A NEW SPIN ON THE OPTIMIZATION OF PLANT-PROTEIN-BASED DIETS FOR RED DRUM, *Sciaenops ocellatus* L.

A Dissertation

by

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ABSTRACT

Red drum, *Sciaenops ocellatus*, is a highly prized marine teleost whose supply largely depends on aquaculture. Commercial diets for red drum still contain considerable amounts of fishmeal (FM), which has become a scarce and increasingly costly protein source. Therefore, the evaluation of alternative-protein feedstuffs and the optimization of low-FM and growth-promoting aquafeeds can add sustainability to red drum aquaculture.

Five feeding trials were conducted to assess the nutritional value of a range of plant-protein (PP) feedstuffs as partial substitutes for dietary FM in early-stage juvenile red drum. Results from Trial I indicated that soy protein concentrate (SPC) and barley protein concentrate can replace 50% of dietary FM protein without compromising the production performance of red drum. Test diets high in PP reduced the growth performance of red drum in Trial II, but a set of promising PP feedstuffs was screened. The digestibility of a commodity and an enzymatically treated soybean meal (ESBM) was assessed in Trial III. Results from Trials IV and V revealed that ESBM can replace up to 70% of FM digestible protein (DP) in the diet of red drum.

Two soy products (SP) were evaluated as partial substitutes for FM DP in the diet of advanced juvenile red drum in Trial VI. Results revealed that 86% of FM DP can be substituted with a combination of SPC and either SP, while the supplementation of a yeast-based prebiotic (GroBiotic®-A; GBA) in a SP-based diet did not improve fish performance.

In Trials VII and VIII, effects of both diet type and GBA supplementation on the gut microbiota composition of red drum were observed. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA and principal component analyses of MiSeq-sequenced genome indicated differences and similarities in microbiota diversity. An overwhelming dominance of the phylum Proteobacteria or Cyanobacteria was observed in distinct treatments.

The present research indicates that the aquafeed industry may utilized PP feedstuffs to replace dietary FM protein from 50 to 86% without compromising production performance of early-stage as well as advanced juvenile red drum. Further research is recommended to elucidate the role of prebiotics in the nutrition of red drum.

DEDICATION

To my brother Giovani, my mother Maria Celia (*in memorian*), and my father Waldemar.

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NOMENCLATURE

ADC	Apparent digestibility coefficient
ANF	Anti-nutritional factor (s)
ANOVA	Analysis of variance
BPC	Barley protein concentrate
BW	Body weight
CP	Crude protein
CPC	Corn protein concentrate
DE	Digestible energy
DP	Digestible protein
ER	Energy retention
ESBM	Enzymatically treated soybean meal
FE	Feed efficiency
FIFO	Fish in-fish out ratio
FMR	Fishmeal replacement
FM	Fishmeal
GBA	GroBiotic®-A
GIT	Gastrointestinal tract
HSI	Hepatosomatic index
IPF	Intraperitoneal fat
MAX	Maximum

NR	No relationship
PC	Principal component
PP	Plant protein
PPC	Plant protein concentrate (s)
PR	Protein retention
PSE	Pooled standard error
REF	Reference
REPL	Replacement
SBM	Soybean meal
SD	Standard deviation
SE	Standard error
SP	Soybean product (s)
SPC	Soy protein concentrate
TAN	Total ammonia nitrogen
TI	Trypsin inhibitor

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CHAPTER I

INTRODUCTION

Aquaculture is the fastest growing agribusiness globally with an average growth rate of 8 – 10% per year (Tacon et al., 2011; Troell et al., 2014). Such a remarkable growth resulted in the production of 66 million tons of food-use fish in 2012, overtaking beef production with a record not seen since 1950 (www.earth-policy.org). World fisheries production for human consumption was estimated at 136 million tons in 2012, and from this total, about half (66.6 million tons) originated from aquaculture (FAO, 2014).

In view of the negligible growth in capture-fisheries production over the past two decades, aquaculture represents the only mean of meeting the ever-increasing demand for fish. However, the continued growth of global aquaculture will depend on the sustained supply of external nutrients as more than 50% of farmed fish and crustaceans rely on aquafeeds (Tacon and Metian, 2015). Concurrently, it is estimated that by 2025 approximately 87 million tons of aquafeeds will be necessary for the production of around 71 million tons of fed fish and crustaceans, a nearly two-fold increase in production from both ends compared to 2012 (Tacon and Metian, 2015).

Regarding nutrient inputs, the fast growth of aquaculture has become a concern not only because it may compete with other animal production sectors for feed ingredients, but also because there is still a considerable dependence upon natural stocks for the supply of protein for aquafeeds. Most of the fishmeal (FM) produced globally has

been utilized by the aquaculture sector (Tacon et al., 2011); while FM production has remained steady since 2006 at around five million metric tons per year (FAO, 2014). Concurrently, in response to both increased demand and seasonal environmental singularities, FM prices have been escalating since 2005 and in 2014 surpassed US$ 2000.00 per ton (prime FM; www.globefish.org/); a wide discrepancy of prices relative to other protein ingredients used in the animal feed industry, such as soybean meal (SBM).

The awareness that the expansion of the aquaculture industry depends on the utilization of cost-effective and more sustainable protein sources entailed a substantial intensification of research devoted to reducing the use of FM and increasing the use of alternative-protein feedstuffs in aquafeeds. Examples of conjunct initiatives for this end include the establishment of the Aquaculture Protein Centre (APC) in Norway and the Plant Products in Aquafeed (PPA) working group in the United States. The APC was initiated in 2002 with the primary goal to generate knowledge necessary to replace FM in aquafeeds (http://apc.umb.no/). The PPA working group was launched in 2005 and its goal was to define a research roadmap for increasing the utilization of plant-protein (PP) feedstuffs in diets for carnivorous fish (Barrows et al., 2008; Gatlin et al., 2007). Concurrently, a substantial increase in the number of publications on FM replacement with PP feedstuffs occurred after 2005, most of which was comprised of studies on marine finfish, salmonids, and crustaceans (Fig. 1).

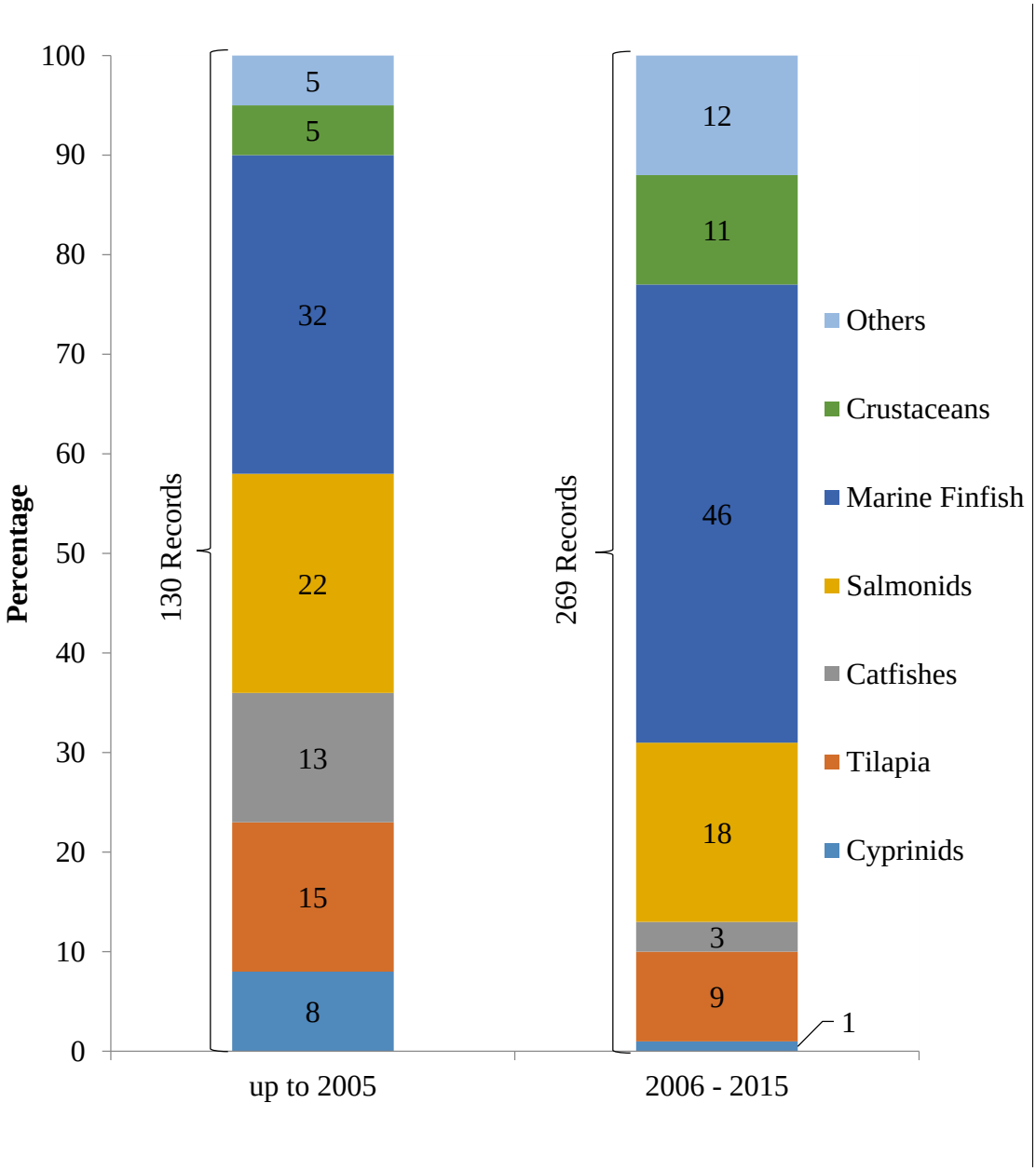

Figure 1 Peer-reviewed publications on the utilization of plant-protein feedstuffs for fishmeal replacement in aquafeeds for aquacultured species up to 2005 and from 2006 to March, 2015. Keywords used: fishmeal/fish meal + soybean; fishmeal/fish meal + soy; fishmeal/fish meal + plant; fishmeal/fish meal + vegetable.

Among the PP feedstuffs with potential for use in aquafeeds, SBM is the most studied and widely utilized partial substitute for FM. Soybean meal is a widely available feedstuff with a reasonably balanced amino acid profile. However, largely attributed to the presence of anti-nutritional factors (ANF) and their adverse effects on the integrity of the gastrointestinal tract and the overall health status of a range of carnivorous fish species, high dietary levels of SBM are usually impractical (Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2003, 2010). Consequently, the development and evaluation of other alternative ingredients with potential for replacing FM in aquafeeds is necessary.

A range of novel PP feedstuffs with potential for use in aquafeeds destined for carnivorous fish species are already in use in other animal production systems or are becoming increasingly available to the aquaculture industry (Gatlin et al., 2007). These feedstuffs include products derived from the processing of soybeans (e.g., soy protein concentrate (SPC)), corn (e.g., corn gluten meal and corn protein concentrate (CPC)), and cereal grains (e.g., wheat gluten and barley protein concentrate (BPC)). Additionally, newly developed SBM products resulting from selective breeding programs or biotechnological processing may lead to substantial advances in the optimization of PP-based aquafeeds.

Plant protein concentrates with high protein and low carbohydrate content have considerable potential as alternatives to FM in the diet of carnivorous fish species. With more than 60% crude protein (CP; NRC, 2011) and lower levels of ANF relative to SBM, SPC has been evaluated and demonstrated to be a high-quality alternative to FM

in the diet of carnivorous fish, including salmonids (Kaushik et al., 1995; Storebakken et al., 2000), Japanese flounder, *Paralichthys olivaceous* (Deng et al. 2006), red sea bream, *Pagrus major* (Takagi et al., 2001), Atlantic halibut, *Hippoglossus hippoglossus* (Berge et al., 1999), and cobia, *Rachycentron canadum* (Salze et al., 2010).

Corn protein concentrate is produced by either a dry or wet milling technique used to extract and separate the endosperm of the corn kernel. After refined and dried, CPC may contain up to 80% CP and less than 1% starch (AAFCO, 2007). Different from oilseeds such as soybeans, CPC contain fewer AFN and is rich in methionine and cysteine but deficient in lysine (Hardy, 2010; Phillips and Sternberg, 1979). Thus, blending CPC with other protein feedstuffs is an alternative to optimize the amino acid profile of aquafeeds and potentially reduce FM levels.

Recent advances in the dehulling of barley as well as fermentation processes have allowed for the development of BPC, another attractive candidate for aquafeeds. Fermentation can produce BPC with approximately 60% CP, which is highly digestible and palatable to fish (Burr et al., 2011; Gaylord et al., 2008). While having a balanced nutritional profile, barley also has a reduced level of ANF in comparison to some other plant feedstuffs (Gatlin et al., 2007).

A summary of selected studies on FM replacement with PP feedstuffs in the diet of salmonids (rainbow trout, *Oncorhynchus mykiss,* and Atlantic salmon, *Salmo salar*), and marine finfish species for the last ten years is presented in Table 1. In general, these studies have evaluated both conventional and novel PP feedstuffs and the primary response criteria included growth, feed efficiency, protein efficiency parameters, and

health status. In salmonids, dietary FM replacement with PP feedstuffs ranged from 35 to 100% in rainbow trout and 100% in Atlantic salmon. Studies evaluating a gradual replacement of FM in Atlantic salmon were limited for this period, which is probably due to an increased focus on the dual replacement of FM and fish oil and/or the effects of specific ANF, both of which were not included. Nevertheless, a minimum dietary FM level of 25% in aquafeeds for Atlantic salmon has been preconized and levels below 25% were found to be detrimental (Pratoomyot et al., 2010). Results for marine finfish reveled that PP feedstuffs are able to replace dietary FM in a range varying from 40% in cobia, *Rachycentron canadum*, and golden pompano, *Trachinotus ovatus*, to 100% in Senegalese sole, *Solea senegalensis*, and yellowtail, *Seriola quinqueradiata*; although most studies have reported replacement levels intermediate to these values. Overall, it is undeniable that considerable progress has been made in decreasing FM inclusion in aquafeeds, with substantial reductions in the weight-equivalent use of wild fish for the production of cultured fish (fish in-fish out ratio, FIFO) being reported (Jackson 2009; Tacon and Metian, 2008).

Studies evaluating alternative-protein feedstuffs in the diet of red drum, *Sciaenops ocellatus*, were conducted earlier (McGoogan and Reigh, 1996; Gaylord and Gatlin, 1996). However, to the best of this author's knowledge, information on the nutritional value of novel PP feedstuffs for this species was very limited up to this point.

Red drum is a carnivorous and highly prized marine teleost that is among the major fed finfish species in aquaculture. Global production of red drum was estimated to be over fifty-three million pounds in 2008 (Tacon et al., 2011). From this total, 95.2%

Table 1 Fishmeal replacement level with plant protein feedstuffs in the diet of selected carnivorous fish species from 2005 to March 2015*.

Species	Size range (g)	Plant-protein feedstuff [2]	Response criteria[3]	FMR (%)	Reference
		Salmonids			
Rainbow trout *Oncorhynchus mykiss*	19.2 – 138.3	CGM, WGM, EP, RM	G	50	Santigosa et al. (2008)
	19.2 – 142.4	CGM, WGM, EP, RM	G, FI, FE, PER, NR, ER	50	Gómez-Requeni et al. (2005)
	39.2 – 97.0	SCSM	G, FI, PER, S	50	Luo et al., (2006)
	14.7 – 65.9	FSBM, CGM	G, FE, FI	100	Yamamoto et al. (2010)
	4.02 – 16.93	PtSBM	G, FCR, PER, S	60	Yang et al. (2011)
	49.6 – 102.1	SBM, FFSBM, CGM	G, FCR, PPV, EPV, S	35	Harlíoğlu (2011)
	6.1 - ~ 30.0	PSG	G, FCR, S	60	Barnes et al. (2012)
Atlantic salmon *Salmo salar*	1214 – 4592	SPC, SE, WGM, PM, CGM	G, FI, FCR, S	50 from 20	Johnsen et al. (2011)
	31.5 – 109.7	CPC, SPC	G, FCR, TGC, S	100 from 38.6	Burr et al. (2012)
		Marine finfish			
Gilthead sea bream *Sparus aurata*	16.7 – 190.9	CGM, WGM, EP, RM, SWL	G, FE, H	50	Sitjà-Bobadilla et al. (2005)
	99.7 – 427.1	CGM, WGM, EP, RM	G, S	75	De Francesco et al. (2007)
Atlantic cod *Gadus morhua*	1652 – ~3200	SBM, SPC, WGM	G, FCR, S	50	Hansen et al. (2007)

Table 1 continued

Species	Size range (g)	Plant-protein feedstuff [2]	Response criteria[3]	FMR (%)	Reference
	1652 - ~3000	SBM, SPC, WGM	H	75	Olsen et al. (2007)
Senegalese sole *Solea senegalensis*	105.6 – 204.8	SBM, SPC, PtC, WGM, Aquatex G2000	G, FCR, PER, S	75	Cabral et al. (2013)
	9.5 – 38.4	SBM, SPC, CGM, WGM	G, FCR, PER, S	100	Silva et al. (2009)
Cobia *Rachycentron canadun*	18.0 – 280.6	N3010	G, FCR, CF	60	Watson et al. (2014)
	8.4 – 37.5	SBM	G, FCR, PER, S	40	Zhou et al. (2005)
	25.9 – 92.6	SBM	G, FI, FCR	40	Romarheim et al. (2008)
Golden Pompano *Trachinotus ovatus*	14.1 – 84.9	SPC + Tau	G, FCR, NRE	40	Wu et al. (2015)
Yellowtail *Seriola quinqueradiata*	482.0 - 1283	SPC + Tau	G, FCR, S	100	Takagi et al. (2008)
California yellowtail *Seriola lalandi*	4.0 – 75.0	N-3032	G, FCR, PER, PR, S	60	Buentello et al. (2015)

*Studies on dual replacement (fishmeal and fish oil) or evaluating the effect of specific ANF were not included.
FMR = fishmeal replacement.
[2] CGM = corn gluten meal; WGM = wheat gluten meal; EP = extruded peas; RM = rapeseed meal; SCSM = solvent extracted cotton seed meal; FSBM = fermented soybean meal; PtSBM = phytase treated soybean meal; SBM = soybean meal; FFSBM = full fat SBM; SPC = soy protein concentrate; PSG = pepsoygen; CPC = corn protein concentrate; SE = sunflower expeller; PM = peas meal; EP = extruded peas; SWL = sweet white lupin; PtC = potato concentrate; N3010 = non-genetically modified (non-GM) SBM; Tau = taurine; N-3032 = non-GM SBM.
[3] G = Growth; FI = feed intake; FE = feed efficiency; PER = protein efficiency ratio; NR = nitrogen retention; ER = energy retention; S = survival; FCR = feed conversion ratio; PPV = protein productive value; EPV = energy productive value; TGC = thermal-unit growth coefficient; H = health; CF = condition factor; NRE = nitrogen retention efficiency; PR = protein retention.

was produced in China and 4.2% (almost five million pounds) was produced in the United States by commercial operations mostly concentrated in south Texas. Nearly all red drum consumed in the United States originates from aquaculture wherein FM is still utilized as source of dietary protein. Therefore, the development of cost-effective and growth-promoting diets for red drum with reduced FM inclusion is a premise for the continued and more sustainable development of this industry.

Another important aspect relative to the shift in protein origin for use in aquafeeds is the increased utilization of feed additives to improve production and/or health of aquaculture species and the overall biosecurity of aquaculture operations. Prebiotics are examples of feed additives increasingly reported to have beneficial effects in fish, including red drum. As recently reviewed by Ringø et al. (2014), responses to prebiotic supplementation in the diet of red drum have included increased apparent nutrient digestibility of a SBM-based diet (Burr et al., 2008), potentiation of non-specific immunity (Li and Gatlin, 2005), improved weight gain (Zhou et al., 2010), and enhanced gut structure (Anguiano et al., 2013). Therefore, in view of the importance of prebiotics in the nutrition of red drum, their continued evaluation may facilitate the optimization of PP-based diets for this species.

The ultimate goal of the current research was to enhance seafood security - ensuring a constant supply of safe and health-promoting seafood for consumers - through the optimization of PP-based aquafeeds for marine finfish, using red drum as a model. To achieve this goal, this research was conducted based on the following objectives:

1) Optimize low-FM, PP-based diets for red drum through the evaluation of novel plant feedstuffs as substitutes for FM.
2) Evaluate the effects of prebiotic supplementation on the best-performing PP-based diets selected in objective 1.
3) Validate the effectiveness of the PP-based diets compared to FM-based diets in an extended cage grow-out trial.
4) Conduct a preliminary evaluation of the potential effects of PP-based diets and prebiotic supplementation on the gut microbiota composition of red drum.

CHAPTER II

ASSESSING THE NUTRITIONAL VALUE OF NOVEL PLANT FEEDSTUFFS IN THE DIET OF RED DRUM*

II.1 Introduction

Fishmeal (FM) has traditionally been the most nutritious feedstuff provided in aquafeeds. In 2006, the aquaculture sector consumed approximately 3.7 million tons of FM and 0.84 million tons of fish oil (Tacon and Metian, 2008). Although the fish in:fish out ratio has been substantially reduced in the production of a large range of species, the continued growth of aquaculture has increased the demand for FM. The steady production and increased demand for FM has caused prices to increase by as much as tripling in price between 2000 and 2008 (Tacon and Metian, 2008). Thus, the continued expansion of aquaculture depends on the identification and development of alternatives for FM.

A range of plant-feedstuffs that are already being utilized or becoming available to the feed industry were identified as potential candidates for FM replacement in the diet of carnivorous fish species (Barrows et al., 2008; Gatlin et al., 2007). These potential surrogates includes soybean products (SP) such as soybean meal (SBM) and soy protein concentrate (SPC), co-products of ethanol production (corn gluten meal and

* Reprinted (with additions): Rossi, W., Moxley, D., Buentello, A., Pohlenz, C. Gatlin, D.M., 2013. Replacement of fishmeal with novel plant feedstuffs in the diet of red drum, *Sciaenops ocellatus*: an assessment of nutritional value. Aquacult. Nutr. 19: 72 – 81. Copyright 2015, with permission from John Wiley and Sons, Inc., Wiley Company.

corn protein concentrate (CPC)), and products derived from cereal grains (e.g., barley protein concentrate (BPC)). In addition, new SP produced from soybean varieties that are selectively bred for increased protein content and lower concentration of anti-nutritional factors (ANF) are becoming available for evaluation in practical diets for animals (Baker and Stein, 2009; Baker et al., 2011), including fish.

Although a limited number of reports on the utilization of novel plant feedstuffs in diets for carnivorous fish species can be found in the literature (Berge et al., 1999; Deng et al., 2006; Gaylord et al., 2008; Kaushik et al., 1995; Salze et al., 2010; Storebakken et al., 2000; Takagi et al., 2001), reports on the applicability of such feedstuffs in the diet of red drum, *Sciaenops ocellatus*, are lacking. Therefore, the objective of the present study was to evaluate the replacement of FM with novel plant feedstuffs in the diet of red drum.

II.2 Material and Methods

II.2.1 Diets

Two feeding trials were conducted to evaluate different plant feedstuffs as alternative replacements for FM in the diet of red drum. In Trial I, diets were formulated to contain 40% crude protein (CP), 10% lipid, and 3.1 kcal of digestible energy (DE) g^{-1}. Digestible energy was calculated based on the physiological fuel values of 4, 4, and 9 kcal/g for carbohydrate, protein and lipid, respectively. While targeting the same CP (40%), the diets in Trial II were formulated to contain 16% lipid and 3.3 kcal DE g^{-1}. Supplementation of DL-methionine and L-lysine was provided to all test diets in excess

of the established requirements of methionine (Craig and Gatlin, 1992) and lysine (Moon and Gatlin, 1991) for red drum, while glycine was added for palatability (McGoogan and Gatlin, 1997). All diets were supplemented with a mineral and vitamin premixes to meet or exceed the established requirements of this species (NRC, 2011).

The reference (designated REF) diet in each trial was formulated to contain all of its protein from menhaden FM, while the test diets were designed to replace 50 or 88% of the FM protein with that from the selected plant feedstuffs. Three plant-protein concentrates (PPC), including CPC, BPC, and SPC, and seven SP, including regular solvent-extracted, commodity SBM (SBM), and six novel meals (N209, N3010, N3010-WF, SG-HP, SG-Trifecta, and SG-ULT) provided by Schillinger Genetics Inc., West Des Moines, Iowa, USA, were evaluated.

All diets were prepared by mixing dry ingredients in a V-mixer. The dry mixture was then blended with oil and water using an industrial mixer with a meat grinding attachment, and pelleted with a 3-mm die. Resulting pellets were dried for 24 h using forced air at 25˚C. The diets were analyzed in duplicate using AOAC (1990) procedures for dry matter, crude protein, lipid, and ash contents.

II.2.2 Trial I

This trial was conducted over an 8-week period in which four test diets were evaluated (Table 2). Three test diets (designated CPC50, BPC50, and SPC50) were formulated to substitute 50% of the FM protein in the REF diet with that from CPC, BPC, or SPC. One additional test diet (designated PPC100) was formulated to provide a

100% replacement of FM protein in the REF diet by equal contributions of each of the PPC. The PPC100 diet was used as the FM-free, negative control diet.

