

THE POTENTIAL INHIBITORY EFFECTS OF MICROALGAE AND MACROALGAE ON PROTEASE ACTIVITIES OF ARGYROSOMUS REGIUS (PISCES, SCIAENIDAE) LARVAE USING IN VITRO ASSAYS

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Abstract

The aim of this research was to gather preliminary data about the potential inhibitory effects of microalgae and macroalgae on protease activities of meagre larvae using in vitro techniques. Three microalgae (*Chlorella* sp., *Spirulina* sp., *Schizochytrium* sp.) and two macroalgae (*Ulva* sp. and *Sargassum* sp.) were tested in the present study. Pooled samples of meagre larvae fed on commercial feeding procedure were collected thirteen times from 3 to 32 days after hatching (DAH). All tests were carried out in triplicates. The differences observed in the total length and weight values were statistically significant ($p < 0.05$). The lowest and highest weights were 0.54 ± 0.02 mg (3 DAH) and 89.21 ± 0.91 mg (32 DAH), respectively. The lowest and highest total length values were 3.22 ± 0.02 mm (3 DAH) and 20.95 ± 0.3 mm (32 DAH). The differences in protease activities of meagre larvae during the sampling period were statistically significant ($p < 0.05$). The highest and lowest protease activities of meagre larvae were 393.97 ± 7.9 U/mg protein (7 DAH) and 9.64 ± 1.25 U/mg protein (20 DAH), respectively. The greatest sensitivity to protease inhibitors present in the assayed algae was observed in *Chlorella* sp. with more inhibitions than 45%. The highest inhibitions of *Schizochytrium* sp., *Spirulina* sp. and *Chlorella* sp. were observed at 3, 5 and 10 DAH, respectively. The lowest inhibitions of *Chlorella* sp., *Schizochytrium* sp. and *Spirulina* sp. were found at 5, 10 and 12 DAH, respectively. The highest and lowest inhibitions of *Ulva* sp. and *Sargassum* sp. were determined at 5 and 20 DAH, respectively. *Spirulina* sp., *Schizochytrium* sp. and *Ulva* sp. had lower inhibitions than 45% except for 3, 5, 7 and 17 DAH. However, the inhibition values of *Chlorella* sp. were more than 45% from 3 to 32 DAH. The inhibitions of *Sargassum* sp. were determined as more than 45% except for 15, 20, 22, 25 and 27 DAH. In conclusion *Spirulina* sp., *Schizochytrium* sp. and *Ulva* sp. can be used as feed ingredient in commercial diets of meagre larvae from 10 to 32 DAH except for 17 DAH. *Chlorella* sp. doesn't seem to be suitable. *Sargassum* sp. can be used as feed ingredient from 15 to 32 DAH except for more inhibitions than 45%. Future researches must be conducted on feed ingredients exhibiting lower inhibitions on protease activities of marine fish larvae.

Key words: microalgae, macroalgae, protease, inhibition, feed ingredient

1. INTRODUCTION

European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) are amongst the most important marine finfish species, especially in the Mediterranean region. Recently, beside the prices of these have decreased to survive economically, aquaculture requires low cost inputs and high productivity. Therefore, aquaculture needs to focus on the introduction of new candidate species. Meagre appears to be one of the potential species for diversification of aquaculture (El-Shebly et al. 2007 ; Monfort 2010). The sciaenid meagre (*Argyrosomus regius*) is found in the Mediterranean and Black Sea and along the Atlantic coasts of Europe and the west coast of Africa (Whitehead et al. 1986; Haffray et al. 2012). Quemener (2002) reported that the most important advantage of meagre suggested as candidate species in aquaculture were high growth rates.

Microalgae are currently used in the culture tanks as well as the zooplankton demanded during larval feeding. There is need for nutritional requirements during critical larval stage, either for direct consumption for filter-feedings or indirectly for the live foods as rotifers and *Artemia* nauplii used in the feeding of larvae. Cahu et al. (1998) showed that the algae act by triggering digestive enzyme production, at both the pancreatic and intestinal level. The dependence on *Artemia* nauplii for larval

feeding is an important problem for hatcheries due to the possibility of a global shortage and the increasing prices of *Artemia* cyst, as well as the variations observed in nutritional compositions. For this reason, there need to sustainable diets capable of to meet the nutritional requirements of fish larvae.

