replacement showing significantly higher FCRs than the fish fed FM, 12% SBM, and 0% SBM (ANOVA, df=6, F=6.83, Pr>F=0.0002, α=0.05, Table 5). Condition factor (K) was relatively constant for all groups, with only the group fed the 36% soybean meal replacement diet having a

condition factor significantly greater than the groups fed the FM control and the 0% SBM replacement diets (ANOVA, df=6, F=3.28, Pr>F=0.0148, α=0.05, Table 5).

Survival to Bacterial Challenge: All of the fish injected with filtered artificial seawater (FASW) as a control survived the 7-day challenge period. Within the groups of fish that were injected with *Vibrio harveyi*, the group fed 12% SBM replacement had significantly higher survival than fish in all other groups except the fish fed 24% soybean meal replacement (Kaplan-Meier log-rank survival analysis, with all pairwise multiple comparison procedures, Holm-Sidak method, df=6, test statistic= 48.64, P=<0.001, Table 5).

DISCUSSION

Overall, this research confirms that using soy protein concentrate as a replacement for 60% of the fish meal in juvenile summer flounder diets can result in equal if not better growth than using fish meal as the sole protein source. Although replacement of fish meal with a combination of soybean meal and soy protein concentrate led to a decrease in fish growth, inclusion of low levels of soybean meal (12 – 24% of the soy protein concentrate in a 60% soy protein concentrate replacement diet) led to a significant reduction in summer flounder mortality following bacterial challenge. This positive effect on survival provided by soybean meal, in addition to the lower cost of soybean meal relative to soy protein concentrate, may balance the negative impacts of soybean meal replacement on fish growth.

Soy protein concentrate does not have many of the antinutritional factors present in soybean meal, and therefore, soy protein concentrate-replacement diets were expected to result in growth similar to the one provided by the traditional fish meal diets (Bengtson et al., in prep.). As expected, in the second trial performed in this research, the group fed the soy protein concentrate replacement level (60% soy protein concentrate and no soybean meal) supported growth similar to that provided by the fish meal diet. These results confirm past reports of soy protein concentrate replacement diets for summer flounder, resulting in at least as good growth rates, if not better, as compared to the control fish meal diet (Bengtson et al. in prep.). In diets for Atlantic cod (*Gadus morhua*) soy protein concentrate can replace 22% of fish meal with no decrease in growth (Refstie et al., 2006), and other studies have shown even higher levels (40-70%) of fish meal replacement with soy protein concentrate may be possible for other carnivorous fish species (Berge et al., 1999; Mambrini et al., 1999; Refstie et al., 2003). In juvenile cod, soy protein concentrate was able to replace 100% of the fish meal in diets, with no observed enteritis or reduction in growth (Walker et al., 2010).

In both trials, replacement of the soy protein concentrate with increasing amounts of soybean meal resulted in a concomitant decrease in specific growth and an increase in the food conversion ratio (FCR). The increase in FCR observed in summer flounder fed increasing amounts of soybean meal also suggests that a decrease in the soybean meal in soybean-based feeds leads to a more efficient use of the diet by the fish and, consequently, a higher specific growth rate. Several authors have investigated the

impact of fish meal replacement with soybean meal in fish diets, which, almost without exception, resulted in a decrease in fish growth (Rumsey et al., 1994; Refstie et al., 1998; Refstie et al., 2006; Hart et al., 2010). Research in salmonids has determined that the replacement of fish meal by soybean meal must be much lower than a total replacement level or the chance of reduced growth and enteritis of the gut is almost guaranteed (Rumsey et al., 1994; Refstie et al., 1998). The decrease in growth from diets where soybean meal replaces fish meal at high levels in carnivorous fish feeds may have to do with the impact of many antinutritional factors present in soybean meal, which may prevent digestion and nutrient absorption (Grisdale-Helland et al., 2008). These include protease inhibitors (trypsin inhibitors), oligosaccharides (stachyose, raffinose, etc.), saponins, isoflavones, antigens (glycinin, conglycinin, lectins), phytate, and tannins (Refstie et al., 2006; Knudsen et al., 2007; Iwashita et al., 2009). Heat processing or alcohol extraction of the soybean meal to produce soy protein concentrate removes many of these factors, and produces a product which, for several species, allows for good growth rates without morphological intestinal changes (*e.g.* enteritis; Francis et al., 2001; Knudsen et al., 2007).

We also investigated the effects of these diets on survival of fish to bacterial challenge, based on previous results indicating that diets containing soybean meal provide protection against bacterial challenge (Lightbourne, 2011). Consistent with these expectations, results from the bacterial challenge following trial 1 demonstrated significantly greater survival for the groups that were fed diets containing soybean meal. Interestingly, higher levels of replacement with soybean meal did not lead to

increased survival from bacterial challenge, since the highest survival rates were observed for the fish fed the 12 and 24% soybean meal replacement. In this work, replacement of 60% of the fish meal with 12% soybean meal and 48% soy protein concentrate provided the best results, by increasing survival after bacterial challenge from 21 to 46% survival with no significant reduction in growth. This may prove to be a working formulation for summer flounder aquaculturists, cheaper than a diet based entirely on fish meal as a protein source, while still providing equal growth and better survival.

We also analyzed the effect of diet on several immune parameters, in order to determine if increased survival to challenge is due to immunostimulation. Immunostimulation from molecules derived from plant sources is not a new concept. There are many molecules known to initiate immunostimulation, such as beta-glucans, molecules of plant or bacterial origin which may directly activate the immune system (for example, Bricknell and Dalmo, 2005). Several of the ANFs have been shown to have immunostimulatory effects, such as oligosaccharides (Grizard and Barthomeuf, 1999) and saponins (Rajput et al., 2007). However the use of soy in fish feeds has mainly focused on the negative aspects of reduced growth and enteritis, instead of potential beneficial immune applications. High levels of protein replacement with soybean meal have been shown to cause an inflammatory response in Atlantic salmon (*Salmo salar*), resulting in both a reduced capacity to fight off bacterial infection (Krogdahl et al., 2000) and an increase in enteritis (Knudsen et al., 2007). In rainbow trout (*Oncorhynchus mykiss*), high (60-70%) replacement of fish meal with soybean

meal was also correlated with a decrease in immune function (macrophage activity and modulation of the intestine), in conjunction with a decrease in growth. The antinutritional factors causing the enteritis in fish fed soybean meal diets, were producing a sustained inflammatory or hypersensitivity response, increasing neutrophil, monocyte and macrophage activity (Rumsey et al., 1994) as well as increased lysozyme and antibody levels (IgM) (Krogdahl et al., 2000).

Our results indicate that replacement of fish meal with either soybean meal (60%) or a mix of soybean meal (30%) and soy protein concentrate (30%) in summer flounder diets fed for 12 weeks did not lead to significant differences in hematocrit, plasma protein, or respiratory burst activity in adherent blood cells of summer flounder, when measured in survivor fish 7 days after challenge with control (filtered artificial sea water) or bacteria. The only parameter that was impacted by diet was lysozyme activity, which was significantly lower after bacterial challenge in the fish fed the SBM/SPC diet. The mechanisms leading to lower levels of lysozyme in these fish are unknown. The lack of significant changes in innate immune function may be due to lack of power or sampling timing (7 days after an acute challenge), so further research needs to be done to determine the mechanisms responsible for increased survival to bacterial challenge in fish fed with diets containing soybean meal, including evaluation of the effect of independent components of soybean meal, and examination of the effect of dose and length of exposure on selected immune responses.

Diet Formulation	FM	SBM	SBM/SPC
Fish Meal	670.0	268.0	268.0
Soybean Meal	-	402.0	201.0
Soy Protein Concentrate	-	0.0	201.0
Fish Oil	35.0	69.3	68.6
Wheat flour	260.5	67.1	113.3
Corn gluten	-	79.5	47.7
Starch	4.5	4.5	4.5
Mineral Premix (URI)	10.0	10.0	10.0
Calcium Phosphate	-	30.0	30.0
Vitamin Premix (URI)	10.0	10.0	10.0
Arginine	-	7.7	0.8
Lysine	-	11.8	8.4
Methionine	-	5.3	4.7
Threonine	-	5.5	3.0
Taurine	-	14.0	14.0
Phytase	-	0.3	0.2
Glycine	10.0	15.0	15.0
Proximate Analysis			
Percent Ash	9.6±0.5	8.2±0.7	9.1±0.4
Percent Protein (DW)	53.1±0.4	50.6±0.5	53.5±0.5
Percent Moisture	17.1±0.4	22.9±0.2	14.7±0.6
Percent Lipid	10.7±0.3	11.4±0.3	9.2±0.7

Table 1. *Diet formulations (1kg; all values for diet ingredients in grams; top part of the table) and proximate analysis (mean ± SD, bottom) for the 3 experimental diets for Trial 1. The fish meal control diet (FM) did not contain any soy products. Soybean meal (SBM) contained 60% replacement of the fish meal with soybean meal. Soybean meal/soy protein concentrate diet (SBM/SPC) contained 60% replacement of the fish meal with soy products, of which that 60% was split 50:50 between soybean meal and soy protein concentrate.*

Diet name	FM	60% SBM	48% SBM	36% SBM	24% SBM	12% SBM	0% SBM
Composition (%)							
Fish Meal	100	40	40	40	40	40	40
Soybean Meal	0	60	48	36	24	12	0
Soy Protein Concentrate	0	0	12	24	36	48	60
Composition (g per kg of diet)							
Fish Meal	670.0	268.0	268.0	268.0	268.0	268.0	268.0
Soybean Meal	-	402.0	321.6	241.2	160.8	80.4	-
Soy Protein Concentrate	-	-	80.4	160.8	241.2	321.6	402.0
Fish Oil	32.0	87.2	69.2	69.2	69.2	69.2	65.2
Wheat flour	238.5	26.1	67.0	87.8	93.3	110.0	120.6
Corn gluten	25.0	122.6	101.7	83.7	79.8	63.4	49.2
Starch	4.5	2.5	4.5	4.5	4.5	6.5	14.5
Mineral Premix (URI)	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Calcium Phosphate	-	30.0	30.0	30.0	30.0	30.0	30.0
Vitamin Premix (URI)	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Arginine	-	2.0	0.5	-	-	-	-
Lysine	-	5.8	4.6	3.3	2.0	-	-
Methionine	-	2.9	2.6	2.4	2.1	1.9	1.6
Threonine	-	1.7	0.8	-	-	-	-
Taurine	-	14.0	14.0	14.0	14.0	14.0	14.0
Phytase	-	0.2	0.2	0.1	0.1	0.0	0.0
Glycine	10.0	15.0	15.0	15.0	15.0	15.0	15.0
Proximate Analysis							
Percent Ash	12.8±0.5	8.9±0.1	8.6±0.4	8.5±0.0	8.6±0.2	8.3±0.1	8.9±0.1
Percent Protein (DW)	51.3±0.3	51.1±0.4	51.2±1.2	53.3±1.6	53.7±1.6	54.3±2.4	53.9±2.4
Percent Moisture	17.8±0.2	22.7±1.6	25.5±0.4	27.5±0.4	33.2±0.2	28.8±0.1	25.7±1.0
Percent Lipid	9.5±0.0	10.7±0.4	10.2±0.2	9.2±0.4	9.8±0.1	10.0±0.6	11.9±0.3

Table 2. *Diet formulations (1kg; all values for diet ingredients in grams) and proximate analysis (mean ± SD) for the 7 experimental diets for Trial 2.*

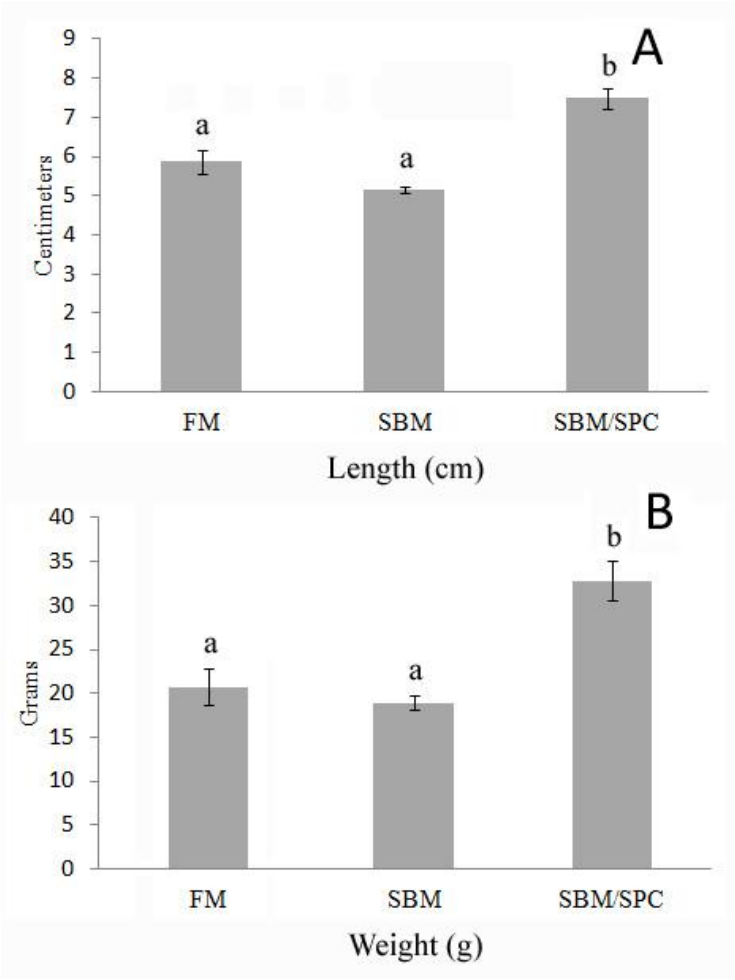

Figure 1.*Growth performance of fish fed for 12 weeks experimental diets with soybean meal (SBM) or soy protein concentrate (SPC) as protein replacement for fish meal (FM). Different superscript letters denote significant differences between column means at p<0.05. A. Average fish length (cm) at the end of the 12 week trial. There was no difference in starting lengths (p<0.05). The SBM/SPC fed group showed significantly longer lengths (ANOVA, df=2, F-value=15.3, p<0.0001, α=0.05 with Tukey's post test) than the other groups. B. Average fish weight (grams) at the end of the 12 week feed trial. Starting weights were not significantly difference between groups (p<0.05). The group fed the SBM/SPC diet displayed significantly more weight gain than the other groups (ANOVA, df=2, F-value=14.5, p<0.0001, α=0.05, with Tukey's post test). Error bars represent SD.*

	FM	SBM	SBM/SPC
FCR	1.21 ± 0.07^{ab}	1.33 ± 0.07^{b}	1.01 ± 0.02^{a}
Condition	1.12 ± 0.01^{a}	1.24 ± 0.02^{b}	1.18 ± 0.02^{ab}
SGR (% d)	2.28 ± 0.07^{a}	2.19 ± 0.02^{a}	2.80 ± 0.07^{b}
Survival (%)	97 ± 2^{a}	87 ± 2^{a}	82 ± 6^{a}

Table 3: *Performance of summer flounder after a 12 week feeding trial. The fish meal control diet (FM) did not contain any soy products. Soybean meal (SBM) contained 60% replacement of the fish meal with soybean meal. Soybean meal/soy protein concentrate diet (SBM/SPC) contained 60% replacement of the fish meal with soy products, of which that 60% was split 50:50 between soybean meal and soy protein concentrate. Values are expressed as mean ± SD. Different superscript letters denote significant differences between row means at $p<0.05$.*