II.2.3 Trial II

Trial II was conducted over a 6-week period to evaluate the seven SP (SBM, N209, N3010, N3010-WF, SG-HP, SG-ULT, and SG-Trifecta) along with BPC as alternative ingredients for FM (Table 3). The SBM, SBM209, N3010, and N3010-WF were dehulled and solvent-extracted, while the SG-HP, SG-Trifecta, and SG-ULT were full-fat, dehulled and heat-treated SP. Compared to SBM, the other SP were higher in CP and/or lower ANF content. The REF diet was formulated to derive protein solely from FM and eight test diets were formulated to provide an 88% replacement of FM protein in the REF. In each of the test diets, a basal level of SPC (10%) was established and the remaining protein necessary to attain the 88% replacement was supplied by each SP.

The diets prepared for Trial I ranged in CP from 40.2 and 44.1% while lipid ranged from 11.2 to 13.6%, compared to the formulated values of 40% and 10%, respectively (Table 2). In Trial II, CP (ranging from 40.1 to 42.7%) and lipid content (ranging from 15.3 to 16.7%) of the diets were very similar to the formulated levels of 40.0% and 16%, respectively (Table 3). Total dietary phosphorus was lower in the N3010 (1.0%) and BPC (1.1%) diets compared to the REF diet (2.0%), but was still above the minimum dietary requirement for red drum (Davis and Robinson, 1987).

Table 2 Composition of diets[1] fed to red drum juvenile for 8 weeks in Trial I (Rossi et al., 2013)

	REF	CPC50	BPC50	SPC50	PPC100
Ingredients	*% of dry matter*				
Menhaden fishmeal[a]	58.0	29.0	29.0	29.0	
Corn protein concentrate[b]		24.2			16.1
Barley protein concentrate[c]			34.7		23.1
Soy protein concentrate[d]				27.7	18.5
Dextrinized starch[e]	14.0	14.0	14.0	14.0	13.0
Menhaden oil[f]	3.2	3.9	2.2	6.0	4.8
Vitamin premix[g]	3.0	3.0	3.0	3.0	3.0
Mineral premix[g]	4.0	4.0	4.0	4.0	4.0
Carboxymethyl cellulose[h]	2.0	2.0	2.0	2.0	2.0
Glycine[h]	1.0	1.0	1.0	1.0	1.0
Lysine HCl[h]		1.1	0.7	0.2	1.3
DL-Methionine[h]		0.1	0.3	0.4	0.5
Celufil[h]	14.8	17.7	9.0	12.7	12.7
Analyzed proximate composition[2]					
Moisture	9.2	9.4	10.0	9.9	10.1
Protein	44.1	43.7	42.1	42.2	40.2
Lipid	11.9	12.9	12.6	11.2	13.6
Ash	13.7	11.5	9.0	10.8	4.3
Analyzed amino acid composition					
Arg	1.9	1.8	1.4	2.5	1.5
His	1.0	1.1	0.8	1.2	0.9
Ile	1.8	2.0	1.4	2.2	1.7
Leu	3.2	5.6	2.6	3.9	4.3
Lys	2.5	2.6	2.1	3.0	2.2

Table 2 continued

	REF	CPC50	BPC50	SPC50	PPC100
Analyzed amino acid composition			*% of dry matter*		
Met	1.0	1.1	1.0	1.2	1.0
Met + Cys	1.1	1.2	1.1	1.3	1.1
Phe	1.7	2.6	1.7	2.3	2.3
Phe + Tyr	3.4	4.4	2.8	3.9	3.7
Thr	1.6	1.7	1.2	1.8	1.3
Val	2.1	2.3	1.7	2.5	2.2

[1] REF = reference; CPC = corn protein concentrate; BPC = barley protein concentrate; SPC = soy protein concentrate; PPC = plant-protein concentrate.

[2] Dry matter basis (except moisture).

[a] Special Select ™ Omega Protein Inc., Abbeville, Louisiana, USA. As dry: Crude protein = 689.8 g kg^{-1}; Lipid = 117.4 g kg^{-1}.

[b] Empyreal 75, Cargill Corn Milling, Blair, Nebraska, USA. As dry: Crude protein = 826.7 g kg^{-1}; Lipid = 113.6 g kg^{-1}.

[c] Montana Microbial Products, Missoula, Montana, USA. As dry: Crude protein = 576.1 g kg^{-1}; Lipid = 127.7 g kg^{-1}.

[d] The Solae Company, St. Louis, Missouri, USA. As dry: Crude protein = 722.3 g kg^{-1}; Lipid = 21.3 g kg^{-1}.

[e] MP Biomedicals, Solon, Ohio, Usa

[f] Omega Protein, Reedville, Virginia, USA.

Table 3 Composition of diets[1] fed to red drum juvenile for 6 weeks in Trial II (Rossi et al., 2013).

	REF	SBM	N209	N3010	N3010-WF	SG-HP	SG-ULT	SG-Trifecta	BPC
Ingredients	*% of dry matter*								
Menhaden FM[a]	57.1	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Soy protein concentrate[b]		10.0	10.0	10.0	10.0	15.0	15.0	15.0	10.0
Commodity SBM[c]		54.4							
N209[d]			46.3						
N3010[d]				48.5					
N3010-WF[d]					44.1				
SG-HP[d]						53.3			
SG-ULT[d]							51.4		
SG-Trifecta[d]								58.0	
Barley protein concentrate[e]									49.1
Dextrinized starch[e]	3.8	2.8	2.8	3.1	2.9	2.8	2.7	3.0	2.1
Menhaden oil[f]	7.4	11.4	12.6	13.5	13.5	3.2	6.1	0.5	6.2
Vitamin premix[g]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Mineral premix[g]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Carboxymethyl cellulose[h]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Glycine[h]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Table 3 continued

	REF	SBM	N209	N3010	N3010-WF	SG-HP	SG-ULT	SG-Trifecta	BPC
Ingredients	*% of dry matter*								
Taurine[h]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lysine HCl[h]		0.4	0.3	0.04	0.07	0.2	0.4	0.2	1.0
DL-methionine[h]		0.8	0.8	0.6	0.8	0.7	0.7	0.7	0.7
Celufil[e]	20.7	2.3	9.3	6.3	10.7	6.7	5.7	4.7	12.9
Analyzed proximate composition[2]									
Moisture	8.5	8.9	10.9	9.3	9.3	12.8	11.0	12.0	9.8
Protein	42.4	41.2	41.3	40.1	41.5	42.7	42.7	42.7	42.1
Lipid	16.0	15.3	16.1	16.4	16.7	15.8	16.4	15.6	15.3
Ash	13.9	9.0	8.1	8.1	8.2	8.7	8.8	8.6	6.5
Total phosphorus	2.0	-	-	1.0	-	-	-	-	1.1
Analyzed amino acid composition									
Arg	2.3	2.5	2.9	2.8	2.6	2.8	2.7	2.6	2.7
His	1.0	1.0	1.1	1.0	0.9	0.9	0.9	0.9	0.9
Ile	1.6	1.6	1.8	1.7	1.6	1.6	1.6	1.5	1.6
Leu	2.9	2.8	3.2	3.1	2.8	2.9	2.7	2.6	2.7
Lys	3.3	3.0	3.7	3.8	3.1	2.9	2.5	2.5	2.6

Table 3 continued

	REF	SBM	N209	N3010	N3010-WF	SG-HP	SG-ULT	SG-Trifecta	BPC
Analyzed amino acid composition	*% of dry matter*								
Met	1.0	1.0	1.1	1.1	1.0	1.1	0.9	1.1	1.0
Met + Cys	1.1	1.1	1.3	1.2	1.1	1.2	1.0	1.2	1.1
Phe	1.5	1.7	2.0	1.9	1.8	1.8	1.7	1.7	1.7
Phe + Tyr	2.4	2.6	3.2	3.1	2.8	2.9	2.7	2.7	2.8
Tau	1.6	1.3	1.2	1.2	1.1	1.1	1.0	1.0	1.0
Thr	1.8	1.7	1.8	1.7	1.6	1.6	1.5	1.5	1.5
Val	1.9	1.8	2.0	1.9	1.7	1.7	1.7	1.6	1.7

[1] REF = reference; SBM = soybean meal; BPC = barley protein concentrate.

[2] Dry matter basis (except moisture).

[a,b, e-h] as in Table 1.

[c] Producers Coop. Association, Bryan, Texas, USA. As dry: crude protein = 51.2%; lipid = 6.2%.

[d] Schillinger Genetics Inc., West Des Moines, Iowa, USA. As dry for crude protein and lipid (%, respectively): N209 = 60.2, 4.6; N3010 = 57.5, 2.6; N3010-WF = 63.2, 2.9; SG-HP= 46.0, 21.4; SG-ULT = 47.2, 16.6; SG-Trifecta = 41.8, 24.3.

For both Trials I and II, the amino acid concentration of experimental diets was fairly constant, and the levels of lysine, methionine, and threonine were in excess of the established requirements for red drum (Boren and Gatlin, 1995; Craig and Gatlin 1992; Moon and Gatlin, 1991).

II.2.4 Fish and Feeding Trials

The feeding trials took place at the Texas A&M Aquacultural Research and Teaching Facility. Red drum were obtained from the Sea Center Texas Marine Aquarium, Fish Hatchery and Nature Center operated by Texas Parks and Wildlife Department in Lake Jackson, TX. Fish were maintained in quarantine for two weeks acclimating to local conditions until adequate size for the feeding trials was attained. Over the quarantine period, red drum were fed at a rate approaching apparent satiation with a 40% CP and 12% crude fat commercial diet (Rangen, Inc., Angelton, TX).

Trial I was conducted in 38-L aquaria while 100-L aquaria were used in Trial II. Both systems operated independently as recirculating systems, whereby waste water gravity-flowed to a settling chamber, then to a biological filter and was pumped through a sand filter before being returned to the aquaria. Water quality was maintained within acceptable levels for red drum. Synthetic sea water was prepared using well water mixed with stock salt and Fritz brand synthetic sea salts to provide culture water of 5-7 g L^{-1} salinity. Water temperature was maintained at 26 ± 2˚C by conditioning ambient air. Dissolved oxygen was maintained near air saturation using supplemental aeration supplied by a regenerative blower and air diffusers. A 12h light:12h dark photoperiod was maintained using fluorescent lighting controlled by timers.

Twenty red drum juveniles (mean weight of 1.5 ± 0.5 g fish^{-1}) were stocked in each aquarium for Trial I and 25 red drum juveniles (mean weight of 3.3 ± 0.1 g fish^{-1}) were stocked in each aquarium for Trial II. A 1-week conditioning period was given prior to the commencement of each feeding trial.

Each of the test diets was randomly assigned to three replicate aquaria (n=3). Fish were fed twice daily for 8 (Trial I) or 6 weeks (Trial II) at a rate approaching apparent satiation. The feeding rate was adjusted weekly after group-weighing all fish in each aquarium and maintained at a level that maximized intake without overfeeding. At the start of each trial, 15 fish were collected from the remaining population and frozen for subsequent analyses of whole-body composition.

II.2.5 Data Acquisition and Analyses

At the end of each trial fish were weighed and sampled 15 h after the last feeding. Six (Trial I) or eight (Trial II) representative fish per aquarium were collected and euthanized with an overdose of tricaine methanesulfonate (MS-222; Western Chemical, Inc., Ferndale, WA, USA). Composite samples of three fish per aquarium were homogenized for proximate analysis to determine crude protein, lipid, moisture, and ash in whole-body tissue (AOAC, 1990). Three additional fish per aquarium were weighed and dissected to obtain liver, intraperitoneal fat (IPF), and muscle weights for computing hepatosomatic index (HSI), IPF ratio, and muscle yield values. In addition, for Trial II, two fish per aquarium within the treatments FM, SBM, N209, N3010, and BPC, were dissected and had their entire gastrointestinal tract (GIT) removed for subsequent histological and morphological analysis of the intestine. The procedures of

GIT removal, fixation, staining and subsequent measurements of fold length, enterocyte height and microvillus height of five different cross sections of intestine from each of two fish per aquarium were performed as previously described (Anguiano et al., 2012).

II.2.6 Calculations and Statistical Analyses

The responses utilized to compare treatments in this study were calculated as follow:

- Weight gain, % = [(Final weight – initial weight)/(initial weight)] × 100;
- Feed efficiency ratio (FE) = [weight gain (g)/ dry feed consumed (g)]
- Protein retention efficiency, PR % = {[(final body weight (g) × final body protein (%)) – (initial body weight (g) × initial body protein (%))]/(protein intake (g)} x 100
- Energy retention efficiency, ER % = {[(final body weight (g) × final body energy (%)) – (initial body weight (g) × initial body energy (%))]/(energy intake (g)} x 100
- Muscle yield, % = [fillet muscle weight (g)/body weight (g)] x 100
- Viscerosomatic indices (HSI & IPF ratio), % = [Liver or intraperitoneal fat weight (g)/body weight (g)] x 100

All data were analyzed for normality (Shapiro-Wilk test) and for homogeneity of variances (Levene's test). The one-way analysis of variance was performed to determine significant ($P < 0.05$) differences among treatment means. When significant differences were identified, Duncan's multiple range test was used for the separation of treatment

means. All Statistical analyses were performed using the SAS® software package (SAS Institute Inc., Cary, NC USA).

II.3 Results

The growth performance, feed efficiency ratio (FE), survival, muscle yield, and condition indices (HSI and IPF ratio) of red drum in Trials I are presented in Table 4. Among all dietary treatments, the SPC50 supported the highest ($P < 0.05$) weight gain (2,112%) and FE (1.03), while a significantly lower performance was observed in fish fed the PPC100 diet. While fish fed the CPC50 diet had lower weight gain, those fed the BPC diet supported weight gain (1,709%) and FE (0.92) similar to the fish fed the REF diet. Red drum fed the REF diet had the lowest survival (86.7%) among all dietary treatments ($P < 0.05$). Highest ($P < 0.05$) HSI values were observed in fish fed the BPC50 (2.5%) and PPC100 (2.7%) diets, while no differences were observed for muscle yield and IPF ratio among treatments.

In Trial II, the combination of SPC with each of the SP or BPC significantly reduced the final weight (15.0 - 74.8%) and weight gain (15.6 – 87.0%) of red drum; whereas, similar FE was observed among fish fed the N209, N3010, and the REF (Table 5). The SG-Trifecta treatment supported the lowest ($P < 0.05$) growth, feed efficiency, and survival. The latter did not differ among the other dietary treatments. No significant differences were observed among the other dietary treatments. No significant differences were observed for muscle yield between each of the SP or BPC treatments relative to the REF diet, while the SBM, N209, N3010, SG-HP, SG-ULT, and the BPC treatments all

Table 4 Final weight, growth, feed efficiency, survival, muscle yield, hepatosomatic index, and intraperitoneal fat ratio of red drum (mean initial weight ± SD = 1.5 ± 0.5 g) after 8 weeks of feeding the experimental diets in Trial I (Rossi et al., 2013)

Treatments[1]	Final weight	Weight gain	FE	Survival	Muscle yield	HSI	IPF ratio
	g	%		%	%	%	%
REF	29.7^{a}	1,876^{b}	0.95^{ab}	86.7^{b}	29.3	1.7^{b}	0.6
CPC50	25.1^{b}	1,632^{c}	0.95^{ab}	95.0^{a}	30.4	2.0^{b}	0.7
BPC50	26.2^{b}	1,709^{bc}	0.92^{b}	93.3^{a}	33.2	2.5^{a}	0.7
SPC50	31.9^{a}	2,112^{a}	1.03^{a}	91.7^{a}	30.5	1.7^{b}	0.6
PPC100	15.5^{c}	962^{d}	0.80^{c}	91.7^{a}	27.9	2.7^{a}	0.4
PSE	*1.010*	*66.287*	*0.033*	*2.107*	*1.671*	*0.120*	*0.100*
Pr > F	*<0.001*	*<0.001*	*0.002*	*0.010*	*0.309*	*<0.001*	*0.817*

[1] REF = reference; CPC = corn protein concentrate; BPC = barley protein concentrate; SPC = soy protein concentrate; PPC = plant-protein concentrate. SD = standard deviation; FE = feed efficiency; HSI = hepatosomatic index; IPF = intraperitoneal fat; PSE = pooled standard error of treatment means (n = 3). Mean values within each column with different superscripts are statistically different ($P < 0.05$).

Table 5 Final weight, growth, feed efficiency, survival, muscle yield, hepatosomatic index, and intraperitoneal fat ratio of red drum (mean initial weight ± SD = 3.3 ± 0.1 g) after 6 weeks of feeding the experimental diets in Trial II (Rossi et al., 2013).

Treatments[1]	Final weight	Weight gain	FE	Survival	Muscle yield	HSI	IPF ratio
	g	%		%	%	%	%
REF	24.7^{a}	639^{a}	0.96^{a}	82.0^{a}	26.1^{abc}	1.6^{b}	0.3^{bc}
SBM	19.8^{bc}	496^{b}	0.84^{b}	79.0^{a}	24.7^{bc}	2.4^{a}	0.5^{ab}
N209	20.0^{bc}	523^{b}	0.88^{ab}	80.3^{a}	28.0^{ab}	2.7^{a}	0.6^{ab}
N3010	21.0^{b}	539^{b}	0.88^{ab}	81.5^{a}	26.3^{abc}	2.5^{a}	0.7^{a}
N3010-WF	12.5^{e}	280^{d}	0.62^{c}	81.5^{a}	25.5^{abc}	1.7^{b}	0.3^{bc}
SG-HP	16.5^{d}	388^{c}	0.72^{c}	80.3^{a}	29.3^{a}	2.4^{a}	0.5^{b}
SG-ULT	11.9^{e}	259^{d}	0.51^{d}	70.0^{a}	24.5^{bc}	2.3^{a}	0.3^{bc}
SG-Trifecta	6.2^{f}	81^{e}	0.16^{e}	46.9^{b}	22.3^{c}	1.3^{b}	0.0^{c}
BPC	18.8^{c}	480^{b}	0.82^{b}	80.3^{a}	29.8^{ab}	2.3^{a}	0.2^{bc}
PSE	*1.067*	*32.880*	*0.047*	*2.367*	*0.510*	*0.100*	*0.066*
Pr > F	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.030*	*<0.001*	*0.009*

[1] REF = reference; BPC = barley protein concentrate.

SD = standard deviation; FE = feed efficiency; HSI = hepatosomatic index; IPF = intraperitoneal fat; PSE = pooled standard error of treatment means (n = 3). Mean values within each column with different superscripts are statistically different (P < 0.05).

had higher ($P < 0.05$) HSI compared to the REF diet. The N3010 treatment also supported a significantly higher IPF ratio relative to the REF. The dietary treatments also affected whole-body proximate composition, as well as protein and energy retention efficiency values of red drum in Trial I (Table 6). Whole-body moisture increased with the inclusion of plant feedstuffs in the diet and the PPC100 diet exhibited the highest ($P < 0.05$) whole-body moisture (77.7%) among all treatments. Red drum fed the BPC50 and PPC diets exhibited lower ($P < 0.05$) whole-body crude protein and lipid compared to those fed the REF diet. The lowest ($P < 0.05$) whole-body ash (3.1%) was observed in red drum fed the PPC100, while no significant differences were observed among the other dietary treatments. Red drum fed the SPC50 and PPC100 diets exhibited the highest (39.3%) and the lowest (30.6%) protein retention efficiency (respectively), while those fed the BPC50 and the PPC100 diets exhibited lower energy retention efficiency relative to the other treatments ($P < 0.05$).

In Trial II, significantly higher whole-body moisture, and lower whole-body protein and lipid were observed in red drum fed the SG-Trifecta diet (Table 7). The N3010 and N209 diets supported the lowest ($P < 0.05$) whole-body ash (3.1 and 3.4, respectively) among all dietary treatments. The N209, N3010 and BPC diets supported similar ($P < 0.05$) protein retention and energy retention efficiency of red drum compared to the REF. The intestinal micromorphology of red drum was unaffected ($P > 0.05$) by the high inclusion levels of SBM, N209, N3010 or BPC in the diets evaluated in Trial II, except for a higher (577.4 μm) fold height in fish fed the REF diet (Table 8).

Table 6 Whole-body proximate composition, protein and energy retention values of juvenile red drum in Trial I (Rossi et al., 2013).

Treatments[1]	Moisture	Protein	Lipid	Ash	PR	ER
			%			
REF	75.4^{c}	16.8^{a}	6.1^{a}	4.4^{a}	36.1^{b}	36.9^{a}
CPC50	76.6^{b}	16.3^{abc}	5.3^{ab}	4.1^{a}	34.4^{b}	35.1^{a}
BPC50	76.5^{b}	16.2^{bc}	3.8^{b}	4.3^{a}	34.4^{b}	29.7^{b}
SPC50	76.0^{bc}	16.5^{ab}	4.7^{ab}	4.2^{a}	39.3^{a}	36.5^{a}
PPC100	77.7^{a}	15.8^{c}	4.0^{b}	3.1^{b}	30.6^{c}	26.5^{b}
PSE	*2.116*	*1.855*	*0.461*	*0.199*	*1.005*	*1.667*
Pr > F	*<0.001*	*0.029*	*0.049*	*<0.001*	*0.002*	*0.004*

[1] REF = reference; CPC = corn protein concentrate; BPC = barley protein concentrate; SPC = soy protein concentrate; PPC = plant protein concentrate.

PR = protein retention; ER = energy retention; PSE = pooled standard error of treatment means (n = 3). Mean values within each column with different superscripts are statistically different ($P < 0.05$).

Table 7 Whole-body proximate composition, and protein and energy retention efficiency values of juvenile red drum in Trial II (Rossi et al., 2013).

Treatments[1]	Moisture	Protein	Lipid	Ash	PR	ER
			%			
REF	75.3^{bc}	17.9^{abc}	3.7^{b}	4.2^{ab}	38.9^{a}	30.3^{ab}
SBM	74.9^{bc}	17.7^{abc}	5.1^{a}	3.7^{bc}	33.4^{bc}	29.5^{bc}
N-209	75.5^{bc}	18.4^{a}	5.6^{a}	3.4^{cd}	36.7^{ab}	33.3^{a}
N3010	73.5^{c}	17.8^{abc}	5.6^{a}	3.1^{d}	36.8^{ab}	33.2^{a}
N3010WF	76.6^{b}	16.6^{c}	3.3^{b}	3.8^{bc}	23.2^{d}	17.5^{e}
SG-HP	75.0^{bc}	18.2^{ab}	4.4^{ab}	3.9^{abc}	28.6^{c}	26.3^{d}
SG-ULT	76.3^{b}	16.8^{bc}	3.4^{b}	4.2^{ab}	18.9^{d}	16.0^{e}
SG-Trifecta	78.8^{a}	15.1^{d}	1.7^{c}	4.5^{a}	5.4^{e}	2.9^{f}
BPC	76.4^{b}	17.7^{abc}	3.6^{b}	3.7^{ab}	31.8^{ab}	27.2^{b}
PSE	*3.387*	*2.271*	*0.261*	*0.955*	*2.050*	*1.880*
Pr > F	*0.005*	*0.001*	*<0.001*	*0.002*	*<0.001*	*<0.001*

[1] REF = reference; SBM = soybean meal; BPC = barley protein concentrate.
PR = protein retention; ER = energy retention; PSE = pooled standard error of treatment means (n=3).
Mean values within each column with different superscripts are statistically different ($P < 0.05$).

Table 8 Histological parameters of the red drum's intestine in Trial II (Rossi et al., 2013).

	Treatments[1]						
	REF	SBM	N209	N3010	BPC	*PSE*	*Pr > F*
Pyloric caeca			*µm*				
Fold height	604.6	397.1	439.0	539.6	609.9	*17.64*	*0.27*
Total enterocyte height	35.6	32.4	33.1	34.9	35.9	*0.33*	*0.40*
Microvillus height	4.1	4.1	3.8	3.8	3.0	*0.06*	*0.11*
Proximal intestine							
Fold height	577.4[a]	498.1[b]	446.1[b]	469.0[b]	486.1[b]	*5.06*	*0.02*
Total enterocyte height	34.3	33.2	33.2	34.2	40.7	*0.45*	*0.12*
Microvillus height	3.9	3.3	3.2	3.4	2.5	*0.08*	*0.24*
Mid intestine							
Fold height	373.7	274.5	261.6	301.4	440.6	*10.59*	*0.11*
Total enterocyte height	31.8	28.8	28.6	28.7	32.1	*0.40*	*0.43*
Microvillus height	3.2	3.2	3.0	2.8	2.6	*0.04*	*0.21*
Distal intestine							
Fold height	268.1	213.4	166.1	240.1	279.8	*10.62*	*0.59*
Total enterocyte height	29.7	30.9	28.8	28.5	30.0	*0.46*	*0.93*
Microvillus height	2.3	2.5	2.4	2.0	1.8	*0.06*	*0.36*

[1] REF = reference; SBM = soybean meal; BPC = barley protein concentrate.

PSE = pooled standard error of treatment means (n=3).

Mean values within each column with different superscripts are statistically different ($P < 0.05$).

