Dried Distillers Grains with Solubles (DDGS): a potential protein source in feeds for aquaculture.

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/______
“With earth’s burgeoning human population to feed we must turn to the sea with understanding and new technology. We need to farm it as we farm the land.”

Jacques Cousteau
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Abstract

In the last decades, fish meal has been the main protein source in aquafeeds for marine fish species. However, the increased demand, price, limited availability, market supply fluctuations lead nutrition research to search for alternative protein sources to fish meal. Within these alternative feedstuffs, distillers dried grains with solubles (DDGS) have been emerging as an interesting protein source to include in the diets for sparing fishmeal. However, few studies have been conducted in the area. The exact levels of DDGS that can be included into the feeds for different cultivated species aren’t known yet, neither the effects of its inclusion at physiological, inflammatory or immunity levels. As a first approach for the evaluation of the potential of DDGS in marine fish diets, in this study two digestibility trials were conducted to evaluate apparent digestibility coefficient (ADC) of nutrients in DDGS from different sources (DDGS$_1$ - Biofuels de Castilla y Leon, Spain and DDGS$_2$ - Pannonia Gold, Hungry, respectively) in sea bass ($D_{icentrarchus~labrax}$) and meagre ($A_{gyrosomus~regius}$). Thereby, the objective of the present study was to evaluate the nutrient availability of two different sources of corn DDGS and their effect on digestive enzyme activities of two carnivorous species.

The ADC of protein in the tested ingredients was very high (92-98%) as well as the ADC of lipids (82-89%). The ADC of dry matter and energy were moderate (57-66% and 58-68%, respectively). The DDGS high fiber content can explain these moderate energy digestibility values. The ADC of protein and phosphorus of the DDGS$_1$ diet were higher than the ADC of the reference diet, both in sea bass (92.85% and 55.63%, respectively) and in meagre (90.96% and 60.36%, respectively). Different ADC values of dry matter, protein, energy and lipids were obtained between the two tested ingredients and the DDGS$_1$ recorded the higher ADC values for the measured parameters.

In both species, the highest activity of lipase was observed in animals fed the diet containing DDGS$_2$. In sea bass, amylase activity was higher in fish fed the diet including DDGS$_2$ than DDGS$_1$ but did not differ significantly to fish fed the reference diet. In meagre, animals fed the DDGS$_2$ diet also showed a higher amylase activity than those fed the DDGS$_1$ diet, but the values were not significantly different. In the two species, proteases activity didn’t differ significantly between diets. There was no effect of diet on the proteolytic activity tested at various pH values. These activity record peaks in the pH values tested (8,9 and 10) along the three analyzed intestine sections.

In sea bass, the highest digestive enzymes activity was recorded in the medium and
distal intestine while in meagre maximum activity was registered in the medium intestine.

Comparing the two species, proteolytic activity was higher in meagre while the highest activity of amylase was recorded in sea bass. Overall, lipase activity was higher in sea bass than in meagre, except in meagre juveniles fed diet 2. Based on the results of this work, it is concluded that distillers dried grains with solubles (DDGS) seem to be a raw ingredient with high potential to be included in diets for marine fish.

Keywords: Distillers dried grains with solubles (DDGS); European Sea Bass (Dicentrarchus labrax); Meagre (Argyrosomus regius); Digestibility; Digestive enzymes

Resumo
A farinha de peixe tem sido a principal fonte de proteína utilizada no fabrico de rações para a alimentação de espécies marinhas durante a última década. Contudo, o aumento da sua procura e preço, a sua reduzida disponibilidade, as flutuações do seu fornecimento aos mercados e a imprevisibilidade destes, assim como as restrições do uso de proteínas provenientes de fontes animais na formulação de rações, direccionou uma grande parte da investigação efectuada em nutrição de animais aquáticos para a procura de outras fontes proteicas abundantes, disponíveis e rentáveis. Deste modo, os grãos secos de destilaria com solúveis (DDGS) perfilam-se como uma alternativa proteica real e interessante à tão problemática farinha de peixe.

No entanto, poucos estudos foram efectuados na área, não se sabendo ainda quais os níveis exactos que podem ser integrados em rações destinadas às diferentes espécies cultivadas, nem quais poderão ser os efeitos que esta integração pode provocar a nível imunitário. Assim, dois ensaios de digestibilidade foram efectuados para avaliar o coeficiente de digestibilidade aparente dos nutrientes de DDGS 1 e DDGS 2 provenientes de fontes diferentes (Biocarburantes de Castilla y Leon, Spain e Pannonia Gold, Hungry, respectivamente) e o seu efeito nas enzimas digestivas em robalo (Dicentrarchus labrax) e corvina (Argyrosomus regius, Asso, 1801). Os resultados deste estudo mostraram que estas duas espécies carnívoras digerem muito bem os ingredientes vegetais.

A digestibilidade da proteína nos ingredientes testados foi bastante elevada (92-98%) assim como a digestibilidade dos lípidos (82-89%). Os valores de digestibilidade da matéria seca e da energia foram moderados (57-66% e 58-68%, respectivamente). O elevado conteúdo em fibra dos DDGS pode explicar estes valores não tão elevados da digestibilidade da energia. Os coeficientes de digestibilidade aparente (CDA) da
proteína (92,85%) e do fosforo (55,63%) em dietas contendo os DDGS₁ foram superiores aos CDA verificados na dieta de referência no robalo (91,88% e 31,65%, respectivamente). Na corvina, os resultados obtidos foram semelhantes, com o CDA da proteína (90,96 %) e do fosforo (60,36%) das dietas contendo DDGS₁ a serem superiores aos verificados em animais alimentados com as dietas controlo (88,99% e 23,63 %, respectivamente). Foram obtidos valores diferentes de CDA de matéria-seca, proteína, energia e lípidos nos ingredientes testados, sendo os valores mais elevados observados no ingrediente DDGS₁.

Nas duas espécies, a actividade mais elevada da lipase foi verificada em animais alimentados com a dieta contendo DDGS₂. No robalo, a actividade da amilase foi mais elevada em peixes alimentados com esta dieta não diferindo significativamente dos peixes alimentados com a dieta de referência. No entanto, na corvina, os animais alimentados com a dieta com incorporação de DDGS₂ apresentaram uma maior actividade da amilase, mas esta não foi significativamente diferente da actividade registada nos animais alimentados com DDGS₁. Nas duas espécies não foi verificado, entre dietas, diferenças significativas na actividade proteolítica.

No robalo a actividade mais elevada das enzimas digestivas foi registada no intestino médio e distal enquanto na corvina esta foi registada majoritariamente, no intestino médio.

Comparando as duas espécies, a actividade proteolítica foi superior na corvina enquanto a actividade mais elevada da amilase foi registada no robalo. Globalmente, a actividade da lipase foi superior nos juvenis de robalo mas esta actividade foi superior em juvenis de corvina alimentados com DDGS₂.

Por fim, não se verificou qualquer influência da dieta na actividade proteolítica testada a vários valores de pH. Sendo que esta registou picos de actividades nos valores de pH testados (8,9 e 10) ao longo das três secções do intestino analisadas.

Concluo, com base nos resultados obtidos neste trabalho, que os grãos secos de destilaria com solúveis (DDGS) se perfilam como uma óptima matéria-prima para ser incluída nas ração de aquacultura e que juntamente com outras matérias-primas de origem vegetal, podem ser uma boa fonte proteica para a substituição parcial ou completa da farinha de peixe nas ração utilizadas neste sector de produção animal.

Palavras-chave: Grãos secos de destilaria com solúveis (DDGS); Robalo (Dicentrarchus labrax); Corvina (Argyrosomus regius); Digestibilidade; Enzimas digestivas.
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Abbreviations

ADC(s) - Apparent Digestibility Coefficient(s)
ANF - Antinutritional Factor(s)
BW - Body Weight
DDGS - Distillers dried grains with solubles
DE - Digestible Energy
DM - Dry Matter
DP - Digestible Protein
EFA - Essential Fatty Acid(s)
FA - Fat Acids
FM - Fish Meal
G - grams
HUFA - Highly Unsaturated Fatty Acids
Kg - kilograms
KJ - Kilojoules
L - Litre
Mg - miligrams
MJ - Milojoules
NSP - Non Starch Polysaccharides
P - Phosphorus
PUFA - Polyunsaturated Fatty Acids

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**Introduction**

**Aquaculture development**

Aquaculture activity was originated in several Asian countries and introduced into Europe in the middle ages by the common carp culture (*Cyprinus carpio*). Aquaculture involves all human activities that use natural and/or artificial water bodies to produce aquatic species consumed by man (Martín et al. 2005). Global aquaculture production has been steadily growing in the new millennium, but at a slower rate than in the 80’s and 90’s (Gjedrem et al. 2012). Since 1980, annual global production has increased at an average rate of around 8% and this growth remains higher than any other major animal food production sector (Campbell and Pauly 2012). This global increase in production together with slowly declining of marine fisheries catches (Watson and Pauly 2001) led to declare that aquaculture represents around half of the seafood consumed by developed country’s markets (Loder et al. 2003). The growth rate in farmed food fish production in these years outpaced the population growth by 1.5 percent, resulting in a 7-fold increase in the average annual per capita consumption of farmed fish, from 1.1 kg in 1980 to 8.7 kg in 2010 (FAO 2012). Indeed, aquaculture is expected to resolve worldwide food issues (Cunningham et al. 2005). In 2010, total aquaculture production reached the historic mark of 79 million tons ($125 billion U.S dollars, approximately, 95 billion euros) or 60 million tons ($119 billion US dollars, approximately 90 billion euros), excluding algae, aquatic plants and non-food products (FAO 2012).

About 600 aquatic species are cultivated worldwide in a diverse variety of culture systems, rearing densities and technological sophistication, in fresh, brackish or salt water (FAO 2012). There is a global trend, driven mainly by demand from western countries, on increasing the intensive production of omnivorous and carnivorous species farmed in marine coastal environments (Campbell and Pauly 2012). The farming of these species, like salmon, sea bass and prawns is reliant on inputs of fish meals and oils, water, land and energy (Trujillo et al. 2007). So, aquaculture has been associated with negative impacts on marine and coastal ecosystem health (Liu et al. 2010).

The development and distribution of aquaculture production is not uniform in all regions of the world (Gjedrem et al 2012). In developed countries, growth rate of aquaculture production decreased from 2.1 percent in 1990 to 1.5 in 2000, while in developing countries it was observed a strong growth. Some countries in Asia, Pacific, sub-Saharan Africa and South America have made great technological progress, in recent
years, and are dominating the production in these regions of the globe. In 2010, there were 181 countries with record production (FAO 2012).

This global aquaculture distribution and production is vulnerable to several factors such as natural, environmental, technological and socio-economic constraints. Countries that are subject to natural disasters such as tropical storms, floods and earthquakes often suffer considerable losses. Water pollution is also another threat of total production loss in intensively urbanized areas. For instance, in 2010, China suffered an output fall of 1.7 million tons with a value of U.S. $ 3.3 billion (2.5 billion euros) caused by diseases (295 000 tonnes), natural disasters (1.2 million tonnes) and pollution (123 000 tonnes) (FAO 2012). In recent years, disease outbreaks are also causing substantial or total losses of, for example, shrimp production in several countries (Walker and Winton 2010).

Adequate feed supply is considered the major constraint to aquaculture development. One third of all farmed fish food production, representing circa 20 million tons, is achieved without providing artificial food (FAO 2012). However, the percentage of non-fed aquaculture is declining gradually. This production represented 45% of world aquaculture production, in 2005 (Tacon and Metian 2008), and currently represents only 33.3%, mainly due to changes in Asia cultivation procedures (FAO 2012). This rapid growth of fed species production is also due to the development, improvement and availability of formulated feed for aquaculture finfishes and crustaceans.

**Aquaculture in Portugal**

Fisheries have been an important activity in Portugal since Neolithic period and suffered a steady development along the several civilizations that inhabited the Iberian Peninsula (Birmingham 2010). Fish culture may have been introduced in the Iberian Peninsula by the Romans, who developed simple rearing techniques associated with sea salt (Birmingham 2010). Salines may have been used for growing larvae and juveniles, and oyster production (FAO 2012).

Aquaculture activity in Portugal has mainly been made on a familiar basis, never reaching the economic importance of the fisheries industry. However, the production of clams in Ria Formosa (Algarve) and oysters in Sado River estuary represents an important incoming source for local communities. Oysters have been exported to France since the 1950s, but in late 1970s its production declined due to an outbreak of gills disease caused by poor water quality and iridovirus (Henriques 1998).
In Portugal, the first large scale industrial facility was built in late 1960s, in Paredes de Coura to produce rainbow trout (Oncorhynchus mykiss). After 1986, economic incentives from Portuguese government and European Community, enabled a great aquaculture development complementing fisheries industry (INE 1998). At this time, large number of salines was transformed into aquaculture facilities in Aveiro, Figueira da Foz, Vale do Tejo, Setúbal and Algarve (Dinis 2010). Initially, fish production in these salines was focused on two species: sea bass and sea bream.

Between 1986 and 1996, Portuguese fisheries production declined over 65.9%, due to an increased interest in aquaculture (FAO 2013). Initially, producers possessed no knowledge about aquaculture and many didn’t survive. In the late 80s, producers have more business knowledge and adopted a professional approach. Consequently, the production increased 27% between 1990 and 1997 (INE 1998).

In 2000, with massive quantities of fish coming from Greece, fish prices suffered a significant decline. National producers faced major problems because of the low competitiveness of production costs. Thus, Portuguese production has not grown between 2000 and 2008 (FAO 2013).

In 2010, marine and brackish species production represents about 88% of total production and bivalve molluscs (clams and oysters), gilthead sea bream (Sparus aurata) and sea bass (Dicentrarchus labrax) are the most produced species (INE 2010).

