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Effects of alternative and sustainable ingredients, insect meal, microalgae and protein and lipid from tuna cooking water, on meagre (*Argyrosomus regius*) growth, food conversion and muscle and liver composition

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Abstract

This study aimed to evaluate the effects of alternative feed ingredients: 1) insect meal (*Acheta domesticus*, DI); 2) a mixture of four marine microalgae species (DM); 3) protein and lipid fraction recovered from cooking water from canned tuna manufacturing processes (DP&L) and 4) a mix of the three ingredients (DMix) on the growth, feed utilisation, digestibility and composition of meagre juveniles, and the results obtained were compared with a feed similar to a commercial one used as a control (DC). Results show that the formulated alternative feeds had different effects on the growth of the fish. DMix have a similar growth performance than the control, whereas the other two treatments show similar values. Hepatosomatic and viscerosomatic indexes did not show differences among the treatments. Muscle protein content was higher for fish fed with DMix group whereas lipids were significantly higher in DI. In the case of the liver, protein was higher in the liver of fish fed with DP&L, whereas lipids were higher in fish fed with DI and DM, a result that was confirmed with the results obtained in hepatocyte size and lipid accumulation.
The nutritional value of the meagre muscle at the end of the study show that meagre fed with DM and DI diets contained a significantly higher content of monounsaturated and n-6 PUFA, whereas fish from the groups fed with DP&L and DMix had a significantly higher content of DHA and n-3 PUFA with the liver showing similar results. In view of the results obtained, the ingredients assayed in this study might be used as alternative sources of protein and lipids in aquafeeds since no negative effects were detected neither on fish growth, muscle composition, fish health nor final nutritional value, except in the case of the diet with microalgae (DM) included, which inclusion rate in the feed must be adjusted and needs more research.

Key words: Alternative ingredients, insect meal, microalgae, by-products, canning industry, tuna water cooking, meagre

Introduction

Fish meal (FM) and fish oil (FO) have been the predominant protein and lipid ingredients used in aquafeeds (Gatlin et al., 2007) due to their high protein content, amino acid and fatty acid profiles, and palatability (Bandara, 2018). Nevertheless, the global FM and FO production is not sustainable because it relies on over-exploited pelagic marine fish (FAO, 2018). Although FM and FO continue to be critically important as feed ingredients and vital to the aquaculture industry (Konar et al., 2019), several developments have helped to reduce the dependence on wild fish resources since 2000. These developments include an increase in omnivorous fish production, improved feed conversion ratios for all fed species, higher use of alternative protein and oil ingredients in aquafeeds, and an increase in production and use of FM and FO from fish-processing wastes and bycatches (Naylor et al., 2021)

Plant-based ingredients have been successfully used to replace part of FM and FO in a number of farmed fish due to their higher availability and lower price (Kalhoro et al., 2018). However, the complete replacement of marine-derived ingredients by plant-based ingredients is hindered by the presence of anti-nutritional factors, causing digestive tract inflammation problems in fish, poor nutritional composition (low protein content and imbalances in the essential amino acid and fatty acid profiles) and low feed palatability. Likewise, they have a high environmental impact due to the amounts of energy, water and land needed for their production (FAO, 2018; Samuelsen et al., 2018; Gong et al., 2019).

In the last decade, research efforts looking for FM and FO alternatives have been mostly focused in soybean-derived products (Berge et al., 1999; Aksnes et al., 2006; Deng et al., 2006; Kalhoro et al., 2018) with satisfactory results on growth at different substitution levels in the feeds for marine and freshwater species. However, the academy and industry have not ceased in their endeavour to look for other ingredients. Among them, insect, micro- and macroalgae, and microbial meals are emerging ingredients for aquafeeds (Henry et al., 2015; Biancarosa et al., 2019; Sarker et al., 2020; Shaikhiev et al., 2020). Other ingredients based on food industry by-products are also being considered due to the needs to boost the circular economy actions implemented by the EU and the availability of untapped huge quantities of these by-products and their protein and lipid quality (García-Sanda et al., 2003; Nazzaro et al., 2021).

Although the wastes generated in tuna processing plants, especially heads, fins, bones and meat are mostly processed to obtain FM for the animal feed industry (Garrido et al., 2013), the tuna canning industry for human consumption may also represent a valuable source of ingredients for aquafeeds. In this sense, cooking is an indispensable step in canning industry and the stickwater (SW) generated represents approximately 60% of the processed fish weight (Bechtel, 2015, Valdez-Hurtado et al., 2018), resulting in approximately 4% water-soluble protein in the cooking juice (Jao and Ko, 2002). Tuna cooking water accounts for more than 1,500,000 m^3 in Spain, being managed as effluents in the processing plants. Only a few studies have been interested in the recovery and valorisation of the biomolecules contained in these effluents (Tremblay et al., 2020). According to Martinez-Montaño et al. (2020), the SW contains 6% of protein and 1.8 % of oil, and more than 70 % of the protein and 12 % of the lipids can precipitate using HCl. Recuperating only 10% of the tuna cooking water from Galicia, one of the main sites of tuna canning in Europe,

would mean recovering about 60,000 litres of oil and 450,000 kg of organic matter that can be reused as new feed ingredients.

Meagre is one of the species selected for Mediterranean aquaculture diversification. It has potential for large-scale farming due to its easy adaptation to captivity, fast growth, good feed conversion ratio, high nutritional value and processing yield, low fat content, and excellent taste and texture (Grigorakis et al., 2011; Monfort, 2010). Meagre is a carnivorous marine fish feeding essentially on fish and crustaceans and due to these characteristics was selected for this experiment. Considering the growing interest of the European aquaculture industry on this species, it is of special relevance evaluating new feed ingredients in order to formulate diets less dependent on the classical sources of dietary proteins and lipids.

The use of novel aquaculture feed ingredients is growing (Cottrell et al., 2020) and the needs to study the efficiency of these alternative ingredients is essential for their industrial implementation. Therefore, the present study aims to evaluate the effects of several sustainable ingredients for meagre production: (1) insect meal as a high-quality protein source, (2) microalgae biomass as a source of lipids rich in omega-3 fatty acids, (3) protein and lipid fractions recovered from the cooking water of tuna canning processes, and (4) a diet with a mix of the three previous ingredients. These diets were evaluated in terms of different key performance indicators such as growth and feed performance, apparent nutrient digestibility of nutrients, and muscle quality in terms of proximate composition and fatty acid profile.

Material and methods

Manipulations of fish were carried out in compliance with the Guidelines of the European Union Council (2010/63/UE) and Spanish legislation for laboratory animal use.