		Control	SBM	SBM/SPC
Survival				
(percent)	FASW	100±0.0 [a]	100±0.0 [a]	100±0.0 [a]
	Bacteria	25.0±7.1 [a]	35.0±7.1 [a]	35.0±21.2 [b]
Hematocrit				
(percent)	FASW	29.6±0.9 [a]	29.7±1.4 [a]	29.6±1.3 [a]
	Bacteria	26.1±1.6 [b]	24.6±1.9 [b]	25.7±0.9 [b]
Plasma Protein				
(mg/ml)	FASW	92.7±17.1 [a]	138.0±21.3 [a]	98.0±19.3 [a]
	Bacteria	89.9±24.3 [a]	108.2±22.5 [a]	142.4±8.3 [a]
Lysozyme				
(units/ml)	FASW	38.7±13.2 [a]	27.0±15.6 [a]	43.3±2.8 [a]
	Bacteria	28.0±0.0 [a]	24.0±5.7 [a]	14.0±8.5 [a]
Bactericidal				
(abs)	FASW	0.4±0.1 [a]	0.7±0.4 [a]	0.6±0.0 [a]
	Bacteria	0.4±0.2 [a]	0.8±0.6 [a]	0.7±0.2 [a]
Respiratory Burst				
(abs)	FASW	77.0±3.7 [a]	74.3±1.7 [a]	59.0±1.0 [a]
	Bacteria	91.5±25.8 [a]	78.3±6.0 [a]	78.3±11.0 [a]

Table 4. *Effect of diet on survival to bacterial challenge and selected blood parameters. All parameters were measured in survivor fish 7 days after challenge. (Survival n=60, hematocrit n=9, plasma protein n=9). Different superscript letters denote significant differences between groups for each parameter at $p<0.05$. mean±SD.*

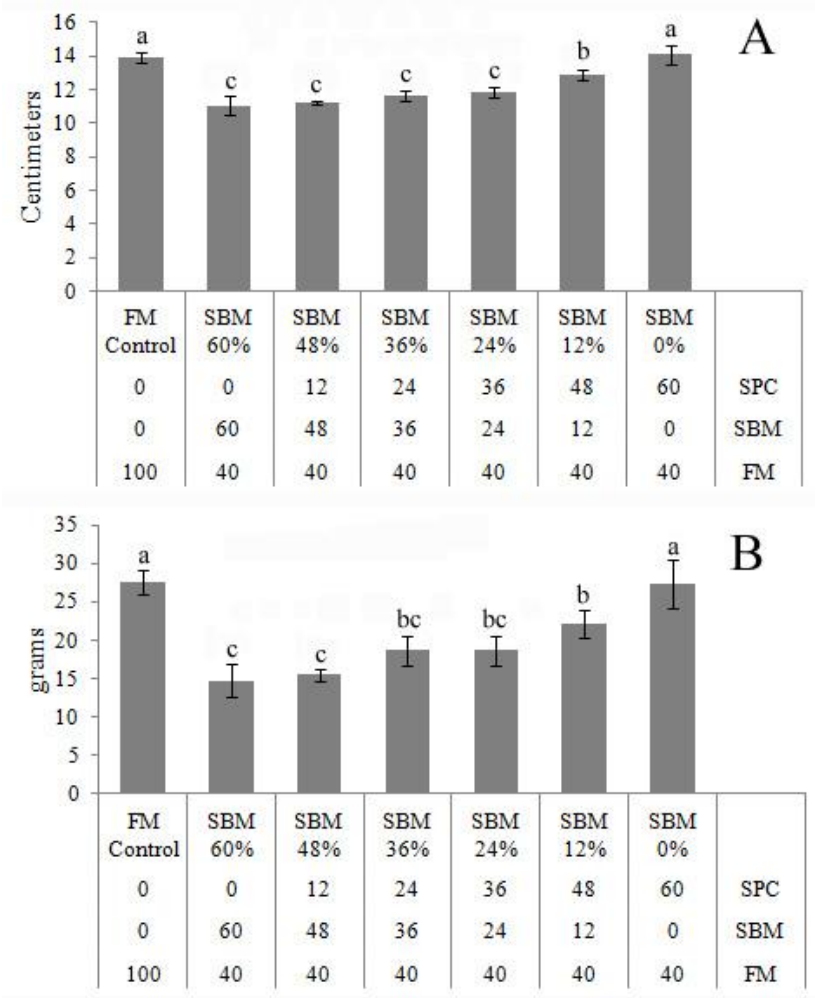

Figure 2. *Final length and weight of summer flounder at the conclusion of the second 12 week feed trial with diets containing different ratios of soybean meal (SBM) soy protein concentrate (SPC) replacement of fish meal (FM). Across the x-axis of both graphs is the total percent content of each diet protein source. A. Final length (cm), B. Final weight (g). Different superscript letters denote significant differences between groups at $p<0.05$. mean ± SD.*

Diet	FM	60% SBM	40% SBM	36% SBM	24% SBM	12% SBM	0% SBM
FCR	1.2±0.1^{a}	2.7±0.5^{b}	2.9±0.2^{b}	2.2±0.2^{ba}	2.1±0.1^{ba}	1.4±0.2^{a}	1.4±0.1^{a}
Condition (K)	1.0±0.0^{b}	1.1±0.0^{ab}	1.1±0.0^{ab}	1.2±0.1^{a}	1.1±0.0^{ab}	1.0±0.0^{ab}	1.0±0.0^{b}
SGR (% d)	2.7±0.0^{a}	1.7±0.1^{bc}	1.7±0.0^{bc}	2.0±0.1^{b}	2.0±0.1^{b}	2.3±0.1^{b}	2.7±0.1^{a}
Survival to Challenge	21±7^{bc}	24±12^{bc}	14±6^{c}	30±8^{bc}	37±8^{b}	46±4^{ac}	18±11^{bc}

Table 5. *Performance of summer flounder after a 9 week feeding trial with diets with different protein sources (fish meal, soybean meal, soy protein concentrate). Different superscript letters denote significant differences between row means at $p<0.05$. Mean ± SD*

CHAPTER 2

IMPACTS OF SOYBEAN MOLASSES AND SUB-FRACTIONATED SOYBEAN MOLASSES ON GROWTH AND SURVIVAL FOLLOWING CHALLENGE IN DIETS FOR SUMMER FLOUNDER (*Paralichthys dentatus*)

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ABSTRACT

Replacing fish meal with soy products may be a viable method to reduce the reliance on fish meal which currently constitutes the primary protein source in aquaculture diets for carnivorous finfish. However, depending on how the raw soybeans are processed, there may be many antinutritional factors (ANF) in soybean meal diets causing reduced growth in certain species. Previous work suggests that products present in soybean meal-based diets, but not in soy protein concentrate, decrease growth but also increase survival of summer flounder to experimental bacterial challenge. Two feeding trials were designed to identify which fractions in soybean are responsible for decreased growth and increased survival to bacterial challenge in summer flounder. Summer flounder were fed diets in which soy protein concentrate replacement diets were supplemented with increasing amounts of soybean molasses

(concentrated, desolventized, aqueous alcohol extract of defatted soybean flakes) at 0.5%, 1.2% or 1.9% (w/w) inclusion levels. The groups fed the fish meal control diet grew significantly more than any group fed the diets supplemented with soybean molasses (ANOVA, df=4, F-value=177.55, Pr>F=<0.0001, α=0.05). There was no difference between groups in survival following bacterial challenge. In the subsequent trial, soybean molasses was phase-separated using *n*-butanol into water, interphase, and butanol fractions. These fractions were added to a soy protein concentrate-based diet and fed to summer flounder for six weeks. The group fed the fish meal control gained significantly more weight than any of the groups fed the diets containing soy protein concentrate, and the group fed the soy protein concentrate control gained significantly more weight than any of the groups fed the soy molasses fractions (ANOVA, df=4, F-value=44.92, Pr>F=<0.0001, α=0.05). Survival following bacterial challenge was significantly lower for the group fed the fish meal control (Kaplan-Meier Survival Analysis: Log-Rank; df=4, statistic=31.77, p<0.001), as compared to all groups fed diets containing fractions from soy molasses. Further research is necessary to identify and quantify the compounds responsible for both the decrease in growth and increase in survival following challenge for summer flounder fed diets supplemented with ANF.

INTRODUCTION

Aquaculture diets, especially for carnivorous fishes, are primarily based on fish meal as a protein source, and therefore, regardless of species, are often still heavily dependent on capture fisheries for fish meal (Tacon and Metian, 2008). This reliance on fish meal limits aquaculture expansion, reduces investment, and contributes to increasing feed costs (Naylor et al., 2009; Hardy, 2010). Widespread incorporation of soy products into diets for marine aquaculture not only opens up a new market for soy products, it is also one way to make carnivorous-finfish aquaculture more sustainable (Naylor et al., 2009; Jackson and Shepard, 2010). However, although the amino acid profile of soy protein is quite similar to FM, and represents a viable possibility for fish meal replacement, soy contains several antinutritional factors (ANF) which have variable though significant effects on growth and fish health (Sitja-Bobadilla et al., 2005; Gatlin et al., 2007; Sun et al., 2007; Salah Azaza et al., 2009).

Soybeans are processed through various means depending on the nutritional requirements of the intended market. Soybean meal is created by removing the hulls, defatting the beans through hexane extraction, and then steam toasting the resultant flakes to denature the proteins and increase digestibility and nutrient availability (Dourade et al., 2011). This process leaves many of the ANF present in raw soybeans intact, including protease inhibitors (trypsin inhibitors), oligosaccharides (stachyose, raffinose, etc.), saponins, isoflavones, antigens (glycinin, B-conglycinin, lectins), phytate, and tannins (Refstie et al., 2005; Knudsen et al., 2007; Iwashita et al., 2009). These compounds are known to cause morphological changes in the intestines of

many species, such as Atlantic salmon (*Salmo salar*) (Knudsen et al., 2007; Yamamoto et al., 2008), rainbow trout (*Oncorhynchus mykiss*) (Ramsey et al., 1994; Yamamoto et al., 2008), and Atlantic cod (*Gadus morhua*) (Olsen et al., 2007), leading to decreased growth.

Alcohol extraction to produce soy protein concentrate, removes or inactivates many of these factors, and produces a product which may result in good growth rates without pathological changes in the intestine (Francis et al., 2001; Knudsen et al., 2007). In feed for summer flounder specifically, fish meal can be replaced by soy protein concentrate at up to a 60% level with no decrease in growth in juvenile fish (Ward, Chapter 1), and throughout the growout cycle over longer six-month trials as well (Bengtson et al. in prep.). Interestingly, while the ANF present in soybean meal reduces growth in many species (Francis et al., 2001; Knudsen et al., 2007), past research at URI has shown that depending on the level, some inclusion of ANF (through fish meal replacement with soybean meal) in the diet can reduce mortality of summer flounder when challenged with pathogenic bacteria (Lightbourne, 2011; Ward, Chapter 1). Juvenile summer flounder fed a 12% soybean meal, 48% soy protein concentrate replacement diet for 12 weeks survive challenge with *Vibrio harveyi* significantly better than fish fed a fish meal control diet, with no significant impact on growth (Ward, Chapter 1). This health benefit of diets containing soybean meals (but not soybean protein concentrate) may be due to immunomodulatory effects of compounds present in soybean meal that are removed when soy protein concentrate is prepared (Rumsey et al., 1994, Ward, Chapter 1).

Two feed trials were initiated to systematically evaluate which fractions of soybean meal may be responsible for decreased growth and increased survival to bacterial challenge in summer flounder. When producing soy protein concentrate, the alcohol-extraction of soy white flake process removes many of the alcohol-soluble ANF into a separate product known as soy molasses (concentrated, desolventized, aqueous alcohol extract of defatted soybean flakes). In this study, we first investigated the effect of adding increasing amounts of soy molasses to soy protein concentrate replacement diets on summer flounder growth and survival to bacterial challenge. Then, in order to evaluate which compounds within the soy molasses may be responsible for the reduction in growth and/or increase in survival to bacterial challenge in summer flounder, the soy molasses was fractionated into three subfractions by phase separation using *n*-butanol, and these fractions were incorporated on summer flounder feeds.

MATERIALS AND METHODS

Diet Preparation: All diets were manufactured at the Food Science and Nutrition Center, at the University of Rhode Island (West Kingston, RI). Diets were formulated to be isocaloric and isonitrogenous and were analyzed for proximate composition as described below. All diets were formulated to contain 50% total crude protein and diets with soy protein included were fortified with taurine (1%; all chemicals purchased from Sigma-Aldrich Co, St. Louis, MO, unless otherwise noted). Fish meal was donated by the USDA fish laboratory, Bozeman, MT, USA (International Protein Corp.; anchovy, 69.6% protein, 9% oil), and fish oil was donated by Omega Protein

(Houston, TX, USA). Soy protein concentrate and soy molasses were produced from soy white flake from a commercial soybean processor (Creston Bean Processing, LLC., Creston, IA).

Specific commercial soy protein concentrate and soy molasses preparation methods are considered intellectual property, and are not commonly disseminated. Therefore, US patents by Hayes et al., 1973 (3,734,901), Pass, 1975 (3,897,574), and Campbell et al., 1981 (42659250), were consulted as well as the methods described by Wang et al. (2004). Briefly, deionized water was mixed with 95% ethanol to create 60% w/w aqueous ethanol. This mixture was heated to 50°C while mixing, at which point soy white flakes were added to produce a 7:1 solvent to soy ratio. At the conclusion of a 30-minute extraction process, most of the liquid was removed by filtration through cheesecloth, and the remaining solids were removed by centrifugation (14,000 x *g*; 15°C for 30 min.). The supernatant from the centrifugation was pooled with the filtered liquid, heated 70°C while mixing to remove the ethanol and concentrate it to 10% of the original volume, and stored at 4°C until used as soy molasses (SM). The solids were spread thin on a tray and desolventized in an oven at 90°C for 45 minutes. At the conclusion of drying, the solids were ground, passed through a 1mm sieve and stored at 4°C until used as soy protein concentrate (SPC).

Diet analysis:

Crude protein, crude lipid, moisture, and ash of all diets were analyzed using AOAC (1995) methods. Oligosaccharide content (mg/g) was determined by the

Raffinose/Sucrose/D-Glucose assay kit (Megazyme, Wicklow, Ireland) (Knudsen et al. 2007). Trypsin inhibitor content was determined through the method of Kaskade et al. (1974), with modifications by Hammerstrand et al. (1981). Quantification of saponin content was determined by the method of Berhow et al. (2002; 2006). Briefly, HPLC analysis was conducted on a Hitachi analytical HPLC system with L-7100 low pressure gradient pump, 4-channel degasser, L-7200 sequential autosampler, high sensitivity diode array detector (190-800 nm), and an L-7485 fluorescence detector (emission: 200-850 nm; excitation 250-900 nm), controlled by a D-700 HPLC System Manager software package. The column used was an Inertsil ODS-3 reverse phase C-18, 5µm, 250 mm x 4.6 mm i.d., with a guard column (Varian, Torrance, CA). For saponin analysis, the initial conditions were 30% acetonitrile and 0.025% trifluoroacetic acid (TFA) in water at a flow rate of 1 mL/ min. The effluent was monitored at 210 nm on the variable wavelength detector. After injection (20 µL), the column was developed to 50% acetonitrile and 0.025% TFA in a linear gradient over 45 min. Before the samples were run, an extinction coefficient for the saponins was determined from a linear standard curve based on mAbs units *vs.* nanomoles injected. This curve was prepared from a dilution series of pure soyasaponin A and B standards that had been supplied by Dr. Mark Berhow (USDA, ARS, NCAUR; Peoria, IL). All samples were run in triplicate, and an average and standard deviation were determined.

Trial 1:

Five diets were formulated as follows (Table 1): FM: no soy protein, all protein from fish meal; SPC: Partial replacement of 60% FM with SPC; SPC/0.5%SM: 60% SPC replacement of fish meal plus 0.5% soy molasses (SM); SPC/1.2%SM: 60% SPC replacement of fish meal plus 1.2% SM; SPC/1.9%SM: 60% SPC replacement of fish meal plus 1.9% SM. The level of SM inclusion in each diet (0.5, 1.2, and 1.9% corresponds to the quantity of antinutritional factors present in soybean meal replacement diets in previous trials (12%, 24% or 26% soybean meal replacement diet, respectively; Ward, Chapter 1).