In present, commercial diets are quite expensive due to inclusion of high cost fish meal. Intensive production of aquaculture species depends heavily on feed ingredients such as fish meal and fish oil. To provide a sustainable aquaculture, there is an urgent need to decrease this dependence and the potential use of feed ingredients such as vegetable meals instead of high cost fish meal. Therefore, an intensive effort has been made in order to evaluate the potential of alternative protein sources in feeds (Alexis 1997).

Studies on determining inhibitory effects of feed ingredients induced the protease activity are the key tools to understand and solve nutritional problems. For this reason, trials have been carried out to evaluate cheap and sustainable alternative protein sources such as soybean meal. However, the main obstacles to the use of high amounts of vegetable protein sources in fish diets are low protein quality due to the amino acid imbalances and the presence of antinutritional factors reducing the activity of fish digestive enzymes (Tacon 1997; Huisman and Tolman 1992 ; Krogdahl et al. 2003).

Researcher reveal a different sensitivity of fish proteases to inhibitors present in feeds, suggesting the need of a preliminary evaluation of such effects when feed ingredients such as vegetable protein source in formulations are used (Moyano et al. 1998; Alarcón et al. 1999). In this sense, the effects of feed ingredients used in the production of microdiets on protease activities of seabream larvae and shrimps were studied (Alarcón et al. 1997, Alarcón et al. 1999).

Global seaweed production is increasing from day to day and is mainly used as feed ingredient and vegetable production. Seaweeds are good source of proteins, vitamins and minerals (Buschman et al. 2001; Burtin 2003). The *in vitro* digestibility of algal proteins can change according to the species and seasonal variations observed in antinutritional factors. The compounds such as phenolic molecules and polysaccharides effect on *in vitro* digestibility of algal proteins. Studies reveal the strong inhibitory action of soluble fibres on *in vitro* pepsin activity as well as their negative effects on protein digestibility (Horie et al. 1995). For this reason, a biotechnological treatment of seaweeds by enzymatic degradation of algal fibres could be attempted to improve protein digestibility. Amano and Noda (1992) showed that an enzymatic pretreatment of *Ulva pertusa* and *Undaria pinnatifida* could be an alternative way to limit the effects of algal fibres as antinutritional factors. On the other hand, previous studies indicated that dietary macroalgae meals are improved the growth, lipid metabolism, physiological activity, stress response, disease resistance and carcass quality of fish species (Ergün et al. 2009; Güroy et al. 2011; Güroy et al. 2013). Macroalgae and microalgae such as *Ulva sp.*, *Sargassum sp.*, *Spirulina sp.* and *Chlorella sp.* have been evaluated as feed ingredients in earlier studies. Results showed that the addition of small amounts of algae meal to feeds resulted in considerable effects on growth, feed utilization body composition and carcass quality (Hashim et al. 1992; Wassef et al. 2005; Valente et al. 2006; Diler et al. 2007; Güroy et al. 2007; Emre et al. 2013). Emre et al. (2013) revealed that dietary inclusion of 4% *Ulva* meal could be used without negative effects on the growth performance, nutrient utilization, and body composition. The other researches confirmed that a 5% or 10% inclusion of dietary *Ulva* meal had no negative effects on the growth performance of juvenile European seabass *Dicentrarchus labrax* (Valente et al. 2006), tilapia (Güroy et al. 2007), and rainbow trout, *Oncorhynchus mykiss* (Güroy et al. 2013; Dantagnan et al. 2009; Soler-Vila et al. 2009)

Until now, researchers have focused on growth, survival and larval rearing of meagre (Pastor et al. 2013; Vallés and Estévez 2013; Vallés and Estévez 2015), the ontogeny of digestive system of meagre (Papadakis et al. 2013) and the effects of different levels of vegetable proteins on juvenile meagre (Estévez et al. 2011). In addition, digestive enzymes of marine fish larvae such as *D. labrax*, *S. aurata*, *Solea senegalensis*, *Diplodus sargus*, *Pagrus auriga*, *A. regius* were investigated by some authors (Zambonino Infante and Cahu 1994; Moyano et al. 1996; Ribeiro et al. 1999; Cara et al. 2003; Moyano et al. 2005; Süzer et al. 2013). We could only found study digestive enzymes (Süzer et al.

