# Optimization of solid state fermentation of dried distillers grains with solubles (DDGS) and its effect on performance and nutrient digestibility in European sea bass

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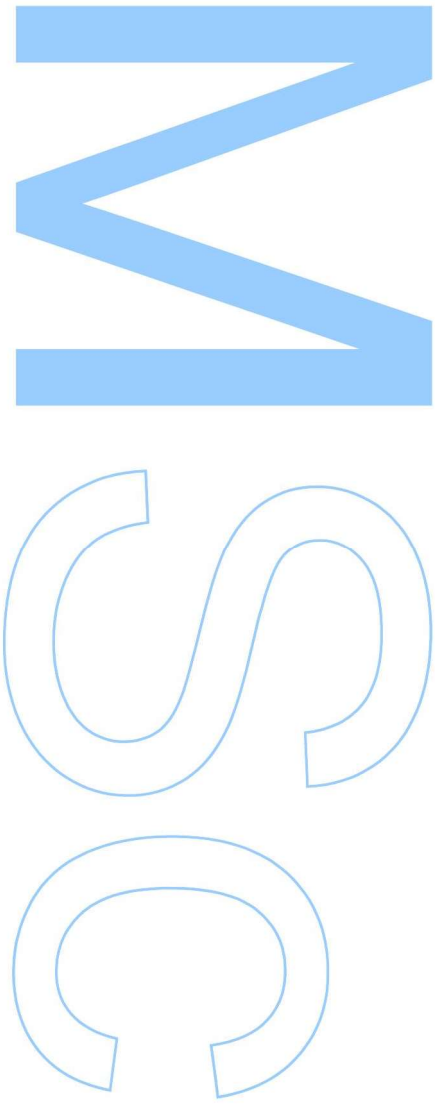

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O Presidente do Júri,

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*“There are a thousand paths to go down, and the one that's a little different and a little awkward is often the best one (...) Go your own way, whatever that way is”*

Zlatan Ibrahimović

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Move forward when we are facing difficulties becomes much easier when we have people on our side willing to help and support is to them that follows my thanks.

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# Resumo

Para aumentar o valor de um subproduto resultante da fermentação do etanol de milho, os grãos secos de destilaria com solúveis (DDGS) foram utilizados como substrato para fermentação em estado sólido (SSF). Inicialmente, a otimização das condições para a fermentação em estado sólido do DDGS foi realizada utilizando diferentes fungos: *Aspergillus ibericus*, *A. Niger* e *A. uvarum* que foram escolhidos com base nas suas capacidades de segregar exo-enzimas e por apresentarem boas taxas de crescimento em condições de SSF. Todos os fungos tiveram um bom desempenho, no entanto A. ibericus mostrou ser mais eficiente em aumentar o teor em proteína e exo-enzimas (celulase e xilanase) do DDGS fermentado, sendo assim escolhido para a realização de SSF do DDGS, numa escala piloto, para produzir um DDGS fermentado (SSF-DDGS) de valor aumentado. A eficácia do DDGS e do SSF-DDGS como ingredientes para rações foram testados em ensaios de crescimento e de digestibilidade em juvenis de robalo (*Dicentrarchus labrax*).

O ensaio de digestibilidade foi realizado para avaliar o coeficiente de digestibilidade aparente (ADC) dos nutrientes e da energia de ambos os ingredientes, DDGS não fermentado e DDGS fermentado (SSF-DDGS), e a atividade das enzimas digestivas. A digestibilidade foi determinada com base na substituição de 30% de uma dieta referência (47% proteína bruta; 15% gordura bruta) para cada ingrediente a testar. Comparado com o DDGS, o SSF-DDGS teve maior ADC da proteína (92.4 vs. 98.3%), lípidos (87.9 vs. 98.3%) e energia (71.7 vs. 89.6%), enquanto a digestibilidade da matéria seca e da matéria orgânica não foram afetadas. A atividade das lípases, tripsinas e quimiotripsinas digestivas foi significativamente maior com as dietas à base de DDGS ou de SSF-DDGS do que na dieta referência. A atividade da amílase foi significativamente maior na dieta à base de DDGS quando comparada com a dieta à base de SSF-DDGS.

O ensaio de crescimento foi realizado para avaliar o efeito da inclusão na dieta do DDGS ou do SSF-DDGS. Cinco dietas experimentais foram formuladas para ser isoproteicas (45% proteína bruta) e isolipídicas (18% gordura bruta), incluindo 20% de farinha de peixe. As dietas teste foram formuladas de forma semelhante à dieta controlo, mas substituindo a mistura de proteína vegetal (farinha de soja + farinha de trigo) da dieta controlo por 10% ou 20% de DDGS ou de SSF-DDGS.

O peso corporal final, o ganho de peso e o índice de crescimento diário foram significativamente inferiores nas dietas que continham 20% de DDGS ou de SSF-DDGS comparadas com a dieta controlo, enquanto não foram observadas diferenças significativas para a ingestão de ração e para a utilização de ração ou proteína. Comparando o efeito do DDGS fermentado contra o não fermentado; a inclusão de 10% de SSF-DDGS promoveu um maior ganho de peso e um maior índice de crescimento diário que a dieta que continha 10% de DDGS, mas não afetou a ingestão e a utilização de ração. O rácio de utilização de proteína também foi menor nas dietas que continham 20% de DDGS ou de SSF-DDGS do que na dieta que continha 10% SSF-DDGS.

No geral, conclui-se que a SSF com A.ibericus é uma técnica adequada para aumentar o valor nutricional e adicionar exo-enzimas ao DDGS, aumentado significativamente a sua digestibilidade. Além disso, a substituição de 10% de farinha de soja + farinha de trigo por DDGS na dieta é possível, se o DDGS for previamente submetido à SSF. De outro modo a sua incorporação vai afetar negativamente o desempenho do crescimento de juvenis de robalo. Sendo assim, podemos concluir que o SSF-DDGS tem um valor nutricional superior ao DDGS não fermentado, o que aumenta o seu potencial como ingrediente para rações para robalo.

**Palavras-chave**: Grãos secos de destilaria com solúveis (DDGS); Fermentação em estado sólido (SSF); *Aspergillus ibericus*; Digestibilidade; Enzimas digestivas; Performance de crescimento.

# Abstract

To increase the nutritional value of coproducts from corn ethanol fermentation, distiller's dried grains with solubles (DDGS) was used as a substrate for solid state fermentation (SSF). Firstly, the optimization of solid state fermentation condition of DDGS was carried out using different fungi: *Aspergillus ibericus*, *A. niger*, and *A. uvarum*; that were chosen based on its capacity to segregate exo-enzymes and good growth rates under SSF conditions. All the fungi performed well, although *A. ibericus* proved to be more efficient in upgrading the protein and exoenzymes (cellulase and xylanase) content of fermented DDGS, being chosen to perform SSF of DDGS, at a pilot scale, to produce an upgrading value fermented DDGS (SSF-DDGS). The efficacy of both DDGS and SSF- DDGS as feed ingredients were then tested in a digestibility and growth trials with European seabass (*Dicentrarchus labrax*) juvenile.

The digestibility trial was conducted to evaluate the apparent digestibility coefficients (ADC) of nutrients and energy of both ingredients, unfermented DDGS and fermented DDGS (SSF-DDGS), and the digestive enzymes activity. Digestibility was determined based on the substitution of 30% of a reference diet (47% CP; 15%CL) by each test ingredient. Compared to the unfermented DDGS, SSF-DDGS had higher ADC of protein (92.4 vs 98.3%), lipids (87.9 vs 98.3%) and energy (71.7 vs 89.6%) while dry matter and organic matter digestibility were not affected. Digestive lipases, trypsin and chymotrypsin activities were significantly higher with the DDGS or SSF-DDGS based diets that with the reference diet. Amylase activity has significantly higher for DDGS diet when compared with SSF-DDGS diet.

The growth trial was conducted to evaluate the effect of dietary inclusion of DDGS or SSF-DDGS. Five experimental diets were formulated to be isoproteic (45% crude protein) and isolipidic (18% crude fat), including 20% of fishmeal. Test diets were formulated similarly to the control diet, but replacing the mixture of plant protein (soybean meal + wheat meal) of control diet by 10 or 20% of DDGS or SSF-DDGS.

Final body weight, weight gain, and daily growth index were significantly lower in 20% DDGS or SSF-DDGS diets than in the control diet, while no differences were observed for feed intake and feed or protein utilization. Comparing the effect of fermented versus unfermented DDGS; the inclusion of 10% SSF-DDGS promoted higher weight gain and daily growth index that 10% DDGS diets, but did not affect feed intake and feed utilization. Protein efficiency ratio has also lower in 20% DDGS or SSF-DDGS than with the 10%SSF-DDGS diet.

Overall, it's concluded that SSF with *A. ibericus* is a suitable technique to upgrading the nutrient content and added exo-enzymes to DDGS, increasing significantly its digestibility. Moreover, the dietary replacement of 10% of soybean meal + wheat meal by DDGS is feasible, if the DDGS is previously submitted to the SSF. Otherwise, its incorporation will negatively affect growth performance of European sea bass juveniles. Therefore, it can be concluded that SSF-DDGS as a higher nutritional value than the unfermented DDGS, which increase its potential as a feedstuff for European sea bass.

**Keywords**: Distillers Dried Grains with Solubles (DDGS); Solid state Fermentation (SSF); *Aspergillus ibericus*; Digestibility; Growth performance; Digestive enzymes.

# Contents:

# Abbreviations

AA- Amino Acid

ANF- Antinutritional Factor(s)

ADC(s) - Apparent Digestibility Coefficient(s)

ADF- Acid Detergent Fibre

ANF's- Anti-nutritional factors

APA- Aquaculture Production Area

DDGS- Distillers Dried Grains with Solubles

EAA- Essential Amino Acids

EFA- Essential Fatty Acids

FAO- United Nations Food and Agriculture Organization

FM- Fish Meal

FO- Fish Oil

LC-PUFA- Long-Chain Polyunsaturated Fatty Acids

MUM- Micoteca of University of Minho

NDF- Neutral Detergent Fibre

NSP- Non-starch polysaccharides

PER- Protein Efficiency Ratio

SEM- Standard Error of the Mean

SmF- Submerged Fermentation

SSF- Solid State Fermentation

WG- Weight Gain

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# Introduction

## Aquaculture

### Aquaculture development

Aquaculture is a global industry, being difficult to precise the beginning of this activity, although it has been proposed some interesting theories about it (Stickney and Treece, 2012). It is important to stress that all of these possibilities could be correct but are perhaps applicable in different areas of the world, where aquaculture may have initiated independently from each other. More recently, with the development of transportation and communication facilities, aquaculture become more unified, intensified and integrated (Rabanal, 1988). As defined by the United Nations Food and Agriculture Organization (FAO), aquaculture is the "farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies some sort of intervention in