Experimental procedure

Meagre juveniles (N = 750) obtained from Alevines del Sureste S.L. (Murcia, Spain) with an initial weight of 12.51 ± 1.48 g (mean ± standard deviation, SD) were transported by road to IRTA San Carlos de la Rápita (Tarragona, Spain). Fish were kept in quarantine for 2 weeks and distributed in 15 tanks of 200 L (50 fish per tank) connected to a water recirculation system (RAS; IRTAMAR®) that maintained adequate water quality through UV, biological, and mechanical filtration. Each tank was provided with continuous aeration and automatic oxygen injection. Water conditions were maintained at 24.3 ± 1.9 ºC, 36 ‰ salinity and 6.2 ± 0.4 mg/L dissolved oxygen, under 12h L: 12h D photoperiod. RAS parameters were maintained stable during all the trial and ammonia (0.30±0.12mg/l) and nitrite (0.18±0.08 mg/l) within the safe levels for the species. Fish were fed manually 3 times per day at 4.5% feeding ratio and 7 days a week. Feed amounts were adjusted each week with an estimation of theoretical growth and uneaten feed was daily recorded and subtracted from the supplied feed in order to calculate feed intake per tank. The trial lasted for 60 days. All the fish were individually weighted at the beginning, mid and at the end of the experiment. Prior to manipulation, fish were anesthetised with tricaine methane sulfonate (MS-222, Sigma-Aldrich, Madrid, Spain). Faeces for digestibility determination were collected by abdominal stripping of the fish of each tank in alternate days during 2 weeks before final sampling.

Faecal samples per tank were freeze dried and stored at -20ºC until chemical analyses. At the end of the experiment, growth performance was assessed using the following parameters: specific growth rate (SGR, % body weight/day = (ln final weight - ln initial weight)/days) x100); feed conversion ratio (FCR = feed intake / increase in biomass); protein efficiency ratio (PER = increase in biomass / total protein intake); relative growth rate (RGR, Final weight-initial weight / initial weight) and Fish In Fish out ratio (FIFO = FCR * (% fish meal + % fish oil in feed)/ (FM ratio + FO ratio), Kok et al., 2020).

Feed formulation

Five experimental diets were formulated by DIBAQ Aquaculture and manufactured by the Technological Center CARTIF (Valladolid, Spain) using the same facilities and extrusion parameters for all of them. Diets were as follows: (1) microalgae diet (DM) containing 10% of a mix of four marine microalgae (*Nannochloropsis gaditana*, *Tisochrysis lutea* (CCAP 927/14), *Rhodomonas lens* (ECC030), *Isochrysis galbana* (CCAP927/1) included at 26%, 33%, 20% and 21%, respectively, and produced by ANFACO-CECOPESCA (Vigo, Spain); (2) insect diet (DI) in which a non-defatted meal obtained from *Acheta domesticus* produced by Nutrinsect (Navarra, Spain) was included at 15%; (3 and 4) protein and oil from water cooking diet (D P&L) containing 7% and 11% of SW recovered by ANFACO-CECOPESCA (Vigo, Spain); and (5) mix diet (DMix) based in the inclusion of the three ingredients (10% microalgae meal, 15% insect meal, 2% protein and 9.4% lipid fraction from tuna canning). A diet with a formulation similar to a commercial feed (DC) was used as a control. The formulation of experimental feeds and their proximate composition are detailed in Table 1.

Muscle and liver composition analysis

Ten fish from each tank were sacrificed with an overdose of anesthetic. The liver and viscera of each fish were dissected and weighted in order to calculate the hepatosomatic index (HSI, % = (100 x [liver weight (g)] / [total body weight (g)]) and viscerosomatic index (VSI, % = (100 x [viscera weight (g)] / [total body weight (g)])). Samples of dorsal muscle and liver of the fish were kept at -20ºC until biochemical analysis.

The chemical analysis of the diets, muscle and liver, and faeces were carried out in duplicates. Muscle and liver protein content was analysed following the method described by Lowry et al. (1951) and lipids extracted by the method of Folch et al. (1957) and quantified by gravimetric analysis. Protein content in extruded diets and faeces was carried out by the Dumas method using a nitrogen/protein analyzer (LECO FP-528). Water content was calculated after oven-drying at 105ºC for 12 h. The results are presented as percentage (%) of the dry weight (DW) as mean ± SD. Fatty acid (FA) methyl esters were prepared by acid-catalysed transmethylation (Christie, 1982), and extracted and purified by TLC following the method described by Tocher and Harvie (1988). Methyl esters were separated and quantified by gas-liquid chromatography (Thermo Trace GC, Thermo Finningan, Milan, Italy) using a 30 m x 0.25 mm ID capillary column (BPX 70, SGE Europe Ltd., UK) with on-column injection and flame ionization detection using helium as carrier gas (1.2 mL min-1 constant flow rate). Individual methyl esters were identified by comparison with known standards (Supelco Inc., Madrid) and a well-characterized fish oil, and quantified by the response factor to the internal standard, 21:0. The results are presented as percentage of the total fatty acids (% TFA) as mean ± SD.

Histological analysis

Liver samples (5 fish per tank; n = 15 per diet) were fixed in 4% buffered formalin (pH = 7.4), dehydrated in a graded series of ethanol (70-96%), embedded in and cut into serial sagittal sections (2-3 um) with a microtome (Leica RM2155, Germany). Sections were stained with hematoxylin and eosin (H&E) (Casa Alvarez, S.A, Madrid, Spain) for general histomorphological observations. All section were observed under a microscope Leica DMLB (Leica Microsystems, Spain) equipped with a digital camera Olympus DP70 (Olympus España SAU, Spain). Digital images (600 dpi) were analyzed using the digital image analysis software ANALYSIS^{TM} (Soft Imaging Systems GmbH, Germany). In particular, the general organization of the hepatic parenchyma was evaluated as well as the size of lipid inclusions within hepatocytes. The surface of lipid inclusions was calculated on a total of 40 hepatocytes from five fish per tank following the formula S = ¼ π *a b*; where *a* and *b* were the minimum and maximum diameters of lipid inclusion.

Digestibility

Faecal samples obtained by manual stripping were freeze dried and stored at -20ºC until chemical analyses. Ytrium oxide content in diets and faeces was determined according to Garantun-Tjeldsto et al. (2006) by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent Technologies 7700x, Madrid, Spain). Protein content was carried out by Dumas method using a nitrogen/protein analyzer (LECO FP-528) whereas crude fat was extracted using a Büchi Extraction System B-811 (Büchi, Switzerland, AOAC 920.39), lipid content was quantified gravimetrically after evaporation of the solvent under a stream of nitrogen followed by vacuum desiccation overnight.

The protein and lipid apparent digestibility coefficients (ADCs) of the experimental diets were calculated according to Maynard et al., (1979):

ADC (%) = 100 × (1 − (dietary Y_2O_3 level/faeces Y_2O_3 level) × (faeces nutrient/dietary nutrient).

