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Research Article

Dietary inclusion of *Schizochytrium limacinum* meal can maintain key productive parameters of pink cusk-eel (*Genypterus blacodes*) juveniles with a reduction in fish oil

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ABSTRACT. The pink cusk-eel (*Genypterus blacodes*) is a promising new candidate species to diversify cold-water aquaculture further. However, there are several knowledge gaps to establish a commercial-scale cultivation strategy, with optimum feed formulation being one of the main remaining challenges. This study aimed to assess the dietary inclusion of *Schizochytrium limacinum* heterotrophic microalgae meal as a sustainable source of docosahexaenoic acid on fish growth and the condition status of pink cusk-eel juveniles. Three extruded diets were formulated to contain 0, 5 and 10% of dried algae and fed to duplicate groups of fish twice daily until apparent satiation during the 12-week trial. On termination of the feeding trial period, the average live weight gain did not show statistically significant differences between the three groups of juvenile fish, achieving an increase in weight of about two times at the end of the trial. Similarly, the protein efficiency and feed conversion ratios did not show significant differences among the experimental diets. On the contrary, a significant reduction in feed intake was observed as the dietary inclusion of microalgae meal increased. These results indicate that *S. limacinum* meal is a sustainable alternative ingredient for the dietary substitution of fish oil in commercial feed for *G. blacodes* at limited inclusion.

Keywords: *Genypterus blacodes*; *Schizochytrium*; microalgae; growth performance; feed efficiency; aquaculture

INTRODUCTION

Pink cusk-eel (*Genypterus blacodes*) is a demersal cold water carnivorous marine fish that inhabits depths between 100 and 800 m associated with rocky substrates (Tascheri et al. 2003, Paredes & Bravo 2005) and preys on pelagic organisms such as fish, cephalopods, and small crustaceans (Tascheri et al. 2003, Nyegaard et al. 2004, Dunn et al. 2010). Good growth performance rates for *G. chilensis* juveniles in captivity (specific growth rate (SGR) mean values of 0.77 after five months feeding of commercial pellets; Vega et al. 2015), as well as for wild *Genypterus* species (Horn 1993, Wiff et al. 2007), tolerance for high stocking density (Vega et al. 2018) and resistance to stress and disease (Uribe et al. *unpubl. data*) make the *Genypterus* species promising candidates for diversification of aquaculture in the South Pacific. Currently, studies on *G. blacodes* are focused on solving scientific and technological challenges to scale up the sustainable production of this species. However, little is known regarding the nutritional requirements and feed ingredients, which has been recognized as one of the main critical limitations to be overcome for aquafeed

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development (Salinas et al. 2020). Traditionally, marine fish diets have been formulated to contain fish oil as the most important source of dietary n-3 long-chain polyunsaturated fatty acids (LC-PUFAs), comprising between 5 and 20% of the total ingredients (Tacon & Metian 2008, Tocher 2015). Feeding n-3 LC-PUFA in particular eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) have been shown to improve productivity along with enhancing fish health (Glencross 2009, Trushenski et al. 2012). Horn et al. (2019) reported the effects of both DHA and EPA as modulating influences on key regulatory genes associated with pro-inflammatory and anti-inflammatory pathways in salmon with effects measured on associated gene expression.

