Partially replacing fish oil with microalgae (Schizochytrium limacinum and Nannochloropsis oceanica) in diets for rainbow trout (Oncorhynchus mykiss) reared in saltwater with reference to growth performance, muscle fatty acid composition and liver ultrastructure

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Microalgae (*Schizochytrium limacinum* and *Nannochloropsis oceanica*) can partially replace fish oil in diets for rainbow trout (*Oncorhynchus mykiss*) reared in saltwater sustaining growth performance, optimum muscle fatty acid composition with enhanced liver ultrastructure.

Running title: Omega-3 rich microalgae meal in diets for trout

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Abstract

The effect of dietary inclusion of a mixture of microalgae meal (*Schizochytrium limacinum* and *Nannochloropsis oceanica*) (1:1 ratio) on growth performance, gene expression, histology and muscle fatty acids composition of rainbow trout (*Oncorhynchus mykiss*) reared in saltwater was investigated. Three experimental extruded diets containing 0%, 9% and 17% of a mixture of microalgae meal were evaluated in triplicate groups during 10-week bioassay. The results showed that growth performance and feed conversion ratio were significantly reduced by

increasing the dietary inclusion of a mixture of microalgae meal. No significant trends were observed with respect to feed intake, and relative gene expression of hsp70, scarb1, il12 and myod. Conversely, increasing dietary levels of a mixture of microalgae meal led to a decrease in vacuolar degeneration of hepatocytes. A reduction of 23% on the amount of 20:5 n-3 fatty acid in the muscle was found between the fish fed control and the highest level of the microalgae meal blend diets. Nevertheless, 22:6 n-3 fatty acid content did not vary among dietary treatments.

These results demonstrate that a mixture of microalgae meal has a potential to be included up to 90 g/kg within diets for rainbow trout as a sustainable replacement of fish oil.

Keywords: *Schizochytrium limacinum*, *Nannochloropsis oceanica*, Rainbow trout, feed performance, n-3 fatty acids

1. INTRODUCTION

The salmon industry is the second largest productive sector in Chile, after copper mining. During 2018, this economic activity accounted for production of around 830.000 tonnes and earned profits of about US $ 5.2 billion. In order to support this production, the salmon farming industry requires high nutritional quality extruded feeds (Glencross, Booth, & Allan, 2007), which represents approximately 50% of the total production cost (Rana, Siriwardena, & Hasan, 2009). The high cost of feeding salmon is attributable to the use of marine ingredients in commercial feeds particularly fish oil (Hardy, 2010; Tacon & Metian, 2008). This industry consumed approximately 0.6 million tonnes of fish oil globally, with an average dietary inclusion of 100 g fish oil per kg diet in commercial feeds for salmon and trout (Shepherd & Bachis, 2014; Ytrestøyl, Aas, & Åsgård, 2015). Traditionally, this ingredient has been used as the main source of highly polyunsaturated fatty acids long-chain omega-3 series (n-3 HUFAs), such as ecoisapentanoic acid (EPA, 20: 5n-3) and docosahexaenoic acid (DHA, 22: 6n-3) (Turchini, Torstensen, & Ng, 2009). Dietary n-3 HUFAs are vital to fish health and also to the nutritional value of the salmon products to consumers (EFSA, 2010; Gebauer, Psota, Harris, & Kris-Etherton, 2006; Jensen et al., 2012).

As worldwide production of fish oil is predicted to remain stable at around 1 million tonnes per annum (Shepherd & Bachis, 2014) and the demand for salmon feed is expected to double to around 6 million tonnes by 2030 (Kobayashi et al., 2015), the development of alternative sources of n3-HUFA is an urgent issue for salmon industry (Turchini et al., 2009). Indeed, various alternative n3-HUFA rich ingredients have been identified as potential replacement of fish oil in salmon feeds, including fish by-products and trimmings, discards and by-catch, krill, mesopelagic fish, genetically modified organisms, and marine microalgae (Miller, Nichols, & Carter, 2008).

Among the above mentioned ingredients, marine microalgae of the genus *Schizochytrium* and *Nannochloropsis* are interesting natural and sustainable ingredients to be used as a source of DHA and EPA for aquafeeds (Shah et al., 2018). The n-3 HUFAs derived from these marine microalgae have been shown to be well accepted and nutritionally valuable feed ingredients for several species. *Schizochytrium* sp meal has been successfully included as a source of DHA in diets for olive flounder (*Paralichthys olivaceus*) (Qiao et al., 2014), longfin yellowtail (*Seriola rivoliana*) (Kissinger, García-Ortega, & Trushenski, 2016), red seabream (*Pagrus major*) (Seong, Matsutani, Haga, Kitagima, & Satoh, 2019), giant grouper (*Epinephelus lanceolatus*) (Garcia-Ortega, Kissinger, & Trushenski, 2016), tilapia (*Oreochromis niloticus*) (Sarker et al., 2016) and channel catfish (*Ictalurus punctatus*) (M. H. Li, Robinson, Tucker, Manning, & Khoo, 2009), without negative effects on growth performance. The optimal level of incorporation of *Schizochytrium* sp in these fish species has not exceeded 50 g kg^{-1}. In Salmonids, investigations regarding the effect of dietary inclusion of *Schizochytrium* sp have demonstrated that it is possible to include between 50 and 100 g kg^{-1} of this ingredient in diets for Atlantic salmon (*Salmo salar*) (Kousoulaki et al., 2020; Kousoulaki et al., 2015; Sprague et al., 2015) and rainbow trout (*Oncorhynchus mykiss*) (Betiku, Barrows, Ross, & Sealey, 2016; Lyons, Turnbull, Dawson, & Crumlish, 2017; Zhang, 2013) without affecting growth rates, digestibility and flesh quality. However, dietary inclusion of *Schizochytrium* sp meal above 100 g kg^{-1} has been reported to lower lipid digestibility, constraining the application of this raw material in salmonid feeds (Kousoulaki et al., 2015; Sprague et al., 2015; Zhang, 2013).

