Alternative ingredients in practical diets for Pacific white shrimp (*Litopenaeus vannamei*)

by

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ABSTRACT

It is a well-known that fish meal supplies will not increase as most fisheries are beyond sustainable limits. If aquaculture and shrimp production in particular is expected to expand, the industry must move away from fish meal as a primary protein source, particularly in the production diets. As shrimp are a major aquaculture product and a primary use of fish meal it is critical that we expand our knowledge of novel protein source which could be used to reduce fish meal levels in production diets. Towards this goal the present study was dedicated to explore the potential of novel ingredients (flash dried yeast, non-genetically modified soy cultivars, bacterial biomass, fish meal analogue, and Ulva meal) as protein sources in practical diets for Pacific white shrimp, *L. vannamei*.

The first study was design to evaluate the potential of a novel yeast product flash dried yeast produced by low pH fermentation of *Saccharomyces Pombe* as a feed supplement in practical shrimp feed. Under the conditions of this study, the energy and protein digestibility of flash dried yeast are significantly lower than FM and SBM. Amino acids digestibility of FDY was lowest among the three ingredients tested. The use of FDY at 60 g kg^{-1} caused significant negative impacts on growth, feed conversion ratio and protein retention. Dietary flash dried yeast supplementation in the practical diets for Pacific white shrimp had no effects on the proximate composition of the whole shrimp body. Based on these results, further research regarding the effects of the low levels ($\leq$ 40 g kg^{-1}) inclusion of FDY in practical diets on immune responses of Pacific white shrimp is warranted.

The second study explored non-genetically modified soy cultivars as protein sources in commercial type shrimp feed formulations. The results indicate that breeding technology and novel soy processing has the potential to increase the nutritional values of SBM for shrimp feeds. Observed trends on immune indicators of shrimp to both independent and combined effects of soy ingredients and fermented yeast were not easily discernible. The variable response may be related to the difficult in working with shrimp or a suboptimal exposure period.

The third study investigated the effects of a dried fermented biomass as alternative ingredients for fish meal or soy protein concentrate. Under the reported conditions of the study, the use of dried fermented biomass can partially replace fish meal up to 50 g kg^{-1} without causing negative effects on the growth performance of Pacific white shrimp. However, completely replacement of fish meal (100 g kg^{-1}) by dried fermented biomass resulted in growth depression. These results were confirmed in a second trial, which replaced soy protein concentrate with fermented biomass dried under two methods. The granular dried fermented biomass worked well as a substitution for soy protein concentrate, however, the inclusion of spray dried dry fermented biomass at 60 and 120 g kg^{-1} decreased the growth of shrimp. This data demonstrates that granular dried fermented biomass is a good nutrient sources and can be incorporated in practical shrimp feed formulations.

The aim of the fifth study was to evaluate a novel bacterial biomass as a replacement for soybean meal in practical shrimp feeds. Under the conditions of the present study bacterial biomass can be utilized up to 4% in shrimp feed without causing growth depression. However, supplementations ($\geq$ 6%) of bacterial biomass can result in negative effects on growth response, FCR, and protein as well as amino acids retention efficiency. Negative result of dry matter digestibility as well as no improvements in the treatments balanced on digestible protein basis

infers that something other than protein is influencing performance. Given that this is a new technology, there is a need to evaluate BB in term of possible immune stimulation as well as a nutrient source.

The sixth study was designed to evaluate a fish meal analogue as a replacement for fish meal in practical shrimp feed. Results indicated that in a practical diet containing 20% fish meal, fish meal analogue can replace all of the fish meal as long as the diets are supplemented with inorganic phosphorus without compromising the growth of shrimp. The improvement of growth when fish meal analogue was incorporated at 4.95% across three trials was not able to be defined. Given the good growth across the range of inclusion without any indication of a growth depression, the digestibility of the protein of fish meal analogue would be similar to that of the fish meal for which it was substituted. Hence, the low nutrient digestibility of fish meal analogue may due to an atypical response or the product simply does not work with the testing technique.

The seventh study evaluated the potential of Ulva meal *Ulva* sp. as an alternative protein source to fish meal and soybean meal in practical shrimp feed. Results demonstrated a clear depressing in growth as fishmeal was replaced. This data also demonstrated significant difference between batches of Ulva with the second batch producing the poorest results. To elucidate if digestible protein was limiting growth, a trial was initiated for which feeds were formulated on a digestible protein basis. In this trial, growth and survival were significantly reduced as the level of Ulva meal (Batch 2) was increased. Although, growth and survival was depressed this was less than that of previous trials, indicating that protein quality may be part of the problem. However, given the level of protein replacement other components of Ulva meal are likely to be causing poor performance. To survey possible problems caused by high levels of minerals the meals and select diets were analyzed for mineral content. Clearly there are shifts in

mineral profiles; however, there is no obvious correlation to a mineral and this research team feels that it is unlikely a mineral toxicity. Other possible reasons which are beyond the scope of this project but would include anti-nutrients present in the algae. If Ulva meals are to be use to their full potential, e.g. as a primary protein source, the anti-nutritional components will need to be identified, specific lines of plants with enhanced nutrient value need to be developed and of course processing technologies evaluated to produce a high quality commercial product.

There is a clear need to develop sustainable ingredient sources for aquaculture, the use of soybean meal is clearly a viable option. Besides soybean meal, there are abundant alternative sustainable ingredients such as yeast, bacterial biomass, seaweed meals, and etc, which exhibited great potential as both immune enhancer and protein source. Therefore, it is vital for us to evaluate these potential alternative ingredients and promote a sustainable, environmentally friendly, and economical aquaculture.

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CHAPTER I

Introduction

Pacific white shrimp, *Litopenaeus vannamei* is native to the Eastern Pacific coast from Gulf of California, Mexico to Tumbes, North of Peru (PeÂrez Farfante and Kensley, 1997). It is well known for its ability to grow and survive well under a wide range of environmental and artificial culture conditions, Adaptability to commercial culture has made it the primary cultured shrimp species as well as atop aquaculture commodity (Roy et al., 2009). The production of Pacific white shrimp increased from 0.15 million metric tons in 2000 to 4.5 million metric tons in 2016 (Anderson, 2016), and was estimated to increase to 9.2 million tonnes in 2020 (Tacon and Metian, 2008). The USA was the top global import market for imported shrimp in 2015, with 0.59 million tonnes imported, and the import value reached 5.46 billion dollars in 2015 (FAO, 2015).

Feed represents one of the largest variable costs associated with fed culture systems; reducing feed costs can produce considerable savings (Davis and Sookying, 2009). Manufacturing of marine shrimp feed uses 24% to 27% of the world's fish meal (Tacon and Metian, 2008) making them one of the prime consumers. Commercial shrimp feed formulations historically contain from 25% to 50% fish meal, which represents the primary and most expensive protein ingredient (Dersjant-Li, 2002; Tacon and Metian, 2008). Fish meal (FM) is preferred among other protein sources because it is an excellent source of essential nutrients such as protein and indispensable amino acids, essential fatty acids, cholesterol, vitamins, minerals attractants and unidentified growth factors (Samocha et al., 2004; Swick et al., 1995). The cost of FM has increased over time because of increased demand, limitations of availability, and growing social and environmental concerns regarding wild fish extraction practices (Tacon and

Metian, 2008). Due to limited supply and increasing prices, we must shift our emphasis and use this ingredient only when nutrient requirements of the animal demand its use (Davis and Sookying, 2009).

To develop sustainable and environmentally friendly aquaculture, various terrestrial plant-based ingredients that contain high protein content are potential alternative sources for fish meal (NRC, 2011). Terrestrial plant-based protein sources such as soybean meal, canola meal, corn gluten meal are available worldwide and have a relatively low cost compared to fish meal. Among plant protein sources, soybean meal (SBM) is the most abundant and has received considerable attention as a replacement for fish meal in aquatic animal feeds because of its availability, balanced amino acid profile and consistent composition (Akiyama, 1989; Akiyama et al., 1991; Amaya et al., 2007; Hardy, 1999; Samocha et al., 2004; Swick et al., 1995; Tacon, 2000). However, the presence of anti-nutritional factors (ANF) such as lectins, oligosaccharides, saponins, and trypsin inhibitors, can limit the inclusion levels of SBM in some aquaculture feeds. Moreover, as the popularity of SBM as a feed ingredient increased, its price continued to increase from 185.02 dollars per metric ton in 2001 to 443.41 dollars per metric ton in 2016. In addition, application of terrestrial plant protein such as SBM in aquaculture feeds may affect the food costs for human communities in developing countries (Delgado et al., 2002; 2003).

As shrimp culture has become an expanded and intensified economic activity, the demand for more cost-effective and sustainable protein sources continues to increase. In this instance, incorporation of marine plant protein (i.e. macro-algae) in aquaculture feeds would be an alternative strategy to reduce the reliance on FM and terrestrial plant protein sources such as SBM. The chemical composition of macro-algae can be influenced by both physical and chemical factors such as temperature, salinity, light (Lobban and Harrison, 1994) and nutrient

concentrations (Björnsäter and Wheeler, 1990; Floreto et al., 1996; García-Ferris et al., 1996) during cultivation. Therefore, the product quality including protein content, lipid content, and tissue pigmentation of macro-algae can be somewhat manipulated by controlling the main parameters of cultivation (i.e. nutrient loading, stocking density, mixing regime, etc.) (Guerin and Bird, 1987; Neori, 1996; Shpigel et al., 1999). Nitrogen-enriched conditions like the effluents of fish or shrimp farms, where seaweeds are used as bio-filters, can enhance their protein contents (Lahaye et al., 1995; Pinchetti et al., 1998). Lipid content can be raised by manipulations of nutrient supply and other growth parameters. Macro-algae are also rich sources of minerals (7% to 38% dry weight basis) with a broad mineral composition including Bo, Ca, Cl, Co, Cu, F, Fe, I, K, Mg, Mn, Mo, Na, Ni, P, S, Se, Zn.

Macro-algae can benefit from recycling waste carbon dioxide (CO_2) from combustive energy production (that otherwise pollutes the atmosphere) and waste nutrients produced by intensive aquaculture operations, agriculture, intensive animal operations, municipal waste treatment, and the like (Kaushik and Troell, 2010; Sargent and Tacon, 1999). In an integrated cultivation system, the macro-algae use the metabolic residues of animals as nutrients, absorb CO_2 and produce O_2 for the environment (Marinho-Soriano et al., 2008). The interaction allows the excretion of an organism to serve as food for another (Qian et al., 1996). Presently, significant improvements in growth and survival rate have been observed when Pacific white shrimp, *Litopenaeus vannamei* (Cruz-Suárez et al., 2010), tiger shrimp, *Penaeous monodon* Fabr (Izzati, 2012; Tsutsui et al., 2010), and yellowtail shrimp, *Farfantepenaeus californiensis* (Portillo-Clark et al., 2012) are co-cultured with macro-algae.

Macro-algae have been demonstrated to replace small fractions (1 to 4%) of FM content in diets of Sea bass, *Dicentrarchus labrax* (Valente et al., 2006), Rainbow trout, *Oncorhynchus*

mykiss (Soler-Vila et al., 2009; Yıldırım et al., 2009) and Pacific white shrimp (Rodríguez-González et al., 2014). Growth responses when high levels of algae are used to replace FM content in diets of aquatic animals vary. Xu et al. (2011) reported that weight gain of teleost fish, *Siganus canaliculatus* was significantly decreased when using seaweed *Gracilaria lemaneiformis* to replace 10% FM. However, Stadtlander et al. (2013) indicated that 7.5 and 15% FM replacement by red alga Nori *Porphyra yezoensis* Ueda did not significantly affect the growth performance of Nile tilapia, *Oreochromis niloticus*.

Besides macro-algae, bacteria biomass is another potential feed ingredient as a replacement for FM and SBM in aquaculture feeds. Rapid growth and high protein content are well known properties of bacteria in protein production (Anupama and Ravindra, 2000; Kuhad et al., 1997; Stringer, 1982). Methane, the main component of natural gas, which is found widely in nature (Hanson and Hanson, 1996; Dalton, 2005) is an attractive substrate for bacterial protein production. The abundant supply, cheap transportation, and reasonable cost of natural gas, indicate that protein production from natural gas could be realistic on a large scale (Overland et al., 2010).

The naturally occurring methanotroph *Methylococcus capsulatus* (Bath) has shown high efficiency in production of bacterial protein from methane (Bothe et al., 2002). Considerable researches have been carried out on the bacterial meal, produced from mainly methane by natural gas fermentation, as a protein source for a number of fish species, including Atlantic salmon, *Salmo salar* (Aas et al., 2006a; Berge et al., 2005), rainbow trout, *Oncorhynchus mykiss* (Aas et al., 2006b; Kiessling and Askbrandt, 1993; Storebakken et al., 2004), and Atlantic halibut, *Hippoglossus hippoglossus* (Aas et al., 2007), and Florida pompano, *Trachinotus carolinus* (Melanie et al., 2015). However, the growth responses of the fish in these studies to the bacterial

meal are not consistent, which may due to the different fish species and various bacterial strains. Information about the nutrient digestibility values of bacterial meal is still limited. However, information about protein digestibility of the diets contained bacterial meal is available. Storebakken et al., 1998 reported that the apparent protein, fat and energy digestibility of the diet that contained 20% bacterial meal replacing 20% FM were 89.7%, 88.3%, and 82.1%, respectively, which were close to those of FM-based diet (91.5%, 90%, and 86%, respectively).

Given the great potential of the alternative ingredients provided above, it is important for us to explore the nutritional value of these products in order to ensure sustainability and options for feed formulator. Therefore the purpose of this research is to evaluate the utilization of potential alternative ingredient in practical diets for Pacific white shrimp, *L. vannamei*.

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CHAPTER II

EVALUATION OF FLASH DRIED YEAST AS A NUTRITIONAL SUPPLEMENT IN PLANT BASED PRACTICAL DIETS FOR PACIFIC WHITE SHRIMP *Litopenaeus vannamei*

1. Introduction

As shrimp culture has become an expanded and intensified economic activity, bacterial and viral diseases are considered to threaten the further progress of semi-intensive and intensive shrimp culture (Pérez-Sánchez *et al*. 2014). Antibiotics have been utilized to supplement in the shrimp feeds for prevention and treatment of diseases (Cabello 2006; Taylor *et al*. 2011). However, the use of antibiotics may develop bacterial strains that are more resistant to antibiotic treatment, meanwhile the antibiotic residues in cultured animals may pose negative impacts on human health (Sharifuzzaman & Austin 2009). To tackle disease problems and avoid potential disadvantages of antibiotics, a number of alternative strategies such as the use of vaccine, immunostimulants, probiotics, and prebiotics have received considerable attention (Li & Gatlin 2005).

The probiotics are usually members of the healthy microbiota associated with the host (Pérez-Sánchez *et al*. 2014). They can prevent bacterial diseases by producing inhibitory compounds to create a hostile environment for pathogens, competing for essential nutrients and adhesion sites or modulating the immune response (Merrifield *et al*. 2010). Probiotic effects in aquaculture are not only limited to intestinal tract of aquatic animals, but also can improve the health of the host by controlling pathogens and improving water quality (Verschuere *et al*. 2000; Zheng *et al*. 2012).

Yeast is one of the probiotics, which is commonly used in aquaculture either alive to feed live food organisms, or after processing, as a feed ingredient (Stones & Mills 2004). Yeast cells contain β-glucans, nucleic acid, oligosaccharides, as well as polyamines, which may help to improve the immune response and growth performance as well as metabolism in fish and shrimp (Gatesoupe 2007). Yeasts are frequently used as a dietary supplement in fish and shrimp feeds to increase growth performance, feed intake, survival and disease resistance (Deng *et al*. 2013; Essa *et al*. 2011; Hoseinifar *et al*. 2011; Li & Gatlin 2003; 2004; 2005; Sheikhzadeh *et al*. 2012; Shen *et al*. 2010; Vechklang *et al*. 2012; Yang *et al*. 2010) as well as an alternative protein sources for fishmeal (Gause & Trushenski 2011a; b; Hauptman *et al*. 2014; Lunger *et al*. 2006; Oliva-Teles & Gonçalves 2001; Peterson *et al*. 2012).

The two common yeasts that used in aquaculture feeds are brewers yeast *Saccharomyces cerevisiae* (BY), which is a natural product of the brewing industry and grain distillers dried yeast (GDDY), which is a co-product obtained from the bioethanol industry. The yeast product utilized in the present study is flash dried yeast (FDY) that is a novel product produced by low pH fermentation of *Saccharomyces Pombe*.

Albeit there are some studies demonstrating positive effects of various yeast supplements in Pacific white shrimp diets on the disease tolerance and growth performance, there is limited data in apparent digestibility coefficients of yeast products. Hence, The objectives of this project was to determine the growth response of Pacific white shrimp juveniles to increasing FDY levels in a soybean meal based feed formulation and determined apparent digestibility values for yeast as compared to other protein sources.

2. Materials and Methods

2.1 Experimental diets

All test diets were formulated to be isonitrogenous and isolipidic (350 g kg^{-1} protein and 80 g kg^{-1} lipid). For the growth trial, five experimental diets were formulated. The first diet or basal diet did not contain FDY. Whereas the next four diets contained FDY at increasing levels (10, 20, 40, and 60 g kg^{-1}) (Table 1). The reference diet (Table 4) for ingredient digestibility trial was formulated to include 10 g kg^{-1} chromic oxide as inert marker. Test diets were made using a 70:30 mixture of the reference diet and test ingredients.

Primary ingredients (Table 2) were analyzed for proximate composition in the diets formulated. Pre-ground dry ingredient and oil were mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 min. Hot water was then blended into the mixture to obtain a consistency appropriate for pelleting. Diets were pressure-pelleted using a meat grinder with a 3-mm die, air-dried (< 50 °C) to a moisture content of 80-100 g kg^{-1}. Pellets were crumbled, packed in sealed plastic bags and stored in a freezer until needed. The diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate composition (g kg^{-1} as is) and amino acid profile (% as is) (Table 3).

2.2 Growth trial

The growth trial utilized 5 treatments with 5 replicates in each treatment. It was conducted in a semi-closed recirculation system. Juvenile shrimp were obtained from the nursery system and selected by hand-sorting to a uniform size. Juvenile shrimp (initial weight 1.78±0.03 g) were stocked into 25 tanks with 10 shrimps in each aquarium (80 L). A sub-sample of shrimp from the initial stocking was retained for whole body samples to be utilized for later protein

retention analysis. As shrimp are difficult to handle, intermittent weights were not taken. However, shrimp were counted to readjust daily feed input on a weekly basis. Based on historical results, a fixed ration was calculated assuming a 1.8 feed conversion ratio and 0.8 to 1.4 g $week^{-1}$. Consequently, for each tank a fixed ration of 2.31 g day^{-1} for the first week, 2.83 g day^{-1} for the second week, 2.90 g day^{-1} for the third week, and 3.09 g day^{-1} for the forth week, 3.34 g day^{-1} for the fifth week, and 3.6 g day^{-1} for the six week was offered over 4 feedings.

Dissolved oxygen (DO), temperature, and salinity were measured twice daily by using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH, USA). pH was measured twice weekly by using a waterproof pHTestr30 (Oakton instrument, Vernon Hills, IL, USA). Water samples were taken to measure total ammonia-nitrogen (TAN) and nitrite every week. TAN and nitrite were determined by using the methods described by Solorzano (1969) and Spotte (1979), respectively. During the experiment period DO, temperature, salinity, pH, TAN, and nitrite were maintained within acceptable ranges for *L. vannamei* at 5.36±0.26 mg L^{-1}, 29.0±1.2 °C, 13.1±0.3 g L^{-1}, 7.45±0.33, 0.066±0.0048 mg L^{-1}, and 0.040±0.037 mg L^{-1}, respectively.

Shrimps were counted to readjust daily feed input on a weekly basis. At the conclusion of 6-week growth trial, shrimps were counted and group weighted. Mean final weight, feed conversion ratio (FCR), weight gain (WG), biomass, and survival were determined. After obtaining the final total weight of shrimps in each aquarium, 4 shrimps were randomly selected and frozen at -20 °C for subsequent determination of whole body composition. Proximate composition of whole shrimp was analyzed by University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory (Columbia, MO, USA). Protein retention was calculated as follows:

Protein retention (%) = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein offered.

2.3 Digestibility trial

The digestibility trial was conducted in the mentioned recirculation system and utilized six shrimp per aquaria with six aquaria per dietary treatment. Once acclimated for three days to the test diets, feces from two aquaria were pooled (n=3) and collected over a five-day period or until adequate samples were obtained. To obtain fecal samples, the aquaria were cleaned by siphoning before each feeding with the first collection of the day discarded. After cleaning, the shrimp were offered an excess of feed and then about 1 hour later feed was removed and feces were collected by siphoning onto a 500 μm mesh screen. Collected feces were rinsed with distilled water, dried at 105 °C until a constant weight was obtained, and then stored in freezer (−20 °C) until analyzed. Apparent digestibility coefficients for dry matter, protein, energy, and amino acids were determined by using chromic oxide (Cr_2O_3, 10 g kg^{-1}) as an inert marker. Chromium concentrations were determined by the method of McGinnis and Kasting (1964) in which, after a colorimetric reaction, absorbance is read on a spectrophotometer (Spectronic genesis 5, Milton Roy Co., Rochester, NY, USA) at 540 nm. Gross energy of diets and fecal samples were analyzed with a Semi micro-bomb calorimeter (Model 1425, Parr Instrument Co., Moline, IL, USA). Protein of diets and fecal samples were determined by micro-Kjeldahl analysis (Ma and Zuazaga, 1942). Amino acids were analyzed by University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory. The apparent digestibility coefficient of dry matter (ADMD), protein (ADP), energy (ADE), and amino acids (ADAA) were calculated according to Cho et al. (1982) as follows:

ADMD (%)= 100 − [100 × (% Cr_2O_3 in feed / % Cr_2O_3 in feces)]

ADP, ADE, and ADAA (%)=100 − [100 × (% Cr_2O_3 in feed / % Cr_2O_3 in feces) × (% nutrient in feces / % nutrient in feed]

The apparent digestibility coefficients of the test ingredients for dry matter, energy, protein and amino acids were calculated according to Bureau & Hua (2006) as follows:

$ADC_{test\ ingredient} = ADC_{test\ diet} + [(ADC_{test\ diet} - ADC_{ref.\ diet}) \times (0.7 \times D_{ref} / 0.3 \times D_{ingr})]$

where D_{ref} = % nutrient (or KJ/g gross energy) of reference diet mash (as is); D_{ingr} = % nutrient (or KJ/g gross energy) of test ingredient (as is).

2.4 Statistical analysis

All the data were analyzed using SAS (V9.3. SAS Institute, Cary, NC, USA). Data from the growth and digestibility trial were analyzed using one-way ANOVA to determine significant differences (P<0.05) among treatments followed by the Tukey's multiple comparison test to determine difference between treatments. Arcsine square root transformation was used prior to analysis for the proportion data in growth and digestibility trial. False discover rate (FDR) controlling procedures were used to adjust the P-value in order to control the FDR for the amino acids digestibility data.

3. Results

Performances and protein retention efficiency of Pacific white shrimp, *L. vannamei* offered diets contained different FDY levels are presented in Table 5. Final biomass, final mean weight, WG, FCR, and PRE of Pacific white shrimp were not significantly influenced when

FDY was utilized up to 40 g kg^{-1} of the diet. However, significantly reduced growth, feed utilization, and PRE were observed when FDY was supplemented at 60 g kg^{-1} feed.

Proximate analysis of whole shrimp body offered diets with FDY levels are presented in Table 6. Supplementation of FDY in the practical diets of Pacific white shrimp did not affect protein (791.9 to 810.9 g kg^{-1}), moisture (767.5 to 776.4 g kg^{-1}), lipid (50.0 to 65.3 g kg^{-1}), crude fiber (52.8 to 56.9 g kg^{-1}), and ash (119.9 to 124.7 g kg^{-1}) content of whole shrimp body.

ADMD, ADE, and ADP for the diet (D) and ingredient (I) using 70:30 replacement technique offered to Pacific white shrimp are presented in Table 7. The digestibility trial contained a range of ingredients; hence, we have provided a few other ingredients as a reference. The analyzed proximate composition of FDY as compares to fishmeal was lower in total protein and lipid content (Table 2). In order to confirm the results, fecal samples for basal diet and FM diet were recollected. FM1 and FM2 represent the first collection and second collection, respectively. Basal diet 1 and Basal diet 2 represent first collection and second collection basal diet, respectively. The results turned out to be quite similar, which indicated that the feces collection and samples analysis methods we utilized in the digestibility study are consistent. The energy and protein digestibility of FDY were 38.20% and 53.47%, respectively, which are significantly lower than fishmeal (FM) and soybean meal (SBM).

Apparent amino acids (AA) digestibility values for the SBM, FM, and FDY using 70:30 replacement technique offered to Pacific white shrimp are presented in Table 8. The analyzed amino acids composition of FDY as compared to FM was lower in concentrations of individual amino acids especially the two common limiting amino acids (methionine and lysine) for Pacific white shrimp (Table 2). Apparent digestibility coefficients of alanine, arginine, aspartic acid, cysteine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline,

serine, threonine, tryptophan, tyrosine, valine and total amino acids of FDY were similar to those of FM but significantly lower than those of SBM. Glycine and hydroxylysine digestibility of FDY were significantly lower than both those of FM and SBM.

4. Discussion

Yeast is a feed ingredient that can originate from a wide range of sources and has shown its potential to be used as nutritional supplement and protein source in production diets (Achupallas *et al*. 2015; Gause & Trushenski 2011a). The digestibility of a feed ingredient can provide estimates of nutrient availability in the ingredient, which helps to select ingredients that optimize the nutritional value and cost of the formulated feed (Brunson *et al*. 1997). Unfortunately, nutrient digestibility may show a high inconsistency due to the feeding practices, environmental conditions, feed processes as well as diet digestibility approaches (Brunson *et al*. 1997). Protein digestibility of FM in the current study for the two collected samples (FM1 and FM2) are 67.07% and 71.30%, respectively. Terrazas-Fierro *et al*. (2010) reported that protein digestibility of various FMs for Pacific white shrimp ranged from 62.7% to 84.9%. Brunson *et al*. (1997) indicated that protein digestibility of FM for white shrimp *Penaeus setiferus* is 75.85%. The protein availability of FM in the current study falls into the range of protein digestibility of FMs tested by other researchers. The similar results of basal diet and FM diet from the two fecal samples collection points to consistency in the feces collection and sample analysis methods. With regards to the protein digestibility of SBM, it was tested to be 97.03%, which falls into the ranges of protein digestibility (80.3% to 98.3%) of various SBMs for Pacific whites shrimp (Cruz-Suarez *et al*. 2009; Fang *et al*. 2015; Liu *et al*. 2012; Yang *et al*. 2009; Zhou *et al*. 2015).

In the present study, protein and energy digestibility of FDY were significantly lower than those of FM and SBM. Amino acids digestibility of FDY was lowest among the three ingredients tested. Yeast is not typically utilized as protein source in the production diets, hence only a few studies have evaluated the digestibility values of the yeasts. Hauptman *et al*. (2014) reported that protein digestibility (97.6%) of GDDY for rainbow trout *Oncorhynchus mykiss* was similar to anchovy fishmeal (97%), soy protein concentrate (99%) and wheat gluten meal (100%), however, digestibility coefficients for dry matter and energy of GDDY were significantly lower than those of fishmeal which most likely due to the relatively high nitrogen free extract content of the ingredient. Amino acids digestibility of the GDDY for rainbow trout *O. mykiss* were lower than those of menhaden fishmeal (Hauptman *et al*. 2014). Rumsey *et al*. (1991) indicated that energy and protein digestibility of intact BY and its fractions for rainbow trout *O. mykiss* ranged from 62.6 to 77.9% and 63.2 to 84.7%, respectively. Energy and protein digestibility coefficients of FDY for Pacific white shrimp were lower compared to digestibility coefficients of GDDY and BY for rainbow trout, which most likely due to the different species of aquatic animals and various kinds of yeasts utilized in the experiments.

Yeast can serve as a source of dietary nucleotides, which have been shown to stimulate the immune response, metabolism, and growth in aquatic animals (Gatesoupe 2007). Dietary yeast supplementation at low levels (no more than 20 g kg^{-1}) as a nutritional supplement in aquatic animals diets has been demonstrated to improve the growth performance and immune response in many aquaculture species including African catfish *Clarias gariepinus* (Essa *et al*. 2011), hybrid striped bass *Morone chrysops* × *M. saxatilis* (Li & Gatlin 2004; 2005), rainbow trout *O. mykiss* (Sheikhzadeh *et al*. 2012), channel catfish *Ictalurus punctatus* (Li *et al*. 2011),

beluga *Huso huso* (Hoseinifar *et al*. 2011), and Pacific white shrimp *L. vannamei* (Deng *et al*. 2013; Yang *et al*. 2010).

With regards to the present growth trial, WG, FCR, and survival of Pacific white shrimp were not significantly influenced when FDY was utilized up to 40 g kg^{-1} of the diet. However, significantly reduced WG and FCR were detected when FDY was incorporated at 60 g kg^{-1} feed. The results of present study are in agreement with Li & Gatlin (2003), who reported that WG, feed efficiency and survival of hybrid stripped bass (*Morone chrysops* × *M. saxatilis*) were not significantly affected when brewer yeast was utilized up to 40 g kg^{-1} of the diet. Similarly, Vechklang *et al* (2012) indicated that WG, feed intake, and survival of Nile tilapia *Oreocbromis niloticus* were not significantly influenced when brewer yeast or GroBioti-A were utilized up to 20 g kg^{-1} of the diet. By contrast, the use of GDDY at the inclusion level up to 300 g kg^{-1} could be utilized in diets without causing adverse effects on growth performance of Pacific white shrimp *L. vannamei* in a clear water system without natural foods (Achupallas *et al*. 2016). Also, Achupallas *et al*. (2015) demonstrated that in a pond and outdoor green water trial growth performance of Pacific white shrimp *L. vannamei* was not significantly affected when GDDY was used up to 150 g kg^{-1} feed. GDDY has the potential to be utilized as a protein source and included at a high level in the diets, which has been confirmed by Gause & Trushenski (2011a;b) that GDDY at the inclusion level up to 413.3 g kg^{-1} could be used in diets without causing negative impacts on the growth performance of sunshine bass and Hauptman *et al*. (2014) that WG and FCR of rainbow trout *O. mykiss* was not affected when GDDY was used up to 112.0 g kg^{-1} of the diet.

Variations in the outcome of present study and study conducted by Achupallas *et al*. (2016) and Achupallas *et al*. (2015) may result from different yeasts types. In the present

digestibility trial, the protein digestibility of FDY is significantly lower than protein digestibility of soybean meal. However, the differences of the digestible protein levels between the basal diet and the diet contains 60 g kg^{-1} FDY is around 10 g kg^{-1}, which cannot result in the reduced growth performance of Pacific white shrimp. Also amino acids levels in all the diets (Table 2) are above the requirements of Pacific white shrimp, thus amino acids deficiency should not be the factor that caused the negative impact on growth response in the current study. As the inclusion levels of FDY increased in the diet formulation, we observed the stickiness of the diets increased during the extrusion, which may increase digesta viscosity and reduce palatability of the experimental diets. Another possible reason for the reduced growth response may because the over stimulation of immune response as the inclusion levels of FDY increased.

PRE was not significantly affected when FDY was utilized up to 40 g kg^{-1} of the diet in the present study. However, significantly reduced PRE was observed when FDY supplemented at 60 g kg^{-1} feed. Hauptman *et al*. (2014) reported that no significant differences were observed in PRE when rainbow trout fed with diets contain up to 295.0 g kg^{-1} GDDY. By contrast, PRE of sea bass *Dicentrarchus labrax* was significantly improved when BY was supplemented up to 548 g kg^{-1} in the diet (Oliva-Teles & Gonçalves 2001). PRE was determined by a number of factors including dietary protein levels, feed intake, final weight and initial weight of animals as well as the final and initial protein content of animals (Halver & Hardy 2002). In the current study, no significant differences were found in dietary protein levels, feed offered to the shrimp, and protein content of whole shrimp body. The significantly reduced PRE should be caused by the decreased weight gain of shrimp in the current study.

In the current study, no significant differences were observed in whole body composition (moisture, protein, lipid, ash, crude fiber) of Pacific white shrimp when fed with diets contain up

to 60 g kg^{-1} FDY. Similarly, Li & Gatlin (2003) reported that whole body composition of juvenile stripped bass was not affected when brewers yeast was utilized up to 40 g kg^{-1} of the diet. Vechklang *et al*. (2012) indicated that no significant differences were observed in whole body composition (moisture, protein, lipid and ash) of tilapia fed with diets supplemented with 10 and 20 g kg^{-1} BY. In addition, many other studies also reported that dietary yeast supplementation had no significant effects on whole body composition of fish (Essa *et al*. 2011; Hauptman *et al*. 2014; Hoseinifar *et al*. 2011; Li *et al*. 2005; Sheikhzadeh *et al*. 2012). However, it should be noted that in the present study lipid content in the whole shrimp body decreased from 65.3 g kg^{-1} to 50.0 g kg^{-1} on a dry weight basis (P=0.0794) as the FDY supplementation levels increased from 0 to 60 g kg^{-1}, which indicates that FDY may have a potential to reduce lipid content of Pacific white shrimp.

5. Conclusion

The energy and protein digestibility of flash dried yeast are significantly lower than FM and SBM. Amino acids digestibility of FDY was lowest among the three ingredients tested. With regards to the growth trial, the use of FDY at 60 g kg^{-1} caused significant negative impacts on growth, feed conversion ratio and protein retention. Dietary flash dried yeast supplementation in the practical diets for Pacific white shrimp had no effects on the proximate composition of the whole shrimp body. Based on these results, further research regarding the effects of the low levels ($\leq$ 40 g kg^{-1}) inclusion of FDY in practical diets on immune responses of Pacific white shrimp is warranted.

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Table 1 Formulation and chemical composition of test diets used in the growth trial.

Ingredient (As is basis g kg^{-1} feed)	Diet code				
	D_1	D_2	D_3	D_4	D_5
Menhaden fish meal[1]	60.0	60.0	60.0	60.0	60.0
Soybean meal[2]	472.0	463.8	455.6	439.5	423.2
Corn protein concentrate[3]	60.0	60.0	60.0	60.0	60.0
Whole wheat[4]	280.0	280.0	280.0	280.0	280.0
Flash dried yeast[10]	0.0	10.0	20.0	40.0	60.0
Menhaden fish oil[2]	55.7	54.9	54.1	52.6	51.0
Trace mineral premix[5]	5.0	5.0	5.0	5.0	5.0
Vitamin premix[6]	18.0	18.0	18.0	18.0	18.0
Choline chloride[4]	2.0	2.0	2.0	2.0	2.0
Stay C[7]	1.0	1.0	1.0	1.0	1.0
Mono-dicalcium Phosphate[8]	18.0	18.0	18.0	18.0	18.0
Lecithin[9]	10.0	10.0	10.0	10.0	10.0
Cholesterol[4]	0.5	0.5	0.5	0.5	0.5
Corn starch[4]	17.8	16.8	15.8	13.4	11.3

[1] Omega Protein Inc., Houston, TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[4] MP Biomedicals Inc., Solon, OH, USA.
[5] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[6] Vitamin premix (g kg^{-1} premix): Thiamin.HCl, 4.95; Riboflavin, 3.83; Pyridoxine.HCl, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[9] The Solae Company, St. Louis, MO, USA.
[10] Archer Daniels Midland Company, Chicago, IL, USA.

Table 2 Proximate composition, phosphorus content, and amino acid profile of the ingredients used in the growth and digestibility trials.

Proximate composition[1] (As is g kg^{-1})	Flash dried yeast	Fish meal	Soybean meal
Crude protein	387.8	627.8	448.9
Moisture	43	79.9	109.7
Crude fat	68.5	105.6	37.8
Crude fiber	62.7	0	32
Ash	36.6	187.5	66.7
Phosphorus	8.6	31.5	6.6
Amino acids profile (As is %)			
Alanine	2.21	3.91	2.04
Arginine	2.00	3.68	3.35
Aspartic acid	3.39	5.34	5.10
Cysteine	0.43	0.47	0.62
Glutamic acid	4.36	7.47	8.24
Glycine	1.74	4.88	2.04
Histidine	0.94	1.63	1.20
Isoleucine	1.96	2.42	2.17
Leucine	3.43	4.21	3.57
Lysine	2.39	4.67	3.06
Methionine	0.80	1.61	0.66
Phenylalanine	1.94	2.39	2.35
Proline	1.89	3.08	2.39
Serine	1.77	2.11	1.90
Taurine	0.15	0.73	0.13
Threonine	1.83	2.41	1.75
Tryptophan	0.44	0.62	0.62
Tyrosine	1.48	1.67	1.64
Valine	2.20	2.99	2.34

[1] Ingredients were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Table 3 Proximate composition (g kg^{-1} as is) and amino acid profile (% as is) of the test diets used in the growth trial.

Proximate composition[1] (As is g kg^{-1})	D_1	D_2	D_3	D_4	D_5
Crude protein	362.0	364.6	358.7	367.4	363.5
Moisture	79.2	74.1	85.2	67.7	68.2
Crude fat	79.7	94.6	85.9	95.8	103.8
Crude fiber	37.2	37.8	37.3	35.7	34.1
Ash	63.4	63.3	61.8	62.3	61.4
Amino acids profile (As is %)					
Alanine	1.84	1.84	1.84	1.86	1.89
Arginine	2.19	2.2	2.19	2.19	2.21
Aspartic acid	3.34	3.33	3.32	3.32	3.35
Cysteine	0.49	0.49	0.50	0.50	0.50
Glutamic acid	7.12	7.03	6.94	6.97	7.02
Glycine	1.58	1.58	1.59	1.60	1.61
Histidine	0.88	0.88	0.88	0.89	0.90
Isoleucine	1.61	1.64	1.64	1.65	1.67
Leucine	3.19	3.20	3.18	3.21	3.25
Lysine	2.00	2.00	1.99	2.00	2.02
Methionine	0.60	0.60	0.60	0.61	0.62
Phenylalanine	1.82	1.82	1.81	1.82	1.84
Proline	2.36	2.32	2.31	2.33	2.34
Serine	1.67	1.59	1.58	1.58	1.64
Taurine	0.15	0.15	0.15	0.15	0.15
Threonine	1.31	1.30	1.30	1.31	1.34
Tryptophan	0.43	0.45	0.43	0.44	0.43
Tyrosine	1.26	1.27	1.27	1.28	1.29
Valine	1.77	1.80	1.80	1.82	1.83

[1] Diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Table 4 Composition of reference diet for the determination of digestibility coefficients of flash dried yeast (FDY), fishmeal (FM), and soybean meal (SBM).

Ingredients	g kg^{-1} as is
Menhaden fish meal[2]	100.0
Soybean meal[1]	325.0
Menhaden fish oil[2]	32.0
Whole wheat[3]	476.0
Trace mineral premix[4]	5.0
Vitamin premix w/o choline[5]	18.0
Choline cloride[5]	2.0
Stay C[6]	1.0
Corn starch[3]	10.0
Lecithin[7]	10.0
Chromic oxide[7]	10.0

[1] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[2] Omega Protein Inc., Houston, TX, USA.
[3] MP Biomedicals Inc., Solon, Ohio, USA.
[4]Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[5] Vitamin premix (g/kg premix): Thiamin.HCl, 4.95; Riboflavin, 3.83; Pyridoxine.HCl, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[6] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ,USA.
[7] The Solae Company, St. Louis, MO, USA.

Table 5 Performance of juvenile Pacific white shrimp *L. vannamei* (1.78±0.03g) offered diets with different flash dried yeast (FDY) levels (0, 10, 20, 40, and 60 g kg^{-1}) for six weeks.

Diet	Final biomass (g)	Final mean weight (g)	WG (%)[4]	FCR[2]	Survival (%)	PRE (%)[3]
D_1	103.04[a]	10.74[a]	503.84[a]	1.40[b]	96.0	36.93[a]
D_2	105.03[a]	10.99[a]	516.94[a]	1.38[b]	96.0	37.32[a]
D_3	102.70[a]	10.48[a]	498.64[a]	1.44[b]	98.0	37.08[a]
D_4	97.16[a]	10.35[a]	489.64[a]	1.48[b]	94.0	35.44[a]
D_5	86.38[b]	9.61[b]	441.09[b]	1.64[a]	90.0	32.52[b]
P-value	0.0007	0.0018	0.0076	<0.0001	0.4909	0.0124
PSE[1]	1.228	0.0928	5.986	0.0131	1.4422	0.9773

[1] PSE: Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] PRE: Protein retention efficiency = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein intake.
[4] WG: Weight gain = (Final weight-initial weight)/initial weight × 100%.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 6 Proximate analysis of whole shrimp body offered diets with different flash dried yeast (FDY) levels (0, 10, 20, 40, and 60 g kg^{-1}) for six weeks.