The global production of fish oil has already reached its limit of sustainability, jeopardizing the further expansion of aquaculture activity (Shepherd & Bachis 2014). Thereby, the dietary substitution of fish oil with alternative sources of LC-PUFAs such as single-cell microalgae is being investigated with promising results (Shah et al. 2018, Glencross et al. 2020). *Schizochytrium limacinum* is a heterotrophic microalgae commercially available and sustainably produced that can produce high levels of lipids rich in DHA (Barclay et al. 2010). This microalga has been assessed successfully as a replacement for fish oil in aquaculture feeds of Atlantic salmon (*Salmo salar*) (Miller et al. 2007, Kousoulaki et al. 2015, 2020, Sprague et al. 2015), rainbow trout (*Oncorhynchus mykiss*) (Lyons et al. 2017, Bélanger-Lamonde et al. 2018), olive flounder (*Paralichthys olivaceus*) (Qiao et al. 2014), longfin yellowtail (*Seriola rivoliana*) (Kissinger et al. 2016), red seabream (*Pagrus major*) (Seong et al. 2019), giant grouper (*Epinephelus lanceolatus*) (García-Ortega et al. 2016), red drum (*Sciaenops ocellatus*) (Perez-Velazquez et al. 2018), tilapia (*Oreochromis niloticus*) (Sarker et al. 2016), and channel catfish (*Ictalurus punctatus*) (Li et al. 2009). Incorporation between 5 and 10% of *S. limacinum* meal into diets for several marine species has been recorded as the optimal level of inclusion in terms of growth rates, digestibility, and flesh quality (Qiao et al. 2014, García-Ortega et al. 2016, Kissinger et al. 2016, Seong et al. 2019). Nevertheless, cell-wall hardness and high levels of tripalmitin of this microalgae meal could affect nutrient bioavailability (Bogevik et al. 2018, Sevgili et al. 2019) and therefore impair the production performance of finfish (Kousoulaki et al. 2020).

Since the biological effects of *S. limacinum* meal are largely dose-dependent and species-specific, feeding studies to assess the nutritional value of this novel ingredient for *G. blacodes* are required to prepare balanced commercial feed formulations.

The present investigation aimed to evaluate the effects of the dietary inclusion of *S. limacinum* meal as a sustainable source of docosahexaenoic acid on fish growth, feed efficiency, and condition status of *G. blacodes* juveniles.

MATERIALS AND METHODS

Diets

Three isoproteic (54% average protein) and isoenergetic (21.5 MJ kg^{-1} average energy) extruded experimental diets were evaluated: a control diet (S0) and two diets containing 5% (S5) and 10% (S10) of *Schizochytrium limacinum* meal. These diets were formulated to replace 0, 28 and 56% of total dietary fish oil with microalgae using feed formulation software (DAPP N-utrition 2.0, Colon, Argentina).

Commercial *S. limacinum* meal was produced heterotrophically by Alltech Inc. (Nicholasville, KY, USA) in closed photobioreactors and sprayed dried before inclusion within the diets. All ingredients were ground (300 μm sieve), mixed, extruded through a 1.5 mm die, and oil-coated in the Center of Studies in Sciences and Food Technology of the University of Santiago (CECTA, Llanquihue, Chile). Diets were packed and stored at 4°C until used. Feed ingredients and the proximate and fatty acid composition of the experimental diets are shown in Tables 1 and 2, respectively.

Fish, feeding experiment, and sampling

The investigation was approved by the ethics and animal welfare committee of the University of Los Lagos and conducted following laws and regulations controlling experiments with live animals in Chile. Pink cusk-eel (*Genypterus blacodes*) juveniles (mean weight 20 g) were obtained from captive broodstock at the Aquaculture Research Center of Fundación Chile (Puerto Montt, Chile). They were transported to the Experimental Center for Aquaculture and Marine Sciences at the University of Los Lagos (CEACIMA-Metri, Puerto Montt, Chile), where they were randomly distributed (10 fish per tank; initial fish density of 2.5 kg m^{-3}) into six 80 L raceway tanks supplied with seawater (temperature 15.2 ± 1.9°C; salinity 28.7 ± 2.0; dissolved oxygen 7.7 ± 0.4 mg L^{-1} and flow rate 2 L min^{-1}) and acclimated to the laboratory conditions for seven weeks. A 12 h light: 12 h dark photoperiod was established throughout the trial. The experimental diets were tested in duplicate groups and fed by hand to apparent visual satiety twice daily over 12 weeks. Daily feed intake and fish mortality were recorded.