Statistical analysis

Growth, feed conversion, biochemical composition of fillet and liver and apparent digestibility coefficients data were tested for normality of variances using Levene's test before being submitted to a one way analyse of variance (ANOVA) using Sigma Plot 12.0 program (Systat Software Inc. USA). The differences were considered statistically significant when $P < 0.05$ and the Holm-Sidak post hoc test was used to perform pair wise comparisons of means between experimental groups.

Results

Table 2 shows the proximate composition and the most important fatty acids of the new ingredients assayed. The content of protein and fat was very high for insect meal and canning byproducts whereas in the case of mixed microalgae their contents were quite low. Insect meal was very rich in monounsaturated fatty acids (MUFA) and omega-6 polyunsaturated fatty acids (N-6 PUFA) derived from the high presence of linoleic acid (18:2n-6, LA), mixed microalgae were very rich in omega-3 PUFA (N-3 PUFA) mostly due to the high presence of Eicosapentaenoic acid (20:5n-3, EPA), and canning by-products were very rich in N-3 PUFA due to the high content of

docosahexaenoic acid (22:6n-3, DHA). Tables 3 and 4 show the fatty acid composition of the feeds, and a summary of the main nutritional components, respectively.

Effect of experimental diets on growth performance:

At the beginning of the experiment, the juveniles weighted 12.51 ± 1.48 g. No significant differences in the initial weight of the fish were observed among tanks or treatments. At the end of the experiment, significant differences were observed in the final weight among diets (Table 5). Thus, meagre fed the DMix and DC diets showed statistically significant higher final weight values than the rest of the groups (ANOVA, $P < 0.05$). The same trend was observed in the results of RGR ($P < 0.05$), whereas SGR values did not show differences among the groups ($P > 0.05$).
PER and FCR ratios showed similar values for all the groups, although FCR was slightly higher in the fish fed DMix diet. FI:FO values varied among experimental diets; in particular, the DM diet had the highest ratio with almost 0.72 kg of feed needed to produce 1 kg of meagre, whereas the best ratio was observed in the DP&L and DMix groups with values of 0.50 and 0.58, respectively.
HSI and VSI indices did not show significant differences among the groups

Effect of experimental diets on muscle and liver composition:

Experimental diets affected the proximate composition of the muscle of meagre (Table 6; $P < 0.05$). In terms of moisture content, higher values were found in fish from the DP&L group. Protein content was higher for DMix and lower for DC and DM groups, and lipids were higher for DI and lower for DMix groups (Table 6). An antagonistic result in muscle composition was observed in DMix group that showed the highest protein (20.42%) and lowest lipid (0.75%) content. DI showed the highest lipid content (1.09%) and intermediate protein levels (13.92%) whereas the muscle of the rest of the groups (DC, DM and DP&L) showed intermediate values. Similar results were obtained in the proximate composition of the liver (Table 7), with DP&L, DI and DM groups showing the highest moisture and protein content and the DI and DM groups the highest lipid values ($P < 0.05$).
Concerning the fillet FA composition (Table 6), significant differences were observed among the groups with fish fed DMix showing the highest content in saturated fatty acids (SFA) due to their high content in stearic acid (18:0). Meagre fed the DI and DM diets had the highest content of monounsaturated fatty acids (MUFA) and n-6 polyunsaturated fatty acids (PUFA), due to their high content in oleic acid (18:1n-9) and linoleic acid (18:2n-6). The DP&L fish had the highest content in total PUFA, n-3 PUFA and docosahexaenoic acid (22:6 n-3, DHA), whereas the DC showed the highest content in eicosapentaenoic acid (20:5 n-3, EPA).
The FA composition of the liver is presented in Table 7. As in the case of muscle, significant differences were detected among the groups being the differences similar to those found in the muscle, especially in terms of SFA, being highest in the liver of fish fed the DMix diet. The levels of total PUFA and n-3 PUFA were the highest in DP&L fish, and MUFA and n-6 PUFA were the highest in fish fed the DI diet followed by the DM and DC groups ($P < 0.05$). The highest content of EPA was found in the liver of meagre fed with DC and DMix diets, whereas the highest content of DHA was found in DP&L fed fish.

Lipid health indexes such as ΣPUFA/ΣSFA and Σn3/Σn6 ratios, show that the fillet of DP&L and DMix groups stand out with the highest Σ n3/Σ n6 ratio (2.74 and 2.30, respectively), whereas DP&L and DM show the highest Σ PUFA/ΣSFA ratio in the fillet (2.18 and 2.22, respectively). In the liver Σ n3/Σn6 ratio was also higher for DP&L and DMix whereas Σ PUFA/Σ SFA ratio was higher for DC and DP&L fish.

Fig 5 shows the correlations found between the fatty acid composition of the experimental feeds and the composition of meagre liver and muscle showing the close relationship between them, and the high nutritional quality of fish fillet (in terms of omega 3 fatty acid content, total N-3 and DHA) using tuna canning by-products (DP&L) or the mix (DMix) of all the ingredients assayed.

Histological organization of the liver

The general histological organization of the liver in meagre juveniles consisted of polyhedral hepatocytes with central nuclei and arranged in tightly packed anastomosed laminae around veins. The hepatic parenchyma was surrounded by a thin capsule of fibro-connective tissue. Liver histological evaluation of the samples taken at the end of the study from the fish fed experimental feeds revealed a high level of hepatocytes vacuolation due to lipid accumulation (Fig. 1 and image b) in fish fed the DI diet, whereas the rest of the groups showed a normal hepatocyte appearance (see Fig 1 image a)

Digestibility of feeds and ingredients

Table 8 shows the protein and lipid apparent digestibility coefficients (ADC) of the feeds used in the experiment and formulated using these sustainable ingredients and by-products. Protein ADC values were in all the cases higher than 70%, whereas lipid ADC varied between 78 and 86 %. However, no statistically significant differences were found in ADC values for proteins and lipids among experimental diets ($P > 0.05$).

