EVALUATING THE EFFECTS OF FERMENTED AND NON-FERMENTED COTTONSEED FLOUR ON GROWTH PERFORMANCE OF JUVENILE RED DRUM *SCIAENOPS OCELLATUS* AND HYBRID STRIPED BASS *MORONE CHRYSOPS* × *M. SAXATILIS*

A Thesis

by

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Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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December 2021

Major Subject: Ecology and Conservation Biology

ABSTRACT

There continues to be a pressing need to develop alternative protein feedstuffs to reduce dependence on fishmeal (FM) and other costly ingredients and thereby increase the sustainability and potential expansion of global aquaculture. In this study, three feeding trials were conducted to evaluate the effects of substituting FM, soybean meal (SBM), or a combination of both with a premium cottonseed flour (CSF) included at incremental levels in the diets of red drum and hybrid striped bass. Feeding trial 1 utilized juvenile red drum, and the remaining two trials were conducted with juvenile hybrid striped bass. Each trial was conducted in 38-L aquaria operated as a recirculating system, and each experimental diet was fed to triplicate groups of juvenile fish for 8 weeks. Red drum in trial 1 were able to readily consume diets in which up to 50% of FM protein was replaced with CSF without observing an appreciable decline in weight gain, feed efficiency or survival. Replacing 75% of FM protein with two different CSF products did result in reduced weight gain and feed efficiency of red drum. Hybrid striped bass in trial 2 were able to readily consume diets in which up to 50% of SBM protein was replaced with either fermented or non-fermented CSF without observing an appreciable decline in weight gain, feed efficiency or survival, while 75% replacement produced lower responses, and replacing 37.5% of protein from both FM and SBM resulted in the lowest responses. Hybrid striped bass in trial 3 were able to readily consume diets in which 30% of SBM and FM protein was replaced with either fermented or non-fermented CSF without observing an appreciable decline in weight gain, feed efficiency or survival. Fermentation of CSF did not substantially improve its nutritional value to hybrid striped bass. Additionally, hybrid striped bass were more sensitive to the inclusion of CSF when substituted for protein from both SBM and FM rather than SBM alone. In conclusion,

diets for red drum and hybrid striped bass could conservatively include one third of its protein provided by CSF without negatively affecting their growth performance or body composition.

ACKNOWLEDGMENTS

I would like to thank my committee chair, Dr. Gatlin, for the continued support and guidance throughout my time here at Texas A&M University. I would also like to thank Dr. Sink and Dr. Hume for serving on my committee and for their instruction that helped me in completing my thesis work.

I would also like to thank my friends and colleagues of the Fish Nutrition Lab at Texas A&M University for always being willing to help me when I needed it and for all the memories we have made over the past two years. I would like to thank my family who have supported me in what has been a difficult time for us. The love of family never fails. Lastly, I would like to thank my girlfriend for her support and encouragement over the years.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a thesis committee consisting of Dr. Gatlin as advisor (Department of Ecology and Conservation Biology), Dr. Sink of the Department of Rangeland, Wildlife and Fisheries Management and Dr. Hume of the U.S. Department of Agriculture Research Service. All other work for the thesis was completed by the student independently.

FUNDING SOURCES

This graduate study was made possible and funded by Texas A&M AgriLife Research and Cotton Incorporated.

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1. INTRODUCTION

For decades, the practice of aquaculture around the world has continued to expand. As the industry continues to grow, so does the demand for resources required to produce a diversity of organisms in various types of culture systems. One of those strained resources in the intensive production of various fish and crustacean species that are fed compounded diets is the protein component which is derived from various protein-rich feedstuffs. That protein component is widely known to be the costliest component of prepared diets (NRC, 2011). Fishmeal is a prime example of a finite ingredient used extensively in aquafeeds due to its high protein and balanced amino acid composition, as well as its high lipid content and palatability to various species, especially carnivorous ones (Minjarez-Osorio et al., 2016). Fishmeal is typically produced by processing wild-caught pelagic marine fish that constitute reduction fisheries, or in other situations it may be produced from bycatch that is deemed unfit for human consumption (Olsen & Hasan, 2012). Various types of fishmeal have seen dramatic increases in price over the last 30 years (IndexMundi, 2021), primarily due to heightened demand from the expansion of aquaculture. For example, the price of a metric ton of Peruvian fishmeal has gone from $476 US in 1991 to over $1,400 US in 2021 (IndexMundi, 2021; Shepherd & Jackson, 2013). This price escalation has resulted not only from the increasing demand for fishmeal primarily from the growing aquaculture sector but also the relatively static supply from global capture fisheries (FAO, 2020; Naylor et al., 2009). As the cost of fishmeal has increased, it has been more extensively substituted by plant feedstuffs and other alternative ingredients in the diets of omnivorous species, which typically require diets of lower protein content. Even for various carnivorous species, there have been considerable efforts in recent decades to reduce the dependence on fishmeal and fish oil due to their increases in price and concerns over sustainability (Hua et al., 2019; Tacon & Metian, 2008).

A multitude of studies have been conducted throughout the years to evaluate a wide variety of feedstuffs as potential alternatives to fishmeal. It is generally agreed that aquaculture's future will gravely depend on the continued development and identification of cost-effective ingredients to potentially spare fishmeal and fish oil inclusion in aquafeeds without hindering the growth performance of various cultured species. Two prominent fish which have a long history of feedstuff evaluation due to their production in aquaculture for both stock enhancement and seafood production are the red drum (*Sciaenops ocellatus*) and hybrid striped bass (*Morone chrysops* × *M. saxatilis*).

The red drum is a native species that can be found all along the eastern coast of the United States from Massachusetts to the Gulf of Mexico. Its euryhaline adaptiveness is one of the reasons why the red drum has been a favorite for aquaculture production. In the 1980s, a significant decline in the wild stocks of red drum occurred due to overfishing which resulted in the closure of the commercial red drum fishery found in the federal waters of the Gulf of Mexico (Gatlin, 2002). In addition, recreational fishing for red drum became more heavily regulated. Such events triggered the acceleration of research to refine methods of aquaculture production of red drum for stock enhancement as well as human consumption (Gatlin, 2002). Nutrient requirements for the red drum were initially studied as early as the 1980s and have continued to be researched to this day (Klett et al., 2020; Webster & Lim, 2009; Yamamoto et al., 2020). A wide variety of alternative protein feedstuffs including those of animal, plant (including algae), and microbial origin have been evaluated with this species (McGoogan & Gatlin, 1997; Perez-Velazquez et al., 2018; Rosales et al., 2017; Rossi et al., 2015b; Whiteman & Gatlin, 2005).

The hybrid striped bass is a carnivorous fish that has become both a lucrative and a significant component of the aquaculture industry in countries such as the United States, Taiwan, and Israel (FAO, 2021). The genetic cross between the white bass and the striped bass produces a hybrid with very favorable characteristics for aquaculture (Hayes & Hodson, 1989). This hybrid has demonstrated greater resistance to disease when compared to either parental stock, has a higher growth rate in their first two years of life, and is able to readily adapt to a formulated diet (Kohler, 2004). As of 2008, production of the hybrid striped bass was considered an important part of U.S. aquaculture with an estimated 14 million pounds of fish being produced (Hodson & Hayes, 1989). The great demand for this popular food fish has driven the growth of this sector by over 600 percent since its early years of production beginning in 1988 (Hayes & Hodson, 1989). However, starting in 2018 U.S., production of hybrid striped bass began to decline because a large producer in California was unable to remain in business due to high costs of production and competition with foreign imports(Andersen et al., 2021). Similar to the red drum, the hybrid striped bass also is cultured for stock enhancement as they are a popular fish targeted by recreational fisheries. This hybrid also is carnivorous in its feeding habits such that their dietary protein requirements tend to be higher than that of herbivorous and omnivorous fish species. Although the dietary protein needs of carnivorous fish such as hybrid striped bass have typically been provided to a large extent by fishmeal, considerable progress also has been made in finding alternatives to fishmeal for hybrid striped bass including a variety of feedstuffs of animal (Rawles et al., 2006), plant (de Cruz et al., 2018), and microbial (Li & Gatlin, 2003) origin.

