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PATHOLOGICAL EFFECTS OF SOYBEAN ANTI-NUTRITIONAL FACTORS ON SUMMER FLOUNDER (PARALICHTHYS DENTATUS) TISSUES

BY

RACHEL MICHELLE BONE

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ABSTRACT

Summer flounder (*Paralichthys dentatus*), a popular carnivorous fish in New England, is an important candidate for aquaculture development. The inclusion of plant proteins as a replacement for fish meal in the diets of marine carnivorous fish may lead to economical advantages and increased sustainability. Anti-nutritional factors, organic molecules that cannot be digested and may inhibit digestion of other molecules present in soybean meal, but not in soy protein concentrate, may limit the inclusion of soybean meal into carnivorous fish diets by impacting fish growth rates or immune function. In order to determine the mechanisms by which soybean meal impacts growth or immune function, it is important to analyze the effect of anti-nutritional factors on the morphology of important digestive and immune organs: liver, spleen, and intestine. The goal of this project was to determine: 1) If pathological change was occurring in selected summer flounder organs when fish were fed diets in which a portion of fish meal was replaced with soy protein concentrate and varying amounts of anti-nutritional factors; and 2) Which fractions of soybeans (either as saponin-containing or oligosaccharide-rich), led to pathological changes. Feeding of summer flounder for eight weeks with diets in which 60% of fish meal was replaced with soy protein concentrate supplemented with increasing amounts of a fraction of soybean flakes containing anti-nutritional factors (corresponding to the amounts present in a 5%, 14%, and 27% soybean meal replacement diet) led to a significant decrease in growth in all diets compared to that with a fish meal control diet. Fish fed diets containing anti-nutritional factors at levels as low as those present in a 5% soybean meal replacement diet showed significant pathological changes in liver,

spleen, and anterior intestinal morphology as early as two weeks into the trial. These changes included: a decrease in the storage of nutrients in liver, a relative increase in the amount of white pulp *versus* red pulp and the presence of fibrosis in the spleen, and a decrease in the amount of goblet cells in the anterior intestine, accompanied by an increase in the thickness of the lamina propria and fusion and shortening of the mucosal folds. Fish fed the 27% diet had the worst overall growth and the most apparent change in tissue morphology, suggesting that anti-nutritional factors in soybean meal have a dose-dependent impact on the liver, spleen, and anterior intestine of summer flounder. A second six-week feeding trial was conducted in order to determine the impact of soy saponins and oligosaccharides on fish growth and tissue morphology. There were no statistically significant changes in morphology in all parameters evaluated except in the thickness of the lamina propria in the anterior intestine. Therefore, low levels of soy saponins and oligosaccharides may not significantly impact the morphology of summer flounder spleen, liver and anterior intestinal tissue. Pathological changes observed in fish fed the soybean meal equivalent replacement diets may be due to higher amounts of anti-nutritional factors in these diets or to additive or synergistic impacts of several anti-nutritional factors.

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DEDICATION

I would like to dedicate this thesis to my mother and father, Pamela Sue Bone and Michael Jay Bone. Your unending support throughout my childhood and into my adult years has irrefutably changed the trajectory of my life. Your meticulous, yet trusting care during my childhood gave me the confidence to question the world. Through probing discussions, and exposure to the natural world, you instilled in me the value of using critical thought to evaluate my surroundings. By encouraging me to seek answers to my own questions you subconsciously prepared me for a life of science. As I further delve into the world of science and uncertainty, I can be certain of your continued support. I am forever grateful for your love, forever thankful for your support, and forever indebted to your care.

PREFACE

This thesis has been prepared in manuscript format. The thesis is in keeping with the requirements of the journal Aquaculture.

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CHAPTER 1

REVIEW OF LITERATURE

As the world population is expected to reach 9.1 billion by 2050, it is vital to enhance the production of high quality, sustainable protein sources (USBC, 2001). Aquaculture, the farming of aquatic species, is an efficient way to produce high-quality protein compared to the traditional farming of cows, pigs, and chickens, because of the fish's ability to efficiently convert food fed to weight gained (Wilkinson, 2011).

Since the 1970's, aquaculture has grown at a rate of 9.2% per year compared to capture fisheries' 1.4% annual growth (FAO, 2009). Many popular aquaculture fish include carnivorous fish such as salmon, trout, sea bass, flounders, and tuna, which all require large amounts of fish meal to fulfill dietary protein requirements (over 40% protein for optimal growth) (Serrano et al., 1992; Chen and Tsai, 1994; Chou et al., 2001). As carnivorous fish aquaculture has expanded over the past decades, the added demand for fish meal has been met through the increased capture of specific fish species. However, as many fisheries species used for fish meal, such as anchoveta, Alaska pollock, Atlantic herring, mackerel, and blue whiting are currently fully exploited or overexploited, there is little to no room for capture fisheries to provide the extra fish meal needed for aquaculture expansion (FAO, 2010). It has been estimated that the demand for fish meal will outpace the supply within the next decade (Gatlin et al., 2007).

Plant protein sources are an important alternative protein source for carnivorous fish (Gatlin et al., 2007). Soybean meal is a promising plant protein replacement because of the high level of protein (48.5%), and an essential amino acid profile that meets all but three dietary amino acid requirements of summer flounder (methionine, lysine and threonine). The amino acids not adequately available in soybean meal may be supplemented in the diet (Fowler,1980; Cheng et al., 2003; Shiau et al., 1988). Soybean meal is also readily available in the United States and currently costs $1000 less per metric ton than fish meal (Index Mundi, 2012). Soybean products would not only provide a cheaper source of protein, but also provide a new market for US soybean farmers.

Although soybean is cheaper than fish meal (Index Mundi, 2012), the biggest limitations facing the field of soybean meal inclusion in fish diets are the anti-nutritional factors contained in this product (Francis et al., 2001). These substances, including soybean trypsin inhibitor (which prevents the use of trypsin, an important digestive enzyme), soya saponins, and oligosaccharides, could limit the growth of carnivorous fish by preventing digestion, inhibiting feeding, and inducing pathological changes in the intestine of fish (Francis et al., 2001). In one study, purified soya saponin, hypothesized to inhibit feeding, induced enteritis when fed to Atlantic salmon (Francis et al., 2001). Similarly, trout and salmon fed 30% soybean meal replacement diets developed enteritis in their distal intestine (Refstie et al., 2000). Soybean meal has also been shown to cause compounding degenerative effects on the integrity of the hindgut structure in common carp fed a 20% soybean meal replacement diet over a five week feeding trial (Urán et al., 2008).

Prior research at the University of Rhode Island has shown that soybean meal can be used to replace fish meal in diets for summer flounder at up to 40% fish meal replacement without affecting growth (Enterria et al., 2011; Lightbourne, 2011), suggesting that certain fish species may be able to handle higher levels of anti-nutritional factors. Interestingly, replacement of fish meal with soybean meal at levels of 40 – 70% has been shown to reduce mortality in summer flounder due to challenge with the bacterial pathogen *Vibrio harveyi* (Lightbourne, 2011; Ward personal communication). This bacterium causes the disease Flounder Infectious Necrotizing Enteritis (FINE), which resulted in mass mortalities at a hatchery in New Hampshire and a grow-out facility in Rhode Island (Soffientino et al., 1999; Gauger et al., 2006). The mechanisms responsible for increased survival and decreased growth in fish fed with diets in which 40 – 70% of fish meal has been replaced with soybean meal are unknown.

An alternative to the use of soybean meal in carnivorous finfish diets that addresses the problems caused by anti-nutritional factors is the use of a more purified product, soy protein concentrate. Soy protein concentrate is produced from a precursor to soybean meal, called defatted soybean white flake. Instead of toasting the defatted soybean white flake, which would produce soybean meal, the white flake is subjected to an ethanol extraction. This ethanol extraction, which produces a solid form (soy protein concentrate) and a liquid form (soy molasses), removes many of the anti-nutritional factors that are present in defatted soy white flake. The result is soy protein concentrate, a promising fish meal alternative that is highly proteinaceous (68%). In a previous study at URI, summer flounder growth did not significantly differ when the

fish were fed a 60% soy protein concentrate replacement diet versus a control fish meal diet. However, these fish did not show increased survival when challenged with *Vibrio harveyi* (Ward, personal communication). Because products in soy molasses are not present in soy protein concentrate, but are present in soybean meal, the substances responsible for both poor growth and better survival to bacterial challenge are likely contained in soy molasses. Thus, one or more of these anti-nutritional factors in the soy molasses fraction may also act as an immunostimulant or immunomodulator, providing protection against bacterial infection in fish. Oligosaccharides and saponins are two known anti-nutritional factors that may also have an immunostimulatory effect. Previous research spanning mice, buffalo, and fish has shown that oligosaccharides may modulate the immune system, and even have antitumor properties (Yuan et al., 2006; Saksena et al., 1999; Geraylou et al., 2012). Saponins, which are secondary metabolites most likely used as anti-feedants by plants to decrease herbivory, are also known to have an immunostimulatory effect (Wagner, 1998)

This research revolves around the idea that soybean-based diets for cultured summer flounder can be optimized by balancing the negative and positive effects of components in soybean meal. This optimization can occur by determining at what level soy molasses causes poor growth and/or enhanced survival during bacterial challenge or by identifying the specific products responsible for these effects. By adding varying proportions of soy molasses to a 60% fish meal replacement diet with soy protein concentrate (a diet shown to provide optimal growth as compared to fish meal diets; Ward, unpublished results), it may be possible to find a point where there

is no negative impact on growth and enhanced survival occurs. Furthermore, further fractionation of soy molasses into a water, butanol, and precipitate portion (Knudsen et al., 2007) would help identify which fractions are responsible for decreased growth (my study) and increased survival to bacterial challenge (Ward's thesis research). If fractions promoting increased survival to bacterial challenge are different from those decreasing growth, the fractions promoting increased survival could be used at appropriate levels to supplement diets with soy protein concentrate.

Although from an economic perspective alone it may appear advantageous to use plant protein sources as an alternative to fish meal diet, it is imperative to consider any long term tissue or organ level damage that may occur. Three organs that are relevant to growth and immune functions in fish in are the liver, spleen, and intestine. The liver is important for lipid and glycogen storage. The spleen in teleost fish is the primary site of lymphoid tissue production. It is also the site of blood storage and, in some instances, hematopoiesis (Fänge and Nilsson, 1985). Therefore, the spleen is important for immune functions. The intestine and more specifically the anterior intestine is important in the digestion and absorption of nutrients. A damaged intestine could reduce the ability to digest and absorb nutrients, and hence lower growth rate.

Pathological changes due to replacement of fish meal with soybean meal may be cumulative, as shown in previous studies (Urán et al., 2008). When red snapper were fed a 48% soybean meal replacement diet, the fish had excessive lipid deposition in the liver as well as necrotic hepatocytes (Catacutan and Pagador, 2004). However, in another study, fish fed a 90% soybean meal replacement diet had significantly lighter and smaller livers, indicating less capacity of storage (McGoogan and Gatlin, 1997).

Previous studies have shown that soya saponins cause soybean-induced enteritis, compromising the long term viability of the intestine (Knudsen et al., 2007). It is clear that soybean meal anti-nutritional factors can impact the morphology of fish tissues. Further research needs to be done to determine at what level of soybean meal replacement anti-nutritional factors cause pathological changes in summer flounder tissues and organs, and what specific substances in soybean products are causing these changes.

CHAPTER 2

Pathological Effects Of Soybean Anti-nutritional Factors On Summer Flounder (*Paralichthys dentatus*) Tissues

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INTRODUCTION

Summer flounder (*Paralichthys dentatus*), a popular fish in New England, USA, has variable annual catch rates (Shepherd and Terceiro, 1994). Because of the fishery fluctuations and the popularity of summer flounder, the University of Rhode Island has actively researched summer flounder aquaculture techniques (Bengtson, 1999). One potential obstacle to the sustained growth of summer flounder large-scale aquaculture is the fact that, as a carnivorous fish, their diets heavily rely on fish meal as a protein source. Fluctuations in the cost of fish meal due to increased demand and a limited and variable supply may inhibit the expansion of aquaculture in general and summer flounder in particular (Stickney and McVey, 2002). In order for aquaculture to expand in a sustainable way, replacing fish meal with a widely available sustainable plant protein source is necessary (Gatlin et al., 2007).

Due to their high protein content, soybeans are a promising candidate for fish meal replacement. Soybeans have a good essential amino acid profile for fish. The use of soy protein as a partial replacement of fish meal has shown encouraging results for many species of flatfish, such as Japanese flounder (*Paralichthys olivaceus*) (Kikuchi, 1999; Sun et al., 2007) and Atlantic halibut (*Hippoglossus hippoglossus*) (Murray et al., 2010). Research performed at the University of Rhode Island has shown that feeding summer flounder with diets in which fish meal is replaced with 40% or less of soybean meal does not cause a major impact on fish growth when compared to a fish meal diet (Enterria et al., 2011). Interestingly, while summer flounder growth was negatively affected upon feeding 40-70% soybean meal replacement diets, fish

survival rates to a bacterial challenge increased, suggesting that soybean meal enhances resistance to bacterial infection (Lightbourne, 2011). Additional research showed that fish fed a 60% fish meal replacement diet with soy protein concentrate, a more purified soy product, had similar growth to fish fed a control fish meal diet, but no increase in survival to bacterial challenge (Ward et al., in prep).