Discussion

Global aquaculture production more than doubled in live-weight volume from 1999 to 2019 (FAO, 2020). FM and FO have been until now the main sources of protein and lipid in aquafeeds but the decrease in captures of forage fish and the increase in the price of these products (Tacon et al., 2011) have driven aquaculture producers to look for alternatives to these marine ingredients by plant-based ingredients and animal by-products (Davies et al., 2019; Pelletier et al., 2018). Novel feed ingredients such as insect meal (Belight et al., 2018, Stamer, 2015, IPIFF, 2019), micro- and macroalgae (Brown et al., 1997, Kiron et al., 2012, Roy and Pal, 2015), industry derived byproducts, such as those from breweries (Oliva-Teles and Gonçalves, 2001, Nazzaro et al., 2021, Estévez et al., 2021, Zhang et al., 2018) among others, have been recently considered also as aquafeed ingredients. Most of the published studies have examined the use of these alternative ingredients in an individual way or in side-by-side comparisons (Trushenski and Gause, 2013; Roques et al., 2018) whereas none have considered combining all these ingredients in the same diet. In the present study two consolidated novel ingredients such as insect meal and microalgae were used not only alone but also combined with new by-product alternatives derived from the canning industry: protein and lipid recuperated from tuna water cooking

Insect meal

The results obtained in growth and feed efficiency are different to previous studies carried out with meagre (Guerreiro et al., 2020, 2021; Coutinho et al, 2021) using *Hermetia illucens* (Diptera, Stratiomyidae, HI) and *Tenebrio molitor* (Coleoptera, Tenebrionidae, TM) included in the feed at different levels. According to the results obtained in the present study, the inclusion of insect meal in DI at 15% level did not lead to any adverse effect on meagre growth or performance. In our study, *Acheta domesticus* (Orthoptera, Gryllidae) was the species selected as the source of insect meal due to its easier and standardised breeding, and its high protein and fat content (Table 2). The results obtained can be considered new and innovative because no previous publication including *Acheta* in meagre feeds or in any other cultured marine fish was found in the literature.

Comparing the growth performance with that obtained using the control diet, no negative effects were observed in growth and feed efficiency except for a tendency for a higher level of fat accumulation in the fillet and the liver (Table 6 and Fig.1) and a lower content of omega 3 PUFA, including EPA and DHA, in both, fillet and liver (Fig.2). Guerreiro et al., (2021) and Coutinho et al., (2021) results showed a negative effect of insect meal inclusion in meagre feeds in fish growth, conversion, digestive enzyme activity and digestibility and recommended not to replace more than 15-17% of FM with insect meal (HI or TM). The results obtained in the present study in FCR (0.6 to 0.79) and PER (2.9 to 3.8) are much better than those indicated by Guerreiro et al., (2020) showing a good feed utilisation by the fish without any negative effect of insect meal inclusion. This good feed utilisation is reflected in the ADC values obtained. Protein digestibility was similar to that found for control feed whereas lipid digestibility was the second highest and similar to the control.

Regarding the final composition of the fillet and liver, Guerreiro et al., (2020) also found a slightly higher fat content, higher levels of SFA and lower n-3 PUFA and DHA in muscle when the fish were fed the highest inclusion level of HI.

Accumulation of fat in fish livers is a common morphological alteration when the amounts of dietary lipid/energy exceed the capacity of hepatocytes to oxidize fatty acids or when protein synthesis is impaired, resulting in excessive deposition of triglycerides in the vacuoles (Spisni et al., 1998). This condition (steatosis) can vary in severity from mild fat accumulation that does not compromise hepatocyte function to cell degeneration and impaired liver function that can ultimately result in fish death (Spisni et al., 1998). In cultivated fish, mild steatosis is a common finding due to the shift from a natural to an artificial diet, often containing high lipid levels (Spisni et al., 1998). More severe cases of steatosis have been described as the result of essential fatty acid deficiency (Montero et al., 2001) or inclusion of vegetable oils in fish diets (Caballero et al., 2004). In the present study, the inclusion of insect meal in the feed slightly affected meagre liver histomorphology and liver lipid deposition (Table 7) whereas did not show effects on HSI.

Microalgae

Previous studies carried out with fish and crustaceans (Sarker et al., 2020; Kiron et al., 2012; Gong et al., 2019) have shown the potential of microalgae for FM and FO full and/or partial substitution in aquafeeds due to their high protein content, optimal fatty acid composition, and equilibrated mineral profile. Various species of *Spirulina*, *Nannochloropsis*, *Chlorella*, *Isochrysis*, *Tetraselmis*, *Secenedesmus*, and *Schizochytrium* have shown their viability as ingredients in aquaculture feeds (Skali et al., 2020; Yarnold et al., 2019, Shah et al., 2018). In the present study a mix of species selected to get a final product rich in protein, EPA and DHA, amino acids, lipid, and various minerals, was used.

The results obtained with fish fed microalgae (DM) showed a significant lower final weight compared to the control group, although SGR was not different and RGR was slightly lower. Only few previous studies with meagre have used micro or macroalgae as FM substitution ingredients (Dos Santos, 2019) or as supplements in the feed to improve health status (Peixoto et al., 2017). DosSantos (2019) used *Fucus vesiculosus* and *Nannochloropsis gaditana* included at 1% or in a mixture of both species at 0.5% of feeds for meagre juveniles without any significant effect on growth performance. Peixoto et al., (2017) used seaweeds *Gracilaria* sp. and *Alaria* sp. included at 5% in the feeds without any effect on growth performance and health status. In the present study microalgae were included at 10% and a slightly lower growth was detected but both FCR and PER were similar to those obtained with the other ingredients, showing a good feed utilization by the fish. Protein digestibility (see Table 8) was higher than the control whereas lipid ADC was similar to that obtained using the protein and lipids derived from tuna canning industry or the mixture of all these sustainable ingredients.

Other publications using microalgae as feed for cultured fish at different inclusion levels such as *Arthrospira* at 7.5% (Teimouri et al., 2013), *Scenedesmus* spp at 5% (Skalli et al., 2020) and *Chlorella* combined with *Spirulina* at 12.5 % (Dallaire et al., 2007), showed no effect on fish growth or feed efficiency. Higher inclusion levels might have a negative effect on these parameters due to a lower feed intake either by the fish or to a lower digestibility. The results available in the literature suggest that the percentage of microalgae meal inclusion in the aquafeed might be changed depending on the microalgae used and the fish species (Shah et al., 2018), although more studies are needed. On the other hand, no negative effects of DM were observed in HSI or VSI or in the fat accumulation in hepatocytes. Final muscle and liver composition of the fish fed this diet showed a higher MUFA and n-6 fatty acid content (Fig.2) and a significantly higher fat accumulation in the liver that was not observed at histological level.

P&L diet

The effects of tuna by-products included in aquafeeds either as meal or oil have been previously studied in several fresh and marine water species (Goddard et al., 2008; Saïdi et al., 2010; Hernández et al., 2011). However, this is the first study using protein and oil recovered from tuna water cooking as ingredients in feeds for meagre ongrowing. The results obtained in terms of growth and feed conversion showed a similar performance than that observed in the DM and DI fed groups and slightly lower than the fish from control group (DC) without any difference in HSI or VSI. Tekinay et al., (2009) observed a reduction of SGR and PER of rainbow trout juveniles fed diets with 50, 60 and 70% inclusion of a meal elaborated with tuna by-products, as a consequence of a lower palatability and feed acceptance. Depending on the process used for tuna by-product obtention and/or the inclusion used in fish diet, differences on the effect of these ingredients were observed in the proximal composition of fish. Oncul et al., (2019) showed no significant differences in proximate body composition of juvenile olive flounder fed different inclusion levels of fermented tuna by-product meal. Kim et al., (2018), Bae et al., (2019) and Tekenay et al., (2009) also observed no differences in moisture, crude protein and ash of Korean rockfish and in rainbow trout fed with tuna by-product meal, whereas lipid content was affected. In the present study the results of final muscle and liver composition of meagre show that the fish fed this diet had the highest PUFA, total n-3, DHA, and DHA+EPA content and the highest ∑PUFA/∑SFA and n-3/n-6 ratios (Fig. 2) and in the case of the liver the highest protein content. As a consequence, meagre fed with these products have a better nutritional quality. Furthermore,

this diet gave the best results in terms of FIFO a very positive result assuming the use of half of FM and FO to guarantee the same biomass of fish produced compared to the control. Nowadays, it is more and more necessary to promote a sustainable aquaculture, producing more farmed fish with less resources and avoiding over-exploitation of wild fish.