Protein feedstuffs of plant origin have received a considerable amount of attention as potential ingredients in the diets of various fish species due to the diversity of products derived from plants such as barley, corn, cotton, soybean, and wheat to name a few. However, most of the feedstuffs derived from these plants have some nutritional limitations or anti-nutritional factors which limit their acceptance and/or utilization by various monogastric species including fish (Table 1) (Daniel, 2018; Gatlin et al., 2007).

Table 1: Common anti-nutritional factors found in plant feedstuffs, detrimental effects, and treatment methods to reduce biological activity[1].

ANF[2]	Source	Effect	Treatment
Proteinase Inhibitor	Legumes	Inhibit enzyme activity in the gastrointestinal tract	Heat, methionine supplementation
Lectins	All plant seeds	Disruption of metabolism in the small intestine, and morphological damage to villi	Heat, specific carbohydrate supplementation
Phytic Acid	All plants	Inhibit mineral absorption	Mineral supplementation
Tannins	Rape seeds, beans	Interference with nutrient digestion, reduction in vitamin B_{12} absorption	Dehulling, restriction of heat treatment
Saponins	Legumes	Uptake reduction of glucose and cholesterol	Alcohol extraction
Gossypol	Cottonseed	Toxicity may cause organ damage, cardiac damage, and possibly death	Non-polar extraction, iron supplementation
Oligosaccharides	Legumes	Interference with nutrient digestion	Alcohol extraction

[1]Modified from Krogdahl et al. (2010), Kokou and Fontoulaki (2018), and Vikas et al. (2012)
[2]Anti-nutritional factor

Soybean-derived products such as soybean meal (SBM) and soy protein concentrate (SPC) have continued to be evaluated as potential replacements for fishmeal in the diet of various species. It is well established that SBM can largely replace fishmeal in the diet of omnivorous species such as common carp (*Cyprinus carpio*) and channel catfish (*Ictalurus punctatus*) (NRC, 2011). However, the substitution of fishmeal with SBM and SPC in the diets of carnivorous species such as Japanese flounder (*Paralichthys olivaceus*) (Ye et al., 2011), white snook (*Centropomus viridis*) (Arriaga-Hernández et al., 2021), and hybrid striped bass (*Morone chrysops* × *M. saxatilis*) (Rossi et al., 2015a) generally has been more challenging. Nevertheless, some studies have reported that health and growth of some carnivorous species were not adversely affected when utilizing higher inclusion levels of SBM. For example, Arriaga-Hernandez et al. (2021) reported with white snook that diets containing 15-45% of SBM in place of fishmeal did not adversely affect the growth performance of the fish. Rossi et al. (2015) reported that 88% of the protein provided by fishmeal was able to be replaced by soy-derived products (70% SBM & 18% SPC) without negatively impacting the growth performance of the hybrid striped bass.

Cotton constitutes another plant from which protein feedstuffs can be derived. Cottonseed products hold considerable potential for inclusion in the diets of various aquatic species although some recently developed products have not been adequately evaluated. Unlike corn, soybeans, and other plant products, cottonseed is typically not destined for human consumption after oil extraction. The main limitation to the use of cottonseed-derived products has been the presence of the anti-nutritional factor gossypol which is toxic to various monogastric animals in its free form. Gossypol, which is stored in the glands of cotton for protection against insect predators, has its highest concentration in cotton seeds (Kumar et al., 2021). Gossypol levels in traditional cottonseed meal have been known to be higher (0.773%) than that of more recently developed products. Nevertheless, traditional solvent-extracted cottonseed meal (CSM) has been effectively incorporated in the diets of omnivorous fish species at up to 20% of diet without causing gossypol toxicity (Li & Robinson, 2006).

However, solvent-extracted CSM contains only 41% crude protein and 11.3% fiber compared to 48.5% crude protein and 3.4% crude fiber for solvent-extracted, dehulled SBM. Therefore, various means have been explored to produce other cottonseed products, such as cottonseed protein concentrate (CPC) and cottonseed flour (CSF), to reduce free gossypol and crude fiber, as well as increase protein concentrations of such products. Beginning as early as the 1970s, various physical and chemical methods were studied extensively with the aim of removing the pigment glands in cottonseed to reduce gossypol (Rathore et al., 2020).

Use of fermentation, enzyme treatment, selective breeding, and genetic modification all have been reported as effective in reducing the levels of anti-nutritional factors of cottonseed products. Physical means of removing gossypol from the cottonseed products included a method recognized as the Air Classification Process (ACP) (Decossas et al., 1982; Rathore et al., 2020). Along with removing gossypol, ACP also reduced the fiber content of cottonseed which in turn increased protein content (Challa et al., 2010). Chemical attempts to remove gossypol have been explored utilizing either acetone, ethanol, or isopropyl alcohol, although these methods have never been used in a commercial setting in the U. S. (Rathore et al., 2020).

Several genetic means of reducing gossypol concentrations in cottonseed products also have been developed, including production of glandless plant varieties by traditional breeding methods and producing transgenic varieties which only contain gossypol in the plant and not its seeds. Biotechnology most recently was used to reduce gossypol in cottonseed with an RNA interference (RNAi)-based method through selective silencing of specific genes to prevent gossypol production in the seed (Rathore et al., 2020). The resulting product known as ultra-low gossypol cottonseed (ULGCS) was created, field tested, and prepared to be commercially available as it has been approved by the FDA for human consumption and for animal feed (Rathore et al., 2020).

To date, only a limited number of investigations with various carnivorous fish species have evaluated such cottonseed products with lower free gossypol and higher crude protein. Therefore, further studies are needed to examine the potential of substituting commercially available cottonseed products for fishmeal in diet formulations of carnivorous fish species.

In recent years, a limited number of carnivorous fish species have been researched with the aim of potentially substituting fishmeal, soybean meal, or other protein feedstuffs with cottonseed-derived products. Such studies have explored the use of a variety of cottonseed products in carnivorous fish species including black sea bass (*Centropristis striata*) (Anderson et al., 2016)*,* red drum (Wang et al., 2020)*,* and hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) (Chen et al., 2020). It was found with juvenile red drum that low-gossypol CSF was able to successfully substitute for up to 50% of the protein component provided by menhaden fishmeal. Anderson et al. (2016) demonstrated that two variations of CSM produced by either acidic ethanol extraction or the use of a glandless seed were able to replace fishmeal by 75% and 100%, respectively, without causing adverse effects on survival, growth, or feed utilization of black sea bass (Anderson et al. 2016). Various other studies have reported similar results with the use of a cottonseed-derived product treated to reduce the amounts of free gossypol. For example, in southern flounder (*Paralichthys lethostigma*)*,* the inclusion of both the genetically improved (GMI) (glandless) and the genetically modified

(GMO) CSF were able to substitute for up to 75% of the fishmeal in the diet without negatively affecting growth performance or health of the fish (Alam et al., 2018). Other researchers have explored alternative methods to reduce the anti-nutritional factors in CSM through the use of fermentation in which live yeast cultures were mixed with the CSM and incubated for some period of time before drying. Such a method of fermentation was utilized by Sun et al. (2015) in a study with black sea bream (*Acanthopagrus schlegelii*) in which they reported notably higher crude protein levels in the fermented cottonseed meal (FCM) as compared to regular CSM, and also a clear decrease in phytic acid and free gossypol concentrations.