On the other hand, there is limited documentation available about the use of *Nannochloropsis* sp in extruded feed for fish. Nutritional assays conducted with rainbow trout (*O. mykiss*) (Sevgili

et al., 2019) and Atlantic salmon (*S. salar*) (Sørensen et al., 2017) have shown that is possible to include in the feed a maximum of 10% of *Nannochloropsis* sp biomass without negative consequences on growth. Nevertheless, no information about the impact on flesh fatty acid profile has been reported. Several genes are known to be affected by omega-3 and omega-6 levels in the diets of fish and are involved in many important metabolic and physiological processes. Namely, those affecting anti-inflammatory and pro-inflammatory pathways respectively and metabolic roles concerning glucose mobilization and metabolism have been reported by Horn et al. (2019) in an assessment for Atlantic salmon. Their functional roles in muscle cell recruitment and development and lipid storage were also discussed by these researchers. Considering that salmonids are recognized by consumers and the retailers as a good source of n-3 HUFA, it is imperative to understand and evaluate the use of these marine microalgae as feed ingredients in order to assure nutritional quality of fillets in terms of omega-3 balance and in particular deposition of DHA and EPA. It is also imperative to promote good growth performance, immune integrity and to enhance fish welfare by supporting immune competence.

Consequently, the present study was conducted to investigate the effects of dietary substitution of fish oil with a mixture of microalgae meal (*Schizochytrium limacinum* and *Nannochloropsis oceanica*) on growth performance, muscle fatty acid composition, liver and distal intestine histology and the expression of selected genes related to inflammatory and physiological responses of post-smolt rainbow trout (*O. mykiss*) under optimum sea rearing conditions.

2. MATERIALS AND METHODS

2.1. Diets

Three isoproteic, isoenergetic and isolipidic diets containing 0%, 9% and 17% of a mixture of heterotrophic microalgae meal (*S. limacinum* and *N. oceanica*; 1:1 ratio) were formulated to replace dietary n-3 HUFA from fish oil and to meet the minimum requirements of essential amino acids and other nutrients by using a feed formulation software (DAPP N-utrition 2.0, Colon,

Argentina). Ingredients and chemical composition of the feeds are presented in Table 1, while fatty acid composition of feeds are shown in Table 2.

The experimental diets were manufactured by extrusion at the feed technology center of the University of Santiago (Llanquihue, Chile) All ingredients were ground into a fine powder through a 300-µm mesh in a hammer mill and then mixed in a twin shaft paddle mixer. Subsequently, the blend was extruded in a twin screw extruder, dried for about 2 h in a ventilated oven at 60°C to approximately 920 g kg^{-1} dry matter and oil coated in a vacuum coater. The feed pellets (1.5×3.0 mm) were packed and stored at 4°C until used.

2.2. Fish, Experimental Condition and Sample Collection.

The experiment was carried out at the aquaculture research station at the University of Los Lagos (Puerto Montt, Chile). A total of 270 rainbow trout (*O. mykiss*) post-smolt with 0.19 kg initial weight were randomly distributed (30 fish per tank) into nine 500 L circular fibreglass tanks supplied with seawater (15 °C; flow rate 12 L min^{-1}). The experimental diets were tested in triplicate groups and fed by hand, to apparent visual satiety twice a day over a period of 10 weeks. Before the beginning of the experiment, all fish were fed the control diet for 7 days as an acclimation period to adjust to tank conditions. Fish were weighed initially and at the end of the experiment in order to determine weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR).

At the end of the experiment, three fish from each tank (nine per treatment) were randomly sampled and euthanized with a lethal concentration of tricaine methanesulfonate (MS-222) according with the animal welfare protocols approved by the Bioethics Committee at University of Los Lagos. From these fish, dorsal muscle samples were dissected, deboned skinned and stored at -20°C for fatty acid analysis. Additionally, liver and distal intestine tissue samples were dissected and fixed in 10% phosphate-buffered formalin for histological examination. Samples of muscle, liver, head kidney and distal intestine tissues were also collected and immediately placed in 1 mL RNALater for muscle growth, stress, inflammatory response and carotenoid transport gene expression analyses respectively.

2.3. Calculations

Fish growth performance were determined using weight gain (WG) and specific growth rate (SGR). These variables were calculated using the following equation: SGR (% day^{-1}) = 100 x (ln(Wf/Wi)) x d^{-1} and WG = (Wf-Wi), where Wi and Wf are the weights (g) at the initial and the final weights and d the number of days. Feed utilization were evaluated through feed conversion ratio (FCR) and calculated according to the following formula: FCR=FI × WG^{-1}, where FI is consumption of dry matter from feed and WG is the weight gain. Organosomatic index of the liver (HSI) was calculated as the percentages of the tissue weight relative to fish body weight using the following formula: HSI = (LW × BW^{-1}), where LW and BW are weight of liver and weight of body, respectively. Spleen-somatic indices (SSI) were calculated in the same manner.

2.4. Histological analysis

The processing of tissues was performed at the veterinary histology center (VEHICE, Puerto Montt, Chile). Tissues were routinely dehydrated in ethanol, equilibrated in xylene, and embedded in paraffin according to standard histological techniques. Samples were sectioned (4–6 µm thick), stained with haematoxylin and eosin and blindly evaluated by light microscopy (Leica Microsystems model DM750, Leica, Bannockburn, IL, USA).