II.4 Discussion

This study provides substantial information on the nutritional value of different plant feedstuffs for red drum. The 50% replacement of FM protein with that from SPC or BPC resulted in performance parameters comparable to the REF diet. Thus, these PPC can be used to replace up to 50% of the FM protein in the diet of red drum without adversely affecting production performance. In addition, despite of the overall reduction in the production performance of red drum when 88% replacement of FM protein with SP or BPC was evaluated, the results indicated the potential of using N209 and N3010 as partial substitutes for dietary FM in red drum.

Evidences linking dietary SBM to alterations in GIT morphology of salmonids have been reported (Baeverfjord and Krogdahl, 1996; Ingh et al., 1996; Merrifield et al., 2009; Van den Ingh et al., 1991), and detrimental effects on growth performance and health status of affected fish have been observed (Krogdahl et al., 2003; Rumsey et al., 1994). In this study, a reduction in fold height of the proximal intestine of red drum fed the SBM-, N209-, N3010-, and BPC-based diets was noticed, but no other histological alterations were evidenced. Therefore, although the extent to which such alteration may have affected the production performance of the fish and merits further evaluations, our results reinforce the increasing evidence of a higher tolerance of red drum to dietary SP.

Salmonid species have been noticed to respond differently when various levels of SBM are incorporated in diets (Refstie et al., 2000), and such may be the case for other marine fish species. Juvenile cobia, *Rachycentron canadum,* were shown to tolerate up to 40% FM protein replacement with solvent-extracted SBM in a 48% crude protein diet

(Chou et al., 2004), but reduced weight gain and FE was observed as the level of SBM increased. Davis et al. (1995) reported similar results in which diets replacing large amounts of FM with SBM significantly reduced growth of red drum. Although these findings are consistent with our current observations, the production performance of red drum fed the N209 or N3010 based diets were promising, indicating their potential as substitutes for FM.

In contrast to SBM which is typically hexane-extracted, SPC is further refined using an ethanol extraction technique or enzyme hydrolysis. Once refined SPC has several advantages over SBM including higher protein content and lower concentration of ANF. The applicability SPC as an alternative for FM in the diet of red drum was evidenced in this study. With adjusted levels of lysine and methionine in the diet, the 50% FM protein replacement with SPC resulted in fish out-performing those fed the REF diet in Trial I. Similarly, Mambrini et al. (1999) reported optimum growth of rainbow trout, *Oncorhynchus mykiss*, when 50% of FM protein was replaced with that provided by SPC with the addition of DL-methionine. Despite of the successful results with SPC observed in this study, its inclusion in the diet replacing 75% of FM protein resulted in reduced production performance of red drum (Moxley et al., 2014).

Corn protein concentrate has been studied only to a limited extent regarding its use in aquafeeds; however, some evaluations have been conducted with corn gluten meal, which is relatively similar. These corn protein isolates have been determined to be highly digestible in juvenile cobia with 94% CP digestibility (Zhou et al., 2004), and in rainbow trout with 96% CP digestibility (Morales et al., 1994). Regost et al. (1999) also

examined corn gluten meal as a replacement for FM and found that it could replace up to 33% of FM protein in the diet of turbot, *Psetta maxima*. Pereira and Oliva-Teles (2003) also found corn gluten meal to be a valuable alternative replacing up to 60% of the FM protein in diets of juveniles gilthead sea bream, *Sparus autatus*. Finally, Kikuchi (1999a) found that corn gluten meal in the diets of Japanese flounder, *Paralichthys olivaceus,* replaced up to 40% of the protein from FM but not 60% or higher. Results from the present study showed that replacing 50% of FM protein with CPC can negatively affect the weight gain, but not FE.

Although only limited information regarding the utilization of BPC in aquafeeds has is currently available, a few studies have demonstrated its potential. The apparent CP digestibility coefficient of BPC (92%) was found to be higher than that of FM (90%) in rainbow trout, suggesting it is a good candidate for use in aquafeeds (Gaylord et al. 2008). Morken et al. (2011) also observed an increase in protein digestibility when BPC was incorporated into the diets of rainbow trout replacing FM. Research with other species such as Atlantic salmon, *Salmon salar,* and Arctic charr, *Salvelinus alpinus,* also reported relatively high apparent digestibility coefficients for protein with values of 96.3% and 85.1%, respectively (Burr et al., 2011). Our results in Trial I corroborate with previous studies confirming the applicability of BPC as a partial (up to 50%) substitute for FM in the diet of red drum.

Protein blends have been suggested as an alternative to produce diets with more suited amino acid profile for fish (Hardy, 2010). Recently, the utilization of protein blends resulted in the complete replacement of FM in the diet of advanced juvenile

Atlantic salmon (Burr et al., 2012). After 12 weeks of feeding, advanced Atlantic salmon juveniles receiving diets containing all-plant protein had similar weight gain and feed conversion ratio to those receiving the REF, which is in contrast with the currently observations with red drum fed the PPC100 diet. A potential explanation for these differences is the differences in growth stages (early vs. late juvenile) among these studies.

Based on results of the present study, all evaluated plant feedstuffs are good candidates for partial FM replacement in the diet of red drum. The SPC and BPC can readily replace 50% of FM protein without affecting the growth performance, condition indices, and whole-body composition of red drum.

CHAPTER III

ASSESSING THE NUTRITIONAL VALUE OF AN ENZYMATICALLY PROCESSED SOYBEAN MEAL IN RED DRUM

III.1 Introduction

Approximately 70% of the fishmeal (FM) produced globally has been destined for the manufacture of aquafeeds that are largely utilized in the production of carnivorous teleosts and shrimp (Tacon and Metian, 2015). The fast growth rate of global aquaculture aligned with steady production and skyrocketing prices of FM have challenged the aquaculture industry in finding sustainable protein sources for the production of cost-effective aquafeeds (Barrows et al., 2008; Gatlin et al., 2007).

Plant-protein (PP) feedstuffs are potential surrogates for fishmeal and their utilization in aquafeeds has been intensified in recent years (Tacon and Metian, 2015). Among the plant feedstuffs commercially available, soybean meal (SBM) is the foremost ingredient substituting for FM in aquafeeds (Troell et al., 2014). Approximately 190 million metric tons (mmt) of SBM was produced globally in 2013-2014 (www.fas.usda.gov/commodities) and it comprises the main PP source used in the animal feed industry. Soybean meal is a high quality protein feedstuff with a reasonably balanced amino acid profile. Nevertheless, a series of bioactive compounds broadly categorized as anti-nutritional factors (ANF) present in commodity SBM may cause adverse effects on the digestive system and the overall physiological status of fish (Francis et al., 2001), thereby limiting its inclusion in aquafeeds.

To overcome the limitations imposed by the presence of ANF in SBM, novel processing technologies are being incorporated into the manufacturing of SBM to improve its nutritional value. Examples of such processing technologies include fermentation and enzymatic treatment. In either case, the ANF content of SBM is reduced in the breakdown of the carbohydrate fraction aided by microorganisms during fermentation, or by the incorporation of a blend of enzymes during processing. These biotechnologically processed SBM also have higher crude protein (CP) compared to the original commodity SBM. Overall, these new technologies have improved the utilization of SBM by terrestrial animals such as swine (Cervantes-Pahm et al., 2010), and increasing evidence suggest their benefit as partial substitutes for FM in aquafeeds for carnivorous teleosts that are highly sensitive to ANF (Refstie et al., 1998).

Although red drum, *Sciaenops ocellatus*, have been demonstrated to be relatively more tolerant to ANF present in SBM compared to some carnivorous teleosts, the performance of early stage juvenile fish fed diets high in SBM can be negatively affected as shown in Chapter II. Therefore, the objective of the present study was to evaluate the nutritional value of an enzymatically treated SBM in the diet of early stage juvenile red drum.

III.2 Material and Methods

III.2.1 Fish

Two batches of red drum were obtained in different time periods from the Sea Center Texas Marine Aquarium, Fish Hatchery and Nature Center operated by Texas

Parks and Wildlife Department in Lake Jackson, TX. Fish were maintained in quarantine for 2 weeks acclimating to local conditions until adequate size for the feeding trials was attained. Over this period, red drum were fed at a rate approaching apparent satiation with a 40% crude protein and 12% crude fat commercial diet (Rangen, Inc., Angleton, TX).

III.2.2 Trial III - Digestibility

A digestibility trial was conducted at the Aquacultural Research and Teaching Facility of Texas A&M University to determine the apparent digestibility coefficient (ADC) of organic matter, crude protein, lipid, and energy of two soybean products (SP) in red drum. The SP evaluated were a conventionally processed, commodity SBM (designated SBM) and an enzymatically treated SBM (HP300, Hamlet Protein Inc., Findlay, Ohio, USA; designated ESBM), both of which were dehulled and solvent-extracted meals. The ESBM was higher in CP and lower in lipid and gross energy content relative to SBM (Table 9). The former also had lower levels of trypsin inhibitor (TI), beta-conglycinin, clycinin, and oligosaccharides than the latter (Table 10).

An indirect method was utilized for the determination of digestibility coefficients and chromic oxide (Cr_2O_3) was used and the non-digestible marker. A pre-existing FM reference diet (Rossi et al., 2015a) containing 46% crude protein (CP) and 12.2% lipid was utilized as the digestibility reference (REF) diet. In order to manufacture the REF diet for the digestibility evaluation, the original diet was hammer-milled followed by the addition of chromic oxide as a non-digestible marker at 1% and carboxymethyl cellulose at 4% for appropriate binding. These dietary components were then mixed altogether in

Table 9 Composition of the soybean products evaluated.

	Moisture	Organic Matter	Crude Protein	Lipid	Gross Energy
	%	%	%	%	kcal g^{-1}
Ingredients		*dry matter basis*			
ESBM[a]	7.0	93.5	59.8	4.1	3.8
SBM[b]	7.5	93.8	53.8	4.7	4.7

[a] ESBM = enzymatically treated soybean meal; Hamlet Protein Inc., Findlay, OH, USA. Crude protein = 581.4 g kg^{-1}; Lipid = 41.1 g kg^{-1} dry-matter basis.

[b] SBM = soybean meal; Producers Coop. Association, Bryan, Texas, USA. Crude protein = 500.8 g/kg; Lipid = 60.2 g/kg on a dry-matter basis.

Table 10 Trypsin inhibitor, beta-conglycinin, glycinin, oligosaccharides and galactose content of the soybean products evaluated[1].

	TI	Beta-conglycinin	Glycinin	Oligosaccharides	Galactose
	mg g^{-1}	ppm	ppm	%	%
Ingredients	*dry matter basis*				
ESBM[a]	1.4	1.1	2.2	0.9	0.6
SBM[b]	3.4	9885.9	41063.2	8.5	-

[1]Analyses were performed by Hamlet Protein, Inc., 5289 Hamlet Drive, Findlay, Ohio, USA. TI = trypsin inhibitor;

[a] ESBM = enzymatically treated soybean meal; Hamlet Protein Inc., Findlay, OH, USA. Crude protein = 581.4 g kg^{-1}; Lipid = 41.1 g kg^{-1} dry-matter basis.

[b] SBM = soybean meal; Producers Coop. Association, Bryan, Texas, USA. Crude protein = 500.8 g/kg; Lipid = 60.2 g/kg on a dry-matter basis.

an industrial mixer for 40 min and then divided equally into three equal fractions for the manufacture of the REF and each of the SP test diets. The test diets were manufactured using a 70:30 percent ratio of the REF and each of the SP being evaluated according to previously described methodologies (Cho et al., 1982; Wilson and Poe, 1985). The dry mixtures were then blended with water using a secondary industrial mixer with a meat grinder attachment, and pelleted with a 4-mm die. The resulting pellets were dried for 24 h using forced air at 25° C and stored at – 20° C until utilized. The estimated ingredient composition of the REF diet and its analyzed composition are presented in Table 11. The reformulation of this diet and the inclusion of chromic oxide at 1% and of carboxymethyl cellulose at 4% slightly reduced final CP and lipid values relative to the original diet. The analyzed chromic oxide level (0.98%) was close to the formulated level of 1%.

Groups of 35 advanced juvenile red drum (~200 g) were stocked in six, 1000-L circular-fiberglass tanks operating as a recirculating system, which maintained water quality within adequate ranges for red drum (mean ± standard deviation (SD)): salinity = 6.0 ± 0.5 g L^{-1}; dissolved oxygen = 6.1 ± 0.3 mg L^{-1} ; temperature = 27.4 ± 1.0° C; pH = 7.8 ± 0.1; total ammonia nitrogen (TAN) = 0.2 ± 0.1 mg L^{-1}). Each diet was randomly assigned to two tanks and the fish were fed their assigned diet once daily to satiation. A total of three fecal collections were performed in each replicate tank at 1-week intervals allowing the fish to recover from handling stress. On the day fecal samples were collected, feed was provided to each group of fish 10-min apart in sequential tanks to ensure that fecal collection occurred at approximately 5 h postprandial in all tanks. Fecal

Table 11 Composition of the reference and test diets[1] used in Trial III.

	REF	ESBM	SBM
Ingredients	*% of dry matter*		
Menhaden fishmeal[a]	55.4	70	70
Wheat starch[b]	10.8		
Menhaden oil[a]	3.3		
Vitamin premix[g]	2.9		
Mineral premix[g]	3.9		
Carboxymethyl cellulose[h]	4.0		
Chromic oxide[i]	1.0		
Celufil[h]	18.7		
ESBM[j]		30	
SBM[k]			30
Analyzed composition[2]			
Moisture	12.5	14.6	12.8
Organic matter	85.8	87.6	88.1
Protein	44.0	49.3	47.3
Lipid	10.6	9.2	9.5
Gross energy (kcal g^{-1})	4.3	4.4	4.6
Ash	15.9	14.4	13.4
Chromic oxide	0.98	0.72	0.72

[1] REF = reference, ESBM = enzymatically treated soybean meal, SBM = soybean meal.

[2] Dry matter basis (except moisture).

[a – h] Rossi et al. (2015a).

[i] Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.

[j] Hamlet Protein Inc., Findlay, OH, USA. Crude protein = 581.4 g kg^{-1}; Lipid = 41.1 g kg^{-1} dry-matter basis.

[k] Producers Coop. Association, Bryan, Texas, USA. Crude protein = 500.8 g/kg; Lipid = 60.2 g/kg on a dry-matter basis.

contents from the lower intestine was manually stripped (Austreng, 1978). Before being returned to their respective culture tanks, fish were immersed for 5 min in a bath containing nitrofurazone (5-nitro-2-furaldehyde semicarbazone, Sigma-Aldrich, Co., St. Louis, MO, USA) at 8 mg L^{-1}, as a preventive measure against bacterial infections. Fecal samples from each fish were pooled by tank in each of the three samplings. Immediately following collection, all fecal samples were partially dried at 60° C for 24 h, and for an additional 3 h at 135° C before chemical analyses were conducted.

III.2.3 Trials IV and V

Trials IV and V were conducted to: 1) evaluate the nutritional value of the ESBM in the diet of red drum by estimating its maximum replacement value (MAX REPL) for dietary FM and 2) validate the MAX REPL of ESBM for FM in the diet of red drum and compare it against a SBM test diet at the same level of FM replacement. The MAX REPL was defined as the maximum replacement of digestible protein (DP) from FM with ESBM at which growth performance of the fish would not change relative to those fed the FM reference diet (designated FM-100).

All experimental diets were formulated to contain 32% DP and 12% lipid. Seven diets were formulated for Trial IV including the FM-100, designed to contain all its protein from menhaden FM, and six ESBM test diets (Table 12). The latter were designed to replace the DP from FM in the FM-100 diet on an isonitrogenous basis, at 15% incremental levels (ESBM-15 to ESBM-90). Three experimental diets were utilized in Trial V: the FM-100 diet, re-manufactured from Trial IV; an ESBM test diet, formulated to validate the estimated MAX REPL of ESBM for dietary FM in Trial IV;

and a SBM test diet, designed to compare both SP as FM substitutes (Table 13). All experimental diets were supplemented with glycine at 2% for enhanced palatability (McGoogan and Gatlin, 1997), and with taurine at 1%. In addition, the supplementation of lysine-HCl, DL-methionine, and dicalcium phosphate was performed to all test diets to ensure that lysine, total sulfur amino acids, and total phophorus requirements were met (Craig and Gatlin 1992; Davis and Robinson, 1987; Moon and Gatlin, 1991). The analyzed values of moisture, crude protein, lipid, ash, total phosphorus, gross energy, and amino acids were all very consistent across treatments, except for a slightly lower lipid level in the SBM-70 diet in Trial V.

Trials IV and V were conducted in sequence for 6 or 8 weeks, respectively, utilizing 110-L aquaria operated as a recirculating system. All monitored water quality parameters in Trials IV and V were maintained within adequate ranges for red drum (respectively, mean ± SD): salinity (g L^{-1}) = 6.8 ± 0.2 and 7.7 ± 0.6; dissolved oxygen (mg L^{-1}) = 6.2 ± 0.3 and 7.41 ± 0.5; temperature (°C) = 27.2 ± 0.7 and 26.3 ± 0.2; pH = 7.9 ± 0.1 and 8.0 ± 0.1; and total ammonia nitrogen (TAN, mg L^{-1}) = 0.5 ± 0.33 and 0.2 ± 0.1). A 12h light:12h dark cycle was maintained using fluorescent lighting controlled by timers. On the day red drum were transferred from the quarantine tanks to the aquaria, a sample of 15 fish was collected from the population and frozen (- 20° C) for subsequent analyses of initial whole-body composition. Twenty red drum juveniles initially weighing (mean ± SD) 5.7 ± 0.04 and 1.7 ± 0.04 g were stocked in each aquarium of Trial IV and Trial V, respectively. After a 1-week conditioning period prior to the commencement of each feeding trial, each experimental diet was randomly

Table 12 Composition of experimental diets[1] fed to red drum for 6 weeks in Trial IV.

	FM-100	ESBM-15	ESBM-30	ESBM-45	ESBM-60	ESBM-75	ESBM-90
Ingredients	*% of dry matter*						
Menhaden fishmeal[a]	56.0	47.6	39.2	30.8	22.4	14.0	5.6
ESBM[b]		10.1	20.1	30.2	40.3	50.3	60.4
Menhaden oil[c]	5.5	6.4	7.3	8.2	9.1	10.0	11.0
Dextrinized corn starch[d]	13.2	11.5	10.0	8.4	6.8	5.2	3.6
Carboxymethyl cellulose[e]	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin premix[d,e]	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Mineral premix[d,e]	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Dicalcium phosphate[f]		0.03	1.4	2.9	4.3	5.7	6.6
Glycine[g]	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Taurine[g]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lysine HCl[g]		0.08	0.13	0.19	0.24	0.30	0.35
DL-Methionine[g]		0.09	0.16	0.23	0.29	0.36	0.43
Celufil[g]	13.3	12.2	9.6	7.1	4.6	2.1	0.0
Analyzed proximate composition[2]							
Moisture	12.5	11.0	10.7	11.3	10.8	11.3	11.9
Protein	44.5	44.0	43.8	44.0	43.9	44.1	44.2
Lipid	14.1	13.8	14.3	14.2	14.2	14.1	14.2
Ash	14.6	13.5	13.9	14.0	14.0	14.3	14.1

Table 12 continued.

	FM-100	ESBM-15	ESBM-30	ESBM-45	ESBM-60	ESBM-75	ESBM-90
Total phosphorus	2.8	2.4	2.5	2.6	2.7	2.8	2.6
Gross energy (kcal g^{-1})	4.8	4.8	4.8	4.8	4.9	4.7	4.9
Analyzed amino acid composition							
Arg	3.1	3.3	3.7	3.1	3.2	3.7	3.6
His	1.4	1.4	1.6	1.3	1.3	1.5	1.5
Ile	2.4	2.5	2.7	2.2	2.2	2.6	2.4
Leu	4.2	4.4	4.7	3.8	3.9	4.5	4.2
Lys	2.9	3.2	3.4	2.6	2.5	2.9	2.4
Met	1.5	1.4	1.5	1.1	1.0	1.1	0.9
Phe	2.3	2.4	2.7	2.3	2.4	2.9	2.9
Tau	1.8	1.9	1.9	1.4	1.4	1.3	1.4
Thr	2.2	2.3	2.4	1.9	1.9	2.2	2.0
Val	2.7	2.8	3.0	2.4	2.5	2.8	2.6

[1] FM = fishmeal; ESBM = enzymatically treated soybean meal.

[2] Dry matter basis (except moisture).

[a] Special Select ™ Omega Protein Inc., Abbeville, LA, USA. Crude protein = 700.8 g kg^{-1}; Lipid = 116.4 g kg^{-1} on a dry-matter basis.

[b] Hamlet Protein Inc., Findlay, OH, USA. Crude protein = 581.4 g kg^{-1}; Lipid = 41.1 g kg^{-1} on a dry-matter basis.

[c] Omega Protein, Reedville, VA, USA.

[d] MP Biomedicals, Solon, OH, USA

[e] Moon and Gatlin (1991).

[f] Fisher Scientific, Pittsburg, PA, USA

[g] USB, Cleveland, OH, USA

Table 13 Composition of experimental diets[1] fed to juvenile red drum for 8 weeks in Trial V.

	FM-100	ESBM-70	SBM-70
Ingredients	*% of dry matter*		
Menhaden fishmeal[a]	57.7	17.1	17.1
ESBM[b]		47.0	
SBM[c]			50.0
Menhaden oil[d]	5.3	9.2	7.2
Dextrinized corn starch[e]	13.2	5.7	5.2
Carboxymethyl cellulose[f]	2.0	2.0	2.0
Vitamin premix[e,f]	3.0	3.0	3.0
Mineral premix[e,f]	4.0	4.0	4.0
Dicalcium phosphate[g]		5.7	5.0
Glycine[h]	2.0	2.0	2.0
Taurine[h]	1.0	1.0	1.0
Lysine HCl[h]		0.3	0.5
DL-Methionine[h]		0.5	0.5
Celufil[h]	11.7	2.6	2.6
Analyzed proximate composition[2]			
Moisture	6.2	5.7	6.0
Protein	43.7	43.6	42.0
Lipid	12.9	13.1	10.7
Ash	15.6	15.7	15.0
Analyzed amino acid composition			
Arg	3.0	4.1	3.6
His	1.4	1.7	1.5
Ile	2.3	2.8	2.5
Leu	4.1	4.9	4.4

Table 13 continued.

	FM-100	ESBM-70	SBM-70
Lys	3.0	3.1	3.1
Met	1.4	1.4	1.3
Phe	2.3	3.1	2.7
Tau	1.9	1.6	1.6
Thr	2.2	2.4	2.1
Val	2.7	3.1	2.8

[1] FM = fishmeal; ESBM = enzymatically treated soybean meal; SBM = soybean meal.

[2] Dry matter basis (except moisture).

Superscripts a, b, d – h = a, b – g in Table 1.

[c] Producers Coop. Association, Bryan, Texas, USA. Crude protein = 500.8 g/kg; Lipid = 60.2 g/kg on a dry-matter basis.

assigned to duplicate aquaria in Trial IV and to triplicate aquaria in Trial V. In Trial IV, fish were feed to complete satiation in the morning and at a fixed rate approaching apparent satiation in the afternoon. In the complete satiation feeding, each aquarium was visited (by the same person) three times within a 30- to 45-min period and fish were fed in accordance to the visual assessment of their appetite. Red drum in Trial V were fed twice daily according to the fixed feeding regimen used in the afternoon feedings of Trial IV.

III.2.4 Data Acquisition and Analyses

III.2.4.1 Trial III - Digestibility

All samples of test ingredients, diets, and fecal material were stored at -20 C and were dried (135° C for 3 h) prior to the analyses. Chromic oxide concentrations in diets and fecal samples was determined according to the method described by Furukawa Tsukahara (1966) in which, after a colorimetric reaction, marker concentrations were measured spectrophotometrically as values of absorbance at 540 nm. Dry matter content was determined after drying samples in 135° C for 3 h, and ash and organic matter were determined after ashing samples at 650° C for 3 h (AOAC, 1990). Crude protein (N × 6.25) was determine by the Dumas method (AOAC, 2005), and crude lipid was determined according to Folch et al. (1957). Gross energy was determined in a Semi-micro-bomb calorimeter (Parr 6200; Parr Instrument Company, Moline, IL). The apparent digestibility coefficient (ADC) of a nutrient in the diets (1) and in the test ingredients (2) was calculated using standard formulas (NRC, 2011):

1)

$$\text{ADC} = \frac{\text{Cr}_2\text{O}_3 \text{ in feed}}{\text{Cr}_2\text{O}_3 \text{ in feces}} \times \frac{\text{Nutrient in feces}}{\text{Nutrient in feed}}$$

2)

$$\text{ADC}_{\text{test ingredient}} = \text{ADC}_{\text{test diet}} + ((\text{ADC}_{\text{test diet}} - \text{ADC}_{\text{ref. diet}})$$

$$\times (0.7 \times \text{D}_{\text{ref.}} \div 0.3 \times \text{D}_{\text{ingredient}}))$$

where $\text{D}_{\text{ref.}}$ is the percent of nutrient or kcal/g gross energy of the reference diet, and $\text{D}_{\text{ingredient}}$ is the percent of nutrient or kcal/g gross energy of the test ingredient (NRC, 2011)

III.2.4.2 Trials IV and V

At the end of Trials IV and V, fish in each aquarium were group-weighed and sampled after overnight fasting. Three representative fish from each tank were euthanized with an overdose of tricaine methanesulfonate (MS-222; Western Chemical, Inc., Ferndale, WA), frozen at -20° C and subsequently homogenized for proximate analysis to determine crude protein, lipid, moisture, and ash in whole-body tissue (AOAC 1990). Three additional representative fish from each aquarium were anesthetized in approximately 2 g L^{-1} MS-222 for blood collection and subsequently euthanized. Approximately 0.5 – 1.0 mL of blood from each fish was collected from the hemal arch in the caudal peduncle using 1-mL syringe with 26-gauge, heparinized needles. These bled fish were then dissected to obtain liver and intraperitoneal fat (IPF) for computing hepatosomatic index (HSI) and IPF ratio values, respectively, and then

filleted to obtain muscle for computing muscle yield values. The gastrointestinal tract (GIT) from each fish was separated into stomach and intestine and flash frozen in liquid nitrogen for the determination of pepsin and trypsin activity according to the methods described by Anson (1938) and Erlanger et al. (1961), respectively. Enzyme extraction procedure prior to quantification was performed as described by Castillo et al. (2014).