Soybean meal, but not soy protein concentrate, contains anti-nutritional substances that may cause pathological changes in the digestive tissues of fish and prevent growth (Knudsen et al. 2008, Knudsen et al., 2007; Krogdahl et al. 2003). Substances that are considered anti-nutritional factors, or factors that inhibit the absorption of nutrients, include protease inhibitors, oligosaccharides, saponins, lectins, phytate (which can sequester phosphate), and anti-feedants such as tannins (Refstie et al., 2005; Knudsen et al., 2007; Iwashita et al., 2009). One or more of these anti-nutritional factors in soybean meal may act as immunostimulants or immunomodulators, providing protection against bacterial infection (Francis et al., 2001).

In order to optimize soybean-based diets that maximize growth while also providing good levels of disease resistance, it is imperative to determine the mechanisms by which anti-nutritional factors present in soybean meal affect growth and survival in summer flounder. The goal of this research was to investigate the effect of soybean-based products on the morphology of important digestive and immune organs (liver, spleen, and intestine) by determining: 1) the effect of feeding fish with diets in which fish meal was replaced with soy protein concentrate supplemented with varying levels of a fraction of soybean enriched in anti-nutritional

factors on the morphology of these organs (trial 1); and 2) which fractions of soybean (enriched in either saponins or oligosaccharides), lead to pathological changes (trial 2).

METHODOLOGY

Trial 1

1.1 Production of Soy Protein Concentrate and Soy Molasses:

Soy protein concentrate was prepared by subjecting defatted soy white flake to an ethanol extraction, following the method of Hayes and Simms (1973) with slight modifications. This alcohol extraction yields a liquid (called soy molasses, containing most of the anti-nutritional factors) and a solid (soy protein concentrate) fraction. Briefly, 100g defatted soy white flake was suspended in 60% ethanol (w/v). The mixture was heated to 50˚C for 30 min while stirring and the resulting solution was centrifuged for 30 min at 14,000 x g. This allowed the solid soy protein concentrate to be separated from the liquid portion comprised of soy molasses, ethanol, and water. The soy protein concentrate (solid fraction) was then desolvantized in a forced-air convection drying oven for 30 min at 90˚C. The ethanol was removed from the resulting soy molasses by evaporation by heating while stirring to 80°C for 60min.

1.2 Formulation of diet

Five diets were prepared at the Food Science and Nutrition Research Center at the University of Rhode Island (West Kingston, RI). The diets were formulated as follows (Table 1): 1) a control diet with no soy protein, all protein from fish meal (Control-FM); 2) a soy protein concentrate diet in which 60% of the fish meal was replaced with soy protein concentrate (diet 1-SPC); 3 - 5) three diets with 60% of the fish meal replaced with soy protein concentrate to which soy molasses has been added at levels

corresponding to the levels of anti-nutritional factors present in a 12% (diet 2), 24% (diet 3), or 36% (diet 4) soybean meal equivalent (SBME) replacement diet. These levels were based on results from previous studies showing impacts on summer flounder growth (Enterria et al., 2011; Ward et al. in prep). Measurement of the levels of oligosaccharides (used as an indicator of the levels of anti-nutritional factors) in each of the prepared diets indicated that these diets corresponded to a 5% (diet 2), 14% (diet 3), and 27% (diet 4) SBME replacement. These latter numbers will be used to refer to each diet.

1.3 Production of diet

Diets were formulated to be isoenergetic and isonitrogenous (Table 1). All ingredients were mixed together in an electric mixer (A-12, Hobart Manufacturing Company, Troy OH). Once the dry ingredients had been mixed, the liquid components (fish oil and the different amounts of soy molasses, all adjusted to 200 ml with water) were then added to the corresponding diets. Once all the ingredients had been mixed, an extruder (Prep-Center VD-52, C.W. Brabender Instruments, So. Hackensack, NJ) with a 1.2mm die was used in order to produce pellets. These pellets were then dried at 95˚C for 30 minutes, using a forced-air convection drying oven, in order to ensure equal moisture levels (average range of 17.3-26.7%). Proximate analysis and determination of oligosaccharide concentration in each diet (as an indicator for the amount of anti-nutritional factors) was performed by Daniel Ward, PhD student at The University of Rhode Island (Appendix 1).

1.4 Fish, rearing conditions and sampling protocol

Fish were handled and maintained following a protocol approved by the URI Institutional Animal Care and Use Committee (Protocol Number: AN10-10-008). Juvenile summer flounder were obtained from Great Bay Aquaculture (Portsmouth, NH). The fish were transported to the URI Ann Gall Durbin Marine Research Aquarium (Narragansett Bay Campus). Fish (range: 1.2 - 5.9 g, average ± SD: 2.4 ± 0.7 g) were allowed to acclimate to the flow-through seawater tanks for two weeks while being fed a commercial diet (Skretting Gemma Diamond 0.8 mm, Stavanger, Norway). After the acclimation period, twenty fish were placed at random in individual 75 liter aquaria at the Blount Aquaculture Laboratory. The fish were once more allowed to acclimate for one week, while being fed a commercial diet. Each aquarium had a separate flow-through system fed with sand-filtered, aerated and UV-treated water. Triplicate aquaria were used for each of the five diet types, resulting in a total of 15 aquaria. Fish were fed to satiation twice daily for a total of eight weeks. All uneaten food was siphoned out daily. Fish were held at an average water temperature between 17 - 19ºC, a salinity of 28 – 32 ‰, and a 12:12 light/dark cycle.

At the start of the feeding trial all fish were measured and weighed individually. During weeks 2, 4, and 6 after the start of the feeding trial, the whole tank mass was weighed in order to determine tank average. During the 8th and final week, fish were measured and weighed individually.

Trial 2

2.1 Production of Soy Protein Concentrate and sub fraction of soy molasses

The soy protein concentrate and soy molasses were produced as described previously. Soy molasses was fractionated using a butanol extraction following a modification of the method of Knudsen et al. (2007), which separates anti-nutritional factors present in soy molasses into three fractions depending on density. The dense lower phase contains the oligosaccharides, while the light upper phase contains saponins. The intermediate phase (precipitate phase) contains a mixture of both the dense and light molecules, meaning both saponins and oligosaccharides are present. Briefly, butanol and water were mixed together (1:1) and allowed to separate until there was a water-saturated butanol phase and a butanol-saturated water phase. The water-saturated butanol was removed by pipette, and added to soy molasses at a 1:1 ratio (100mls of both water saturated n-butanol and soy molasses). This solution was inverted several times, poured into a separatory funnel and allowed to separate for 24 hours. The mixture inside the separatory funnel formed three distinct fractions (butanol, water, and precipitate). The three fractions were all heated individually in a rotary evaporator at 70˚C until dryness to evaporate any remaining butanol. The remaining solids (which contained anti-nutritional factors) were suspended in water three times and again evaporated to dryness. The resulting fractions were suspended in deionized water to reach the original volume (100mls for each fraction).

2.2 Formulation of diet

Five diets were manufactured at the Food Science and Nutrition Research Center at the University of Rhode Island (West Kingston, RI). The diets were formulated as follows (Table 2): 1) a control diet with no soy protein, all protein from fish sources (Control-FM); 2) a soy protein concentrate diet in which 60% of the fish meal was replaced with soy protein concentrate (Diet 1-SPC); 3 - 5) three diets containing 60% soy protein concentrate replacement of fish meal and enriched with a specific subfraction of soy molasses, a saponin-enriched fraction (diet 2, saponin), a mixed-fraction containing both oligosaccharides and saponins (diet 3, Mixed), and an oligosaccharide-enriched fraction (diet 4, Oligosaccharide).

2.3 Production of diet

Diets were formulated to be isoenergetic and isonitrogenous. In some instances, additional ingredients were added to the soy protein concentrate diet, in order to reach levels present in fish meal (Table 4). All dry ingredients were mixed together in an electric mixer (A-12, Hobart Manufacturing Company, Troy OH). Once the dry ingredients had been mixed, the liquid components (fish oil and the different subfractions, all adjusted to 200ml with water) were then added to the corresponding diet. Once all the ingredients had been mixed, an extruder (Prep-Center VD-52, C.W. Brabender Instruments, So. Hackensack, NJ) with a 1.6mm die was used, in order to produce pellets. These pellets were then dried for 30min at 90°C in order to ensure equal moisture levels. Proximate analysis and determination of the oligosaccharide

concentration in each diet were performed by Daniel Ward, PhD student at The University of Rhode Island (Appendix 1).

2.4 Fish, rearing conditions and sampling protocol

Juvenile summer flounder (range: 5 - 23 g, average +SD: 11.2 ± 3.5 g) that had been obtained from Great Bay Aquaculture (Portsmouth, NH) and held in the URI Blount Aquaculture Laboratory were transferred to 75 liter aquaria. Prior to the move and during a one-week acclimation period, the juvenile summer flounder were fed a commercial diet (Skretting Gemma Diamond 1.2 mm, Stavanger, Norway). Twenty-five 75 liter tanks (5 replicates for 5 diets) were equipped with a separate flow-through system, fed UV-treated water, and aerated using an air bubbler. Fish were fed to satiation twice daily for a total of 6 weeks. All uneaten food was siphoned out daily. The aquaria temperatures were between 17 - 19ºC, a salinity of 28 – 32 ‰, and a 12:12 hour light/dark cycle.

At the start of the feeding trial all fish were measured and weighed individually. During Week 2, the whole tank mass was weighed in order to determine tank average. During Weeks 4 and 6 each fish was weighed and the length was recorded.

Trial 1 and 2

3.1 Histological Sample Collection and Preparation (Trials 1 and 2)

Histology was used in order to determine any tissue abnormalities or differences in structure integrity between fish in the different experimental treatments. In Trial 1, fish (2 fish per tank, n = 6 fish per diet and time point) were collected for histological examination of tissues during weeks 1, 2, 4, and 8 after the start of the feed trial. With the exception of Week 1, fish were starved for a 24-hour period before any dissections were performed. In Trial 2, fish (1 fish per tank, n = 5 fish per diet and time point, except fish meal control which only had two samples processed per time point) were starved for 24 hours. Only fish from the fish meal control, SPC control, saponin-containing, and oligosaccharide-rich diets were processed for histological examination in this study.

Depending on the fish size, fish were processed for histology as follows. Fish less than 5 g were euthanized using a triple overdose of Tricaine MS222 and the peritoneal cavity was opened so that neutral buffered formalin could rapidly reach the internal organs, and properly fix all tissues. Fish were immediately transferred to a container with a solution of 10% neutral buffered formalin. The fish remained in the fixative for a minimum of 4 days. Once the fish had been fixed they were transferred to a decalcifying solution (16.7% EDTA, 2.8 % sodium hydroxide). This decalcifying solution was chosen because it is less harsh on the tissue than some other current solutions. Thus, specialized stains such as antibody staining could be performed in the future. Once the tissues had been decalcified, they were washed several times with tap

water and placed in a solution of 70% ethanol. Because fully fixed tissue may remain in ethanol for prolonged periods of time without tissue damage, the fish remained in the 70% ethanol solution until processing. The fish were then removed from the ethanol and cut lateral-medially into three cross-sections including a section of the intestine, liver, and spleen. Fixed and decalcified samples were placed into cassettes and sent to Mass Histology, Inc. (Worcester, MA) for embedding in paraffin and preparation of 7 μm sections using a microtome. The tissues were stained using a routine Hematoxylin and Eosin (H&E) stain. Fish larger than 5 grams were processed as above, however, once the peritoneal cavity was opened, a paper towel soaked in formalin was placed under the organs, between the organs and the ventral surface. This ensured that all portions of the organs were exposed to formalin, and was a preventative measure against degradation of the tissues during necropsy. Then, fish were placed in a container filled with formalin and fixed as above. Once completely fixed the specimens were removed from the formalin and placed under a fume hood for dissection. Tissues were taken from the spleen, liver, and intestine and processed as above.

3.2 Histological examination

All slides were randomly numbered and evaluated blindly. The cassettes for six representative samples (three control and three experimental) were sent to a commercial histology service (Mass Histology, Worcester, MA) for the preparation of section stained with the following special stains: PAS, Trichrome, and Alcian Blue pH 2.5. The PAS special stain was selected because of its ability to highlight

mucopolysaccahrides. The Trichrome stain was selected because connective tissue is stained a deep blue color. The Alcian Blue pH 2.5 was chosen in order to determine the presence of goblet cells, which produce mucus. Liver, spleen, and anterior intestine tissues were evaluated using light microscopy (Nikon Eclipse 50i) and rated using a semi-quantitative scale that was developed based on previous research (Knudsen et al., 2007) and on our observations (Table 3, Table 4, Figure 1, and Figure 2).

3.3 Statistical Analysis

Growth data from Week 8 (Trial 1) or 6 (Trial 2) were analyzed using a One-way ANOVA. Because significant differences were found, a post hoc Holm-Sidak's multiple comparisons test was run to compare each diet. A significance level of 0.05 was used for the p-value. The histological lesion scoring results were analyzed using a Two-way ANOVA on ranks, with diet and time as factors and an alpha of 0.05. A post hoc Tukey's multiple comparisons test was used in order to determine differences between groups.

RESULTS

1.1 Growth Performance

At the conclusion of the eight-week feeding trial 1, there was a statistically significant difference in weight between fish fed the fish meal control diet and fish fed with the experimental diets (Figure 3, $p < 0.0001$, One-way ANOVA). Fish in the experimental diets containing soybean meal products had a weight less than half of the weight of fish fed the control fish meal diet. There were no statistically significant differences among any of the experimental diets ($p > 0.05$). At the conclusion of the second six-week feed trial 2, there was a significant difference between fish fed a fish meal diet, and all other diets. There was also a significant between the SPC diet and all three experimental diets (Figure 4, $p < 0.0001$, One-way ANOVA).