2.5. Gene expression analysis

Total RNA was extracted from 10 mg of tissue (liver, head kidney, distal intestine and muscle) using Trizol (Invitrogen, Carlsbad, CA, USA) followed by phase separation with chloroform then precipitated with isopropanol. The isolated RNA concentration and purity was assessed by electrophoresis in agarose 1,0% and spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, Waltham, MA, USA) respectively. 1 µg of RNA was treated with DNAse I to avoid possible interference from contaminating DNA, and then reverse transcribed using the SuperScript III reverse transcriptase to synthesize cDNA following manufacturer's instructions. Subsequently, the expression genes related to muscular growth (myoblast determination protein 1, myod), stress (heat shock protein 70, hsp70), inflammatory response (interleukinI 12, il12) and pigment transportation (scavenger receptor class B type 1, scarb1) were studied by quantitative

real time PCR (Stepone Plus, Applied Biosystems, Carlsbad, CA, USA). Specific primer pairs and probe were designed using the Primer3Plus software (Applied Biosystems, Carlsbad, CA, USA) based on the gene sequences for rainbow trout (Table 3). Elongation factor alpha (eEF1α) was used as a housekeeping gene to normalize the expression of the genes studied.

The efficiency of amplification of all genes was evaluated by generating serial dilution curves based on 10, corroborating their efficiency of approximately 100%. Each gene sample was analyzed once per gene and each assay was performed in duplicate.

The PCR conditions for all the genes studied were the followings: 95 °C for 3 min and 30 s, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 ° C for 30 s; and final step 60 °C for 10 s. The 2- △△ Ct method was used to calculate the expression of mRNAs as described by Livak & Schmittgen (2001).

2.6. Chemical Analysis

Proximate composition of the dietary ingredients and experimental diets were performed in duplicate according to the methods of AOAC (2012). Dry matter was calculated by drying at 105°C overnight. Protein was determined by Kjeldahl digestion, based on N × 6.25. Fat was determined using HCl hydrolysis followed by diethylether extraction. Ash was determined by combustion at 550°C for 16 h. Gross energy was measured by bomb calorimetry.

Total lipids from diets and muscle issues was extracted based on the method of Folch, Lees and Stanley (1957). A sample of 0.5 g was homogenized in a chloroform/methanol solution (1:1; v/v). Fatty acids methyl esters (FAME) were prepared using an acidic (methanolic HCl) and basic (sodium methoxide) reagents and analyzed by gas chromatography using a flame ionization detector (GC-2010 Plus; Shimadzu®, Kyoto, Japan) and a fused silica capillary column (SP-2560, 100 m, 0.25 mm i.d. with 0.2-µm film thickness; Supelco Inc Bellefonte, PA, USA). Helium was used as a carrier gas (flow rate of 1 mL min^{-1}) and the injector and detector temperature were set at 250 °C. Fatty acids were identified by comparison with fatty acid standards (Supelco 37 component FAME mix, Supelco, Bellefonte, PA, USA) and expressed as mg 100 g^{-1} dry matter.

2.7. Statistical methods

The results were analyzed using one-way analysis of variance (ANOVA) at a significance level of 0.05, following confirmation of normality and homogeneity of variance. Significant differences among dietary treatments was calculated using Tukey's test.

Statistical analyses were performed using the program Statistica (Statsoft Inc., Tulsa, USA), and data are showed as mean ± standard error of mean.

3. RESULTS

3.1. Fish Growth rate and feeding parameters

Fish survival was not affected by the experimental diets. Growth, assessed as weight gain, final body weight and SGR, was significantly decreased by increasing dietary level of a mixture of microalgae above 90 g kg^{-1}. Similarly, the FCR was also affected by the levels of microalgae in the feed ($P<0.05$), showing an increase trend as high as three-fold in comparison to the control group. By contrast, no differences were found in feed intake and hepatosomatic and spleensomatic indices among the three dietary treatments. The results of growth performance and feed utilization are presented in Table 4.

3.2. Histological examination

Dietary inclusion of a mixture of microalgae did not cause observable histopathological effects on liver or distal intestine. Fish fed microalgae inclusion up to 90 g kg^{-1} showed a slight decrease in vacuolation of hepatocytes, resulting in a lesser degree of abnormal tissue findings (Figure 1). The distal intestine displayed no enteritis or morphological changes in the enterocytes related to the dietary treatments. Similar intestinal fold length was observed in all groups, reaching a maximum average value of 818 µm in the group fed the control diet and a minimum average value of 714 µm in the group fed 90 g kg^{-1} of microalgae meal (Figure 2).

3.3. Gene expression

Relative mRNA abundance of studied genes is presented in the figure 3. The mRNA level of myod in muscle has showed a slight decrease tendency as the dietary inclusion of microalgae increase, although this was no statistically significant. In addition, the expression of hsp70 in the liver, il12 in the head kidney and scarb1 in the distal intestine were similar among all feeding treatments studied ($P>0.05$).

3.4. Muscle fatty acid composition

The fatty acid profiles in muscle of rainbow trout mostly reflects their respective dietary treatment (Table 5). There were no significant differences in total saturated (SAFA), and total polyunsaturated fatty acids (PUFA). However, as the level of microalgae increased, fish fillets exhibited a decrease ($P<0.05$) in total monounsaturated fatty acids (MUFA) due to a slight reduction in the amount of palmitoleic acid (16:1n-7), eicosenoic acid (20:1n-9) and gadoleic acid (20:1n-11).