Diet	Proximate composition (g kg^{-1})				
	Protein[2]	Moisture	Lipid[2]	Crude fiber[2]	Ash[2]
D_1	810.9	776.4	65.3	56.1	120.2
D_2	802.1	775.5	59.7	55.1	120.3
D_3	807.3	773.0	59.8	56.9	124.7
D_4	791.9	767.5	56.1	55.5	120.6
D_5	807.7	771.8	50.0	52.8	119.9
P-value	0.2649	0.5580	0.0801	0.5548	0.7707
PSE[1]	2.8031	1.7898	1.6074	0.7855	1.3021

[1] PSE: Pool standard error.
[2] Dry weight basis.

Table 7 Apparent dry matter (ADMD), apparent energy (ADE) and apparent protein (ADP) digestibility values for the diet (D) and ingredient (I) using 70:30 replacement technique offered to Pacific white shrimp *L. vannamei*.

Diet	ADMDD (%)	ADED (%)	ADPD (%)	ADMDI (%)	ADEI (%)	ADPI (%)
Basal diet 1	76.38[a]	82.66[a]	92.08[a]			
Flash dried yeast	62.18[c]	69.18[c]	79.47[b]	29.04[c]	38.20[b]	53.47[c]
Soybean meal	75.42[a]	81.42[a]	94.00[a]	73.18[a]	78.20[a]	97.03[a]
Fish meal 1	68.21[b]	78.31[ab]	80.86[b]	49.15[b]	69.77[a]	67.07[b]
Basal diet 2	75.93[a]	82.10[a]	92.10[a]			
Fish meal 2	67.99[b]	76.44[b]	82.34[b]	49.45[b]	65.78[a]	71.30[b]
PSE[1]	0.5580	0.5601	0.3655	2.2552	1.9425	1.1743
P-value	<0.0001	<0.0001	<0.0001	0.0004	0.0002	<0.0001

[1] PSE: Pool standard error.
FM1 and FM2 represent the first collection and second collection, respectively.
Basal diet 1 and Basal diet 2 represent first collection and second collection basal diet, respectively.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 8 Apparent amino acids (AA) digestibility value for the soybean meal (SBM), fish meal (FM) and flash dried yeast (FDY) using 70:30 replacement technique offered to Pacific white shrimp *L. vannamei*.

AA digestibility coefficients (%)	SBM	FM	FDY	PSE[1]	*P*-value	Adjust *P*-value
Alanine	93.75[a]	69.09[b]	51.31[b]	2.6712	0.0019	0.004
Arginine	96.91[a]	75.35[b]	67.83[b]	2.3361	0.0056	0.0069
Aspartic acid	95.39[a]	69.23[b]	62.40[b]	1.9463	0.0010	0.0029
Cysteine	91.29[a]	54.39[b]	34.80[b]	4.1270	0.0039	0.0055
Glutamic acid	95.69[a]	70.84[b]	56.83[b]	2.1174	0.0008	0.0028
Glycine	95.06[a]	66.55[b]	38.81[c]	3.1679	0.0011	0.0029
Histidine	94.33[a]	74.26[ab]	61.75[b]	2.9546	0.0115	0.0127
Hydroxylysine	97.28[a]	64.83[b]	39.89[c]	1.5132	<0.0001	<0.0001
Isoleucine	93.23[a]	68.72[b]	64.80[b]	2.1605	0.0034	0.0051
Leucine	92.23[a]	71.29[b]	60.02[b]	2.5186	0.0055	0.0069
Lysine	95.03[a]	76.97[ab]	66.88[b]	2.4289	0.0089	0.0104
Methionine	95.20[a]	70.63[b]	68.55[b]	1.4766	0.0006	0.0028
Phenylalanine	93.41[a]	65.28[b]	60.29[b]	2.4329	0.0029	0.0047
Proline	94.68[a]	67.21[b]	50.53[b]	2.8633	0.0022	0.0042
Serine	93.11[a]	58.31[b]	48.19[b]	2.4971	0.0008	0.0028
Taurine	50.56[ab]	90.28[a]	30.54[b]	6.0375	0.0180	0.0189
Threonine	91.99[a]	66.33[b]	59.45[b]	2.0842	0.0016	0.0037
Tryptophan	95.37[a]	80.31[b]	80.39[b]	0.6973	0.0002	0.0021
Tyrosine	95.28[a]	73.62[b]	72.85[b]	1.3263	0.0007	0.0028
Valine	90.78[a]	67.06[ab]	59.88[b]	3.5325	0.0271	0.0271
Total AA	94.31[a]	69.91[b]	58.33[b]	2.4113	0.0024	0.0042

[1] Pooled standard error

Values within a row with different superscripts are significantly different based on Tukey's multiple range test.

CHAPTER III

EVALUATION OF THREE NON-GENETICALLY MODIFIED SOYBEAN CULTIVARS AS INGREDIENTS AND A YEAST-BASED ADDITIVE AS A SUPPLEMENT IN PRACTICAL DIETS FOR PACIFIC WHITE SHRIMP *Litopenaeus vannamei*

1. Introduction

Soybean meal (SBM) is usually considered as the most reliable ingredient and cost-effective protein source in shrimp feed because of its worldwide availability, low price, relatively balanced amino acid profile, and consistent composition (Amaya *et al*. 2007b; Davis & Arnold 2000). There have been a number of studies demonstrating the successful use of conventional SBM in practical diets for Pacific white shrimp, *Litopenaeus vannamei* (Amaya *et al*. 2007b; Roy *et al*. 2009; Sookying & Davis 2011; Zhu *et al*. 2013). Albeit commodity SBM is still an acceptable protein source with good digestibility for shrimp, it also has some potential limitations associated with insufficient levels of essential amino acids (EAA) such as methionine and lysine, presence of anti-nutritional factors (ANFs) for example trypsin inhibitors, and poor palatability, which may limit its potential use in feed formulations (Dersjant-Li 2002). Traditionally, supplementation methionine and lysine and inclusion of attractants or palatability enhancers in SBM-based diets to meet the shrimp requirement is recommended to provide a good growth response (Akiyama 1989). Also, ANFs such as trypsin inhibitors present in raw soybean can negatively affect nutrient digestion and availability but this can be reduced or eliminated through heat processing (Dersjant-Li 2002; New 1987).

Alternatively, novel soy breeding technology can be used to develop new soybean cultivars, which selectively decrease certain heat-resistant ANFs (such as oligosaccharides), while increasing the protein content of the resulting meals. Building on this progress, unconventional, non-thermal (energy-saving), nutrient-preserving processing methods can be used to produce these novel meals. The resulting meals are of considerable interest in aquaculture because they may potentially provide improved feed ingredients with no need for genetic modifications of the native soy DNA, therefore, resulting in nutritionally superior products (Fang *et al*. 2016). In addition, selective soy breeding allows for a tight control on some functional compounds that may promote immunogenicity in shrimp. As shrimp culture has become an expanded and intensified economic activity, bacterial and viral diseases are considered a threat to the future progress of semi-intensive and intensive shrimp culture (Pérez-Sánchez *et al*. 2014). Traditional antibiotics supplementation can prevent or treat diseases but it may also develop resistant bacterial strains immune to antibiotic treatment. In addition, the antibiotic residues in cultured animals may act in detriment of human health (Cabello 2006; Sharifuzzaman & Austin 2009; Taylor *et al*. 2011). To tackle disease problems and avoid potential disadvantages of antibiotic use, alternative strategies, for example the use of probiotics (e.g., yeast) and prebiotics has received considerable attention in recent years (Li & Gatlin 2005).

Products of various fermented yeast, not containing live/viable cells, often contain primary metabolites, such as nucleotides, polysaccharides, small peptides, organic acids and lipids. Some of these materials have been demonstrated to improve the growth, survival, and immune response of *L. vannamei* in several studies (Deng *et al*. 2013; Tipsemongkol *et al*. 2009).

Our laboratory has already evaluated digestibility values of some new non-genetically modified (non-GM) soybean cultivars and the effects on the growth performance of *L.vannamei*

(Fang *et al*. 2016; Zhou *et al*. 2015). However, information about the impacts of novel processing on selected soybean cultivars on immune response is still needed. Therefore, the purposes of this study were to investigate the effects of three new, non-GM soybean cultivars processed with novel technologies on the growth performance and immune responses of *L. vannamei* and to verify the effects of a fermented yeast product on growth and immunity of *L. vannamei*.

2. Materials and Methods

2.1 Ingredients preparation

Four sources of SBM, including three new, non-GM soybean cultivars, were obtained for evaluation of their potential use as ingredients in *L. vannamei* feeds. Commodity SBM soy was obtained from Bunge Limited, Decatur, AL, USA. The non-GM ingredients were processed via proprietary technology and donated by Navita^{TM} Premium Feed Ingredients (NPFI), West Des Moines, IA, USA. They were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate composition, phosphorus content, trypsin inhibitor, urease activity, acid detergent fiber (ADF), neutral detergent fiber (NDF), protein dispersibility index (PDI), starch, and amino acid profiles in the diets were formulated (Tables 2).

2.2 Experiment design and diets

Two growth trials were conducted to evaluate biological response of shrimp to three novel soybean cultivars and a fermented yeast commercial product in practical diets with regards

to growth and feed utilization. All test diets were formulated to be iso-nitrogenous and iso-lipidic (35% protein and 8% lipid). A total of 9 experimental diets were formulated (Table 1). The basal diet (1) was primarily composed of a commodity SBM, fishmeal (FM), whole wheat, corn protein concentrate, poultry by-product meal (PM, pet food grade) and corn starch. For the experimental diets, either low (L) or high (H) inclusion levels of three novel soybean ingredients (N1-N3) were utilized to replace all the conventional SBM and to partially substitute FM and PM in the basal diet. Additionally, the last two diets (8 and 9) were essentially the same as diets 1 and 3 but supplemented with a commercially available fermented yeast product.

Pre-ground dry ingredient and oil were mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 minutes. Hot water was then blended into the mixture to obtain a consistency appropriate for pelleting. Diets were pressure-pelleted using a meat grinder with a 3 mm die, air-dried (<50 °C) to a moisture content of 8-10%. Pellets were crumbled, packed in sealed plastic bags and stored in a freezer (-20 °C) until needed. As the ingredients, the diets were also analyzed at University of Missouri for proximate composition (g 100 g^{-1} as is) and amino acid profile (g 100 g^{-1} as is) (Table 3).

2.3 Growth trials

Two trials were conducted at the E.W. Shell Fisheries Research Station, Auburn University (Auburn, AL, USA). Pacific white shrimp post larvae (PL) were obtained from Shrimp Improvement Systems (Islamorada, Florida) and nursed in an indoor recirculating system. PLs were fed a commercial feed (Zeigler Bros., Inc., Gardners, Pennsylvania, USA) using an automatic feeder for ~1 week, and then switched to crumbled commercial shrimp feed (Rangen® Inc., Buhl, Idaho, USA) for ~1- 2 weeks.

At the end of the nursery phase, juvenile shrimp (initial mean weight 0.15 g for trial 1; 0.20 g for trial 2) were obtained from the nursery system and hand-sorted to uniform size. Both growth trials utilized 9 treatments with 6 replicates in each treatment. In trial 1, juvenile shrimp were stocked into 54 tanks with 15 shrimp per aquarium in a semi-recirculation system. In trial 2, juvenile shrimp were stocked into 54 tanks with 10 shrimp per aquarium in the same semi-recirculation system as the trial 1. The semi-recirculation system consisted of 54 aquaria (60 L) connected to a common reservoir, biological filter, bead filter, fluidized biological filter and recirculation pump.

During the two trials, shrimp were fed four times daily over 46 days for trial 1 and 35 days for trial 2. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality. Based on the historic results in our laboratory, daily feed rations were initially calculated assuming a 1.8 FCR and doubling in size the first three weeks for trial 1, first two weeks for trial 2 and an increment of 0.8 g/week thereafter. Consequently, for each tank in trial 1, a fixed ration of 0.58 g day^{-1} for the first week, 1.16 g day^{-1} for the second week, 2.31 g day^{-1} for the third week, and 3.09 g day^{-1} for the remaining culturing period was offered, partitioned in 4 feedings each day. For each tank in trial 2, a fixed ration of 0.51 g day^{-1} for the first week, 1.03 g day^{-1} for the second week, 1.85 g day^{-1} for the third, forth, and fifth week was also allotted in 4 portions per day. At the conclusion of each growth trial, shrimp were counted and group-weighted. Mean final weight, FCR, WG, biomass, and survival were determined (Table 4).

2.4 Physiological assessment

A second recirculation system was used to condition sub-adult shrimp for the collection of physiological samples. The culture system was equivalent to the previously described system but utilized a series of 130 L aquaria which were stocked with 6 sub-adult shrimp (14.8 g mean weight). Shrimp were offered diets 1, 3, 8, and 9 to slight excess over a 15 days period. At the conclusion of the conditioning period the shrimp were sacrificed and hemolymph samples were collected for assessment of total hemocyte count (THC), hyaline cells count (HCC), granular cells count (GCC), semi-granular cells count (SGCC), hemolymph glucose (HG), hemolymph packed cells volume (HPCV), hemolymph protein (HP), hemocyte phagocytic capacity (HPC), and hemocyte respiratory burst activity (HRBA) (Table 8). A sample of hemolymph was obtained from each shrimp using a 25 gauge needle through the dorsal surface, and the glucose level was determined using a handheld glucometer (Abbott Diabetic Care, Inc., Alameda, CA). The remainder of each sample was loaded into a hematocrit tube, capped and centrifuged at 10,000 rpm for 5 minutes, and the packed hemocyte volume was read using a Micro-Hematocrit Capillary Tube Reader (Monoject Scientific, St. Louis, MO). After this step, the hemolymph protein content was determined by placing a drop of the supernatant onto a handheld protein refractometer (VEEGEE Scientific Inc. Kirkland, WA). An additional sample of hemolymph was collected into an equal volume of anticoagulant and a 20 µl aliquot of the hemolymph and anticoagulant mixture was added to an equal volume of Trypan Blue and allowed to sit for 1 minute. Cells were counted using a hemocytometer, and one corner of 16 squares was read. Cell number per ml for both total and differential counts was determined by the following formula: cell number x dilution factor (4) x 10^6. After cell counting was completed, the cell numbers were standardized using PBS to dilute the samples. The respiratory burst activity of the cells was

assessed in the following manner using the standardized samples: 100 µl of sample and 100 µl of zymosan solution were added in triplicate to a 96-well plate and incubated at room temperature for 30 minutes. The zymosan solution was then removed, the wells were rinsed three times with 100 µl PBS, and 100 µl of NBT solution was added and incubated for 30 minutes and removed. The wells were then fixed with absolute methanol, and rinsed three times with 70% methanol. Subsequently, 120 µl of KOH and 140 µl DMSO were added to dissolve the DBT diforazon precipitate. Plates were then read in a spectrophotometer at 600 nm for comparison of absorbance (Yeh *et al*. 2004). To determine phagocytic capacity, an additional sample of 50 µl of hemolymph with anticoagulant was loaded induplicate on Esco Fluor glass slides and incubated at room temperature for 90 minutes. After incubation, 50 µl of zymosan solution was added and incubated at room temperature for 60 minutes. The slides were then washed with PBS, air dryed, and fixed in methanol, and stained with Wright stain. At least 100 hemocytes per slide were microscopically examined at 100 x magnification for evidence of phagocytic activity, and phagocytic capacity was determined by the percentage containing 5 or more zymosan particles (Mustafa *et al*. 2000).

2.5 Water quality monitoring

For both trials, dissolved oxygen (DO), water temperature and salinity were measured twice daily by using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH, USA). Hydrogen potential (pH) was measured twice weekly by using a waterproof pHTestr30 (Oakton instrument, Vernon Hills, IL, USA). Total ammonia-nitrogen (TAN) and nitrite were evaluated every week by using the methods described by Sororzano (1969) and Spotte (1979).

2.6 Statistical analysis

All data was analyzed using SAS (V9.3. SAS Institute, Cary, NC, USA). For the growth trial data was analyzed using one-way ANOVA to determine significant differences ($P < 0.05$) among treatments followed by Tukey's multiple comparison test to determine difference between treatment means. Principle component analysis (PCA) was performed to reduce the dimensions. Multiple linear regression was utilized to detect the relationship of WG or FCR with principle components selected from PCA. Correlation coefficient analysis was utilized to identify the relationships between trypsin inhibitor, methionine and lysine levels in the diets and the shrimp's biological responses. Data from the physiological assessment was analyzed using two-way ANOVA to evaluate soy sources across fermented yeast product supplementation levels.

3. Results

3.1 Water quality

In trial 1, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 6.01±0.38mg L^{-1}, 27.8±1.1°C, 7.4±0.3ppt, 7.21±0.20, 0.068±0.037mg L^{-1}, and 0.201±0.090mg L^{-1}, respectively. In trial 2, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 5.63±0.28mg L^{-1}, 29.3±0.4°C, 5.1±0.1ppt, 7.67±0.22, 0.030±0.023mg L^{-1}, and 0.023±0.016mg L^{-1}, respectively. Water quality conditions in both of the trials were suitable for normal growth and survival of this species.

3.2 Growth trials

Growth performance of juvenile *L. vannamei* offered diets with different ingredients are presented in Table 4. In trial 1, significant differences were detected in final biomass, final mean

weight, WG, FCR, and survival when shrimp were fed with various diets. Highest final biomass (65.74 g) was observed in shrimp fed with the basal diet. Shrimp fed diets 2 and 3 (LN1 and HN1, respectively) exhibited the highest final mean weight (5.08 g) and WG (3,463%), while shrimp fed diet 5 (HN2) had the lowest values for those two indicators (4.06 g and 2,627%, respectively). The FCR range spanned from 1.38 to 1.75 and the lowest value was observed in shrimp fed diet 2 (LN1), while the highest FCR was found in shrimp fed diet 3 (HN2). Survival ranged from 65.6 to 91.1% and the lowest survival was observed in shrimp fed diets 2 and 3. In contrast, the highest survival was documented for shrimp fed the basal diet.

Similarly, trial 2 revealed that final biomass, final mean weight, WG, and FCR were significantly affected by dietary treatment. Shrimp fed diet 2 had the highest final biomass, final mean weight, and WG (36.72 g, 4.02 g, and 2,050%, respectively), while shrimp fed diet 3 exhibited the lowest values for these indices (27.32 g, 3.29 g, 1,519%, respectively). In trial 2 FCR ranged from 1.34 to 1.74 and the lowest value was observed in shrimp fed diet 8, while the highest FCR was found in animals fed diet 3. In this second trial survival ranged from 78.3 to 93.3% but no significant differences were observed among shrimp fed the various diets.

Principle component analysis (PCA) of trypsin inhibitors and essential amino acids and their loadings of experimental diets are presented in Table 5. The first principle component (PC) explained 71.1% of total sample variance. Collectively, the first three PCs explained 92.86% of total samples variance. Loadings of essential amino acids are similar in PC1 (ranged from 0.234 to 0.347). The only negative loading in PC1 is trypsin inhibitor (-0.153). In PC2, loadings of methionine and lysine are -0.495 and -0.205 respectively. Trypsin inhibitor (loading=0.836) is in charge of the third PC.

The results of multiple linear regression of WG and FCR on the first three PCs (PC1, PC2, and PC3) are presented in Table 6. *P*-value for all the models are less than 0.05. In trial 1, PC3 has a significantly negative impact on WG while it has positive effect on FCR. In trial 2, PC2 and PC3 have significantly negative influence on WG, however they have positive effect on FCR. The first PC has significantly positive effect on WG while it has negative effect on FCR. Combined the results of PCA and multiple linear regression, we may conclude that the significantly reduced growth may be attributed to the high trypsin inhibitor level and relatively insufficient essential amino acids (mainly methionine and lysine) levels as well as their combined effects.

Pearson correlation coefficients of growth performances (final biomass, final mean weight, and WG), FCR and survival with dietary trypsin inhibitor, methionine or protein levels are presented in Table 7. In trial 1 and 2, dietary trypsin inhibitor levels negatively correlated with growth performance, while positively correlating with FCR. Similarly, dietary lysine positively correlated with WG while negatively correlating with FCR. Finally, methionine levels positively correlated with WG while negatively correlating with FCR in trial 2. However, no correlations of methionine levels were found for WG or FCR in trial 1.

3.3 Physiology trial

The two-way ANOVA analysis for stress and immune responses of *L. vannamei* fed different soy ingredients and fermented yeast are presented in Table 8. The combined effect soy*yeast was significant ($P = 0.035$) for hemolymph glucose (HG) but the most significant effect ($P = 0.003$) was found for the fermented yeast significantly reducing granular cells counts (GCC). Another important effect was identified when soy ingredients significantly reduced the

hemolymph pack cells volume (HPCV). No significant differences were observed in total hemocyte counts (THC), hyaline cells counts (HCC), semi-granular cells counts (SGCC), hemolymph protein (HP), hemocyte phagocytic capacity (HPC), or hemocyte respiratory burst activity (HPBA).

4. Discussion

Dietary protein is one of the most important factors affecting growth performance of shrimp and feed cost (Hu *et al*. 2008). Reducing or replacing costly animal protein sources through the use of more economical plant protein sources such as SBM could result in substantial saving in feed cost. However, the presence of ANFs in SBM may affect the digestion and reduce nutrient availability to shrimp (Dersjant-Li 2002), thus limiting the inclusion level of SBM in practical shrimp diets. By using novel soybean breeding technology, improved soybean varieties with reduced levels of ANFs and enhanced protein and lipid content could improve nutrient bioavailability, and therefore allow for the supplementation of these ingredients in practical shrimp feeds at higher concentrations preventing adverse effects on the digestive physiology of shrimp. An added benefit of these novel ingredients, compared to commodity SBM is that these typically have higher protein content which could help meet the shrimp's dietary protein requirement at a lower inclusion level; thus, opening more formulation space in the diet (Fang *et al*. 2016).

SBM has been successfully utilized as a protein source in practical diets for Pacific white shrimp. Amaya *et al*. (2007b) demonstrated that FM could be completely replaced with plant protein by including 39.52% SBM in combination with 16% PM in practical shrimp feeds without compromising production or economic performance of *L. vannamei* reared in ponds. In a

different study, Amaya et al. (2007a) also confirmed that good performance can be attained when shrimp is fed a plant protein-based feed with 56.46% solvent extracted SBM in combination with 1% squid meal (SM). Moreover, SBM levels up to 58% have been demonstrated to be feasible in practical shrimp diets without adversely affecting WG, survival, or FCR (Roy *et al*. 2009; Sookying & Davis 2011). The apparent success of such high dietary SBM levels indicates that this product can serve as the primary protein source in practical shrimp diets and thus, the further evaluation of novel soy cultivars and processing technologies is paramount to further enhance shrimp aquaculture.

Protein quality of SBM is linked to both the reduction of ANFs and the optimization of protein digestibility. Of tests commonly used, the evaluation of urease activity (UI) and trypsin inhibitors are especially useful to identify under processed SBM (Căpriţă *et al*. 2010). PDI measures the dispersibility of proteins in water and is generally used as qualitative parameter of protein denaturation upon thermal processing of SBM (Qin *et al*. 1996). Manufacturers of soy-based fish and shrimp feeds require low PDI values to prevent losses of valuable protein into the aquatic environment (Reinitz 1984). Combining results from PDI and urease tests could become a useful tool to monitor SBM quality. In the current study, protein contents of ingredients N1-3 (55.33, 47.61, and 49.38 % versus 45.98 %) and lipid contents of N2 and N3 were enhanced (4.98 and 6.66 % versus 1.54 %) compared to the commodity SBM by using the novel soy breeding technology (Table 2). However, the trypsin inhibitor levels, urease activity, and PDI of N2 and N3 were also higher than the commodity SBM (18,423 and 16,943 TIU g^{-1} vs 8,656 TIU g^{-1}, 2.27 and 2.20 vs 0.04, and 49.17 and 85.19 vs 26.69) (Table 2). This is due to the fact that N1 originates in a different cultivar than N2 and N3 and although the second cultivar has significantly less trypsin inhibitors, the novel processing options utilized were not able to

sufficiently reduce these indices to levels comparable to traditional roasting of SBM. Because as oil is extracted a concomitant concentration of protein occurs in soy products and due to the fact that both the Kunitz and Bowman-Birk protease inhibitors are proteins, more research is needed to fine tune the non-thermal novel processing options such that these are able to attain similar levels of ANFs inactivation as traditional heat treatments (DiPietro & Liener 1989). The suboptimal inactivation resulted in higher trypsin inhibitors levels in the diets (Table 4).

Trypsin inhibitors have been demonstrated to result in growth depression and pancreatic hypertrophy in numerous experimental animals (Lim & Akiyama 1992), such as Atlantic salmon *Salmo salar* (Olli *et al*. 1994) and Rainbow trout *Oncorhynchus mykiss* (Kaushik *et al*. 1995) as well as Pacific white shrimp (Fang *et al*. 2016; Zhou *et al*. 2015). Negative effects on protein digestibility have been attributed to trypsin inhibitors due to their capacity to bind digestive enzymes (Francis *et al*. 2001), which may negatively affect growth performance of the Pacific white shrimp. In the present study, shrimp fed diet 5 (HN2) showed significantly reduced WG compared to the shrimp fed diets 2 (LN1) in trial 1 and 2, as well as compared to shrimp fed with diets 1, 3 and 8 in trial 2. The FCR was significantly increased when shrimp consumed diet 3 (HN2) in contrast with shrimp fed with diets 1, 2, 3, and 8 in trial 1 and 2 as well as compared to shrimp fed diet 6 (LN3) in trial 2. The trends of the WG and FCR in trial 1 were similar to those in trial 2, which points to consistency between results of the two trials. There were slight differences in the statistics analysis results, which may have resulted from data variation.

In both of the trials, shrimp fed diet 5 exhibited lowest growth across all the treatments. As diet 5 contained highest trypsin inhibitor level and lowest methionine and lysine levels, we suspect if trypsin inhibitor level and methionine and lysine levels caused the depressed growth. In order to demonstrate our thoughts, PCA and multiple regression analysis were performed. In

trial 1, the multiple regression results showed that PC3 had a significantly negative impact on WG while it had positive effect on FCR. From the loading of PC3 (Table 5), PC3 is positively dominated by trypsin inhibitor level (loading=0.836). Combined the results of PCA and multiple regression analysis in trial 1, we can infer that trypsin inhibitor pose negative effect on WG but positive effect on FCR. In trial 2, PC2 and PC3 have significantly negative influence on WG, however they have positive effect on FCR. PC2 is negatively dominated by lysine (loading=-0.495) followed by methionine (-0.205). Combined the results of PCA and multiple regression analysis in trial 2, we can infer that trypsin inhibitor pose negative effect on WG but positive effect on FCR. Methionine and lysine levels had positive effect on WG but negative effect on FCR. Therefore, from the results of PCA and multiple regression analysis, we may infer that the significantly reduced growth may be attributed to the high trypsin inhibitor level and relatively insufficient essential amino acids (typically methionine and lysine) levels as well as their combined effects. Also, the results of correlation analysis in the present experiment confirmed that protease inhibitor levels in the diets negatively correlated with shrimp growth while positively correlated with FCR. Similarly, Zhou *et al*. (2015) and Fang *et al*. (2016) reported that the growth performance of Pacific white shrimp was significantly improved when shrimp fed with diets contained different soybean cultivars with a reduced level of trypsin inhibitors. Based on correlation analysis, trypsin inhibitors levels in the soybean meal cultivars were confirmed to be negatively correlated with protein digestibility (Zhou *et al*. 2015) and shrimp growth (Fang *et al*. 2016).

Methionine and lysine are generally the most limiting amino acids in the plants and rendered animal byproducts. The correlation analysis in the current study demonstrated that lysine level in the diets positively correlates with growth performance while negatively

correlating with FCR in both trial 1 and 2. No correlation of methionine with WG and FCR were observed in trial 1, while methionine positively correlated with WG while negatively correlated with FCR in trial 2. The dietary lysine requirement for the Pacific white shrimp estimated by the broken-line model based on specific growth rate (SGR) was 2.05% of diet (dry weight basis) or 4.93% of dietary protein (Xie *et al*. 2012). With regards to the methionine requirement, the information for Pacific white shrimp is still limited. In the present study, dietary lysine and methionine levels (Table 4) were both lowest for diets 5 (lysine: 1.89% of diet, methionine: 0.51% of the diet) which might partially explained relatively reduced WG and increased FCR in this treatment.

In the current study, fermented yeast supplementation at 0.13% of diet did not affect the WG of the shrimp. Similarly, Tipsemongkol *et al*. (2009) reported that 0.125% supplementation of a fermented yeast product did not affect the WG of Pacific white shrimp. However, WG of shrimp was significantly improved when the supplementation increased to 0.25%. By contrast, Deng *et al*. (2013) indicated that final mean weight of Pacific white shrimp was significantly improved under pond conditions when a fermented yeast product was included at both 1.0 and 1.5 g kg^{-1}. In pond trials, natural food is available to shrimp, which may probably mask the effects of fermented yeast products on the growth performance of shrimp, thus causing the differences among these studies.

As for the physiological trial, independent and combined effects of the fermented yeast product and the soy ingredients were evaluated in a 2 × 2 factorial design (Table 7). An elevation of hemolymph glucose (HG) is considered as indicator of stress responses in invertebrates (Chang *et al*. 2016). Hemolymph packed cells volume (HPCV) measures the volume percentage of red blood cells in vertebrates and hemocytes in shrimp. A higher HPCV level is indicative of

an increased capacity of the hemocoel to transport oxygen (Birchard 1997). In the current study, the shrimp fed diets 3, 8 and 9 (HN1, and yeast product) exhibited a significantly higher HG and lower HPCV content, which indicates that the shrimp in these treatments were stressed. No other independent or combined effects of these particular diets were observed on immune responses (THC, HC, SGC, and HPC) of shrimp. However, GC in shrimp fed diets containing the yeast product was significantly lower than those fed yeast-free diets. Since no decreasing trends were observed in other immune responses indicators, the reduction of GC in the current study might be odd or due to the testing errors. Similarly, Tipsemongkol *et al*. (2009), reported that immune responses (THC, percentage phagocytosis, superoxide dismutase, and phenol oxidase activity) of Pacific white shrimp were not affected after 10, 20, and 30 days of feeding with a fermented yeast product at 0.125%. However, in the same study 0.125% supplementation of a fermented yeast product in shrimp diets significantly enhanced immune responses after 40 and 50 days of feeding with the product at 0.125% (Tipsemongkol *et al*. 2009). The time frame for the present physiological evaluation was only 15 days, which may be insufficient for immune system of shrimp to develop detectable responses to the inclusion of dietary fermented yeast.

5. Conclusion

In the present study, the growth performance of shrimp fed N1 diets was highest among the novel soy ingredients and the commodity SBM. This indicates that breeding technology and novel soy processing has the potential to increase the nutritional values of SBM for shrimp feeds. Observed trends on immune indicators of shrimp to both independent and combined effects of soy ingredients and fermented yeast were not easily discernible and may be related to a suboptimal exposure period.

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Table 1 Formulation and chemical composition of test diets used in the growth trial

Ingredient (As is basis g kg^{-1} feed)	Diet code								
	1	2	3	4	5	6	7	8	9
	Basal	LN1	HN1	LN2	HN2	LN3	HN3	Basal+yeast	HN1+yeast
Fish meal[1]	120.0	120.0	60.0	120.0	60.0	120.0	60.0	120.0	60.0
Poultry meal[5]	20.0	20.0	5.0	20.0	5.0	20.0	5.0	20.0	5.0
Soybean meal[2]	434.0	-	-	-	-	-	-	434.0	-
Navita 1[6]	-	322.0	416.0	-	-	-	-	-	416.0
Navita 2[6]	-	-	-	367.0	474.0	-	-	-	-
Navita 3[6]	-	-	-	-	-	372.0	480.0	-	-
Corn protein concentrate[3]	20.0	20.0	8.0	20.0	8.0	20.0	8.0	20.0	8.0
Whole wheat[4]	300.0	300.0	300.0	300.0	300.0	300.0	300.0	300.0	300.0
Fermented yeast product	-	-	-	-	-	-	-	1.3	1.3
Menhaden fish oil[2]	48.7	52.6	60.1	38.0	41.3	28.2	28.5	48.7	60.1
Trace mineral premix[7]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin premix[8]	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0
Choline chloride[4]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Stay C[9]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Mono-dicalcium phosphate[10]	19.0	19.0	25.0	19.0	25.0	19.0	25.0	19.0	25.0
Lecithin[11]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Cholesterol[4]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Corn starch[4]	1.8	109.9	89.4	79.5	50.2	84.3	57.0	0.5	88.1

[1] Omega Protein Inc., Houston TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[4] MP Biomedicals Inc., Solon, Ohio, USA.
[5] Tyson Foods, Inc., Springdale, AR, USA.
[6] Navita Premium Feed Ingredients,West Des Moines, IA, USA.
[7] Trace mineral premix (g/100 g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[8] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[9] Stay C®, (L-ascorbyl-2-polyphosphate 25% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[10] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[11] The Solae Company, St. Louis, MO, USA.

Table 2 Proximate analysis (as is g 100 g^{-1}), trypsin inhibitors levels (TIU g^{-1}), acid detergent fiber (ADF) and neutral detergent fiber (NDF) (as is g 100 g^{-1}), phosphorus (as is g 100 g^{-1}), urease activity (pH increase), protein dispersibility index (PDI), starch (as is g 100 g^{-1}) and amino acid (AA) profiles (as is g 100 g^{-1}) of solvent extracted soybean meal (SBM), navita 1, 2, and 3 utilized in the trials.

Composition[1]	Solvent extracted SBM	Navita 1	Navita 2	Navita 3
Crude Protein	45.98	55.33	47.61	49.38
Moisture	13.61	9.94	11.35	7.49
Crude Fat	1.54	0.58	4.98	6.66
Crude Fiber	3.18	4.44	4.46	4.78
Ash	5.73	6.28	5.64	5.91
ADF	6.93	14.9	10.86	15.09
NDF	7.24	13.87	14.31	10.82
Phosphorus	0.63	0.75	0.56	0.58
Trypsin inhibitor	8656	9396	18423	16943
Urease activity	0.04	0.06	2.27	2.20
PDI	26.69	15.22	49.17	85.19
Starch	2.23	0.00	0.74	0.14
Alanine	1.88	2.33	2	2.08
Arginine	3.18	4.1	3.55	3.64
Aspartic Acid	4.84	6.24	5.33	5.41
Cysteine	0.60	0.76	0.64	0.65
Glutamic Acid	7.51	9.66	8.34	8.51
Glycine	1.83	2.29	1.98	2.03
Histidine	1.27	1.44	1.24	1.25
Hydroxylysine	0.15	0.05	0.18	0.08
Hydroxyproline	0.08	0.06	0.06	0.06
Isoleucine	2.06	2.54	2.17	2.21
Leucine	3.56	4.26	3.67	3.77
Lysine	2.89	3.49	2.93	3.06
Methionine	0.59	0.75	0.65	0.65
Ornithine	0.07	0.06	0.04	0.05

Phenylalanine	2.34	2.78	2.42	2.46
Proline	2.30	2.75	2.36	2.41
Serine	1.92	2.42	2.13	2.15
Taurine	0.12	0.08	0.08	0.07
Threonine	1.70	2.1	1.82	1.86
Tryptophan	0.75	0.73	0.58	0.62
Tyrosine	1.85	1.86	1.78	1.81
Valine	2.05	2.69	2.3	2.36

[1] Ingredients were analyzed at University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory.

Table 3 Proximate analysis (as is g 100 g^{-1}), trypsin inhibitors levels (TIU/g), and amino acid (AA) profiles (as is g 100 g^{-1}) of the test diets utilized in the trials.

Composition[1] (As is %)	Diet code								
	1	2	3	4	5	6	7	8	9
	Basal	LN1	HN1	LN2	HN2	LN3	HN3	Basal+yeast	HN1+yeast
Crude Protein	35.42	34.17	32.73	34.20	34.02	33.54	33.70	35.51	32.91
Moisture	9.83	6.61	9.95	6.46	6.28	9.30	7.71	9.04	9.46
Crude Fat	8.42	9.17	7.82	8.72	8.72	7.80	7.61	8.74	8.32
Crude Fiber	3.45	2.93	3.23	4.22	4.09	3.31	3.58	3.43	3.12
Ash	7.59	7.19	6.68	7.19	6.92	6.93	6.86	7.48	6.70
Trypsin Inhibitor	2244	2185	2189	7358	8112	6848	7953	3207	2189
Alanine	1.67	1.60	1.44	1.61	1.45	1.54	1.46	1.65	1.42
Arginine	2.10	2.07	2.13	2.04	2.16	2.03	2.11	2.17	2.12
Aspartic Acid	3.15	3.08	3.12	3.02	3.16	2.93	3.13	3.22	3.12
Cysteine	0.43	0.43	0.43	0.42	0.44	0.40	0.42	0.45	0.44
Glutamic Acid	6.33	6.20	6.22	6.10	6.28	5.92	6.28	6.35	6.16
Glycine	1.79	1.66	1.46	1.72	1.48	1.59	1.48	1.71	1.46
Histidine	0.93	0.91	0.89	0.90	0.89	0.87	0.88	0.93	0.89
Hydroxylysine	0.09	0.08	0.07	0.11	0.12	0.09	0.07	0.08	0.07
Hydroxyproline	0.25	0.17	0.10	0.22	0.10	0.17	0.11	0.16	0.10
Isoleucine	1.54	1.49	1.45	1.49	1.48	1.42	1.49	1.57	1.47
Leucine	2.62	2.57	2.47	2.56	2.53	2.49	2.52	2.70	2.48

Lysine	2.05	1.99	1.93	1.92	1.89	1.89	1.92	2.07	1.89
Methionine	0.58	0.57	0.50	0.57	0.51	0.55	0.50	0.61	0.51
Ornithine	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Phenylalanine	1.64	1.59	1.59	1.59	1.62	1.54	1.61	1.68	1.60
Proline	1.96	1.74	1.67	1.92	1.70	1.81	1.68	1.83	1.78
Serine	1.53	1.49	1.49	1.44	1.45	1.38	1.47	1.49	1.41
Taurine	0.20	0.22	0.18	0.21	0.16	0.20	0.16	0.20	0.16
Threonine	1.27	1.22	1.18	1.20	1.20	1.17	1.19	1.27	1.17
Tryptophan	0.44	0.46	0.40	0.43	0.44	0.41	0.47	0.48	0.46
Tyrosine	1.01	0.98	1.07	0.96	1.10	1.06	1.01	1.12	1.08
Valine	1.59	1.55	1.52	1.55	1.53	1.49	1.52	1.65	1.50

[1] Diets were analyzed at University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory.

Table 4 Response of juvenile *Litopenaeus vannamei* (Initial weight 0.15±0.01g, 46 days in trial 1; initial weight 0.20±0.01g, 35 days in trial 2) offered diets with different soybean ingredients at varying levels and w/o fermented yeast.

Trial		Diet	Final Biomass (g)	Final Mean Weight (g)	WG[3] (%)	FCR[2]	Survival (%)
Trial 1 n = 6	1	Basal	65.74a	4.83ab	3332ab	1.46bc	91.1a
	2	LN1	53.51b	4.98ab	3463a	1.42bc	72.2bc
	3	HN1	49.79b	5.08a	3359ab	1.38c	65.6c
	4	LN2	49.98b	4.33bc	2831ab	1.65ab	77.8abc
	5	HN2	49.37b	4.06c	2627b	1.75a	81.1abc
	6	LN3	57.20ab	4.58abc	3128ab	1.54abc	83.3abc
	7	HN3	58.60ab	4.34bc	3032ab	1.62abc	90.0ab
	8	Basal+yeast	53.89b	4.75ab	3136ab	1.48bc	75.6abc
	9	HN1+yeast	55.01ab	4.61abc	2926ab	1.53abc	80.0abc
		P-value	0.0009	0.0001	0.0136	0.0002	0.0008
		PSE[1]	1.0492	0.0592	66.1983	0.0215	1.6183
Trial 2 n = 6	1	Basal	36.25a	4.05ab	2005a	1.39cd	90.0
	2	LN1	36.72a	4.02abc	2050a	1.41cd	91.7
	3	HN1	31.78abc	3.90abc	1880ab	1.45bcd	81.7
	4	LN2	27.72bc	3.60bcd	1731abc	1.59abc	78.3
	5	HN2	27.32c	3.29d	1519c	1.74a	83.3
	6	LN3	35.03ab	3.76abcd	1793abc	1.50bcd	93.3
	7	HN3	31.98abc	3.49cd	1652bc	1.63ab	91.7
	8	Basal+yeast	34.95ab	4.24a	1981ab	1.34d	83.3
	9	HN1+yeast	33.68abc	3.68bcd	1733abc	1.54abcd	91.7
		P-value	0.0003	<0.0001	<0.0001	<0.0001	0.2750
		PSE[1]	0.6483	0.0479	31.2517	0.0192	1.9902

[1] PSE: Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] WG: Weight gain = (Final weight-initial weight)/initial weight*100%.

