Project Number: 1830-352-0501b

Project Title: Testing the replacement of fish meal and fish oil in *Seriola rivoliana* diets with soy-based protein and oil

Organization: University of Nebraska-Lincoln

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Project Status-What key activities were undertaken and what were the key accomplishments during the life of this project? Please use this field to clearly and concisely report on project progress. The information included should reflect quantifiable results (expand upon the KPIs) that can be used to evaluate and measure project success. Technical reports, no longer than 4 pages, may be included in this section

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This program was a multi-year effort, which brought together a transdisciplinary team of researchers with expertise in aquaculture, aquafeed formulations, agribusiness, and agriculture biotechnology, to develop and test aquafeed formulations designed to displace fishmeal and fish oil with soybean-based protein and oil in the diet of Kampachi, the Hawaiian yellow tail (*Seriola rivoliana*). A synopsis of this effort was communicated in the 2017 peer reviewed publication entitled, "Towards the development of a sustainable soya bean-based feedstock for aquaculture", which appeared in the Plant Biotechnology Journal in 2017, volume 15 pages 227-236. This communication summarizes our studies on an aquafeed formulation, designated 40-SPC, which has a 40% inclusion of soybean protein concentrate (SPC), effectively displacing the majority of fishmeal with SPC, and 50% of the fish oil with a soybean oil enriched in an omega-3 fatty acid stearidonic acid (SDA).

Working with a group of Ag business researchers, is was demonstrated that the 40-SPC formulation is cost competitive with current commercial diets used to produce Kampachi. This latter research was communicated in the journal Aquaculture Economics and Management in 2016, entitled, "Economic feasibility of high omega-3 soybean oil in mariculture diets: A sustainable replacement for fish oil" (DOI: 10.1080/13657305.2016.1228711).

A key ingredient in the 40-SPC formulation is the requirement for taurine supplementation, which was first demonstrated in the Kampachi relative, the Japanese yellow tail (*Seriola quinueradiata*), wherein a 5% inclusion level is necessitated to avoid a deadly nutritional deficiency called, green liver disease, when attempting to displace fishmeal with plant-based proteins. Through a series of feeding trials with Kampachi this program has shown that levels of taurine, which costs between $4-$10 per kg, can be reduced from 5% to 1% in the 40-SPC formulation, without compromising growth performance or fatty acid levels within the harvested flesh. As can be gleaned from Table 1, Kampachi fed on the 40-SPC formulation coated with 50% SDA enriched soybean oil, for approximately 78 days, contain high percentage of total omega-3 fatty acids (ALA, ETA, SDA, EPA, DHA) relative to Kampachi fed the commercial diet.

Table 1: Evaluation of taurine inclusion levels in 40-SPC formulation coated with SDA/Fish oil (2012)

Sample	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	LA	GLA	ALA	SDA	ETA	EPA	DHA
EWOS-F	3.9±0.1	15.6±0.5	7.1±0.1	3.6±0.1	19.6±1.0	6.5±0.0	0.3±0.0	1.6±0.1	1.8±0.2	1.0±0.0	9.6±0.7	10.2±2.6
Soy-1%	2.6±0.3	15.2±0.5	5.2±0.6	5.1±0.1	18.4±1.8	6.7±0.4	2.8±0.1	5.1±0.0	4.4±0.0	1.7±0.1	6.7±0.1	9.2±1.2
Soy-3%	2.5±0.0	15.1±0.1	5.0±0.0	5.4±0.1	19.8±0.2	7.1±0.0	3.5±0.1	6.2±0.1	5.2±0.1	1.4±0.1	6.2±0.1	7.8±0.3
Soy-5%	2.5±0.1	15.7±0.1	4.8±0.1	5.2±0.0	18.5±0.0	7.7±0.0	3.4±0.0	6.1±0.0	5.1±0.0	1.3±0.1	5.8±0.0	8.6±1.2
Soy-7%	2.3±0.1	15.4±0.1	4.7±0.1	5.7±0.0	21.8±0.2	7.1±0.2	3.2±0.1	5.7±0.2	4.8±0.2	1.4±0.0	5.1±0.1	7.2±0.4

Sample column indicates material analyzed: EWOS-F-harvested flesh from Kampachi fed commercial diet: Soy- harvested flesh from Kampachi fed 40SPC-SDA/Fish oil-coated diet, with 1%, 3%, 5%, and 7% taurine inclusion in the pellet, respectively. Numbers within each column refer to mean percentage of the respective fatty acid in the sample ±SEM.