The total concentration of n-3 PUFA in the muscle showed no significant changes among the respective treatments. Muscle concentration of docosahexaenoic acid (C22:6n-3, DHA) remained steady in all groups. On the contrary, eicosapentaenoic acid (20:5n-3, EPA) is significantly decreased in fish fed the diet containing 170 g kg^{-1} microalgae compared to those fed the other experimental diets. Surprisingly, EPA + DHA levels were no affected by the dietary treatments.

Likewise, the total concentration of n-6 PUFA in the muscle was reduced, but no significant difference was observed among the groups fed experimental diets. The content of linoleic acid (C18:2n-6), eicosadienoic acid (20:2n-6) and di-homo-g-linolenic acid (20:3n-6) decreased in response to the increasing contribution of microalgae lipids to dietary crude fat ($P<0.05$).

As a result of the decrease in the above mentioned n-6 PUFA, the n-3/n-6 ratio in the muscle was significantly increased ($P<0.05$) from 1.6 in the control diet to 1.84 in the diet containing 170 g kg^{-1} microalgae.

4. DISCUSSION

Several species of microalgae meal have been successfully included in diets for marine and freshwater fish species as a promising alternative that can effectively substitute for both fishmeal and fish oil and ensure sustainability standards in aquaculture are met (Shah et al., 2018; Tocher, Betancor, Sprague, Olsen, & Napier, 2019; Turchini et al., 2009). The use of these alternative ingredients sources, however, have shown some issues related to their biochemical and morphological characteristics that can reduce the inclusion in aquafeed formulations (Popper, Ralet, & Domozych, 2014; Rashidi & Trindade, 2018). These can be related to the constraints of the cell wall in algae inhibiting complete digestion, and the often higher level of saturated fatty acids such as palmitic (C16:0) in *Schizochytrium* sp. These algal biomasses can be difficult to include at higher levels in extruded feeds due to mechanical slippage in feed manufacture.

In the current study with rainbow trout, the results provide evidence that the combined inclusion of *S. limacinum* and *N. oceanica* up to 90 g kg^{-1} in feeds is possible without affecting growth performance and feed intake. Similar results were obtained by Qiao et al. (2014) using a mixture of *S. limacinum* and *N. oceanica* meal in olive flounder (*P. olivaceus*) inclusion levels of 227 g kg^{-1}, by Kissinger et al. (2016) using a mixture of *Haematococcus pluvialis* and *S. limacinum* meal at the inclusion levels of 194 g kg^{-1} in diets for longfin yellowtail (*S. rivoliana*) and by Cardinaletti et al. (2018) using a mixture of *Tisochrysis lutea* and *Tetraselmis suecica* at the inclusion levels of 180 g kg^{-1} in diets for European seabass (*Dicentrarchus labrax*). According with these authors, the use of a blend of microalgae allowed a dietary inclusion level higher than the one observed when the two microalgae were used as single ingredients. Nevertheless, in our experiment was noted a tendency towards reduced feed utilization as the level of mixture of microalgae increased in the diet. Such results suggested that marine fish species are able to utilize higher dietary concentration of microalgae compared to salmonids. Therefore, the species and concentrations of microalgae used to elaborate the blend are relevant to obtain optimal nutritional performance of the salmonid diet. Incorporation of 100 g kg^{-1} of *S. limacinum* meal into diets for salmonids

290 has been considered as the maximum level of inclusion in terms of growth and nutrient utilization
291 (Kousoulaki et al., 2020; Kousoulaki et al., 2015). The main constraint identified to further dietary
292 inclusion is the high level of saturated lipid especially palmitic acid, which could affect growth
293 performance, feed conversion ratio and physical feed quality (Kousoulaki et al., 2015; Sprague et
294 al., 2015). Considering that the highest concentration of *S. limacinum* evaluated in this study (85
295 g kg^{-1}) is within the acceptable range to be used as feed ingredient, the poor performance of this
296 dietary treatment could be associated with the presence of *N. oceanica* into the microalgae
297 blend. Indeed, the use of *N. oceanica* over 100 g kg^{-1} within diets for salmonids tend to result in
298 reduced fish growth rate and feed conversion ratio (Sørensen et al., 2017) as a result of the
299 presence of complex indigestible carbohydrates in the microalga cell wall which decrease the
300 digestibility of protein and lipids (Gong, Guterres, Huntley, Sørensen, & Kiron, 2018). Reported
301 digestibility values of *N. oceanica* are lower compared to others species of microalgae when fed
302 to salmonids (Sevgili et al., 2019). More recently Santigosa, Constant, Prudence, Wahli, &
303 Verlhac-Trichet (2020) tested a novel marine algal oil for rainbow trout that contained a more
304 balanced ratio of both EPA and DHA with results showing that up to 9% fish oil could be
305 satisfactorily replaced in the formulation. These workers used a new commercial algal oil
306 (Veramaris®) with 39.8% DHA and 15.7% EPA within the oil in their experimental trial. As
307 expected muscle tissue fatty acid composition reflected that of the diets. Digestibility of oil was
308 uniform and high at above 99% for trout.
309 Dietary inclusion of microalgae can trigger morphological alterations in the intestinal and liver
310 structures of fish and other animal species due to their nutritional components (Atalah et al.,
311 2007; Messina, Bulfon, Beraldo, Tibaldi, & Cardinaletti, 2019; Ringø et al., 2016). In the present
312 study, the histological analyses did not reveal histopathological effects on the liver and distal
313 intestine samples of rainbow trout, confirming previous observations in Atlantic salmon
314 (Sørensen et al., 2017), gilthead sea bream (Valente, Custódio, Batista, Fernandes, & Kiron, 2019;
315 Vizcaíno et al., 2014), longfin yellowtail (Kissinger et al., 2016) and rainbow trout (Lyons et al.,
316 2017).
317 Although it should be noted that in the current investigation, the amount of lipid vacuoles in liver
318 samples of fish fed diets containing mixture *of S. limacinum* and *N. oceanica* meal was slightly