Values within a column with different letters are significantly different based on Tukey's multiple range test.

Table 5 Principle component analysis of trypsin inhibitors and essential amino acids of diets.

	PC1	PC2	PC3
TrypsinInhibitor	-0.153	-0.013	0.836
Histidine	0.342	-0.095	-0.185
Isoleucine	0.346	0.105	0.145
Leucine	0.341	-0.152	0.219
Lysine	0.334	-0.205	-0.140
Cysteine	0.234	0.572	-0.225
Methionine	0.270	-0.495	0.095
Phenylalanine	0.316	0.333	0.093
Tryptophan	0.215	0.444	0.318
Threonine	0.342	-0.163	0.038
Valine	0.347	-0.089	0.084
Eigenvalue	7.821	1.302	1.093
% total variance	71.100	11.830	9.930
Cumulative eigenvalue	7.821	9.123	10.216
Cumulative %	71.10	82.93	92.86

Table 6 Multiple linear regression of weight gain (WG) and feed conversion ratio (FCR) with first three principle components (PC1 PC2 and PC3).

Trial		Model *P*-value and R^2	Parameter estimate			*P*-value for each variable		
			PC1	PC2	PC3	PC1	PC2	PC3
1	WG	*P*=0.0095 R^2=0.203	23.85	-96.10	-148.75	*P*=0.2433	*P*=0.0579	*P*=0.0082
	FCR	*P*<0.0001 R^2=0.368	-0.011	0.029	0.085	*P*=0.1161	*P*=0.0798	*P*<0.0001
2	WG	*P*<0.0001 R^2=0.418	35.39	-69.78	-86.83	*P*=0.0004	*P*=0.0035	*P*=0.0010
	FCR	*P*<0.0001 R^2=0.463	-0.026	0.045	0.063	*P*<0.0001	*P*=0.0033	*P*=0.0003

Table 7 Pearson correlation coefficients of growth performances (final biomass, final mean weight, and weight gain), feed conversion ratio (FCR), and survival with trypsin inhibitors, methionine and protein levels of the diets. The first line of each cell is the value of correlation coefficient and the second line of each cell is *P*-value.

Trial		Final biomass	Final mean weight	Weight gain	FCR	Survival
Trial 1	Trypsin inhibitors	-0.1350	-0.6063	-0.4081	0.5971	0.2647
		0.3304	<0.0001	0.0022	<0.0001	0.0530
	Methionine	0.1603	0.1850	0.1767	-0.1828	0.0064
		0.2468	0.1805	0.2013	0.1858	0.9633
	Lysine	0.2743	0.3561	0.3206	-0.3529	0.0080
		0.0448	0.0082	0.0181	0.0089	0.9542
Trial 2	Trypsin inhibitors	-0.4540	-0.5655	-0.5478	0.5964	-0.0661
		0.0006	<0.0001	<0.0001	<0.0001	0.6351
	Methionine	0.2926	0.5183	0.4694	-0.4950	-0.0493
		0.0318	<0.0001	0.0003	0.0001	0.7232
	Lysine	0.3851	0.6114	0.5536	-0.5915	-0.0233
		0.0040	<0.0001	<0.0001	<0.001	0.8273

Table 8 Two-way analysis output of stress and immune response of Pacific white shrimp *L. vannamei* fed with different soy sources and fermented yeast product levels.

Soy sources	DVA (%)	THC[1]	HCC[2]	GCC[3]	SGCC[4]	HG[5]	HPCV[6]	HP[7]	HPC[8]	HRBA[9]
AU soy	0	5.891	4.040	0.560	1.573	30.643	12.667	12.229	44.270	0.0252
Schillnger1	0	5.779	3.512	0.504	1.336	30.286	6.900	12.478	44.860	0.0247
AU soy	0.13	6.117	3.670	0.200	1.450	30.067	11.545	12.317	53.462	0.0230
Schillnger1	0.13	5.767	2.290	0.300	1.160	37.938	5.000	13.117	47.838	0.0155
PSE		0.1344	0.2241	0.0277	0.1437	0.4852	0.6090	0.3271	1.1782	0.0003
Soy sources		0.6510	0.0814	0.3114	0.4141	0.0384	0.0028	0.5982	0.4489	0.5534
Fermented yeast		0.8364	0.0842	0.0004	0.6069	0.0631	0.4374	0.6702	0.1220	0.3988
Soy sources *Fermented yeast		0.8221	0.3684	0.1915	0.9297	0.0346	0.8407	0.7567	0.4249	0.6072
Model		0.9595	0.0941	0.0022	0.8009	0.0089	0.0233	0.9023	0.3067	0.7134

[1] THC: Total hemocyte count (x10^6/ml).
[2] HCC: Hyaline cells count (x10^6/ml).
[3] GCC: Granular cells count (x10^6/ml).
[4] SGCC: Semi-granular cells count (x10^6/ml).
[5] HG: Hemolymph glucose (mg/dl).
[6] HPCV: Hemolymph packed cells volume (%).
[7] HP: Hemolymph protein (mg/ml).

[8] HPC: Hemocyte phagocytic capacity (%).
[9] HRBA: Hemocyte respiratory burst activity (OD).

CHAPTER IV

EVALUATION OF DRIED FERMENTED BIOMASS AS A FEED INGREDIENT IN PLANT-BASED PRACTICAL DIETS FOR JUVENILE PACIFIC WHITE SHRIMP *Litopenaeus vannamei*

1. Introduction

As shrimp consumption is expected to continue to increase globally, it is important to develop sustainable alternative ingredients in shrimp diets to support the rapid expansion of the shrimp industry (Achupallas *et al*., 2016). A range of traditional plant protein products from agricultural production such as soybean meal (SBM) and corn gluten meal (CGM) have been identified as appropriate alternative ingredients to complement or replace fish meal (FM) in shrimp feeds. To further enhance the protein composition of SBM and CGM, various processes have been utilized to create concentrates which have been used with good success in practical shrimp feed formulations (Alam *et al*. 2005; Bauer *et al*. 2012; Fang *et al*. 2016; Paripatananont *et al*. 2001; Qiu & Davis 2016a; b; c; d; Sookying *&* Davis 2012; Zhou *et al*. 2015). The successful replacement of FM by soy- and corn-based products can result in reduced cost of feed to a certain extent, however, the higher protein products are also somewhat expensive.

Coproducts of manufacturing processes have been gaining interests as alternative protein sources in shrimp feeds as they have high nutritional value for less cost, because these are derived from secondary streams of various commodity processing applications. Corn and wheat coproducts such as distiller's dried grains with solubles, fermented bacterial biomass, and ethanol yeast are the most popular coproducts from the biofuels and ethanol industries. Fermented bacterial biomass received considerable attention because of its well-known

properties of rapid growth and protein accretion in protein production (Kuhad *et al*. 1997; Stringer 1982). Various strains of bacteria with a carbohydrate sugar source such as molasses, sucrose, or glucose were utilized in the fermentation process for L-threonine (Melanie *et al*. 2015). This process results in a dried fermented biomass which is derived from the manufacture of L-thereonine by fermentation using *Escherichia coli*. It has a high protein content (up to 800 g kg^{-1}) and good amino acid (AA) composition, which may serve as a potential alternative protein ingredient in the aqua feed industry.

This DFB product has been confirmed to successfully replace FM and SBM in nursery diets for pigs (Perez *et al*. 2011) and served as a substitution for FM in practical diets for Florida pompano, *Trachinotus carolinus* (Melanie *et al*. 2015). However, information about the application of DFB in practical shrimp feed is still limited. Therefore, the purposes of this study were to determine the biological responses of Pacific white shrimp, *L. vannamei* to dietary DFB supplementation as a replacement for FM or soy protein concentrate (SPC) w/o corn protein concentrate (CPC). Additionally, the effects of two drying processes on nutritional value of the meals to shrimp were assessed.

2. Material and Methods

2.1. Experimental design and diets

All test diets were formulated to be iso-nitrogenous and iso-lipidic (350 g kg^{-1} protein and 80 g kg^{-1} lipid). In trial 1, the basal diet was primarily composed of FM, SBM, corn gluten meal, and whole wheat. Four experimental diets (DF0, DFB25, DFB50, and DFB100) were formulated to utilize increasing levels (0, 25, 50, and 100 g kg^{-1}) of DFB as a replacement of FM (Table 1). In trial 2, the basal diet was primarily consisted of FM, SBM, corn protein concentrate (CPC),

and soy protein concentrate (SPC). Nine experiment diets were formulated to be supplemented with increasing levels (0, 20, 40, 60, and 120 g kg^{-1}) of SDFB and GDFB) to replace SPC w/o CPC (Table 2).

Primary ingredients were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate and amino acid composition (Table 3). All experimental diets were produced at the Aquatic Animal Nutrition Laboratory at the School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University (Auburn, AL, USA), using standard procedures for shrimp feeds. Briefly, diets were prepared by mixing the pre-ground dry ingredients in a food mixer (Hobart, Troy, OH, USA) for 10–15 minutes. Hot water was then blended into the mixture to obtain a consistency appropriate for pelleting. Diets were pressure-pelleted using a meat grinder with a 3-mm die in trial and a 2.5-mm die in trial 2. The moist pellets were then placed into a fan-ventilated oven (< 50 °C) overnight in order to attain a moisture content of less than 10%. Dry pellets were crumbled, packed in sealed bags, and stored in a freezer until use. The diets from trial 2 were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate and amino acid composition (Table 4)

2.2. Growth trials

Two trials were conducted at the E.W. Shell Fisheries Research Station, Auburn University (Auburn, AL, USA). Pacific white shrimp post larvae (PL) were obtained from Shrimp Improvement Systems (Islamorada, Florida) and nursed in an indoor recirculating system. PLs were fed a commercial feed (Zeigler Bros., Inc., Gardners, Pennsylvania, USA) using an automatic feeder for ~1 week, and then switched to crumbled commercial shrimp feed (Zeigler Bros., Inc., Gardners, Pennsylvania, USA) for ~1- 2 weeks.

In trial 1, the recirculating system consisted of 16 square tanks (340 L) connected to a common reservoir, biological filter, bead filter, fluidized biological filter and recirculation pump. Four replicate groups of shrimp (0.59 g initial mean weight; 10 shrimp / tank) were offered diets using our standard feeding protocol over 6 weeks. Based on historic results, feed inputs were pre-programmed assuming the shrimp would double their weight weekly up to one gram then gain 0.8 g weekly with a feed conversion ratio (FCR) of 1.8. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality. Consequently, for each tank in trial 1, a fixed ration of 1.5 g day^{-1} for the first and second week and 2.1 g day^{-1} for the remaining culturing period was offered, partitioned in 4 feedings each day.

In trial 2, the recirculating system consisted of 45 aquaria (80 L) connected to a common reservoir, biological filter, bead filter, fluidized biological filter and recirculation pump. Four replicate groups of shrimp (2.34 g initial mean weight, 10 shrimp / tank) were offered diets using a standard feeding protocol over 6 weeks. Based on historic results, feed inputs were pre-programmed assuming the shrimp would double their weight weekly up to one gram then gain 0.8-1.4 g weekly with a feed conversion ratio (FCR) of 1.8. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality. Consequently, for each tank in trial 2, a fixed ration of 2.1 g day^{-1} for the first week, 2.6 g day^{-1} for the second week, 2.8 g day^{-1} for the third week, 3.3 g day^{-1} for the fourth and fifth week, and 3.6 g day^{-1} for the last week was allotted in 4 portions per day.

At the conclusion of each growth trial, shrimp were counted and group-weighed. Mean final weight, FCR, WG, biomass, and survival were determined (Table 5 and Table 6). After obtaining the final total weight of shrimps in each aquarium, four shrimps from each tank in trial

2 were frozen at -20 °C for subsequent determination of whole body composition. Proximate composition of whole shrimp was analyzed by Midwest Laboratories (Omaha, NE, USA). Protein retention was calculated as follows:

Protein retention efficiency (PRE, %) = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein offered.

2.3. Water quality monitoring

For both trials, dissolved oxygen (DO), water temperature and salinity were measured twice daily using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH, USA). Hydrogen potential (pH) was measured twice weekly using a waterproof pHTestr30 (Oakton instrument, Vernon Hills, IL, USA). Total ammonia-nitrogen (TAN) and nitrite were evaluated every week using the methods described by Solorzano (1969) and Spotte (1979).

2.4. Statistical analysis

All the data were analyzed using SAS (V9.3. SAS Institute, Cary, NC, USA). Data from trial 1 and 2 were analyzed using one-way ANOVA to determine significant differences ($P < 0.05$) among treatments followed by Tukey's multiple comparison test to determine difference between treatment means in each trial. The pooled standard errors were used across growth trials, as the variance of each treatment is the same. Analysis of covariance (ANCOVA) was performed to determine the importance of the processing methods (covariate) in trial 2.

3. Results

3.1. Water quality

In trial 1, DO, temperature, and salinity were maintained at 6.18 ± 0.41 mg L^{-1}, 29.7 ± 0.8 °C, and 2.8 ± 0.4 ppt, respectively. In trial 2, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 6.94 ± 0.47 mg L^{-1}, 28.5 ± 0.7 °C, 8.6 ± 0.7 ppt, 7.2 ± 0.4, 0.05 ± 0.05 mg L^{-1}, and 0.05 ± 0.05 mg L^{-1}, respectively. Water quality conditions in both of the trials were suitable for normal growth and survival of this species.

3.2. Growth trials

Performances of Pacific white shrimp offered diets with various DFB levels in Trials 1 and 2 are presented in Tables 5 and 6, respectively. In trial 1, final mean weight, WG and FCR were not negatively affected when shrimp fed with diets contained up to 50 g kg^{-1} DFB as a replacement of FM. However, a level of 100 g kg^{-1} DFB significantly decreased WG but increased FCR. No significant differences were detected in final biomass (57% to 62.7%) or survival (100%) across all the treatments.

In trial 2, shrimp fed with diet contained 20 g kg^{-1} GDFB performed the best across all the treatments in terms of final mean weight, WG, and FCR. GDFB can be utilized up to 120 g kg^{-1} to replace SPC and CPC without causing negative effects on growth performance and FCR. However, dietary SDFB supplementation at 60 and 120 g kg^{-1} significantly reduced final mean weight and WG of shrimp, while increased FCR compared to the treatment contained 20 g kg^{-1} GDFB. No significant impacts were detected in final biomass (80.4 to 92.2 g), survival (84 to 90 %), and PRE (27.5 to 35.9 %).

3.3. Whole body composition

Whole body proximate composition of shrimp when animals were offered diets contained various DFB levels from two sources are presented in Table 7. Shrimp fed with diets supplemented with 120 g kg^{-1} GDFB exhibited significantly lower body lipid level than those fed with diets containing 40 and 120 g kg^{-1} SDFB. Significant differences were detected in carcass moisture content among dietary treatments, however, Tukey's multiple comparison did not determine significant differences between the treatments, which may due to the variance. No significant differences were observed in protein (676.2 to 756.4 g kg^{-1}) or ash (83.1 to 119.4 g kg^{-1}) contents of whole shrimp body.

3.4. ANCOVA output

ANCOVA output of performance and proximate composition of whole shrimp body is presented in Table 8. The main effect (DFB inclusion levels) and the covariate effect (processing methods) had significant effects on final mean weight, WG, and FCR. A combined effect of DFB inclusion levels and processing methods was observed on the lipid content of whole shrimp body. No differences were detected in survival, PRE, and moisture, protein, and ash content of whole shrimp body.

4. Discussion

Feed represents over 50% of the cost for aquaculture producers, which is an important economic part that can be improved upon. Identification of suitable protein sources as FM substitutes in shrimp feeds may result in reduced feed costs and foster an economically feasible, expanded, and sustainable shrimp industry. The analyzed proximate composition indicates that

DFB has a higher protein content (790 and 805.1 g kg^{-1}) than FM (627.8 g kg^{-1}), SPC (649.3 g kg^{-1}), and CPC (780.7 g kg^{-1}). When FM is replaced with alternative protein sources, it is critical to balance the amino acid profile in the diets. Methionine and lysine are two essential amino acids (AA) that are typically most limiting AA in the plant-derived byproducts. Concentrations of these and other AA in DFB are similar to FM and much higher than in SPC and CPC (Table 3). As a consequence, AA levels in diets supplemented with DFB were higher than those of the basal diet (Table 4).

In trial 1, data indicates that DFB can be utilized up to 50 g kg^{-1} in shrimp diets to replace FM without compromising the growth. However, a significant reduction in WG and increase in FCR were observed when shrimp were fed with the diet supplemented with 100 g kg^{-1} DFB. A number of studies have evaluated the feasibility of inclusion of bacterial biomass as a substitute for FM. Only a few of them were conducted on coproducts from AA production such as fermented bacterial biomass. The same product (DFB) has been demonstrated to be suitable for complete replacement of FM (120 g kg^{-1}) in Florida pompano *Trachinotus carolinus* diets containing 8% poultry byproduct meal (Rhodes *et al*. 2015) and nursery pig diets (Perez *et al*. 2011), without resulting in negative effects on growth of experiment animals or FCR. A similar dried bacterial biomass from L-lysine manufacture using fermenting bacteria, *Micrococcus glutamicus* was used up to 100 g kg^{-1} as a replacement of FM without causing negative effects on the growth of tilapia, *Oreochromis mossambicus*; however, significant reduction on growth and increase in FCR were detected when lysine biomass was supplemented at higher levels (150 and 200 g kg^{-1}) in tilapia diets (Davies & Wareham 1988).

Aside from AA bacterial byproducts, using methane-oxidizing bacteria as protein and AA sources in aquatic animal nutrition recently received considerable attention due to the abundant

supply, cheap transportation, and reasonable cost of natural gas (Øverland *et al*. 2010). A bacterial protein meal (BPM) consisting of *M. capsulatus*, *Alcaligenes acidovorans*, *Bacillus brevis* and *B. firnus* was produced in a process that converts natural gas into protein (Aas *et al*. 2007). Aas *et al*. (2006a) reported that BPM can be utilized up to 360 g kg^{-1} to replace FM in the diets for Atlantic salmon, *Salmo salar,* without compromising growth. Similar results were demonstrated by Berge *et al*. (2005) and Storebakken *et al*. (2004) who documented that BPM can be supplemented in *S. salar* diets at 200 and 193 g kg^{-1}, respectively. The inclusions of BPM up to 270 g kg^{-1} were also confirmed to be successfully applied in the diets for rainbow trout *Oncorhynchus mykiss* as a substitute for FM (Aas *et al*. 2006b). In contrast, Aas *et al*. (2007) indicated that BPM can be used up to 90 g kg^{-1} in the diets for Atlantic halibut *Hippoglossus hippoglossus* but supplementation of BPM at 180 g kg^{-1} caused growth depression in this species.

Results from the researches listed above demonstrated that partial replacement of FM by bacterial biomass proteins are appropriate for several aquatic species. However, the effects of high inclusion levels of bacterial biomass proteins in diets for aquatic animals are still contradictory among those studies. Variations among these researches may be attributed to various strains utilized to produce the bacterial biomass proteins, processing of the meal, and different species of experimental animals. The failure of complete FM replacement by DFB in the present study may due to the palatability or nutritional imbalances in the diet devoid of FM.

Soy protein concentrate is produced through a series of different extraction and precipitation process from high-quality de-hulled soybeans, and its crude protein content can reach up to 700 g kg^{-1} (Sookying & Davis 2012). Corn protein concentrate (CPC) is produced through wet milling and refined to contain up to 800 g kg^{-1} crude protein (AAFCO 2007). The

application of SPC in shrimp feeds were demonstrated to be feasible in multiple studies (Alam *et al*. 2005; Bauer *et al*. 2012; Paripatananont *et al*. 2001; Sookying *&* Davis 2012). Inclusion levels of CPC at 47-60 g kg^{-1} were widely applied in practical diets for Pacific white shrimp as a good source for protein and methionine (Fang *et al*. 2016; Zhou *et al*. 2015; Qiu & Davis 2016a; b; c; d). Based on the information provided by trial 1 in the present study, a follow up growth trial (trial 2) further explored different inclusion levels (0, 20, 40, 60, 120 g kg^{-1}) of DFB products produced by two different processing methods (SDFB and GDFB) as a substitution for SPC w/o CPC in a low FM-based diet. Results indicated that GDFB can be utilized up to 120 g kg^{-1} to replace SPC and CPC without causing negative effects on growth performance and FCR. However, significantly reduced final mean weight and WG as well as increased FCR were detected when dietary SDFB were supplemented at 60 and 120 g kg^{-1}. Analysis of covariance detected a significant effect of the processing method on the final biomass, final mean weight, WG of shrimp and FCR. Shrimp fed with GDFB performed significantly better in terms of growth than those fed with SDFB. The research on bacterial biomass proteins for utilization as replacements for SPC and CPC in aquatic animal feeds is still limited. Based on information provided in the FM replacement researches conducted in the current study and by many other researchers, the successfully replacement of SPC w/o CPC by GDFB could be easily understood because of the balanced amino acid profile and suitable palatability. However, the significant reduction on the shrimp performances resulted from 60 and 120 g kg^{-1} SDFB supplementation indicated that spray drying method may not be the most appropriate processing option for proteinaceous bacterial meal.

In the present study, PRE was not significantly affected when either SDFB or GDFB were utilized up to 120 g kg^{-1}. Similarly, Aas et al. (2006) documented that inclusion of BPM up

to 270 g kg^{-1} did not affect PRE in rainbow trout. Also in agreement, Aas *et al*. (2007) and Berge *et al*. (2005) reported that PRE in Atlantic salmon was not significantly affected when BPM was supplemented up to 180 and 200 g kg^{-1} as a replacement of FM. PRE was determined by a number of factors including dietary protein level, feed intake, final weight and initial weight of animals as well as the final and initial protein content of animals (Halver & Hardy, 2002). In the current study, no significant differences were found in dietary protein levels, feed offered to the shrimp, initial weight of shrimp, and protein content of shrimp carcass. The significantly reduced final weight of shrimp fed with the diet supplemented with 60 g kg^{-1} SDFB resulted in comparatively lower PRE observed in this treatment.

With regards to whole body proximate composition, lipid content in shrimp fed the diet containing 120 g kg^{-1} GDFB was significant lower than those fed with diets supplemented with 40 and 120 g kg^{-1} SDFB. No significant differences were detected in carcass protein or ash content. Likewise, Aas *et al*. (2006b) reported that no significant differences were detected in the contents of crude lipid, crude protein, or ash in carcass of rainbow trout when BPM were utilized up to 270 g kg^{-1} in the diet. In addition, other published work also documented that dietary bacteria protein meal had no significant effect on proximate composition (protein, lipid, or ash) of Atlantic halibut (Aas *et al*. 2007) and Atlantic salmon (Aas *et al*. 2006a; Berge *et al*. 2005; Storebakken *et al*. 2004). In the present experiment, analysis of covariance indicated that the combined effect of the DFB source and level significantly affected the whole lipid content of shrimp. Generally, shrimp fed with diets containing SDFB had a relatively higher lipid level than those fed with GDFB. The carcass lipid content decreased as the inclusion level of GDFB increased, which may be attributed to relatively lower energy availability in this ingredient.

5. Conclusion

Under the reported conditions of the study, the use of DFB can partially replace FM up to 50 g kg^{-1} without causing negative effects on the growth performance of Pacific white shrimp. However, completely replacement of FM (100 g kg^{-1}) by DFB resulted in growth depression which may due to palatability or nutrient imbalances. The use of GDFB as a substitution for SPC w/o CPC did not significantly affect growth of shrimp. However, the inclusion of SDFB at 60 and 120 g kg^{-1} decreased growth of shrimp indicating a negative effect of spray drying. The results in the current study demonstrate that GDFB is a good protein source which can be incorporated in practical shrimp feed formulations.

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Table 1 Composition (g kg^{-1} as is) of test diets utilized in trial 1.

Ingredient	Diet code			
	DFB0	DFB25	DFB50	DFB100
Soybean meal[1]	350.0	350.0	350.0	350.0
Fish meal[2]	160.0	129.0	98.0	37.0
Corn gluten meal[3]	50.0	50.0	50.0	50.0
Whole wheat[4]	340.5	340.5	340.5	340.5
Dried fermented biomass[5]	0	25.0	50.0	100.0
Fish oil[2]	47.3	49.2	51.1	54.7
Trace mineral premix[6]	5.0	5.0	5.0	5.0
Vitamin premix[7]	18.0	18.0	18.0	18.0
Choline chloride[4]	2.0	2.0	2.0	2.0
Stay C[8]	1.0	1.0	1.0	1.0
Lecithin[9]	10.0	10.0	10.0	10.0
Cholesterol[4]	0.5	0.5	0.5	0.5
Corn starch[4]	5.7	6.3	7.4	8.3

[1] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[2] Omega Protein Inc., Huston TX, USA.
[3] Grain Processing Corporation, Muscatine, IA, USA.
[4] MP Biomedicals Inc., Solon, OH, USA.
[5] Proplex T, Archer Daniels Midland Company, Chicago, IL, USA.
[6] Trace mineral premix (g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[7] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[8] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[9] The Solae Company, St. Louis, MO, USA.

Table 2 Composition (g kg^{-1} as is) of test diets utilized in trial 2.

Ingredient	Diet code								
	Basal	SDFB20	SDFB 40	SDFB 60	SDFB 120	GDFB20	GDFB40	GDFB60	GDFB120
Fish meal[1]	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0
Soybean meal[2]	382.0	382.0	382.0	382.0	382.0	382.0	382.0	382.0	382.0
Whole wheat[3]	280.0	280.0	280.0	280.0	280.0	280.0	280.0	280.0	280.0
Corn protein concentrate[4]	60.0	60.0	60.0	60.0	0.0	60.0	60.0	60.0	0.0
Soy protein concentrate[5]	74.0	50.0	26.0	0.0	0.0	50.0	26.0	0.0	0.0
Dried fermented biomass[5]	0.0	20.0	40.0	60.0	120.0	20.0	40.0	60.0	120.0
Fish oil[1]	53.5	52.6	51.6	50.6	48.9	52.6	51.6	50.6	48.9
Trace mineral premix[6]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin premix[7]	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0
Choline chloride[8]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Stay C[9]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Mono-dicalcium phosphate[10]	25.0	25.5	26.5	27.5	28.0	25.5	26.5	27.5	28.0
Lecithin[11]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Cholesterol[8]	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Corn starch[8]	28.7	33.1	37.1	43.1	44.3	33.1	37.1	43.1	44.3

[1] Omega Protein Inc., Huston, TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Bobs Red Mill, Milwaukie, OR, USA.
[4] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[5] Proplex T, Archer Daniels Midland Company, Chicago, IL, USA.

[6] Trace mineral premix (g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[7] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[8] MP Biomedicals Inc., Solon, OH, USA.
[9] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[10] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[11] The Solae Company, St. Louis, MO, USA.

Table 3 Proximate composition, and amino acid profile of dried fermented biomass used in trial 1 (DFB) and 2 (SDFB and GDFB have identical nutrient compositions), fish meal (FM), soy protein concentrate (SPC), and corn protein concentrate (CPC).

Composition[1] (g kg^{-1} as is)	DFB	S/GDFB	FM	SPC	CPC
Crude protein	805.1	790.0	627.8	649.3	780.7
Moisture	47.8	34.5	79.9	85.5	81.6
Crude fat	10.0	49.0	105.6	0	2.0
Crude fiber	-	8.0	0	3.5	0.9
Ash	-	27.0	187.5	6.3	1.0
Alanine	60.6	55.3	39.1	26.3	64.2
Arginine	52.3	49.5	36.8	44.4	22.5
Aspartic acid	85.5	78.5	53.4	68.4	42.9
Cysteine	8.2	9.0	4.7	8.0	13.0
Glutamic acid	98.4	92.4	74.7	104.4	146.8
Glycine	39.1	35.9	48.8	25.5	19.5
Histidine	18.8	17.8	16.3	17.7	14.8
Isoleucine	42.4	39.6	24.2	29.5	29.6
Leucine	76.5	70.9	42.1	50.8	129.7
Lysine	53.3	49.8	46.7	38.8	11.4
Methionine	22.2	20.9	16.1	8.3	18.0
Phenylalanine	37.6	33.3	23.9	33.3	48.0
Proline	30.9	30.1	30.8	32.9	73.1
Serine	26.9	24.3	21.1	27.1	38.0
Threonine	41.4	36.7	24.1	23.8	24.8
Tryptophan	10.8	9.9	6.2	8.6	3.7
Tyrosine	30.3	28.4	16.7	25.0	42.4
Valine	53.3	50.8	29.9	29.4	32.3

[1] Ingredients were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Table 4 Proximate composition and amino acid profile of the test diets used in trial 2.

Composition[1] (g kg^{-1} as is)	Basal	SDFB20	SDFB 40	SDFB 60	SDFB 120	GDFB20	GDFB40	GDFB60	GDFB120
Crude protein	364.9	369.6	365	365.6	365.7	362.1	365	365.4	369
Moisture	54.9	50.8	63.7	63.9	72.7	51.3	51.9	59.1	55.6
Crude fat	102	86.1	89.5	90.6	86.1	88.3	85.4	91.4	90.2
Crude fiber	31.7	33.1	31.3	31.1	27.5	35.2	33.3	32.3	33.1
Ash	67.7	68.7	66.8	67.2	68.4	67.1	67.2	63.9	68.5
Alanine	18.2	19.1	19.2	19.5	19.0	18.2	19.2	19.6	19.2
Arginine	22.3	22.6	21.8	21.4	23.1	21.9	22.0	21.8	23.0
Aspartic Acid	33.3	34.2	32.9	32.3	34.6	32.8	33.1	32.9	34.7
Cysteine	5.0	5.0	4.8	4.7	4.5	4.6	4.8	4.7	4.3
Glutamic Acid	70.8	72.0	69.5	68.1	64.9	69.0	69.8	68.9	64.9
Glycine	16.8	17.1	17.2	17.7	19.3	16.7	17.1	17.3	19.0
Histidine	8.7	9.0	8.6	8.4	8.6	8.5	8.6	8.5	8.6
Isoleucine	16.2	16.8	16.3	16.0	16.7	16.0	16.2	16.0	16.3
Leucine	31.0	32.5	32.1	31.6	28.8	30.8	32.2	32.1	28.8
Lysine	20.7	21.0	20.5	20.5	23.0	20.6	20.8	20.8	22.8
Methionine	6.0	6.4	6.4	6.6	6.7	6.2	6.4	6.5	6.6
Phenylalanine	18.5	19.0	18.4	18.0	17.5	18.2	18.7	18.3	17.4
Proline	23.0	22.9	22.3	22.3	20.0	22.1	23.7	22.0	20.0
Serine	15.3	15.5	15.1	15.1	15.0	15.6	15.8	16.1	15.8
Threonine	12.8	13.5	13.3	13.4	14.5	13.3	13.7	13.9	15.0
Tryptophan	4.7	4.5	4.7	4.8	5.2	4.8	4.7	5.0	5.2
Tyrosine	12.7	13.1	13	12.7	12.4	12.6	13.2	13.1	12.3

Valine	18.0	18.8	18.4	17.7	18.6	17.8	18.1	17.6	19.3

[1] Diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Table 5 Performance of juvenile shrimp *L. vannamei* (Initial weight 0.59 g) offered diets with different dried fermented biomass (DFB) levels (0, 25, 50, and 100 g kg^{-1}) for six weeks in trial 1.

Diet	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)
DFB0	62.7	6.3	970.0[a]	1.52[b]	100
DFB25	61.8	6.2	944.6[ab]	1.55[ab]	100
DFB50	58.5	5.9	884.9[ab]	1.64[ab]	100
DFB100	57.0	5.7	845.4[b]	1.70[a]	100
PSE[1]	0.8047	0.0737	14.0715	0.0212	
P-value	0.0812	0.0493	0.0337	0.0395	

[1] PSE: Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] WG: Weight gain = (Final weight - initial weight) / initial weight × 100%.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 6 Performance of juvenile shrimp *L. vannamei* (Initial weight 2.34 g) when shrimp were offered diets with different levels (0, 20, 40, 60, and 120 g kg^{-1}) of spray dry (S) and granular (G) dried fermented biomass (DFB) for six weeks in trial 2**.**

Diet	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)	PRE[4]
Basal	89.0	10.4^{a}	338.9^{ab}	1.61^{b}	86.0	34.9
SDFB20	82.6	9.6^{ab}	314.2^{abc}	1.78^{ab}	86.0	31.2
SDFB40	80.9	9.7^{ab}	319.6^{abc}	1.76^{ab}	84.0	30.4
SDFB60	80.4	8.9^{b}	281.7^{c}	1.97^{a}	90.0	27.5
SDFB120	78.6	9.4^{ab}	302.1^{bc}	1.84^{ab}	84.0	30.4
GDFB20	89.6	10.7^{a}	359.3^{a}	1.53^{b}	84.0	35.9
GDFB40	86.3	10.3^{ab}	338.0^{abc}	1.65^{ab}	84.0	33.2
GDFB60	92.2	10.5^{a}	347.1^{ab}	1.57^{b}	88.0	32.5
GDFB120	85.0	9.9^{ab}	316.9^{abc}	1.70^{ab}	86.0	31.1
PSE[1]	1.6804	0.1324	5.4717	0.0313	1.5348	0.7987
P-value	0.1777	0.0021	0.0022	0.0019	0.9257	0.0704

[1] PSE: Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] WG: Weight gain = (Final weight - initial weight) / initial weight × 100%.
[4] PRE: Protein retention efficiency = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein intake.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 7 Proximate analysis (g kg^{-1}) of whole shrimp body when shrimp were offered diets with different levels (0, 20, 40, 60, and 120 g kg^{-1}) of spray dry (S) and granular (G) dried fermented biomass (DFB) for six weeks in trial 2.

Diet	Protein[2]	Moisture	Lipid[2]	Ash[2]
Basal	737.0	757.2	83.1[ab]	106.0
SDFB20	726.2	750.8	84.2[ab]	111.0
SDFB40	744.4	768.8	93.6[a]	107.7
SDFB60	704.5	755.2	75.8[ab]	102.5
SDFB120	756.4	769.2	95.6[a]	83.1
GDFB20	715.4	749.4	88.0[ab]	102.1
GDFB40	723.5	754.2	81.5[ab]	94.6
GDFB60	676.2	750.8	72.2[ab]	118.3
GDFB120	718.4	756.4	67.4[b]	119.4
PSE[1]	10.4656	2.1546	2.4667	4.3119
P-value	0.4519	0.0390	0.0129	0.2364

[1] PSE: Pool standard error.
[2] Dry weight basis.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 8 Analysis of covariance (ANCOVA) output of performance and proximate composition of whole shrimp body data in trial 2.

Parameters	Adjusted LSmeans		*P*-value			
	SDFB	GDFB	Processing method	Level	Processing method*Level	Model
Performances						
Final biomass	82.3	88.4	0.0081	0.0673	0.4306	0.0014
Final mean weight	9.6	10.3	0.0003	0.0106	0.5979	0.0003
WG[1]	311.3	340.0	0.0011	0.0103	0.7981	0.0010
FCR[1]	1.79	1.61	0.0003	0.0180	0.4874	0.0004
Survival	86.0	85.6	0.8459	0.9699	0.6781	0.9750
PRE[1]	31.8	32.6	0.5000	0.8541	0.5727	0.8445
Proximate composition						
Moisture	760.2	753.7	0.0544	0.1421	0.2434	0.0706
Protein	733.7	714.1	0.2164	0.9737	0.4229	0.5325
Lipid	86.5	78.5	0.0353	0.3680	0.0089	0.0090
Ash	102.1	108.1	0.3588	0.6944	0.0219	0.0992

[1] WG: weight gain. FCR: feed conversion ratio. PRE: protein retention efficiency.

CHAPTER V

EVALUATION OF A NOVEL BACTERIAL BIOMASS AS A SUBSTITUTION FOR SOYBEAN MEAL IN PLANT-BASED PRACTICAL DIETS FOR PACIFIC WHITE SHRIMP *Litopenaeus vannamei*

1. Introduction

Soybean meal (SBM) is usually considered as the most reliable ingredient and cost-effective protein source in shrimp feed because of its worldwide availability, low price, relatively balanced amino acid profile, and consistent composition (Amaya *et al*., 2007; Davis and Arnold, 2000). The dietary SBM inclusions in practical shrimp feeds have been well defined in a number of studies (Amaya *et al*., 2007; Roy *et al*., 2009; Sookying and Davis, 2011). However, as the popularity of SBM utilized as a primary protein ingredient in animal feed formulation increased, the price of SBM has risen from $175 per metric ton in the year 2000 to over $500 per metric ton in 2014 (Index Mundi, 2015). Therefore, it would be necessary for us to explore novel ingredients that are more economical and sustainable as shrimp culture has become an expanded and intensified economic activity.

The rapid growth and high protein content of bacteria in protein production has led to considerable attention on the potential of the application of microbial protein in animal production. One of the attractive substrates used to produce bacterial protein is methane, which is the main component of natural gas and distributed widely in nature (Dalton, 2005; Hanson and Hanson, 1996). The abundant supply, cheap transportation, and reasonable cost of natural gas, indicated that protein production from natural gas could be realistic on a large scale (Øverland *et al*., 2010). Moreover, owing to the low water and no fertile soil requirements, the cultivation of

bacterial protein does not compete with agriculture lands and even can be achieved in dry climates.

A number of researches have been conducted to evaluate a bacterial protein produced from mainly methane by natural gas fermentation as a protein ingredient for several fish species including Atlantic salmon *Salmo salar* (Aas *et al*., 2006a; Berge *et al*., 2005; Storebakken *et al*., 1998; 1999; 2004), rainbow trout *Oncorhynchus mykiss* (Aas *et al*., 2006b; Øverland *et al*., 2006), and Atlantic halibut *Hippoglossus hippoglossus* (Aas *et al*., 2007). The feeding experiments in most of these publications recommended that the bacterial protein derived from natural gas can be utilized as a sustainable protein source in aquaculture production.

The bacterial biomass evaluated in the present study is derived from *Methylobacterium extorquens*. The application of this product as a protein source in commercial type of shrimp feed is still limited. Therefore, the purposes of this project are to determine the growth response of Pacific white shrimp juveniles to increasing bacterial biomass inclusion levels as a substitution for soybean meal and determine apparent digestibility values for bacterial biomass as compared to other traditional protein sources.

2. Material and Methods

2.1. Experimental design and diets

Three trials were conducted to evaluate the biological response of shrimp to BB in soy-based diets in terms of the growth. In the trial 1 and 2, test diets were formulated to be isonitrogenous and isolipidic (35% protein and 8% lipid). In trial 1, three experimental diets (T_1D_1 - T_1D_3) were formulated to contain increasing levels (0, 6, and 12%) of BB as a replacement of SBM (Table 1). In trial 2, to confirm the results in trial 1 and investigate the

effects of low inclusion levels of BB, six experimental diets (T_2D_1 - T_2D_6) were formulated to supplement with increasing levels (0, 1, 2, 4, 6, and 12%) of BB as a replacement of SBM (Table 2). In trial 3, five experimental diets (T_3D_1 - T_3D_5) were formulated (Table 3). T_3D_1, T_3D_2, and T_3D_4 are the same as diets in trial 2 that utilized 0, 6, and 12% BB to replace SBM. Whereas T_3D_3 and T_3D_5 utilized BB to replace the same ratio of SBM as T_3D_2 and T_3D_4, respectively, on a digestible protein basis. Additionally, a reference diet (Table 4) was utilized to determine digestibility coefficients in conjunction with 1% chromic oxide as an inert marker and 70:30 replacement strategy.

Primary ingredients were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate and amino acid composition (Table 5). All experimental diets were produced at the Aquatic Animal Nutrition Laboratory at the School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University (Auburn, AL, USA) using the standard procedures for the shrimp feeds described by Qiu and Davis, (2016). Briefly, diets were prepared by mixing the pre-ground dry ingredients in a food mixer (Hobart, Troy, OH, USA) for 10–15 minutes. Hot water was then blended into the mixture to obtain a consistency appropriate for pelleting. Diets were pressure-pelleted using a meat grinder with a 2.5-mm die. The wet pellets were then placed into a fan-ventilated oven (< 50 °C) overnight in order to attain a moisture content of less than 10%. Dry pellets were crumbled, packed in sealed bags, and stored in a freezer until use. The diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate and amino acid composition in trial 1 and 2 (Table 6 and Table 7) and at Midwest Laboratories (Omaha, NE, USA) for proximate and mineral composition in trial 3(Table 8).

2.2. Growth trials

Three trials were conducted at the E.W. Shell Fisheries Research Station, Auburn University (Auburn, AL, USA). Pacific white shrimp post larvae (PL) were obtained from Shrimp Improvement Systems (Islamorada, Florida) and nursed in an indoor recirculating system. PLs were fed a commercial feed (Zeigler Bros., Inc., Gardners, Pennsylvania, USA) using an automatic feeder for ~1 week, and then switched to crumbled commercial shrimp feed (Zeigler Bros., Inc., Gardners, Pennsylvania, USA) for ~1- 2 weeks.