Mix diet

The Mix diet has shown the best results in terms of fish growth and fillet composition showing the highest protein content and the second highest content of PUFA, n-3, EPA+DHA and n-3/n-6 ratio. This MIX diet is the diet with the second FI: FO value. Digestibility was also very high with protein and lipid ADC values around 80 %.

No negative effects were observed derived from the inclusion of alternative ingredients in the nutritional value of the fish and, indirectly, on human health. The fillet composition of DP&L and DMix fed fish stands out with 10% more total omega 3 than the other groups, probably due to the inclusion of microalgae and the oil recovered from the cooking water, both rich in omega 3. This increase in total omega 3 fatty acids was accompanied by a decrease in total omega 6, which affected significantly the n-3/n-6 index. The rest of the alternative ingredients showed similar nutritional value as the control, with the exception of the highest MUFA and lowest n-3 PUFA content in the fish fed DM and DI feeds. Similar results were found by Guerreiro et al., (2020) in a study using HI at different inclusion levels with a consistent increase of n-6 and a decrease of n-3 FAs in fish muscle and a decrease in n-3/ n-6 and Σ PUFA/Σ SFA ratios. According to these authors, one of the main concerns of replacing FM with insect meals in aquafeeds is its potential negative effect on fillet FA profile. This may be overcome by adding additional FO in the HI diets to compensate the EPA and DHA that was removed by the replacement of FM by HI. Such strategy was successfully applied in Atlantic salmon fed HI diets (Belghit et al., 2019). Another strategy may be the modulation of HI lipid content and FA composition that can be achieved by changing growth diets, since it will directly affect HI final composition.

The use of a mixture of different new ingredients such as this Mix diet can also be a good solution to compensate negative effects of insect meal and/or microalga inclusion, taking into account the results obtained with meagre fed Mix diet

Conclusion

This study provides a comparison between the effects of the inclusion of new alternative ingredients in meagre ongrowing diets. The results obtained using *Acheta domesticus* meal, P&L recuperated from water cooking in tuna canning factories and a mix of marine microalgae in diets for meagre juveniles were very good and, according to the results obtained, it seems that any of these ingredients might be used as alternative sources of protein and lipids in aquafeeds, since there was no negative effect on growth, feed conversion, muscle composition, fish health or final nutritional value. In the case of the diet using microalgae more research is needed in order to adjust the inclusion levels and/or different combinations of species. These new alternative ingredients showed a higher degree of sustainability as they present a lower Fish In: Fish Out ratio than the control diet used in the study. These results are quite promising because they integrate zootechnical efficiency together with environmental sustainability. The formulation of a more balanced Mix diet with an adequate percentage of microalgae can be a viable alternative to the combination of

alternative ingredients, since DMix was the second in FIFO ratio, gave good results in fish growth and conversion and provided very good final nutritional values.

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Tables and figures

Table 1. Formulation (%) and crude protein and lipid composition of the experimental diets used in the study.
DC: Control diet, DI: Insect meal diet, DM: Microalgae meal diet, DP&L: Protein and lipid from tuna water canning (TWC) diet, DMix: diet with all the ingredients mixed

Ingredients	**DC**	**DI**	**DM**	**DP&L**	**D Mix**
Squid meal	-	-	-	-	4.41
Fish meal	23.76	21.46	20.00	21.63	20.00
Insect meal	-	15.00	-	-	15.00
Microalgae	-	-	10.00	-	10.00
Protein (TWC)	-	-	-	7.00	2.00
Oil (TWC)	-	-	-	10.89	9.43
Pea starch	6.68	7.97	6.32	7.14	1.14
Wheat gluten	3.57	-	15.54	4.23	15.00
Wheat	6.00	6.00	6.00	6.00	17.12
Soy bean	6.16	5.18	4.63	6.18	0.15
Gluten meal	15.00	8.32	15.00	10.57	1.61
Guar meal	6.00	6.00	-	6.00	-
Salmon Oil	7.20	8.55	10.92	-	-
Krill Oil	4.00	-	-	-	-
AA mix (Aminopro)	5.00	5.00	5.00	5.00	2.29
Lysine	0.18	0.27	0.50	0.50	0.50
Threonine	0.00	0.07	0.32	0.29	0.50
Methionine	0.07	0.14	0.05	0.18	0.09
Choline	0.16	0.16	0.16	0.16	0.16
Taurine	0.16	0.18	0.17	0.18	0.15
Butyric acid	0.10	0.10	0.10	0.10	0.10
Antibacterian	0.15	0.15	0.15	0.15	0.15
Antifungal	0.015	0.015	0.015	0.015	0.015
Antioxidant	0.07	0.07	0.07	0.07	0.07
Attractant	0.10	0.10	0.10	0.10	0.10
Anhydrous betaine	0.07	0.07	0.07	0.07	0.07
Organic mineral conc.	0.10	0.10	0.10	0.10	0.10
Vitamin Conc.	0.10	0.10	0.10	0.10	0.10
Moisture (%)	7.07	11.99	9.38	6.22	5.98
Crude Protein (% DW)	47.08	47.18	47.11	47.63	46.83
Crude Fat (% DW)	14.88	15.55	16.14	14.87	16.81

Table 2. Composition (% of dry weight, DW) of the experimental ingredients used in the formulation of the feeds. Saturated (SFA), Monounsaturated (MUFA) and Polyunsaturated (PUFA) fatty acids are expressed in % total fatty acids (%TFA)
I: Insect meal, M: Microalgae meal, P&L: Protein and lipid from tuna water canning (TWC)

	I (*Acheta domesticus*)	**M** (mixed microalgae)	**P&L** (TWC)
Moisture (%)	6.4	10.0	6.2
Protein	62.2	25.8	56.3
Fat	24.4	9.0	43.7
SFA (%TFA)	37.0	28.0	30.4
MUFA	27.8	28.0	21.2
PUFA	35.3	42.0	42.1
EPA	0.1	34.9	7.4
DHA	0.1	0.1	26.5
n-3 PUFA	0.41	37.57	38.67
n-6 PUFA	35.67	5.43	6.28
n-3/n-6	0.01	6.55	6.16