2013) none study on the inhibitory effects of feed ingredients on protease activities of meagre larvae. In this point, inhibitory effects of the potential feed ingredients in the larvae rearing must be investigated to solve the nutritional problem. Therefore the aim of this research was to called preliminary data about the potential inhibitory effects of feed ingredients such as three microalgae (*Chlorella sp.*, *Spirulina sp.*, *Schizochytrium sp.*) and two macroalgae (*Ulva sp.* and *Sargassum sp.*) on protease activities of meagre larvae using *in vitro* techniques.

2. MATERIAL AND METHODS

2.1. Larvae rearing and sampling

Sampling stage of the present study was carried out at the EGEMAR Aquaculture Food Industry and Commercial Incorporated Company. Eggs were obtained with hormone injection from meagre broodstocks (GnRH; 20 µg/kg ♀ and 10 µg/kg ♂). Fertilized eggs of meagre were collected from the broodstock tanks and incubated in conical fiberglass tanks at a temperature of 22.0 ± 0.2 °C. Newly hatched larvae were transferred from the incubators to fiber glass 7 m³ ellipsoidal fiberglass tanks with black walls until 15 days after hatching (DAH). From 15 to 32 DAH, larvae were stocked to concrete raceway 15 m³ tanks (stocking density; at 0-15 DAH 75-80 larvae/L and at 16-32 DAH 10-12 larvae/L). The rearing tanks supplied with running sea water that had been filtered through a sand, bag and UV filters. Temperature, salinity, oxygen levels, and pH were 20.8-22.2 °C, 27.0-40.0 g/L, 7.8-14.7 mg/L, and 7.7-8.1, respectively. Air and fresh sea water were introduced into the surface of the tanks to prevent water stratification until 15 DAH. Rearing tanks were exposed to a photoperiod (18:6).

Nannochloropsis oculata were used for green water technique from 3 to 15 DAH. Rotifer (*Brachionus plicatilis*) was cultured with Algamac Protein Plus (Aquafaune Bio-Marine Inc. Hawthorne USA) and Sparkle (Inve Aquaculture). The average water temperature and salinity during the culture were 25 °C and 25 g/L, respectively. Rotifer was enriched with Spresso (INVE Aquaculture) prior transfer to the larval feeding tanks. The average water temperature and salinity during the enrichment were 26 °C and 28 g/L, respectively.

Artemia nauplii (**Artemia Cysts**; Vinh Chau-Bac Lieu Artemia Co.O) were cultured at 29 °C and 28 g/L. *Artemia* metanauplii (**Artemia EG**; *Artemia* SepArt EG >250.000 npl/g INVE Aquaculture Salt Lake City Utah/USA) were cultured at 29 °C and 28 g/L. *Artemia* metanauplii were enriched with enrichments (Spresso-INVE Aquaculture) for 24 h at 26°C and 28 g/L.

The feeding regime consisted of *B. plicatilis* from 3 to 8 DAH, reaching a maximum concentration of 10-15 prey/mL, *Artemia* nauplii from 7 to 11 DAH, with a maximum density of 4-6 prey/mL, *Artemia* metanauplii from 10 to 15 DAH, with a maximum density of 2-4 prey/mL, *Artemia metanauplii* from 16 to 32 DAH with a maximum density of 1,5-5 prey/mL. From 16 to 26 DAH, with a maximum density of 2 prey/mL and *Artemia metanauplii* from 27 to 32 DAH, with a maximum density of 4.5 prey/mL. Commercial diets such as **Gemma Micro 150** (100-200µ; **Skretting AS**) from 17 to 22 DAH, **Caviar** (200-300µ; **BernAqua**) from 21 to 24 DAH, **Caviar** (300-500µ; **BernAqua**) from 24 to 29 DAH and Perla Larva Proactive 4.0 (300-500µ; **Skretting AS**) from 28 to 32 DAH were used in the commercial feeding procedure of meagre larvae. Proximate compositions of commercial diets used in the present study were given in Table 1. Also, *Nannochloropsis oculata* was added into the growth tanks from 16 to 26 DAH. Samples of meagre larvae fed on commercial feeding procedure were collected in triplicates from 3 to 32 DAH. Larvae were taken before the morning feeding and immediately stored in liquid nitrogen (-196 °C) to prevent protein autolysis.