1.2 Effect of soybean anti-nutritional factors on digestive and immune organs

The microscopy analysis of summer flounder tissues from Trials 1 and 2 revealed changes in histological patterns in all tissues examined (liver, spleen, and anterior intestine). The morphological changes were most evident in fish that were fed the 27% SBME diet during Trial 1; however, changes were also noted in fish fed the 5% SBME diet and SPC diets (Tables 5-7). During Trial 2, morphological changes were also observed in liver and intestine samples from fish fed both the saponin-containing and the oligosaccharide-enriched subfractions (Tables 5-6, 8). Morphological changes in each tissue are described in more detail below.

1.2a Liver

Fish fed the fish meal control diet in both trials had livers showing normal morphology, characterized by full hepatocytes that had evenly centered nuclei and no signs of vacuolization (Figure 5a). During Trial 1, fish fed a 27% SBME diet for 4 and 8 weeks showed several abnormalities in liver tissue morphology (Figure 5b,c,f). The hepatocytes appeared shrunken and there were large eosin-staining inclusions throughout the liver tissue, but more highly concentrated toward the lateral edges of the liver. Pyknotic nuclei were present, suggesting single cell necrosis, and some mitotic figures were evident (Figure 5c). Samples taken from fish fed 27% SBME for eight weeks also showed a proliferation of unpigmented melanomacrophage centers (Figure 5f). None of the morphological changes described above were observed in fish from Trial 2. However, there was a decrease in hepatocyte size of fish fed both saponin-containing and oligosaccharide-rich fractions, suggesting a decrease in the ability to store nutrients (Figure 5e).

Results from scoring the severity of morphological changes in the liver of fish from Trial 1 are shown in Table 5. Two weeks after the initiation of the feeding trial, fish fed the 27% SBME diet showed statistically significant changes in liver morphology as compared to fish fed a fish meal control diet (Table 5, $p < 0.0032$). Four and 8 weeks after the initiation of the feeding trial, there was a significant difference in liver ratings between fish in the control diet compared to fish fed the 5% and 27% SBME ($p < 0.0001$) and between fish in the SPC diet compared to the 27% SBME diet.

1.2b Spleen

Fish fed the fish meal control diet in both trials had spleens that demonstrated normal morphology, characterized by a higher abundant red pulp and only a small portion of white pulp (Figure 6a). During Trial 1, fish fed a 27% SBME diet for four and eight weeks showed altered morphology as compared to control fish. These fish had spleens that were characterized by a higher percentage of white pulp than red pulp and thickening of the connective tissue (Figure 6b). Trichrome staining, which stains collagen blue, of the histological sample showed that collagen is mostly restricted to the walls of the blood vessels in spleens of fish fed a control diet. Spleens of fish fed a 27% SBME diet showed an increase in white pulp and collagen, indicating that there had been a proliferation of connective tissue into the parenchyma of the spleen (Figure 6b). During Trial 2 an increase in the relative amount of white pulp was observed in the spleens of fish fed the soy protein concentrate and oligosaccharide-rich diets for two weeks, compared to the saponin-containing and fish meal diets. These changes were less severe than those observed in trial 1 in the 27% SBME diet, since there were no signs of connective tissue proliferation (not shown).

Results from scoring the severity of morphological changes in the spleen of fish from Trial 1 are given in Table 6. Two weeks after the initiation of the feeding trial, only fish fed the 27% and the 5% SBME diets showed significant differences in rating (Table 6 $p < 0.031$). Four weeks after the initiation of the feed trial, there were significant differences in spleen ratings between fish in the control diet compared to fish fed the 27% SBME diet, with control fish having the lowest rating (Table 6, $p <$

0.0005). There were also significant differences between the SPC and 27% SBME, with the latter having a higher rating (Table 6, $p < 0.0342$). During Week 8, there was a significant increase in spleen rating between fish in the control and 27% SBME diets (Table 6, $p < 0.0051$). During Trial 2 there were no significant differences between any of the diet types.

1.2b Anterior Intestine

Fish fed the fish meal control diet in both trials showed anterior intestines with a normal morphology, characterized by abundant goblet cells, a thin lamina propria, and mucosal folds that were long and not fused to one another. The anterior intestines of these fish also showed an intact brush border, and an intact mucosal lining of the brush borders and enterocytes with an absence of vacuoles (Figure 7a).

During Trial 1,fish fed diets with soybean products showed several abnormalities, which were more evident in the fish fed the highest levels of anti-nutritional factors (27% SBME diet). These abnormalities included a thickening of the lamina propria as demonstrated by trichrome and PAS staining (Figures 8e-f), a decrease in goblet cells and little to no mucus on the microvilli lining, as demonstrated by PAS and Alcian blue staining (Figures 8 d, f), and a shortening on the mucosal folds (Figure 7b, Figure 8f).

Results from scoring the severity of morphological changes in the anterior intestine of fish from Trials 1 and 2 are given in Tables 7 and 8. Significant decreases in the amount of goblet cells in the anterior intestine were observed in fish fed soybean products for 2, 4, and 8 weeks compared to fish fed fish meal ($p < 0.0001$; Table 7).

Fish fed the 27% SBME diet also showed a significant increase in the severity of the thickening of the lamina propria that was evident at all time points sampled (2, 4, 8 weeks, $p < 0.0065$, $p < 0.0001$, $p < 0.0001$; Table 7). In addition, these fish showed a statistically higher level of the thickening of the lamina propria than fish fed the SPC and 5% SBME diets at 4 and 8 weeks (Table 7). Fish fed the SPC and the 5% diets had significantly more thickening of the lamina propria than did control fish during Week 8 (Table 7, $p < 0.0005$). Regarding the severity of the abnormalities in the mucosal folds, fish fed the highest levels of anti-nutritional factors showed an increased severity in these lesions compared to fish fed control and 5% SBME diets at all weeks, and to fish fed an SPC diet at weeks 4 and 8 (Table 7). There was also a significant increase in the severity of abnormalities in mucosal folds in fish fed the 27% SBME diet between weeks 2 and 8.

No significant differences in liver morphology were observed between fish in the different treatments in Trial 2. However, there was a trend showing increased ratings for the SPC, saponin-containing, and oligosaccharide-rich diets compared to the fish meal control diet (Table 7). For Trial 2, there were no significant differences between the diets; however, there was a trend of increased rating for the Oligosaccharide-enriched diet (Table 7). During Trial 2, there were morphological differences in the anterior intestine between control and experimental fish. Fish fed diets enriched in saponins and oligosaccharides tended to have less goblet cells, a thicker lamina propria and more mucosal fold fusion when compared to fish meal control-fed fish (Table 7). Although trends in the scoring of the morphological changes observed in anterior intestine were observed, suggesting that fish fed the saponin-containing and

oligosaccharide-rich diets showed an increase in morphological changes during Trial 2, there was only a significant difference between these diets and the fish meal control diet in the lamina propria thickness ratings at both weeks 2 and 6 (Table 8). There was also a significant increase in rating between the SPC and the oligosaccharide-rich diets (Table 7).

DISCUSSION

In this study we used a combination of growth data and morphological observations to determine that when summer flounder are fed with increasing amounts of a fraction of soybean containing the anti-nutritional factors was added to diets in which 60% of fish meal was replaced with soy protein concentrate there is a significant decrease in growth and an increase in pathological changes in the liver, spleen, and anterior intestine. This study also demonstrated that morphological change in the anterior intestine, albeit to a lesser degree, can occur when diets containing low amounts of soy saponins and oligosaccharides are fed to summer flounder.

Previous research has demonstrated that feeding of soybean meal diets, or diets enriched in specific anti-nutritionals leads to enteritis in fish (Knudsen et al., 2007; Iwashita et al., 2009), suggesting that enteritis is a contributing factor to the decreased growth observed in fish fed soybean meal replacement diets. This present study determined what impact anti-nutritional factors present in soybean meal have on the morphology of different tissues and organs relevant to digestion, food processing, and immunity in summer flounder. I demonstrate here that anti-nutritional factors in soybean, when added to a 60% replacement diet of fish meal with soy protein concentrate to levels as low as those present in a 5% soybean meal replacement diet, in addition to leading to morphological changes in the intestine of summer flounder, caused pathological changes in the spleen and liver. These changes may indicate an inability to deal with anti-nutritional factors at even small concentrations. Higher amounts of anti-nutritional factors, such as the level present in the 27% SBME, diet may even cause a maladaptive

mechanism by which non-nutritional substances are stored (as evidenced in the large eosin-staining inclusion seen in the liver) and nutritional elements are not stored, thereby exacerbating poor growth.

These trials confirm that the presence of anti-nutritional factors present in soybean meal lead to decreased growth in summer flounder compared to fish fed on fish meal. An unexpected finding was that fish fed the 60% soy protein concentrate replacement diet also showed a significantly lower final weight during these two trials than fish in the fish meal diet. In previous studies, fish fed a 60% soy protein diet had good growth compared to a fish meal control diet (Ward, personal communication). Two explanations could explain the discrepancy between these two studies. During trial one, defatted soy white flake was used in order to make soy protein concentrate. A partial extraction may have left some anti-nutritional factors in the SPC, as suggested by the amount of oligosaccharides present in this diet (Appendix 1). Another potential explanation for the reduced growth observed in fish fed a Control vs. SPC diet in trial 1 may relate to the sampling protocol used to collect fish for histology. In order to prepare fish for histology, fish were starved for a 24 hour period of time prior to sample collection during weeks 2 - 8. Observational data on animal behavior taken during these time points revealed that fish in all tanks except fish meal control fish had a decreased appetite the day after starvation. The decreased appetite could have led to decreased growth rates in these fish. Previous studies have shown that feeding Atlantic salmon SPC at high replacement rates leads to decreased feed intake (Médale et al., 1998). This second hypothesis is supported by the fact that fish in tanks from which no fish were sampled for histology (and therefore were not starved prior to sample collection), belonging to a parallel trial performed by Daniel

Ward, a URI PhD student at URI, did not show this behavior. In this parallel trial, fish fed the SPC diet showed similar growth to that of fish fed the fish meal diet. Because of the potentially confounding factors present in my growth trial, I would be unwilling to conclude from my study alone that a 60% replacement SPC diet is an unsatisfactory diet for summer flounder. However additional studies have shown that high levels of SPC can impact the growth of Japanese flounder and rainbow trout, even when food additive were used to enhance the flavor (Mambrini et al., 1999; Deng et al., 2006).

Changes in the liver of fish fed the highest concentrations of soybean meal anti-nutritional factors in this study (27% SBME) are indicative of single-cell necrosis of hepatocytes occurring in this organ. Cell death can occur in many different ways. External blunt trauma or the consumption of toxic chemicals could lead to necrotic patches of tissue that must be repaired. Apoptosis, or programmed cell death, may lead to the destruction of a patch of tissues as well as an individual cell (Elmore, 2007). The presence of pyknotic nuclei and strongly eosin-stained hepatocytes, caused by the breakdown of RNA combined with the denaturation of protein (Kuntz and Kuntz, 2008), suggests that single-cell necrosis is occurring. These alterations, and in particular the presence of pyknotic nuclei, commonly seen in fish that have been exposed to pollutants (Mishra and Mohanty, 2008; Jiraungkoorskul et al., 2003), in fish fed diets containing soybean anti-nutritional factors suggest that these products can cause liver pathology similar to that observed in cases of serious chemical intoxication. Liver damage, may lead to a decrease in bile production, as well as necrosis, thereby reducing the ability to digest and store future feed, and contributing to poor growth.

The most prevalent morphological changes in the liver, including a decrease in hepatocyte size, often occurred during the earlier time points and improved by the later time point. Although these changes in rating are not statistically different, this trend suggests that potentially the liver can heal after being damaged by the lower concentrations of soybean meal anti-nutritional factors (not the 27% SBME). Previous studies in juvenile tilapia have described the negative impacts that soybean products have on liver structure or function (Lin and Luo, 2011); however no studies have taken multiple time points in order to determine on what time scale liver damage occurs. Several of the fish fed the diets containing anti-nutritional factors for 4 weeks showed the presence of mitotic figures, a sign of cell replication commonly seen in tissues that are being repaired. The regenerative capability of a mammal liver is quite comprehensive (Michalopoulos and DeFrances, 1997). However, research on the regenerative capability of piscine livers where a hepatomactomy has not been performed is very limited.

To my knowledge, this study is the first to show that the inclusion of soybean meal anti-nutritional factors cause pathological changes in the spleen of summer flounder. The proliferation of white pulp and connective tissue observed in fish fed a 27% SBME diet may indicate of a chronic inflammatory response and fibrosis (the proliferation of connective tissue in order to repair damaged tissue). The white pulp of a fish spleen is where lymphogenesis occurs, and where lymph tissue is stored. Therefore, a diet that enhances the production of white blood cells may indicate that the diet is causing an allergic reaction. Previous researchers have demonstrated that fibrosis is characteristic of wound healing (Friedman, 2000). However, since fibrosis of the spleen has been rarely

reported in fish, it would be hard to predict the impacts of fibrosis on spleen functionality.