The present study was conducted to evaluate a commercially available cottonseed-derived flour known as Proflo which is marketed by Archer Daniel Midland (ADM; Chicago, IL). Proflo is considered by ADM to be a premium CSF created via heating, oil extraction, and an ACP to contain a minimum of 58% crude protein, 3.19% crude fiber, and approximately 0.063% free gossypol (ADM, 2021). These qualities, along with many others, have made this a desirable product to research as a potential substitute for other protein ingredients in the diets of carnivorous fish species. Therefore, the present study aimed to examine the effects of Proflo and fermented Proflo as potential alternatives to fishmeal and soybean meal in diets of red drum and hybrid striped bass.

2. MATERIALS AND METHODS

Feeding trial 1 was conducted with juvenile red drum in which a reference diet was formulated to contain 42% crude protein (dry-matter basis) contributed by menhaden fishmeal at 28% of dry weight, along with 20% soybean meal and 16.6% soy protein concentrate (Table 2). Diets were formulated to contain incremental levels of the commercial cottonseed product, Proflo, provided by ADM, and used to substitute for incremental levels of menhaden fishmeal. There were three experimental diets designated as PF-FM25, PF-FM50, and PF-FM75 that contained the Proflo product added at inclusion levels to replace 25%, 50%, and 75% of fishmeal protein, respectively. An additional diet was formulated to include a cottonseed flour derived from GMO cottonseed that had reduced levels of gossypol and was included to replace 75% of the protein provided by menhaden fishmeal. All five diets were supplemented with the necessary amounts of lysine, methionine, and taurine in order to meet the amino acid requirements of the red drum (NRC, 2011).

Table 2: Formulation and proximate composition of diets for juvenile red drum.

Ingredient[1]	Reference	PF-FM25[2]	PF-FM50	PF-FM75	GMO75
GMO cottonseed flour	0.00	0.00	0.00	0.00	19.85
Proflo cottonseed meal	0.00	7.55	15.15	22.70	0.00
Menhaden fishmeal	28.05	21.05	14.00	7.00	7.00
Dehulled soybean meal	20.05	20.05	20.05	20.05	20.05
Wheat flour	11.55	11.55	11.55	11.55	11.55

Soy protein concentrate	16.60	16.60	16.60	16.60	16.60
L-Lysine	0.00	0.20	0.40	0.60	0.40
Taurine	1.00	1.00	1.00	1.00	1.00
DL-Methionine	0.50	0.50	0.50	0.50	0.50
Dextrinized starch	2.00	2.00	2.00	2.00	2.00
Celufil	7.00	6.05	5.05	4.05	6.90
Menhaden oil	5.95	6.15	6.40	6.65	5.30
Cottonseed oil	0.00	0.00	0.00	0.00	1.55
Vitamin premix	3.00	3.00	3.00	3.00	3.00
Mineral premix	4.00	4.00	4.00	4.00	4.00
CMC	0.30	0.30	0.30	0.30	0.30
Proximate composition[3]					
Dry matter	91.8	91.9	91.9	91.8	91.2
Protein	46.9	46.3	45.3	44.8	43.8
Lipid	8.5	8.2	8.7	8.3	8.9
Ash	13.6	12.0	11.0	10.4	9.9

[1]Ingredients are presented in g/100 g of dry weight.

[2]PF-FM25 = Proflo at 25% replacement of fishmeal (FM); PF-FM50 = Proflo at 50%; PF-FM75 = Proflo at 75%; GMO 75 = Genetically modified cottonseed flour at 75% replacement; CMC = Carboxymethyl cellulose.

[3]Mean values, n = 3. Proximate composition values are percentage dry weight.

Feeding trial 2 was conducted with hybrid striped bass and included a reference diet formulated to contain approximately 40% crude protein provided by 19.1% menhaden fishmeal along with 42% soybean meal and 14% meat and bone meal (Table 3). In this trial, Proflo cottonseed flour was incrementally added to replace 25%, 50%, and 75% of the protein provided by soybean meal and designated PF-SBM25, PF-SBM50, and PF-SBM75, respectively. Two additional diets designated as FPF-SBM50 and FPF-SBM75 were formulated with the Proflo product subjected to a fermentation process and were included in place of the soybean meal protein at rates of 50% and 75%, respectively. The fermentation process was carried out by following the solid-state fermentation method described by Hassan et al. (2015) with a few modifications as follows. A 2-kg batch of Proflo was blended in one liter of distilled water containing 0.1 g of *Saccharomyces cerevisiae*/100 g of Proflo. The resulting dough was stored in a sealed plastic bag then incubated for 24 h at 36°C. Upon completion of the 24-h incubation, the dough was allowed to dry for 2 days at 65°C then ground and stored at -20°C until included in the diet formulations. A final experimental diet labelled INT37.5 was formulated at an intermediate rate of inclusion and partially replaced the protein provided by both the soybean meal and fishmeal in the reference diet at 37.5%. All the diets used in this trial were supplemented with the required amounts of lysine, methionine, and taurine to satisfy the amino acid requirements of hybrid striped bass (NRC, 2011).

Table 3: Formulation and proximate composition of diets for juvenile hybrid striped bass in trial 2.

Ingredient[1]	Control	PF-SBM25[2]	PF-SBM50	PF-SBM75	FPF-SBM50	FPF-SBM75	INT37.5
Proflo cottonseed meal	0.00	8.60	17.15	25.75	0.00	0.00	25.75
Fermented Proflo cottonseed meal	0.00	0.00	0.00	0.00	16.60	24.85	0.00

Menhaden fishmeal	19.10	19.10	19.10	19.10	19.10	19.10	11.95
Dehulled soybean meal	42.10	31.60	21.05	10.55	21.05	10.55	26.30
Wheat flour	9.40	9.40	9.40	9.40	9.40	9.40	9.40
Meat and bone meal	13.95	13.95	13.95	13.95	13.95	13.95	8.70
L-Lysine	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Taurine	0.50	0.50	0.50	0.50	0.50	0.50	0.50
DL-Methionine	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Celufil	0.20	2.05	4.05	5.95	4.85	7.20	1.70
Menhaden oil	4.25	4.30	4.30	4.30	4.05	3.95	5.20
Vitamin premix	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Mineral premix	4.00	4.00	4.00	4.00	4.00	4.00	4.00
CMC	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Proximate Composition[3]							
Dry matter	91.26	91.47	91.73	91.85	91.27	91.46	92.12
Crude protein	44.25	43.45	43.87	43.88	43.07	42.91	42.87
Crude lipid	8.27	8.64	9.31	8.79	7.64	9.08	8.22
Ash	12.50	12.28	13.54	12.39	12.53	12.74	10.98

[1]Ingredients are presented in g/100 g of dry weight.
[2]PF-SBM 25 = Proflo at 25% replacement of fishmeal; PF-SBM50 = Proflo at 50%; PF-SBM75 = Proflo at 75%; FPF-SBM50 = Fermented Proflo at 50%; FPF-SBM75 = 75%; INC37.5 = inclusion of soybean meal + fishmeal at 37.5%; CMC = Carboxymethyl cellulose.
[3]Mean values, n = 3. Proximate composition values are percentage dry weight.

Feeding trial 3 was conducted as a follow-up study to feeding trial 2 with juvenile hybrid striped bass. This feeding trial utilized different diet formulations than trial 2 and served to further validate the effects of the fermented and non-fermented cottonseed products. Feeding trial 3 included a reference diet formulated to contain approximately 40% crude protein provided by menhaden fishmeal included at 15.25% of diet along with soybean meal at 55.1% of diet and meat and bone meal at 2.8% of diet (Table 4). In this trial, Proflo cottonseed flour was incrementally added to replace 30%, 60%, and 90% of the protein provided by fishmeal and soybean meal and designated PF-30, PF-60, and PF-90, respectively. Three additional diets designated as FPF-30, FPF-60, and FPF-90 were formulated with the Proflo product subjected to fermentation and included in place of the protein provided by fishmeal and soybean meal at rates of 30%, 60% and 90%, respectively.