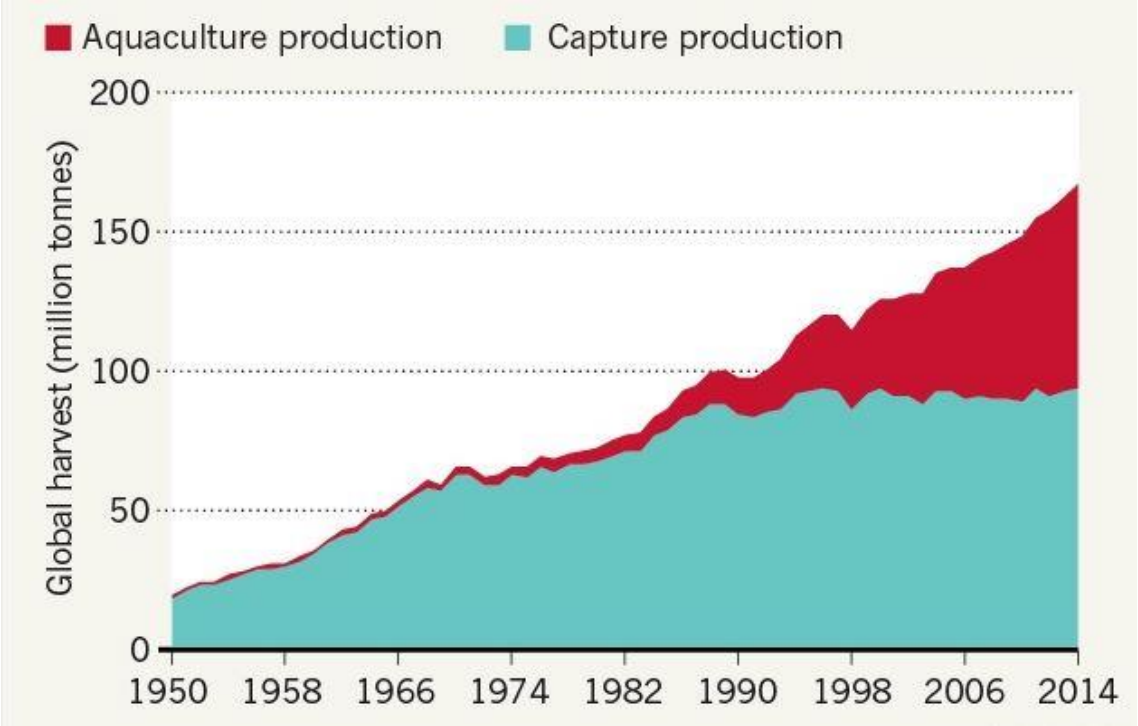



**Figure 1**- World aquaculture and capture production. Adapted from FAO. (2016b).

the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated…"

According to the last database from FAO, the world aquaculture production continued to grow in 2014, reaching 73.8 million tonnes (live weight) of fish production with an estimated value of 160.2 billion US dollars, approximately 150 billion euros. Almost all fish produced in aquaculture are for human consumption purposes, even though by-products may be used for non-food purposes. Besides fish production, aquaculture produces considerable quantities of aquatic plants and in 2014 the production of fish and plants combined reached the mark of 101.1 million tonnes in live weight (FAO., 2016b).

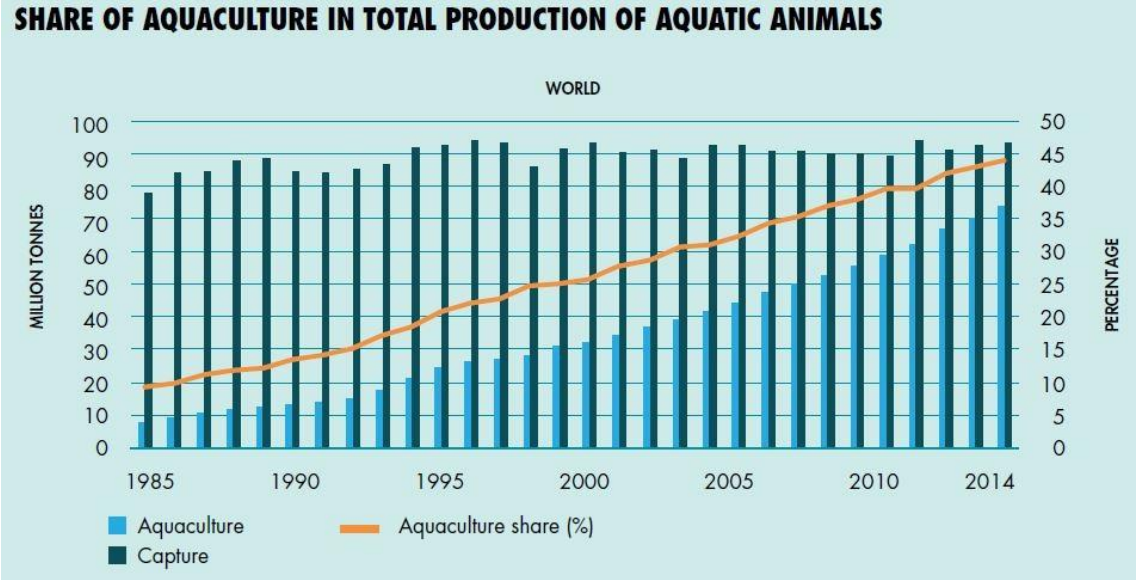



**Figure 2**-Share of aquaculture in total production of aquatic animals. Adapted from FAO. (2016b)

A decade ago, the contribution of aquaculture to the world total fish production was 30.6 percent, in 2014 it reached 44.1 percent, up from 42.1 percent in 2012, making aquaculture the fastest growing food production industry in the world, as it may be seen in the figures above.

In a more national level, in 2014, 35 countries produced more farmed fish than wild-caught fish, representing 45 percent of the world's population (FAO., 2016b). Although in 2014 was the first year in which more fish for consumption came from farming than from capture fisheries. In that year, people ate an average of 20.1 kilograms of fish, up from an average of 9.9 kg in the 1960s (FAO., 2016b). That is related to the lower price of aquaculture fish compared with other food sources, as well as all the benefits it represents for human health (Cole et al., 2009).

All continents have shown a general trend of an increasing share of aquaculture production in total fish production, even if in Oceania this share has declined in the last three years (FAO., 2016b).

As the amount of farmed seafood produced rises, it is critical to minimize the negative impacts of aquaculture on the environment and society. Indeed, the fast grows of intensive aquaculture productions, in some cases, not well planned has caused increased concerns about environmental impacts. The opposition to aquaculture development is strongest in the western world, where modern aquaculture is still a relatively new industry that competes with already well-stablished activities. Most of the issues concerning consumers are related to health, environment and safety aspects of farmed products (Hofherr et al., 2015; Kawasaki et al., 2016).

With the constant grow of aquaculture industry it is crucial to increase production without increasing the costs, solutions may be selective breeding (Gjedrem, 2012) or the use of alternative protein sources.

Aquaculture long term potential is big through different pathways: by increasing the availability of fish as a human food, by generating jobs, by facilitating better utilization of high-quality protein, by providing more efficient opportunities for efficient transformation of agriculture and fisheries resources. But for that, it is required a shared vision between the public and private sectors (Morgan et al., 2016; Naylor et al., 2000; Troell et al., 2014)

## Aquaculture in Portugal

In Portugal, the number of licensed aquaculture facilities in 2014 was 1521, a number relatively low for the potential that the Portuguese coast represents. In 2014 aquaculture produced 10791 tons of fish, generating receipts of 50.3 million euros. However, in 1990 the production in Portugal was restricted to two species, trout, and clam. At present, Portugal produces various species either fish either bivalves. The 90's were characterized by the strong growth and modernization of marine species aquaculture, in the beginning, focused in seabass and seabream, between the most produced species. Actually, seabass, seabream, turbot, sole, clams and oysters are the most produced species. Marine aquaculture started developing in the estuaries and wetlands, like Ria de Aveiro and Ria Formosa. In the last one, there are nearly 1200 production units that work in an extensive or semi-intensive system and produce mostly bivalves. In these areas, infrastructures previously occupied by the salt industry were reutilized, however, the tank dimension, the bottom characteristics, and the water flow are limiting factors of productivity. In the last years, some facilities settled on the coastline working in an intensive regime, pulling water from the sea and producing turbot and sole (DGRM, 2014).

In the open sea, the most favourable zones in continental Portugal are the south coast and the sheltered bays, and in the rest of the coast, the sea conditions are more adverse, especially in the winter which will demand advanced technology for the installation of structures. The installation of open sea facilities along the Algarve coast has been encouraged and some facilities are already producing and it is foreseen the installation of new ones in the Aquaculture Production Area (APA) of Armona and APA of Monte Gordo. APA are areas destined especially to aquaculture and have special conditions

for implementation of projects, including simplified licensing. With the new ‘’Plano de Ordenamento do Espaço Marítimo’’ were identified potential zones for aquatic means in the open sea (DGRM, 2014).

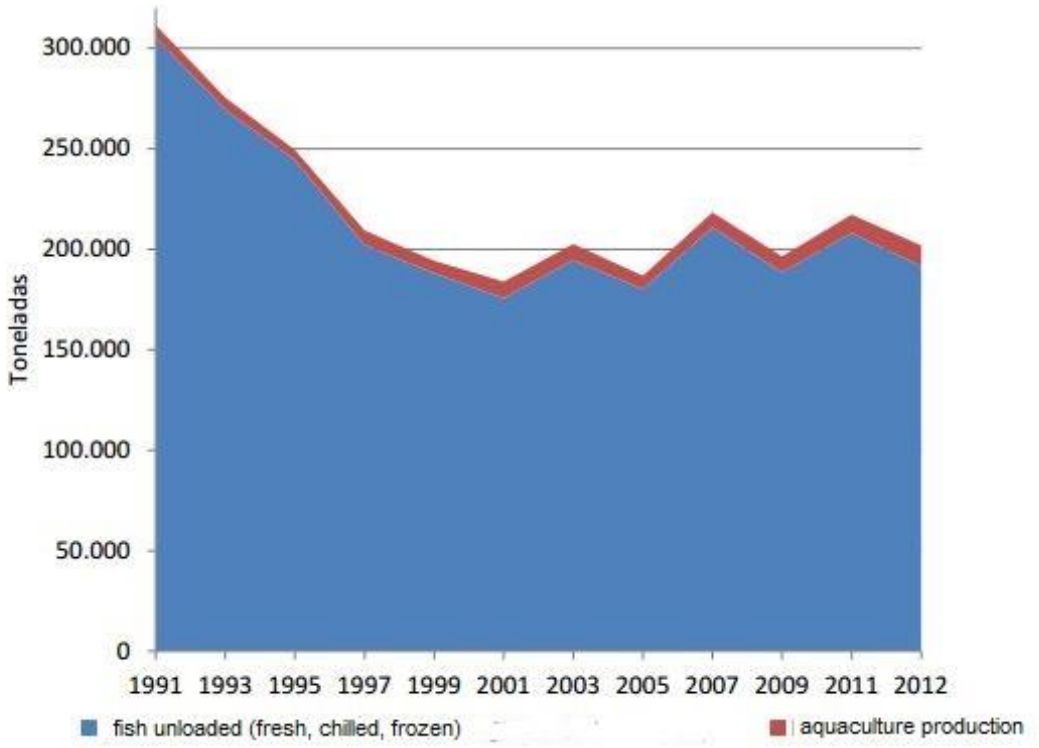
Freshwater aquaculture activity has more than 120 years in Portugal; began in 1896 with the opening of ‘’Estação Aquícola do Rio Ave’’. In this unit and others 11 facilities, built by the state until 1980, were produced several species of salmonids, cyprinids, and carps destined to the repopulation of national rivers, the supply of other aquacultures and for selling. Since 1980, a dramatic reduction of aquaculture stations was registered as well as the species diversity, existing only 3 stations in activity nowadays: Torno, Castrelos, and Manteigas, were are produced 2 species, rainbow trout and brown trout (DGRM, 2014).

