SOY AQUACULTURE ALLIANCE (2018 - 2020)

Project final report

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Project Title: Indirect criteria to select the farmed fish lines to enhance the efficiency of soybean meal utilization in their diet

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Introduction

Aquaculture is the fastest growing food-producing sector and it comprises half of the total fish supply sector in both the U.S. and the world. In recent years with increased production of carnivorous fish such as rainbow trout, aquaculture industry has experienced a massive expansion worldwide. This increment is mainly expedited by the use of fishmeal which is the most important protein ingredient owing to high nutritional and palatability value. Utilization of alternative protein and oil sources to replace fishmeal in feeds is critical for continued and sustainable aquaculture industry. However, literature shows suboptimal protein retention efficiency and lower growth performance when fish are fed high plant protein (low fishmeal) even if all known essential nutrients are provided in the diet above required levels.

Selective breeding of fish species offers a substantial opportunity for increasing production efficiency, health, production quality and, ultimately, profitability in aquaculture industries. Growth and feed utilization traits are of particular economic and importance to the aquaculture industry. To improve production efficiency and quality of the US aquaculture industry, a selective breeding program for rainbow trout has been established in Aquaculture Research Institute (ARI) at University of Idaho. USDA Agriculture Research Service (USDA-ARS) in collaboration with University of Idaho ARI have developed rainbow trout families that exhibit high growth rates when fed all plant protein. The selected rainbow trout strain is a unique model to identify genetic and physiological parameters associated with sustainable plant protein utilization in fish. They grow rapidly when fed all plant-protein based diets, unlike un-selected trout that exhibited 10-15% lower growth than selected trout. In addition to genetic selection, improvement of diet plays an important role in increasing the fish health and growth, which will result in advances in aquaculture sustainability and profitability. Development of rainbow trout lines that are capable of consuming the all plant protein as well as all plant oil diet is the ultimate goal of trout production industry.

Like other agriculture sectors, aquaculture industry plans to improve according to production system changes (e.g. replacement of fishmeal with plant meal) through genetic improvement programs. Breeding programs exist for various commercial traits like growth, health improvement, feed efficiency and substitution of feed sources in carnivorous fish (Henryon et al., 2002; Abernathy et al., 2017. Selective breeding of fish species offers a substantial opportunity for increasing production efficiency, health, production quality and, ultimately, profitability in aquaculture industries. Growth and feed utilization traits are of particular economic importance to the aquaculture industry (Henryon et al., 2002). Although many studies investigated the effect of genetics in growth and nutrient retention when the fish were fed plant-based diet, Overturf et al. claimed that they were the first group to select rainbow trout on a fishmeal-free plant-based diet for several generations (Overturf et al., 2013). To improve production efficiency and quality of the US aquaculture industry, a selective breeding program for rainbow trout has been

established in Aquaculture Research Institute at University of Idaho, based on the genetic variability for growth related trait observed in rainbow trout families when fed plant-based diets. Starting in year 2000, University of Idaho Aquaculture Research Institute in collaboration with the USDA Agriculture Research Service have been developing rainbow trout families (ARS/UI strain) that exhibit high growth rates when fed all plant protein. The weight gain and length differences in a five month feeding trial, with initial fish weight of 30 ∓ 1.6 g in each year of selection is visualized in figure 1. The ARS/UI selected rainbow trout strain is an unprecedented and outstanding model to study genetic and physiological parameters in regards to sustainable plant protein utilization in fish. Unlike unselected trout with 10-15% lower growth rate when fed all-plant protein diet, UI strain grows rapidly and efficiently (Overturf et al., 2013).

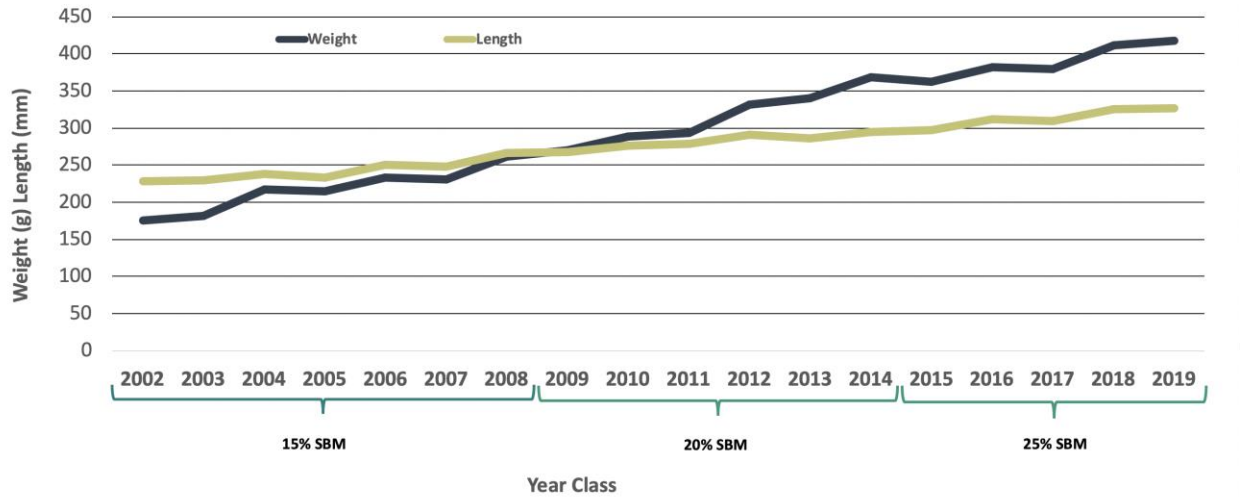

Figure 1: USDA-ARI trout families weight gain and length improvements in a 5 month feeding trial during a long-term selection program, initial fish weight of 30 ∓ 1.6 g.