lower than those fed the control diet. This may be attributable to bioactive compounds contained in these microalgae, which could have hypocholesterolemic properties (De Jesus Raposo, De Morais, & De Morais, 2013). Similar changes in lipid metabolism has been previously reported in several marine fish species fed graded levels of microalgae, which include a reduction of blood triglycerides and cholesterol (M. Li et al., 2014; Yeganeh, Teimouri, & Amirkolaie, 2015). However, further studies are necessary to test this hypothesis.

Several studies have reported that omega-3 polyunsaturated fatty acids and bioactive compounds, such as pigments, fibers and phytosterols, contained in microalgae are involved in promoting fish health, growth and nutrient metabolism (Chauton, Reitan, Norsker, Tveterås, & Kleivdal, 2015; De Jesus Raposo et al., 2013; Shah et al., 2018). In fish species, hyperplasia muscle growth occurs continuously during the entire life and is regulated by myogenic regulatory factors (MRFs) genes in particularly myod (Johnston, Bower, & Macqueen, 2011). Studies carried out by Shi et al.(2017) using *Chlorella* meal in crucian carp (*Carassius auratus*) diets have demonstrated an improvement in the expressions of MRFs genes (including myod, myog, mrf4, myf5) promoting the growth and the development of muscle. Conversely, in the present study, dietary inclusion of mixture of microalgae meal has tended to decrease myod expression, which could be linked to the reduction in fish growth rate probably due to reduced nutrient digestibility. On the other hand, incorporation of ingredients rich in omega-3 fatty acids into salmonid feed can enhance pigment uptake and resistance to stress and diseases (Glencross, 2009; Sargent, Tocher, & Bell, 2002). In particular, the dietary substitution of fish oil with mixture of microalgae meal did not affect the expression of selected genes involved in carotenoid transport metabolism (scarb1), stress response (hsp70) and immune response (Il12). The above responses were also observed by Kousoulaki et al. (2015) and Sørensen et al. (2017), who found no sign of transcriptomic changes related with stress or inflammation from *Schizochytrium* sp. and *N. oceania* supplementation in diets for Atlantic salmon. Improvement of fillet color have been demonstrated in terms of increased redness, reduced paleness, and reduced melanin spots in Atlantic salmon fed *Schizochytrium* sp meal (Kousoulaki et al., 2020), which were attributable to pigment intestinal uptake facilitation and antioxidant effect provided by the nutritional components within microalgae whole cells, specially DHA and vitamin E. However, no data is

available on the effects of dietary microalgae on genes involved in carotenoid metabolism in salmonids.

The microalgae mixture used in the current experiment had relatively high lipid content rich in n-3 PUFA and SAFA, and as they were substituting the dietary fish oil, the concentration of several fatty acids were altered among the experimental diets. These changes in dietary fatty acid composition were mirrored in the fatty acid composition in rainbow trout muscle. Similar results have been previously noted in cultured fish species using microalgae as feed ingredients (Cardinaletti et al., 2018; Garcia-Ortega et al., 2016; Kissinger et al., 2016; Kousoulaki et al., 2020; Kousoulaki et al., 2015; Qiao et al., 2014; Sevgili et al., 2019; Sprague et al., 2015).

Increasing the dietary inclusion of microalgae mixture caused a slight but non-significant decreasing trend in the total SAFA of muscle tissue. This could indicate that these fatty acids are catabolized as an energy source, improving deposition of long chain polyunsaturated fatty acids (LC-PUFA) in the fish flesh (Sargent et al., 2002; Turchini et al., 2009). The same results have been obtained with the use of *S. limacinum* in diets for *S. salar* (Kousoulaki et al., 2020; Kousoulaki et al., 2015), *E. lanceolatus* (Garcia-Ortega et al., 2016), *S. rivoliana* (Kissinger et al., 2016), *P. major* (Seong et al., 2019) and *P. olivaceus* (Qiao et al., 2014).

Moreover, in the present study with trout the substitution of dietary oil with microalgae mixture significantly reduced MUFA concentrations in muscle. This is due to a decrease in the dietary concentration of 18:1n9, oleic fatty acid, found in appreciable amounts in rapeseed oil and used as energy sources by the fish (Stubhaug, Frøyland, & Torstensen, 2005; Turchini et al., 2009).

The inclusion of a microalgae mixture in diets for rainbow trout also causes a slight decrease in the muscle content of other unsaturated 18-C fatty acids (linoleic acid and linolenic acids). Kousoulaki et al. (2015) and Qiao et al. (2014) reported similar results in marine fish fed diets containing microalgae meal. These latter changes can be explained by the reduction of dietary vegetable oils and by the low amount of linoleic acid and linolenic acids present in *Schizochytrium* sp (Kousoulaki et al., 2015) and *Nannochloropsis* sp (Sevgili et al., 2019). However, in the current study with rainbow trout, the vegetable oil (rapeseed) inclusion was maintained and only fish oil replaced with algal oil mixture. It was interesting to record a reduction in dihomo-gamma-linolenic acid (20:3, n-6) in trout flesh with increasing algal mixtures. Hassam & Crawford (1978)

have shown that this fatty acid can be more potent than gamma-linolenic acid (18:3, n-6), the latter being superior to that of linoleic acid (alpha18:2, n-6) in meeting n- 6 requirements for EFA deficient rats. Whether this is also the case in salmonids remains unresolved.