In the fifty years emerged, some private aquacultures initiated activity. They mostly explore two species: rainbow trout and brown trout and the number of this facilities increase significantly between 1984 and 1995, and stabilized after that.

In 2013 were registered 28 units of freshwater aquaculture in activity, producing, besides trout, eel, and ornamental cyprinids. In the last decade arose the aquaculture of endemic cyprinids of the Iberian Peninsula, in the ambit of *ex-situ* conservation projects. It was reproduced with success 8 species to reinforce the natural populations with individuals created in captivity (DGRM, 2014).

In 2014, the Portuguese aquaculture production (10791 tons), continued to be far for the demand of 57 kg of fish / year per capita, but to point out, some of the most consumed species aren’t produced in Portugal, like cod or hake. In the past, Portuguese aquaculture had several strains to its development like bad planning and long bureaucratic process involving the licensing of aquaculture facilities, but it’s expected that these problems are resolved and the aquaculture in Portugal grows to fulfil the demand (DGRM, 2014; INE., 2015).

**Figure 3**– Origin of fish products. Adapted from DGRM (2014)




## European Sea Bass (*Dicentrarchus labrax*, Linnaeus, 1758)

European sea bass belongs to Actinopterygii class, Teleostei 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 (Fischer et al., 1984; Fishbase., 2016; Paugy et al., 2003).

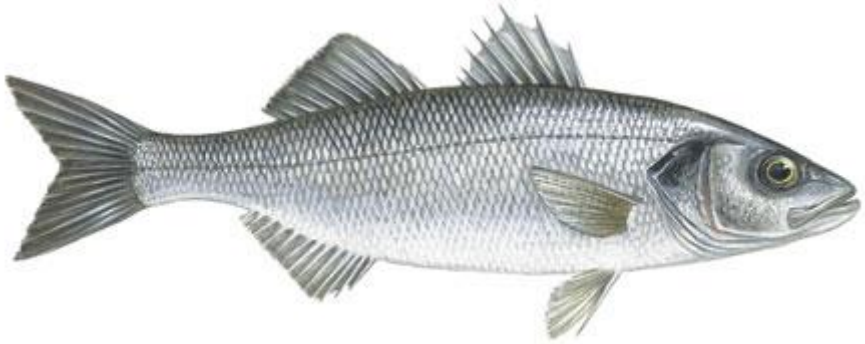


**Figure 4**- *Dicentrarchus labrax*. Adapted from Commission. (2016)

## Habitat and biology

European sea bass is a euryhaline marine teleost species (Varsamos et al., 2001). It has a geographical distribution in the Eastern Atlantic, from Norway to Morocco, the Canary Islands and Senegal. As well-known from the Mediterranean and the Black Sea. Is absent from White, Barents, Baltic and Caspian Seas (Kottelat and Freyhof, 2007).

Adults inhabit coastal waters down to about 100m depth but are more common in shallow waters and manifest demersal behaviour. Mostly found in the bottom of estuaries, lagoons and occasionally rivers in the littoral zone. Juveniles form school but adults appear to be less gregarious, they enter coastal waters in summer and migrate offshore in cold weather. They feed essentially on shrimps and molluscs, also on small fishes. Juveniles feed on invertebrates, taking gradually more fish with age (Fishbase., 2016).

Spawn in groups and eggs are pelagic, the sexual maturity depends on where it occurs, in the Mediterranean occurs generally between 2 and 4 years old while in the Atlantic happens a little later, females are larger than males, they can be 10 to 40% larger (Haffray et al., 2007). Spawning happens only once a year and normally in winter, embryo development lasts about three days at 13-14 ºC and larval development about 40 days at 19 ºC (Fishbase., 2016).

European Sea Bass have a high commercial value, both from capture and for aquaculture production (Haffray et al., 2007).

## Production

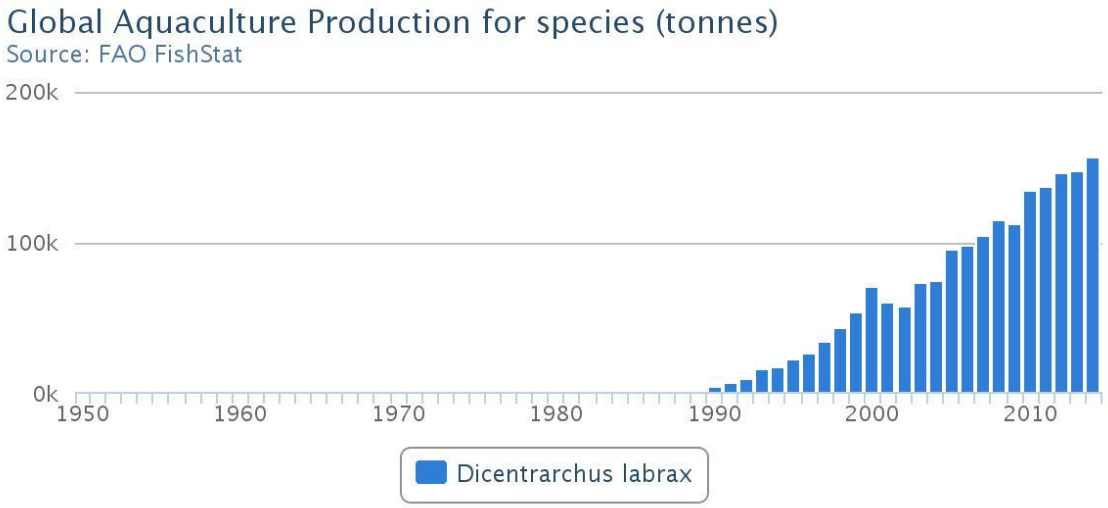



**Figure 5**- Global aquaculture production of European sea bass. Adapted from FAO. (2016a)

Domestication of the European Sea Bass initiated in the mid 80's by some pioneering companies in France (Haffray et al., 2007). Later this knowledge was dispersed to all Mediterranean countries to develop mass production techniques (Fishbase., 2016). It is one of the most important commercial species produced in the Mediterranean Sea, mainly in Greece, Turkey, Italy and Spain and it is marked mostly fresh or frozen and sometimes smoked (FAO., 2016a; Fishbase., 2016). In 2014, the global aquaculture production of this species reached 156 449 tonnes and it is expected to keep increasing for the next years (FAO., 2016a).

As a result of being a very important species for the industry, the nutritional requirements of this species has been roughly studied. The optimum dietary protein level for juveniles was established to be around 50% (Ballestrazzi et al., 1994; Hidalgo and Alliot, 1988; Oliva-Teles, 2000; Peres and Oliva-Teles, 1999b). Nevertheless, other authors reported that sea bass performed well with 43% of the protein in diets with at least 21 kJ/g (Dias et al., 1999) or with a diet including 45% protein (Pérez et al., 1997). Differences may be attributed to the different feed formulation, including the nature and level of each ingredient and nutritional composition, mainly dietary digestible energy level. Moreover, this difference may also be related to the existence of three genetically distinct zones from where the sea bass may originate (Haffray et al., 2007), which may lead to different performances (Perez-Sanchez and Le Bail, 1999; Saillant et al., 2001). Water temperature does not influence optimum dietary protein concentration, temperatures

close to the optimum of the species increase voluntary feed intake, resulting in a higher growth, feed efficiency, protein and energy retention (Hidalgo and Alliot, 1988; Moreira et al., 2008).

Concerning the optimum dietary lipid level, it was observed that an increase of dietary lipid level from 12 to 24% did not improve growth performance and feed efficiency of sea bass juveniles, while the increase up to 30% depresses growth rate, it significantly reduced protein and energy retention efficiencies (Oliva-Teles, 2000; Peres and Oliva-Teles, 1999a). Lanari et al. (1999) and Dias et al. (1998) reported higher optimum dietary lipid level for seabass around 15-18%. Due to the possible sparing effect of high dietary lipid on protein utilization (Dias et al., 1998) the increase of dietary lipid level is desired. However, it seems that seabass did not utilize high lipid levels as efficiently as salmonids (Oliva-Teles et al., 2015).

Carbohydrates are an excellent energy source for mammals and birds, but fish have limited capability to digest and metabolize carbohydrates and because that diets shouldn't include more than 20% digestible carbohydrates (Enes et al., 2011; Wilson, 1994). Factors as nature of starch, the level of dietary inclusion and molecule complexity had been found to affect the digestive and metabolic utilization of carbohydrates in sea bass (Enes et al., 2011). For sea bass, crude starch digestibility decreases with the increase of dietary inclusion levels (Dias et al., 1998). Gelatinized starch is adequately digested by seabass and significantly increases feed efficiency and digestive utilization of diets (Enes et al., 2011; Peres and Oliva-Teles, 2002) and sea bass performs better with starch than with glucose (Enes et al., 2011). Moreover, dietary incorporation of carbohydrates contributes to improving the mechanical properties of pellets (Lanari et al., 1999).

There are few studies about the requirement of minerals and vitamins, (Kaushik et al.) demonstrate that the data present in NRC (2011) on vitamin requirements of rainbow trout are sufficient to other finfish species like sea bass. To maintain the maximum growth of juveniles sea bass a minimum of 5 mg of ascorbic acid per kg of diet should be included (Fournier et al., 2000).

## Ingredients used in aquaculture feeds

Ingredients are sources of protein (amino acids), fats (fatty acids), carbohydrates, vitamins and minerals (NRC, 2011). Although energy is not independent of nutrients,

ingredients are also a source of energy. The feeds manufacture industry may use ingredients that are directly produced to be that purpose or may be by-products, the outputs from a manufacturing process or chemical reaction.

The most important source of protein in aquafeeds is fish meal (FM) that, depending on its quality, has a protein level ranging from 56% to 76%, with an adequate essential amino acids (EAA) profile. It is as well a good source of energy, essential fatty acids (EFA) and minerals and is highly palatable and digestible for most fish (NRC, 2011).FM is the crude flour obtained after milling and drying fish or fish parts (FAO., 2016b). Due to its unique characteristics, FM is considered the reference ingredient in aquafeeds manufacture. However, with annual oscillations (mainly caused by El Ninõ phenomena) FM production has declined progressively since 2005, but overall demand has continued to grow, pushing prices to historic highs through late 2014. FM prices are expected to remain high in the long term because of sustained demand (FAO., 2016b). So, this particular ingredient usually contributes to the high final price of fish feeds, which typically represents the largest variable cost for aquaculture producers (Naylor et al., 2009). Given the advantages that FM possesses as a feed ingredient, a compelling reason is needed to give the aquafeeds industry an incentive to decrease its use, and that incentive his that low-cost FM appears to be ending (Drew et al., 2007).