After nine generations of selection for optimal plant protein consumption, the goal of the present project was to identify the best lines among the selected families of rainbow trout, which can utilize the plant protein (soy-based ingredients) more efficiently and investigate potential molecular markers and microbial symbiosis to improve the future selection programs. The hypothesis is that genetic selection and feed improvement strategies can be used to increase the efficiency of the soy-based diet consumption. The specific aim of this proposal is to focus on the indirect criteria by which we can improve the feed efficiency to fulfill the sustainability and profitability of the aquaculture industry.

Objectives: to develop an indirect benchmark to select the families of rainbow trout to enhance the efficiency of soybean-based diets utilization:

1- to determine phenotypic relationship between residual feed intake and body weight variations using compensatory feeding regimes.

2- developing alternative method to improve feed efficiency of Soybean Meal Diet (SBMD).

Feed formulation, proximate composition of experimental diet:

We utilized an in-house formulated Soybean-based feed with 41.23% crude protein and 21.52% crude lipid. Feed formulation and proximate composition are presented in Table1 and Table 2 respectively.

Table 1: Feed Formulation (%) of diet developed at the Aquaculture research Institute (Hagerman)

Ingredient name	**g/100 g**
Soybean Meal, Solvent extracted	25.00
Wheat flour	13.3
Wheat gluten meal	2.24
Fish Oil, Whitting trimmings oil	17
Soybean Protein Concentrate, Profine	23
Vitamin Premix, ARS702	1.00
Trace min pre mix ARS 1520	0.1
Stay-C	0.2
Lecithin	2.00
Taurine	0.5
Astazanthin, pink	0.08
Corn Protein Concentrate, E75	10.23
Dicalcium Phosphate	2.85
Lysin-HCL	1.67
DL-Methionine	0.6
Threonine	0.23
Formula total:	100

Table 2: Analyzed nutrient profile of feed

Nutrient	Amount with Unit
Dry matter	92.97%
Moisture	6.9%
Protein, Crude	41.23%
Fat, Crude	20.52%
Fiber, Crude	1.9%
Calcium	0.73%
Phosphorus, total	0.91%
Ash	5.36%
Phosphorus, Digestible	0.39%
Protein, digestible	4.11%
Fat, digestible	16.84%
Methionine	1.21%
Cystine	0.6%
Lysine	3.33%
Tryptophan	0.46%
Threonine	1.75%
Isoleucine	1.77%
Histidine	0.96%
Valine	1.9%
Leucine	3.64%
Arginine	2.37%
Phenylalanine	2.06%
Taurine	0.5%
Sodium	0.04%
Potassium	2.98%
Magnesium	0.18%
Sulphur	0.17%
Manganese	35.59 PPM
Iron	407.13 PPM
Copper	22.37 PPM
Zinc	61.68 PPM
Selenium	0.53 PPM
Cobalt	0 PPM
Flourine	0.01%
Chloride	0%
14:0	0.68
16:0	3.15

16:1	1.53
18:0	0.68
18:1	5.44
18:2 n-6	0.34
18:3 n-3	0.17
18:4 n3	0.17
20:1	0.34
20:4 n-6	0.22
20:5 n-3	1.45
22:1	0.07
22:5 n-3	0.27
22:6 n-3	0.77
Total n-6	1.11
Total n-3	2.38
n3/n6 ratio	0.37

Experimental phase I: Individual performance and separation into groups

General approach and proposed project activities with time frame are shown in Figure 2. We have used 12 families of Rainbow Trout from Hagerman Fish Culture Experiment Station (HFCES) of University of Idaho for this study. The twelve families have been selected which can tolerate high quantities of plant protein based feed including soy protein in their diet for seven generations. Fish was stocked in a flow through system in spring water (15^{o}C), and fed with our selected SBMD at satiation level. We reared the fish in the same condition in four tanks (400 in each tank) at HFCES at University of Idaho as early as possible. SBMD is chosen based on our previous genetic selection experiments. Major ingredients for ***SBMD (crude protein: 41% and lipid: 21%) are fishmeal: 0%, soybean meal: 25%, soy protein concentrate: 23%, corn protein concentrate: 10%.*** Please refer table 1 for feed formulation and their chemical composition.

