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Modification of Plant Proteins and Their Potential Application as Fish Meal Replacements in Rainbow Trout *Oncorhynchus mykiss* Feeds

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MODIFICATION OF PLANT PROTEINS AND THEIR POTENTIAL APPLICATION AS FISH MEAL REPLACEMENTS IN RAINBOW TROUT *Oncorhynchus mykiss* FEEDS

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DISSERTATION ACCEPTANCE PAGE

Miran J. Hama Salh

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

RBT: rainbow trout

FM: Fish meal

PPM: Parts-Per-Million

SBM: Soybean meal

FSBM: Fermented soybean meal

FFSBM: Full fat soybean meal

SPI: Soy protein isolate

SPC: Soy protein concentrate

DVSE: Davison variety solvent extracted

DVEXT: Davison variety extruded

TNSE: Triple null solvent extracted

TNEXT: Triple null extruded

RG: Relative growth

SGR: Specific growth rate

FCR: feed conversion ratio

Fulton's K: Fulton's condition factor K

FY: fillet yield

VSI: viscerosomatic index

SSI: spleensomatic index

HSI: hepatosomatic index

VFSI: visceral fat somatic index

HCT: Hematocrit

BPC: Barley protein concentrate

MS-222: Tricaine methane sulfonate

F75SBM:25BAR: Fermented co-blend of 75% soybean meal and 25% pearl barley

F75SBM:25BAR: Fermented co-blend of 50% soybean meal and 50% pearl barley

W75SBM:25BAR: Washed co-blend of 75% soybean meal and 25% pearl barley

W75SBM:25BAR: Washed co-blend of 50% soybean meal and 50% pearl barley

ABSTRACT

MODIFICATION OF PLANT PROTEINS AND THEIR POTENTIAL APPLICATION AS FISH MEAL REPLACEMENTS IN RAINBOW TROUT *Oncorhynchus mykiss* FEEDS

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Soybean meals (SBM) and its by-products are the most common plant feedstuffs that are used in feeds for a variety of fish species of fish over the last three decades due to high protein levels and good amino acid profile, in comparison with other plant protein sources. In the last two decades the production of soybean was around zero metric tons (Index, 2020). Barley is another plant protein that has been tested in some species diets. While barley is used in many areas such as brewing industry, animal feeding, human consumption, it is mainly used for animal feeding in Kurdistan. Including barley in aqua-feed is potentially important to develop aquaculture because of the high production and low price of barley in Kurdistan. The production of fish meal (FM) is non-existent in Iraq, but annual aquaculture production is growing. For example, global aquaculture production in 2011 was 61.8 million tons and this number increased to 80 million tons in 2016 (FAO, 2018). Fermentation, genetic modification, and extrusion are techniques that have been used to enhance ingredient nutrient values and reduce undesirable compounds in plant-based feedstuffs. In this study, performance (growth and digestibility) trials were conducted to evaluate barley and soybean meals, following additional processing to improve nutritional value, in Rainbow trout (RBT) diets. In Kurdistan the barley production is second top grain production after wheat. The Kurdistan requirement of food fish is 6,700

tons in 2011, however; only 21% of this demand is produced inside this region (IIG, 2013).

A 70-day experiment was conducted to evaluate two fermented, co-blends (75% washed soybean meal with 25% washed barley and 50% washed soybean meal with 50% washed barley) to replace fish meal (FM) in juvenile RBT feed. One reference and four treatment diets were fed. Three treatment diets were used to replace 25%, 35%, and 100% of FM with a fermented co-blend (75% of washed soybean with 25% washed barley). An additional diet was formulated to replace 25% of FM with fermented co-blend (50% of washed soybean with 50% washed barley). Fish were stocked in 25 tanks (16 fish/tank) providing 320 g (18$\pm$ 1 g) of biomass per tank in a recirculating aquaculture system (RAS). Non-significant differences were found in survival and growth metrics among all diet treatments. There were no significant differences observed among fish fed treatment diets for organosomatic indices such as the spleen somatic index (SSI), viscerosomatic index (VSI), visceral fat somatic index (VFSI) and hepatosomatic index (HSI). The VFSI value of reference diet was differed significantly in comparing with 100% replacing diet. The results of growth study presented that significant differences were only found between reference and 100% replacing diet. The values of HSI were significantly differed in comparing reference diet with both 100% replacing diet and 25% replacing diet by fermented co-blend (50% of washed soybean with 50% washed barley). In conclusion, FM can be replaced up to 35 % by fermented co-blend (75% of washed soybean with 25% washed barley) and 25% by fermented co-blend (50% of washed soybean with 50% washed barley) without any significant

negative in growth performance. Non-significant effects realized between all diets for intestine morphology tests.

A second study (155 days) was done to compare performance of novel soybean (termed Triple Null, TN) and a conventional variety (Davison, DV) in RBT diets as potential ingredients for the RBT aqua feed industry. Twenty age-0 RBT (~24 g each) were reared in 187 L, semi-square tanks of a 7,500 L RAS. The number of replication per diet was 5. Four diets were formulated to include 20% of TN solvent-extracted and extruded SBMs and similarly processed conventional SBM (DV), along with reference diets. All diets were included almost same level of protein, lipid, energy and ash. Rearing conditions, such as pH, oxygen, and temperature, were optimum for RBT. There was no significant difference among treatments for each of gain rates such as Specific growth rate (SGR) % and relative growth (RG) %, and feed efficiency. In addition, all diets did not significantly differ for each of SSI, VSF, VFSI and HSI indices, and distal intestine histological scores. In conclusion, all ingredients tested in this study can be included in RBT diets up to 20% without any significant effects of fish growth and health performance. Further, a 21-d digestibility trial was conducted to determine to know the level of protein digestibility in RBT diets. One hundred RBT (300 g each) were stocked into each of the six 756-L tanks of ~6000-L RAS. The duration of study was 21 day. Fecal samples were collected three times per diet. No significant difference was found among all diets for protein digestibility.

In conclusion, plant protein such as soybean and barley can be used in the carnivorous fish like RBT as an economical protein sources compare to FM. Using some processes such as using extrusion with temperature and fermentation can be a proper way

to improve plant protein as a protein source or FM replacement in RBT diet. Using genetic modified to reduce anti-nutritional factors was not results in improving using soybean in RBT diet in comparing with conventional soybean. Fermentation is a process used to improve the nutrition value of soybean and barley due to increasing the protein content and reducing carbohydrate content in barley and soybean meal.

CHAPTER 1. SOYBEAN AND BARLEY INGREDIENTS IN AQUAFEEDS

Introduction

Per capita global fish consumption has increased 3.1 % per year from 1961 to 2017 and the human population is growing by 1.6 % per year (FAO, 2020). Increased consumption has been attributed urbanization and income in the rising population (FAO, 2020). The total aquaculture production has increased from 59.7 million tons of live weight as average per year between 1986 – 1995 to 76.5 million tons in 2016 and 82.1 million tons in 2018 (FAO, 2020). In fish production, the price of feed is typically the highest cost and accounts for more than half of operating expenses. Thus, finding excellent quality and low price feed ingredients is very important to improving and sustaining aquaculture production (Cho and Slinger, 1979; Dadgar et al., 2010). Fish meal (FM) is a main protein source in the aquafeed industry but its production has decreased and price increased in the past decades (FAO, 2020). Most fish meal is mostly prepared from whole body of small, pelagic species which are not considered desirable for human consumption such as anchovies, menhaden, and sardine (Jobling et al., 2001).

Due to the high cost and limited supply of fish meal, many studies have been carried out to find suitable alternative proteins (e.g., cereal grains, legumes). Soybean meal (SBM) is the most commonly used plant protein that is considered a viable replacement for FM in aquafeed. Also, it has lower and more stable prices than FM. In the US Midwest, soy is be produced locally with more availability and cheaper prices than FM. In the past, soy products have shown excellent performance in many fish species diets through the application of byproducts such as soy protein concentrate

(SPC), soybean meal (SBM), fermented soybean meal (FSBM), soy protein isolate (SPI), and soy flour (USDA, 2012; Forster, 2002).

The presence of anti-nutritional factors in plant meals are a large consideration for using plant protein source in aquafeed. SBM includes several types of ANFs such as protein inhibitors, phytic acid, saponins, lectins, allergens, phytonutrients, and anti-vitamins (NRC, 2011). These ANFs have negative impacts of fish growth and health parameters in different aspects (Gatlin et al., 2007). Several processes are used to reduce and limit the amount of ANFs in plant sources. Utilizing high temperature during extrusion process is useful to reduce some types of ANFs such as trypsin inhibitors, lectin, and anti-vitamins. However, the heat process must be performed carefully because using very high temperatures and longer durations than the recommended levels leads to the reduction of protein bio-availability for fish (Francis et al., 2001; Drew et al., 2007). Fermentation is another option to increase protein digestibility and eliminate some ANFs in plant protein sources including oligosaccharides, trypsin inhibitor, and phytic acid. Fermentation increases the value of plant nutrients by the addition of single-cell proteins and essential amino acids derived from the micro-organisms (Francis et al., 2001; Barnes et al., 2012; Gatlin et al., 2007). Utilizing enzymes is another technique to reduce ANFs such as phytic acid. For example, phytase is used to eliminate phytic acids to increase the availability of phosphorus and other elements in soybean for fish (Storebakken et al., 2000).

Thus, there has been considerable research on replacing FM with plant feedstuffs to reduce cost and use of FM in feeds. For example, RBT (RBT; *Oncorhynchus mykiss*) feed prices have rapidly increased in the last few years due to FM prices (Gaylord and

Barrows 2008). Several studies have been conducted on replacing FM with plant protein, especially SBM in RBT diets, because of high protein content and appropriate amino acids are necessary to meet nutritional requirements (Barnes et al., 2013; Chainark et al., 2006; Mambrini et al., 1999; Heikkinen et. al. 2006; Gomes et al., 1995; Thiessen et al., 2004; Barnes et al 2014). A few researchers have also used barley protein as alternative to FM in fish diets (Degani et al., 1997; Burr et al., 2011; Burr et al., 2013).

The primary objectives of the present study were: (1) to investigate the effects of fermented co-blended protein concentrates (CPC) as FM replacements on growth performance and health assessments of juvenile RBT; and (2) to investigate the use a genetically modified soybean (reduced soybean agglutinin [lectin] and trypsin inhibitor) in RBT feed.

Background

Fish Meal and Plant Protein Feedstuffs

Fisheries production is utilized for human nutrition or non-nutrition purposes. Human consumption has been on the rise from 71% of fisheries production in 1980 to 86% in 2012 (FAO, 2014). FM is a part of the non–food use market, and production has declined since 2005. Also, the price of FM, the "main protein component and essential amino acids source in fish feeds," has increased because of growth in aquaculture, more than doubling over the past decade (FAO, 2020). For example, Figure 1 depicts the price of Peruvian FM (anchovy, 65% protein) from 1980 to 2016. The trends shown in Figures 2 and 3 indicate that FM production of cannot be sustained at same rates as aquaculture production growth. Because of this difference, researchers must find sustainable, cost-

effective alternatives for FM such as plant protein for commercial production of fish feeds (Hardy, 2010). Several studies have indicated that plant proteins can replace at least a portion of FM in carnivorous fish diets (Chou et al., 2004; Lim et al., 2004; Hernandez et al., 2007; Pham et al., 2007; Lim and Lee, 2008).

Plant protein sources must possess a number of characteristics to be a feasible alternative to FM, such as wide availability, economical price, ease of handling, and storage. In addition, it must include a low amounts of fiber and starch, mostly non-soluble carbohydrates and anti-nutrients, and also must contain a relatively a large amount of protein and essential amino acids with a high level of digestibility and palatability (Gatlin et al., 2007).

Protein utilization must be boosted and the anti-nutritional factor effects must be minimized to increase the level of plant protein inclusions in carnivorous fish diets (Lim et al., 2008). In addition, protein utilization and growth in carnivorous fishes often decreases when replacing FM by plant protein in the feeds, such as SBM (Refstie et al., 2006; Aslaksen et al., 2007). When conducting research in replacing animal feedstuffs in fish diets with plant ingredients, it is important to consider the complete nutritional requirements and carbohydrate tolerance of a particular species (Wilson, 1994). Also, it is necessary to consider the amount and type of anti-nutrients that may have a negative effect on protein and energy digestibility and fish health (Francis et al., 2001; Brinker & Reiter, 2011). The main concern for replacing FM with plant protein is reduced feed conversion which is typically commensurate with the decrease in protein utilization (Robaina et al., 1995; 2000; Opstvedt et al., 2003). Plant protein concentrates and isolates contain higher protein levels and reduced carbohydrates than base plant meals, thus

providing better alternatives for replacing FM in aquaculture diets (NRC, 2011, as cited by Rossi JR, 2013). For example, Slawski et al. (2013) indicated that canola protein isolate could be a promising FM alternative in juvenile RBT diet.

Alternatively, Hernande and Roman (2016) utilized blends of defatted SBM, lupin meal and corn gluten meal as a partial FM replacement in RBT feed. Gaylord et al., (2008) indicated that apparent protein and energy coefficients are higher in plant protein concentrate than in base plant meals due to the processing methods used to make protein concentrates. Feed containing concentrate has a positive impact on protein and energy digestibility, which leads to improved nutritional value for fish and better feed efficiency. Protein levels and amino acid profiles found in FM are more balanced in regards to nutrient requirements when compared to amino acids profile in plant protein. Hence, it is often necessary to supplement amino acids to diets when a high ratio of plant protein is used in feed (Gaylord and Barrows, 2009; Lim et al., 2008; as cited by Burr et al., 2012). In general, when fish are fed high plant protein diets, there is a decrease in production performance (Gomes et al., 1995; Kaushik et al., 1995; 2004; Mambrini et al., 1999; Dias et al., 2005; Torstensen et al., 2008). This may be the result of their diets having an imbalanced amino acid content and / or ANFs present in the plant feedstuff. But even with a balanced amino acid profile, the performance often decreases with increasing plant components in diets (Fournier et al., 2003, 2004, cited by Espe et al., 2012). Francesco et al., (2007) indicated that it is possible to replace (75%) of FM in the Sea Bream (*Sparus aurata*) diet with a mixture of plant protein ingredients such as wheat and corn gluten, wheat and rapeseed meals, without any change in fish weight gain and with a small impact on fish quality. As a result of replacing FM with plant ingredients, there is often

decreased weight gain in RBT (Pongmaneerat & Watanabe, 1992; Gomes et al., 1995; Kaushik et al., 1995). However, with sufficient processing, plant protein (Table 1) maybe the superior choice to replace FM because of the nutritional value and lower cost.

Rainbow Trout Production

Global RBT aquaculture production has expanded from 752.4 tons in 2010 to reach 848.1 tons in 2018, (FAO, 2020). Currently, there are no accurate records on whether fishes that are sold in the Kurdistan market are cultured fish or wild capture fish. However, production in the Kurdistan Region was estimated to be 2,462 tons in 2013; however, the Kurdistan region requires 6,700 tons of fish per year for human consumption (Kurdistan border investment, 2013). Today, most of the RBT in the Kurdistan market are imported from Iran except for a small percentage that is produced in Kurdistan. The Kurdistan Region has several natural, cold-water resources, such as streams and large springs, in the mountainous territories that may be suitable for RBT production. However, these resources are not abundant, therefore the Kurdistan Regional Government needs to identify better culture systems to utilize these resources efficiently as well as facilitating the develop development of economical RBT feed manufacturing and distribution.

Farmers in the Kurdistan region attempt to rear RBT as a commercial product. However, trout rearing is a new venture for most of the farmers. They do not have the culture experience, equipment, or life cycle feeds for RBT production. The production facilities, such as hatcheries to produce fingerlings and aquaculture feed plants to manufacture pellets, are not readily available in the Kurdistan Region. Consequently, the

farmers depend on Iran to obtain these resources for RBT production (Kurdistan Regional government – Ministry of Agriculture report, 2013).

Rainbow Trout Nutrition

Known RBT nutrients requirement are given in Table 2. For RBT and other salmonids, the common, desirable protein source is FM (Satia, 1974; Cheng and Hardy, 2004). Cheng and Hardy (2003) explained that apparent digestibility coefficient (ADC) of barley meal for protein in RBT was not different than the ADC for SBM. Using barley for RBT production in Kurdistan may be the best option because it is readily available at an economical price, has a good nutrient profile, and is widely cultivated in the region. In addition, it is not used widely for human nutrition and would not compete with a human food commodity. Another plant protein available is wheat (Randal and Drew, 2010).

A number of issues exist (e.g., low protein, unbalanced amino acids, and anti-nutritional factors) with the use of unprocessed cereal grains and oilseeds (Storebakken, et al.,2015; Tusche, et al., 2012; Santigosa et al., 2008; Monentcham et al., 2010; Hansen, et al., 2007). Hence, economical processing techniques must be applied to plant-based meals to upgrade and secure the highest nutritional value (OECD, 2015) Common approaches include extrusion (Barrows et al., 2007), and microbial conversions, such as fermentation (Skrede, 2002).

Fermentation

Fermentation is a process applied to plant feedstuffs to improve aroma, test, texture, and nutritional value of food. Fermentation is commonly used to prepare and preserve food as well as provides safer food for consumers (Mensha, 1997; Steinkraus,

2002). Fermentation has several effects on the nutritional value of food such as the reduction of non-digestible carbohydrate (oligosaccharides and polysaccharides), the microbial production of certain amino acids and the improvement of the use of B group vitamins (Nout and Ngoddy, 1997).

The traditional fermentation of cereal is mostly conducted by adding bacteria, yeast or both to the cereal food (Table 3). The common genera of fermenting bacteria are *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Pediococcus*, *Micrococcus* and *Bacillus.* The main fermentation bacteria are Lactic Acid Bacteria (LAB) while alcoholic fermentation is occurred by yeast fermentation. Additionally, a number of fungal types such as *Aspergillus, Penicillium, Fusarium,* and *Cladosporium* may be used in some aerobic production. In cereal fermentation, lactic acid fermentation has been indicated as the main fermenting mechanism. The genus *Lactobacillus* is the most important member in the LAB using in food processing (Davidson and Hoover, 1998; Olasupo et al., 1997; Opere et al., 2012). *Lactobacillus* spp. are an important bacteria used for in most cereal grain fermentative (Sanni, 1993). Lactic acid fermentation of some aquatic plants reduced some anti-nutrition and fiber content and had a positive impact on the nutritional quality (Cruz et al., 2011). Fermentation of barley at low pH increases the genus CFU, and decreases fiber, soluble β-glucans and non-starch polysaccharide (NSP) (Kianfar et al., 2013). Pedersen and Lindberg (2003) showed increasing digestibility of organic matter and crude protein in vitro as result of fermentation.

During fermentation, the pH encourages activity of existent proteolytic enzyme, converting long polypeptide proteins to shorter polypeptides making it more digestible (Monawar, 1983 as cited by Alka et al., 2012). Ali et al., (2003) indicates that

fermentation in food grains results in the decrease of anti-nutrients and the improvement of protein and starch digestibility. Kianfar et al., (2013) showed that fermentation led to the reduction of the (NSP).

Fermentation alters long chain proteins to smaller chain proteins to make proteins easier to digest and metabolize in young animals. Also, the fermentation process reduces trypsin inhibitor and indigestible oligosaccharides (e.g., raffinose and stachyose) that can decrease nutrient digestibility and have a negative impact on gut health (Barnes et al., 2012). Fermentation may also lead to improved content and quality of cereal proteins (Wang and Fields 1978; Cahvan et al., 1988). Alka et al., (2012) showed that fermentation significantly improved digestibility of in vitro protein and starch for sorghum, maize and pearl millet. Kohajadova and Karovicova (2007) explained fermentation has a nutritional value advantage in cereals by increasing protein digestibility and mineral bioavailability. Fermentation of maize led has been shown to increase protein digestibility and improve nutritional value of maize grain (Yousif and El Tinay, 2000). Skrede et al., (2002) reported that fermentation enhances protein digestibility in wheat and barley whole meal flours when used in Atlantic salmon *Salmo salar* diets. Fermentation also improves the availability of lysine and nutritional value in cereals (Hamad and Fields 1979).