662 Table 3.Fatty acid composition (% Total Fatty Acids, TFA) of the experimental feeds used in the
663 study. Different letters indicate significant differences (ANOVA; P<0.05)
664 DC: Control diet, DI: Insect meal diet, DM: Microalgae meal diet, DP&L: Protein and lipid from
665 tuna water canning (TWC) diet, DMix: diet with all the ingredients mixed
666

	DC	DI	DM	DP&L	Dmix
14:0	2.05 ± 0.40	0.93 ± 0.04	1.19 ± 0.05	1.83 ± 0.22	1.89 ± 0.10
15:0	0.18 ± 0.00	0.20 ± 0.01	0.20 ± 0.01	0.54 ± 0.01	0.48 ± 0.01
16:0	14.51 ± 0.21	16.63 ± 0.05	14.06 ± 0.24	18.47 ± 0.87	22.49 ± 0.53
18:0	3.12 ± 0.21	5.58 ± 0.06	3.11 ± 0.09	4.90 ± 0.72	5.09 ± 0.21
20:0	0.82 ± 0.00	0.53 ± 0.04	0.74 ± 0.06	0.33 ± 0.04	0.57 ± 0.00
Total saturated	**21.96 ± 0.39d**	**23.88 ± 0.00c**	**19.30 ± 0.46e**	**26.07 ± 0.34b**	**30.52 ± 0.41a**
16:1	3.22 ± 0.40	1.87 ± 0.03	3.28 ± 0.04	2.78 ± 0.01	4.55 ± 0.11
18:1n-9	23.75 ± 0.51	30.72 ± 0.15	31.43 ± 0.29	19.57 ± 0.33	16.67 ± 0.55
18:1n-7	3.81 ± 0.26	1.94 ± 0.08	3.13 ± 0.61	2.83 ± 0.38	2.59 ± 0.07
20:1	1.33 ± 0.12	1.61 ± 0.01	2.16 ± 0.00	1.23 ± 0.06	0.88 ± 0.04
Total monounsaturated	**34.81 ± 0.01c**	**36.42 ± 0.19b**	**40.37 ± 0.31a**	**26.84 ± 0.07d**	**24.99 ± 0.58d**
18:2n-6	21.04 ± 0.20	26.84 ± 0.03	23.41 ± 0.00	18.13 ± 0.12	16.95 ± 0.17
18:3n-6	0.25 ± 0.06	0.28 ± 0.05	0.30 ± 0.04	0.21 ± 0.03	0.21 ± 0.00
20:4n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.10 ± 0.04	1.21 ± 0.04
Total n-6 PUFA	**22.81 ± 0.13c**	**27.12 ± 0.03a**	**23.71 ± 0.05b**	**19.45 ± 0.12e**	**18.37 ± 0.13d**
18:3n-3	3.02 ± 0.18	3.43 ± 0.05	3.90 ± 0.09	2.38 ± 0.19	1.09 ± 0.01
20:5n-3	8.19 ± 0.05b	2.19 ± 0.01e	4.43 ± 0.07d	5.39 ± 0.15c	8.75 ± 0.03a
21:5n-3	0.57 ± 0.03	0.65 ± 0.06	0.71 ± 0.06	0.66 ± 0.09	0.62 ± 0.00
22:5n-3	0.78 ± 0.02	0.77 ± 0.01	0.88 ± 0.05	1.07 ± 0.05	0.84 ± 0.04
22:6n-3	6.11 ± 0.36c	5.39 ± 0.04d	6.45 ± 0.09c	18.13 ± 0.09a	14.82 ± 0.08b
Total n-3 PUFA	**20.43 ± 0.53c**	**12.58 ± 0.16e**	**16.62 ± 0.19d**	**27.62 ± 0.39a**	**26.12 ± 0.05b**
Total PUFA	**43.23 ± 0.40b**	**39.70 ± 0.19c**	**40.33 ± 0.15d**	**47.07 ± 0.27a**	**44.49 ± 0.18b**
Total FAs (mg/g Lipids)	610.94 ± 8.43c	654.27 ± 6.92a	636.95 ± 4.25b	611.91 ± 2.80c	632.94 ± 2.08b
PUFA/SFA	1.93 ± 0.05a	1.66 ± 0.01c	2.09 ± 0.06a	1.81 ± 0.03b	1.46 ± 0.01d
n-3/n-6	0.88 ± 0.03b	0.46 ± 0.01d	0.70 ± 0.01c	1.42 ± 0.03a	1.42 ± 0.01a
EPA+DHA	15.37 ± 0.30b	7.58 ± 0.03d	10.89 ± 0.16c	23.52 ± 0.23a	23.57 ± 0.11a

667 Table 4. Composition (% of dry weight, mean±SD) of the experimental diets used for meagre.
668 Different letters indicate significant differences (ANOVA P<0.05).
669

	DC	**DI**	**DM**	**DP&L**	**DMix**
Moisture (%)	7.07±0.01 c	11.99±0.19 a	9.38±0.06 b	6.22±0.09 d	5.98±0.03 e
Protein (%)	47.08±0.08 a	44.18±0.16 b	47.11±0.16 a	47.63±0.24ba	46.83±0.22 a
Fat (%)	14.88±0.34	15.55±0.81	16.14±1.24	14.87±1.28	16.81±0.90
SFA (%TFA)	21.96± 0.39 d	23.88±0.00 c	19.30±0.46 e	26.07±0.34 b	30.52±0.41 a
MUFA	34.81±0.01 c	36.42±0.19 b	40.37±0.31 a	26.84±0.07 d	24.99±0.58 d
PUFA	43.23±0.40 b	39.70±0.19 c	4..33±0.15 d	47.07±0.27 a	44.49±0.18 b
EPA	8.19±0.05 b	2.19±0.01 e	4.43±0.07 d	5.39±0.15 c	8.75±0.03 a
DHA	6.11±0.36 c	5.39±0.04 d	6.45±0.09 c	18.13±0.09 a	14.82±0.08 b
n-3 PUFA	20.43±0.53 c	12.58±0.16 e	16.62±0.16 d	27.62±0.39 a	26.12±0.05 b
n-6 PUFA	22.81±0.13 c	27.12±0.03 a	23.71±0.05 b	19.45±0.12 e	18.37±0.13 d
∑PUFA/∑SFA	1.93±0.05 a	1.66±0.01 c	2.09±0.06 a	1.81±0.03 b	1.46±0.01 d
n-3/n-6	0.88±0.03 b	0.46±0.01 d	0.70± 0.01 c	1.42±0.03 a	1.42±0.01 a
EPA+DHA	15.37±0.30 b	7.58±0.03 d	10.89±0.16 c	23.53±0.23 a	23.57±0.11 a
Total Fatty Acids (TFA, mg/g Lipids)	610.94±8.43 c	654.27±6.92 a	636.95±4.25 b	611.91±2.80 c	632.94±2.08 b