However, carnivorous fish species have high protein requirements and only a few selected ingredients with high protein content may be used as an alternative protein source to FM (Hardy, 2008). These selected ingredients essentially fall into three categories: animal rendered by-products, plant feedstuffs (mostly concentrates) and single-cell organisms (Naylor et al., 2009; Oliva-Teles, 2012). Relatively to FM, alternative feedstuffs should possess certain characteristics including competitive prices, low levels of fibre, non-starch polysaccharides, and anti-nutrients, plus have a high protein content, favourable amino acid (AA) profile, relatively high palatability and high nutrient digestibility (Gatlin et al., 2007; Naylor et al., 2009). The removal of the fibrous material present in the outer hull of plant feedstuffs led to significant improvement of the nutritional quality of these feedstuffs, particularly to those with carnivorous feeding habits (Booth et al., 2013).

Partial replacement of FM by alternative protein sources has been achieved at different levels in various fish species. Nevertheless, a dietary formulation with total or almost total FM replacement generally leads to a depression of voluntary feed intake and growth performance in carnivorous fish species (Kaushik et al., 2004).

In aquafeeds, the major alternative to FM is soybean meal, a plant feedstuff which presents high protein content and a relatively good amino acid profile for the majority of the produced species (Gatlin et al., 2007). Up till now, the increasing prices of soybean meal has driven the decrease its use and search for alternatives. Protein concentrates obtained from cereals, like wheat gluten and maize gluten, and from oilseeds, such as rapeseed and cottonseed are also good potential alternatives to FM (Gatlin et al., 2007; Mbahinzireki et al., 2001). Their use is limited due to unbalance of the amino acids profiles, although supplementation of the diets with the limiting amino acids may allow a higher replacement level (Pereira and Oliva-Teles, 2003).

Incorporation of lipids and carbohydrates in diets is necessary to spare protein for plastic purposes rather than 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 long-chain polyunsaturated fatty acids (LC-PUFA), which are EFA for marine fish spices (NRC, 2011). Fish oil (FO) is usually a clear brown/yellow liquid obtained through the pressing of the cooked fish and is the richest available source of LC-PUFA (FAO., 2016b).

FO is the main lipid source in aquafeeds but like FM, their high prices result in exploring alternative sources of LC-PUFA and with the expected increase of aquaculture industry (FAO., 2016b) its actual levels of incorporation are considered unsustainable (Turchini et al., 2009). Its incorporation level is also limited by the possible presence of contaminants, for instance, dioxin or dioxin-like polychlorinated biphenyls arise from man-made products (Bell et al., 2005). Undeniably, FO is considered the main source of persistent organic pollutants in aquaculture industry (Hixson, 2014; Turchini et al., 2009). Alternatives may be plant oils like soybean, canola (Glencross et al., 2003), linseed (Bell et al., 2004) and rapeseed oil (Bell et al., 2001; Bell et al., 2003) that have been used to successful partially replace FO, although their fatty acid composition differ significantly due to their low levels of LC-PUFA (Oliva-Teles, 2012). However, the use of FO in specific stages of production, particularly hatchery, brood stock and finishing diets, will be the ideal complement to a diet using plant oils. According to Bell et al. (2004) diets containing 50 or 100% of linseed oil followed by a period of 16-24 weeks on FO finishing diet provide a carcass with an EFA composition highly beneficial. Although without an FO finishing diet, high inclusion levels of vegetal oils reduce nutritional benefits of the fish to human consumption (Bell et al., 2001).

Aquafeeds do not include high dietary carbohydrate levels, mostly in carnivorous species, because fish do not have specific dietary carbohydrate requirements and use

diets with no carbohydrates as efficiently, or even more efficiently depending on the type and the quantity of the carbohydrate source, as those containing carbohydrates (Enes et al., 2009; Oliva-Teles, 2012; Peres and Oliva-Teles, 2002). Although it is of great importance to afford an adequate level of carbohydrates in the diet, due to the low commercial price of these commodities as well as to decrease catabolism of protein for energy purposes and to improve physical properties of pelleted feed (Peres and Oliva-Teles, 2002; Wilson, 1994). Cereal grains have 62-72% starch and are important binders in steam-pelleted and extruded feeds (NRC, 2011). A lot of by-products of grain industry like wheat, oat, corn, rice or rye are available as ingredients for animal feeds (Gatlin et al., 2007; Naylor et al., 2009; Sapkota et al., 2007). Grain legumes such as peas, beans and chickpeas also contain large amounts of starch that is used by fish as an energy source (Booth et al., 2001). Raw starch is considered a poor energy source, however, processing technologies involving heat and pressure increase nutrient availability and consequently may increase its digestibility (Peres and Oliva-Teles, 2002).

Supplementation with nutrients or exogenous enzymes can compensate for anti-nutritional factors and improved the bioavailability of nutrients (Pariza and Cook, 2010). Other diet supplements include functional amino acids, prebiotics, probiotics, mineral and vitamin premixes, feed binders, carotenoid supplements, drugs, antibiotics, preservatives and flavourings (Abowei and Ekubo, 2011) that may be used at low incorporation levels, but will not be further detailed here.

## The importance of new alternatives in aquaculture feeds

The crescent growth of the aquaculture industry and consequent increase demand of ingredients like FM, FO, soybean meal, corn, and wheat meal result in high prices of feeds manufacture. To keep aquaculture sustainable their incorporation in feeds have to be reduced. Also for a more economic sustainable growth, industry should explore feedstuffs that can be cultivated in the near zone, decreasing the dependence of imported ingredients to produce feeds. In our case, soybean production is very limited due to Europe climate and geographical constraints as well as to the restriction of using genetically modified products.

Besides alternative feedstuffs, new techniques of improving its nutrient availability and digestibility of ingredients should be explored. This step is extremely important for

aquaculture industry to maintain its growing process that may allow the use of low cost-low nutritional value ingredients for carnivorous fish species.

## Distillers Dried Grains with Solubles (DDGS)

Distillers 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 DDGS. The yeast from the fermentation process remains in the finished co-product.

In the United States, most of the ethanol produced is made from corn. Each 1.54 Kg of corn fermented in a dry-mill ethanol plant produces about 1.02 L of ethanol, 0.28 Kg of carbon dioxide and 0.82 Kg of DDGS (Lim and Yildirim-Aksoy, 2008).

In fact, global production of ethanol is increasing every year due to the fact that ethanol can be used as vehicle fuel. As ethanol can be blended with gasoline, this represents an attempt to reduce the use of petrol.

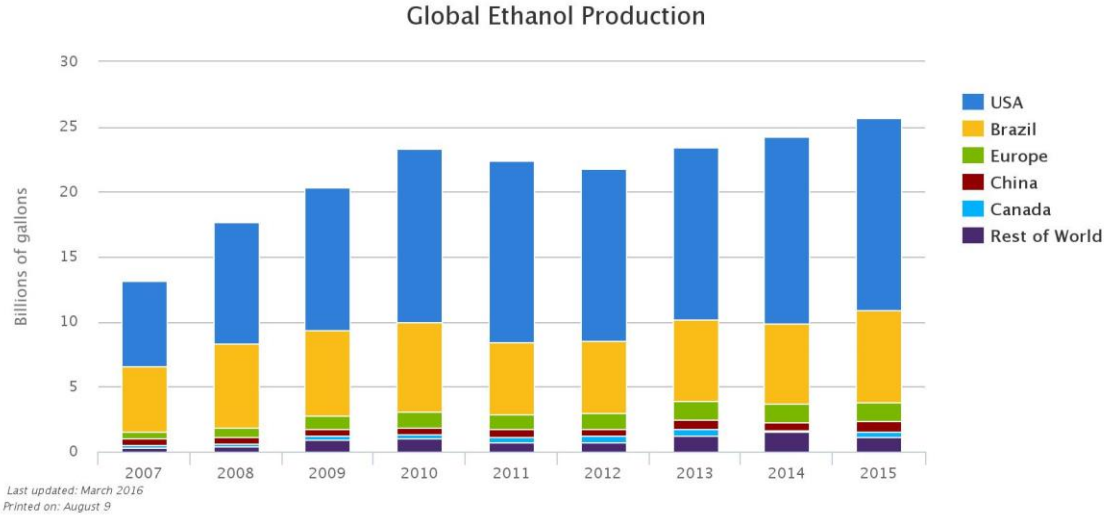



**Figure 6**- Global ethanol production. Adapted from Association. (2016)

Therefore, the availability of by-products like DDGS has increased considerably and its production is expected to keep growing. With this strong growth, DDGS became readily available and competitively priced, relative to other conventional sources (Lim and Yildirim-Aksoy, 2008), that has enhanced its use in animal feeds. Per unit of protein,

DDGS are much less expensive than conventional protein sources (Beckman et al., 2011). Also, the use of by-products like DDGS help mitigate the environmental consequences of expansion by the biofuel industry (Taheripour et al., 2010).

However , their use in fish feeds is still limited due to its nutritional composition and some authors have pointed out that the inclusion of DDGS should only be as a complement to another protein source (Thompson et al., 2008). However, several studies have shown that DDGS is a promising ingredient to be used in fish feeds as for omnivorous species as for carnivorous species.

## Physical characteristics

Corn DDGS is a free-flowing granular product with colour ranging from yellow/tan to dark brown (Lim and Yildirim-Aksoy, 2008). Quite a few factors can influence this colour, including the amount of distiller's solubles together with distiller's grains before drying, the drying temperature and duration (Rosentrater, 2006).

The dark coloured DDGS is associated with a lower nutritional value as it is the result of overheating during the drying process, which lead to binding and/or destruction of nutrients such as lysine (Cromwell et al., 1993; Fastinger et al., 2006).

The main obstacle to the inclusion of DDGS in diets for animals is the wide variation in the nutritional content of DDGS, which may be due to different production techniques or different sources and quality of grain used to produce ethanol (Beckman et al., 2011; Lim and Yildirim-Aksoy, 2008; Świątkiewicz and Koreleski, 2008). A key component to using DDGS as a protein source in aquaculture feeds is information on processing (Kannadhason et al., 2009). Periodically analyses should be done in DDGS sources (Świątkiewicz and Koreleski, 2008) because the market price depends on is the nutritional composition of DDGS (Belyea et al., 2004; Nuez Ortín and Yu, 2009).

## Chemical composition

Corn DDGS contains between 28 and 33% crude protein, although new technologies are being used to remove fibre from DDGS, increasing its protein content to 40% or more (Hardy, 2008; Lim et al., 2011). DDGS does not contain the anti-nutritional factors found in most plant proteins sources and has a reasonable amount of fat (amongst 10 and 13%) (Lim and Yildirim-Aksoy, 2008). DDGS also have very low starch levels because

most starch is converted to ethanol during fermentation making the nutrient content of DDGS about 2 to 3 times more concentrated than corn (Lim and Yildirim-Aksoy, 2008). Corn DDGS have a higher crude fibre content than wheat DDGS and the neutral detergent fibre may represent 29 to 39% of the weight (Lim et al., 2011). Due to fermentation, it is estimated that 3.9% of DDGS biomass is yeast (Ingledew, 1999). Besides contributing to 5.3 % of the protein content of DDGS, yeast as also been reported to modulate the immune response in fish (Lim and Yildirim-Aksoy, 2008; Oliva-Teles and Gonçalves, 2001). The ash content is higher in wheat than in corn DDGS.

