Afonso Manuel Roberto Valente

Optimizing the nutritional composition of commercial diets for flatfish larvae

University of Algarve

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Abstract

Aquaculture industry is the food sector with higher contribution to recent growth in global food supply. Atlantic salmon (*Salmo salar*) is the most commercialized species in Europe. Mediterranean aquaculture is dominated by seabass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*). Senegalese sole (*Solea senegalensis*) and turbot (*Scophthalmus maximus*) are two flatfish identified with potential in aquaculture whose production recently increased significantly in Portugal, Spain and France especially due to research effort made for these countries, in subjects such as weaning strategy, larvae nutrition and disease control. There are important improvements still needed for these species because they are cultivated in recirculation systems and water quality should be kept under control. It is necessary to find formulations that comply with larval necessary nutritional requirements and allow reduce nutrient leaching into the water and adapted to the flatfish species-specific feeding behaviour. The type of binder and/or binding process selected in a diet formulation and feed production technology will influence the leaching and physical characteristics of the pellets. Ingredients that provide microdiets a higher capacity of sinking of the pellets are more suitable for Senegalese sole that feed on the bottom, while binders that will slow-down sinking should be used for turbot diets that feeds on the water column.

This thesis aims to perform adaptations in microdiets formulation in order to improve their physical properties and leaching and evaluate how the new ingredients affect the Senegalese sole larvae growth performance. New binders and different combinations of them were used to reduce Turbot microdiets nutrient leaching, adjust the dispersion and sinking capacity and also correlate with the biological efficiency which was evaluated in two trials.

On trial 1 Senegalese sole larvae were fed with four different microdiets. The used diets were a commercial microdiet as control, and 3 diets with different ingredients. B/C was a diet with high benefit/cost ratio composed of high quality fish and squid meals and a mixture of plant-proteins including wheat gluten and pea protein concentrates, LFAT a diet with a similar composition to diet B/C, but where the reduction of the crude fat content was targeted, and PL was a diet also with a similar composition to diet B/C, but where the inclusion of a higher phospholipid content was targeted. The growth performance from all treatments was not significantly different

except on LFAT which had a significantly lower growth performance, but regarding the microdiets properties PL was the diet with lower protein leaching, B/C and PL were the diets with higher sinking capacity and B/C was the diet with higher dispersion capacity. Furthermore, the results suggest that PL and B/C can be considered the best treatments used on trial 1 essentially due to the growth performance results which are similar to the control treatment and represent a higher benefit/cost ratio, but because they also fulfil important requirements on physical properties.

On the second trial four different microdiets were tested in Turbot larvae, a diet used as control and three experimental diets. CTRL was a commercial-like diet, being composed of high quality fish and squid meals and plant-proteins including wheat gluten. MIX was produced with a similar composition to CTRL but using a mixture of binders in its dietary composition, LOW was produced with a similar composition to CTRL, but using a novel binder at a low inclusion level, and HIGH was produced with a similar composition to CTRL, but using a novel binder at a high inclusion level. At the end of trial CTRL and HIGH were the diets with best results for growth performance. Despite HIGH and CTRL does not have the best results on leaching reduction and the physical properties tests were not the ones who best fit into the Turbot feeding behaviour, the growth performance obtained with this dietary treatment was higher than the remaining treatments. When compared with other treatments, MIX can be a good solution to reduce leaching but will result in a with lower growth performance. CTRL and HIGH were, therefore, the most advantageous solutions.

In summary, B/C and PL microdiet for Senegalese sole was the best solution due to it high benefit/cost ratio, combined with a good growth performance and reduced protein leaching, regarding Turbot, the best treatments were CTRL and HIGH due to its best growth performance results.

Keywords: Senegalese sole, Turbot, microdiets, binders, leaching, physical properties

Resumo

A aquacultura é o setor da indústria alimentar com maior contribuição para o fornecimento global de alimentos. O Salmão do atlântico (*Salmo salar*) é a espécie mais comercializada da Europa. A aquacultura mediterrânica é dominada pelo Robalo (*Dicentrarchus labrax*) e pela Dourada (*Sparus aurata*). O Linguado (*Solea senegalenses)* e o pregado (*Scophthalmus maximus*) são dois peixes planos cuja produção aumentou recentemente e de forma significativa em Portugal, Espanha e França, essencialmente devido ao esforço na investigação feito por estes países, sobretudo em temas como a estratégia de desmame, nutrição larvar e controlo de doenças. Ainda assim, são necessárias melhorias na produção destas espécies devido ao seu tipo de cultivo, pois são cultivadas em sistemas de recirculação e a qualidade da água deve ser controlada. É importante encontrar dietas formuladas de modo a reduzir a lixiviação e que em simultâneo sejam adaptadas ao comportamento predatório dos peixes planos sem que os requisitos nutricionais das espécies sejam prejudicados. O tipo de ligante ou a combinação de ligantes utilizada na formulação de uma dieta vai influenciar a lixiviação e as propriedades físicas dos pellets. Ingredientes que forneçam uma maior capacidade de afundamento às partículas são mais adequados para microdietas formuladas para Linguado que se alimenta no fundo, por outro lado, ligantes que proporcionem uma menor taxa de afundamento devem ser utilizados em dietas para pregado.

Esta tese tem como objetivo testar adaptações na formulação de dietas para Linguado, com o intuito de reduzir a lixiviação e melhorar as suas propriedades físicas, e em simultâneo avaliar se o crescimento é afetado. Novos ligantes e diferentes combinações dos mesmos, foram utilizados em microdietas para Pregado com o objetivo de avaliar a sua capacidade de reter nutrientes e ajustar a capacidade de dispersão e afundamento dos pellets, verificando se existe uma correlação com o crescimento larval que foi avaliado em dois ensaios.

No primeiro ensaio, as larvas de Linguado foram alimentadas com quatro dietas diferentes. Foi utilizada uma dieta comercial como controlo e 3 dietas com ingredientes diferentes. A dieta B/C foi uma dieta formulada com uma mistura de proteínas vegetais e concentrados de proteína de ervilha, a dieta LFAT foi formulada com uma composição semelhante à B/C em que o seu conteúdo foi direcionado para a redução do

teor de gordura, e a dieta PL que também foi formulada com uma composição semelhante à dieta B/C, mas direcionada para a adição de um maior teor em fosfolípidos. Relativamente ao desempenho, os resultados obtidos para o crescimento não foram significativamente diferentes excepto na microdieta LFAT que apresentou piores resultados que os restantes tratamentos, mas relativamente às propriedades fisicas das dietas, a PL foi o tratamento com menor percentagem de lixiviação. B/C e PL foram as dietas com maior capacidade para afundar e a dieta B/C foi a dieta que obteve uma maior área de dispersão. Os resultados sugerem que as dietas B/C e PL podem ser consideradas as melhores dietas devido aos bons resultados de crescimento quando comparados com a microdieta utilizada como controlo e à sua elevada relação benefício / custo, mas também por cumprirem requisitos importantes das propriedades físicas.

Relativamente ao segundo ensaio todas as dietas foram testadas em Pregado, sendo a dieta controlo uma dieta com proteínas vegetais e as restantes dietas três dietas em que na MIX o ligante existente foi substituído por uma mistura de dois ligantes, na dieta LOW o ligante foi substituído por um novo ligante, mas em pequena concentração, e na dieta HIGH o ligante foi substituído pelo mesmo ligante que na dieta LOW, mas com concentrações mais elevadas. No fim do ensaio apesar de as microdietas HIGH e CTRL não apresentarem os melhores resultados na redução da lixiviação, e de não serem as microdietas cujo as propriedades físicas melhor correspondem ao comportamento alimentar das larvas de pregado foram as dietas com melhores resultados de crescimento. Quando comparadas com as outras microdietas testadas como a MIX que apresenta melhores resultados. na prevenção da lixiviação. As dietas HIGH e CTRL foram as hipóteses mais vantajosas para o crescimento larvar.

Para concluir, a dieta B/C e PL para Linguado foram as melhores opções, devido ao seu elevado beneficio/custo combinado com uma boa performance de crescimento e uma lixiviação reduzida, relativamente ao Pregado as dietas testadas que apresentaram melhores resultados foram as dietas CTRL e HIGH devido aos seus bons resultados para o crescimento.

Palavras-Chave: Linguado, Pregado, microdietas, ligantes, lixiviação, propriedades físicas

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DAH: Days after hatching

TL: Total length

DW: Dry weight

FCR: Feed conversion ratio

RGR: Relative growth rate

1. Introduction

1.1. Aquaculture industry global overview

Aquaculture industry is an important animal food sector to supply the global food demand. Aquaculture is considered a fast-growing sector, and became an alternative for fisheries industry that has been relatively stable in volumes since 1980. Food and Agriculture Organization of the United Nations (FAO) estimates that combined, fisheries and aquaculture production in 2018 reached 179 million tonnes. Since 1990, total fish production suffered some fluctuations in Europe and has almost doubled in Asia. In the year of 2018, total fish production in Asia represented a significant share in global fish production with approximately 69 % of global fish captured and produced. China was the country with highest contribution for Asian total fish production (35 %). Aquaculture represents 46 % of the global fish production, according to the latest statistics on aquaculture compiled by FAO. In 2018, a total of 622 species were farmed for reporting countries, although FAO also admits that sometimes the reporting country is not capable of counting the total number of aquatic species produced (FAO, 2020).

In 2018, European aquaculture production reached 3082 thousand tonnes, being most of that production from Norway (1.354 thousand tonnes) essentially from Atlantic salmon production (APROMAR, 2020; FAO, 2020). Heavily dependent on imported fish, European market is constantly looking to find new species with potential to balance the market where the production fails to fully satisfy the demand. Evaluate the potential of a new species requires knowledge about biological and economical aspects. Meagre (*Argyrosomus regius* Asso, 1801), the greater amberjack (*Seriola dumerili* Risso, 1810), the wreckfish (*Polyprion americanus* Bloch and Schneider, 1801), the Atlantic halibut (*Hippoglossus hippoglossus* Linnaeus, 1758), the grey mullet (*Mugil cephalus* Linnaeus, 1758) and the pikeperch (*Sander lucioperca* Linnaeus, 1758) are some of the species identified with potential to balance the market. Senegalese sole and turbot are also considered promising species for aquaculture diversification and its production has gradually increased over the past few decades (Mylonas and Robles, 2014; Morais *et al*., 2016; APROMAR, 2020).

1.2. Aquaculture in Portugal

Portuguese coastline represents 1.8% of the European Union coastline. Ranked as one of highest per capita aquatic products consumption countries in Europe, Portuguese fisheries and aquaculture industry have low contribution to the economy but a large cultural and social importance especially in traditional fishing towns (FAO, 2020). In 2018, Portuguese aquaculture industry produced nearly 14 thousand tonnes, which represents an increase of 11.5% relatively to 2017 and a 96.8 million Euros income, 18.5% more than in 2017 (INE, 2019). Portugal was considered the second European country with highest production of turbot (*Scophthalmus maximus* Linnaeus, 1758) with 2.3 thousand tonnes. Besides turbot, gilthead seabream (*Sparus aurata* Linnaeus, 1758) and European seabass (*Dicentrarchus labrax* Linnaeus, 1758) were the most produced species in 2018. Regarding Senegalese sole, Portugal is considered one of the European countries with highest production in 2018, with 145 tonnes produced. (APROMAR, 2020; INE, 2019).

1.3. Senegalese sole and Turbot

1.3.1. Senegalese Sole

Senegalese sole is a marine flatfish well adapted to warm climates and commonly raised in shallow raceway systems combined with re-circulation aquaculture systems (Bjorndal *et al*, 2016). The spawning season in nature occurs during spring. In farming conditions spawning season can be extended from March to November by temperature manipulation and photoperiod, so far mostly using wild broodstocks. Temperature is an important factor for egg emission because for temperatures below 16 ºC egg emission stops (Morais *et al*., 2016).

Adequate culture conditions to the complex metamorphosis of Senegalese sole will enhance larvae survival and quality. Mouth opening in Senegalese sole occurs relatively early at 2 DAH, at this stage the incipient stomach is distinguishable, and the mouth gape is wide enough for zooplanktonic preys as enriched rotifers, although small *Artemia* nauplii can also be used as first feeding (Conceição *et al*., 2007; Morais *et al*., 2016). Despite live-feeds enhance growth performance and survival of Senegalese sole larvae during early stages, they may also act as vectors for diseases and its nutritional

value is difficult to manipulate. On the other hand, inert microdiets provide nutritional consistency. To improve larval performance in this stage, decades of research were necessary, focusing essentially in feeding regimes and microdiet quality. At mouth opening, a co-feeding strategy with live-feed and inert microdiets will bring benefits for the development of the species in the long term (Engrola *et al*., 2009). In long-term co-feeding with moderate replacement of live-feeds at mouth opening, a co-feeding regime with live-feed (65% of *Artemia* provided) and inert microdiet is initially established. During this period live-feed is gradually replaced for inert feed, at 25 DAH diet is only composed by inert feed. After settling, a short-term co-feeding with high replacement of live-feeds may also bring benefits in terms of growth and survival. In short-term co-feeding with high replacement of live-feeds immediately after larval settling strategy, only 20% of the Artemia is provided which leads to high replacement of live-feed by inert feed. Both strategies have acceptable results, however a short period co-feeding after settling led to higher survival rates (Pinto *et al*., 2018).

1.3.2. Turbot

Turbot is a marine flatfish with asymmetric body and well adapted to shallow waters and sandy or muddy bottoms (Aydin *et al*., 2020). It has characteristics such as fast growth rate and high tolerance to water quality changes. Turbot production mainly occurs in recirculation and flow-through systems (Liu *et al*., 2019). Although turbot is a species quite tolerant to changes in water quality, culture conditions must be continuously monitored, in captivity oxygen should be kept at approximately 9.5 mg/L, pH level must be set between 7.5 and 8.5 while temperature can be settled between 16ºC and 19ºC (Aydin *et al*., 2020).

Generally, fertilization and spawning are artificially induced, and becomes more effective if spawners were reared in hatcheries, although the spawns from wild broodstocks may occur without hormonal help, a successful fertilization is not totally ensured (Aydin *et al*., 2020). Under well-established culture conditions, sperm of a male can be collected from november to september while female eggs can be collected from May to August. Larval development of turbot lasts 70 days, at 16-19 ºC. Until 2 DAH, mouth and anus are not developed, pelagic larvae are about 2.5 mm long and have transparent eyes. Between 3 and 29 DAH mouth and anus start developing, one of the former lateral side becomes the ventral side, the dorsal and anal fin rays are complete at

25 DAH. The eyes coloration becomes visible, and one eye migrate to other side of the body, and it becomes a benthonic post-larvae. From 30 to 70 DAH larvae become juveniles. Turbot first feeding take place at 3 DAH (Person-Le Ruyet, 2002; Aydin *et al*., 2020). Rotifers are generally used as first feed combined with phytoplankton species, this combination assists rotifers to be identified by larvae and stimulate the immune system and improve the water quality of the system. Artemia nauplii can be given between 12 DAH and 30 DAH and at 21 DAH Artemia metanauplii can also be used at turbot larvae diet (Neori, 2011; Aydin *et al*., 2020). Despite the benefits of using live feed as enriched rotifers and *Artemia* to achieve the nutritional larvae requirements, this usage may also be the cause of larvae malpigmentation and other metamorphosis problems due to *Artemia* content limitation of iodine and possibly other minerals (Bruno *et al. 2017*; Conceição *et al.*,2010). To attenuate this problem inert feed can be included on larvae diet to provide the specific compounds that are not provided through the live-feed (Conceição *et al.*,2010). Inert microdiets can be included on Turbot feeding plan at 25 DAH with initial granulometry of 200- 400 µm that will gradually increase according to the size of the larvae (Aydin *et al*., 2020).