Table 4: Formulation and proximate composition of diets for juvenile hybrid striped bass in trial 3.

Ingredient[1]	Reference	PF-30[2]	PF-60	PF-90	FPF-30	FPF-60	FPF-90
Pro-Flo	0	20.6	41.2	61.75	0	0	0
Fermented Pro-Flo	0	0	0	0	20.2	40.35	60.5
Menhaden fishmeal	15.25	10.5	5.7	0.95	10.5	5.7	0.95
Dehulled Soybean meal	55.1	37.85	20.65	3.45	37.85	20.65	3.45
Wheat flour	9.4	9.4	9.4	9.4	9.4	9.4	9.4
Meat and bone meal	2.8	2.8	2.8	2.8	2.8	2.8	2.8
L-Lysine	0	0.3	0.6	0.95	0.3	0.55	0.8
DL-Methionine	0.5	0.55	0.6	0.65	0.55	0.6	0.65
Taurine	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Celufil	1.35	2.3	3.15	4.05	3.2	5.25	7.15
Menhaden Oil	6.1	6.2	6.4	6.5	5.7	5.2	4.8
Vitamin Premix	3	3	3	3	3	3	3
Mineral Premix	4	4	4	4	4	4	4
CMC	2	2	2	2	2	2	2

Proximate Composition[3]							
Dry matter	90.49	91.49	93.23	89.74	92.94	92.56	92.04
Crude Protein	40.34	41.28	41.89	42	41.45	41.94	43.43
Crude Lipid	8.07	8.16	7.77	7.73	9.93	9.25	10.01
Ash	10.41	10.8	9.19	8.22	10.17	9.53	8.56

[1]Ingredients are presented in g/100 g of dry weight.
[2]PF-30 = Proflo at 30% replacement of soybean meal + fishmeal; PF-60 = Proflo at 60%; PF-90 = Proflo at 90%; FPF-30 = Fermented Proflo at 30%; FPF-60 = 60%; FPF-90 = 90%; CMC = Carboxymethyl cellulose.
[3]Mean values, n = 3. Proximate composition values are percentage dry weight.

The diets in trials 1, 2, and 3 were prepared utilizing the same preparation protocol. All dry ingredients for each diet formulation were weighed then homogenized in a V-mixer (Blendmaster; Patterson-Kelly, Stroudsburg, PA, USA) after which the mixture was thoroughly blended with the respective amounts of menhaden fish oil (Table 2) then tap water in a food mixer to create a dough that was passed through a meat grinder attachment to the food mixer and fitted with a 2-mm die to produce strands. The resulting strands were placed on a metal tray drying rack and dried by forced air overnight at 25°C. The following day, dried strands of diet were broken up then sieved to yield appropriately sized pellets ranging from 1-3 mm that could be readily consumed by the fish. The diets were stored at -20°C with smaller quantities maintained at 4°C prior to feeding.

All three feeding trials were conducted at the Aquacultural Research and Teaching Facility (ARTF) located at Texas A&M University. The red drum (*Sciaenops ocellatus*) used in trial 1, and the hybrid striped bass (*Morone chrysops* x *M. saxatilis*) that were used in trials 2 and 3 were cultured at the ARTF in a recirculating system containing 30 glass aquaria each filled with 38 L of brackish water. The brackish water was produced by mixing well water and a commercial synthetic sea salt (Instant Ocean, Blacksburg, VA). The salinity of the culture system for the red drum was kept at 4 g/L, and hybrid striped bass were maintained at a salinity of 3 g/L. As a part of the recirculating system, the aquaria were connected to both biological and mechanical filters in order to maintain satisfactory water quality parameters that were measured a minimum of two times a week. As another layer of protection, a UV filter was included as part of the recirculating system for germicidal treatment. Every aquarium was equipped with an air stone through which low pressure air produced by a blower was pushed to maintain dissolved oxygen at levels approaching air saturation. Water temperature was maintained at 27°C by conditioning the ambient air and a 12h:12h light:dark cycle was achieved with fluorescent lights controlled by time. Before commencing each feeding trial, fish in each aquarium were fed the reference diet twice a day to apparent satiation while undergoing an acclimation period of 1 week.

Feeding trial 1 occupied 21 of the 30 aquaria with juvenile red drum stocked as groups of 14 fish per aquarium with an initial average weight of approximately 1 g per fish. Feeding trial 2 utilized 28 of 30 aquaria and was conducted with juvenile hybrid striped bass initially averaging 17 g each which were stocked as groups of 14 fish per aquarium. Feeding trial 3 was also conducted with juvenile hybrid striped bass initially averaging 6.3 g per fish which were stocked as groups of 12 fish per aquarium. Upon completion of the acclimation period, each aquarium was randomly assigned one of the experimental diets ensuring that each diet was fed to triplicate groups of fish. During the first week of the trial, any observed mortalities were weighed and recorded before being replaced by fish of similar weight. Once each trial passed the first week, any further mortalities were not replaced but weighed and recorded to be taken into account when calculating feed efficiency and survival. Before the initiation of each trial, a representative sample of red drum and hybrid striped bass was taken to later be analyzed for proximate composition of whole-body tissues.

Fish from trials 1, 2, and 3 were fed twice a day throughout an 8-week period. The fish from every aquarium were group weighed weekly to monitor weight gain, and to properly adjust feed quantities according to total biomass. The initial feeding rate for red drum in trial 1 was set at 7% of the total biomass, whereas, the initial feeding rate for hybrid striped bass in trial 2 was set at 4%. As for trial 3, the initial feeding rate was set at 5% of the total biomass. The feeding rates in all three trials were adjusted weekly so that the fish were fed close to apparent satiation without overfeeding.

When each trial reached the end of their respective trial period, fish from each aquarium were counted and weighed to determine final weight along with the total number of fish. Three fish were randomly selected from each aquarium and dissected to compute body condition indices including condition factor, hepatosomatic index, muscle ratio, and intraperitoneal fat ratio according to the following formulas:

Condition Factor (CF) = $(W_t \times 100)/$ (fish body length^3)

Hepatosomatic index (HSI) = (liver weight $\times$ 100)/W_t

Muscle Ratio (MR) = (fillets weight $\times$ 100)/W_t

Intraperitoneal fat (IPF) ratio = (IPF weight $\times$ 100)/W_t

W_t represents final body weight in grams, and length is total length in centimeters.

Samples consisting of three additional fish were collected from each aquarium in trials 1, 2, and 3 and homogenized to form a composite sample that was analyzed for whole-body proximate composition utilizing the procedures specified by the AOAC Official Methods 942.05 (AOAC, 2005). In addition to measuring moisture and ash in each sample, crude lipid was analyzed using the methods of Folch et al. (1957), while crude protein was measured by the Dumas method (AOAC, 2005; Method 968.06). Each analysis was carried out in triplicate.

Remaining fish from trial 3 were fed their respective experimental diets for an additional 4 days prior to collecting digesta samples from microbial analysis. For that digesta collection, each aquarium of fish was fed its morning ration staggered by 10-minute intervals to ensure that the digesta was collected 5 h after being fed. The three randomly selected fish from each tank were euthanized with an overdose of 300 mg/L of MS-222 (tricaine mesylate; Western Chemical, Ferndale, Washington) at which point they were individually weighed followed by an external disinfection utilizing 70% ethanol. Upon completion of the disinfection, the fish had their gastrointestinal tracts (GITs) excised, and the intestinal contents squeezed using forceps into a sterile microfuge tube. The microfuge tubes were flash frozen in liquid nitrogen then stored at -80°C until microbial analyses were performed at a later date using 16S rRNA Illumina MiSeq (Illumina, San Diego, CA) sequencing.