The anterior intestine is an important organ for digestion and absorption of nutrients. Therefore, changes to the enterocytes, which are the functional absorptive unit of the anterior intestine, could cause a decrease in fish growth. During this study, observations of vacuolated enterocytes in fish fed diets soybean anti-nutritional factors were few (Figure 7b). Fish fed high levels of anti-nutritional factors, however, had a significant decrease in goblet cells, and had less mucus on the brush border (Figure 8d). Mucus, which is produced by the goblet cells throughout the length of the intestine, is an important physical defense mechanism against pathogens. Because mucus is important for immune protection, it would seem that fish fed the high levels of anti-nutritional factors would be at greater risk of disease if pathogens were present in the water than fish fed the control diet. The amount of goblet cells in the anterior intestines of fish fed most experimental diets, however, was lowest during the earliest time point, and increased to a higher level at 8 weeks. This may suggest that, although the presence of soybean anti-nutritional factors in the diet may initially decrease the amount of goblet cells and/or mucus in the anterior intestine, fish may be able to adapt to these diets and increase the production of mucus to normal levels.

The lamina propria in the anterior intestine, which is the connective tissue center of intestinal plicae, thickened when any level of anti-nutritional factor was added to the diets (Figure 7b, Figure 8e-f). The lamina propria is important in tissue support rather than absorption and digestion. It is composed of fibrous connective tissue. A thickening of this tissue, which did not improve or resolve over time, could be further indication of a

chronic inflammatory response in the anterior intestine caused by exposure to anti-nutritional factors, which could be responsible for decreased nutrient absorption, contributing to the observed decrease in summer flounder growth.

Lastly, the mucosal folds of the anterior intestine were found to have changed morphology as early as Week 2 when fish were fed a 5% and a 27% SBME replacement diets. The mucosal folds of fish fed the 27% SBME diet for 8 weeks showed a large amount of fusing (Figure 8b). This could be because the folds were in the process of maturing after recovery from damage (Uni et al., 2000). During Trial 2, no significant differences in the morphology of the mucosal folds in the anterior intestine were found between diet types; however as the trial progressed, fish fed saponin-containing, and oligosaccharide-rich subfractions had more morphological changes occurring in the mucosal folds than control fish (Figure 8f). These results suggest that small amounts of soy anti-nutritional factors can cause significant pathological changes in the integrity of the anterior intestine. Although our results suggest that fish tissues may be able to adapt and recover from these changes, not all tissues may respond in the same manner.

No investigators have shown that soy oligosaccharides cause a change in tissue morphology; however, many studies have shown that soya-saponin can cause intestinal change (Knudsen et al., 2008;Zhang et al., 2013). Research by Cai et al., (2012) demonstrated that a mixed saponin/oligosaccharide-rich diet caused a decrease in growth and a change in intestinal morphology in silver crucian carp, whereas an oligosaccharide-specific diet did not induce the same changes. The conclusion the authors drew was that saponins were in fact responsible for a decrease in growth and change in intestinal morphology, and not the oligosaccharides. Although it may appear that my results

showing that some morphological changes were evident in both the saponin-containing and oligosaccharide-rich fractions appear to contradict the results of Cai et al. (2012), this is most likely due to the fact that extraction efficiency of butanol was lower than expected and that our oligosaccharide diet probably contained some levels of saponins (Appendix 1). This is based on the fact that diets that should not have contained oligosaccharides, which was used as a marker for anti-nutritional factor, contained some level of oligosaccharides. Conversely, it could be inferred that the diet containing the oligosaccharide-rich subfraction may have had some saponins. Therefore, rather than conclude that this research provides evidence that soybean's oligosaccharides cause pathologies in digestive and immune tissue, it is more prudent to conclude that a butanol extraction may not have a high enough level of efficiency to be a useful mechanism of separation. Further research needs to be done to determine the effect of purified saponins and oligosaccharides on tissue and organ morphology in summer flounder.

During Trial 1 there were significant pathological changes in all tissue types examined for fish fed the 27% diet. However, in Trial 2, there were only differences in the lamina propria thickness of oligosaccharide and saponin-fed fish, as compared to a fish meal control. During Trial 2 the oligosaccharide-rich diet was equivalent to about a 10% SBME diet, or less than 50% of the anti-nutritional factors in the 27% diet. The lack of significant pathological changes in Trial 2 suggests that low levels of soy saponins and oligosaccharides may not significantly impact the morphology of summer flounder spleen, liver and anterior intestinal tissue. Pathological changes observed in fish fed the soybean meal equivalent replacement diets from Trial 1 may be due to higher amounts of

anti-nutritional factors in these diets or to additive or synergistic impacts of several anti-nutritional factors.

Conclusions

This study confirmed that adding relatively low levels of soybean anti-nutritional factors to summer flounder diets (as low as 5% SBME) led to pathological changes in summer flounder tissues and organs that increase in severity as the level of anti-nutritional factors increase. By investigating morphological changes in the liver and spleen, rather than simply sampling the intestine (a commonly sampled tissue used to determine impacts of soybean-based diets) I was able to further elucidate why soybean meal's anti-nutritional factors cause a decrease in growth. Liver damage may be just as important for limiting the summer flounder growth as changes in the anterior intestine. Similarly, by determining that the morphology of an immune tissue, the spleen, was changed through the addition of anti-nutritional factors, the mechanism by which oligosaccharides and saponins serve as an immunostimulant or immunomodulator may be further explored. This study concludes that soy protein concentrate may prove to be an adequate fish meal replacement, predicated on the fact that a more efficient extraction method is devised. Therefore, future research should concentrate on two main areas: 1) Devising large scale efficient extraction methods, that may in one fell swoop make soy protein concentrate a more nutritious and more economical protein source 2) Determining how saponins and oligosaccharides modulate the immune system. By accomplishing the above goals, researchers will be one step closer to providing optimal carnivorous fish nutrition that both maintains or improve growth and provides better survival over typical fish-meal based diets.

TABLES

Table 1. Formulation of the diets for Trial 1. Soy molasses was prepared by ethanol extraction of soybean white flakes as described in the methodology section.

Ingredients	FM Control	SPC Control	5% SBME	14% SBME	27% SBME
Fish Meal (g)	670	268	268	268	268
Soybean molasses (ml)	0	0	52.6	123.3	194
SPC (g)	0	402	402	402	402
Fish Oil (ml)	32	65.2	65.2	65.2	65.2
Wheat flour (g)	238.5	120.6	120.6	120.6	120.6
Corn gluten (g)	25	49.2	49.2	49.2	49.2
Starch	4.5	14.5	14.5	14.5	14.5
Mineral Premix-URI (g)	10	10	10	10	10
Calcium Phososphate-21%P (g)	0	30	30	30	30
Vitamin Premix-URI (g)	10	10	10	10	10
DL-Met 99% (g)	0	1.6	1.6	1.6	1.6
Taurine 95% (g)	0	14	14	14	14
Glycine 100% (g)	10	15	15	15	15
Total weight (g)	1000	1000	1000	1000	1000

Table 2- Formulation of diets for Trial 2. The saponin, mixed, and oligosaccharide diets were prepared by butanol extraction of soy molasses onto an upper phase (mostly saponins), a precipitate phase (a mixture of saponins and oligosaccharides) and a lower phase (mostly oligosaccharides).

Ingredients	Fish Meal Control	SPC Control	Saponin	Mixed	Oligo-saccharide
Fish Meal (g)	670	268	268	268	268
Upper Phase (ml)	0	0	101.7	0	0
Precipitate Phase (ml)	0	0	0	101.7	0
Lower phase (ml)	0	0	0	0	101.7
SPC (g)	0	402	402	402	402
Fish Oil (ml)	32	65.2	65.2	65.2	65.2
Wheat flour (g)	238.5	120.6	120.6	120.6	120.6
Corn gluten (g)	25	49.2	49.2	49.2	49.2
Starch	4.5	14.5	14.5	14.5	14.5
Mineral Premix-URI (g)	10	10	10	10	10
Calcium Phososphate-21%P (g)	0	30	30	30	30
Vitamin Premix-URI (g)	10	10	10	10	10
DL-Met 99% (g)	0	1.6	1.6	1.6	1.6
Taurine 95% (g)	0	14	14	14	14
Glycine 100% (g)	10	15	15	15	15
Total weight (g)	1000	1000	1000	1000	1000

Table 3. Histological scoring system for morphological changes in the liver and spleen of summer flounder

	Score	Appearance
Liver	1	The hepatocyte appears to be round and storing lipid or glycogen.
	2	The hepatocyte appears to be not quite as full, but still storing lipid or glycogen. The location of the nucleus has not changed.
	3	The hepatocyte seems to be flattened; the overall staining of the hepatocyte is darker because there is a reduction in lipid/glycogen storage.
	4	The hepatocyte seems to only appear as a nucleus because there is a total loss of lipid/glycogen storage.
	5	The hepatocyte is not recognizable. The location, appearance, and stain all indicate there is no organization or lipid/glycogen storage.
Spleen	1	The spleen appears to consist mostly of red pulp; normal white pulp is present.
	2	The spleen appears to consist mostly of red pulp; there seems to be an increase in the amount of white pulp.
	3	The spleen appears to have an even percentage of white pulp and red pulp. There appears to be slight fibrosis.
	4	The spleen appears to consist mostly of white pulp; there appears to be moderate fibrosis around the white pulp.
	5	The spleen appears to consist mostly of white pulp; there are signs of extensive fibrosis around the white pulp.

Table 4. Histological scoring system for morphological changes in the anterior intestine of summer flounder

	Score	Appearance
Goblet Cells	1	Large, well-developed goblet cells are seen throughout the entirety of the tissue.
	2	Medium sized goblet cells are seen throughout the tissue
	3	Medium and small sized goblet cells are seen in the tissue, however there is a noticeable decrease in total number
	4	Small sized goblet cells are infrequently scattered throughout the tissue
	5	No mucus-filled goblet cells are present
Lamina Propria	1	There is a thin layer of connective tissue in each simple fold
	2	The lamina propria seems slightly increased in some of the simple folds
	3	There is a clear increase of connective tissue in most of the simple folds
	4	There is a thick lamina propria in many folds
	5	There is a very thick lamina propria in many folds
Mucosal Folds	1	Simple and complex mucosal folds (those with branching) appear long, thin and discrete
	2	Simple mucosal folds are still long and then, but complex mucosal folds appear to be thicker
	3	Simple folds appear to be of medium length, and have thickened. Complex mucosal folds appear even more thick
	4	Thick simple mucosal folds are seen, and the villi of the complex mucosal folds begin fusing
	5	Thick simple mucosal folds are seen, and the villi of the complex mucosal fold are extensively fused

Table 5. Histological Evaluation of Liver Pathological Change*

Trial 1	Week	Fish Meal Control	Soy Protein Concentrate	Diet 2 5% SBME	Diet 4 27% SBME
	2	2.6 ± 1.1^{b}	3.4 ± 0.6^{ab}	3.4 ± 0.6^{ab}	4.4 ± 0.9^{a}
	4	1.4 ± 0.9^{c}	2.6 ± 0.6^{bc}	3.0 ± 0.0^{ab}	4.0 ± 0.8^{a}
	8	1.4 ± 0.9^{c}	2.6 ± 0.9^{bc}	3.4 ± 0.9^{ab}	4.6 ± 0.6^{a}

Trial 2	Week	Fish Meal Control	Soy Protein Concentrate	Saponin-Containing Diet	Oligosaccharide-rich diet
	2	1.5 ± 0.7^{a}	2.6 ± 0.9^{a}	2.8 ± 0.8^{a}	2.8 ± 1.1^{a}
	6	2.0 ± 1.4^{a}	2.0 ± 0.6^{a}	2.6 ± 0.6^{a}	2.2 ± 0.8^{a}

** Histological sections were scored according to the criteria listed in **Table 3 (liver)**. A score of "1-2" represents normal morphology while a score of "5" represents severe morphological change. Reported data are mean values from ~5 fish ± SD.*

Table 6. Histological Evaluation of Spleen Pathological Changes*

Trial 1	Week	Fish Meal Control	Soy Protein Concentrate	Diet 2 5% SBME	Diet 4 27% SBME
	2	1.8 ± 1.0^{ab}	2.0 ± 1.0^{ab}	1.3 ± 0.6^{b}	3.0 ± 0.0^{a}
	4	1.3 ± 0.6^{b}	2.3 ± 0.5^{b}	2.4 ± 0.9^{ab}	3.6 ± 0.6^{a}
	8	2.4 ± 0.9^{b}	3.8 ± 0.5^{ab}	3.0 ± 0.8^{ab}	4.0 ± 0.0^{a}

Trial 2	Week	Fish Meal Control	Soy Protein Concentrate	Saponin-Containing diet	Oligosaccharide-rich diet
	2	2.5 ± 2.1^{a}	3.4 ± 1.5^{a}	2.5 ± 0.6^{a}	3.4 ± 1.5^{a}
	6	2.0 ± 0.0^{a}	2.2 ± 1.3^{a}	2.0 ± 1.2^{a}	3.0 ± 0.7^{a}

Histological sections were scored according to the criteria listed in **Table 3 (spleen). A score of "1-2" represents normal morphology while a score of "5" represents severe morphological change. Reported data are mean values from ~5 fish ± SD.*