The fermentation by bacteria is expected to increase the bioavailability of essential amino acids that are higher than provided by yeast fermentation because bacterial fermentation includes proteolytic activity (Chaven and Kadam 1989). Additionally, fermentation typically causes positive developments in amino acid balance [Au and Fields, 1981]. In general, aerobic conversion or fermentation improves protein

digestibility and bioavailability of minerals such as zinc and iron in sorghum due to decreases in anti-nutritional factor particularly tannins, phytate and enzyme inhibitor during fermentation (Afify et al., 2012). Also, Sindhu & Khetarpaul (2001) indicated significantly reduced of anti-nutritional factors (phytates, trypsin inhibitor activity) in food mixtures that included barley during fermentation. Also, Osman (2004) noted that fermentation reduce anti-nutrients such as phytic acid and tannin. Marklinder et al., (1996) explained that lactic acid fermentation decreases phytic acid in barley flour because of bacterial phytase. The decrease of phytic acid occurred via fermentation in both barley and wheat (Skrede et al., 2002). Teleost fishes have limited ability to use organic phosphorus that exists in bound phytic acid, which also impacts the availability of minerals such as zinc, magnesium, and iron (Storebakken et al., 1998).

Barley Products as a Potential Aqua-feed Ingredient in Kurdistan

The production of barley was about 11,584,060 tons in 2011 in Kurdistan Region-Iraq; the price of one ton of whole barley is 500 USD (Kurdistan regional government-ministry of agriculture and water source report, 2013). Barley is mainly used for animal in Iraq; for example, in 2019, the production of barley was 1600 tons, and the total supply of barley was 1.611million metric tons. Approximately 1.200 million metric tons was used for animal feeding (USDA, 2020). The Barley grain has a good composition profile (Tables 4, 5) that has applications for animal feeds. Barley is also a grain source used to produce ethanol in the Northern Plains and Pacific North-west. Barley was using ethanol production before came in fish feed industry. For example, barley protein concentrate is utilized in aquaculture diets because of quality and amount of its protein (Flores et al.,

2004; Gatlin et al., 2007). Barley protein concentrate is likely a suitable choice to use as a FM replacement (Table 6, 7). Wheat, another popular feed ingredient, is the top cereal crop in Kurdistan, Iraq. The estimated production of wheat in Kurdistan was 2,262.996 tons in 2019. The production of wheat in Iraq increased from 3,000,000 tons in 2018 to 6,000,000 tons in 2020. The price of wheat in Iraq is between $352 to 468/ton USD. Wheat is mainly used for human consumption in Iraq; for example, in 2019 imported wheat was 2,300,000 metric tons and the total supply of wheat was 9,787,000 metric tons. Approximately 7,200 tons was used for human consumption and 1,400 for animal feeding and seed (residual) (USDA, 2020). In the last two decades, the soybean production in Iraq was near zero metric tons. All soybean meal used in animal feed is imported from other countries; for example, the volume of imported soybean meal to Iraq was 275,000 metric tons in 2019 (Index, 2020).

The recent developments in de-hulling and fermentation processing of barley have led to improvements in making protein concentrate. Barley protein concentrate can be produced that includes 600g/kg of the protein that is highly digestible and palatable in fish (Gaylord et al., 2008; Burr et al., 2011). Barley concentrate contains good nutritional content and contains a lower level of anti-nutritional factors (Table 8) than a number of plant feedstuffs (Gatlin et al., 2007 as cited by Rossi et al., 2013). Also, Rossi et al., (2013) indicated in their research that barley protein concentrate or soy protein concentrate that used in their study were suitable for replacing FM in Red Drum *Sciaenops ocellatus* diets; 50% of fish meal protein was replaced with barley protein concentrate or soy protein concentrate without any negative effect in weight gain and whole body composition. Sealey et al. (2008) explained that barley from select genotypes

can be used in place of wheat ground in RBT feed without any significant negative effect of growth, also Sealey et al. (2008) indicated that β-glucans contained in barley may improve disease resistance in RBT.

Barley Chemical Contents and Nutrient Value

The major content of barley contains 80% carbohydrates. Starch consists of 55% of the whole carbohydrate in barley. The primary content of endosperm is starch which mainly contains amyloseis (75%) and (25%) of amylose.

Amylose is a long strain of the glucose without branches while Amylospectin contains huge branches of glucose. Starch is the main energy source in animal feed. The rate of non-starch polysaccharides and lignin is 10-20% of the entire carbohydrate contents.

In general, the amylose rate is 25% of total starch and amylopectin is 75% of total starch in plants, these rates vary between different plants and different strains of single cereal; for example, the percentage of amylopectin is 100% of starch in barley wax and in some strains of barley, such as amylotypes barley, the percentage of total starch is 70%.

Lignin builds up 75% of cell walls in barley endosperm. However, arabinoxylans rate in cell walls are only 25% (Macewic Zetat, 2006). Acid detergent fiber (ADF) is a known test for measuring the amounts of cellulous and lignin (soluble fiber acid) barley. The unit of mixed biding of fiber is mixed bound. (1-3, 1-4) B glucans which creates 4.8% as an average. Also, the arabinoxlans content in barley fiber is between 4% and 8%.

Another study showed gluten percentage is between 2% and 10% of whole barley grain (Henry 1987).

The lipid content of barley is between 1.73% and 2.13%. The first abandon fatty acid in barley is linolenic. Lipids are linoleic acid in rate 56.6% followed by the oleic 19.94% then palmitic 8.53% (Ozcan et al., 2018). The primary content of lipid in barley is triglycerides which makes up 77.9% of the whole lipids. The barley lipid includes other components such as sterols, diglycerides, free fatty acid, hydrocarbons and sterol esters. The largest part of lipids is in the endosperm part which consist of 77% of total lipid followed by the embryo, 18%, then the hull is 5% of total lipids. Polyphenolic compounds such as proanthocyanidins and anthocyamin present in barley lipids.

The protein content of barley is between (8- 13) % (Pomeranz and Shands, 1974). There are four types of protein found in barley, which are albumin, globulin, prolamines, and glutelins. The amount of albumin and globulin is low in barley. Albumin and globulin are rich in lysine contents and they are sat/soluble proteins. The protein in endosperm is mainly prolamine which controls lower than 2% of lysine, present in endosperm part it is glutome present in endosperm part, it is structural protein which includes 4% of lysine. In general, the amount of lysine and threonine are limited in barley (Kulp, 2000).

Use of Barley Protein in Rainbow Trout and Atlantic Salmon Diets

A feeding trial was conducted to evaluate using BPC in Atlantic salmon diets. Twelve tanks were used. Each tank included 40 fish weight 123 gm weight. Three diets were introduced to fish, one reference diet and tow experimental diets which contain 11% and 22% of BPC. The duration of the study was 16 weeks. Temperature and salinity of the water were 12 C^0 and around 2, respectively. No significant difference were found in specific growth rate, feed conversion, weight gain, and proximate composition of the

salmon for protein, lipid, moisture, and ash among all diets. However, there was significant difference between the diets contain 22%v of BPC and other tow diets which include 11% and 0% of BPC. The study results concluding that the BPC can be used by under level 22% in Atlantic salmon diets and performing as reference diet (Burr et al., 2013).

Eighty four days study was carried out to show the influence of inclusion barley in diet of RBT mainly on fish meat quantity and, secondary on digestibility and growth performance. The study used four treatment diets to used barley from 40 gm/kg up to 319 g/kg of diets and reference diet. The inclusion different amount of barley up to 32% of whole diet had not significant negative effects on growth performance, and digestibility of lipid and carbohydrate in comparing with reference diet. The features of fish meat such as the water activity, texture and the color of meat were improving with increasing the barley quantity in diet. However, the amount of digestible protein was decreased in treatment diets. The blood glucose, indictor of stress, was significantly increasing with higher inclusion of barley in diets (Pinedo-Gil et al., 2017).

A feeding experiment was conducted to test the influence of including three genotypes of barley variety amounts of β-glucan (low, average, and high β-glucan contain) on growth performance, disease resistance and immune response of RBT. Three treatment diets were formulate. Each diet was used to replace all amount wheat in control diet by one of three barley genotypes. One control diet was inclusion zero barley and added by yeast β-glucan product in rate 2gm per Kg. the duration of study was 9 weeks. Each diet was introduced to on tank which was included 50 RBT with average initial weight 14.3 gm. The study results indicated that in the end of the 9 weeks study the

growth performance was not statistically changed among all diets and the fish resistance for viral disease was increased by using barley β-glucan in diets (Sealey et al., 2008).

Soybean Chemical Contents and Nutrient Value

The average oil and protein composition is about 60% of the dry matter in whole soybeans. The carbohydrate fraction is about 35% of dried soybeans (Liu, 2012). The range of water in whole soybean is between (5.6% - 11.5%), and for crude protein between (32%-43.6%). The oil content is between 15.5% and 24.7%. Ash percentages typically range from 4.5% to 6.4% and the carbohydrate content ranges between 31.7% to 31.85% (Ensminger et al., 1990; NRC, 1998; Poultry Feeding Standards, 2005: Banaszkiewicz, 2011). (Table 11) shows the average content of soybean seed and by-products. The nutritional content in soybeans is affected by genetics, geographic location, environmental and seasonal differences. (Liu, 1997).

Full-fat soy is de-hulled and cleaned after harvest. The dried de-hulled soybean seed contents consist of 30.1% of protein and 23.3% of oil. The majority of soy is used for oil products, the byproduct of this process (solvent extracted or extruded) is SBM which includes a higher quantity of protein and around 1% of oil. Researchers, feed ingredient and feed manufacturers endeavor to make soybeans richer in nutritional value as a protein source (table 11). These soybean byproducts include soy protein concentrate, soy protein isolate, and antigen-free soybean protein concentrate. In the production of these soy protein sources, many improved processes are utilized such as biological, enzymatic, and thermal and pressure processes (Mukherjee et al., 2016; Brane et al., 2014; Biswas et al., 2007; Lewis et al., 1973). SBM is a by-product which is obtained after extracting the oil from full fat soybean by a solvent (typically hexane) (Cheng and

Rosentrater 2017). SPC is manufactured by extracting soluble carbohydrate (stachyose and raffinose) from SBM using isoelectric leaching, aqueous alcohol, or degrading carbohydrases (Phillips and William 2011; Peisker 2001). SPI is made through several process steps, starting with extraction at pH 9, acid precipitation, and then followed by separating the precipitated proteins by centrifuging and washing, and drying the protein (Barbosa et al., 2006; Wang et al., 1998).

Carbohydrates

In SBM, the carbohydrates appear mainly as two groups. First, as sugars such as mono-, di- and oligosaccharides and second as non-starch poly saccharides (NSP); although there is 1% of starch content in carbohydrate in soy (Choct, 1997). The rate of free sugar in soy NSPs is nearly 10% and appear as three groups, including 4% stachyose, 5% sucrose, and 1% raffinose. Nutrient analyses of soy seeds show that sugar and protein are inversely proportional (Hartwig et al., 1997). The second group, NSP, consists of 8% cellulose, 20%-30% non-cellulosic, and the remainder is pectin. Two types of carbohydrate are found in soybean based on compound structure as structural carbohydrate, for example, cellulose and hemi-cellulose and non-structural carbohydrate like sugars and oligosaccharide (Grieshop et al., 2003). Based on water solubility, the polysaccharides are divided into soluble and non-soluble contents such as dietary fiber. The quantity of soluble and non-soluble, the structure of the NSP and its physiochemical features vary extensively between different soybean varieties. Soybean NSP contains pectic polysaccharides such as types I and II of rhamnogalacturonans, xylogalacturonan, and I-arabinogalactan (Fransen, 1999). The levels of the NSP's compounds in soybeans vary with geographic location, harvest conditions, and processing after harvesting (Karr-

Lilienthal et al., 2005). Alpha-galactosidic bond is used to bind saccharose and galactose to create oligosaccharides and constitutes alpha- galactosidic compounds.

Protein

Soybean seed averages around 40% protein, but ranges from 32% to 43.6% (El-shemy, 2011) and the protein level in SBM can range from 40% to 49% with an average of 44%. Two types of SBM protein are soy protein globulin and albumin. Generally, the globulin content is higher than albumin. The globulins are characterized as salt extractable while the albumin is soluble in water (Derbyshire et al., 1976). The globulins, based on their sedimentation coefficients can be divided more into 7s globulins and 11s globulins. The 7s globulins can be classified into other compounds based on their different functions (Utsumi et al., 1997). The 11s globulin, glycinin, contain disulfide-linkage and essential amino acids in soy protein (Adachi et al., 2003). Soybeans, as a leguminous plant, contain a low level of sulfur amino acids such as methionine, tryptophan, threonine and cysteine. However, soybean includes a significant level of lysine which is limited in cereal grains. About 19 to 20% of total amino acids in soybean are hydrophobic acids while 9.1% to 9.8% are aromatic acids (Liu, 2012).

Lipid

The mature cell of soybean grain doesn't include mitochondria, nucleus and other organelles which are replaced by large mass of protein bodies with oil mass. The majority of soy oil is consists of triglyceride (95-97) %. Triglyceride is formed through the binding of three fatty acids and single glycerol. The nutritional benefits of soybean oil are based on the type of the lipid acids in triglyceride and other soybean properties. Unsaturated fatty acids constitute the majority of soybean fatty acids. The levels of

different unsaturated lipid acids in soybean from highest to lowest sequence are linoleic acid, oleic acid, palmitic acid, linolenic acid, and stearic acid (Liu, 1997). The range of phospholipids in soy oil is between (1.5-2.5) percent. Most of soy phospholipids consist of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, and phosphatidic acid (Pryder, 1980).

Ash

The amount of ash based on a dry matter basis is around 5% in soybeans. Most of ash weight is coming from its oxygen content while the main mineral forms in ash consist of sulfates, phosphates, and carbonates. In total, the remaining minerals, which includes potassium, magnesium, calcium, sodium, and sulfur, in soybean ash ranges between 0.2 to 2.1%. Iron, magnesium, and copper form minor levels of soybean minerals, only 0.01 to 140 ppm. Certain amounts of calcium, magnesium, and sulfur are extracted during the oil extraction process with phospholipid separating with the soybean oil (liu, 2012).

Vitamins

Soybean includes both groups of vitamins, water- and oil-soluble vitamins. The majority of water-soluble vitamins existing in soybeans are riboflavin, thiamin, folic acid and niacin. The oil extraction process does not cause the loss of water-soluble vitamins in soybeans. The amount of vitamin C in mature seed is high but in immature is a trace. Vitamin A and E are oil-soluble vitamins. However, the quantity of vitamin D and K in soybeans is not mentionable. Vitamin K is found in beta-carotenes forms. Vitamin E and A as Vitamin C is abundant in mature soybean grain. The quantity of vitamin E is different in each soybean variety. Vitamin E has four different isomers which are alfa, beta, gamma, and tetra. The amount of beta is low. Vitamin E-gamma, folate, and

thiamine in soybean seed ranges between 10.2 to 28.4, 150 to 191, and 24.6 to 72.5 mg/kg^{-1}, respectfully (Liu, 2012).

Anti-nutritional Factors

Anti-nutritional factors (ANFs) in plant seeds limit the use of plant protein sources as an economical replacement for fish meal protein, particularly in animals with simple digestion system such as fish and poultry. ANFs are chemical compounds that can interact with nutrient ingredients in animals directly or indirectly through ANF's metabolic by-products. Several ANF'S were indicated in raw and processed soybean (Table 12).

Anti-nutrients are classified according to several features, such as their chemical characteristics, biological activities, or their endurance of heat treatment (Tacon, 1985, Francis 2001) The classification of ANFs according to their biological activities include: 1) reducing protein bio-availability in fish feeding via protease inhibitors and lectins; 2) factors that affect mineral digestibility such as phytic acid, gossypol and glucosinolates; 3) anti-vitamins for B12 and A; and 4) miscellaneous factors such as estrogenic compounds.

Trypsin Inhibitors

Trypsin inhibitors consist of two groups of proteins, Bowman-Birk and Kunitz. Bowman-Birk inhibits powerfully tryptic and chymotryptic enzyme. Kunitz inhibits trypsin highly and lightly chymotrypsin (Takii et al., 2001)). These inhibitors act against protein enzymes such as protease and making them inactive reducing ingredient protein digestibility. Also, these inhibitors can have a negative impact on pancreatic function through effects on enzyme feedback. The activity of trypsin inhibitors can be eliminated

by 90% using heat treatment (Wolf, 1976; Lucus and Rhee, 1995; Refstie and Storebakken, 2001).

Genetic modification has successfully reduced a portion of the inhibitors from soybeans. Friedman and Brandon (1991) showed around 20% of Kunitz and Bowman-Brink inhibitors remained active after using heating treatment on soybeans. The structure of Bowman-Brink, having seven disulfides in its structure, is more stable than Kunitz inhibitor during heat and acid treatment (Wolf, 1976).

Lectins (agglutinin)

Lectins are chemically complex between protein and sugar (glycoproteins) which includes one or more non-catalytic site. They are strongly linked to terminal N-acetyl-D-galactosamine to a smaller extent, D-galactose. Lectins can be classified based on their level of its denaturation to agglutinating non-agglutinating lectins. Lectins cannot be digested in fish (Fasina et al., 2003) because of the quaternary structure that can bind to carbohydrates and agglutinates cell membranes. Lectin binding feature in soybeans can cause damage of internal cells of fish intestines and morphological damage (Hendriks et al., 1990; Van der Ingh et al., 1991)

Saponins

Saponins are a class of bitter class of compounds generally found in plants. Several different monosaccharides are in the structures of saponins consisting of: glucose, glucuronic acid, galactose, rhamnose, arabinose, xylose and fucose. Saponins structure includes different compounds of aglycones and sugars; thus, saponins group has large number of chemical, physical, and biological features (Guclu-Ustundog and Mazza, 2007). Rickert et al., (2004) showed that saponin may interact with some storage proteins

in soybeans such as glycinin and B-conglycinin. The amount of saponins compounds in soybeans and soybean by products are slightly high (Hu et al., 2002). The amount of saponins differs in soybean based on processing, seed age, storage conditions, geographic location and breed (Mastrodi Salgado and Donado-Pestana, 2011). Saponins can be altered with different processing and storage methods. Saponins compounds are excellent foaming agents because they have both polar and non-polar side compounds (Mastrodi Salgado and Donado-Pestana, 2011; MacDonald et al., 2005). Two groups of saponins exist in soybeans. The A and B group contains the majority of saponins in soybean seeds and SBM (Hu et al., 2002; Rickert et al., 2004). Saponin bitterness decreases palatability and feed intake (Chen et al., 2011). It also has a negative effect on fish growth by reducing protein digestibility (Chen et al., 2011 and Francis et al., 2001). Saponins may have toxic effects on fish and invertebrates because it is ability to form foamy solutions in water and changing the intestinal histology and decreasing growth (Lin et al., 2006; Salgade and Pestane, 2011; Bureau et al., 1998).

Phytic Acid

Phytic acid is created by the ester bond between phosphate molecules and six hydroxyl groups which exist in the compound myo-inositol. The molecules bind to F^2 Mn^{2+} Zn^{2+} and K^4 due to releasing the H4 ion from phosphate group to form phytic acid. Phytates are stored 70% in the total amount of the phosphorus in soybeans (Smith and Rackis, 1956). In addition, phytates can be a factor of reducing protein digestibility by introducing protein produced phytates-mineral-protein compounds (Morales et al., 2012; Refstie and Storebakken, 2001)**.** Phytase is used for breaking the phytic acid and releases

the phosphorus, protein and other elements improving availability to fish (Kumar et al., 2012).

Non-starch Polysaccharides (NSPs)

Non-starch polysaccharides cause a high level of velocity in digested nutrient in the intestine which results in reducing the digestibility of nutrient ingredients. The amount of NSP- degrading Enzymes such as hemicellulase, cellulase, xylanase, B-glucanase, pectinase and a-galactosidase are low in the fish's digestive system and thus fish can't degrade all NSP in soybean, when NSP- degrading Enzymes were added in fish diets, a positive effect on using nutrient ingredients in fish is showed (Sinha et al., 2011; Glencross et al., 2012). Some oligosaccharides can be digested by bacteria which commonly lives in the intestine in poultry (Patterson and Burkholder, 2003). Soy bean Oligosaccharides is part of NSP, are soluble which is detected in soybeans that includes stachyose and sucrose that are bound by mono and oligosaccharide bonds (Zhou et al., 2012).

Antigen

Glycine, and B-conglycinin's are the main antigens in soybean. They are indigestible proteins that stimulate immune system to produce antigen-specific antibodies which damages intestinal mucosal and villi (Hel et al., 2015, Kumar et al., 2012). These non-esteroidal estrogenic substances continue to be widely distributed in plant. They have estrogen activities and properties, and can bind to estrogen respecter or can be converted to some compounds that mimic estrogen. Estrogen has a role in a several physiological actions in fish such as stimulate vitellogenesis synthesis and rise concentrate of vitellogenin protein in blood plasma (Hajra et al., 2013; Kumar et al., 2012).