In trial 1, the recirculating system consisted of 12 aquaria (160 L) connected to a common reservoir, biological filter, bead filter, fluidized biological filter and recirculation pump. Four replicate groups of shrimp (1.51 g initial mean weight; 8 shrimp / tank) were offered diets using our standard feeding protocol over 6 weeks. Based on historic results, feed inputs were pre-programmed assuming the shrimp would double their weight weekly up to one gram then gain 0.8-1.3 g weekly with a feed conversion ratio (FCR) of 1.8. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality. Consequently, for each tank in trial 1, a fixed ration of 1.65 g day^{-1} for the first and second week, 1.85 g day^{-1} for the third week, 2.26 g day^{-1} for the fourth week, 2.47 g day^{-1} for the fifth week, and 2.67 g day^{-1} for the remaining culturing period was offered, partitioned in 4 feedings each day.

In trial 2 and trial 3, the recirculating system consisted of 24 aquaria (135 L) connected to a common reservoir, biological filter, bead filter, fluidized biological filter and recirculation pump. Four replicate groups of shrimp (In trial 2: 0.98 g initial mean weight, 10 shrimp / tank; In trial 3: 0.15 g initial mean weight, 10 shrimp / tank) were offered diets using our standard feeding protocol over 6 weeks. Based on historic results, feed inputs were pre-programmed

assuming the shrimp would double their weight weekly up to one gram then gain 0.8-1.3 g weekly with a feed conversion ratio (FCR) of 1.8. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality. Consequently, for each tank in trial 2, a fixed ration of 2.06 g day^{-1} for the first week, 2.31 g day^{-1} for the second week, 2.57 g day^{-1} for the third week, 2.83 g day^{-1} for the fourth week, 3.09 g day^{-1} for the fifth week, and 3.34 g day^{-1} for the last week was allotted in 4 portions per day. For each tank in trial 3, a fixed ration of 0.39 g day^{-1} for the first week, 0.83 g day^{-1} for the second week, 1.66 g day^{-1} for the third week, 2.24 g day^{-1} for the fourth week, 2.53 g day^{-1} for the fifth week, and 3.05 g day^{-1} for the last week was also allotted in 4 portions per day.

At the conclusion of each growth trial, shrimp were counted and group-weighted. Mean final weight, FCR, WG, biomass, and survival were determined (Table 9, Table 10, and Table 11). After obtaining the final total weight of shrimps in each aquarium, 4 shrimps from trial 2 and trial 3 were randomly selected and frozen at -20 °C for subsequent determination of whole body composition. Proximate composition and amino acid profile (Table 12 and Table 14) of whole shrimp was analyzed by University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory (Columbia, MO, USA). Protein and amino acids retention efficiencies were calculated as follows:

Protein retention (%) = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein offered.

Amino acids (AA) retention (%) = (final weight × final AA content) - (initial weight × initial AA content) × 100 / AA offered.

2.3. Water quality monitoring

For all trials, dissolved oxygen (DO), water temperature and salinity were measured twice daily by using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH, USA). Hydrogen potential (pH) was measured twice weekly by using a waterproof pHTestr30 (Oakton instrument, Vernon Hills, IL, USA). Total ammonia-nitrogen (TAN) and nitrite were evaluated every week by using the methods described by Sororzano (1969) and Spotte (1979).

2.4. Digestibility trial

The digestibility trial was conducted in the mentioned recirculation system and utilized six shrimp per aquaria with six aquaria per dietary treatment. Once acclimated for three days to the test diets, feces from two aquaria were pooled (n=3) and collected over a five-day period or until adequate samples were obtained. To obtain fecal samples, the aquaria were cleaned by siphoning before each feeding with the first collection of the day discarded. After cleaning, the shrimp were offered an excess of feed and then about 1 hour later feed was removed and feces were collected by siphoning onto a 500 μm mesh screen. Collected feces were rinsed with distilled water, dried at 105 °C until a constant weight was obtained, and then stored in freezer (−20 °C) until analyzed. Apparent digestibility coefficient for dry matter, protein, energy, and amino acids were determined by using chromic oxide (Cr_2O_3, 1%) as an inert marker. Chromium concentrations were determined by the method of McGinnis and Kasting (1964) in which, after a colorimetric reaction, absorbance is read on a spectrophotometer (Spectronic genesis 5, Milton Roy Co., Rochester, NY, USA) at 540nm. Gross energy of diets and fecal samples were analyzed with a Semi micro-bomb calorimeter (Model 1425, Parr Instrument Co., Moline, IL, USA). Protein were determined by micro-Kjeldahl analysis (Ma and Zuazaga, 1942). Amino acids were

analyzed by University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory (Columbia, MO, USA). The apparent digestibility coefficient of dry matter (ADMD), protein (APD), energy (AED), and amino acids (AAAD) were calculated according to Cho et al. (1982) as follows:

ADMD (%) = 100 − [100 × (% Cr_2O_3 in feed / % Cr_2O_3 in feces)]

APD, AED, and AAAD (%) = 100 − [100 × (% Cr_2O_3 in feed / % Cr_2O_3 in feces) × (% nutrient in feces / % nutrient in feed)

The apparent digestibility coefficients (ADC) of the test ingredients for dry matter, energy, protein and amino acids were calculated according to Bureau and Hua (2006) as follows:

$ADC_{test\ ingredient} = ADC_{test\ diet} + [(ADC_{test\ diet} - ADC_{ref.\ diet}) \times (0.7 \times D_{ref} / 0.3 \times D_{ingr})]$

where D_{ref} = % nutrient (or KJ/g gross energy) of reference diet mash (dry weight); D_{ingr} = % nutrient (or KJ/g gross energy) of test ingredient (dry weight).

2.5. Statistical analysis

All the data were analyzed using SAS (V9.3. SAS Institute, Cary, NC, USA). Data from three growth trials were analyzed using one-way ANOVA to determine significant differences (P<0.05) among treatments followed by the Tukey's multiple comparison test to determine difference between treatments in each trial. The pooled standard errors were used across growth trials, as the variance of each treatment is the same. Arcsine square root transformation was used prior to analysis for the proportion data. False discover rate (FDR) controlling procedures were used to adjust the P-value to control the FDR for data from nutrient contents of whole body and amino acid retention. Data from digestibility trial were analyzed using non-parametric (kruskal-

wallis) one-way ANOVA to determine significant differences (P<0.05) among treatments followed by the Tukey's multiple comparison test to determine differences between treatments.

3. Results

3.1. Water quality

In trial 1, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 6.15 ± 0.49 mg L^{-1}, 28.1 ± 1.6 °C, 9.6 ± 0.7 ppt, 7.4 ± 0.3, 0.17 ± 0.05mg L^{-1}, and 0.16 ± 0.07 mg L^{-1}, respectively. In trial 2, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 6.20 ± 0.73 mg L^{-1}, 29.5 ± 0.9 °C, 8.4 ± 1.0 ppt, 7.5 ± 0.3, 0.09 ± 0.10 mg L^{-1}, and 0.05 ± 0.04 mg L^{-1}, respectively. In trial 3, temperature, salinity, pH, TAN, and nitrite were maintained at 6.96 ± 0.31 mg L^{-1}, 28.1 ± 0.3 °C, 8.2 ± 0.6 ppt, 7.0 ± 0.3, 0.05 ± 0.04 mg L^{-1}, and 0.12 ± 0.12 mg L^{-1}, respectively. Water quality conditions in all three trials were suitable for normal growth and survival of this species.

3.2. Growth trials

Performances of Pacific white shrimp offered diets with various BB levels in trial 1, 2 and 3 are presented in Table 9, 10, and 11, respectively. In trial 1, shrimp fed with diets incorporated with BB exhibited significantly improved survival. However, final mean weight and WG were significantly reduced when shrimp fed with diets contained 12% BB. Whereas FCR was significantly increased when shrimp fed with diets supplemented with both 6 and 12% BB. No significant difference was observed in final biomass (45.70 to 53.85 g) across the treatments

In trial 2, final biomass was significantly reduced when 12% BB was included in the practical shrimp diet. Significant improvements in final mean weight and WG whereas dramatically reduced FCR were determined when shrimp fed with the diet concluded 1% BB compared to those fed with diet supplemented with 6 and 12% BB. No significant difference was found in survival (92.5 to 100%) across all the treatments.

In trial 3, final biomass was significantly reduced when 26.6% BB was added in the diet compared to the diet without BB supplementation. Shrimp fed with diets contained 12% and 26.6% BB exhibited significantly lower WG than those fed with diet did not contain BB. Significant increment in FCR was determined in the diet incorporated with 12 and 26.6% BB compared to the treatment supplemented with 0 and 13.3% BB. No significant difference was detected in survival (90 to 100%) across all the treatments.

3.3. Whole body composition

Proximate composition and amino acids profile of whole shrimp body in trial 2 and trial 3 are presented in Table 12 and Table 14. Dietary BB inclusion at 12% significantly improved protein content while decreased lipid content of whole shrimp body. Moisture content of shrimp in the treatment fed with 12% BB was significantly lower than the treatment contained 4% BB. Arginine and glycine contents were significantly improved when BB was supplemented at 12% than other treatments. Significant improvement was detected, whereas reduction was also observed in the diet contained 12% BB compared to the treatment supplemented with 0, 1, and 2% BB. Threonine content of shrimp body fed with diet contained 1% BB was significantly higher than other treatments. No significant differences were detected in alanine, aspartic acid, cysteine, glutamic acid, histidine, hydroxylysine, hydroxyproline, isoleucine, leucine, lysine,

phenylalanine, serine, tryptophan, tyrosine, and valine concentrations of whole shrimp body across all the treatments.

In trial 3, protein and ash content of shrimp fed with diet contained 26.6% BB was significantly improved compared to those fed with diet supplemented with 0 and 6% BB. Lipid content of shrimp fed with diet included with 13.3 and 26.6% BB was dramatically reduced in contrast with those offered with diet contained 0 and 6% BB. No significant effects were detected in moisture (76.1 to 77.4%) and fiber (5.25 to 5.68%) contents.

3.4. Protein and amino acid retentions

Protein and amino acids retention efficiencies of Pacific white shrimp in trial 2 and trial 3 are shown in Table 13 and Table 14. In trial 2, PRE was significantly reduced when shrimp fed with diets contained 12% BB compared to other treatments. There were reasonable correspondences of total AAs and individual AAs retention efficiencies to PRE. In general, total AA and most of individual AAs retention efficiencies except hydroxylysine, hydroxyproline, and tyrosine retention efficiency were significantly depressed when shrimp fed with diet contained 12% BB compared to other treatments. Hydroxyproline retention efficiency was significantly higher in the diet incorporated with 4% BB than other treatments. Tyrosine retention efficiency was significantly reduced in the treatment supplemented with 12% BB compared to the treatments contained 0, 1, 2, and 4% BB. No significant difference was detected in the multiple comparison of hydroxylysine, which may due to the variance. In trial 3, PRE was significantly depressed when 26.6% BB was incorporated in the diet.

3.5. Digestibility trial

ADMD, APD, and AED for the diet (D) and ingredient (I) using 70:30 replacement technique offered to Pacific white shrimp are presented in Table 15. The digestibility trial contained a range of ingredients; hence, we have provided a few other ingredients as a reference. To confirm the digestibility results, faecal samples for basal diet, FM diet, and BB diet were recollected. Basal1 and Basal2, FM1 and FM2, and BB1 and BB2 represent first collection and second collection of basal diet, FM diet, and BB diet, respectively. ADMD, AED, and APD of BB were significantly lower than those of FM and SBM.

AAAD values for the SBM, FM and BB using 70:30 replacement technique offered to Pacific white shrimp are presented in Table 16. Apparent digestibility coefficients of alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysing, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, and total amino acids of BB were significantly lower than those of FM and SBM. Total AA and individual AA digestibility coefficients were reasonably corresponded to APD.

4. Discussion

The nutrient digestibility of a feed ingredient is an important factor to evaluate the overall nutritive value of the ingredient because it is related to the quantity of the nutrient absorbed by the animals. SBM had the highest APD (97.03%), AED (82.56%), and AAAD (90.78 – 96.91%) among the ingredients tested in the current study. Similar ranges of results for APD, AED, and AAAD were reported in multiple shrimp studies (Cruz-Suárez *et al*., 2009; Fang *et al*., 2016; Liu *et al*., 2013; Yang *et al*., 2009; Zhou *et al*., 2015). APD and AED of FM1 were 67.07% and 69.77%, respectively. Similar results were acquired in FM2 (APD and AED: 71.3% and 65.78%,

respectively). The analogous results of basal diet and FM diet from the collections under two occasions pointed to the consistency in the feces collection and sample analysis methods. APD of FM has been reported to be ranged from 62.7% to 91.6% in numerous studies (Lemos *et al*., 2009; Liu *et al*., 2013; Terrazas-Fierro *et al*., 2010; Yang *et al*., 2009). ADP of FM in our study were in the lower range of the results documented among those researches. The differences in digestibility of FM among various studies could be attributed to several factors, such as different raw materials, location or processing methods used to produce the products, and unknown factors related to different production batches.

Physiologically, ADCs should not below 0% or above 100%. However, ADMD of BB1 was negative, and the same phenomenon was confirmed in the recollected faecal sample (BB2). The negative ADMD resulted in very low values of APD, AED, and AAAD of BB for Pacific white shrimp. Similarly, Akiyama *et al*. (1989) also documented negative ADMD values of some dietary fillers (diatomaceous sand, chitin, and cellulose) for Pacific white shrimp. Unexpected negative ADMD value may be attributed to an unidentified interaction among the ingredients of the feed or endogenous losses by the animal from processes such an enzymatic secretion, sloughing of gut epithelial cells, formation of the chitinous peritrophic membrane, and the secretion of other lubricative substances (Akiyama *et al*., 1989). Another explanation of the negative ADMD value may be due to the leaching of chromic oxide of the feed before consumption or from the fecal samples. However, in the present study leaching of chromic oxide would be negligible for short exposure time to the seawater. Hence, the negative ADMD may indicate that BB cannot be digested by Pacific white shrimp. Ingredient digestibility data of BB for shrimp is still limited. Two digestibility studies of BB for rainbow trout and Atlantic salmon were available (Øverland *et al*., 2006; Storebakken *et al*., 1998). However, neither of these

researches documented negative values, all the digestibility values they reported were in the normal ranges. Differences among these studies may be attributed to different aquatic animals and bacterial proteins.

Using methane-oxidising bacteria as protein and amino acid sources in aquatic animal nutrition recently received considerable attention due to the abundant supply, cheap transportation, and reasonable cost of natural gas (Øverland *et al*., 2010). In the current study, significantly reduced growth performance was detected when the diet was supplemented with 12% BB in trial 1. FCR was significantly increased when the diets were incorporated with both 6 and 12% BB. To demonstrate the results from trial 1 and explore the effects of BB supplementation at low levels, the basal diet was incorporated with 0, 1, 2, 4, 6, and 12% BB in trial 2. Our findings indicated that no significant differences were observed in terms of growth performance and FCR when the diets supplemented with BB up to 6%. However, dietary BB incorporation at 12% dramatically reduced the WG and increased FCR, which were in accordance with the results documented in trial 1. Similarly, Aas *et al*. (2007) indicated that bacterial protein meal (BPM) can be used up to 9% in the diets for Atlantic halibut but supplementation of BPM at 18% caused depression in growth. By contrast, Aas *et al*. (2006a) reported that BPM can be utilized up to 36% in the diets for Atlantic salmon without compromising the growth. Similar results were demonstrated by Berge *et al*. (2005) and Storebakken *et al*. (2004) who documented that BPM can be supplemented in Atlantic salmon diets at 20% and 19.3%, respectively. The inclusions of BPM up to 27% were also confirmed to be successfully applied in the diets for rainbow trout (Aas *et al*., 2006b). Factors caused the inconsistent results with supplemental BB may be different strains utilized to produce BB and various aquatic animal species investigated among the experiments.

To elucidate if the digestible protein is the cause of the depressed growth and increased FCR, another growth trial (trial 3) was initiated to essentially use the same diets that contained 0, 6 and 12% BB in trial 2. Additionally, two more diets were formulated to replace the same ratio of SBM as diets contained 6 and 12% BB, respectively, but on a digestible protein basis. Dietary 12% BB supplementation significantly reduced WG but increased FCR in trial 3, which has been proven in both trial 1 and trial 2. The diets balanced on digestible protein basis performed essentially the same as those did not balanced for digestible protein in terms of WG and FCR, which may reveal that BB evaluated in the current study is not digestible by shrimp or the digestibility of this product is still in question.

With regards to the proximate composition of whole shrimp body, lipid level was dramatically reduced, while protein content was significantly enhanced when shrimp fed with diet contained 12% BB in trial 2. Similar trends of protein and lipid content of whole body were determined in trial 3. In trial 3, shrimp fed with diets contained 13.3 and 26.6% BB exhibited significantly improved protein and depressed lipid contents. By contrast, no significant effects of dietary BB supplementation on the protein and lipid content of fish body were detected in many other studies (Aas *et al*. 2006a; b; 2007; Berge *et al*., 2005, Storebakken *et al*., 2004). The significantly reduced lipid content of shrimp body in trial 2 and trial 3 may be caused by low digestible energy in the diet contained 12% BB as this ingredient had significantly lower energy digestibility than SBM. The improvements in protein content of whole body in trial 2 and trial 3 might be indirectly response to the decreased lipid content.

In the present study, PRE was significantly reduced when the diet was supplemented with 12% BB, and there was a reasonable correspondence to the total AA and individual AA amino acids retention efficiency in trial 2. In general, total AA and most of individual AAs except

hydroxylysine, hydroxyproline, and tyrosine retention efficiencies were significantly depressed when shrimp fed with diet contained 12% BB. Similarly, Aas *et al*. (2006b) reported that significantly reductions were determined in PRE and most individual AA retention efficiency when rainbow trout was fed with diet supplemented with 18% or 27% BB. In addition, Aas *et al*. (2007) documented that PRE and indispensable AA retention efficiency were significantly decreased when BB was supplemented at 18% in Atlantic halibut diet. PRE was determined by a number of factors including dietary protein levels, feed intake, final weight and initial weight of animals as well as the final and initial protein content of animals (Halver and Hardy, 2002). In trial 2, no significant differences were detected in dietary protein levels, feed offered to the shrimp, and initial weight of shrimp. Although protein content of whole shrimp body was significantly improved in the diet contained 12% BB, its effect cannot counteract with the significantly reduced final mean weight of shrimp in the same treatment, which is the primary cause of reduced PRE. In trial 3, a similar decreasing trend was observed when BB was included at 12% and 26.6%.

Survival of shrimp was significantly enhanced in trial 1 when diets were incorporated with both 6 and 12% BB. However, no significant differences were observed in survival across all the treatments in trial 2 and 3. Similarly, no mortality problems were reported in multiple researches investigated BB as protein sources in different kinds of fish (Aas *et al*., 2006a; b; 2007; Berge *et al*., 2005; Storebakken *et al*., 2004). Survival is not repeatable as it is impossible to conduct experiments under the same conditions in all three trials. The significantly improved survival by BB supplementation in trial 1 indicated that BB might induce immune responses in shrimp. Hence, further study considering the immune effects of BB supplementation at low levels is warranted.

5. Conclusion

Under the conditions of the present study BB can be utilized up to 4% in shrimp feed without causing growth depression. However, supplementations (≥ 6%) of BB can result in negative effects on growth response, FCR, and protein as well as amino acids retention efficiency. Given the negative result of dry matter digestibility of BB and no improvements in the treatments balanced on digestible protein basis by using BB, we may infer that the negative effects caused by high incorporation levels of BB may due to low nutrient digestibility of this ingredient for shrimp. Based on dramatically enhanced survival in the treatment with BB supplementation in trial 1, further research regarding the immune effects low inclusion levels of BB in practical shrimp feed is warranted.

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Table 1 Composition (% as is) of test diets utilized in trial 1.

Ingredient	Diet code		
	T_1D_1	T_1D_2	T_1D_3
Soybean meal[1]	54.10	47.40	40.50
Corn protein concentrate[2]	8.00	8.00	8.00
Whole wheat[3]	25.00	25.00	25.00
Bacterial biomass[4]	0.00	6.00	12.00
Fish oil[2]	6.05	6.14	6.24
Trace mineral premix[5]	0.50	0.50	0.50
Vitamin premix[6]	1.80	1.80	1.80
Choline chloride[3]	0.20	0.20	0.20
Stay C[7]	0.10	0.10	0.10
Mono-dicalcium phosphate[8]	2.50	2.50	2.50
Lecithin[9]	1.00	1.00	1.00
Cholesterol[3]	0.05	0.05	0.05
Corn starch[3]	0.70	1.31	2.11

[1] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[2] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[3] MP Biomedicals Inc., Solon, OH, USA.
[4] KnipBio Inc., Lowell, MA, USA.
[5] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[9] The Solae Company, St. Louis, MO, USA.

Table 2 Composition (% as is) of test diets utilized in trial 2.

Ingredient	Diet code					
	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6
Fish meal[1]	6.00	6.00	6.00	6.00	6.00	6.00
Soybean meal[2]	53.00	51.90	50.80	48.60	46.50	40.10
Corn protein concentrate[3]	8.00	8.00	8.00	8.00	8.00	8.00
Bacteria biomass[4]	0.00	1.00	2.00	4.00	6.00	12.00
Fish oil[2]	5.92	5.93	5.94	5.95	5.97	6.01
Trace mineral premix[6]	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix[7]	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride[5]	0.20	0.20	0.20	0.20	0.20	0.20
Stay C[8]	0.10	0.10	0.10	0.10	0.10	0.10
Mono-dicalcium phosphate[9]	2.50	2.50	2.60	2.60	2.80	2.90
Lecithin[10]	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol[5]	0.08	0.08	0.08	0.08	0.08	0.08
Methionine[11]	0.05	0.05	0.04	0.04	0.04	0.02
Lysine[11]	0.00	0.01	0.01	0.03	0.04	0.07
Corn starch[5]	20.85	20.93	20.93	21.10	20.97	21.22

[1] Omega Protein Inc., Huston TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[4] KnipBio Inc., Lowell, MA, USA.
[5] MP Biomedicals Inc., Solon, OH, USA.
[6] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[7] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[8] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[9] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[10] The Solae Company, St. Louis, MO, USA.
[11] Aldrich-Sigma, St. Louis, MO, USA.

Table 3 Composition (% as is) of test diets utilized in trial 3.

Ingredients	Diet code				
	T_3D_1	T_3D_2	T_3D_3	T_3D_4	T_3D_5
Fish meal[1]	6.00	6.00	6.00	6.00	6.00
Soybean meal[2]	53.00	46.50	46.50	40.10	40.10
Corn protein concentrate[3]	8.00	8.00	8.00	8.00	8.00
Bacteria biomass[4]	0.00	6.00	13.30	12.00	26.60
Fish oil[2]	5.92	5.97	5.81	6.01	5.70
Trace mineral premix[6]	0.50	0.50	0.50	0.50	0.50
Vitamin premix[7]	1.80	1.80	1.80	1.80	1.80
Choline chloride[5]	0.20	0.20	0.20	0.20	0.20
Stay C[8]	0.10	0.10	0.10	0.10	0.10
Mono-dicalcium phosphate[9]	2.50	2.80	2.80	2.90	2.90
Lecithin[10]	1.00	1.00	1.00	1.00	1.00
Cholesterol[5]	0.08	0.08	0.08	0.08	0.08
Methionine[11]	0.05	0.04	0.03	0.02	0.02
Lysine[11]	0.00	0.04	0.05	0.07	0.09
Corn starch[5]	20.85	20.97	13.83	21.22	6.91

[1] Omega Protein Inc., Huston TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[4] KnipBio Inc., Lowell, MA, USA.
[5] MP Biomedicals Inc., Solon, OH, USA.
[6] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[7] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[8] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[9] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[10] The Solae Company, St. Louis, MO, USA.
[11] Aldrich-Sigma, St. Louis, MO, USA.

Table 4 Composition of reference diet for the determination of digestibility coefficients of bacteria biomass (BB)

Ingredients	% as is
Soybean meal[1]	10.00
Fish meal[2]	32.50
Fish oil[2]	3.20
Whole wheat[3]	47.60
Trace mineral premix[4]	0.50
Vitamin premix[5]	1.80
Choline cloride[6]	0.20
Stay C[7]	0.10
Corn starch[3]	1.00
Lecethin[8]	1.00
Chromic oxide[9]	1.00

[1] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[2] Omega Protein Inc., Houston TX, USA.
[3] MP Biomedicals Inc., Solon, OH, USA
[4] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[5] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[6] VWR, Radnor, PA, USA.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] The Solae Company, St. Louis, MO, USA.
[9] Alfa Aesar, Haverhill, MA, USA.

Table 5 Proximate composition (% as is), phosphorus content (% as is), and amino acid profile (% as is) of the ingredients used in the growth and digestibility trials.

Composition[1]	Bacterial biomass	Fish meal	Soybean meal
Crude protein	52.42	62.78	44.89
Moisture	3.50	7.99	10.97
Crude fat	1.29	10.56	3.78
Crude fiber	0.00	0.00	3.20
Ash	4.25	18.75	6.67
Phosphorus	1.00	3.15	0.66
Alanine	3.81	3.91	2.04
Arginine	3.23	3.68	3.35
Aspartic acid	3.72	5.34	5.10
Cysteine	0.30	0.47	0.62
Glutamic acid	6.13	7.47	8.24
Glycine	2.73	4.88	2.04
Histidine	0.80	1.63	1.20
Isoleucine	1.80	2.42	2.17
Leucine	3.24	4.21	3.57
Lysine	2.79	4.67	3.06
Methionine	0.86	1.61	0.66
Phenylalanine	2.04	2.39	2.35
Proline	2.25	3.08	2.39
Serine	1.21	2.11	1.90
Taurine	0.08	0.73	0.13
Threonine	2.00	2.41	1.75
Tryptophan	0.10	0.62	0.62
Tyrosine	1.34	1.67	1.64
Valine	2.84	2.99	2.34

[1] Ingredients were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Table 6 Proximate composition (% as is) and amino acid profile (% as is) of the test diets used in trial 1.

Composition[1]	T_1D_1	T_1D_2	T_1D_3
Crude Protein	37.67	36.37	36.77
Moisture	5.41	8.34	6.66
Crude Fat	9.54	8.71	8.49
Crude Fiber	4.05	3.48	3.05
Ash	6.06	5.65	5.51
Alanine	1.90	1.92	2.01
Arginine	2.26	2.17	2.16
Aspartic Acid	3.51	3.26	3.16
Cysteine	0.55	0.52	0.49
Glutamic Acid	7.40	6.88	6.76
Glycine	1.48	1.46	1.50
Histidine	0.92	0.86	0.83
Isoleucine	1.69	1.59	1.56
Leucine	3.48	3.29	3.23
Lysine	1.92	1.83	1.81
Methionine	0.60	0.65	0.62
Phenylalanine	1.99	1.90	1.87
Proline	2.40	2.27	2.25
Serine	1.58	1.46	1.44
Taurine	0.10	0.10	0.10
Threonine	1.34	1.30	1.31
Tryptophan	0.39	0.40	0.36
Tyrosine	1.53	1.47	1.43
Valine	1.80	1.73	1.74

[1] Diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Table 7 Proximate composition (% as is) and amino acid profile (% as is) of the test diets used in trial 2.

Composition[1]	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6
Crude protein	36.33	35.52	36.42	34.29	34.48	36.10
Moisture	7.15	8.57	7.34	9.47	9.42	8.08
Crude fat	9.39	9.44	8.94	9.36	9.83	8.15
Crude fiber	3.21	3.28	3.01	2.99	2.73	2.69
Ash	6.86	6.75	6.70	6.62	6.60	6.55
Alanine	1.87	1.88	1.97	1.85	1.91	2.01
Arginine	2.18	2.14	2.14	2.09	2.10	2.08
Aspartic Acid	3.44	3.42	3.42	3.24	3.22	3.16
Cysteine	0.48	0.47	0.46	0.43	0.43	0.40
Glutamic Acid	6.33	6.26	6.39	5.93	5.96	5.88
Glycine	1.56	1.54	1.59	1.52	1.55	1.54
Histidine	0.86	0.85	0.86	0.80	0.80	0.78
Isoleucine	1.60	1.61	1.63	1.53	1.53	1.53
Leucine	3.28	3.25	3.37	3.09	3.14	3.21
Lysine	2.01	2.00	2.00	1.94	1.94	1.92
Methionine	0.64	0.63	0.68	0.61	0.60	0.58
Phenylalanine	1.85	1.84	1.87	1.75	1.76	1.77
Proline	2.13	2.04	2.14	2.00	2.04	2.07
Serine	1.48	1.43	1.47	1.37	1.39	1.37
Taurine	0.16	0.14	0.14	0.15	0.14	0.14
Threonine	1.29	1.28	1.30	1.24	1.26	1.27
Tryptophan	0.47	0.49	0.45	0.43	0.44	0.45
Tyrosine	1.33	1.25	1.27	1.26	1.25	1.23
Valine	1.73	1.75	1.78	1.67	1.70	1.80

[1] Diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Table 8 Proximate composition[1] (% as is) and mineral composition[1] (g kg^{-1}: phosphorus, sulfur, potassium, magnesium, calcium, sodium; mg kg^{-1}: iron, manganese, copper, zinc) of the test diets used in trial 3.

Composition[1]	T_3D_1	T_3D_2	T_3D_3	T_3D_4	T_3D_5
Crude protein	35.7	33.7	38.4	34.7	41.1
Moisture	8.7	11.71	8.39	9.7	10.46
Crude fat	6.71	7.57	8.2	7.64	7.26
Crude fiber	3.1	2.47	7.1	2.62	8.3
Ash	7.08	6.67	7.08	6.61	6.56
Sulfur	0.40	0.36	0.44	0.36	0.43
Phosphorus	1.36	1.36	1.29	1.47	1.37
Potassium	1.33	1.16	1.23	1.13	1.11
Magnesium	0.18	0.16	0.19	0.15	0.17
Calcium	1.31	1.27	1.37	1.25	1.37
Sodium	0.10	0.11	0.14	0.13	0.16
Iron (ppm)	149	127	166	126	161
Manganese (ppm)	40.1	38.1	67.8	43.4	69.4
Copper (ppm)	16.8	15.9	16.9	14.6	15.8
Zinc (ppm)	183	168	213	166	177

[1] Diets were analyzed at Midwest Laboratories (Omaha, NE, USA).

Table 9 Performance of juvenile shrimp *L. vannamei* (Initial weight 1.51g) offered diets with different bacterial biomass levels (0, 6, and 12 %) for six weeks in trial 1.

Diet	BB levels (%)	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)
T_1D_1	0	49.25	8.26[a]	440.04[a]	1.65[a]	75.0[b]
T_1D_2	6	53.85	6.96[ab]	370.55[ab]	1.99[b]	96.9[a]
T_1D_3	12	45.70	5.72[b]	280.94[b]	2.61[b]	100.0[a]
PSE[1]		1.1211	0.1646	1.7274	0.0603	12.4145
P-value		0.0831	0.0014	0.0012	0.0010	0.0047

[1] PSE: Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] WG: Weight gain = (Final weight - Initial weight) / Initial weight × 100%.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 10 Performance of juvenile shrimp *L. vannamei* (Initial weight 0.98g) offered diets with different bacteria biomass levels (0, 1, 2, 4, 6, and 12 %) for six weeks in trial 2.

Diet	BB levels (%)	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)
T_2D_1	0	79.3^{a}	8.4^{ab}	766.6^{ab}	1.64^{bc}	95.0
T_2D_2	1	84.9^{a}	9.2^{a}	836.8^{a}	1.50^{c}	92.5
T_2D_3	2	84.0^{a}	8.6^{ab}	811.1^{ab}	1.56^{bc}	97.5
T_2D_4	4	85.3^{a}	8.5^{ab}	765.3^{ab}	1.63^{bc}	100.0
T_2D_5	6	75.3^{a}	7.7^{b}	697.5^{b}	1.83^{b}	97.5
T_2D_6	12	58.1^{b}	5.8^{c}	493.70^{c}	2.50^{a}	100.0
PSE[1]		1.2191	0.1031	13.7258	0.0303	1.0623
P-value		<0.0001	<0.0001	<0.0001	<0.0001	0.1458

[1] PSE: Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] WG: Weight gain = (Final weight - Initial weight) / Initial weight × 100%.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 11 Performance of juvenile shrimp *L. vannamei* (Initial weight 0.15g) offered diets formulated to partially replace soybean meal on a digestible protein basis for six weeks in trial 3.

Diet	BB levels (%)	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)
T_3D_1	0	42.68^{a}	4.74^{a}	3160.39^{a}	1.72^{c}	90.0
T_3D_2	6	43.15^{ab}	4.30^{ab}	2813.38^{ab}	1.90^{bc}	100.0
T_3D_3	13.3	45.38^{ab}	4.54^{a}	2732.16^{abc}	1.73^{c}	100.0
T_3D_4	12	38.48^{ab}	3.84^{bc}	2438.14^{bc}	2.11^{ab}	100.0
T_3D_5	26.6	35.05^{b}	3.60^{c}	2304.94^{c}	2.26^{a}	97.5
PSE[1]		1.1420	0.0710	57.1783	0.0338	1.9084
P-value		0.0406	0.0002	0.0008	0.0001	0.3194

[1] PSE: Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] WG: Weight gain = (Final weight - Initial weight) / Initial weight × 100%.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 12 Proximate composition[2] (moisture: % as is; protein and lipid: % dry weight) and amino acid profile[2] (% dry weight) of whole shrimp body offered diets with different bacteria biomass levels (0, 1, 2, 4, 6, and 12 %) for six weeks in trial 2.

Diet	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6	PSE[1]	*P*-value	Adjust *P*-value
BB levels (%)	0	1	2	4	6	12			
Moisture	75.65^{ab}	75.48^{ab}	75.79^{ab}	75.17^{b}	76.93^{ab}	77.23^{a}	0.2091	0.0117	0.0351
Protein	75.08^{b}	74.97^{b}	74.39^{b}	74.80^{b}	75.28^{b}	77.77^{a}	0.1807	<0.0001	0.0003
Lipid	6.37^{b}	6.54^{ab}	7.92^{a}	7.26^{ab}	5.76^{b}	3.62^{c}	0.1706	<0.0001	<0.0001
Alanine	4.27	4.33	4.44	4.28	4.40	4.31	0.0365	0.5092	0.5555
Arginine	5.45^{bc}	5.23^{c}	5.43^{c}	5.47^{bc}	5.72^{b}	6.17^{a}	0.0325	<0.0001	<0.0001
Aspartic Acid	6.76	6.94	6.79	6.71	6.84	6.86	0.0322	0.2140	0.3425
Cysteine	0.60	0.61	0.61	0.60	0.62	0.63	0.0039	0.0673	0.1614
Glutamic Acid	10.18	10.41	10.16	10.06	10.22	10.31	0.0559	0.3439	0.4855
Glycine	5.01^{cd}	4.83^{d}	5.01^{cd}	5.19^{bc}	5.49^{b}	6.08^{a}	0.0350	<0.0001	<0.0001
Histidine	1.49	1.52	1.49	1.48	1.50	1.52	0.0115	0.6861	0.7159
Hydroxylysine	0.14	0.15	0.17	0.18	0.17	0.17	0.0070	0.5032	0.5555
Hydroxyproline	0.20	0.22	0.23	0.21	0.21	0.20	0.0051	0.4981	0.5555
Isoleucine	2.95	3.00	2.95	2.93	2.97	2.95	0.0111	0.3829	0.5105
Leucine	4.95	5.03	4.94	4.92	4.97	5.01	0.0162	0.1896	0.3250
Lysine	4.92	5.04	4.98	4.93	5.04	5.11	0.0239	0.0874	0.1907
Methionine	1.46^{c}	1.49^{c}	1.50^{bc}	1.51^{abc}	1.55^{ab}	1.57^{a}	0.0065	0.0002	0.0010
Phenylalanine	3.16	3.23	3.20	3.18	3.20	3.27	0.0169	0.3398	0.4855
Proline	4.09^{a}	4.15^{a}	4.18^{a}	4.00^{ab}	3.88^{ab}	3.67^{b}	0.0415	0.0036	0.0143
Serine	2.35	2.38	2.35	2.34	2.36	2.36	0.0195	0.9934	0.9934
Threonine	2.62^{b}	2.76^{a}	2.62^{b}	2.60^{b}	2.64^{b}	2.61^{b}	0.0135	0.0049	0.0169

Tryptophan	0.87	0.85	0.85	0.85	0.85	0.88	0.0048	0.1132	0.2263
Tyrosine	2.51	2.33	2.49	2.52	2.45	2.57	0.0405	0.4536	0.5555
Valine	4.10	4.19	4.12	4.16	4.17	4.00	0.0257	0.1632	0.3014
Total	68.05	68.66	68.49	68.08	69.23	70.23	0.2581	0.0629	0.1614

[1] PSE: Pool standard error.

[2] Proximate composition and amino acid profile of whole body samples were analyzed at University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory (Columbia, MO, USA).

Values within a row with different superscripts are significantly different based on Tukey's multiple range test.

Table 13 Protein[2] and amino acids[3] retention efficiencies of Pacific white shrimp at the conclusion of a 6-week growth trial 2 in which shrimp were offered diets formulated to contain different bacteria biomass levels (0, 1, 2, 4, 6, and 12 %).

Retention efficiencies	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6	PSE[1]	*P*-value	Adjust *P*-value
BB levels (%)	0	1	2	4	6	12			
Protein	34.2^{ab}	39.0^{a}	35.2^{ab}	38.3^{a}	31.6^{b}	22.1^{c}	0.6018	<0.0001	<0.0001
Alanine	37.5^{ab}	42.4^{a}	38.9^{a}	40.4^{a}	33.2^{b}	21.7^{c}	0.5532	<0.0001	<0.0001
Arginine	41.5^{a}	45.1^{a}	43.9^{a}	46.1^{a}	39.8^{a}	31.1^{b}	0.7623	<0.0001	<0.0001
Aspartic Acid	32.6^{ab}	37.6^{a}	34.4^{ab}	36.4^{a}	30.8^{b}	22.3^{c}	0.5675	<0.0001	<0.0001
Cysteine	20.5^{b}	24.0^{ab}	22.9^{ab}	24.6^{a}	20.9^{b}	16.3^{c}	0.3868	<0.0001	<0.0001
Glutamic Acid	26.7^{ab}	30.9^{a}	27.5^{ab}	29.9^{a}	24.9^{b}	18.0^{c}	0.4745	<0.0001	<0.0001
Glycine	53.4^{ab}	58.0^{ab}	54.7^{ab}	60.6^{a}	52.1^{b}	42.1^{c}	0.8645	<0.0001	<0.0001
Histidine	28.6^{ab}	33.2^{a}	30.0^{ab}	32.4^{ab}	27.1^{b}	19.9^{c}	0.5900	<0.0001	<0.0001
Hydroxylysine	29.3	40.4	36.5	39.4	31.2	22.5	2.0246	0.0445	0.0445
Hydroxyproline	16.9^{b}	18.3^{b}	21.1^{b}	36.3^{a}	11.9^{c}	9.2^{c}	0.5395	<0.0001	<0.0001
Isoleucine	30.5^{ab}	34.5^{a}	31.3^{ab}	33.7^{a}	28.2^{b}	19.8^{c}	0.5290	<0.0001	<0.0001
Leucine	25.0^{ab}	28.7^{a}	25.4^{ab}	28.1^{a}	23.0^{b}	16.1^{c}	0.4198	<0.0001	<0.0001
Lysine	40.7^{bc}	46.9^{a}	43.3^{abc}	45.0^{ab}	38.0^{c}	27.6^{d}	0.6348	<0.0001	<0.0001
Methionine	38.1^{b}	43.9^{a}	38.5^{ab}	43.8^{a}	38.0^{b}	28.2^{c}	0.6263	<0.0001	<0.0001
Phenylalanine	28.3^{ab}	32.5^{a}	29.7^{ab}	32.1^{a}	26.4^{b}	19.0^{c}	0.5253	<0.0001	<0.0001
Proline	32.4^{bc}	38.4^{a}	34.5^{ab}	35.9^{ab}	28.0^{c}	18.4^{d}	0.6150	<0.0001	<0.0001
Serine	26.3^{bc}	30.8^{a}	27.7^{abc}	30.1^{ab}	24.6^{c}	17.6^{d}	0.4686	<0.0001	<0.0001
Threonine	33.6^{bc}	40.1^{a}	34.9^{bc}	36.8^{ab}	30.3^{c}	21.1^{d}	0.5590	<0.0001	<0.0001
Tryptophan	30.5^{ab}	31.9^{ab}	32.3^{ab}	34.6^{a}	27.9^{b}	20.1^{c}	0.5820	<0.0001	<0.0001
Tyrosine	31.2^{a}	34.3^{a}	34.0^{a}	35.2^{a}	28.3^{ab}	21.4^{b}	0.9020	0.0003	0.0003

Valine	39.4^{ab}	44.5^{a}	40.1^{ab}	43.9^{a}	35.7^{b}	22.7^{c}	0.6802	<0.0001	<0.0001
Total	32.3^{ab}	36.9^{a}	33.7^{ab}	36.3^{a}	30.1^{b}	21.7^{c}	0.5415	<0.0001	<0.0001

[1] Pooled standard error.
[2] Protein retention (%) = (Final weight × Final protein content) - (Initial weight × Initial protein content) × 100 / Protein offered.
[3] Amino acids (AA) retention (%) = (Final weight × Final AA content) - (Initial weight × Initial AA content) × 100 / AA offered.
Values within a row with different superscripts are significantly different based on Tukey's multiple range test.