Table 1. Proximate compositions of commercial diets used in the present study.

Microdiets	Size (µm)	Proteins (%)	Lipids (%)	Ash (%)	Fibre (%)
SKRETTING					
Gemma Micro 150	100-200	59,0	14,0	15,0	0,2
Perla LP 4.0	300-500	62,0	11,0	8,0	1,2
BERNAQUA					
Caviar 200-300	200-300	55,0	15,0	15,0	2,0
Caviar 300-500	300-500	55,0	15,0	15,0	2,0
Σn-3 HUFA 25,0 mg/g DHA 1,0 mg/g EPA 10,0 mg/g					

2.2. Analytical methods

2.2.1. Extracts of larvae

The samples were rinsed in distilled water after thawing and then the extracts of whole larvae were homogenized and centrifuged (16,000 g, 30 minute 4°C).

2.2.2. Extracts of algae

Five algae as feed ingredients (*Chlorella sp.*, *Spirulina sp.*, *Schizochytrium sp.*, *Ulva sp.* and *Sargassum sp.*) were tested with *in vitro* techniques. Extracts of five algae prepared by homogenization (100 mg/mL in distilled water) followed by centrifugation (15,000 g, 10 minute) were used in protease inhibition analyses.

2.2.3. Determination of protease activities of larvae

Total protease activities of meagre larvae were measured as described by Walter (1984), using casein (10 mg/mL) in 50 mM Tris-HCl buffer at pH 8.5 as the substrate. The mixtures including extracts of larvae and substrate were incubated and then the reaction was stopped by addition of 500 µL trichloroacetic acid (TCA) (120 g/L). One unit of enzyme activity was defined as 1 µg of tyrosine release per minute. The soluble protein concentrations of meagre larvae were determined according to Bradford (1976).

2.2.4. Effects of algae on protease activities of larvae

The inhibitory effects of five algae on protease activities of meagre larvae were determined by measuring the reduction in protease activity of extracts using a modification of the method described by García-Carreno (1996). The method is based on the measurement of residual protease activity remaining after preincubation with different algae such as *Chlorella sp.*, *Spirulina sp.*, *Schizochytrium sp.*, *Ulva sp.* and *Sargassum sp.*

2.2.5. Statistical methods

All measurements were carried out in triplicates. The experimental data were subjected to one-way (ANOVA) and mean±standard error (SE) differences were made by Duncan test at $P=0.05$ content level by using SPSS 15.0 statistical package (SPSS 2006).

3. RESULTS

Table 2 shows the changes observed in growth rate of meagre larvae. The differences determined in the total length and weight values from 3 to 32 DAH were statistically significant ($p < 0.05$). The lowest and highest weight values were 0.54 ± 0.02 mg (3 DAH) and 89.21 ± 0.91 mg (32 DAH), respectively. Larval weight remained relatively constant until 10 DAH and followed by a sharp

increase in larval weight continued until 32 DAH ($p < 0.05$). The lowest and highest total length values were 3.22 ± 0.02 mm (3 DAH) mm and 20.95 ± 0.3 mm (32 DAH). The total length value of meagre larvae remained relatively up to 5 DAH. After 5 DAH, the total length value tended to increase until 32 DAH ($p < 0.05$).

Table 2. The total length and the weight values of meagre (*Argyrosomus regius*) larvae (mg) observed during the study. Results are expressed as mean $\pm$ standard error (a pool of 30 larvae).