Relatively to soybean meal, corn, and wheat DDGS are deficient in several EAA, for example, lysine and methionine. Among the ten EAA, lysine is the most limiting EAA in DDGS from various grain sources, compared to soybean meal and FM (Lim and Yildirim-Aksoy, 2008). The concentration of minerals and vitamins varies among sources and batches of DDGS. Corn DDGS is a rich source of vitamin A, niacin, choline and several minerals, including a high level of highly available phosphorus (Dale and Batal, 2005; Lim et al., 2011).

Mycotoxins are defined as toxic or carcinogenic chemicals that are secondary metabolites of fungi that colonize crops. Several mycotoxins can potentially be found in grains, including aflatoxins, fumonisins, deoxynivalenol, T-2 toxin and zearalenone (Liu, 2011). When grains containing mycotoxins are used for bioethanol production, the mycotoxins are accumulated in DDGS in concentrations that can be 3 to 3.5 times higher than values found in corn (Zhang and Caupert, 2012). Mycotoxin-producing fungi and their mycotoxins are found primarily in the pericarp of the kernel, therefore separation of these components preceding fermentation could be advantageous for increasing the value of coproducts (Liu, 2011). However, according to Zhang et al. (2009) concentrations of mycotoxins could fall well below any harmful concentration when the DDGS are blended with other ingredients to make up the overall animal diet.

## DDGS utilization in aquaculture

Several studies had been made for the inclusion of DDGS in diets for omnivorous species. For channel catfish (*Ictalurus punctatus*) it was observed that DDGS may be used as a substitute of FM and/or soybean meal up to 35% of diet, without lysine supplementation (Webster et al., 1993; Webster et al., 1992; Webster et al., 1991) and with no negative impacts on growth performance. Higher dietary replacement levels may

be reached by using amino acids supplements or combining different protein sources to fulfil the adequate dietary EAA profile (Cheng and Hardy, 2004; Thompson et al., 2008; Webster et al., 1991). Subsequently, for catfish, dietary lysine supplementation allowed an increased incorporation of DDGS in the diet up to 70% (Webster et al., 1991), although some authors defend that the inclusion level with lysine supplementation is about 30-40% for DDGS to be considered a suitable ingredient for catfish diets formulation (Li et al., 2011; Lim et al., 2009; Robinson and Li, 2008). According to Webster et al. (1992) a diet formulated with 0% FM, 35% DDGS and 49% soybean meal lead to similar results to diets with a higher percentage of FM (12%) for channel catfish juveniles.

In hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) diets containing 30% DDGS gave provided good growth when complimented with animal-based protein sources (i.e. FM or meat and bone meal) (Coyle et al., 2004). In Nile tilapia (*Oreochromis niloticus*) DDGS can be incorporated in diets at a level of 40% (Schaeffer et al., 2009), however an inclusion of 20% DDGS provided the highest growth response, more closely related to commercial diets (Lim et al., 2007; Magdy et al., 2015; Schaeffer et al., 2009, 2012). Whereas DDGS appears to be deficient in lysine for Nile Tilapia, a supplementation with this amino acid to a diet containing 40% DDGS lead to weight gain (WG) and protein efficiency ratio (PER) levels comparable to those of a control diet without DDGS (Lim et al., 2007), therefore diets containing 40% DDGS may be used for Nile tilapia when supplemented with lysine. According to Herath et al. (2016), DDGS is one of the best choices among different corn co-products to replace FM in diets for Nile tilapia juveniles.

For rainbow trout (*Oncorhynchus mykiss*), DDGS appears to be a good protein source. It can be used at 15% inclusion level, without AA supplementation, or at 22.5% when supplemented with lysine and methionine (Cheng and Hardy, 2004). Although, the extent of FM protein replacement in diets for rainbow trout seems to be dependent on the ratio of DDGS to corn gluten meal used (Stone et al., 2005). According to Overland et al. (2013), diets including DDGS improved phosphorus digestibility and did not affect the relative weight of the distal intestine, nor intestinal enzyme activity of rainbow trout.

In yellow perch (*Perca flavescens*) diets including 40% DDGS presented good results, without negative impacts on growth, nonetheless, plant proteins should not exceed 50% of incorporation levels in diets for yellow perch (Schaeffer et al., 2011).

In sunshine seabass (*Morone chrysops* x *Morone saxatilis*) diets containing no FM, 29% soybean meal, 29% meat and bone meal and 10% DDGS had similar final height, percentage weight gain, survival, specific growth rate and feed conversion ratio compared to fish fed a diet containing 30% FM (Webster et al., 1999). According to Thompson et al. (2008), the use of DDGS in sunshine base diets appears to be desirable even though it should be complemented with other protein sources.

Recently, the inclusion of DDGS in diets for carnivorous fish has been tested, in particular for European sea bass (*Dicentrarchus labrax*) and meagre (*Argyrosomus regius*). The digestibility of this ingredients, from two different sources, was evaluated and it was concluded that corn DDGS is well digested and seems to have a high potential for inclusion in diets of these two species (Magalhães et al., 2015). Apparent digestibility of protein and lipids ranged 98 to 82% while that of energy (58–68%) was low, reflecting the high indigestible fibre content of DDGS. It is exactly its high fibre contents, along with variation in chemical and physical properties, that may limit the use of DDGS for some fish species (Kannadhason et al., 2009; Liu, 2011).

Published data on AA availability if DDGS for aquatic species is limited. One disadvantage of using DDGS in fish diets is that the most limiting amino acids are lysine and methionine, which are usually lower than in FM (Cheng and Hardy, 2004). Metts et al. (2011) reported amino acid availabilities in DDGS higher than 50% for sunshine bass except for cysteine, histidine, and valine. For European seabass and meagre AA digestibility of two different sources of DDGS was high, ranging from 75% to 92% (Magalhães et al., 2016), however, lysine, glutamic acid, alanine, and proline digestibility were different depending on the DDGS source.

DDGS contains a substantial percentage of yeast cells, which correspond approximately to 4 to 7% of DDGS. Yeast at may be incorporated in the diets as a protein source or an immune stimulant. Studies have shown that the inclusion of yeast as protein sources lead to good results in several species (Gause and Trushenski, 2011; Hoseinifar et al., 2011; Oliva-Teles and Gonçalves, 2001; Øverland et al., 2013; Vidakovic et al., 2015). Besides, yeast has also proven to improve immune response (Dawood and Koshio, 2016). For catfish, a 40% DDGS based diet improved disease resistance to *Edwardsiella ictaluri* and likely it increased haemoglobin, haematocrit, total serum immunoglobulin, and antibody titers (Lim et al. 2009). Similarly, in Nile tilapia, feeding 40% DDGS diets improved resistance to *Streptococcus iniae*, but it did not affect haematological parameters as white blood cell count, red blood cells, haemoglobin and haematocrit or

immune responses, such as serum proteins, lysozyme activity and antibody production against *Streptococcus iniae* (Lim et al. 2007). There is limited knowledge of the biologically active compounds of DDGS, but it is assumed that it derives from yeast present in DDGS. DDGS β-glucans content may be as high as 8% (Shurson, 2012).

It is recognized that diet modification can positively or negatively affect the fish immune status and disease resistance (Lim et al., 2007). Yeast is rich in protein, B-complex vitamins, and β-glucans. These compounds, either present in living forms or in purified forms, appear to stimulate the immune response of fish and may have great potential for disease prevention (Chen and Ainsworth, 1992; Li and Gatlin, 2004). Numerous studies have shown the potential of β-glucans administration on fish growth performance, immune response and potential to increase survival to disease (Oliva-Teles, 2012). However, some contradictory results have been reported for Atlantic salmon (*Salmo salar*) and gilthead sea bream (*Sparus aurata*) that increased the susceptibility to bacterial infections after a prolonged feeding period with high levels of β- glucans (Couso et al., 2003; Robertsen et al., 1990).

According to Rumsey et al. (1992) nucleotides correspond to 12-20% of total N in yeast. Nucleotides administered in the diet are capable of increasing immune responsiveness, disease resistance, and recovery of organs that have undergone a metabolic or inflammatory insult (Li and Gatlin, 2004; Ringø et al., 2012). 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 (Burrells et al., 2001). As a result, the surface of the intestine is increased and the nutrients will be absorbed more efficiently.

DDGS contains up to three times of the amount of yellow pigments lutein and zeaxanthin found in yellow corn. The high level of yellow pigments may limit DDGS use in fish diets because high dietary yellow pigment levels may result in pigment deposition in the flesh, making it less appealing to the general consumer (Li et al., 2010; Li et al., 2011). Ethanol-extracted DDGS has lower pigment levels as it removed most of the fat and yellow pigments in DDGS (Li et al., 2011). For channel catfish, it was observed that dietary incorporation DDGS above 30% may result in a pronounced deposition of yellow pigments may reduce its commercial value (Li et al., 2011). The same authors also observed that fresh pigmentation level was dependent on the origin of DDGS. The use of ethanol extracted DDGS limits the excessive deposition of these pigments and so it can be incorporated at higher levels without causing excessive pigmentations owing to the pigments are successfully removed by ethanol extraction (Li et al., 2011).

As it was aforementioned, new technologies are being used improve the nutritional value of DDGS, as high-protein DDGS. High-protein DDGS is produced through a fractionation technology, increasing the protein level and reducing the lipids and fibre content, making it more adequate to be used as a feed ingredient for carnivorous fish. This technology is expensive and so it is needed to search for a new process to make this ingredient more suitable for the incorporation at high levels in aquafeeds for carnivorous fish. Solid state fermentation (SSF) has been equated.

## Solid State Fermentation

With the increasing of human population the industry has to grow to keep up with the basic demands of people. In addition, some activities as agriculture and agro-industries produce thousands of tons of dry material by-products (as DDGS) per year (Graminha et al., 2008). Some of these residues are already being used directly as feeds or as a vegetable matter to formulate fish diets due to the need to find good quality and reliable alternative resources to FM in diets formulation (Kaur and Saxena, 2004).To increase digestibility of agricultural by-products it is vital to destroy the linkage between cellulose, hemicellulose, and lignin (Murad and Azzaz, 2010).

Solid State Fermentation (SSF) is a process that occurs in the absence or near absence of any free water in the space between particles (Lonsane et al., 1985) and has gained interest in biotechnology industry due to its possible applications for the productions of value added products like enzymes and the enrichment of the nutritive value of these by-products. (Bhargav et al., 2008; Graminha et al., 2008; Singhania et al., 2009). Also, these processes use agro-industrial residues providing an alternate way of recovery and them (Bhargav et al., 2008). Along with this, it reduces environmental impacts that may be caused by the direct elimination of residues (Singhania et al., 2009). The advantages of SSF compared to the most common submerged process are the higher yield and high concentrations of products which facility their purification. In addition, energy and water consumption is low, reducing the generation of wastewaters and it has less risk of bacterial contamination (Ali and Zulkali, 2011). Oppositely there are some disadvantages like problems with scale up production; complications in control and monitoring of parameters like heating, nutrient supply, pH, air flow and humidity (Archana and Satyanarayana, 1997; Falony et al., 2006; Graminha et al., 2008; Lonsane et al., 1985).