Table 1. Ingredients and proximate composition of experimental diets containing different levels of microalgae (*Schizochytrium limacinum)* as a replacement for fish oil. [a]Lota protein S.A., Talcahuano, Chile. [b]Aker BioMarine, Oslo, Norway. [c]Alltech Inc., Nicholasville, KY, USA. [d]Graneles Chile S.A., Santiago, Chile. [e]BioMar Chile S.A., Puerto Montt, Chile. [f]Montana Chile S.A., Puerto Montt, Chile. [g]Calculated as the remainder 100-[crude protein + crude lipid + ash] (inclusive of fiber).

	Experimental diets		
	S0	S5	S10
Ingredient (%)			
Fishmeal[a]	45.5	45.5	45.5
Krill meal[b]	5.0	5.0	5.0
Whole cell *S. limacinum* meal[c]	0.0	5.0	10.0
Fish oil[a]	9.0	6.5	4.0
Corn gluten meal[d]	11.0	11.0	11.0
Wheat gluten[d]	11.0	11.0	11.0
Rapeseed meal[d]	5.0	2.5	0.0
Wheat flour[d]	10.0	10.0	10.0
Tapioca starch[d]	2.0	2.0	2.0
Vitamin and mineral premix[e]	0.5	0.5	0.5
Calcium phosphate[f]	1.0	1.0	1.0
Chemical composition (%)			
Dry matter	93.96	95.72	94.92
Crude protein	54.50	54.63	54.85
Crude lipid	14.40	17.08	16.11
Ash	10.76	8.68	11.03
Carbohydrates[g]	14.29	15.34	12.93
Gross energy (kJ kg^{-1})	20.94	22.22	21.47

All fish were individually weighed (scale Excell model ESW-6, readability of 0.01 g), and total length (ichthyometer, 0.1 cm precision) was recorded at the beginning and the end of the experiment in order to estimate weight gain (WG), SGR, protein efficiency ratio (PER) and feed conversion ratio (FCR). Before samplings, fish were fasted for a day and anesthetized with a non-lethal dose of benzocaine (1.5 mL BZ-20 diluted in 10 L; BZ-20 from Veterquímica Laboratories, Santiago, Chile).

Calculations

Growth performance and nutrient utilization were estimated using the following equation: weight gain, WG (g) = (final mean wet weight (FW) (g) - initial mean wet weight (IW) (g)). Feed conversion ratio, FCR = dry feed intake (g) / WG (g). Protein efficiency ratio, PER (g/g) = WG (g) / protein intake (g). Specific growth rate, SGR (% body weight d^{-1}) = [(ln FW (g) - ln IW (g)) / time (d)] × 100. Survival rate, SR (%) = (number of fish in each group remaining / initial number of fish) × 100. Condition factor, K = (fish weight (g) / fish total length^3 (cm)) × 100.

Table 2. Fatty acid composition of the experimental diets containing different levels of microalgae (*Schizochytrium limacinum)* as a replacement for fish oil. [a]Includes unlisted fatty acids: SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Fatty acids	Experimental diets		
(mg 100 g^{-1} of the sample)	S0	S5	S10
C14:0	833	962	977
C16:0	2733	4808	6507
C18:0	460	466	443
C20:0	44	39	33
Total SAFA[a]	4199	6552	8357
C16:1n-9	910	868	697
18:1n-9	4928	3870	2328
18:1n-11	603	537	403
20:1n-9	55	47	25
Total MUFA[a]	6916	5686	3709
18:2n-6	2069	1838	1422
18:3n-6	17	18	15
20:4n-6	64	68	60
Total n-6 PUFA[a]	2172	1951	1521
18:3n-3	648	504	302
20:5n-3	1670	1629	1412
22:5n-3	214	200	167
22:6n-3	708	1628	2501
Total n-3 PUFA[a]	3300	4038	4459
Total PUFA[a]	5472	5989	5980
EPA + DHA	2378	3257	3913
n-3:n-6	1.52	2.07	2.93

Chemical analysis

The proximate composition of experimental diets was determined according to the Official Association of Analytical Chemists (AOAC 2005). The moisture and ash were obtained by further drying at 105 and 550°C, respectively. The crude protein content was determined using the Kjeldahl- N method (N × 6.25) and crude fat with the Soxhlet extraction. The crude fiber was analyzed by acid hydrolysis, followed by the alkali extraction method, and carbohydrates were calculated by difference, as usual for proximate analyses. The gross energy was determined using 23.4 kJ g^{-1} crude lipid, 39.8 kJ g^{-1} crude protein, and 17.2 kJ g^{-1} carbohydrates.