1.4. Microdiets for flatfish

Aspects as reproductive and life cycle, rearing conditions, behaviour and nutrition are important to establish a suitable production of a given species. The success of producing new species depends heavily on a well establish nutritional plan during the early stages of development (Toomey *et al*., 2020). Totally or partial replacement of live-feed to inert microdiets will provide nutritional advantages to fish development, despite weaning fish larvae onto inert microdiets also bring some challenges for fish nutrition (Kolkovski, 2013). For inert microdiet formulation aspects as larval feeding behaviour should be considered, during larval growth pellet physical characteristics as sinking capacity should be adjusted according to the flatfish larvae feeding behaviour, that can occur in the bottom as it happens in sole or a feeding behaviour where the larvae remain in the water column due to its swimming capacity what correspond into the Turbot feeding behaviour. (Bruno *et al*., 2018; Debnath *et al*., 2020; Wu *et al*., 2020). These microdiets should be also well formulated and produced with a suitable technology to ensure that several steps involving chemical and visual stimulation on larval process of finding and ingesting feed are complete and satisfy the energetic and nutritional

requirements for larval development. Furthermore, after immersed on water, feed particles must be able to avoid disintegration and ensure the retention of water-soluble nutrients which are an important energy source and are considered a requirement to optimize the growth and survival of the larvae (Ribeiro *et al*., 1999; Kolkovski, 2008; Hamre *et al*.,2013). Another limitation that should be considered is the high nutrient leaching, that occurs when the pellets are in contact with the water and results on leakage of water-soluble nutrients from the microdiet, and consequently the diet will have a sub-optimal concentration of these nutrients that are important for a proper larval development (Langdon, 2003; Kvåle *et al*., 2006). During the first minutes of immersion microdiets can lose 26 and 28 % of water-soluble proteins, which is an high value considering the importance of this nutrients for fish development. Losing proteins on water will not only influence the larvae growth performance as also will negatively influence the water quality due to the increment of ammonia and the development of microorganisms that will deteriorate the water and possibly cause infections on larvae (Kvåle *et al*., 2006; Hamre *et al*.,2013).

Binders are ingredients which provide more stability and firmness to the pellet, they can be divided in 3 groups, binders of protein origin as gelatine and collagen, carbohydrate source binders as different starches or molasses, and binders without nutritional value as carboxymethylcellulose and alginates (Cuzon *et al*. 1994; Palma *et al*., 2007). Some possible binders that can be used on diet formulation are agar, gelatine, carrageen, carboxymethylcellulose, and depending on the type of the used binder, microdiets stability will change (Palma *et al*., 2007). Binders can influence the pellet stability through the reduction of void spaces which results in more compact and durable pellets, by acting as adhesives and also altering the nature of the feed through chemical action (Ruscoe *et al*., 2005; Palma *et al*., 2007).

Considering that binders are able to provide stability to the pellets which is defined by the capacity of reduce the physical disintegration, binders are important to substantially reduce the nutrient leaching, and improve the efficacy and durability of a microdiet (Obaldo *et al*., 2002; Palma *et al*., 2007; Haetami and Abun, 2021). Diet composition is one of the aspects that affect the leaching, microbound diets, formulated with gelatine binders have higher leaching when compared with micro-encapsulated diets (Kvåle *et al*., 2006; Kolkovski, 2008). Research efforts in this specific area of larval feeding and nutrition are particularly important in species cultured in RAS

systems such as flatfish. These efforts have influenced positively the aquaculture production of Senegalese sole (*Solea senegalensis* Kaup, 1858) and turbot (*Scophthalmus maximus* Linnaeus, 1758) that recently increase in Europe specially in Portugal, Spain and France (APROMAR, 2020).

1.5. Objectives

This thesis aims to perform adaptations to optimize microdiets of Senegalese Sole and Turbot. Ingredient replacement by new ingredients was performed Senegalese sole microdiets and new binders and different combinations of them were used on Turbot. Microdiets were produced with the aim of reduce the leaching and adapt the dispersion and sinking capacity to both species feeding behaviour, providing the adequate nutrition to the larvae.. Two trials tested the biological efficiency with objective of evaluate how different ingredient replacement affects growth performance, survival, and feed conversion of Senegalese sole and how the usage of different inclusion levels of a binder and also a mixture of binders affect the same parameters on Turbot larvae. Microdiet sinking, dispersion and leaching analysis tested how the experimental diets are adequate for Senegalese sole and turbot feeding behaviour. Leaching test evaluated the amount of protein lost on water. Further analysis investigated if the microdiets sinking and dispersion capacity, and leaching was related with fish larvae growth performance, survival and feed conversion

2. Trial 1 – Effects of ingredient replacement on the performance of Senegalese sole larvae

2.1. Material and methods

2.1.1. Dietary treatments

In trial 1 a total of four dietary treatments were tested: a commercial diet (COMM) as control, and three experimental diets, B/C, LFAT and PL. The COMM diet was a commercial diet for flatfish produced by SPAROS Lda. containing high quality fish, squid and krill meals. Diet B/C was a commercial-like diet with a high benefit/cost ratio, being composed of high quality fish and squid meals and a mixture of plant-proteins including wheat gluten and pea protein concentrates. Diet LFAT was formulated and produced with similar composition to B/C diet, but targeting the reduction of the crude fat content. The diet PL was formulated and produced with similar composition to diet B/C but targeting the inclusion of a higher phospholipid content. Diets were produced at SPAROS Lda (Olhão, Portugal) where ingredients were mixed according to the established formulation in a double-helix mixer, and then ground twice in a micropulverizer hammer mill. The oil fraction of the formulation was added followed by humidification and agglomeration through low-shear extrusion. Posteriorly, diets were dried for 4 h at 60 ºC and subsequently crumbled and sieved in different size ranges.

A short-term co-feeding period with high replacement of live feeds after larval settling was the weaning strategy used on larvae (Pinto *et al*, 2018). From 27 to 28 DAH, larvae were fed with enriched frozen *Artemia*. From 29 to 32 the larvae were co-feed with frozen *Artemia* and experimental diets (200-400 µm). The initial diet amount for each tank was 1 g per day divided in 8 meals per day distributed by automatic feeders and 0.5-1.0 million/tank of frozen *Artemia* per day in 2 meals. After this period, the enriched frozen *Artemia* was fully replaced by inert microdiets which range size was 400-600 µm from 33 to 50 DAH and 600-800 µm from 51 until 68 DAH.

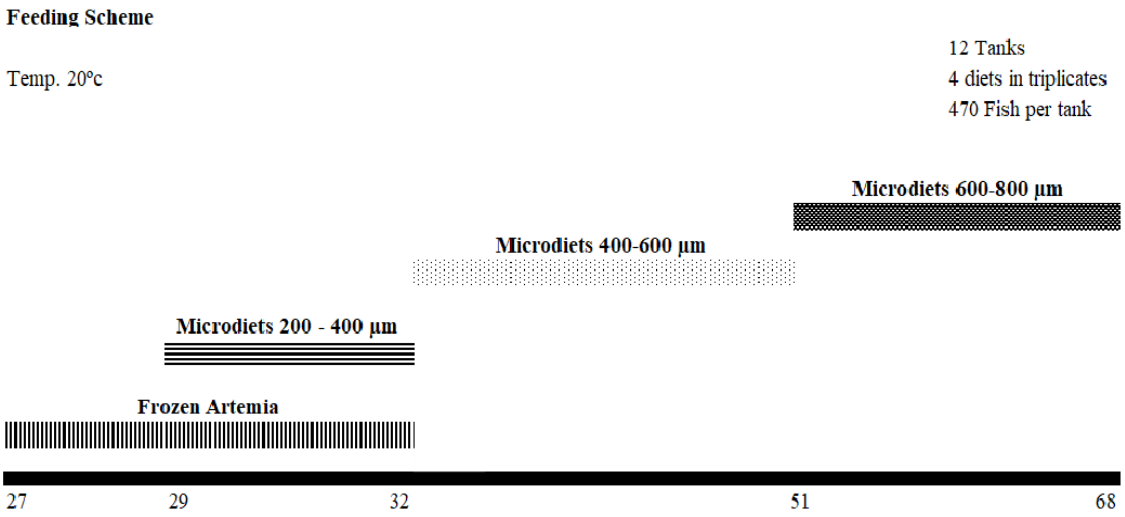

Figure 1 – Trial 1 experimental design. Initially larvae were fed with frozen *Artemia*, following a co-feeding period where the experimental diets were introduced, frozen *Artemia* was fully replaced by inert microdiets at day 32.

2.1.2. Fish rearing

Trial 1 was performed with Senegalese sole post-larvae at SPAROS Lda. Research facility (Olhão, Portugal). Senegalese sole post-larvae were shipped from the EPPO facility of Instituto Português do Mar e da Atmosfera (IPMA, Olhão, Portugal) where they were reared until 27 DAH. The larvae were reared from 27 to 68 DAH and distributed by treatments in triplicates for 12 tanks each one with 8L. A total of 470 larvae were placed in each tank at a larvae density of 4500 larvae per m^2. Light intensity and photoperiod were settled at 105 lux (9h00-18h00) and 0 lux- (19h00-9h00). The rearing system was set to a semi-open recirculating system with a water renewal of 2 times per day and 2 times per hour in each 8L tank. Water renewals were adjusted according to the amount of nitrogen compounds that were controlled to stay at minimum values. Water parameters such as dissolved oxygen, salinity, temperature and nitrogen compounds were measured and recorded daily. Dissolved oxygen was kept in a concentration higher than 90% ± 1%, salinity at 36 g/L, and temperature 20ºC ± 0.2 ºC. Cleaning and siphoning of each tank was part of the daily routines that were adjusted according to the fish growth during the trial. Initially, post-larvae were exclusively fed with frozen Artemia which was supplied in 4 meals per day, with the daily routine presented in Table 1. During the co-feeding period which daily routines were as

described in Table 2, the daily amount of frozen *Artemia* was divided in four meals, while the daily amount of inert diet was supplied with automatic feeders set to provide 8 meals per day. Each meal took a duration of 3 hours, in which the automatic feeder provided the feed for 2 hours and stopped for 1 hour. Feeders were checked and cleaned daily to ensure proper functioning, and check if significant amounts of feed were not discharged, these were quantified and registered. After the co-feeding period and until the end of the trial, Senegalese sole larvae were fed exclusively with inert microdiets, and the daily routine was adjusted as presented in Table 3.

Table 1- Daily routines from 27 to 28 DAH.

Hours	Daily routine (27 to 28 DAH)
9:00h	Behaviour observation, mortality check and removal of dead fish
9:30h	Meal: *Artemia* (33.3 %)
10:30h	Measure of environmental parameters
12:00h	Meal: *Artemia* (16.6 %)
14:00h	Meal: *Artemia* (16.6%)
15:00h	Filter/tank cleaning and mortality check
18:00h	Meal: *Artemia* (33.3%)

Table 2- Daily routines from 29 to 32 DAH.

Hours	Daily routine (29 to 32 DAH)
9:00h	Behaviour observation, mortality check and removal of dead fish
9:30h	Charge Feeders
10:00h	Measure of environmental parameters
10:15h	Meal: *Artemia* (33.3 %)
12:00h	Clean the skimmer and wall filters
12:30h	Meal: *Artemia* (16.6 %)
14:00h	Filter/tank cleaning and mortality check
15:00h	Meal: *Artemia* (16.6%)
16:00h	Sump cleaning
18:00h	Meal: *Artemia* (33.3%)

Table 3- Daily routines from 33 to 68 DAH.

Hours	Daily routine (33 to 68 DAH)
9:00h	Behaviour observation, mortality check and removal of dead fish
9:30h	Measure of environmental parameters
10:00h	Tank cleaning (including tank filters)
12:00h	Cleaning feeders/quantification of remnants / charge the feeder
14:00h	If necessary, filter/tank cleaning and mortality check
14:30h	Clean skimmer and wall filters
15:30h	Sump cleaning

2.1.3. Sampling

At 27 DAH, 100 Senegalese sole post-larvae were sampled from the initial pool of larvae. For total length (TL) and dry weight (DW) determination, 50 individuals were sampled at 51 and 68 DAH. To determine TL, all larvae sampled were photographed and measured the software ImageJ. Regarding DW, the sampled individuals were freeze-dried and weighted on a digital scale. For TL, DW and relative growth rate (RGR), only larvae from the two upper quartiles were considered, to simulate the selection process performed by the commercial hatcheries to ensure that only the individuals with better development go on production. Feed conversion ratio (FCR) was calculated in each treatment from 27 to 68 DAH. Survival and condition factor (K) were accessed in the end of the trial. During the sampling the larvae were harvested from the tank with a mesh sieve and then euthanized lethal doses of anaesthetics, after that the larvae were washed in distilled water and stored at -20ºC in a white sheet where they will remain until analysis. To reduce the effect of feed on the post-larvae digestive tract and ensure that dry weight was not affected, fish were only fed until 00h00 on previous day to sampling. The survival was estimated through a daily check for mortality and a final count of individuals at the end of the trial.

2.1.4 Leaching

A nutrient leaching test was performed at SPAROS Lda. facility to quantify the amount of soluble protein lost by COMM, B/C, LFAT and PL microdiets at different time periods. Each microdiet was tested in three different range sizes, 200-400 µm, 400-600 µm, 600-800 µm. To perform this test, sieves with different sizes, seawater and distilled water and a kiln were required. Leaching test was performed in duplicates for a period of 2 and 30 minutes. Sieve size was lower size than the pellet size in order to retain the sample inside the sieve.

After selected the adequate sieve size two buckets were filled, one with salt water (6L) and other with distilled water (6L), 1g of the sample was weighted and placed into the sieves. First the sample was immersed during the period of 2 or 30 minutes at seawater and then the sieves were vertically removed to ensure that water leave the sieves and sample stay in the sieve. In order to remove the salt from the sample, sieves were immersed in distilled water. After checking if the sample have lumps that were counted and removed, sample was collected and stored in a kiln for 24 hours at 105 ºC. After 24 hours the dry samples were mashed and weighted. Furthermore, to calculate the percentage of leaching, the protein content of the dry and leached samples was quantified and compared with the protein content of the same microdiet that was not immersed on water and consequently leaching did not occur.