Days after Hatching (DAH)	Total length (mm)	Larval weight (mg)
3	3.22 ± 0.02	0.54 ± 0.02
5	3.42 ± 0.03	0.79 ± 0.02
10	5.24 ± 0.07	1.49 ± 0.36
15	6.54 ± 0.05	3.86 ± 0.37
20	9.49 ± 0.09	11.67 ± 0.56
25	14.18 ± 0.14	29.91 ± 2.93
30	18.44 ± 0.19	64.63 ± 0.12
32	20.95 ± 0.3	89.21 ± 0.91

The changes measured in protease activities of meagre larvae are given in Table 3. The differences observed in protease activities from 3 to 32 DAH were statistically significant ($p < 0.05$). The highest and lowest protease activities of meagre larvae were 393.97 ± 7.9 U/mg protein (7 DAH) and 9.64 ± 1.25 U/mg protein (20 DAH), respectively.

Table 3. The changes determined in protease activities of meagre (*Argyrosomus regius*) larvae during the study (U/mg protein). Results are expressed as mean $\pm$ standard error.

Days After Hatching (DAH)	U/MG PROTEIN
3	345.66 ± 1.45
5	184.25 ± 0.46
7	393.97 ± 7.9
10	16.64 ± 0.96
12	17.46 ± 0.59
15	32.81 ± 1.76
17	245.95 ± 5.59
20	9.64 ± 1.25
22	16.63 ± 0.75
25	64.01 ± 1.27
27	35.1 ± 1.34
30	51.38 ± 2.13
32	68.66 ± 2.52

Protease activities of larvae tended to decrease from 3 to 5 DAH. After 5 DAH, a sharp increase until 7 DAH and then, a sharp decrease from 7 to 10 DAH was observed. Protease activities of larvae tended to increase from 10 to 17 DAH and then, followed by a sharp decrease up to 20 DAH. After 20 DAH, protease activities of meagre larvae tended to increase until 25 DAH and followed by a decrease at 27 DAH and then, increased from 27 to 32 DAH.

The inhibitory effects of algae on protease activities of meagre larvae are given in Figure 1 and Figure 2, respectively. The high inhibitions of protease activities were obtained when extracts were incubated in the presence of solutions prepared with algae used in the present study. The digestive proteases of meagre larvae showed the greatest sensitivity to protease inhibitors present in *Chlorella sp.*

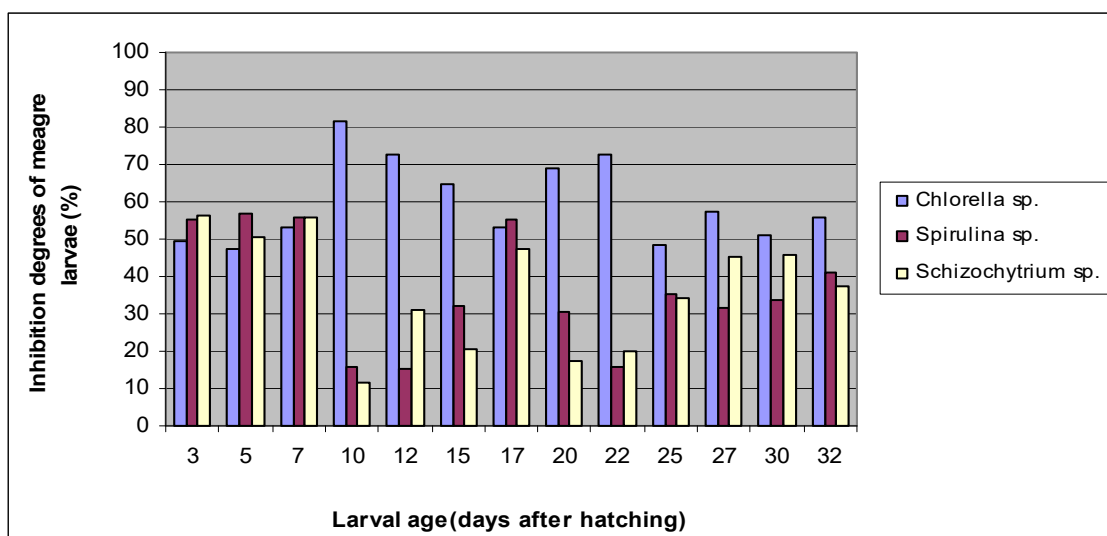


Figure 1. The inhibitory effects of microalgae such as *Chlorella sp.*, *Spirulina sp.* and *Schizochytrium sp.* on protease activities of meagre (*Argyrosomus regius*) larvae.