Aquaculture represents only 3% of fish national production, a much lower number than the European average (20%) (INE 2010). Indeed, total fisheries catch in Portugal ensures consumption levels per capita of 23 kg / year, which is identical value to the European average, but insufficient to meet the demand of 57 kg of fish / year per capita. Several constrains to the development of Portuguese aquaculture production has been contributing to this panorama. For instance, incorrect location of fish farms which limits production expansion; licensing of aquaculture facilities involving a long bureaucratic process; high land costs and competition with other users of the coastal areas, such as tourism, urban development or environment protection are the main constraints to aquaculture production.

In 2011, Portuguese aquaculture production was 9165 tons (FAO 2013). The operational program for fisheries 2007-2013 predicted that in 2013 the sector would reach 15000 tons, representing 8% of national fish production. These values were not achieved but for this increase it would be necessary to promote intensive production.
models, especially in offshore cages. As a consequence, in March 2008 was created a zone designated Pilot Area of aquaculture production in Armona (APPA), located on the southern coast of the country. This project consists in the installation of cages for fish production and shellfish in long-lines. Thus, it is expected to achieve the goals that were established initially for Portuguese aquaculture, though not until 2013.

**Meagre (Argyrosomus regius, Asso, 1801)**

Meagre belongs to Sciaenidae family, which includes 270 species in 70 genera (Ono et al. 1982; Chao 1986). Meagre has a large head with an elongated and almost fusiform body (Whitehead et al., 1984/1986). Mouth is located in a terminal position, it has no barbells and buccal cavity is golden-yellow. Eyes are small and the lateral line is black and evident, extending to the caudal fin (FAO 2013). The second dorsal fin is much longer than the first one and the anal fin has a first short and spiny ray and a second one very thin (FAO 2013). The caudal fin is truncated to s-shape and large. Scales are mostly ctenoids except in the anterior area, nose and below the eyes, which present some cycloid scales (Whitehead et al. 1984/1986). Various branched appendages and a pair of muscles are present in the gas bladder and are associated with the lateral musculature of the body (Tower 1908; Takemura et al. 1978). Those appendages can vibrate producing a characteristic sound (a typical "grunt") that can be heard up to 30 meters (Tavolga 1971). Meagre has very large otoliths, its body color in vivo is silvery-gray, with bronze dorsally traits and a reddish-brown fin base. The coloration becomes brown after death.

Adult meagre has a relatively short digestive tract, typical of carnivorous fish, representing about 70% of its body length. The esophagus is short and broad, with muscular walls. The stomach has secretory function; it is muscular and with small bag shape, in which the anterior portion fits both the esophagus and the intestines, forming a posterior stomach that allows the storage of large size preys. The intestine is short and has a wall of varying thickness. In the anterior portion of the intestine, near the pyloric area of the stomach, exists 9 pyloric caeca (Gil et al. 2009), which together with
the intestine have secretory and absorptive functions. Meagre can reach 2 meters in length and a weight of 100 kg (Froese and Pauly 2011).

**Habitat and Biology**

Meagre can be found in the Mediterranean Sea, Eastern Atlantic Ocean between Senegal and the English Channel, in southern Norway, Denmark, Iceland and the Black Sea (FAO 2013; González-Quirós et al. 2011). They migrate through the Suez Canal to the Red Sea and can make the reverse Lessepsian migration (Chao 1990). They live at moderate depths (15-300 m) over sand and rocks (Froese and Pauly 2011) and are euryhaline and benthopelagic.

Meagre is a carnivorous fish and in the wild it feeds on Mysidacea, Decapoda and Teleostei (Cabral and Ohmert 2001). The species reaches sexual maturity at 2-3 years of age, depending on where they live, and make reproductive migrations towards the coast (Maybank 2008) between April and July in the southern Mediterranean (Whitehead et al., 1984/1986). Generally, they congregate inshore (Froese and Pauly, 2011), penetrate the estuaries (FAO 2013) and salt-marshes, and form large agglomerates in muddy waters, aided by long grunts emitted by males (González-Quirós et al., 2011).

This species spawning is seasonal, with a peak between April and June, and egg fertilization is external (Chao, 1990). Matting elapses during an in-pair courtship in the presence of typical deep sounds produced by the male by compression of the abdominal muscles against the gas bladder (Lagardère and Mariani, 2006; González-Quirós et al. 2011). From mid-June until the end of July they proceed from the estuaries to coastal waters, where they feed in shallow waters until early autumn. During winter they return to deep waters (FAO 2013). The major spawning places for meagre in the North Atlantic and Mediterranean Sea are: Nile delta (Egypt), the Levrier Bay (Mauritania) and the Gironde estuary (France) (Quéro 1989a and 1989b). A female can reach 19 years of age and the male 16. A female of 1.2 meters can produce 800,000 eggs per year.

Meagre is a gonochoric species that remains sexually undifferentiated up to nine months of age. Histologically, sexual differentiation begins to be notorious from six months of age (Schiavone et al. 2008) and usually occurs in females first than in males. In captivity there is no record of natural spawns and viable egg production is achieved only by artificial induction of reproduction with hormones administration (Duncan et al. 2008). Then, spawning occurs spontaneously, at a temperature between 17° and 22°C, without requiring stripping or artificially fertilizing eggs, and eggs are not
released simultaneously but in multiple small batches (Mañanós et al. 2008). During larval growth survival rates are encouraging (Pousão-Ferreira et al. 2010).

**Production**

Aquaculture of meagre has begun in the late 90s due to a consensus between Italian and French producers. The first commercial production was performed in France, in 1997, with fingerlings produced in Sète. These fingerlings were then grown near Orbetello lagoon, in Tuscany, on the west coast of Italy. This species production has spread to other Mediterranean countries and increased rapidly. Global production was 4000 tons in 2008, more than 10000 tons in 2010 (Monfort 2010) and 13742 tons in 2011 (FAO 2013).

The intensive production of meagre is made in land-based tanks and in sea cages. The juveniles supply comes from hatcheries located across the Mediterranean area. Countries like Spain, France, Greece, Italy and Egypt stand out as main juvenile’s producers (Suquet et al. 2009). Juveniles are stocked in small ponds or in cages with a weight between 3 and 20 g and are maintained there for three months until reaching 100 g.

Rearing techniques are very similar to those for European sea bass and gilthead sea bream (Chatzifotis et al. 2012). Nowadays, meagre on-growing is done mainly in sea cages and the animals are fed extruded diets with 45-50% protein and 17-20% lipid (Monfort 2010; Chatzifotis et al. 2012). Due to its high growth rate and its excellent feed conversion ratio (FCR) (Calderón et al. 1997; Pastor et al. 2002) meagre is considered one of the most promising species for aquaculture, being an attractive alternative to diversify production. Meagre can increase 1 kg per year depending on growing conditions, with FCR values from 0.9 to 1.6, depending on the feed used (Chatzifotis et al. 2012).

Meagre is highly tolerant to environmental conditions changes and has good resistance against environmental stress factors (Monfort 2010). In fact, they can tolerate wide temperature (2-38 °C) and salinity (5-39‰) variations and consequently can adapt to different latitudes and farming conditions (Cittolin et al. 2008; Suquet et al. 2009; Chatzifotis et al. 2010). However, more studies are needed to get further knowledge on the characteristics and quality of the meat in relation to different diets (Piccolo et al. 2008), nutritional requirements and protocols for producing larvae (Roo et al. 2010; Chatzifotis et al. 2010).
European Sea Bass (*Dicentrarchus labrax*, Linnaeus, 1758)

European sea bass belongs to Actinopterigii class, Teleostea superorder, Perciformes order and Moronidae family. It has an elongated body with 8 to 10 dorsal spines, 12 to 13 dorsal soft rays, 3 anal spines and 10 to 12 anal soft rays. The posterior edge of the operculum is finely serrated, and the lower part possesses strong denticles directed forward. It has 2 flat opercular spines and the mouth is moderately protractile. Vomerine teeth are present anteriorly in a crescent band (Fishbase 2013). The juveniles have black spots in the upper body.

**Habitat and Biology**

European sea bass is a euryhaline marine teleost species (Varsamos et al. 2001). It has a geographic distribution that extends from Eastern Atlantic to Morocco, Canary Islands and Senegal, to Black and Mediterranean Sea (FAO 2013). Sea bass inhabits coastal zones, estuaries with various types of bottoms, ponds and even rivers. In the summer months it enters the mouths of rivers; however, when the water temperature drops it migrate to offshore and remains in deep waters during winter (FishBase 2013). Seabass is a gonochoric species and spawning takes place between December and March in the Mediterranean, to June in the Atlantic Ocean (Haffray et al. 2006). The eggs, larvae and juveniles in the first 3 months are widely distributed and adults migrate hundreds of kilometers along the coast (Haffray et al. 2006).

They are predators feeding on shrimp, mollusks and fish (Smith 1990). Some species of nekton including zoobenthos (amphipods), benthic copepods, insects, fish eggs, larvae, crabs, mysids, cladoceran, planktonic crustaceans, are also part of their diet (FishBase 2013).

**Production**

The first mass-production techniques for this species were developed in France and Italy in the late 1960s and 1970s. These techniques were then dispersed and further developed by all Mediterranean countries (FAO 2013). European seabass is,
nowadays, one of the most important commercial species cultivated in the Mediterranean Sea and was one of the first non-salmonid marine species to be cultivated and commercialized in Europe. The main producers are: Greece, Italy, Turkey and Spain (FAO 2013). The global aquaculture production of this species reached 144 365 tonnes in 2011 (FAO 2013).

There are numerous studies on this species nutritional requirements. The optimal dietary protein level for juvenile was established to be around 50% and is not affected by water temperature (Hidalgo and Alliot 1988; Peres and Oliva-Teles 1999a). In fingerlings, optimal growth was achieved with 45% (Perez et al. 1997) and no growth differences were found with diets including either 43 or 52 % (Dias et al. 1998).

The optimum level of dietary lipids for juveniles was estimated to be 12.5% (Alliot et al. 1974). Growth performance was not affected by a dietary lipid range between 12 and 24%; however, incorporation of 30% of lipids in diets depressed growth rate (Peres and Oliva-Teles 1999b). On the contrary, Lanari et al. (1999) observed higher growth performance with 19% of lipids in diets than with 11 % or 15%. Similar results were observed by Dias et al. (1998).

Starch may be used for partial replacement of protein and fat as energy source in feed formulations, and it also contribute to improve the mechanical properties of pellets (Lanari et al. 1999). Fish have limited capability to digest and metabolize carbohydrates (Wilson 1994). In sea bass, crude starch digestibility decreases with the increase of dietary carbohydrate level (Oliva-Teles 2000). Gelatinized starch is adequately digested by seabass (Enes et al. 2011). No differences were found in the growth of animals fed diets with 15 or 25 % crude or gelatinized starch (Gouveia et al. 1995) and with 16-28% of gelatinized starch, but there was suppression of growth with 33% starch (Perez et al. 1997).

Studies about requirement of minerals and vitamins are very scarce. A requirement of vitamin C was demonstrated but not quantified (Alexis 1997; Henrique et al. 1998). It is recommended to incorporate a minimum of 5 mg of ascorbic acid per kg of diet for maximum growth and maintenance of normal skin and optimal collagen concentration (Fournier et al. 2000).

The optimum protein to energy ratio of sea bass diets were estimated to be 19 mg/kJ in diets with at least 21 MJ DE/kg (Dias et al. 1998). Lupastsch et al (2001) estimated the requirements for maintenance to be 43.6 kJ DE  BW (kg)^{-0.79} day^{-1} and 0.66 g DP BW (kg)^{-0.69} day^{-1}.
Ingredients used in aquaculture feeds
In feeds manufacture, the ingredients are by-products from human food or are directly produced for using in animal feeds. The ingredients are sources of proteins (amino acids), fats, carbohydrates, vitamins and minerals (NRC 1993). They are also source of energy although energy is not independent from nutrients.

The most important source of protein is fish meal which provides, depending on its quality, a protein level ranging from 56% to 76%, with an appropriate essential amino acids profile. It is also a good source of energy, essential fatty acids (EFA) and minerals and is highly palatable and digestible for most fish (NRC 1993). However, fish meal availability is limited due to fluctuations of production. Due to its high demand (SOFIA 2006), it is also an expensive ingredient that usually contributes to the high final price of fish feeds (Josupeit 2008). However, for carnivorous fish species only selected ingredients with high protein content may be used as alternative protein sources for fish, due to their high protein requirements (Hardy 2008). Among these ingredients there are by-products of animal production, vegetable feedstuffs and single-cell organisms. Comparatively to fish meal, alternative protein sources have some nutritional disadvantages such as inadequate amino acid (AA) profile, low digestibility, low palatability, and the presence of several anti-nutritional factors (Gatlin et al. 2007; Lim et al. 2008b). The presence of contaminants like mycotoxins may also limit the use of vegetable feedstuffs in aquafeeds (Hendricks 2002).

Partial replacement of fish meal by alternative protein sources has been achieved at different levels in various species. However, dietary formulation with total or almost total fish meal replacement usually leads to a depression of growth performance in carnivorous species (Kaushik et al. 2004).

Soybean meal presents high protein content and a good amino acid profile and it is a major alternative to fish meal in aquafeeds (Mohsen 1989). However, the increasing prices in recent years tend to decrease its use (Josupeit 2008). Protein concentrate obtained from oilseeds, such as rapeseed, cottonseed and sunflower, and from cereals, like wheat gluten and maize gluten are also potential alternatives to fish meal (Aslaksen et al. 2007). Their use is however limited due to incorrect balances of amino acids profile (Pereira and Oliva-Teles 2003).