The extraction of total lipids from diets was carried out according to Aldai et al. (2012). A sample of 0.5 g was extracted using a chloroform/methanol solution (1:1, v/v). Fatty acid methyl esters were prepared using an acidic (methanolic HCl) and basic (sodium methoxide) reagents and analyzed by gas chromatography equipped with a flame ionization detector (GC-2010 Plus; Shimadzu®, Kyoto, Japan) and fused silica capillary column (SP-2560, 100 m, 0.25 mm i.d.

with 0.2 μm film thickness; Supelco Inc. Bellefonte, PA, USA). Hydrogen was used as carrier gas with a constant flow rate of 1 mL min^{-1}, and the injector and detector temperature were set at 250°C. Fatty acids were identified by reference to external standards (GLC-603, GLC-463, UC-59M, and Nu-Chek. Prep. Inc., Elysian, MN, USA) expressed as mg 100 g^{-1} dry matter.

Statistical analysis

All statistical analyses were performed using the software Statistica (Statsoft Inc., Tulsa, USA). The growth performance and nutrient utilization results were tested for normality and homogeneity by the Shapiro-Wilk's and Bartlett's tests, respectively, before comparing dietary treatments with one-way analysis of variance (ANOVA) at a significance level of 0.05. Tukey's *post-hoc* test was used to identify any differences. Data were presented as the mean ± the standard error of the mean.

RESULTS

All experimental diets have similar contents in most fatty acids (SAFA's, MUFA's, and PUFA's) (Table 2). There was a decrease in oleic acid, linoleic acid, linolenic acid, and EPA as the dietary levels of fish oil were replaced with the algal product.

On the other hand, in the diets with increasing microalgae levels, the content of palmitic acid and DHA were increased markedly. The content of DHA increased from 0.7% (S0) to 2.5% (diet S10).

During the experiment, all experimental diets were well accepted by fish and the survival rate was 97%. The mortality of two fish was recorded during the experimental period, but they were not related to the inclusion level of *S. limacinum* algal meal. The results of growth performance and feed utilization are presented in Table 3.

Feeding *S. limacinum* meal to pink cusk-eel juveniles, regardless of the inclusion levels, showed a growth performance similar to that obtained by the control diet. The final mean body weight varied from 44.49 ± 0.89 g (S10) to 45.83 ± 1.53 g (S0), displaying no significant differences among groups.

The WG of fish ranged from 24.19 ± 0.56 g (S10) to 25.03 ± 1.43 g (S0), representing 119.2 to 120.3% of the initial weight, which was not deemed significantly different. Correspondingly, the increasing level of algal biomass in the feeds resulted in no significant differences in SGR between fish (Table 3).

Feed intake was significantly affected by the increasing dietary microalgae inclusion, decreasing from 26.15 ± 0.20 g $fish^{-1}$ in fish fed the control diet to 23.50 ± 0.25 g $fish^{-1}$ in fish fed diet S10. However, this was not seen to be restrictive to overall growth performance under these experimental conditions. The feed utilization indices, FCR and PER, ranged from 0.97 ± 0.01 (S10) to 1.05 ± 0.05 (S0) and 1.76 ± 0.09 (S0) to 1.88 ± 0.02 (S10), respectively, without significant differences among treatments. In terms of morphometric measurements, K did not change owing to dietary treatments, and this fluctuated from 0.363 ± 0.027 (S10) to 0.390 ± 0.043 (S0) (Table 3).