Table 14 Proximate composition of whole shrimp body and protein retention efficiency (PRE) offered diets formulated to utilize bacterial biomass (BB) partially replace soybean meal on a digestible protein basis for six weeks in trial 3.

Diet	BB levels (%)	Protein[2] (%)	Moisture (%)	Lipid[2] (%)	Fiber[2] (%)	Ash[2] (%)	PRE[3] (%)
T_3D_1	0	70.83[b]	76.1	8.40[a]	5.25	11.50[c]	30.50[a]
T_3D_2	6	70.68[b]	76.7	7.89[a]	5.26	11.80[c]	29.37[a]
T_3D_3	13.3	72.52[ab]	76.6	5.07[b]	5.30	12.56[bc]	27.97[a]
T_3D_4	12	72.59[ab]	77.0	6.00[ab]	5.48	13.35[ab]	25.43[ab]
T_3D_5	26.6	73.55[a]	77.4	4.07[b]	5.68	14.01[a]	20.11[b]
P-value		0.0107	0.2379	0.0003	0.3623	<0.0001	0.0002
PSE[1]		0.2819	0.1932	0.2803	0.0849	0.1399	0.6234

[1] PSE: Pool standard error.
[2] Dry weight basis.
[3] Protein retention (%) = (Final weight × Final protein content) - (Initial weight × Initial protein content) × 100 / Protein offered.

Table 15 Apparent dry matter (ADM), apparent energy (AED) and apparent protein (APD) digestibility values for the diet (D) and ingredient (I) using 70:30 replacement technique offered to Pacific white shrimp *L. vannamei*.

	ADMDD	AEDD	APDD	ADMDI	AEDI	APDI
Basal1	76.38 ± 0.37^{a}	82.66 ± 1.20^{a}	92.08 ± 0.55^{a}			
SBM	77.02 ± 0.87^{a}	82.63 ± 1.05^{ab}	94.76 ± 0.49^{a}	78.51 ± 2.89^{a}	82.56 ± 3.79^{a}	97.03 ± 0.83^{a}
FM1	68.21 ± 3.80^{b}	78.31 ± 3.21^{bc}	80.86 ± 1.80^{b}	49.15 ± 12.67^{b}	69.77 ± 9.51^{ab}	67.07 ± 4.02^{b}
BB1	41.12 ± 2.03^{c}	59.86 ± 1.25^{d}	73.09 ± 1.50^{c}	-40.11 ± 6.78^{c}	13.33 ± 3.85^{c}	42.55 ± 3.92^{c}
Basal2	75.69 ±0.52^{a}	81.51 ± 0.41^{ab}	92.04 ± 0.03^{a}			
FM2	67.99 ± 0.17^{b}	76.44 ± 0.78^{c}	82.34 ± 0.31^{b}	49.45 ± 0.56^{ab}	65.78 ± 2.23^{b}	71.30 ± 0.68^{ab}
BB2	38.54 ± 1.49^{c}	53.98 ± 0.27^{e}	67.48 ± 1.48^{d}	-48.16 ± 4.96^{c}	-0.94 ± 0.82^{c}	29.26 ± 3.79^{c}

Values from each treatment are means and standard deviation of triplicate tanks. Values within a column different superscripts are significantly different ($P < 0.05$).

Table 16 Apparent amino acids (AA) digestibility value for the soybean meal (SBM), fish meal (FM), bacteria biomass (BB) using 70:30 replacement technique offered to Pacific white shrimp *L. vannamei*.

AA digestibility coefficients (%)	SBM	FM	BB
Alanine	93.75 ± 2.02^{a}	69.09 ± 4.09^{b}	51.05 ± 3.55^{c}
Arginine	96.91 ± 1.44^{a}	75.35 ± 3.78^{b}	51.33 ± 3.43^{c}
Aspartic acid	95.39 ± 1.36^{a}	69.23 ± 3.70^{b}	33.82 ± 4.79^{c}
Cysteine	91.29 ± 1.68^{a}	54.39 ± 7.06^{b}	-50.61 ± 16.31^{c}
Glutamic acid	95.69 ± 1.52^{a}	70.84 ± 3.70^{b}	40.00 ± 4.08^{c}
Glycine	95.06 ± 2.05^{a}	66.55 ± 6.26^{b}	16.82 ± 5.30^{c}
Histidine	94.33 ± 1.69^{a}	74.26 ± 2.86^{b}	20.70 ± 6.02^{c}
Isoleucine	93.23 ± 1.72^{a}	68.72 ± 3.99^{b}	37.95 ± 5.92^{c}
Leucine	92.23 ± 1.96^{a}	71.29 ± 3.16^{b}	35.17 ± 5.32^{c}
Lysine	95.03 ± 1.84^{a}	76.97 ± 2.24^{b}	40.13 ± 3.88^{c}
Methionine	95.20 ± 1.54^{a}	70.63 ± 3.30^{b}	48.31 ± 3.06^{c}
Phenylalanine	93.41 ± 1.90^{a}	65.28 ± 4.13^{b}	34.43 ± 5.37^{c}
Proline	94.68 ± 1.92^{a}	67.21 ± 5.39^{b}	4.86 ± 7.02^{c}
Serine	93.11 ± 1.91^{a}	58.31 ± 4.65^{b}	20.24 ± 5.47^{c}
Threonine	91.99 ± 1.94^{a}	66.33 ± 3.35^{b}	40.16 ± 3.84^{c}
Tryptophan	95.37 ± 1.92^{a}	80.31 ± 1.53^{b}	-34.14 ± 24.88^{c}
Tyrosine	95.28 ± 1.22^{a}	73.62 ± 3.40^{b}	44.04 ± 7.25^{c}
Valine	90.78 ± 2.39^{a}	67.06 ± 3.75^{b}	49.27 ± 6.00^{c}
Total	94.31 ± 1.67^{a}	69.91 ± 3.89^{b}	34.87 ± 4.71^{c}

Values from each treatment are means and standard deviation of triplicate tanks. Values within a row different superscripts are significantly different ($P < 0.05$).

CHAPTER VI

EVALUATION OF A FISH MEAL ANALOGUE AS A REPLACEMENT FOR FISH MEAL IN PRACTICAL DIETS FOR PACIFIC WHITE SHRIMP *Litopenaeus vannamei*

1. Introduction

Fish meal (FM) is the most important and preferred protein source in most shrimp feeds, because it is an excellent source of essential nutrients such as protein and indispensable amino acids, essential fatty acids, cholesterol, vitamins, minerals, attractants and unidentified growth factors (Samocha *et al*., 2004; Swick *et al*., 1995). Because of FM's comparatively high nutritional value and limitation of availability, it has a high demand and limit supply resulting in a corresponding high market value. Hence, we must shift our emphasis and use this ingredient only when nutrient requirements of the animal demand its use (Davis and Sookying, 2009).

As commercial shrimp culture has become an expanded and intensified economic activity, the demand for cost effective protein sources continues to increase because there is a considerable cost saving in shifting protein sources when it is economical and nutritionally viable. Partial replacement of FM in the diets for Pacific white shrimp were demonstrated to be feasible in many studies (Cruz-Suárez *et al*., 2007; Goytortúa-Bores *et al*., 2006; Ju *et al*., 2012; Liu *et al*., 2012; Oujifard *et al*., 2012; Tan *et al*., 2005). If the substitution strategy considers shifts in essential nutrients, it also appears that FM can be removed from shrimp formulations if suitable alternative sources of protein and lipids are provided to meet the nutrient requirements of the animal (Davis *et al*., 2004). The use of complementary ingredients is a practice used to obtain a more balanced nutrient profile in the feeds (i.e. essential amino acids, fatty acids) and to increase nutrient utilization and facilitate feed processing (Samocha *et al*., 2004). Based on

numerous studies with Pacific white shrimp reared under a variety of culture conditions and densities, FM can be successfully removed from shrimp feed formulations in properly balanced production diets without compromising the growth of shrimp (Amaya *et al*., 2007a;b; Browdy *et al*., 2006; Roy *et al*., 2009; Samocha *et al*., 2004; Sookying and Davis, 2011).

Aqua-Pak Pro-Cision is a blend of animal and plant protein products supplemented with encapsulated essential amino acids and some other essential nutrients that designed for shrimp industry as a substitution for FM. Because it has a similar balanced nutrient profile in terms of protein, lipid, and amino acids to that of FM and has a lower price it may be suitable for inclusion in the shrimp diets. The information of this product as a FM replacement in practical diets for the Pacific white shrimp is still limited. Hence, the objectives of this study were to determine the nutrient digestibility values of the ingredient and evaluate the potential effects of this product as a replacement for FM in practical diets for Pacific white shrimp, *L. vannamei*.

2. Materials and Methods

2.1. Experimental design and diets

All test diets were formulated to be isonitrogenous and isolipidic (35% protein and 8% lipid). In the growth trial 1 and 2, five experimental diets were formulated to contain increasing level of FMA (0, 4.85, 9.70, 14.55, and 19.44%) as a replacement of FM (0, 5, 10, 15, and 20%) (Table 1). In trial 3, five experimental diets were essentially the same as the diets used in trial 1 and 2, but balanced for P (Table 2). Additionally, a reference diet (Table 3) was utilized to determine digestibility coefficients in conjunction with 1% chromic oxide as an inert marker and 70:30 replacement strategy.

Primary ingredients were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate composition, P content, and amino acid profile (Table 4). All experimental diets were produced at the Aquatic Animal Nutrition Laboratory at the School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University (Auburn, AL, USA) using the standard procedures for the shrimp feeds. Briefly, diets were prepared by mixing the pre-ground dry ingredients in a food mixer (Hobart, Troy, OH, USA) for 10–15 minutes. Hot water was then blended into the mixture to obtain a consistency appropriate for pelleting. Diets were pressure-pelleted using a meat grinder with a 2.5-mm die. The wet pellets were then placed into a fan-ventilated oven (< 50 °C) overnight in order to attain a moisture content of less than 10%. Dry pellets were crumbled, packed in sealed bags, and stored in a freezer until use. The diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) and Midwest Laboratories (Omaha, NE, USA) for proximate composition, amino acid profile, and mineral composition (Table 5 and 6).

2.2. Growth trials

Three trials were conducted at the E.W. Shell Fisheries Research Station, Auburn University (Auburn, AL, USA). Pacific white shrimp post larvae (PL) were obtained from Shrimp Improvement Systems (Islamorada, Florida) and nursed in an indoor recirculating system. PLs were fed a commercial feed (Zeigler Bros., Inc., Gardners, Pennsylvania, USA) using an automatic feeder for ~1 week, and then switched to crumbled commercial shrimp feed (Zeigler Bros., Inc., Gardners, Pennsylvania, USA) for ~1- 2 weeks.

In trial 1 and 2, the recirculating system consisted of 30 aquaria (135 L) connected to a common reservoir, biological filter, bead filter, fluidized biological filter and recirculation pump. Six replicate groups of shrimp (0.47 g initial mean weight, 10 shrimp/tank for trial 1; 0.40 g initial mean weight, 10 shrimp/tank for trial 2) were offered diets using our standard feeding protocol over 6 weeks. Based on historic results, feed inputs were pre-programmed assuming the shrimp would double their weight weekly up to one gram then gain 0.8-1.1 g weekly with a feed conversion ratio (FCR) of 1.8. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality. Consequently, for each tank in trial 1, a fixed ration of 1.21 g day^{-1} for the first week, 2.06 g day^{-1} for the second week, 2.31 g day^{-1} for the third week, 2.57 g day^{-1} for the fourth week, and 2.83 g day^{-1} for the remaining culturing period was offered, partitioned in 4 feedings each day. For each tank in trial 2, a fixed ration of 1.03 g day^{-1} for the first week, 2.06 g day^{-1} for the second to fourth week, 2.31 g day^{-1} for the fifth week, and 2.57 g day^{-1} for the last week was also allotted in 4 portions per day.

In trial 3, the recirculating system consisted of 20 aquaria (80 L) connected to a common reservoir, biological filter, bead filter, fluidized biological filter and recirculation pump. Four replicate groups of shrimp (0.25 g initial mean weight, 10 shrimp/tank) were offered diets using our standard feeding protocol over 6 weeks. Based on historic results, feed inputs were pre-programmed assuming the shrimp would double their weight weekly up to one gram then gain 0.8-1.1 g weekly with a feed conversion ratio (FCR) of 1.8. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality. Consequently, for each tank in trial 3, a fixed ration of 0.68 g day^{-1} for the first week, 1.36 g day^{-1} for the second week, 2.18 g day^{-1} for the third and fourth week, and 2.73 g day^{-1} for the remaining culturing period was offered, partitioned in 4 feedings each day.

At the conclusion of each growth trial, shrimp were counted and group-weighted. Mean final weight, FCR, WG, biomass, and survival were determined (Table 7). After obtaining the final total weight of shrimps in each aquarium, 4 shrimps from trial 2 and trial 3 were randomly selected and frozen at -20 °C for subsequent determination of whole body composition. Proximate and mineral compositions of whole shrimp body in trial 2 and trial 3 (Table 9 and Table 10) were analyzed by University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory (Columbia, MO, USA) and Midwest Laboratories (Omaha, NE, USA), respectively. Protein and mineral retention was calculated as follows:

Protein retention (%) = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein offered.

Mineral retention (%) = (final weight × final mineral content) - (initial weight × initial mineral content) × 100 / mineral offered.

2.3. Water quality monitoring

For all trials, dissolved oxygen (DO), water temperature and salinity were measured twice daily by using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH, USA). Hydrogen potential (pH) was measured twice weekly by using a waterproof pHTestr30 (Oakton instrument, Vernon Hills, IL, USA). Total ammonia-nitrogen (TAN) and nitrite were evaluated every week by using the methods described by Sororzano (1969) and Spotte (1979).

2.4. Digestibility trial

The digestibility trial was conducted in the mentioned recirculation system and utilized six shrimp per aquaria with six aquaria per dietary treatment. Once acclimated for three days to

the test diets, feces from two aquaria were pooled (n=3) and collected over a 5-day period or until adequate samples were obtained. To obtain fecal samples, the aquaria were cleaned by siphoning before each feeding with the first collection of the day discarded. After cleaning, the shrimp were offered an excess of feed and then about 1 hour later feed was removed and feces were collected by siphoning onto a 500μm mesh screen. Collected feces were rinsed with distilled water, dried at 105 °C until a constant weight was obtained, and then stored in freezer (−20 °C) until analyzed. Apparent digestibility coefficient for dry matter, protein, energy, and amino acids were determined by using chromic oxide (Cr_2O_3, 10 g kg^{-1}) as an inert marker. Chromium concentrations were determined by the method of McGinnis and Kasting (1964) in which, after a colorimetric reaction, absorbance is read on a spectrophotometer (Spectronic genesis 5, Milton Roy Co., Rochester, NY, USA) at 540nm. Gross energy of diets and fecal samples were analyzed with a Semi micro-bomb calorimeter (Model 1425, Parr Instrument Co., Moline, IL, USA). Protein were determined by micro-Kjeldahl analysis (Ma and Zuazaga, 1942). Amino acids were analyzed by University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory (Columbia, MO, USA). The apparent digestibility coefficient of dry matter (ADMD), protein (APD), energy (AED), and amino acids (AAAD) were calculated according to Cho *et al*. (1982) as follows:

ADMD (%) = 100 − [100 × (% Cr_2O_3 in feed / % Cr_2O_3 in feces)]

APD, AED, and AAAD (%) =100 − [100 × (% Cr_2O_3 in feed / % Cr_2O_3 in feces) × (% nutrient in feces / % nutrient in feed]

The apparent digestibility coefficients (ADC) of the test ingredients for dry matter, energy, protein and amino acids were calculated according to Bureau, Hua (2006) as follows:

$ADC_{test\ ingredient} = ADC_{test\ diet} + [(ADC_{test\ diet} - ADC_{ref.\ diet}) \times (0.7 \times D_{ref} / 0.3 \times D_{ingr})]$

where D_{ref} = % nutrient (or KJ/g gross energy) of reference diet mash (as is); D_{ingr} = % nutrient (or KJ/g gross energy) of test ingredient (as is).

2.5. Statistical analysis

All the data were analyzed using SAS (V9.3. SAS Institute, Cary, NC, USA). Data from were analyzed using one-way ANOVA to determine significant differences (P<0.05) among treatments followed by the Tukey's multiple comparison test to determine difference between treatments in each trial. Pearson correlation analysis was performed to determine if there is a correlation between dietary P levels and biological responses of shrimp. The pooled standard errors were used across growth trials, as the variance of each treatment is the same.

3. Results

3.1. Water quality

In trial 1, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 6.04±0.42mg L^{-1}, 27.9±0.4°C, 9.7±0.3ppt, 7.8±0.2, 0.083±0.088mg L^{-1}, and 0.020±0.019mg L^{-1}, respectively. In trial 2, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 5.86±0.31mg L^{-1}, 28.5±0.8°C, 8.2±1.4ppt, 7.5±0.3, 0.041±0.050mg L^{-1}, and 0.058±0.102mg L^{-1}, respectively. In trial 3, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 6.66±0.3 mg L^{-1}, 28.8 ± 2.4 °C, 8.0 ± 0.9 ppt, 7.0 ± 0.3, 0.029 ± 0.036 mg L^{-1}, and 0.121 ± 0.128 mg L^{-1}, respectively. Water quality conditions in all trials were suitable for normal growth and survival of this species.

3.2. Growth trial

Growth performance of juvenile Pacific white shrimp fed with experimental diets are presented in Table 7. In trial 1, shrimp offered diets incorporated with 4.85% FMA replacement exhibited significantly higher WG than treatment contained 14.55% FMA. No significantly differences were observed in final biomass (34.68g to 37.10g), final mean weight (4.29 g to 4.85g), FCR (2.21 to 2.55), and survival (75% to 86.7%). In trial 2, shrimp fed with diets contained 4.85 and 9.7% FMA showed significantly higher final biomass, final mean weight, and WG, but lower FCR than shrimp offered diets supplemented with 19.4% FMA. No significant difference was found in survival (81.7% to 91.7%). In trial 3, final mean weight and WG were significantly improved in the treatment contained 4.85% FMA compared to the treatment supplemented with 14.55% FMA. No significant differences were determined in biomass (35.28 to 42.35 g), FCR (1.68 to 2.20), and survival (80.0 to 87.5%).

Pearson correlation coefficients of growth performance, FCR, and survival with dietary P levels are presented in Table 8. In trial 1 and trial 2, dietary phosphorus levels had significant positive correlation with weight gain while negatively correlated with FCR. No correlations of phosphorus levels with survival were observed.

Proximate and mineral compositions of whole shrimp body as well as protein and P retention in trial 2 and trial 3 are presented in Table 9 and Table 10. In trial 2, shrimp fed with diet containing 4.85% FMA exhibited significantly higher lipid content compared to treatment included with 19.4% FMA. Protein content was significantly improved when shrimp was fed with diet contained 19.4% FMA in contrast with treatments contained 0 and 4.85% FMA. Shrimp fed with diets containing 4.85 and 9.7% FMA exhibited significantly improved protein retention (PR) compared to those fed with the diet supplemented with 19.4% FMA. No

significant differences were detected in moisture (72.28 to 75.97%), crude fiber (5.71 to 6.25%), ash (11.85 to 12.45%), and P (1.03 to 1.08%) contents of whole shrimp body and P retention (11.49 to 14.09%) across the treatments.

In trial 3, significantly improved PR was detected in the shrimp fed diets containing 4.85% FMA. P retention in the treatment supplemented with 19.4% FMA was significantly higher than that in the treatment containing 14.55% FMA. No significant differences were determined in protein (74.23 to 75.8%), moisture (76.81 to 78.26%), lipid (4.65 to 5.82%), ash (11.40 to 12.07%), sulfur (0.84 to 0.9%), phosphorus (0.99 to 1.06%), potassium (1.33 to 1.46%), magnesium (0.26 to 0.3%), calcium (3 to 3.61%), sodium (1.07 to 1.19%), iron (17.1 to 26.03 mg kg^{-1}), manganese (2.68 to 3.53 mg kg^{-1}), copper (69.4 to 75.85 mg kg^{-1}), zinc (74 to 77.2 mg kg^{-1}) contents in the whole shrimp body.

3.3. Digestibility trial

Apparent dry matter (ADM), apparent energy (AED), and apparent protein (APD) digestibility values for the diet (D) and ingredient (I) using 70:30 replacement technique offered to shrimp are presented in Table 11. The digestibility trial contained a range of ingredients; hence, we have provided a few other ingredients as a reference. In terms of the proximate composition, FMA contains a higher protein and lipid content, but lower phosphorus level than FM (Table 2). In order to confirm the results, fecal samples for basal diets and FM diet were recollected. The results turned out to be quite similar, which indicated that the feces collection and samples analysis methods we utilized in the digestibility study are consistent. The energy and protein digestibility of FMA were 53.5% and 32.4%, respectively, which were significantly lower than those of FM.

Apparent amino acids (AA) digestibility values for the SBM, FM, and FMA using 70:30 replacement technique offered to Pacific white shrimp are presented in Table 12. Most individual amino acids compositions in FMA are higher than those in FM. In terms of two of the most limiting AAs in shrimp feeds, FMA shared similar methionine and lysine levels as FM (4.99% vs 4.67% and 1.69% vs 1.61%, respectively). The AAs digestibility corresponded to the protein digestibility. Apparent digestibility coefficients of alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine were of FMA were significantly lower than those of FM and SBM.

4. Discussion

The nutrient composition of FMA compared to FM exhibited higher crude protein (68.28% vs 62.78%) and lipid (13.89% vs 10.56%) contents, but lower P level (1.68% vs 3.15%). Generally, both essential and non-essential amino acids concentrations in FMA are superior to FM. When FM is replaced with alternative protein sources, it is crucial to balance essential nutrients especially amino acids in the diets. Proximate compositions, amino acids profiles, and mineral contents (excluding P levels) are consistent in all the treatments of the growth trials (Table 5 and 6). There is a decreasing trend of P level as the inclusion level of FMA increases in the diets for trial 1 and trial 2 (Table 5) due to the comparatively lower P content in this ingredient. All diets in trial 3 shared a similar P content as P level was balanced by adding inorganic P in formulation (Table 6).

The nutrient digestibility of a feed ingredient is an important factor to evaluate the overall nutritive value of the ingredient because it is related to the quantity of the nutrient absorbed by

the animals. In the current study, APD and AED of FMA were significantly lower than those of SBM and FM. The comparatively lower APD of FMA translated to lower AAAD. In general, there was a reasonable correspondence of the AAAD to APD of FMA. SBM had the highest APD (97.03%), AED (82.56%), and ADAA (90.78 – 96.91%) among the ingredients tested in the current study. Similar ranges of results for APD, AED, and AAAD were reported in multiple shrimp studies (Cruz-Suarez *et al*., 2009; Fang *et al*., 2016; Liu *et al*., 2013; Yang *et al*., 2009; Zhou *et al*., 2015). ADP and ADE of FM1 were 67.07% and 69.77%, respectively. Similar results were acquired in FM2 (ADP and ADE: 71.3% and 65.78%, respectively). The analogous results of basal diet and FM diet from the collection of two fecal samples pointed to the consistency in the feces collection and sample analysis methods. APD of FM has been reported to be ranged from 62.7% to 91.6% in numerous studies (Lemos *et al*., 2009; Liu *et al*., 2013; Terrazas-Fierro *et al*. 2010; Yang *et al*., 2009). ADP of FM in the present study were in the lower range of the results documented among those researches. The differences could be attributed to several factors, such as different raw materials, location or processing methods used to produce the products, and unknown factors related to different production batches.

With regards to the growth trials, significantly reduced growth was observed in the diet contained 14.55% FMA compared to the one supplemented with 4.85% FMA. If the growth depression resulted from the FMA inclusion levels, the diet contained highest inclusion of FMA should also be negatively affected. However, no difference in terms of growth was detected in the treatment contained 19.4% FMA. Given the relatively lower survival in trial 1, the growth results may be masked. Hence, trial 2 was initiated by applying the same diets to confirm the results. At the end of trial 2, shrimp fed with diet contained 19.4% FMA exhibited significantly lower WG and higher FCR than those fed with diets supplemented with 4.85 and 9.7% FMA.

There is a clear decreasing trend when inclusion level of FMA increased to 14.55% of the diet. Given the balanced proximate composition and amino acid profile across the treatments, the reduced P level might be attributed to depressed growth at the high inclusion levels of FMA. Correlation analysis outputs (Table 8) also indicated that P level in the diets positively correlated with WG and negatively correlated with FCR in both trial 1 and trial 2, which demonstrated our nutritional assumption from statistical standpoint.

P is an important constituent of nucleic acids and cell membranes, a major constituent of structural components of structural tissues, and is directly involved in all energy-producing cellular reactions (NRC, 2011). Dietary P deficiency impairs intermediary metabolism, which results in reduced growth and increased feed conversion. P requirement of Pacific white shrimp is affected both by P and calcium (Ca) levels as well as presence of inhibitors (Cheng *et al*., 2006; Davis *et al*., 1993). Cheng *et al*. (2006) indicated diets contained 0.93% total P in the absence of Ca were adequate for optimal growth of Pacific white shrimp. In the current study, P levels in diets supplemented with 14.55% and 19.4% FMA are 0.82% and 0.78%, respectively, which may suggest P limitation in these two diets. To elucidate if decreased P was the cause of the growth depression in trial 1 and trial 2, the third trial was conducted to essentially utilize the same diets in trial 1 and 2, but balanced for P. Results indicated that shrimp fed with 4.95% FMA performed significantly better in terms of growth than those fed with diets contained 14.55% FMA. No clear decreasing trend of growth was detected when diets contained FMA were compared to the reference diet (20% FM based diet). At a conclusion of the growth trials, P levels in the diets may limit the growth when shrimp fed with diets contained 14.55% and 19.4% FMA in trial 1 and 2. However, reasons for the improvements in growth when FMA was supplemented at 4.95% in all trials are not able to be defined.

There are numerous studies looking at the potential of alternative ingredients as FM replacement in shrimp feeds. The results varied as different ingredient and reference diets utilized. In general, many researches documented that partial FM replacement by alternative ingredients in shrimp feeds was applicable (Cruz-Suárez *et al*., 2007; Goytortúa-Bores *et al*., 2006; Ju *et al*., 2012; Liu *et al*., 2012; Oujifard *et al*., 2012; Suárez *et al*., 2009; Tan *et al*., 2005; Yue *et al*., 2012). The failure of complete FM replacement can be attributed to many factors such as palatability problem, imbalanced essential nutrients (protein, lipid, essential amino acids, P, and etc.), and relatively lower nutrient availability than FM. Hence, choosing suitable alternative ingredients and considering shifts in essential nutrients in the substitution strategy would be the key factors to the success of complete FM replacement. In proper balanced shrimp diets, complete FM can be replaced by suitable alternative ingredients which were reported by multiple researchers (Amaya *et al*., 2007a;b; Bauer *et al*., 2012; Browdy *et al*., 2006; Roy *et al*., 2009; Samocha *et al*., 2004; Sookying and Davis, 2011).

In the present study, lipid content of whole shrimp body was significantly decreased in the diet contained 19.4% FMA compared to the one supplemented with 4.95% FMA in trial 2, which may due to the comparatively lower energy availability in FMA than FM. Whereas the protein content of whole shrimp body was dramatically improved in the diet supplemented with 19.4% FMA in contrast with the ones incorporated with 0 and 4.95% FMA, which may be a result of reduced lipid content of shrimp as no significant difference were observed in moisture, crude fiber, and ash content of shrimp body. Similarly, Hernández *et al*. (2008) indicated that protein content of shrimp body was significantly enhanced while lipid level was significantly reduced when porcine meat meal was incorporated in the diet to replace FM. On the contrary, no significant differences were detected in the protein and lipid contents of shrimp body in trial 3

when shrimp were fed with the essentially same diets except for different P levels as in trial 2. As energy digestibility of FMA was not dramatically lower than FM, its effect on the lipid content may be marginal. Accordingly, no significant differences were detected in protein and lipid contents of shrimp body in FM replacement studies (Oujifard *et al*., 2012; Ju *et al*., 2012; Yue *et al*., 2012).

Although there is a decreasing trend of P content in the diets, no significant difference was observed in the P level in shrimp body in trial 2. Similarly, Ju *et al*. (2012) documented that P level of shrimp body was not affected when there was a decreasing trend of P content in the diets using a defatted microalgae meal to replace FM. Hepatopancreas and carapace are two good indicator tissues of the mineral status of shrimp (Davis *et al*., 1993). As the concentrations of these two tissues were diluted in the whole body samples, the reflection of P status in shrimp might be masked. In trial 3, no significant difference was detected in the P content of shrimp body as the diets were balanced for P level.

Nutrient retention was determined by a number of factors including dietary nutrient levels, feed intake, final weight and initial weight of animals as well as the final and initial nutrient content of animals (Halver and Hardy, 2002). In trial 2, shrimp fed diets containing 4.85 and 9.7% FMA exhibited improved PR in contrast with those offered the diet containing 19.4% FMA. No differences were detected in dietary protein levels, feed offered to the shrimp, and initial weight of shrimp. Although protein content of whole shrimp body was significantly improved in the diet contained 19.4% FMA, its effect cannot counteract with the significantly reduced final weight of shrimp in the same treatment, which would be the primary cause of reduced PR. In trial 3, results demonstrated that PR in the treatment supplemented with 4.95% FMA was significantly improved, resulting by the significantly enhanced final mean weight.

Similarly, Hernández *et al*. (2008) reported PR was significantly reduced when shrimp fed with diets supplemented with high levels of porcine meat meal to replace FM, resulting from the significantly depressed final weight even though protein content of shrimp body was enhanced. In both trial 2 and trial 3, no significant differences were detected in the P retention.

5. Conclusion

Under the conditions of this work, FMA can replace FM up to 20% in practical shrimp diets supplemented with inorganic P without compromising the growth of shrimp. P limitation is likely the reason for growth depression in the treatment devoid of FM when diets were not balanced for P. The improvement of growth when FMA was incorporated at 4.95% across three trials was not able to be defined. Given the good growth across the range of inclusion without any indication of a growth depression, the digestibility of the protein of FMA would be similar to that of the FM for which it was substituted. The low nutrient digestibility of FMA may due to an atypical response or the product simply does not work with the testing technique.

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Table 1 Composition (% as is) of test diets used in the growth trial 1 and 2.

Ingredient	Diet code				
	D_1	D_2	D_3	D_4	D_5
Fish meal[1]	20.00	15.00	10.00	5.00	0.00
Soybean meal[2]	37.20	37.20	37.20	37.20	37.20
Whole wheat[3]	32.00	32.00	32.00	32.00	32.00
Fish meal analogue[4]	0.00	4.85	9.70	14.55	19.40
Fish oil[2]	4.39	4.26	4.13	4.00	3.87
Trace mineral premix[5]	0.50	0.50	0.50	0.50	0.50
Vitamin premix[6]	1.80	1.80	1.80	1.80	1.80
Choline chloride[3]	0.20	0.20	0.20	0.20	0.20
Stay C[7]	0.10	0.10	0.10	0.10	0.10
Lecithin[8]	1.00	1.00	1.00	1.00	1.00
Corn starch[3]	2.81	3.09	3.37	3.65	3.93

[1] Menhaden fish meal, special select. Omega Protein Inc., Houston, TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] MP Biomedicals Inc., Solon, OH, USA.
[4] Aqua-Pak Pro-Cision, H. J, Baker Brother Inc., Shelton, CT, USA.
[5] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] Enhanced D97, The Solae Company, St. Louis, MO, USA.

Table 2 Composition (% as is) of test diets used in the growth trial 3.

Ingredient	Diet code				
	D_{1p}	D_{2p}	D_{3p}	D_{4p}	D_{5p}
Fish meal[1]	20.00	15.00	10.00	5.00	0.00
Soybean meal[2]	37.30	37.30	37.30	37.30	37.30
Whole wheat[3]	34.00	34.00	34.00	34.00	34.00
Fish meal analogue[4]	0.00	4.85	9.70	14.55	19.40
Fish oil[2]	4.54	4.37	4.19	4.02	3.87
Trace mineral premix[5]	0.50	0.50	0.50	0.50	0.50
Vitamin premix[6]	1.80	1.80	1.80	1.80	1.80
Choline chloride[3]	0.20	0.20	0.20	0.20	0.20
Stay C[7]	0.10	0.10	0.10	0.10	0.10
Lecithin[8]	1.00	1.00	1.00	1.00	1.00
Cholesterol[3]	0.05	0.05	0.05	0.05	0.05
Mono-dicalcium phosphate[9]	0.00	0.50	0.75	1.20	1.50
Corn starch[3]	0.51	0.33	0.41	0.28	0.48

[1] Menhaden fish meal, special select. Omega Protein Inc., Houston, TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] MP Biomedicals Inc., Solon, OH, USA.
[4] Aqua-Pak Pro-Cision, H. J, Baker Brother Inc., Shelton, CT, USA.
[5] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] Enhanced D97. The Solae Company, St. Louis, MO, USA.
[9] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.

Table 3 Composition of reference diet for the determination of digestibility coefficients of fish meal analogue (FMA), fish meal (FM), and soybean meal (SBM).

Ingredients	% as is
Soybean meal[1]	10.0
Fish meal[2]	32.5
Fish oil[2]	3.2
Whole wheat[3]	47.6
Trace mineral premix[4]	0.5
Vitamin premix[5]	1.8
Choline cloride[6]	0.2
Stay C[7]	0.1
Corn starch[3]	1.0
Lecethin[8]	1.0
Chromic oxide[9]	1.0

[1] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[2] Menhaden fish meal, special select. Omega Protein Inc., Houston TX, USA.
[3] MP Biomedicals Inc., Solon, Ohio, USA
[4] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[5] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[6] VWR, Radnor, PA, USA.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] Enhanced D97. The Solae Company, St. Louis, MO, USA.
[9] Alfa Aesar, Haverhill, MA, USA.

Table 4 Proximate composition[1], phosphorus content[1], and amino acid profile[1] of the ingredients used in the growth and digestibility trials.

Composition (% as is)	Fish meal analogue	Fish meal	Soybean meal
Crude protein	68.28	62.78	44.89
Moisture	4.95	7.99	10.97
Crude fat	13.89	10.56	3.78
Crude fiber	0.80	0.00	3.20
Ash	12.54	18.75	6.67
Phosphorus	1.68	3.15	0.66
Alanine	4.38	3.91	2.04
Arginine	4.21	3.68	3.35
Aspartic acid	5.22	5.34	5.1
Cysteine	1.4	0.47	0.62
Glutamic acid	7.46	7.47	8.24
Glycine	5.15	4.88	2.04
Histidine	1.9	1.63	1.2
Isoleucine	2.7	2.42	2.17
Leucine	5.47	4.21	3.57
Lysine	4.99	4.67	3.06
Methionine	1.69	1.61	0.66
Phenylalanine	3.19	2.39	2.35
Proline	4.17	3.08	2.39
Serine	3.12	2.11	1.9
Taurine	0.22	0.73	0.13
Threonine	2.87	2.41	1.75
Tryptophan	0.39	0.62	0.62
Tyrosine	2.04	1.67	1.64
Valine	4.11	2.99	2.34

[1] Ingredients were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).

Table 5 Proximate composition[1] (% as is), mineral composition (% as is: phosphorus, sulfur, potassium, magnesium, calcium, sodium; mg kg^{-1} as is: iron, manganese, copper, zinc)[2] and amino acid profile[1] (% as is) of the test diets used in the growth trial 1 and 2.

Composition	D_1	D_2	D_3	D_4	D_5
Crude protein	36.82	36.66	36.70	36.36	36.90
Moisture	6.76	7.36	7.37	7.61	7.49
Crude fat	7.62	7.66	7.31	7.14	8.00
Crude fiber	4.57	3.96	4.51	3.89	4.05
Ash	7.42	6.78	6.38	5.93	5.48
Phosphorus	1.10	1.01	0.92	0.84	0.78
Sulfur	0.43	0.42	0.43	0.43	0.46
Potassium	1.29	1.19	1.15	1.11	1.12
Magnesium	0.22	0.21	0.21	0.20	0.20
Calcium	1.33	1.32	1.11	0.93	0.80
Sodium	0.20	0.17	0.15	0.13	0.10
Iron	270	278	299	314	336
Manganese	57.7	57.0	55.6	55.0	56.3
Copper	16.4	18.7	15.4	15.8	16.5
Zinc	227	210	204	212	209
Alanine	1.74	1.71	1.74	1.80	1.80
Arginine	2.2	2.15	2.22	2.27	2.29
Aspartic Acid	3.22	3.11	3.16	3.21	3.21
Cysteine	0.42	0.45	0.51	0.57	0.61
Glutamic Acid	6.27	6.13	6.23	6.23	6.23
Glycine	1.98	1.94	1.98	1.93	1.91
Histidine	0.89	0.88	0.90	0.94	0.98
Isoleucine	1.60	1.56	1.59	1.66	1.65
Leucine	2.61	2.6	2.64	2.84	2.86
Lysine	2.21	2.17	2.22	2.25	2.33
Methionine	0.63	0.65	0.66	0.69	0.70
Phenylalanine	1.64	1.66	1.71	1.81	1.82
Proline	2.13	2.15	2.22	2.29	2.28
Serine	1.13	1.18	1.19	1.26	1.29
Taurine	0.24	0.22	0.20	0.17	0.15
Threonine	1.22	1.21	1.22	1.28	1.3
Tryptophan	0.40	0.39	0.38	0.34	0.34

Tyrosine	1.08	1.09	1.13	1.17	1.18
Valine	1.78	1.78	1.84	1.99	2.00

[1] Proximate composition and amino acid profiles of test diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).
[2] Mineral composition was tested at Midwest Laboratories (Omaha, NE, USA).

Table 6 Proximate composition[1] (% as is), mineral composition (% as is: phosphorus, sulfur, potassium, magnesium, calcium, sodium; mg kg^{-1} as is: iron, manganese, copper, zinc)[2] and amino acid profile[1] (% as is) of the test diets used in the growth trial 3.

Composition[1]	D_{1p}	D_{2p}	D_{3p}	D_{4p}	D_{5p}
Moisture	6.92	6.73	7.61	7.58	7.90
Protein	36.60	37.40	36.80	36.20	36.20
Fat	7.86	8.06	7.70	7.41	7.42
Fiber	5.10	6.70	5.60	5.90	6.50
Ash	7.34	7.59	7.28	7.25	6.88
Sulfur	0.41	0.43	0.43	0.4	0.38
Phosphorus	1.09	1.29	1.17	1.12	1.03
Potassium	1.17	1.19	1.15	1.05	0.97
Magnesium	0.21	0.22	0.22	0.19	0.18
Calcium	1.44	1.82	1.55	1.44	1.27
Sodium	0.19	0.19	0.15	0.12	0.09
Iron	214	276	269	285	288
Manganese	65.1	62.1	61.8	67.6	57.6
Copper	13.1	16.1	14.1	25.3	12.7
Zinc	270	188	177	173	144
Alanine	1.78	1.77	1.83	1.77	1.77
Arginine	2.25	2.22	2.24	2.22	2.21
Aspartic Acid	3.26	3.23	3.27	3.19	3.18
Cysteine	0.44	0.46	0.53	0.52	0.55
Glutamic Acid	6.43	6.30	6.22	6.18	6.08
Glycine	2.01	2.05	2.06	2.02	2.00
Histidine	0.88	0.90	0.98	0.95	0.98
Isoleucine	1.50	1.48	1.43	1.41	1.36
Leucine	2.59	2.60	2.75	2.73	2.81
Lysine	2.33	2.38	2.41	2.36	2.37
Methionine	0.67	0.70	0.70	0.67	0.68
Phenylalanine	1.62	1.63	1.69	1.68	1.72
Proline	2.02	2.06	2.13	2.17	2.14
Serine	1.49	1.43	1.53	1.56	1.65
Threonine	1.33	1.29	1.31	1.30	1.31
Tryptophan	0.46	0.45	0.44	0.42	0.41

Tyrosine	1.12	1.09	1.09	1.07	1.06
Valine	1.74	1.80	1.93	1.96	1.99

[1] Amino acid profiles of test diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).
[2] Proximate composition and mineral content were tested at Midwest Laboratories (Omaha, NE, USA).

Table 7 Performance of juvenile Pacific white shrimp *L. vannamei* (Initial weight: 0.47, 0.40, 0.25 g in trial 1, 2, and 3, respectively) offered diets with different fish meal analogue (FMA) levels (0, 4.85, 9.70, 14.55, and 19.44%) replacing different levels of fish meal for six weeks.