2.1.5 Microdiet suspension profile

Suspension profiles of the 4 experimental microdiets were evaluated at SPAROS LDA. facility, this test was performed in duplicates for a period of 5 minutes with the help of a dark chamber, designed to fit an orbital agitator, a video camera and a 0.8 L beaker were required. Dark chamber was designed with three holes to limit the external light input but allowing the samples introduction and recording. One hole was placed on top where the feed was dropped, other one on the bottom where the lightbulb was placed and other on one side of the chamber where video camera was placed. Microdiets COMM, B/C, LFAT and PL were tested in three different range sizes, 200-400 µm, 400-600 µm, 600-800 µm, and microdiets Initially, a video camera was set to record at a rate of 30 frames per second (FPS), then each sample with 0.1g of the tested microdiet

was dropped in the centre of the beaker filled with seawater and the video recording was initiated and the pellet sinking movement was recorded.

After recorded, the video was transformed in frames trough the software VLC Media Player. The obtained frames were then analysed trough the software ImageJ, with this analysis it was possible to quantify the number of pellets that sink in the period of 5 minutes.

2.1.6 Microdiet Dispersion profile

Dispersion capacity of the 4 experimental diets was tested Sparos LDA. facility, this test was performed in duplicates for a time period of approximately 15 seconds. The test was performed using all range sizes used on trial 1. To perform the test a 5L recipient with a black background and 750 Cm^2 of surface, and a video camera was required. Initially, a video camera was set to record at a rate of 30 frames per second (FPS) and 0.2g of each sample was weighted and dropped at a 10 cm distance in the recipient filled with seawater. The pellet movement into the water surface was record during approximately 15 seconds and then the video was transformed in frames using the software VLC Media Player. The frames were analysed with the software ImageJ and the dispersion area was measured for each frame, creating the dispersion profile for the 15 second period.

2.1.7 Data analysis

Relative growth rate (RGR) was calculated as: $RGR\ (\%) = (e^{g} - 1) * 100$, where g = (lnWt – lnW0) x t^{-1}. Wt and W0 represent the final and initial dry weights, respectively, at a time period t. Feed conversion ratio (FCR) was calculated as: $FCR = \frac{Fi}{Ww}$, where Fi corresponds to feed supplied (g) and Ww to the mean of wet weight gained (g). Condition factor (K) was calculated as: $K = 100 * \frac{W}{l^3}$, where w is the wet weight and L is the total length. Percentage of survival was calculated as: $S = Li - \frac{Lf}{Li} * 100$, where Li and Lf correspond to the starting and remaining larvae in the tanks, respectively. The number of sampled individuals was counted, but not considered to determine the percentage of survival.

Levene's test was conducted to access the variance homogeneity and verify if the data complied with the one-way ANOVA assumptions. Differences in the total length, dry weight, relative growth rate, condition factor, feed conversion ratio and survival between dietary treatments were evaluated. One-way ANOVA tests were performed, with differences being considered significant when $\alpha<0.05$. When significant differences were found, Tukey multiple comparison tests were performed to determine what dietary treatments were significantly different between them. When the one-way ANOVA assumptions were not met, non-parametric tests for Kruskal Wallis independent samples were conducted. Mann Whitney U Test for testing the equality of means in two independent samples was used when significant differences were found in the Kruskal Wallis. Results were expressed as means ± standard deviation (SD).

2.2. Results

2.2.1. Experimental diets

The proximal composition of the dietary treatments used on trial 1 is presented on Table 4. It is presented the wet matter of each compound, in each dietary treatment, namely protein, fat, ash, phosphorus, and also the energy and the relative cost.

Table 4 - Proximal composition of experimental diets used in the experimental trial 1.

% WM	COMM	B/C	PL	LFAT
Protein	65.7	64.7	63.7	66.4
Fat	19.4	16.4	16.3	14.4
Ash	9.4	9.8	10.6	10.0
Phosphorous	1.9	1.8	1.9	1.9
Energy (MJ/Kg)	21.9	21.9	21.2	21.5
Cost (relative %)	100.0	56.3	57.1	56.8

Results are expressed as means ± standard deviation (n=2). WM— wet matter

2.2.2. Growth performance and survival

Senegalese sole total length (mm) values obtained on trial 1 are represented in table 4, It was observed that at 51 DAH senegalese sole feed with COMM had the lowest total length values and they were significantly lower than the remaining treatments ($p<0.001$). At 68 DAH, total length values for all treatments were not significantly different.

Table 5- Senegalese sole post-larvae total length (mm) values at 51 and 68 DAH.

Total length (mm)	COMM	B/C	PL	LFAT
51 DAH	15.01 ± 2.44^{a}	16.44 ± 2.44^{b}	16.52 ± 2.20^{b}	17.28 ± 3.65^{b}
68 DAH	30.50 ± 5.59	31.33 ± 4.44	30.42 ± 5.26	32.04 ± 4.60

Values presented as mean ± standard deviation. At 51 and 68 DAH, n=50 observed individuals. Statistical differences ($p<0.05$) between larvae from different treatments at same age represented with different superscript letters.

The Senegalese sole dry weight (mg) values obtained on trial 1 are shown on table 5. At 51 DAH, it was observed that dry weight values from all treatments were not significantly different. At 68 DAH, it was observed that LFAT had the lowest values,

and they were significantly lower than the remaining treatments ($p<0.017$), furthermore, were not observed significantly differences between treatments LFAT, PL and B/C.

Table 6- Senegalese sole post-larvae dry weight (mg) values at 51 and 68 DAH.

Dry weight (mg)	COMM	B/C	PL	LFAT
51 DAH	18.95 ± 7.50	20.24 ± 6.13	19.33 ± 5.96	19.23 ± 7.82
68 DAH	103.67 ± 32.19^{b}	98.90 ± 36.27^{b}	97.82 ± 33.13^{b}	89.73 ± 31.50^{a}

Values presented as mean ± standard deviation. At 51 and 68 DAH, n=50 observed individuals. Statistical differences ($p<0.05$) between larvae from different treatments at same age represented with different superscript letters.

Senegalese sole post-larvae RGR, FCR and K values obtained at 51 DAH, can be observed in table 6. No significant differences were found between treatments in RGR, FCR and K values.

Table 7- Senegalese sole post-larvae RGR, FCR and K values at 51 DAH.

	COMM	B/C	PL	LFAT
RGR (% DW day^{-1})	9.03 ± 0.57	9.34 ± 0.37	9.14 ± 0.37	9.03 ± 1.12
FCR	2.48 ± 0.40	2.00 ± 0.31	2.11 ± 0.11	2.41 ± 0.89
K (g/cm^{3})	2.85 ± 0.54	2.32 ± 0.57	2.16 ± 0.32	2.01 ± 0.79

Values presented as mean ± standard deviation. RGR values refer to larvae from the two upper quartiles. n=50 observed individuals.

At 68 DAH, senegalese sole post-larvae RGR, FCR, K and survival are shown in table 7. The treatments did not present significative differences in RGR, FCR, K and survival.

Table 8- Senegalese sole post-larvae RGR, FCR, K and survival values at 68 DAH.

	COMM	B/C	PL	LFAT
RGR (% DW day^{-1})	9.67 ± 0.43	9.49 ± 0.49	9.48 ± 0.64	9.26 ± 0.22
FCR	1.08 ± 0.19	0.98 ± 0.22	1.03 ± 0.14	0.90 ± 0.13
K (g/cm^{3})	2.04 ± 0.87	1.69 ± 0.54	1.89 ± 0.77	1.42 ± 0.42
Survival (%)	57.52 ± 3.63	65.75 ± 6.69	61.27 ± 2.88	60.85 ± 0.42

2.2.3. Microdiet protein leaching

The percentages of protein leaching for microdiets range sizes of 200-400 µm used on trial 1 during 2 and 30 minutes of immersion are shown and Figures 2 and 3. It was observed that the treatment with higher percentage of protein leaching on both immersion times was the diet COMM. In contrast diet PL was the diet with lower protein leaching. When compared by time, from 2 minutes for 30 minutes of immersion B/C was the diet with lowest percentage of leaching increment.

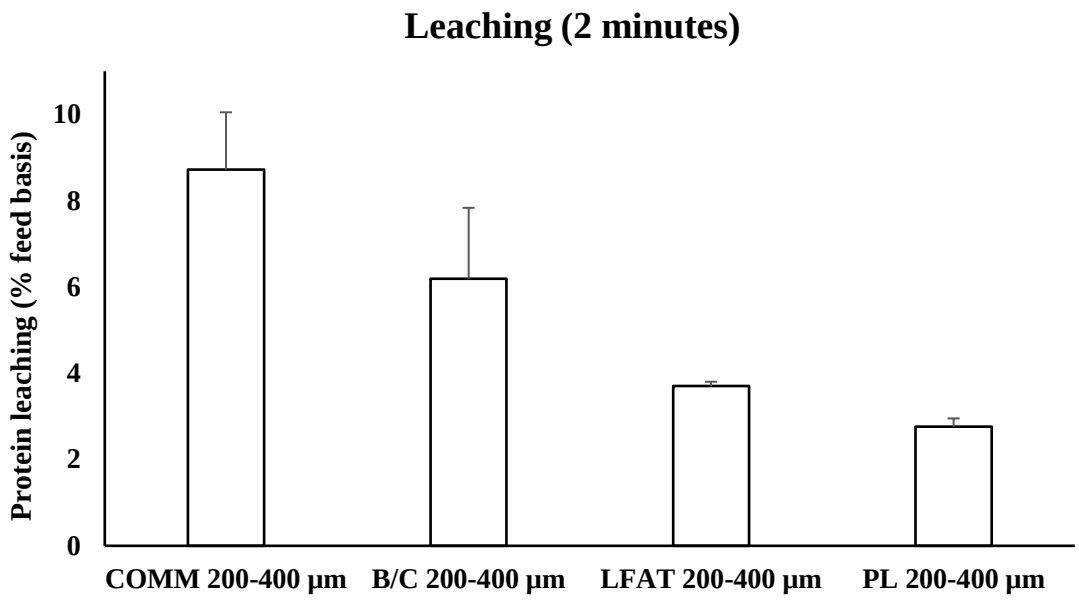

Figure 2- Protein leaching (% feed basis) of microdiets used on trial 1. Values presented as mean ± standard deviation. Sample weight= 1g, microdiet range size= 200-400 µm, immersion time= 2 minutes.

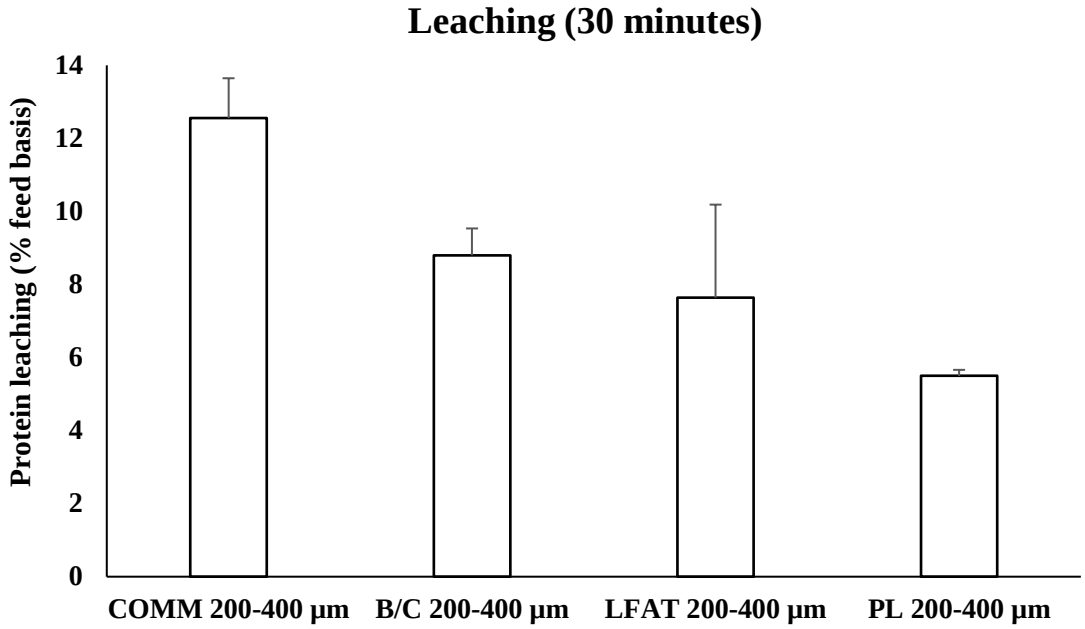

Figure 3 - Protein leaching (% feed basis) of microdiets used on trial 1. Values presented as mean ± standard deviation. Sample weight= 1g, microdiet range size= 200-400 µm, immersion time= 30 minutes.

Protein leaching percentage for microdiets range sizes of 400-600 µm used on trial 1 during 2 and 30 minutes of immersion are shown and Figures 4 and 5. commercial control treatment was the diet with higher leaching on both immersion times, in contrast, LFAT was the dietary treatment with lower percentage of protein leaching. From 2 for 30 minutes the treatment with lower leaching percentage increment was PL.

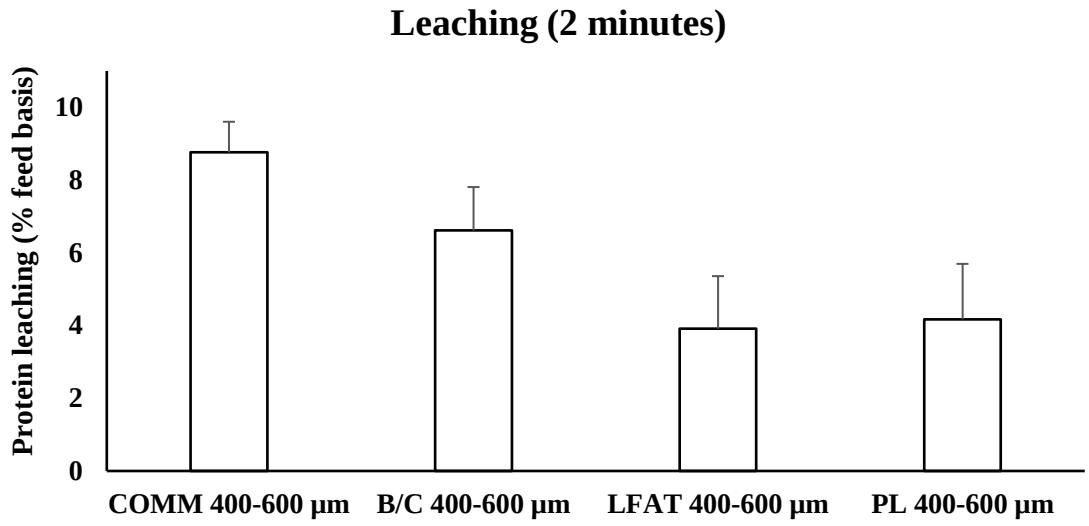

Figure 4- Protein leaching (% feed basis) of microdiets used on trial 1. Values presented as mean ± standard deviation. Sample weight= 1g, microdiet range size= 400-600 µm, immersion time= 2 minutes.