DISCUSSION

The findings regarding growth performance and feed utilization achieved in the present study have demonstrated the potential use of whole-cell *S. limacinum* meal in extruded diets for pink cusk-eel (*G. blacodes*), displaying better growth rates compared to previously published data from juveniles of *Genypterus chilensis* in culture conditions, which has reported SGR values of 0.77 (Vega et al. 2015). Microalgae biomass, such as *S. limacinum* meal, represent a source of renewable and sustainable lipids that is high in essential fatty acids for marine fish species, particularly DHA. Indeed, this fatty acid is reported to be more essential than EPA in some fish species, improving growth and survival during larval and juvenile development in farm-raised conditions (Glencross 2009, Trushenski et al. 2012), as it plays an important structural and physiological function in cell membranes of nerve and muscle tissues (Sargent et al. 2002) as well as specific immune response mechanisms in different aquatic species as was reported by Horn et al. (2019).

The replacement of fish oil with *S. limacinum* meal exhibited no growth detriment of fish in terms of WG or SGR and agreed with other studies, including similar or highest levels of this ingredient in experimental diets. Kissinger et al. (2016) reported that 5% inclusion of *S. limacinum* meal in low marine ingredients diets for longfin yellowtail (*S. rivoliana*) did not affect growth and feed utilization parameters. Likewise, García-Ortega et al. (2016) found that diets containing up to 15% of *S. limacinum* did not compromise giant grouper (*E. lanceolatus*) performance. In the same way, feeding studies carried out by Qiao et al. (2014) on olive flounder (*P. olivaceus*) have shown that growth rates of fish fed 9% *S. limacinum* were comparable to those of fish fed a fish oil-based diet. More recently, Perez-Velazquez et al. (2018) and Seong et al. (2019) observed that dietary inclusion of 10 and 11% *S. lima-*

Table 3. Growth performance and feed utilization of pink cusk-eel (*Genypterus blacodes*) juveniles fed diets with different levels of inclusion of microalgae meal (*Schizochytrium limacinum*). Values are means ± standard error of the mean of two replicate. Means in the same column with different superscript letters differ significantly ($P < 0.05$). SGR: specific growth rate, FCR: feed conversion ratio, PER: protein efficiency ratio, K: condition factor.

Parameter	Experimental diets			*P*-value
	S0	S5	S10	
Initial weight (g)	20.8 ± 0.10	20.78 ± 0.98	20.30 ± 1.45	0.9270
Final weight (g)	45.83 ± 1.53	45.54 ± 1.24	44.49 ± 0.89	0.7511
Weight Gain (g)	25.03 ± 1.43	24.76 ± 0.26	24.19 ± 0.56	0.8174
Feed intake (g fish^{-1})	26.15 ± 0.20^{a}	24.55 ± 0.55^{ab}	23.50 ± 0.25^{b}	0.0326
SGR	1.08 ± 0.04	1.08 ± 0.03	1.08 ± 0.07	0.9965
FCR	1.05 ± 0.05	0.99 ± 0.03	0.97 ± 0.01	0.3561
PER	1.76 ± 0.09	1.85 ± 0.06	1.88 ± 0.02	0.4511
Initial K	0.381 ± 0.038	0.378 ± 0.043	0.377 ± 0.031	0.9430
Final K	0.390 ± 0.043	0.370 ± 0.030	0.363 ± 0.027	0.0550

cinum could substitute fish oil completely without significant differences in growth of red drum (*S. ocellatus*) and red seabream (*P. major*) respectively. Also, Santigosa et al. (2020) successfully replaced the fish oil in diets for rainbow trout (*O. mykiss*) with up to 10% inclusion with a marine algal oil with no detrimental effects on overall DHA and EPA and associated metabolism and physiological responses. According to these authors, it satisfied the known requirements for optimal fish health for salmonids of around 1.6% of the diet. For the present study, we attained 3.9% of the content as EPA + DHA with the higher algal meal inclusion, thus meeting the likely essential fatty acid requirement for pink cusk-eel, although not yet established for this species from the scientific literature.