Trial	Diet	FMA levels (%)	Biomass (g)	Final mean weight (g)	WG (%)[2]	FCR[3]	Survival (%)
Trial 1 n=6	D_1	0	35.62	4.56	881^{ab}	2.35	78.3
	D_2	4.85	36.35	4.85	978^{a}	2.21	75.0
	D_3	9.70	37.10	4.32	854^{ab}	2.51	86.7
	D_4	14.55	35.63	4.29	774^{b}	2.55	83.3
	D_5	19.40	34.68	4.37	839^{ab}	2.47	80.0
	PSE[1]		1.8373	0.1545	15.6790	0.0953	4.6904
	P value		0.9116	0.0915	0.0163	0.1046	0.467
Trial 2 n=6	D_1	0	37.10^{ab}	4.36^{a}	973^{ab}	2.22^{b}	85.0
	D_2	4.85	38.20^{ab}	4.60^{a}	1027^{a}	2.10^{b}	83.3
	D_3	9.70	41.55^{a}	4.53^{a}	1013^{a}	2.10^{b}	91.7
	D_4	14.55	33.88^{bc}	3.92^{ab}	883^{ab}	2.54^{ab}	86.7
	D_5	19.40	29.70^{c}	3.64^{b}	812^{b}	2.73^{a}	81.7
	PSE[1]		0.8008	0.0730	19.6751	0.0498	1.2397
	P value		0.0032	0.0030	0.0184	0.0027	0.2063
Trial 3 n=4	D_{1p}	0	37.70	4.38^{ab}	1689.0^{ab}	2.11	87.5
	D_{2p}	4.85	42.35	5.32^{a}	2039.4^{a}	1.68	80.0
	D_{3p}	9.70	38.80	4.73^{ab}	1848.4^{ab}	1.99	82.5
	D_{4p}	14.55	36.25	4.13^{b}	1515.5^{b}	2.20	87.5
	D_{5p}	19.40	35.28	4.43^{ab}	1634.8^{ab}	2.08	80.0
	PSE[1]		1.2521	0.1228	55.5090	0.0621	3.0448
	P value		0.3511	0.0350	0.0382	0.0779	0.8142

[1] PSE: Pooled standard error.
[2] WG: Weight gain = (Final weight-initial weight)/initial weight*100.

[3] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 8 Pearson correlation coefficients of final biomass, final mean weight, weight gain (WG), feed conversion ratio (FCR), and survival in trial 1 and trial 2 with phosphorus levels of the diets. The first line of each cell is the value of correlation coefficient and the second line of each cell is *P*-value.

Trial		Final biomass	Final mean weight	WG	FCR	Survival
Trial 1	Phosphorus	0.0758	0.3393	0.3840	-0.3481	-0.1627
		0.6905	0.0666	0.0362	0.0594	0.3904
Trial 2	Phosphorus	0.4220	0.5251	0.4662	-0.5335	0.0343
		0.0202	0.0029	0.0094	0.0024	0.8571

Table 9 Whole body proximate composition (% dry weight basis), phosphorus content (P, % dry weight basis), and protein and phosphorus retention (%) when shrimp were offered diets supplemented with different fish meal analogue levels replacing fish meal without balanced for phosphorus for six weeks in trial 2.

Diet	Protein	Moisture	Lipid	Fiber	Ash	P	PR[2]	PHR[3]
D_1	72.84[b]	75.83	8.22[ab]	6.25	12.45	1.07	23.58[ab]	11.49
D_2	72.99[b]	75.28	8.65[a]	5.84	11.97	1.08	25.80[a]	13.84
D_3	73.76[ab]	75.97	7.72[ab]	6.09	12.14	1.03	25.49[a]	14.09
D_4	73.99[ab]	75.94	7.64[ab]	5.71	11.85	1.06	21.88[ab]	13.43
D_5	74.53[a]	76.79	7.09[b]	5.73	12.17	1.03	18.91[b]	12.17
P-value	0.0051	0.0909	0.0348	0.4326	0.8888	0.2026	0.0029	0.2921
PSE[1]	0.1314	0.1460	0.1383	0.0981	0.1759	0.0073	0.4995	0.0689

[1] PSE: Pool standard error.
[2] PR: Protein retention = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein offered.
[3] PHR: Phosphorus retention = (final weight × final phosphorus content) - (initial weight × initial phosphorus content) × 100 / phosphorus offered.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 10 Whole body proximate[1] composition, mineral composition[1], and protein and phosphorus retention when shrimp were offered diets supplemented with different fish meal analogue levels replacing different levels of fish meal balance for phosphorus for six weeks in trial 3.

Diet	D_1	D_2	D_3	D_4	D_5	PSE[2]	*P*-value
Proximate composition (% dry weight basis)							
Protein	74.57	75.80	75.13	75.53	74.23	0.5048	0.8028
Moisture	77.68	76.81	76.98	78.26	77.33	0.5145	0.8629
Lipid	5.32	5.82	5.80	4.65	5.78	0.2273	0.3272
Ash	12.07	11.95	11.45	11.93	11.40	0.1652	0.5337
Macro elements (% dry weight basis)							
Sulfur	0.90	0.89	0.87	0.85	0.84	0.0072	0.0751
Phosphorus	1.01	1.03	0.99	1.00	1.06	0.0111	0.2544
Potassium	1.46	1.45	1.37	1.40	1.33	0.0161	0.0725
Magnesium	0.3	0.29	0.27	0.27	0.26	0.0052	0.2387
Calcium	3.61	3.57	3.18	3.36	3.00	0.1026	0.2641
Sodium	1.19	1.10	1.08	1.11	1.07	0.0161	0.2048
Trace elements (mg kg^{-1} dry weight basis)							
Iron	21.70	17.10	18.40	26.03	24.6	1.3880	0.1636
Manganese	3.53	2.68	3.10	2.73	3.10	0.2136	0.6902
Copper	70.63	69.40	69.88	69.63	75.85	1.2789	0.3866
Zinc	77.2	75.53	74.00	74.40	74.83	0.7098	0.6344
Retention (%)							
Protein[3]	22.95^{b}	28.38^{a}	23.78^{b}	20.92^{b}	22.91^{b}	0.4442	0.0006
Phosphorus[4]	10.37	11.03	9.77	8.81	11.40	0.2670	0.2288

[1] Body samples were analyzed at tested at Midwest Laboratories (Omaha, NE, USA).
[2] PSE: Pool standard error.
[3] Protein retention = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein offered.
[4] Phosphorus retention = (final weight × final phosphorus content) - (initial weight × initial phosphorus content) × 100 / phosphorus offered.
Values within a row with different superscripts are significantly different based on Tukey's multiple range test.

Table 11 Apparent dry matter (ADM), apparent energy (AED) and apparent protein (APD) digestibility values for the diet (D) and ingredient (I) using 70:30 replacement technique offered to Pacific white shrimp *L. vannamei*.

	ADMD	AEDD	APDD	ADMDI	AEDI	APDI
Basal1	76.38 ± 0.37[a]	82.66 ± 1.20[a]	92.08 ± 0.55[a]			
Basal2	75.69 ±0.52[a]	81.51 ± 0.41[a]	92.04 ± 0.03[a]			
SBM	77.02 ± 0.87[a]	82.63 ± 1.05[a]	94.76 ± 0.49[a]	78.51 ± 2.89[a]	82.56 ± 3.79[a]	99.00 ± 1.27[a]
FM1	68.21 ± 3.80[b]	78.31 ± 3.21[ab]	80.86 ± 1.80[b]	49.15 ± 12.67[b]	69.77 ± 9.51[a]	67.07 ± 4.02[b]
FM2	67.99 ± 0.17[b]	76.44 ± 0.78[bc]	82.34 ± 0.31[b]	49.45 ± 0.56[b]	65.78 ± 2.23[ab]	71.30 ± 0.68[b]
FMA	66.06 ± 1.67[b]	72.34 ± 1.67[c]	64.00 ± 2.14[c]	41.98 ± 5.58[b]	53.53 ± 4.71[b]	32.39 ± 4.55[c]

[1] PSE: Pool standard error.

Values from each treatment are means and standard deviation of triplicate tanks. Values within a column different superscripts are significantly different ($P < 0.05$).

Table 12 Apparent amino acids (AA) digestibility value for the soybean meal (SBM), fish meal (FM), poultry meal (PM), and fish meal analogue (FMA) using 70:30 replacement technique offered to Pacific white shrimp *L. vannamei*.

AA digestibility coefficients (%)	SBM	FM	FMA
Alanine	93.75 ± 2.02[a]	69.09 ± 4.09[b]	39.23 ± 2.89[c]
Arginine	96.91 ± 1.44[a]	75.35 ± 3.78[b]	42.66 ± 2.71[c]
Aspartic acid	95.39 ± 1.36[a]	69.23 ± 3.70[b]	34.38 ± 3.57[c]
Cysteine	91.29 ± 1.68[a]	54.39 ± 7.06[b]	23.73 ± 5.72[c]
Glutamic acid	95.69 ± 1.52[a]	70.84 ± 3.70[b]	43.48 ± 3.86[c]
Glycine	95.06 ± 2.05[a]	66.55 ± 6.26[b]	50.14 ± 2.93[c]
Histidine	94.33 ± 1.69[a]	74.26 ± 2.86[b]	32.72 ± 3.35[c]
Isoleucine	93.23 ± 1.72[a]	68.72 ± 3.99[b]	31.55 ± 3.41[c]
Leucine	92.23 ± 1.96[a]	71.29 ± 3.16[b]	30.58 ± 3.24[c]
Lysine	95.03 ± 1.84[a]	76.97 ± 2.24[b]	54.13 ± 2.40[c]
Methionine	95.20 ± 1.54[a]	70.63 ± 3.30[b]	72.49 ± 2.01[c]
Phenylalanine	93.41 ± 1.90[a]	65.28 ± 4.13[b]	29.28 ± 3.66[c]
Proline	94.68 ± 1.92[a]	67.21 ± 5.39[b]	38.77 ± 3.13[c]
Serine	93.11 ± 1.91[a]	58.31 ± 4.65[b]	37.23 ± 2.99[c]
Threonine	91.99 ± 1.94[a]	66.33 ± 3.35[b]	34.41 ± 3.37[c]
Tryptophan	95.37 ± 1.92[a]	80.31 ± 1.53[b]	61.88 ± 6.20[c]
Tyrosine	95.28 ± 1.22[a]	73.62 ± 3.40[b]	48.02 ± 3.60[c]
Valine	90.78 ± 2.39[a]	67.06 ± 3.75[b]	26.77 ± 2.23[c]
Total	94.31 ± 1.67[a]	69.91 ± 3.89[b]	40.42 ± 3.14[c]

[1] Pooled standard error.
Values from each treatment are means and standard deviation of triplicate tanks. Values within a row different superscripts are significantly different ($P < 0.05$).

CHAPTER VII

UTILIZATION OF GREEN SEAWEED *ULVA* sp. AS A PROTEIN SOURCE IN PRACTICAL DIETS FOR PACIFIC WHITE SHRIMP *Litopenaeus vannamei*

1. Introduction

Traditionally, fish meal (FM) is the most important and preferred protein source in most shrimp feeds, because it is an excellent source of essential nutrients such as protein and indispensable amino acids, essential fatty acids, cholesterol, vitamins, minerals, attractants and unidentified growth factors (Samocha *et al.*, 2004). As most fisheries are beyond sustainable limits, fish meal supplies will not increase. Hence, the high demand and limit supply resulted in a corresponding high market value of FM. The price of FM has risen from around $400 per metric ton in 2000 to more than $1700 per metric ton in 2016, and the highest price reached to $2388 per metric ton in 2014 (Index Mundi, 2016).

Shrimp nutritionists have investigated numerous FM alternatives to reduce the reliance on this costly and potential limiting resources in shrimp feed formulation. Among the potential alternative ingredients, soybean meal (SBM) is usually considered as the most reliable, cost-effective, and nutritionally valuable protein source in shrimp feed. The popularity of soybean meal as a protein source is the result of a well-balanced nutrient profile, high digestibility, stead supply, expandable production and reasonable price (Davis and Arnold, 2000, Amaya *et al.*, 2007a, Amaya *et al.*, 2007b). The successful application of SBM in practical feed formulation for Pacific white shrimp were well documented in many studies (Amaya *et al.*, 2007a, Amaya *et al.*, 2007b, Davis and Arnold, 2000, Sookying and Davis, 2011, Roy *et al.*, 2009, Samocha *et al.*, 2004, Qiu and Davis, 2016b, Qiu and Davis, 2016a).

Although FM and SBM will continue to be a mainstay in shrimp formulation in terms of their nutritional preponderances, it is also critical to explore alternative ingredients to these two conventional protein sources that may be more economical and sustainable to support the rapid expansion of the shrimp industry as shrimp consumption is expected to continue to increase. Marine macro-algae, commonly referred to seaweeds, are categorized by their pigmentation, morphology, anatomy, and nutritional composition as red, brown or green seaweeds (Dawczynski *et al.*, 2007). They can benefit from recycling waste carbon dioxide (CO_2) from combustive energy production and waste nutrients produced by intensive aquaculture operations, intensive animal operations, and municipal waste treatment (Kaushik, 2010, Sargent and Tacon, 1999). In an integrated cultivation system, the macro-algae use the metabolic residues of animals as nutrients, absorb CO_2 and produce O_2 for the environment (Marinho-Soriano *et al.*, 2007). The interaction allows the excretion of an organism to serve as food for another. Presently, significant improvements in growth and survival rate have been observed when Pacific white shrimp, *Litopenaeus vannamei* (Cruz-Suárez *et al.*, 2010, Brito *et al.*, 2014a, Brito *et al.*, 2014b), giant tiger shrimp, *Penaeous monodon* Fabr (Tsutsui *et al.*, 2010, Izzati, 2012), and yellowleg shrimp, *Farfantepenaeus californiensis* (Portillo‐Clark *et al.*, 2012) are co-cultured with seaweeds.

Ulva spp belongs to the green seaweed. A number of studies have demonstrated that dietary Ulva meal inclusion at low levels (<5% of the diet) did not affect the growth performance in a variety of species including African catfish, *Clarias gariepinus* (Abdel-Warith *et al.*, 2016), gilthead seabream *Sparus aurata* (Emre *et al.*, 2013), Pacific white shrimp (Cárdenas *et al.*, 2015, Rodríguez-González *et al.*, 2014), Nile tilapia *Oreochromis niloticus* (Güroy *et al.*, 2007, Ergün *et al.*, 2009), rainbow trout *Oncorhynchus mykiss* (Güroy *et al.*, 2013). However, the

growth response of different fish species to the moderate and high supplementation levels of Ulva meal are somewhat inconsistent.

The information about green seaweed *Ulva* sp. as a protein source in shrimp feed formulation is still limited. Therefore, the purposes of this study were to evaluate the potential of *Ulva* sp. as a protein source in comparison to FM and SBM in practical diets for Pacific white shrimp and investigate the nutrient availability in this ingredient.

2. Materials and Methods

2.1. Experimental diets

Primary ingredients and four pooled batches of sun dried *Ulva pertussa* meal were analyzed for proximate composition, amino acids and minerals (Table 1 and 2) and the diets were formulated. Additionally, prior to pooling batch two, the 7 individual daily collections of Ulva meal were sampled and analyzed individually (Table 3). Upon completion of analysis diets were made by weighing pre-grounding dry ingredients and oil which was then mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 min. Hot water was then blended into the mixture to obtain a consistency appropriate for pelleting. Diets were pressure-pelleted using a meat grinder with a 3-mm die, air-dried (< 45 °C) to a moisture content of 8-10%. Pellets were crumbled, packed in sealed plastic bags and stored in a freezer until needed.

In trial 1, 2, 3 and 4, diets were formulated to be isonitrogenous and isolipidic (35% protein and 8% lipid). In most diets substitution was done on a protein to protein basis however in trial 3, ingredient replacement was done on a digestible protein basis. In trial 1, five experimental diets were formulated to contain increasing levels (0, 6.35, 12.70, 19.05, and 25.40%) of the first batch of Ulva meal (UM1) as a replacement for fish meal (Table 4a). In trial

2, nine experimental diets were formulated (Table 5a). The basal diet for this and subsequent trials was designed to have 6% fishmeal in all formulations to help stabilize nutrients as well as palatability. The first seven diets utilized increasing levels of the second batch of Ulva meal (UM2) (0, 5, 10, 15, 20, 25, and 30%) to replace soybean meal. Diet 8 and Diet 9 utilized high incorporation of Ulva meal from the first and third batch, respectively. This allowed a comparison of all three meals at equivalent levels of protein replacement. In trial 3, four experimental diets were formulated using the first three batches of Ulva meal replacing soybean meal on a digestible protein basis (Table 6a). In trial 4, five experimental diets were designed utilizing different levels (0, 4.75, 9.5, 12, and 24%) of fourth batch of Ulva meal (high protein content~38%) replacing fish meal and soybean meal. Digestibility values for the meals were determined using standard methods and 1% chromic oxide as an inert marker (Table 8a). Test diets were made on a dry matter basis using a 70:30 mixture of the reference diet and test ingredients.

The ingredients (Table 1) were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate composition and amino acid profile and mineral profiles (Table 2) by the Soil Lab (Auburn, AL, USA). Daily collections of UM2 samples (Table 3) collected across seven different dates were analyzed individually at Midwest laboratories (Omaha, NE, USA) for proximate and mineral composition. Diets (Table 4a, Table 5a, Table 6a, and Table 7a) were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate composition and amino acid profile and Soil Lab (Auburn, AL, USA) for mineral composition.

2.2. Growth trials

The trial 1 utilized 5 treatments with 7 replicates in each treatment. It was conducted in a semi-closed recirculation system. Juvenile shrimp were obtained from the nursery system and selected by hand-sorting to a uniform size. Juvenile shrimp (initial weight 0.26 ± 0.02 g) were stocked into 35 tanks with 10 shrimp in each aquarium (135L). A sub-sample of shrimp from the initial stocking was retained for whole body analysis to be utilized for later protein retention analysis. As shrimp are difficult to handle, intermittent weights were not taken. However, shrimp were counted to readjust daily feed input on a weekly basis. Based on historically results, a fixed ration was calculated assuming a 1.8 feed conversion ratio and a doubling in size the first two weeks and 0.8-0.9 g $week^{-1}$. Therefore, for ten shrimp in a given tank, a fixed ration of 0.67 g day^{-1} for the first week, 1.45 g day^{-1} for the second week, 2.06 g day^{-1} for the third week, and 2.31 g day^{-1} for the fourth week, 2.57 g day^{-1} for the fifth week, and 2.83 g day^{-1} for the six week was offered over 4 feedings.

The trial 2 was conducted in the same semi-closed recirculation system which is mentioned above. It utilized 9 treatments with 4 replicates in each treatment. Juvenile shrimp (initial weight 0.24 ± 0.01 g) were stocked into 36 tanks with 10 shrimp in each aquarium (135L). A sub-sample of shrimp from the initial stocking was retained for whole body samples to be utilized for later protein and phosphorus retention analysis. Shrimp were counted to readjust daily feed input on a weekly basis. Based on historically results, a fixed ration was calculated assuming a 1.8 feed conversion ratio and a doubling in size the first two weeks and 0.8-0.9 g $week^{-1}$ thereafter. Consequently, for each tank a fixed ration of 0.62 g day^{-1} for the first week, 1.23 g day^{-1} for the second week, 2.06 g day^{-1} for the third and fourth week, and 2.31 g day^{-1} for the fifth week was offered over 4 feedings.

The trial 3 was conducted in the same semi-closed recirculation system which is mentioned above. It utilized 4 treatments with 4 replicates in each treatment. Juvenile shrimp (initial weight 0.98 ± 0.01 g) were stocked into 16 tanks with 10 shrimp in each aquarium (135L). A sub-sample of shrimp from the initial stocking was retained for whole body samples to be utilized for later protein and phosphorus retention analysis. As shrimp are difficult to handle, intermittent weights were not taken. However, shrimp were counted to readjust daily feed input on a weekly basis. Based on historically results, a fixed ration was calculated assuming a 1.8 feed conversion ratio and a doubling in size the first two weeks and 0.8-1.3 g $week^{-1}$ thereafter. Consequently, for each tank of 10 shrimp a fixed ration of 2.1 g day^{-1} for the first week, 2.3 g day^{-1} for the second week, 2.8 g day^{-1} for the third week, 3.1 g day^{-1} for the fourth week, 3.4 g day^{-1} for the fifth week, and 3.7 g day^{-1} for the sixth week was offered over 4 feedings.

The trial 4 was conducted in a similar semi-closed recirculation system to what was previously described. It utilized 5 treatments with 4 replicates in each treatment. Juvenile shrimp (initial weight 0.15 ± 0.01 g) were stocked into 20 tanks with 10 shrimp in each aquarium (135L). Based on historically results, a fixed ration was calculated assuming a 1.8 feed conversion ratio and a doubling in size the first two weeks and 0.8-1.3 g $week^{-1}$ thereafter. Consequently, for each tank of 10 shrimp a fixed ration of 2.1 g day^{-1} for the first week, 2.3 g day^{-1} for the second week, 2.8 g day^{-1} for the third week, 3.1 g day^{-1} for the fourth week, 3.4 g day^{-1} for the fifth week, and 3.7 g day^{-1} for the sixth week was offered over 4 feedings.

At the end of each growth trials, shrimp were counted and group weighted. Mean final weight, feed conversion ratio (FCR), weight gain (WG), biomass, and survival were determined (Table 4c, 5d, 6c, 7c). After obtaining the final total weight of shrimps in each aquarium, 4 shrimps were randomly selected and frozen at -20 °C for subsequent determination of whole

body composition. Proximate composition (Table 4d, 5e, 6d, 7d) of whole shrimp was analyzed by Midwest Laboratories, Inc (Omaha, NE, USA) or University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA). Mineral profiles of whole shrimp and select algae meals were analyzed by Soils lab (Auburn, AL, USA). Apparent net protein and amino acids retention were calculated using the following equations

Protein retention (%) = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein offered.

Amino acids (AA) retention (%) = (final weight × final AA content) - (initial weight × initial AA content) × 100 / AA offered.

2.3. Water quality monitoring

Dissolved oxygen (DO), temperature, and salinity were measured twice daily by using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH, USA). The pH was measured twice weekly by using a waterproof pHTestr30 (Oakton instrument, Vernon Hills, IL, USA). Water samples were taken to measure total ammonia-nitrogen (TAN) and nitrite every week. TAN and nitrite were determined by the methods described by (Solorzano, 1969) and (Spotte, 1979), respectively.

2.4. Digestibility trial

The digestibility trial was conducted in the mentioned recirculation system and utilized 6 shrimp per aquaria with 6 aquaria per dietary treatment. Once acclimated for 3 days to the test diets, feces from two aquaria were pooled (n=3) and collected over a 5-day period or until adequate samples were obtained. To obtain fecal samples, the aquaria were cleaned by siphoning

before each feeding with the first collection of the day discarded. After the aquaria were cleaned, the shrimp were offered an excess of feed and then about 1 hour later feed was removed and feces were collected by siphoning onto a 500μm mesh screen. Collected feces were rinsed with distilled water, dried at 105 °C until a constant weight was obtained, and then stored in freezer (-20 °C) until analyzed. Apparent digestibility coefficient for dry matter, protein, energy and amino acids were determined by using chromic oxide (Cr_2O_3, 10 g kg^{-1}) as an inert marker. Chromium concentrations were determined by the method of (McGinnis and Kasting, 1964) in which, after a colorimetric reaction, absorbance is read on a spectrophotometer (Spectronic genesis 5, Milton Roy Co., Rochester, NY, USA) at 540nm. Gross energy of diets and fecal samples were analyzed with a Semi micro-bomb calorimeter (Model 1425, Parr Instrument Co., Moline, IL, USA). Protein were determined by micro-Kjeldahl analysis (Ma and Zuazaga, 1942). The apparent digestibility coefficient of dry matter (ADMD), protein (ADP), energy (ADE) and amino acids (ADAA) were calculated according to (Cho *et al.*, 1982)as follows:

ADMD (%) = 100 − [100 × (% Cr_2O_3 in feed / % Cr_2O_3 in feces)]

ADP, ADE, and ADAA (%) = 100 − [100 × (% Cr_2O_3 in feed / % Cr_2O_3 in feces) × (% nutrient in feces / % nutrient in feed).

The apparent digestibility coefficients (ADC) of the test ingredients for dry matter, energy, protein and amino acids were calculated according to Bureau *et al.* (2006) as follows:

$ADC_{test\ ingredient} = ADC_{test\ diet} + [(ADC_{test\ diet} - ADC_{ref.\ diet}) \times (0.7 \times D_{ref} / 0.3 \times D_{ingr})]$

where D_{ref} = % nutrient (or KJ/g gross energy) of reference diet mash (as is); D_{ingr} = % nutrient (or KJ/g gross energy) of test ingredient (as is).

2.5. Statistical analysis

All the data were analyzed using SAS (V9.3. SAS Institute, Cary, NC, USA). Data from both the growth trial and digestibility trial were analyzed using one-way ANOVA to determine significant differences ($P<0.05$) among treatments followed by the Tukey's multiple comparison test to determine difference between treatments. Arcsine square root transformation was used prior to analysis for the proportion data. False discover rate (FDR) controlling procedures were applied to adjust the *P*-value to control the FDR for amino acid data. Linear, second- or third-order polynomial regressions were performed to investigate the relationship between the supplemental Ulva meal levels and weight gain, FCR, survival, and lipid content of whole shrimp body. To identify the most appropriate regression model, we compared *P*-value of the model components, R^2 value, adjust R^2 value, and the sum of squares for error (SSE) with different regression models.

3. Results

3.1. Water quality

In trial 1, DO, temperature, salinity, pH, TAN, and nitrite were maintained within acceptable ranges for *L. vannamei* at 6.19 ± 0.25 mg L^{-1}, 28.4 ± 0.8 °C, 11.8 ± 0.4 ppt, 7.23 ± 0.22, 0.079 ± 0.041 mg L^{-1}, and 0.039 ± 0.021 mg L^{-1}, respectively. In trial 2, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 5.82 ± 0.26 mg L^{-1}, 29.7 ± 0.8 °C, 8.6 ± 0.4 ppt, 7.5 ± 0.5, 0.052 ± 0.107 mg L^{-1}, and 0.003 ± 0.004 mg L^{-1}, respectively. In trial 3, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 6.20 ± 0.72 mg L^{-1}, 29.5 ± 0.9 °C, 8.4 ± 1.0 ppt, 7.5 ± 0.3, 0.092 ± 0.103 mg L^{-1}, and 0.050 ± 0.039 mg L^{-1}, respectively. In trial 4, DO, temperature, salinity, pH, TAN, and nitrite were maintained at 6.96 ± 0.31 mg L^{-1}, 28.1 ±

0.3 °C, 8.2 ± 0.6 ppt, 7.0 ± 0.3, 0.05 ± 0.04 mg L^{-1}, and 0.12 ± 0.12 mg L^{-1}, respectively. Water quality conditions in all the trials were suitable for normal growth and survival of this species.

3.2. Growth performances

Growth performances of juvenile Pacific white shrimp offered diets contained different levels of Ulva meal in trial 1-4 are presented in Table 4c, 5d, 6c, and 7c. In trial 1, final biomass was significantly reduced when UM1 was included at 25.4% compared to the treatment supplemented with 0 and 6.35% UM1. Significant reductions in final mean weight and increments in FCR were detected when 19.05 and 25.4% UM1 was incorporated in the diet in contrast with diets contained 0 and 6.35% UM1. Shrimp fed with diets contained more than 12.7% UM1 exhibited significantly reduced WG than those offered with diets supplemented with 0 and 6.35% UM1. No significant difference was determined in survival across all the treatments (82.9% to 92.9%).

In trial 2, final biomass was significantly reduced when more than 5% Ulva meal was included in the diet. Final mean weight and WG were significantly decreased when more than 10% Ulva meal was supplemented in the diet. Significant increment in FCR was determined when the diet contained 25% UM2. Shrimp fed with diets contained 25% and 30% UM2 exhibited significantly lower survival than those fed with diets supplemented with 0, 5, and 15% UM2.

In trial 3, in general shrimp fed UM2 exhibited poorest performance in terms of growth, FCR, and survival. Significant reductions in final biomass, final mean weight, WG, and survival were determined when UM2 was supplemented in the diet compared to other treatments. FCR was significantly increased in the treatment contained UM2 in contrast with other treatments.

In trial 4, shrimp fed with diets supplemented with different levels of UM4 showed significantly reduced final biomass, final mean weight, and WG as well as increased FCR. Survival was significantly reduced when 9.5% UM4 was included in the diet to replace 6% FM.

3.3. Proximate composition and amino acid profile of whole shrimp body

Proximate composition and amino acids profile of whole shrimp body in trial 1-4 are presented in Table 4d, 5e, 6d, and 7d. In trial 1, shrimp fed with diets supplemented with different levels of UM1 exhibited significantly reduced crude lipid of whole body. No significant effects were detected in moisture (76.29% to 76.99%) and crude protein content (72.77% to 74.27%) across the treatments.

In trial 2, crude lipid content was significantly reduced when more than 5% Ulva meal was incorporated in the diets. Shrimp fed with diets contained more than 5% UM2 exhibited significantly improved crude protein content. No significant difference was detected in the moisture content (75.66% to 78.05%) across all the treatments.

Shrimp fed with diet contained 25% and 30% exhibited significantly higher arginine and glycine content than the one fed with reference diet. Cysteine and lysine contents in the treatment fed with 15% to 30% UM2 were significantly higher than those fed with the reference diet. Histidine content in the treatment fed with diet contained 10% to 25% UM2 and 23.6% UM3 was significantly higher than the ones fed with reference diet. Shrimp fed with diet contained 30% UM2 exhibited significantly higher methionine content than those fed with diets contained 0, 5, and 10% UM2. Phenylalanine in the treatment contained 20% UM2 was significantly higher than the treatments contained 0 and 5% UM2 as well as 26.3% UM1. No significant differences were observed in alanine, aspartic acid, glutamic acid, hydroxylysine,

hydroxyproline, isoleucine, leucine, proline, serine, threonine, tyrosine, tryptophan, valine, and total amino acids levels in whole shrimp body across all the treatments.

In trial 3, significant reduction in crude lipid content was detected when shrimp was fed with diet contained UM2. Shrimp fed with diet contained UM2 exhibited significantly higher body moisture content than those fed with reference diet and diet contained UM4. No significant difference was detected in protein content (75.08% to 76.98%) across all the treatments.

Methionine content was significantly improved when UM1, UM2, UM3 were included in the diets. No significant differences were observed in alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, hydroxylysine, hydroxyproline, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, and total amino acids contents of whole shrimp body across all the treatments.

In trial 4, moisture content in the treatment fed with diets contained 12% and 24% UM4 was significantly higher than those fed with reference diet and diet contained 4.75% UM4. Crude protein was significantly enhanced when shrimp were fed with diet contained 24% UM4 compared to the reference diet and diet supplemented with 4.75% UM4. Crude lipid was dramatically decreased when more than 9.5% UM4 was included in the diet. Significant increment in ash content was observed when the diet was incorporated with 9.5% and 24% UM4 in contrast with the reference diet.

3.4. Protein and amino acids retention

Protein and amino acids retention in trial 1-4 are presented in Table 4c, 5f, 6e, and 7c. In trial 1, shrimp fed diet contained 6.35% UM1 showed significantly improved PRE compared to

those fed with diets supplemented with 19.05 and 25.4% UM1. In trial 4, PRE was significantly depressed when more than 9.5% UM4 was include in the diet

In trial 2, PRE was significantly reduced when shrimp was fed with diets supplemented with 15 to 30% UM2, 26.3% UM1, and 23.6%UM3 compared to the one offered with reference diet. In general, total and individual amino acids retention corresponded to PRE. Alanine, arginine, aspartic acid, hydroxyproline, isoleucine, phenylalanine, proline, threonine, and valine retention were significantly lower in the treatments contained 15 to 30% UM2, 26.3% UM1, and 23.6%UM3 than the reference diet. Total amino acids, cysteine, glutamic acid, glycine, leucine, and serine retention were significantly reduced in the treatments contained 15 to 30% UM2 and 23.6% UM3. Shrimp fed with diets contained 15 to 30% UM2 exhibited significantly higher histidine, hydroxylysine, lysine, methionine, tryptophan, and tyrosine retention than those fed with reference diet.

In trial 3, PRE was significantly reduced when UM2 and UM3 were supplemented in the diets. In general, total and individual amino acids retention corresponded to PRE. Arginine and hydroxyproline retention were significantly lower in treatments incorporated with UM1-3. Total amino acids, cysteine, serine, threonine, and valine retention in treatments supplemented with UM2 and UM3 were significantly reduced compared to the treatments fed with reference diet. Alanine, aspartic acid, glycine, histidine, hydroxylysine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, tryptophan, and tyrosine retention were significantly depressed when UM2 was supplemented in the diet.

3.5. Regression analysis

Dietary Ulva meal levels significantly correlated with WG, FCR, and lipid content in trial 1, 2, and 4 (Figure 1, 2, and 3). In general, the trends of the relationships between WG/FCR/lipid content of shrimp body and dietary Ulva meal levels are consistent in trial 1, 2, and 4. WG is negatively correlated with dietary Ulva meal levels. In trial 1, 2, and 4, the regression lines are described by $y = 0.1686x^3 - 6.2114x^2 + 34.409x + 1797.8$ ($R^2 = 0.4922$, $P < 0.0001$), $y = 1.1925x^2 - 62.699x + 1713.1$ ($R^2 = 0.6815$, $P < 0.0001$), and $y = 2.6848x^2 - 119.17x + 3049.3$ ($R^2 = 0.6934$, $P < 0.0001$), respectively (y = WG, x = Ulva meal levels). FCR is positively associated with Ulva meal levels. In trial 1, 2, and 4, the regression lines are exhibited as $y = 0.0276x + 1.7708$ ($R^2 = 0.3582$, $P = 0.0001$), $y = -0.0703x + 1.5451$ ($R^2 = 0.4766$, $P < 0.0001$), and $y = 0.06x + 1.8532$ ($R^2 = 0.721$, $P < 0.0001$), respectively (y = FCR, x = Ulva meal levels). Lipid content of shrimp body is negatively correlated with Ulva meal levels. In trial 1, 2, and 4, the regression lines are described by $y = -0.0962x + 7.4$ ($R^2 = 0.3503$, $P = 0.0002$), $y = 0.0078x^2 - 0.4095x + 7.8033$ ($R^2 = 0.9051$, $P < 0.0001$), and $y = -0.1903x + 7.9555$ ($R^2 = 0.7224$, $P < 0.0001$), respectively. Survival is negatively correlated with dietary Ulva meal levels in trial 2 and 4. However, no relationship is determined in trial 1. In trial 2 and 4, the regression lines are described by $y = -1.1607x + 100.27$ ($R^2 = 0.6113$, $P < 0.0001$) and $y = -0.7625x + 90.105$ ($R^2 = 0.2479$, $P = 0.0156$).

3.6. Digestibility trial

Apparent dry matter (ADM), apparent energy (AED), and apparent protein (APD) digestibility values for the diet (D) and ingredient (I) using 70:30 replacement technique offered to shrimp are presented in Table 8b. The digestibility trial contained a range of ingredients;

hence, we have provided a few other ingredients as a reference. In order to confirm the results, fecal samples for basal diets and FM diet were recollected. The results turned out to be quite similar, which indicated that the feces collection and samples analysis methods we utilized in the digestibility study are consistent. The energy and protein digestibility of UM1 and UM2 were 40.39% and 15.17%, and 19.11% and 43.51%, respectively, which were significantly lower than those of FM and SBM.

Apparent amino acids (AA) digestibility values for the SBM, FM, UM1, and UM2 using 70:30 replacement technique offered to Pacific white shrimp are presented in Table 8c. In general, the AAAD corresponded to the APD. Apparent digestibility coefficients of alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine of UM1 and UM2 were significantly lower than those of FM and SBM.

4. Discussion

4.1. Ingredient composition

Seaweeds are valuable sources of protein, fiber, vitamins, macro and trace elements, as well as important bioactive compounds (Ortiz *et al.*, 2006). Protein content of seaweeds is tied to the variations in seasons (Fleurence, 1999). In the current study, differences in protein contents were detected among the four pooled batches of Ulva meal (Table 1) and individual Ulva meal samples collected from seven dates within the UM2 (Table 2). The protein content of seaweed can be somewhat manipulated by controlling the nutrient loading during cultivation (Floreto *et al.*, 1996). In this instance, nitrogen enriched conditions like the effluents of fish or shrimp farms, where seaweeds are utilized as bio-filters, can enhance their protein contents (Lahaye *et*

al., 1995, Pinchetti *et al.*, 1998). Owing to the manipulation of nitrogen loading during cultivation, the protein content of seaweed *Ulva* sp. can be improved up to 38.16% in the present study, which is superior to the upper limit of the protein range (10% to 26%) of this species reported by several researchers (Benjama and Masniyom, 2011, Fleurence, 1999, Lee *et al.*, 2014).

As a result of the enhanced protein content, the amino acid concentrations in the *Ulva* sp. reported in the present study were improved at least by around 50% in contrast with those documented by Lee *et al.* (2014) or by even more than 300% compared to those described by Benjama and Masniyom (2011). For most seaweed species, aspartic acid and glutamic acid constitute a large portion of the total amino acids (Lourenço *et al.*, 2002). Similarly, the sum of these two amino acids in UM1 to UM4 represented 22.27% to 28.6% of total amino acids in the present study. In terms of the two most limiting amino acids (methionine and lysine) in ingredients for shrimp, their concentrations in UM1-4 ranged from 0.26% to 0.63% and 0.82% to 1.51%, respectively. Methionine level in UM1-4 were similar to that in SBM (0.66%) but lower than that in FM (1.61%). Lysine level in UM1-4 were low compared to both SBM (3.06%) and FM (4.67%).

Lipid content in most seaweeds was reported less than 4% dry weight (DW), whereas some seaweeds such as *Dictyota acutiloba* and *D. sandvicenis* contained a high level of lipid (16.1% and 20.2 % DW, respectively) (McDermid and Stuercke, 2003). In the current study, lipid content in UM1-4 and individual UM2 samples ranged from 0 to 0.62%. Similarly, Lee *et al.* (2014) reported that Lipid content of *Ulva* sp. was 0.1% DW. By contrast, Benjama and Masniyom (2011) documented that lipid content of *Ulva* sp. in summer and rainy season were

7.4% and 2.1% DW, respectively. The differences among these researches would be mainly attributed to different nutrient loadings during the seaweed cultivation.

Seaweeds are rich sources of minerals with a broad mineral composition including Al, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, S, Se, Si, and Zn, which has not been observed in edible terrestrial plants (Lee *et al.*, 2014). In the present study, the macro elements (Ca, K, Mg, Na, P, and S) and trace elements (Al, As, B, Ba, cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, Si, Zn, and Zr) of pooled UM1-3 ranged from 0.31-4.79% and 0.6-9086.7 mg kg^{-1}, respectively. Within the individual UM2 samples collected from seven dates, the macro elements (Ca, K, Mg, Na, P, and S) and trace elements (Cu, Fe, Mn, and Zn) ranged from 0.31-4.38% and 7.6-6780 mg kg^{-1}, respectively. Considerable variations were detected in the Ca, Na, and Fe levels in the pooled UM1-3 and individual UM2 samples, which would be mainly attributed to the nutrient loadings, temperature, and salinity during cultivation.

4.2. Nutrient digestibility

The nutrient digestibility of a feed ingredient is an important factor to evaluate the overall nutritive value of the ingredient because it is related to the quantity of the nutrient absorbed by the animals. SBM had the highest APD (97.03%), AED (82.56%), and AAAD (90.78 – 96.91%) among the ingredients tested in the current study. Similar ranges of results for APD, AED, and AAAD were reported in multiple shrimp studies (Cruz-Suárez *et al.*, 2009, Fang *et al.*, 2016, Liu *et al.*, 2013, Yang *et al.*, 2009, Zhou *et al.*, 2015). APD and AED of FM1 were 67.07% and 69.77%, respectively. Similar results were acquired in FM2 (APD and AED: 71.3% and 65.78%, respectively). The analogous results of basal diet and FM diet from the collections under two occasions pointed to the consistency in the feces collection and sample analysis methods. APD

of FM has been reported to be ranged from 62.7% to 91.6% in numerous studies (Lemos *et al.*, 2009, Liu *et al.*, 2013, Terrazas-Fierro *et al.*, 2010, Yang *et al.*, 2009). ADP of FM in our study were in the lower range of the results documented among those researches. The differences in digestibility of FM among various studies could be attributed to several factors, such as different raw materials, location or processing methods used to produce the products, and unknown factors related to different production batches.