Incorporation of lipids and carbohydrates in diets is necessary to spare protein for plastic purposes instead of being used for energetic purposes. Fats and oils are the major sources of energy and also of EFA, with marine fish oils containing 10-25% of HUFA (NRC 1993). Marine oils are derived from marine animals and are classified as
processed fish oils (menhaden oil), fish liver oils and marine mammal oils (Hertrampf and Piedad-Pascal 2000). Fish oil is the main lipid source in aquafeeds but due to the expected rates of aquaculture growth and fish oil decreased availability (SOFIA 2006) its actual levels of incorporation will not be economically sustainable (Turchini et al. 2009). Its dietary incorporation is also limited to the possible presence of contaminants such as dioxins or dioxin-like polychlorinated biphenyls (Bell et al. 2005). Indeed, in farmed fish, fish oil is considered the main source of persistent organic pollutants (Turchini et al. 2009). Plant oils such as soybean, canola (Glencross et al. 2003), linseed (Bell et al. 2004), sunflower (Abowei and Ekubo 2011) and rapeseed or palm oil (Karalazos 2007) have been used to replace fish oil, although their fatty acid composition differ significantly because they contain low levels of n-3 HUFA (Oliva-Teles 2012). As muscle FA profile tend to reflect the dietary FA profile, the dietary incorporation of vegetal oils may affect the fatty acid composition of farmed fish (Bell et al. 2001, 2003). This effect can be overcome by using diets rich in fish oil during the finishing period as this, at least partially, allow to recover the “fishy” composition of fats (Bell et al. 2004).

Carbohydrates are relatively inexpensive sources of energy that may spare protein (more expensive) from being used as an energy source (Abowei and Ekubo 2011). Fish use diets with no carbohydrates as efficiently as those including carbohydrates, because fish do not have specific dietary carbohydrate requirements (Enes et al. 2009). Cereal grains have 62-72% starch and are important binders in steam-pelleted and extruded feeds (NRC 1993). Many by-products of grain industry like wheat, oat, corn, rice or rye are available as ingredients for animal feeds (Hardy and Barrows 2002). Plant feedstuffs such as peas, beans and chickpeas also contain large amounts of starch that are used for fish as energy source (Booth et al. 2001). Raw starch is considered a poor energy source (Peres and Oliva-Teles 2002). However, technological treatment, involving heat and pressure, may increase its digestibility (Peres and Oliva-Teles 2002).

Other ingredients used in low amounts in feeds are mineral and vitamin premixes, feed binders, carotenoid supplements, drugs, antibiotics, probiotics, enzyme supplements, preservatives, fiber, flavorings and water (Abowei and Ekubo 2011), but will not be further detailed here.

The importance of new ingredients in aquaculture feeds
The rising prices due to increased demand and supply fluctuations of fishmeal, fish oil, soybean meal, corn and wheat meal in global markets emphasizes the need to reduce
their incorporation in feeds. Production of fishmeal and fish oil has stabilized in the last decades and natural phenomena like El Nino in 1997-1998 showed that availability of these ingredients is unpredictable and therefore industry cannot rely solely on them.

Soybean production is limited in Europe due to climate and geographical constraints and also due to the restriction of using genetically modified products adopted by the EU countries in 2003. So, it is necessary to explore other plants cultivated in Europe and around the Mediterranean zone and thereby decrease dependence on soybeans and other ingredients produced outside the European continent.

**Dried Distillers Grains with Solubles (DDGS)**

Currently we are witnessing a concerted effort by nutritionists and feed formulators to reduce aquafeeds costs by replacing fish meal and other expensive protein sources for other lower cost vegetable sources.

Distiller's dried grains with solubles (DDGS) are the dry residue that remains after fermentation of grain (corn, wheat, sorghum and barley) mash by selected enzymes and yeasts to produce ethanol and carbon dioxide. When corn is used to produce ethanol, approximately two thirds of the grain weight, corresponding to starch, is fermented by yeast. The by-product will be used to produce distiller's dried grains with solubles (DDGS). The yeast from the fermentation process remains in the finished co-product.

Global production of ethanol to be used as vehicle fuel has rapidly increased in recent years, in an attempt to reduce the use of petrol, since ethanol can be blended with gasoline. According to the United States Department of Agriculture approximately 700 billion liters of starch-based ethanol (mostly from corn) will be used in the United States by 2015 (Linwood and Baker 2011). As a result, the availability of these by-products has increased considerably, and according to the Agricultural Marketing Resource Center, in 2012/2013 its production in the United States amounted to 38.89 million tons, more than doubling the 2007 production (14.5 million tons). Thus, as availability increases, prices of distiller by-products become more competitive and this has enhanced its use in animal feeds. Per unit of protein, DDGS are much less expensive than conventional protein sources. However, their use in fish feeds is still limited, although some studies showed that this ingredient can be a promising ingredient to be used in fish feeds, namely for omnivore species.
Physical characteristics
Corn DDGS is a granular product with a color ranging from light yellow to dark brown. Several factors can influence this color, including the amount of distiller's solubles added to distiller's grains before drying, the drying temperature and duration. The color of the raw material has little effect on the final color of DDGS (Rosentrater 2006; Liu 2009).

The main obstacle to the use of DDGS in diets for animals is the wide variation in the nutritional content of DDGS produced in different manufactories due to differences in ethanol processing methods (Liu 2009). The color is a strong indicator of the nutritional value of DDGS, particularly of corn DDGS. An incorrect processing method (i.e. higher drying temperatures) results in darker DDGS which have lower nutritional value conditioning their use in animal feeds (Fastinger et al. 2006).

Chemical Composition
Corn DDGS have a protein content between 28 and 33%; lack of anti-nutritional factors that are commonly found in most plant protein sources and have a reasonable amount of fat (10.9 to 12.6%) (Lim et al. 2011). DDGS also have very low starch levels as most of it is converted to ethanol during the fermentation process. Corn DDGS have a higher crude fiber content than wheat DDGS and the neutral detergent fiber may represent 29-39% of the weight (Lim et al. 2011). The ash content is higher in wheat than in corn DDGS.

Expressed as a percentage of crude protein, and relatively to soybean meal, corn and wheat, DDGS are deficient in several essential amino acids (EAA), including lysine, threonine, tryptophan, arginine, isoleucine, phenylalanine and methionine (Lim and Yildirim-Aksoy 2008). The concentration of vitamins and minerals differs between sources and batches of DDGS. Corn DDGS are rich in vitamin A, niacin and choline, and contain several minerals, including phosphorus that is high bioavailable and present in high levels (Lim et al. 2011).

When contaminated corn with mycotoxins is used for bioethanol production the mycotoxins are accumulated in DDGS. The mycotoxins concentration in DDGS may be 3 to 3.5 times higher than values found in corn. Aflatoxins (B1, B2, G1 and G2), deoxynivalenol (DON), fumonisins (B1, B2, B3) and T-2 may also be found in DDGS. However, mycotoxins do not seem to be a major problem when corn is produced under normal climacteric conditions (Zhang and Caupert 2012).
**DDGS utilization in aquaculture**

For omnivorous species, such as channel catfish (*Ictalurus punctatus*), DDGS may be used to replace fish meal and soybean meal, up to 40% of diet, without lysine supplementation (Webster et al. 1991, 1992, 1993; Lim et al. 2009) and with no negative repercussions on growth performance. Higher dietary replacement levels may be achieved with the adequate restoration of dietary essential amino acid profile, by using amino acid supplements or combination among different protein sources (Webster et al. 1991; Cheng and Hardy 2004b). Concordantly, for catfish, the dietary lysine supplementation allowed an increased used of DDGS in the diet up to 70% (Webster et al. 1991; Robinson and Li 2008). Also for catfish, Webster et al. (1992a) states that a combination of DDGS with soybean meal (35% DDGS and 49% soybean meal) can be used to totally replace fish meal in the diet, with or without lysine supplementation and methionine.

In rocky mountain white tilapia (*O. niloticus x O. aureus*) a diet containing 30 % DDGS, 26 % meat and bone meal, and 16 % soybean meal provided good growth (Coyle et al. 2004). In Nile tilapia (*Oreochromis niloticus*), DDGS can be incorporated in diets at a level of 20 % as a substitute of soybean meal and corn meal without affecting growth performance and body composition (Schaeffer et al. 2009). For this species, this feedstuffs can be also replaced in diets for 40 to 60 % of DDGS plus lysine supplementation (Lim et al. 2007; Shelby et al. 2008). In the same species, Li et al. (2011) found the same weight gain, feed efficiency ratio (FER), protein efficiency (PER) in fish fed diets with up to 30% wheat DDGS or up to 40% with lysine supplementation.

In rainbow trout diets, Cheng and Hardy (2004b) states that DDGS can be used at 22.5% inclusion level or at 75 % with lysine and methionine supplementation. In the same species a replacement of 25 % of fish meal can be achieved with a mixture of corn DDGS and corn gluten meal (Stone et al 2005).

In yellow perch (*Perca flavescens*) a mixture of DDGS and soybean meal can be incorporated up to 49.5 % without negative effects on growth (Schaeffer et al. 2011).

The same author (Webster et al. 1999) reported that sunshine sea bass (*Morone chrysops x M. saxatilis*) can be fed with a diet of 29% soybean meal, 29% meat and bone meal and 10% DDGS without negative effects on final weight, percent weight gain, survival, specific growth rate and feed conversion ratio. High fiber content, phytate and pigments, along with variation in chemical composition and physical properties of DDGS may limit its use for some species (Belyea et al. 2004).
DDGS contains a substantial percentage of yeast cells. Ingledew (1999) estimated that 3.9% of the total biomass of this ingredient is yeast, representing 5.3% of total protein present in DDGS. Li et al. (2010) showed improved weight gain and feed efficiency in animals fed diets with 30% DDGS and attributed it to yeasts. Studies with sea bass (Oliva-Teles and Goncalves 2001) and sunshine sea bass (Gause and Trushenski 2011) also showed growth improvement with diet yeast inclusion.

Few data has been published on amino acid availability of DDGS for aquatic species (Webster et al. 2008). Cheng and Hardy (2000) were the first authors to assess the bioavailability of amino acids and protein digestibility of DDGS. Results indicate for rainbow trout an apparent digestibility of more than 80% for both amino acids and protein, with the exception of cysteine (75.9%). In sunshine sea bass, bioavailability of amino acids in DDGS was greater than 50% except for cysteine, histidine and valine; protein digestibility was moderate (65%) compared to that reported for rainbow trout (90%, Cheng and Hardy 2004; Metts et al. 2011).

Few studies have been performed to assess DDGS effects in fish immunity, although it is recognized that diet modifications can positively or negatively affect fish immune status and disease resistance (Lim et al. 2007). Yeasts are rich in protein, B vitamins, β-glucans and nucleotides. These compounds, either in its purified form, as byproducts of yeast, or present in living forms appear to stimulate the immune response in humans and animals, including fish (Chen and Ainsworth 1992; Robertson et al. 1994; Li et al. 2004). Several studies point to an enhancement of non-specific immune response when animals are fed with diets including β-glucans (Gatlin 2002; Oliva-Teles 2012). However, a prolonged feeding with high levels of β-glucan seems to increase Atlantic salmon and gilthead sea bream susceptibility to bacterial infections (Robertsen et al. 1990; Couso et al. 2003).

Nucleotides correspond to 12-20% of total N in yeasts (Oliva-Teles 2012). Nucleotides are associated to increase innate defense mechanisms and disease resistance in fish (Li et al. 2004). A supplementation with a mix of nucleotides seems to increase the number of villi in the intestines of mice (Uauy et al. 1990) and Atlantic salmon (Salmo salar) (Burrells et al. 2001). Thus, the surface of the intestine is increased and the nutrients seem to be absorbed more efficiently.

However, DDGS inclusion in Nile tilapia diets do not seems to affect hematological parameters (white blood cell count, red blood cells, hemoglobin and hematocrit) or immune responses, such as serum proteins, lysozyme activity and antibody production against Streptococcus iniae (Lim et al. 2007). However, diets with 10 to 40% DDGS
DDGS: a potential protein source in feeds for aquaculture

significantly increased immunoglobulin and hematocrit percentage compared to animals fed with control diet (0% DDGS) (Lim et al. 2009).

Lim et al. (2009) noticed that juvenile catfish fed diets incorporating between 20 and 40% DDGS (1.14 g to 2.28 g glucans/kg diet) showed a significant increase in plasma immunoglobulins but no changes in serum proteins, lysozyme activity, alternative complement, production of superoxide anion and rate of macrophages chemotaxis. An increase in plasma immunoglobulins may be responsible for a decrease or delay of mortality in fish fed diets containing DDGS. Shoemaker et al. (2007) refers that fish immunoglobulins are capable of specifically binding to epitopes of the bacterial surface. Further, the same author asserts that these are potential activators of the complement system and are effective opsonins and agglutinins that facilitate the removal of pathogens. Consequently, more research is necessary to evaluate the potential of incorporating DDGS in diets and its influence on animal's immunity.

Some studies indicate that diets with DDGS may have high levels of pigments. Levels of lutein and zeaxanthin over 7-10 mg per feed kg can be deposited as visible pigments in catfish fillets (Lee 1987; Lim et al. 2009). Li et al. (2011) state that DDGS levels above 30% may result in a pronounced deposition of yellow pigments that may reduce the commercialization value of fish. However, DDGS obtained as byproducts of ethanol production can be incorporated at higher percentages without causing excessive pigmentation since the pigments are effectively removed by ethanol extraction (Li et al. 2011).

**Digestibility**

The bioavailability of nutrients and energy in feeds for fish may be defined in terms of digestibility. Digestibility indicates the fraction of nutrient or energy in the ingested diet that is not excreted in feces. Apparent digestibility does not take into account nutrient losses of endogenous origin which are part of feces. “True” or “correct” digestibility excludes the endogenous losses from the feces. The apparent digestibility has a more practical importance than true digestibility because the endogenous losses are minor if the animal is not fed (Lovell 1998).