Phytoestrogens are mainly isoflavones which present commonly as glucosides (Francis et al., 2001).

Other ANF's

As antivitamin B12 and antivitamin E are part of soybean's ANFs (Shurtleff and Aoyagi, 2010; Norton, 2013).

Use of Soybean Protein in Rainbow Trout Diets

Several studies have investigated the use of soybean products in diets for RBT and other species. In a study showed that FM replaced partially 69.4% of protein. The soy protein was obtained from a combined protein, which is made from soy concentrated protein and SBM protein in the rate of 4:1. The study is conducted on large fish (250) g for 24 weeks (Vilma et al., 2000).

In another study that was conducted for 8 weeks in two different trails to observe the possibility of using SBM and SDI in florid pompano (*Trachinotiz carolinues*) 80 % of FM protein can be replaced by SBM without having negative impact on growth performance in first trial. In another trial 40% of FM protein is replaced by SPI successfully without effects of the growth parameters of fish (Rich and Williams, 2019).

A study was carried out for 8 weeks on RBT to utilize extracted soy with full fat soybean. Six with full fat soybean. Six diets were formulated to include FSB extracted soybean from 7.5 to 32.5 of the total diet percentage. There was no significant changes among all six diets (reference and treatment diets) in rearing performance and mortality rates. However, decreasing was happening in yield of carcass. Proximate and color of fillet were no significantly changed by adding SBM and FFSM. Total Omega-3 fatty

acids and fatty acid 22:6n3 relative were not affected by adding FFSB with ES. The recorded results of histological hind-gut exam were not different in the end of the study (Morris et al., 2005).

A study has been conducted to show the effect of using Methionine and taurine diet with replacing 49% of FM by soy protein concentrate. Fish are fed those diets for 12 weeks. The results show that the 49% can be replaced by soy protein concentrate with using methionine supplements. Adding taurine did not show positive effects on fish growth (Boonyoung et al., 2012).

A research had been performed to evaluate 100% FM replaced protein diet by soy protein concentrate on liver and intestinal histology in RBT fish was fed 0.1 percent of its body weight for 90 days. Every 14 days, 10 fish were sampled for gut morphology exam. The results of the study explain which generally the morphology of the fish was not changed due to using replacement FM protein by SPC protein (Escaffre et al., 2007).

In a previous project two blends of plant protein (soy protein based and wheat based) were tested in a diet of RBT (weight 19.5 gm) and Atlantic salmon as a replacement of fish meal protein over a 12 week period. Three treatment diets were used for feeding RBT to replace 63%, 82% and 100 % of fish meal by blend-based soy protein. The growth performance and feed efficiency of diet 63% and 82% of plant protein were not significantly changed compared to the reference diet. The soy protein blend was improved and used in the juvenile Atlantic salmon as a replacement of fish meal three treatment were formulated to replace 50% and 60%, and 64% of fish meal. The diets were an 18-week experiment, all of the treatments significantly reduced the weight gain and feed efficiency in fish compared to reference diet. The same soybean

protein blends were improved more and used in larger Atlantic salmon 31.5 grams. The whole fish meal was replaced by this protein blend and introduced to the diet for 12 weeks. There were no changes in growth performance between experimental diets. (Burr et al., 2012).

A previous project of two trials were performed to use SPC and SPI as a possible FM replacement in Hybrid striped Bass. Both SPC and SPI were used to replace more protein, FM in two different study and growth in the diet which contain maxim in tolerance SBM protein (10% FM & 65% SBM). The results show that using less that 10 percent of FM in hybrid striped bass diet reduce of feed like intake because of reduction of palatability of diet (Blaufuss and Trushensky, 2012).

SPI was examined as an optional FM replacement in juvenile Amur Sturgeon (26, 38 $\pm$ 0.24g weight). For this purpose, performance experiment was conducted, 8 weeks, eight treatment diets were formulated to replace a variety of ratios (25, 50, 62.5, 75, 87.5, and 100) as percent of FM by SPI with one diet 100% FM replacement with addition of cysteine amino acid. The result growth parameters was same reference diet up to replacing 62.5% of fish meal. However, the diet which included replaced 62.5% FM failed to work as a reference diet for digestive enzymes activities. The conclusion of study was 57.64% of the fish meal can be replaced by SPI in Armor Sturgeon diets (Xu et al., 2012).

A study was conducted to examine PepSoyGen (fermented SBM) five treatment diets formulated to include (10, 20, 30, 40, 50) percent of PSG as a direct FM protein replacement. The experimental diets were administered over two periods, the initial 70 days followed by an additional 40 days to assess the different PSG ratios. The study

concluded with three main findings: first, there were no health assessment differences observed due to any of the diets. Second, there were no significant differences in weight gain and feed conversion when the diets included up to 30% PSG. Finally, weight gain was significantly reduced when the diets included 40% or higher PSG. (Barnes et al., 2012).

A study was conducted to evaluate bioprocessed SBM as a meal replacement in RBT for the purpose. Three diets, one reference, two treatment diets to replace 60% and 80% of fish meal protein were fed for 125 days. The result of study observed that replacing 80% of FM by bioprocessed SBM does not have any negative effects on the fish performance in growth and feed conversion. Also, changes in gut morphology and health assessment were not noted between all diets. (Voorhees et al., 2019).

PepSoyGen is a fermented soybean which evaluated in a previous study for trout feeding. Three experimental diets were formulated, one reference diet and two treatment diets. Two treatment diets include: 35% and 50% of PSG, to replace 37.5 and 100% of fish meal. The duration of the experiment was 205 days. The weight of the diet which includes 35% of PSG was similar to the reference diet weight gain. Also the average of the individual weight and length of those fish receiving 35% of PSG diet was higher than fish that were fed the reference diet. The record of the viscerosomatic index was lower in the fish that received 35% PSG of the diet. There were no significant changes of the health assessment between diets. There were no significant differences noted among the diets for morphology of distal intestine. Inclusion 35% of PSG can be used in RBT diet, without any negative impact on fish growth and health (Barnes et al., 2014).

Bioprocessed SBM were examined in study on RBT as a possible FM protein replacement for fish rearing in two different velocities (2-3 cm/s -1 and 18.7cm s-1). 2 treatment diets were formulated to replace 60 and 85 percent of fish meal. The two treatment diets were not significantly different with reference diet in weight gain and SGR. However, reference diet had significantly lower value of FCR and higher hepatosomatic index than diet which includes 85% BSM. The fish was rearing in the velocity 17-8cm/5 1 recorded significantly higher rearing performance and feed efficiency in all diets compared to fish that were reared in 2-3 cm/5 1 velocity (Voorhees et al., 2018).

A study was carried out for five months to evaluate including of SBM and full fat soybean (FFSBM) in trout diets. Five experimental diets were formulated to increase using SBM and FFSB more than 25 % (control diet) to replace more FM protein in RBT diets. Study results exhibited that up to 40% soybean and 11% of FFSBM were used as FM replacement in RBT diet without having negative effects on growth metric and feed efficiency (Harlioglu, 2011).

Palatability

Formally, palatability is defined as “acceptable to the taste or sufficiently agreeable in flavor to be eaten.” Feed characteristics that affect palatability include: the food content of toxins and nutrients, the animal nutritional requirements, and the historic experience of the animal with particular foods (Provenza, 1995). In nutrition research, determining whether a fish finds a flavor palatable, or not, can be difficult. However, feed intake can be determined by measuring the difference between feed fed and feed

consumed. The amount consumed is used as an inference to palatability by fish. This is a key in that regardless of whether an ingredient has acceptable nutrient profile and digestibility; if the palatability of the ingredient is not sufficient then the use of that ingredient will be limited in formulated diets. While several ways may be used to prevent issues of feedstuff palatability such as using feeding stimulants or additional processing, it is more economical if these approaches are unnecessary. One of the key principles of excellent nutritional research is determining the amount of feed intake of any ingredient used for formulation of aquafeed pellets (Glencross et al., 2007).

It was observed that pellet stability, and the gustatory response and feed intake of the Giant Freshwater Prawn *Macrobrachium rosenbergii* was improved with protein hydrolysate from tuna viscera (Sae-alee and Tantikitti, 2008). Research indicates that lipids should not play a major role in palatability of aqua feed, due to the fact that fish are primarily attracted to low-molecular weight compounds that are nitrogen containing, nonvolatile, and water soluble (Turchini et al., 2009a). Yet, there is research that indicates that dietary lipid source has an effect on voluntary feed intake of farmed fish, or at least in RBT (Geurden et al., 2005, 2007). The ingredients that contain an amount of a small peptide and nucleotide are relatively associated with the efficacy of the ingredients that are used to enhance attractiveness in shrimp diets (Suresh et al., 2011). Krill meal and krill hydrolysate contain high levels of small peptides and free amino acids, so using them in the diets increased weight gain of the juvenile Giant Tiger Prawn *Penaeus monodon* (Smith et al., 2005). The palatability of soybean-based diets was enhanced by krill hydrolysate that resulted in increased diet acceptance by the American lobster, *Homarus americanus* (Floreto et al., 2001). Also, the palatability of diet is affected by PH

factor, thus naturalization of diets is recommended prior to mixing the ingredients (Raa and Gildberg, 1982; Riuero and Vianu, 1996).

Apparent Digestibility

An indirect method of determining nutrient digestibility of an ingredient is by apparent digestibility. "Apparent digestibility is estimated by subtracting nutrients contained in the feces from nutrients contained in the dietary intake" (ILCK, 1990) providing an estimate of nutrition utilization. This does not consider the secretions that are added to the food that will raise the weight of the feces that would provide a slight decrease in digestibility. However, in calculation of true digestibility, the endogenous materials are separated from diet compounds in the feces.

Endogenous materials primarily consist of nitrogenous complexes such as epithelial cells, peptides, and enzymes, and digestibility can be estimated by using a nitrogen-free diet. Most of study use apparent digestibility because the amount of endogenous materials insignificantly affect the percentage of nutrient digestibility (Lovell, 1989). Glencross et al. (2007) explained that there are five major steps to determine value of ingredients for aqua feed. These are ingredient digestibility, ingredient characterization, palatability, nutrient utilization, and functionality. There have been many studies conducted to determine digestibility of plant ingredients in RBT, as shown in Tables 9 and 10.

Conclusion

Soybean meal is the most popular plant protein among cereal and legumes either to be used as a FM replacements or as part of diet formulation. The protein level and the

amino acid profile of soybean are very high compering with other plant sources. However, soybean and its by-products as other plant sources include several types of ANF's which cause undesirable effects on fish growth and health. Barley content lower level of protein than the soya product and the protein quality of barley is not good as much as soya products. Using barley in aqua-feed is slightly limited because of a lower protein level, amino acid profile, and ANF's. Several studies have used different techniques to limit the ANF's and improve the nutrient values in soybeans and barley such as fermentation, using high temperature, genetic modification and germination. These techniques showed acceptable results on including soya and barley products in aqua-feed.

Research Objective

The objectives of this research were:

1) To test barley in a RBT feed as a potential FM replacement, two fermented co-blend of soybean and barley will be used to investigate replacing part of FM by F75SBM:25BAR and F50SBM:50BAR on RBT growth and health;

2) To assess the digestibility of both F75SBM:25BAR and F75SBM:25BAR;

3) To evaluate using triple null soybean extruded from full fat soybean and solvent- extracted soybean as plant protein source in the whole diet of RBT; and

4) To detect digestible protein in both full fat triple null soybean and full fat triple null soybean and solvent- extracted triple null soybean meal.

In general, enhancing the plant protein source to be more suitable for use in aqua feed as a FM replacement, or as a part of whole diets, is important to develop the aquaculture sector. Formulating economically efficient diets which can meet fish nutrient requirements will reduce production costs and replace scarce ingredients such as FM.

Using barley with soybean protein in RBT feed is important for Kurdistan – Iraq because the price of both ingredients are more affordable relative to FM prices. Barley production capacity and supply is abundant in Kurdistan and the required soybeans can be imported to Kurdistan.

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Table 1. Plant-based feedstuffs used in fish nutrition research as a protein sources.

Plant stuffs name	Fish Species	Protein percentage of plant protein	Replace Percentage of FM protein	Replace Percentage of FM	Include Percentage of Whole Diets	References
Soybean meal	Atlantic salmon, (*Salmo salar* L.)	-----	33		------	(Carter et al., 2000)
Fermented soybean meal	Chinese sucker (*Myxocyprinus asiaticus*)	-----	30			(Yu et al., 2014)
Soybean meal	Nile tilapia (*Oreochromis niloticus*)	-----		100		(A-S Goda et al., 2007)
Soybean meal	Tilapia, Oreochromis niloticus × O. aureus		75			(Lin and luo, 2011)
Fermented soybean meal	Chinese sucker (*Myxocyprinus asiaticus*)	50.86	35			(Yuan et al., 2012)
Soybean meal	Black sea bass	47.5	70	-----		(Sullivan, 2008)
Soy Protein Concentrate	Red Drum (*Sciaenops Ocellatus*	72.23 (on dry matter	50			(Rossi JR et al., 2013)

Soybean meal	Nile tilapia (*Oreochromis niloticus L.)*	-----	62.5		(Kumar et al., 2011)
Australian soybean meal	Australian short-finned eel (*Anguilla australis australis*)	-----	23		(Engin and Carter, 2005)
PPB* (soybean meal and wheat gluten meal)	Siberian sturgeon (*Acipenser baerii)*	-----	100		(Yun et al., 2014)
PPB (Soy cake, corn gluten, wheat gluten and beans)	Meagre (*Argyrosomus regius*)			31.5	(ESTÉVEZ et al., 2011)
Barley Protein Concentrate with Wheat gluten meal and Corn protein concentrate	Atlantic salmon (*Salmo salar* L.)	53.1, 77.8 75.6		21.54 7.5	(Bell et al., 2014)
Non-genetically modified soybean meals (3010 solvent extracted meal	Cobia (*Rachycentron canadum)*	54.9	70		(Watson et al., 2014)
PPB (Corn gluten meal, Peptide meal and Feather meal) with the addition of 5 g kg^{1}	Yellowtail (*Seriola quinqueradiata*)	-----	70		(Sarker et al., 2012)

PPB (Squid meal, Soy protein concentrate, Soybean meal 48, Corn gluten and Wheat gluten with rate 3.0, 14.0, 22.0, 15.0 and 17.9, respectively)	Senegalese sole juveniles (*Solea senegalensis Kaup*, 1858)		100	-------	-------	(Silva et al., 2009)
PPB (Corn gluten meal, Wheat gluten, Extruded peas, Rapeseed meal and Extruded whole wheat)	Gilthead sea bream (*Sparus aurata, L.*)	------ ------			75	(De Francesco et al., 2007)
PPB (krill meal and pea protein concentrates)	Atlantic salmon (*Salmo salar*)				59.80 15.20	(Hansen et al., 2011)
Protein blend containing SPC	RBT(*Oncorhynchus mykiss*)	61.0, 49.6	100			(Burr, 2012)
Mixture of soybean and cottonseed meals	African bonytongue (*Heterotis niloticus*)	62.9	50	87		(Monentcham et al., 2010)
PPB (Wheat Gluten meal 8.6%, Corn Gluten meal 42.22% and Soybean meal 23.14%)	Rainbow trout (*Oncorhynchus mykiss*)	43.9	100 -----		73.96 -----	(Barrows et al., 2007)
Plant protein	Senegalese sole	42.5	-----	75	-----	(Cabral et al., 2013)

Mixture (Pea Protein Concentrate (PPC) and Rice Protein Concentrate (RPC))	Gilthead sea bream	71.5 61.5		-------	 60		(Sánchez-Lozano et al., 2009)
Lupine protein concentrates and Pea Protein Concentrate	RBT(*Oncorhynchus mykiss*)	50.0	54.9			30	(Zhang et al., 2012)
Corn gluten meal	Chinese sucker (*Myxocyprinus asiaticus*)	-----		30			(Yu et al., 2014)
Distiller's dried grains with soluble (DDGS)	RBT(Oncorhynchus mykiss)	------		------- ------	------ ------	30	(Welker et al., 2014)
High protein distillers dried grains	RBT(Oncorhynchus mykiss)	48.1			------	15	(Prachom et al., 2013)
Wheat DDGS	Nile tilapia, Oreochromis niloticus	34.4				40	(Li et al., 2011)
DDGS or HPDDG	RBT(*Oncorhynchus mykiss)*	27.5	44.7			50 45	(Øverland et al., 2013)
Distiller's dried grains with solubles (DDGS)	Nile tilapia, Oreochromis niloticus					20	(Lim et al., 2007)

Lipid-extracted algae (LEA) meals	Red Drum (*Sciaenops ocellatus*)	13.3	------	------	30	(Patterson and Gatlin, 2013)
Jatropha platyphylla kernel meal	Nile tilapia (Oreochromis niloticus L.)	-----	62.5	------	-------	(Kumar et al., 2011)
Cottonseed meal	Chinese sucker (*Myxocyprinus asiaticus*)	-----	30			(Yu et al., 2014)
3032 cold-pressed cake meal	Cobia (*Rachycentron canadum)*	49.2	70			(Watson et al., 2014)
Peanut (A. hypogaea) leaf meal	Nile tilapia (Oreochromis niloticus L.)	22.3	20	-------	--------	(Garduno-Lugo and Olvera-Novoa, 2008)
Lupine meal	Rainbow trout (*Oncorhynchus mykiss*)	41.4	-----	-----	25	(Hernández et al., 2013)
Corn gluten meal	Australian short-finned eel (*Anguilla australis australis*)	------	23	-----	------	(Engin and Carter, 2005)
Pea Protein Concentrate	Atlantic salmon, (*Salmo salar* L.)	-----	33			(Carter et al., 2000)

Pea Protein Concentrate	Gilthead sea bream (*Sparus aurata, L.*)	55.4		32		(SÁNCHEZ-LOZANO et al., 2011)
Pea Protein Concentrate	Atlantic salmon (*Salmo salar*)	50 and 30	20			(Øverland et al., 2009)
Barley Protein Concentrate	Red Drum (*Sciaenops Ocellatus)*	57.6	50			(Rossi JR et al., 2013)
Canola protein concentrate	Atlantic salmon (*Salmo salar*)	60.4	-------	-------	10	(Burr et al., 2013)
Rice protein concentrate	RBT(*Oncorhynchus mykiss*)	------	-------	-------	20	(Gasco et al., 2005)
Barley meal	RBT(*Oncorhynchus mykiss*)	-----			26.24	(Barrows et al., 2007)

Table 2. Known RBT nutritional requirements.

Nutrient level	Fry	Fingerling	Juvenile	Grower	Broodstock
Crude protein, % min	45-50	45	43	42	35-40
Amino acids, min %, dry matter basis					
Arginine	2.00	2.00	2.00	2.00	2.00
Histidine	0.70	0.7	0.70	0.70	0.70
Isoleucine	0.80	0.80	0.80	0.80	0.80
Leucine	1.40	1.40	1.40	1.40	1.40
Lysine	1.80	1.80	1.80	1.80	1.80
Methionine	1.00	1.00	1.00	1.00	1.00
Phenylalanine	1.20	1.20	1.20	1.20	1.20
Threonine	0.80	0.80	0.80	0.80	0.80
Tryptophan	0.20	0.20	0.20	0.20	0.20
Valine	1.30	1.30	1.30	1.30	1.30
Crude lipid, % min					
Essential fatty acids, % min					
18:2n-6					
20:4n-6	0.5	0.5	0.5	0.5	0.5
18:3n-3					
20:5n-3	1	1	1	1	1
22:6n-3	0.5	0.5	0.5	0.5	0.5
Carbohydrate, % max	12	12	12	12	12
Crude fiber, % max	3	3	3	3	3
Gross energy, min kJ/g	15.5	15.5	15.5	15.5	15.5
Digestible energy, min kJ/g	15.5	15.5	15.5	15.5	15.5
Protein to energy ratio, mg/kJ	25	25	25	25	25
Minerals					
Macroelements (%)					
Calcium, maximum	1	1	1	1	1
Phosphorus, minimum	0.8	0.7	0.6	0.6	0.6

Magnesium, minimum	0.05	0.05	0.05	0.05	0.05
Sodium, minimum	0.06	0.06	0.06	0.06	0.06
Microelements, min mg/kg dry diet					
Potassium	0.7	0.7	0.7	0.7	0.7
Iron	60	60	60	60	60
Copper	3	3	3	3	3
Manganese	13	13	13	13	13
Zinc	30	30	30	30	30
Selenium	0.3	0.3	0.3	0.3	0.3
Iodine	1.1	1.1	1.1	1.1	1.1
Vitamins, min IU/kg					
Vitamin A	2500	2500	2500	2500	2500
Vitamin D	2400	2400	2400	2400	2400
Vitamins, min mg/kg					
Vitamin E	25-100	25-100	25-100	25-100	25-100
Vitamin K	1	1	1	1	1
Thiamine	10	10	10	10	10
Riboflavin	5	5	5	5	5
Pyridoxine	6	6	6	6	6
Pantothenic acid	20	20	20	20	20
Niacin	10	10	10	10	10
Folic acid	2	2	2	2	2
Vitamin B12	0.02	0.02	0.02	0.02	0.02
Choline	800	800	800	800	800
Inositol	300	300	300	300	300
Biotin	0.15	0.15	0.15	0.15	0.15
Ascorbic acid	40	40	40	40	40

Source: (NRC, 2011

Table 3. Common fermentative genera of bacteria fungi and yeasts used in food and feedstuffs processing.