The highest inhibitions of *Schizochytrium sp.*, *Spirulina sp.* and *Chlorella sp.* were observed at 3, 5 and 10 DAH, respectively. The lowest inhibitions of *Chlorella sp.*, *Schizochytrium sp.* and *Spirulina sp.* were found at 5, 10 and 12 DAH, respectively. The highest and lowest inhibitions of *Ulva sp.* and *Sargassum sp.* were determined at 5 and 20 DAH, respectively. *Spirulina sp.*, *Schizochytrium sp.* and *Ulva sp.* had lower inhibitions than 45% except for 3, 5, 7 and 17 DAH. However, the inhibition values of *Chlorella sp.* were more than 45% from 3 to 32 DAH. The inhibitions of *Sargassum sp.* were determined as more than 45% except for 15, 20, 22, 25 and 27 DAH.

Chlorella sp. had more inhibitions than 45% in the critical larval stage (from 3 to 15 DAH). However, *Spirulina sp.* and *Schizochytrium sp.* had good performance with lower inhibitions than 45% except for 3, 5 and 7 DAH than *Chlorella sp.* From 20 to 32 DAH, *Spirulina sp.*, *Schizochytrium sp.* exhibited better performance than *Chlorella sp.* *Sargassum sp.* had more inhibitions than 45% in the critical larval stage except for 15 DAH while *Ulva sp.* shows better performance than *Sargassum sp.* except for 10, 12 and 15 DAH. Also, *Ulva sp.* had lower inhibitions than 45% except for 17 DAH. The inhibitions of *Sargassum sp.* were lower than 45% except for 17, 30 and 32 DAH.

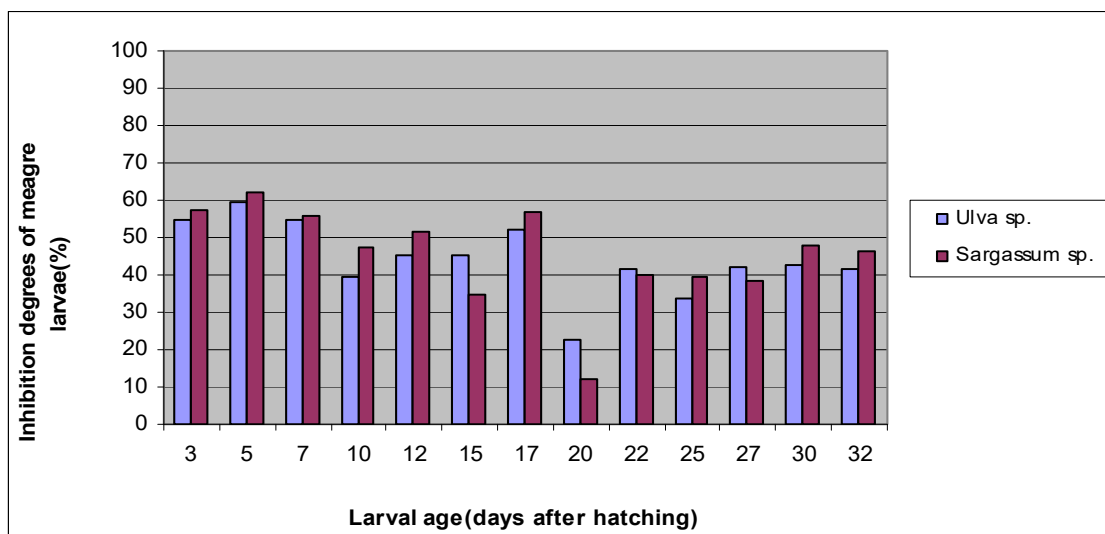


Figure 2. The inhibitory effects of macroalgae such as *Ulva sp.* and *Sargassum sp.* on protease activities of meagre (*Argyrosomus regius*) larvae.