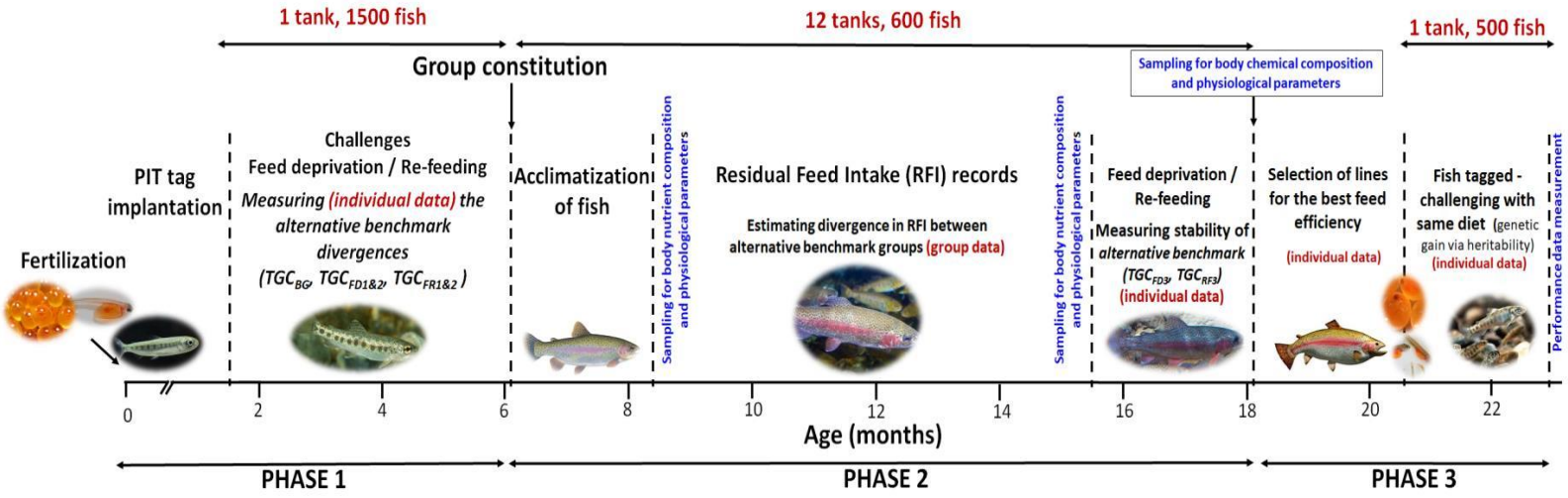

Figure 2: Project activities with time frame.

***This study was comprised of three main experimental phases and 5 steps*:**

Phase I:

At day 120-post fertilization (pf), 1600 fish were randomly chosen from 12 selected families of HFCES, individually tagged with a Passive Integrated Transponder and individually weighed. Fish were fed ad libitum with previously described diet, containing 41% protein and 20% lipid. Individual weight was recorded at day 174 and day 273 pf for assessing the basic growth (BG). Fish were then submitted to two consecutive periods, each consisting of four weeks of feed deprivation (FD1 day 285 to day 313 pf, and FD2 day 342 to day 365pf) followed by four weeks of ad libitum re-feeding (RF1 day 318 to day 337 pf, and RF2 day 371 to day 393 pf). To record individual weight, free access to food was stopped 24 h before the measurements; fish were anaesthetized (Tricaine mesylate, MS-222), individually identified using a PIT-Tag reader and weighed to the nearest 0.1 g. They were re-fed the day after the measurements, except when the following period was FD.

***Step 1:** individual growth, feed deprivation (FD) and re-feeding period (RF) performance was recorded on the largest number of fish representative of the families and reared in a common tank;*

Growth rate was expressed as the Thermal Growth Coefficient (TGC) in each period, and is referred to as TGCBG, TGCFD1, TGCRF1, TGCFD2, and TGCRF2 for the different periods. Separation into groups: We sorted the fish according to the TGC values measured during the feed deprivation and re-feeding periods: low or high weight loss during feed deprivation (FD- and FD+, respectively), low or high weight gain during re-feeding (RF- and RF+, respectively)

and constitute four groups: FD-/RF-, FD+/RF+, FD-/RF+ and FD+/RF-. The four groups were constructed at the end of the second RF period. TGCFD and TGCRF were each regressed and the residuals of the regressions were standardized and their values (FDcorr and RFcorr) were used to constitute the groups.

Step 2: *fish performance was classed as FD-, FD+, RF- and RF+ for fish exhibiting loss (FD) and gain (RF) of weight relatively lower (-) and higher (+) than the population mean resulted in clustering of the fish into four triplicate groups FD-/RF-, FD+/RF+, FD-/RF+ and FD+/RF-.*

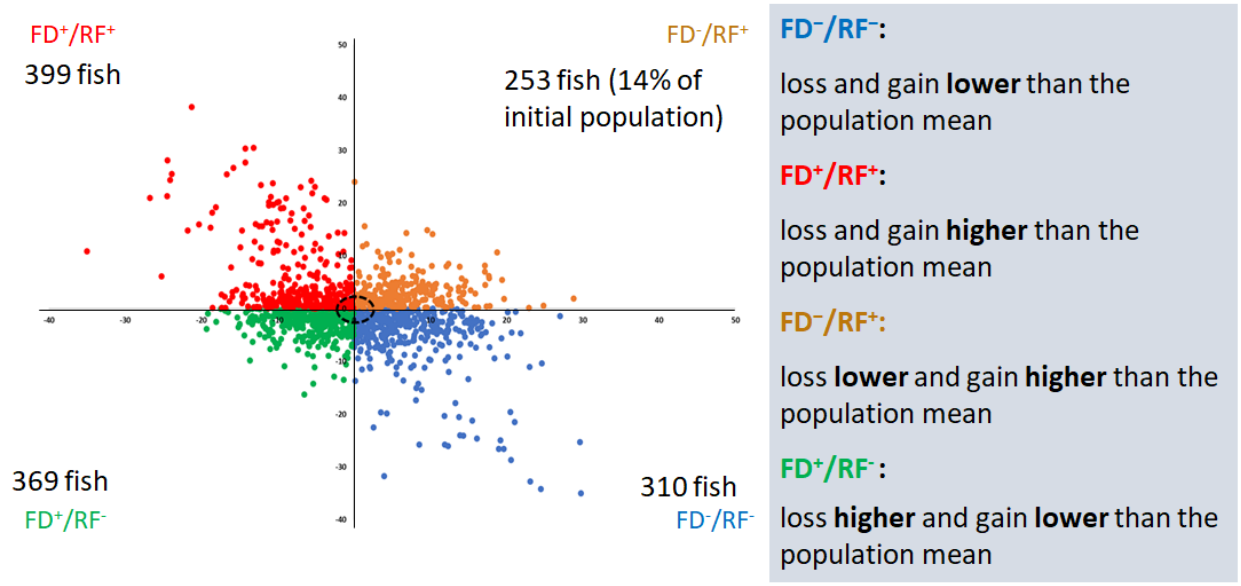

Figure 3: Fish shows variation in terms of weight gain and loss in response to FD and RF. Grouping of fish. Total fish were divided into four groups based on weight gain and loss of fish during feeding regime.