The first task to assess the potential of any new ingredient for inclusion in aquaculture diets is the determination of its apparent digestibility (Cho et al. 1982; Bureau et al. 2002). The apparent digestibility coefficients (ADC) of ingredients are necessary to formulate commercial and experimental diets on a digestible basis rather than on a gross basis.
Several methods of direct or indirect quantification of digestibility have been used with fish. The direct method involves measuring directly all of food ingested and all the feces excreted. The indirect method eliminates the need to quantitatively collect all the feces the fish produce (Vandenberg and Noûé 2001). It involves the quantification of the ratios of nutrient to some indigestible indicator in the feed and feces. This digestion indicator must be indigestible, unaltered chemically, nontoxic to the fish, easily quantified and capable to pass through the gut uniformly with other ingesta (Ward et al. 2005). As the dietary ingredients are absorbed in the gut, the ratio of nutrient to the indicator will be lower in feces than in fed. Some internal indicators used are ash, crude fiber or plant chromagens, while external indicators are additives such as chromium oxide or yttrium oxide (Carter et al. 2003).

The collection of fecal material in fish is a difficult process and can substantially influence apparent digestibility coefficients for nutrients in feed ingredients (Amirkolaie et al. 2005). The methods of fecal collection must ensure that the results are accurate, repeatable and harmless to the fish (Austreng 1978; Cho et al. 1982; Allan et al. 1999). Methods for collecting feces include suctioning of fecal material, dissection (Windell et al. 1978), manual stripping (Glencross et al. 2005), settling columns (Cho et al. 1982) and netting (fecal matter is sieved continuously by a net present at the water outlet) (Choubert et al. 1979).

The removal of chyme before being completely digested and the contamination of feces with physiologic fluids and intestinal epithelium, that would have been reabsorbed by the fish before natural defecation, are the main disadvantages of direct feces collection from the intestine. This affects the reliability of these methods and in general leads to underestimation of digestibility (Amirkolaie et al. 2005)

ADC in which feces came in contact with water before collection may present overestimated values than those obtained by stripping or dissection. This is due to disintegration or separation of feces, or leaching of nutrients and/or marker from the fecal matter (Cho and Kaushik 1990). However, these methods are widely accepted because they facilitate repeated measures and lower handling stress. Specific devices to collect fecal material were created by Ogino et al. (1973), Cho et al.(1975) and Choubert et al. (1979). Ogino et al. (1973) collected feces by passing the effluent water from the tanks through a filtration column. The Guelph system uses sedimentation columns without significant nutrient leaching (Cho and Slinger, 1979). Choubert et al. (1979) developed a mechanically rotating screen to filter fecal material from the water (INRA system).
Smith (1971) developed a metabolic chamber to collect fecal material voided naturally into the water. In this method the fish is restrained and need to be force-fed. This technique imposes a high stress level on the fish and the digestibility values were questionable (Cho et al. 1982).

A major problem to evaluating the digestibility of ingredients in fish is their refusal to eat most of these ingredients separately or, in some cases, the reduced voluntary feed intake and nutrient utilization due to nutrient unbalances, amino acids deficiencies, presence of anti-nutritional factors and reduced palatability (Tacon 1995; Booth et al. 2001). To avoid these problems, the most appropriate approach is the substitution method, where the test diet comprises a portion of a reference diet (usually 70%) and a portion of the test ingredient (usually, 30%) (Glencross et al. 2007).

**Digestive enzymes**

Digestive enzymes are crucial for digestive processes, allowing protein, carbohydrate and fat degradation into smaller and simple molecules. These molecules can then be absorbed and transported into tissues, by the circulatory system, and used for growth, tissues repair and reproduction (Furné et al. 2005).

There are several factors that affect the activity of digestive enzymes. These include diet composition (Debnath et al. 2007; Santigosa et al. 2008; Chatzifotis et al. 2008; Cedric 2009), age (Kuz’mina 1996b) and environment conditions (Zhi et al. 2009). The structure of digestive tract and digestive enzymes distribution are closely related (i.e. the gastrointestinal tract of herbivorous species is longer than the carnivorous ones) (De Almeida et al. 2006). Quantifying the activity of digestive enzymes is a useful way to provide information on the nutritional value of diets and possible interaction between anti-nutritional factors and digestive enzymes of fish when fed formulated diets (Refstie et al. 2006, Corrêa et al. 2007).

**Amylase**

All fish species seem to possess the enzymatic apparatus necessary to hydrolyze and absorb simple and complex carbohydrates. Digestion and absorption takes place by the same routes in herbivores, omnivores and carnivores species. The α-amylase (EC 3.2.1.1) is a key enzyme for carbohydrate digestion. It acts on complex polysaccharides, like starch and glycogen, hydrolyzing them up into maltotriose and maltose, a combination of branched oligosaccharides and some glucose (Papoutsoglou and Lyndon 2003).
In mammals, amylase is produced by the salivary and pancreatic cells, while in fish it has been identified in pancreatic juice, in the stomach and in the intestines but the main producers seems to be the pancreas and the liver (Klahan et al. 2009). Amylase activity has been found in most tested tissues, including the heart (Hokari et al. 2003). Together with other pancreatic enzymes, α-amylase activity is detected in the lumen of the intestine, in the chyme and connected to mucosal membranes (Hoehne-Reitan et al. 2001; Krogdahl 2004). The highest values of enzyme activity are usually reported in the pyloric caeca (Correa et al. 2007, Gai et al. 2012). Pérez-Jiménez et al. (2009) found increased activity in pyloric caeca of juvenile dentex (Dentex dentex) fed increasing levels of carbohydrates and low levels of lipids in the diet. This is plausible since starch digestion and glucose absorption occur mainly in the anterior part of the intestine (Lundstedt et al 2004).

The pancreatic tissue is considered to be the source of amylase detected in the medium and distal intestine; however, the origin of amylase present in the proximal portions of the gastrointestinal tract is not properly documented (Krogdahl 2004). Amylase present in the proximal regions may however be of pancreatic origin because the species in question mostly lack distinct stomach pouches. Thus, it was not possible to identify the origin of amylase and distinguish the contribution of dietary amylase or the reflux of lower intestine. Moreover, some enzymes may be supplied by the diet or produced by intestinal bacteria, and these exogenous contributions can be substantial (Caruso et al. 2009).

Surprisingly, little is known about amylases and other carbohydrases and even less about its regulation. Herbivorous and omnivorous species appear to digest starchy components of vegetable feedstuffs more efficiently than carnivorous species (De Almeida et al. 2006; Al-Tameemi et al. 2010). In herbivorous (Boops boops) and omnivorous species (Cyprinus carpio, Carassius auratus, Tinca tinca and Pagellus erythrinus), α-amylase activity is higher than in carnivores species (Oncorhyncus mykiss, Sparus aurata, Anguilla anguilla) (Fernandez et al. 2001; Hidalgo et al. 1999; De Almeida et al. 2006). Thus, feeding habits have great impact on amylase activity (Horn et al. 2006). As demonstrated in European sea bass, rainbow trout and paddlefish (Polyodon spathula), amylase activity in fish is directly related to the level of carbohydrates in the diet and feeding intensity (Corrêa et al. 2007; Caruso et al. 2009; Ji et al. 2012). However, it appears that some ingredients such as rice protein concentrate included in large quantities in the feed may exert an inhibitory effect on amylase activity, probably due to the presence of anti-nutritional factors (Gai et al. 2012).
The characteristics of amylase differ among species in relation to optimum pH for maximum activity and temperature stability. The optimum pH for maximum activity of intestinal amylase has been shown to be 6.0-7.5 in different species (Li et al. 2006). Papoutsoglou and Lyndon (2005) observed in vitro that higher levels of carbohydrates and lower temperatures decrease amylase activity. Gilthead sea bream seems to have the ability to regulate the amylolitic activity to compensate differences in temperatures (Couto et al. 2012). Fish amylases also show differences in the dependence on ions and ion concentrations (Munilla-Moran and Saborido-Rey 1996a).

**Lipase**

The general digestive process of lipids involves their extracellular hydrolysis in the stomach, intestine and cecal lumen by a variety of lipases and colipases (Higgs and Dong 2000).

Lipase (E.C.3.1.1.3) catalyses the breakdown of triacylglycerol into diacylglycerol and monoacylglycerol (Savona et al. 2011). Lipase activity in fish has been found in pancreas extracts, pyloric ceca and upper intestine but can extend to the distal part of the intestine, decreasing progressively its activity (Klahan et al. 2009). This lipase activity may have pancreatic or gastric origin, resulting in adsorption of enzyme in the intestinal mucosa or can be secreted by intestinal cells (Wong 1995). However, the pyloric ceca and anterior intestines seem to be the primordial sites of lipid hydrolysis (Halver and Hardy 2002). Nevertheless, lipase activity can increase distally in intestine of some fish species perhaps as a lipid-scavenging mechanism (German and Bittong, 2009). Despite of all fat-digestive enzymes are known to act in alkaline media (7-9) (Tramati et al. 2005; Klahan et al. 2009), in some species like the Siberian sturgeon there is hydrolysis by lipase in the stomach (Halver and Hardy 2002). It is improbable that lipolytic activity found in the stomach to be of pancreatic origin, suggesting that this organ is also a source of lipases; also, it can never be excluded any possible lipolytic activity from bacteria present in the digestive tract of fish (Caruso 2009). In *Diplodus puntazzo*, lipase activity was detected in all regions of the gut, indicating a uniform distribution in the entire gut system (Tramati et al. 2005). Lipase may play a relatively minor role in lipid digestion in some fish species (Savona et al. 2011).

Several authors suggested that lipase presence is greater in carnivorous than in omnivores or herbivores fish because carnivorous species consume fat-rich food (Tengjaroenkul et al., 2000; Furné et al., 2005). This fact might suggest that the type of diet could influence the production of lipases in adult fish (Ji et al. 2012; Kuz’mina et al. 2008). The largest class of lipids present in fish diets is triacylglycerol class. The lipase
activity is directly related to triglycerides (TG) and phospholipids (PL) levels present in the diet as shown in sea bass larvae fed diets which contain different levels of these lipid fractions (Cahu et al. 2003; Zambonino Infante and Cahu 2007; Savona et al. 2011).

**Proteases**

The digestive proteases are polyfunctional enzymes that catalyze the hydrolytic degradation of proteins (García-Carreno and Hernández-Cortes 2000). Proteases also activate the released zymogens of a large number of digestive enzymes into their active form (Bureau et al. 2002). In the EC system for the enzymatic nomenclature, all proteases (peptide hydrolases) belong to the subclass 3.4, which is divided into 3.4.11-19 (exoproteases) and 3.4.21-24 (endoproteases) (Nissen, 1993). Endoproteases disrupt in the middle of polypeptide chain, while exoproteases hydrolyze the free ends of peptide chains (Bureau et al. 2002). The exopeptidases, particularly aminopeptidases, are mostly intracellular or membrane bound and cuts amino acids from the amino end of a peptide chain one at a time (Bureau et al. 2002). The high catalytic efficiency at low temperatures and low thermal stability are some of the differences in properties of proteases from marine and terrestrial animals (Klomklao et al. 2005).

In fish digestive organs have been found proteases such as pepsin, gastricsin, trypsin, chymotrypsin, collagenase, elastase, carboxypeptidase and carboxyl esterase (Dimes 1994; Simpson 2000). In the fish digestive system, the two major groups of proteases are pepsin and trypsin. Pepsin has been identified as the major acidic protease in fish stomach and is the first proteolytic enzyme to break large peptide chains (Tengjaroenkul et al. 2000). Trypsin and chymotrypsin are the major alkaline proteases in the intestine (Caruso 2009; Natalia et al. 2004).

The distribution of proteases varies between species and organs. Several studies show high activity in the acidic pH region, in the stomach, and in the alkaline pH region, in the intestine (Alarcón 1998). Proteolytic activities at low pH (1-3) have been reported in species with a clear stomach region and high pepsin secretion (Chong et al. 2002). Pepsins from several important species like tilapia (Yamada et al. 1993), dentex (Jimenez et al. 2009) or European seabass have been documented (Eshel et al. 1993). Pyloric caeca may be related to the need of retaining feed for the acid secretion neutralization. However, this gastrointestinal region is also associated to alkaline proteases storage until the acid bolus is neutralized by other pancreatic secretions (Alarcón et al. 1998). In fact, high proteases activities at alkaline pH (8-10) have also
been reported in several fish species like rainbow trout (Kristjansson and Nielsen 1992) and seabass (Eshel et al. 1993)

The expression of proteases in higher trophic level species increases with age (Kuz’mina et al. 2008) and in lower trophic level species this enzymatic activities are brought down (Ugolev and Kuz’mina 1993a). Usually, carnivorous fish species have a short intestine, with higher protease activity than herbivores (Lazzari et al. 2010) and the level of proteolytic activity seems to be related with the fish species growth rate (Hidalgo et al. 1999). Some authors suggested that vegetable protein sources included in the diet can decrease proteolytic activity (Venou et al. 2003; Santigosa et al. 2008).

The pancreas secretes enzymes with alkaline protease activity as inactive proenzymes mixed with digestive juice with a basic pH. A peptide located in the active site need to be released for the activation of this enzyme. In fish, this family of alkaline enzymes includes the serine proteases (trypsin, chymotrypsin, elastase and collagenase). In pancreatic tissue most of the trypsin and chymotrypsin are present as trypsinogen and chymotrypsinogen. Trypsinogen is activated by enterokinase with a release of a peptide located at the active site. The active trypsin then activates other digestive enzymes like chymotrypsin (Bureau et al. 2002). These enzymes become active in existing pyloric caeca and proximal intestine and hydrolyze the protein (Santigosa et al. 2008).

Trypsin, specifically hydrolyses the carboxyl side of arginine and lysine peptide bond (Klomklao et al. 2006a). This digestive enzyme, contributes to 40-50 % of the overall protein digestion activity in carnivorous fishes (Eshel et al. 1993). The optimal pH range for this enzyme is between 7.0 and 9.0 (Jimenez et al., 2009). At low pH values the enzyme denatures, alters its conformation and, therefore, cannot bind correctly to the substrate (Klomklao et al. 2006a).

Chymotrypsin has much broader specificity than trypsin. This digestive enzyme hydrolyses peptide bonds close to hydrophobic amino acids such tyrosine, phenylalanine and tryptophan (Neurath 1989). Although the activation pH is similar to trypsin, chymotrypsin seems to be present at pH values between 7.0 and 8.0 in several fish species (Jimenez et al. 2009).