Microbe	Common genera	Common feedstuffs	References
Bacteria	*Lactobacilus*	Barley	(Song, et al., 2007)
		Wheat	(Zhang et al., 2015)
		Soybean	(Zhang et al., 2014)
		Corn	(Olsen et al., 1995)
	Lactococcus	Barley	(Booysen, 2002)
		Wheat	(Dal Bello et al., 2007)
		Soybean	(Lee, 2004)
		Corn	(Hounhouigan et al., 1993)
	Leuconostoc	Barley	(Booysen, 2002)
		Wheat	(Robert et al., 2006)
		Soybean	(Kanekar et al., 1992)
		Corn	(Nwachukwu et al., 2010)
Fungi	*Aspergillus*	Barley	(Farnworth, 2008)
		Wheat	(Deshpande, 2000)
		Soybean	(Farnworth, 2008)
		Corn	(Farnworth, 2008)
	Penicillium	Barley	(Shurtleff and Aoyagi, 2009)
		Wheat	(Mehta et al., 2012)
		Soybean	(Mehta et al., 2012)
		Corn	(Bamforth, 2008)
Yeast	*Saccharomyces*	Barley	(Gibreel et al., 2009)
		Wheat	(Verheyen et al., 2014)
		Soybean	(Hassan et al., 2015)
		Corn	(Halm et al., 2004)
	Candida	Barley	(Spencer et al., 2013)
		Wheat	(Montet and Ray, 2016)
		Soybean	(Watanabe et al., 2002)

Table 3. Basic composition of barley flour or meal.

Constituent	Measurement	Amount
Water	g	12.11
Energy	kcal	345
Protein	g	10.50
Total lipid (fat)	g	1.60
Carbohydrate, by difference	g	74.52
Fiber, total dietary	g	10.1
Sugars, total	g	0.80
Minerals		
Calcium, Ca	mg	32
Iron, Fe	mg	2.68
Magnesium, Mg	mg	96
Phosphorus, P	mg	296
Potassium, K	mg	309
Sodium, Na	mg	4
Zinc, Zn	mg	2.00
Vitamins		
Vitamin C, total ascorbic acid	mg	0.0
Thiamin	mg	0.370
Riboflavin	mg	0.114
Niacin	mg	6.269
Vitamin B-6	mg	0.396
Folate, DFE	µg	8
Vitamin B-12	µg	0.00
Vitamin A, RAE	µg	0
Vitamin A, IU	IU	0
Vitamin E (alpha-tocopherol)	mg	0.57
Vitamin D (D2 + D3)	µg	0.0
Vitamin D	IU	0
Vitamin K (phylloquinone)	µg	2.2

Fatty acids, total saturated	g	0.335
Fatty acids, total monounsaturated	g	0.205
Fatty acids, total polyunsaturated	g	0.771
Cholesterol	mg	0

*USDA National Nutrient Database for Standard Reference (Release 27).

Table 4. Chemical composition of barley grain on a dry matter (DM) basis (DMB) (Saeed 2011).

Component (%)	Barley grain
DM	92.31
Organic matter	90.32
Crude protein	8.40
Crude fiber	6.24
EE	3.20
NFE	72.48
NDF	25.22
ADF	5.78
Cellulose	4.76
Hemicellulose	19.44
ADL	1.02

Table 5. Comparison between fish meal and barley protein composition.

Component	***Fish meal**	***Barley protein concentrate**	****Barley protein concentrate**
Dry Matter	91.55	90.81	93.3
In Dry matter			
Lipid	9.31	12.66	-----
Ash	23.16	3.83	3.58
Starch	0.7	4.22	
Crude protein	72.43	57.76	56.88
Energy (kJ g-1)	------	-------	24.28

*(Morken et al., 2011).

**Montana Microbial Products, Butte, MT, USA

Table 6. Composition of xena barley and xena barley distiller's grains.

Components	***Xena Barley**	****Xena Barley DDGS (with hull)**	** **Xena Barley DDGS (dehulled)**
Moisture (%)	12.3	5.5	4.4- 8.8
Dry Matter (%)	87.7	----	-----
Crude Protein (%)	14.4	37.2	39.3- 43.3
Ash (%)	3.2	6.0	5.0- 6.5
Starch (%)	66.9	-----	-----
Crude Fiber	-----	7.1	2.0- 2.7
Fat (%)	3.2	7.5	4.9- 7.9

*(Silveira et al., 2007)

** (Gibreel et al., 2009).

Table 7. Types, amount of anti-nutrient and reduction method in barley processing.

Anti-nutrient	Concentration	Reference	Method to reduce	Amount	Reference
Tannins	625-mg/100g	(Jood and Kalra, 2001)	*Soaking, cooking, germination and fermentation **Dehulling, soaking and germination	-----	*(Afify et al., 2012) **(Mubarak, 2005)
Lectin	0.08 HU (g/kg)	(Trugo et al., 1999)	*Fermentation ** Germination	-------	*(Cuadrado, et al., 2002) ** (Trugo et al., 1999)
Beta-glucan	1.6-8%	(De Boer and Bickel 1988)	Fermentation	(37.2 to 30.7) g/kg dry matter	(Skrede et al., 2002)
Phytic acid	4 and 7 mg/g	(Garcõ a-Estepa et al., 1999)	*Malting 24 hours (germination) **Soaking, cooking, and fermentation	*(256 to 217) g/100g,dry basis	*(Trugo et al., 1999) **(Afify et al., 2012)
Trypsin inhibitor	0.45 mg/g	(Mikola and Suolin, 1969) (Casaretto et al., 2004)	*Dehulling, soaking and germination **Fermentation	-----	*(Mubarak, 2005) **(Chaven and Kadam, 1989)

			***Using high temperature		***(Saini, 1989)
Arabinoxylas	(4.4-6.7 g/kg)	(Xue, 1992)	Fermentation	-----	(Lambo et al., 2005)
Alpha -amylase inhibitor	(169 AIU) Activity of Alpha - amylase inhibitor	(Jood and Kalra, 2001) (Nielsen et al., 2004)	*Fermentation **Heat treatment and germination	------	*(Chaven and Kadam, 1989) **(Mulimani And Rudrappa, 1994)
Chymotrypsin inhibitors		(Casaretto et al., 2004)	Fermentation	-----	(Chaven and Kadam, 1989)
Alpha-galactoside	(75 g/100g, dry basis)	(Trugo et al., 1999)	Malting 48 hours (germination)	(75 to 12) g/100g,dry basis	(Trugo et al., 1999)

Table 8. Apparent digestibility coefficients (Dry matter, Energy, Protein and Total amino acids) of plant protein sources tested in RBT.

	Apparent digestibility coefficients (%)				
Ingredients	**Dry matter**	**Energy**	**Protein**	**Total Amino acids**	**Reference**
Soybean meal	79.0	83.0	95.0	---	(Mansfield et al., 2010)
Fish meal	---	---	90.8	---	
Soybean meal	---	---	93.2	---	
Fermented soybean meal-1 (fermented for 7 hours)	---	---	93.8	---	(Yamamoto et al., 2010)
Fermented soybean meal-2 (fermented for10 hours)	---	---	94.3	---	
Fish meal	---	---	82.4	88.2	(Morken et al., 2011)
Barley Protein Concentrate	---	---	84.2	88.2	
Wheat distiller's dried grains	66.0	75.0	90.0	---	(Randall and Drew, 2010)
Porphyra dioica	---	66.8	79.5	---	
Gracilaria vermiculophylla	---	62.4	87.8	---	(Pereira et al., 2012)
Ulva spp.	---	72.7	75.6	---	
Sargassum muticum	---	58.0	65.5	---	
Luteus lupin kernel meal	65.0	78.5	92.5	---	
Luteus protein concentrate	88.5	90.2	96.5	---	
Angustifolius lupin kernel meal	49.3	69.6	92.2	---	(Glencross, 2011)
Angustifolius protein concentrate	90.7	88.8	97.5	---	
Soybean meal	64.5	70.5	97.0	---	

Canola meal	26.0	68.8	99.6	---	
Solvent-extracted soybean meal	68.5	64.8	93.3	---	
L. angustifolius kernel meal	47.8	59.5	99.2	---	
L. angustifolius protein concentrate	40.5	58.5	91.7	---	
L. angustifolius protein isolate	90.1	88.4	97.7	---	(Glencross et al., 2010)
L. luteus protein concentrate.	62.0	76.2	90.3	---	
L. luteus protein isolate	85.3	85.5	92.1	---	
L. mutabilis protein concentrate	78.9	84.8	93.1	---	
Cottonseed meal, variety Pak	69.2	---	87.3	---	(Dadgar et al.,
Soybean meal	62.7	---	82.4	---	2010)
Wheat distillers' dried grains and soluble	70.0	77.0	85.0	---	(Reveco and
Wheat distillers' dried grains and soluble- fractionated	72.0	74.0	88.0	---	Murray, 2012)
Dehulled raw pea seed meal	47.33	46.59	81.74	---	(Hernández et
*5' Autoclaved pea seed meal	59.29	68.58	86.72	---	al., 2010)
Indian mustard protein concentrate	74.0	83.0	90.0	---	(Chowdhury
Indian mustard meal	83.0	90.0	99.0	---	et al., 2012)
Soy protein concentrate	87.0	930	98.0	---	
Wheat gluten meal	---	99	100	---	(Gaylord et
Corn gluten meal	---	85	92	---	al., 2008)

Table 9. Apparent digestibility coefficients of amino acids for different plant protein ingredients in RBT.

Plant protein feedstuff **Amino acids**	**Barley Protein Concentrate***	**Wheat distillers' dried grains****	**Mustard protein concentrate*****	**Indian mustard meal*****	**Soy protein concentrate *****
Essential amino acids					
Arginine	90.1	88.0	97.0	98.0	97.0
Histidine	86.7	83.0	94.0	96.0	95.0
Isolucine	91.6	79.0	91.0	96.0	95.0
Lucine	91.5	83.0	86.0	100	94.0
Lysine	88.6	80.0	95.0	98.0	100
Methionine	88.7	82.0	----	---	---
Phenylalanine	83.7	89.0	90.0	100	100
Therionine	85.5	83.0	90.0	90.0	91.0
Valine	90.0	83.0	90.0	92.0	93.0
Non-essential amino acids					
Alanine	86.6	---	---	---	---
Aspargine	78.1	---	---	---	---

Cysteine	78.1	---	---	---	---
Glutamine	92.7	---	---	---	---
Glysine	77.0	---	---	---	---
Proline	89.0	---	---	---	---
Serine	86.5	---	---	---	---
Tyrosine	95.3	---	---	---	---

*(Morken, 2011)

**(Reveco and Drew,2012)

***(Chowdhury et al.,2012)

Table 10. Proximate composition of soybean and its products (Van Eys et al., 2004).

	RFFSB	SBMME	SBMSE 44	SPC	SPI
Dry matter %	89.44	89.80	88.08	91.83	93.38
Crude protein %	37.08	43.92	44.02	68.60	85.88
Crude fiber %	5.12	5.50	6.26	1.65	1.32
Ether extracts %	18.38	5.74	1.79	2.00	.62
Ash %	4.86	5.74	6.34	5.15	3.41
NDF %	12.98	21.35	30.05	13.50	-
ADF %	7.22	10.02	8.76	5.38	-
ADL %	4.30	1.17	.75	0.40	-
Starch %	4.66	7.00	5.51	-	-
Total sugar %	-	-	9.06	-	-
Gross energy Kcal/ kg	5013	-	4165	4280	5370
Lysine %	2.34	3.50	2.85	4.59	5.26
Threonine %	1.53	2.21	1.80	2.82	3.17
Methionine %	0.52	0.80	0.62	.87	1.01
Cystine %	0.55	0.77	0.68	.89	1.19
Tryptophane %	0.49	0.74	0.56	.81	1.08
Calcium gm/Kg	2.62	2.96	3.12	2.37	1.50
Phosphorus gm/Kg	5.70	6.64	6.37	7.63	6.50
Magnesium gm/Kg	2.80	2.84	2.72	1.85	.80
Potassium gm/Kg	15.9	20.28	19.85	12.35	2.75
Sodium gm/Kg	0.29	0.33	0.18	.55	2.85
Linoleic acid C18:2 %	9.70	2.87	0.64	-	-

RFFSB: roasted full fat soybean,

SBMME: Soybean meal mechanical extracted,

SPC: Soy protein concentrate,

SPI: soy protein isolate.

Table 11. The anti-nutritional factors profile in soybean, SBM, and SPC (Peisker, 2001).

Anti-nutritional factors	Raw SB	SBM	SPC
Trypsin (mg/g)	45-50	1-8	1-2
Glycinin ppm	180	66	<100
B-glycinin ppm	>60	16	<10
Agglutinin	3.5	10- 200	<1

Figure 1. Fish meal price (USD$ per ton) from 1980 to 2020 (Indexmundi, 2020).

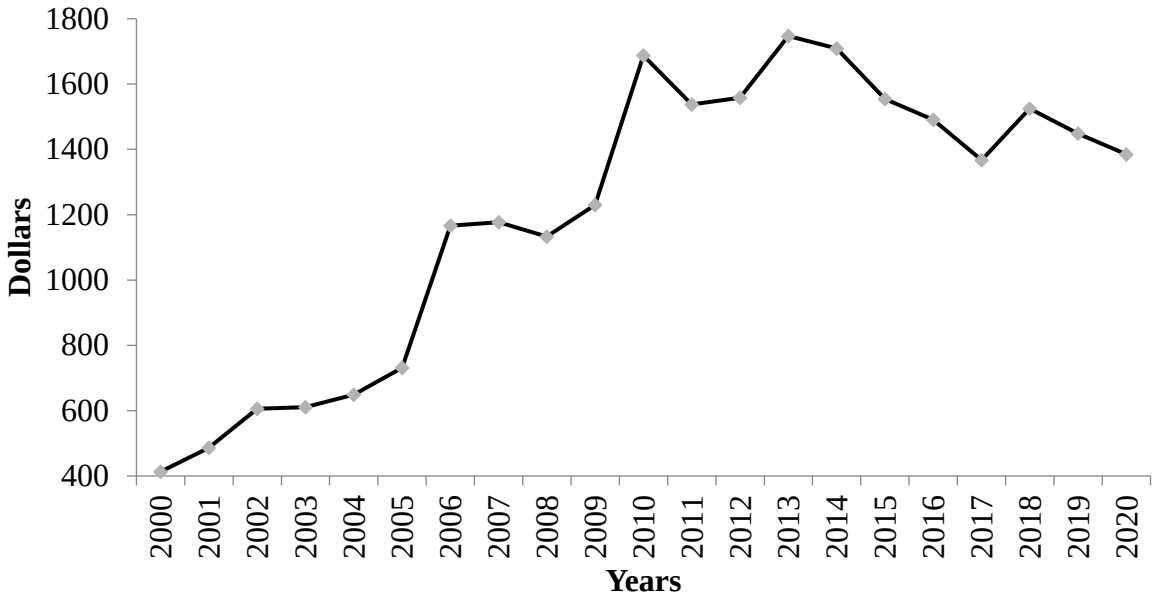

Figure 2. Global aquaculture production from 1980 to 2018(FAO, 2019).

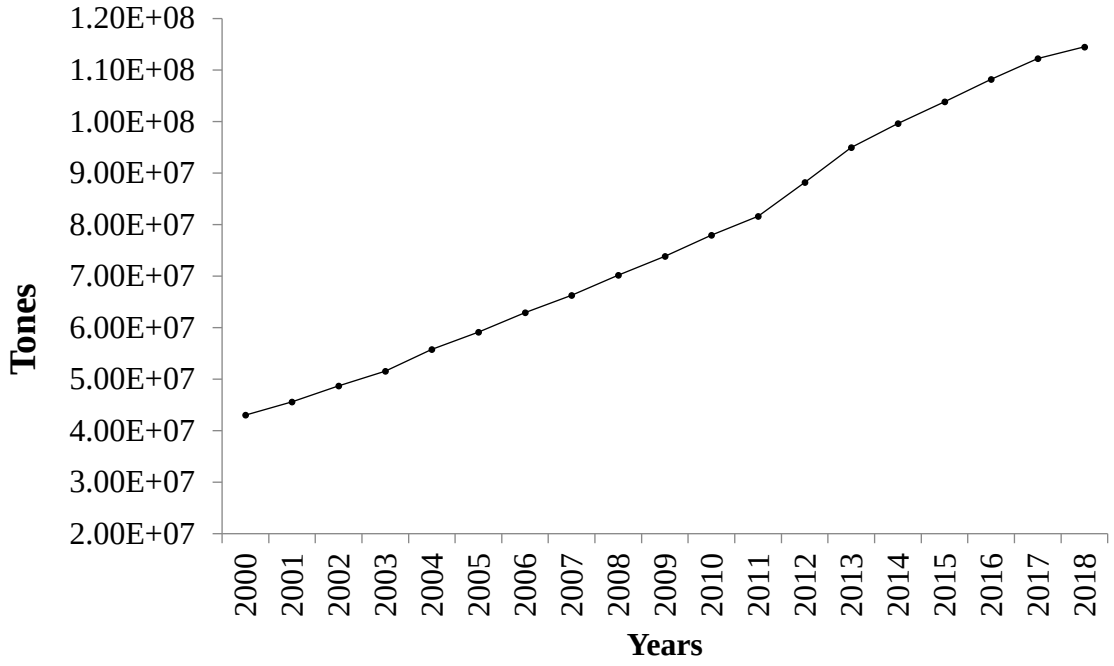

Figure 3. Annual global fish meal production between 1995 -2017(IFFO, 2018).

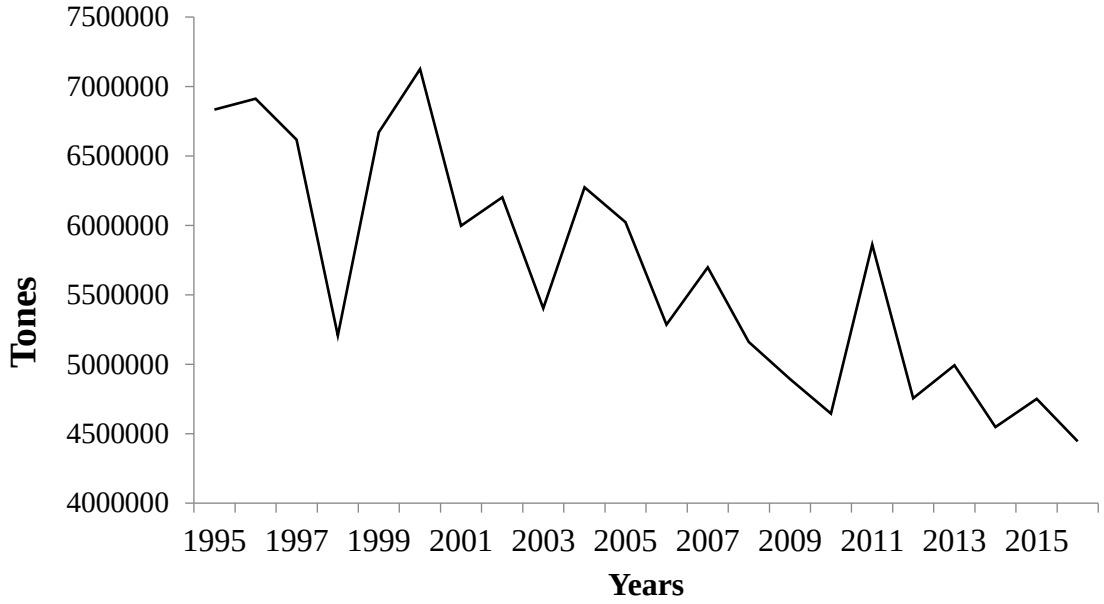

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Figure 4. Soybean processing and products (USSEC, 2008).