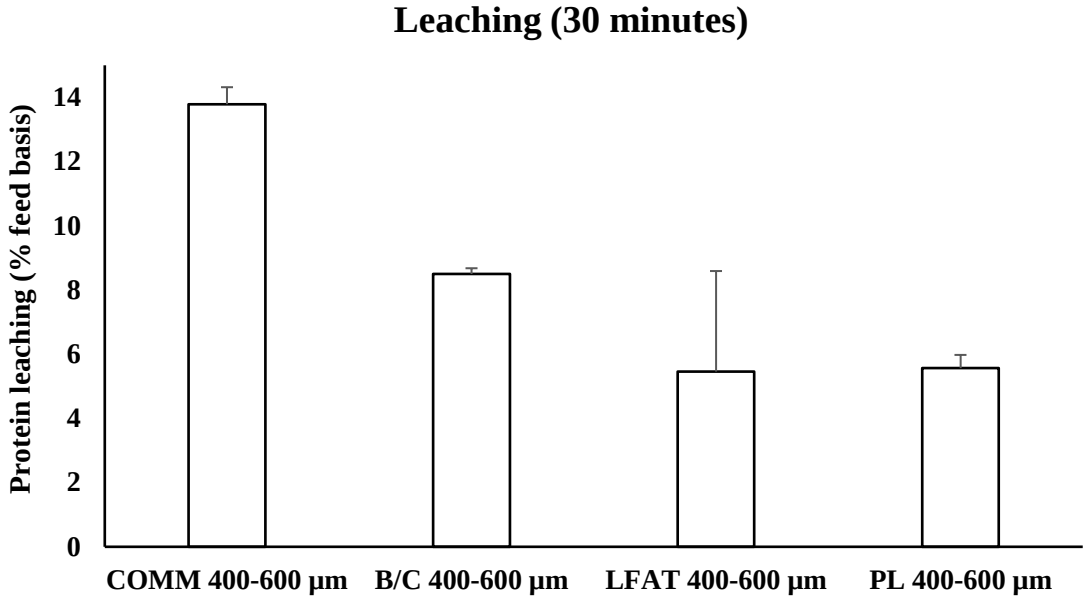

Figure 5 - Protein leaching (% feed basis) of microdiets used on trial 1. Values presented as mean ± standard deviation. Sample weight= 1g, microdiet range size= 400-600 µm, immersion time= 30 minutes.

Microdiets range sizes of 600-800 µm used on trial 1 percentage of protein leaching during 2 and 30 minutes of immersion are shown and Figures 6 and 7. Microdiet COMM was the diet with higher leaching percentage for both immersion time, in contrast, during 2 minutes of immersion B/C was the diet with lower protein leaching, and for 30 minutes of immersion time, PL was the treatment with lower percentage of leaching. When compared by time, from 2 minutes for 30 minutes of immersion LFAT and PL were the diets with lowest percentage of leaching increment.

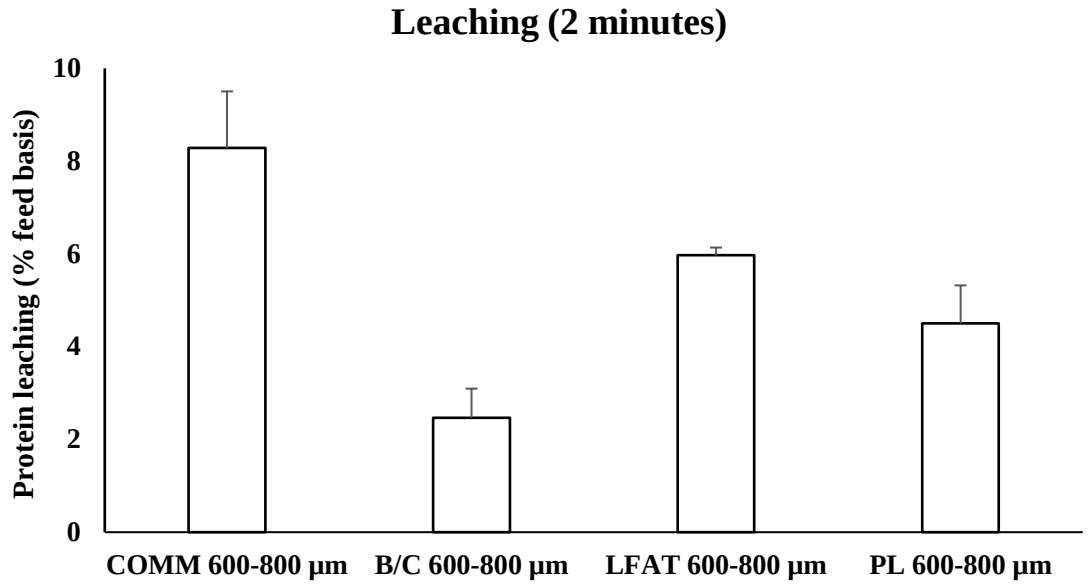

Figure 6- Protein leaching (% feed basis) of microdiets used on trial 1. Values presented as mean ± standard deviation. Sample weight= 1g, microdiet range size= 600-800 µm, immersion time= 2 minutes.

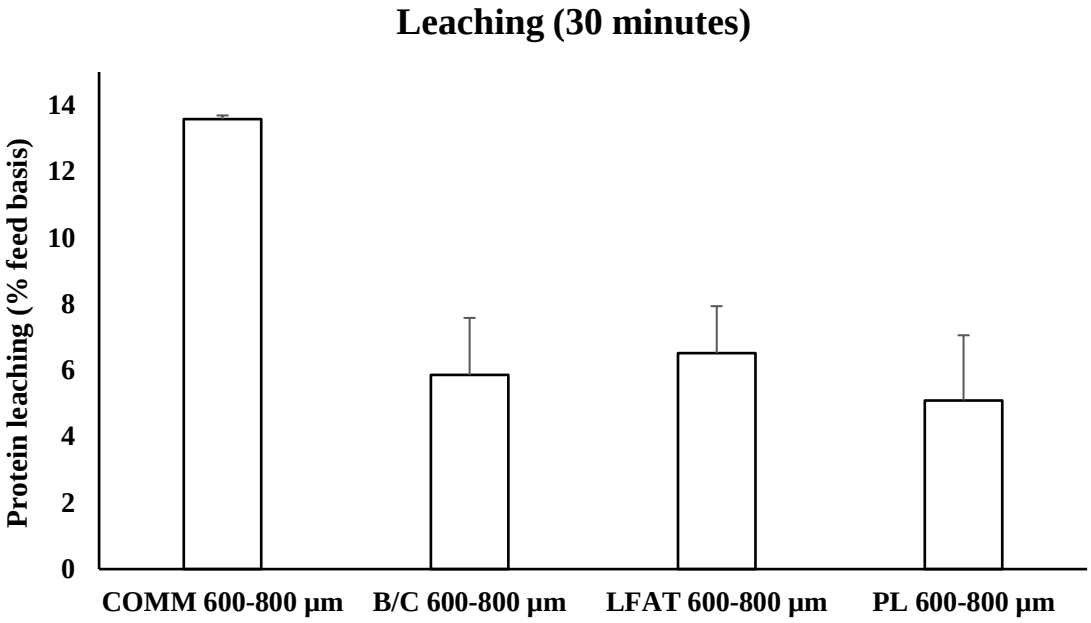

Figure 7 - Protein leaching (% feed basis) of microdiets used on trial 1. Values presented as mean ± standard deviation. Sample weight= 1g, microdiet range size= 600-800 µm, immersion time= 30 minutes.

When compared by size, it was observed that on both immersion times, treatments LFAT and PL were the diets with lower percentage of protein leaching, except on 600-800 µm where B/C was the treatment with lower percentage of leaching. In

contrast, on both immersion times, COMM treatment was the treatment with higher percentage leaching on all range sizes.

2.2.4. Microdiet Suspension profile

The microdiets with range sizes of 200-400 µm, 400-600 µm and 600-800 µm used on trial 1 suspension profiles are shown in Figures 8, 9 and 10. It was verified that COMM and LFAT diets pellet availability had similar behaviour for all pellets range sizes. It was also verified that COMM and LFAT were the diets with lower cumulative availability on 400-600 µm and 600-800 µm sizes. Furthermore, it was shown that PL microdiet had higher pellet cumulative availability for pellet size 200-400 µm and 400-600 µm, while the B/C microdiet was the diet with higher pellet availability for 600-800 µm range size and the diet with lower availability for 200-400 µm size microdiets.

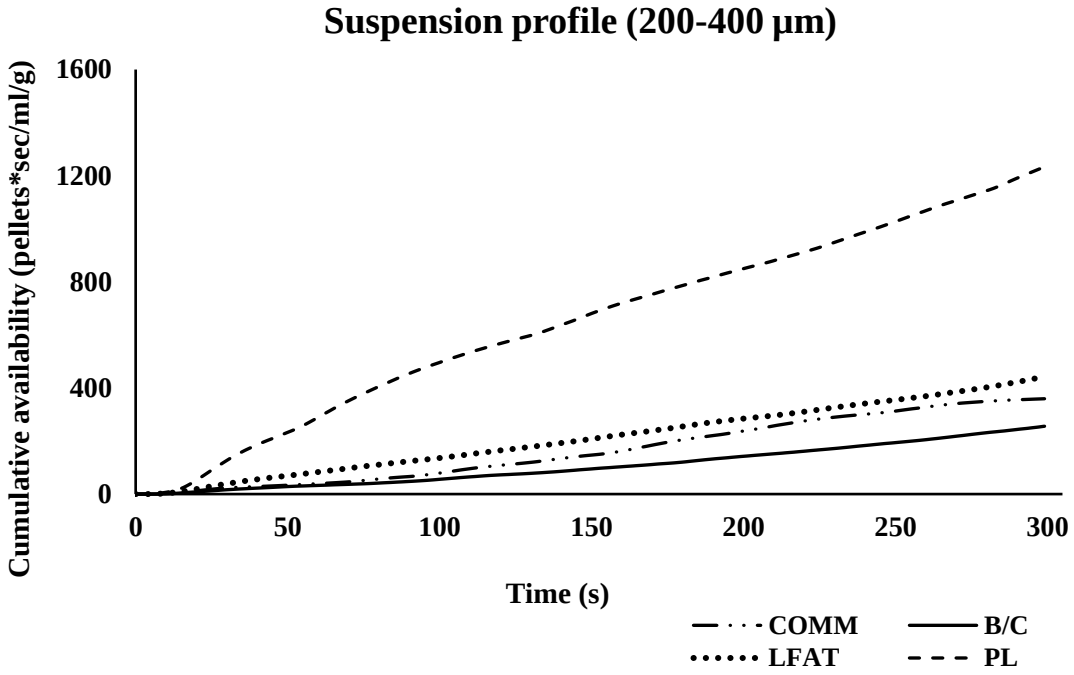

Figure 8 - Cumulative availability (pellets*sec/ml/g) of microdiets used on trial 1. Values presented as mean. Sample weight = 0.1g, microdiet range size= 200-400 µm, precipitation time = 300 s.

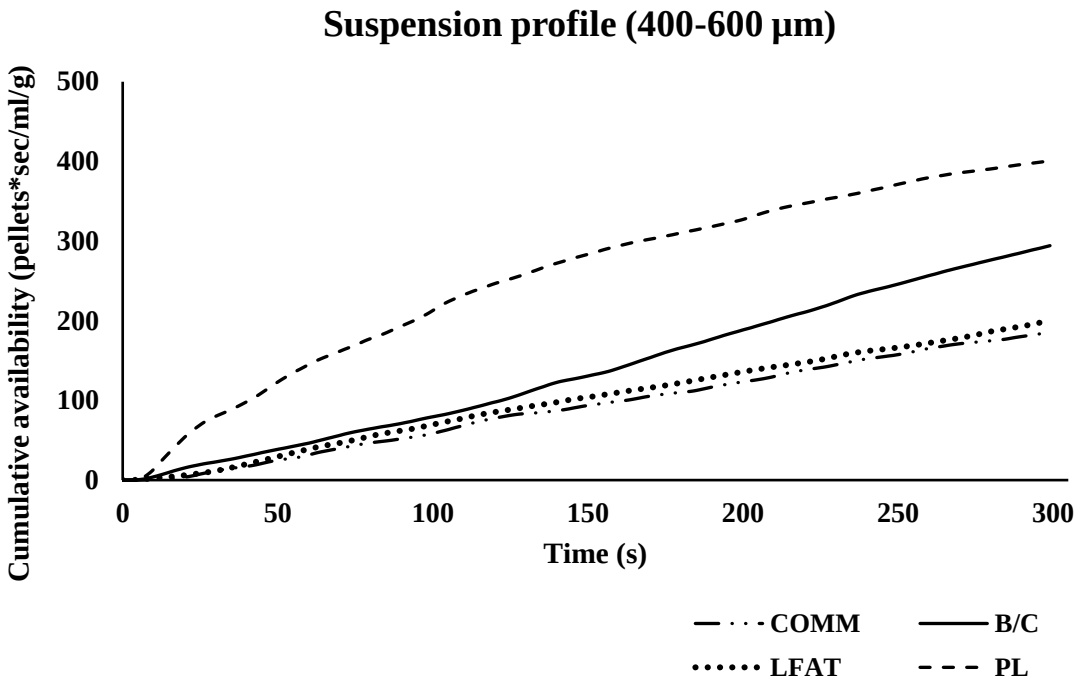

Figure 9 - Cumulative availability (pellets*sec/ml/g) of microdiets used on trial 1. Values presented as mean. Sample weight = 0.1g, microdiet range size= 400-600 µm, precipitation time = 300 s.

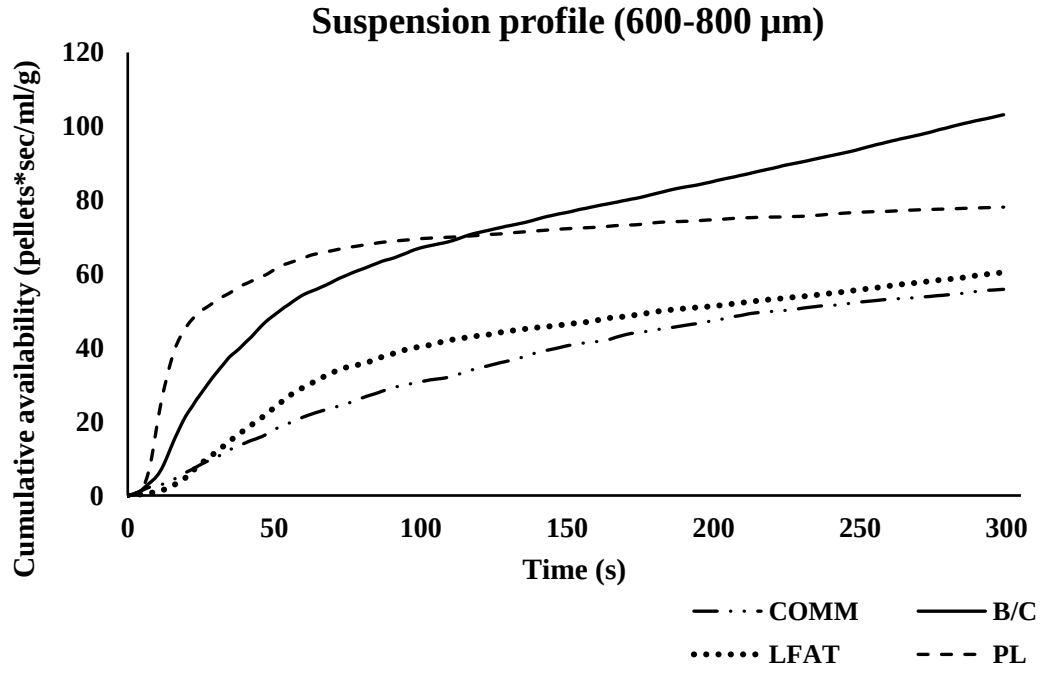

Figure 10 - Cumulative availability (pellets*sec/ml/g) of microdiets used on trial 1. Values presented as mean. Sample weight = 0.1g, microdiet range size= 600-800 µm, precipitation time = 300 s.