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671

672 Table 5. Initial and final weight and length, growth (SGR and RGR, %), hepatosomatic (HSI) and
673 viscerosomatic (VSI) indices, feed conversion and protein efficiency (FCR and PER ratios) and
674 FIFO rate of meagre fed the experimental diets. Different letters indicate significant differences
675 (ANOVA $P<0.05$)
676

	DC	**DI**	**DM**	**DP&L**	**DMix**
Initial weight (g)	12.61±1.55	12.50±1.38	12.44±1.44	12.52±1.58	12.47±1.46
Final weight (g)	87.24±16.83a	79.75±17.17b	79.04±14.06b	80.17±17.17b	89.63±15.75a
SGR (% d^{-1})	2.76±0.02	2.65±0.01	2.68±0.06	2.65±0.03	2.11±1.41
RGR (%)	5.92±0.09a	5.38± 0.03b	5.35±0.10b	5.41±0.12b	6.20±0.34a
HSI (%)	2.89±1.25	3.26±1.52	2.49±1.45	2.00±1.50	1.98±0.46
VSI (%)	5.58±1.20	5.78±1.46	5.16±1.56	4.64±1.29	4.19±0.57
PER (%)	3.52±0.11a	3.77±0.04a	3.76±0.15a	3.61±0.04b	2.92±0.18b
FCR	0.60±0.02b	0.65±0.01b	0.64±0.03b	0.64±0.01b	0.79±0.05a
FIFO	0.68±0.02b	0.71±0.04a	0.72±0.04a	0.50±0.03e	0.58±0.03c

677

678 Table 6. Muscle composition (% of dry weight, mean±SD) of meagre fed the experimental diets.
679 Different letters indicate significant differences (ANOVA P<0.05).
680

	DC	DI	DM	DP&L	DMix
Moisture (%)	77.41±0.34 b	77.03±0.36 b	77.55±0.23 b	78.64±0.14 a	77.35±0.16 b
Protein (%)	17.82±0.41 c	19.32±0.50 b	17.75±0.40 c	18.46±0.37b	20.42±0.72 a
Fat (%)	0.85±0.01 b	1.09±0.00 a	0.81±0.01 b	0.85±0.03 b	0.75±0.02 c
SFA (%TFA)	25.98± 0.17 b	26.02±0.83 b	22.68±0.00 d	24.76±0.30 c	28.48±0.50 a
MUFA	25.15±0.17 b	28.12±0.39 a	27.85±1.00 a	20.00±0.17 c	18.60±0.18 d
PUFA	48.86±0.02 c	45.87±0.44 d	49.46±0.99 c	54.99±0.13 a	52.60±0.32 b
EPA	8.27±0.13 a	3.92±0.08 d	5.12±0.06 c	4.99±0.00 c	6.80±0.01 b
DHA	16.74±0.14 c	14.80±0.19 d	17.74±0.41 c	32.16±0.14 a	27.29±0.45 b
n-3 PUFA	31.18±0.10 c	22.79±0.12 e	28.54±0.71 d	40.29±0.03 a	36.65±0.45 b
n-6 PUFA	17.68±0.11b	23.08±0.32 a	20.93±0.28 a	14.71±0.17 d	15.95±0.13 c
∑PUFA/∑SFA	1.89±0.01 b	1.67±0.06 d	2.18±0.04 b	2.22±0.03 a	1.85±0.04 c
n-3/n-6	1.78±0.02 c	0.99±0.01 e	1.36±0.02 d	2.74±0.03 a	2.30±0.05 b
EPA+DHA	26.91±0.27 c	18.72±0.27 e	22.86±0.35 d	37.15±0.14 a	34.09±0.47 b
Total Fatty Acids (TFA, mg/g Lipids)	607.03±6.23 b	620.97±0.01 a	495.38±51.30 c	586.73±12.57b	591.41±6.63 b

681

682

683 Table 7. Liver composition (% of dry weight, mean±SD) of meagre fed the experimental diets.
684 Different letters indicate significant differences (ANOVA P<0.05).

685

	DC	DI	DM	DP&L	DMix
Moisture (%)	60.17±0.14 c	59.22±0.19 e	59.86±0.13 d	65.38±0.06 a	61.76±0.0.4 b
Protein (%)	7.80±0.30 b	9.34±0.42 a	9.98±0.29 a	9.44±0.21 a	8.01±0.23 b
Fat (%)	14.98±0.56 b	17.56±0.29 a	16.90±0.22 a	12.34±0.19 b	13.85±0.85 b
SFA (%TFA)	26.14± 0.78 c	25.12±0.14 c	24.14±0.15 d	28.41±0.53 b	32.95±0.73 a
MUFA	37.94±0.159 b	42.53±0.02 a	43.35±0.24 a	31.67±0.16 c	31.25±0.58 c
PUFA	35.93±0.18 b	32.35±0.11 c	32.50±0.39 c	39.80±0.70 a	35.77±0.15 b
EPA	4.36±0.13 a	1.58±0.02 d	2.15±0.09 c	3.02±0.15 b	4.07±0.15 a
DHA	5.04±006 c	3.48±0.05 d	3.52±0.20 d	13.00±0.70 a	9.58±0.20 b
n-3 PUFA	14.72±0.06 c	8.73±0.11 e	10.84±0.44 d	19.95±1.00 a	16.35±0.30 b
n-6 PUFA	21.21±0.24 b	23.61±0.00 a	21.66±0.05 b	19.85±0.30 c	19.43±0.16 c
∑PUFA/∑SFA	1.41±0.05 a	1.03±0.38 c	1.35±0.02 b	1.40±0.05 a	1.09±0.03 c
n-3/n-6	0.63±0.01 c	0.37±0.00 e	0.50±0.02 d	1.01±0.07 a	0.84±0.02 b
EPA+DHA	9.98±0.06 c	5.05±0.08 d	5.67±0.29 d	16.02±0.85 a	13.64±0.35 b
TFA (mg/g Lipids)	729.67±12.31 c	774.45±1.48 b	825.44±7.28 a	690.66±4.18 d	784.68±9.10 a