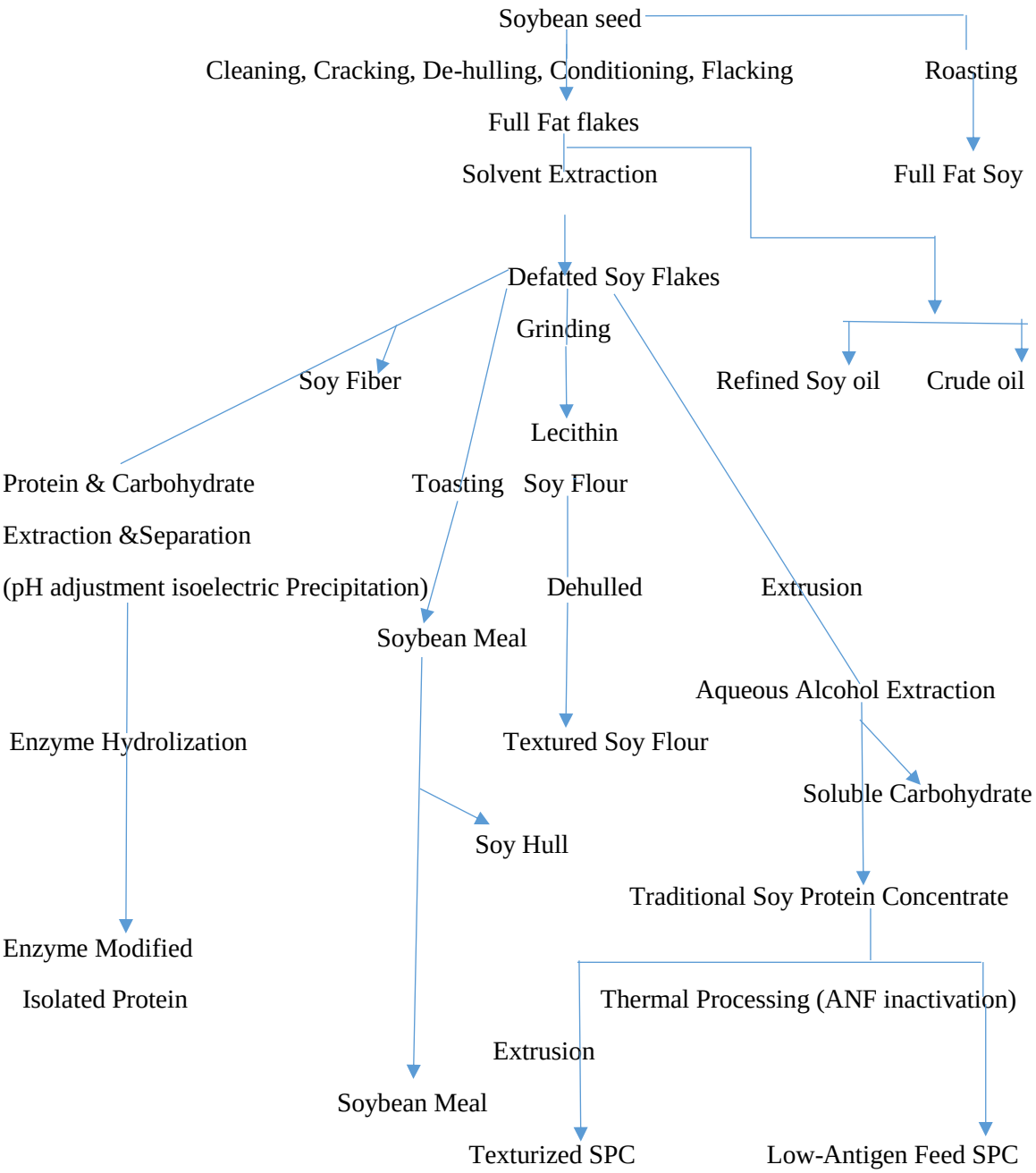


Figure 5. Trypsin inhibitor chemical structure (from Voss et al., 1996).

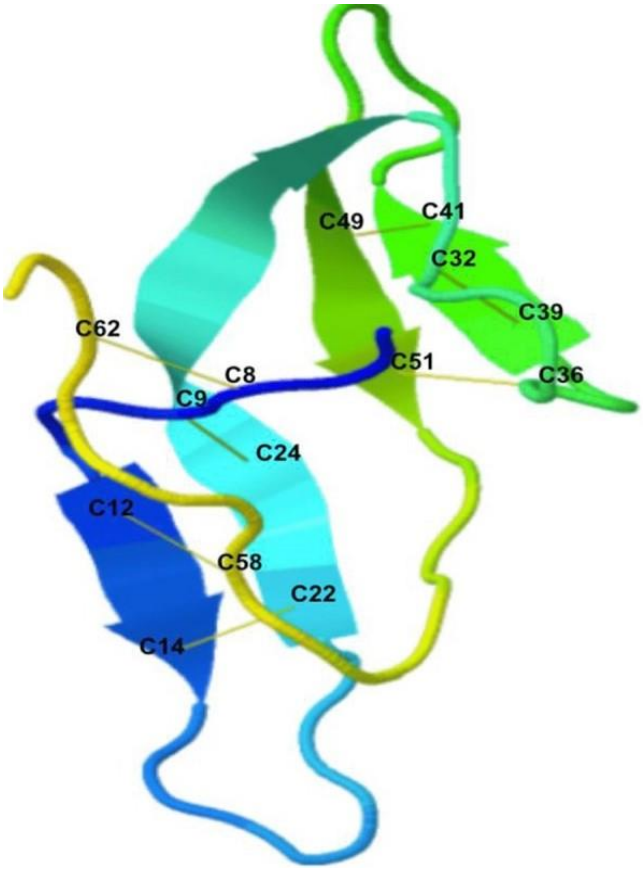


Figure 6. Chemical structure of phytic acid **(**Ingelmann, et al., 1993).

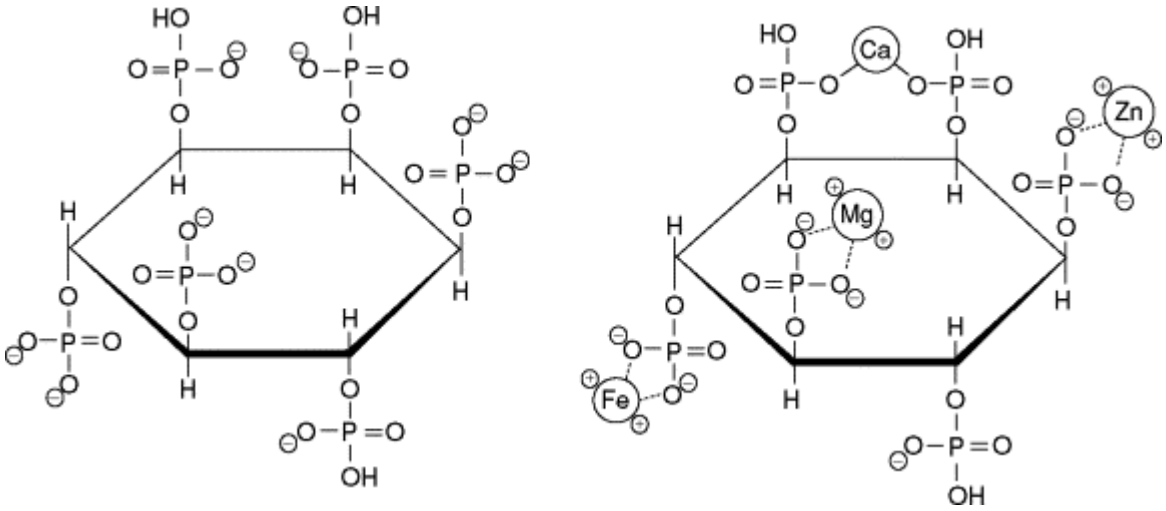
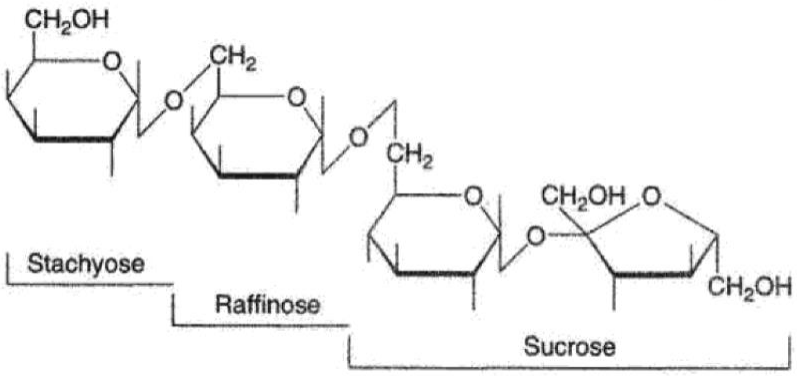


Figure 7. Carbohydrate structures in soybean seed (Erickson, 2015).



CHAPTER 2. USING FERMENTED CO-BLENDED PLANT PROTEIN IN RAINBOW TROUT *Oncorhynchus mykiss* FEED

Abstract

A 70-d experiment was conducted to investigate two fermented co-blends (75% of washed soybean with 25% washed barley and 50% of washed soybean with 50% of washed barley) as a fish meal (FM) replacement in juvenile RBT feed. Five experimental diets were formulated, one reference diet and four treatment diets. Three of the treatment diets were used to replace 25%, 35%, and 100% of FM with fermented co-blended meals (75% of washed soybean with 25% washed barley) F75SBM:25BAR. One diet was used to replace 25% of FM by fermented co-blend (50% of washed soybean with 50% washed barley) F50SBM:50BAR. Fish averaging 18 $\pm$1 g were stocked in 25 tanks at a rate of 16 fish per 110-L tank. All growth-related metrics, including feed conversion, were significantly different between the reference and 100% F75SBM:25BAR treatments. However, there were not significant performance differences among the reference and remaining treatments. No significant difference was observed between diets for gut health indices, except visceral fat (VFSI) and hepatosomatic (HSI) indices. The VFSI values differed significantly between the reference and 100% F75SBM:25BAR treatments. The values of HSI were significantly different between a reference diet and the 100% and 25% F50SBM:50BAR treatments. The intestinal histology was not statistically different among used diets in experiment.

In conclusion, the growth trial results showed that the FM can be replaced up to 35 % with F75SBM:25BAR and 25% with fermented co-blend F50SBM:50BAR without any negative effects on growth performance.

Introduction

Barley *Hordeum vulgare* is an annual plant, grown in both irrigated and non-irrigated land. In the US, barley is mainly planted in the Northwestern region. In barley production, the United States is ranked the fifth country in the world. Barley is used in the brewing industry and food animal feed ingredients but hasn't been highly utilized in aquafeed (Gatlin et al., 2007). The barley production in Iraq is increasing. For example, the average barley production in 2008 was 431 thousand metric tons and in 2019 this amount increased to 1,600 thousand metric tons (Knoema, 2020). New genetically improved barley provides lower levels of phytic acid, and hull-less barley contains lower fiber content (Bregitzer, 2005). Barley contains around 9 to 15 percent protein depending on geographic location; however, barley protein is low in lysine content. The level of lysine is 3.6% of protein; it is one of the negative attributes of barley for use in aqua-feed. However, this issue in barley protein can be solved by using a lysine supplement or other ingredients that include high level of lysine. Arginine is also present in slightly low levels in barley protein. The ratio of methionine and cysteine in barley is sufficient for fish diets but using barley protein in large quantity in fish diets requires the addition of methionine Wang et al., 2017). The fiber level in barley is 6% (NRC, 1994). Since carnivorous fish have a limited ability to digest fiber, utilizing de-hulled barley in fish diet is beneficial due to low levels of fiber (Gatlin et al., 2007). Overall, the use of barley in aquafeed is impeded by a number of reasons such as having high amount of ANFs such as phytic acid and β-glucan, low level of protein and high level of fiber. However, these factors may be controlled by using processes to improve protein level and limit ANFs in barley, such as genetic modification and fermentation (Gatlin et al., 2007).

Soybean is one of the most common oilseeds used for human and animal nutrition in the world. A large amount of this oilseed is used to obtain oil (soybean oil) for human and animal consumption, and biodiesel more recently. The by-product of oil extraction from soybean meal (SBM) is known as an excellent source of protein for aquafeed, given the high level of protein 44% - 50%. The soybean amino acid profile is considered one of the richest among all of the plant protein sources in the feed industry. However, amino acids lysine, methionine, and threonine are the most limited amino acids in SBM that must be supplemented in aquafeed, especially for carnivorous species. However, the level of essential amino acids, except cysteine, in SBM as compared with fish meal protein is low. This lower level can be partially solved by using fermentation to produce fermented SBM and SPC or it can be improved by adding amino acids as supplements (Liu, 2011; Gatlin et al., 2007; Barnes et al., 2015). The used land for plant soybean is increased from 74.3 to 83.4 million acres between 2000 and 2016. The production of soybean in the same time was expanded by 1.5% from 75.1 to 117.2 million metric tons (USDA, NASS, 2017). Soybean, as with other grains, contains various types of ANFs which have negative impacts on fish growth and health. Several methods have been developed to reduce or limit the amount and activity of these ANFs such as utilization of fermentation, heat, and enzymes (Gatlin et al., 2007).

Over the past two decades, several studies have been successfully conducted on the use of barley in aquafeed, such as in RBT diets. These studies showed the suitability of barley as a potential supplemental ingredient in aquafeed industry (Cheng et al., 2002; Morken et al., 2011; Sealey et al., 2008; Sugiura et al., 1999; Barrows et al., 2007). In contrast to barley, more research has been done to evaluate the use of soybean and its

byproducts in RBT diets (Bruce et al., 2014; Bruce et al., 2018; Voorhees et al., 2019, Voorhees et al., 2018; Yamamoto et al., 2010; Glencross et al., 2005; Sealey et al., 2009; Barnes et al., 2015, Cheng et al., 2003; Morris et al., 2005; Barrows et al., 2008 Barnes et al., 2014).

Fermentation a biological process that has desirable influence on plant meals used in animal feed. It is environmentally friendly due to require low energy and lower waste. Fermentation is easy to apply to different plant sources. This approach converts larger and complex molecules to smaller and simpler molecules through hydrolyzing protein and breaking down bonds in the protein compound, and also decreasing the amount of ANFs. The resulting product has higher protein and amino acid digestibility, and improved palatability for fish. In addition, it may enhance the health and immune response in fish. Increasing the bioavailability of ingredients leads to less stress on the environment, and less environmental contamination. Applying fermentation processes to plant protein sources such as soybean improves utility of plant protein as a fish meal replacement (Barnes et al., 2015; Barnes et al., 2014; Fagbenro et al., 1994; Felix and Brindo, 2008; Hong et al., 2004 Bruce et al., 2014; Bruce et al., 2018; Voorhees et al., 2019, Voorhees et al., 2018). Plant ingredients in aquafeed are restricted due to variety of adverse ANF components. The most relevant of these ANFs on fish growth and health are protease inhibitors, glucosinolates, phytates, saponins lectins, oligosaccharides tannins, non-starch polysaccharides, phytoestrogens, alkaloids, antivitamins, antigenic compounds, gossypols, and phorbol esters. Several processes such as biological (fermentation), mechanical, thermal (high temperature), and extrusion have been used to

reduce ANFs in feed ingredients. (Hong et al., 2004; Arndt et al., 1999; Yamamot0 et al., 2010; Francis et al., 2001).

Fermentation is a promised technique which has been used to improve barley and soybean meal through reducing the amount and effect on the ANF's and raise the bioavailability of dietary ingredients (Refstie et al., 2005; Skrede et al., 2002; Mukherjee; Mukhopadhyay and Ray, 1999) et al., 2016. In the present study, two-fermented co-blends of SBM and barley (F75SBM:25BAR and F75SBM:25BAR) were used to in RBT feed as a fish meal replacement after improving the protein sources using a fermentation process. Assessment of the co-blends were done to assess any negative impacts on fish growth and fish health.

Materials and Methods

Cultures, Maintenance, and Inoculum Preparation

In the present study, the *Aureobasidium pullulans* (NRRL-Y-2311-1) was used for fermentation. This strain was supplied by the USDA-National Center for Agricultural Utilization Research (NCAUR, Peoria, IL, USA). For short-term preservation, Potato dextrose agar (PDA) plates and slants were used to store the strain at 4°C temperature. For long-term storage, lyophilized stock was used. Seed cultures were prepared in 1-L Erlenmeyer flasks including 500 ml of glucose yeast extract (GYE) media (5% glucose and 0.5% yeast extract). The pH of culture media was adjusted to 5.0 either with 10M sulfuric acid or 10M sodium hydroxide before inoculation. The seed cultures were

inoculated with isolated colonies of *A. pullulans*. Shake flasks were incubated at 30°C in a rotary shaker at 150 rpm for 72 h.

70L Bioreactor Fermentation Trials

The SBM was obtained from Prairie AquaTech (Brookings, South Dakota). Pearled barley was obtained from Valley Grain Milling Inc. (Casselton, North Dakota). The pearled barley was ground (model DAS06 Fitzmill, Fitzpatrick Co., Elmhurst, Illinois) to a consistent particle size of 0.05. Both ingredients of the co-blend were separately washed; 10 L of municipal water were used to wash each kilogram of barley or SBM. The slurry was centrifuged at 4000 RPM for 12 minutes (Model J-6B, Beckman, Palo Alto, California) to separate the liquid and the washed co-blend was then dried at $60C^0$ for 24 hours using a lab oven dryer (1960, VWR International, Cornelius, Oregon).

After finalizing the best conditions and formative microbe for fermenting both co-blend ingredients, batch fermentations commenced November 1, 2019 continued until February 1, 2020. During that time, nine batches were fermented in 70 L bioreactors at South Dakota State University. However, only seven batches were successfully completed, primarily due to contamination events. The fermented co-blends completed were SBM with barley at ratios of 75:25 and 50:50, respectively. Generally, the reactor was sterilized through steam and then cooled to ambient room temperature. Washed co-blended material as combined with water at a 12% solids to liquid ratio (6 kg solids in 50L water) in a mixing container and then added to the reactor. The slurry was sterilized for 60 mins at 121°C and then the slurry was cooled to 30°C. A 48-h seed culture of *A. pullulans* was transferred through a reactor port at 1% of the reactor volume. After inoculation the reactor was operated at 30°C and 60 rpm for two days. During the

incubation period, a small amount of sample were taken daily to check the quality of the fermentation trial (i.e., plating and gram staining techniques). Upon the completion of the fermentation process, samples were transferred and centrifuged (Beckman, Model J-6B, Palo Alto, California) to separate the liquid and solid fractions. The solid fraction was then oven dried (VWR International, Cornelius, OR), ground and stored in the refrigerator prior to use. Samples were taken from F75SBM:25BAR and F50SBM:50BAR, then were sent to University of Missouri (Experiment Station Chemistry Lab, ESCL) for proximate composition analysis. ESCL used standard methods to determine crude protein (AOAC 2006, method 990.03), crude fat (AOAC 2006, method 990.03), crude fiber (AOAC 2006, method 978.10), moisture (AOAC 2006, method 934.01), and ash (AOAC, method 942.05).

Growth Trial

A 3,000-L recirculating aquaculture system (RAS), composed of 30, 110-L circular tanks were used for rearing fish. The system contained a fluidized bio-filter, UV filter, radial flow settler, bead filter, heat pump, and secondary sump, and was equipped with water level control and water quality monitoring**.** Fish averaging 18 $\pm$1 g were stocked in 25 tanks at a rate of 16 fish per tank. Five replicate tanks were used for each treatment and control. The duration of the trial was 70 days. The RAS provided optimum operating conditions with aerated water at a temperature of 15°C (total hardness as $CaCO_3$, 360 mg/L; alkalinity as $CaCO_3$, 210 mg/L).