Phase II:

Step 3: *Residual feed intake (RFI) of each group (FD-/RF-, FD+/RF+, FD-/RF+ and FD+/RF-) was measured based on body weight gain and feed intake for over a period of 2 months.*

Feed Conversion Ratio (FCR), and stability of the indirect criteria

Fish was rearing for Feed Conversion Ratio (FCR) measurement: After sorting the fish into four groups, the 253 fish from each group was randomly distributed into three replicated tanks (1500 l; 84 fish per tank). Tanks are supplied with flow-through water and temperature is maintained at ~15°C. Fish were fed at satiation level with the same plant-based diet (SBMD) as the one used in the experimental phase I. After a 3-week adaptation period, all the fish were fed continually for 8 weeks and feed conversion ratio (FCR= Feed fed (g)/ body weight variations (g)) was recorded.

Results of recorded FCR showed that the FD-/RF+ (loss lower and gain higher than the population mean) shows the lowest FCR of 0.99 and at the other end of the spectrum, group FD+/RF- (loss higher and gain lower than the population mean) showed the highest FCR (1.4) which translates to the lowest feed efficiency. These results can be observed in figure 4.

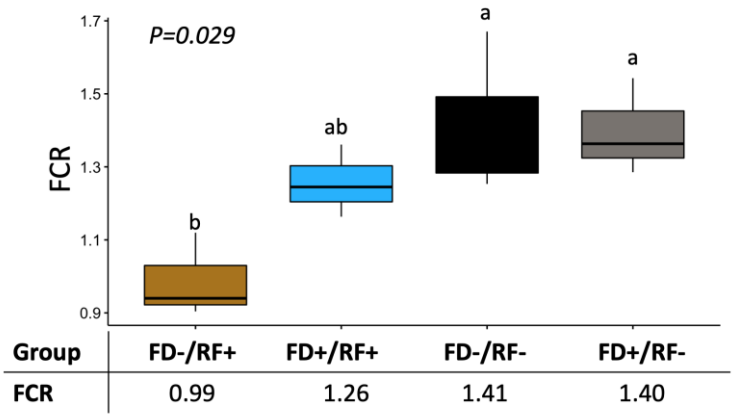

Figure 4: Feed Conversion Ratio of the Four Groups

Step 4: at the end of the experiment *step 3*, individual FD and RF performance has been re-evaluated as *done in step 2*, and measured the consistency of weight gain and loss thereafter. Using the same criteria and methodology from step 2 of the experiment fish was grouped for the second time and the performance of individuals was re-evaluated in each group.

Consistency of FD/RF growth performance:

Traits measurement and statistical analyses: Body weight gain (BWG) and feed intake recorded for each replicated tank during fish rearing is used to estimate RFI. For each replicate, feed intake is calculated as the difference between feed distributed and feed waste over the period lasting, and the mean metabolic body weight (MMWT) is calculated as the weight at the midpoint of the test period. The expected FI is estimated using the following model (Grima et al., 2008, Grima et al., 2010a,b and Crews et al., 2005):

$Yij = \mu + groupi + eij$

where Yi is the performance of an individual fish, μ is the population mean, group i is the fixed effect of the group i (i=1,..4), and eij is the random residual. The effect of the groups is assessed on each variable recorded during the second phase. In addition, to test the effect of feed deprivation performance alone, we merged the data obtained for the FD-/RF- group with that from FD-/RF+, and do the same for FD+/RF- and FD+/RF+. In the same way, we merged the data obtained for FD-/RF- with that for FD+/RF-, and data for FD-/RF+ with that for FD+/RF+, to test the contrast between fish with low (RF-) and high (RF+) weight gain during re- feeding. Group effects on FI and FCR is analyzed using tank replicate as the experimental unit (as FI and FCR cannot be estimated on individuals) with the model: Yij = μ + groupi + eij,

where Yij is the performance of the replicate, μ is the population mean, groupi is the fixed group effect, and eij is the random residual. BWG, TGCFD3 and TGCRF3 analyses is performed (Grima et al., 2010a) using individual fish as the experimental unit (n=1012) and the following mixed model: Yijk = μ + groupi + replicate(group)ij + eijk,
where Yijk is the performance of an individual fish, μ is the population mean, replicate(group)ij is the random tank replicate effect nested within group, and eijk is the random residual. The correlations between RFI and the other variables is examined with the CORR procedure of SAS, using tank as the experimental unit. The consistency of fish response to feed deprivation and re-feeding periods is assessed by the correlations between TGCFD1&2 and TGCFD3 and between TGCRF1&2 and TGCRF3 using the CORR procedure of SAS (Grima et al., 2008, Grima et al., 2010a,b).

The result of grouping after the second feeding challenge can be observed in figure 5A. The individuals that have showed the same pattern of weight gain and loss during both challenges are shown by green asterisk, and their distribution among families is illustrated in figure 5B.