Fish, rearing conditions, and sampling protocol: Both feed trials took place at the Blount Aquaculture Research Laboratory (BARL), University of Rhode Island Narragansett Campus (Narragansett, RI, USA). Juvenile summer flounder for both trials were transported from Great Bay Aquaculture (Portsmouth, NH, USA), to holding tanks at the BARL in May 2012 and maintained on flow-through, sand-filtered seawater with constant aeration. Five-hundred summer flounder (2.30±0.1g, 6.06±0.1 cm) were moved from the holding tanks and randomly distributed into 25 flow through 75 L aquaria at twenty fish per tank (five replicates per diet), with temperature (18.2±0.9°C), lighting (12L:12D), salinity (30-32 psu), and aeration controlled throughout the experiment. All fish were fed a commercial diet (Skretting Gemma Diamond 0.8mm, Stavanger, Norway) twice daily by hand during the 2-week acclimation period. Fish were then fed the experimental diets twice daily (08:00-10:00 and 15:00-17:00) to satiation for a period of eight weeks, and each tank was cleaned via siphoning daily (08:00). Individual fish wet weight and total length were determined at the beginning and end of the trial, with total tank biomass calculated

every 2 weeks in between. Weight gain, feeding efficiency, specific growth rate, condition factor, and survival were calculated at the end of the feeding trial (Hopkins, 1992).

Bacterial challenge: Following the conclusion of the feeding trial, fish from three randomly selected tanks per group were injected with *Vibrio harveyi* (a known pathogen of summer flounder; Soffientino et al., 1999), and fish from two tanks were injected with filtered artificial seawater (FASW). Bacterial cell concentration for the challenge (LD_{50}, dose lethal to 50% of the fish) was determined prior to the challenge by the Karber method (Barros et al., 2002). Fish in the bacterial challenge groups were injected intraperitoneally with $2.27x10^7$ cells *Vibrio harveyi* (in 100 µl), while the fish in the control groups were injected intraperitoneally (i.p.) with 100 µl FASW. Mortality was monitored and recorded twice daily for seven days following injection. Post-challenge, bacteria were isolated from the peritoneal fluid, and confirmed to be *V. harveyi* by amplification and sequencing of a portion of the 16S rDNA gene using polymerase chain reaction (Gauger et al., 2006). At the conclusion of the challenge (day 7) all remaining fish were anesthetized with tricaine methane sulphonate (MS-222 (100 mg/L for 5 min).

Trial 2:

The soy molasses was fractionated into three subfractions by phase separation using *n*-butanol (Knudsen et al., 2007). Briefly, the soy molasses produced through the aqueous-ethanol extraction process was mixed with water-saturated *n*-butanol 1:1

(v/v), and allowed to form three distinct layers in a separation funnel for 24 hours. The lower "water phase" was removed by opening the valve at the bottom of the separation funnel, the upper "butanol phase" was removed via pipette, and the remaining "interphase" in the middle was again removed via collection below by opening the valve (Fig 1). The three subfractions were mixed with deionized water (1:1, v/v) and evaporated to dryness in a rotary evaporator at 70°C under reduced pressure. The resulting residues were resuspended in deionized water three times and evaporated to dryness to ensure no *n*-butanol remained. Following the evaporation process, each subfraction was again rehydrated to the original pre-fractionated volume and analyzed for antinutritional factors.

Five diets were formulated to evaluate the impact of each subfraction on growth and survival following challenge. Each subfraction was added to the diet corresponding to the 0.5% SM inclusion level, based on results from trial 1. The diets were designed as follows (Table 3): FM: (no soy protein, all protein from fish meal); SPC: Partial replacement of 60% FM with SPC; SPC/Butanol: 60% SPC replacement diet with *n*-butanol-phase from soy molasses; SPC/Interphase: 60% SPC replacement diet with the interphase fraction from soy molasses; SPC/Water: 60% SPC replacement diet with water-phase from soy molasses. All diets were analyzed for proximate analysis and antinutritional factor content prior to the start of the feed trial as described above.

Fish, rearing conditions, and sampling protocol: The second feed trial was also performed at the BARL within a temperature controlled (19.0±0.4°C), flow-through system, with 12L:12D lighting. Five-hundred juvenile Summer flounder (*Paralichthys*

dentatus) (length 10.37±0.3cm, weight 11.16±0.8g) were moved from the holding tanks and randomly distributed into 25 flow-through 75 L aquaria at 20 fish per tank (5 replicates per diet). All fish were fed a commercial diet (Skretting Gemma Diamond 1.2mm, Stavanger, Norway) twice daily by hand during the 2 week acclimation period, then fish were fed twice daily (08:00-10:00 and 15:00-17:00) to satiation for a period of six weeks, and each tank was cleaned via siphoning daily (08:00). Individual fish wet weight and total length were determined at the beginning and end of the trial, with total tank biomass calculated every 2 weeks in between. At the conclusion of the growth trial, a bacterial challenge was administered, following the same protocol as outlined above. Weight gain, feeding efficiency (FCR), specific growth rate (SGR), condition factor (K), and survival were calculated at the end of the feeding trial (Hopkins, 1992).

Statistics:

All growth and condition data were analyzed using the General Linear Model procedure using SAS computer software (SAS 9.2 TS Level 2M2, Cary, NC, USA). Mean results were subjected to one-way analysis of variance (ANOVA), and significant results were further analyzed using Tukey's post test. Bacterial challenge survival data were analyzed using the Kaplan-Meier log rank survival analysis, with all pairwise multiple comparison procedures (Holm-Sidak method) using SigmaStat (Systat Software, San Jose, CA, USA). The significance level of 0.05 was chosen, and all data are presented as mean ± SEM.

RESULTS

Trial 1:

Proximate analysis revealed no differences between diets in nutritional composition (Table 8). Antinutritional factors differed between diets based on the amount of SM added back into the diet (Table 8). Much of the trypsin inhibitors (TI) are typically denatured through the SPC production process, so TI content was much lower in the SPC-based diet which was not supplemented with any additional SM. Levels of TI ranged from 15.9±0.3 TIU/ml in SPC Control diet to 17.4±0.2 TIU/ml in the SM-supplemented diets. Saponin content also followed a trend of increasing concentration in the soy-containing diets (0.6±0.0mg/g in the SPC diet to 4.8±0.2mg/g in the SPC/1.9%SM diet) as the amount of SM added to the diet increased. The level of non-starch oligosaccharides in the diet also increased with the amount of SM added back into the diet, ranging from 1.0±1.0 mg/g in the FM diet to 6.9±2.7 mg/g in the SPC/1.9%SM diet (Table 8).

Over the 8-week feed trial, gains in both length and weight were greatest for the FM group (Fig. 4). All groups which were fed diets containing the experimentally produced SPC gained significantly less weight than the groups fed the FM Control (ANOVA, df=4, F-value=311.93, Pr>F=<0.0001, α=0.05, Fig. 4), and grew to reach significantly shorter lengths than the group fed the FM diet (ANOVA, df=4, F-value=177.55, Pr>F=<0.0001, α=0.05, Fig. 4). Feed conversion ratio (FCR) varied little between groups fed the SPC-produced diets (ranging from 2.24±0.8 to 2.67±0.9), and were similar to the FCR for the group fed the FM Control diet (0.99±0.3; ANOVA, df=4, F-value=2.80, Pr>F=0.0641; Fig 4).

Survival following bacterial challenge was lowest for the group fed the FM diet and the diets with the highest proportion of soy molasses, however, due to high variability between tanks, there was no significant difference between groups (Kaplan-Meier Survival Analysis: Log-Rank; df=4, statistic=6.176, p=0.186) (Fig 5). All of the fish which were injected with filtered artificial seawater (FASW) as a control survived the 7-day challenge period.

Trial 2: Proximate analysis revealed no differences between diets in nutritional composition (Table 9). Antinutritional factors differed between diets based on the amount and composition of subfractionated SM added back into the diet (Table 9). Trypsin inhibitor content was stable between SPC-based diets, ranging from15.6±0.4 to 15.9±0.2 TIU/ml. Saponin content was low among all SPC diets (Table 9). The ANF that showed the highest variation between the SPC diets was the soy oligosaccharides, which ranged from a background level of 1.0±1.0 mg/g found in the SPC control diet to 3.8±0.1 mg/g for the SPC/Interphase (Table 9).

Gains in both length and weight at the conclusion of the second feed trial were significantly higher for the group fed the FM diet (Fig. 6), the only diet that did not contain ANFs. In addition, fish fed the SPC diet were also significantly longer than fish fed diets supplemented with fractions from the SM *n*-butanol extraction (ANOVA, df=4, F-value=24.64, $Pr>F\leq0.0001$, $\alpha=0.05$). Gains in weight followed the same trend, with the group fed the FM diet gaining significantly more weight than any of the groups fed the diets containing SPC, and the group fed the SPC diet gaining

significantly more weight than any of the groups fed with diets supplemented with fractions from the SM *n*-butanol extraction (ANOVA, df=4, F-value=44.92, Pr>F=<0.0001, α=0.05). Feed conversion ratio was again significantly lower for the FM-fed group (0.91±0.08) than all other diets, and the groups fed the SPC diet was significantly lower than the groups fed the diet containing the SM sub-fractions (ANOVA, df=4, F-value=10.50, Pr>F=<0.0001, α=0.05) (Fig. 6).

Survival of summer flounder to an acute bacterial challenge performed after the feeding trial was lowest for the group fed the FM diet; significantly lower than that of the groups fed either the SPC/Butanol, SPC, or SPC/Water diets (Kaplan-Meier Survival Analysis: Log-Rank; df=4, statistic=31.77, p<0.001, Fig. 7). Survival was also significantly higher in the groups fed either the SPC/Water or SPC/Butanol compared to the SPC/Interphase diet (Fig. 7). All of the fish injected with filtered artificial seawater (FASW) as a control survived the 7-day challenge period.

DISCUSSION

Replacing fish meal in feeds for carnivorous marine finfish with protein from soy has the potential to create a more sustainable aquaculture industry by reducing costs due to high feed prices. Our results confirm previous work (Ward, Chapter 1) suggesting that diets containing soybean meal can reduce mortality in summer flounder following an acute bacterial challenge. We have shown that, although adding soy molasses or subfractions of soy molasses to a diet in which fish meal has been replaced by 60% soy protein concentrate reduced growth in summer flounder, the presence of small levels of products resulting from the fractionation of soy molasses to an soy protein

concentrate replacement diet also led to increased survival to bacterial challenge. Based on the results showing that the highest survival to bacterial challenge was observed in fish fed the diet containing relatively higher levels of oligosaccharides (3.8 mg/g, the amount present in the water fraction from soybean molasses), we hypothesize that oligosaccharides are probably responsible for the health benefits provided by these diets.

The intent of the current feed trials was to try narrow down which products in soybean meal, but not soy protein concentrate, were responsible for the decrease in growth and increase in survival to bacterial challenge observed in previous feeding trials with summer flounder (Lightbourne, 2011, Bengtson et al., in prep., Ward, Chapter 1). There is no consensus on the causative agent of pathological changes observed in the intestine of fish fed with soybean meal replacement diets (Knudsen et al., 2007). Several authors have added a single antinutritional factor (eg. saponins, Francis et al., 2001) but no studies, to our knowledge, have produced both soy protein concentrate and soy molasses from the same precursor product in order to determine effects on both growth and survival to bacterial challenge. In order to do so consistently, we started with one initial product (soy white flake), and produced both soy protein concentrate and soy molasses from the same batch to eliminate potential variability due to differences in source of the products. By eliminating that variability, it was possible to be certain the results we find are due to only the soy molasses (or sub-fractionated sections) added back into the diet, as the SPC produced from the soy white flake was identical for each diet.

In previous studies, a 60% soy protein replacement diet provided similar growth than a fish meal diet in summer flounder (Bengtson et al., in prep.; Ward, Chapter 1). Given the high protein content of the SPC produced in this study, the decrease in growth seen in summer flounder fed the SPC diet is probably due to some leftover antinutritional factors present in our SPC diet, primarily undenatured trypsin inhibitors. Previous studies have shown decreased growth in studies where trypsin inhibitor content was either not denatured or only partially denatured, with results similar to those found in this study (Wilson and Poe, 1985; Refstie et al., 1998; Hart et al., 2010; Santigosa et al., 2010; Kumar et al., 2011). There were also further significant reductions in both length and weight gain as the amount of whole or fractionated soy molasses added to the diet increased, showing that small increases in levels of ANFs present in soy molasses further contribute to the negative effects in growth.

In addition, the SPC replacement diet, as well as diets containing small amounts of the water and butanol fractions from soy molasses, provided significant increases in summer flounder survival to bacterial challenge. These results suggest that the relatively small levels of antinutritional factors present in these diets, which are absent from the fish meal, are responsible for these the positive effects of these diets on survival to bacterial challenge. Lack of statistical power was probably the reason why a similar significant effect on survival from bacterial challenge was not observed in fish fed the SPC diet in the first trial.

Given that compounds found in soy molasses have been shown to reduce growth, in order to increase survival following bacterial challenge while providing good growth rates, the optimal amount of soy molasses required to cause an increase in survival should be determined. Based on the results from these 2 trials, the two diets providing the best survival were the SPC/water fraction, followed by the SPC diets, which, in addition to trypsin inhibitors, contained levels of saponins of about 0.6 mg/g of diet and between 1.0 and 3.8 mg of oligosaccharides per g of diet.

Further research needs to be done to determine which compounds may be responsible for the protective effect of diets containing soybean meal products. Saponins have been shown to have many antibacterial, antifungal, and antiviral qualities (Francis et al., 2001; Francis et al., 2002; Sparg et al., 2004; Knudsen et al., 2007). However, saponins in particular have also been shown to have growth reducing and pathological effects on the intestine of many cultured finfish species (Refstie et al., 2005; Knudsen et al., 2007; Iwashita et al, 2009). Oligosaccharides have many known and hypothesized immunomodulatory properties which may have beneficial impacts on survival (Francis et al., 2001; Staykov et al., 2007; Torrecillas et al., 2007).). The inclusion of oligosaccharides in diets for finfish have any not shown the same enteritis or growth reducing qualities that saponins have (Hart et al., 2010; Sorensen et al., 2011). Oligosaccharides are known to have immunomodulatory properties in cultured fish such as an increase in lysozyme concentration, alternative complement pathway, and improved disease resistance (Staykov et al., 2007; Torrecillas et al., 2007). Oligosaccharides in the diet are not digestible by summer flounder; therefore, their

effects on gut cells may translate to systemic effects. They may also act as substrate for beneficial bacterial growth within the gut (Gatesoupe, 1999; He et al., 2009; Ringo et al., 2010). Based on the analysis of ANF composition of our diets, the only (observed) difference between the SPC/water fraction diet, which provided the highest levels of survival to bacterial challenge, and the other soy-based diets was on the levels of oligosaccharides, suggesting that oligosaccharides (and/or unknown products present in the water fraction of soybean molasses) may be responsible for the positive impact on survival to bacterial challenge.

In conclusion, our results confirm that low levels of supplementation with soy molasses or soybean meal of a diet in which 60% of fish meal has been replaced with soy protein concentrate provides increased survival to bacterial challenge in summer flounder, a benefit that may balance the negative impacts on growth of these diets. Furthermore, this study suggests that small levels of oligosaccharides present in these diets may be responsible for the increased survival to bacterial challenge. Future work should focus on evaluating the impact of supplementation of soy protein replacement diets with purified oligosaccharides in order to determine if the potential beneficial effects of these compounds on survival to bacterial challenge in summer flounder can be achieved without a concomitant decrease in growth.