Table 7. Histological Evaluation of Anterior Intestine Pathological Changes*

Trial 1	Week	Fish Meal Control	Soy Protein Concentrate	Diet 2 5%SBME	Diet 4 27% SBME
Goblet Cells	2	1.8 ± 0.5^{b}	3.6 ± 0.6^{a}	3.2 ± 0.8^{a}	3.6 ± 0.9^{a}
	4	1.6 ± 0.6^{b}	2.8 ± 0.8^{a}	3.4 ± 0.6^{a}	3.6 ± 0.6^{a}
	8	1.0 ± 0.0^{b}	3.0 ± 0.7^{a}	3.0 ± 0.0^{a}	2.6 ± 0.9^{a}
Lamina Propria	2	1.8 ± 0.5^{b}	2.6 ± 0.6^{ab}	3.0 ± 0.7^{ab}	3.4 ± 0.9^{a}
	4	1.4 ± 0.6^{b}	2.6 ± 1.1^{b}	2.6 ± 0.6^{b}	4.2 ± 0.5^{a}
	8	1.2 ± 0.5^{b}	2.4 ± 0.9^{b}	2.4 ± 0.9^{b}	4.4 ± 0.9^{a}
Mucosal Folds	2	1.8 ± 0.7^{b}	2.8 ± 0.5^{ab}	3.2 ± 0.5^{a}	3.4 ± 0.9^{a}
	4	2.0 ± 0.7^{c}	2.6 ± 0.6^{bc}	2.6 ± 0.9^{bc}	3.6 ± 0.6^{a}
	8	1.6 ± 0.6^{c}	2.4 ± 0.6^{bc}	2.6 ± 0.6^{bc}	4.4 ± 0.6^{a}
Trial 2	Week	Fish Meal Control	Soy Protein Concentrate	Saponin-Containing Diet	Oligosaccharide -rich diet
Goblet Cells	2	2.0 ± 0.0^{a}	3.2 ± 0.8^{a}	3.6 ± 0.5^{a}	3.0 ± 1.2^{a}
	6	1.0 ± 0.0^{a}	2.8 ± 0.5^{a}	2.4 ± 0.9^{a}	2.8 ± 0.8^{a}
Lamina Propria	2	1.5 ± 0.7^{a}	2.8 ± 0.8^{ab}	2.8 ± 0.5^{bc}	3.6 ± 0.6^{c}
	6	1.5 ± 0.7^{a}	2.5 ± 0.6^{ab}	3.2 ± 0.5^{bc}	3.8 ± 0.5^{c}
Mucosal Folds	2	2.0 ± 0.0^{a}	2.6 ± 0.6^{a}	2.6 ± 0.6^{a}	2.6 ± 0.9^{a}
	6	2.0 ± 0.0^{a}	2.0 ± 0.8^{a}	3.2 ± 0.8^{a}	3.2 ± 0.5^{a}

Histological sections were scored according to the criteria listed in **Table 4. A score of "1-2" represents normal morphology while a score of "5" represents severe morphological change. Reported data are mean values from ~5 fish ± SD.*

FIGURES

Figure 1. Scale used to score the severity of liver changes **(A) Rating 1-**Normal liver morphology characterized by large hepatocytes **(B) Rating 2-**Normal liver morphology, however the hepatocytes have less storage capacity **(C) Rating 3-**Loss of hepatocyte storage **(D) Rating 4-**No apparent storage capacity **(E) Rating 5-**Loss of organization, no apparent storage capacity and eosin staining inclusions are present. All photos taken at 20X, scale bar represents 100µm (H & E stain).

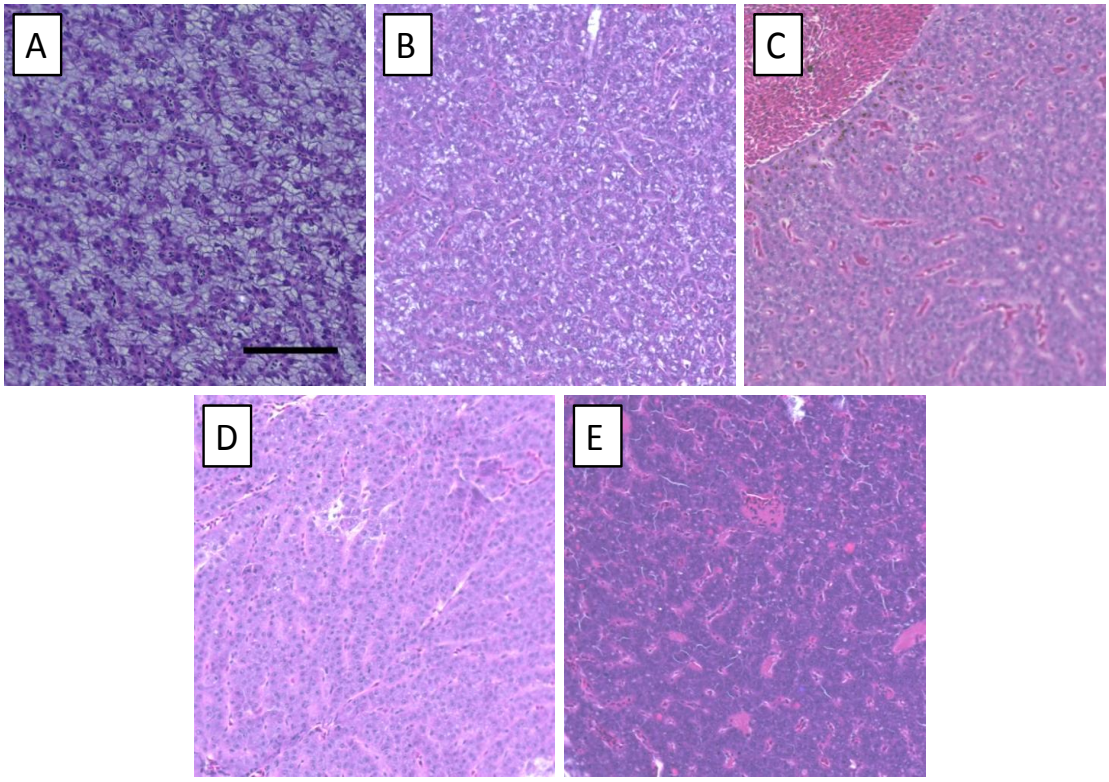


Figure 2. Scale used to score the severity of spleen changes **(A) Rating 1**-Normal spleen characterized by a majority of red pulp, however some white pulp is present **(B) Rating 2**-Normal spleen morphology characterized by a majority of red pulp, however a higher proportion of white pulp is present **(C) Rating 3**- Red pulp and white pulp are present in a relatively even proportion **(D) Rating 4**- Red pulp is still present, however the majority of cells are those belonging to white pulp **(E) Rating 5**- White pulp dominates the tissue, and pink staining connective tissue related to fibrosis is present. All photos taken at 20X, scale bar represents 100µm (H & E Stain).

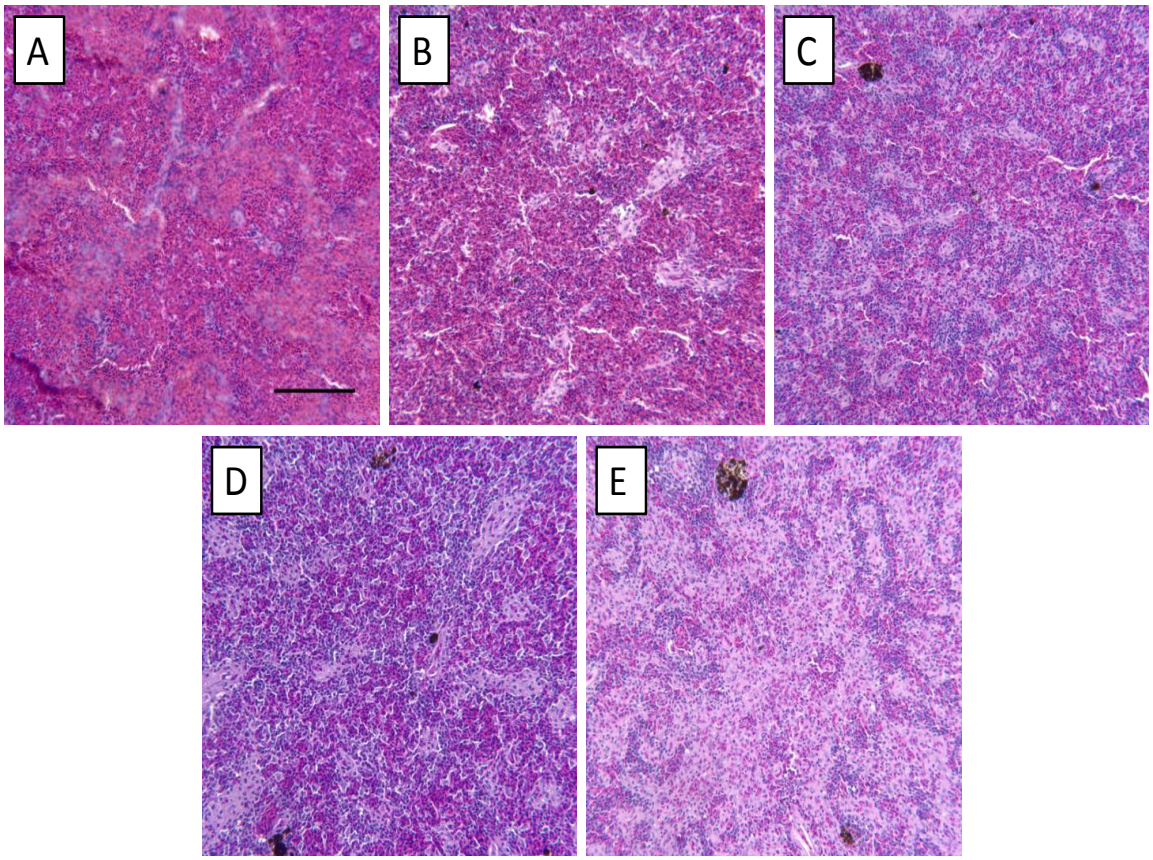


Figure 3. Effect of soybean anti-nutritional products on summer flounder growth Trial 1. Final weight (g) at the conclusion of an 8 week feeding trial with diets: Control (fish meal); SPC (60% replacement of fish meal with soy protein concentrate); and 5%, 14%, and 27% SBME (supplementation of the SPC diet with amounts of a fraction of soybean flakes containing anti-nutritional factors to levels corresponding to a 5%, 14%, or 27% soybean meal replacement diets). Different letters indicate statistical significance between experimental groups ($p \leq 0.0001$).

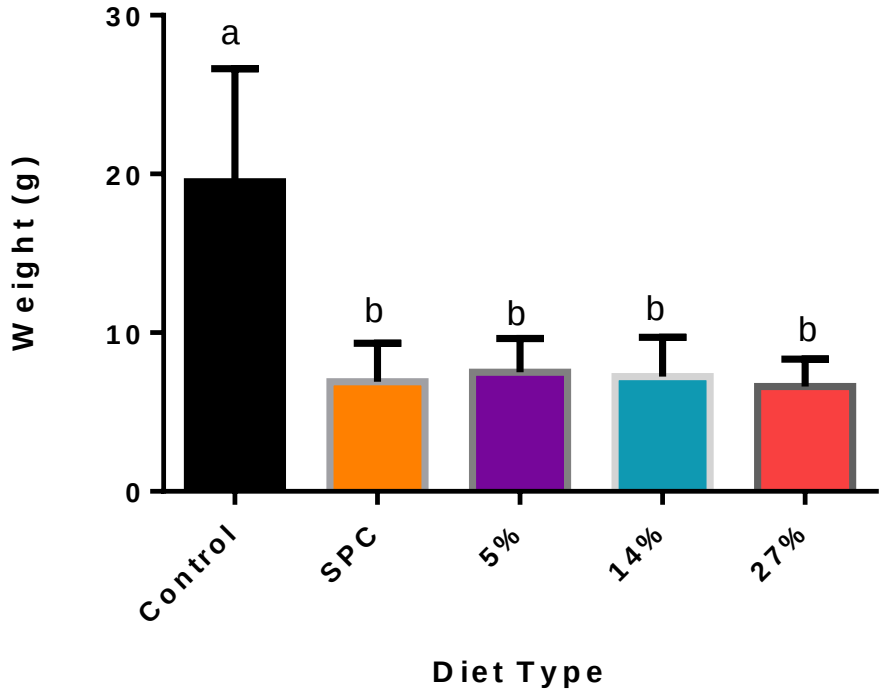


Figure 4. Effect of soybean anti-nutritional products on summer flounder growth Trial 2. Final weight (g) at the conclusion of a 6 week feeding trial with diets: Control (fish meal); SPC (60% replacement of fish meal with soy protein concentrate); Saponin (supplementation of the SPC diet with a saponin-containing subfraction of soy molasses); Mixed (supplementation of the SPC diet with a subfraction of soy molasses that contains both saponins and oligosaccharides); and Oligosaccharide (supplementation of the SPC diet with a oligosaccharide-rich subfraction of soy molasses). Different letters indicate statistical significance between experimental groups *(p*≤0.0001).