It is known that trypsin and chymotrypsin secretion occur in response to food ingestion and they complete the protein hydrolysis when the food reaches the intestine (Savona et al. 2011). Trypsin activities were found generally higher in carnivorous species while chymotrypsin activities were higher in omnivorous and herbivorous species (Jonas et
al. 1983; Chong et al. 2002). Nevertheless, both types of proteases play a cooperative role in protein digestion at intestinal tract (Chong et al., 2002).

**Aims**
Currently, the aquafeed industry seeks to reduce the inclusion of fishmeal in diets. This approach is justified by the high price of this raw material, the fluctuations of its availability in the market and environmental concerns. Thus, feedstuffs such as Dried Distillers Grains with Solubles (DDGS) emerged as potential substitutes. This study has the following main objectives:

- Evaluate the apparent digestibility coefficient of dry matter, protein, lipids and energy of DDGS in European sea bass and meagre.
- Assess the effect of DDGS incorporation in the diets in digestive enzymes activity: amylase, lipase and total proteases.
- Determine if the DDGS inclusion in the diet might have some influence on proteolytic activity at different pH (8,9 and 10).

**Materials and methods**

**Ingredient composition**
The proximate composition of ingredients tested in this trial is present in table 1. Proximate composition and energy content of both sources of DDGS were very similar. Dry matter content was similar in all raw materials, but protein content was considerably higher in fishmeal that in DDGS. Lipid and gross energy content was higher in DDGS than in fishmeal. Ash content was markedly higher in fishmeal and concurrently phosphorus content was also higher. Crude fiber and starch in both DDGS was quite similar, but slightly higher in DDGS$_2$. On the contrary, acid and neutral detergent fibers contents were higher in DDGS$_1$. 


Table 1: Proximate composition (% dry matter) of the experimental ingredients

<table>
<thead>
<tr>
<th></th>
<th>Dry matter</th>
<th>Protein</th>
<th>Lipids</th>
<th>Gross Energy (kJ/g)</th>
<th>Ash</th>
<th>Phosphorus</th>
<th>Crude fiber</th>
<th>Acid detergent fiber</th>
<th>Neutral detergent fiber</th>
<th>Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal¹</td>
<td>89.0</td>
<td>70.7</td>
<td>9.2</td>
<td>17.9</td>
<td>19.2</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DDGS₁²</td>
<td>89.2</td>
<td>30.4</td>
<td>11.8</td>
<td>20.0</td>
<td>4.7</td>
<td>0.5</td>
<td>7.2</td>
<td>14.6</td>
<td>42.4</td>
<td>0.5</td>
</tr>
<tr>
<td>DDGS₂³</td>
<td>88.7</td>
<td>29.4</td>
<td>12.8</td>
<td>19.6</td>
<td>4.9</td>
<td>0.78</td>
<td>7.8</td>
<td>13.8</td>
<td>39.3</td>
<td>2.9</td>
</tr>
</tbody>
</table>

¹ Steam Dried LT, Pesquera Diamante, Peru
² DDGS₁: corn dried distillers grains with soluble from Biocarburantes de Castilla y Leon, Spain
³ DDGS₂: corn dried distillers grains with soluble from Pannonia Ethanol, Hungry

Experimental diets
A reference diet was formulated containing 45% protein, 16% lipids, and 1% chromium oxide, included as an inert digestibility marker. Two experimental diets were then formulated containing a mixture of 70% of the reference diet and 30% of the test ingredient, i.e. corn DDGS obtained from two different producers. DDGS were supplied by Pannonia Gold, Hungary (www.pannoniagold.com), and by Biocarburantes Castilla y Leon, Spain (www.abengoabioenergy.com). All dietary ingredients were finely ground and well mixed. Mixtures were then dry pelleted without steam using a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA) through 3 mm die. After dried in an oven for 24h at 35°C, pellets were sieved and stored in a freezer until use. The ingredients and proximate composition of the experimental diets are presented in table 2.

Due to inherent feed formulation, diet composition of reference and test diets was considerably different. The protein content was higher in the reference diet than that in the experimental diets (DDGS₁ and DDGS₂). Reference and DDGS₂ diets had similar lipid content, which was higher than in DDGS₁ diet. Gross energy content was similar among diets. Ash content was higher in reference diet and by consequence also its phosphorus content. Crude fiber content was almost equal in DDGS₁ and DDGS₂ diets. Chromium oxide and starch were higher in reference diet than in the experimental diets.
Table 2: Composition of the experimental diets.

<table>
<thead>
<tr>
<th>Ingredients (% dry weight)</th>
<th>Reference</th>
<th>DDGS₁</th>
<th>DDGS₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal¹</td>
<td>63.2</td>
<td>44.2</td>
<td>44.2</td>
</tr>
<tr>
<td>Pre-gelatinized corn starch²</td>
<td>22.1</td>
<td>15.4</td>
<td>15.4</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>10.2</td>
<td>7.2</td>
<td>7.2</td>
</tr>
<tr>
<td>Vitamin premix³</td>
<td>1.0</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Choline chloride (50%),</td>
<td>0.5</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Mineral premix⁴</td>
<td>1.0</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Binder⁵</td>
<td>1.0</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Chromic oxide</td>
<td>1.0</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>DDGS₁</td>
<td>–</td>
<td>30</td>
<td>–</td>
</tr>
<tr>
<td>DDGS₂</td>
<td>–</td>
<td>–</td>
<td>30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Proximate composition (% dry matter)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td>95.5</td>
<td>93.8</td>
<td>94.6</td>
</tr>
<tr>
<td>Protein</td>
<td>45.6</td>
<td>41.2</td>
<td>41.3</td>
</tr>
<tr>
<td>Lipids</td>
<td>15.9</td>
<td>13.6</td>
<td>15.4</td>
</tr>
<tr>
<td>Chromium oxide (Cr₂O₃)</td>
<td>0.82</td>
<td>0.53</td>
<td>0.59</td>
</tr>
<tr>
<td>Energy (kJ/g)</td>
<td>20.0</td>
<td>20.7</td>
<td>20.8</td>
</tr>
<tr>
<td>Ash</td>
<td>14.8</td>
<td>11.5</td>
<td>11.6</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>1.77</td>
<td>1.58</td>
<td>1.49</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>-</td>
<td>2.0</td>
<td>2.1</td>
</tr>
<tr>
<td>Starch</td>
<td>13.6</td>
<td>12.7</td>
<td>9.0</td>
</tr>
</tbody>
</table>

¹ Steam Dried LT, Pesquera Diamante, Peru.
² Cerestar, France.
³ Vitamins (mg kg⁻¹ diet): retinol, 18,000 (IU kg⁻¹ diet); calciferol, 2000 (IU kg⁻¹ diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
⁴ Minerals (mg kg⁻¹ diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.9 (g kg⁻¹ diet); potassium chloride, 1.15 (g kg⁻¹ diet); sodium chloride, 0.4 (g kg⁻¹ diet).
⁵ Binder (Aquadube, Agil, England).
⁶ DDGS₁: corn dried distillers grains with soluble from Biocarburantes de Castilla y Leon, Spain
⁷ DDGS₂: corn dried distillers grains with soluble from Pannonia Gold, Hungry.
Experimental animals

Two digestibility trials were performed with European sea bass (*Dicentrarchus labrax*) and meagre (*Argyrosomus regius*) at the Marine Zoology Station, Porto University. Both fish species were provided from IPMA, Algarve. After arriving at the experimental unit, fish were kept in the quarantine system for 3 weeks. Then, fish were transferred to the experimental system and acclimatized to the rearing conditions for 15 days. During this period fish were fed a commercial diet two times a day.

Experiment design

Experimental system consisted in a thermo-regulated recirculation water system, equipped with twelve 60 L fiberglass tanks, designed according to Cho et al. (1982). A feces settling column was connected to the outlet of each tank. A continuously water-flow was established, at a rate of about 4.5 L/min. During the trial, water temperature averaged 22 ºC, salinity averaged 38 ‰ and dissolved oxygen was kept above 90% of saturation.

Nine groups of six European sea bass, with an average weight of 206 g, were established. Diets were randomly assigned to triplicate tanks and fish were fed to apparent satiation, twice a day (9.30 a.m and 16.30 p.m). The first 7 days of the experimental period were used for fish adaption to the diets and then feces were collected once a day for 24 days. Before the morning meal, feces accumulated in each settling column were collected, centrifuged (3000 g), pooled for each tank and stored at -20 º C until analysis. Thirty minutes after the afternoon meal, tanks, water pipes and settling columns were thoroughly cleaned to remove excess feed and feces.

After the experimental period with sea bass juveniles, groups of nine meagre juveniles, with an average body weight of 78.8 g, were randomly distributed to the same tanks and the same experimental protocol used for sea bass was utilized.
Apparent digestibility coefficients (ADC) of protein, lipid, dry matter, energy and phosphorus was determined by the following formula (Maynard and Loosli 1979):

\[
ADC_{diet} = \left( 1 - \frac{\text{dietary CR}_2O_3 \text{ level} \times \text{feces nutrient or energy level}}{\text{feces CR}_2O_3 \text{ level} \times \text{dietary nutrient or energy level}} \right) \times 100
\]

The apparent digestibility coefficients of the test ingredients were calculated according to Bureau et al. (1999) as follows:

\[
ADC_{test \ ingredient} = ADC_{test \ diet} + [(ADC_{test \ diet} - ADC_{control \ diet}) \times (0.7 \times D_{control} \div 0.3 \times D_{ing})]
\]

Where \( D_{control} \) = % nutrient (or kJ/g) of control diet mash (dry matter basis) and \( D_{ing} \) = % nutrient (or kJ/g) of test ingredient (dry matter basis).

![Fig 5: Sampling collection of biological material.](image)

At the end of each digestibility trial, and to ensure a full intestine at the sampling time fish were fed in a continuous manner during the sampling collection day. Then, two fish per tank were randomly sampled, euthanized with a sharp blow to the head and immediately eviscerated. Digestive tract was excised, and adherent adipose and connective tissue were carefully removed. The intestine was divided in three different portions: anterior, middle and distal. The distal part was distinguished from the mid intestine by the increase in intestinal diameter, darker mucosa and annular rings. The anterior and medium portions were obtained by division of the remaining intestine into two parts. The anterior intestine represents the portion, with the pyloric caeca, directly after the stomach. The different portions of intestine were immediately frozen in liquid nitrogen and stored at -80°C until measurement of enzyme activity.
Chemical analyzes performed in ingredients, diets and feces

Sample Preparation
Prior to analysis, ingredients, diets and feces were dried, in an oven at 105 °C until constant weight and, then, finely ground to obtain a homogeneous sample.

Crude protein
The protein content (N x 6.25) of ingredients, diets and feces were determined by Kjeldahl method following acid digestion, using a Kjeltec digester and distillation units (Tecator Systems, Höganäs, Sweden; model 1015 and 1026, respectively). Crude protein was calculated by multiplying the total nitrogen content by the factor 6.25 (16gN/100 protein). Crude protein was determined by estimating the total nitrogen content of the material, assuming that all nitrogen is proteinaceous in origin. Approximately 400 mg of sample was added to the tubes of digestion. Two tablets containing 1 g of sodium sulphate (Na₂SO₄) plus 0.05 g of selenium (Se) were added to each sample as catalyst. The samples were digested for one hour at 450 °C with 15 ml concentrated sulfuric acid (H₂SO₄) which converts organic nitrogen to ammonium sulfate. After cooling, 20 ml of distilled water was added to each tube and the samples were distilled in the Kjeltec distillation unit. The solution was neutralized with 50 ml sodium hydroxide (40%) and ammonium salt converted to ammonia. Ammonia reacts with the boric acid and the amount of ammonia was determined by titration with hydrochloric acid (HCL) (0.2 N), in the presence of a methyl orange pH indicator.

Crude lipids
Soxtec method
The lipid content of ingredients and diets was determined by the Soxtec method, involving a continuous extraction with petroleum ether in a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046). Approximately 500 mg of sample was placed in a thimble and positioned in the extraction unit. Samples were boiled for 30 min in petroleum ether, rinsed for 2 hours and the extracted lipids were completely collected in the extraction cups. After extraction the solvent was evaporated and the extracted material weighed after drying. Lipid content was estimated through the difference in weight of the cups before and after extraction.

Folch method
Lipid content in feces was determined according to the gravimetric method described by Folch et al. (1957). 200 mg of feces were weighed to a tube and 5ml of chloroform / methanol (2:1, v / v) added; mixed in a vortex for 3 minutes and then centrifuged for 5
minutes at 2000 rpm. The upper phase was discarded in a pre-weighted centrifuge tubes, 1 ml of water was added and homogenized in a vortex. The tubes were then centrifugated at 3000 r.p.m. during 10 minutes. The top layer was removed and about 1 ml of Folch reagent (chloroform / methanol / NaCl 0.9%, 3:48:47) was added. The top layer was discarded again. Then, 0.2ml of methanol was added to the remaining solution and it was placed in an oven at 50 °C overnight. In the morning, the samples were cooled in a desiccator and then weighed. The lipid content was calculated as the difference in weight between the tubes before and after extraction.

**Dry matter**
Approximately 500 mg of sample was placed in pre-weighed crucibles. The moisture content was determined by total weight loss of the sample, expressed as a percentage of the original weight, after drying at 105°C until constant weight.

**Ash content**
After determining the moisture content, samples were placed in a muffle furnace and the ash content was determined as the inorganic residue obtained after incineration at 450°C for 16 hours.

**Gross energy**
The gross energy content in a sample is defined by -Δ Uc, which is the combustion energy at constant volume (kJ / g) (Rossini, 1956). Energy content of the samples was determined using an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261). Approximately 200-500 mg of sample, depending of the predicted caloric value, was weighed, pelletized and combusted under a pressurized (2.53x10^6 Pa) oxygen atmosphere in the bomb. After combustion, the temperature in the 2 L water jacket surrounding the stainless steel bomb rised and it was measured and used to calculate the energy content in the sample. The apparatus was calibrated with benzoic acid, the conversion factor of 1 cal = 4.1814 Joule was applied.