	FM Control	SPC Control	SPC + 0.5% SM	SPC + 1.2% SM	SPC + 1.9% SM
Fish Meal	670	268	268	268	268
Soybean Molasses	0.0	0.0	5.3	12.3	19.4
Soy Protein Concentrate	0.0	402	402	402	402
Fish Oil	32	65.2	65.2	65.2	65.2
Wheat flour	238.5	120.6	115.3	108.3	101.2
Corn gluten	25.0	49.2	49.2	49.2	49.2
Starch	4.5	14.5	14.5	14.5	14.5
Mineral Premix (URI)	10	10	10	10	10
Calcium Phososphate	0	30	30	30	30
Vitamin Premix (URI)	10	10	10	10	10
Methionine	0	1.6	1.6	1.6	1.6
Taurine	0	14.0	14.0	14.0	14.0
Glycine	10	15.0	15.0	15.0	15.0
g/kg diet					

Table 6. *Diet formulations for the 5 experimental diets (1kg; all values for diet ingredients in grams) for Trial 1. Diet 1: Control: (no soy protein, all protein from fish meal); Diet 2: SPC control (Partial replacement of 60% FM with SPC); Diet 3: SPC control with 0.5% SM; Diet 4: SPC control with 1.2% SM; Diet 5: SPC control with 1.9% SM.*

Figure 3. *Water saturated* n*-butanol and soy molasses mixture (1:1, v/v). The three subfractions from top to bottom are butanol phase, interphase and water phase.*

	FM Control	SPC Control	SPC + Upper	SPC + Interphase	SPC + Lower
Fish Meal	670	268	268	268	268
Upper (butanol) Phase	0	0	1.5	0	0
Precipitate Phase	0	0	0	3.6	0
Lower (water) Phase	0	0	0	0	5.1
Soy Protein Concentrate	0.0	402	402	402	402
Fish Oil	32	65.2	65.2	65.2	65.2
Wheat flour	238.5	120.6	119.1	117.0	115.5
Corn gluten	25.0	49.2	49.2	49.2	49.2
Starch	4.5	14.5	14.5	14.5	14.5
Mineral Premix (URI)	10	10	10	10	10
Calcium Phososphate	0	30	30	30	30
Vitamin Premix (URI)	10	10	10	10	10
Methionine	0	1.6	1.6	1.6	1.6
Taurine	0	14.0	14.0	14.0	14.0
Glycine	10	15.0	15.0	15.0	15.0
g/kg diet					

Table 7. *Diet formulations for the 5 experimental diets for Trial 2 (1kg; all values for diet ingredients in grams). The FM Control did not contain any soy products. The other four experimental diets we formulated to have 40% of the protein from FM and 60% from SPC, with varying levels of antinutritional factors added.*

Proximate Analysis (dry weight basis)	FM	SPC	SPC/0.5% SM	SPC/1.2% SM	SPC/2% SM
Ash	13.7±0.6	10.5±0.7	10.3±0.6	10.0±1.0	10.0±0.0
Protein	52.6±0.5	45.0±0.3	51.7±0.1	49.2±0.5	44.8±0.5
Lipid	10.2±0.9	10.1±0.7	8.2±0.1	9.5±0.6	8.8±0.3
Trypsin inhibitors (TIU/ml)	0.0±0.0	15.9±0.3	17.4±0.2	17.3±0.1	17.2±0.1
Saponins (mg/g of diet)	0.0±0.0	0.6±0.0	3.0±0.0	4.7±0.2	4.8±0.2
Oligosaccharides (mg/g of diet)	0.1±0.1	0.9±0.1	2.7±0.1	4.4±0.9	6.9±2.7
Soybean Molasses as a % of diet	0	0	0.53	1.23	1.94

Table 8. *Proximate analysis and antinutritional factor content for the 5 diets. The three diets with soybean molasses added; SPC + 0.5%, SPC + 1.2%, and SPC +1.9% correspond to the level of antinutrional factors present in a 12%, 24% or 36% fish meal replacement diet, respectively. Saponins are all type A and B saponins quantified per sample. Oligosaccharide content refers to all non-starch (ie. not including glucose and sucrose), content per diet. All values mean ± SD, unless otherwise noted.*

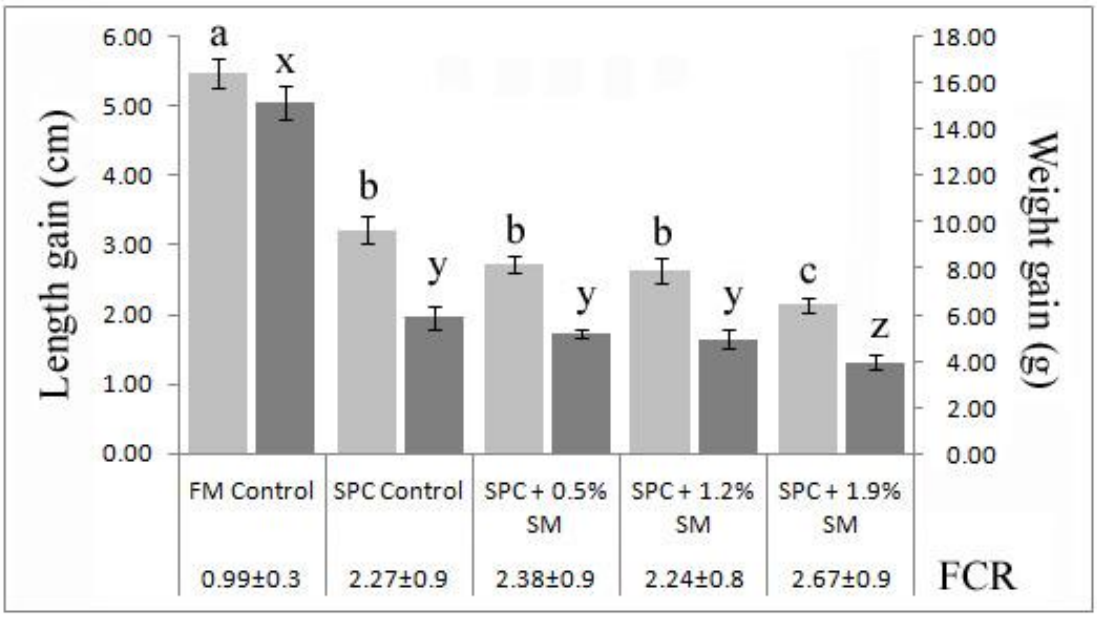

Figure 4. *Length gain (grey bars) and weight gain (black bars) over eight week feeding trial 1. Bars denote mean, and error bars standard deviation. Feed conversion ratios (FCR) for each group listed under growth averages, mean±SD. Different letters denote significant differences (one-way ANOVA, α=0.05).*

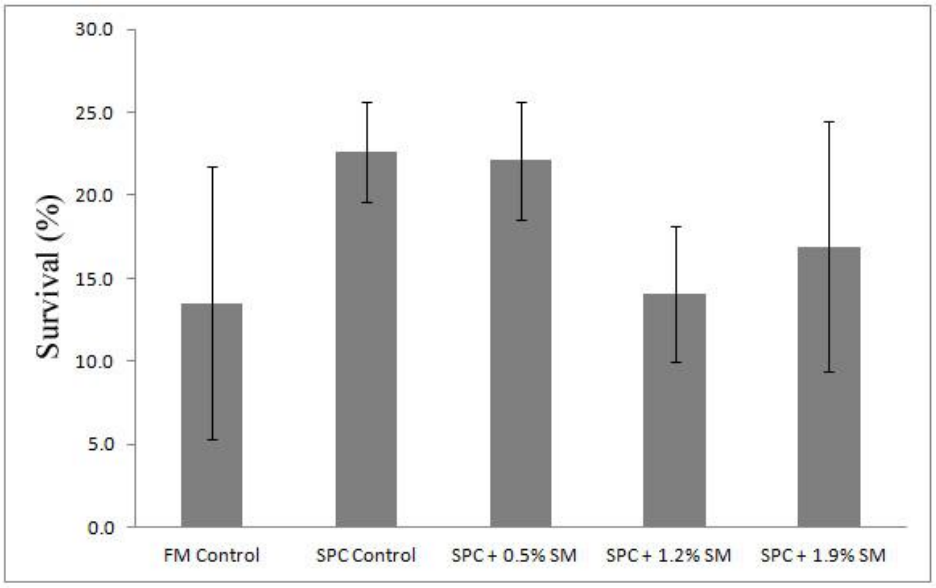

Figure 5. *Percent survival following the seven-day bacterial challenge in trial 1, mean±SD. Due to high variability between tanks, there was no significant difference between groups (Kaplan-Meier Survival Analysis: Log-Rank; df=4, statistic=6.176, p=0.186)*

	FM Control	SPC Control	SPC + Upper	SPC + Interphase	SPC + Lower
Ash	16.0±0.0	11.0±0.0	10.3±0.6	10.7±0.6	11.7±0.6
Protein	52.6±0.6	56.5±0.4	53.6±3.5	56.0±0.1	55.4±0.3
Lipid	11.7±1.0	10.7±0.4	10.2±0.6	10.9±2.1	8.6±0.4
Trypsin inhibitors (TIU/ml)	0.0±0.0	15.9±0.2	15.6±0.4	15.8±0.4	15.8±0.5
Saponins (mg/g)	0.0±0.0	0.6±0.2	0.8±0.3	0.6±0.2	0.6±0.2
Oligosaccharides (mg/g)	0.3±0.5	1.0±1.4	1.6±0.5	1.5±0.3	3.8±0.1

proximate analysis on a dry weight basis

Table 9. *Proximate analysis and antinutritional factor content for 5 diets; Trial 2. The three diets with subfractionated soybean molasses added; SPC + Butanol, SPC + Interphase, and SPC +Water correspond to the level of antinutrional factors present in the 0.53% SM diet from Trial 1 (or a 12% fish meal replacement diet). Saponins are all type A and B saponins quantified per sample. Oligosaccharide content refers to all non-starch (ie. not including glucose and sucrose), content per diet. All values mean ± SD.*

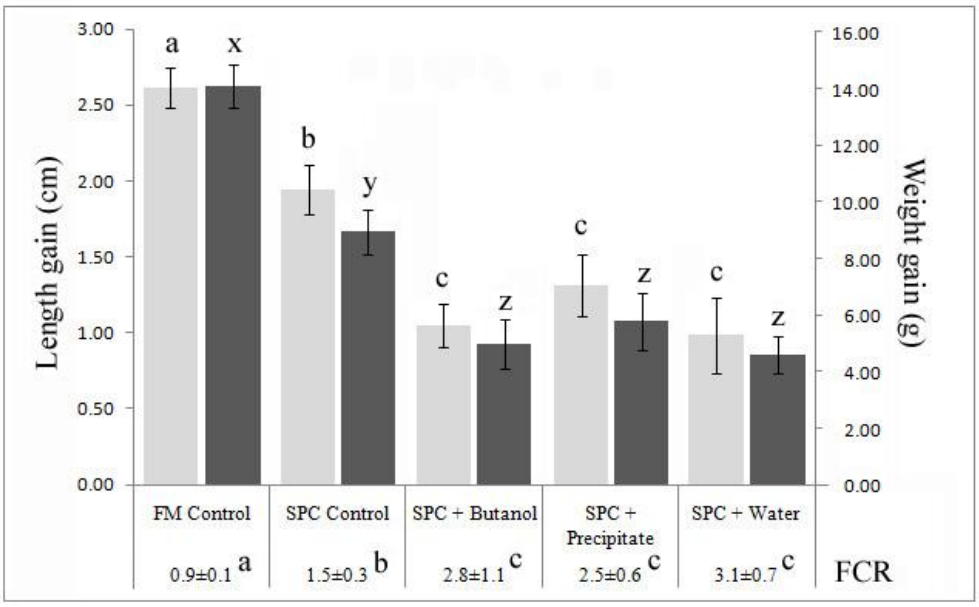

Figure 6. *Length gain (grey bars) and weight gain (black bars) over six week feeding trial 2. Bars denote mean, and error bars standard deviation. Feed conversion ratios (FCR) for each group listed under growth averages, mean±SD. Different letters denote significant differences (one-way ANOVA, α=0.05).*

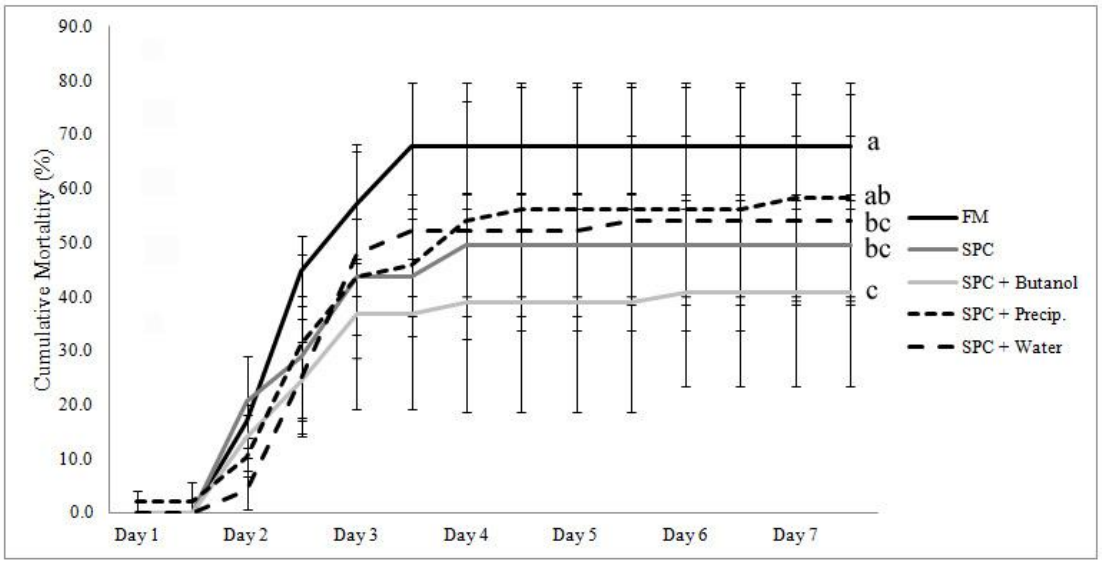

Figure 7. *Cumulative mortality curves for the seven-day bacterial challenge in trial 2, mean ± SD. Different letters denote significant differences between group (Kaplan-Meier Survival Analysis: Log-Rank; df=4, statistic=31.77, p<0.001).*

CHAPTER 3

INFLUENCE OF OLIGOSACCHARIDE SUPPLEMENTATION OF SOY PROTEIN CONCENTRATE REPLACEMENT DIETS ON GROWTH AND SURVIVAL TO BACTERIAL CHALLENGE OF SUMMER FLOUNDER (*Paralichthys dentatus*)

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ABSTRACT

Aquaculture diets have progressed to a point where plant protein sources can partially replace fish meal (FM) for many species of carnivorous fishes. Soybean meal (SBM) can only replace some of the FM due to the presence of antinutritional factors (ANF), whereas soy protein concentrate (SPC) can be incorporated at much higher levels due to the removal of ANF through post-harvest processing. Previous research has demonstrated that, in summer flounder, the negative effects of soybean meal replacement of fish meal on growth may be balanced by a positive effect on survival to bacterial challenge, and that optimal growth and survival to bacterial challenge can be achieved with a 60% replacement diet composed of mostly SPC and some low levels of SBM. Further studies in summer flounder suggest that soy oligosaccharides present in soybean meal may be responsible for the increased survival following

bacterial challenge. The current work was designed to evaluate the impact of supplementing soy protein concentrate replacement diets with either a fraction of soybean meal enriched with soy oligosaccharides (1.9% w/w) or crystalline oligosaccharides (raffinose pentahydrate and stachyose hydrate at either 0%, 0.2%, 0.4% or 0.6% (w/w)) on growth, survival to bacterial challenge, hematological parameters, and tissue morphology in summer flounder. Following a four-week feed trial, there were no significant differences in growth between any of the treatment groups. Survival following challenge was significantly greater for the group fed the 0.4% oligosaccharide supplementation as compared to all other diets (Kaplan-Meier Survival Analysis: Log-Rank; df=4, statistic=53.674, p=<0.001). There were no differences in the morphology liver, spleen or anterior intestine, and no significant differences in blood chemistry. These results demonstrate the possibility of oligosaccharide supplementation to carnivorous fish soy protein concentrate replacement diets to reduce mortality due to bacterial infection.