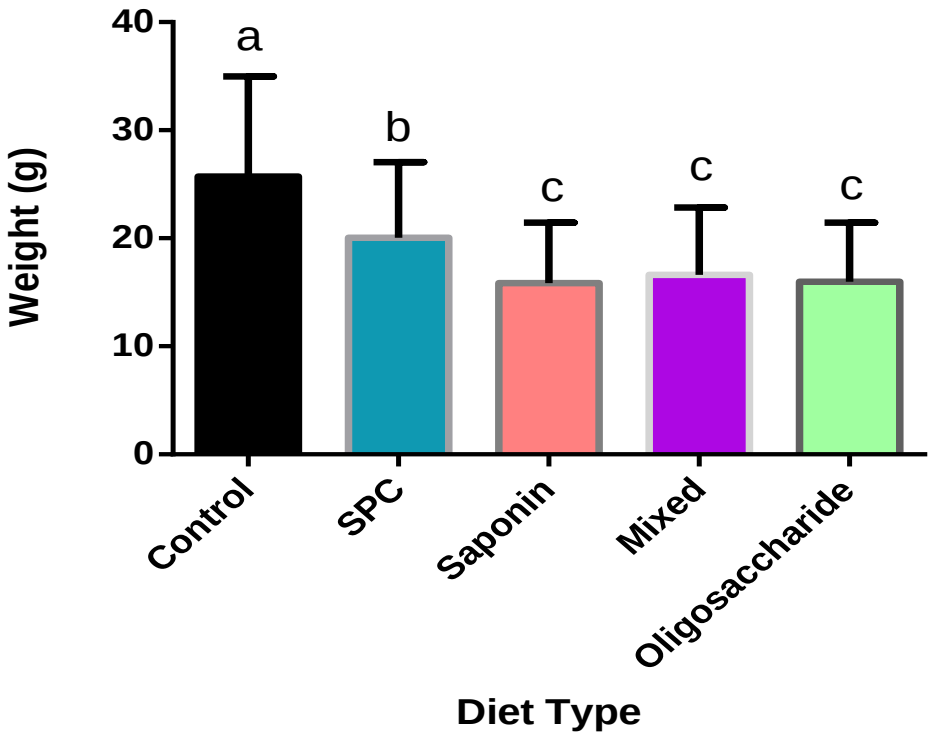


Figure 5. Effect of soybean anti-nutritional factors on liver morphology. **(A)** Trial 1- Representative fish fed the fish meal control diet for 8 weeks. Hepatocytes (HC) are full and the nuclei are visible; **(B)** Trial 1- fish fed the 27% SBME diet for 8 weeks. Individual hepatocytes are barely visible and eosin staining inclusions (I) are abundant; **(C)** Trial 1- Liver section obtained from a fish fed an experimental 27% SBME diet for 4 weeks. Pyknotic nuclei (PN) and mitotic figures (MF) present; **(D)** Trial 2- Fish fed a fish meal control diet for 6 weeks showing normal liver morphology; **(E)** Trial 2 – Fish fed a saponin-containing diet for 6 weeks. There is a decrease in hepatocyte size (HC), but no pronounced morphological changes as in **B. (F)** Trial 1- Fish fed a 27% SBME diet for 8 weeks. In addition to eosinophilic inclusions, these fish showed an increase presence of proliferative unpigmented melanomacrophage centers (UMM). All photos taken at 40X (H & E stain).

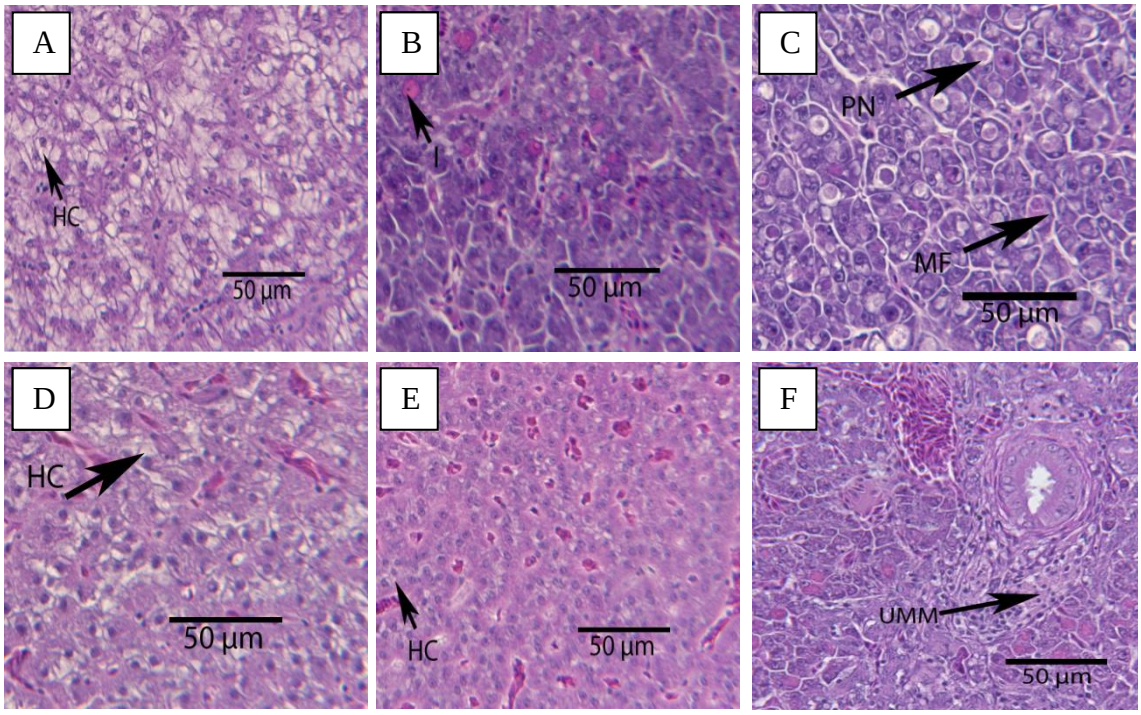


Figure 6. Effect of soybean anti-nutritional factors on spleen morphology. **(A)** Trial 1- Fish fed a fish meal control diet for 8 weeks showing normal spleen morphology with a majority of red pulp and thin bands of extracellular matrix (blue staining) around blood vessels (V) (Rating 1); **(B)** Trial 1- Representative fish fed a 27% SBME diet for 8 weeks showing large portions of blue staining, indicative of fibrosis. All photos taken at 40X (Trichrome stain).

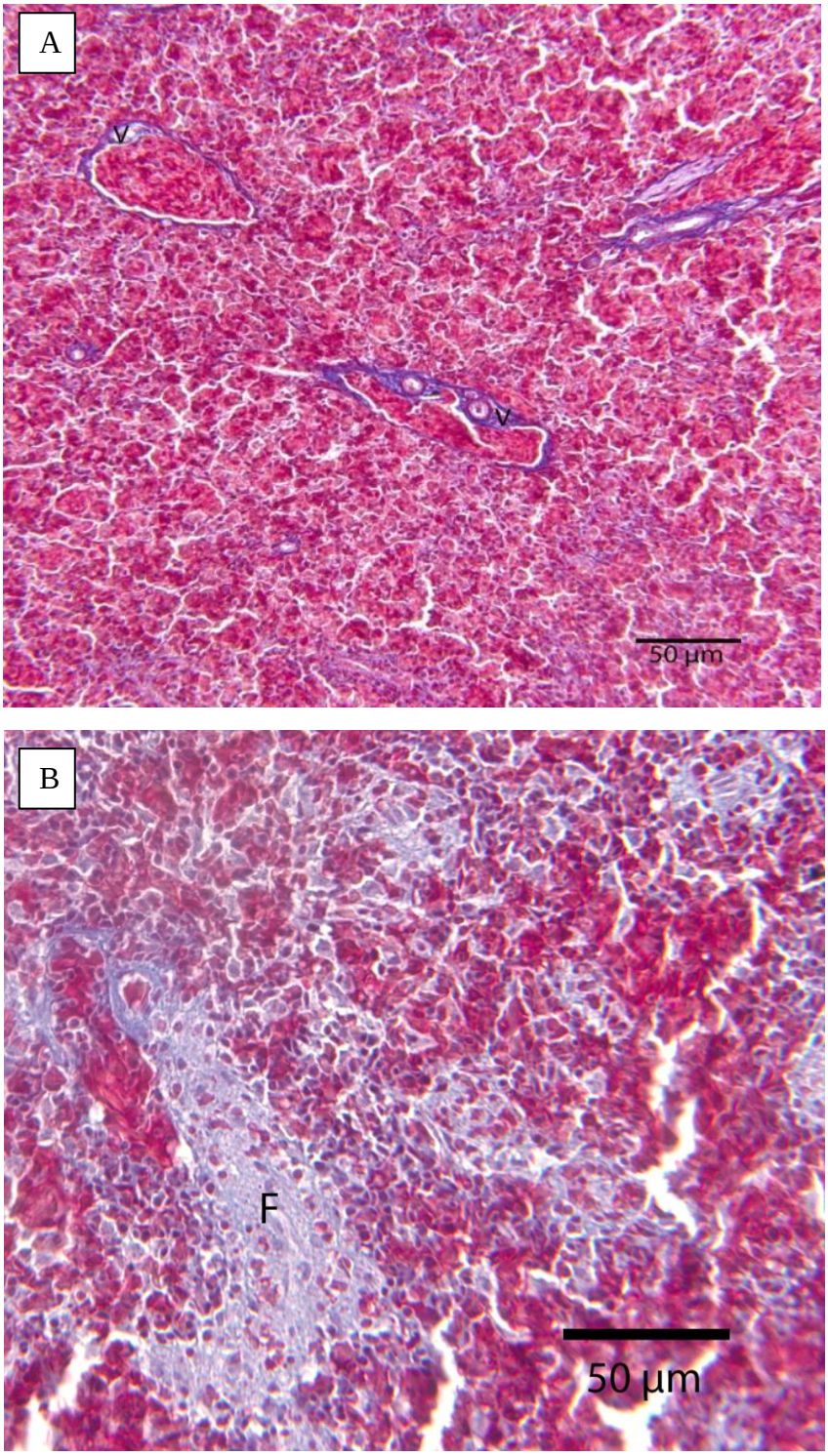


Figure 7. Effect of soybean anti-nutritional factors on intestinal tissue morphology. **(A)** Representative section of the anterior intestine of a fish fed the fish meal diet for 8 weeks, showing normal morphology with prominent goblet cells (GC) a thin layer of lamina propria (LP) very little clubbing or fusion (F) of the mucosal folds, with an intact brush border (BB). **(B)** Anterior intestine tissue from a fish fed a 27% SBME diet for eight weeks, showing vacuolization (VZ) of enterocytes (EC), a thickening of lamina propria (LP), and fusion (F) of mucosal folds. All photos taken at 40X (H & E stain).

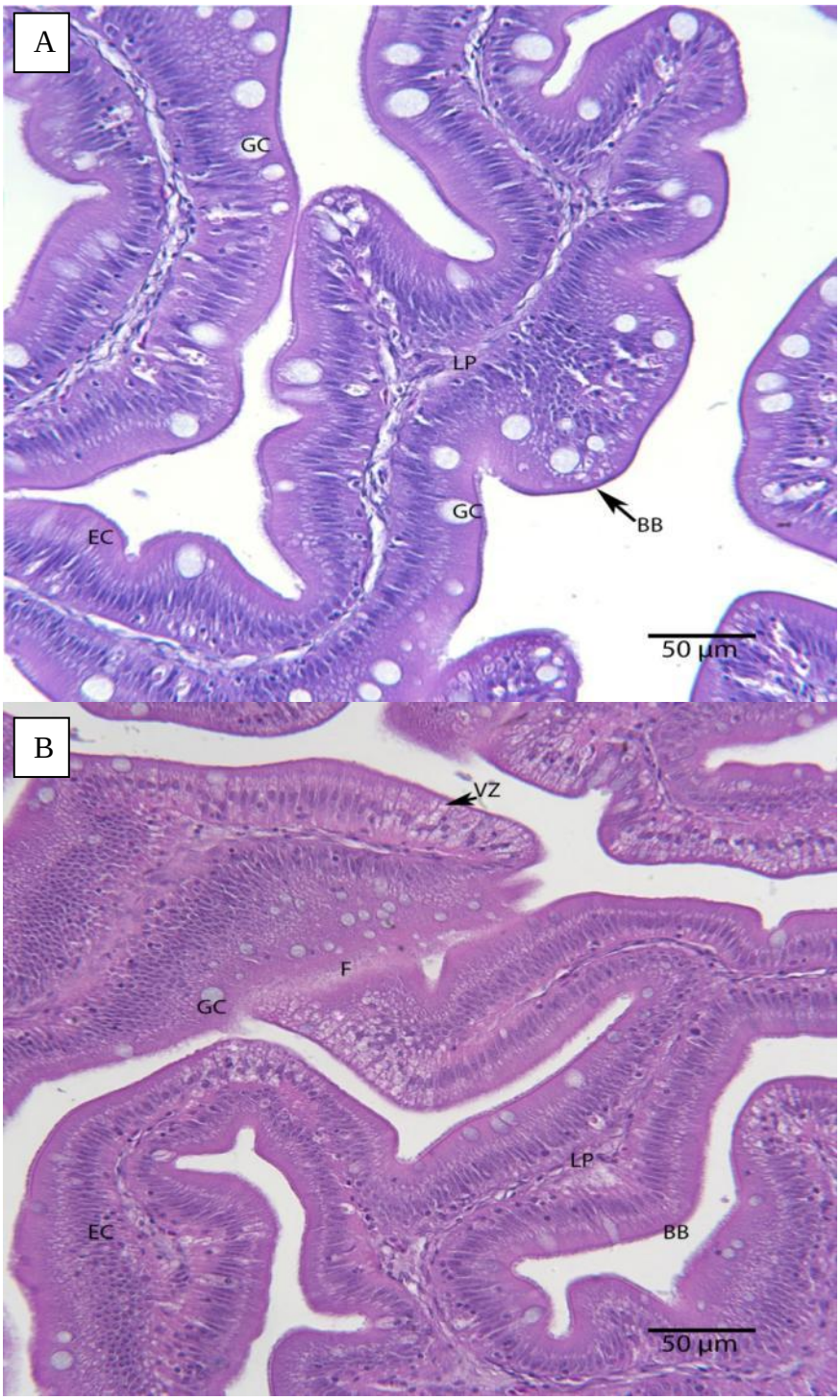
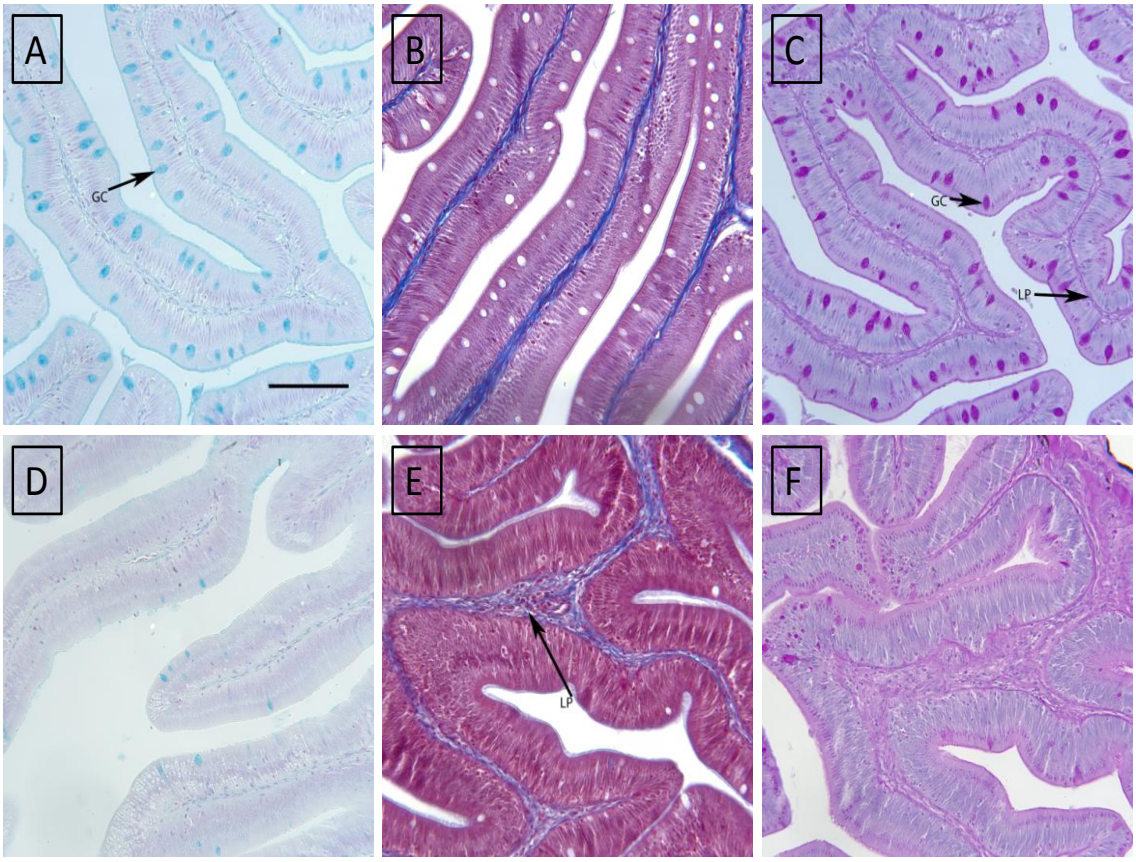