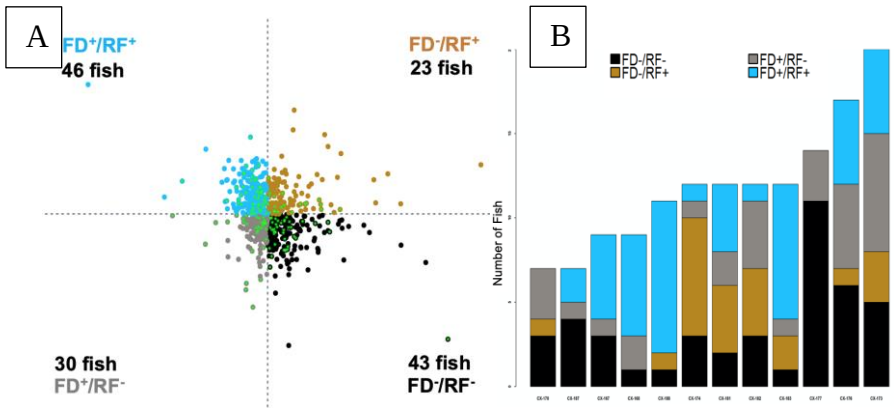

Figure 5: Fish response to compensatory feeding challenge in the 2nd feeding challenge (A), individuals represented in * followed the same body weight variation pattern in two feeding challenges and have been selected for breeding. (B) distribution of selected fish among the 12 families.

Overall message from PHASE III revealed that FD^-/RF^+ contained 23 fish that have best feed efficiency capacity compared to other groups. FD^-/RF^+ could be considered for breeding program as this group has FCR 0.99 whereas other groups exhibited FCR in the range of 1.26 -1.41. Overall, feed efficiency capacity increased from 26 to 41%. Which is great news for fish farmers especially for fish breeder and soy producers.

Investigating the gut microbiome variations in the gut during the second feeding challenge:
After each FD and RF we collected digesta from the distal intestine of 9 fish from each group by opening the gut and scooping the digesta out in a septic way to reduce the chance of contamination. Additionally, water microbiome (using DNeasy PowerWater Kit) and feed microbiome was sampled at each time point. We extracted DNA from all digesta samples using Qiagen QIAamp PowerFecal DNA Kit and sent them to the University of Northern Texas Genome Core for library prep and sequencing of the V4 region of 16S gene. Our data shows that, the microbial community at the distal intestine of these fish loses diversity after each FD period and the dominant genus is *Cetobacterium*. On the other hand, the gut microbiome is more diverse after a re-feeding period, the most abundant genera are: *Cetobacterium, Mycoplasma* and *Clostridium* respectively. These results show that the genus *Cetobacterium* outcompetes all other genera when there are no nutrients available for the microbiota, but the same genera recolonize the gut after the RF period regardless of the variations in water microbiota over time. These results can be viewed in more details in figure 6.

FD-/RF+ and Control group had a similar network.

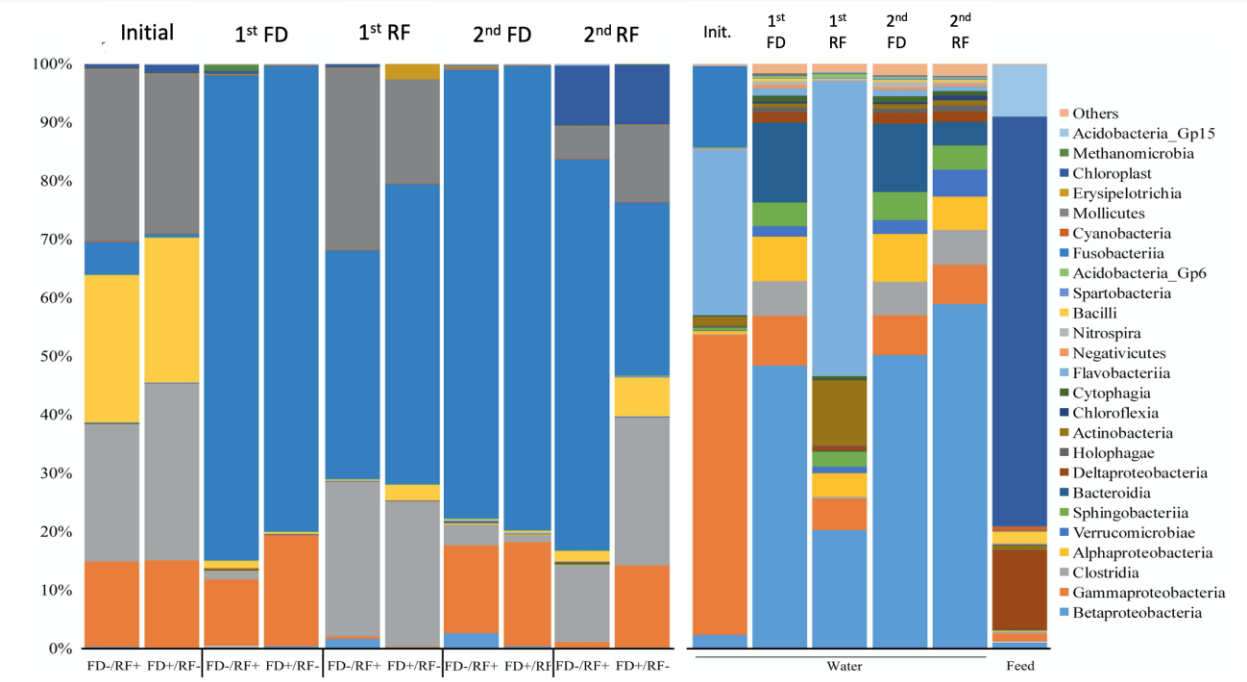

Figure 6: Gut Microbial Abundance at the Genus Level During the Second Feeding Challenge
Ecological network describes the interaction between species of the intestine microbial community. The network graph with submodule structure by the fast-greedy modularity optimization method. Each node in network graph indicates one OTU. Colors of the nodes indicate different major classes. The edges (Blue edge = positive interaction and red edge = negative interaction) inside ecological network represent the interactions between species.