The responses of fish fed the various dietary treatments in all three feeding trials were subjected to an analysis of variance (ANOVA), 2-way ANOVA, or a regression analysis depending on the data sets being evaluated. Statistical significance set at $P < 0.05$ for all analyses. If differences are detected with ANOVA, means were compared with Tukey's HSD or Student's t-test. The statistical analysis for both trials were run via JMP Pro 15 for Mac IOS (SAS Institute Inc., Cary, NC).

3. RESULTS

3.1. Trial 1

The production performance and condition indices of red drum after the 8-week feeding trial are presented below in Table 5. Weight gain was found not to be significantly different amongst treatments although there appeared to be a declining trend as the substitution of fishmeal with cottonseed flour increased (P = 0.0548) (Table 5). Feed efficiency (FE) presented a significant negative quadratic trend with the differences most evident between the PF-FM25 and PF-FM75 dietary treatments. No significant differences (P > 0.05) were detected amongst treatments regarding survival, hepatosomatic index (HSI), intraperitoneal fat (IPF) ratio, muscle ratio (MR), or Fulton's condition factor, although there was an overall declining trend as inclusion levels of cottonseed flour increased. In addition, no significant differences were observed in the whole-body proximate composition of red drum fed increasing levels of cottonseed flour (Table 6). The production performance, condition indices, and whole-body proximate composition of fish fed diets labeled PF-FM75 and GMO75 were compared to evaluate these different cottonseed products, but no significant differences were observed, although GMO75 did show slightly higher values for most responses (Table 7).

Table 5: Production performance and condition indices of red drum fed incremental levels of fishmeal replacement by cottonseed flour.

Diet Designation	Weight[1] Gain (%)	Feed[1] Efficiency	Survival[1] (%)	Hepatosomatic[1] Index (%)	Intraperitoneal Fat Ratio (%)[1]	Muscle[1] Ratio Yield (%)	Fulton[1] Factor (g/cm3)
Reference	1322	0.79	85.7	1.84	0.3	30.9	1.06

Pro-Flo 25	1342	0.85	90.5	1.98	0.28	30.61	1.06
Pro-Flo 50	1126	0.76	83.3	1.78	0.17	29.64	1.05
Pro-Flo 75	756	0.66	69	1.74	0.14	29.34	1.05
PSE[B]	93.66	0.03	6.2	0.08	0.06	0.5	0.02
Regression P value							
Linear Trend (Pr > F)[A]	0.2195	0.0942	0.2538	0.656	0.6225	0.8583	0.9901
Quadratic Trend (Pr > F)[A]	0.0548	0.0307	0.1376	0.5608	0.9702	0.9967	0.9167
Adj. R^2	0.7	0.62	0.33	-0.11	0.41	-0.09	-0.18

[1] values represent mean of three replicate aquariums.
[2] Significance probability associated with the F-statistic.
[3] PSE represents pooled standard error.

Table 6: Proximate composition (% of fresh weight) of whole-body tissues of red drum fed incremental levels of cottonseed flour after the 8-week feeding trial.

	Moisture[1]	Protein[1]	Lipid[1]	Ash[1]

Reference	72.5	18.7	4.1	4.0
Pro-Flo 25	72.6	18.3	4.0	4.1
Pro-Flo 50	72.0	18.7	3.8	4.5
Pro-Flo 75	74.4	17.5	3.4	4.0
PSE[3]	0.52	0.43	0.22	0.16
Regression P value				
[2]Linear Trend (Pr > F)	0.1226	0.5228	0.7682	0.0958
[2]Quadratic Trend (Pr > F)	0.0660	0.3608	0.4641	0.1101
Adj. R^2	0.37	0.12	0.32	0.12

[1] composite samples of three fish from each of three replicate aquaria
[2] Significance probability associated with the F-statistic.
[3] PSE represents pooled standard error.

Table 7: Production performance and condition indices of red drum fed 75% replacement of two different cottonseed flours.

	Weight[1] Gain (%)	Feed[1] Efficiency	Survival[1] (%)	Hepatosomatic Index (%)[1]	Intraperitoneal Fat Ratio (%)[1]	Muscle Ratio (%)[1]	Fulton[1] Factor (g/cm^3)
Pro-Flo 75	756	0.66	69.0	1.74	0.14	29.34	1.05

GMO 75	827	0.73	73.8	1.75	0.14	33.2	1.07
PSE[3]	57.17	0.03	7.53	0.07	0.03	1.78	0.008
ANOVA (Pr > F)[2]	0.4288	0.1506	0.6777	0.9231	0.9351	0.2012	0.1583

[1] values represent mean of three replicate aquariums.

[2] Significance probability associated with the F-statistic.

[3] PSE represents pooled standard error.

Table 8: Proximate composition (% of fresh weight) of whole-body tissues of red drum fed 75% replacement of two different cottonseed flours after the 8-week feeding trial.

	Moisture[1]	Protein[1]	Lipid[1]	Ash[1]
Pro-Flo 75	74.4	17.5	3.4	4.02
GMO 75	74.1	17.7	3.2	3.89
PSE[3]	0.35	0.47	0.14	0.08
ANOVA (Pr > F)[2]	0.5836	0.7474	0.3855	0.2963

[1] composite samples of three fish from each of three replicate aquaria.

[2] Significance probability associated with the F-statistic.

[3] PSE represents pooled standard error.

3.2. Trial 2

The production performance and condition indices of hybrid striped bass fed various diets with incremental levels of cottonseed flour in place of soybean meal for 8 weeks are presented in Table 9. No significant ($P > 0.05$) differences were detected amongst treatments regarding weight gain, FE, survival, HSI, or IPF ratio values, although there was an overall declining trend as inclusion levels of cottonseed flour increased. Muscle ratio percentage was found to have a significant negative linear trend and a significant difference was detected between fish fed the reference and INC37.5 diets. No significant differences were observed in the whole-body proximate composition of hybrid striped bass fed increasing levels of cottonseed flour including fish fed the INC37.5 diet (Table 10). A two-way ANOVA, shown in table 11, was run to compare the reference, non-fermented (PF-SBM50 & PF-SBM75), and the fermented treatments (FPF-SMB50 & FPF-SBM75) regarding inclusion level and fermentation main effects. The only significant differences identified were regarding the condition indices HSI and MR which tended to decrease with increasing levels of cottonseed flour. Whole-body proximate composition did not show any significant differences among treatments when comparing the inclusion and fermentation effects (table 12).

Table 9: Production performance and condition indices of hybrid striped bass fed incremental levels of cottonseed flour to replace soybean meal. This table also includes the experimental diet (combined PF 37.5) that partially replaced both fishmeal and soybean meal with cottonseed flour.

	WG[1]	FE[1]	Survival[1]	HSI[1]	IPF[1]	Fillet[1]
Reference	192	0.58	100	0.95	3.41	33.18
Pro-Flo 25	188	0.57	100	0.96	3.76	32.40
Pro-Flo 50	176	0.54	100	0.95	3.91	31.25
Pro-Flo 75	161	0.51	100	0.89	3.27	32.48
Inclusion PF 37.5	134	0.44	100	0.87	3.50	30.20

PSE[3]	9.29	0.02	-	0.05	0.25	0.65
Regression P value						
Linear Trend (Pr > F)	0.1032	0.1306	-	0.7817	0.1719	0.012
Quadratic Trend (Pr > F)	0.2485	0.2561	-	0.9889	0.1295	0.018
Adj. R^2	0.13	0.08	-	-0.07	0.03	0.24
Contrasts (Pr > F)[2]						
Reference - Inclusion 37.5	0.0005	0.0009	-	0.2694	0.7926	0.0056

[1] values represent mean of three replicate aquariums
[2] Significance probability associated with the F-statistic.
[3] PSE represents pooled standard error.