III.2.5 Calculations and Statistical Analyses

The fish performance parameters utilized to compare treatments in the growth trials were calculated as follow:

- Weight gain, % = [(Final weight – initial weight)/(initial weight)] × 100;
- Feeding rate, % of body weight per day (BW d^{-1}) = [dry feed intake (g)/($\sqrt{\text{initial body weight} \times \text{final body weight (g)}}$)/days on feed] × 100;
- Feed efficiency ratio (FE) = [weight gain (g)/ dry feed consumed (g)];
- Protein retention efficiency, % = {[(final body weight (g) × final body protein (%)) – (initial body weight (g) × initial body protein (%))]}/(protein intake (g) x 100;
- Energy retention efficiency, % = {[(final body weight (g) × final body energy (%)) – (initial body weight (g) × initial body energy (%))]}/(energy intake (g) × 100;
- Muscle yield, % = [fillet muscle weight (g)/body weight (g)] × 100;
- Viscerosomatic indices (HSI or IPF ratio), % = [Liver or intraperitoneal fat weight (g)/body weight (g)] × 100.

All data were analyzed for normality (Shapiro-Wilk test) and for homogeneity of variances (Bartlett's test). Digestibility data were subjected to Student's t-test to detect significant differences among ADC values between ESBM and SBM. Data resulting from Trials IV and V were subjected to one-way ANOVA to detect significant ($P < 0.05$) differences among treatment means. When significant differences were detected, regression analyses were performed for Trial IV, while orthogonal contrasts were used for the separation of treatment or treatment-group means for Trial V. The adjusted R^2 (Adj. R^2) was calculated as previously described by Kvalseth (1985). All statistical analyses were carried out using the SAS® software package (SAS Institute Inc., Cary, NC USA).

III.3 Results

The ADC of organic matter, lipid, and gross energy was significantly higher in the ESBM than in SBM, while the ADC of CP was significant higher in SBM than ESBM (Table 14).

In Trial IV, the relationship between dietary ESBM and the dependent variables final weight, feed efficiency, survival, protein and energy retention ($P < 0.05$) was best explained by second order polynomial models, while no relationship was found for feeding rate (Table 15). The MAX REPL of ESBM for dietary FM was estimated in 71.8% for final weight and weight gain, 63% for feed efficiency, 64.2% for protein retention, and 54.1% for energy retention efficiency. The effect of dietary ESBM on composition indices and whole-body composition of red drum in Trial IV also was best

explained by second order polynomial models ($P < 0.05$) for HSI and whole-body values of moisture, protein, and lipid (Table 16). A linear response was found for IPF ratio, which decreased as the level of ESBM increased in the diets. No relationship ($P > 0.05$) was found for muscle yield and whole-body ash. In Trial V, red drum fed the FM-100 diet showed a significantly higher protein retention efficiency compared to fish fed both SP diets (ESBM-70 or SBM-70), while no differences were found among treatments regarding the other performance parameters (Table 17). The HSI and IPF ratio of red drum fed the FM-100 diet was significantly higher than in fish fed either SP. A significantly higher HSI was displayed by red drum fed the ESBM-70 diet relative to those fed the SBM-70 diet. Whole-body ash content also was significantly higher in fish fed the FM-100 diet compared to either SP diet (Table 17).

Pepsin and trypsin activities were significantly affected by the replacement of FM with ESBM in the diet of red drum in Trial IV. The activity of both enzymes decreased linearly in response to the incremental levels of ESBM in the diet (Fig. 2 and 3, respectively). No significant differences were found for pepsin or trypsin activity among treatments in Trial V (Fig. 4).

Table 14 Apparent digestibility coefficient values of the diets and the soybean products for red drum in Trial III.

	Organic Matter	Crude Protein	Lipid	Gross Energy
Diets	*ADC, % of dry matter*			
REF[1]	57.6	76.1	91.7	63.8
ESBM	80.4	92.1	65.5	60.4
SBM	80.6	91.8	64.7	57.8
Ingredients[2]				
ESBM	59.8	81.3	95.0	62.9
SBM	51.7	82.0	92.3	60.9
PSE	*2.420*	*0.818*	*1.373*	*2.890*
Student's t-test (Pr > t)	*<0.001*	*<0.001*	*<0.001*	*<0.001*

[1] REF = reference.

[2] ESBM = enzymatically treated soybean meal; SBM = soybean meal.

ADC = apparent digestibility coefficient; PSE = pooled standard error.

Table 15 Growth, feed efficiency, survival, protein and energy retention efficiencies of red drum (mean initial weight ± SD = 5.72 ± 0.04 g) after 6 weeks of feeding the experimental diets in Trial IV.

Treatments[1]	Final weight	Weight gain	FE	Feeding rate	Survival	PR	ER
	g	%		% BW d^{-1}	%	%	%
FM-100	33.5	484	0.9	4.8	95.0	33.5	32.0
ESBM-15	37.2	547	1.0	4.8	90.0	35.2	31.7
ESBM-30	40.0	597	1.0	4.8	90.0	40.0	37.2
ESBM-45	37.2	547	1.0	4.9	77.5	35.1	31.1
ESBM-60	38.6	576	0.9	4.8	85.0	35.1	31.5
ESBM-75	32.4	464	0.8	4.8	85.0	29.9	24.1
ESBM-90	24.5	330	0.7	4.9	85.0	22.2	17.6
PSE	*1.616*	*26.975*	*0.035*	*0.018*	*1.817*	*1.619*	*1.947*
Regression (N=2)							
Model	SOP	SOP	SOP	NR	SOP	SOP	SOP
Pr > F	0.006	0.006	0.002		0.029	0.002	0.005
Adj. R^2	0.51	0.50	0.64		0.35	0.86	0.51
MAX REPL (%)	71.8	71.8	63.0		-	64.2	54.1

[1] FM = fishmeal; ESBM = enzymatically treated soybean meal.

SD = standard deviation; FE = feed efficiency; BW d^{-1} = body weight per day; PR = protein retention; ER = energy retention; PSE = pooled standard error of treatment means; SOP = Second order polynomial; NR = no relationship; MAX REPL = maximum replacement.

Table 16 Composition indices and whole-body composition of red drum after 8 weeks of feeding the experimental diets in Trial IV.

	Composition Indices			Whole-body Composition			
Treatments[1]	Muscle yield	HSI	IPF ratio	Moisture	Protein	Lipid	Ash
			%				
FM-100	36.4	2.03	0.92	74.6	16.0	5.8	3.7
ESBM-15	35.5	1.91	0.72	74.8	16.1	5.3	3.8
ESBM-30	37.2	1.68	0.90	73.9	16.8	5.9	3.9
ESBM-45	36.7	1.65	0.87	74.4	17.1	5.5	4.8
ESBM-60	38.0	1.57	0.34	74.7	16.3	5.4	4.0
ESBM-75	36.0	1.58	0.51	76.3	15.9	4.3	4.0
ESBM-90	36.7	1.88	0.24	76.8	15.2	4.3	3.8
PSE	*0.243*	*0.065*	*0.074*	*0.312*	*0.181*	*0.231*	*0.104*
Regression (N=2)							
Model	NR	SOP	L	SOP	SOP	SOP	NR
Pr > F		0.022	< 0.001	0.005	0.004	0.037	
Adj. R^2		0.38	0.60	0.51	0.53	0.33	

[1] FM = fishmeal; ESBM = enzymatically treated soybean meal.

HSI = hepatosomatic index; IPF = intraperitoneal fat; FM = fishmeal; ESBM = enzymatically treated soybean meal; PSE= pooled standard error; NR = no relationship; SOP = second order polynomial; L = linear.

Table 17 Final weight, weight gain, feed efficiency, survival, protein and energy retention efficiencies of red drum (mean initial weight ± SD = 1.69 ± 0.04 g) after 8 weeks of feeding the experimental diets in Trial V.

Treatments[1]	Final weight	Weight gain	FE	Survival	PR	ER
	g	%		%	%	%
FM-100	33.3	1862	0.97	80.0	37.4	36.9
ESBM-70	33.5	1924	1.0	78.3	38.9	36.7
SBM-70	31.5	1764	0.97	81.7	40.7	34.5
PSE	*1.176*	*72.02*	*0.011*	*2.357*	*0.573*	*0.648*
Anova (Pr > F)	*0.795*	*0.718*	*0.560*	*0.880*	*0.037*	*0.277*
Contrasts (Pr > t)						
FM-100 – SP					*0.027*	
ESBM-70 – SBM-70					*0.106*	

[1] FM = fishmeal; ESBM = enzymatically treated soybean meal; SBM = soybean meal.

SD = standard deviation; FE = feed efficiency; PR = protein retention; ER = energy retention; PSE = pooled standard error of treatment means (n = 3).

Table 18 Composition indices and whole-body composition of red drum after 8 weeks of feeding the experimental diets in Trial V.

	Composition Indices			Whole-body Composition			
Treatments[1]	Muscle yield	HSI	IPF ratio	Moisture	Protein	Lipid	Ash
			%				
FM-100	37.9	2.60	0.71	73.5	16.8	6.2	3.7
ESBM-70	36.4	2.10	0.47	74.4	17.0	5.7	3.8
SBM-70	35.1	1.55	0.25	73.8	17.5	5.0	3.8
PSE	*0.561*	*0.168*	*0.008*	*0.548*	*0.306*	*0.173*	*0.040*
Anova (Pr > F)	*0.097*	*0.006*	*0.035*	*2.889*	*1.673*	*2.581*	*0.295*
Contrasts (Pr > t)							
FM-100 – SP		*0.004*	*0.021*				*0.015*
ESBM -70 – SBM -70		*0.046*	*0.147*				*0.585*

[1] FM = fishmeal; ESBM = enzymatically treated soybean meal; SBM = soybean meal.
HSI = hepatosomatic index; IPF = intraperitoneal fat; PSE = pooled standard error.

III.4 Discussion

This study provides additional evidence of the high nutritional value of SP for red drum. According to these results, both ESBM and SBM can be used as partial replacements for dietary FM to provide over half of the dietary DP. Once reproduced under commercial production settings, these results will aid in the development of more sustainable and cost-effective aquafeeds for red drum.

Although the commodity SBM utilized in this study was not exactly the same as that used in the manufacture of the ESBM evaluated, it is reasonable to assume that the enzymatic treatment used in the manufacturing of ESBM can remarkably reduce the level of ANF in commodity SBM. Besides the reduction of important bioactive compounds known for causing adverse physiological effects in the GIT of monogastric animals, including fish (Francis et al., 2001), the enzymatic process also leads to an increased protein and amino acid concentration in the final product, thereby reducing the need for supplementing crystalline amino acids (primarily lysine) in the diet to meet the animal's requirement.

With the exception of CP, the ADC values of organic matter, lipid, and gross energy of ESBM were significantly higher than in SBM. However, despite the statistical significance found, the narrow differences observed may have no practical significance. In addition, regardless of SP type, these results agree fairly well with those reported by Gaylord and Gatlin (1996) with the exception of a higher ADC for lipid in the present study (92.3 and 95% vs. 62.7%). In addition, the low ADC values observed for organic matter and energy agree with those previously found for solvent extracted, dehulled

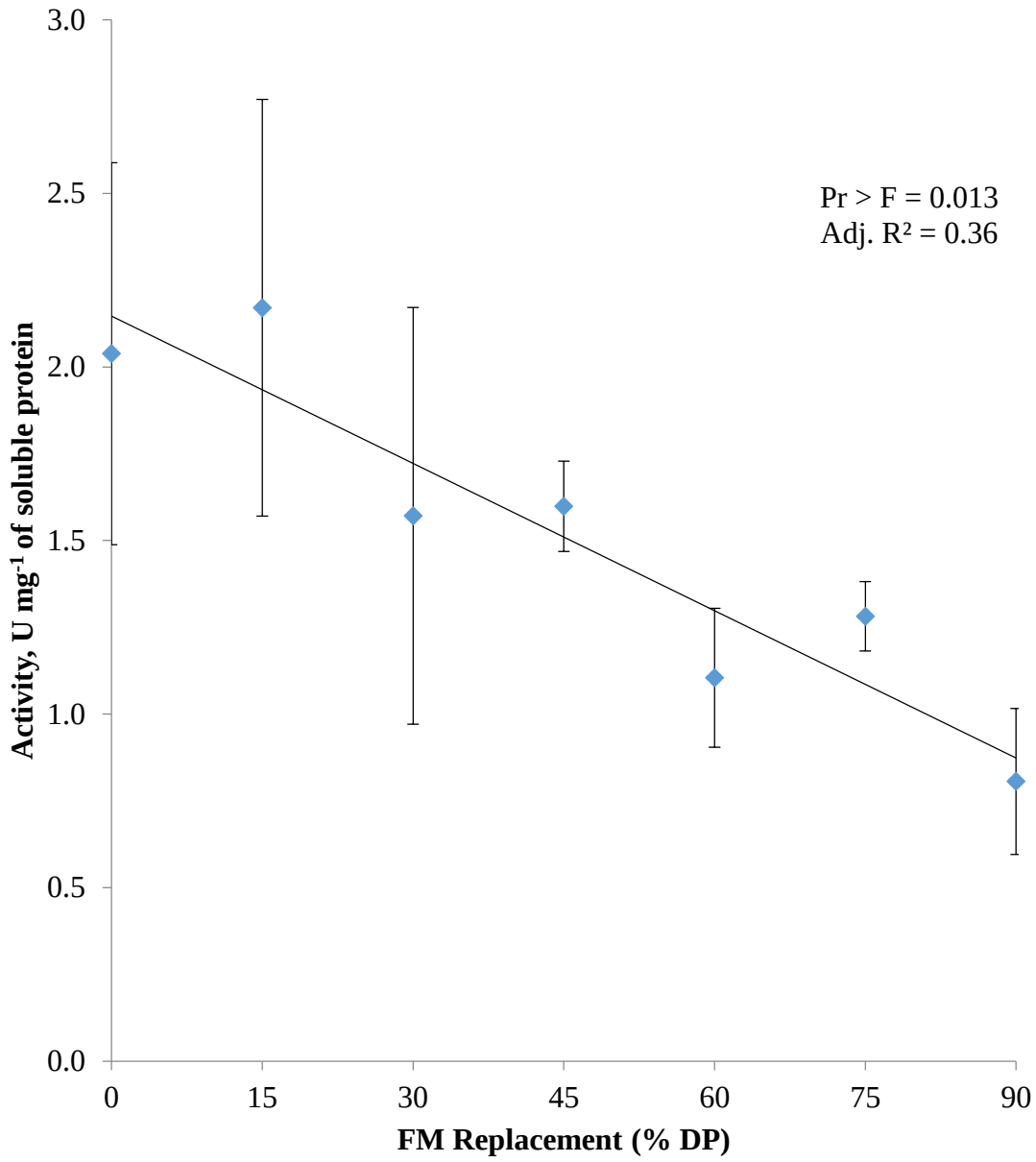

Figure 2 Trypsin activity in red drum fed the experimental diets for 6 weeks in Trial IV. Error bars represent standard error (SE, n = 2). FM = fishmeal; DP = digestible protein.

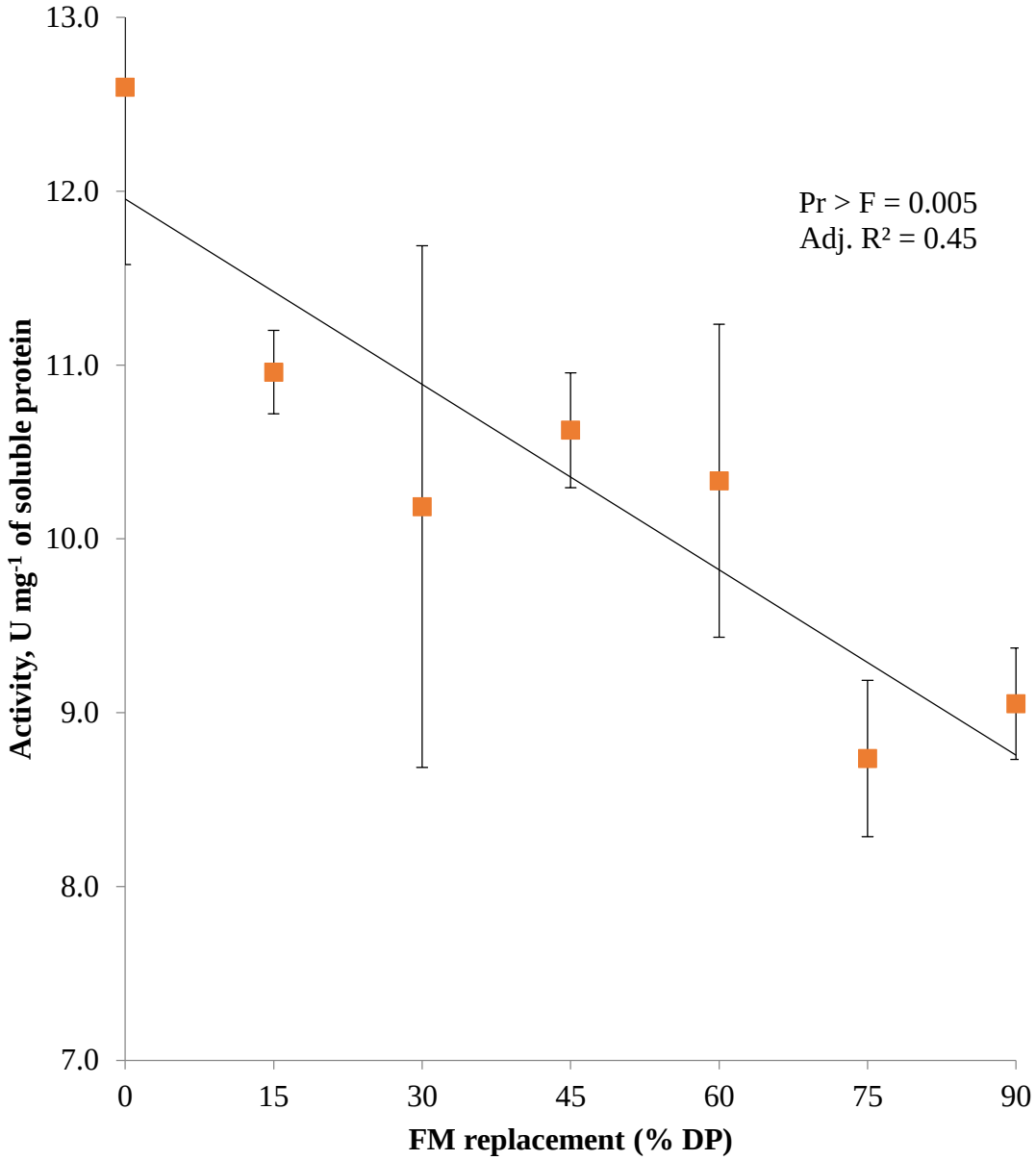

Figure 3 Pepsin activity in the stomach of red drum fed the experimental diets for 6 weeks in Trial IV. Error bars represent standard error (SE, n = 2). FM = fishmeal; DP = digestible protein.

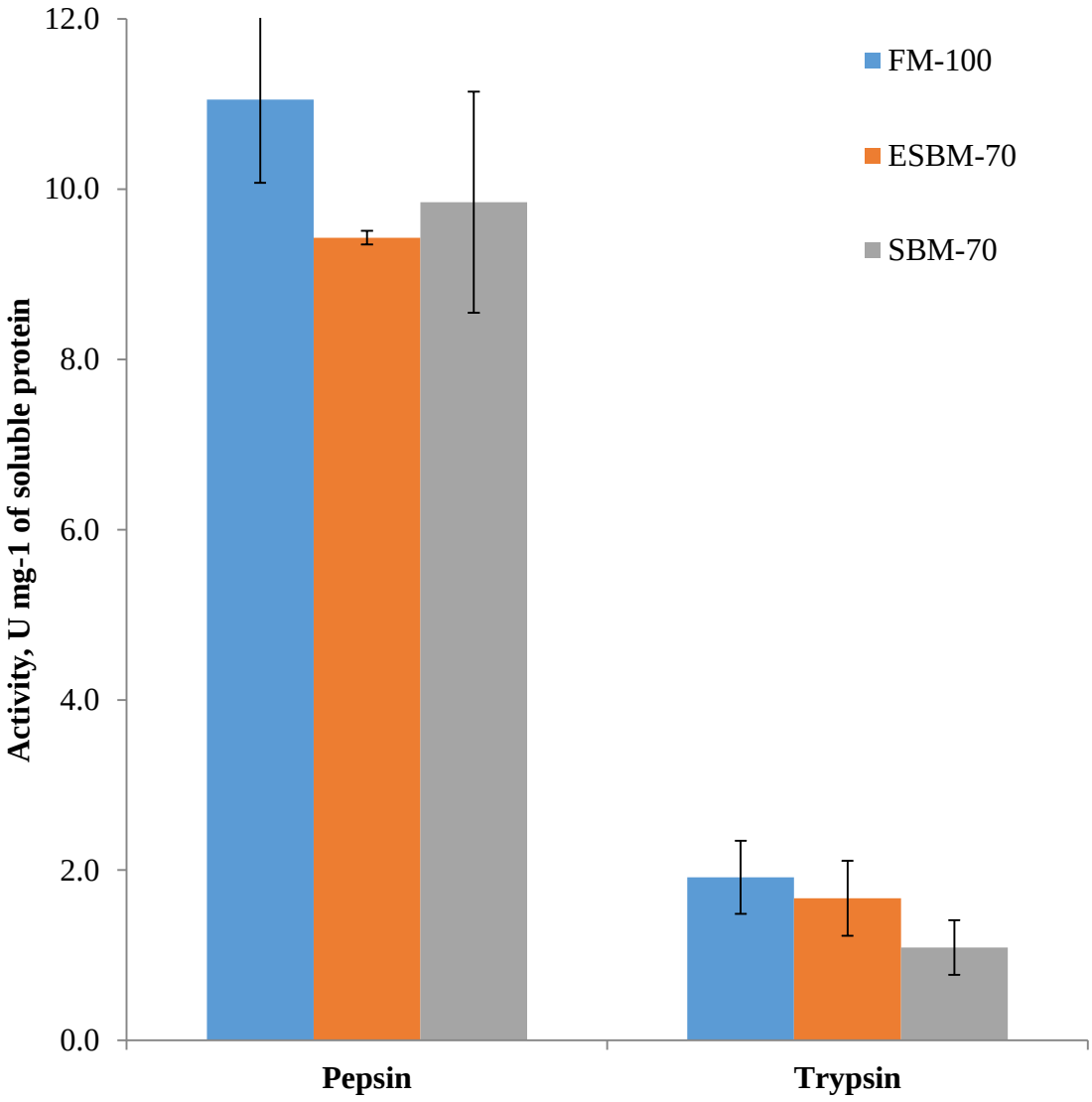

Figure 4 Stomach pepsin and intestinal trypsin activities (Pr > F = 0.498 and 0.49, respectively) of red drum fed the experimental diets for 8 weeks in Trial V. Error bars represent standard error (SE, n = 3). FM = fishmeal; ESBM = enzymatically treated soybean meal; SBM = soybean meal.

SBM (Gaylord and Gatlin, 1996), and reflect the poor digestibility of the carbohydrate fraction by red drum.

According to the second order polynomial model, 72% of the DP provided by FM can be substituted with ESBM in the diet of red drum before reductions in growth occur relative to that supported by the FM-100 diet (Fig. 5). However, when a quadratic broken line model was fitted (Robbins et al., 2006), the MAX REPL was estimated to be 60% of DP. Considering that the performance of red drum was largely unaffected in Trial V wherein ESBM was used to replace 70% of the DP provided by FM in the FM-100 diet, the 60 and 70% MAX REPL values can be taken as a recommended range of DP replacement. In addition, it is important to point out that no differences in performance of red drum fed either an ESBM- or a SBM-based diet replacing 70% of the DP in the FM-100 diet were observed, indicating that in the case of juvenile red drum, either SP can be used. This is potentially due to the fact that red drum may display a higher tolerance to ANF present in SBM than do other carnivorous teleosts such as Atlantic salmon, *Salmo salar*, well-known for being highly sensitive (Francis et al., 2001; Krogdahl et al., 2003). For instance, Atlantic salmon fed a bioprocessed SBM replacing 40% of the FM protein outperformed fish fed diets in which SBM was the alternative protein source (Refstie et al., 1998). Therefore, even though the enzymatic treatment used in the manufacture of ESBM diminishes the content of important ANF in SBM, it may not translate into improved performance in less sensitive fish species.