One commercial reference diet and four treatment diets (Table 1) were formulated to contain 44.5% protein and 17% lipid. In three of treatment diets, 25%, 35%, and 100% of FM were replaced by the F75SBM:25BAR (Table 2). In the remaining

treatment diet, 25% of FM was replaced by F50SBM:50BAR (Table 2). All diets met minimum known nutritional requirements of protein and energy for RBT (NRC 2011). Appropriate amounts of vitamins, mineral premixes, and essential amino acids were supplemented to reach required levels (NRC, 2011). Experimental diets were manufactured using the following processes. First, large particle ingredients were ground (model DAS06 Fitzmill, Fitzpatrick Co., Elmhurst, Illinois) with a 2.4-mm screen. Then the blend was conveyed to a ribbon mixer for 20 minutes (Patterson Equipment, Toronto, Ontario). Diet extrusion was completed by a single-screw extruder (model 325, ExtruTech, Sabetha, Kansas) to produce ~3.5 mm pellets. A conveyor oven drier (HC-1210, Colorado Mill Equipment, canon, Co) was used to reduce pellet moisture to approximately 5%. Next, a Rotex screener (Rotex Inc., Cincinnai, OH) was used to screen-sift fines and a Phlauer vacuum coater (2013106, AandJ Mixing, Oakville, Ontario) was used to apply an additional lipid coating. Finally, the diets were cooled to a room temperature and stored at 4°C.

Fish were fed twice for satiation a day, morning and evening. The time interval between morning and evening feedings was approximately 7.5 hours. Temperature, oxygen content, and pH of water were reported daily. Daily feed rations were estimated weekly. Once every three weeks the total weight of tanks was recorded for growth and feed ration adjustment. Fish mortalities were recorded throughout the trial. Fish sampling and handling were carried out under South Dakota State University Animal Care and Use protocol 1912-065E.

At the end of the study, the total tank weight and the individual weights (± 1 g) and length (±1 mm) of three fish per a tank were measured. Tricaine methanesulfonate

(MS 222, 250 ppm solution) was used to euthanize fish (Neiffer and Stamper, 2009). Blood samples were collected by caudal sever, filling a hematocrit tube, and then plugging one end of the tube to measure hematocrit (HCT). The tubes were centrifuged at 1,500 PRM for 10 minutes to separate the blood cell from serum. The size of red cell blood and blood serum were separately measured (±0.1mm) using a micrometer.

Feed efficiency and performance parameters were estimated with the following equations.

FCR = total feed fed (g) /total weight gain (g) (Wu et al., 1996).

Feed intake per fish were calculated as feed consumed per tank/number fish per tank. Protein retention efficiency were calculated using the equation:

PER = fish protein gained/feed protein intake X 100.

$$\text{Specific Growth Rate (SGR)} = 100 \times \frac{[(\ln \text{final weight g} - \ln \text{initial weight g})/\text{days}]}{\text{Total duration of study (Days)}}$$

Relative growth (%) = Final weight - initial weight/ Initial weight X100

Survival (%) = (Number of fish at end of trial/ Number of fish at beginning of trial) x 100

Total weight gain (g) = final weight (g) - Initial weight (g)

Individual weight gain (g) = total weight of tank (g)/ number of fish in tank

Whole body length and weight body of 30 fish per diet were measured to calculate the Condition Factor (*K*) as:

K = Individual fish weight (g) / (individual fish length (cm) ^{3}X 100,000

Hematocrit was calculated as:

Hematocrit (%) = (Volume of red cells/ Volume of whole blood) x100

Gut Health Indices and Histology

One day after the final day of study, three fish per tank were euthanized using a lethal dose 250 ppm of MS 222 (Neiffer and stamper, 2009). Then the fish were necropsied to measure organosomatic characteristics. Fish livers were weighed to the nearest 0.01 g and the hepatosomatic index (HSI) was estimated as:

HSI = 100 x (liver weight (gm)/whole fish weight (g)) (Strange, 1996).

Whole viscera, minus fecal material, were measured to the nearest 0.01g and the (VSI) were calculated as:

VSI = 100x (viscera weight g/whole fish weight gm)

The intraperitoneal fat was separated from the viscera and weighed to the nearest 0.01g to calculate the visceral fat somatic index as:

VFSI = Weight of viscera fat (g) / Body weight (g) x 100

Spleen were separated and weighed to the nearest 0.01g to calculate the spleen somatic index as:

SSI = Weight of spleen (g)/ Body weight (g) x 100

Lastly, the fish were filleted and weighed to calculate the fillet ratio as:

Fillet ratio (%) =Weight of fillets (g)/ Body weight (g) x 100

To examine if co-blends induced any changes in distal intestine morphology, samples of distal intestine were collected from three fish per tank. A series of 2-mm cross sections of the distal intestine were fixed in 10% buffered formalin, and stained with eosin and hematoxylin using standard histological techniques (Bureau et al, 1998; Burrell

et al., 1999). Digital images were taken via microscopy with 10X magnification (Olympus BX53 microscope). The morphology of the intestine examined by two independent individuals who measured the lamina propria thickness and cellularity, submucosal connective tissue width, and the number of large vacuoles (Knudsen et al., 2007; Colburn et al., 2012). A histological intestine scoring system (Table 3) was used to score the inner surface of distal intestine.

Liver Color Measuring

LanScan XE (No. D38452, South Dakota State University) colorimeter were used to measure the color for fresh liver at Prairie Aqua Tech, Brookings, SD. The Hunter Lab *L, a, b* color scale (Reston, VA). The highest value for *L* is 100, representing a perfect reflecting surface (i.e., white); zero is the minimum value of *L*, which indicate black. Values for a and b have no limits; positive *a* is red, negative *a* is green, positive *b* is yellow, and negative *b* is blue.

Statistical Analyses

The primary statistical comparisons were one-way analysis of variance (ANOVA) or Kruskal-Wallis tests, depending on data type and test assumptions. If treatment differences are detected ($\alpha = 0.05$), comparisons were performed using the Tukey HSD test or Mann-Whitney tests to isolate which treatment responses differ. Kruskal–Wallis test was specifically used to analyze ordinal data (e.g., health assessment and histological data) (Kuehl, 2000).

Result and Discussion

Ingredients and Feeds

There was a difference was detected in protein level (F75SBM:25BAR was 65.8% and F50SBM:50BAR was 60.7%) between fermented co-blends. However, small differences were realized in lipid and ash ratios between fermented co-blends (Table 1). The difference between fermented co-blends was directly related to the ratios of SBM and pearled barley. The protein content is considerably different between barley and soybean. For example, the washed SBM protein is 61.23%, while washed pearled barley content is 12.7 % protein. Lipid and ash content are slightly different between barley and SBM.

Growth Trial

Table 4 shows final weight, total weight gain, average individual weight, relative growth and specific growth rate for dietary treatments. Fish fed diets containing 20% and 35% of FM replacement were not statistically different from the reference diet. However, fish fed the diet containing a 100% FM replacement with F75SBM:25BAR was significantly different from the reference diet. The survival rate was 100% for all diets except for the 100% FM replacement treatment.

RBT are a carnivorous fish. The protein level and quality is critical point for good growth and proper health. All experimental diets met or exceeded the RBT requirements of protein and lipid. The ash content is somewhat lower than recommended amount. The lab results showed ash content is between 6.46% - 7.45% of whole diets (Table 2)

Previous studies have used fermented plant meals, especially fermented SBM, in RBT and other carnivorous species. Those studies provide evidence of replacing a portion of FM by fermented SBM or barley in fish diets. For example, Barnes et al, 2014 successfully replaced 37.5% of FM by fermented soybean in RBT feed. In another study, the replacement of 80% of FM by fermented soy protein did not show any undesirable effects on RBT growth (Voorhees et al., 2018). Fermented soybean was used to replace 85% of FM in RBT with no negative effects on fish growth performance (Voorhees et al., 2019). Rossi et al., (2013) carried out a study where 50% of FM was successfully replaced by fermented SBM in Red Drum *Sciaenops ocellatus* diet. Morken et al., (2011) explained that BPC (produced by fermentation technique) is a desirable potential to be used in RBT feed. In a different study, fermented barley was found to be a promising plant protein source in Atlantic salmon diet (Skrede et al., 2002). Bell et al., (2016) in a study included 21.5 % of BPC in Atlantic salmon diet. Their results showed no significant differences in fish growth performance parameters compared with a reference diet. Burr et al., (2013) reported that BPC can be used in Atlantic salmon diet.

In addition, many studies have found that fermentation leads to increased palatability of ingredients and improved digestibility of energy and protein in aqua-feed. Fermentation improves properties such as low levels of protein and essential amino acid content, and high levels of several ANFs in plant protein sources. The fermentation process results in reducing some types of ANFs as well as adding protein and amino acid content obtained from microorganism's bodies. In fermentation, microorganisms convert a part of the carbohydrate in plant ingredients to protein and then store the protein in their

bodies (Gaylord et al., 2010; Burr et al., 2011; Gatlin et al., 2007; Skrede et al., 2002; Morken et al., 2011; Mukherjee et al., et al., 2016; Hong et al., 2004).

The SGRs for all treatments in the present study were between 2.23 to 2.76. Several studies on fermented soybean or barley showed similar SGR results found in the current study (Barnes et al., 2018; Yamamoto et al., 2012). Other studies had lower SGR results (Voorhees et al., 2019; Bell et al., 2016; Barnes et al., 2014; Voorhees et al., 2018). Bruce et al., (2017) reported higher SGR results.

The FCR also did not differ among fish fed the reference diet and treatment diets except for the 100% FM replacement treatment ($p<0.01$). The FCR results of the present study are similar to other studies on fermented soybean and barley (Barnes et al., 2014; Bell et al., 2016). While a number of studies reported different FCR results (Barnes et al., 2015; Barnes et al., 2013; Voorhees et al., 2018; Voorhees et al., 2019; Yamamoto et al., 2012; Yamamoto et al., 2010). A number of reasons can affect FCR such as body fat content the fish that fed 100% replacement has higher VFSI comparing with other diets. The other effective reasons on FCR are fish size, the balance of energy to protein in diet, the digestibility of ingredient, or having some ANF in the feed (Lovell, 2012).

Non-significant differences were observed for SSI and VSI among the dietary treatments (Table 5). However, a significant difference was detected between the reference and the 100% FM treatment for VFSI may due to unbalance protein and energy ratio in diet (Lovell, 2012).

Some previous studies on fermented soybean found non-significant differences or obtained similar values for VSI (Barnes, et al., 2014; Barnes et al., 2015; Voorhees et al., 2019; Voorhees et al., 2018), and SSI (Voorhees et al., 2019). VFSI results from the

THIS study are recognized by a number previous studies which found significant differences between experimental diets or reported close values (Bruce et al., 2016; Barnes et al., 2013; Barnes, et al., 2012; Yamamoto et al., 2012; Barnes, et al., 2014). Unbalanced energy to protein ratio in a diet may lead to an increased amount of fat in the viscera (Mjoun et al., 2012).

Significant difference was only found between reference and **25%** F50SBM:50BAR diets for HIS. HSI results from the current study are approved by several previous studies which showed significant differences between dietary treatments or reported close values (Bruce et al., 2016; Barnes et al., 2013; Barnes, et al., 2012; Yamamoto et al., 2012; Barnes, et al., 2014). High HSI values may be attributed to a high level of carbohydrate in the diet, as there is a positive correlation between carbohydrate level in diet and accumulation of lipid and glycogen in liver (Hemre et al., 2002; Zhang et al., 2019; Lin et al., 2018; Hilton and Dixon, 1982; Hung and Storebakken, 1994; Bergot, 1979).

Condition (K) and fillet ratio values were not significantly different among the treatments. Many studies obtain lower values of K than current study K values (Bruce et al., 2016; Barnes et al., 2013; Barnes et al., 2012; Barnes et al., 2015; Barnes et al., 2014). However, Voorhees et al. (2019) reported higher K values.

The fillet ratios observed in the current study is slightly lower in comparison with values reported in a previous study that was conducted by Bruce et al. (2016).

There was no significant difference among all diets for all three-color characteristics. The color of liver may differ between fish due to insufficient absorption

of lipid by fish or imbalance amount of n-3/n-6 fatty acids inside the feed diet (Takeuchi, 1982); and the content of carotenoid in diet (Rossouw, 1978). The color of liver may be a welfare indicator for fish (Eliasen et al., 2019).

Non-significant differences were found between the diets in HCT parameters. HCT parameter can be a stress indicator in fish, for example, relations were found between the acute stress and fatigued swimming. Also in other studies, the HCT were changed with some dietary supplements such as vitamin C, arginine, and inulin (Gallaugher et al., 1995; Ibrahem et al., 2010; Buentello et al., 2007; Biron and Benfey, 1994; Wells and Weber, 1991).

Histology Results

In the current study, up to 21.7% of F75SBM:25BAR was used to replace 100% of FM in RBT diet, and non-significant differences were found among all treatments for distal intestine morphology (lamina propria thickness, connective tissue quantity, and vacuolization) (Table 6). The results of intestine morphology are also supported by findings of Barnes et al., 2014 which showed that up to 35% of fermented SBM in RBT diet did not change the morphology of distal intestine after 90 and 205 days of feeding, and Yamamoto et al., 2010 used fermented SBM up to 47.6% in RBT diet without causing any significant change in intestine morphology. Saponins in SBM and soy lectins (protein) induces the morphological changes in intestine of fish (Knudsen et al., 2007; Iwashita et al., 2009, Krogdahl et al., 2015). Fermentation reduces ANF's in SBM such as lectin, saponins and improves using SBM in aqua-feed (Wang et al, 2016; Mukherjee et al., 2016; Barnes et al., 2015 ; Zhang et al., 2018).

In conclusion, in RBT diets the FM can be replaced up to 35% using F75SBM:25BAR and up to 25% of FM in RBT diets can be replaced using F50SBM:50BAR without having any negative effects on growth performance, most of the health indicators and intestine morphology.

In Iraq, barley has economical pricing and a high production level. It is the second most highly produced grain after wheat production. Barley is mostly used for animal feeding no human consumption in Iraq, from 1,611 metric tons supply of barley in 2019, a total of 1,200 metric tons was used for animal feeding (USDA, 2020). Availability supports the use of barley in aquafeed in Iraq.

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Table 1. Proximate composition of fermented co-blended soybean and barley meals. 75% soybean & 25% barley = F75SBM:25BAR and 50% soybean & 50% barley F50SBM:50BAR.

Component	F75SBM:25BAR	F50SBM:50BAR
Moisture (%)	1.62	2.06
Crude protein (%)	65.80	60.70
Crude Fiber (%)	7.90	6.40
Crude Fat (%)	4.30	6.00
Ash (%)	1.80	2.70

Table 2. Diet formulations (g/100g, dry matter basis) used in the RBT feeding trial for 70 days. FCB = fermented co-blend of soybean and barley meals.

	Diets				
Ingredients	**Reference**	**25%** F75SBM:25BAR	**35 %** F75SBM:25BAR	**100 %** F75SBM:25BAR	**25 %** F50SBM:50BAR
Fish meal (Special Select)[a]	20.00	15.00	13.00	---	15.00
F75SBM:25BAR[b]	---	5.13	07.19	20.53	---
F50SBM:50BAR[c]	---	---	---	---	5.57
Pork blood meal [d]	3.00	3.00	3.00	3.00	3.00
Poultry byproduct meal [e]	15.00	15.00	15.00	15.00	15.00
Hydrolyzed feather meal[f]	2.00	2.00	02.00	02.00	02.00
Whole cleaned wheat[g]	21.77	20.90	19.90	18.08	20.00
Yellow whole corn[h]	09.00	09.17	10.03	7.82	10.40
Wheat gluten [k]	12.30	11.70	11.50	11.80	11.70
Carboxymethyl cellulose	00.50	00.30	00.20	---	0.15
Vitamin premix [l]	01.00	1.20	01.40	1.70	1.00
Trace mineral premix [m]	00.20	1.00	01.30	4.00	1.00
Lysine [n]	00.70	0.85	0.93	1.33	0.83
Tryptophan [o]	00.10	0.10	0.10	0.20	0.10

Methionine [p]	00.50	0.55	0.65	0.55	0.60
Histidine	00.00	0.10	0.10	0.14	0.05
Taurine [q]	00.50	0.50	0.50	0.50	0.50
Betaine	00.50	0.50	0.50	0.50	0.50
Menhaden, VA prime gold[r]	08.83	9.00	8.80	8.30	8.50
Soybean NRC[s]	02.50	2.40	2.30	3.00	2.50
Lecithin [t]	01.00	1.00	1.00	1,00	1.00
Proximate analysis					
Protein	46.40	46.10	46.40	46.90	46.30
Lipid	18.40	17.40	18.00	17.50	17.90
Fiber	1.27	1.08	1.15	2.25	1.32
Ash	7.45	7.34	7.22	6.46	7.12
NFE	24.44	26.07	25.10	25.01	25.57

Pearl barley. NFE= Nitrogen free extract (100-protein-lipid-ash-crude fiber-moisture)

a Special Select, Omega Protein, Houston, TX; b South Dakota State University, Brookings, SD; c South Dakota State University, Brookings, SD; d Mason City By-Products, Mason City, IA; f Wilber Ellis, Aurora, CO; g Prairie Ag Partners, Lake Perston, SD; h Rancher's Choice, North Sioux City, SD; k Cargill, Minneapolis, MN; l Nutra blend LLC, Neosho, MO; m Nutra blend LLC, Neosho, MO; n CJ BioAmerica Inc, Fort Dodge, IA; o ChemSol, Minnetonka, MN; p DSM Nutritional Products, Parsippany, NJ,; r Daybrook Fisheries, Empire, LA; s South Dakota Soybean Processors, Volga, SD; t Archer Daniels Midland Co, Chicago, IL.

Table 3. Histological intestine scoring system used to assess RBT distal intestines after 70 days of feeding experimental diets (modified from Knudsen et al., 2007; Colburn et al., 2012, Barnes et al., 2014)

Scores	Appearance
Lamina propria of simple folds	
1	Thin and delicate core of connective tissue in all simple folds
2	Lamina propria slightly more distinct and robust in some folds
3	Clear increase in lamina propria in most simple folds
4	Thick lamina propria in many folds
5	Very thick lamina propria in many folds
Connective tissue between base of folds and stratum compactum	
1	Very thin layer of connective tissue between base of folds and stratum compactum
2	Slightly increased amount of connective tissue beneath some mucosal folds
3	Clear increase of connective tissue beneath most mucosal folds
4	Thick layer of connective tissue beneath many folds
5	Extremely thick layer of connective tissue beneath some folds
Vacuolization	
1	Large vacuoles abundant and present in most epithelial cells
2	Large vacuoles numerous
3	Increased number of large vacuoles
4	Very few large vacuoles present
5	Large vacuoles absen

Table 4. Performance metrics (means ±standard errors) of RBT after 70 days of feeding experimental diets that used to used F75SBM:25BAR and F50SBM:50BAR as a FM replacement.

Treatments	**Reference**	**%25** F75SBM:25BAR	**%35** F75SBM:25BAR	**%25** F50SBM:50BAR	**%100** F75SBM:25BAR	**P-Value**
Parameters						
Initial weight (g)	18± 2	18± 2	18± 2	18± 2	18± 2	
T. Feed Cons. (g)	2,057± 16^{a}	1,961± 27.7^{ab}	1,914± 64^{ab}	1,906± 24.6^{ab}	1,690± 25.9^{b}	0.000
Final Wt. (g)	2,217± 71^{a}	2,013± 72^{ab}	1,879± 73^{ab}	1,828± 74^{ab}	1,514± 72^{b}	0.000
T. Wt. gain (g)	1897± 69^{a}	1695± 67^{ab}	1565± 71^{ab}	1514± 73^{ab}	1197± 69^{b}	0.000
Ave. indv. Wt. (g)	139± 4.33^{a}	126± 4.07^{ab}	117± 4.54^{ab}	114± 4.61^{ab}	95± 4.34^{b}	0.000
RG (%)	593± 26.7^{a}	532± 23.4^{ab}	498± 20.4^{ab}	482± 22.4^{ab}	377± 21.4^{b}	0.000
SGR (%)	2.76± 0.06^{a}	2.63± 0.05^{ab}	2.55± 0.05^{ab}	2.51± 0.05^{ab}	2.23± 0.06^{b}	0.000
FCR	1.09± 0.04^{a}	1.17± 0.03^{ab}	1.23± 0.02^{ab}	1.27± 0.05^{ab}	1.43±0.07^{b}	0.001
Survival (%)	100	100	100	100	96.7± 0.39	0.068

Cons= Consumed, T= Total, Wt. = Weight, Ave= Average, RG=Relative growth, SGR=Specific growth rate, FCR=feed conversion ratio.