**Starch content**
The starch content was measured by an enzymatic procedure, according to Thivend et al. (1972). Quantitative enzyme hydrolyzes of starch by amylglucosidase was performed in dimethyl sulfoxide (DMSO) buffer solvent and the glucose released was measured using a Spinreact commercial kit (ref: 1001200). In this kit hexoquinase (HK) catalyzes the phosphorylation of glucose to glucose-6-phosphate (G6P) by ATP. The glucose-6-phosphate is then reduced to 6-phosphogluconate in the presence of glucose-6-phosphate dehydrogenase (G6P-DH) with the subsequent reduction of NAD^+ to NADH.
**Glucose + ATP → Glucose-6-phosphate + ADP**

**G6P + NAD → G6P - DH**

**Phosphogluconate + NADH + H+**

**Chromic oxide**

Chromium oxide content in diets and feces was quantified by acid digestion, according to Furukawa and Tsukahara (1966). 50 mg of sample were weighed and placed in 100 ml volumetric flasks. Then, 5 ml of HNO₃ was added and flasks content was digested in a heating mantle at 220 ° C until the acid volume was reduced to half (approximately 30 min). Then, another 5 ml of HNO₃ was added to ensure a complete digestion of the organic matter. The flasks were allowed to cool and subsequently,3 ml of perchloric acid (HClO₄) were added to the cold flasks. If flasks are still hot and/or an incomplete digestion of organic matter has occurred, an explosive reaction may happen. The flasks are placed again on the heating mantle until the green solution becomes lemon yellow. Then, flasks were cooled and its content was poured into 25 ml volumetric flasks. The spectrophotometer was adjusted with the blank and then reading of the samples at 350 nm was performed. With the standard line obtained by oxidation with the acid technique, chromium oxide content of the sample was calculated using the following standard line:

\[ y = 0.2089 \times + 0.0032 \]

Where \( y \) represents optical density at 350 nm and \( x \) represents the content of chromium oxide of the sample in mg/100 ml.

**Crude fiber**

Crude fiber is the organic residue that is obtained after treating the samples with diluted sulfuric acid, diluted sodium hydroxide and a lipid diluent. For that purpose, 0.5 g of sample (C) was weighed and placed in a digestion flask. Thereafter 50 ml of boiling sulfuric acid was added and the flask placed in a heating mantle for 30 minutes. Then, the flask content was transferred to centrifuge tubes and centrifuged for 15 minutes. The precipitate was again placed in the digestion flasks and 50 ml of boiling sodium hydroxide solution was added and the flask placed in a heating mantle for another 30 minutes. Thereafter, the flask content was filtered with a filter funnel and the flask walls washed twice with boiling water. The filtered sample was washed with 30 ml of boiling petroleum ether. The filter was placed in the oven for 24 hours and in the next day was cooled in the desiccator (A), weighed and placed in muffle furnace for 16 hours. In the next morning, was cooled in the desiccator and weighed again (B). The crude fiber content was determined with the following equation:
Phosphorus
Total phosphorus content in ingredients, diets and feces were determined based on a colorimetric method (Silva and Queiroz 2002). Briefly, phosphorus content was dissolved in aqueous solution and in contact with ammonium molybdate produced ammonium phosphomolybdate. This compound was reduced in the presence of ascorbic acid, forming colloidal oxides proportionally to the concentration of phosphorus in the solution. Excess ammonium molybdate does not react with ascorbic acid. The quantity of phosphorus was determined by measuring the intensity of blue color, which is produced by the formation of colloidal oxides. The color intensity developed by phosphomolybdate depends on the acidity and temperature of the solution, being stable in acidic solution.

Digestive Enzymes activities
For the enzymatic activity measurement, each intestine was homogenized in ice, with an Ultra Turrax, and centrifuged at 3,300 g, for 10 min at 4°C. The supernatant was collected and stored at -80°C, until analyses. All enzyme activities were determined using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA).

Total protein activity
Total protease activity was measured by the casein-hydrolysis method according to Hidalgo et al. (1999) at three different pH values, within the physiological range of digestive tract (Hidalgo et al. 1999; Furné et al. 2005). For each pH, the following buffers were used: 0.1M tris HCL buffer, for pH 8.0 and 9 and 0.1 M glycine-NaOH for

**Crude fiber content (%)** = \( \frac{(A - B)}{C} \times 100 \)
pH 10. The reaction mixture containing casein (1% w/v; 0.125 ml), buffer (0.125 ml) and homogenate supernatant (0.05 ml) was incubated for 1 hour at 37°C and stopped by adding 0.6 ml trichloroacetic acid (8% w/v) solution. After being kept for 1 h at 2°C, samples were centrifuged at 1800 g for 10 min and the supernatant absorbance was read at 280 nm against blanks. A control blank for each sample was prepared adding the supernatant from the homogenates after incubation. Tyrosine solution was used to establish a calibration curve. One unit of enzyme activity was defined as the amount of enzyme needed to catalyze the formation of 1.0 µmol of tyrosine per min.

**Amylase activity**

Amylase (E.C.3.2.1.1) activity was measured using a Spinreact kit (ref. 41201). The method comprises in the hydrolysis of 2-chloro-4-nitrophenyl-α-D-maltotrioside by α-amylase; this reaction releases 2-chloro-4-nitrophenol(CNP) and forms 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNPG₂), maltotriose (G₃) and Glucose(G). The rate of 2-chloro-4-nitrophenol formation, measured photometrically, is proportional to the catalytic concentration of α-amylase present in the sample.

\[
\text{Amylase} \quad 10 \text{ CNPG}_3 \rightarrow 9 \text{ CNP} + 1 \text{ CNPG}_2 + G_3 + G
\]

The reaction mix consisted of 200 µl of amylase reagent (2-chloro-4-nitrophenyl-α-D-maltotrioside, CNPG3) and 10 µL of sample homogenate. This mixture was incubated at 37 °C during 30 seconds and absorbance (ΔDO/min) was read at 1 minute intervals during 3 minutes at 405 nm and 37°C.

**Lipase activity**

Lipase (EC 3.1.1.3) activity was measured using a Spinreact Kit (Ref. 1001274). This is a new procedure at the Nutrimu laboratory, so it was needed to validate and adjust this method to fish intestine samples. Validation was accomplished by ensuring linearity of lipase activity in the same sample with different dilutions and comparing it to the quantification of the calibrator activity using the molar extinction coefficient of the reaction product (Methylresorufin) and the theoretical activity expected in the kit calibrator.

In this method, the pancreatic lipase, along with the colipase, desoxycholate and calcium ions, hydrolyses the substrate 1-2-O-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin)-ester.
1-2-O-dilauryl-rac-glycerol-3-glutaric-6'-methylresorufin-ester \[ \xrightarrow{\text{Lipase}} \]

1-2-O-dilauryl-rac-glycerol + Glutaric-6'-methylresorufin-ester (no stable) \[ \xrightarrow{\text{OH}^{-}} \]

Glutaric acid + Methylresorufin

The rate of methylresorufin formation was quantified photometrically and it is proportional to the concentration of catalytic lipase present in the sample homogenate. The reaction mix consists in 200µl of R1 (TRIS pH 8.3, colipase, desoxycholate and taurodesoxycholate), 40 µl of R2 (tartrate pH 4.0, lipase substrate and calcium chloride (CaCl\(_2\)) and 20 µl of sample. This mixture was incubated for 30 seconds and the sample absorbance (ΔDO/min) was then read at 10 seconds intervals, during 11 minutes, at 580 nm and 37°C.

**Specific enzymatic activity**

Enzyme activity of total proteases, amylase and lipase was expressed as specific activity (units per milligram of soluble protein; one unit (U) of activity was defined as µmol of product generated per minute). Soluble protein concentration was determined using Bradford's method (1976), with bovine serum albumin solution as standard. Amylase and lipase activities were determined using the formula:

\[
mU \ mg \ protein^{-1} = \frac{(\Delta \ DO/\Delta \ t) \times V_t \times f}{E_x \times 10^{-3} \times 10^{-9} \times V_e \times d \times P}
\]

Where \((\Delta \ DO/\Delta \ t)\) is the decrease or increase of optical density / minute, \(V_t\) is the total reaction volume, \(f\) is the correction factor for the dilution of the extract, \(E_x\) is the molar extinction coefficient, \(10^3\) is the conversion factor of liter to milliliter, \(10^9\) is the conversion factor from mol to nmol, \(V_e\) is the volume of added extract in ml, \(d\) is the length of the light beam through the microplate (0.79 for lipase activity and 0.675 for amylase activity) and \(P\) is the mg of protein per ml.
**Statistics analyses**

All statistical analyses were done using the SPSS 21.0 software package for Windows. Data are presented as means ± standard error of the mean (S.E.M). Before analysis, ADC values and enzymatic activity were subjected to arcsin square root transformation and ln or $\sqrt{x}$ transformation, respectively, to meet ANOVA requirements (homoscedasticity and normality).

Differences in ADC data was accomplished by one-way analysis of variance (ANOVA) for each species. Differences in ADC between species, diets and ingredients were performed by two-way ANOVA. For each species, differences between diets and intestine section, in digestive enzymes, were analyzed using a two-way ANOVA. If interaction was significant, one-way ANOVA was performed for diets and intestine sections. To test differences on enzyme activities between species and dietary treatment two-way ANOVA was performed. For each species and each intestine section, differences on total protease activity between pH and dietary treatment diet were analyzed by a two-way ANOVA. When $p$-values were significant ($P<0.05$), means were compared with Tukey´s HSD test (Tukey, 1949).
Results

Diets and ingredient digestibility

Digestible basis ingredient composition
On a digestible basis the experimental ingredients have a much lower dry matter and gross energy composition but the protein and lipids content remain almost the same (table 3). The table data were obtained from the nutrient digestibility values media of the two tested species.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Dry matter</th>
<th>Protein</th>
<th>Lipids</th>
<th>Gross Energy (kJ/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDGS₁</td>
<td>57.5</td>
<td>27.6</td>
<td>10.4</td>
<td>13.5</td>
</tr>
<tr>
<td>DDGS₂</td>
<td>50.5</td>
<td>27.0</td>
<td>10.8</td>
<td>11.9</td>
</tr>
</tbody>
</table>

¹DDGS₁: corn dried distillers grains with soluble from Biocarburantes de Castilla y Leon, Spain
²DDGS₂: corn dried distillers grains with soluble from Pannonia Ethanol, Hungry

Experiment 1 – Sea bass
The ADC of diets and ingredients for sea bass are presented in table 4. In this species, ADC of dry matter, energy and lipids of the reference diet was significantly higher than in the experimental diets (Table 4). ADC of protein and phosphorus was significantly higher in DDGS₁ diet (92.85% and 55.63%, respectively) than in the reference diet (91.88% and 31.65%, respectively). Regarding ingredient digestibility, DDGS₁ showed higher ADC for all analysed parameters than DDGS₂.
Table 4: Apparent digestibility coefficients (ADC %) of nutrients and energy of the experimental diets and test ingredients in sea bass.

<table>
<thead>
<tr>
<th>Sea Bass</th>
<th>Ref. diet</th>
<th>DDGS₁ diet</th>
<th>DDGS₂ diet</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td>83.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.24</td>
</tr>
<tr>
<td>Protein</td>
<td>91.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>91.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.31</td>
</tr>
<tr>
<td>Energy</td>
<td>93.93&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>84.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.33</td>
</tr>
<tr>
<td>Lipids</td>
<td>98.93&lt;sup&gt;b&lt;/sup&gt;</td>
<td>96.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.43</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>31.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.34&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.32</td>
</tr>
</tbody>
</table>

One-way ANOVA: Means in the same row with different superscript letters are significantly different (P < 0.05).

Experiment 2 - Meagre

The ADC of diets and ingredients for meagre is presented in table 5. ADC of dry matter, energy and lipids of the reference diet was significantly higher than that of the experimental diets. ADC of protein was higher in DDGS₁ diet (90.96%) than in the reference diet (88.99%). ADC of phosphorus was higher in the experimental diets (60.36% and 52.04%) than in the reference diet (23.6%). Regarding ingredient digestibility, DDGS₁ showed higher ADC for all parameters analysed than DDGS₂.

Ingredient digestibility – comparison between species

The ADC of dry matter, protein and energy of the reference diet were significantly higher in seabass than in meagre. Regarding ADC of DDGS, no significant differences were observed between species.
Table 5: Apparent digestibility coefficients (ADC%) of nutrients and energy of experimental diets and test ingredients in meagre\(^1\).

<table>
<thead>
<tr>
<th></th>
<th>Meagre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ref. diet</td>
</tr>
<tr>
<td><strong>ADC diets</strong></td>
<td></td>
</tr>
<tr>
<td>Dry matter</td>
<td>79.91(^b)</td>
</tr>
<tr>
<td>Protein</td>
<td>88.99(^a)</td>
</tr>
<tr>
<td>Energy</td>
<td>91.96(^b)</td>
</tr>
<tr>
<td>Lipids</td>
<td>98.35(^b)</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>23.63(^a)</td>
</tr>
<tr>
<td><strong>ADC ingredient</strong></td>
<td></td>
</tr>
<tr>
<td>Dry matter</td>
<td>-</td>
</tr>
<tr>
<td>Protein</td>
<td>-</td>
</tr>
<tr>
<td>Energy</td>
<td>-</td>
</tr>
<tr>
<td>Lipids</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\)One-way ANOVA: Means in the same row with different superscript letters are significantly different (P <0.05).

\(^2\)DDGS\(_1\): corn dried distillers grains with soluble from Biocarburantes de Castilla y Leon, Spain and Pannonia Gold, Hungry, respectively.