The Kunitz soybean trypsin inhibitor (TI) reduces the activity of trypsin, and to a lesser extent chymotrypsin, by combining with the enzymes and forming irreversible

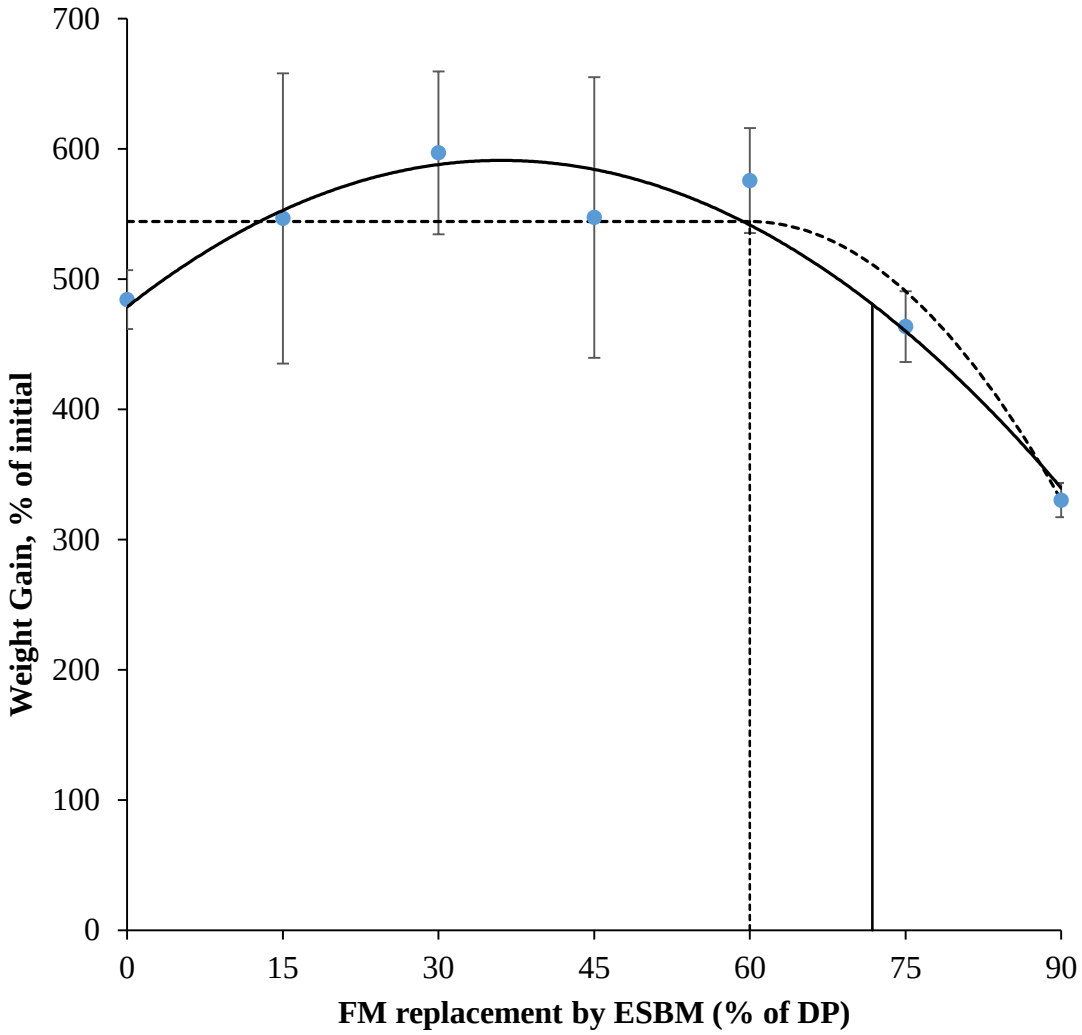

Figure 5 Second order polynomial (solid line, Pr > F = 0.006, Adj R^2 = 0.50) and quadratic broken line (dashed line, Pr > F = 0.025, R^2 = 0.51) regression of weight gain as a function of the gradual replacement of fishmeal (FM) with enzymatically treated soybean meal (ESBM) in the diet of red drum in Trial IV. The maximum replacement value of ESBM for FM was estimated in 60 or 72% of digestible protein by each regression model, respectively. Error bars represent standard error (SE). FM = fishmeal; ESBM = enzymatically treated soybean meal; DP = digestible protein.

stoichiometric compounds whose concentration are directly related to the amount of TI (Kunitz, 1947). On the other hand, a molecule of Bowman soybean TI can inhibit one molecule of trypsin and one molecule of chymotrypsin simultaneously (Birk, 1985). Although heat treatment can effectively reduce TI activity in SBM (Friedman et al., 1991; Wilson and Poe, 1985), not much heat was provided during the cold-pelleting of the experimental diets evaluated in the current study. Thus, assuming that the TI present in the SP evaluated was not deactivated during diet manufacture, its estimated concentration in the diets of Trial IV ranged from 0.14 (ESBM-15) to 0.85 mg g^{-1} (ESBM-90), and 0.7 and 1.7 mg g^{-1} in the ESBM-70 and SBM-70 diets of Trial V, respectively. In either case, the estimated TI level was lower than those found detrimental to the performance of channel catfish, *Ictalurus punctatus* (Wilson and Poe, 1985), or Atlantic salmon (Olli et al., 1994), suggesting that TI concentration was not the primary factor limiting red drum performance fed diets high in SP.

Despite the relative low levels of TI in the experimental diets evaluated in the present study, a significant reduction in trypsin activity was observed in red drum as the level of ESBM increased in the diets of Trial IV. These findings are in agreement with other studies showing a reduction in trypsin activity when soybean proteinase inhibitors were present in the diet of carnivorous teleosts such as rainbow trout, *Oncorhynchus mykiss* (Krogdahl et al., 1994), gilthead sea bream, *Sparus aurata* (Robaina et al., 1995), and Atlantic salmon (Lilleeng et al., 2007). Likewise, a reduction of pepsin activity has been observed in Japanese sea bass, *Lateolabrax japonicus*, fed SBM-based diets replacing 45% or 60% of protein from FM in the control diet (Li et al., 2014). Contrary

to the differences in enzyme activity observed in Trial IV, a significant effect of the ESBM-70 and SBM-70 diets on the activity of these enzymes was not observed in Trial V, which may possibly be attributed to the high variability in the data.

Both β-conglycinin and glycinin are major storage proteins in soybean and are known for being allergenic in animals and humans (Garcia et al., 1997), and their detrimental effects also have been observed in fish (Gu et al., 2014; Rumsey et al., 1994). However, as in the case of TI, the levels of β-conglycinin and glycinin evaluated in those studies are much higher than those estimated for the current study; ranging from < 0.01 mg g^{-1} in all ESBM diets to 5 (β-conglycinin) and 20.5 (glycinin) in the SBM-70 diet. Therefore, considering the similar performance observed for red drum fed either the ESBM-70 or the SBM-70 diet, the extent to which the observed decrease in red drum performance when more than 70% of the DP from FM was replaced by either ESBM or SBM can be attributed to the combined effects of all measured ANF is unclear.

It is also important to note that, even though FM replacement was performed on a DP basis, the MAX REPL of ESBM for FM in terms of protein retention was estimated in 64.2%. Even though replacing 70% of FM with ESBM did not alter protein retention in the Trial V, assuming constant protein digestibility across a range of dietary SP based on the previous digestibility assessment may be incorrect. This is suggested based on the fact that the inclusion level of ESBM in the diets of Trial IV surpassed that used in the digestibility evaluation (30%) beyond the ESBM-45 treatment (see Table 12), from which digestibility may have changed substantially.

In summary, the present study demonstrated that ESBM or SBM can be used in the diet of juvenile red drum replacing the DP provided by FM in a range of 60 – 70% without compromising the overall production performance of the fish.

CHAPTER IV

PRODUCTION PERFORMANCE AND NON-SPECIFIC IMMUNITY OF CAGE-RAISED RED DRUM FED SOYBEAN-BASED DIETS*

IV.1 Introduction

The aquaculture industry has long relied on fishmeal (FM) as the primary protein source in aquafeeds for carnivorous fish species (Tacon and Metian, 2008), but limited supply and increasing prices have prompted the utilization of alternative protein feedstuffs. Soybean meal (SBM) is currently the foremost feedstuff substituting for FM (Tacon et al., 2011; Troell et al., 2014). However, different degrees of tolerance to dietary SBM have been reported in a range of aquacultured fish species (Francis et al., 2001), largely attributed to the presence of anti-nutritional factors (ANF).

Soy-protein concentrate (SPC) is an example of an highly refined soybean product (SP) with relatively higher protein content (NRC, 2011) and lower levels of ANF compared to commodity SBM (Barrows et al., 2008); however, processing costs may limit its inclusion in aquafeeds (Gatlin et al., 2007). On the other hand, the selective breeding of non-genetically modified (non-GM) soybean varieties has led to the introduction of novel SP to the feed industry (Schillinger et al., 2012). Because they contain higher protein content and lower levels of ANF, such novel ingredients may be more cost-effective for use in plant-based diets for aquacultured species.

* Reprinted: Rossi, W., Tomasso, J.R., Gatlin, D. M., 2015. Production performance and non-specific immunity of cage-raised red drum, *Sciaenops ocellatus*, fed soybean-based diets. Aquaculture 443, 84-89. Copyright 2015, with permission from Elsevier Science.

Red drum, *Sciaenops ocellatus*, is a carnivorous and highly prized marine teleost to which plant-based diets have been evaluated (McGoogan and Gatlin, 1997; Moxley et al., 2014; Reigh et al., 1992). These studies have shown that the red drum is able to tolerate relatively high dietary levels of plant feedstuffs without showing detrimental effects on production performance. However, these evaluations were restricted to early stage juveniles. Considering that the response to plant-based diets can be growth-stage specific (Burr et al., 2012), plant-based diets must be evaluated in larger fish.

With the increased utilization of alternative feedstuffs, the application of various feed additives in aquaculture also has increased (NRC, 2011). Particularly in the case of prebiotics, increasing evidence points out their beneficial effects in the diet of aquacultured species, as reviewed in Ringø et al. (2014). Responses of red drum to prebiotic supplementation have included increased apparent nutrient digestibility of SBM-based diets (Burr et al., 2008a), modulation of gut microbiota community (Burr et al., 2008b), and enhanced weight gain, feed efficiency, non-specific immunity, and gut morphology (Buentello et al., 2010). Enhanced gut morphology resulting from prebiotic supplementation in red drum also was evidenced in a later study (Anguiano et al., 2013).

The objective of this study was to evaluate the performance and health status of advanced juvenile red drum in response to the effects of: i) a FM- or SP-based diets; ii) a SBM- or a novel SP-based diet; and iii) a yeast-based prebiotic supplement in a SBM-based diet. The study was conducted in cages suspended in aquaculture ponds to allow the use of larger fish than used in previous studies and incorporate the variable conditions of the pond environment.

IV.2 Material and Methods

IV.2.1 Diets

A 15-week feeding trial (Trial VI) was conducted to evaluate the replacement of FM with SP in the diet of red drum. The SP evaluated included SBM, and Navita Premium Feed Ingredients 3010 (designated as N3010), a SP produced from non-GM, selectively bred soybean varieties. All diets were formulated to contain 35% digestible protein (DP), 12% lipid, and an estimated digestible energy (DE) level of 3.5 kcal g^{-1} (Table 19). Digestible protein was calculated based on a previous assessment in this laboratory (unpublished), while DE was calculated based on the physiological fuel values of 4, 4, and 9 kcal/g for carbohydrate, protein and lipid, respectively. The reference diet (designated REF) was formulated such that 71% of the DP was provided from FM, and the remaining DP (29%) was provided from corn protein concentrate (CPC). Two test diets (designated SBM and N3010) were formulated to replace approximately 72% of the DP from FM in the REF diet with SBM or N3010. An additional inclusion of SPC at 5.7% into each of the test diets resulted in a total of 86% replacement of DP from FM compared to the REF diet. Additionally, in order to evaluate the potential additive effect of a yeast-based prebiotic (GroBiotic®-A; GBA) on the overall performance of red drum, a third test diet (designated SBM+GBA) was formulated identically to the SBM diet with the addition of GBA at 2.0%, at the expense of the same amount of wheat flour. All diets were supplemented with mineral and vitamin premixes to meet or exceed the established requirements for red drum (NRC, 2011), along with the supplementation of taurine (1.0 or 1.5%). Supplementation of

Table 19 Composition of experimental diets[1] fed to advanced juvenile red drum for 15 weeks in Trial VI (Rossi et al., 2015b).

	REF	SBM	SBM+GBA	N3010
Ingredients	*% of dry matter*			
Menhaden fishmeal[a]	44.6	6.0	6.0	6.0
Corn protein concentrate[b]	15.0	15.0	15.0	15.0
Soy protein concentrate[c]		5.7	5.8	5.8
Soybean meal[d]		40.0	40.0	
Navita 3010[e]				35.6
Wheat flour [f]	20.5	7.5	5.5	11.8
Menhaden oil[g]	9.8	13.3	13.3	13.6
Vitamin premix[h,i]	3.0	3.0	3.0	3.0
Mineral premix[h,i]	4.0	4.0	4.0	4.0
Dicalcium phosphate[j]		1.0	1.0	1.0
GroBiotic®-A[k]			2.0	
Glycine[l]	2.0	2.0	2.0	2.0
Taurine[l]	1.0	1.5	1.5	1.5
Lysine HCl[l]		0.5	0.5	0.2
DL-Methionine[l]		0.5	0.5	0.5
Analyzed proximate composition[2]				
Moisture	2.4	2.7	3.1	3.3
Protein	46.6	47.6	46.9	46.9
Lipid	14.7	12.5	12.8	11.6
Ash	11.8	6.5	6.5	6.2
Total phosphorus	1.9	1.0	1.0	1.0
Crude fiber	2.9	2.1	2.0	1.6
Analyzed amino acid composition				

Table 19 continued.

	REF	SBM	SBM+GBA	N3010
Arg	2.3	3.0	2.4	3.0
His	0.9	1.0	1.0	1.1
Ile	1.9	2.3	1.9	2.2
Leu	4.3	5.2	4.3	5.0
Lys	2.4	2.7	2.2	2.4
Met	1.1	1.2	0.9	1.3
Phe	2.2	2.9	2.4	2.9
Tau	1.1	1.7	1.5	1.7
Thr	1.7	1.9	1.5	1.8
Val	2.2	2.6	2.1	2.4

[1] REF = reference; SBM = soybean meal; GBA = GroBiotic®-A;

[2] Dry matter basis (except moisture).

[a] Special Select ™ Omega Protein Inc., Abbeville, LA, USA. Crude protein = 68.4%; Lipid = 10.2% on a dry-matter basis.

[b] Empyreal 75, Cargill Corn Milling (Blair, NE, USA). Crude protein = 81.3%; Lipid =4.2% on a dry-matter basis.

[c] The Solae Company, St. Louis, MO, USA. Crude protein = 71.9%; Lipid = 0.44% on a dry-matter basis.

[d] Producers Coop. Association, Bryan, TX, USA. Crude protein = 53.9%; Lipid = 10% on a dry-matter basis.

[e] Navita Premium Feed Ingredients, West Des Moines, IA, USA. Non-GM. Crude protein = 60.6%, crude lipid = 0.44%, on a dry-matter basis.

[f] Manildra Milling. Crude Protein = 18.4%; Lipid = 1.6% on a dry-matter basis.

[g] Omega Protein, Reedville, VA, USA.

[h] Moon and Gatlin (1991).

[i] MP Biomedicals, Solon, OH, USA

[j] Fisher Scientific, Pittsburg, PA, USA

[k] International Ingredient Corporation, St. Louis, MO, USA.

[l] USB, Cleveland, OH, USA.

DL-methionine and L-lysine was provided to the test diets in excess of the established requirements for methionine (Moon and Gatlin, 1991) and lysine (Craig and Gatlin, 1992) by red drum, while glycine was supplemented at 2.0% for palatability enhancement (McGoogan and Gatlin, 1997).

All diets were manufactured at the U.S. Fish and Wildlife Service, Bozeman Fish Technology Center - Fish Feed Laboratory (Bozeman, MT). The manufacturing procedures, including ingredient grinding, mixing, and extruding, were performed as described by Read et al. (2014). All proximate composition analyses were performed in triplicate using AOAC (1990) procedures. The analyzed composition of the diets fed to advanced red drum juvenile closely matched the targeted formulation values for dietary CP of approximately 45% (35% DP), while lipid was slightly lower (11.6 – 12.8%) in the soybean-based diets (Table 19). The dietary phosphorus ranged from 1.0% in the SBM, SBM+GBA, and N3010 diets to 1.9% in the REF diet and was in excess of the 0.8% established as the requirement for red drum (Davis and Robinson, 1987). Supplementation of DL-methionine and L-lysine was provided to the test diets in excess of the established requirements for red drum (Boren and Gatlin, 1995; Brown et al., 1988; Moon and Gatlin, 1991), while glycine was supplemented at 2.0% for palatability enhancement (McGoogan and Gatlin, 1997).

IV.2.2 Fish and Feeding Trial

Red drum were obtained from the Sea Center Texas Marine Aquarium, Fish Hatchery and Nature Center operated by Texas Parks and Wildlife Department in Lake Jackson, TX, and transported by truck to the Texas A&M Aquacultural Research and

Teaching Facility. In an indoor setting (water temperature = ~ 26° C; dissolved oxygen (DO) = near air saturation; pH = between 7 and 8; salinity = ~ 6 g L^{-1}, and 12h light: 12 h dark photoperiod), the fish were cultured in 6, 1200-L circular, fiberglass tanks operating as a recirculating system until over 60 g of mean weight was attained. During this period, red drum were fed at a rate approaching apparent satiation with a 40% crude protein and 12% crude fat commercial diet (Rangen, Inc., Angleton, TX).

At the commencement Trial VI, a sample of 15 fish was collected from the population and frozen (- 20° C) for subsequent analyses of initial whole-body composition. Thirty red drum juveniles were then stocked into each of 16, 1-m^3 floating-cages divided equally between two, 0.05-ha, rubber-lined ponds. Constant mechanical aeration was provided to each pond and well water was added as necessary to cope with evaporation. In each cage, red drum were fed their assigned diet for a few days until active feeding was observed, then fish in each cage were group weighed for the commencement of Trial VI. Fish [mean initial weight ± standard deviation (SD) = 68 ± 5.1 g] in each cage were fed their assigned diet once daily to apparent satiation during the entire feeding trial, which began on June 14, and ended on September 27, 2013 (15 weeks). Two cages in each pond were assigned either the REF or a test diet, resulting in a generalized randomized block (GRB) design with four replicate cages per dietary treatment.

Average morning temperature was 28.0 ± 1.1 (mean ± SD) and morning DO was 7.1 ± 0.4, with total ammonia-nitrogen averaging 0.4 ± 0.1 mg L^{-1}. Water salinity was increased by the addition of sea salt (Fritz Industries, Dallas, TX, USA) and food-grade

NaCl (Producers Coop. Association, Bryan, TX, USA) at a 1:3 ratio to the well water and was maintained at 5.8 ± 0.26 g L^{-1}.

IV.2.3 Data Acquisition and Analyses

At the end of Trial VI, fish in each cage were group weighed and sampled ~ 24 h after the last feeding. Three representative fish from each cage were euthanized with an overdose of tricaine methanesulfonate (MS-222; Western Chemical, Inc., Ferndale, WA), frozen at – 20° C and subsequently homogenized for proximate analysis to determine crude protein, lipid, moisture, and ash in whole-body tissue (AOAC, 1990). Five additional representative fish from each cage were anesthetized in approximately 2 g L^{-1} MS-222 for blood collection and subsequently euthanized. Approximately 1.0 mL of blood from each fish was collected from the hemal arch in the caudal peduncle using 1-mL syringe with 26-gauge, heparinized needles. A portion of the collected blood was used to determine hematocrit and neutrophil oxidative radical production. Plasma was separated from the remaining blood by centrifugation and frozen for subsequent determination of osmolality, glucose concentration, and lysozyme activity. These various blood and plasma parameters were measured to generally assess homeostasis and health status of the fish.

Neutrophil oxidative radical production was determined spectrophotometrically by the means of nitroblue tetrazolium (NBT) reduction, as described by Siwicki et al. (1994). Osmolality was determined by means of a vapor pressure osmometer (Vapro 5520, Wescor, Inc.; Logan, UT). Glucose concentrations in plasma were determined spectrophotometrically by the glucose-oxidase method (Point Scientific, Inc.; Canton,

MO) and lysozyme activity as described by Engstad et al. (1992). A lysozyme activity unit was defined as the amount of enzyme required to produce a decrease in absorbance of 0.001 min^{-1} at 450 nm. The bled and euthanized fish were then dissected to obtain liver, intraperitoneal fat (IPF), and muscle weights for computing hepatosomatic index (HSI), IPF ratio, and muscle yield values. The gastrointestinal tract (GIT) of these fish also was removed and the intestine fixed with buffered formalin (10%, v/v) for subsequent histological and morphological analysis as previously described by Anguiano et al. (2013). Head-kidney samples were collected for macrophage isolation and assay of extracellular superoxide anion (ECSA) production following the procedures of Sealey and Gatlin (2002). The amount of ECSA was calculated according to Pick and Mizel (1981) using the following formula: nmol of ECSA/well= $[(\Delta_{\text{absorbance after 60 min}} \times 100)/6.3]$.

IV.2.4 Calculations and Statistical Analyses

The fish performance parameters utilized to compare treatments in this study were calculated as follow:

- Weight gain, % = [(Final weight – initial weight)/(initial weight)] × 100;
- Feeding rate, % of body weight per day (BW d^{-1}) = [dry feed intake (g)/($\sqrt{\text{initial body weight} \times \text{final body weight (g)}}$)/days on feed] × 100;
- Feed efficiency ratio (FE) = [weight gain (g)/ dry feed consumed (g)];
- Protein retention efficiency, % = {[(final body weight (g) × final body protein (%)) – (initial body weight (g) × initial body protein (%))]/(protein intake (g)]} x 100;

- Energy retention efficiency, % = {[(final body weight (g) × final body energy (kcal)) – (initial body weight (g) × initial body energy (kcal))]/(energy intake (kcal)} × 100;
- Muscle yield, % = [fillet muscle weight (g)/body weight (g)] × 100;
- Viscerosomatic indices (HSI or IPF ratio), % = [Liver or IPF weight (g)/body weight (g)] × 100.

The normality and homogeneity of variances of the resulting data were assessed by using Shapiro-Wilk and Levene's tests, respectively. In the GRB design, diet represented treatment and pond the blocking factor. When significant ($P < 0.05$) differences were identified, treatment or treatment-group means were compared using orthogonal contrasts. All statistical analyses were performed using the SAS® software package (SAS Institute Inc., Cary, NC USA). Cage means were entered into all analyses.

IV.3 Results

Red drum showed prompt acclimatization to the cages and were feeding readily within a couple days of stocking. The growth, feeding rate, feed efficiency, survival, and muscle yield of red drum after the 15 weeks of feeding revealed no significant differences attributable to the dietary treatments, while significant differences were observed for HSI and IPF ratio (Table 20) and whole body ash (Table 21). Orthogonal contrasts showed that the HSI and IPF ratio of red drum fed the REF diet were significantly lower relative to those fed the SBM or N3010 diet, while whole-body ash content was significantly higher in fish fed the REF diet. In addition, the orthogonal

Table 20 Final weight, weight gain, feed intake, feed efficiency, survival, muscle yield, hepatosomatic index, and intraperitoneal fat ratio of red drum (mean initial weight ± SD = 68 ± 5.1 g) after 15 weeks of feeding the experimental diets in Trial VI (Rossi et al., 2015b).

Treatments[1]	Final weight g	Weight gain %	Feeding rate % BW d^{-1}	FE	Survival %	Muscle yield, %	HSI %	IPF ratio %
REF	245.1	253.4	2.3	0.56	98.3	34.0	1.1	0.11
SBM	247.9	267.4	2.3	0.57	97.5	35.1	1.7	0.29
SBM+GBA	237.8	272.3	2.4	0.56	99.2	34.0	1.6	0.21
N3010	246.7	249.4	2.3	0.53	95.0	34.0	1.7	0.26
PSE	*9.100*	*9.925*	*0.100*	*0.018*	*1.250*	*0.475*	*0.175*	*0.018*
Anova (Pr > F)	*0.655*	*0.298*	*0.720*	*0.452*	*0.222*	*0.330*	*0.009*	*< 0.001*
Contrasts (Pr > t)								
REF – SBM – N3010							*0.001*	*< 0.001*
N3010 – SBM							*0.599*	*0.340*
SBM+GBA – SBM							*0.867*	*0.011*

[1] REF = reference; SBM = soybean meal; GBA = GroBiotic®-A. Values are mean of four replicate cages.
BW d^{-1} = body weight per day; FE = feed efficiency; HSI = hepatosomatic index; IPF = intraperitoneal fat; PSE = pooled standard error.