The SSF also appears to be a financial attractive alternative to the submerged fermentation (SmF). In SSF is at or near the surface of the solid substrate containing low moisture levels that the microbial growth and the product formation occurs. Water is present in the solid substrate whose capacity for liquid retention varies with the type of material (Graminha et al., 2008). In SSF, the moisture content is a crucial factor that determines the success of the process, it may alter the physical properties of the solid substrate and influence on microbial growth and product biosynthesis (Archana and Satyanarayana, 1997).

Usually, the substrate is a non-soluble solid material that can serve as a solid support and as a source of nutrients and carbon to sustain microbial growth or only as an inert physical support to which nutrients and the carbon source are added (Pandey, 2003).

Whenever solid substrate serve both as a support and a nutrient source, a pre-treatment may be necessary to allow the growth of microorganism and to enhance the bio digestibility of the residues (Salgado et al., 2014a; Taherzadeh and Karimi, 2008). The industrial processes sometimes serve as pre-treatment as milled or thermal treatments. The presence of lignin in lignocelluloses leads to a protecting barrier that prevents plant cell destruction by fungi and bacteria. The cellulose and hemicellulose must be broken down into their resultant sugars so that microorganisms can utilize them (Kumar et al., 2009). Size reduction by grinding, rasping or chopping; physical, chemical or enzymatic hydrolysis; homogenization, cooking or vapour treatments are the most common pre-treatments (Salgado et al., 2014a).

SSF is attractive due to its numerous advantages as a process used for enzyme production. The use of enzymes in animal feeding has been recognized for partial degradation of agro-industrial residues (Graminha et al., 2008; Roopesh et al., 2006).

### Microorganisms

The filamentous fungi are microorganisms better adapted to the SSF due to the hyphal mode of growth and their good tolerance to the low water activity. In addition, they are particularly interesting due to their high production of extracellular enzymes (Díaz et al., 2007; Soccol et al., 1994). In SSF, the fungi grow in conditions very close to their natural habitats, in favour of being able to produce certain enzymes and metabolites which are usually not produced in SmF (Jecu, 2000).

The filamentous fungi can grow in conditions with a high concentration of nutrients making these microorganisms efficient and competitive for bio-conservation of solid substrates microorganisms.

Black aspergilli (*Aspergillus* section *Nigri*) is used in industry due to the production of metabolites such as hydrolytic enzymes (Bhargav et al., 2008). These microorganisms are one of the most problematic groups concerning classification and identification and several taxonomic schemes have been proposed. New molecular approaches have revealed that there is a high biodiversity but species are difficult to recognize based exclusively on their phenotypic characters (Samson et al., 2007).

Among them, *A. niger* is one of the most studied species and is known as one of the best to produce extracellular enzymes (Falony et al., 2006; Hosseinpour et al., 2012; Pothiraj et al., 2006). In previously work, Leite et al. (2016) evaluated the ability to produce cellulose and xylanases of several fungi in agar plates, and they observed that *A. uvarum*, *A. ibericus* and some strain of *A. niger* are potential producers. *A. uvarum* is a fungus isolated from grape berries in the Mediterranean area (Perrone et al., 2008) and considered suitable for lipase production (Salgado et al., 2014a). *A. ibericus* is a species isolated from grape berries in the Iberian Peninsula that do not produce any relevant mycotoxins (Serra et al., 2006), therefore is safe for biotechnological applications to produce many metabolites with commercial value, like lipases (Oliveira et al., 2016; Salgado et al., 2014a).

## Enzymes produced by SSF

The production of enzymes is one of the most important applications of SSF and the agroindustrial substrates are considered good substrates for the enzymes production due its low cost. These residues have bring in good results in the production of enzymes as well as cellulases (Pothiraj et al., 2006), xylanases (Haltrich et al., 1996), amylases (Francis et al., 2003; Ramachandran et al., 2004), proteases (Bhargav et al., 2008), lipases (Oliveira et al., 2016; Salgado et al., 2014a), among others.

Cellulase is of a great significance among the industrially important hydrolytic enzymes. Cellulase are enzymatic complexes which act in concert to hydrolyse fibre of plant cell wall to glucose, cellobiose or cello-oligosaccharides. The microbial conversion of cellulosic/lignocellulosic biomass into useful products is a complex process (Murad and Azzaz, 2010). Fungal cellulase is produced only in the presence of cellulose (Suto and

Tomita, 2001). Cellulose biodegradation by cellulases is very important to improve feed utilization and animal performance by enhancing fibre degradation (Murad and Azzaz, 2010).

Xylanases are enzymes that can hydrolyze the bonds in xylan chain from lignocellulosic materials such as corn, hull, sugarcane and fruit and oil processing residues (Beg et al., 2001). Filamentous fungi are normally chosen as xylanases producers due to the fact that they excrete xylan-degrading enzymes into the medium, thus eliminating the need for cell disruption (Haltrich et al., 1996).

## The use of SSF products in aquaculture feeds

The process of SSF has been reported to upgrade the nutritional quality of agro-industrial products that can be used in animal feed industry (Chiang et al., 2010; Graminha et al., 2008), however in aquafeeds industry are few studies about the impact of including SSF products.

However, the use of enzymes resulting from SSF as feed exoenzyme additives has been studied with promising results. Recently, in Nile tilapia, the inclusion of 150 ppm of enzymes from SSF in pellet diets improved fish performance due to a higher bioavailability of nutrients (Moura et al.). Similarly, results were obtained for rainbow trout and carp (Anwar, 2013; Bowyer et al., 2015).

Besides the use of the exoenzymes obtained through SSF, some authors have studied the impact of SSF in the nutritional profile of some important aquafeeds ingredients like soybean meal and wheat bran. It was observed that SSF converted complex carbohydrates to simpler molecules and supplement the feedstuffs with microbial protein (Imelda et al., 2008). Similarly, it was demonstrated that SSF may enhance the nutritional value of vegetable waste (dried vegetable waste powder and oil cake mixture (soybean flour, wheat flour, groundnut oil cake and sesame oil cake) decreasing the lipid and fibre content and increasing the protein content (Rajesh and Raj, 2010).

In *Labeo rohita* fingerlings diet, a de-oiled groundnut oil cake was bio- processed through SSF reducing crude fibre content and antinutritional factors, whereas enhancing crude protein, lipid, ash, total free amino acids and fatty acids and in general. Comparatively to the unfermented feedstuffs, this SSF product produced significantly better performance in terms of growth, feed utilization, nutrient digestibility, carcass composition and digestive enzyme (Ghosh and Mandal, 2015).

Hassaan et al. (2015) tested the inclusion of SSF soybean meal in diets for Nile tilapia and showed that SSF could improve the nutritive values of soybean meal and that up to 37.4% of FM could be replaced by the SSF soybean meal without any adverse effect on growth performance, nutrient digestibility and physiological condition.

Even though not well explored, the SSF process of low economic value ingredients may open the possibility of using new ingredients in aquafeeds.

## Evaluation of Digestibility

The bioavailability of nutrients and energy in feeds for fish may be defined in terms of digestibility. The determination of digestibility is one of the first steps to test the potential of a new ingredient for inclusion in aquafeeds. The apparent digestibility coefficients (ADC) of ingredients are required for accurate diet formulation, on a digestible basis rather than on a gross basis, therefore contributing to decreasing aquafeeds cost and for the reduction of feed wastes.

Digestibility specifies the portion of nutrients or energy in the ingested diet that is not excreted in faeces. Apparent digestibility does not take into account nutrient losses of endogenous origin which are part of faeces. ‘’True’’ digestibility excludes the endogenous losses from the faeces. The apparent digestibility has a more practical importance than true digestibility because the endogenous losses are a minor portion of faeces if the animal is not fed (Lovell, 1998). Apparent digestibility generally provides a good indication of the availability of energy and nutrients (Bureau et al., 1999).

Digestibility in fish can be quantified using several direct or indirect methods. The direct method involves the complete account of feed ingested and faeces excreted. The indirect method eliminates the need to quantitatively collect all the faeces produced by the fish but requires representative sample of both the feed and the faeces (Vandenberg and De La Noüe, 2001). The ratio of nutrient and an indigestible marker in the feeds and faeces is used to determine ADC. This digestion marker to be effective must be inert, not be absorbed or interact with the fish digestive system or other components of the feed, ease of analysis, and not alter the passage of nutrients through the gastrointestinal tract or the metabolism of fish (Alan Ward et al., 2005; Atkinson et al., 1984; Austreng et al., 2000; Fernandez et al., 1999). With the absorption of dietary ingredients in the intestine, the ratio of nutrient to the indicator is lower in faeces than in feed. Some internal indicators used are ash, crude fibre or plant chromagens, while external indicators are additives

such as chromium oxide, yttrium oxide or cholestane (Carter et al., 2003; Vandenberg and De La Noüe, 2001). The chromic oxide and yttrium oxide are the most widely used digestibility external marker for aquatic animals, while cholestane has been used specifically for the evaluation of fatty acid digestibility (Martins et al., 2009).

In fish, digestibility measurements are difficult because collecting the faecal material is a hard process. An incorrect collection of faeces significantly influence the ADC for nutrients in feed ingredients, as nutrients and organic matter can leach from the faeces into the water before collection (Amirkolaie et al., 2005). The methods for faecal collection must guarantee that the results are precise, repeatable and harmless to the fish (Allan et al., 1999; Austreng, 1978; Cho, 1992). Methods for collection faeces include dissection or anal suction technique (Windell et al., 1978), manual stripping (Glencross et al., 2005), settling columns (Cho et al., 1982) and by automatic faeces collector (faecal matter is sieved continuously by a net present at the water outlet) (Choubert Jr et al., 1979).

Direct faeces collection from the intestine displays some disadvantages that affect the reliability of this method and in general, it leads to underestimation of digestibility. Besides the need to sacrifice animals to dissect the intestine and for intestinal content removal, another, there is an inherent risk of sampling the chime before being completely digested and of contamination of faeces with endogenous material, as body fluids (like blood or sperm) and intestinal epithelium, that would have been reabsorbed by the fish before natural defecation (Amirkolaie et al., 2005).

Contrarily, the collection of faeces from the water column or following settlement may potentially overestimate the ADC values. This is due to disintegration or separation of faeces, or leaching of nutrients and/or marker from the faecal matter (Cho and Kaushik, 1990). However, due simplify repeated measures and lower fish handling stress, these methods are far and wide accepted.

Smith (1971) developed a metabolic chamber to collect faecal matter voided naturally into the water. However, in this metabolic chamber, the fish is restrained and need to be force-fed, consequently, they often regurgitate, and are highly stressed, resulting in questionable digestibility values (Cho et al., 1982).

To prevent these problems specific devices to collect faecal material were created by Ogino et al. (1973) and Choubert Jr et al. (1979). Ogino et al. (1973) device collects faeces by passing the effluent water from the tanks trough a filtration column (TUF

column). Later, Cho and Slinger (1979) developed a settling column to separate the faeces from the effluent water (Guelph system) and Choubert Jr et al. (1979) developed a mechanically rotating screen to filter out the faecal matter (Choubert, St. Pée system). The adequacy of one method will depend on the fish species, mainly if it is a seawater of freshwater species, figs size, and the consistency of the faecal material (Peres et al., 2013).