The content of n-3 highly unsaturated fatty acids (HUFAS) found in the muscle of rainbow trout given diets included graded levels of microalgae mixture was similar to that of control group, indicating selective retention of these fatty acids as is typically observed in fish (Glencross, 2009; Sargent et al., 2002). These findings confirm that n-3 HUFAS supplied by the microalgae were actually deposited in the flesh of rainbow trout. The above was also reported in studies conducted with *Schizochytrium* sp. (Kissinger et al., 2016; Kousoulaki et al., 2020; Kousoulaki et al., 2015; Qiao et al., 2014), *Crypthecodinium cohnii* (Eryalçin, Ganuza, Atalah, & Hernández Cruz, 2015), *Nannochloropsis* sp (Qiao et al., 2014; Walker & Berlinsky, 2011), *Phaeodactylum tricornutum* (Atalah et al., 2007; Sørensen, Berge, Reitan, & Ruyter, 2016), *T. lutea* (Cardinaletti et al., 2018) and *Isochrysis galbana* (He et al., 2018) based diets for commercial fish.

The retention of EPA and DHA in fish tissues is important both for fish growth and health (Bou et al., 2017; Glencross, 2009). In the present study the deposition of DHA in the muscles of all algal treatment groups was high compared to EPA. This corresponds with fact that DHA are preserved in the membrane of body tissues meanwhile EPA are selectively used as for β-oxidation (Bou et al., 2017; Sargent et al., 2002). Furthermore, the physical characteristics of microalgae cell wall consisting complex networks of polysaccharides and glycoproteins can affect the nutrient apparent digestibility coefficient (ADC) of fish feed (Baudelet, Ricochon, Linder, & Muniglia, 2017), since carnivorous fishes do not have the capacity to digest non-starch polysaccharides (Krogdahl, Hemre, & Mommsen, 2005).

Indeed, feeding studies using *Nannochloropsis* species as ingredient for salmonids have reported a poor digestibility of n-3 HUFAS as a result of their tough cell wall (Sevgili et al., 2019). This is in agreement with our findings in which the content of EPA in rainbow trout muscle was decreased as the dietary level of *N. oceanica* increased. Nevertheless, the reduction in the concentration of EPA did not cause any adverse effects on fish performance, indicating that the requirement for this fatty acid could be covered by the content of dietary fish oil and fish meal (Cho & Kim, 2011; Tocher et al., 2019; Turchini et al., 2009) or by the capacity of fish to metabolically retro-convert

DHA into EPA as the dietary inclusion of *S. limacinum* increased (Glencross, 2009; Kousoulaki et al., 2015).

From a consumer point of view, salmonids are recognized as a unique source of EPA and DHA, providing several benefits to human health (Gebauer et al., 2006; Jensen et al., 2012). In our study, replacing fish oil with a mixture of *N. oceanica* and *S. limacinum* achieved similar EPA+DHA levels among dietary treatment and the consumption of 100 g portion of rainbow trout fillet is able to provide 23% of the weekly intake recommended by European Food Safety Authority (EFSA, 2010) for these n-3 HUFAS.

To conclude, this study shows that a blend of *N. oceanica* and *S. limacinum* can be included up to 9% in diets for seawater farmed rainbow trout as a fish oil substitute without negative effects on fish growth, health and lipid composition. These results confirm that n-3 HUFAS rich microalgae may be utilized as alternative ingredients to reduce the forage fish dependency ratio of aquafeed and therefore improve the sustainability of the salmon farming industry. Nevertheless, the high production cost of algal biomass and the scalable physical-mechanical and biochemical pretreatment for algal cell wall disruption are the major remaining problems to be addressed in future research. This will allow economically and reliable strategies to optimize the utilization of these ingredients in formulated diets for salmonids. It will be important to compare the use of whole algal biomass suitably processed or the extracted and stabilized oils in feed formulations to reduce the fish oil dependency. This will be essential for high value marine fish species such as salmon but will also be an important consideration to enhance the omega-3 fillet composition of other species prior to harvest in conditioning feeds. Further studies should be undertaken on rainbow trout grown to larger harvest size in sea pens to develop bespoke conditioning diets using algal mixtures for lipid enrichment to meet with consumer expectations for a premium product.

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CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest to declare concerning the submission of this manuscript.

AUTHOR CONTRIBUTIONS

Edison Serrano involved in designing the experiment, analyzing statistical data and writing original draft of the manuscript. Robert Simpfendorfer designed and performed the experiment. Alberto Medina obtained funding for this project and assisted with carrying out experiments. Carlos Sandoval carried out histological analysis. Alexis Martínez performed gene expression analysis. Rodrigo Morales conducted fatty acids analysis. Simon J Davies reviewed and edited the final version of the manuscript. All authors read and approved the final manuscript.