Table 10: Proximate composition (% of fresh weight) of whole-body tissues of hybrid striped bass fed incremental levels of non-fermented cottonseed flour for 8 weeks. This table also includes the experimental diet (combined PF 37.5) that partially replaced both fishmeal and soybean meal with cottonseed flour.

	Moisture[1]	Protein[1]	Lipid[1]	Ash[1]
Reference	70.25	17.62	7.46	4.39
Pro-Flo 25	68.33	18.76	8.37	4.35
Pro-Flo 50	68.24	18.54	8.62	4.59
Pro-Flo 75	67.96	18.78	8.24	4.17
Inclusion PF 37.5	68.69	18.63	8.33	4.31
PSE[3]	0.75	0.4	0.48	0.22
Regression P value				
Linear Trend (Pr > F)[2]	0.1153	0.0937	0.0982	0.6571
Quadratic Trend (Pr > F)[2]	0.3267	0.2314	0.1756	0.5363
Adj. R^2	0.17	0.14	0.08	-0.08
Contrasts (Pr > F)[2]				
Reference - Inclusion 37.5	0.1602	0.0957	0.217	0.7962

[1] composite samples of three fish from each of three replicate aquaria.
[2] Significance probability associated with the F-statistic.
[3] PSE represents pooled standard error.

Table 11: Production performance and condition indices of hybrid striped bass fed incremental levels of non-fermented cottonseed flour and fermented cottonseed in place of soybean meal for 8 weeks.

	Weight Gain (%)[1]	Feed Efficiency[1]	Survival (%)[1]	Hepatosomatic Index (%)[1]	Intraperitoneal Fat Ratio (%)[1]	Muscle ratio (%)[1]
Reference	192	0.58	100	1.0	3.4	33.2
Pro-Flo 50	176	0.54	100	1.0	3.9	31.3
Pro-Flo 75	161	0.51	100	0.9	3.3	32.5
Fermented PF 50	179	0.54	100	0.9	3.6	30.4
Fermented PF 75	196	0.58	100	0.9	3.6	32.8
Main effects – Inclusion[2]						
0%	192	0.58	100	1.0^{A}	3.4	33.2^{A}
50%	178	0.54	100	1.0^{A}	3.8	30.8^{A}
75%	179	0.55	100	0.9^{B}	3.4	32.6^{B}
Main effects – Fermentation[3]						
Fermented	189	0.55	100	0.9	3.5	32.3

Non-fermented	176	0.57	100	0.9	3.5	32.1
PSE	9.59	0.02	-	0.05	0.24	0.79
Two-way ANOVA						
P-values						
Inclusion	0.2122	0.2521	-	0.0441	0.2738	0.0119
Fermentation	0.0966	0.202	-	0.5062	0.9451	0.7434
Fermentation x Inclusion	0.12	0.1188	-	0.8065	0.449	0.7152

[1] values represent mean of three replicate aquariums
[2] values represent mean of means of respective inclusion levels. Means followed by the same letter are not significantly different.
[3] values represent mean of means of either fermented or non-fermented treatments.

Table 12: Proximate composition (% of fresh weight) of whole-body tissues of hybrid striped bass fed incremental levels of fermented cottonseed flour and non-fermented cottonseed flour for 8 weeks.

	Moisture[1]	Protein[1]	Lipid[1]	Ash[1]
Reference	70.3	17.6	7.5	4.4
Pro-Flo 50	68.2	18.5	8.6	4.6

Pro-Flo 75	68.0	18.8	8.2	4.2
Fermented PF 50	68.6	18.5	8.5	4.4
Fermented PF 75	70.5	17.5	7.5	4.3
Main effects Inclusion[2]				
0%	70.3	17.6	7.5	4.4
50%	68.4	18.5	8.6	4.5
75%	69.2	18.1	7.9	4.4
Main effects Fermentation[3]				
Non-fermented	68.8	18.3	8.1	4.4
Fermented	69.8	17.9	7.9	4.4
PSE				
Two-way ANOVA				
P-values				
Inclusion	0.2664	0.2871	0.1179	0.6381
Fermentation	0.2917	0.3536	0.5498	0.8715
Fermentation x Inclusion	0.48	0.4195	0.7658	0.7056

[1] composite samples of three fish from each of three replicate aquaria.
[2] values represent mean of means of respective inclusion levels.
[3] values represent mean of means of either fermented or non-fermented treatments.

3.3. Trial 3

A second trial with hybrid striped bass was conducted to further evaluate the effects of graded levels of Proflo, both regular and fermented, on responses of hybrid striped bass. Table 13 shows there were significant main effects associated with Proflo inclusion level for weight gain, feed efficiency, fillet yield, and Fulton condition factor of hybrid striped bass in this trial. Fish fed diets with a lower inclusion rate of cottonseed flour had significantly higher percentage weight gain compared to those fish fed diets with a higher level of inclusion of cottonseed flour. Feed efficiency was observed to be significantly higher in fish fed the reference diet than fish fed diets with inclusion levels of cottonseed flour at 60 and 90%. The fish fed diets that had 30% of the protein substituted with a cottonseed flour product also had higher feed efficiency than fish fed diets with 90% of the protein substituted with a cottonseed flour. Furthermore, it was observed that the fillet yield percentage was significantly higher in fish fed the reference diet compared to the fish fed the diet that had 90% of the protein substituted with a cottonseed flour. Lastly, the Fulton factor for the fish fed the diet with 90% of the protein substituted with a cottonseed flour product was significantly lower when compared to all other inclusion levels at 0% (reference), 30%, and 60%. Table 14 presents the two-way ANOVA of whole-body proximate composition. It was found that crude protein in whole-body tissues was significantly lower in fish fed higher levels of cottonseed flour.

Table 13: Production performance and condition indices of hybrid striped bass fed diets with incremental levels of regular and fermented cottonseed flour to partially replace soybean meal and fishmeal for 8 weeks

	Weight Gain (%)[1]	Feed Efficiency[1]	Survival (%)[1]	Hepatosomatic Index (%)[1]	Intraperitoneal Fat Ratio (%)[1]	Muscle ratio Yield (%)[1]	Fulton Condition Factor (g/cm^3)[1]
Reference	463	0.54	100	1.3	3.9	34.3	1.3
Pro-Flo 30	446	0.52	97.2	1.2	3.6	32.2	1.3
Pro-Flo 60	365	0.48	100	1.2	4.1	31.9	1.3
Pro-Flo 90	367	0.48	80.6	1.0	4.1	29.8	1.2
Fermented PF 30	416	0.51	100	1.2	4.4	32.3	1.3
Fermented PF 60	345	0.45	97.2	1.2	4.5	30.6	1.3
Fermented 90	292	0.43	88.9	1.3	4.3	26.6	1.1
Main effects Inclusion[2]							
0%	463^{A}	0.54^{A}	100^{A}	1.3	3.9	34.3^{A}	1.3^{A}
30%	431^{A}	0.52^{A}	98.6^{A}	1.2	4.0	32.2^{A}	1.3^{A}
60%	355^{B}	0.47^{B}	98.6^{A}	1.2	4.3	31.3^{AB}	1.3^{A}
90%	329^{B}	0.45^{B}	84.7^{B}	1.1	4.2	28.3^{B}	1.2^{B}
Main effects[3] Fermentation							
Fermented	379	0.48	96.5	1.2	4.3	30.9	1.2
Non-fermented	410	0.51	94.5	1.2	3.9	32.0	1.2
PSE							
Two-way ANOVA							

P-values							
Inclusion	0.0005	0.0031	0.0038	0.1992	0.6995	0.009	0.0098
Fermentation	0.1321	0.1936	0.4639	0.3825	0.2019	0.3242	0.58
Fermentation x Inclusion	0.5953	0.5985	0.5497	0.2973	0.8169	0.6846	0.3401

[1] values represent mean of three replicate aquariums.
[2] values represent mean of means of respective inclusion levels. Means followed by the same letter are not significantly different.
[3] values represent mean of means of either fermented or non-fermented treatments.