In the present study, APD and AED of UM1 and UM2 were significantly lower than those of FM and SBM. The low APD of UM1 and UM2 translated to poor AAAD. Total amino acids and most individual amino acids availability in UM1 and UM2 were significantly lower than those of FM and SBM. With regards to the two batches of Ulva meal, UM1 exhibited significantly higher AED than that of UM2. On the contrary, APD of UM1 was significantly lower than that of UM2. Because of comparatively low APD in UM1, ADC of total amino acids and many individual amino acids in UM1 were significantly lower than those in UM2. No differences were detected in ADMD of UM1 and UM2. The variations in APD, AED, and AAAD of UM1 and UM2 indicated that nutrient availability in Ulva meal would also be affected by the conditions such as temperature, salinity, nutrient loadings during cultivation.

There are relatively few studies looking at the nutrient availability of seaweed meals in aquatic animal feeds particularly with regards to shrimp. Cárdenas *et al.* (2015) documented that APD of Nutrikelp (a brown seaweed meal is comprised of mixtures of *Macrocystis*, *Lessoniaceae* and *Lessonia*) and Nutrigreen (a green seaweed meal contains mixtures of *Ulva*, *Caulerpa*, and *Enteromorpha*) for *L. vannamei* were 85.37% and 86.81%, respectively, which may be masked by their high ADMD (80.97% and 80.33%, respectively) accordingly. Moreover, Pereira *et al.* (2012) reported that AED, and APD of four seaweeds (*Ulva spp*, *Porphyra dioica*,

Gracilaria vermiculophylla, and *Sargassum muticum*) in rainbow trout *Oncorhynchus mykiss* were 72.7% and 75.6%, 66.8% and 79.5%, 62.4% and 87.8%, and 58% and 65.5%, respectively. In the same study, the AED and APD of the four seaweeds in Nile tilapia *Oreochromis niloticus* were 57.1% and 63.4%, 39.6% and 58.5%, 27.8% and 51.4%, and 54.9% and 65.1%, respectively (Pereira *et al.*, 2012). The variations in the nutrients availability results presented in the researches could be mainly attributed to the use of multiple seaweed species and different aquatic animals in the experiment.

There are several other studies investigated the nutrient availability of the test diets supplemented with seaweed meals. Valente *et al.* (2006) indicated that supplementation of three seaweeds (*Gracilaria bursa-pastoris*, *Ulva rigida*, and *Gracilaria cornea*) up to 10% in the diets for European sea bass *Dicentrarchus labrax* did not affect the APD and ADMD of the test diets. Moreover, Marinho *et al.* (2013) documented that utilization of *Ulva spp* up to 20% as a replacement for 0-20% FM did not influence the APD and AED of the test diets for Nila tilapia *Oreochromis niloticus*. In addition, Cyrus *et al.* (2015) summarized that 5% inclusion of *Ulva spp* in the diets for sea urchin *Tripneustes gratilla* significantly improved APD, whereas significantly reduced APD and AED were determined when *Ulva spp* was supplemented at 15%.

4.3. Growth performance and survival

In trial 1, UM1 can be included at 6.35% as a replacement for 2% FM without compromising WG and FCR in a reference diet contained 10% FM. However, significant reductions in WG and increment in FCR were determined when UM1 was supplemented from 12.7% to 25.4% as a replacement for 4% to 8% FM. Regression results demonstrate WG of shrimp decreases as the inclusion level of UM1 increases, whereas FCR increases with

enhancing UM1 levels (Figure 1a and 1b). Similarly, many studies demonstrated that low inclusion levels (≤ 5%) of seaweed meals generally did not result in poor growth performance in African catfish *Clarias gariepinus* (Abdel-Warith *et al.*, 2016, Al-Asgah *et al.*, 2016), European sea bass (Valente *et al.*, 2006), gilthead seabream *Sparus aurata* (Emre *et al.*, 2013), Nile tilapia (Marinho *et al.*, 2013, Güroy *et al.*, 2007, Valente *et al.*, 2016), red tilapia *Oreochromis Sp*. (El-Tawil, 2010), Pacific white shrimp (Rodríguez-González *et al.*, 2014, Cárdenas *et al.*, 2015), and rainbow trout (Soler-Vila *et al.*, 2009, Güroy *et al.*, 2013).

However, the results of the moderate and high inclusion levels of seaweeds meals are somewhat inconsistent. Rodríguez-González *et al.* (2014) indicated that dietary supplementation of *Ulva lactuca* meal at both 10% and 15% as a substitution for FM significantly reduced the WG of Pacific white shrimp, whereas shrimp fed with diets contained 10% and 15% *Gracilaria parvispora* meal did not exhibit growth depression. Furthermore, Felix and Brindo (2014) reported that dietary inclusion of raw *Ulva lactuca* meal at 10%, 20%, and 30% resulted in depressed growth performance in giant freshwater prawn *Macrobrachium rosenbergii*, nevertheless the supplementation of fermented *Ulva lactuca* meal at the same levels did not affect the growth response. Moreover, Güroy *et al.* (2013) indicated that no difference in terms of growth performance was determined when 10% raw or autoclaved *Ulva rigida* meal were supplemented in the diet as a supplement for rainbow trout. However, significant reductions in WG was detected in rainbow trout fed with diets contained 10% *Ulva lactuca* and *Enteromorpha linza* meal (Yildirim *et al.*, 2009). In addition, several studies demonstrated that Nile tilapia fed with diets supplemented with *Ulva spp.* (a mixture of *Ulva rigida* and *Ulva lactuca*) or *Ulva rigida* meal up to 10% did not exhibit difference with regards to the growth response, whereas significant reduced WG was observed when inclusion level of those seaweed meals rose to 15%

(Güroy *et al.*, 2007, Marinho *et al.*, 2013, Valente *et al.*, 2016). Another two publications also confirmed that dietary inclusion of *Ulva lactuca* and *Gracilaria arcuata* meals at 9% and 13.5% significantly reduced the WG of African catfish and increased FCR (Abdel-Warith *et al.*, 2016, Al-Asgah *et al.*, 2016). Variations among these researches could be attributed to the utilization of different kinds of seaweed meal species and aquatic animal species as well as the over-formulation of reference diet. The result might be masked as the reference diets in some of the studies list above contained a high FM content resulting in over satisfaction of the nutrient requirement of the target animals. As the protein, lipid, phosphorus, and amino acids compositions are balanced in trial 1 (Table 4b), nutrient imbalances should be not counted as the problem caused growth depression. The potential problems resulted in the reduced growth of shrimp in trial 1 might be palatability shifts due to the reduced FM levels and the low nutrients availability and high mineral concentrations in UM1.

As the replacement of FM results in shifts in numerous nutrients as well as possible palatability changes of the diet, we chose to shift the nutrition model to replace a graded level of SBM and compare the effects of three batches Ulva meal (UM1, 2, and 3) at high inclusion levels in the trial 2. To compared the variations of Ulva meal from the three batches (UM1-3), additional two diets (T_2D_8 and T_2D_9) basically formulated by using high levels of UM3 and UM1, respectively, to replace the same amount of SBM as T_2D_5 which supplemented with UM2. Results indicated that there was a decreasing trend of WG as the UM2 inclusion level increased (Figure 2a). UM2 can be included up to 10% in the shrimp diet without causing growth depression, whereas significant reduction in WG was determined when more than 15% UM2 was supplemented in the diet. This phenomenon has been confirmed by trial 1 and multiple studies cited above. Besides reduced growth performance, there was a decreasing trend of survival when

more than 20% UM2 was included in the diet (Figure 2c). Significant reduction in survival was detected at 25% and 30% inclusion level. Similarly, a number of studies have demonstrated that low and moderate inclusion levels (≤20%) of various seaweed meals had no effects on the survival of a variety of fish or shrimp species including Nile tilapia (Güroy *et al.*, 2007), Pacific white shrimp (Rodríguez-González *et al.*, 2014), rainbow trout (Soler-Vila *et al.*, 2009, Yildirim *et al.*, 2009), and red tilapia (El-Tawil, 2010). By contrast, Felix and Brindo (2014) reported that survival of freshwater prawn was not affected when *Ulva lactuca* was used up to 30%. In addition, Al-Asgah *et al.* (2016) documented that dietary *Gracilaria arcuata* meal inclusion up to 30% did not affect the survival of African catfish. The different results were associated with both seaweed and aquatic animal species. Shrimp fed with UM1 (T_2D_9) and UM3 (T_2D_8) replacing the same levels of SBM as UM2 (T_2D_5) exhibited higher WG and lower FCR clearly demonstrating differences across batches. Although UM2 produced the poorest result, UM3 also resulted in significant reduction in growth.

Given the balanced nutrient composition of the test diets (Table 5b) and identical FM level across all the treatments, nutrient imbalances and palatability shift can be eliminated as the problems caused growth depression. The diet supplemented with UM1 (T_2D_9) contained highest mineral contents among the test diets, whereas the shrimp fed this diet performed the best compared to those fed with UM2 and UM3 replacing the same levels of SBM, which pointed out high mineral composition was not the problem led to the reduced growth and survival. Low nutrient availability of Ulva meal should be one of the potential problem result in growth reduction, which can be mediated by formulating diets on a digestible basis.

To elucidate if digestible protein was limiting growth, a third trial was initiated for which feeds were formulated on a digestible protein basis. In trial 3, four diets utilized high levels of

Ulva meal from three batches to replace SBM on the digestible basis. Since methionine and lysine are typically the two most limiting amino acids in shrimp feeds, they are also balanced on the digestible basis. Results indicated that no significant differences in terms of WG, FCR, and survival were detected in the diets contained UM1, however, significantly reduced growth and survival as well as increased FCR were determined when shrimp fed with the diet contained UM2. With regards to the supplementation of UM3, no differences were detected in WG and FCR, however, survival was significantly reduced. The same trend for the WG was observed in trial 2, in which reduced growth and survival as well as increased FCR were also observed for shrimp fed with the diet supplemented with UM2. Although UM1 contained the highest level of minerals among the three batches of Ulva meal (UM1-3), shrimp fed with UM1 performed the best in terms of WG, FCR and survival, which also help to exclude minerals as one of the problem. There must be some other factors affecting the growth of shrimp. The limited reduction in performance of shrimp offered high levels of UM1 and UM3 indicated that part of the problem is probably due to low digestibility of Ulva meal. However, this did not solve the problem for UM2 which has both poor survival and growth. One theory that has been advanced is that the *Ulva* sp. contains certain anti-nutritional factors or secretes certain secondary poisonous metabolites which are detrimental for shrimp. However, identification of the specific ANF or secondary poisonous metabolites was not available and beyond the scope of the current study.

As the protein content of seaweed is tied to culture conditions, the high protein content of Ulva meal can be achieved by culturing Ulva meal in water contains high nitrogen. The trial 4 evaluated the fourth batch Ulva meal which exhibited higher protein content (38.16%) than UM1-3 as a replacement for FM and SBM. In general, there was a decreasing trend of WG and

survival as the inclusion levels of UM4 increased (Figure 3a and 3d), which was in accordance with previous trials. Shrimp fed with diets contained UM4 exhibited significantly reduced WG as inclusion level of UM4 increases. Survival was significantly reduced when 9.5% UM4 was incorporated in the diet to replace 6% FM.

4.4. Body compositions

In the current study, there was a consistently decreasing trend of whole body lipid content as inclusion rates of Ulva meal increased in trial 1, 2, and 4. Significant difference in lipid content was detected when UM1-3 were supplemented at more than 6.35, 10, and 9.5%, respectively, in trial 1, 2, and 4. Similarly, Al-Asgah *et al.* (2016) recorded lipid content of carcass was significantly reduced when more than 20% *Gracilaria arcuata* meal was supplemented in the diets for African catfish. However, other authors did not report differences in lipid content of whole body (Abdel-Warith *et al.*, 2016, Emre *et al.*, 2013, Felix and Brindo, 2014, Güroy *et al.*, 2013, Güroy *et al.*, 2007, Marinho *et al.*, 2013, Rodríguez-González *et al.*, 2014, Soler-Vila *et al.*, 2009, Valente *et al.*, 2016, Valente *et al.*, 2006, Yildirim *et al.*, 2009). The significantly reduced lipid content in the present study would be a result of comparatively lower energy availability in the Ulva meal in contrast with the that in the FM and SBM.

Among the first three batches of Ulva meal, shrimp fed with UM2 exhibited significantly lower lipid content of whole body compared to those fed with diets contained UM1 and UM3 replacing the same levels of SBM in trial 2. Similarly, shrimp fed with the diet contained UM2 formulating on the digestible protein basis exhibited significantly lower lipid content than those fed with diets contain UM1 and UM3 in trial 3. The relatively lower lipid content in shrimp fed with UM2 would be attributed to the significantly lower energy digestibility in UM2.

Protein content was significantly improved when more than 10% UM2 was included in the diets in trial 2. Similarly, in trial 4, significantly improved protein content was detected when 24% UM4 was incorporated in the diet. However, no significantly differences were observed in the protein content in trial 1 and trial 3. A number of studies did not observe differences in the protein content of whole body in a variety of aquatic animals (Emre *et al.*, 2013, Felix and Brindo, 2014, Güroy *et al.*, 2013, Güroy *et al.*, 2007, Valente *et al.*, 2016, Valente *et al.*, 2006, Yildirim *et al.*, 2009). By contrast, improvements in protein content were detected in African catfish (Al-Asgah *et al.*, 2016) and rainbow trout (Soler-Vila *et al.*, 2009) when they are fed with diets contained seaweed meals. The significantly improved protein content in the present study might be an indirectly response to the dramatically reduced lipid content of shrimp body. As a result of the enhanced protein content in trial 2, total amino acids and most individual amino acids concentrations in shrimp body were improved in the treatments supplemented with Ulva meal. Significant improvements were detected in the contents of arginine, cysteine, glycine, histidine, lysine, methionine, and phenylalanine. In trial 3, no differences were determined in total amino acids and most of individual amino acids except methionine contents in shrimp body, which was in accordance with the unaffected protein content.

4.5. Protein and amino acid retention

In the present study, there were clearly decreasing trends of protein retention as Ulva meal levels increased in trial 1, 2, and 4. Protein retention was determined by a number of factors including dietary protein levels, feed offered, final weight, and initial weight of animals as well as the final and initial protein content of animals (Halver and Hardy, 2002). In trial 1, 2, and 4, the diets were balanced on an isonitrogenous basis. No difference was detected in the dietary

protein levels, feed offered and initial weight of shrimp. Although in trial 2 and trial 4, shrimp fed with diets contained Ulva meal exhibited higher protein content of whole body, the improved protein content cannot counteract the negative effects resulted by the significantly reduced growth. Consequently, the significantly reduced protein retention in trial 1, 2, and 4 followed the trends of decreased final mean weight as inclusion rates of Ulva meal enhanced. Similarly, a number of studies have documented negative effects of seaweed meals on the protein retention in Nile tilapia (Marinho *et al.*, 2013), rainbow trout (Güroy *et al.*, 2013, Soler-Vila *et al.*, 2009, Yildirim *et al.*, 2009) and European sea bass (Valente *et al.*, 2006).In trial 2, the dramatically depressed protein retention translated to the significantly reduced amino acids retention. In general, total amino acids and individual amino acid retention followed the decreasing trend of protein retention as UM2 inclusion rate increased.

In trial 3, protein retention was significantly reduced when shrimp fed with diets contained UM2 and UM3 compared to those fed with the reference diet. Among the diets contained three batches of Ulva meal, shrimp fed diets contained UM2 performed the worst with regards to protein retention compared to those fed with the diets supplemented with UM1 and UM3. The test diets in trial 2 were formulated on the digestible basis. As protein digestibility of Ulva meal is lower than that of SBM for which it substituted, more Ulva meal were included in the diets resulting in higher protein contents in the diets supplemented with Ulva meal than the reference diet. No differences were detected in feed offered, initial weight of shrimp, and final and initial protein content of shrimp body. The reduced protein retention would be attributed to the reduced final mean weight. There were reasonable correspondences between the amino acids retention and protein retention. Total amino acids and individual amino acids retention in the treatment contained UM2 were significantly depressed than other treatments.

5. Conclusions

Under the reported conditions of this study, the results demonstrated a clear depressing in the growth of shrimp when fish meal or soybean meal was replaced by Ulva meal. Among the first three batches of Ulva meal (UM1-3), the second batch Ulva meal produced the worst result. The low nutrient availability of Ulva meal in contrast with fish meal and soybean meal would be part of the problems resulting in the growth depression. Other possible reasons which are beyond the scope of this project but would include anti-nutrients present in the algae.

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Table 1 Proximate composition[1], phosphorus content[1], and amino acid profile[1] of the primary protein sources used in diet formulations.

Composition (% as is)	Ulva meal 1	Ulva meal 2	Ulva meal 3	Ulva meal 4	Fish meal	Soybean meal
Crude Protein	20.64	27.24	26.80	38.16	62.78	44.89
Moisture	8.89	13.74	11.19	8.41	7.99	10.97
Crude Fat	0.53	0.12	0.42	0.10	10.56	3.78
Crude Fiber	5.17	2.93	4.07	5.57	0.00	3.20
Ash	46.01	22.18	20.31	13.49	18.75	6.67
Phosphorus	0.43	0.30	-	0.42	3.15	0.66
Alanine	1.64	2.03	1.89	2.68	3.91	2.04
Arginine	0.99	1.39	1.01	1.77	3.68	3.35
Aspartic Acid	2.12	2.67	3.23	3.46	5.34	5.10
Cysteine	0.34	0.39	0.46	0.49	0.47	0.62
Glutamic Acid	2.02	2.59	3.02	3.35	7.47	8.24
Glycine	1.17	1.59	1.29	2.00	4.88	2.04
Histidine	0.25	0.40	0.22	0.45	1.63	1.2
Hydroxylysine	0.17	0.12	0.10	0.21	0.2	0.05
Hydroxyproline	0.2	0.30	0.38	0.35	1.03	0.05
Isoleucine	0.8	1.06	0.92	1.39	2.42	2.17
Leucine	1.22	1.87	1.50	2.43	4.21	3.57
Lysine	0.95	1.22	0.82	1.51	4.67	3.06
Methionine	0.26	0.44	0.46	0.63	1.61	0.66
Phenylalanine	0.98	1.37	1.16	1.78	2.39	2.35
Proline	0.76	1.17	1.02	1.50	3.08	2.39
Serine	0.91	1.05	0.93	1.47	2.11	1.90
Taurine	0.15	0.18	0.18	0.18	0.73	0.13
Threonine	0.94	1.17	1.13	1.56	2.41	1.75
Tryptophan	0.16	0.20	0.22	0.266	0.62	0.62
Tyrosine	0.48	0.77	0.49	0.94	1.67	1.64
Valine	1.17	1.56	1.40	2.13	2.99	2.34

[1] Diets were analyzed at University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory (Columbia, MO, USA).

Table 2 Mineral composition[1] of the three batches Ulva meal (UM1, UM2, and UM3) utilized in feed formulations.

Minerals	UM1	UM2	UM3
Quantity elements (% as is)			
Calcium	2.29	0.49	0.36
Potassium	1.99	2.21	3.9
Magnesium	2.57	2.93	1.11
Sodium	4.79	1.63	2.82
Phosphorus	0.4	0.32	0.31
Sulfur	3.46	4.54	2.96
Trace elements (mg kg^{-1} as is)			
Aluminum	4173.2	380.5	31.7
Arsenic	1.6	1.3	0.6
Boron	76.2	38.8	70.0
Barium	13.8	2.6	1.6
Cadmium	50.4	8.3	9.6
Cobalt	3.0	0.8	0.6
Chromium	9.7	1.8	0.7
Copper	26.5	17.5	56.9
Iron	9086.7	581.6	70.0
Manganese	112.4	21.1	17.8
Nickel	7.7	2.1	3.3
Lead	10.8	2.0	1.2
Selenium	5.3	3.9	2.8
Silicon	70.3	68.4	16.4
Zinc	63.1	34.6	18.9
Zirconium	1.0	1.0	1.0

[1] Three batches Ulva meal were analyzed at Auburn University, Soils Laboratory (Auburn, AL, USA)

Table 3 Proximate[1] and mineral[1] composition of Ulva meal 2 (UM2) utilized in trial 2 and 3 collected from seven different dates.

Proximate composition (% as is)	Dates (2015)						
	7/21	7/30	8/16	8/20	8/23	8/25	8/30
Moisture	83.99	87.14	85.59	82.78	83.32	82.44	85.07
Crude protein	28.2	19.4	29	28.3	27.3	28	26.9
Crude fat	0.46	n.d.	0.2	n.d.	n.d.	0.62	n.d.
Fiber	10	13.9	13.4	10.9	11.3	10.5	10.5
Ash	17.3	39.2	19.8	15.6	17.1	18.4	20.9
Quantity elements (% as is)							
Calcium	0.42	2.01	0.86	0.47	0.44	0.42	0.46
Magnesium	3.12	3.07	3	3.25	3.21	3.25	3.12
Phosphorus	0.32	0.37	0.38	0.35	0.31	0.31	0.31
Potassium	2.71	2.26	1.82	2.2	1.95	2.31	2.49
Sodium	1.26	2.74	1.46	0.89	1.55	1.96	2.05
Sulfur	4.38	3.64	3.78	4.19	4.16	4.33	4.24
Trace elements (mg kg^{-1} as is)							
Copper (ppm)	7.7	28.2	11	7.8	7.6	8.9	8.8
Iron (ppm)	331	6780	2040	424	450	356	510
Manganese (ppm)	21.1	99.2	47	22.9	22.2	21.9	24.3
Zinc (ppm)	37.6	79.3	64	49	38.4	38.9	38.8

[1]Analyses conducted by Midwest Laboratories (Omaha, NE, USA).
n.d.: not detected.

Table 4a Formulation of test diets designed to evaluate Ulva meal 1 (UM1) as a replacement for fish meal on an iso-nitrogenous basis (Trial 1).

Ingredient (% as is)	T_1D_1	T_1D_2	T_1D_3	T_1D_4	T_1D_5
Fish meal[1]	10.00	8.00	6.00	4.00	2.00
Soybean meal[2]	48.70	48.70	48.70	48.70	48.70
Corn protein concentrate[3]	8.00	8.00	8.00	8.00	8.00
Ulva meal 1[10]	0.00	6.35	12.70	19.05	25.40
Fish oil[2]	5.65	5.75	5.86	5.97	6.07
Trace mineral premix[5]	0.50	0.50	0.50	0.50	0.50
Vitamin premix[6]	1.80	1.80	1.80	1.80	1.80
Choline chloride[4]	0.20	0.20	0.20	0.20	0.20
Stay C[7]	0.10	0.10	0.10	0.10	0.10
Mono-dicalcium phosphate[8]	1.62	1.90	2.15	2.40	2.65
Lecithin[9]	1.00	1.00	1.00	1.00	1.00
Cholesterol[4]	0.05	0.05	0.05	0.05	0.05
Corn starch[4]	22.54	17.78	13.05	8.31	3.58

[1] Omega Protein Inc., Huston, TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[4] MP Biomedicals Inc., Solon, OH, USA.
[5]Trace mineral premix (g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[9] The Solae Company, St. Louis, MO, USA.
[10] Ulva meal first batch experimentally produced.

Table 4b Proximate composition[1] and amino acid profile[1] of the test diets used in the trial 1.

Composition (% as is)	T_1D_1	T_1D_2	T_1D_3	T_1D_4	T_1D_5
Ulva levels (%)	0	6.35	12.70	19.05	25.40
Crude Protein	36.83	36.52	36.60	36.28	35.65
Moisture	5.46	6.56	5.12	7.15	8.70
Crude Fat	10.09	8.94	9.06	8.22	7.51
Crude Fiber	2.92	3.08	3.48	3.22	3.33
Ash	6.54	8.92	11.80	14.40	16.58
Alanine	2.03	2.00	2.08	2.08	2.04
Arginine	2.24	2.21	2.23	2.19	2.14
Aspartic Acid	3.56	3.53	3.62	3.58	3.53
Cysteine	0.48	0.47	0.48	0.49	0.49
Glutamic Acid	6.39	6.18	6.32	6.11	6.01
Glycine	1.65	1.63	1.63	1.61	1.55
Histidine	0.92	0.89	0.89	0.85	0.82
Hydroxylysine	0.04	0.06	0.08	0.09	0.06
Hydroxyproline	0.13	0.12	0.11	0.10	0.09
Isoleucine	1.68	1.64	1.70	1.68	1.6
Leucine	3.43	3.29	3.41	3.34	3.21
Lysine	2.13	2.08	2.06	1.99	1.91
Methionine	0.66	0.64	0.63	0.62	0.63
Phenylalanine	1.85	1.86	1.94	1.92	1.81
Proline	2.09	2.08	2.12	2.09	1.98
Serine	1.51	1.50	1.53	1.47	1.51
Taurine	0.18	0.17	0.16	0.14	0.13
Threonine	1.36	1.35	1.38	1.37	1.35
Tryptophan	0.42	0.39	0.39	0.39	0.37
Tyrosine	1.45	1.44	1.49	1.47	1.39
Valine	1.78	1.77	1.82	1.80	1.76

[1] Diets were analyzed at University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory (Columbia, MO, USA).

Table 4c Protein retention efficiency and growth performance of juvenile Pacific white shrimp (0.26±0.02g) offered diets with different Ulva meal 1 levels (0, 6.35, 12.70, 19.05, and 25.40%) as a fish meal replacement over a six-week growth trial (Trial 1).

Diet	Ulva levels (%)	Final Biomass (g)	Final Mean Weight (g)	WG[3] (%)	FCR[2]	Survival (%)	PRE[4] (%)
T_1D_1	0	44.63^{a}	5.01^{a}	1792.8^{a}	1.83^{b}	88.6	25.70^{ab}
T_1D_2	6.35	45.45^{a}	5.09^{a}	1830.9^{a}	1.81^{b}	88.6	27.16^{a}
T_1D_3	12.70	39.58^{ab}	4.30^{ab}	1555.1^{b}	2.15^{ab}	91.4	23.07^{ab}
T_1D_4	19.05	36.10^{ab}	3.88^{b}	1389.1^{b}	2.36^{a}	92.9	20.20^{b}
T_1D_5	25.40	32.26^{b}	3.87^{b}	1407.4^{b}	2.43^{a}	82.9	20.36^{b}
P-value		0.0175	0.0006	0.0003	0.0039	0.2451	0.0073
PSE[1]		1.1253	0.0868	28.9568	0.0491	1.2074	0.5699

[1] Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] WG: Weight gain = (Final weight - Initial weight) / Initial weight × 100%.
[4] PRE: Protein retention efficiency = (Final weight × Final protein content) - (Initial weight × Initial protein content) × 100 / Protein intake.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 4d Proximate[3] analysis of whole shrimp body offered varying Ulva meal 1 levels (0, 6.35, 12.70, 19.05, and 25.40%) as a replacement of fish meal over a six-week growth trial (Trial 1).

Diet	Ulva Levels (%)	Moisture (%)	Crude protein[2] (%)	Crude lipid[2] (%)
T_1D_1	0	76.88	72.77	8.04[a]
T_1D_2	6.35	76.29	73.63	6.12[b]
T_1D_3	12.70	76.99	74.27	5.73[b]
T_1D_4	19.05	76.37	72.83	5.99[b]
T_1D_5	25.40	76.83	74.11	5.09[b]
P-value		0.7933	0.2576	0.0006
PSE[1]		0.1340	0.2240	0.1613

[1] Pooled standard error.
[2] Dry weight basis.
[3] Body samples were analyzed at University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory (Columbia, MO, USA).
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 5a Formulation of test diets designed to evaluate three batches of Ulva meal (UM1, UM2, and UM3) as a replacement for soybean meal on an iso-nitrogenous basis (Trial 2).

Ingredient (% As is basis)	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6	T_2D_7	T_2D_8	T_2D_9
Fish meal[1]	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Soybean meal[2]	55.55	52.55	49.60	46.60	43.75	40.80	37.80	43.75	43.75
Corn protein concentrate[3]	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Ulva meal 2[11]	0.00	5.00	10.00	15.00	20.00	25.00	30.00	0.00	0.00
Ulva meal 3[11]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	23.60	0.00
Ulva meal 1[11]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	26.30
Fish oil[2]	5.84	5.88	5.92	5.96	6.00	6.04	6.08	6.00	5.89
Trace mineral premix[5]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix[6]	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride[4]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Stay C[7]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Mono-dicalcium phosphate[8]	1.85	1.85	1.90	1.90	1.95	1.95	2.00	1.90	1.70
Lecithin[9]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol[4]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Methionine[10]	0.07	0.06	0.06	0.06	0.05	0.05	0.04	0.03	0.07
Corn starch[4]	15.04	13.01	10.87	8.83	6.60	4.51	2.43	3.07	0.64

[1] Omega Protein Inc., Huston TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[4] MP Biomedicals Inc., Solon, OH, USA.
[5] Trace mineral premix (g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[9] The Solae Company, St. Louis, MO, USA.
[10] Aldrich-Sigma, St. Louis, MO, USA.

[11] Three batches of Ulva meal experimentally produced.

Table 5b Proximate composition[1], phosphorus content[1], and amino acid profile[1] of the test diets used in the trial 2.

Composition[1] (% as is)	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6	T_2D_7	T_2D_8	T_2D_9
Ulva levels (%)	0	5	10	15	20	25	30	23.6	26.3
Crude Protein	36.46	36.67	35.91	36.78	36.64	36.69	37.46	37.08	36.34
Moisture	7.32	7.92	9.44	7.56	8.29	8.03	6.46	7.74	8.48
Crude Fat	10.02	8.49	8.68	9.71	8.90	8.28	6.29	6.35	7.01
Crude Fiber	3.54	3.43	3.65	3.64	3.79	4.10	4.09	3.86	3.86
Ash	6.49	7.61	8.47	9.02	10.17	10.47	11.48	10.56	16.77
Phosphorus	0.99	1.01	1.00	1.03	1.01	1.06	1.02	1.00	1.02
Alanine	1.86	1.90	1.91	2.00	2.11	2.05	2.18	2.03	1.99
Arginine	2.30	2.26	2.20	2.22	2.19	2.18	2.24	2.12	2.12
Aspartic Acid	3.68	3.62	3.56	3.62	3.61	3.60	3.68	3.72	3.50
Cysteine	0.50	0.47	0.47	0.48	0.48	0.47	0.47	0.50	0.47
Glutamic Acid	6.68	6.48	6.25	6.29	6.26	5.98	6.07	6.14	5.95
Glycine	1.64	1.68	1.67	1.69	1.84	1.77	1.86	1.73	1.62
Histidine	0.91	0.88	0.85	0.86	0.84	0.82	0.84	0.79	0.80
Hydroxylysine	0.05	0.05	0.05	0.06	0.07	0.07	0.07	0.06	0.07
Hydroxyproline	0.11	0.10	0.11	0.12	0.18	0.37	0.16	0.22	0.17
Isoleucine	1.64	1.60	1.56	1.60	1.58	1.57	1.61	1.56	1.54
Leucine	3.22	3.14	3.09	3.16	3.20	3.08	3.22	3.03	3.00
Lysine	2.04	1.99	1.95	1.95	1.91	1.92	1.94	1.86	1.86
Methionine	0.70	0.67	0.66	0.67	0.68	0.66	0.67	0.63	0.61
Phenylalanine	1.85	1.82	1.80	1.84	1.86	1.82	1.89	1.79	1.76
Proline	2.09	1.97	2.06	1.96	2.13	2.02	1.99	2.03	2.00
Serine	1.53	1.53	1.47	1.52	1.51	1.49	1.54	1.45	1.46
Taurine	0.14	0.16	0.15	0.15	0.15	0.16	0.17	0.17	0.14
Threonine	1.35	1.35	1.34	1.37	1.38	1.39	1.44	1.37	1.34
Tryptophan	0.50	0.50	0.47	0.48	0.48	0.47	0.49	0.44	0.45
Tyrosine	1.19	1.19	1.14	1.21	1.19	1.17	1.21	1.10	1.13
Valine	1.83	1.81	1.80	1.88	1.88	1.86	1.91	1.84	1.80

[1] Diets were analyzed at University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory (Columbia, MO, USA).

Table 5c Mineral profile[1] of the test diets used in the trial 2.

Mineral	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6	T_2D_7	T_2D_8	T_2D_9
Quantity elements (g kg^{-1})									
Calcium	8.5	9.7	9.0	9.5	9.1	8.9	9.5	9.3	13.3
Potassium	12.7	13.5	13.7	14.6	14.9	14.9	15.9	18.8	15.5
Magnesium	1.9	3.4	4.7	6.2	7.5	8.7	9.6	4.8	8.3
Sodium	0.8	1.7	2.4	3.3	4.1	4.8	5.7	7.4	14.0
Phosphorus	10.3	10.8	10.2	10.7	10.3	9.9	10.4	10.5	8.3
Sulfur	3.8	5.9	7.7	10.0	11.8	13.6	16.2	10.4	12.0
Trace elements (mg kg^{-1})									
Aluminum	97.8	119.5	133.1	160.8	173.8	185.6	199.1	99.7	1175.4
Arsenic	0.7	0.6	0.4	0.6	0.8	0.9	0.4	0.4	1.0
Boron	17.6	18.6	19.3	20.5	21.5	21.5	23.8	29.7	34.5
Barium	5.1	5.3	5.7	5.1	5.0	4.5	4.6	4.4	8.2
Cadmium	4.5	0.9	13.2	1.2	14.3	12.6	6.5	1.4	13.7
Cobalt	1.1	1.3	1.2	1.2	1.2	1.0	1.1	1.0	1.7
Chromium	1.0	1.0	1.1	1.1	1.1	1.1	1.1	0.9	3.6
Copper	39.4	110.3	21.0	22.9	18.6	23.9	27.1	28.8	23.9
Iron	59.2	43.8	69.4	74.6	66.9	74.4	66.2	48.7	904.5
Manganese	34.2	35.4	34.5	33.6	33.5	32.7	34.1	33.0	61.4
Molybdenum	3.9	4.0	3.4	3.3	3.5	2.5	2.7	3.1	0.1
Nickel	3.1	3.2	3.1	2.6	2.7	2.4	2.2	2.7	4.5
Lead	1.0	1.3	1.7	0.5	1.4	0.5	0.6	0.2	4.0
Selenium	3.3	4.6	2.3	3.1	3.8	4.4	4.8	4.5	4.2
Silicon	57.9	81.0	107.0	120.8	119.9	131.7	131.4	59.9	61.8
Zinc	158.1	165.1	145.8	145.8	135.6	155.4	153.9	152.6	174.1
Zirconium	0.9	1.0	0.9	0.9	1.0	0.9	0.9	0.9	0.7

[1] Diets were analyzed at Auburn University, Soils Laboratory (Auburn, AL, USA)

Table 5d Growth performance of juvenile Pacific white shrimp (0.24±0.01g) offered diets with different levels of three batches Ulva meal (UM1, UM2, and UM3) for five weeks (Trial 2).

Diet	Ulva levels (%)	Final Biomass (g)	Final Mean Weight (g)	WG[3] (%)	FCR[2]	Survival (%)
T_2D_1	0	43.31^{a}	4.55^{a}	1734.21^{a}	1.46^{b}	95.0^{a}
T_2D_2	5	36.19^{ab}	3.70^{ab}	1398.22^{ab}	1.83^{ab}	97.5^{a}
T_2D_3	10	28.40^{bc}	3.25^{ab}	1241.46^{ab}	2.23^{ab}	87.5^{ab}
T_2D_4	15	23.89^{cd}	2.58^{b}	948.74^{b}	2.82^{ab}	92.5^{a}
T_2D_5	20	18.98^{cd}	2.53^{b}	990.26^{b}	2.96^{ab}	75.0^{ab}
T_2D_6	25	16.34^{cd}	2.56^{b}	943.46^{b}	3.53^{a}	67.5^{b}
T_2D_7	30	15.50^{d}	2.45^{b}	864.67^{b}	3.37^{ab}	65.0^{b}
T_2D_8	23.6	26.14^{bcd}	2.96^{b}	1131.07^{b}	2.61^{ab}	87.5^{ab}
T_2D_9	26.3	27.10^{bcd}	3.09^{b}	1226.92^{ab}	2.36^{ab}	87.5^{ab}
P-value		<0.0001	0.0002	0.0008	0.0201	0.0006
PSE[1]		1.2872	0.1392	61.8604	0.2020	2.6131

[1] Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] WG: Weight gain = (Final weight - Initial weight) / Initial weight × 100%.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 5e Proximate composition[2] and amino acid profile[2] of whole shrimp body offered diets contain different levels of three batches Ulva meal (UM1, UM2, and UM3) levels in trial 2.

Diet	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6	T_2D_7	T_2D_8	T_2D_9	PSE[1]	*P*-value	Adjust *P*-value
Ulva levels (%)	0	5	10	15	20	25	30	23.6	26.3			
Moisture	756.6	780.2	760.5	773.3	768.3	771.6	780.5	770.4	767.6	0.3204	0.1925	0.3300
Protein	725.6^{d}	728.1^{cd}	743.3^{abc}	751.8^{ab}	755.0^{a}	747.7^{ab}	754.3^{a}	741.5^{abcd}	736.2^{bcd}	0.1802	<0.0001	0.0001
Lipid	77.2^{a}	62.2^{ab}	45.4^{bc}	27.5^{d}	29.0^{cd}	29.7^{cd}	22.0^{d}	47.4^{b}	52.7^{b}	0.1779	<0.0001	0.0001
Alanine	4.30	4.50	4.40	4.37	4.36	4.20	4.28	4.36	4.34	0.0256	0.0298	0.0650
Arginine	4.98^{c}	4.91^{c}	4.95^{c}	5.21^{abc}	5.22^{abc}	5.43^{ab}	5.51^{a}	5.13^{bc}	5.18^{abc}	0.0390	<0.0001	0.0003
Aspartic Acid	6.68	6.85	6.87	6.93	6.87	6.79	6.85	6.89	6.85	0.037	0.5257	0.6309
Cysteine	0.57^{c}	0.58^{c}	0.60^{bc}	0.62^{ab}	0.62^{ab}	0.62^{ab}	0.64^{a}	0.60^{abc}	0.60^{abc}	0.0037	<0.0001	0.0002
Glutamic Acid	9.96	10.10	10.21	10.24	10.25	10.09	10.06	10.11	10.27	0.0694	0.7825	0.8412
Glycine	4.53^{c}	4.68^{bc}	4.85^{bc}	5.28^{abc}	5.10^{abc}	5.45^{ab}	5.68^{a}	4.98^{abc}	4.98^{abc}	0.0842	0.0011	0.0050
Histidine	1.48^{b}	1.52^{ab}	1.58^{a}	1.59^{a}	1.60^{a}	1.59^{a}	1.55^{ab}	1.60^{a}	1.56^{ab}	0.0103	0.0031	0.0105
Hydroxylysine	0.14	0.13	0.16	0.16	0.16	0.14	0.14	0.16	0.15	0.0007	0.3939	0.5252
Hydroxyproline	0.21	0.20	0.20	0.21	0.20	0.21	0.20	0.21	0.21	0.0051	0.9706	0.9706
Isoleucine	2.87	2.90	2.95	2.96	2.96	2.93	2.90	2.92	2.91	0.0129	0.2423	0.3635
Leucine	4.80	4.87	4.92	4.96	4.99	4.93	4.90	4.92	4.90	0.0227	0.2309	0.3635
Lysine	4.72^{b}	4.86^{ab}	4.92^{ab}	5.02^{a}	5.05^{a}	5.06^{a}	5.06^{a}	4.99^{ab}	4.94^{ab}	0.0312	0.0095	0.0253
Methionine	1.40^{b}	1.41^{b}	1.42^{b}	1.47^{ab}	1.46^{ab}	1.48^{ab}	1.52^{a}	1.44^{ab}	1.46^{ab}	0.0095	0.0028	0.0105
Phenylalanine	3.10^{b}	3.12^{b}	3.21^{ab}	3.24^{ab}	3.28^{a}	3.20^{ab}	3.14^{ab}	3.23^{ab}	3.12^{b}	0.0159	0.0044	0.0132
Proline	3.80	3.74	3.85	3.73	3.95	3.78	3.55	3.65	3.63	0.0639	0.5216	0.6309
Serine	2.29	2.34	2.33	2.37	2.37	2.35	2.38	2.35	2.42	0.0237	0.8062	0.8412

Threonine	2.64	2.68	2.71	2.71	2.74	2.68	2.64	2.72	2.70	0.0136	0.1540	0.2844
Tryptophan	0.84	0.87	0.88	0.90	0.89	0.89	0.89	0.89	0.88	0.0054	0.0272	0.0650
Tyrosine	2.28	2.23	2.50	2.52	2.54	2.48	2.16	2.50	2.47	0.0643	0.3161	0.4462
Valine	4.05	4.09	4.07	4.09	4.22	4.12	4.12	4.11	4.20	0.0367	0.7681	0.8412
Total	65.61	66.55	67.56	68.58	68.82	68.41	68.15	67.74	67.71	0.3262	0.0373	0.0746

[1] Pooled standard error.
[2] Body samples were analyzed at University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory (Columbia, MO, USA).
Values within a row with different superscripts are significantly different based on Tukey's multiple range test.