It has been conversely reported that high inclusions of *S. limacinum* in diets for salmonids, in general, have been associated with a reduction in the growth rate attributable to energy and nutrient digestibility limitations caused by the thickness of the algal cell wall and the content of saturated fatty acids such as palmitate (C16:0) (Sevgili et al. 2019, Tibbetts et al. 2020). We do, however, see a rise of the saturated fatty acid, palmitic acid (C16:0) from 2.7 in the control fish oil diet (S0) to reach 6.5% in the 10% algal meal inclusion (S10 diet; Table 2), typical of *S. limacinum*, but ultimate effects on fish carcass lipid composition remain to be evaluated for pink cusk-eel.

Use of other microalgae species (*Nannochloropsis* sp., *Phaeodactylum tricornutum*, and *Isochrysis galbana*) as a replacement of fish oil in diets for Atlantic salmon (*S. salar*) and Atlantic cod (*Gadus morhua*) have also resulted in a reduced digestibility. However, appetite remained unaffected (Reitan et al. 2012).

In this regard, *G. blacodes* presents a digestive tract typical of a carnivorous benthonic fish adapted to feeding on fish and crustaceans (Salinas et al. 2020). Therefore the digestibility of vegetable feedstuffs rich in non-starch polysaccharides may be limited, as reported for several carnivorous marine fish species (Nengas et al. 1995, Hansen & Hemre 2013, Bai et al. 2019).

Interestingly, in our study, a decrease in feed intake was observed at dietary concentrations of *S. limacinum* of 10% or 100 g kg^{-1} of diet, but there were no significant differences in FCR and PER among treatments. They are possibly related to palatability issues associated with the presence of anti-nutritional factors in some microalgae meals (Güven et al. 2010, Kim-Hue et al. 2018), which in plant protein feedstuffs are reported to provoke similar responses in rainbow trout (Glencross et al. 2006, Serrano et al. 2012). Walker & Berlinsky (2011) had observed a reduction in feed intake due to poor diet palatability when *Nannochloropsis* sp. and *Isochrysis* sp. were included at levels of 15 and 30% within the diet of *G. morhua*. However, there are no known reports regarding the feeding deterrent effects of *S. limacinum* on other marine fish species (Kissinger et al. 2016, Seong et al. 2019, Salinas et al. 2020) and is worthy of further investigation. It is worthwhile pointing out that all diets employed in this study containing 5% krill meal as feed attractant, considering that *Genypterus* sp. species prey on different species of decapod crustaceans (Dunn et al. 2010).

The condition factor of *G. blacodes* juveniles, on the other hand, was not affected by the presence of microalgae meal in the diet and were comparable to those reported for *G. chilensis* juveniles fed commercial diets (Vega et al. 2015). Similar results were

obtained by Perez-Velazquez et al. (2018), who found no changes in the condition factor of *S. ocellatus* fed increasing levels of *S. limacinum* meal.

In this context, the dietary inclusion of whole-cell *S. limacinum* meal increased the proportion of dietary DHA presented to this species under controlled feeding trial conditions. Although no direct effects on performance were recorded, there will likely be benefits to fish's flesh nutritional quality due to the increased deposition of omega-three lipids such as DHA. Further studies are being conducted to assess this potential in cusk-eel grown to harvestable weight class and to determine the effects of whole-cell *S. limacinum* meal on nutrient digestibility and fatty acid concentrations of muscle tissue in the diet of pink cusk-eel over a more defined range of inclusion level. It is envisaged that a bespoke fish can be produced to meet consumer expectations for high nutritional value and more sustainable practice by reducing marine ingredient dependency in crucial stages of production. To conclude, our results demonstrate that whole-cell *S. limacinum* meal is a promising sustainable ingredient to be included up to 10% as partial replacement of fish oil in diets for pink cusk-eel juveniles without compromising growth performance, body condition, and feed utilization.

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