ETHICAL APPROVAL

The experiment was approved by the ethics and animal welfare committee of University of Los Lagos and conducted in accordance with the regulations and guidelines for the care of experimental animals established by Chilean laws.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request

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669 **TABLES**

670 Table 1. Ingredients and proximate composition of experimental diets

	Dietary treatments		
	Control	A 9	A 17
Ingredient composition (g kg^{-1})			
Fishmeal*	150.0	150.0	150.0
Fish oil*	60.0	28.0	0.0
Rapeseed oil ¶	140.0	146.0	150.0
Schizochytrium limacinum meal‡	0.0	45.0	84.0
Nannochloropsis oceanica meal†	0.0	45.0	84.0
Hydrolyzed feather meal §	70.0	70.0	70.0
Poultry by-product meal §	80.0	80.0	80.0
Blood meal §	30.0	30.0	30.0
Corn gluten meal**	50.0	50.0	50.0
Soy protein concentrate**	57.0	50.0	40.0
Wheat gluten**	50.0	50.0	50.0
Defatted rapeseed meal¶	130.0	73.0	29.0
Wheat flour**	152.2	152.2	152.2
Vitamin and mineral premix¶¶	5.5	5.5	5.5
L-Lysine‡‡	3.0	3.0	3.0
DL-methionine‡‡	2.0	2.0	2.0
Monocalcium phosphate††	20.0	20.0	20.0
Carophyll pink§§	0.3	0.3	0.3
Proximate composition (g kg^{-1} DM)			
Dry matter	971.0	973.2	961.9
Crude protein	427.6	427.7	423.9
Crude lipid	257.9	263.1	241.6
Ash	78.7	84.9	93.5
Carbohydrates***	235.8	224.3	241.0
Gross energy (kJ kg^{-1})	24.3	24.3	23.7

671 *Lota protein S.A., Talcahuano, Chile

672 ¶Oleotop S.A., Freire, Chile
673 ‡Alltech Inc., Nicholasville, KY, USA.
674 †Allmicroalgae Natural Products S.A., Lisboa, Portugal
675 §Agrosuper S.A., Doñihue, Chile.
676 **Graneles Chile S.A., Santiago, Chile.
677 ¶¶BioMar Chile S.A., Puerto Montt, Chile.
678 ‡‡Evonik Nutrition & Care GmbH, Hanau, Germany.
679 ††Montana S.A., Lima, Perú.
680 §§DSM Nutritional Products Ltd., Basel, Switzerland
681 ***Calculated as the remainder of crude protein crude +lipid + ash.

682

683 Table 2. Fatty acid composition of the experimental diets

	Dietary treatments		
Fatty acids (mg 100 g^{-1} of sample)	Control	A 9	A 17
C14:0	362.28	310.23	270.12
C16:0	1707.48	2475.64	3267.82
C18:0	414.45	406.92	381.97
C20:0	101.42	71.17	71.92
Total SAFA‡	2757.96	3489.53	4290.22
C16:1n-7	34.73	28.35	17.34
C16:1n-9	469.90	352.40	283.61
18:1n-9	6614.64	6703.93	6307.33
18:1n-11	490.94	438.43	380.98
20:1n-9	218.85	181.88	142.00
Total MUFA‡	8338.48	8027.77	7303.90
18:2n-6	2467.16	2613.73	2553.04
18:3n-6	12.80	8.15	10.50
20:2n-6	9.97	11.06	12.00
20:3n-6	5.84	5.30	6.90
20:4n-6	27.38	39.67	57.46
Total n-6 PUFA‡	2584.72	2719.31	2661.82
18:3n-3	918.73	973.39	934.77
20:3n-3			
20:5n-3	669.25	493.83	349.25
22:5n-3	113.91	57.14	32.84
22:6n-3	283.92	686.39	1091.63
Total n-3 PUFA‡	2136.39	2299.49	2455.93

Total PUFA‡	4721.11	5018.80	5117.75
EPA + DHA	953.17	1180.22	1440.88
n-3:n-6	0.83	0.85	0.92

‡Includes unlisted fatty acids: SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 3. Primer sequences used for the quantification of the mRNA expression by qPCR of selected genes and accession numbers for *Oncorhynchus mykiss*

Gen	Reference Sequence	Forward (5′ – 3′)	Reverse (5′ – 3′)	Probe (FAM)
myod	NM001124720	GGACTCGGATGCGTC CAGTC	TCTCCGTCTTGGTGG ACAAGAC	TCCAACTGCTCAGACG GAATGATGGATTTCAA
hsp70	AB062281	CATGGTCCTGGTGAA GATGAGGG	GCGTCCTTAGTGGCC TGTCTCTGT	TGGGCCAGAAGGTGT CCAATG
il12	NM001124392	TGGTCTCACCTCCTTC CATGAA	GAGAATGCCGTGGG ACATGTC	TGATAAGGGGGACAG TTTGGTGACTC
scarb1	XM021590608	CGGCTGATTCACAAA GTGAACTC	GTTGATCATGTTACA CTGGGGAGTC	ATGGAACGGCTTGACC AAGTTGATATATTGGA

Abbreviations: myod, myoblast determination protein 1; hsp70, heat shock protein 70; scarb1, scavenger receptor class B type 1; il12, interleukin 12.

Table 4. Growth performance, and somatic parameters of rainbow trout (*Oncorhynchus mykiss*) fed increasing dietary mixture of microalgae meal*.