2.2.5. Microdiet Dispersion profile

Microdiets used on trial 1 dispersion profile are shown in Figures 11,12 and 13. It was possible to verify that microdiet PL had the lowest dispersion area for all pellet sizes. It was also verified that microdiet B/C had higher dispersion area for pellet sizes 200-400 µm and 400-600 µm while for 600-800 µm, LFAT was the microdiet with higher dispersion area.

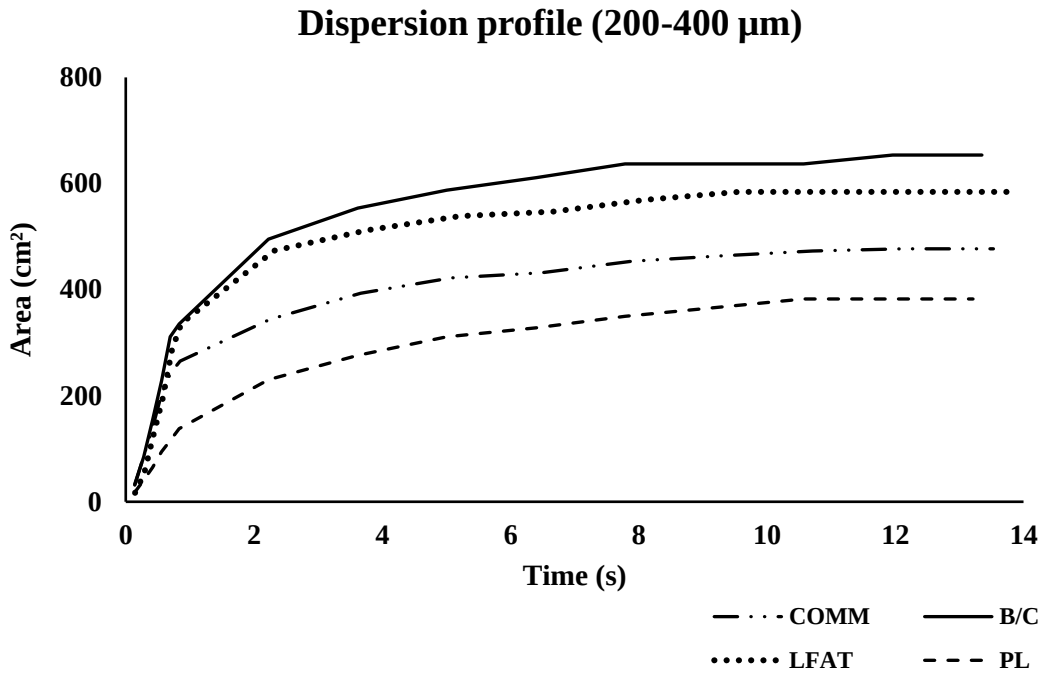

Figure 11- Dispersion area (cm²) of microdiets used on trial 1. Values presented as mean. Sample weight = 0.2g, microdiet range size= 200-400 µm, precipitation time = 14 s.

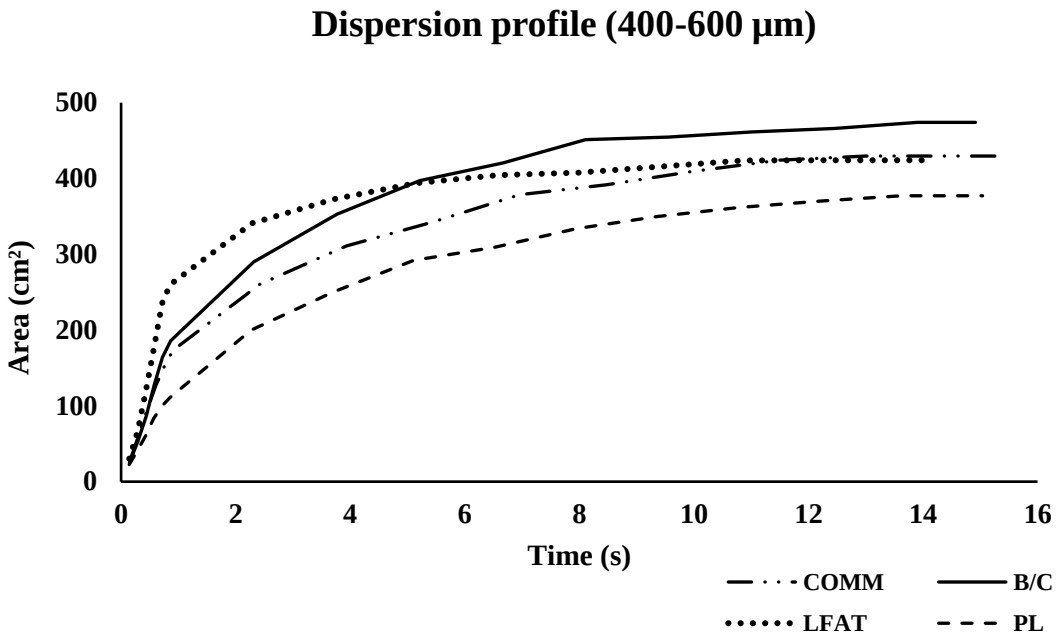

Figure 12- Dispersion area (cm²) of microdiets used on trial 1. Values presented as mean. Sample weight = 0.2g, microdiet range size= 400-600 µm, precipitation time = 15 s.

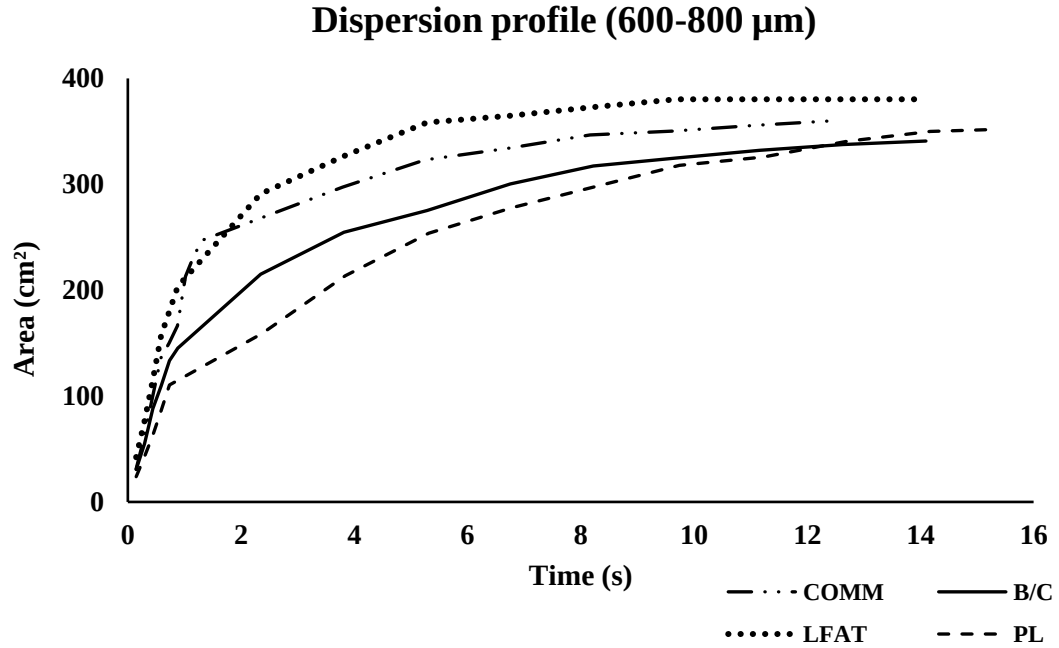

Figure 13- Dispersion area (cm²) of microdiets used on trial 1. Values presented as mean. Sample weight = 0.2g, microdiet range size= 600-800 µm, precipitation time = 14 s.

2.3. Discussion

This trial aimed to test the use of different ingredients in microdiets for Senegalese sole larvae in criteria as growth performance and physical properties. Senegalese sole post-larvae at 68 DAH achieved total length values of 32.04 ± 4.60 mm, dry weight values of 103.67 ± 32.19 mg. RGR values around 9.48 % day^{-1}; FCR values around 1, K values around 2 and percentage of survival between 57.5 and 65.8%. When compared with some authors, growth performance values are an improvement, considering dry weight, Engrola *et al*. (2009) obtained dry weight values of 63.2 mg at 68 DAH, and Pinto *et al*. (2016) obtained 37,5 mg at 74 DAH, and considering RGR, Engrola *et al*. (2009) obtained a value of 6 %.day^{-1} and Pinto *et al*. (2016) obtained values 5 %.day^{-1}. In contrast, Pinto *et al.* (2018) that obtained dry weight values of 184.5 mg and 12.9 %.day^{-1} of RGR, represents a better growth performance when compared with the obtained values for growth performance on trial 1. Regarding the percentage of survival, the obtained results were lower when compared with other authors as Engrola *et al.* (2005) that obtained 69.8%, Engrola *et al.* (2007) that obtained 99%, Pinto *et al*. (2016) that obtained 86% and Pinto *et al.* (2018) that obtained 73.9% of survival. In contrast the result obtained for survival was an improvement when compared with Dâmaso-Rodrigues *et al.* (2010) that obtained 30%.

Senegalese sole post larvae from all treatments exhibited similar performance results at 64 DAH for total length, RGR, FCR, K and survival. Regarding dry weight, despite the LFAT had a significantly lower value, the remaining treatments were not significantly different. The results obtained on the trial 1, demonstrate that the commercial control, the ingredient replacement for ingredients which provide high benefit/cost ratio performed on B/C, and dietary treatment PL, where ingredient replacement also represent a high benefit/cost ratio due to the similarity with B/C where the difference is the inclusion of the higher phospholipid content, obtained the best growth performance results. Considering the higher relative cost of COMM when compared with the remaining treatments which represent a lower benefit/cost ratio and knowing that LFAT diet growth performance results were significantly lower when compared with the remaining treatments, for growth performance criteria, the results suggest that B/C and PL are the most suitable diets.

Microdiet leaching, suspension and dispersion profile evaluation aimed to test how different ingredients affect characteristics as leaching, sinking and dispersion of experimental microdiets used on trial 1. Treatment COMM present highest protein leaching values for both immersion times when compared with the remaining treatments, was also one of the treatment with lower sinking capacity which does not represent an advantage because higher capacity to sink represent a higher feed availability for Senegalese sole larvae that feed on the bottom (Imsland *et al*.,2003). This treatment was also one of the treatments with lower dispersion capacity, what reinforces that this treatment is not a very good one. B/C treatment leaching results are higher on small particle sizes and decrease on larger pellet sizes. Regarding the sinking capacity, this treatment presents lower sinking capacity on lower particle sizes and higher results on larger particle sizes. In contrast, the treatment presents higher capacity of dispersion at lower sizes. These results suggest that this treatment is also not a good option essentially due to the fact that the leaching percentage is higher on small particles that can be also justified by a lower sinking capacity which will result in a higher contact time between the pellet and the water. PL was the treatment with lower protein leaching and higher sinking capacity, and in addition was the dietary treatment with lower dispersion capacity. These results suggest that PL was the best treatment on physical properties criteria andlower leaching percentage. PL presented best results when compared with Kvåle *et al*. (2006) that obtained leaching percentages of 26-28 %.

B/C and PL are the dietary treatments with better growth performance results, also have a lower relative production cost which provide them a high benefit/cost ratio. Considering the percentage of protein leaching of PL treatment represent the lower leaching percentage, and B/C perform the best in terms of physical properties, these diets can be considered the best treatments used on trial 1 essentially for the growth performance results and high benefit/cost ratio, but also because they fulfil important requirements on physical properties.

3. Trial 2 – Effects of the use of different binders on the performance of Turbot larvae

3.1. Material and methods

3.1.1. Dietary treatments

Trial 2 tested four different microdiets: A commercial-like diet (CTRL) as control, and 3 experimental microdiets, MIX, LOW and HIGH. CTRL diet was a commercial-like diet, being composed of high quality fish and squid meals and plant-proteins including wheat gluten. Diet MIX was formulated and produced with a similar composition to CTRL, but using a mixture of binders in its dietary composition. Diet LOW was formulated and produced with a similar composition to CTRL, but using a novel binder at a low inclusion level. Diet HIGH was formulated and produced with a similar composition to CTRL, but using a novel binder at a high inclusion level.

All dietary treatments for Turbot larvae were produced at SPAROS Lda. (Olhão, Portugal) with the same production process as the one described for trial 1. Weaning occur from 23 to 29 DAH, in this period larvae were co-fed with *Artemia* metanauplii and the experimental microdiets. The initial amount of *Artemia* was 2 x 10^4 *Artemia*/meal per tank and the initial diet amount was 0.5g per day which distributed by hand during the day and the remaining feed through automatic feeds until the 0 lux period. At 30 DAH live feed was fully replaced by inert microdiets which range size was 200-400 µm, from 37 until 50 DAH the microdiets range size was 400-600 µm as demonstrated on Figure 14.