**Digestive enzymes**

The proteolytic, amylolytic and lipolytic activities (mU mg protein\(^{-1}\)) in the anterior, medium, distal and total intestine of seabass and meagre juveniles fed the experimental diets are present in tables 6 and 7, respectively. These tables express proteolytic activity measured at pH 8. Total intestine activity was obtained by the sum of the activity in anterior, medium and distal intestine.

Irrespectively of dietary treatment and species, digestive enzymes activity was higher in medium intestine than in the other intestine regions. In sea bass, total protease activity was not different among groups, while total lipase activity was higher in fish feed with DDGS\(_2\) diet than the other diets. Total amylase activity was higher in fish fed DDGS\(_2\) diet than DDGS\(_1\) diet but did not differ from that of fish fed the reference diet.

In meagre juveniles there were no differences between diets in total proteolytic activity. Total lipase activity was higher in fish fed DDGS\(_2\) diet than the other diets, while total amylase activity was higher in fish fed DDGS\(_2\) diet than the reference diet.
Table 6: Specific activities of protease, lipase and amylase (mU mg protein\(^{-1}\)) in different intestine sections (anterior (IA), medium (IM), distal (ID) and total) of sea bass fed the experimental diets\(^1\).

<table>
<thead>
<tr>
<th>Sea bass</th>
<th>Ref. diet</th>
<th>DDGS(_1) diet</th>
<th>DDGS(_2) diet</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>55.18</td>
<td>72.16</td>
<td>54.72</td>
<td>5.76</td>
</tr>
<tr>
<td>IM</td>
<td>152.6</td>
<td>58.98</td>
<td>139.75</td>
<td>13.34</td>
</tr>
<tr>
<td>ID</td>
<td>51.13</td>
<td>37.79</td>
<td>61.31</td>
<td>5.67</td>
</tr>
<tr>
<td>Total(^2)</td>
<td>258.92</td>
<td>168.94</td>
<td>250.71</td>
<td>15.58</td>
</tr>
<tr>
<td><strong>Lipase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total(^2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Amylase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total(^2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Two-way ANOVA: N.S: non-significant (P > 0.05); ** P < 0.01; *** P < 0.001. If interaction was significant, a One-Way ANOVA was performed for each factor (diet and section). Means in same row with different superscript letters represent significant differences between diets and in the same column means with different capital letters represent significant differences between intestine sections (P < 0.05).

\(^1\)Two-way ANOVA: Total intestinal tract: sum of the activity in anterior, medium and distal intestine sections.

\(^2\)DDGS\(_1\): corn dried distillers grains with soluable from Biocarburantes de Castilla y Leon, Spain and Pannonia Gold, Hungry, respectively.
**Table 7**: Specific activities of protease, lipase and amylase (mU mg protein\(^{-1}\)) in different intestine sections (anterior (IA), medium (IM), distal (ID) and total) of meagre fed the experimental diets\(^1\)

<table>
<thead>
<tr>
<th>Meagre</th>
<th>Ref. diet</th>
<th>DDGS(_1) diet</th>
<th>DDGS(_2) diet</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>(^{A})147.89(^{a})</td>
<td>(^{A})185.50(^{ab})</td>
<td>(^{A})223.33(^{b})</td>
<td>12.58</td>
</tr>
<tr>
<td>IM</td>
<td>(^{B})348.34</td>
<td>(^{B})398.72</td>
<td>(^{A})345.65</td>
<td>33.6</td>
</tr>
<tr>
<td>ID</td>
<td>(^{A})170.03</td>
<td>(^{A})133.74</td>
<td>(^{A})166.18</td>
<td>23.64</td>
</tr>
<tr>
<td>Total(^2)</td>
<td>(^{C})666.25</td>
<td>(^{C})717.96</td>
<td>(^{B})798.65</td>
<td>36.07</td>
</tr>
<tr>
<td><strong>Lipase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>1.27</td>
<td>1.60</td>
<td>1.76</td>
<td>0.19</td>
</tr>
<tr>
<td>IM</td>
<td>3.06</td>
<td>6.03</td>
<td>9.23</td>
<td>0.74</td>
</tr>
<tr>
<td>ID</td>
<td>2.25</td>
<td>1.52</td>
<td>3.76</td>
<td>0.35</td>
</tr>
<tr>
<td>Total(^2)</td>
<td>6.58</td>
<td>9.16</td>
<td>14.75</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>Amylase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>(^{AB})17.32</td>
<td>(^{A})15.30</td>
<td>(^{A})22.17</td>
<td>2.24</td>
</tr>
<tr>
<td>IM</td>
<td>(^{B})21.72(^{a})</td>
<td>(^{B})37.07(^{b})</td>
<td>(^{B})42.64(^{b})</td>
<td>2.93</td>
</tr>
<tr>
<td>ID</td>
<td>(^{A})10.25</td>
<td>(^{A})7.35</td>
<td>(^{A})14.25</td>
<td>1.31</td>
</tr>
<tr>
<td>Total(^2)</td>
<td>(^{C})49.29(^{a})</td>
<td>(^{C})59.72(^{ab})</td>
<td>(^{C})79.06(^{b})</td>
<td>4.34</td>
</tr>
</tbody>
</table>

**Two-way ANOVA**

<table>
<thead>
<tr>
<th>Section</th>
<th>Diet</th>
<th>interaction</th>
<th>Section</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>IM</td>
<td>ID</td>
<td>Total</td>
<td>Diet</td>
</tr>
<tr>
<td>Proteases</td>
<td>***</td>
<td>**</td>
<td>***</td>
<td>-</td>
</tr>
<tr>
<td>Lipase</td>
<td>***</td>
<td>**</td>
<td>N.S</td>
<td>A</td>
</tr>
<tr>
<td>Amylase</td>
<td>***</td>
<td>**</td>
<td>*</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\)Two-way ANOVA: N.S: non-significant (P > 0.05); ** P < 0.01; *** P < 0.001). If interaction was significant, a One-Way ANOVA was performed for each factor (diet and section). Means in same row with different superscript letters represent significant differences between diets and in the same column means with different capital letters represent significant differences between intestine sections (P < 0.05).

\(^2\)Total intestinal tract: sum of the activity in anterior, medium and distal intestine sections.

\(^3\)DDGS\(_1,2\): corn dried distillers grains with soluble from Biocarburantes de Castilla y Leon, Spain and Pannonia Gold, Hungry, respectively.
A comparative analysis of total proteases, amylase and lipase activity (mU mg protein\(^{-1}\)) between sea bass and meagre fed the experimental diets is present in the table 7. This table expresses proteolytic activity measured at pH 8.

Independently of diet, proteolytic activity was higher in meagre than in sea bass while amylase activity was higher in sea bass. Lipase activity was also higher in sea bass than in meagre, though differences between species were not very important.

Overall, and independently of species, lipase and amylase activity was higher in fish fed diet DDGS\(_2\) diet than the other diets, and no differences among diets was observed for protease activity.

**Table 8: Two-way ANOVA analysis of effect of intestine section and diet on the specific activities of protease, lipase and amylased (mU mg protein\(^{-1}\)) in seabass and meagre\(^1\).**

<table>
<thead>
<tr>
<th></th>
<th>Sea Bass</th>
<th>Meagre</th>
<th>Sea bass / Meagre</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reference Diet</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteases</td>
<td>258.92</td>
<td>666.25</td>
<td>0.39</td>
</tr>
<tr>
<td>Lipase</td>
<td>11.31</td>
<td>6.58</td>
<td>1.72</td>
</tr>
<tr>
<td>Amylase</td>
<td>100.81</td>
<td>49.29</td>
<td>2.05</td>
</tr>
<tr>
<td><strong>DDGS(_1) Diet</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteases</td>
<td>168.94</td>
<td>717.96</td>
<td>0.24</td>
</tr>
<tr>
<td>Lipase</td>
<td>7.26</td>
<td>9.16</td>
<td>0.79</td>
</tr>
<tr>
<td>Amylase</td>
<td>77.68</td>
<td>59.72</td>
<td>1.30</td>
</tr>
<tr>
<td><strong>DDGS(_2) Diet</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteases</td>
<td>250.71</td>
<td>798.65</td>
<td>0.31</td>
</tr>
<tr>
<td>Lipase</td>
<td>27.59</td>
<td>14.75</td>
<td>1.87</td>
</tr>
<tr>
<td>Amylase</td>
<td>168.58</td>
<td>79.06</td>
<td>2.13</td>
</tr>
</tbody>
</table>

**Two-Way ANOVA**

<table>
<thead>
<tr>
<th></th>
<th>Reference diet</th>
<th>DDGS(_1) diet</th>
<th>DDGS(_2) diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>Interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteases</td>
<td>*** N.S</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lipase</td>
<td>* *** N.S</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>Amylase</td>
<td>*** ** N.S</td>
<td>a</td>
<td>a</td>
</tr>
</tbody>
</table>

\(^1\)Two-way ANOVA: N.S: non-significant (P>0.05); ** P<0.01; *** P<0.001.

\(^2\)Total intestinal tract comprises the sum of anterior intestine, medium intestine and distal intestine activities.

\(^3\)DDGS\(_1\): corn dried distillers grains with soluble from Biocarburantes de Castilla y Leon, Spain and Pannonia Gold, Hungry, respectively.
In both species a similar profile activity of proteases relating to pH values was observed in the anterior intestine (Fig. 8). In the anterior intestine of sea bass higher protease activity occurred at lower pH values (8 and 9). However, in the medium intestine the highest proteolytic activity was found at pH values of 8 and 10. In the anterior intestine of meagre the highest proteolytic activity was observed at pH 8 and 9, while in the medium intestine that was found at pH 9 and 10. In both species, protease activity in the distal intestine was not affected by pH.

Diet influenced protease activity in the two species, except in the distal intestine of sea bass and the medium intestine of meagre. In the anterior intestine of sea bass the highest proteolytic activity was observed in DDGS\textsubscript{1} diet, while in the medium intestine fish fed this diet showed the lowest protease activity. On the distal intestine there are no longer significant differences in proteolytic activity between diets. In the anterior intestine of meagre proteolytic activity was higher in DDGS\textsubscript{2} diet but not significantly different from proteolytic activity of DDGS\textsubscript{1} diet while in the distal intestine there was a decrease in activity in the DDGS\textsubscript{1} diet. There were no interactions between pH and diet in the protease activity in any intestine section.
Fig 8. Variation in the activity of proteases in different sections of the intestine (anterior intestine (IA), medium intestine (IM) and distal intestine (ID)) and different pH (8, 9 and 10).
Discussion
Fishmeal is the most commonly protein source used in aquafeeds due to its high protein content, essential amino acid profile, n-3 HUFA contents, high digestibility and palatability and no anti-nutritional factors. The aquaculture industry urgently needs to find alternative ingredients to reduce or eliminate the use of fish meal in aquafeeds. New feedstuffs are required due to fish meal production fluctuations, constant demand (SOFIA 2006), restrictions on the use of animal origin protein in feed formulation and elevated costs that most contribute to the fish feeds final price (Josupeit 2008). Evaluation of diets and ingredients digestibility has a huge relevance for the development of a new aquaculture feed and to determine the potential of new ingredients to incorporate those diets (Glencross et al. 2007). Currently, the modern fish diets are formulated on digestible nutrient and energy basis, and it is impossible to do this if accurate and precise digestibility data is not available (Glencross 2008).

The nutritional value and nutrient content of corn DDGS may be greatly affected by source, quality of the grains, fermentation efficiency, temperature and time of expose to drying process and quantity of distiller’s solubles added (Lim et al. 2011). Thus, it is imperative to evaluate the nutritional value of this feedstuff, particularly its digestibility value, rather than simply applying standard values for the raw materials before considering their incorporation in fish diets. Indeed, although in this study the two sources of corn DDGS used had similar proximal composition, it is known that chemical composition of DDGS is more variable that in the original cereal, depending on the DDGS processing technology (Schaeffer et al. 2011).

To evaluate DDGS digestibility, it was followed a method, previously suggested by Cho et al. (1982), in which 30% of the test ingredient (DDGS) is mixed with 70% of a reference diet. This procedure assumes that diet digestibility is additive, i.e. it is equal to the sum of the digestibility of individual diet ingredients, and don’t exist any interaction between ingredients or nutrients. Thus, it is presupposed that the ADCs of the ingredients are constant, regardless of its inclusion level and that they are not affected by other ingredients or the inclusion levels of the other ingredients. In practice, there are interactions between ingredients and effects of these interactions on diet digestibility and ingredients availability may be difficult to predict (Gregory et al. 2012). In the present study, to reduce potential interference of these factors and others, such as temperature, feed transit, fecal digestion volume and evacuation time, the reference diet was formulated with very low content of indigestible constituents (Refstie et al.
2006; Adamidou et al. 2009). Indeed, a reference diet with high content of indigestible fraction may compromise the applicability of this method for the evaluation of ingredients digestibility. The adequacy of the formulated reference diet used is attested by its high nutrient and energy ADC in both species. In this study, it was used a low-temperature fishmeal, which preserves protein structure and amino acid profile (Davies et al. 2011), leading to a high protein and energy digestibility of the reference diet.

Comparatively to the reference diet, DDGS diets had lower ADCs, except for protein which was higher in DDGS1 diet. This lower ADC may be related to the carbohydrate fraction of DDGS, particularly non starch polysaccharides (NSPs) and fiber. Many fish species, especially carnivorous species, have a moderate capacity to digest and metabolize carbohydrates and cannot digest fiber or NSP (Enes et al. 2011). Non-digestible carbohydrates, if present in high quantity, may also impair digestibility of other nutrients by reducing gut-retention time of feed and time available for nutrients absorption (Stone 2003; Enes et al. 2011). The mechanism responsible for this effect is primarily physical. Nutrient absorption is maximized when food has sufficient gut-retention time to complete digestion and properly nutrients absorption (Gregory et al. 2012). However, nutrients can be absorbed only if they come into contact with enterocytes.