Through interactions between species, intestinal bacteria can form a complex ecological network and rely on this network to maintain its dynamic balance. The dominant microbiota is the main component of the network structure. A network connection between two OTUs describes the co-occurrence of the OTUs, which may be caused by similar or complementary functions or shared environmental conditions of the species. A recent study has shown that ecological competition could improve microbiome stability (Coyte et al., 2015). In this study, the intestinal microbiota from FD+/RF- group was dominated by cooperative interactions. Coyte et al. (2015) demonstrated that the cooperation could destabilize the system due to its positive feedbacks. Hence, the stability of the sub-modules in FD+/RF- network would be impressionable, and the ecological network of the FD+/RF- group tended to be destabilizing.

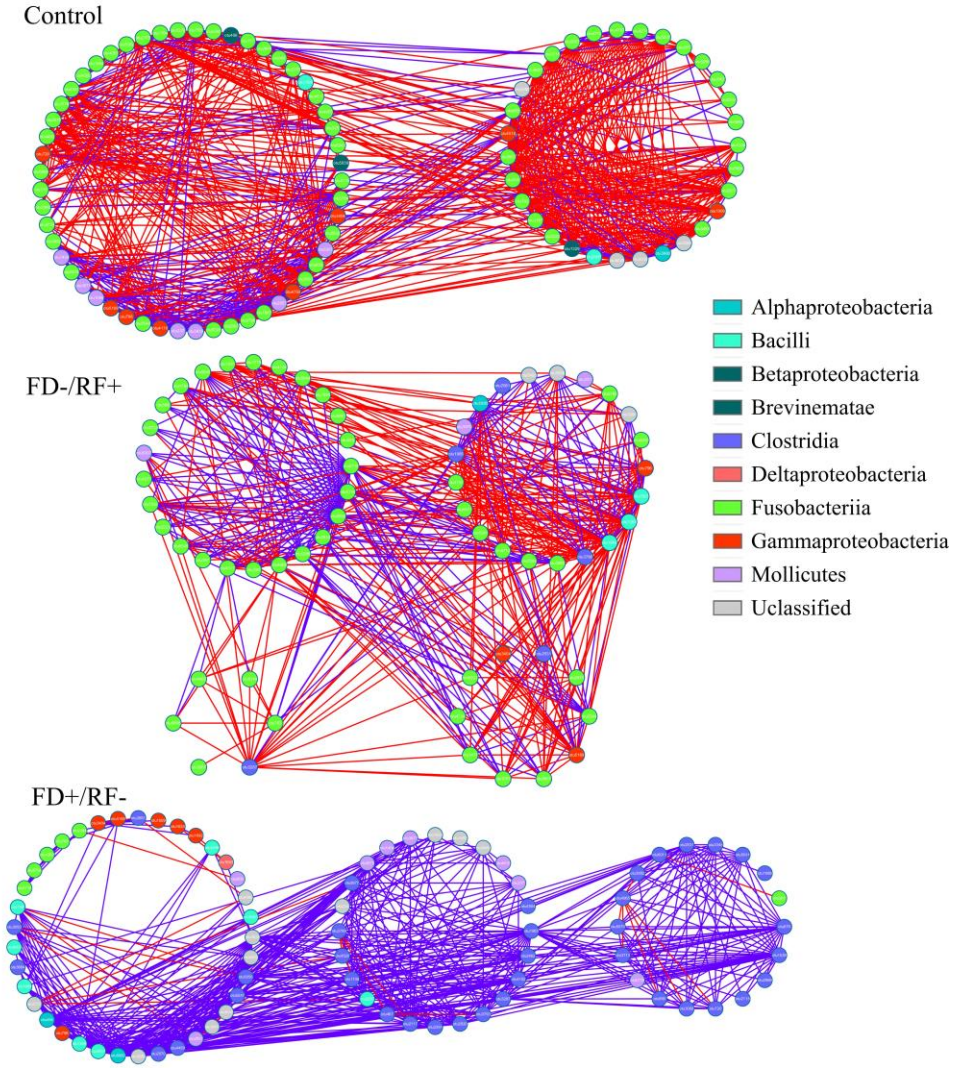

Figure 7: Interactions between species of the intestine microbial community.

Investigating muscle composition throughout during the second feeding challenge:
Liver and muscle samples were collected at each sampling point for nutrient composition. At the end of each period, fish were individually weighed as previously described and 9 fish were sampled from each group to collect fillet (2 from each fish). Fillet were pooled by tank, pureed, and homogenized using an industrial food processor, dried in a convection oven at 105°C for 24 hours (to determine moisture level), and ground with mortar and pestle for further analysis. Crude lipid was assessed with an ANKOM XT15 extraction apparatus (ANKOM Technology, Macedon, NY) per manufacturer's instructions with petroleum ether as the extracting solvent. Additionally, the crude protein content (total nitrogen x 6.25) of the fillet was measured using a LECO FP-428 nitrogen analyzer (LECO Instruments, St. Joseph, MI) per manufacturer's instructions.

The result of moisture, protein and fat content variations during the second feeding challenge can be viewed in figure 8 A-C respectively.