	Dietary treatments			
Growth parameters	Control	A 9	A 17	*P value*
Initial weight (g)	189.7 ± 0.1	184.7 ± 2.3	193.1 ± 1.9	0.1308
Final weight (g)	281.4 ± 15.4^{a}	244.7 ± 2.5^{ab}	230.7 ± 4.9^{b}	0.0221
Gain (g)	91.6 ± 15.4^{a}	60.0 ± 4.8^{ab}	37.6 ± 4.5^{b}	0.0210
Feed intake (g/fish)	122.3 ± 19.5	138.5 ± 10.4	117.4 ± 11.8	0.5861
SGR	0.58 ± 0.08^{a}	0.42 ± 0.03^{ab}	0.26 ± 0.03^{b}	0.0190
FCR	1.34 ± 0.02^{a}	2.31 ± 0.02^{b}	3.14 ± 0.07^{c}	<0.0001
HSI	1.68 ± 0.15	1.52 ± 0.14	1.22 ± 0.02	0.0836
SSI	0.21 ± 0.03	0.19 ± 0.03	0.14 ± 0.01	0.2356

*Each value is the mean ± SEM of three replicates. Different superscripts letters in a row indicate statistically significant differences ($P < 0.05$) among groups. SGC, specific growth rate; FCR, feed conversion ratio; HSI, hepatosomatic index: SSI, spleen somatic index

Table 5. Fatty acid composition in muscle of rainbow trout (*Oncorhynchus mykiss*) fed diets with increasing dietary dietary mixture of microalgae meal levels after 70 days of feeding (as mg fatty acid 100 g^{-1} of wet fillet tissue)

Fatty acids(mg 100 g^{-1} of sample)	Dietary treatments			*P-value*
	Control	A9	A17	
C14:0	29.75 ± 2.83^{a}	25.4 ± 0.30^{ab}	20.14 ± 0.88^{b}	0.0214
C16:0	207.50 ± 13.19	190.30 ± 5.99	174.25 ± 7.96	0.1228
C18:0	51.79 ± 2.82	46.67 ± 1.53	42.05 ± 2.79	0.0813
C20:0	3.17 ± 0.32	2.70 ± 0.07	2.40 ± 0.18	0.1071
Total SAFA‡	302.59 ± 19.85	274.53 ± 7.61	248.42 ± 11.87	0.0892
C16:1n-7	4.53 ± 0.33^{a}	3.30 ± 0.11^{ab}	2.83 ± 0.36^{b}	0.0144
C16:1n-9	39.42 ± 3.99^{a}	32.25 ± 0.61^{ab}	25.62 ± 1.23^{b}	0.0202
18:1n-9	494.57 ± 53.78	399.53 ± 3.89	351.06 ± 19.98	0.0561
18:1n-11	41.11 ± 4.24^{a}	33.37 ± 0.29^{ab}	28.19 ± 1.34^{b}	0.0327
20:1n-9	3.59 ± 0.37^{a}	2.89 ± 0.08^{ab}	2.23 ± 0.10^{b}	0.0155
20:1n-11	20.73 ± 2.00^{a}	16.75 ± 0.07^{ab}	14.07 ± 0.40^{b}	0.0197
Total MUFA‡	622.11 ± 66.54^{a}	502.22 ± 3.58^{ab}	435.91 ± 23.63^{b}	0.0465
18:2n-6	180.76 ± 19.24	157.11 ± 2.19	140.77 ± 7.76	0.1393
18:3n-6	3.67 ± 0.10^{a}	2.49 ± 0.12^{b}	2.35 ± 0.21^{b}	0.0015
20:2n-6	7.60 ± 0.65^{a}	6.28 ± 0.24^{ab}	5.71 ± 0.15^{b}	0.0412
20:3n-6	6.78 ± 0.40^{a}	4.67 ± 0.47^{b}	4.04 ± 0.21^{b}	0.0050
20:4n-6	10.13 ± 0.18	9.95 ± 0.30	9.79 ± 0.70	0.8706
Total n-6 PUFA‡	211.36 ± 20.27	182.43 ± 1.40	164.42 ± 8.87	0.1018
18:3n-3	51.51 ± 6.23	43.97 ± 0.44	39.50 ± 1.91	0.1541
20:3n-3	1.91 ± 0.21	1.81 ± 0.11	1.63 ± 0.12	0.4714
20:5n-3	56.27 ± 1.29^{a}	50.69 ± 2.62^{ab}	42.81 ± 2.54^{b}	0.0151
22:5n-3	18.02 ± 0.74^{a}	16.93 ± 0.60^{ab}	14.35 ± 0.66^{b}	0.0204
22:6n-3	186.42 ± 6.50	195.64 ± 9.51	196.30 ± 10.80	0.7074

Total n-3 PUFA‡	314.13 ± 14.60	309.04 ± 12.99	294.59 ± 15.09	0.6266
Total PUFA‡	525.49 ± 34.78	491.48 ± 12.12	459.01 ± 21.20	0.2387
EPA + DHA	242.69 ± 7.70	246.33 ± 12.12	239.11 ± 13.26	0.9044
n-3:n-6	1.57 ± 0.04[a]	1.71 ± 0.08[ab]	1.84 ± 0.05[b]	0.0497

*Each value is the mean ± SEM of three replicates. Different superscripts letters in a row indicate statistically significant differences ($P < 0.05$) among groups.

‡Includes unlisted fatty acids: SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

FIGURE LEGENDS

Figure 1. Hepatocytes of rainbow trout (*Oncorhynchus mykiss*) fed control diet (a) 9% mixture of microalgae meal (b) and 17% mixture of microalgae meal (c) (H&E x40).

Figure 2. Morphology of distal intestine in rainbow trout (*Oncorhynchus mykiss*) fed control diet (a) 9% mixture of microalgae meal (b) and 17% mixture of microalgae meal (c) (H&E x10).

Figure 3. Relative mRNA levels of myod in muscle, hsp70 in the liver, il12 in the head kidney and scarb1 in the distal intestine of (*Oncorhynchus mykiss*) after feeding the experimental diets. Bars represent mean ± and line the SEM, n = 9. The statistical significance was determined using One-way ANOVA and Tukey's test ($P < 0.05$). Abbreviations: myod, myoblast determination protein 1; hsp70, heat shock protein 70; scarb1, scavenger receptor class B type 1; il12, interleukin 12.