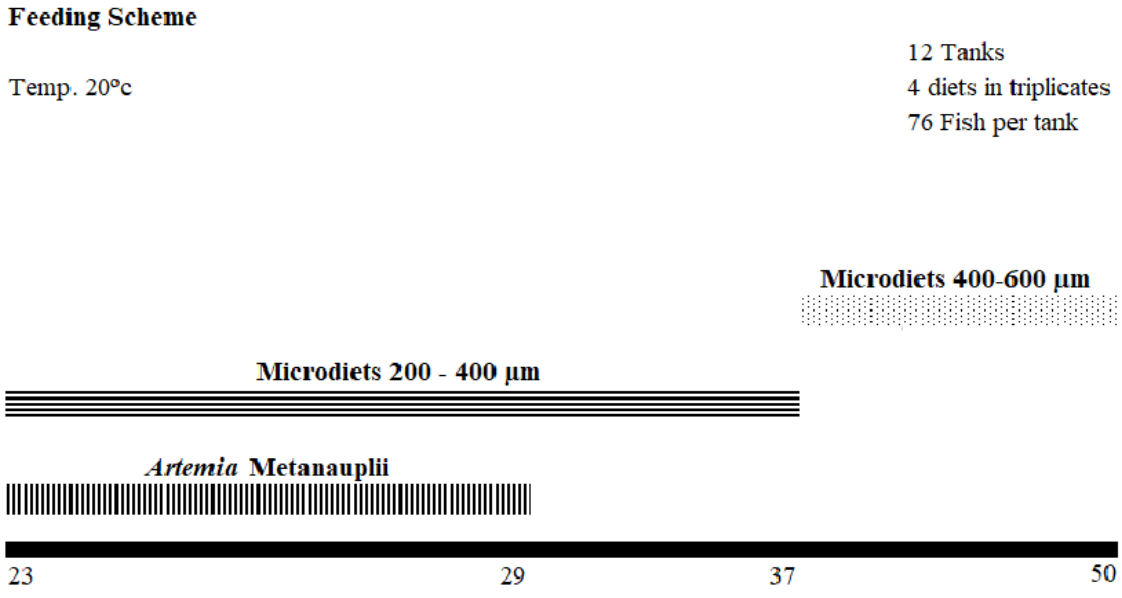

Figure 14 – Trial 2 experimental design. Initially larvae were fed a co-feeding period with experimental diets *Artemia* metanauplii, which was fully replaced by inert microdiets at day 29.

3.1.2. Fish rearing

Trial 2 was performed with Turbot larvae at SPAROS Lda. Research facility (Olhão, Portugal). Turbot larvae were shipped from Acuinova- Atividades piscícolas, S.A. (Praia de Mira, Portugal), where they were reared until 23 DAH. The larvae were reared from 23 to 50 DAH and distributed in triplicates for 12 tanks each one with 8L. A total of 76 larvae was placed in each tank. Light intensity and photoperiod were settled at 1200 lux (8h30-23h30) and 0 lux- (23h30-8h30). The water temperature was kept at 18 ºC ± 0.2 ºC and the remaining water parameters were the same as described in section 2.1.3. Cleaning and siphoning of each tank was part of the daily routines that were adjusted according to the fish growth during the trial as described on Tables 8 and 9.

Table 9 - Daily routines from 29 to 32 DAH.

Hours	Daily routine (23 to 29 DAH)
9:00h	Behaviour observation, mortality check and removal of dead fish
9:15h	Meal: Inert microdiet (feeding by hand)
9:30h	Meal: *Artemia* (reduce water lever during the meal)
10:15h	Measure the environmental parameters
11:00h	Meal: Inert microdiet (feeding by hand)
11:30h	Meal: *Artemia* (reduce water lever during the meal)
12:00h	Meal: Inert microdiet (feeding by hand)
13:00h	Meal: *Artemia* (reduce water lever during the meal)
14:00h	Sump cleaning
14:30h	Meal: Inert microdiet (feeding by hand)
15:00h	Meal: *Artemia* (reduce water lever during the meal)
15:30h	Meal: *Artemia* (reduce water lever during the meal)
16:00h	Filter/tank cleaning
17:00h	Meal: Inert microdiet (feeding by hand)
17h30	Charge automatic feeders
18:00h	Meal: *Artemia* (reduce water lever during the meal)

Table 10 - Daily routines from 30 to 50 DAH.

Hours	Daily routine (30 to 50 DAH)
9:00h	Behaviour observation, mortality check and removal of dead fish
9:15h	Meal: Inert microdiet (feeding by hand)
10:00h	Measure the environmental parameters
11:00h	Meal: Inert microdiet (feeding by hand)
11:30h	Sump cleaning
12:30h	Meal: Inert microdiet (feeding by hand)
14:00h	Meal: Inert microdiet (feeding by hand)
15:00h	Meal: Inert microdiet (feeding by hand)
16:00h	Filter/tank cleaning
17:00h	Meal: Inert microdiet (feeding by hand)
17:30h	Charge automatic feeders

3.1.3 Sampling

At 23 DAH 60 Turbot larvae were sampled form the initial pool of larvae. For dry weight determination a total of 30 individuals were sampled at 50 DAH. To determine the dry weight, the sampled larvae were freeze- dried and weighted on a digital scale. For dry weight, RGR, only larvae from the two upper quartiles were considered, to simulate the selection process performed by the commercial hatcheries to ensure that only the individuals with better development go on production. Feed conversion ratio was accessed in each dietary treatment from 23 DAH to 50 DAH. The method of sampling was the same described in section 2.1.3. A daily check for mortality and a final count at 50 DAH was performed to access the survival.

3.1.4 Microdiet protein leaching

A nutrient leaching test was performed at SPAROS Lda. facility to quantify the amount of soluble protein leached by CTRL, MIX, LOW and HIGH diets at time periods of 2 and 30 minutes. Each microdiet was tested in three different range sizes, 200-400 µm, 400-600 µm, 600-800 µm. The test was performed in duplicates and the process was the

same explained in section 2.1.4. To calculate the percentage of nutrient leaching, the protein content of the leached samples was quantified and compared with the protein content of the same microdiet that was not immersed on water and consequently the leaching did not occur.

3.1.5 Microdiet suspension profile

Suspension profiles of the 4 experimental microdiets used on trial 2 were evaluated at SPAROS Lda. facility. The microdiets tested range size were 100-200 µm, 200-400 µm and 400-600 µm. The method used to access the microdiet sinking capacity was the method used to access the sinking capacity of microdiets used on trial 1. Each treatment suspension profile was later analysed, and compared by range size.

3.1.6 Microdiet dispersion profile

The dispersion profiles from 4 dietary treatments used on trial 2 were analysed on SPAROS Lda. facility. The evaluated range sizes were the same tested in the previous test. This test methodology was already described on section 2.1.6. The obtained dispersion profiles were analysed and compared by range size.

3.1.7 Data analysis

Relative growth rate, feed conversion ratio and survival were calculated as mentioned in section 2.1.7. Levene's test was conducted to access the variance homogeneity and verify if the data complied with the one-way ANOVA assumptions. Differences in the dry weight, relative growth rate, feed conversion ratio and survival between dietary treatments were evaluated. One-way ANOVA tests were performed, with differences being considered significant when $\alpha<0.05$. When significant differences were found, Tukey multiple comparison tests were performed to determine what dietary treatments were significantly different between them. When the one-way ANOVA assumptions were not met, non-parametric tests for Kruskal Wallis independent samples were conducted. Mann Whitney U Test for testing the equality of means in two independent

samples was used when significant differences were found in the Kruskal Wallis. Results were expressed as means ± standard deviation.

3.2. Results

3.2.1. Experimental diets

The proximal composition of the dietary treatments used on trial 1 is presented on Table 4. It is presented the wet matter of each compound, in each dietary treatment, namely protein, fat, ash, phosphorus, and also the energy and the relative cost.

Table 11 - Proximal composition of experimental diets used in the experimental trial 2.

% WM	CTRL	MIX	LOW	HIGH
Protein	62.2	61.0	60.3	60.5
Fat	18.0	18.0	17.6	18.0
Ash	8.7	8.7	8.8	8.9
Phosphorous	1.9	1.9	1.9	1.9
Energy (MJ/Kg)	21.1	21.3	21.5	21.7
Cost (relative %)	100.0	93.8	88.9	89.0

Results are expressed as means ± standard deviation (n=2). WM— wet matter

3.2.2. Growth performance and survival

Turbot post larvae dry weight (mg) values obtained during trial 2 are shown in table 12. At the end of the trial (50 DAH), it was observed that LOW dry weight values were significantly smaller when compared with other treatments ($p<0.001$). In contrast CTRL and HIGH had significantly highest dry weight values ($p< 0.001$).

Table 12- Turbot post-larvae dry weight (mg) values at 50 DAH.

Dry weight (mg)	CTRL	MIX	LOW	HIGH
50 DAH	210.81 ± 33.56^{b}	186.61 ± 38.96^{ab}	175.01 ± 27.95^{a}	195.99 ± 26.26^{b}

Values presented as mean ± standard deviation. At 50 DAH, n=30 observed individuals. Statistical differences ($p<0.05$) between larvae from different treatments at same age represented with different superscript letters.

Turbot larvae RGR, FCR and survival from trial 2 can be observed in table 6. No significant differences were found between treatments for FCR and survival values. Regarding RGR, treatments CTRL and HIGH were the treatments with highest growth performance.

Table 13 - Turbot post-larvae RGR, FCR and Survival values at 50 DAH.

	CTRL	MIX	LOW	HIGH
RGR (% DW day^{-1})	13.45 ± 0.20 ^{b}	12.90 ± 0.09 ^{a}	12.70 ± 0.24 ^{a}	13.10 ± 0.1 ^{b}
FCR	1.04 ± 0.08	1.31 ± 0.09	1.13 ± 0.33	1.13 ± 0.14
Survival (%)	69.01 ± 8.97	57.09 ± 3.81	65.79 ± 23.39	60.35 ±3.81

Values presented as mean ± standard deviation. RGR values refer to larvae from the two upper quartiles.

3.2.3. Microdiet nutrient leaching

Protein leaching of microdiets used on trial 2, with range size 200-400 µm during 2 and 30 minutes of immersion are shown in Figures 15 and 16. It was observed that microdiet MIX was the diet with lower percentage of protein leaching. It was also verified that diet HIGH was the diet with higher percentage of protein leaching during 2 minutes of immersion and CTRL was the diet with higher protein leaching during 30 minutes of immersion. When compared by time, HIGH presents a decrease from 2 minutes for 30 minutes of immersion, and MIX was the diet with the lowest percentage of leaching increment.

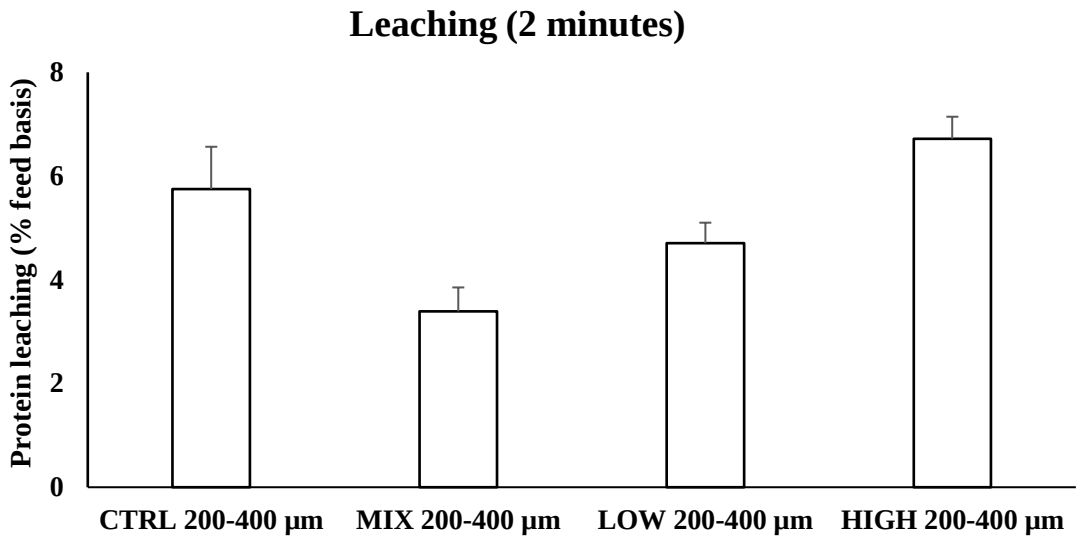

Figure 15 - Protein leaching (% feed basis) of microdiets used on trial 2. Values presented as mean ± standard deviation (n=2). Sample weight= 1g, microdiet range size= 200-400 µm, immersion time= 2 minutes.

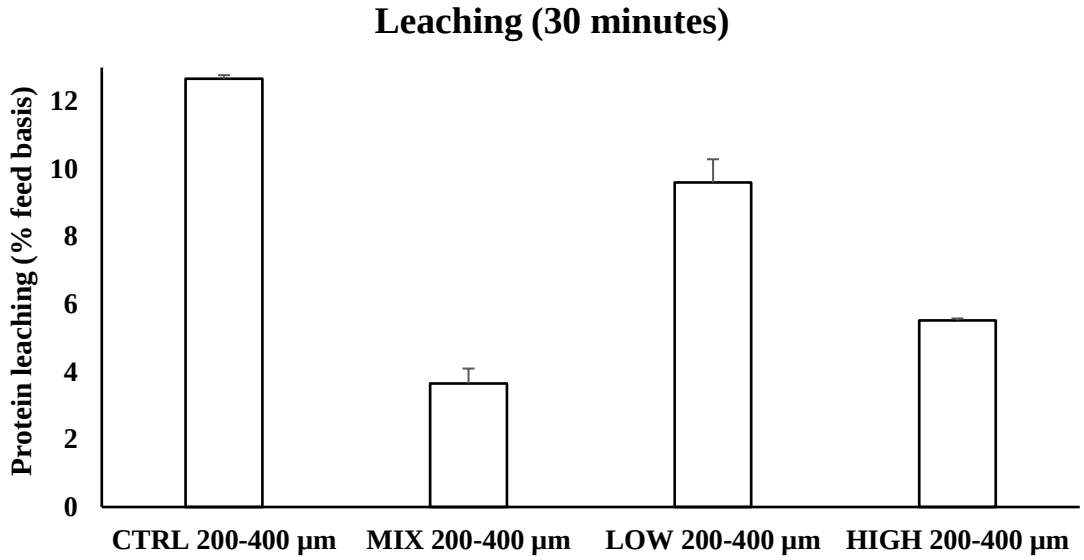

Figure 16 - Protein leaching (% feed basis) of microdiets used on trial 2. Values presented as mean ± standard deviation (n=2). Sample weight= 1g, microdiet range size= 200-400 µm, immersion time= 30 minutes.

Microdiets used on trial 2 percentage of leaching, with range size 400-600 µm during 2 and 30 minutes of immersion are shown in Figures 17 and 18. It was verified that MIX was the treatment with lower percentage of leaching, in contrast CTRL was the diet with higher leaching during 2 minutes of immersion and LOW was the

treatment with higher leaching during 30 minutes of immersion. When compared by time MIX was the diet with lower percentage of protein leaching increment.