This outcome led to the further refinement and reduction in cost of the 40-SPC formulation through reduction in taurine inclusion level from 5% down to 1% of the pellet. The team's next step was to evaluate the 40-SPC formulation (1% taurine) coated with 50/50 SDA enriched soybean oil/fish oil in a grow-out trial (app 272 days). The outcome of this grow-out mirrored the short feeding trials, wherein, no impact on growth performance of the fish, and higher percentage of omega-3 fatty acids in the flesh, relative to the fish fed the commercial diet. Importantly, a taste tasting conducted on sashimi prepared from this grow-out demonstrated that the consumer could not differentiate sashimi derived from the 40-SPC formulation and commercial diet, in appearance, smell, or flavor.

As a means to push more terrestrial-based ingredients into the diet, the 40-SPC formulation was coated with varying ratios of SDA enriched oil/fish oil. Feeding trial conducted with Kampachi supported our earlier findings that Kampachi is evidently missing delta-5 desaturase activity and the outcome is a predominate build-up of ETA in the flesh, and a reduction in EPA and DHA, as the SDA/fish oil ratio is increased beyond 50/50.

In keeping with this approach, to push more terrestrial-based ingredients into the diet, a finishing feed strategy was implemented, wherein, the 40-SPC formulation was coated with SDA/fish oil ratio of 90/10, until the last month of trial, then the coated of the pellet was flipped 10/90. The outcome of this feeding trial was the FCR was excellent in the 40-SPC fed fish, but with a slight reduction in growth, which was attributed to the use of Alaskan Pollack fish oil, instead of the anchovy. Nonetheless, the fatty acid data (Table 2) revealed that with a slight tweaking of the timing of introduction of the finishing feed equivalent percentages, relative to commercial diet, of the omega-3 fatty acids, EPA and DHA, can be achieved.

Table 2: **Fatty Acid profile of flesh and feed samples derived from Ziegler 2017 SDA finishing feed trial**

Sample	Palmitic	Palmitoleic	Stearic	Oleic	LA	GLA	ALA	SDA	EPA	DHA	% oil
EWOS-F	17.6±0.3	6.3±0.7	3.8±0.1	14.4±0.9	4.9±0.2	0.6±0.1	0.8±0.1	1.2±0.1	7.6±0.3	14.2±0.8	2.4±0.7
Soy-90	15.6±0.4	3.6±0.6	5.1±0.4	21.4±1.6	9.9±0.1	4.1±0.3	8.7±0.6	7.1±0.8	1.23±0.8	4.4±1.1	2.8±0.4
Soy-Fin	15.0±0.7	2.7±0.2	4.5±0.3	18.4±1.5	8.4±0.2	2.5±0.2	6.8±1.0	6.2±1.1	3.4±0.6	7.4±0.9	2.7±0.5
					AquaFeed						
EWOS	16.9±0.1	9.6±0.1	3.6±0.0	13.1±0.1	7.8±0.1	0.4±0.0	1.6±0.0	2.4±0.0	11.6±0.0	8.6±0.1	15.8±1.3
Soy-90	11.0±0.1	1.2±0.0	3.0±0.0	23.8±0.5	9.5±0.1	4.8±0.0	17.2±0.2	20.2±0.4	1.1±0.1	1.1±0.1	23.2±0.2
Soy-Fin	12.6±0.1	5.0±0.1	2.6±0.0	17.1±0.1	8.1±0.3	1.0±0.0	4.6±0.0	7.0±0.1	8.0±0.1	6.6±0.1	18.5±0.6

Sample column indicates material analyzed: EWOS-F-harvested flesh from Kampachi fed commercial diet: Soy-90 harvested flesh from Kampachi fed 40SPC formulation coated with 90/10 SDA enriched soybean oil/ fish oil, respectively. Soy-Fin (finishing feed) indicates harvested flesh from Kampachi fed 40SPC formulation coated with 10/90 SDA enriched soybean oil/ fish oil. Samples beneath the "Feed" refer to fatty acid profile of the respective aquafeeds. Numbers within each column refer to mean (6 samples) percentage of the respective fatty acid in the sample ±SEM.