Table 5f Protein[2] and amino acids[3] retention efficiencies of Pacific white shrimp offered varying Ulva meal 2 levels diets in trial 2 for five weeks.

Diet	T_2D_1	T_2D_2	T_2D_3	T_2D_4	T_2D_5	T_2D_6	T_2D_7	T_2D_8	T_2D_9	*P*-value	PSE[1]	Adjust *P*-value
Ulva levels (%)	0	5	10	15	20	25	30	23.6	26.3			
Protein	36.4^{a}	26.5^{ab}	25.6^{ab}	19.0^{b}	18.1^{b}	17.1^{b}	15.3^{b}	21.4^{b}	22.9^{b}	1.1928	<0.0001	0.0002
Alanine	42.3^{a}	31.8^{ab}	28.5^{bc}	20.2^{bcd}	18.1^{cd}	17.2^{cd}	14.8^{d}	23.0^{bcd}	24.7^{bcd}	1.3131	<0.0001	<0.0001
Arginine	39.8^{a}	29.2^{ab}	28.1^{ab}	21.9^{b}	21.1^{b}	21.1^{b}	19.0^{b}	26.1^{b}	27.9^{ab}	1.3498	0.0003	0.0005
Aspartic Acid	33.2^{a}	25.3^{ab}	23.9^{abc}	17.7^{bc}	16.7^{bc}	15.9^{bc}	14.1^{c}	19.9^{bc}	22.1^{bc}	1.1108	<0.0001	0.0002
Cysteine	20.8^{a}	16.5^{ab}	15.6^{ab}	11.9^{b}	11.3^{b}	11.0^{b}	10.2^{b}	12.8^{b}	14.4^{ab}	0.7296	0.0004	0.0005
Glutamic Acid	27.3^{a}	20.9^{ab}	20.3^{ab}	15.1^{b}	14.4^{b}	14.3^{b}	12.6^{b}	17.7^{b}	19.6^{ab}	0.9825	0.0004	0.0005
Glycine	50.5^{a}	37.3^{ab}	36.1^{ab}	29.1^{b}	24.4^{b}	25.5^{b}	23.4^{b}	31.0^{b}	34.9^{ab}	1.6381	<0.0001	0.0002
Histidine	29.7^{a}	23.0^{ab}	23.0^{ab}	17.1^{b}	16.7^{b}	16.3^{b}	13.9^{b}	21.7^{ab}	21.9^{ab}	1.1148	0.0010	0.0010
Hydroxylysine	50.3^{a}	31.6^{abc}	38.6^{ab}	25.2^{bc}	20.3^{bc}	16.6^{bc}	14.2^{c}	29.3^{abc}	23.1^{bc}	2.4299	0.0004	0.0005
Hydroxyproline	34.0^{a}	26.6^{ab}	22.7^{bc}	15.9^{cd}	9.8^{de}	4.9^{e}	9.6^{de}	10.4^{de}	14.0^{d}	0.8258	<0.0001	<0.0001
Isoleucine	32.1^{a}	24.3^{ab}	23.4^{abc}	17.2^{bc}	16.5^{bc}	15.8^{bc}	13.7^{c}	20.1^{bc}	21.3^{bc}	1.1065	0.0001	0.0002
Leucine	27.3^{a}	20.7^{ab}	19.8^{abc}	14.5^{bc}	13.7^{bc}	13.5^{bc}	11.5^{c}	17.4^{bc}	18.5^{abc}	0.9449	<0.0001	0.0002
Lysine	42.2^{a}	32.7^{ab}	31.2^{ab}	23.8^{b}	23.2^{b}	22.2^{b}	19.8^{b}	28.7^{ab}	30.0^{ab}	1.5286	0.0006	0.0007
Methionine	36.4^{a}	28.2^{ab}	26.8^{ab}	20.4^{b}	18.9^{b}	18.9^{b}	17.2^{b}	24.5^{ab}	27.0^{ab}	1.3455	0.0005	0.0006
Phenylalanine	30.6^{a}	22.9^{ab}	22.1^{abc}	16.3^{bc}	15.5^{bc}	14.8^{bc}	12.6^{c}	19.3^{bc}	20.0^{bc}	1.0502	<0.0001	0.0002
Proline	33.3^{a}	25.5^{ab}	23.3^{abc}	17.8^{bc}	16.4^{bc}	16.4^{bc}	13.6^{c}	19.2^{bc}	20.5^{bc}	1.2344	0.0002	0.0004
Serine	27.4^{a}	20.4^{ab}	19.6^{ab}	14.4^{b}	13.8^{b}	13.3^{b}	11.8^{b}	17.3^{b}	18.8^{ab}	0.9469	0.0001	0.0002
Threonine	35.7^{a}	26.5^{ab}	25.0^{ab}	18.3^{bc}	17.4^{bc}	16.3^{bc}	13.9^{c}	21.3^{bc}	22.8^{bc}	1.1486	<0.0001	0.0001
Tryptophan	30.7^{a}	23.0^{ab}	23.1^{ab}	17.3^{b}	16.2^{b}	15.9^{b}	13.8^{b}	21.7^{ab}	21.9^{ab}	1.1080	0.0004	0.0005
Tyrosine	35.2^{a}	24.7^{abc}	27.1^{ab}	19.4^{bc}	18.7^{bc}	17.9^{bc}	13.1^{c}	24.4^{abc}	24.6^{abc}	1.4386	0.0008	0.0008

Valine	40.4[a]	30.1[ab]	28.0[abc]	20.2[bc]	19.7[bc]	18.7[bc]	16.4[c]	24.0[bc]	26.5[bc]	1.3655	<0.0001	0.0002
Total	33.5[a]	25.3[ab]	24.2[ab]	18.1[b]	17.0[b]	16.5[b]	14.5[b]	21.0[b]	22.7[ab]	1.1483	0.0001	0.0002

[1] Pooled standard error.
[2] Protein retention efficiency = (Final weight × Final protein content) - (Initial weight × Initial protein content) × 100 / Protein offered.
[3] Amino acids (AA) retention efficiency = (Final weight × Final AA content) - (Initial weight × Initial AA content) × 100 / AA offered.
Values within a row with different superscripts are significantly different based on Tukey's multiple range test.

Table 6a Formulation of test diets designed to evaluate Ulva meal 1, 2, and 3 as a replacement for soybean meal on a digestible protein basis (Trial 3).

Ingredient (% as is)	T_3D_1	T_3D_2	T_3D_3	T_3D_4
Fish meal[1]	6.00	6.00	6.00	6.00
Soybean meal[2]	53.00	46.30	49.90	46.30
Corn protein concentrate[3]	8.00	8.00	8.00	8.00
Ulva meal 2[10]		22.00		
Ulva meal 1[10]			25.00	
Ulva meal 3[10]				25.00
Fish oil[2]	5.92	5.98	5.85	5.91
Trace mineral premix[5]	0.50	0.50	0.50	0.50
Vitamin premix[6]	1.80	1.80	1.80	1.80
Choline chloride[4]	0.20	0.20	0.20	0.20
Stay C[7]	0.10	0.10	0.10	0.10
Mono-dicalcium phosphate[8]	2.50	2.50	2.50	2.50
Lecithin[9]	1.00	1.00	1.00	1.00
Cholesterol[4]	0.08	0.08	0.08	0.08
Methionine	0.05	0.04	0.04	0.04
Lyisine	0.00	0.07	0.00	0.11
Corn starch[4]	20.85	2.43	2.03	2.46

[1] Omega Protein Inc., Huston TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[4] MP Biomedicals Inc., Solon, OH, USA.
[5]Trace mineral premix (g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[9] The Solae Company, St. Louis, MO, USA.
[10] Three batches Ulva meal experimentally produced.

[11] Aldrich-Sigma, St. Louis, MO, USA.

Table 6b Proximate composition[1], mineral composition[2], and amino acid profile[1] of the test diets used in trial 3.

Composition	T_3D_1	T_3D_2	T_3D_3	T_3D_4
Proximate composition (% as is)				
Crude protein	36.33	38.40	39.66	39.13
Moisture	7.15	7.59	8.93	8.34
Crude fat	9.39	9.03	9.01	8.68
Crude fiber	3.21	3.84	4.42	4.13
Ash	6.86	15.93	11.44	11.22
Quantity elements (% as is)				
Phosphorus	1.36	1.25	1.24	1.37
Sulfur	0.4	1.06	1.27	1.08
Potassium	1.33	1.73	1.65	2.13
Magnesium	0.18	0.76	0.86	0.52
Calcium	1.31	1.79	1.17	1.30
Trace elements (mg kg^{-1} as is)				
Sodium	0.1	1.16	0.51	0.77
Iron (ppm)	149	1240	286	169
Manganese (ppm)	40.1	71.6	39.1	40.1
Copper (ppm)	16.8	22.9	20.2	28.7
Zinc (ppm)	183	215	187	194
Amino acid profile (% as is)				
Alanine	1.87	2.15	2.24	2.14
Arginine	2.18	2.26	2.34	2.21
Aspartic Acid	3.44	3.66	3.78	3.79
Cysteine	0.48	0.49	0.50	0.51
Glutamic Acid	6.33	6.43	6.33	6.24
Glycine	1.56	1.69	1.82	1.68
Histidine	0.86	0.86	0.89	0.80
Isoleucine	1.60	1.70	1.71	1.65
Leucine	3.28	3.49	3.50	3.32
Lysine	2.01	2.03	2.16	2.05
Methionine	0.64	0.62	0.67	0.65
Phenylalanine	1.85	2.00	2.05	1.90
Proline	2.13	2.16	2.22	2.22

Serine	1.48	1.61	1.68	1.59
Taurine	0.16	0.13	0.14	0.16
Threonine	1.29	1.43	1.50	1.44
Tryptophan	0.47	0.48	0.45	0.44
Tyrosine	1.33	1.38	1.44	1.33
Valine	1.73	1.95	2.01	1.94

[1] Proximate composition and amino acid profiles of test diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA).
[2] Mineral composition was tested at Midwest Laboratories (Omaha, NE, USA).

Table 6c Growth performance of juvenile Pacific white shrimp *L. vannamei* (Initial weight 0.98g) offered diets formulated to partially replace soybean meal on a digestible protein basis with three different batches of Ulva meal over six weeks (Trial 3).

Diet	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)
T_3D_1	79.3[a]	8.4[a]	766.6[a]	1.64[b]	95.0[a]
T_3D_2	66.8[a]	7.8[a]	689.7[a]	1.87[b]	85.0[ab]
T_3D_3	30.6[c]	4.9[b]	397.3[b]	3.58[a]	62.5[c]
T_3D_4	57.3[b]	7.2[a]	618.1[a]	2.09[b]	80.0[b]
PSE[1]	1.7351	2.3457	18.1043	0.1167	1.5427
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1] Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] WG: Weight gain = (Final weight - Initial weight) / Initial weight × 100%.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 6d Proximate composition[2] and amino acids profile[2] of shrimp at the conclusion of a 6-week growth trial in which shrimp were offered diets formulated to partially replace soybean meal on a digestible protein basis with three different batches of Ulva meal (Trial 3).

Diet	T_3D_1	T_3D_2	T_3D_3	T_3D_4	PSE[1]	*P*-value	Adjust *P*-value
Moisture	75.65[b]	76.32[ab]	77.88[a]	75.56[b]	0.2029	0.0054	0.0432
Protein	75.08	76.70	76.98	75.24	0.2774	0.0675	0.1800
Lipid	6.37[a]	5.04[a]	2.68[b]	5.31[a]	0.2479	0.0015	0.0180
Alanine	4.27	4.21	4.21	4.36	0.0416	0.5419	0.5612
Arginine	5.45	5.89	5.67	5.31	0.0543	0.0126	0.0756
Aspartic Acid	6.76	6.94	7.01	6.96	0.0440	0.2679	0.4559
Cysteine	0.60	0.63	0.64	0.61	0.0045	0.0387	0.1327
Glutamic Acid	10.18	10.51	10.47	10.40	0.0708	0.3940	0.4728
Glycine	5.01[b]	5.41[ab]	5.90[a]	5.07[b]	0.0962	0.0246	0.0984
Histidine	1.49	1.56	1.58	1.54	0.0156	0.2442	0.4508
Hydroxylysine	0.14	0.17	0.16	0.14	0.0067	0.2203	0.4406
Hydroxyproline	0.20	0.22	0.22	0.21	0.0034	0.3229	0.4559
Isoleucine	2.95	3.00	2.95	3.01	0.0150	0.3048	0.4559
Leucine	4.95	5.08	5.00	5.03	0.0264	0.3920	0.4728
Lysine	4.92	5.20	5.13	5.10	0.0281	0.0234	0.0984
Methionine	1.46[b]	1.58[a]	1.53[a]	1.53[a]	0.0071	0.0007	0.0168
Phenylalanine	3.16	3.28	3.26	3.19	0.0314	0.5019	0.5551
Proline	4.09	4.14	3.79	4.11	0.0750	0.3737	0.4728
Serine	2.35	2.42	2.47	2.38	0.0286	0.5088	0.5551
Threonine	2.62	2.71	2.73	2.71	0.0136	0.0453	0.1359
Tryptophan	0.87	0.88	0.88	0.89	0.0046	0.5612	0.5612
Tyrosine	2.51	2.59	2.59	2.36	0.0459	0.3004	0.4559
Valine	4.10	4.21	4.36	4.23	0.0322	0.0935	0.2040
Total	68.05	70.61	70.52	69.08	0.3708	0.0884	0.2040

[1] Pooled standard error.

[2] Body samples were analyzed at University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory (Columbia, MO, USA).

Values within a row with different superscripts are significantly different based on Tukey's multiple range test.

Table 6e Protein[2] and amino acid[3] retention efficiency of Pacific white shrimp at the conclusion of a 6-week growth trial in which shrimp were offered diets formulated to partially replace soybean meal on a digestible protein basis with three different batches of Ulva meal (Trial 3).

Retention	T_3D_1	T_3D_2	T_3D_3	T_3D_4	*P*-value	PSE[1]	Adjust *P*-value
Protein	34.2^{a}	29.4^{ab}	14.4^{c}	26.0^{b}	0.8054	<0.0001	<0.0001
Alanine	37.5^{a}	28.5^{a}	13.6^{b}	27.3^{a}	0.6848	<0.0001	<0.0001
Arginine	41.5^{a}	38.8^{b}	18.2^{c}	32.5^{b}	1.1379	<0.0001	<0.0001
Aspartic Acid	32.6^{a}	28.0^{a}	13.9^{b}	24.9^{a}	0.7763	<0.0001	<0.0001
Cysteine	20.5^{a}	18.9^{ab}	9.6^{c}	16.2^{b}	0.5597	<0.0001	0.0001
Glutamic Acid	26.7^{a}	24.2^{a}	12.4^{b}	22.6^{a}	0.6799	<0.0001	<0.0001
Glycine	53.4^{a}	47.9^{a}	25.0^{b}	41.3^{a}	1.5060	<0.0001	0.0002
Histidine	28.6^{a}	26.9^{a}	13.3^{b}	26.2^{a}	0.8115	<0.0001	0.0001
Hydroxylysine	29.3^{ab}	25.7^{b}	11.2^{b}	44.7^{a}	2.1886	0.0015	0.0015
Hydroxyproline	16.9^{a}	13.4^{b}	7.6^{c}	11.3^{b}	0.3435	<0.0001	<0.0001
Isoleucine	30.5^{a}	26.1^{a}	12.9^{b}	24.8^{a}	0.6891	<0.0001	<0.0001
Leucine	25.0^{a}	21.6^{a}	10.7^{b}	20.6^{a}	0.5786	<0.0001	<0.0001
Lysine	40.7^{a}	38.1^{a}	18.0^{b}	33.9^{a}	0.9340	<0.0001	<0.0001
Methionine	38.1^{a}	38.1^{a}	17.3^{b}	32.2^{a}	0.9552	<0.0001	<0.0001
Phenylalanine	28.3^{a}	24.3^{a}	11.9^{b}	22.7^{a}	0.7167	<0.0001	<0.0001
Proline	32.4^{a}	28.8^{a}	13.2^{b}	25.5^{a}	0.8271	<0.0001	<0.0001
Serine	26.3^{a}	22.2^{ab}	11.0^{c}	20.3^{b}	0.6670	<0.0001	<0.0001
Threonine	33.6^{a}	28.1^{ab}	13.7^{c}	25.5^{b}	0.7495	<0.0001	<0.0001
Tryptophan	30.5^{a}	27.2^{a}	14.5^{b}	27.3^{a}	0.7670	<0.0001	<0.0001
Tyrosine	31.2^{a}	27.7^{a}	13.5^{b}	23.8^{a}	0.9244	0.0001	0.0001
Valine	39.4^{a}	32.0^{ab}	16.4^{c}	29.7^{b}	0.9798	<0.0001	<0.0001
Total	32.3^{a}	28.4^{ab}	14.1^{c}	25.8^{b}	0.7669	<0.0001	<0.0001

[1] Pooled standard error.

[2] Protein retention efficiency = (Final weight × Final protein content) - (Initial weight × Initial protein content) × 100 / Protein offered.

[3] Amino acids retention efficiency = (Final weight × Final amino acids content) - (Initial weight × Initial amino acids content) × 100 / Amino acids offered.

Values within a row with different superscripts are significantly different based on Tukey's multiple range test.

Table 7a Formulation of test diets designed to evaluate Ulva meal 4 as a protein source in trial 4.

Ingredient (% as is)	T_4D_1	T_4D_2	T_4D_3	T_4D_4	T_4D_5
Fish meal[1]	6.00	6.00	6.00	3.00	0.00
Soybean meal[2]	53.00	43.00	33.00	53.00	53.00
Corn protein concentrate[3]	8.00	8.00	8.00	8.00	8.00
Ulva meal 4[11]	0.00	12.00	24.00	4.75	9.50
Fish oil[2]	5.92	6.05	6.18	6.19	6.45
Trace mineral premix[5]	0.50	0.50	0.50	0.50	0.50
Vitamin premix[6]	1.80	1.80	1.80	1.80	1.80
Choline chloride[4]	0.20	0.20	0.20	0.20	0.20
Stay C[7]	0.10	0.10	0.10	0.10	0.10
Mono-dicalcium phosphate[8]	2.50	2.60	2.60	2.90	3.10
Lecithin[9]	1.00	1.00	1.00	1.00	1.00
Cholesterol[4]	0.08	0.08	0.08	0.08	0.08
Lyisine [10]	0.00	0.11	0.22	0.07	0.13
Methionine[10]	0.05	0.11	0.17	0.10	0.15
Corn starch[4]	20.85	18.45	16.15	18.31	15.99

[1] Omega Protein Inc., Huston TX, USA.
[2] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[3] Empyreal® 75, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA.
[4] MP Biomedicals Inc., Solon, OH, USA.
[5] Trace mineral premix (g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[6] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] J. T. Baker®, Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
[9] The Solae Company, St. Louis, MO, USA.
[10] Fourth batch Ulva meal experimentally produced.
[11] Aldrich-Sigma, St. Louis, MO, USA.

Table 7b Proximate composition[1], mineral composition[2] and amino acid profile[1] of the test diets used in trial 4.

Composition	T_4D_1	T_4D_2	T_4D_3	T_4D_4	T_4D_5
Proximate composition (% as is)					
Crude protein	35.70	35.20	35.00	34.30	33.40
Moisture	8.70	9.93	10.2	9.89	10.22
Crude fat	6.71	8.37	8.65	8.03	8.21
Crude fiber	3.10	7.30	6.40	5.80	8.40
Ash	7.08	7.67	9.19	7.22	7.24
Quantity elements (% as is)					
Sulfur	0.40	0.74	1.19	0.56	0.72
Phosphorus	1.36	1.03	1.08	1.09	1.10
Potassium	1.33	1.14	1.20	1.24	1.35
Magnesium	0.18	0.40	0.66	0.29	0.40
Calcium	1.31	1.27	1.36	1.32	1.36
Sodium	0.10	0.23	0.40	0.13	0.17
Trace elements (mg kg^{-1})					
Iron	149	165	193	125	136
Manganese	40.1	50.9	54.9	54.4	59.9
Copper	16.8	16.7	15.2	16.3	16.1
Zinc	183	173	266	292	212
Amino acid profile (% as is)					
Alanine	1.87	2.03	1.85	1.86	1.88
Arginine	2.18	2.01	1.67	2.08	2.07
Aspartic Acid	3.44	3.29	2.82	3.30	3.35
Cysteine	0.48	0.45	0.39	0.45	0.47
Glutamic Acid	6.33	5.91	4.71	6.06	6.06
Glycine	1.56	1.56	1.42	1.46	1.39
Histidine	0.86	0.78	0.62	0.79	0.78
Hydroxylysine	0.08	0.07	0.08	0.05	0.05
Hydroxyproline	0.20	0.09	0.10	0.06	0.03
Isoleucine	1.60	1.53	1.27	1.51	1.51
Leucine	3.28	3.24	2.67	3.16	3.17
Lysine	2.01	2.02	1.78	2.01	2.00
Methionine	0.64	0.73	0.67	0.66	0.69

Phenylalanine	1.85	1.79	1.51	1.76	1.79
Proline	2.13	1.97	1.62	1.93	1.92
Serine	1.48	1.51	1.30	1.52	1.56
Threonine	1.29	1.31	1.16	1.28	1.29
Tryptophan	0.47	0.40	0.38	0.43	0.44
Tyrosine	1.33	1.25	1.02	1.27	1.25
Valine	1.73	1.81	1.54	1.74	1.72

[1] Proximate composition and mineral composition was tested at Midwest Laboratories (Omaha, NE, USA).
[2] Mineral composition was tested at Midwest Laboratories (Omaha, NE, USA).

Table 7c Performance of juvenile Pacific white shrimp *L. vannamei* (Initial weight 0.15g) offered diets formulated to evaluate Ulva meal 4 as a replacement for soybean meal and fish meal on an iso-nitrogen basis in juvenile shrimp over six weeks (Trial 4).

Diet	Ulva Levels (%)	Final biomass (g)	Final mean weight (g)	WG[3] (%)	FCR[2]	Survival (%)	PRE[4] (%)
T_4D_1	0	42.68^{a}	4.74^{a}	3160.39^{a}	1.72^{c}	90.0^{ab}	30.50^{a}
T_4D_2	12	27.85^{bc}	3.41^{bc}	2057.91^{bc}	2.52^{b}	82.5^{ab}	20.68^{bc}
T_4D_3	24	20.08^{d}	2.72^{c}	1718.67^{c}	3.26^{a}	74.0^{ab}	15.91^{c}
T_4D_4	4.75	34.60^{b}	3.69^{b}	2335.98^{b}	2.23^{bc}	94.0^{a}	25.11^{ab}
T_4D_5	9.5	24.84^{cd}	3.52^{bc}	2254.04^{bc}	2.51^{b}	72.0^{b}	22.31^{b}
PSE[1]		0.9041	0.1135	76.79	0.0826	2.4965	<0.0001
P-value		<0.0001	<0.0001	<0.0001	<0.0001	0.0107	0.7882

[1] Pooled standard error.
[2] FCR: Feed conversion ratio = Feed offered / (Final weight - Initial weight).
[3] WG: Weight gain = (Final weight - Initial weight) / Initial weight × 100%.
[4] PRE: Protein retention efficiency = (Final weight × Final protein content) - (Initial weight × Initial protein content) × 100 / Protein offered.
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 7d Proximate[2] composition of shrimp at the conclusion of a 6-week growth trial in which shrimp were offered diets formulated to evaluate Ulva meal 4 as a replacement for soybean meal and fish meal on an iso-nitrogen basis in juvenile shrimp (Trial 3).

Diet	Moisture (%)	Crude protein (%)	Crude lipid (%)	Crude fiber (%)	Ash (%)
T_4D_1	76.1[b]	70.83[b]	8.40[a]	5.25	11.50[c]
T_4D_2	77.9[a]	73.02[ab]	5.07[bc]	4.98	12.69[bc]
T_4D_3	78.2[a]	73.76[a]	3.65[c]	5.57	14.26[a]
T_4D_4	76.1[b]	70.95[b]	6.90[ab]	5.34	12.11[bc]
T_4D_5	76.9[ab]	71.73[ab]	6.17[b]	5.45	12.94[ab]
P-value	0.0006	0.0027	<0.0001	0.2712	0.0001
PSE[1]	0.1937	0.3056	0.2541	0.0976	0.1669

[1] Pooled standard error.
[2] Body samples were analyzed at University of Missouri-Columbia, Agriculture Experiment Station Chemical Laboratory (Columbia, MO, USA).
Values within a column with different superscripts are significantly different based on Tukey's multiple range test.

Table 8a Composition of reference diet for the determination of digestibility coefficients of Ulva meal 1 and 2.

Ingredients	% as is
Soybean meal[1]	10.00
Fish meal[2]	32.50
Fish oil[2]	3.20
Whole wheat[3]	47.60
Trace mineral premix[4]	0.50
Vitamin premix[5]	1.80
Choline cloride[6]	0.20
Stay C[7]	0.10
Corn starch[3]	1.00
Lecethin[8]	1.00
Chromic oxide[9]	1.00

[1] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.
[2] Omega Protein Inc., Houston TX, USA.
[3] MP Biomedicals Inc., Solon, OH, USA
[4] Trace mineral premix(g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.550; Ferrous sulfate, 2.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 69.664.
[5] Vitamin premix (g/kg premix): Thiamin.HCL, 4.95; Riboflavin, 3.83; Pyridoxine.HCL, 4.00; Ca-Pantothenate, 10.00; Nicotinic acid, 10.00; Biotin, 0.50; folic acid, 4.00; Cyanocobalamin, 0.05; Inositol, 25.00; Vitamin A acetate (500,000 IU/g), 0.32; Vitamin D3 (1,000,000 IU/g), 80.00; Menadione, 0.50; Alpha-cellulose, 856.81.
[6] VWR, Radnor, PA, USA.
[7] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), DSM Nutritional Products., Parsippany, NJ, USA.
[8] The Solae Company, St. Louis, MO, USA.
[9] Alfa Aesar, Haverhill, MA, USA

Table 8b Apparent dry matter (ADM), apparent energy (AED) and apparent protein (APD) digestibility values for the diet (D) and ingredient (I) using 70:30 replacement technique offered to Pacific white shrimp (*L. vannamei*)

Means	ADMD	AEDD	APDD	ADMI	AEDI	APDI
Basal diet 1	76.38 ± 0.37^{a}	82.65 ± 1.20^{a}	92.08 ± 0.55^{a}			
Soybean meal	77.02 ± 0.87^{a}	82.63 ± 1.05^{ab}	94.76 ± 0.49^{a}	78.51 ± 2.89^{a}	82.56 ± 3.79^{a}	97.03 ± 0.83^{a}
Fish meal 1	68.21 ± 3.80^{b}	78.31 ± 3.21^{bc}	80.86 ± 1.80^{b}	49.15 ± 12.67^{b}	69.77 ± 9.51^{a}	67.07 ± 4.02^{b}
Ulva meal 1	62.19 ± 1.26^{c}	71.96 ± 0.89^{d}	75.14 ± 1.19^{d}	29.10 ± 4.19^{c}	40.39 ± 3.52^{b}	15.17 ± 5.41^{d}
Basal diet 2	75.69 ± 0.52^{a}	81.51 ± 0.41^{ab}	92.04 ± 0.03^{a}			
Fish meal 2	67.99 ± 0.17^{b}	76.44 ± 0.78^{c}	82.34 ± 0.31^{b}	49.45 ± 0.56^{b}	65.78 ± 2.23^{a}	71.30 ± 0.68^{b}
Ulva meal 2	64.63 ± 1.08^{bc}	69.99 ± 0.64^{d}	78.33 ± 0.42^{c}	38.26 ± 3.61^{bc}	19.11 ± 3.33^{c}	43.51 ± 1.49^{c}

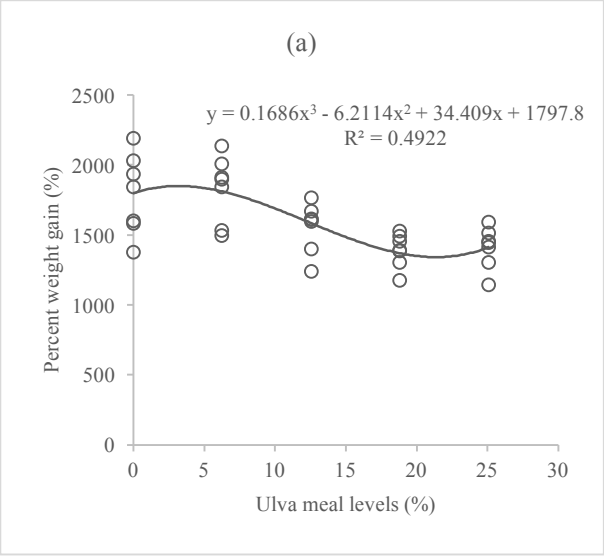
Ulva meal 1 and Ulva meal 2 represent first and second batch Ulva meal.
Fish meal 1 and Fish meal 2 represent the first and second collection of fish meal diet, respectively.
Basal diet 1 and Basal diet 2 represent the first and second collection of basal diet, respectively.
Values are presented as mean ± standard deviation.
Values within a column with different superscripts are significantly different on Tukey's multiple range test.

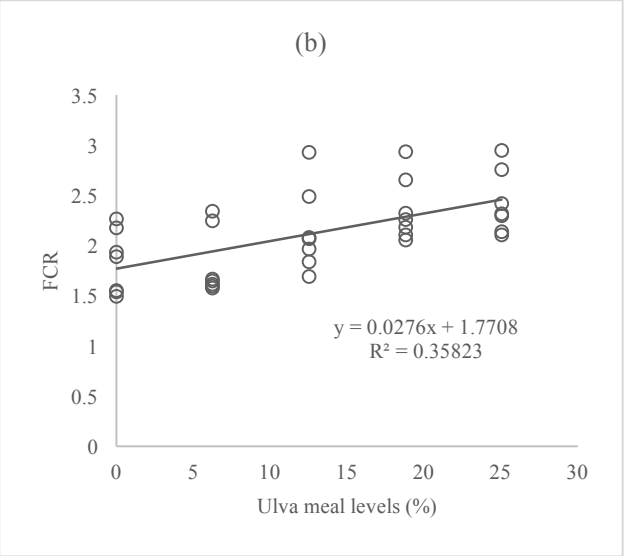
Table 8c Apparent amino acids (AA) digestibility value for the soybean meal (SBM), fish meal (FM), Ulva meal 1 (UM1) and Ulva meal 2 (UM2) using 70:30 replacement technique offered to Pacific white shrimp (*L. vannamei*)

AA	SBM	FM	UM1	UM2
Alanine	93.75 ± 2.02^{a}	69.09 ± 4.09^{b}	36.90 ± 6.56^{d}	50.77 ± 1.10^{c}
Arginine	96.91 ± 1.44^{a}	75.35 ± 3.78^{b}	42.20 ± 6.60^{c}	47.21 ± 0.77^{c}
Aspartic Acid	95.39 ± 1.36^{a}	69.23 ± 3.70^{b}	35.87 ± 5.69^{c}	38.09 ± 1.70^{c}
Cysteine	91.29 ± 1.68^{a}	54.39 ± 7.06^{b}	13.44 ± 10.85^{c}	6.66 ± 7.45^{c}
Glutamic Acid	95.69 ± 1.52^{a}	70.84 ± 3.70^{b}	33.85 ± 7.24^{c}	23.25 ± 3.17^{c}
Glycine	95.06 ± 2.05^{a}	66.55 ± 6.26^{b}	29.84 ± 8.78^{c}	34.04 ± 4.96^{c}
Histidine	94.33 ± 1.69^{a}	74.26 ± 2.86^{b}	7.10 ± 1.87^{d}	43.52 ± 0.22^{c}
Isoleucine	93.23 ± 1.72^{a}	68.72 ± 3.99^{b}	39.15 ± 5.74^{c}	46.33 ± 0.79^{c}
Leucine	92.23 ± 1.96^{a}	71.29 ± 3.16^{b}	34.65 ± 8.50^{d}	50.43 ± 0.80^{c}
Lysine	95.03 ± 1.84^{a}	76.97 ± 2.24^{b}	40.65 ± 6.50^{c}	38.07 ± 3.04^{c}
Methionine	95.20 ± 1.54^{a}	70.63 ± 3.30^{b}	44.13 ± 5.12^{c}	40.89 ± 3.18^{c}
Phenylalanine	93.41 ± 1.90^{a}	65.28 ± 4.13^{b}	27.23 ± 7.02^{d}	47.25 ± 0.76^{c}
Proline	94.68 ± 1.92^{a}	67.21 ± 5.39^{b}	15.81 ± 10.45^{c}	18.20 ± 2.42^{c}
Serine	93.11 ± 1.91^{a}	58.31 ± 4.65^{b}	10.76 ± 11.00^{c}	43.41 ± 0.82^{b}
Threonine	91.99 ± 1.94^{a}	66.33 ± 3.35^{b}	32.83 ± 6.84^{c}	42.57 ± 0.26^{c}
Tryptophan	95.37 ± 1.92^{a}	80.31 ± 1.53^{b}	65.58 ± 2.46^{c}	70.84 ± 3.26^{c}
Tyrosine	95.28 ± 1.22^{a}	73.62 ± 3.40^{b}	36.51 ± 4.10^{d}	59.02 ± 0.45^{c}
Valine	90.78 ± 2.39^{a}	67.06 ± 3.75^{b}	29.94 ± 6.89^{d}	54.20 ± 0.42^{c}
Total AA	94.31 ± 1.67^{a}	69.91 ± 3.89^{b}	29.80 ± 6.68^{d}	41.67 ± 0.51^{c}

Values are presented as mean ± standard deviation.
Values within a row with different superscripts are significantly different on Tukey's multiple range test.

Figure 1 (a) In trial 1, relationship between weight gain (y) of shrimp and incorporation levels of Ulva meal 1 levels (x) in the diets. The regression line is described by $y = 0.1686x^3 - 6.2114x^2 + 34.409x + 1797.8$ ($R^2 = 0.4922$, $P < 0.0001$). (b) In trial 1, relationship between FCR (y) and supplemental Ulva meal levels (x) in the diets. The regression line is described by $y = 0.0276x + 1.7708$ ($R^2 = 0.3582$, $P = 0.0001$). (c) In trial 1, relationship between lipid content (y) of shrimp body and supplemental Ulva meal levels (x) in the diets. The regression line is described by $y = -0.0962x + 7.4$ ($R^2 = 0.3503$, $P = 0.0002$).





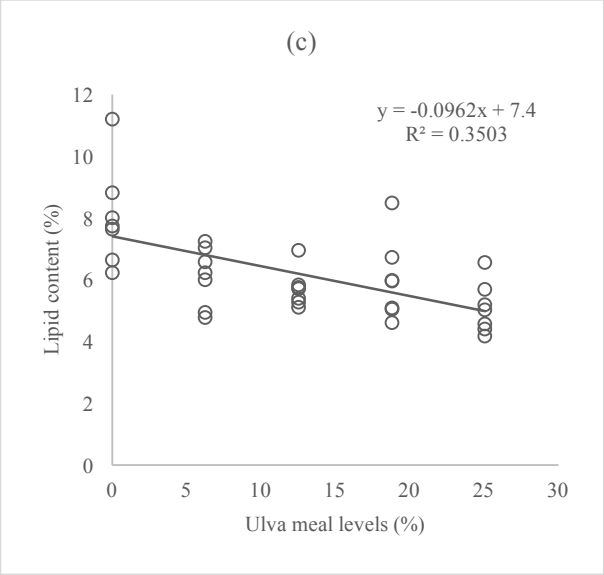


Figure 2 (a) In trial 2, relationship between weight gain (y) and Ulva meal 2 levels (x) in the diets. The regression line is described by $y = 1.1925x^2 - 62.699x + 1713.1$ ($R^2 = 0.6815$, $P < 0.0001$). (b) In trial 2, relationship between FCR (y) and supplemental Ulva meal 2 levels (x) in the diets. The regression line is described by $y = -0.0703x + 1.5451$ ($R^2 = 0.4766$, $P < 0.0001$). (c) In trial 2, relationship between survival (y) and supplemental Ulva meal 2 levels (x) in the diets. The regression line is described by $y = -1.1607x + 100.27$ ($R^2 = 0.6113$, $P < 0.0001$). (d) In trial 2, relationship between lipid content (y) of shrimp body and supplemental Ulva meal 2 levels (x) in the diets. The regression line is described by $y = 0.0078x^2 - 0.4095x + 7.8033$ ($R^2 = 0.9051$, $P < 0.0001$).

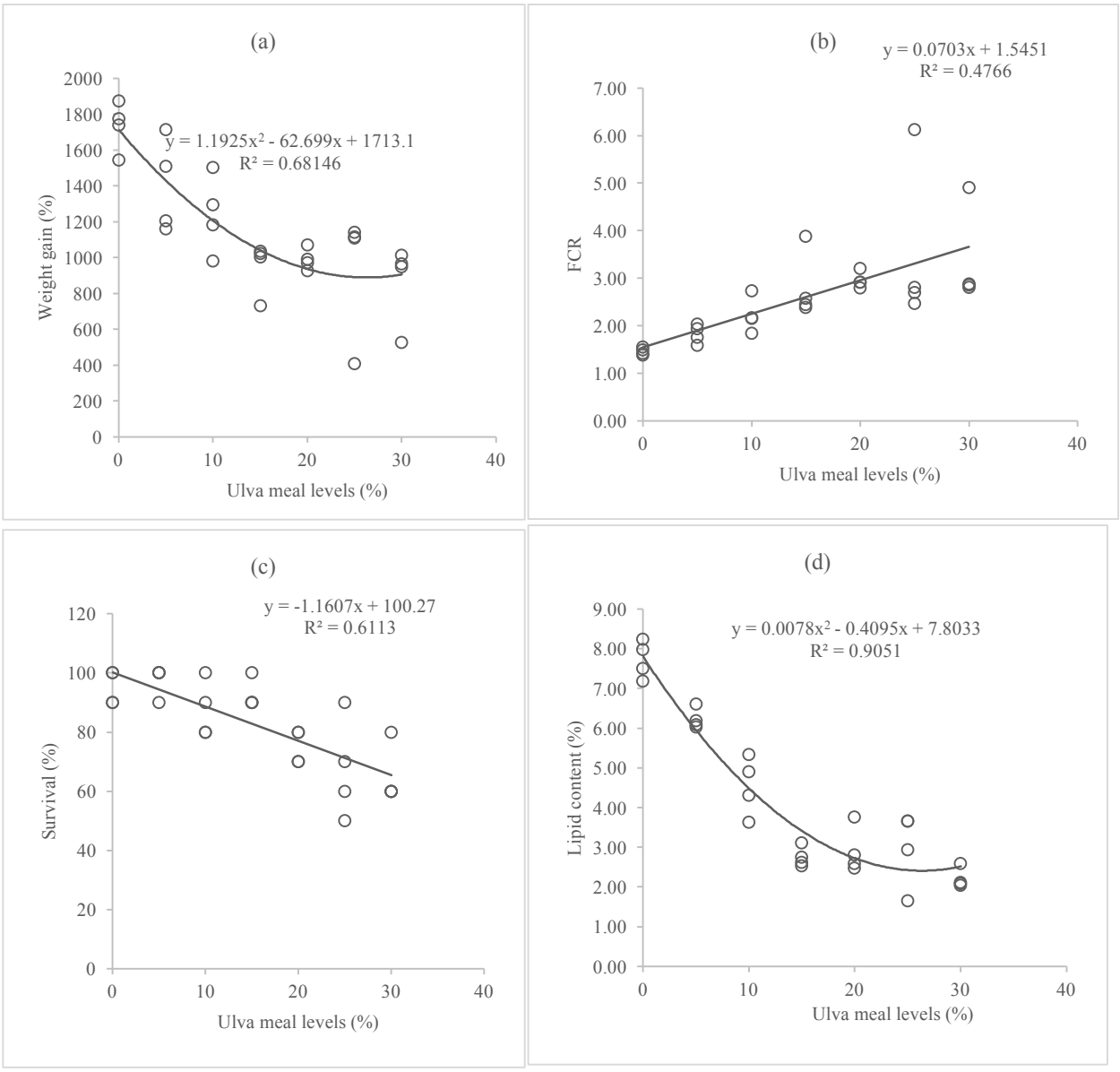


Figure 3 (a) In trial 4, relationship between weight gain (y) of shrimp and incorporation levels of Ulva meal 4 levels (x) in the diets. The regression line is described by $y = 2.6848x^2 - 119.17x + 3049.3$ ($R^2 = 0.6934$, $P < 0.0001$). (b) In trial 4, relationship between FCR (y) and supplemental Ulva meal 4 levels (x) in the diets. The regression line is described by $y = 0.06x + 1.8532$ ($R^2 = 0.721$, $P < 0.0001$). (c) In trial 4, relationship between lipid content (y) of shrimp body and supplemental Ulva meal 4 levels (x) in the diets. The regression line is described by $y = -0.1903x + 7.9555$ ($R^2 = 0.7224$, $P < 0.0001$).