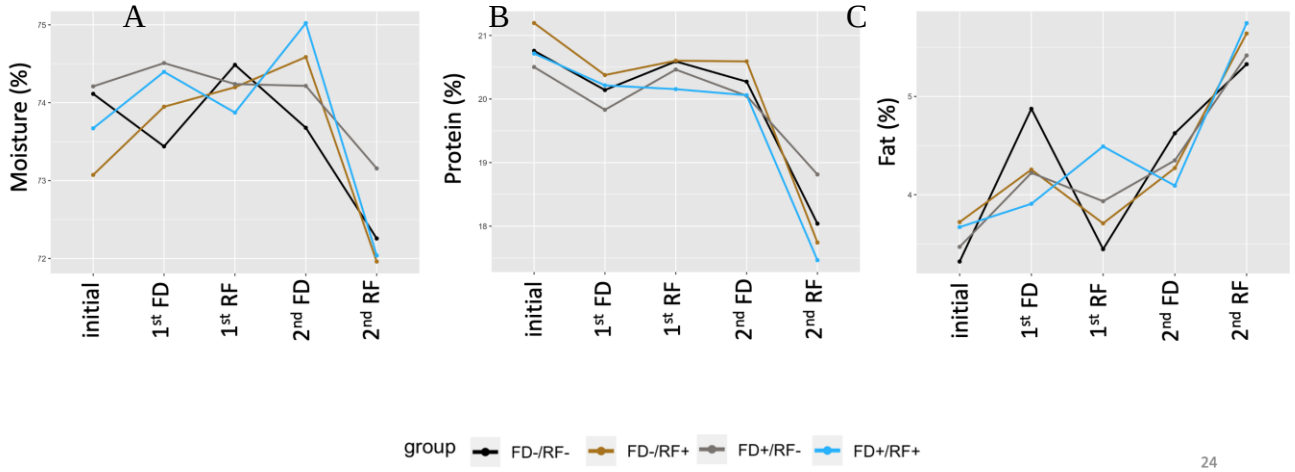

Figure 8: Muscle protein, lipid and moisture variations during the second compensatory feeding regime

Fat content and moisture content of fish fillet was inversely related. Protein content was decreased as the time increased with regards to feed deprivation and refeeding. Fat content increased in the 2nd feeding challenge as experiment proceed further which revealed that fish was approaching towards the breeding season. Fish were getting mature, so they are storing fat for spawning.

Gene expression analysis of the four groups during the second challenge:
This part of the study is to understand the underlying molecular mechanism on how some animal can conserve energy more efficiently compared to others and loose less weight over a feed deprivation period (FD^-) and gain more weight over a re-feeding period (RF^+). We examined the expression of three major categories of genes since weight gain and weight loss variations are affiliated with growth, protein turn over and energy consumption in the body.

We investigated the expression pattern of some genes involved in growth such as growth hormone receptor 1 and 2 (*ghr1* and *ghr2*) in liver. Our analysis showed that *ghr1* has shown a significantly higher expression during feed deprivation periods. Additionally, some genes involved in proteolysis such as *fbxo32* and *atg4* were studied and a down regulation was observed during FD. A summary of gene expression events during the FD period is presented in figure 10. An opposite pattern is expected during the re-feeding time however more details are still under investigation.

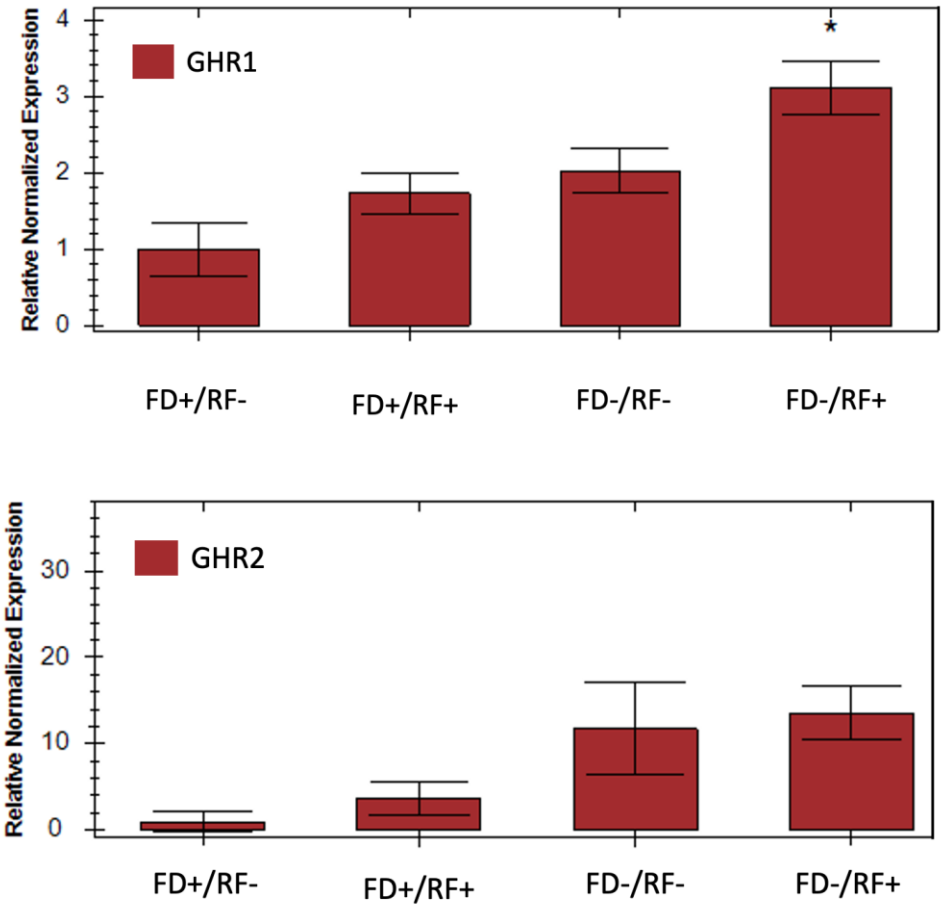

Figure 9: Gene expression analysis of growth hormone receptor 1 and 2 in the liver tissue during the feed deprivation period.