The challenge the team faced at this point in the program was how to get this formulation produced at scale, which would require all ingredients be readily available on the market. Given the SDA enriched soybean was produced at the University of Nebraska-Lincoln back in 2004, and is considered regulated by the global regulatory agencies, the team attempted to reach out to Monsanto who developed a soybean referred to as SoyMega™ that accumulated SDA, using different transgenic alleles than the event developed in Nebraska, but with same enzymatic activities. The SoyMega™ event has been de-regulated in the USA, and classified under the category of GRAS (Generally Regarded as Safe) by the FDA. However, approval is for human consumption not fish, hence, some additional regulatory hurdles would have to be addressed if this soybean is to be used in the future for aquaculture. Unfortunately, our efforts did not materialize, and the team was left to come up with other options to pursue for the lipid component of the 40-SPC formulation.

To this end, the team decided to evaluate the finishing feed strategy, with high oleic acid soybean oil, as a means to have a complete formulation with all ingredients readily available on the market. This feeding trial brought in Prairie Aquatech a company that developed a feed grade SPC that was earlier evaluated in the 40-SPC formulation with Kampachi. The 40-SPC diet was formulated, however, with Solae's food grade SPC for this trial, and if successful the next round was to re-evaluate Prairie Aquatech's product given its significant lower cost relative to Solae's product. The 40-SPC pellets were coated with high oleic acid soybean oil (Plenish) at a 90/10 ratio with fish oil. The goal being to demonstrate that the 40-SPC formulation is suitable for Kampachi in a production type scenario and to use this information to attract commercial aquafeed companies to produce this diet to scale to meet the annual needs of Kampachi Farm's operations in Kona and Mexica. However, during the first few months of the trial it was apparent that the fish on the 40-SPC diet were under performing, relative to the fish on the commercial diet, wherein the commercial diet fed fish had an average weight of 541±128 grams and those on the 40-SPC diet with an average mass of 357±107 grams. The culprit appears to be that the level of inclusion of high oleic acid soybean oil rendered the diet deficient in omega-3 fatty acids, which are critical for *Seriola* species development (see Aquaculture 2016. 463:123-134). This is highlighted from the data shown in Table 3, wherein the percentage of omega-3 fatty acids in the flesh of the fish fed the pellet coated with high oleic acid soybean oil, possessed approximately 16% omega-3 fatty acids, while fish fed the commercial formulation accumulated over 37% omega-3 fatty acids. While this outcome negatively impacted this past year of the program, it reinforced the importance to consider both the protein and lipid components of aquafeed when attempting to displace marine-based ingredients with terrestrial-based sources.

As a means to confirm our suspicions regarding insufficient level of omega-3 fatty acids in this last 40-SPC formulation, a final diet was formulated last Fall, wherein the 40-SPC pellet was coated with fish oil. The Kampachi feeding trial for this final test will be initiated this Feb with funds provided by Prairie Aquatech.

Table 3: Fatty Acid profile of flesh and feed samples derived from Kona 2018 high oleic inclusion trial

Sample	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	LA	ALA	SDA	EPA	DHA
EWOS-F	1.3±0.1	12.5±1.0	3.18±0.1	5.3±0.3	12.9±0.4	7.1±7	0.7±0.1	0.6±0.0	12.1±0.9	24.8±1.2
Soy-HO	0.4±0.1	10.7±1.0	1.7±0.2	5.8±0.3	31.4±1.4	22.2±1.1	2.1±0.1	0.1±0.0	4.3±0.5	9.6±0.1
					Aquafeeds					
EWOS	6.2	15.0	9.5	2.6	14.6	5.8	1.3	2.6	13.3	11.0
Soy-4mm	0.8	10.8	1.8	3.6	53.3	15.7	3.2	0.3	1.2	1.8
Soy-6mm	0.9	11.1	2.0	3.3	51.8	17.0	3.5	0.3	1.5	1.9
Soy-9mm	1.1	13.2	2.6	3.4	39.8	25.0	4.2	0.3	1.75	2.4