INTRODUCTION

Aquaculture is currently growing faster than any other animal-producing sector (8.9% annually) (FAO, 2008). Two of the major challenges to the expansion of marine aquaculture are the cost of feeds and disease. Fish meal (FM) is the primary source of protein in carnivorous fish feeds (Murray et al., 2010) and the demand for FM available for aquafeeds is expected to exceed supply within the next decade (Gatlin et al., 2007). One of the major issues is formulating sustainable diets for popular marine carnivorous fish like flounders, seabasses, and tunas, due to the variable cost and questionable sustainability of FM, which depends on capture fisheries. The continued growth of sustainable aquaculture production depends upon the development of reliable, ecologically sound protein sources to replace FM in aquaculture feeds.

Fish meal has become prohibitively expensive as a primary protein source of protein; as of August 2013 the price was $2088 per metric ton. This is in comparison to the bulk price of soybean meal (SBM) as a primary protein source at $456 per metric ton (www.indexmundi.com). Although soy products are currently included in many diets for carnivorous fishes, soy protein is not included at a very high level due to uncertainty about antinutritional factors (ANFs) present in certain soy products such as SBM (Ziegler representative, per comm.). Soy protein concentrate (SPC) is a product which has significantly lower levels of ANFs, which can replace FM in diets for finfish at much higher amounts; however, the price is higher as well.

The primary issue with including SBM at high levels in diets for finfish is that the ANFs contained in SBM cause a reduction in growth, health, and therefore overall reduction in economic viability for the farmer (Ramsey et al., 1994; Francis et al., 2001; Knudsen et al., 2007; Olsen et al., 2007; Yamamoto et al., 2008; Ward, 2014, Chapters 1 and 2). Many researchers have sought to determine the compounds responsible for the reduction in growth, and have found soyasaponins to be at least partially responsible, as well as trypsin inhibitors (Wilson and Poe, 1985; Refstie et al., 1998; Hart et al., 2010; Santigosa et al., 2010; Kumar et al., 2011), isoflavones, antigens (glycinin, B-conglycinin, lectins), phytate, and tannins (Refstie et al., 2005; Knudsen et al., 2007; Iwashita et al, 2009). Heat processing of the soybean meal, or alcohol extraction to produce soy protein concentrate, removes many of these factors, and produces a product allowing for good growth rates without intestinal morphological changes (Francis et al., 2001).

While SPC as a FM replacement results in growth rates comparable to FM-based diets, the inclusion of low levels of SBM (and therefore ANFs), but not SPC, in diets for finfish also has the potential to impact disease resistance in finfish (Lightbourne, 2011; Bengtson et al., in prep.; Ward, 2014; Chapters 1 and 2). There have been outbreaks of vibriosis at the only marine finfish hatchery in the northeast which has caused widespread mortality of summer flounder (George Nardi per. comm.), and recent outbreaks of disease at the only summer flounder grow-out site in the northeast has demonstrated similar issues. In light of the impact of disease on production,

commercial aquaculture companies are particularly interested in any opportunities to strengthen immune function and prevent disease outbreaks.

Some of the products included in soybean meal may have immunomodulatory effects (Rumsey et al., 1994; Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2000). Several antigenic compounds present in soybeans have been hypothesized to cause immunostimulation in fish (Francis et. al, 2001). Saponins have been shown to have many antibacterial, antifungal, and antiviral qualities (Francis et al., 2001; Francis et al., 2002; Sparg et al., 2004; Knudsen et al., 2007). Oligosaccharides have many known and hypothesized immunomodulatory properties which may have an effect on growth and survival (Hart et al., 2010; Sorensen et al., 2011). Previous studies have showed reduced mortality to bacterial challenge in summer flounder fed SBM-based diets while no protection was provided by replacement diets with soybean protein concentrate (Bengston et al. in prep.; Ward, Chapters 1 and 2, 2014). These results suggest that this health benefit (protection against bacterial challenge) of diets containing SBM (but not SPC) may be due to immunomodulatory effects of ANFs present in SBM that are removed when SPC is prepared (Rumsey et al., 1994; Grisdale-Helland et al., 2008).

In our previous studies, we have investigated which fraction from soybean molasses (concentrated, desolventized, aqueous alcohol extract of defatted soybean flakes; SM) is responsible for increased survival to bacterial challenge in summer flounder. We determined that fish fed subfractions of SM enriched in oligosaccharides led to the

highest survival following bacterial challenge compared to other subfractions of SM and fish meal diets (Ward, Chapters 1 and 2, 2013). Oligosaccharide supplementation has not been shown to have a negative impact on growth in aquacultured species (Gatesoupe, 1999; He et al., 2009; Ringo et al., 2010), whereas there has been an increase in beneficial immunological function due to oligosaccharide supplementation (Staykov et al., 2007; Torrecillas et al., 2007).

In order to determine if oligosaccharides can provide improved resistance to bacterial challenge in summer flounder, different levels of crystalline oligosaccharide supplementation of a 60% soy protein concentrate replacement diet were evaluated in the current experiment. If it can be conclusively shown that these supplemented replacement diets can reduce mortality due to disease, while having no measurable impact on growth, aquaculturists can use that knowledge to design soy-based diets which are economically advantageous with the knowledge that disease impacts can be mitigated while promoting a more sustainable primary protein source for finfish diets.

METHODS

Diet Preparation:

All diets were manufactured at the Food Science and Nutrition Center, University of Rhode Island (West Kingston, RI). Diets were formulated to be isocaloric and isonitrogenous and to contain 50% total crude protein (Table 10). Diets with soy protein included were fortified with taurine (1%) (Sigma-Aldrich Co, St. Louis, MO). Fish meal was donated to the project by the USDA fish laboratory, Bozeman, MT,

USA (International Protein Corp. (anchovy, 69.6% protein, 9% oil)), fish oil was donated by Omega Protein (Houston, TX, USA), and soy protein concentrate was donated by Solae LLC (St. Louis, MO, USA). Soy molasses was produced from soy white flake from a commercial soybean processor (Creston Bean Processing, LLC., Creston, IA). The water fraction of soy molasses was prepared as described by Ward et al. (Chapter 2). Stachyose hydrate and raffinose pentahydrate (Sigma-Aldrich Co, St. Louis, MO), the more predominant oligosaccharides found within soybeans (Hart et al., 2010), were combined at a 3.16:1 ratio (w/w), and added to three oligosaccharide-supplemented diets at 0.2%, 0.4% and 0.6% of the total diet weight.

Five diets were formulated as follows (Table 10): SPC Control (Replacement of 60% FM with SPC); SPC/WFSM (water fraction of soy molasses at a 0.5% SM inclusion level; from chapter 2); SPC/0.2% Oligo (SPC control diet with 0.2% oligosaccharide (w/w) added); SPC/0.4% Oligo (SPC control diet with 0.4% oligosaccharide (w/w) added); SPC/0.6% Oligo (SPC control diet with 0.6% oligosaccharide (w/w) added).

Diet analysis:

Crude protein, crude lipid, moisture, and ash of all diets were analyzed using AOAC (1995) methods. Oligosaccharide content (mg/g) was determined by the Raffinose/Sucrose/D-Glucose assay kit (Megazyme, Wicklow, Ireland) (Knudsen et al., 2007). Trypsin inhibitor content was determined through the method of Kaskade et al. (1974), with modifications by Hammerstrand et al. (1981). Quantification of saponin content was determined by the method of Berhow et al. (1996). Briefly,

HPLC analysis was conducted on a Hitachi analytical HPLC system (L-7100 low pressure gradient pump, 4-channel degasser, L-7200 sequential autosampler, high sensitivity diode array detector (190-800 nm), and a L-7485 fluorescence detector (emission: 200-850 nm; excitation 250-900 nm), controlled by a D-700 HPLC System Manager software package. The column used was an Inertsil ODS-3 reverse phase C-18, 5µm, 250 mm x 4.6 mm i.d., with a guard column (Varian, Torrance, CA). For saponin analysis, the initial conditions were 30% acetonitrile and 0.025% trifluoroacetic acid (TFA) in water at a flow rate of 1 mL/ min. The effluent was monitored at 210 nm on the variable wavelength detector. After injection (20 µL), the column was developed to 50% acetonitrile and 0.025% TFA in a linear gradient over 45 min. Before the samples were run, an extinction coefficient for the saponins was determined from a linear standard curve based on mAbs units vs nanomoles injected. This curve was prepared from a dilution series of pure soyasaponin A and B standards (mg/g) that had been supplied by Dr. Mark Berhow (USDA, ARS, NCAUR; Peoria, IL). All samples were run in triplicate, and an average and standard deviation were determined.

Fish, rearing conditions, and sampling protocol:

The feed trial took place at the BARL (Narragansett, RI). Juvenile summer flounder were transported from Great Bay Aquaculture (Portsmouth, NH, USA), to holding tanks at the Blount Aquaculture Research Facility in May 2012. Three-hundred summer flounder (28.3±1.2 g, 14.1±0.2 cm) were moved from the holding tanks and randomly distributed into twenty flow-through 75 L aquaria at fifteen fish per tank

(four replicates per diet), with temperature (19.0±1.5°C), lighting (12L:12D), salinity (30-32 psu), and aeration controlled throughout the experiment. All fish were fed a commercial diet (Skretting Gemma Diamond 0.8mm, Stavanger, Norway) twice daily by hand during the 2 week acclimation period. Fish were fed twice daily (08:00-10:00 and 15:00-17:00) to satiation for a period of eight weeks, and each tank was cleaned via siphoning daily (08:00). Individual fish wet weight and total length was determined at the beginning and end of the trial, with total tank biomass calculated every 2 weeks in between. Weight gain, feeding efficiency, specific growth rate, condition factor, and survival will be calculated at the end of the feeding trial (Hopkins, 1992).

Histology, blood sampling and bacterial challenge:

Following the conclusion of the feeding trial, one fish was randomly selected from each replicate tank for histological examination (four fish per treatment). Fish were euthanized with an overdose of tricaine methane sulphonate (MS-222; 250 mg/L for 10 min). Samples for histological examination of the proximal and distal intestine were fixed in 10% buffered formalin for seven days. The samples were then rinsed, repackaged in 95% ethyl alcohol and shipped to Mass Histology (Worcester, MA, USA) for processing onto slides. All samples were prepared according to the procedure of Merrifield et al. (2009); and ultrathin sections (7 µm) stained with hematoxylin and eosin were mounted on slides for each treatment. Slides were examined using light microscopy for structure integrity of the intestinal mucosa,

number and size of absorptive vacuoles in the mucosal enterocytes and the appearance of the submucosa and lamina propria.

At the conclusion of the feed trial, and prior to the initiation of the bacterial challenge, another subset of fish was selected from the treatments for evaluation of hematological parameters after a sub-lethal bacterial challenge. One fish was removed from each tank, and grouped by treatment into 5, 75 L aquaria (4 fish per tank). These fish were first anesthetized with tricaine methane sulphonate (MS-222; 100 mg/L for 5 min) and then injected with the pathogenic bacteria *Vibrio harveyi* at a dose (2.27×10^5 cells/fish), two orders of magnitude less than the LC_{50} (calculated as described below). The fish were monitored for 48 hrs with normal husbandry (feedings, aeration, siphoning once daily). At the conclusion of the 48 hr period, the fish were again anesthetized (MS-222; 100 mg/L for 5 min), and a minimum 500 µl blood sample was drawn from the caudal vein using a heparinized syringe. Approximately 250 µl blood was immediately transferred to a BD Microtainer K2EDTA blood collection tube (BD, Franklin Lakes, NJ, USA), immediately inverted 20 times (to reduce clotting), and stored on ice until shipping for analysis of hematological parameters. The other half of the collected blood (250 µl) was transferred to a BD Microtainer tube with lithium heparin and plasma separator additive, and was then centrifuged at 1500 rpm for 20 mins. to separate the plasma. The plasma was then pipetted into an Eppendorf tube and all samples (blood and plasma) were sent overnight (cold) to Idexx Laboratories (Groton, MA, USA) for complete blood count and blood chemistry analysis.

The remaining fish were grouped by treatment and then split back into five tanks per treatment. Optimum bacterial cell concentration (LC_{50}, concentration lethal to 50% of the fish) was determined prior to the challenge by the Karber method (Barros et al., 2002). Fish in the bacterial challenge groups (3 randomly selected tanks) were injected intraperitoneally with $2.27x10^7$ cells *Vibrio harveyi* (100 µl) (a known pathogen of summer flounder; Soffientino et al., 1999), while the fish in the control groups (2 tanks) were injected intraperitoneally with 100 µl filtered artificial sea water (FASW). Mortality was monitored and recorded twice daily for seven days following injection. Post-challenge, bacteria were isolated from the peritoneal fluid, and confirmed to be *V. harveyi* by amplification and sequencing of a portion of the 16S rDNA gene using polymerase chain reaction. The fish were monitored twice daily for mortality, and at the conclusion of the challenge (day 7) all remaining fish were euthanized with tricaine methane sulphonate (MS-222; 250 mg/L for 5 min).

Statistics:

All growth and condition data were analyzed using the General Linear Model procedure using SAS computer software (SAS 9.2 TS Level 2M2, Cary, NC, USA). Mean results were subjected to one-way analysis of variance (ANOVA), and significant results were further analyzed using Tukey's post test. Bacterial challenge survival data were analyzed using the Kaplan-Meier survival analysis: log-rank, with all pairwise multiple comparison procedures (Holm-Sidak method) using SigmaStat (Systat Software, San Jose, CA, USA). The significance level of 0.05 was chosen, and all data are presented as mean ± SEM.

RESULTS

All five diets were formulated to be isocaloric and isonitrogenous. Trypsin inhibitor and oligosaccharide content is highest for the diet which has the water sub-fraction of soy molasses added. All other diets follow trend of increasing oligosaccharide content based on the supplemental sugars added. Proximate analysis revealed that trypsin inhibitor, saponin and oligosaccharide content were low (Table 10).

Over the four-week feed trial, all groups regardless of diet showed similar growth in both length (1.85±0.07 cm average length gain) and weight (15.82±0.91 g average weight gain). Although the group fed the SPC Control with no added antinutritional factors grew slightly more (1.95±0.39 cm length gain and 17.25±2.57 g weight gain) than the groups fed the supplemented diets, there were no significant differences between groups in either length or weight gain (ANOVA, df=4, F-value=0.87, $Pr>F=0.5071$, $\alpha=0.05$, and ANOVA, df=4, F-value=0.81, $Pr>F=0.5404$, $\alpha=0.05$, respectively) Feed conversion ratios (FCR) were low for all diets (<1), and were not significantly different between groups (ANOVA, df=4, F-value=0.57, $Pr>F=0.6857$) (Fig. 8).

All of the fish injected with FASW as a control survived the seven-day challenge period. The lowest survival among all treatments was seen in the group fed the SPC diet. Survival following the seven-day bacterial challenge was significantly greater for the group fed the SPC diet supplemented with 0.4% oligosaccharides compared to all

other treatments. It was also significantly higher in the SPC 0.6% diet than in the SPC and SPC diet supplemented with the water fraction from soy molasses (SPC/WFSM) (Kaplan-Meier Survival Analysis: Log-Rank; df=4, statistic=53.674, p=<0.001) (Fig. 9).

No intestine, liver, or spleen tissue abnormalities or differences among fish in the different diets treatments were noticed in the examination of the histological samples (Figs. 11-13). Qualitative analysis of anterior intestine samples revealed regular-shaped morphology of the columnar epithelium, normal microvilli, and consistent vascularization throughout all samples. Hepatocytes showed well-developed internal compartmentalization with a roundish or circular shape. Spleen cells were homogeneously distributed with regular shape without any noticeable inclusions or distinct pathological features.

No significant differences in blood chemistry and cell counts were observed among treatments (Table 11). Fish in the groups fed the 0.2% and 0.4% oligosaccharide-supplemented diets were generally similar in blood chemistry profile, with the lowest sodium and lowest potassium levels among all groups. The 0.2% and 0.4% supplementation groups were also lowest in cholesterol, total protein, globulin, and alkaline phosphotase. All other parameters measured varied among groups with no trend or significant difference.