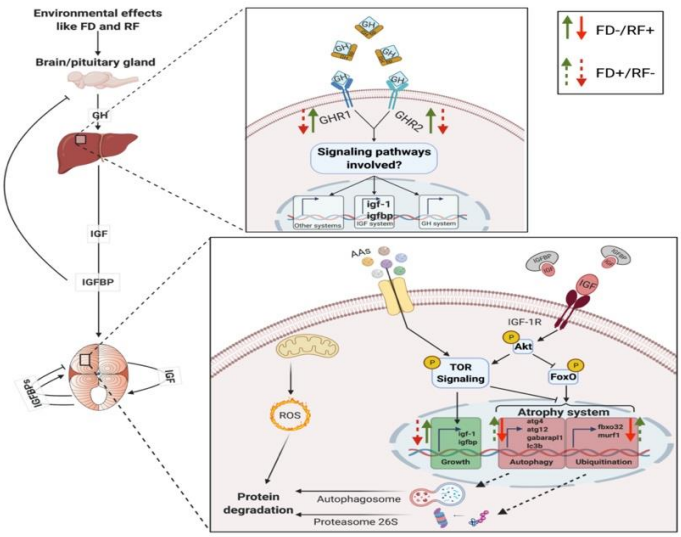

Figure 10: Schematic for gene expression pattern of several genes involved in growth and protein turnover in liver and muscle during a feed deprivation period. Genes involved in growth such as Growth Hormone Receptor 1 and 2 (GHR1 and GHR2) showed a higher expression in the FD-/RF+ group relative to the FD+/RF-. On the other hand, genes involved in proteolysis such as Autophagy and Ubiquitination-related genes are down regulated in FD-/RF+ in comparison to FD+/RF- .

Concluding remarks:

- Our results revealed that selected strain (*University of Idaho – USDA-ARS*: UI-ARS) of rainbow trout exhibited different feed efficiency among 12 families (1600 fish). Those selected trout are vegetarian trout, and their diet is soybean meal protein based diet (SBMD), which is composed of 50% soy ingredients. Our indirect benchmarks criteria were able to select families/individual fish, which showed significantly great feed efficiency. One of the four groups from 12 families (1600 fish) displayed feed conversion ratio (FCR) 0.99 whereas FCR for other three groups were in the range of 1.26 to 1.41 (please see figure 4). Overall feed efficiency for selected group (FD^{-}/RF^{+}) was improved more than 25% that is great news for aquaculture industry. Soy protein based diets utilization can be improved by using the current indirect benchmarks criteria for rainbow trout and it can be applied for other fish species.

- The outcomes of this project is to start the genetic improvement program by producing brood stock of rainbow trout with the traits of best SBMD utilization efficiency which is good news for the aquaculture industry including the fish farmers and feed industry especially the soybean producer.

- Additionally, application of this study in the aquaculture industry helped to improve the sustainability the most nutritious animal production. Besides the industrial applications, this project also help us to understand the molecular mechanism involved in feed efficiency as well as the bacterial dynamics in the gut during a compensatory growth and we understood the basics of how feed efficiency can be improved in animals.

- Procedure for selection for fish lines to enhance SBM utilization efficiency is a bit complicated therefore it will be little challenging to transfer the technology from lab to fish farm because traditional fish breeder may not like to accept the new technology.

- The alternative methods to improve the feed efficiency could be used for to improve the fish lines for other fish species such as Atlantic salmon, tilapia, large-mouth bass, hybrid bass and catfish etc. The long-term impact of this proposed project is to develop a method to produce rainbow trout families with best performance in terms of growth and feed efficiency (SBMD) to US and the global aquaculture industry. Finally, cost of fish production can be decreased by enhancing the utilization of soybean meal via improving the feed efficiency by 15% per generation. Furthermore, this information has application to all cultured fin-fish species and would assist in increasing US soy meal in aqua-feeds.

References:

Coyte, K.Z., Schluter, J., and Foster, K.R. (2015). The ecology of the microbiome: Networks, competition, and stability. Science 350, 663.

Crews, D.H. Jr., 2005. Genetics of feed utilization and national cattle evaluation: a review. Genet Mol Res. 4:152–65.

Douirin et al., 1998. Determination of the lipid content of rainbow trout (*Oncorhynchus mykiss*) fillets with the Torry Fish Fat Meter(R). Sci. Aliment. 18, 527–535.

Grima L., et al., 2008. Genetic variability in residual feed intake in rainbow trout clones and testing of indirect selection criteria. Genet. Sel. Evol. 40, 607–624.

Grima et al., 2010a. In search for indirect criteria to improve residual feed efficiency in sea bass (*Dicentrarchus labrax*) Part I. Aquaculture 300, 50-58

Grima et al., 2010b. In search for indirect criteria to improve feed utilization efficiency in sea bass (*Dicentrarchus labrax*). Part II: Heritability of weight loss during feed deprivation and weight gain duringre-feeding periods. Aquaculture 302, 169–174.

Jason Abernathy et al. (2017). Integrative functional analyses using rainbow trout selected for tolerance to plant diets reveal nutrigenomic signatures for soy utilization without the concurrence of enteritis. PLoS One 12.7, 0180972.

Ken Overturf, Frederick T Barrows, and Ronald W Hardy. (2013). Effects and interaction of rainbow trout strain (*Oncorhynchus mykiss*) and diet type on growth and nutrient retention." Aquaculture Research, 44.4 pp. 604-611.

Mark Henryon et al. (2002). Genetic variation for growth rate, feed conversion efficiency, and disease resistance exists within a farmed population of rainbow trout. Aquaculture, 209.1-4, pp. 59-76.