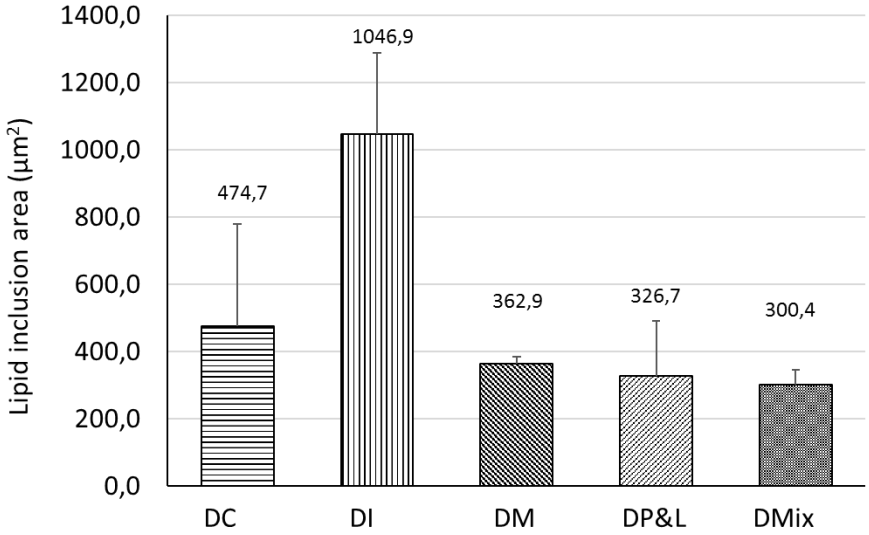
686

687 Table 8. Apparent digestibility coefficient (ADC, %) of protein and lipids of the different diets
688 used.
689

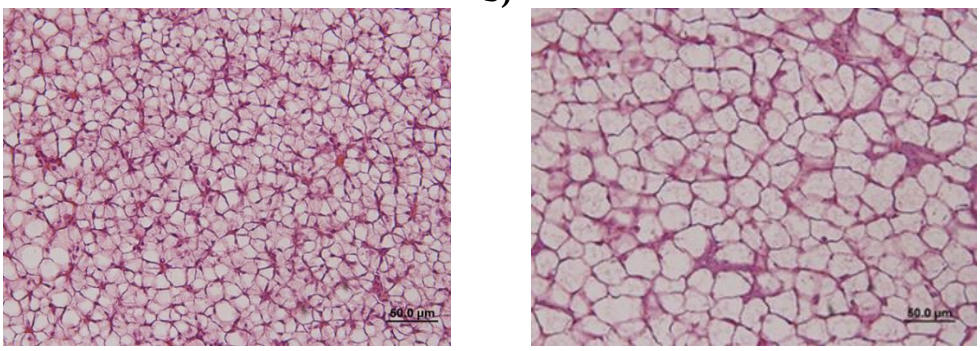
	DC	**DI**	**DM**	**DP&L**	**DMix**
ADC Protein	72.76	73.76	77.07	74.67	79.96
ADC Lipids	86.11	84.83	79.86	78.15	80.72

690

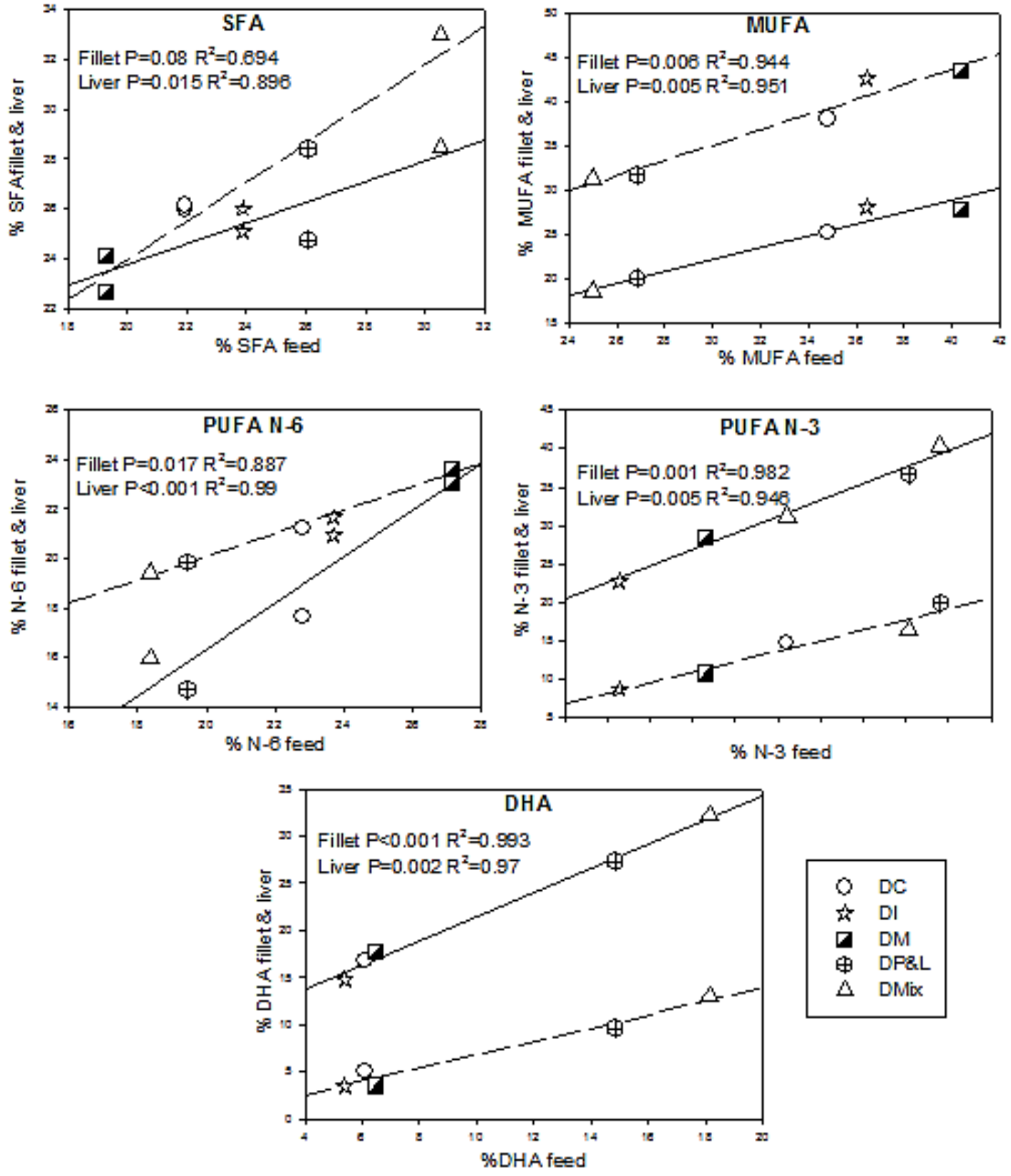
Figure 1. Lipid inclusion area (µm2) of the fish fed the experimental diets: DC (control diet), DM (Microalgae diet), DP&L diet (Protein and lipid from tuna water cooking), Insect diet (DI) and Mix diet (DMix). The photographs included show an image of the liver of DC (a) and DI (b) fed fish



a) **b)**



703 Figrure 2.- Regressions (R^2 and P values) between the main fatty acids in the feeds (DC, DI, DM,
704 DP&L, DMix) and in the muscle (solid line) and liver (long dash line).
705



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