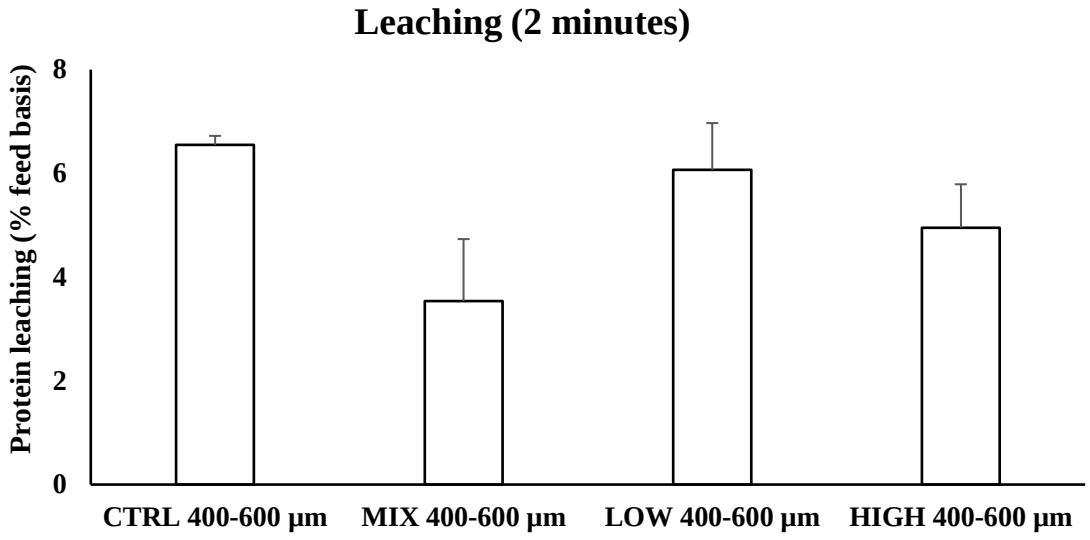

Figure 17 - Protein leaching (% feed basis) of microdiets used on trial 2. Values presented as mean ± standard deviation (n=2). Sample weight= 1g, microdiet range size= 400-600 µm, immersion time= 2 minutes.

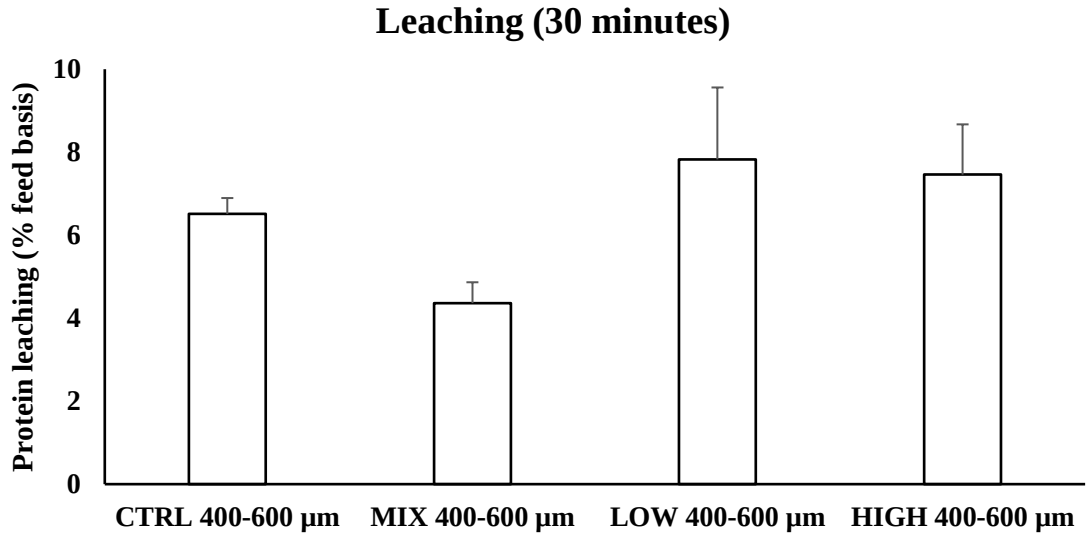

Figure 18 - Protein leaching (% feed basis) of microdiets used on trial 2. Values presented as mean ± standard deviation (n=2). Sample weight= 1g, microdiet range size= 400-600 µm, immersion time= 30 minutes.

Percentage of leaching of microdiets used on trial 2, with range size 400-600 µm during 2 and 30 minutes of immersion are shown in Figures 19 and 20. It was observed that MIX was the dietary treatment with lower percentage of leaching on both immersion times, in contrast, CTRL was the diet with higher percentage of protein leaching. When compared by time HIGH was the treatment with lower protein leaching increment from 2 to 30 minutes of immersion.

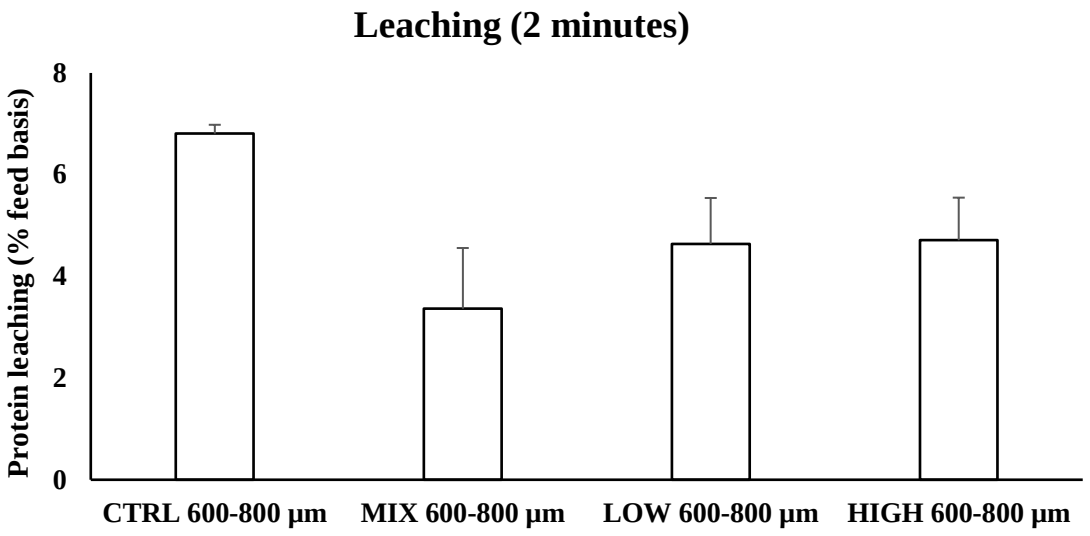

Figure 19 - Protein leaching (% feed basis) of microdiets used on trial 2. Values presented as mean ± standard deviation (n=2). Sample weight= 1g, microdiet range size= 600-800 µm, immersion time= 2 minutes.

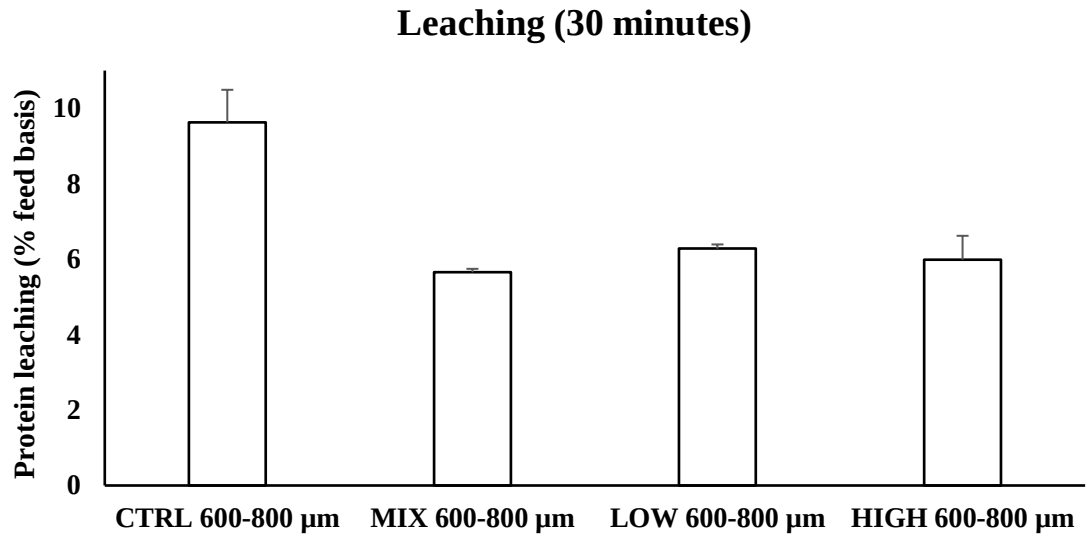

Figure 20 - Protein leaching (% feed basis) of microdiets used on trial 2. Values presented as

mean ± standard deviation (n=2). Sample weight= 1g, microdiet range size= 600-800 µm, immersion time= 30 minutes.

3.2.4. Suspension profile

Suspension profile of microdiets used on trial 2 for range sizes of 100-200 µm, 200-400 µm and 400-600 µm can be observed in Figures 21, 22 and 23. It was observed that microdiet CTRL was the diet with higher availability of pellets for 300 seconds. It was also demonstrated that microdiet HIGH was the diet with lower cumulative availability in 100-200 µm and 200-400 µm range sizes, while MIX microdiet was the diet with lower availability on 400-600 µm size. Furthermore, it was observed that diets LOW and HIGH perform similar sinking behaviours in all range sizes.

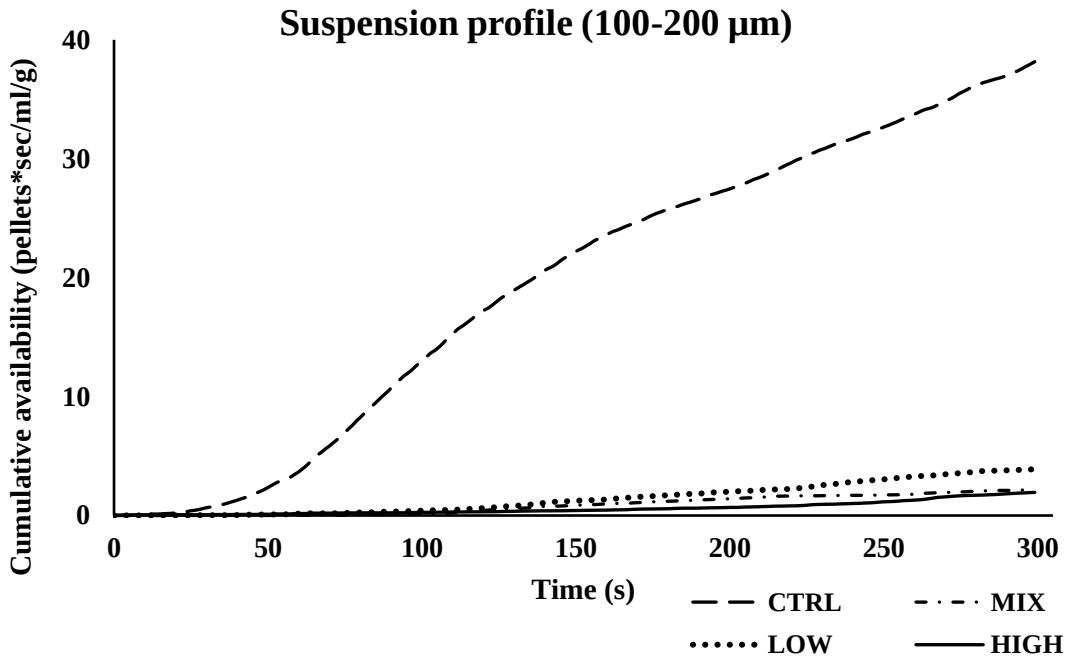

Figure 21 - Cumulative availability (pellets*sec/ml/g) of microdiets used on trial 2. Values presented as mean (n=2). Sample weight = 0.1g, microdiet range size= 100-200 µm, precipitation time = 300 s.

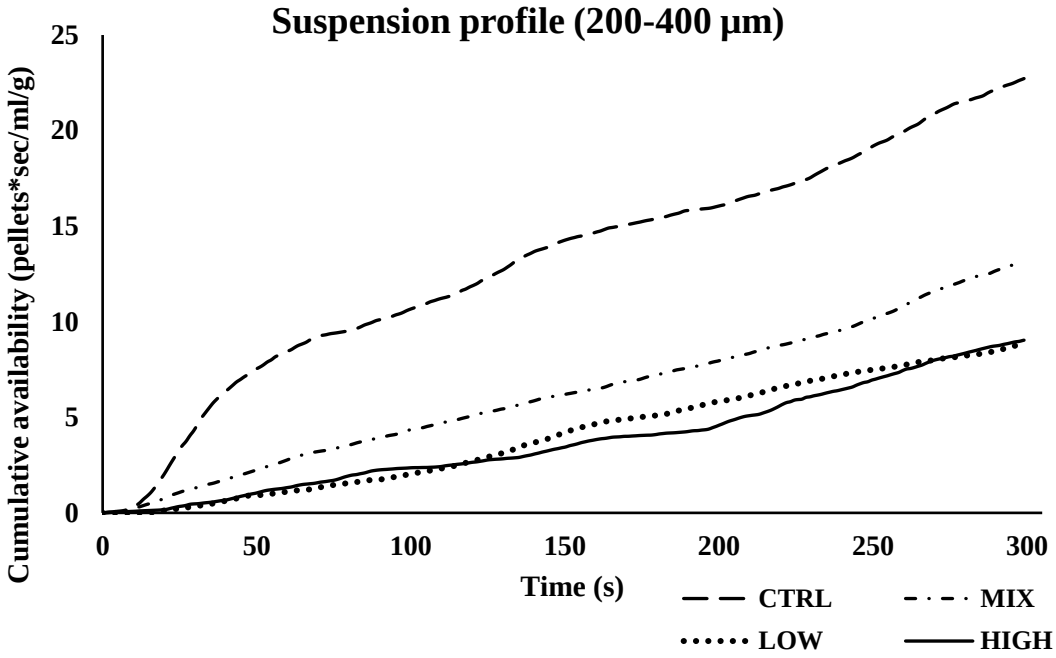

Figure 22 - Cumulative availability (pellets*sec/ml/g) of microdiets used on trial 2. Values presented as mean (n=2). Sample weight = 0.1g, microdiet range size= 200-400 µm, precipitation time = 300 s.