In most carnivorous fishes, the intestinal tract is short, less than the length of the body, which limit the retention time of the food. This is the case of the two target species of this study, seabass and meagre, that have carnivorous feeding habits and short intestine length. The high DDGS fiber content, containing more that 15% of acid and 40% of neutral detergent fiber, probably explain the decrease of dry matter and energy digestibility in both species. Also, the high amount of NSPs present in corn DDGS (averaging 19% soluble, and more than 17% insoluble NSP; Widyaratne and Zijlstra 2007), may also contribute to the low digestibility of dry matter and energy in DDGS diets. Indeed, NSPs are not digested by fish and have been associated to low diet digestibility (Francis et al. 2001). This is related with a mechanism that involves the binding of the nutrient with bile salts, changes in viscosity and the digesta rate of passage and / or digestive enzymes obstruction. Dietary NSP may also affect lipid digestibility as it interfere with the micelle formation in the gastrointestinal tract (Enes et al. 2012). The impact of NSP in digestibility depends of its functional properties on the intestine microbiota, as NSP may be partially digested by the intestinal bacteria (Ringo et al. 2010). A limited incorporation of low molecular weight NSP may act as prebiotic with beneficial proprieties in fish through the stimulation of growth and/or activity of intestine bacteria (revised by Ringo et al. 2010). In white sea bream, for instance, it
was observed that dietary incorporation of guar gum up to 12% did not compromise growth, feed utilization or intestine health (Enes et al. 2012). Also in red drum, it was observed that an adequate amount on NSP in the diet acted as prebiotic enhancing nutrient and energy digestibility (Burr et al. 2008).

The inclusion of vegetable oils in carnivorous fish diets can affect the digestive and absorptive processes (Santigosa et al. 2010). Thus, modifications in the enterocyte membranes composition have been described in fish fed with vegetable oils (Sitjà – Bobadilla et al. 2005). These modifications can compromise the intestinal function, reducing lipid digestibility (Geurden et al. 2009). These facts can explain the lower lipids digestibility found in the tested diets since part of fish oil lipids were replaced by vegetable oils from DDGS.

Comparatively to the reference diet, dietary incorporation of DDGS increased protein digestibility. This may be due to yeast fermentation process, as circa 4% of DDGS biomass or 5.5% of DDGS protein has yeast origin (Ingledew et al. 1999; Zhou and Davies 2010).

Approximately 60 to 70% of phosphorus (P) in cereals is bound to phytate, which is poorly available for fish (Oliva-Teles et al. 1998). The high digestibility of P in DDGS indicates that the availability of P in this ingredient is improved due to the phytate-P hydrolysis. During the fermentation process of DDGS, phyatate-P may be partially degraded, increasing P availability (Widyaratne and Zijlstra 2007). The higher P digestibility in DDGS diets can also be related to the total dietary P content, as it is known that ADC of phosphorus is influenced by dietary inclusion level (Buyukates et al. 2000). This also suggests that fishmeal replacement by DDGS may improve P excretion management, preventing eutrophication of water courses. Li et al (2008a,b) documented that several marine yeast strains isolated from the gut of sea cucumber (Holothuria scabra) and marine fish (Hexagrammos otakii and Synecogobius hastis) had the ability to produce large amount of extra-cellular phytase. They claimed that such marine yeasts might play an important role in phytate degradation within marine animals gut. By consequence, the yeasts present in DDGS may have helped to degrade the phosphorus contained in the form of phytate and thereby increase its digestibility.

DDGS digestibility studies in fish are relatively scarce, as this is a new ingredient for aquafeeds, even though DDGS has long been used in terrestrial animal feeds. The present study was the first to evaluate the digestibility of DDGS in sea bass and meagre. DDGS digestibility was similar for both species, averaging 94.5% for protein,
86.5% for lipids and 64% for energy. These values are close to those reported for rainbow trout (ADC of 90 and 82% for protein and lipids, respectively; Cheng and Hardy 2004) and for Florida Pompano (63-66% energy digestibility; William et al. 2008). However, other authors reported much lower digestibility values for DDGS in hybrid sea bass (*Morone saxatilis x M chrysops*) (ADC of 65 and 69 % for protein and lipids, respectively; Thompson et al. 2008; Metts et al. 2011). Differences in DDGS digestibility may depend of species (Thodesen et al. 2001), DDGS processing methods or composition of the diets.

Comparatively to raw corn meal, DDGS seems to have higher digestibility. For instance, Venou et al. (2003) in gilthead sea bream, found ADC values of protein, lipid and energy of diets incorporating raw corn were much lower than those obtained in this study, but ADC values were similar with extruded corn. In rainbow trout fed 10, 20 and 30% of corn meal, protein digestibility was 87.6, 90.1 and 90.2%, respectively (Ufodike and Matty 1989). In seabass, ADC of DDGS seems to be similar to that of soybean meal (ADC of dry matter, protein and energy averaging 65.5, 89.8 and 69.3% respectively; Gomes da Silva and Oliva-Teles 1998). In meagre juveniles, the ADC of protein of corn meal, corn gluten meal and soybean meal (99.6%, 89% and 92.9%, respectively) were similar to that obtained with DDGS in this study. However, comparatively to DDGS, the ADC of energy and dry matter were higher in corn gluten meal (77.8% and 78.9%, respectively) and soybean meal (73.6% and 65.1%, respectively) but lower in corn meal (40.6 % and 49.8 %, respectively) (Olim 2012). Even though seabass and meagre are species of a high trophic level, their digestive capacity to utilize DDGS seems very promising.

Enzymatic investigations are crucial to clarify the effect of fishmeal replacement by plant protein feedstuffs in fish diets, allowing evaluating if the metabolic functionality of intestine was modified by DDGS incorporation (Palmegiano et al. 2006; Corrêa et al. 2007) as any changes in digestive enzyme level may influence digestion and absorption of the food (Lemieux et al. 1999). Several factors may affect digestive enzyme production in fish, such as feeding habits, food preferences, diet formulations and anti-nutritional factors (ANFs) (Pavasovic et al. 2007).

A bibliographic comparison of digestive enzymatic activity is not easy due to different protocols used (Hidalgo et al. 1999) and different distribution patterns of digestive enzymes among species (Corrêa et al., 2007). In fish, nutrient absorption is known to take place in anterior intestine and, to a lower extent, in the posterior intestine (Gai et al. 2012). In this study, results showed generally higher levels of proteases, lipase and
Amylase activity in medium and distal intestine. This uncommon elevated digestive enzyme activity in the mid and distal intestine observed both in seabass and meagre may be due a possible drag of the secreted mucous to this parts of the digestive tract, as previously observed in two other carnivorous species, the rainbow trout (Gai et al. 2012) and common dentex (Pérez-Jiménez et al. 2009).

In seabass, total proteases activity was not significantly affected by the dietary treatment, but in meagre protease activity in the anterior intestine was significantly increased by dietary DDGS incorporation. In other species like Scylla serrate (Pavasovic et al. 2004), Gadus morhua (Refstie et al. 2006) and Litopenaeus vannamei (Rivas-Vega et al. 2006) proteases activity was not affected by dietary soy bean meal at different inclusion levels. Proteases activity may be also influenced by nutrient quality and quantity (Le Moullac et al. 1996). For instance, in seabream and rainbow trout, fishmeal replacement by plant protein mixtures decreased trypsin and chymotrypsin activity (Santigosa et al. 2008). However, fish may develop compensatory mechanism and adapt its digestive physiology in response to changes in dietary profile (Daprá et al. 2009; Lin and Luo 2011), which are in accordance to the increase of proteases activity observed in meagre.

Amylase activity is directly related to dietary starch levels, as demonstrated in European seabass and rainbow trout (Cahu and Zambonino Infante 1994; Corrêa et al. 2007). In the present study dietary starch was higher in the reference diet than in the DDGS diets. Nevertheless, in meagre, higher amylase activity was observed in DDGS diets. One possible explanation for this result is that the yeasts present in DDGS could have worked as probiotic enhancing amylase activity and improving the starch digestibility.

Indeed it has been suggested that an increase in the dietary lipids amount and composition may lead to an increase in lipolytic activity (Barrington et al. 1962; Ghosh 1976; Borlongan 1990; Bazaz and Keshavanath 1993). Lipase activity is closely linked to dietary triglycerides (TG) and phospholipids content, as previously reported for seabass lavae (Zambonino Infante and Cahu 2007). Thus differences in the fatty acid composition of the diets may contribute to explain differences in lipase activity in DDGS diets (Kenari et al. 2011). Corn oils are mainly composed by complex mixtures of TG (generally 95-97 %), before refining. After the refining process, TG increase up to 99% (Gunstone 2011). In DDGS, a refining product, lipid and TG are concentrated as the starch faction is removed during the fermentation processes, concentrating all other nutrients of the ingredient. Oil is typically present at a concentration of approximately
10% in DDGS, and contains high levels of polyunsaturated fatty acids (PUFA), particularly linoleic acid and unsaturated fatty acids (UFA) like oleic acid (Wang et al. 2007). This high content in free unsaturated fatty acids may be due to the high temperatures of processing, combined with moisture content, acids and bases used for pH adjustment and the high temperatures used during evaporation of thin stillage and grains drying (Jill 2012). Unsaturated fatty acids have lower melting points than saturated fatty acids, resulting in higher digestibility coefficients of unsaturated vegetables oils (Olsen and Ringo 1997). The specificity of pancreatic lipase activity is related to the acyl chain length and the degree of unsaturation, which has been shown to have a higher preference for PUFA as substrate resulting in high lipids digestibility in diets (Iijima et al. 1998).

In this work lipase activity seemed to be higher in mid and distal intestinal in both species. Lipolytic activity in fish is generally greater in the proximal part of intestine and pyloric caeca, deceasing progressively to the end of the gut, but it can be extended into the lower parts of intestine (Tocher 2003). However, in others carnivorous species such as turbot (Koven et al. 1994a) and plaice (Olsen and Ringo 1997), lipolitic activity was also found to be higher in the distal part of intestine. This may be a physiologic adaptation to a short digestive tract and few pyloric caeca as in turbot.

Results of this study strengthen the hypothesis that fishes can modulate digestive enzyme activities in response to changes in dietary composition (Fountoulaki et al. 2005). Several authors argue that the presence of lipases is higher in carnivoures fish than in omnivores and herbivores fish (Tengjaroenkul et al. 2000; Furné et al. 2005). In nature, carnivorous fish ingest large amounts of lipids and thereby lipase is needed in higher amounts for digesting it (Chakrabarti et al. 1995). Concordantly, present results also indicate that the two species have similar capacity to digest lipids. Even though, optimal dietary lipid levels seem to differ between the two species, with values of up to 22% in seabass (Peres and Oliva-Teles 1999; Montero et al. 2005) and up to 17% in meagre (Chatzifotis et al. 2012).

In the wild seas bass and meagre have similar eating habits, being described as carnivorous and predatory species. However, the two species have different trophic level (4.3 for meagre and 3.8 for sea bass according to Fishbase). Most authors consider that proteolytic activity is not strictly dependent on nutritional habits being amylase much more dependent on that (Hidalgo et al.1999). In this study, substantial higher proteolytic activity was however observed in meagre than in seabass. This higher protease activity in meagre can be related to growth rate, as meagre is a fast
growth fish species, reaching 1.2 kg in less than 2 years (Chatzifotis et al. 2012). Direct relationship between digestive proteolytic activity and the growth rate is often observed (Hidalgo et al. 1999).

Generally, the amount of specific enzymes is directly related to the capacity of digesting a nutrient. Sea bass had a higher amylase activity than meagre and this is in accordance to its ability to digest starch. Indeed, seabass digests efficiently processed starch, with a digestibility average of 90% (Enes et al. 2011).

Many studies in teleost fish reported that the pH 8-10 is the optimal range for alkaline proteases in the pyloric caeca and intestine (Chong et al. 2002; Tramati et al. 2005). Present results showed that DDGS dietary incorporation did not influence the optimum pH for protease activity in the intestine. In both species, higher proteolitic activity was attained in anterior intestine at pH 8-9 and in medial intestine at pH 8-10. Similar results were reported for carp (Jonas et al. 1983), rainbow trout and Atlantic salmon (Torrissen 1984), halibut and turbot (Glass et al. 1989), European sea bass and striped sea bass (Eshel et al. 1993), goldfish (Hidalgo et al. 1999), and dentex (Jimenez et al. 2009). These results suggest at least two major groups of alkaline proteases. The high activity of intestinal proteases at pH 7.0-9.0 was related to trypsin activity in carnivorous species like Solea solea (Clark et al. 1985) and S. formosus (Natalia et al. 2004). In contrast, chymotrypsin despite having a similar pH of activation appears to be more active at pH 7.0-8.0. An increase in trypsin activity may occur in animals fed with vegetable feedstuffs due to long term compensation mechanisms, as described in trout (Krogdahl et al. 1994). Changes in trypsin and chymotrypsin activities imply differential availability of oligopeptides and amino acids, which can cause an amino acid imbalance under these feeding circumstances. The high proteolytic activity at pH 10 may be associated with collegenase or elastase activities which are related to the higher pH of 9.5 in Dover sole (Clark et al. 1985).

**Conclusions**

The ADC of the diets, except for phosphorus, was higher in sea bass than in meagre. However, both sea bass and meagre digested well DDGS. ADC of protein (92 % and 98%) and of lipid (82 and 89 %) of the tested ingredients was high. ADC of dry matter and energy were moderate (57 and 66%; 58 and 58%, respectively). The high fibre content of DDGS may explain this moderate dry matter and energy digestibilities.
Due to differences in DDGS processing, it was possible to observe differences in dry matter, protein and energy ADC between the two types of DDGS tested, reinforcing the importance of a good characterization of raw materials before using them in formulated diets. Diets with DDGS have lower ADC of dry matter, energy and lipids than the reference diet. However the protein ADC in DDGS, and phosphorus in both tested diets was higher than in the reference diet.

The enzymatic activity data showed that DDGS affected the digestive enzymatic ability of both species, especially lipase and amylase activity.

In summary, DDGS seems to have high potential to be included in diets for sea bass and meagre.

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