A major problem to estimating the digestibility of ingredients in fish is their refusal to ingest test ingredients separately or, in some cases, the reduction of voluntary feed intake and nutrient utilization due to nutrient unbalances, amino acids deficiencies, the presence of anti-nutritional factors and reduced palatability of feed (Booth et al., 2001).

To avoid these problems, the most recommend approach is the substitution method, which consists including in the test diet 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, the digestion of feed into subunits appropriate for absorption in the digestive tract of fish depends largely on the available enzymes, allowing protein, carbohydrate, and fat degradation into minor and simple molecules. These molecules can then be absorbed and used for maintenance, growth, and reproduction (Blier et al., 1997; Furne et al., 2005).

There are more than a few factors that affect the activity of digestive enzymes. These include diet composition (Chatzifotis et al., 2008; Debnath et al., 2007; Péres et al., 1998; Santigosa et al., 2008; Simon, 2009), age/size (Kuz'mina, 1996; Péres et al., 1998) and environmental factors (Hofer, 1979; Kuz'mina et al., 1996).

The anatomy of the digestive tract and its digestive enzymes profile are closely related, i.e. the gastrointestinal tract of herbivorous species is longer than that of the carnivorous species (De Almeida et al., 2006).Therefore, digestive enzyme pattern can reflect the feeding habit of fish and his digestive capacity (Smith, 1980). Thus, quantifying the activity of digestive enzymes is an advantageous way to offer information on the nutritional value of diets, help resolve nutritional problems (such as the matching of an artificial diet to the nutritive capabilities of the fish) and anticipate possible interactions

between anti-nutritional factors and digestive enzymes (Corrêa et al., 2007; Furne et al., 2005; Refstie et al., 2006).

Besides the digestive enzymes produced by the animal, some enzymes may be supplied by the diet or produced by intestinal bacteria, and these exogenous digestive enzymes may have an important and active role in digestive processes (Caruso et al., 2009).

### Amylase

All fish species seem to own the enzymatic device necessary to hydrolyze and absorb simple and complex carbohydrates. Carbohydrates have a main role in metabolism, offering energy when oxidised. Digestion and absorption follow the same mechanism in herbivores, omnivores, and carnivore 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 maltose, maltotriose, a combination of branched (1:6) oligosaccharides and some glucose (Papoutsoglou and Lyndon, 2003).

In mammals, amylase is produced by the salivary and pancreatic cells, while in fish it is produced in exocrine pancreas (De Silva and Anderson, 1994; Klahan et al., 2009). Amylase is released into the pyloric caeca, and so the so the amylase detected in the medium and distal intestine has a pancreatic origin while the one detected in the posterior intestine is possibly the result of the drag of the secreted mucus (Bakke et al., 2010; Pérez-Jiménez et al., 2009).

Amylase activity largely depends on the feeding habits of the fish species, but besides that little is known about amylases and even less about its regulation. Herbivorous species appear to digest starch components of vegetable feedstuffs more efficiently followed by omnivorous and then carnivorous has the less efficient (Al-Tameemi et al., 2010; De Almeida et al., 2006; Horn et al., 2006). In herbivorous (*Spanisoma cretense,Barbus sharpeyi, Boops boops*) (Al-Tameemi et al., 2010; Fernandez et al., 2001; Papoutsoglou and Lyndon, 2003) and omnivorous species (*Cyprinus carpio, Carassius auratus, Tinca tinca, Pagellus erythrinus, Oreochromis niloticus*) (Al-Tameemi et al., 2010; Fernandez et al., 2001; Hidalgo et al., 1999; Klahan et al., 2009), α-amylase activity is higher than in carnivores species (*Oncorhyncus mykiss, Sparus aurata, Anguilla anguilla, Uranoscopus saber, Dentex dentex, Aspius vorax, Polyodon spathula*) (Al-Tameemi et al., 2010; Fernandez et al., 2001; Hidalgo et al., 1999; Ji et al., 2012; Papoutsoglou and Lyndon, 2003; Pérez-Jiménez et al., 2009).

Amylase activity is up-regulated by dietary carbohydrate level, as it was observed for common dentex (*Dentex dentex*) in the pyloric caeca and posterior intestine (Pérez-Jiménez et al.). Also for other carnivorous species like rainbow trout and European sea bass, amylase activity was straight linked with the dietary carbohydrates level and feeding amount (Caruso et al., 2009; Corrêa et al., 2007). However, for salmonids, other authors did not observe this type of regulation of amylase activity (Bakke et al., 2010).

The characteristics of amylase differ among species in relation to ideal pH for maximum activity and temperature stability. The ideal pH for maximum activity of intestinal amylase has been shown to vary between 6.0 and 7.5 for different species. Also, the temperature for maximum amylase activity is amongst 25 and 35 ºC (Jun-sheng et al., 2006).

According to *in vitro* experiments performed by (Papoutsoglou and Lyndon) higher levels of carbohydrates and lower temperatures decrease amylase activity. Although some species like gilthead sea bream appears to have the ability to regulate the amylolytic activity to compensate variations in temperatures (Couto et al., 2012).

Fish amylases also show differences in the dependence on chloride ions and ion concentrations when working with marine species, in which food digestion must proceed under high chloride concentrations (Munilla-Morán and Saborido-Rey, 1996b).

### Lipase

The wide-ranging digestive process of lipids involves their extracellular hydrolysis in the stomach and intestinal lumen, and pyloric caeca by a variety of lipases and colipases (Halver and Hardy, 2002).

Lipase (E.C.3.1.1.3) catalyses the breakdown of triacylglycerol, first to diacylglycerol and then to monoacylglycerol (Savona et al., 2011). Lipase activity has been found in extracts of the pancreas, pyloric caeca, and upper intestine and decreases at very low levels in the stomach and in the lower intestine; the principal site for lipase activity is the mucosal layer (German, 2009; German and Bittong, 2009; Klahan et al., 2009). However, lipase activity may increase distally in the intestine of some fish species perhaps as a lipid-scavenging mechanism (German and Bittong, 2009). All fat digestive enzymes are known to act in alkaline media (7.0 – 9.0) and lipase activity is in agreement with these values (Klahan et al., 2009). It is unlikely that lipolytic activity found in the stomach to be of pancreatic origin, proposing that this organ is also a source of lipases, not forgetting the possibility of lipolytic activity from bacteria present in the fish digestive tract (Caruso

et al., 2009). Indeed, research indicates that for some fish species the wall of the digestive tract from the foregut to the distal intestine may also be a source of lipases, as highest lipase activity was observed in both proximal and distal intestine, depending on the fish species (Tocher, 2003; Castro et al., 2013; Magalhães et al., 2015; 2016). Experiments realized in *Diplodus puntazzo* by Tramati et al. (2005) showed lipase activity in all regions of the intestine, indicating a uniform distribution in the entire intestinal system. Lipase may play a relatively minor role in lipid digestion in some fish species and according to its life stage (Savona et al., 2011).

Usually, the occurrence of lipase is higher in the digestive tract of carnivorous fish than in omnivorous or herbivorous species, because of their fat-rich diet (Chakrabarti et al., 1995). Recent studies argue that the type of diet could influence the production of lipases in adult fish (Ji et al., 2012).

The largest class of lipids present in fish diets is triglycerides class and the lipase activity is directly related to the levels of triglycerides present in diet (Cahu et al., 2003; Savona et al., 2011), however, fish larvae are way better prepared to digest phospholipids rather than triglycerides (Infante and Cahu, 2007).

### Proteases

Protease is a generic name given to enzymes responsible for hydrolyzing the peptide bond in proteins, they are involved in several physiological functions like food digestion or even response to infections and pathologies (Garcia-Carrenno and Hernández-Cortés, 2000). Digestive proteases are polyfunctional enzymes that catalyse the hydrolytic degradation of proteins (Garcia-Carrenno and Hernández-Cortés, 2000). Proteases can be classified as exoproteases or endoproteases. Exoproteases split terminal amino acids from one end of the chain while endoproteases act on peptide bonds in the interior of peptide chain (Reed, 2012). According to the EC system for the enzymatic nomenclature, all proteases belong to the subclass 3.4, which is divided into 3.4.11-19 for exoproteases and 3.4.21-24 for endoproteases (McDonald, 1985). High catalytic efficiency at low reaction temperatures, lower thermostability, and cold stability are some of the specific characteristics which make marine proteases different from terrestrial (Simpson, 2000). These characteristics have emerged by the adaptation of marine animals to special environment conditions, mostly to cold temperatures (Blanco et al., 2007).

Proteases such as pepsin, gastricsin, trypsin, chymotrypsin, collagenase, elastase, carboxypeptidase and carboxylesterase have been found in fish digestive organs (Simpson, 2000). In the fish digestive system, there are two major groups of proteases, pepsin, and trypsin. Pepsin has been reported as the major acidic protease in the fish stomach and is the first proteolytic enzyme to break large peptide chains (Sabapathy and Teo, 1993; Tengjaroenkul et al., 2000). Trypsin and chymotrypsin are the major alkaline proteases in the intestine and their presence enables the products of pepsin digestion to be hydrolysed (Caruso et al., 2009; Natalia et al., 2004; Sabapathy and Teo, 1993).

Pancreatic enzymes as amylase and lipase are released in the active forms, but alkaline proteases are secreted as inactive pro-enzymes (mainly as trypsinogen and chymotrypsinogen), that are activated in the intestinal lumen. Trypsinogen is activated by enterokinase to form the active trypsin, which in turn activates other digestive enzymes like chymotrypsin (Bureau et al., 2002; Fänge and Grove, 1979). These enzymes become active in the pyloric caeca and proximal intestine (Santigosa et al., 2008).

Trypsin specifically hydrolyses the carboxyl end of lysine and arginine peptide bond (Chong et al., 2002). According to Eshel et al. (1993) in carnivorous fish, this enzyme contributes to 40-50% of the overall protein digestion activity. The optimal pH range for this enzyme is between 7.0 – 10.0 according to the species (Martinez and Serra, 1989). At low pH values, trypsin suffers irreversible denaturation, loss of stability and consequently cannot bind correctly to the substrate (Klomklao et al., 2006).

Chymotrypsin has far wider specificity than trypsin. This digestive enzyme hydrolyses peptide bonds close to hydrophobic amino acids such tyrosine, phenylalanine and tryptophan (Chong et al., 2002). The optimum pH values for chymotrypsin activity is slightly lower than trypsin and varies between 7.0 – 8.0 (Hidalgo et al., 1999; Martinez and Serra, 1989).

Both types of proteases are believed to play a collaborative role in protein digestion at the intestinal tract (Chong et al., 2002). Partial hydrolysis of protein by pepsin is subsequently completed by the combined action of trypsin and chymotrypsin when food reaches the intestine, it is also notorious that the secretion of trypsin and chymotrypsin take place in response to food digestion (Savona et al., 2011). In carnivorous species, trypsin activities were found normally higher while in omnivorous and herbivorous species chymotrypsin were higher (Jónás et al., 1983).

The proteases profile varies among species and organs. Several studies show that protein digestion occurs both in the acid pH region in the stomach and in the alkaline pH region in the intestine, both reveals high protease activity (Alarcón et al., 1998; Chong et al., 2002; Xiong et al., 2011). Proteolytic activities at low pH have been described in species with a clear stomach region and high pepsin secretion (Chong et al., 2002). Pepsin activity from several important species like Atlantic salmon (Torrissen, 1984), rainbow trout (Twining et al., 1983) and European sea bass (Eshel et al., 1993) have been documented.