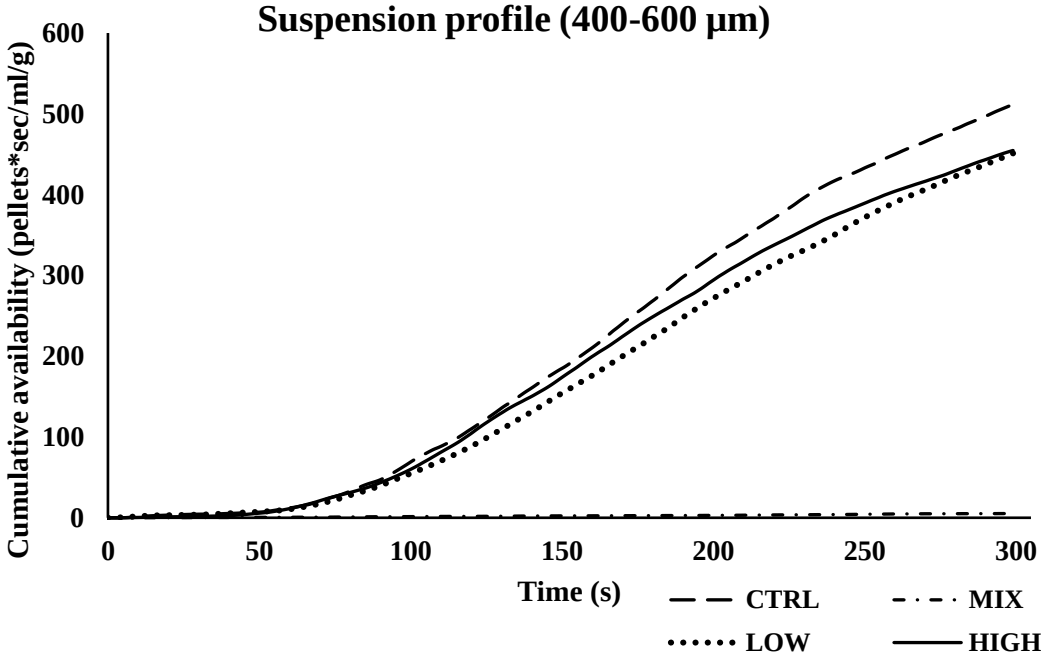

Figure 23 - Cumulative availability (pellets*sec/ml/g) of microdiets used on trial 2. Values presented as mean (n=2). Sample weight = 0.1g, microdiet range size= 400-600 µm, precipitation time = 300 s.

3.2.5. Dispersion profile

Dispersion profiles of microdiets with range sizes 100-200 µm, 200-400 µm and 400-600 µm used on trial 2 are shown in Figures 24, 25 and 26. It was observed that microdiet LOW was the diet with lower dispersion area for range sizes 100-200 µm and 200-400 µm, while MIX microdiet dispersion area was the smallest for range size 400-600 µm. It was also observed that CTRL and MIX microdiets were the diets with higher dispersion area for 100-200 µm, and diet HIGH was the diet with higher dispersion area for 200-400 µm and 400-600 µm sizes.

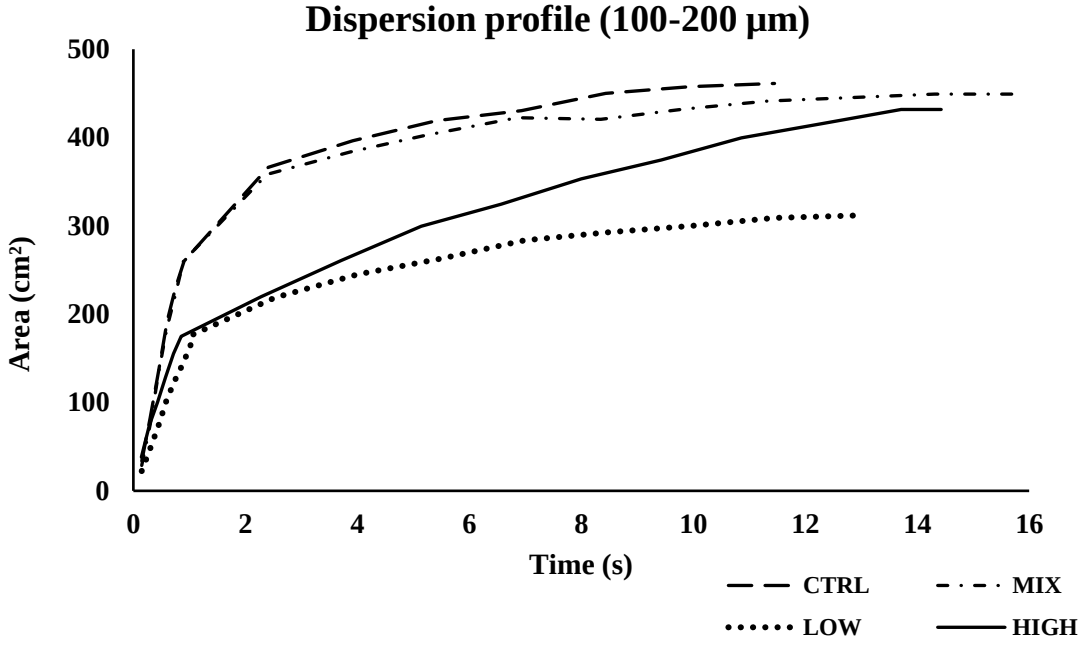

Figure 24 - Dispersion area (cm²) of microdiets used on trial 2. Values presented as mean (n=2). Sample weight = 0.2g, microdiet range size= 100-200 µm, precipitation time = 15 s.

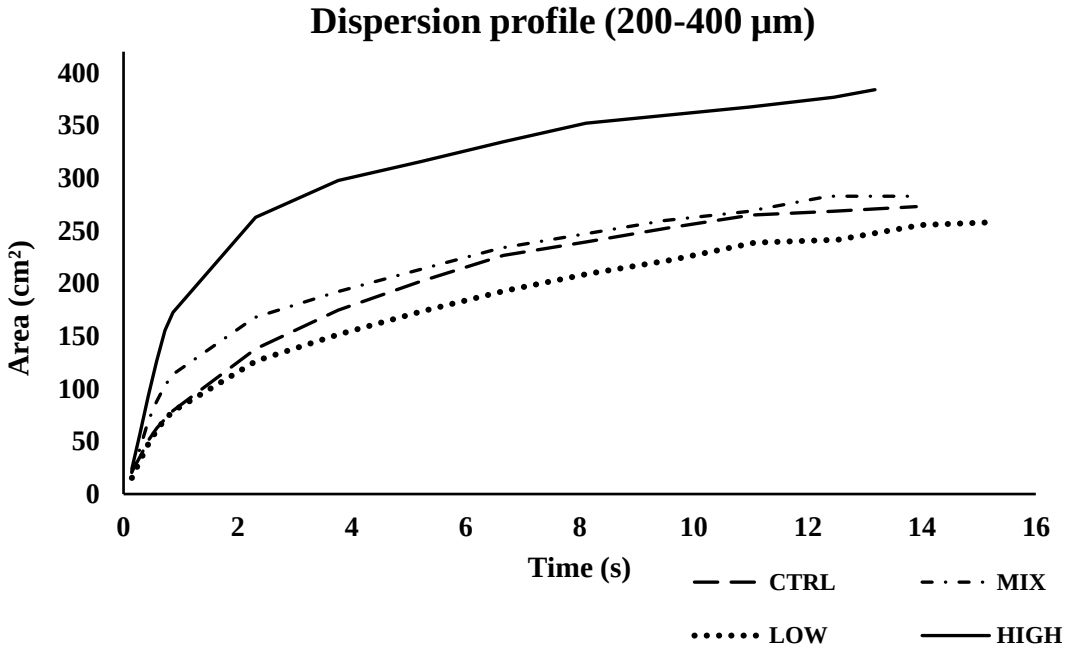

Figure 25 - Dispersion area (cm²) of microdiets used on trial 2. Values presented as mean (n=2). Sample weight = 0.2g, microdiet range size= 200-400 µm, precipitation time = 14 s.

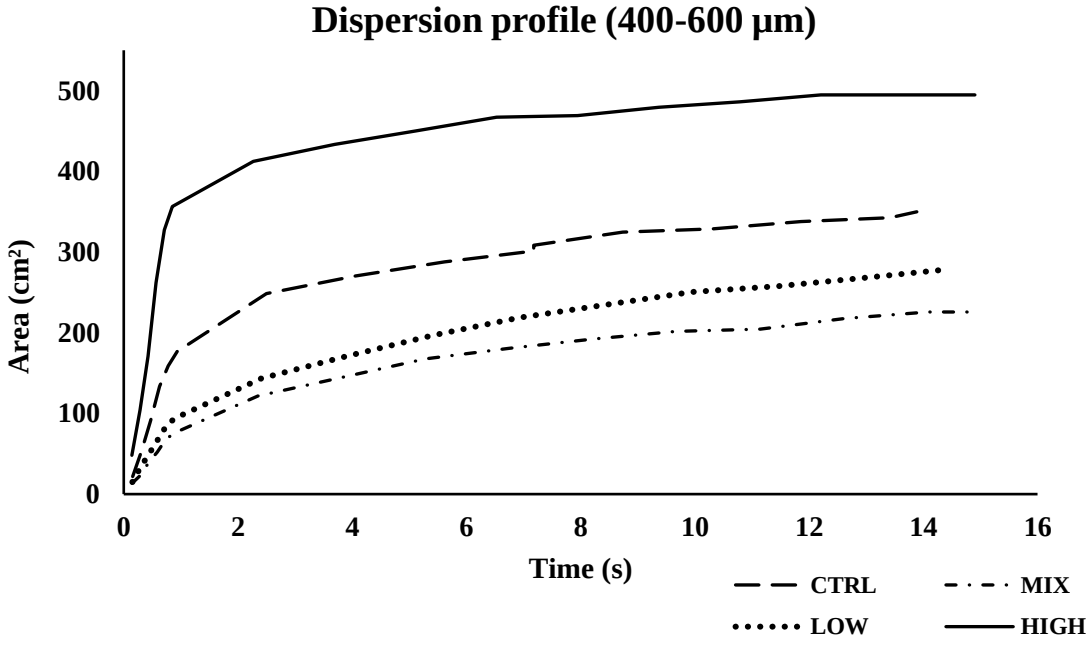

Figure 26 - Dispersion area (cm²) of microdiets used on trial 2. Values presented as mean (n=2). Sample weight = 0.2g, microdiet range size= 400-600 µm, precipitation time = 14 s.

3.3. Discussion

Trial 2 aimed to test the ingredient replacement on Turbot microdiets in two criteria, the growth performance and physical properties. Turbot post-larvae at 50 DAH achieved dry weight values of 210.81 ± 33.56 mg, RGR values around 12.28 % DW day^{-1}, FCR values around 1 and percentage of survival between 57.09 and 69.01 %. Dry weight was lower when compared with Mahious *et al.* (2006) at 55 DAH obtained dry weight values around 410 mg but higher when compared with Sierra-Flores *et al.*, (2016) obtained values around 150 mg at 50 DAH. Regarding the survival values, Mahious *et al.* (2006) obtained between 82.1 and 88.6% at 55 DAH. Therefore, the results in the present study can be considered as reasonable for the species.

Larvae fed with CTRL and HIGH presented the highest performance results, at the end of the trial, the larvae present better results for dry weight. The obtained growth performance results for dietary treatment HIGH where a microdiet was formulated with a high inclusion of the novel binder were similar to the growth results obtained on control,in contrast LOW and MIX presented lowest growth performance results, where the dry weight values obtained were significantly lower when compared with CTRL which demonstrate that a high inclusion of a novel binder is a better option when compared with a low inclusion of the same binder or a mixture of binders. This goes in agreement with Yokoama *et al.*, (2020) that observed that a diet with a mixture of binders appears to negatively influence the growth in juvenile Amberjack (*Seriola dumerili*, Risso, 1810).

Microdiet leaching, suspension and dispersion profile evaluation aimed to test how different inclusion levels of a novel binder and a mixture of binders affect characteristics as leaching, sinking and dispersion of experimental microdiets used on trial 2. CTRL treatment percentage of leaching values in general represent the highest values, except for lower size during 2 minutes of immersion where HIGH treatment presented the highest value and for medium size pellets at 30 minutes of immersion where both LOW and HIGH treatments present higher values when compared with the remaining treatments. In addition, MIX treatment protein leaching presents the lowest values when compared with the remaining treatments. The obtained leaching results were better than Kvåle *et al*., (2006) that obtained leaching percentages of 26-28 %, and when compared with CTRL, all tested microdiets perform better results. This

demonstrates that the low and high inclusion of a new binder and the inclusion of a mixture of binders provides enhancements on microdiets leaching reduction. Compared with the growth performance results, leaching test suggest that the binder inclusion used on HIGH is a good option, because despite not having the lowest leaching percentage, this leaching value remains lower than control (non significant). On the other hand, despite good results on leaching reduction, the usage of a mixture of binders does not present the highest growth performance results, which suggest that probably the performed changes may be limiting the nutrient absorption by the larvae

The results for microdiet suspension profiles shown that CTRL was the diet with higher capacity to sink, and the remaining treatments had lower sinking capacity. The results suggest that the inclusion of a novel binder or a mixture of a binder decrease the sinking capacity of the pellets when compared with control On the other hand for the lowest particle sizes, HIGH treatment sinking capacity was lower when compared with the remaining treatments while for high particle sizes HIGH was the treatment with higher sinking capacity. When relating with the growth performance results and considering small pellet sizes, the obtained results suggests that the high inclusion of the new binder might be an alternative for the control microdiet. On the other hand the low inclusion of a binder was a treatment also with lower sinking capacity but this treatment did not provide good performance results and therefore does not seem to be as adequate for turbot feeding behaviour as the High diet.

Regarding, microdiet dispersion profiles, HIGH was the microdiet with higher dispersion area, except on the lower particle size where CTRL and MIX had higher dispersion areas. Considering that a higher pellet dispersion represents more availability of feed for larvae, and the growth performance, the obtained results suggest that the new binder inclusion at high level performed on dietary treatment HIGH is a good alternative to the control microdiet. On the other hand, LOW was the treatment with lower dispersion area which can helps to justify the lower growth performance due to the fact that this dietary treatment will be less available on water than the remaining microdiets.

When related, this trial growth performance, leaching and the physical properties results demonstrate that high inclusion level of the new binder may potentially be the most suitable alternative for the control microdiet, with the advantage of a reduction on leaching without affecting the growth performance of the larvae. Results also suggest

that a low inclusion level of the new binder cannot be considered a good alternative once the growth performance was the smallest between the treatments, although this experimental treatment had also lower leaching than the control microdiet, it was not possible to find a evident relationship between growth performance and leaching as it happens in all dietary treatments.

In summary, considering that physical characteristics as sinking capacity should be adjusted according to the flatfish larvae feeding behaviour (Bruno *et al*., 2018; Debnath *et al*., 2020), but the most important diet selection criteria is the growth performance, the best dietary treatments tested are CTRL and HIGH, even if there are experimental treatments with lower leaching percentages, what turns out to have a lower relevance once the microdiets normally remain for a shorter time in water in turbot compared to sole.

4. Final Conclusions

The present Thesis supports the following conclusions:

- The thesis demonstrates that a well-established microdiet formulation may represent significative improvements on production cost and simultaneously improve aspects as leaching reduction without affect the growth performance.It is possible to create new microdiets prototypes for Senegalese sole with high cost/benefit ratio.
- There were no benefits for Senegalese sole growth performance by supplementing phospholipids and reduce the fat content on a microdiet, despite that, the phospholipid addition appears to help on reduced protein leaching and pellet sinking capacity.
- Microdiets with better capacity to retain water soluble proteins and similar growth performance to the Control diet used in Senegalese sole were diets B/C and PL.
- Binders inclusion on Turbot microdiets helps on leaching reduction, however it was not an advantage on growth performance and it was not possible to relate both parameters. A diet High in a novel binder resulted in a similar growth performance to the Control.
- Further species-specific research can be done in order to select ingredients with an optimal balance to simultaneously benefit on growth performance, reduce leaching, control of the physical properties.

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