Table 5. Health assessment parameters of Juvenile RBT after 70 days of feeding on experimental diets experimental diets that used to use F75SBM:25BAR and F50SBM:50BAR as a FM replacement.

Treatment	**Reference**	**25%**	**35%**	**25%**	**100%**	**P-Value**
Parameters		F75SBM:25BAR	F75SBM:25BAR	F50SBM:50BAR	F75SBM:25BAR	
VSI (%)	12.63±0.59	13.41±0.49	12.54±0.42	13.84±0.51	13.78±0.48	0.225
Fillet (%)	41.54±1.77	43.19±1.99	43.48±1.86	41.31±2.53	40.37±1.48	0.768
SSI (%)	0.099±0.009	0.071±0.005	0.11±0.039	0.09±0.018	0.121±0.011	0.495
VFSI (%)	2.85±0.29[a]	2.23±0.09[a]	2.54±0.38[a]	2.63±0.19[a]	3.63±0.29 [b]	0.016
HSI (%)	1.66±0.07[a]	1.81±0.09[ab]	2.16±0.13[ab]	2.23±0.09[b]	1.78±0.06[a]	0.001
K	1.36±0.02	1.38±0.02	1.37±0.02	1.35±0.03	1.37±0.02	0.943
HCT %	39.46±0.91	41.3±2.77	40.3±0.30	41.6±1.68	39.5±1.17	0.822
L*	27.04±0.43	27.63±0.64	26.73±0.61	27.28±0.49	26.83±0.20	0.687
a*	4.51±0.07	4.17±0.27	4.42±0.33	4.94±0.16	4.64±0.21	0.214
b*	3.36±o.28	3.27±0.37	3.42±0.28	3.85±0.21	3.77±0.17	0.478

K: condition factor K, FY: Fillet yield, VSI: Viscerosomatic index, SSI: Spleen somatic index, HSI: Hepatosomatic index, VFSI: Visceral fat somatic index, HCT: hematocrit, L*: Lightness, a*: Redness, b*: Yellowness

Table 6. Health scores for RBT distal intestine in feeding after 70 days of feeding on experimental diets experimental diets that used to used F75SBM:25BAR and F50SBM:50BAR as a FM replacement.

Diets	**Lamina propria**	**Connective tissue**	**Vacuolization**
Reference	1.6±0.3	1.7±0.3	1.3±0.0
25% F75SBM:25BAR	1.7±0.3	1.3±0.3	1.9±0.3
35% F75SBM:25BAR	1.3±0.9	1.4±0.7	1.8±0.0
25% F50SBM:50BAR	2±0.7	1.7±0.3	1.7±0.3
100% F75SBM:25BAR	2±0.3	1.6±0.3	1.6±0.0

CHAPTER 3. EVALUATION OF A NOVEL SOYBEAN VARIETY IN RAINBOW TROUT *Oncorhynchus mykiss* FEEDS

Abstract

This study investigated feeding performance of low antigen (termed Triple Null, TN) and conventional soybean (Davison, DV) varieties in RBT *Oncorhynchus mykiss* (RBT) diets to assess their potential as a plant protein ingredient for the aquafeed market. Four diets were formulated to include 20% each of novel solvent-extracted and extruded soybean meals (SBMs) or similarly processed conventional SBM (DV), for comparison to a reference diet. Twenty age-0 RBT (~24 g each) were stocked into 30, 187-L semi-square tanks of a 7,500 L recirculating aquaculture system (RAS) to provide five replicates per diet in the 155 d trial. A separate 21-d digestibility trial was completed to determine *in vitro* protein digestibility of each SBM ingredient. One hundred RBT (300 g each) were stocked into each of six 756-L tanks of a 6000-L RAS. Non-significant differences were found in final weight (P=0.343), weight gain (P=0.39), daily gain (P=0.35), RG (P=0.43), SGR (P=0.26) among the experimental diets. The FCRs of TNEXT and DVSE were significantly different form rest of diets of experiment. VSI (P=0.12), SSI (P=0.14) and HSI (P=0.95), VFSI (P=0.56) were not statically difference among diets. No significant difference was found among all diets for protein digestibility (76.5- 85.6 %). In conclusion, all SBM ingredients compared in this study can be included in RBT diets up to 20% without significant effects of fish growth and health performance. Thus, using conventional soybean is more economical than Triple Null soybean (TN-SBM) because genetic modification processes to produce TN-SBM which cost money are not required to be used.

Introduction

Fish meal (FM) protein is considered as a primary protein source in carnivorous fish such as salmonids. FM contain high quantity of high quality of protein which are important for proper growth of fish (Gatlin et al., 2007; FAO, 2020; Cheng and Hardy 2004). However, due to growing aquaculture production, demand for FM is growing and outpacing production. As a result, price of FM has dramatically increased (Rana, 2009; FAO 2020; Hardy 2010). In consequence, alternative protein sources are required to provide for salmonid diets (Hardy, 2010).

Several different plant protein sources were investigated as an alternative protein source for FM such as soybean and its products in salmonid feed (Harlioglu, 2011; Morris et al., 2005; Barnes et al., 1013). Although, SBM in salmonid feed is restrictive due to several anti-nutritional factors (ANFs) such as trypsin inhibitor (causing low protein digestibility), phytic acid, Oligosaccharides, lectins and other ANFs (Rickert et al., 2004; Gatlin et al., 2007; Kumar et al., 2012; Zhou et al., 2012; Fasina et al., 2003).

Many previous studies have indicated that SBM is a promised plant protein source to replace part of FM protein in salmonid diets without undesirable effect on RBT growth (Aslaksen et al., 2007; Yang et al., 2011; Castro et al., 2011; Oliva-Teles et al., 1994; Refstie et al., 2000; Hernande and Roman, 2016) and morphology of fish distal intestine (Iwashita et al., 2008; Romarheim et al., 2008; Barrows et al., 2008; Merrifield et al., 2009; Sealey et al., 2009). ANFs in SBM can be reduced using a variety of processes such as high temperature, extrusion, or fermentation (Barrows et al., 2007; Francis et al., 2001; Refstie and Roem, 1998; Evans et al., 2005).

This study was conducted to evaluate novel solvent-extracted and extruded SBMs genetically modified (TN) compared to similarly processed conventional SBM (DV) and reference diets. The main goal of this study is adding a new SBM product that contains a low level of each P34 protein (an immuno-dominant allergen), trypsin inhibitor (de-activing trypsin enzyme, decreasing protein digestion), or agglutinin lectin (attaches to intestinal epithelial cells leading to an inflammation). Producing SBM with low level of ANFs results in adding more value to SBM as a protein source in aquaculture feeding.

Materials and Methods

Test Ingredients and Diet Preparation.

Test ingredients and diet preparation. The NCI produced the soybeans used in this study. Soybeans were crashed and de-hulled. Conventional dry extrusion or solvent (hexane) extraction operation were used to processes different batches of each soybean. The steps used to produce defatted soybean is schemed in Figure 1.

Extruded SBM was processed with an Insta-Pro model 600 Jr (Insta-Pro International, Grimes, Iowa). The extruder was basically processed using material soybeans to reach the operating temperature up to 150^0 C. After processing conditions had been obtained, TN or DV SBMs were added to the feed hopper as the remaining commodity beans exited the extruder.

Prepared SBMs were received at SDSU where they were milled to homogenous particle size and samples were weighted and sent to University of Missouri (Experiment Station Chemistry Lab, ESCL) for analyses (proximate composition and amino acids).

ESCL analytics conformed to standard methods for analysis of crude protein (AOAC 2006, method 990.03), crude fat (AOAC 2006, method 990.03), crude fiber (AOAC 2006, method 978.10), moisture (AOAC 2006, method 934.01), ash (AOAC, method 942.05), and amino acids [AOAC 2006, method 982.30 E (a,b,c)] (Table 1.)

Feeding Trial

One reference diet and four treatment diets (Table 2) were formulated using the known approximate composition of test and other feed stuffs. The required dietary protein and lipid percentage for RBT was 46% and 17%, respectively. A commercial diet was also used in the project to allow for comparisons of the feeding performance. Similar levels of mineral and vitamin premixes were added to all treatment diets and the control diet to fulfill nutritive needs. All diets were also supplemented with adequate menhaden fish oil at equal level to reach the fatty acid requirements of RBT and soybean oil was utilized to stabilize the lipid contents of all diets. Feed ingredients were weighed and then blended with ribbon mixer for approximately 5 minutes to form a homogeneous mixture and then milled to obtain similar a particle size ($\leq$250 µm). Pellets with size 3.5 mm diameter were extruded in feed speed 303 Kg per hour, pre-conditioner temperature ~96^0 C, barrel temperature ~138^0 C using a single screw extruder (ExtruTech 325) (Sabetha, Kansas, USA). The pellets were cooled down and dried at 99^0 C (Colorado Mill Equipment, Canon City, Colorado). The cooled diets were moved over a Rotex screener (Rotex Inc., Cincinnati, Ohio) and conveyed to a rotary coater for lipid finishing.

Fish Husbandry and Culture System.

Twenty Age-0 RBT with average weight ~24 g were reared in each 187 L semi-square tank of a 7,500 L recirculating aquaculture system (RAS). The fish were fed on a

commercial diet to acclimate in new RAS for approximately three days. Each diet was introduced to five tanks. The RAS was a closed-loop recirculation system that includes solids separation tanks, a bio-reactor, and UV irradiation filtration. The rate of water flow changes in single tank was 25 time per 24-hour period. A heat/chill pump was used to maintain the system temperature at $14C^0$. Culture tanks were wrapped with double-backed foil insulation to reduce water temperature fluctuations caused by ambient temperature. Fish were fed to satiation twice daily in the morning and afternoon. Fecal and other solid wastes were siphoned from the tanks daily.

Temperature, pH and dissolved oxygen were checked daily and averaged 14.6 ± 0.01^0 C, 8.8 ± 0.2 and 8 ± 0.03 mg/L, respectively. Ammonia and nitrite were checked twice a week. Unionized ammonia and nitrite averaged 0.034 ± 0.006 mg/L and 1.10 ± 0.11 mg/L, respectively. Similar flow rates in each tank, aeration by airstones (supplied from a blower) and liquid oxygen were used to keep dissolved oxygen levels close to saturation (required near the end of the trial at high tank biomass levels). The light exposure was kept at 14h light and 10h semi-dark for the study duration. The total duration of the study was 155 days

Feeding behavior and diet consumption were the initial means of comparing dietary treatments during the first two weeks of the growth trial. Feeds were measured volumetrically using calibrated tablespoons and the mass was back-calculated, based on bulk density. Feed rations were estimated for satiation at individual tank biomass levels and then increased slightly to overfeed. Uneaten pellets were counted and recorded at 30 minutes after each feeding episode. Feed consumed was calculated as the difference of the weight of fish offered and the weight of uneaten pellets. Feed remaining in each

container was weighed every evening to reconcile the daily feed amounts offered. Any fish mortalities were recorded per tank throughout the trial. Fish were sampled every after four weeks to monitor growth.

Gut Health Indices and Histology

At the end of the trial, fish in each tank were counted, and the fish were weighed individually and then bulk- weighed. Three fish per tank were euthanized using an overdose (250 mg/L) of tricaine methane-sulfonate (MS-222). Individual lengths and weights were measured and blood samples were obtained from each fish with severing tails. The blood samples were transferred into heparinized capillary tubes which were centrifuged at 12,000 rpm for 10 minutes to separate red blood cells from plasma for hematocrit calculation. The fish were also necropsied and viscera removed to separate the fat, liver and spleen for calculation of the corresponding indices. A section of the distal intestine was also obtained and fixed in 10% neutral buffered formalin for histological analysis. The fish were also filleted to determine yield. Performance parameters were calculated as follows:

$$\text{Initial weight fish}^{-1}\text{ (g)} = \frac{\text{Biomass at beginning of trial}}{\text{Number at beginning of trial}}$$

$$\text{Final weight fish}^{-1}\text{ (g)} = \frac{\text{Biomass at end of trial}}{\text{Number at end of trial}}$$

$$\text{Relative growth (\%)} = \frac{\text{Final weight - initial weight}}{\text{Initial weight}} \times 100$$

$$\text{Specific growth rate (SGR, \%)} = \frac{((\ln \text{final weight}) - (\ln \text{initial weight})) \times 100}{\text{Days of growth}}$$

$$\text{Survival (\%)} = \frac{\text{Number of fish at end of trial} \times 100}{\text{Number of fish at beginning of trial}}$$

$$\text{Condition factor (K)} = \frac{\text{Weight (g)} \times 100}{\text{Length (cm)}^3}$$

Feed allowance (g) = Sum of feed offered from start to end of trial

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Total dry feed fed for trial (g)}}{\text{Total biomass gained during trial (g)}}$$

$$\text{Viscerosomatic index (VSI, \%)} = \frac{\text{Weight of viscera (g)}}{\text{Body weight (g)}} \times 100$$

$$\text{Hepatosomatic index (HSI, \%)} = \frac{\text{Weight of liver (g)}}{\text{Body weight (g)}} \times 100$$

$$\text{Spleen somatic index (SSI, \%)} = \frac{\text{Weight of spleen (g)}}{\text{Body weight (g)}} \times 100$$

$$\text{Visceral fat somatic index (VFSI, \%)} = \frac{\text{Weight of viscera fat (g)}}{\text{Body weight (g)}} \times 100$$

$$\text{Fillet yield (FY, \%)} = \frac{\text{Weight of fillets (g)}}{\text{Body weight (g)}} \times 100$$

$$\text{HCT (\%)} = \frac{\text{Length of red cells}}{\text{Length of whole blood}} \times 100$$

Histological sections of the intestines were processed by Animal Disease Research and Diagnostic Laboratory at South Dakota State University. Digital images of the sections were recorded with an Olympus BX53 microscope using 10X magnifications. The health status of the gastrointestinal tract was assessed using a scoring system (Table 3) provided by (Barnes et al., 2015) (modified from Knudsen et al., 2007, Colburn et al., 2012 and Barnes et al., 2014).

Digestibility Trial

Diet formulations

Diets were composed of 70% control diet and 30% test ingredient with adding chromic oxide at 0.75% to all diets as an inert marker. Hamlet 300 (Hamlet Protein, Inc., Findlay, Ohio) was used as a commercial soy product for comparison to TN and DV SBMs. Ingredient preparation and diet manufacture were similar to the feeding trial diets.

Fish husbandry and culture system

One hundred, 300 g RBT were stocked into each of the six 756-L tanks of ~6000-L RAS. The temperature, pH and dissolved oxygen for the entire study averaged 13.8 ± 2^0 C, 7.1 ± 0.06 and 1.3 ± 0.4 mg/L, respectively. Fish were conditioned for one week using a reference diet without chromic oxide and then fed with test tracer diets for

seven days prior to fecal stripping. The fish were anesthetized using 50 mg/L of MS-222, wiped with a towel around the abdomen to remove water and slime, and a gentle pressure was applied from the mid-section of the abdomen towards the anus to expel the distal feces. Feces from all fish of a given tank were pooled and frozen at -20^0 C. After stripping, all fish were combined and re-randomized the next fecal collection. The fish were again fed a reference diet without chromic oxide for 7 days and then switched to the test diets for another 7 days before stripping. Each of the stripping phases provided a replicate for a particular feed digestibility treatment. The whole process was repeated until three replicates were obtained per SBM ingredient. The frozen fecal samples were then freeze-dried (Labconco Freezone 2.5, Kansas City, Missouri) for 72 hours. The dry feces were later ground and sent for protein and tracer chromium analysis together with the diets to the Central Analytical Lab, University of Arkansas (Fayetteville, Arkansas).

The apparent digestibility coefficients (ADCs) of crude protein for six diets and test ingredients were determined using indirect methods (NRC, 2011):

$$ADC_{ref\ and\ feed} = 1 - \frac{Cr_2O_3 \text{ in feed}}{Cr_2O_3 \text{ in feces}} \times \frac{\text{Nutrient content of feces}}{\text{Nutrient content of feed}}$$

$$ADC_{ingred} = ADC_{test\ diet} + [(ADC_{test\ diet} + ADC_{ref\ diet}) \times (0.7 \times D_{ref} / 0.3 \times D_{ingred})]$$

Where D_{ref} is the percentage of nutrients or Kcal/g gross of energy of the reference diet, and D_{ingred} is the percentage of nutrients or Kcal/g of gross energy of the ingredient.

Statistical Analysis

. Continuous data (e.g., growth and digestibility) were analyzed with ANOVA

models in R (R Core Team, 2017) followed by Tukey's HSD test to identify pairwise differences among treatments. Kruskal–Wallis and Mann-Whitney U tests were used to analyze distal intestinal histology rank scores. Means or medians were considered statistically different at $\alpha = 0.05$.

Results and Discussion

Ingredients and Feeds.

Ingredient analyses showed a wide range in protein, lipid and total amino acid contents among SBM products given the processing method (Table 1). The effects of the different processes were expected as solvent extraction removes significantly more lipid, thus more lipid was present in the dry extruded meals where lipid content was 15.6 to 17.4% higher in comparison with solventized meals. Also, protein concentrations were raised in the solventized meals by 9.4 to 10.1%. Increased amino acid levels followed the ratio of protein increase (~9 to 10%). Generally, DV SBMs yielded a higher percentage of protein and each amino acids than did TN SBMs. The trypsin inhibitors content in TNEXT SBM was lower than anticipated due to the temperature (friction heat) in the extrusion process to produce the extruded soybean ingredient. However, the effect of temperature on reducing the trypsin inhibitor was lower for DVEXT.

The amount of protein and amino acid in diet are considerably important for salmonids because they require high content protein diet with high quality amino acid profile. Although all formulated diets met amino acids requirements for RBT, with the exception of methionine, individual amino acid was calculated to formulate any aqua-feed. However, choosing protein source(s) that meet species nutritive requirements simplifies feed formulation. The protein source(s) should supply just enough EAA and in

the right proportions necessary for protein accretion. This requires that the amount and composition of EAA and conditional EAA (CEAA) in diets to provide free EAA and CEAA in tissues that are sufficient and of similar composition to the EAA and CEAA of tissue proteins. Lysine is one of the two most limited EAA, especially when plant protein sources. The lysine content of all diets were reached fish requirement.

Feed Palatability

In the first ten days of the growth performance study the amount of consumed diet was same among all treatment diets. It may be due to the fact that all treatment diets include the same levels of dietary ingredients and ant-nutritional factor were low in treatment diet because of using the high temperature and extrusion to create pellet diet. Some of factors, which effect fish acceptability of feed, are protein level, amino acids file and amount of anti-nutrient in diets or used ingredients.

Ingredients Digestibility

Overall, no significant differences (P=0.86) were found in protein ADCs among all SBM types. This may be due to the amount trypsin inhibitors in all diets were not high enough to provide differences in protein ADCs. The amount of trypsin inhibitor in diets may be gone-down more using the diet manufacturing process as observed for the feeding trials. For example, the level of trypsin in unprocessed conventional soybean is 56,700 TIU/g. That amount was decreased to 9,213 TIU/g (%84) by reason of using high temperature 150^0 C for 10 minutes through extrusion processes. Then during making pellets using other high temperature (96 and 138) ^{0} C causes more reducing of trypsin inhibitor amount to 269 TIU/g by (97%).

Feeding Trial

Non statistical differences were found in final weight (P=0.343), weight gain (P=0.39), daily gain (P=0.35), RG (P=0.43), SGR (P=0.26) among the test diets and the reference diet (Table 4). This is due to the nutritional value of diets and consumed feed (P=0.15) was similar among the diets. Also, all diets were covered or exceeded nutrient requirements of RBT (Table 2). The amount of digested protein among diets were not affected by small. Survival rate is considered to be 100%, only the few mortalities were due to jump from the tanks. However, significant differences were found in FCR (P=0.002). The FCRs of TNEXT and DVSE were higher than rest of diets of experiment. FCR is effected by several reasons such as fish spaces, fish size, amount of consumed feed, the body fat content, and the diet nutritional ingredient and the balance of the diet nutrients and apparent digestibility coefficient of diet ingredients. This high level of FCR in DVSE and TNEXT may be due to using part of protein for energy purpose. While part of consumed protein are used for energy purpose is not stored in body and cause slow growth (Lovell, 2012). Also, feed the fish till satiation lead to higher level of FCR (NRC, 2011).