Figure 8. Effect of soybean anti-nutritional factors on intestinal tissue morphology. **(A)** Representative sample from a control fish showing normal morphology, including blue (Alcian Blue pH 2.5) staining of mucus in goblet cells and on the surface of the intestine; **(B)** Control fish showing normal thickness of the lamina propria (stained in dark blue, Trichrome) **(C)** Control fish with prominent purple staining mucus in goblet cells and a normal thin layer of lamina propria (PAS Stain); **(D)** Fish fed a 27% SBME diet for 8 weeks showing a loss of mucus in goblet cells and in the mucosal intestinal barrier (Alcian Blue pH 2.5 stain); **(E)** Fish fed a 27% SBME diet showing a thickened lamina propria (dark blue staining, Trichrome), and widening of the mucosal folds **(F)** Fish fed a 27% SBME diet for 8 weeks showing a thickening of lamina propria and a shortening of the mucosal folds (PAS stain). All photos taken at 40X, scale bar represents 50µm.



APPENDICES

Appendix 1: Oligosaccharide Content in Diets (determined by Dan Ward).

Trial 1	SPC Control	SPC + 12% SoyMol	SPC + 24% SoyMol	SPC + 36% SoyMol
% Oligo Content [projected]	0.23	0.4	0.64	0.87
Corresponding % SBM [projected]	2.67	12	24	36
% Oligo Content [actual]	0.09	0.28	0.44	0.69
Corresponding % SBM [actual]	0	5.3	13.84	26.85

Trial 2		(SPC Control)	(Upper) Saponin	(precip) Mixed	(lower) Oligosaccharide
	Control	Diet 1	Diet 2	Diet 3	Diet 4
% Oligo Content [projected]	0	0.23	0	0	0.4
Corresponding % SBM [projected]	0	2.67	0	0	12
% Oligo Content [actual]	0.03	0.09	0.16	0.15	0.38
Corresponding % SBM [actual]	0	0	Not Calc	Not Calc	10.87

Appendix 2: Trial 1 statistical analyses of growth data.

Table Analyzed	**Trial 1 Growth Data**

ANOVA summary

F	84.28
P value	< 0.0001
P value summary	****
Are differences among means statistically significant? (P < 0.05)	Yes
R square	0.6432

Number of families	1
Number of comparisons per family	10
Alpha	0.05

Holm-Sidak's multiple comparisons test	Mean Diff.	Significant?	Summary	Adjusted P Value
Control vs. SPC	12.59	Yes	****	< 0.0001
Control vs. 5.3%	12.01	Yes	****	< 0.0001
Control vs. 13.8%	12.27	Yes	****	< 0.0001
Control vs. 26.9%	12.89	Yes	****	< 0.0001

SPC vs. 5.3%	-0.5875	No	ns	0.9571
SPC vs. 13.8%	-0.325	No	ns	0.9717
SPC vs. 26.9%	0.2996	No	ns	0.9717
5.3% vs. 13.8%	0.2625	No	ns	0.9717
5.3% vs. 26.9%	0.8871	No	ns	0.8846
13.8% vs. 26.9%	0.6246	No	ns	0.9571

Appendix 3: Trial 2 statistical analyses of growth data.

Table Analyzed	**Trial 2 Growth data**

ANOVA summary

F	35.72
P value	< 0.0001
P value summary	****
Are differences among means statistically significant? (P < 0.05)	Yes
R square	0.2343

Number of families	1
Number of comparisons per family	10
Alpha	0.05

Holm-Sidak's multiple comparisons test	Mean Diff.	Significant?	Summary	Adjusted P Value
Control vs. SPC	5.69	Yes	****	< 0.0001
Control vs. Saponin	9.891	Yes	****	< 0.0001
Control vs. Mixed	9.107	Yes	****	< 0.0001
Control vs. Oligosaccharide	9.739	Yes	****	< 0.0001
SPC vs. Saponin	4.201	Yes	***	0.0002
SPC vs. Mixed	3.417	Yes	**	0.0029
SPC vs. Oligosaccharide	4.049	Yes	***	0.0003
Saponin vs. Mixed	-0.784	No	ns	0.8164
Saponin vs. Oligosaccharide	-0.1521	No	ns	0.8778
Mixed vs. Oligosaccharide	0.6319	No	ns	0.8164

Appendix 4: Trial 1 Histology Statistics

Table Analyzed	**Liver Data**

Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	3.968	0.4745	ns	No
Row Factor	5.79	0.0224	*	Yes

Column Factor	55.92	< 0.0001	****	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	3.366	6	0.5611	F (6, 47) = 0.9418	P = 0.4745
Row Factor	4.913	2	2.457	F (2, 47) = 4.123	P = 0.0224
Column Factor	47.45	3	15.82	F (3, 47) = 26.55	P < 0.0001
Residual	28	47	0.5957		

Number of missing values	1

Number of families	3
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Week 2					
Control vs. SPC	-0.8	-2.100 to 0.5002	No	ns	0.3673
Control vs. 5.3%	-0.8	-2.100 to 0.5002	No	ns	0.3673
Control vs. 26.9%	-1.8	-3.100 to -0.4998	Yes	**	0.0032
SPC vs. 5.3%	0	-1.300 to 1.300	No	ns	> 0.9999
SPC vs. 26.9%	-1	-2.300 to 0.3002	No	ns	0.1852
5.3% vs. 26.9%	-1	-2.300 to 0.3002	No	ns	0.1852

Week 4

Control vs. SPC	-1.2	-2.500 to 0.1002	No	ns	0.0801
Control vs. 5.3%	-1.6	-2.900 to -0.2998	Yes	*	0.0103
Control vs. 26.9%	-2.6	-3.979 to -1.221	Yes	****	< 0.0001
SPC vs. 5.3%	-0.4	-1.700 to 0.9002	No	ns	0.845
SPC vs. 26.9%	-1.4	-2.779 to -0.02098	Yes	*	0.0454
5.3% vs. 26.9%	-1	-2.379 to 0.3790	No	ns	0.2291

Week 8

Control vs. SPC	-1.2	-2.500 to 0.1002	No	ns	0.0801
Control vs. 5.3%	-2	-3.300 to -0.6998	Yes	***	0.0009
Control vs. 26.9%	-3.2	-4.500 to -1.900	Yes	****	< 0.0001
SPC vs. 5.3%	-0.8	-2.100 to 0.5002	No	ns	0.3673
SPC vs. 26.9%	-2	-3.300 to -0.6998	Yes	***	0.0009
5.3% vs. 26.9%	-1.2	-2.500 to 0.1002	No	ns	0.0801

Table Analyzed **Spleen Data**

Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	4.669	0.5944	ns	No
Row Factor	19.42	0.0004	***	Yes
Column Factor	38.59	< 0.0001	****	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2.318	6	0.3863	F (6, 37) = 0.7753	P = 0.5944
Row Factor	9.641	2	4.821	F (2, 37) = 9.676	P = 0.0004
Column Factor	19.15	3	6.385	F (3, 37) = 12.82	P < 0.0001
Residual	18.43	37	0.4982		

Number of missing values	11

Number of families	3
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Week 2					
Control vs. SPC	-0.25	-1.700 to 1.200	No	ns	0.9665
Control vs. 5.3%	0.4167	-1.033 to 1.867	No	ns	0.8662
Control vs. 26.9%	-1.25	-2.700 to 0.2000	No	ns	0.1121
SPC vs. 5.3%	0.6667	-0.8835 to 2.217	No	ns	0.6573
SPC vs. 26.9%	-1	-2.550 to 0.5501	No	ns	0.3205
5.3% vs. 26.9%	-1.667	-3.217 to -0.1165	Yes	*	0.031
Week 4					
Control vs. SPC	-0.9167	-2.367 to 0.5333	No	ns	0.338

Control vs. 5.3%	-1.067	-2.453 to 0.3198	No	ns	0.182
Control vs. 26.9%	-2.267	-3.653 to -0.8802	Yes	***	0.0005
SPC vs. 5.3%	-0.15	-1.424 to 1.124	No	ns	0.9888
SPC vs. 26.9%	-1.35	-2.624 to -0.07644	Yes	*	0.0342
5.3% vs. 26.9%	-1.2	-2.401 to 0.0007265	No	ns	0.0502

Week 8

Control vs. SPC	-0.6	-1.874 to 0.6736	No	ns	0.5891
Control vs. 5.3%	-0.6	-1.801 to 0.6007	No	ns	0.5415
Control vs. 26.9%	-1.6	-2.801 to -0.3993	Yes	**	0.0051
SPC vs. 5.3%	0	-1.274 to 1.274	No	ns	> 0.9999
SPC vs. 26.9%	-1	-2.274 to 0.2736	No	ns	0.1682
5.3% vs. 26.9%	-1	-2.201 to 0.2007	No	ns	0.1313

Table Analyzed **Goblet Cells**

Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	5.414	0.2331	ns	No
Row Factor	7.451	0.0056	**	Yes
Column Factor	56.26	< 0.0001	****	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value

Interaction	3.367	6	0.5611	F (6, 48) = 1.403	P = 0.2331
Row Factor	4.633	2	2.317	F (2, 48) = 5.792	P = 0.0056
Column Factor	34.98	3	11.66	F (3, 48) = 29.15	P < 0.0001
Residual	19.2	48	0.4		

Number of missing values	0

Number of families	3
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Week 2					
Control vs. SPC	-1.8	-2.865 to -0.7355	Yes	***	0.0002
Control vs. 5.3%	-1.4	-2.465 to -0.3355	Yes	**	0.0054
Control vs. 26.9%	-1.8	-2.865 to -0.7355	Yes	***	0.0002
SPC vs. 5.3%	0.4	-0.6645 to 1.465	No	ns	0.7501
SPC vs. 26.9%	0	-1.065 to 1.065	No	ns	> 0.9999
5.3% vs. 26.9%	-0.4	-1.465 to 0.6645	No	ns	0.7501
Week 4					
Control vs. SPC	-1.2	-2.265 to -0.1355	Yes	*	0.0215
Control vs. 5.3%	-1.8	-2.865 to -0.7355	Yes	***	0.0002

Control vs. 26.9%	-2.2	-3.265 to -1.135	Yes	****	< 0.0001
SPC vs. 5.3%	-0.6	-1.665 to 0.4645	No	ns	0.4455
SPC vs. 26.9%	-1	-2.065 to 0.06455	No	ns	0.0727
5.3% vs. 26.9%	-0.4	-1.465 to 0.6645	No	ns	0.7501