Sample column indicates material analyzed: EWOS-F-harvested flesh from Kampachi fed commercial diet: Soy-HO harvested flesh from Kampachi fed 40SPC-HO diet. Samples beneath the "Feed" refer to fatty acid profile of the respective aquafeeds. Numbers within each column refer to mean (3 samples) percentage of the respective fatty acid in the sample ±SEM. Note only one sample run across the respective feed pellets

Another component of this program targeted the development of an algal-based ingredient to compliment the 40-SPC formulation. The motivation for this effort arose when the team formulated a diet, based on the 40-SPC diet, with an algal-inclusion, that resulted in a formulation devoid of marine-ingredients. While the growth performance of Kampachi was excellent, and fatty acid profile of the harvested from this feeding trial were excellent, the cost of the algal component was prohibitive. To address this an ongoing effort has targeted the development of a Tetraselmis strain of alga that was selected due to its inherent ability to produce EPA and taurine, two critical ingredients for aquaculture. The goal being to maximize these two co-products, while optimizing growth conditions, as a way to reduce the cost of this algal component of the modified 40-SPC diet, thereby, making the 40-SPC diet with no marine-based ingredient cost competitive. The key step in meeting this goal is to have a reliable gene transformation system for Tetraselmis, while progress has been made in this area, our team continues to improve upon the system. We were fortunate to secure some funding from the Gordon & Betty Moore Foundation to continue this line of research.

<u>Did this project meet the intended Key Performance Indicators</u>

This program was successful in meeting a key performance indicator, an aquafeed formulation that displaces a significant portion of fishmeal and fish oil, with soybean-based protein and oil, suitable for production of Kampachi. The technology is in hand to produce terrestrial-based sources of EPA and DHA in seeds of commodity crops. Indeed, with funding from the Nebraska Soybean Board, in collaboration with the laboratory of Ed Cahoon (Nebraska), a soybean the co-synthesizes the high value carotenoid used in aquaculture, astaxanthin, coupled with up to 9% of EPA in the oil fraction of the seed has been developed. The information gathered from the past two years of field testing on these novel soybean events, allowed us to re-build genetic constructs to further optimized production of EPA and astaxanthin, along with the DHA in the oil. Hence the technology is in hand to generate a soybean-based feedstock that can serve the aquaculture industry in a sustainable fashion.

<u>Expected Outputs/Deliverables</u>

This program delivered an aquafeed formulation that significantly reduces the inclusion level of marine-ingredients and replaces them with soybean-based ingredients in a cost effective manner. Importantly, over the course of the program lead PI Clemente has contributed to numerous public sector outreach activities discussing this area of research, soybean-based feedstocks for aquaculture, which are always well received, and gives the public a more realistic view of the power of technologies that introduce novel genetic variation into crop plants.

<u>Describe any unforeseen events or circumstances that may have affected project timeline, costs, or deliverables</u>

Progress over this past year was hampered by not recognizing the high oleic acid inclusion level would lead to omega-3 fatty acid deficiency in the diet. Secondly, the inability to get the SDA soybean event SoyMega™ on the radar screen for approval for use in aquafeed formulations.

Note: Approximately 40 kg of SDA enriched soybean oil was shipped to Alex Buentello who will be evaluating this oil in production of yellow fin tuna in Panama.

What, if any, follow-up steps are required to capture benefits for all US soybean farmers?

The outcomes of this program should be used to highlight the power of the tools of biotechnology to add novel genetic variation to crops. In this case, the synthesis of a high omega-3 fatty acid soybean oil with value to the aquaculture industry. Secondly, the soybean industry, as it moves forward in the aquaculture space, will need to strengthen its engagement with commercial sector aquafeed formulators. It is these folks who hold great influence as to what goes into a bag of aquafeed that enters the marketplace.