4. DISCUSSION

The shortage observed in live food stocks together with the increase demand, especially *Artemia* nauplii caused to increase in market prices of live foods commonly used in critical stages of marine fish larvae. For this reason, there has been a growing interest in finding the most suitable feed ingredients for sustainable aquaculture microdiets. We aimed to determine the inhibitory effects of three microalgae (*Chlorella sp.*, *Spirulina sp.*, *Schizochytrium sp.*) and two macroalgae (*Ulva sp.* and *Sargassum sp.*) on protease activities of meagre larvae using *in vitro* assays. Also, growth parameters (total length and weight) of larvae were determined. The results of the study showed that larvae had high growth rates. Quemener (2002) supported that the most important advantage of meagre larvae was high growth rates.

Currently, we could only found study about digestive enzymes of meagre larvae (Süzer et al. 2013) and none about protease activities and the inhibition effects of feed ingredients on protease activities of meagre larvae. The fluctuations observed in protease activities of meagre larvae were high until 10 DAH. Protease activities of larvae tended to increase from 10 to 15 DAH and then, followed by a sharp increase up to 17 DAH. The lowest level of protease activities of meagre larvae was observed at 20 DAH. Then, protease activity values remained relatively constant from 20 to 32 DAH. Zambonino Infante and Cahu (2001) indicated that the fluctuations observed in specific activities of enzymes is not due to a diminution in enzyme synthesis but is the result of an increase in tissue proteins.

Our results showed that feed ingredients such as microalgae and macroalgae used in the present study caused the inhibitions on protease activities of meagre larvae. The highest resistances to protease inhibitors present in *Chlorella sp.*, *Schizochytrium sp.* and *Spirulina sp.* were found at 5,10 and 12 DAH, respectively. However, the resistances of *Ulva sp.* and *Sargassum sp.* were observed at 20 DAH. Results obtained from the *in vitro* inhibition assays also indicated that the negative effects of feed ingredients such as microalgae and macroalgae tested in the present study on the protease activities of meagre larvae could affect whole digestibility of diets.

Moyano et al. (1999) indicated that the negative effects of using protease inhibitor-containing feed ingredients on fish growth may be related to dietary factors such as the type of meal and the sensitivity of a given fish species to the antinutritional compounds. Alarcon et al. (1999) showed that ovalbumin significantly reduced (60%) the activity of proteases in 8 day old seabream larvae. Similar results were found when commercially produced microcapsules containing ovalbumin were tested using shrimp proteases (Alarcon et al. 1997). Reductions in nutritional value of commercial diets are the results of

the presence of antinutritional compounds found in feed ingredients commonly used in the formulation of aquaculture feeds.

The present study indicates a different sensitivity of meagre proteases to inhibitors present in feed ingredients, especially *Chlorella sp.* exhibiting more inhibition than 45% from 3 to 32 DAH. For this reason, *Chlorella sp.* should not be recommended as feed ingredient for microdiets of meagre larvae. The present study indicated that *Schizochytrium sp.*, *Spirulina sp.* and *Ulva sp.* are suggested from 10 to 15 DAH but not until 7 DAH. However, *Sargassum sp.* should not be recommended except 15 DAH in critical larval stage.

Results suggest that the need of a preliminary evaluation of both positive and negative effects of the feed ingredients used in commercial diets in future. Also, the present paper reveals the usefulness of using *in vitro* assays for a preliminary assessment of the effects of feed ingredients used in commercial diets of fish larvae.

In conclusion, results obtained confirm the existence of protease inhibitors in microalgae and macroalgae tested as feed ingredient. In addition, the results of the study provide important contributions to determine the most suitable feed ingredient for using in commercial diets of meagre larvae. *Spirulina sp.*, *Schizochytrium sp.* and *Ulva sp.* can be used as feed ingredient in commercial diets of meagre larvae from 10 to 32 DAH except for 17 DAH. *Chlorella sp.* doesn't seem to be suitable. *Sargassum sp.* can be used as feed ingredient from 15 to 32 DAH except for more inhibitions than 45%. When such data become available, they will serve the regulation of feeding protocol of cultured marine fish larvae. Future researches must be conducted on feed ingredients exhibiting lower inhibitions on protease activities of marine fish larvae.

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