Table 21 Whole-body proximate composition, protein and energy retention efficiency values of red drum after 15 weeks of feeding the experimental diets in Trial VI (Rossi et al., 2015b).

Treatments[1]	Moisture	Protein	Lipid	Ash	PR	ER
			%			
REF	71.8	17.7	6.5	4.9	27.3	21.2
SBM	71.0	18.7	7.7	3.7	28.0	24.2
SBM+GBA	71.7	17.7	6.9	4.0	25.0	21.0
N3010	71.7	17.9	6.5	4.2	25.1	20.9
PSE	*0.650*	*0.375*	*0.550*	*0.275*	*1.225*	*1.200*
Anova (Pr > F)	*0.816*	*0.097*	*0.535*	*0.019*	*0.245*	*0.872*
Contrasts (Pr > t)						
REF – SBM – N3010				*0.007*		
N3010 – SBM				*0.138*		
SBM+GBA – SBM				*0.506*		

[1] REF = reference; SBM = soybean meal; GBA = GroBiotic®-A.
PR = protein retention; ER = energy retention; PSE = pooled standard error of treatment means (n = 4). Values are means of five fish from each of four replicate cages.

Table 22 Hematocrit, plasma values of osmolality, glucose, and lysozyme; neutrophil oxidative radical production, and extracellular superoxide anion production of head-kidney macrophages of red drum after 15 weeks of feeding the experimental diets in Trial VI (Rossi et al., 2015b).

Treatments[1]	Hematocrit %	Osmolality mosm	Glucose µg mL^{-1}	Lysozyme unit mL^{-1}	NBT test mg mL^{-1}	ECSA nmol O_2^-
REF	27.3	347.8	52.8	241.7	9.9	2.2
SBM	27.0	359.3	48.5	242.2	9.5	2.7
SBM+GBA	27.8	355.0	52.0	262.1	9.7	2.3
N3010	24.8	354.0	50.8	242.0	9.3	2.8
PSE	*1.900*	*2.525*	*3.075*	*12.125*	*0.525*	*0.325*
Anova (Pr > F)	*0.737*	*0.920*	*0.805*	*0.656*	*0.891*	*0.577*

[1] REF = reference; SBM = soybean meal; GBA = GroBiotic®-A.

NBT test = neutrophil oxidative radical production; ECSA = extracellular superoxide anion; PSE = pooled standard error of treatment means (n = 4). Values are mean of five fish from each of four replicate cages.

Table 23 Histological parameters of intestinal cross-sections of red drum after 15 weeks of feeding the experimental diets in Trial VI (Rossi et al., 2015b).

	Treatments[1]				*PSE*	*Pr > F*
	REF	SBM	SBM+ GBA	N3010		
Proximal Intestine	*µm*					
Fold height	1273.7	1264.4	1250.9	1386.7	*77.625*	*0.530*
Total enterocyte height	36.2	36.6	35.6	34.8	*0.700*	*0.366*
Microvilli height	2.3	2.6	2.4	2.3	*0.125*	*0.107*
Mid intestine						
Fold height	808.5	772.6	754.6	775.4	*60.875*	*0.950*
Total enterocyte height	32.6	30.4	33.3	30.1	*2.450*	*0.852*
Microvilli height	2.0	2.3	2.2	2.1	*0.100*	*0.101*
Distal intestine						
Fold height	664.9	583.1	608.1	531.5	*38.15*	*0.355*
Total enterocyte height	29.0	27.6	27.2	31.5	*2.850*	*0.755*
Microvilli height	1.8	1.7	1.7	1.8	*0.100*	*0.312*

[1] REF = reference; SBM = soybean meal; GBA = GroBiotic®-A. PSE = pooled standard error of treatment means (n = 4).Values are means of ten observations of three fish from each of four replicate cages.

contrast on the SBM+GBA versus SBM treatments revealed a significant reduction in IPF ratio of red drum fed the former.

Hematocrit, plasma osmolality, plasma glucose concentration, plasma lysozyme activity, NBT test, and ECSA production of head-kidney macrophages did not differ significantly among treatments (Table 22). In addition, no significant differences in the micromorphology (fold height, enterocyte height, and microvilli height) of anterior, middle, or posterior sections of the red drum intestine were found among treatments (Table 23). This showed there were no apparent alterations in the intestinal structure of red drum fed diets predominantly composed of SP for an extended period.

IV.4 Discussion

The results of the present study indicate that both SBM and N3010 can partially substitute for FM in the diet of advanced juvenile red drum raised in cages. Overall, the production performance and health status of red drum were unaffected by the high dietary levels of SBM or N3010 in combination with SPC, replacing 86% of the DP from FM in the REF diet.

In the previous study (Chapter II, Trial I), red drum fed a diet containing CPC at 24.2% showed a slight reduction in weight gain, but other performance parameters including feed efficiency, muscle yield, protein and energy retention were unaffected. Thus, the utilization of CPC at 15.0% in the REF diet was deemed adequate in this study. In addition, resulting from its higher methionine content relative to SP, CPC is a

complementary ingredient that can lessen the need for supplemental methionine in soybean-based formulations.

As expected, the 15 weeks of feeding in Trial VI were not long enough for red drum to attain a typical market size (0.5 – 1.0 kg), but it was long enough to validate the nutritional value of the experimental diets as a minimum of 3-fold increase in size was observed in all treatments, which is acceptable for nutrition studies using advanced juveniles (NRC, 2011). A longer time period was not possible as colder water temperatures in the fall months would limit fish growth.

Advanced juvenile red drum appear to exhibit a higher tolerance to soybean-based diets than younger ones. In our previous study (Chapter II, Trial I), red drum (1.5 g initial weight) fed a diet in which 50% of the FM was replaced with SPC outperformed fish fed the FM control diet, but when SP (including SBM, SPC, or N3010) replaced 88% of FM in the diet of similar size fish (3.3 g initial weight), poor performance was observed (Chapter II, Trial II). Similar results were reported in a later study (Moxley et al., 2014) in which a reduction in red drum performance was observed when SPC substitution for FM was increased from 50 to 75%. In contrast, the performance of advanced juveniles (68 g initial weight) fed soybean-based diets was unaffected in this study. Different responses to plant-based diets at different life stages have been reported in Atlantic salmon, *Salmo salar* (Burr et al., 2012). Reduced performance of young juvenile Atlantic salmon (5.5 g initial weight) was observed when dietary FM was completely replaced with a plant-protein blend, but no differences in performance were observed in later stage juveniles (31.5 g initial weight). These results highlight the

importance of evaluating alternative ingredients for FM at different growth stages to optimize the use of plant-based formulations.

Previous research demonstrated that feeding rates of 4 and 6% of body weight per day (BW d^{-1}) were necessary to maximize feed efficiency and final weight of red drum juveniles, respectively (McGoogan and Gatlin, 1998). Such values are substantially higher than the 2.3 – 2.4% of BW d^{-1} observed in this study with larger fish. However, the observed feeding rate was only 20% lower than that observed by Thoman et al. (1999) when red drum (50 g initial weight) were fed a 44% CP and 13% lipid diet twice daily to apparent satiation at similar water temperature (mean ± SD = 28.8° ± 1.4° C). Therefore, although only one daily ration was administered in the current study, the observed feeding rate is within the range previously reported for advanced juvenile red drum.

As observed in our previous study (Chapter II, Trial II), a significant increase in HSI and IPF ratio was observed in red drum fed most of the soy-based diets. Such findings are in contrast with results from other studies showing a significant reduction in HSI and/or IPF ratio in response to SBM substitution for FM in red drum (McGoogan and Gatlin, 1997) and other carnivorous fish species (Kaushik et al., 1995; Zhang et al., 2014). With regard to fat deposition, red drum have been shown to be highly responsive to dietary lipid (Craig et al., 1999). In addition, both the HSI and IPF ratio of red drum has been shown to increase in response to a greater intake of digestible energy (McGoogan and Gatlin, 1998). Thus, considering that DE was probably higher in the REF diet due to its higher lipid concentration (14.8 to 27.0% higher than the soybean-

based diets), but no differences in feeding rate was found, the observed differences in HSI and IPF ratio might not be attributed to the differences in dietary lipid. As detailed information on specific-tissue composition and/or nutrient metabolism are lacking, the potential causes for the observed results remain unclear.

Although some studies have reported significant effects of dietary SBM or other plant-feedstuffs on the hematological characteristics and/or plasma constituents of fish (Gómez-Requeni et al., 2004; Hansen et al., 2007; Kikuchi, 1999b; Zhou et al., 2005), results of the present study with red drum for hematocrit, osmolality, and glucose are in agreement with the findings from other studies showing no significant effects (Laporte and Trushenski, 2012; Lim et al., 2004). Likewise, the overall health status of red drum, as indicated by the non-specific immunological responses evaluated, was unaffected by the dietary treatments. Responses in lysozyme activity observed in this study are in line with previous studies evaluating plant-based diets (Güroy et al., 2013; Kokou et al., 2012; Sitjà-Bobadilla et al., 2005), but the lack of effects on NBT and ECSA disagrees with the observed increase in the activity of neutrophil and macrophages in response to soybean-based diets in the study of Rumsey et al. (1994). These authors suggested a potential inflammatory or hypersensitivity response of experimental rainbow trout, *Oncorhynchus mykiss,* to dietary SBM or SPC. Salmonids are well known for their relatively high sensitivity to dietary SP (Olli et al., 1995; Rumsey et al., 1994), whereas increasing evidence suggests red drum is relatively more tolerant (McGoogan and Gatlin, 1997; Moxley et al., 2014; Reigh and Ellis, 1992). Therefore, species-specific

sensitivity to SP may potentially explain the observed differences in non-specific immune responses.

Beneficial effects from prebiotic supplementation in fish diets have been demonstrated in an ever-increasing number of studies (Ganguly et al., 2013; Ringø et al., 2010; Ringø et al., 2014). In red drum, effects of prebiotic supplementation have spanned from increased nutrient and energy digestibility of a SBM-based diet (Burr et al., 2008a) to improved weight gain, feed efficiency, and disease protection (Buentello et al., 2010; Zhou et al., 2010). In the study by Buentello et al. (2010), wherein red drum were fed diets in which FM and SBM provided equal amounts of dietary CP, the supplementation of GBA at 1.0% resulted in improved feed efficiency and survival after a parasitic challenge. Although the implementation of a disease challenge may have provided a better evaluation of GBA supplementation to the SBM-based diet, there were no effects resulting from GBA supplementation in any of the various response variables measured in Trial VI. Additionally, the benefits of prebiotic supplementation to fish generally have been more apparent when culture conditions were less than optimal (Gatlin and Peredo, 2012), which did not appear to be the case in this study based on the growth performance and survival of red drum. The relevance of factors such as size of experimental fish, culture environment, and dietary level of SP on the effectiveness of GBA supplementation still remains unclear.

As with other carnivorous fish species including Atlantic cod, *Gadus morhua* (Refstie et al., 2006), cobia, *Rachycentron canadum* (Watson et al., 2014), and hybrid striped bass, *Morone chrysops* × *M. saxatilis* (Rossi et al., 2015a) the soybean-based

diets evaluated in the current study did not promote any adverse effects on the integrity of red drums' intestine, which is in close agreement with a previous report of only minor alterations (Zhou et al., 2010). Such evidences contrast with what has been found in salmonids (Ingh et al., 1996; Merrifield et al., 2009; Van den Ingh et al., 1991), but corroborates to the evidence of red drum's higher tolerance to SP.

The N3010 meal evaluated in Trials II and VI is produced from non-GM varieties of soybean that are selectively bred for relatively higher CP and lower ANF content than in SBM. Despite the limited information about the nutritional value of novel SP, recent studies conducted with fish (Buentello et al., 2015; Suarez et al., 2013; Watson et al., 2014) and Pacific white shrimp, *Litopenaeus vannamei* (Zhou et al., 2014), have reported promising results. Nevertheless, the inclusion of N3010 meal in place of SBM in the diet of red drum has shown non-significant improvements. Again, such findings might result from the lower sensitivity of red drum to the ANF present in SBM.

In conclusion, the combination of SBM or N3010 with SPC was effective at reducing FM dietary component from 44.6 to 6.0%, while supporting similar production performance and health status of advanced juvenile red drum raised in floating cages.

CHAPTER V

EFFECTS OF PLANT-PROTEIN-BASED DIETS ON THE GUT MICROBIOTA COMPOSITION OF RED DRUM: A PRELIMINARY ASSESSMENT

V.1 Introduction

Soybean meal (SBM) and soy protein concentrate (SPC) are high quality plant-protein (PP) feedstuffs with increased utilization in aquafeeds for both omnivorous and carnivorous fish species (Barrows et al., 2008; Tacon et al., 2011; Troell et al., 2014). However, changes in diet formulation have been shown to affect the gut microbiota composition of fish (Dimitroglou et al., 2010; Ingerslev et al., 2014), which in turn may affect the homeostasis of the highly important gut ecosystem and directly affect the productivity and health of these animals.

Important roles of the gut microbiota are continuously being characterized (Prakash et al., 2011). These characterizations include effects on metabolism (such as the synthesis of amino acids, vitamins, and short-chain fatty acids), protection (such as the development and activation of innate and adaptive immune system), structure and morphology (epithelial cell growth and differentiation, microvilli and crypt development). These important functions attributed to the gut microbiota also have been observed in fish as reviewed by Nayak (2010).

Studies dealing with the manipulation of the gut microbiota of fish have been intensified in recent years. Increased regulations upon the use of antibiotics and the need to assure biosecurity in the global aquaculture industry are probably the main reasons

underlying such a trend. Most studies have evaluated feed additives, particularly probiotics and prebiotics, as potential alternatives for beneficial modulation of the gut microbiota (Ringo et al., 2014). Particularly in the case of red drum, *Sciaenops ocellatus*, the utilization of prebiotics have shown to increase the apparent nutrient digestibility of a SBM-based diet (Burr et al., 2008a), modulate the composition of gut microbiota (Burr et al., 2008b), as well as to enhance weight gain, feed efficiency, non-specific immunity, and gut morphology of experimental fish (Anguiano et al., 2013; Buentello et al., 2010). These findings point to the potential relevance of supplementing prebiotics in the diet of red drum as demonstrated in other aquaculture species (Ringo et al., 2014). However, up to this point, a complete characterization of the gut microbiota composition of red drum fed diets differing with respect to primary protein ingredients (FM or plant feedstuffs) and/or prebiotic supplementation is lacking, thereby limiting the understanding of the mechanisms underlying such responses.

In view of the current trend of increased utilization of PP feedstuffs in aquafeeds and the compounding evidence of the importance of prebiotics in the nutrition of red drum, the objective of this study was to characterize the gut microbiota composition of red drum in response to soybean product (SP) -based diets and prebiotic supplementation.

V.2 Material and Methods

V.2.1 Diets

Two feeding trials (VII and VIII) were conducted to assess the effect of SP-based diets on the overall production performance and gut microbiota composition of red drum. The diets used in Trial VII were the same as utilized in the study by Rossi et al. (2015) and those used in Trial VIII were the same as utilized in Trial VI (Chapter IV). Briefly, all diets were formulated to contain 45% CP, 12% lipid, and an estimated digestible energy (DE) level of 3.1 kcal g^{-1} (Table 24). Digestible energy was calculated based on the physiological fuel values of 4, 4, and 9 kcal/g for carbohydrate, protein and lipid, respectively. In both feeding trials, a combination of SBM and SPC was used to replace most of the FM in the reference (designated REF) diet, reducing the FM component in the first test diet (designated SBM) from 57.1 or 46.6% to 7 or 6%, respectively. A second test diet (designated SBM+GBA) was formulated identically to the SBM diet except for the supplementation of 2% yeast-based prebiotic (GroBiotic® - A, GBA; International Ingredient Corporation, St. Louis, MO, USA), in place of a similar amount of wheat flour, and was used to evaluate the potential additive effect of this prebiotic on the overall performance and gut-microbiota composition of red drum fed a SP-based diet.

Table 24 Composition of experimental diets[1] fed to juvenile red drum for 16 and 15 weeks in Trials VII and VIII, respectively.

	Trial VII			Trial VIII		
	REF	SBM	SBM+GBA	REF	SBM	SBM+GBA
Ingredients	*% of dry matter*					
Menhaden fishmeal[a]	57.1	7.0	7.0	44.6	6.0	6.0
Corn protein concentrate[k]				15.0	15.0	15.0
Soy protein concentrate[b]		10.0	10.0		5.7	5.8
Soybean meal[c]		54.4	54.4		40.0	40.0
Wheat flour[d]	11.1	10.1	8.1	20.5	7.5	5.5
Menhaden oil[e]	3.4	7.4	7.4	9.8	13.3	13.3
Vitamin premix[f,g]	3.0	3.0	3.0	3.0	3.0	3.0
Mineral premix[f,g]	4.0	4.0	4.0	4.0	4.0	4.0
Dicalcium phosphate[h]		1.0	1.0		1.0	1.0
GroBiotic®-A[i]			2.0			2.0
Glycine[j]	1.0	1.0	1.0	2.0	2.0	2.0
Taurine[j]	1.0	1.0	1.0	1.0	1.5	1.5
Lysine HCl[j]		0.4	0.4		0.5	0.5
DL-Methionine[j]		0.8	0.8		0.5	0.5
Celufil[j]	19.4					

Table 24 continued.

	REF	SBM	SBM+GBA	REF	SBM	SBM+GBA
Analyzed proximate composition[2]						
Moisture	4.0	4.7	3.8	2.4	2.7	3.1
Protein	44.4	45.0	44.4	46.6	47.6	46.9
Lipid	12.2	12.1	12.0	14.7	12.5	12.8
Ash	13.1	7.3	7.2	11.8	6.5	6.5
Total phosphorus	2.2	0.9	0.9	1.9	1.0	1.0
Crude fiber				2.9	2.1	2.0
Analyzed amino acid composition						
Arg	2.5	3.6	3.0	2.3	3.0	2.4
His	1.0	1.3	1.1	0.9	1.0	1.0
Ile	1.8	2.3	1.9	1.9	2.3	1.9
Leu	3.5	4.4	3.5	4.3	5.2	4.3
Lys	3.2	3.7	3.4	2.4	2.7	2.2
Met	1.3	1.4	1.1	1.1	1.2	0.9
Phe	2.0	2.9	2.4	2.2	2.9	2.4
Tau	1.4	1.3	1.1	1.1	1.7	1.5
Thr	2.0	2.3	1.9	1.7	1.9	1.5

Table 24 continued.

	REF	SBM	SBM+GBA	REF	SBM	SBM+GBA
Val	2.2	2.5	2.1	2.2	2.6	2.1

[1] REF = reference; SBM = soybean meal; GBA = GroBiotic®-A.

[a – j] Rossi et al. (2015a).

[k] Empyreal 75, Cargill Corn Milling, Blair, Nebraska, USA. As dry: Crude protein = 82.7%; Lipid = 11.4%.

V.2.2 Fish and Feeding Trials

Two different batches of red drum were obtained from the Sea Center Texas Marine Aquarium, Fish Hatchery and Nature Center operated by Texas Parks and Wildlife Department in Lake Jackson, TX, and transported by truck to the Aquacultural Research and Teaching Facility of Texas A&M University. Upon arriving at the facility, the fish were acclimated to local water conditions and stocked into 4, 500-L circular-fiberglass tanks operating as a recirculating system. The fish remained in this system (water temperature = ~ 26° C; dissolved oxygen (DO) = near air saturation; pH = between 7 and 8; salinity = ~ 7 g/L, and 12h light:12 h dark photoperiod) until adequate size for the feeding trials was achieved. During this period, red drum were fed at a rate approaching apparent satiation with a 40% CP and 12% crude fat commercial diet (Rangen, Inc., Angleton, TX).

For Trial VII, 90 red drum juveniles (mean initial weight ± standard deviation (SD) = 4.13 ± 0.11 g) were stocked in each of nine, 1200-L circular-fiberglass tanks operating as a recirculating system. A 1-week conditioning period was given prior to the commencement of the feeding trial. Each experimental diet was randomly assigned to three replicate tanks (n=3). Fish in each replicate tank were fed twice daily to satiation for 16 weeks. Satiation was assumed as the maximum amount of feed consumed within 30 min from the initiation of feeding. Water quality was maintained within acceptable ranges for red drum. When necessary, partial water exchanges were performed to maintain total ammonia-nitrogen (TAN) and nitrite-nitrogen below 1.0 mg L^{-1} and 0.1 mg L^{-1}, respectively. Supplemental aeration was supplied to culture tanks by a

regenerative air blower and air stones maintaining dissolved oxygen (DO) near air saturation. The pH was maintained between 7 and 8 with sporadic additions of sodium bicarbonate. Synthetic sea water was prepared using sea salt (Fritz Industries, Dallas, TX, USA) and food-grade NaCl (Producers Coop. Association, Bryan, TX, USA) to provide culture water of 5-7 g L^{-1} salinity. Water temperature was maintained at 26 ± 2°C by conditioning ambient air. A 12 h light:12 h dark photoperiod was maintained using fluorescent lighting controlled by timers.

For detailed information on experimental fish and procedures utilized in Trial VIII, see Chapter IV.

V.2.3 Data Acquisition and Analyses

A few days prior to the termination of each feeding trial, red drum in each replicate tank or cage were fed their assigned experimental diets in the morning, and 6 hours later, samples of intestinal contents were collected. In order to synchronize the time of sample collection between experimental units, the feeding was staggered by 10-min intervals between fish groups. The sample collection for molecular analysis of gut-microbiota composition was performed as described by Burr et al. (2009). Three representative fish from each tank or cage were collected and euthanized with an overdose of tricaine methanesulfonate (MS-222; Western Chemical, Inc., Ferndale, WA). After individually weighing and externally disinfecting with ethanol, each fish had its gastrointestinal tract (GIT) excised and the intestinal contents squeezed into a sterile microfuge tube. All intestinal samples were immediately flash-frozen in liquid nitrogen and then stored under -80° C until microbial analyses were performed.

At the end of each feeding trial, red drum in each tank or cage were group-weighed 24 h after the last feeding for the computation of production performance parameters and body condition indices. Weight gain (percent of initial) = [(Final weight – initial weight)/(initial weight)] × 100; and feed efficiency ratio (FE) = [weight gain (g)/ dry feed consumed (g)] of red drum in Trial I were computed. Detailed information on the growth performance of red drum in Trial II can be seen in Chapter IV.

V.2.4 DNA Isolation and PCR

Triplicate samples of intestinal contents from each dietary treatment were thawed and pelleted by short centrifuging at 5,000 × *g* for 5 min. Approximately 0.7 g of pelleted intestinal contents in each replicate was placed in a sterile microfuge tube for genomic DNA isolation. The pellet was initially suspended in 180 µL lysis buffer (20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1.2% Triton-100; 20 mg lysozyme mL^{-1} (Sigma)) and incubated for 30 min at 37° C. The following steps for genomic DNA isolation were conducted using a QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA) and according to the manufacturer's instructions. Once isolated, DNA was amplified through PCR as described by Hume et al. (2003), using bacteria-specific PCR primers to conserved regions of the variable V3 region of 16S rDNA.

V.2.5 Denaturing Gradient Gel Electrophoresis and MiSeq Sequencing

Denaturing Gradient Gel Electrophoresis (DGGE) was performed as described by Hume et al. (2003) using polyacrylamide gels (8% v/v, acrylamide-bisacrylamide (BioRad Laboratories, Richmond, CA) ratio 37.5:1)). Electrophoresis was performed for

17 h at 60 V using a DCode Universal Mutation Detection System (BioRad). Subsequently, gels were stained with SYBR Green I (1:10,000 dilution; Sigma) and digitalized. Fragment pattern relatedness was determined with Molecular Analysis Fingerprint Software, version 1.6 (BioRad, Hercules, CA, USA), based on Dice similarity coefficient and unweighed pair group method using arithmetic averages (UPGMA) for clustering.

Isolated genomic DNA from pooled intestinal samples per treatment was subjected to sequencing of the 16S rDNA for microbial identification and diversity. The sequencing was performed by an external laboratory (Research and Testing Laboratory, Lubbock, TX, USA) using a MiSeq sequencer.

V.2.6 Statistical Analyses

For both Trials VII and VIII, production performance data were analyzed for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test), then were subjected to the analysis of variance (ANOVA). In Trial VIII, pond was included as the blocking factor according to the generalized randomized block (GRB) design used in Trial VI (Chapter IV). When significant ($P < 0.05$) differences were identified, treatment means were compared using Tukey's HSD test. The ANOVA was performed using the SAS® software package (SAS Institute Inc., Cary, NC USA). Fragment analysis pattern relatedness of DGGE results was determined using Molecular Analysis Fingerprinting software (v 1.6; Bio-Rad, Hercules, CA, USA), using Dice's similarity coefficient (SC, %) for comparison between treatment band patterns. Finally, principal component analyses (PCA) were performed for the microbial diversity data from MiSeq sequencing

using XLSTAT Statistical Software for Excel (v 2015; Addinsoft, New York, NY, USA).