Blood cell count and analysis also demonstrated a relative decrease in certain immunological parameters for groups fed the oligosaccharide-supplemented diets, while the results still remained not statistically significant. Plasma protein, like total blood protein, was lower for the 0.2% and 0.4% oligosaccharide-supplemented groups, and highest for the group fed the SPC-Control diet. Hematocrit varied between groups with no clear trend. All values ranged between 20.4±4.7% for the 0.2% oligosaccharide-supplemented group, to 32.8±5.7% for the group fed the SPC-Control diet. White blood cell count was lowest for the groups fed the 0.2% and 0.4% oligosaccharide-supplemented diets, and highest for the group fed the SPC + Water Fraction. Absolute leukocyte counts (neutrophil, lymphocyte and monocyte) were variable both among and within groups, however, the two groups fed the 0.2% and 0.4% oligosaccharide-supplemented diets consistently resulted in the lowest values (Fig. 13).

DISCUSSION

The results of this study demonstrate that summer flounder fed with SPC replacement diets supplemented with crystalline oligosaccharides showed growth similar to those fed on FM diets. Oligosaccharide supplementation promoted a reduction in mortality when the fish were challenged with a pathogenic bacterium compared to FM or SPC diets. We also show that oligosaccharide supplementation in the diet has no measurable effect on the morphology of the liver, spleen or intestines of summer flounder or on blood parameters after a non-lethal bacterial challenge.

There were no significant differences between groups for length or weight gain throughout the entire feeding study. However, due to time restrictions, the trial was only four weeks, and there is the potential for greater growth in oligosaccharide-supplemented diets if the trial continued longer (He et al., 2009), and future research is needed. The SPC/WFSM diet was previously evaluated in an 8 week feeding trial (Ward, 2014; Chapter 2), resulting in reduced growth and increased survival to bacterial challenge compared to the group fed a fish meal-based diet. In this study, survival following bacterial challenge was significantly greater for the groups fed the diet with the intermediate level of oligosaccharide supplementation (0.4 – 0.6%), consistent with results from previous trials showing that survival to bacterial challenge is highly dependent of the concentration of soybean meal or soybean meal-based products, with a decrease in survival when concentrations of ANFs (including oligosaccharides) in the diet are too low or too high (Ward, 2014; Chapters 1 and 2).

In previous studies, antinutritional factors present in soybean meal, including oligosaccharides, are considered to be responsible for decreased growth seen in fish fed with soybean meal replacement diets (Ramsey et al., 1994; Francis et al., 2001; Knudsen et al., 2007; Olsen et al., 2007; Yamamoto et al., 2008; Enterria et al., 2011; Ward, 2014). This current study, however, demonstrates that in summer flounder, the presence of oligosaccharides at levels below 4.0 mg/g does not lead to a decrease in growth.

The effect of the presence of oligosaccharides in the diet on survival of summer flounder to bacterial challenge could be due to either modification of the intestinal microflora, or via direct stimulation of the innate immune system. Oligosaccharides are known to have immunomodulatory properties in cultured fish such as an increase in lysozyme concentration, alternative complement pathway, and improved disease resistance (Staykov et al., 2007; Torrecillas et al., 2007). Oligosaccharides are not digestible by summer flounder; therefore, they may also act as substrate for beneficial bacterial growth within the gut (Gatesoupe, 1999; He et al., 2009; Ringo et al., 2010). Oligosaccharides can contribute to greater beneficial intestinal flora which can limit pathogenic bacterial growth, reduce infections starting in the gut and result in greater growth for fish fed supplemented diets (Burr et al., 2005).

Regardless of the mechanism of action, the effects of the oligosaccharides in the summer flounder gut must have a systemic effect, since they are able to improve survival to a bacterial challenge delivered through intraperitoneal injection. In an effort to determine the potential effect of oligosaccharide supplementation on systemic responses, we evaluated both blood chemistry and complete blood cell counts in the challenged fish. Blood chemistry and cell count analysis, however, did not differ significantly among groups, probably due to large amount of variation in these parameters, preventing detection of potential differences between the treatments. Further research is needed in this area to define and characterize the exact mechanism behind the immunomodulation due to oligosaccharide supplementation.

The current study first confirms that 60% of the FM in diets for summer flounder can be replaced with SPC with no reduction in growth. Furthermore, diets for summer flounder can be supplemented with crystalline oligosaccharides with no decrease in growth, while significantly reducing mortality due to challenge. Additional research is required to determine the mechanism of action whereby oligosaccharides modulate the immune system to provide beneficial health impacts. Uncertainty surrounding feed costs due to variable primary protein supply and potential impacts from disease outbreaks are two of the most pressing obstacles to aquaculture expansion. The results of the current suite of studies demonstrates that diet supplementation of a 60% soy protein concentrate replacement diet with oligosaccharides shows great promise of reducing farmer risk of disease, while promoting sustainable diets for carnivorous finfish, and therefore sustainable aquaculture expansion.

	SPC Control	SPC + WFSM	SPC + 0.2% Oligo	SPC + 0.4% Oligo	SPC + 0.6% Oligo
Fish Meal	670	268	268	268	268
Raffinose pentahydrate	0	0	0.3	0.5	0.8
Stachyose hydrate	0	0	0.8	1.6	2.4
Lower (water) Phase	0	5.1	0	0	0
Soy Protein Concentrate	0	402	402	402	402
Fish Oil	32	65.2	65.2	65.2	65.2
Wheat flour	238.5	115.5	119.5	118.5	117.4
Corn gluten	25	49.2	49.2	49.2	49.2
Starch	4.5	14.5	14.5	14.5	14.5
Mineral Premix (URI)	10	10	10	10	10
Calcium Phososphate	0	30	30	30	30
Vitamin Premix (URI)	10	10	10	10	10
Methionine	0	1.6	1.6	1.6	1.6
Taurine	0	14	14	14	14
Glycine	10	15	15	15	15

g/kg diet

	SPC Control	SPC + WFSM	SPC + 0.2% Oligo	SPC + 0.4% Oligo	SPC + 0.6% Oligo
Ash	9.2±0.2	9.7±0.2	9.9±0.9	8.7±1.4	8.1±2.4
Protein	55.3±0.6	54.4±0.8	54.1±0.7	56.1±0.2	55.4±0.2
Lipid	7.9±0.3	8.1±0.5	8.8±1.4	9.3±0.8	8.8±0.4
Trypsin inhibitors (TIU/ml)	6.9±0.1 [a]	9.7±0.0 [b]	5.9±0.1 [a]	5.6±0.7 [a]	6.6±0.6 [a]
Saponins (mg/g)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Oligosaccharides (g/100g)	0.1±0.0 [a]	0.4±0.1 [b]	0.2±0.0 [a]	0.3±0.1 [b]	0.4±0.0 [b]

proximate analysis on a dry weight basis

Table 10. *Diet formulations for the 5 experimental diets (1kg; all values for diet ingredients in grams). Diet 1: SPC control (Partial replacement of 60% FM with SPC); Diet 2: SPC + Water Fraction (SPC control diet with the water phase of a 0.53% SM inclusion level added to the diet); Diet 3: SPC control + 0.2% Oligo (SPC control diet with 0.2% oligosaccharide (w/w) added); Diet 4: SPC control + 0.4% Oligo (SPC control diet with 0.4% oligosaccharide (w/w) added); Diet 5: SPC control + 0.6% Oligo (SPC control diet with 0.6% oligosaccharide (w/w) added). Proximate analysis and antinutritional factor content for the 5 diets. All values mean ± SD, unless otherwise noted. Different superscript letters denote significant differences (α=0.05).*

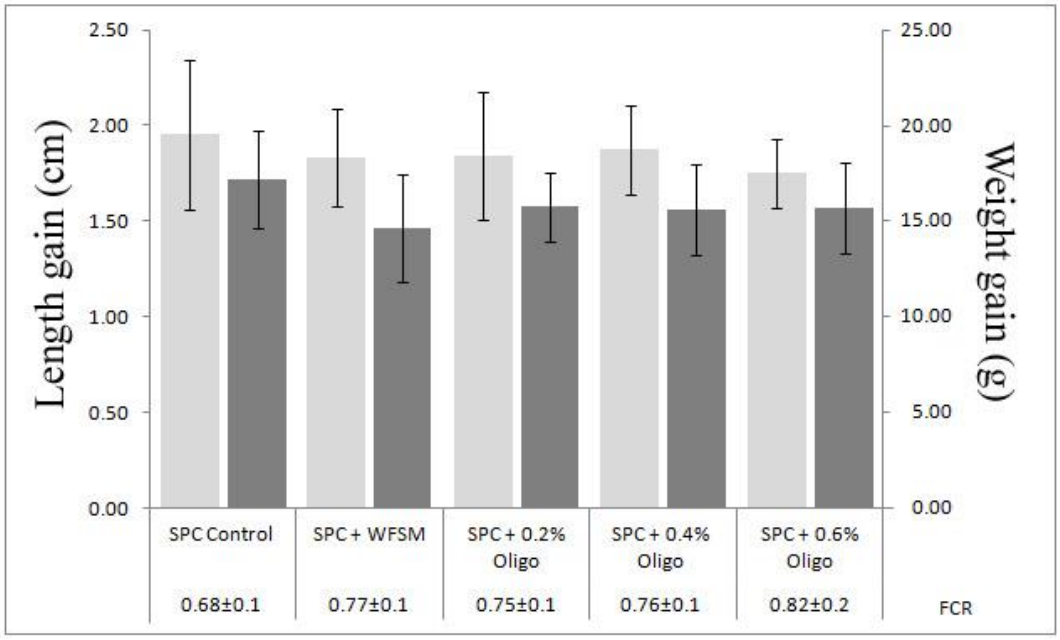

Figure 8. *Length gain (grey bars) and weight gain (black bars) over the four week feeding trial. Bars denote mean, and error bars standard deviation. Feed conversion ratios (FCR) for each group listed under growth averages, mean±SD. No significant differences between any groups in either length or weight (one-way ANOVA, α=0.05).*

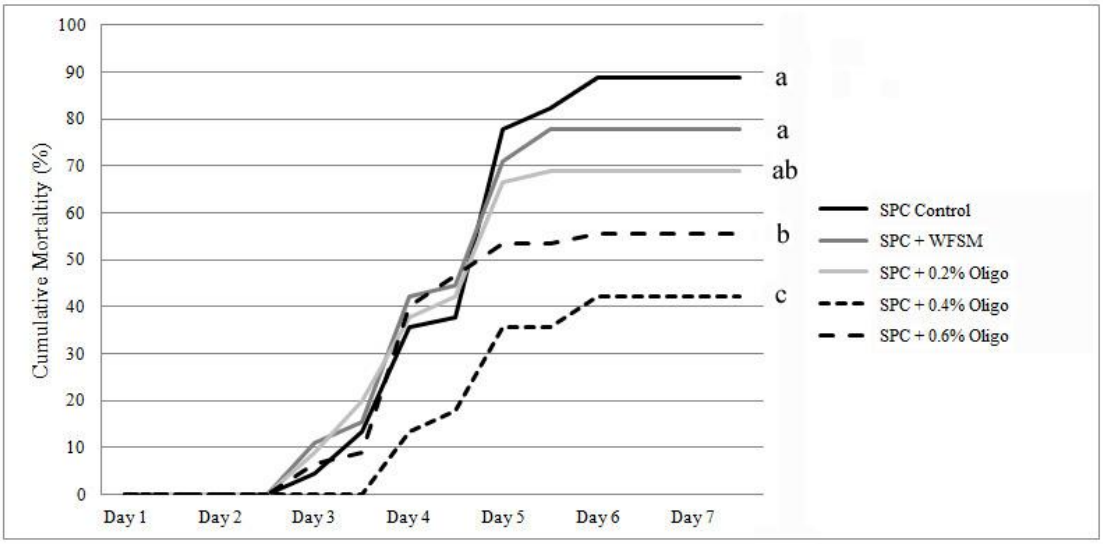

Figure 9: *Cumulative mortality over the seven-day bacterial challenge. Different letters denote significant differences between groups (Kaplan-Meier Survival Analysis: Log-Rank; df=4, statistic=53.674, p=<0.001).*

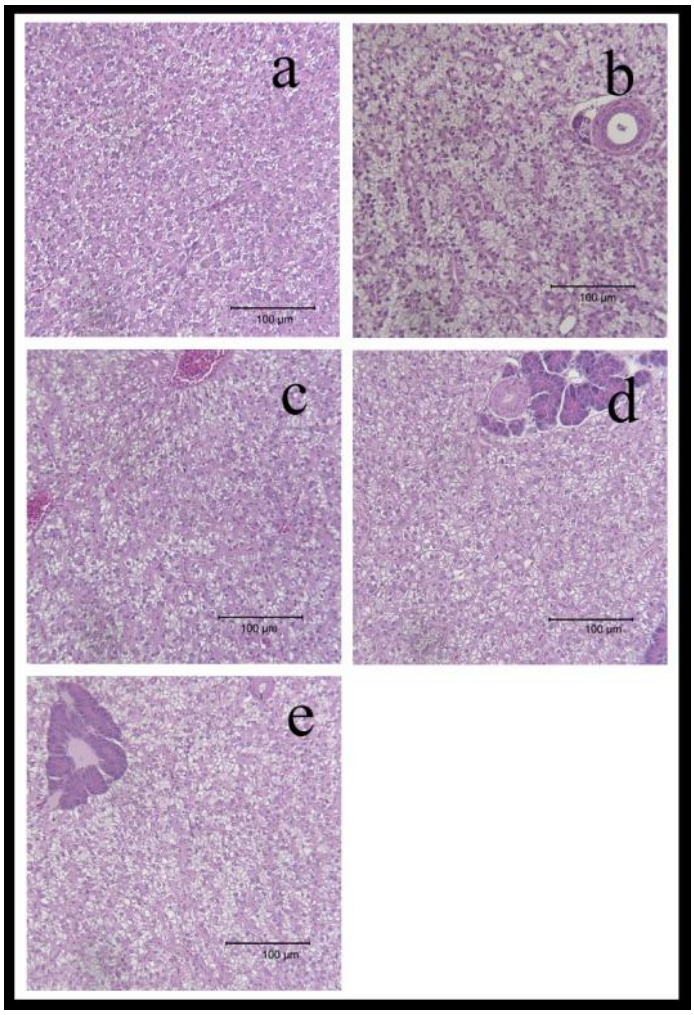

Figure 10: *Liver histology from all five diets showing normal liver appearance and no abnormalities. a) SPC Control, b) SPC + Water Fraction, c) SPC + 0.2% Oligos, d) SPC + 0.4% Oligos, e) SPC + 0.6% Oligos. Scale bar= 100µm.*