Table 14: Proximate composition of whole-body tissues of hybrid striped bass fed incremental levels of regular and fermented cottonseed flour to partially replace soybean meal and fishmeal for 8 weeks.

	Moisture[1]	Protein[1]	Lipid[1]	Ash[1]
Reference	70.9	17.2	6.6	4.1
Pro-Flo 30	70.9	17.2	0.8	4.0
Pro-Flo 60	71.1	16.8	6.7	4.0
Pro-Flo 90	71.3	16.8	7.5	4.2
Fermented PF 30	71.0	17.1	6.9	4.1
Fermented PF 60	70.2	17.0	7.3	4.2
Fermented 90	70.4	16.4	8.0	4.3

Main effects Inclusion[2]				
0%	70.9	17.2^{A}	6.6	4.1
30%	71.0	17.1^{A}	7.0	4.1
60%	70.7	16.9^{A}	7.0	4.1
90%	70.9	16.6^{B}	7.7	4.3
Main effects Fermentation[3]				
Fermented	70.6	16.9	7.2	4.2
Non-fermented	71.1	17.0	7.0	4.1
PSE				
Two-way ANOVA P-values				
Inclusion	0.96	0.01	0.12	0.5358
Fermentation	0.26	0.45	0.44	0.2
Fermentation x Inclusion	0.67	0.38	0.78	0.88

[1] composite samples of three fish from each of three replicate aquaria
[2] values represent mean of means of respective inclusion levels. Means followed by the same letter are not significantly different.
[3] values represent mean of means of either fermented or non-fermented treatments

Table 15: Comparison of parameters between different classes of cottonseed products

Parameter	Regular	GMO	Glandless	Proflo	Fermented Proflo

Gossypol	0.77%	0.09%	0.02%	0.06%	N/A
Crude Protein	41%	56.0%	52.1%	59.0%	59.5%
Crude Lipid	2%	13.5%	11.5%	4.5%	7.2%

4. DISCUSSION

Numerous on-going research efforts are investigating alternative protein feed ingredients for potential inclusion in aquafeeds for a multitude of species (Hua et al., 2019). Cottonseed products have continued to be of interest to many for their relative global abundance, high protein content, and more affordable price compared to other high-protein feedstuffs such as fishmeal (FM) and soybean meal (SBM). Novel technology and improved methods have led to the development of cottonseed products with superior nutritional profiles compared to traditional cottonseed meal as seen in table 15. In this study, the premium cottonseed flour known as Proflo was utilized as a partial substitute for FM, SBM, or a combination of both across three separate feeding trials utilizing red drum and hybrid striped bass.

Along with the study conducted by Wang et al. (2020), trial 1 served to further evaluate the feasibility of partially substituting menhaden FM in red drum diets with Proflo. In addition, trial 1 included a diet that had 75% of the FM substituted with a genetically modified (GMO) cottonseed flour which was also fed at incremental levels to red drum in the study of Wang et al. (2020). Red drum in trial 1 showed a significant decline in percentage weight gain and feed efficiency at the highest level of Proflo inclusion (75% replacement of FM protein), like that of the GMO product. It is important to note that the weight gain percentage in trial 1 did not exhibit a significant linear or quadratic trend as the substitution of FM with cottonseed flour increased; whereas, the weight gain percentage in the study conducted by Wang et. al (2020) with the GMO product showed a significant negative linear trend as the substitution rate increased.

Just as was seen in trial 1, the feed efficiency, feed conversion ratio, or feed intake of various species of fish have been reported to be negatively influenced by increasing levels of cottonseed products in diets (Alam et al., 2018; Cook et al., 2016; Liu et al., 2019; Wang et al., 2020). This decline has been theorized to potentially arise from various factors including gossypol toxicity, reduced palatability, or deficiency in essential amino acids. Wang et al. (2020) theorized that a palatability issue, potentially caused by the heightened presence of cottonseed oil as inclusion levels of CSF increased, could have been the reason behind the reduced growth performance seen in their study. Therefore, Wang et al. (2020) conducted a second trial with red drum and evaluated several palatability enhancers such as inosine monophosphate (IMP) and citric acid (CA) in diets containing the GMO cottonseed product. Although these supplements tended to improve the overall growth performance of red drum compared to those fed the basal diet, differences were not statistically significant.

Although a record quantifying uneaten feed was not kept in trial 1 of the present study, it was apparent that fish fed diets with higher levels of CSF did not consume their feed ration as readily as those fish fed diets with lower levels of CSF. Even an herbivorous fish species such as the grass carp *Ctenopharyngodon idellus*, which can better adapt to high plant-protein diets compared to carnivorous fish, has been observed to have a statistically lower feed intake when fed a diet containing a high level of cottonseed meal compared to a FM-based reference diet (Liu et al., 2019).

Gossypol levels in traditional cottonseed meal have been known to be higher (0.773%) than that of more recently developed products that have been either genetically improved (0.02%) or genetically modified (0.09%) (Table 15). Studies previously have shown how cottonseed products that have been processed to have diminished levels of gossypol allowed for higher inclusion of cottonseed meal in the diets of carnivorous species such as the black sea bass *Centropristis striata* and southern flounder *Paralichthys lethostigma* (Alam et al., 2018; Anderson et al., 2016). Results from Alam et al. (2018) indicated southern flounder were able readily consume genetically improved (glandless) and genetically modified (GMO) diets where up to 75% of the dietary protein provided by FM was substituted by CSF without observing substantial differences in growth performance. Just as reported with southern flounder by Alam et al. (2018), it was concluded that black sea bass was able to consume diets that contained FM substituted at high levels (100%) with a CSF that had been prepared from a genetically improved (glandless) seed (Anderson et al., 2016). Along with the glandless product, juvenile black sea bass were able to readily consume a diet that contained 75% of the FM substituted with another CSF that was produced utilizing acidic ethanol extraction (Anderson et al., 2016). The results from Anderson et al. (2016) provide useful data regarding the developed technology and methods for processing cottonseed products to yield superior growth performance of a carnivorous species of fish.

Different species of fish have been observed in some cases to respond to the inclusion of feed ingredients differently. Responses of hybrid striped bass from trials 2 and 3 of the present study are good examples of this. Even though red drum and hybrid striped bass are both carnivorous species, that does not guarantee that they will respond similarly when including the same feedstuff in their diets. A study previously conducted in our laboratory explored how incremental levels of cottonseed oil (CSO) might affect the growth performance of both red drum and hybrid striped bass (Candelaria, 2021). That study showed that after a 6-week feeding trial there was an overall decrease in weight gain of both red drum and hybrid striped bass with increasing levels of cottonseed oil; however, the effects were much more pronounced in hybrid striped bass, leading us to believe that hybrid striped bass may be more sensitive to the inclusion of cottonseed products than red drum.

Another factor to consider is tolerance and toxicity levels of gossypol which have been shown to vary from species to species as explained by Li & Robinson (2006). Channel catfish *Ictalurus punctatus* for example were reported to have a tolerance level of up to 900 mg of free gossypol /kg diet from traditional cottonseed meal without affecting growth (Dorsa et al., 1982; Li & Robinson, 2006). However, in salmonid species such as the rainbow trout *Oncorhynchus mykiss,* the tolerance level was much lower at 95 mg/kg (Herman, 1970; Li & Robinson, 2006). It is noteworthy to mention that data from a long-term study looking into the effects of a solvent-extracted cottonseed meal in rainbow trout diets reported the possibility of broodstock fish being more resistant to gossypol toxicity than juveniles (K. J. Lee et al., 2006).