V.3 Results

The weight gain of red drum fed the SBM and SBM+GBA in Trial VII was significantly lower relative to the fish fed the REF diet, while reduced FE was only observed in fish fed SBM+GBA diet (Fig. 6). In Trial VIII, the weight gain and FE of red drum were unaffected by the dietary treatments (see Chapter IV).

According to the DGGE dendrograms presented in Fig. 7, the gut microbiota population differed (SC < 80%) among the three dietary treatments in Trial VII. In Trial VIII, the gut microbiota composition of red drum was identical (SC > 95%) in the SBM and SBM+GBA treatments and differed (SC < 80%) relative to the REF treatment.

In Trial VII, a total of eleven phyla were found (Table 25), six of which were present in all treatments (Acidobacteria, Actinobacteria, Bacteriodetes, Cyanobacteria, Firmicutes Proteobacteria), with Protebacteria being the most abundant across treatments. A total of 121 genera were found (data not shown), forty-six of which were present at a relative abundance ≥ 1% in at least one treatment, with *Methylobacterium* and *Pseudomonas* being the most abundant genera in the REF and both the SBM and SBM+GBA treatments, respectively. Sixteen genera were present in all treatments (*Alcaligenes, Pseudonomas, Clostridium, Methylobacterium, Propionobacterium, Staphylococcus, Geobacter, Acidobacterium, Peptoniphilus, Comamonas, Rhodococcus, Sphingomonas, Eubacterium, Hyphomicrobium, Nostoc,* and *Desulfobulbus*) with

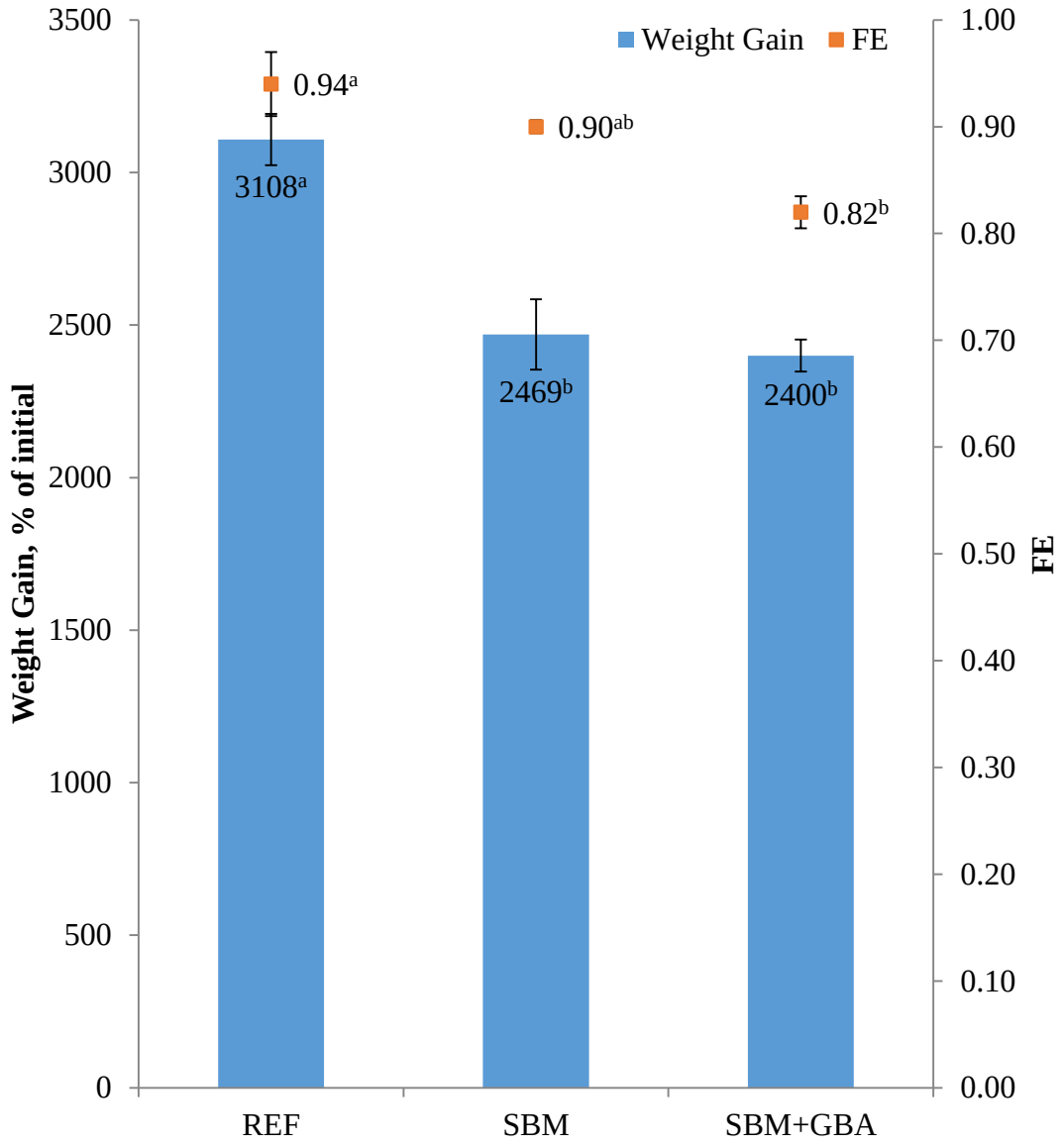

Figure 6 Weight gain (%) and feed efficiency ratio (FE) of red drum fed the experimental diets for 16 weeks in Trial VII. REF = reference; SBM = soybean meal; GBA = GroBiotic®-A. Error bars represent mean ± standard error (SE).

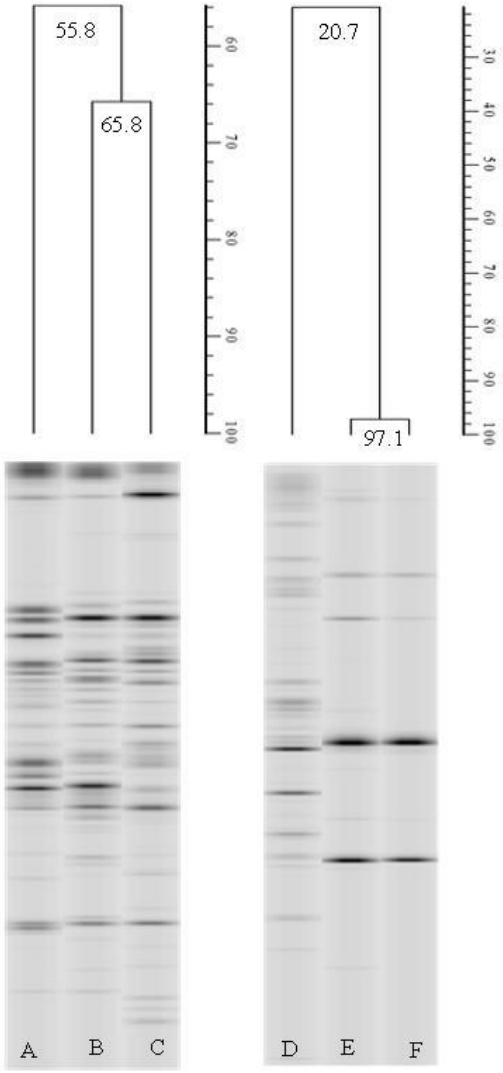

Figure 7 Dendrogram of gut microbiota of red drum fed the experimental diets in Trial VII (A = SBM, B = SBM+GBA, C = REF) and Trial VIII (D = REF, E = SBM, F = SBM+GBA) with Dice's similarity coefficients (SC). SC > 95% = identical populations; 80 ≤ SC ≤ 95% = similar populations; SC < 80% = different populations (SBM = soybean meal, GBA = GroBiotic®-A, REF = reference).

Table 25 Phylum composition of gut bacteria in red drum fed the experimental diets[1] in Trials VII and VIII.

Phylum	Trial VII			Trial VIII		
	REF	SBM	SBM+GBA	REF	SBM	SBM+GBA
	relative abundance %					
Acidobacteria	5.4	2.9	2.0			
Actinobacteria	11.1	8.1	10.1	25.7	0.6	0.7
Bacteroidetes	9.4	2.4	2.4	3.8		
Chloroflexi		1.0	2.5			
Cyanobacteria	6.6	2.5	2.7	12.8	97.2	94.2
Fibrobacteres		1.1	1.2			
Firmicutes	9.3	20.5	17.6	12.8		0.3
Fusobacteria			0.1			0.3
Planctomycetes	0.8				0.2	0.1
Proteobacteria	57.0	61.5	60.7	44.8	2.0	4.4
Spirochaetes	0.3		0.6			

[1] REF = reference; SBM = soybean meal; GBA = GroBiotic®-A.

Methylobacterium being the most abundant in the REF and *Pseudomonas* the most abundant in the SBM and SBM+GBA treatments. Seven genera were present at a relative abundance ≥ 1% in all treatments: *Pseudomonas, Geobacter, Alcaligenes, Clostridium, Staphylococcus, Propionibacterium,* and *Acidobacterium* (Fig. 8). Within this group, *Alcaligenes* was the most abundant genus in the REF treatment, while *Pseudomonas* was the most abundant genus in the SBM and SBM+GBA treatments. A total of 168 bacteria species were found (data not shown), 48 of which were present at a relative abundance ≥ 1% in at least one treatment, with *Methylobacterium jeotgali, Alcaligenes* sp., and *Pseudomonas aeruginosa* being the most abundant in the REF, SBM, and SBM+GBA treatments, respectively. Thirteen species were present in all treatments with *Alcaligenes* sp. and *Pseudomonas aeruginosa* present at the highest relative abundance in the REF and both the SBM and SBM+GBA treatments, respectively (data not shown). Within this group, seven species were found at a relative abundance ≥ 1% in all treatments: *Pseudomonas aeruginosa, Geobacter* sp., *Alcaligenes* sp, *Clostridium* sp., *Staphylococcus arlettae*, *Propionibacterium acnes*, and *Acidobacterium* sp. *Alcaligenes* sp. were the most abundant species in the REF and SBM treatments, while *Pseudomonas aeruginosa* was the most abundant species in the SBM+GBA treatment (Fig. 9). The distance bi-plot for genera within the phylum Proteobacteria (Fig. 10), the most abundant (> 50% relative abundance), and for the species (Fig. 11) found at a relative abundance ≥ 1% for at least one treatment in Trial VII are displayed with the ordination of each treatment according to their respective scores from the PCA analysis. The total variability (100%) in both datasets was

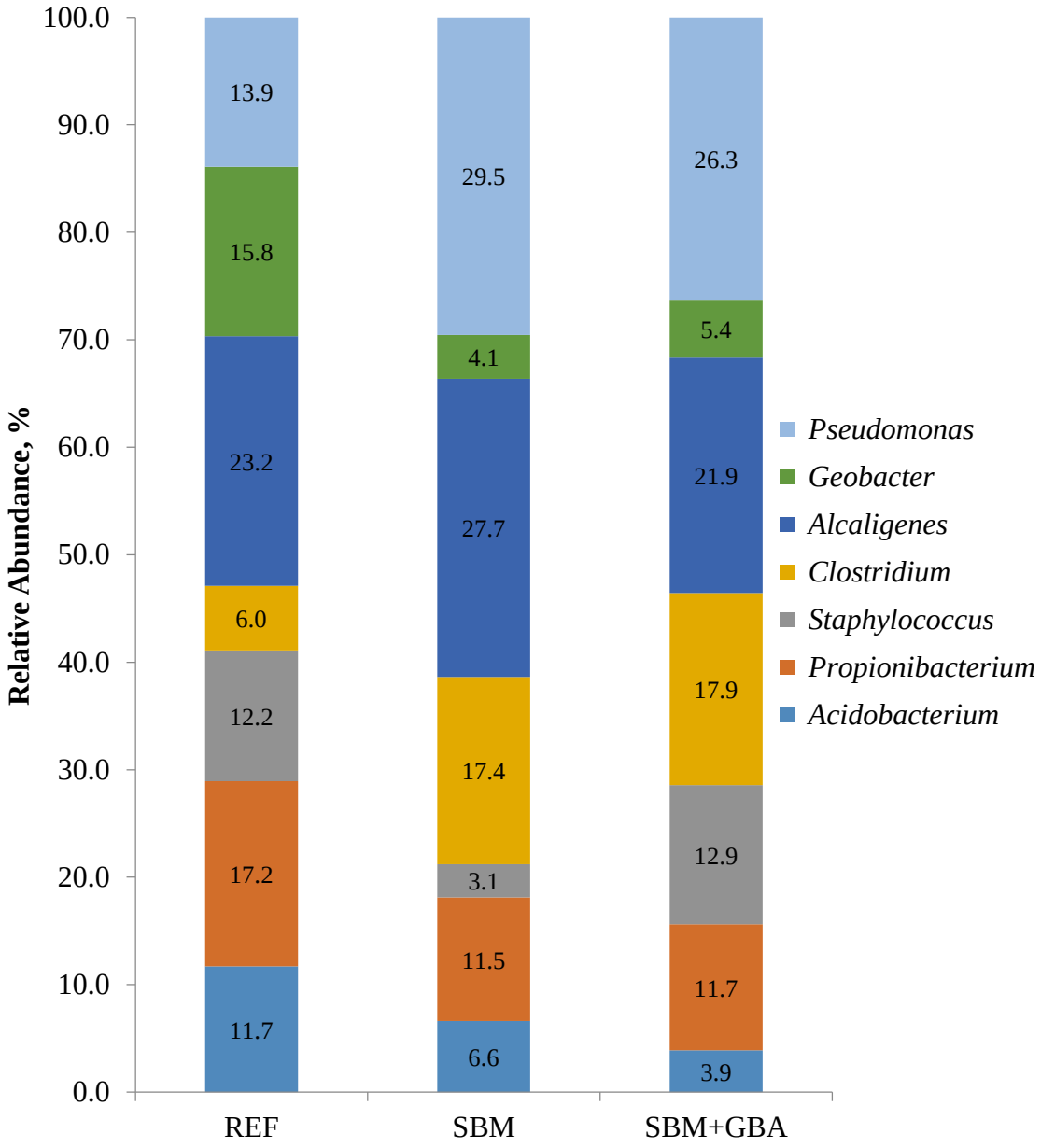

Figure 8 Genera composition (relative abundance ≥ 1% for all treatments) of red drum's gut microbiota after 16 weeks of feeding the experimental diets in Trial VII. REF = reference; SBM = soybean meal; GBA = GroBiotic®-A.

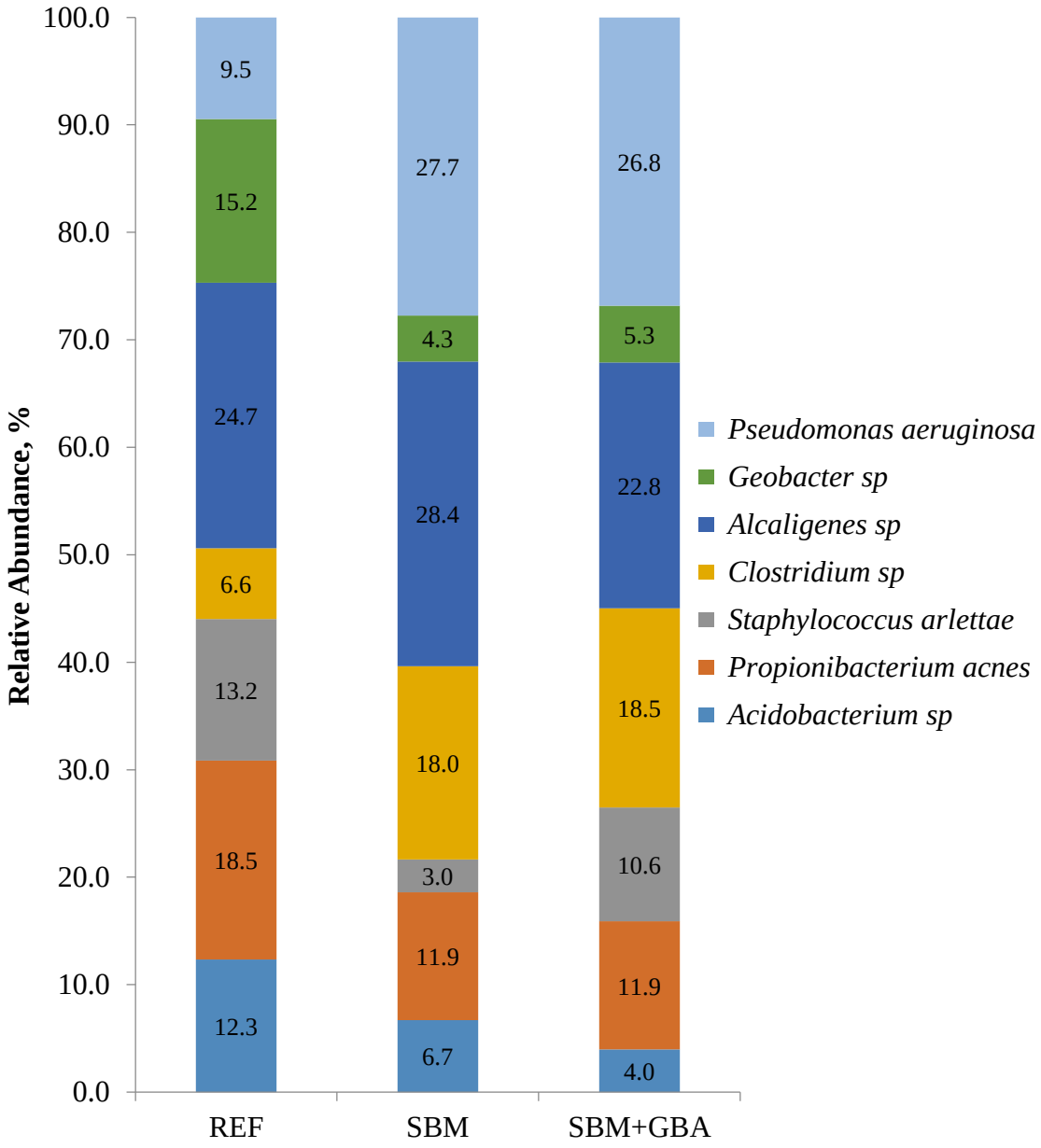

Figure 9 Species composition (relative abundance ≥ 1% for all treatments) of red drum's gut microbiota after 15 weeks of feeding the experimental diets in Trial VII. REF = reference; SBM = soybean meal; GBA = GroBiotic®-A.

explained by two principal components (PC) and the bi-plot showed a distinct ordination of each treatment.

In Trial VIII, a total of six phyla were found, three of which present in all treatments (Actinobacteria, Cyanobacteria, and Proteobacteria), with Proteobacteria being the most abundant in the REF and Cyanobacteria the most abundant in the SBM and SBM+GBA treatments (Table 25). A total of 31 genera were found (data not shown), 19 of which were present at a relative abundance ≥ 1% in at least one treatment, with *Phenylobacterium* being the most abundant in the REF and *Cyanobium* the most abundant in the SBM and SBM+GBA treatments (Fig. 12). Three genera were present in all treatments (*Propionibacterium*, *Streptomyces*, and *Cyanobium*), with *Propionibacterium* and *Cyanobium* being the most abundant in the REF and both the SBM and SBM+GBA treatments, respectively. Within this group, only *Cyanobium* was present at a relative abundance ≥ 1% in all treatments. A total of 57 species of bacteria were found with *Phenylobacterium sp*. and *Cyanobium sp*. being the most abundant in the REF and both the SBM and SBM+GBA treatments, respectively (data not shown). Within this group, 19 were present at a relative abundance ≥ 1% in at least one treatment, with *Phenylobacterium sp*. being the most abundant species in the REF treatment and *Cyanobium sp.* the most abundant species in the SBM and SBM+GBA treatments (Fig. 13). Within this group, only *Cyanobium sp*. was present in all treatments at a relative abundance ≥ 1% (8.6, 98.1 and 96.4% in the REF, SBM, and SBM+GBA treatments, respectively.

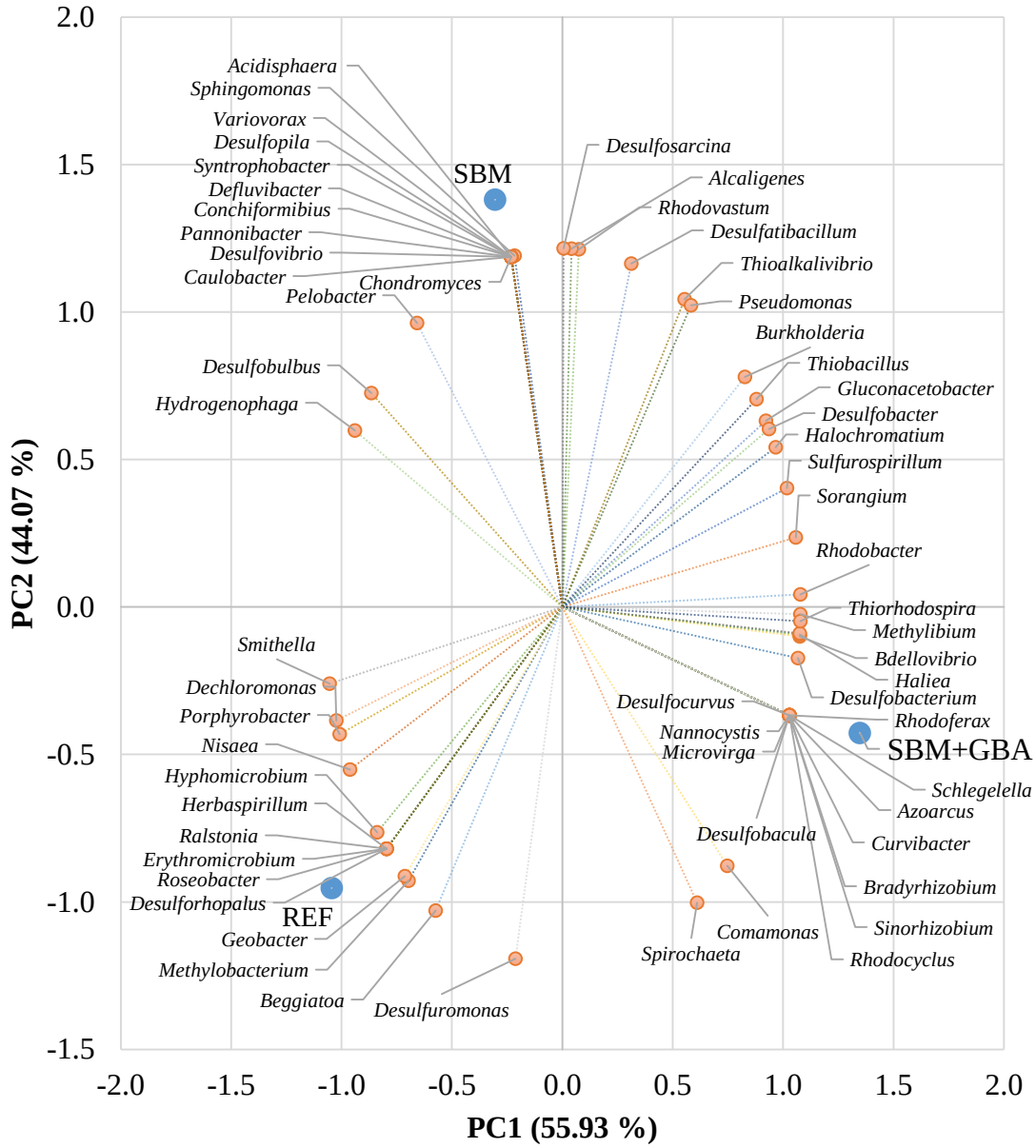

Figure 10 Distance bi-plot for genera within the phylum proteobacteria of red drum gut microbiota in Trial VII. PC = principal component; REF = reference; SBM = soybean meal; GBA = GroBiotic®-A.