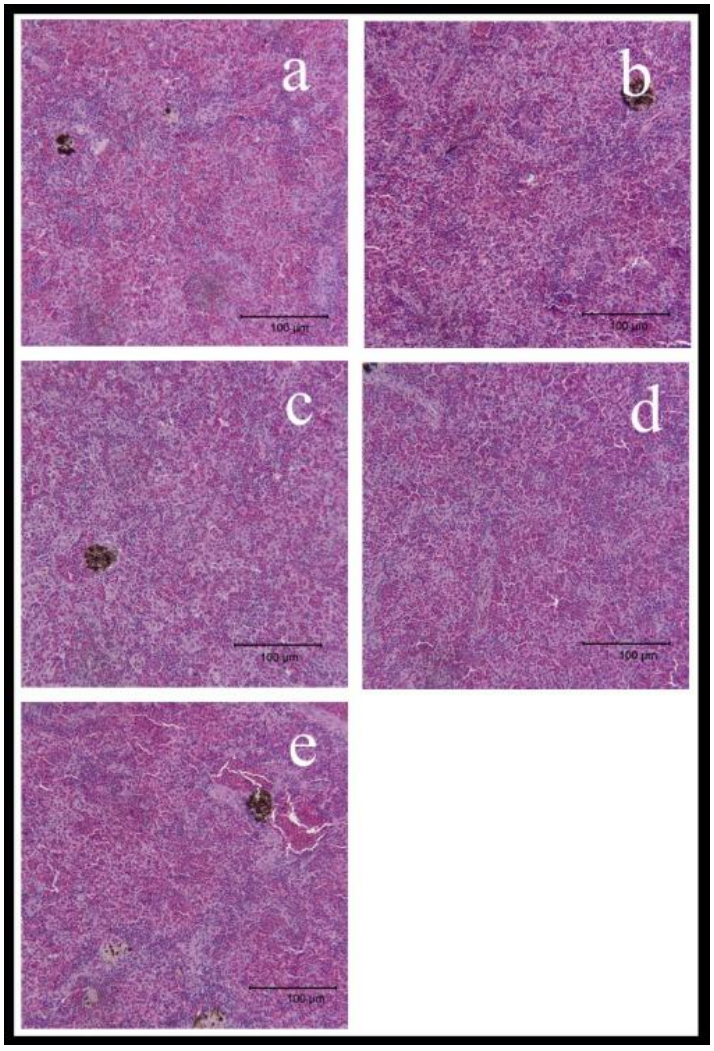

Figure 11: *Spleen histology from all five diets showing normal appearance and no abnormalities. a) SPC Control, b) SPC + Water Fraction, c) SPC + 0.2% Oligos, d) SPC + 0.4% Oligos, e) SPC + 0.6% Oligos. Scale bar= 100µm.*

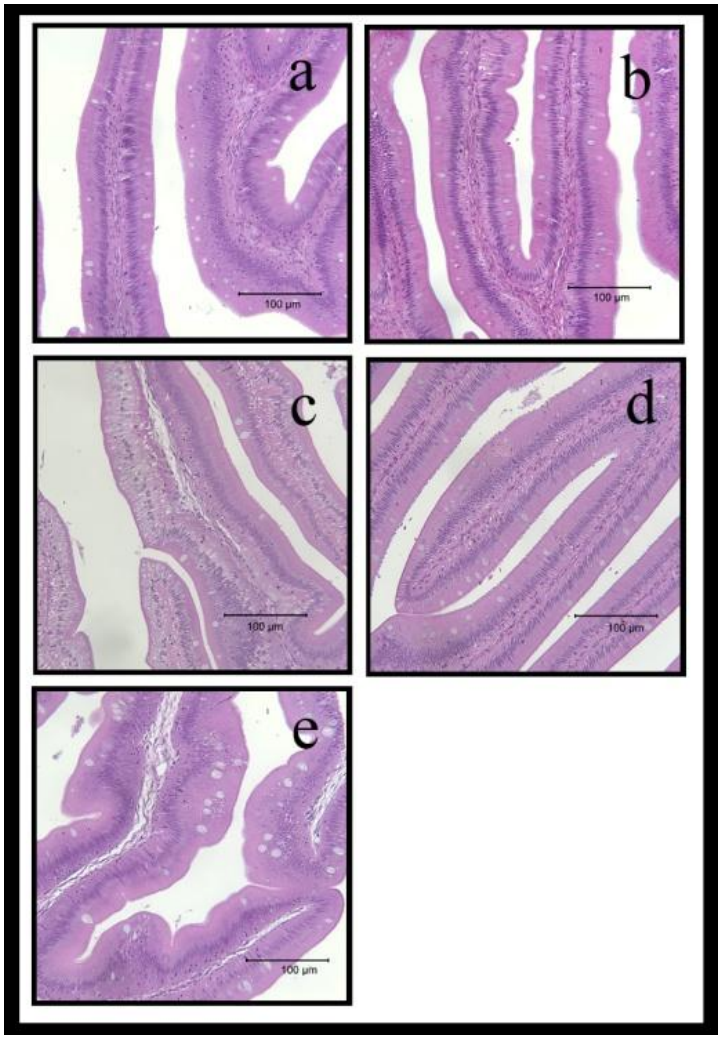

Figure 12: *Anterior intestine histology from all five diets showing normal appearance and no abnormalities. a) SPC Control, b) SPC + Water Fraction, c) SPC + 0.2% Oligos, d) SPC + 0.4% Oligos, e) SPC + 0.6% Oligos. Scale bar= 100µm.*

	SPC Control	SPC + WFSM	SPC + 0.2% Oligo	SPC + 0.4% Oligo	SPC + 0.6% Oligo
Alk. Phosphotase	21.6±23.0	16.0±7.8	13.3±8.2	14.3±7.5	30.5±14.6
AST (SGOT)	42.8±15.7	103.7±104.4	140.8±56.0	52.8±15.5	64.0±42.4
Amylase	1.8±0.4	2.0±1.0	2.3±1.0	2.0±0.8	0.8±1.0
Albumin	1.5±0.2	1.4±0.1	1.0±0.1	1.1±0.1	1.1±0.3
Total Protein	3.9±0.4	3.7±0.2	2.8±0.2	3.2±0.4	3.4±0.8
Globulin	2.4±0.3	2.3±0.1	1.8±0.1	2.1±0.3	2.3±0.5
Cholesterol	98.8±20.5	104.3±19.3	85.8±25.9	86.0±9.6	108.3±30.6
Calcium	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	3.3±4.5
Phosphorus	7.5±1.2	7.0±0.9	6.9±0.6	7.0±0.9	7.9±2.9
Potassium	5.3±1.2	4.5±1.0	3.4±0.4	3.4±0.2	5.1±0.8
Sodium	177.0±20.7	174.3±16.5	172.3±21.0	172.0±9.9	185.8±17.3
A/G Ratio	0.6±0.1	0.6±0.0	0.5±0.0	0.6±0.1	0.5±0.1
Uric Acid	0.3±0.2	0.1±0.1	0.2±0.1	0.2±0.1	0.3±0.1

Alk. Phosphotase (IU/L), AST (SGOT) (IU/L), Amylase (IU/L), Albumin (gm/dL), Total Protein (gm/dL), Globulin (gm/dL), Cholesterol (mg/dL),Calcium (mg/dL), Phosphorus (mg/dL), Potassium (meq/L), Sodium (meq/L), Uric Acid (mg/dL)

Table 11: *Plasma blood chemistry. All values mean±SD. No statistical differences between groups were detected for any of the measured parameters.*

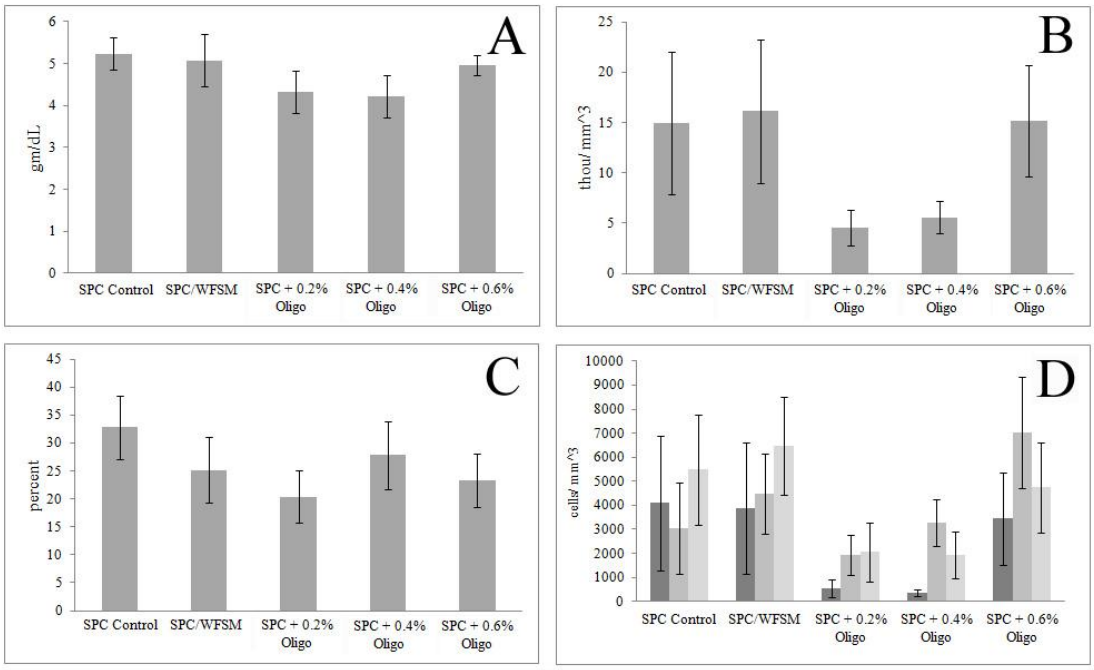

Figure 13: *Blood count and analysis. A) Plasma protein, B) White blood cell count, C) Hematocrit, D) Absolute leukocyte counts: dark grey= absolute neutrophil, medium grey= absolute lymphocyte, light grey= absolute monocyte.*

BIBLIOGRAPHY

Able, K. W., Sullivan, M. C., Hare, J. A., Bath-Martin, G., Christopher Taylor, J., Hagan, R. 2011. Larval abundance of summer flounder (*Paralichthys dentatus*) as a measure of recruitment and stock status. Fishery Bulletin. 109, 1: 68.

Baeverfjord, G., & Krogdahl, Å. 1996. Development and regression of soybean meal induced enteritis in Atlantic salmon, Salmo salar L., distal intestine: a comparison with the intestines of fasted fish. Journal of Fish Diseases. 19, 5: 375-387.

Barros, M.M., Lim, C., Klesius, P.H., 2002. Effect of soybean meal replacement by cottonseed meal and iron supplementation on growth, immune response, and resistance of Channel Catfish (*Ictalurus puctatus*) to *Edwardsiella ictaluri* challenge. Aquaculture. 207, 263-279.

Bengtson D. A. 1999. Aquaculture of summer flounder (*Paralichthys dentatus*): status of knowledge, current research, and future research priorities. Aquaculture 176: 39–49.

Bengtson, D.A., Volson, B., Karayannakidis, P, and Lee, C.M. Soy Protein Concentrate as Fish Meal Replacement in Diets for Summer Flounder. *In Prep.*

Berg A.-G.T., Horsberg T.E. 2009. Plasma concentrations of emamectin benzoate

after Slice(TM) treatments of Atlantic salmon (*Salmo salar*): Differences between fish, cages, sites and seasons. Aquaculture. 288: 22-26.

Berge G.M., Grisdale-Helland B., Helland S.J. 1999. Soy protein concentrate in diets for Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture. 178, 1-2: 139-148.

Berhow, M.A., Cantrell, C.L., Duval, S.M., Dobbins, T.A., Maynes, J., Vaughn, S.F. 2002. Analysis and quantitative determination of group B saponins in processed soybean products. Phytochem Anal. 13, 343-348.

Berhow, M. A., Kong, S. B., Vermillion, K. E., & Duval, S. M. 2006. Complete quantification of group A and group B soyasaponins in soybeans. Journal of agricultural and food chemistry, 54, 6: 2035-2044.

Bonaldo A., Roem A.J., Pecchini A., Grilli E., Gatta P.P. 2006. Influence of dietary soybean meal levels on growth, feed utilization, and gut histology of Egyptian sole (*Solea aqgyptiaca*) juveniles. Aquaculture. 261, 580-586.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72, 1-2; 248-254.

Bricknell I. and Dalmo R.A. 2005. The use of immunostimulants in fish larval aquaculture. Fish and Shellfish Immunology. 19, 5: 457-472.

Brubacher J.L., and Bols N.C. 2001. Chemically de-acetylated 2′,7′-dichlorodihydrofluorescein diacetate as a probe of respiratory burst activity in mononuclear phagocytes. Journal of Immunological Methods. 251, 1-2; 81-91.

Bureau, D. P., Harris, A. M., & Young Cho, C. 1998. The effects of purified alcohol extracts from soy products on feed intake and growth of chinook salmon (Oncorhynchus tshawytscha) and rainbow trout (Oncorhynchus mykiss). Aquaculture. 161, 1: 27-43.

Burr, G., Gatlin, D., and Ricke, S. 2005. Microbial ecology of the gastrointestinal tract of fish and the potential application of prebiotics and probiotics in finfish aquaculture. Journal of the World Aquaculture society. 36, 4: 425-436.

Campbell, M.F., Fiala, R.J., Rasche J.F., Wideman, J.D. 1981. Bland vegetable protein product and method of manufacture US 4265925 A. Patent. May 5, 1981.

Cathcart R., Schwiers E., Ames B.N. 1983. Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. Analytical Biochemistry. 134, 1; 111-116.

Colburn, H. R., Walker, A. B., Breton, T. S., Stilwell, J. M., Sidor, I. F., Gannam, A. L., & Berlinsky, D. L. 2012. Partial replacement of fishmeal with soybean meal and soy protein concentrate in diets of Atlantic Cod. North American Journal of Aquaculture. 74, 3: 330-337.

Dourado, L. R. B., Pascoal, L. A. F., Sakomura, N. K., Costa, F. G. P., & Biagiotti, D. 2011. Soybeans (*Glycine max*) and Soybean Products in Poultry and Swine Nutrition. Krezhova, D. Recent Trends for Enhancing the Diversity and Quality of Soybean Products. Hard cover, 536.

El-Boshy M.E., El-Ashram A.M., Abdel Hamid F.M., Gadalla H.A. 2010. Immunomodulatory effect of dietary *Saccharomyces cerevisiae*, β-glucan and laminaran in mercuric chloride treated Nile tilapia (*Oreochromis niloticus*) and experimentally infected with *Aeromonas hydrophila*. Fish & Shellfish Immunology. 28: 5---6, 802---808.

El-Sayed, A. F. 1998. Total replacement of fish meal with animal protein sources in Nile tilapia, Oreochromis niloticus (L.), feeds. Aquaculture Research. 29, 4: 275-280.

El-Saidy, D. M., & Saad, A. S. 2011. Effects of partial and complete replacement of soybean meal with cottonseed meal on growth, feed utilization and haematological indexes for mono-sex male Nile tilapia, Oreochromis niloticus (L.) fingerlings. Aquaculture Research. 42, 3: 351-359.

Enterria, A., Slocum, M., Bengtson, D. A., Karayannakidis, P. D., and Lee, C. M. 2011. Partial Replacement of Fish Meal with Plant Protein Sources Singly and in Combination in Diets for Summer Flounder, Paralichthys dentatus. Journal of the World Aquaculture Society. 42, 6:, 753-765.

FAO 2008. The State of World Fisheries and Aquaculture 2008. in: Department, F.a.A. (Ed.). Food and Agriculture Organization of the United Nations, Rome.

FAO 2010. The State of World Fisheries and Aquaculture 2010. in: Department, F.a.A. (Ed.). Food and Agriculture Organization of the United Nations, Rome.

FAO 2012. The State of World Fisheries and Aquaculture 2012. in: Department, F.a.A. (Ed.). Food and Agriculture Organization of the United Nations, Rome.

Francis G., Makkar H.P.S., Becker K. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture. 199, 3-4: 197-227.

Francis G., Kerem Z., Makkar H.P., Becker K. 2002. The biological action of saponins in animal systems: a review. Br J Nutr. 88:6, 587-605.

Gauger, E., Smolowitz, R., Uhlinger, K., Casey, J., Gómez-Chiarri, M., 2006. *Vibrio harveyi* and other bacterial pathogens in cultured summer flounder. Aquaculture. 260, 10-20.

Gatesoupe, F.J. 1999. The use of probiotics in aquaculture. Aquaculture. 180, 1: 147-165.

Gatlin D.M.I., Barrows F.T., Brown P., Dabrowski K., Gaylord T.J., Hardy R.W., Herman E., Hu G., Krogdahl A., Nelson R., Overturf K., Rust M., Sealey W., Skonberg D., Souza E.J., Stone D., Wilson R., Wurtele E. 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquaculture Research. 38, 551-579.