It also is well-known that cottonseed meal is most limiting in the essential amino acid lysine, while others such as isoleucine, methionine, and cystine also tend to be lower in cottonseed meal than in some other protein feedstuffs (Li & Robinson, 2006). Fortunately, the nutritional requirements of many fish species have already been evaluated allowing the supplementation of diets with adequate amounts of potentially limiting nutrients. Alam et al. (2018) provided the essential amino acid profiles of GMI, GMO, and traditional cottonseed meals showing that the more recently developed products have a superior amino acid profile when compared to the traditional cottonseed meal. Even with an improved essential amino acid profile, most recent studies have supplemented the diets containing cottonseed products with certain amino acids to ensure that the fish will not experience negative effects due to deficiencies of those essential amino acids.

Trials 2 and 3 of the present study evaluated how the inclusion of a recently developed premium CSF, both regular and fermented, in the diets of hybrid striped bass affected the fish's growth performance over the course of two separate 8-week feeding trials. Each trial had its own diet formulations with the aim of being able to compare the results with one another as well as evaluate the use of fermentation as a method to potentially improve the nutritional profile of CSF. Results from trial 2 demonstrated that incremental levels of fermented and non-fermented cottonseed flour did not significantly affect the weight gain of hybrid striped bass when substituting up to 75% of SBM protein in the diet. It is theorized that this outcome may be because there was still enough protein contributed by the fishmeal to provide the necessary nutrients, such as essential amino acids, required for adequate growth as well as preserving the diets' palatability.

Studies conducted with common carp *Cyprinus carpio* showed similar results to those seen in trial 2 of the present study where the substitution level reached 75% without significantly impeding weight gain percentage (Su et al., 2019). Su et al. (2019) only observed a significantly negative effect on weight gain of fish when SBM was substituted 100% with cottonseed meal. It was theorized that the 100% substitution could have led to an imbalance of essential amino acids as well as a decline in the quality of protein provided by the cottonseed meal (Su et al., 2019). Trial 3 of the current study revealed a significant decline in weight gain percentage of hybrid striped bass as the inclusion level of CSF protein replaced FM and SBM protein from 30% to 90%. An important factor to consider is the differences in the diet formulations between trials 2 and 3 of the current study. Diets in trial 3 not only substituted CSF for SBM, but also partially substituted FM; whereas, only SBM was incrementally substituted in trial 2. Even though the amount of FM substituted in trial 3 was only a third of the amount of SBM, it was potentially enough to cause a reduction in the weight gain and feed efficiency of the fish over time. It is also possible that because trial 3 had higher substitution levels than trial 2, the reduction seen in growth performance may have been more distinct compared to the results in trial 2. The theory that source of substituted protein may have influenced the degree to which CSF could be substituted was further validated by the results in trial 2 regarding the INT37.5 diet which substituted both FM and SBM as opposed to only SBM as in the other diets. While hybrid striped bass fed the INT37.5 diet did not exhibit significantly different values regarding weight gain percentage and feed efficiency, it was evident that by substituting CSF for FM along with SBM, the hybrid striped bass were more sensitive than substituting only SBM.

The average initial weight of fish in trials 2 and 3 may have also potentially influenced how the fish responded to the experimental diets. As observed in tables 11 and 13, the weight gain of fish at the end of each 8-week feeding trial did not reach 200% of initial weight for fish in trial 2 compared to fish fed the experimental diets in trial 3 which approached % of initial weight. Typically, smaller fish such as those from trials 1 and 3 are preferred to be used in feeding trials because of their potential to show more distinct differences in nutritional quality based on their more rapid growth performance compared to larger fish. As such, the responses of hybrid striped bass in trial 3 in which substitution of up to 30% of protein from CSF in place of SBM did not have any appreciable effects on growth performance is a conservative measure of the potential for CSF substitution in the diet of this fish. Comparing results from trial 1 and 3 also support previous observations that hybrid striped bass appear to be more sensitive to cottonseed products (contributing protein and/or oil) compared to red drum in which up to 50% substitution of protein from CSF was possible without reducing growth performance.

As mentioned earlier, techniques such as fermentation have been used as promising tools by researchers when dealing with plant-based diets as this processing method has shown not only to decrease the presence of ANFs, but also has the potential to increase protein content (Daniel, 2018; Hassaan et al., 2015; S. J. Lim et al., 2010; S. J. Lim & Lee, 2011; Olukomaiya et al., 2019; Rombenso et al., 2013; Sun et al., 2015, 2016). Rombenso et al. (2013) presented data in which hybrid striped bass fed diets containing fermented SBM performed better than fish fed diets mainly consisting of regular SBM. It also was reported that the fermented SBM contained a higher protein content than the regular SBM indicating the fermented product had a more superior nutritional profile (Rombenso et al., 2013).

In another study, it was found that black seabream *Acanthopagrus schlegelii* were able to readily consume diets where FM was partially substituted with fermented CSM without observing significant differences in the growth when included at 16% of diet (Sun et al., 2015). Fermentation of plant-protein feedstuffs is not a guarantee that they will outperform fish fed non-fermented products as observed in trials 2 and 3 of the current study with hybrid striped bass. Another example is olive flounder *Paralichthys olivaceus* which were fed diets in which FM was substituted with both fermented CSM and SBM and had significantly lower weight gain percentage when compared to the fish fed diets containing non-fermented CSM (Lim et al., 2010).

Studies looking into the use of fermented cottonseed products are limited, but further research on its use in the diets of economically important species may still provide much needed insight. Even though the fermentation process may decrease the amount of free gossypol found in cottonseed products, the increased substitution rates may still be increasing the overall amounts of free gossypol found in aquafeeds that utilize cottonseed products as a source of protein. On the other hand, like the red drum in trial 1, it is apparent that the hybrid striped bass were more affected by the substitution level of the main protein feedstuff with CSF than the fermentation status of the CSF. Variation in the results from all three trials may demonstrate species-specific differences, primarily in acceptability of the diets containing CSF.

Unfortunately, due to the COVID-19 pandemic, gossypol levels in the diets used in the current study as well as gossypol levels in the liver of fish fed the various diets have not been determined to date. Samples have been sent to the USDA/ARS Laboratory in New Orleans, but gossypol analysis has not been completed. Also, the analysis of digesta samples that were collected at the end of trial 3 to characterize the effects of fermentation on the gastrointestinal tract microbiota will be completed once the USDA/ARD Southern Plains Laboratory in College Station, Texas re-opens.

Even though the Proflo CSF product has been processed to contain lower levels of gossypol than the traditional cottonseed meal, it will be beneficial to have the documented values in order to better determine if gossypol content may have played a more pivotal role in the decline in growth performance of red drum and hybrid striped bass at the higher levels of CSF inclusion.

5. SUMMARY AND CONCLUSION

In conclusion, diets that had up to 50% of the crude protein from FM substituted with cottonseed flour were readily consumed by red drum without impairing growth performance of the fish. Exploring the use of fermented CSF in the diets of red drum could potentially prove to be beneficial as has been seen in other species of aquatic organisms. Hybrid striped bass from trial 2 were able to readily consume diets that had up to 75% of its crude protein provided by SBM substituted with either fermented or non-fermented CSF without observing a significant negative effect on growth performance. Hybrid striped bass from trial 3 demonstrated that fish were able to readily consume diets that had up to 30% of the crude protein provided by SBM and FM substituted with either fermented or non-fermented CSF without impairing the growth performance of the fish. This study has demonstrated how the substitution of one or two feed ingredients (FM and SBM) may affect the growth performance of hybrid striped bass, so it may be of interest to explore the idea of blending other protein feedstuffs to limit palatability issues that were seen in this study. The inclusion of cottonseed products in aquafeeds has shown much promise, but more research should be conducted to further evaluate the inclusion of genetically improved (glandless), genetically modified (GMO), and fermentation-based products in the diets of various fish species.

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