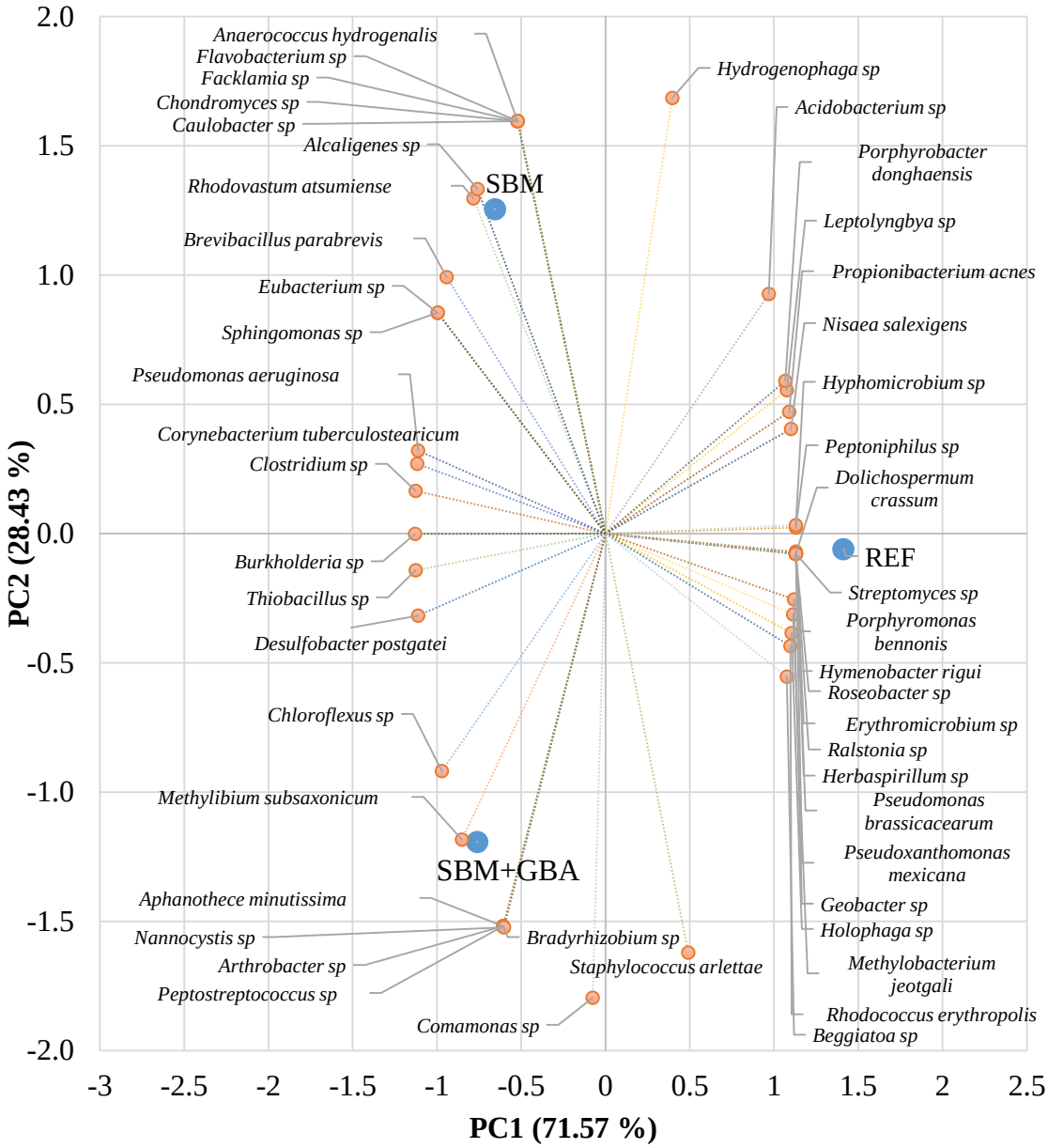

Figure 11 Distance bi-plot of bacteria species (relative abundance ≥ 1% for at least one treatment) of red drum gut microbiota in Trial VII. PC = principal component; REF = reference; SBM = soybean meal; GBA = GroBiotic®-A.

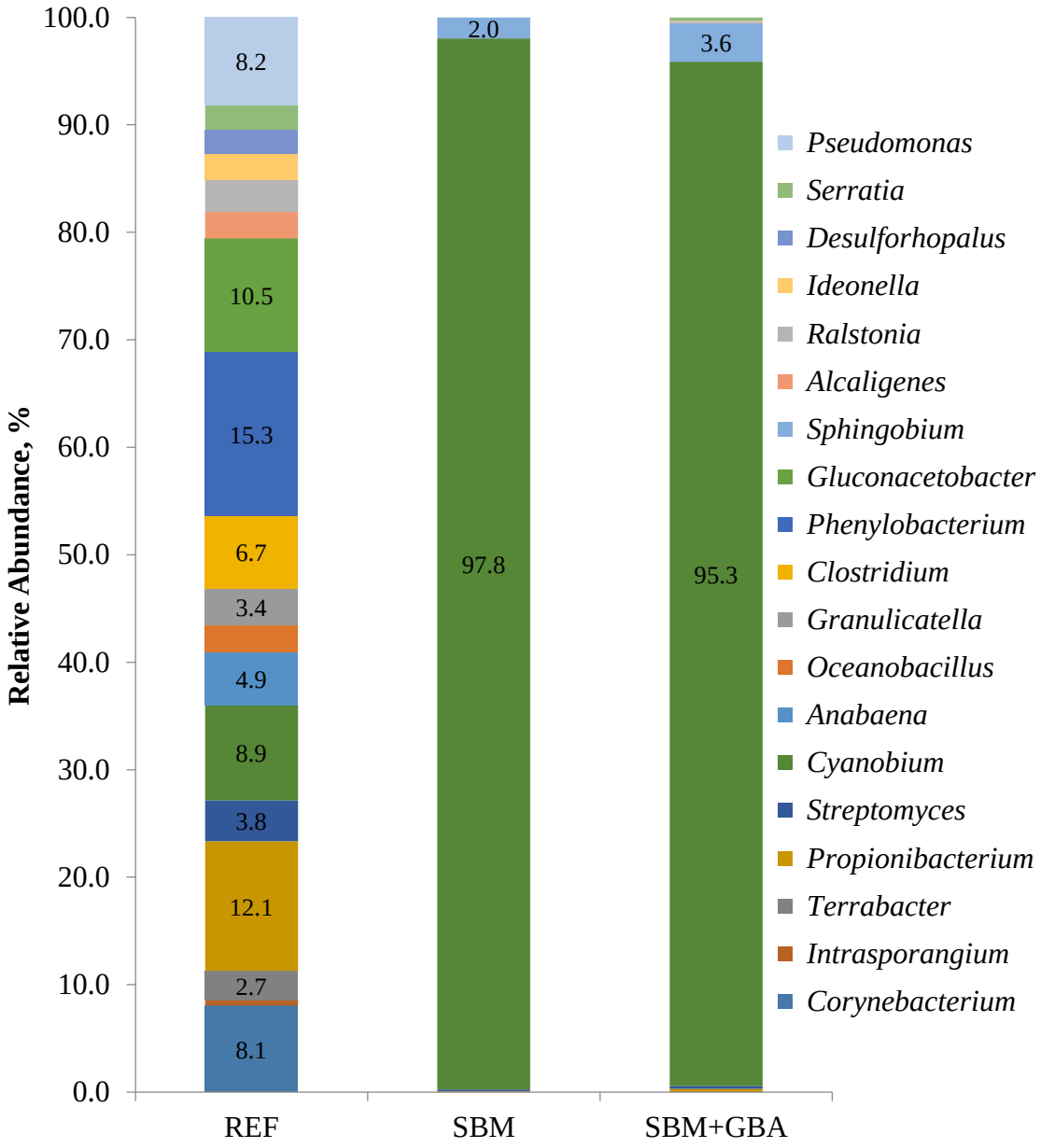

Figure 12 Genera composition (relative abundance ≥ 1% in at least on treatment) of red drum gut microbiota in Trial VIII. REF = reference; SBM = soybean meal; GBA = GroBiotic®-A.

The distance bi-plots for the genera (Fig. 14) and for the species (relative abundance ≥ 1% for at least one treatment (Fig. 15) in Trial VIII are displayed with the ordination of each treatment according to their respective scores from the PCA analysis.

The variability in both datasets was explained by two PC and the bi-plots showed a distinct ordination of each treatment. The PC1 explained most of the variability in genera (81.59%) and in species (98.77%). With respect to PC1, a distinct ordination was observed between the REF treatment in comparison to both SBM and SBM+GBA treatments for both genera and species.

V.4 Discussion

The characterization of the gut microbiota of red drum in Trials VII and VIII consisted of a complementary objective in the evaluation of plant-protein feedstuffs (primarily soybean products) as alternative substitutes for FM in the diet of red drum. Regarding growth performance, these two independent evaluations showed contrasting results: in Trial VII the red drum fed SP-based diets had a poorer performance than fish fed the REF diet, while in Trial VIII no differences were found among dietary treatments (Chapter IV). However, with respect to the supplementation of GBA, no effects on any of the response variables analyzed were found. Potential reasons for the different outcomes regarding growth performance of red drum fed PP-based diets with or without supplemental prebiotic are discussed in the previous chapters.

According to the DGGE and MiSeq results, the microbial diversity in the gut of red drum fed the soy-based diets in both Trials VII and VIII was affected by the almost

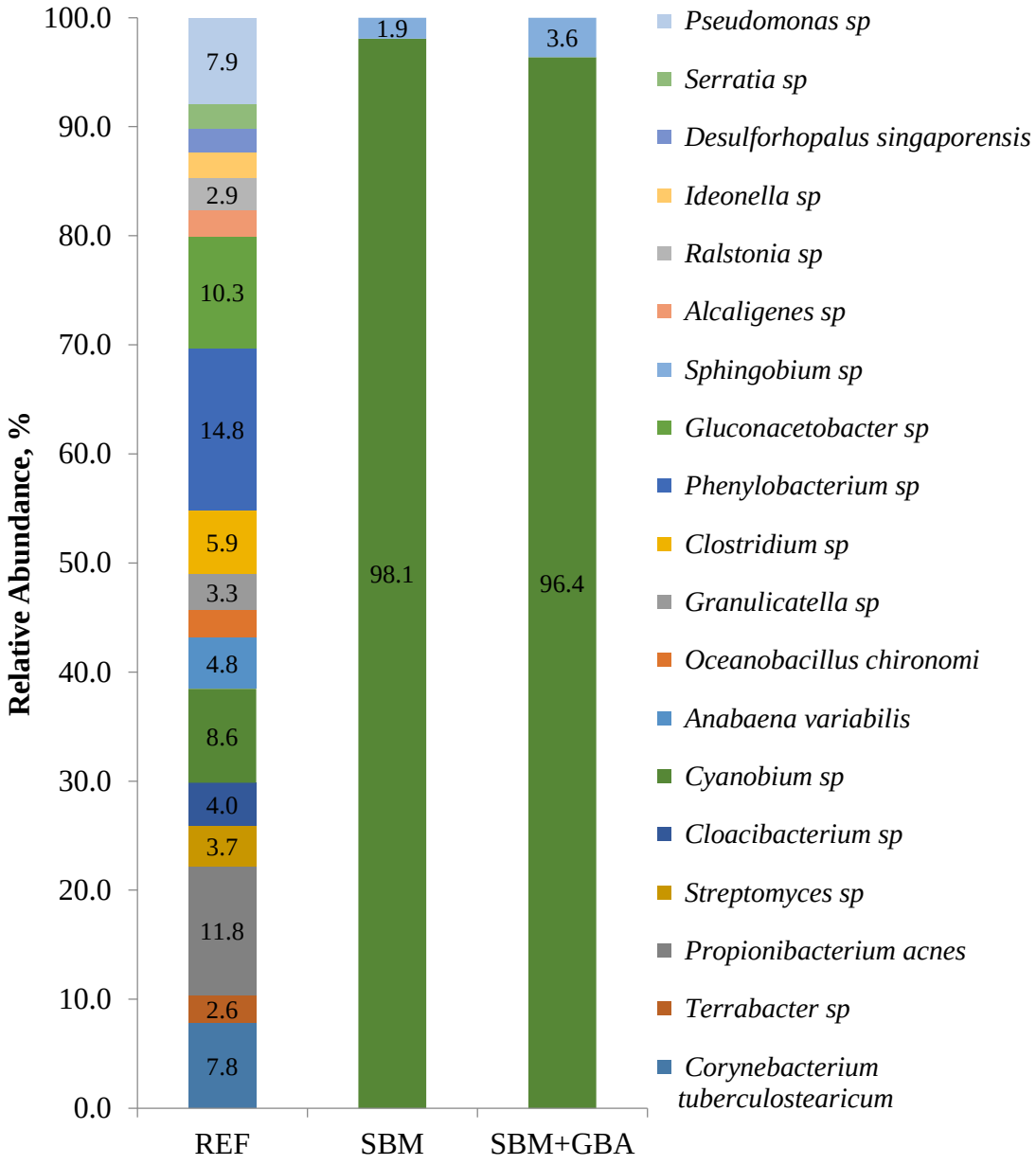

Figure 13 Species composition (relative abundance ≥ 1% in at least one treatment) of red drum gut microbiota in Trial VIII. REF = reference; SBM = soybean meal; GBA = GroBiotic®-A.

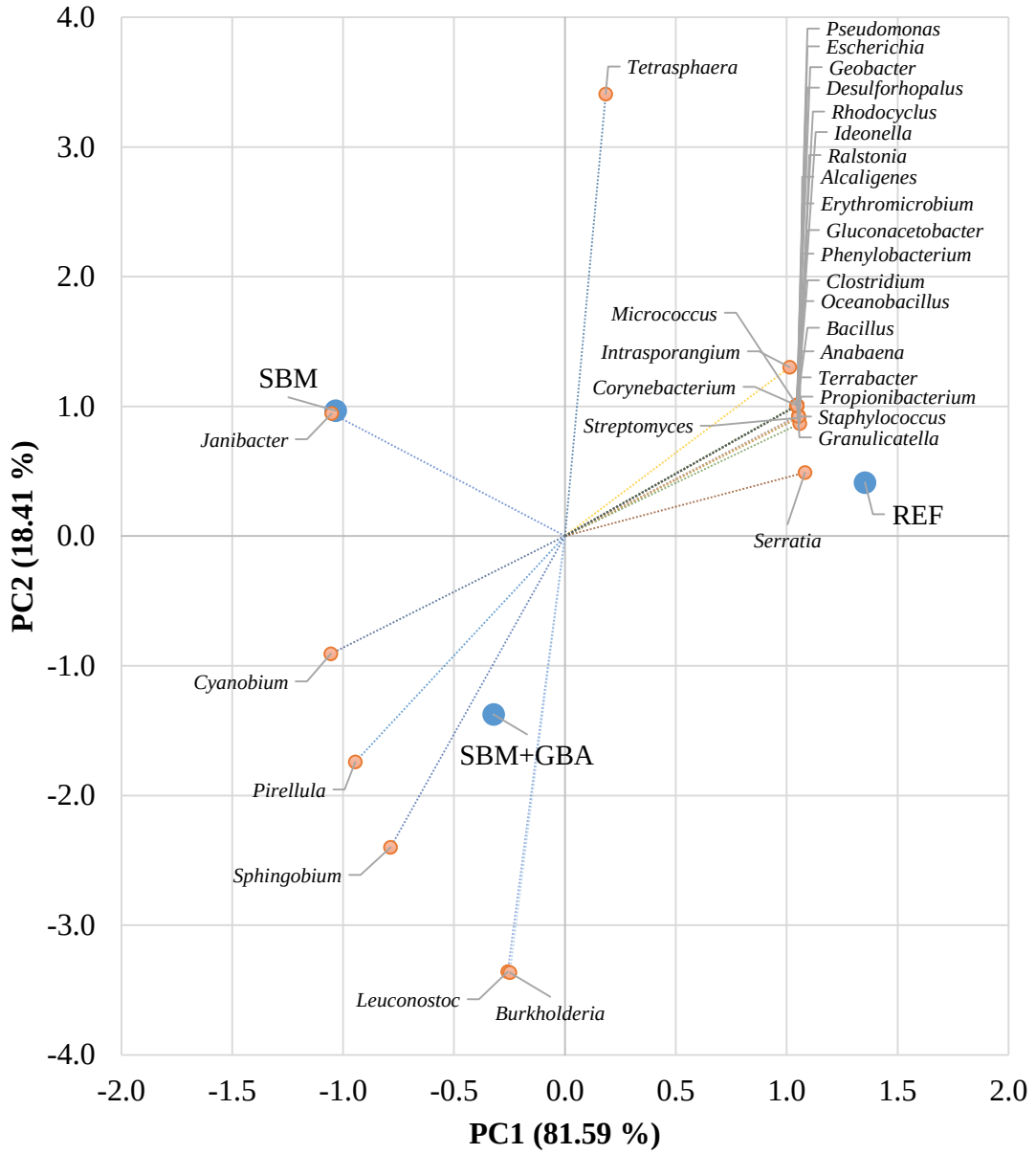

Figure 14 Distance bi-plot of bacteria genera of red drum gut microbiota in Trial VIII. PC = principal component; REF = reference; SBM = soybean meal; GBA = GroBiotic®-A.

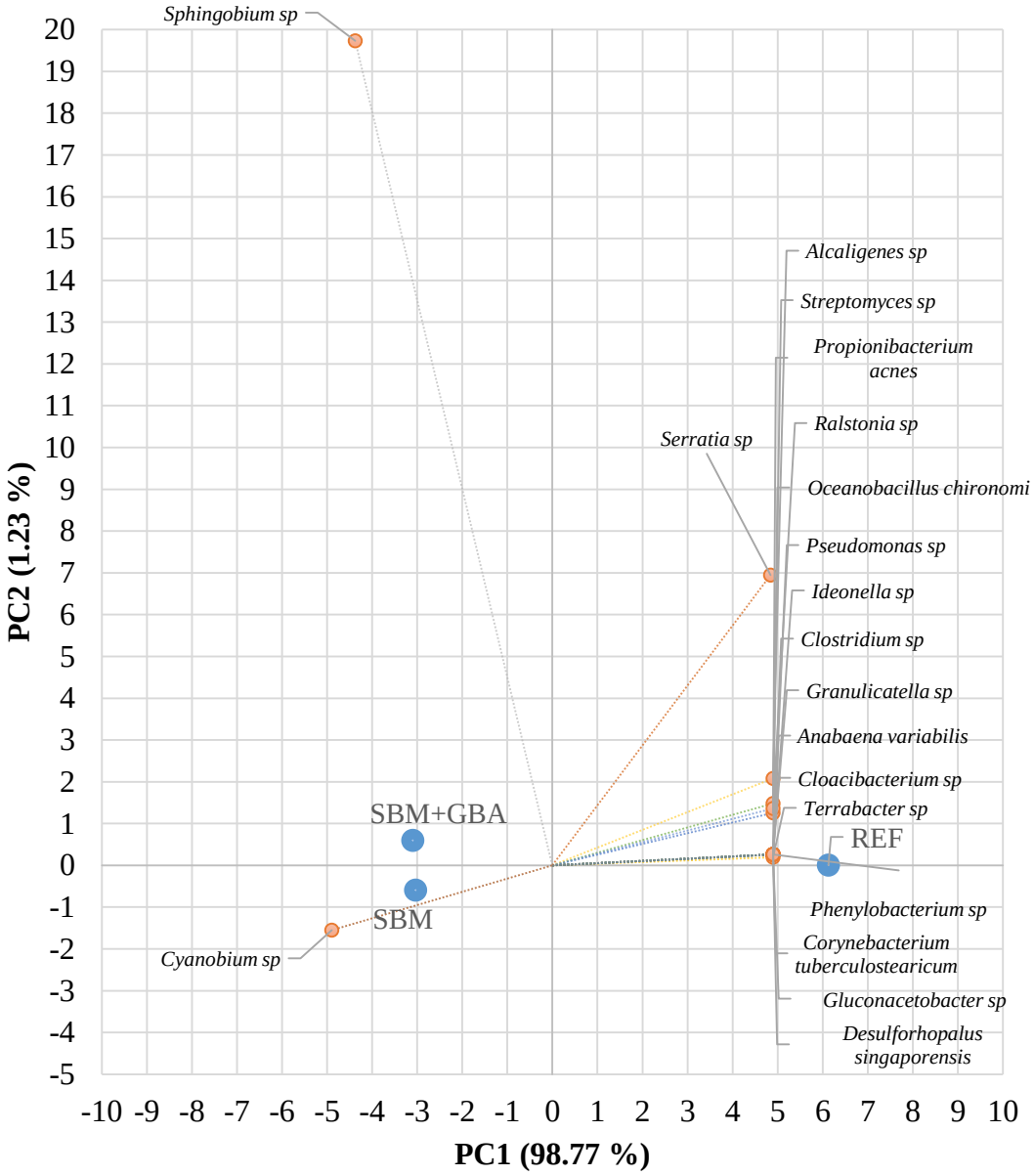

Figure 15 Distance bi-plot of bacteria species (≥ 1% relative abundance in at least one treatment) of red drum gut microbiota in Trial VIII. PC = principal component; REF = reference; SBM = soybean meal; GBA = GroBiotic®-A.

complete replacement of dietary FM with a combination of SBM and SPC. The composition of the gut microbiota in other fish species has been demonstrated to change in response to the inclusion of SBM in the diet (Dimitroglou et al., 2010; Heikkinen et al., 2006). Dimitroglou et al. (2010) demonstrated that red sea bream, *Pagrus major*, fed SBM-based diets had a distinct gut microbiota composition compared to fish fed the FM-based diet. These authors also reported that the modulatory effect of mannan oligosaccharide on the gut microbiota composition was more evident in red sea bream fed FM- rather than SBM-based diets. This is in agreement with the apparent ineffective modulation of red drum's gut microbiota by GBA in Trial VIII, but it disagrees with the results from Trial VII showing a distinct separation of gut microbiota between SBM and SBM+GBA groups (Fig. 7).

The PCA analysis of the gut microbiota indicated a distinct and more diverse bacteria population of red drum in the REF treatment compared to SBM and SBM+GBA treatments in both Trials VII and VIII. However, in Trial VII all three groups showed distinct ordinations at both genus (within the phylum Proteobacteria, the most abundant) and species level; whereas, in Trial VIII no clearly distinct ordination was observed for genus or species between the SBM and SBM+GBA groups (Fig. 14 and 15, respectively). As reported in other studies (Huber et al., 2004; Rawls et al., 2006; Roeselers et al., 2011; Wu et al., 2010), the gut microbiota of red drum in Trial VII was dominated by the phylum Proteobacteria. *Methylobacterium jeotgali*, a facultative methylotrophic bacteria isolated from jeotgal, a traditional Korean fermented seafood (Aslam et al., 2007), was the most abundant species of that phylum in the gut of red

drum fed the REF diet, while *Pseudomonas aureginosa* was the most abundant species in the gut of red drum fed the SBM and SBM+GBA diets. The latter species have been found to be a pathogen in fish (Day et al. 1978), thus it is arguable whether the higher relative abundance of *P. aureginosa* in these treatments would have been enough to promote a disease outbreak under less favorable culture conditions. Proteobacteria also dominated the gut microbiota population of red drum in the REF treatment of Trial VIII; however, an overwhelming dominance of cyanobacteria was observed in the SBM and SBM+GBA treatments. *Phenylobacterium sp*. and *Cyanobium sp*. were the dominant species of Proteobacteria and Cyanobacteria, respectively. Species within the genus *Phenylobacterium* are considered harmless (Kaiser et al., 1980), while toxins produced from cyanobacteria can lead to massive mortality of fish (Rodger et al., 1994). High relative abundance of cyanobacteria in the gut of fish has been indicated to be associated with herbivorous and/or planktivorous feeding behavior (Wu et al., 2012, Ye et al., 2014). Such evidences contrast with the results of the present study showing a massive dominance of this phylum in the gut microbiota of red drum.

In summary, this preliminary evaluation indicates that despite the diversity of red drum's gut microbiota - which was potentially linked to differences in diet composition - it may not influence the production performance and survival of the fish when culture conditions are optimal.

CHAPTER VI

CONCLUSIONS

This dissertation compiled data from a series of feeding trials outlined to optimize low-fishmeal (FM), plant-protein (PP)-based diets for red drum by evaluating a range of feedstuffs that are currently available or becoming increasingly available to the feed industry. It is reasonable to assume that results from this research can substantially impact the aquafeed industry as it strives to reduce FM inclusion in diets for red drum. Although FM is still an important ingredient for early-stage juvenile red drum, it may be further reduced in diets of advanced stages.

Plant-protein feedstuffs with improved nutritional value for carnivorous fish species are playing an important role in reducing FM usage in aquafeeds. Although considerable success has already taken place - as the fish in-fish out ratio has substantially declined compared to two decades ago - aquafeeds for some carnivorous fish still contain FM as a primary or secondary protein source. Henceforth, there is still room for improvement.

The continued technological improvement in the processing of raw materials and the use of breeding programs to augment the expression of favorable nutritional traits in plant feedstuffs may, unquestionably, lessen aquaculture's reliance on FM. However, an important premise for the utilization of new products in aquafeeds is their cost-effectiveness relative to conventional ingredients. On this regard, it is anticipated that the price of novel PP feedstuffs such as barley protein concentrate and non-genetically

modified soybean meal may decrease as these products become more readily available, however, their incorporation in aquafeeds will largely be dictated by their price relative to other high-quality protein feedstuffs such as FM. The price of FM is quite dynamic and is greatly influenced by yearly harvest of reduction fisheries around the world as well as the global demand for FM. As such, with the growth of aquaculture projected to continue increasing, it is quite likely that the price of FM will continue to increase and its substitution with PP feedstuffs will become more economically favorable.

The lack of an effect of prebiotic supplementation in the performance parameters evaluated in the present research must not overshadow the importance of further research on this subject. This perspective is reinforced by a series of important findings on the benefit of prebiotic supplementation in red drum as in other carnivorous fish species. The efficacy of supplementing prebiotics such as GroBiotic®-A into plant-based diets should be investigated in advanced stages of red drum production, as most studies conducted to date used considerable amounts of dietary FM and early-stage juveniles. The potential influence of the culture environment on microbial composition of the gastrointestinal tract also should be more fully characterized.

Lastly, the selection of specimens expressing high genetic and/or phenotypic tolerance to PP feedstuffs may, in the near future, represent a relevant step towards increasing PP utilization in aquafeeds by carnivorous fish. Until then, PP feedstuffs can be used to replace FM in the diet of red drum in a range from 50 to 86%, depending on the stage of production.

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