Glencross, B.D., Booth, M., Allan, G.L., 2007. A feed is only as good as its ingredients: a review of ingredient evaluation strategies for aquaculture feeds. Aquaculture Nutrition. 13: 17-34.

Grizard D and Barthomeuf C. 1999. Non-digestible oligosaccharides used as prebiotic agents: mode of production and beneficial effects on animal and human health. Reprod Nutr Dev. 39(5-6): 563-88.

Grisdale-Helland B., Helland S.J., Gatlin D.M. 2008. The effects of dietary supplementation with mannanoligosaccharide, fructooligosaccharide or

galactooligosaccharide on the growth and feed utilization of Atlantic salmon (*Salmo salar*). Aquaculture. 283, 1-4: 16--167.

Hamerstand, G.E., Black, L.T. and Glover, J.D. 1981. Trypsin inhibitors in soy products: Modification of the standard analysis procedure. Cereal Chemistry. 58: 42-45.

Hart S.D., Bharadwaj A.S., Brown P.B. 2010. Soybean lectins and trypsin inhibitors, but not oligosaccharides or the interactions of factors, impact weight gain of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 306: 310–314.

Hardy R.W. 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquaculture Research. 41, 5: 770-776.

Hayes, L., Simms, R. 1973. Defatted soybean fractionation by solvent extraction US 3734901 A. Patent. May 22, 1973.

He, S., Zhou, Z., Liu, Y., Shi, P., Yao, B., Ringø, E., and Yoon, I. 2009. Effects of dietary *Saccharomyces cerevisiae* fermentation product (DVAQUA) on growth performance, intestinal autochthonous bacterial community and non-specific immunity of hybrid tilapia (*Oreochromis niloticus, O. aureus*) cultured in cages. Aquaculture. 294, 1: 99-107.

Hopkins, K.D., 1992. Reporting fish growth: a review of the basics. J. World Aquacult. Soc. 23, 173-179.

Ibrahem M.D., Fathi M., Mesalhy S., Abd El-Aty A.M. 2010. Effect of dietary supplementation of inulin and vitamin C on the growth, hematology, innate immunity, and resistance of Nile tilapia (*Oreochromis niloticus*). Fish and Shellfish Immunology. 29, 2: 241-246.

Igboeli O.O., Fast M.D., Heumann J., Burka J.F. 2012. Role of P-glycoprotein in emamectin benzoate (SLICE®) resistance in sealice, *Lepeophtheirus salmonis. Aquaculture. 344-349: 40-47.*

Indexmundi. 2013. Internet, online. Soybean Meal Monthly Price. http://www.indexmundi.com/commodities/?commodity=soybean---meal. Site visited 8/25/2013.

Iwashita, Y.; Suzuki, N.; Matsunari, H.; Sugita, T.; Yamamoto, T. 2009. Influence of soya saponin, soya lectin, and cholyltaurine supplemented to a casein-based semipurified diet on intestinal morphology and biliary bile status in fingerling rainbow trout *Oncorhynchus mykiss*. Fisheries Science. 75, 5: 1307-1315.

Jackson, A., & Shepherd, J. 2010. Connections between farmed and wild fish: Fishmeal and fish oil as feed ingredients in sustainable aquaculture. In Advancing the Aquaculture Agenda: Workshop Proceedings–OECD (pp. 331-343).

Kakade, M.L., Rackie, J.E., McGhee, J.E. and Puski, G. 1974. Determination of trypsin inhibitor activity of soy products: A collaborative analysis of an improved procedure. Cereal Chemistry. 51: 376-382.

Kikuchi K. 1999. The use of defatted soybean meal as a substitute for fish meal in diets of Japanese flounder (*Paralichthys olivaceus*). Aquaculture. 179, 3-11.

Knudsen D., Uran P., Arnous A., Koppe W., Frokiaer H. 2007. Saponin-containing subfractions of soybean molasses induce enteritis in the distal intestine of Atlantic salmon. Journal of Agricultural and Food Chemistry 55, 6: 2261-2267.

Krogdahl A., Bakke-Mckellep A.M., Roed K.H., Baeverfjord G. 2000. Feeding Atlantic salmon Salmo salar L. soybean products: effects on disease resistance (*furunculosis*), and lysozyme and IgM levels in the intestinal mucosa. Aquaculture Nutrition. 6, 2: 77–84.

Kumar, V., Makkar, H. P. S., & Becker, K. 2011. Detoxified Jatropha curcas kernel meal as a dietary protein source: growth performance, nutrient utilization and

digestive enzymes in common carp (Cyprinus carpio L.) fingerlings. Aquaculture Nutrition. 17, 3: 313-326.

Lees F., Baillie M., Gettinby G., Revie C. W. 2008. The efficacy of emamectin benzoate against infestations of Lepeophtheirus salmonis on farmed Atlantic salmon (*Salmo salar* L) in Scotland, 2002–2006. PLoS ONE. 3:e1549.

Lightbourne C. 2011. Effects of soybean meal replacement with added taurine in fish meal diets for summer flounder (*Paralichthys dentatus*). Masters Thesis. University of Rhode Island, Kingston RI.

Litwack G. 1955. Photometric Determination of Lysozyme Activity. Journal of Experimental Biology and Medicine. 89, 3; 401-403.

Mambrini, M., Roem, A.J., Cravèdi, J.P., Lallès, J.P. and Kaushik, S.J. 1999. Effects of replacing fishmeal with soy protein concentrate and of DL-Methionine supplementation in high-energy, extruded diets on the growth and nutrient utilization of rainbow trout, *Oncorhynchus mykiss*. Journal of Animal Science. 77: 2990-2999.

Manderson, J. P., Phelan, B. A., Stoner, A. W., Hilbert, J. 2000. Predator–prey relations between age-1+ summer flounder (*Paralichthys dentatus*, Linnaeus) and age-0 winter flounder (*Pseudopleuronectes americanus*, Walbaum): predator diets, prey selection, and effects of sediments and macrophytes. Journal of Experimental Marine Biology and Ecology. 251, 1: 17-39.

Murray H.M., Lall S.P., Rajaselvam R., Boutilier L.A., Blanchard B., Flight R.M., Colombo S., Mohindra V., Douglas S.E. 2010. A nutrigenomic analysis of intestinal response to partial soybean meal replacement in diets for juvenile Atlantic halibut, *Hippoglossus hippoglossus*, L. Aquaculture. 298, 282-293.

Naylor, R. L., Hardy, R. W., Bureau, D. P., Chiu, A., Elliott, M., Farrell, A. P., and Nichols, P. D. 2009. Feeding aquaculture in an era of finite resources. Proceedings of the National Academy of Sciences. 106, 36: 15103-15110.

Olsen R.E., Hansen A., Rosenlund G., Hemre G., Mayhew T.M., Knudsen D.L., Eroldogan O.T., Myklebust R. and Karlsen O. 2007. Total replacement of fish meal with plant proteins in diets for Atlantic cod (Gadus morhua L.) II — Health aspects. Aquaculture. 272: 1-4, 612-624.

Olsen, R. L., and Hasan, M. R. 2012. A limited supply of fishmeal: Impact on future increases in global aquaculture production. Trends in Food Science & Technology. 27, 2: 120-128.

Packer, D.B., Griesbach S.J., Berrien P.L., Zetlin, C.A., Johnson, D.L., Morse W.W. 1999. *Essential Fish Habitat Source Document:* Summer Flounder, *Paralichthys dentatus*, Life History and Habitat Characteristics NOAA Technical Memorandum NMFS-NE-151.

Pass, D.W. 1975. Purification of ethanol extractant in soy protein concentrate process US 3897574 A. Patent. July 29, 1975.

Pratoomyot, J., Bendiksen, E. Å., Bell, J. G., and Tocher, D. R. 2010. Effects of increasing replacement of dietary fishmeal with plant protein sources on growth performance and body lipid composition of Atlantic salmon (*Salmo salar* L.). Aquaculture. 305, 1: 124-132.

Rajput, Z. I., Hu, S. H., Xiao, C. W. and Arijo, A. G. 2007. Adjuvant effects of saponins on animal immune responses. Journal of Zhejiang University-Science B. 8(3): 153-161.

Refstie S., Storebakken T., Roem A.J. 1998. Feed consumption and conversion in Atlantic salmon (*Salmo salar*) fed diets with fish meal, extracted soybean meal or soybean meal with reduced content of oligosaccharides, trypsin inhibitors, lectins and soya antigens. Aquaculture. 162, 3-4: 301-312.

Refstie S., Teikstra H.A.J. 2003. Potato protein concentrate with low content of solanidine glycoalkaloids in diets for Atlantic salmon (*Salmo salar*). Aquaculture. 216: 283-298.

Refstie, S., Sahlstrom S., Brathen E., Baeverfjord G., Krogedal P. 2005. Lactic acid fermentation eliminates indigestible carbohydrates and antinutritional factors in soybean meal for Atlantic salmon (*Salmo salar*). Aquaculture. 246, 1-4: 331-345.

Refstie, S., Landsverk, T., Bakke-McKellep, A. M., Ringø, E., Sundby, A., Shearer, K. D., & Krogdahl, Å. 2006. Digestive capacity, intestinal morphology, and microflora of 1-year and 2-year old Atlantic cod (*Gadus morhua*) fed standard or bioprocessed soybean meal. Aquaculture. 261, 1: 269-284.

Ringø, E., Olsen, R. E., Gifstad, T. Ø., Dalmo, R. A., Amlund, H., Hemre, G. I., and Bakke, A. M. 2010. Prebiotics in aquaculture: a review. Aquaculture Nutrition. 16, 2: 117-136.

Riche, M., & Williams, T. N. 2011. Fish meal replacement with solvent-extracted soybean meal or soy protein isolate in a practical diet formulation for Florida pompano (Trachinotus carolinus, L.) reared in low salinity. Aquaculture Nutrition. 17, 4: 368-379.

Rumsey, G.L., Siwicki, A.K., Anderson, D.P., Bowser, P.R., 1994. Effect of soybean protein on serological response non-specific defense mechanisms, growth, and protein utilization in rainbow trout. Veterinary Immunology and Immunopathology. 41: 323-339.

Salah Azaza M., Kammoun W., Abdelmouleh A., Kraiem M.M., 2009. Growth performance, feed utilization, and body composition of Nile tilapia (*Oreochromis niloticus* L.) fed with differently heated soybean-meal-based diets. Aquaculture International. 17, 507-521.

Salze, G., McLean, E., Battle, P. R., Schwarz, M. H., & Craig, S. R. 2010. Use of soy protein concentrate and novel ingredients in the total elimination of fish meal and fish oil in diets for juvenile cobia, *Rachycentron canadum.* Aquaculture. 298, 3: 294-299.

Santigosa, E., Sáenz de Rodrigáñez, M. Á., Rodiles, A., Barroso, F. G., & Alarcón, F. J. 2010. Effect of diets containing a purified soybean trypsin inhibitor on growth performance, digestive proteases and intestinal histology in juvenile sea bream (Sparus aurata L.). Aquaculture Research. 41, 9: e187-e198.

Sitja-Bobadilla A., Pena-Llopis S., Gomez-Requeni P., Medale F., Kaushik S., Perez-Sanchez J., 2005. Effect of fish meal replacement by plat protein sources on non-specific defense mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture. 249, 387-400.

Smith M.D., Roheim C.A., Crowder L.B., Hallpern B.S., Turnipseed M., Anderson J.L., Asche F., Bourillon L., Guttormsen A.G., Khan A., Liguori L.A., McNevin A., O'Connor M.I., Squires D., Tyedmers P., Brownstein C., Carden K., Klinger D.H.,

Sagarin R., Selkoe K.A. 2010. Sustainability and global seafood. Science 327, 5967:784–6.

Soffientino, B., Gwaltney, T., Nelson, D., Specker, J., Mauel, M., Gomez-Chiarri, M., 1999. Infectious necrotizing enteritis and mortality caused by *Vibrio carchariae* in summer flounder (*Paralichthys dentatus*) during intensive culture. Diseases of Aquatic Organisms. 38, 201-210.

Sørensen, M., Penn, M., El-Mowafi, A., Storebakken, T., Chunfang, C., Øverland, M., & Krogdahl, Å. 2011. Effect of stachyose, raffinose and soya-saponins supplementation on nutrient digestibility, digestive enzymes, gut morphology and growth performance in Atlantic salmon (*Salmo salar*, L). Aquaculture. 314, 1: 145-152.

Sparg S.G., Light M.E., van Staden J. 2004 Biological activities and distribution of plant saponins. Journal of Ethnopharmacology. 94: 2-3, 219-243.

Staykov, Y., Spring, P., Denev, S., and Sweetman, J. 2007. Effect of a mannan oligosaccharide on the growth performance and immune status of rainbow trout (Oncorhynchus mykiss). Aquaculture International. 15, 2: 153-161.

Stentiford, G. D., D. M. Neil, E. J. Peeler, J. D. Shields, H. J. Small, T. W. Flegel, J. M. Vlak et al. 2012. Disease will limit future food supply from the global crustacean fishery and aquaculture sectors. Journal of invertebrate pathology. 110, 2: 141-157.

Sun M., Kim Y.C., Okorie O.E., Lee S., Devnath S., Yoo G., Bai S.C., 2007. Evaluation of fermented soybean curd residues as an energy source in diets for juvenile olive flounder, *Paralichthys olivaceus*. Journal of the World Aquaculture Society. 38, 536-542.

Tacon, A. G., & Metian, M. 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. Aquaculture. 285, 1: 146-158.

Terceiro M. 2012. Stock Assessment of Summer Flounder for 2012. US Dept Commer, Northeast Fish Sci Cent Ref Doc. 12-21; 148 p. Available from: National Marine Fisheries Service, 166 Water Street, Woods Hole, MA 02543-1026, or online at http://www.nefsc.noaa.gov/publications/

Torrecillas, S., A. Makol, M. J. Caballero, D. Montero, L. Robaina, F. Real, J. Sweetman, L. Tort, and M. S. Izquierdo. 2007. Immune stimulation and improved infection resistance in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides. Fish & Shellfish Immunology. 23, 5: 969-981.

Twibell, R. G., Gannam, A. L., Hyde, N. M., Holmes, J. S., and Poole, J. B. 2012. Effects of fish meal-and fish oil-free diets on growth responses and fatty acid composition of juvenile coho salmon (*Oncorhynchus kisutch*). Aquaculture. 360-261: 69-77.

Walker A.B., Sidor I.F., O'Keefe T.O., Cremer M., Berlinksy D.L. 2010. Partial Replacement of Fish Meal with Soy Protein Concentrate in Diets of Atlantic Cod. North American Journal of Aquaculture. 72, 4: 343-353.

Wang, H., Johnson, L. A., & Wang, T. 2004. Preparation of soy protein concentrate and isolate from extruded-expelled soybean meals. Journal of the American Oil Chemists' Society. 81, 7: 713-717.

Wilson, R. P., & Poe, W. E. 1985. Effects of feeding soybean meal with varying trypsin inhibitor activities on growth of fingerling channel catfish. Aquaculture. 46, 1: 19-25.

Yamamoto T., Goto T., Kine Y., Endo Y., Kitaoka Y., Sugita T., Furuita H., Iwashita Y., Suzuki N. 2008. Effect of an alcohol extract from a defatted soybean meal supplemented with a casein-based semi-purified diet on the biliary bile status and intestinal conditions in rainbow trout *Oncorhynchus mykiss* (Walbaum). Aquaculture Research. 39: 9, 986–994.

Ye, J., Liu, X., Wang, Z., and Wang, K. 2011. Effect of partial fish meal replacement by soybean meal on the growth performance and biochemical indices of juvenile Japanese flounder Paralichthys olivaceus. Aquaculture International. 19, 1: 143-153.

Zhou, Q. C., and Yue, Y. R. 2010. Effect of replacing soybean meal with canola meal on growth, feed utilization and haematological indices of juvenile hybrid tilapia, Oreochromis niloticus× Oreochromis aureus. Aquaculture Research. 41, 7: 982-990.