Week 8

Control vs. SPC	-2	-3.065 to -0.9355	Yes	****	< 0.0001
Control vs. 5.3%	-2	-3.065 to -0.9355	Yes	****	< 0.0001
Control vs. 26.9%	-1.6	-2.665 to -0.5355	Yes	**	0.0012
SPC vs. 5.3%	0	-1.065 to 1.065	No	ns	> 0.9999
SPC vs. 26.9%	0.4	-0.6645 to 1.465	No	ns	0.7501
5.3% vs. 26.9%	0.4	-0.6645 to 1.465	No	ns	0.7501

Table Analyzed **Lamina Propria**

Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	5.882	0.2207	ns	No
Row Factor	0.1681	0.8845	ns	No
Column Factor	61.18	< 0.0001	****	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	4.667	6	0.7778	F (6, 48) = 1.436	P = 0.2207
Row Factor	0.1333	2	0.06667	F (2, 48) = 0.1231	P = 0.8845

Column Factor	48.53	3	16.18	F (3, 48) = 29.87	P < 0.0001
Residual	26	48	0.5417		

Number of missing values	0

Number of families	3
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Week 2					
Control vs. SPC	-0.8	-2.039 to 0.4388	No	ns	0.3254
Control vs. 5.3%	-1.2	-2.439 to 0.03880	No	ns	0.0608
Control vs. 26.9%	-1.6	-2.839 to -0.3612	Yes	**	0.0065
SPC vs. 5.3%	-0.4	-1.639 to 0.8388	No	ns	0.8256
SPC vs. 26.9%	-0.8	-2.039 to 0.4388	No	ns	0.3254
5.3% vs. 26.9%	-0.4	-1.639 to 0.8388	No	ns	0.8256
Week 4					
Control vs. SPC	-1.2	-2.439 to 0.03880	No	ns	0.0608
Control vs. 5.3%	-1.2	-2.439 to 0.03880	No	ns	0.0608
Control vs. 26.9%	-2.8	-4.039 to -1.561	Yes	****	< 0.0001
SPC vs. 5.3%	0	-1.239 to 1.239	No	ns	> 0.9999

SPC vs. 26.9%	-1.6	-2.839 to -0.3612	Yes	**	0.0065
5.3% vs. 26.9%	-1.6	-2.839 to -0.3612	Yes	**	0.0065

Week 8

Control vs. SPC	-1.2	-2.439 to 0.03880	No	ns	0.0608
Control vs. 5.3%	-1.2	-2.439 to 0.03880	No	ns	0.0608
Control vs. 26.9%	-3.2	-4.439 to -1.961	Yes	****	< 0.0001
SPC vs. 5.3%	0	-1.239 to 1.239	No	ns	> 0.9999
SPC vs. 26.9%	-2	-3.239 to -0.7612	Yes	***	0.0005
5.3% vs. 26.9%	-2	-3.239 to -0.7612	Yes	***	0.0005

Table Analyzed **Mucosal Fold**

Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	9.074	0.0689	ns	No
Row Factor	0.4263	0.7438	ns	No
Column Factor	56.15	< 0.0001	****	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	4.967	6	0.8278	F (6, 48) = 2.113	P = 0.0689
Row Factor	0.2333	2	0.1167	F (2, 48) = 0.2979	P = 0.7438
Column Factor	30.73	3	10.24	F (3, 48) = 26.16	P < 0.0001
Residual	18.8	48	0.3917		

Number of missing values	0

Number of families	3
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Week 2					
Control vs. SPC	-0.8	-1.853 to 0.2534	No	ns	0.1946
Control vs. 5.3%	-1.2	-2.253 to -0.1466	Yes	*	0.0198
Control vs. 26.9%	-1.4	-2.453 to -0.3466	Yes	**	0.0049
SPC vs. 5.3%	-0.4	-1.453 to 0.6534	No	ns	0.744
SPC vs. 26.9%	-0.6	-1.653 to 0.4534	No	ns	0.4362
5.3% vs. 26.9%	-0.2	-1.253 to 0.8534	No	ns	0.9574
Week 4					
Control vs. SPC	-0.6	-1.653 to 0.4534	No	ns	0.4362
Control vs. 5.3%	-0.6	-1.653 to 0.4534	No	ns	0.4362
Control vs. 26.9%	-1.8	-2.853 to -0.7466	Yes	***	0.0002
SPC vs. 5.3%	0	-1.053 to 1.053	No	ns	> 0.9999
SPC vs. 26.9%	-1.2	-2.253 to -0.1466	Yes	*	0.0198
5.3% vs. 26.9%	-1.2	-2.253 to -0.1466	Yes	*	0.0198

Week 8

Control vs. SPC	-0.8	-1.853 to 0.2534	No	ns	0.1946
Control vs. 5.3%	-0.8	-1.853 to 0.2534	No	ns	0.1946
Control vs. 26.9%	-2.8	-3.853 to -1.747	Yes	****	< 0.0001
SPC vs. 5.3%	0	-1.053 to 1.053	No	ns	> 0.9999
SPC vs. 26.9%	-2	-3.053 to -0.9466	Yes	****	< 0.0001
5.3% vs. 26.9%	-2	-3.053 to -0.9466	Yes	****	< 0.0001

Appendix 5: Trial 2 Histology Statistics

Table Analyzed **Liver**

Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	4.462	0.7102	ns	No
Row Factor	1.496	0.501	ns	No
Column Factor	11.52	0.3314	ns	No

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.074	3	0.3579	F (3, 25) = 0.4636	P = 0.7102
Row Factor	0.36	1	0.36	F (1, 25) = 0.4663	P = 0.5010
Column Factor	2.771	3	0.9236	F (3, 25) = 1.196	P = 0.3314
Residual	19.3	25	0.772		

Number of missing values	7

Number of families	2
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Week 2					
Control vs. SPC	-1.1	-3.122 to 0.9221	No	ns	0.4545
Control vs. Saponin	-1.3	-3.322 to 0.7221	No	ns	0.3116
Control vs. Oligosaccharide	-1.3	-3.322 to 0.7221	No	ns	0.3116
SPC vs. Saponin	-0.2	-1.729 to 1.329	No	ns	0.9837
SPC vs. Oligosaccharide	-0.2	-1.729 to 1.329	No	ns	0.9837
Saponin vs. Oligosaccharide	0	-1.529 to 1.529	No	ns	> 0.9999
Week 6					
Control vs. SPC	0	-2.093 to 2.093	No	ns	> 0.9999
Control vs. Saponin	-0.6	-2.622 to 1.422	No	ns	0.8462
Control vs. Oligosaccharide	-0.2	-2.222 to 1.822	No	ns	0.9928
SPC vs. Saponin	-0.6	-2.221 to	No	ns	0.7406

		1.021			
SPC vs. Oligosaccharide	-0.2	-1.821 to 1.421	No	ns	0.9862
Saponin vs. Oligosaccharide	0.4	-1.129 to 1.929	No	ns	0.8883

Table Analyzed **Spleen**

Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	5.797	0.6165	ns	No
Row Factor	7.435	0.1393	ns	No
Column Factor	8.594	0.4552	ns	No

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2.754	3	0.9178	F (3, 24) = 0.6077	P = 0.6165
Row Factor	3.532	1	3.532	F (1, 24) = 2.338	P = 0.1393
Column Factor	4.082	3	1.361	F (3, 24) = 0.9009	P = 0.4552
Residual	36.25	24	1.51		

Number of missing values	8

Number of families	2
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Week 2					
Control vs. SPC	-0.9	-3.737 to 1.937	No	ns	0.8175
Control vs. Saponin	0	-2.936 to 2.936	No	ns	> 0.9999
Control vs. Oligosaccharide	-0.7	-3.537 to 2.137	No	ns	0.9034
SPC vs. Saponin	0.9	-1.374 to 3.174	No	ns	0.6979
SPC vs. Oligosaccharide	0.2	-1.944 to 2.344	No	ns	0.9939
Saponin vs. Oligosaccharide	-0.7	-2.974 to 1.574	No	ns	0.8305
Week 6					
Control vs. SPC	0.25	-2.686 to 3.186	No	ns	0.9953
Control vs. Saponin	0	-2.837 to 2.837	No	ns	> 0.9999
Control vs. Oligosaccharide	-1	-3.837 to 1.837	No	ns	0.7661
SPC vs. Saponin	-0.25	-2.524 to 2.024	No	ns	0.9901
SPC vs. Oligosaccharide	-1.25	-3.524 to 1.024	No	ns	0.4438
Saponin vs. Oligosaccharide	-1	-3.144 to 1.144	No	ns	0.58

Table Analyzed	**Goblet Cells**

Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	3.597	0.6842	ns	No
Row Factor	11.12	0.0407	*	Yes
Column Factor	25.48	0.0285	*	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.009	3	0.3365	F (3, 25) = 0.5022	P = 0.6842
Row Factor	3.121	1	3.121	F (1, 25) = 4.658	P = 0.0407
Column Factor	7.15	3	2.383	F (3, 25) = 3.557	P = 0.0285
Residual	16.75	25	0.67		

Number of missing values	7

Number of families	2
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Week 2					
Control vs. SPC	-1.2	-3.084 to 0.6837	No	ns	0.3192

Control vs. Saponin	-1.4	-3.284 to 0.4837	No	ns	0.1992
Control vs. Oligosaccharide	-1	-2.884 to 0.8837	No	ns	0.4755
SPC vs. Saponin	-0.2	-1.624 to 1.224	No	ns	0.98
SPC vs. Oligosaccharide	0.2	-1.224 to 1.624	No	ns	0.98
Saponin vs. Oligosaccharide	0.4	-1.024 to 1.824	No	ns	0.866

Week 6

Control vs. SPC	-1.75	-3.700 to 0.1999	No	ns	0.0899
Control vs. Saponin	-1.4	-3.284 to 0.4837	No	ns	0.1992
Control vs. Oligosaccharide	-1.8	-3.684 to 0.08374	No	ns	0.0648
SPC vs. Saponin	0.35	-1.160 to 1.860	No	ns	0.9189
SPC vs. Oligosaccharide	-0.05	-1.560 to 1.460	No	ns	0.9997
Saponin vs. Oligosaccharide	-0.4	-1.824 to 1.024	No	ns	0.866

Table Analyzed **Lamina Propria**

Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total	P value	P value summary	Significant?

	variation			
Interaction	0.6225	0.9256	ns	No
Row Factor	0.1675	0.7266	ns	No
Column Factor	65.66	< 0.0001	****	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.1486	3	0.04955	F (3, 25) = 0.1548	P = 0.9256
Row Factor	0.04	1	0.04	F (1, 25) = 0.1250	P = 0.7266
Column Factor	15.68	3	5.226	F (3, 25) = 16.33	P < 0.0001
Residual	8	25	0.32		

Number of missing values	7

Number of families	2
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Week 2					
Control vs. SPC	-1.1	-2.402 to 0.2018	No	ns	0.1194
Control vs. Saponin	-1.5	-2.802 to -0.1982	Yes	*	0.0196
Control vs. Oligosaccharide	-2.1	-3.402 to -0.7982	Yes	***	0.0009
SPC vs. Saponin	-0.4	-1.384 to	No	ns	0.6821

		0.5841			
SPC vs. Oligosaccharide	-1	-1.984 to -0.01590	Yes	*	0.0454
Saponin vs. Oligosaccharide	-0.6	-1.584 to 0.3841	No	ns	0.3563

Week 6

Control vs. SPC	-1	-2.348 to 0.3475	No	ns	0.2002
Control vs. Saponin	-1.7	-3.002 to -0.3982	Yes	**	0.0072
Control vs. Oligosaccharide	-2.3	-3.602 to -0.9982	Yes	***	0.0003
SPC vs. Saponin	-0.7	-1.744 to 0.3438	No	ns	0.277
SPC vs. Oligosaccharide	-1.3	-2.344 to -0.2562	Yes	*	0.0107
Saponin vs. Oligosaccharide	-0.6	-1.584 to 0.3841	No	ns	0.3563

Table Analyzed **Mucosal Fold**

Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	9.735	0.3123	ns	No
Row Factor	1.638	0.4344	ns	No
Column Factor	20.07	0.0756	ns	No

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.729	3	0.5765	F (3, 26) = 1.249	P = 0.3123
Row Factor	0.2909	1	0.2909	F (1, 26) = 0.6303	P = 0.4344
Column Factor	3.565	3	1.188	F (3, 26) = 2.575	P = 0.0756
Residual	12	26	0.4615		

Number of missing values	6

Number of families	2
Number of comparisons per family	6
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Week 2					
Control vs. SPC	-0.6	-2.159 to 0.9593	No	ns	0.7188
Control vs. Saponin	-0.6	-2.159 to 0.9593	No	ns	0.7188
Control vs. Oligosaccharide	-0.6	-2.159 to 0.9593	No	ns	0.7188
SPC vs. Saponin	0	-1.179 to 1.179	No	ns	> 0.9999
SPC vs. Oligosaccharide	0	-1.179 to 1.179	No	ns	> 0.9999
Saponin vs. Oligosaccharide	0	-1.179 to 1.179	No	ns	> 0.9999

Week 6

Control vs. SPC	-0.2	-1.759 to 1.359	No	ns	0.9847
Control vs. Saponin	-1.2	-2.759 to 0.3593	No	ns	0.1761
Control vs. Oligosaccharide	-1.2	-2.759 to 0.3593	No	ns	0.1761
SPC vs. Saponin	-1	-2.179 to 0.1787	No	ns	0.1177
SPC vs. Oligosaccharide	-1	-2.179 to 0.1787	No	ns	0.1177
Saponin vs. Oligosaccharide	0	-1.179 to 1.179	No	ns	> 0.9999

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