VSI (P=0.12), SSI (P=0.14) and HSI (P=0.95), VFSI (P=0.56) (Table 5) did not differ among dietary treatments, as did the FY (P=0.10). All K values (1.46 to 1.53) were ranged from good to excellent (Barnham and Baxter, 1998), but were not different (P=0.36) among diets.

Gut Health

The three histological parameters tested and scored for the distal intestinal inner morphology were lamina propria, connective tissue and vacuolization. Statistical

difference were not detected all diets in histological exam scores. However, of fish fed TNEXT had lowest score of lamina propria (1.3±0.3). Additional sections will be scored to determine if increased sample size can separate out the treatments. Morphological changes in the inner walls of the distal intestine could be happened due to present saponin and lectins in SBM (Iwashita et al., 2008; Buttle et al., 2001). In the current experiment, the different score of lamia propria may be due to the content of lectins and saponins in the all diets. Additional analysis to determine lectins and saponins may describe this small difference.

In conclusion, the current study shows all four soybean ingredients (DVSE, DVEXT, TNSV and TNEXT) can be used (20%) in RBT without causing significant differences in growth performance such as, SGR, GR, daily weight gain and final weight. However, statistically, DVSE and TNEXT diets are higher than the rest of experimental diets. All treatment diets were palatable to fish. There was not any statistically significant change in distal intestinal histology in compering treatment diets with reference diets. The digestibility coefficient for protein in all ingredients was similar to that of the reference diets.

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Table 1. Proximate composition (g/100g, dry matter basis) of DVSE, TNSE, DVEXT, and TNEXT. Triple Null = TN and Davison (conventional) = DV, and EXT = extruded and SE = solvent extracted

Nutrient	**DVSE**	**TNSE**	**DVEXT**	**TNEXT**
Crude Protein	53.62	48.78	45.57	39.37
Moisture	2.54	4.45	5.31	2.83
Crude Fat	3.46	3.35	20.47	21.75
Ash	6.34	6.19	5.10	4.91
NFE	34.04	37.23	23.55	31.14
Trypsin Inhibitor (TIU/g)	11402	10925	9213	1904
EAA				
Arginine	3.89	3.49	3.35	2.89
Histidine	1.40	1.29	1.19	1.06
Isoleucine	2.55	2.29	2.15	1.86
Leucine	4.09	3.78	3.48	3.15
Lysine	3.51	3.20	2.97	2.56
Methionine	0.76	0.68	0.65	0.56
Phenylalanine	2.74	2.50	2.37	2.08
Threonine	2.09	1.89	1.80	1.59
Tryptophan	0.71	0.60	0.62	0.50
Valine	2.55	2.29	2.17	1.90
Conditionally EAA				
Cysteine	0.84	0.70	0.73	0.54
Tyrosine	1.91	1.69	1.65	1.49
Non-EAA				
Alanine	2.25	2.08	1.91	1.72
Aspartic Acid	6.11	5.31	5.15	4.35
Glutamic Acid	9.42	8.59	7.80	6.91
Glycine	2.25	2.03	1.96	1.71
Proline	2.74	2.51	2.31	2.04

Serine	2.30	2.04	2.03	1.76	
Amino acid-like (sulfonic acid)					
Taurine	0.09	0.10	0.08	0.09	

Table 2. Formulations (g/100g, dry matter basis) of diets used in the RBT feeding trials. Triple Null = TN and Davison (conventional) = DV, and EXT = extruded and SE = solvent extracted

Ingredient	**Referenc e**	**DVSE**	**DVEX T**	**TNSE**	**TNEX T**	**Commercial**
Fish meal (Special Select)	20.00	20.00	20.00	20.00	20.00	
DVSE	0.00	20.00	0.00	0.00	0.00	
DVEXT	0.00	0.00	20.00	0.00	0.00	
TNSE	0.00	0.00	0.00	20.00	0.00	
TNEXT	0.00	0.00	0.00	0.00	20.00	
Pork blood meal	3.00	3.00	3.00	3.00	3.00	
Poultry byproduct meal	15.00	15.00	15.00	15.00	15.00	
Hydrolyzed feather meal	2.00	2.00	2.00	2.00	2.00	
Whole cleaned wheat	22.37	12.57	13.97	11.60	13.00	
Yellow whole corn	5.90	7.00	6.50	7.00	6.42	
Wheat gluten	12.00	1.50	3.50	2.77	5.00	
Carboxymethyl cellulose	0.50	0.30	0.20	0.00	0.15	
Vitamin premix	1.70	1.50	1.50	1.50	1.50	
Trace mineral premix	1.50	1.50	1.50	1.50	1.50	
Lysine	100	1.00	1.00	1.00	1.00	
Tryptophan	0.10	0.10	0.10	0.10	0.10	
Methionine	0.50	0.50	0.50	0.50	0.50	
Proximate analysis						
Protein	45.6	46.7	46.6	46.3	47.3	47
Lipid	17.5	16.4	17.8	17	17.7	18
Fiber	1.9	1.97	2.13	2.15	2.24	2.7
Ash	8.69	9.55	9.39	9.63	9.48	8.93

NFE	26.31	25.38	24.08	24.92	23.28	23.37
Trypsin Inhibitor	ND	1788	269	1691	37	ND

NFE= Nitrogen free extract (100-protein-lipid-ash-crude fiber-moisture)

Table 3. Histological intestine scoring system used on RBT distal intestines in feeding trial after 155 days (Barnes et al., 2015, modified from Knudsen et al., 2007*).*

Score	Appearance
Lamina propria of simple folds	
1	Thin and delicate core of connective tissue in all simple folds.
2	Lamina propria slightly more distinct and robust in some of folds.
3	Clear increase in lamina propria in most of the simple folds.
4	Thick lamina propria in many folds.
5	Very thick lamina propria in many folds
Connective tissue between base of folds and stratum compactum	
1	Very thin layer of connective tissue between base of folds and stratum compactum.
2	Slightly increased amount of connective tissue beneath some of the mucosal folds.
3	Clear increase of connective tissue beneath most of the mucosal folds.
4	Thick layer of connective tissue beneath many folds.
5	Extremely thick layer of connective tissue beneath some of the folds.
Vacuoles	
1	Large vacuoles absent.
2	Very few large vacuoles present.
3	Increased number of large vacuoles.
4	Large vacuoles are numerous.
5	Large vacuoles are abundant in present in most epithelial cells.

Table 4. Performance metrics (mean ± standard error) of RBT in feeding trial after 155 days. Triple Null = TN and Davison (conventional) = DV, and EXT = extruded and SE = solvent extracted.

Metric	**Reference**	**Commercial**	**DVSE**	**DVEXT**	**TNSE**	**TNEXT**
Initial wt (g)	24.4±0.3	24.4±0.1	24.1±0.2	24.0±0.2	24.0±0.1	24.3±0.1
Final wt (g)	535± 23	512± 17	477± 14	513± 24	518± 07	520± 13
Wt gain (g)	511± 23	488± 17	453± 15	489± 24	494± 08	497± 13
Daily gain (g)	3.30± 0.14	3.15± 0.11	2.92± 0.10	3.16± 0.15	3.20± 0.09	3.19± 0.05
RG (%)	2,094±86	1,953±92	1,855±46	1,929±88	1,932±31	1,965±89
SGR (%/day)	1.99±0.03	1.95±0.03	1.92±0.01	1.94±0.03	1.94±0.01	1.95±0.03
Feed /tank (g)	12,630±391	11,584±389	11,812±132	11,922±376	12,038±139	12,689±307
FCR	1.18±0.03	1.18±0.03	1.29±0.02	1.21±0.03	1.24±0.04	1.27±0.01
Survival	100±0.0	97.5±1.4	98.8±1.3	95±0.0	93.8±1.3	96.3±2.4

Wt=weight, RG=Relative growth, SGR=Specific growth rate, FCR=feed conversion ratio

Table 5. Health indices (mean + standard error) of RBT in feeding trial after 155 days. Triple Null = TN and Davison (conventional) = DV, and EXT = extruded and SE = solvent extracted.

Index	**Reference**	**Commercial**	**DVSE**	**DVEXT**	**TNSE**	**TNEXT**
Fulton's K	1.53±0.02	1.52±0.03	1.50±0.2	1.54±0.02	1.47±0.4	1.53±0.02
VSI (%)	12.04±0.27	12.76±0.70	14.05±0.83	12.756±0.89	12.91±0.38	11.88±0.89
SSI (%)	0.063±0.017	0.062±0.009	0.061±0.003	0.063±0.007	0.053±0.003	0.061±0.003
HSI (%)	1.510±0.166	1.628±0.146	1.633±0.179	1.321±0.098	1.430±0.06	1.189±0.056
VFSI (%)	2.8±0.8	3.1±0.7	3.6±0.9	3.24±1.2	3.7±1.1	4.0±1.1
Hct (%)	41.82.1	44.3±3.2	42.7±1.8	46.2±1.0	40.9±0.8	42.2±4.0
FY (%)	48±1.80	49±2.63	48±1.50	46±1.39	45±1.20	51±1.95

Fulton's K= Fulton's condition factor K, FY= fillet yield, VSI= viscero-somatic index, SSI= spleensomatic index, HSI=hepato-somatic index, VFSI= visceral fat-somatic index, Hct= hematocrit.

Table 6. Apparent digestibility coefficients (ADC, mean ± standard errors) of soy products and the Reference diets. Triple Null = TN and Davison (conventional) = DV, and EXT = extruded meal (full fat) and SE = solvent extracted.

	Reference	**DVSE**	**DVEXT**	**TNSE**	**TNEXT**	Commercial
ADC (%)	80.1±1.0	84.6±5.0	76.5±1.0	76.0±8.0	80.3±4.0	85±6

Table 7. Health scores for RBTdistal intestine in feeding trial after 155 days. Triple Null = TN and Davison (conventional) = DV, and EXT = extruded meal (full fat) and SE = solvent extracted.

Diets	**Lamina propria**	**Connective tissue**	**Vacuolization**
Reference	2.7±0.3	1.3±0.3	2±0.0
Commercial	2.7±0.3	1.7±0.3	2.7±0.3
DVSE	2.3±0.9	1.7±0.7	2±0.0
DVEXT	2.7±0.7	1.7±0.3	1.6±0.3
TNSE	2±0.3	1.3±0.3	2±0.0
TNEXT	1.3±0.3	1.3±0.3	2.3±0.3

Figure 1. Processes to prepare Defatted soybean.

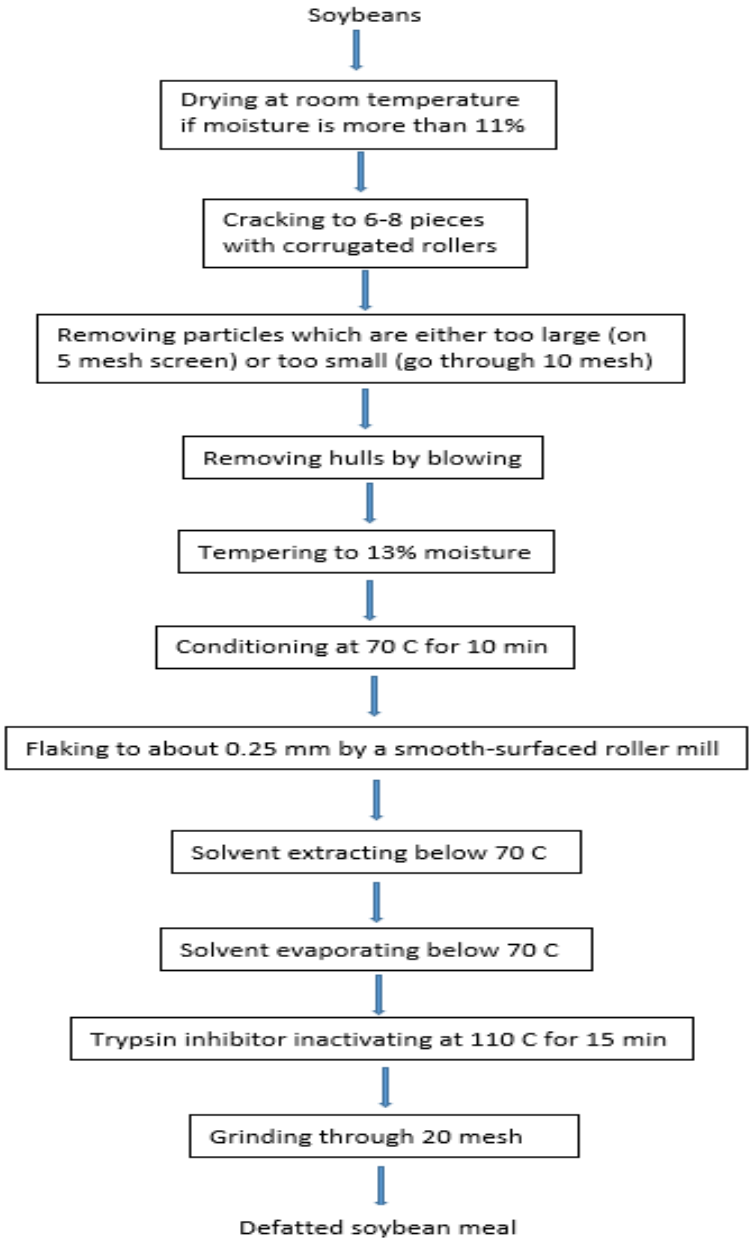
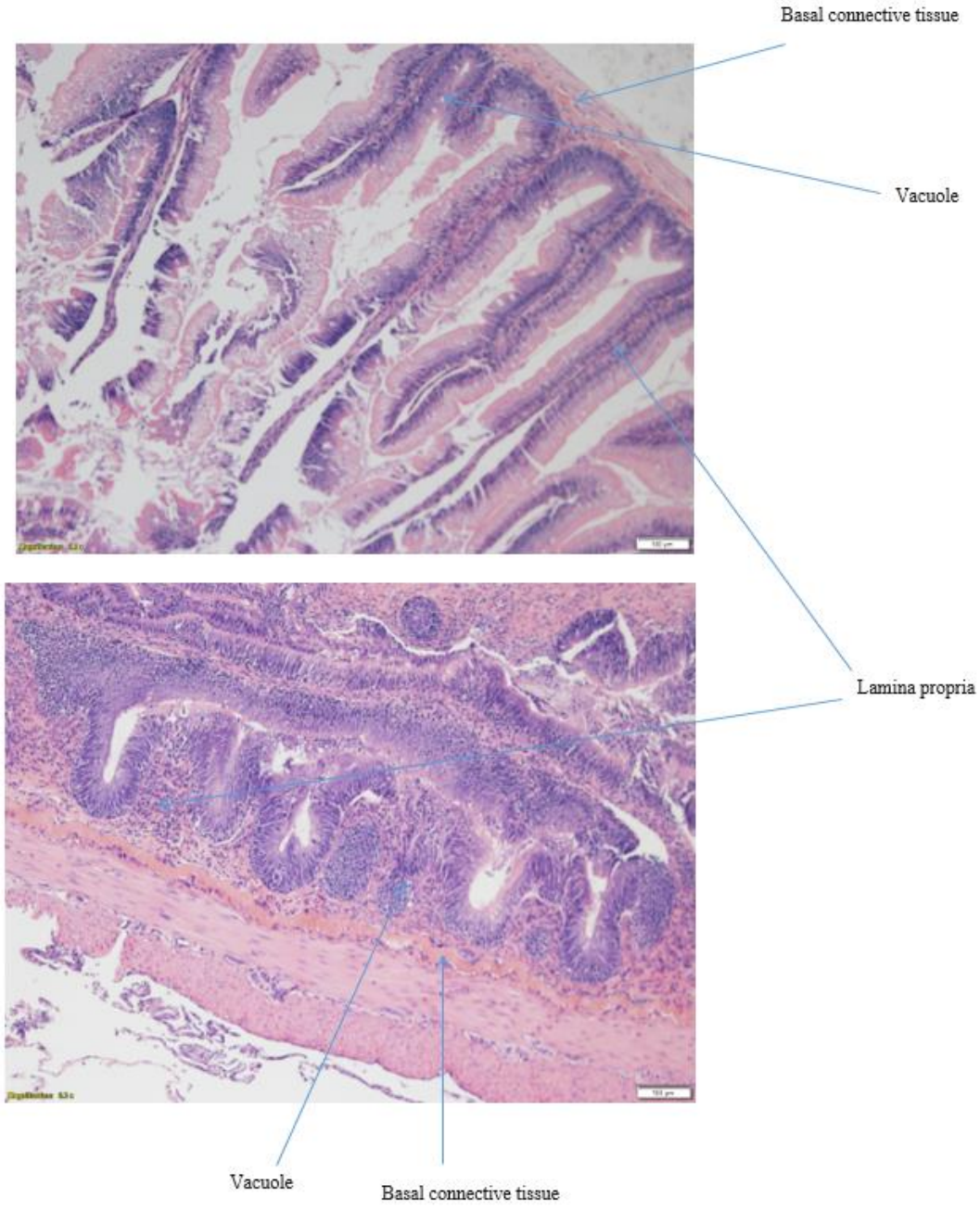


Figure 2. Histological sections of the distal intestines of RBT(x10) showing the lowest (top) and highest (bottom) scores after 155 days of feeding.



CHAPTER 4. RESEARCH NEEDS

This dissertation reports on feeding trial investigations that assessed the impact of plant proteins on fish growth performance, and gut histology and health. The plant materials studied included varieties of SBM and fermented co-blends of SBM and pearled barley. The main driver behind this study is that the aquaculture sector in Kurdistan is expanding and so demand for efficient feed sources is growing; especially for feed that provides all nutritional requirements at a lower price. Feed efficiency is essential to promote growth in the sector as feed costs are a major portion of total aquaculture production costs. In Kurdistan, all FM used in aquaculture is imported. Moreover, the price of FM, main protein source in aqua-feed industry, is commonly higher than other feedstuffs used in fish feed formulations. As such, using an improved co-blend of SBM and barley have the potential to reduce overall feed costs. The results of this study provide important information to the University of Sulaimani in Kurdistan-Iraq because the barley is available at a low price, locally grown, and supply is abundant. Because the primary use of locally grown barley is for animal feed, this adds additional utility of a locally grown crop.

The co-blend products utilized in feeding trials used fermentation to improve digestibility of protein and mineral ingredients and improve the palatability. In addition, fermentation reduces the amounts of some ANFs and converts some carbohydrates to protein. The results of these trials in the dissertation provides more information towards the development and use of plant protein sources in aqua-feed manufacturing.

The following are the conclusions of these studies and suggestions for further research to expand on these results:

1. A new produced soybean that contain minimal of three anti-nutrient factors (trypsin inhibitor, 34P antigen, and agglutinin lectin) was used as a plant protein source in RBT feeding (chapter 3). The SBMs used were in two different process forms: defatted soybean meal and extruded full fat soybean. Two treatment diets were formulated to use 20% of TNSE and TNEXT. These SMB treatment diets were compared with a reference diet and a commercial diet. The study results showed that no significant differences were found among all six experimental diets in whole growth performance parameters (RG%, SGR%, final weight, and average individual weight), health assessments or distal intestine morphology. Feed palatability was assessed during the first 10 days of feeding; all feeds were found to be palatable. The FCR of fish fed all diets was statistically similar. Further, the apparent protein digestibility was not statistically different among dietary treatments. Overall, the results show that an inclusion level of 20% of the new soybean variety in the whole diet was similar to a conventional soybean in RBT performance.

2. Up to 35% of FM was replaced with fermented co-blends of SBM and pearled barley (SBM75:BRL25) in RBT diets without having any significant change in growth parameters such as GR, SGR, final weight, and weight gain. The protein sources used to replace FM were fermented co-blends, SBM75:BRL25 and SBM50:BRL50 (chapter 2). In addition, the health assessments of the fish fed diets containing 25% and 35% of FM were not significantly different from reference diets. Also, feed consumption was statistically similar for all diets. Palatability of treatment diets was similar to a reference diet. Furthermore, the FCR values of 25% and 35% FM

replacements were similar to the reference diet. Thus, up to 35% of FM can be replaced in RBT without reducing feed performance.

These findings suggest that locally grown barley can be used to significantly reduce FM costs without degrading feed performance. For future studies, it would be beneficial to assess the maximum FM replacement ratio, beyond 25% and 35% used in this study, to identify the upper limits of the replacement ratio that will still result in acceptable feed performance. Further fermentation studies also can be done with other microbes to determine if a higher barley to SBM ratio can yield a similar co-blend protein level through the proliferation of single-cell protein. This would provide a feed ingredient at lower cost by reducing the amount of SBM necessary for fermentation and feed production in Kurdistan.