Pyloric caeca may be related to the need to retain feed for the neutralization of acid secretion, however, the pyloric caeca are associated with the storage of alkaline proteases until the acid bolus is neutralized by other pancreatic secretions (Munilla-Morán and Saborido-Rey, 1996a). Indeed, high proteases activities at alkaline pH (8- 10) have also been reported in species like rainbow trout (Kristjánsson and Nielsen, 1992) and sea bass (Eshel et al., 1993).

During early ontogeny, the protease activity has been reported to show an exponential trend with the increasing age of the fish, the ratio of membrane and cavity digestion in fact changes in fishes with oldness (Chakrabarti et al., 2006). Also, the level of proteolytic activity seems to be related with the fish species growth rate (Hidalgo et al., 1999). Typically, carnivorous species have a short intestine, with higher protease activity than herbivorous (Garcia-Carrenno and Hernández-Cortés, 2000; Lazzari et al., 2010). However, high proteolytic activity is also reported in omnivorous fish, since vegetable proteins are hard to ingest and, even though omnivores need less protein than carnivores, protein has to be well used (Hidalgo et al., 1999). According to Santigosa et al. (2008) fish showed a compensatory mechanism for the decreased digestive activity when FM is replaced by a plant protein source.

## Aims

The objective of this study is to evaluate the potential of solid state fermentation in the nutritive value of DDGS. This will allow us to better understand the mechanisms by which SSF can improve the digestibility of DDGS. This work aims at the advancement of SSF application in fish nutrition contributing to produce sustainable and low compound feed for fish.

# Material and Methods:

## Task I. Optimization of Solid Sate Fermentation process of DDGS

The optimization of the SSF process of DDGS was done in Centre of Biological Engineering at the University of Minho.

### Raw material

The Distiller Dried Grains with Solubles (DDGS) was provided in the form of pellet by Aquasoja and preserved at room temperature. To facilitate further chemical analysis and fermentation residue pellets were milled.

### Microorganisms

Black *Aspergillus* species obtained from Micoteca of University of Minho, Braga, Portugal (MUM) culture collection were used (*Aspergillus niger*; code: 01.183, *Aspergillus ibericus*, code: 01.294 and *Aspergillus uvarum*, code: 01.128). They were revived on malt extract agar (MEA) plates (2% malt extract, 2% glucose, 0.1% peptone and 2% agar) from preserved glycerol stocks stored at -80º C. Then subcultured on MEA slants and incubated at 25º C for 7 days to obtain inocula for SSF.

### Solid State Fermentation

The fermentations were carried out in cotton-plugged Erlenmeyer flask of 500 mL with 10g of the dry solid substrate, in our case DDGS. The moisture level was adjusted to 75%. After that, Erlenmeyer with the solid substrate was sterilized at 121º C for 15 minutes. Separate fermentations were performed using *A. niger*, *A. ibericus,* and *A. uvarum*. For the inoculation, spores of the fungus were grown in malt extract agar medium slant tubes and were suspended in a sterilized solution composed by 0.1 % of peptone and 0.01 % of Tween-80. The inoculum spore concentration was adjusted to $10^6$ spores/mL using a Neubauer counting chamber. Each flask was inoculated with 2 mL of the spore suspension and incubated at 30 °C for 6 days. At the end of each fermentation, the fermented solid from each Erlenmeyer flask was placed to dry in an

oven at 60º C, in a petri dish, for further analysis and use. Fermented solid was also use for the extraction of enzymes that was performed with a solution composed of 1 % NaCl and 0.5 % Triton X-100 at 4 °C in a solid/liquid ratio of 1:5, and 1 h of stirring. Following, extracts were filtered through a fine mesh net and centrifuged (10000 rpm, 10 minutes). The solid residue that stayed in the net and the precipitate were placed to dry in the oven at 60º C for further analysis. The supernatants were preserved at -20º C until analysis.

## Scale-up of Solid State Fermentation

Solid state fermentation performed with *A. ibericus* was proven to result in the higher total protein content and enzymes activity of the solid state fermented DDGS (SSF-DDGS) than the other fungi, being chosen to ferment the DDGS that was used as an ingredient in fish diets. To increase the quantity of produced fermented solid, the fermentations were carried out in trays, using 500 g of dry substrate (DDGS), with a moisture level of 75% and previously sterilized at 121 ºC for 15 minutes, as aforementioned. Each tray was inoculated with 50 mL of the spore suspension, covered with a perforated adherent film to keep the moisture level, and incubated at 30 º C for 7 days. At the end of each fermentation, a small part of solid was preserved at 4 ºC until analysis and the remaining fermented product was placed to dry in the oven at 40 ºC until constant weight and then stored to hereafter produce the experimental diets.

# Task II. Apparent digestibility of non-fermented and fermented DDGS for European seabass

## Experimental Diets

The proximate composition of ingredients used in this trial is present in Table 1.

**Table 1**- Composition (% dry weight) of fish meal, DDGS, and SSF-DDGS

| | Ingredients | | |
|---|---|---|---|
| | **Fish Meal** | **DDGS** | **SSF-DDGS** |
| Dry matter | 85.8 | 85.3 | 95.5 |
| Ash | 10.6 | 3.8 | 5.1 |
| Crude Protein | 77.5 | 26.2 | 30.1 |
| Crude Lipids | 7.6 | 3.4 | 4.3 |
| Cellulose | — | 40.5 | 28.3 |
| Hemicellulose | — | 22.9 | 29.2 |
| Lignin | — | 17.6 | 20 |
| Starch | — | 0.5 | 0.5 |
| Phosphorus | 2.4 | 0.6 | 0.6 |
| Gross energy (kJ $g^{-1}$) | 18.2 | 19.8 | 20.1 |
| Enzyme activity (U/g) | | | |
| Cellulase | — | — | 43.4 |
| Xylanase | — | — | 68.0 |

A reference diet was formulated containing 47% protein, 15% lipids, and 1% chromium oxide, was included as an inert digestibility marker. In the reference diet, fish meal and fish oil were used as protein and lipid sources. Two experimental diets were then formulated containing a mixture of 70% of the reference diet and 30% of the test ingredient, i.e., DDGS before (DDGS) and after solid state fermentation (SSF-DDGS). 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) trough 3.5 mm die. Pellets were sleeved and stored after dried in an oven for 48h

at 35 ºC. Composition and proximate analysis of the experimental diets are presented in Table 2.

**Table 2**- Diet formulation and proximate composition of the experimental diets used in the digestibility trial.

| | Diets | | |
|---|---|---|---|
| | **Reference** | **DDGS** | **SSF-DDGS** |
| **Ingredients (% dry weight)** | | | |
| Fish meal | 63.2 | 44.2 | 44.2 |
| DDGS | — | 30 | — |
| SSF-DDGS | — | — | 30 |
| Pre-gelatinized corn starch[1] | 22.1 | 15.4 | 15.4 |
| Fish oil | 10.2 | 7.2 | 7.2 |
| Vitamins premix[2] | 1 | 0.7 | 0.7 |
| Choline chloride (50%) | 0.5 | 0.4 | 0.4 |
| Mineral premix[3] | 1 | 0.7 | 0.1 |
| Chromium oxide | 1 | 0.7 | 0.7 |
| Binder[4] | 1 | 0.7 | 0.7 |
| **Proximate analysis (% dry weight)** | | | |
| Dry matter (%) | 94.3 | 93.3 | 93.5 |
| Crude protein | 47.9 | 40.6 | 42.6 |
| Crude lipids | 14.6 | 12.6 | 12 |
| Ash | 14.6 | 13.3 | 14.2 |
| Phosphorus | 0.8 | 0.7 | 0.7 |
| Cellulose | — | 12.2 | 8.5 |
| Starch | 20.4 | 19.2 | 19.9 |
| Gross energy (kJ $g^{-1}$) | 20.2 | 20.6 | 20.8 |
| Chromic oxide | 0.7 | 0.5 | 0.5 |

[1] Cerestar, France.
[2] 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; mascorbyl monophosphate, 50; inositol, 400.
[3] Minerals (mg $kg^{-1}$ diet): cobalt sulfate, 1.91; copper sulfate, 19.6; iron sulfate, 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).
[4] Binder (Aquacube. Agil, England).

## Animals, experimental conditions, and sampling

A digestibility trial was performed with European sea bass (*Dicentrarchus labrax*) at the Marine Zoology Station, University of Porto.

The experimental system was designed according to Cho et al (1982) and consisted of a thermo-regulated recirculation water system, equipped with twelve 60 L fiberglass tanks. A continuously water-flow was established, at a rate of about 4.5 L/min. During the trial, water temperature averaged 22±1 ºC, salinity averaged 34±1‰; dissolved oxygen was kept above 90% of saturation, nitrogenous compounds were kept below 0.02 mg $l^{-1}$, and photoperiod was controlled to 12 h light and 12 h dark. Temperature, dissolved oxygen, salinity and nitrogenous compounds were monitored during the entire trial.

Nine homogenous groups of five European sea bass were established, with an average weight of 171 g. Diets were randomly assigned to triplicate tanks and fish were fed by hand to apparent satiation, twice a day (8:30 a.m. and 15:30 p.m.), seven days a week. The first 7 days of the experimental period were used for fish adaption to the diets and then faeces were collected once a day for 50 days. Before the morning meal, faeces accumulated in each settling column were collected, centrifuged (3000 g), pooled for each tank and stored at -20 ºC until analysis. Thirty minutes after, tanks, water pipes, and settling columns were thoroughly cleaned to remove excess feed and faeces.

At the end of the trial, and to ensure a full intestine at the sampling time, fish were fed in a continuous manner during the sampling collection day. Then three fish per tank were randomly sampled, euthanized with a sharp blow to the head and immediately eviscerated. Digestive tract was excised, adherent adipose and connective tissue were carefully removed. The intestine, directly after the stomach with the pyloric caeca, was divided into two portions: anterior and distal and stored at -80 ºC until measurement of enzyme activity.

Apparent digestibility coefficients (ADCs) of protein, amino acids, lipid, dry matter, and energy of the diets were determined by the following formula:

$$ADC_{diet} = \left[1 - \left(\frac{\text{dietary } Cr_2O_3 \text{ level} \times \text{feces nutrient or energy level}}{(\text{feces } Cr_2O_3 \text{ level} \times \text{dietary nutrient or energy level})}\right)\right] \times 100$$

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

$$\mathrm{ADC}_{\text{test ingredient}} = \mathrm{ADC}_{\text{test diet}} + \left[(\mathrm{ADC}_{\text{test diet}} - \mathrm{ADC}_{\text{ref.diet}}) \times \left(0.7 \times \mathrm{D}_{\text{ref.}} / 0.3 \times \mathrm{D}_{\text{ingr.}}\right)\right]$$

Where $D_{ref.}$ is the % nutrient (or kJ $g^{-1}$) of reference diet (dry matter basis) and $D_{ingr}$ is the % nutrient (or kJ $g^{-1}$) of test ingredient (dry matter basis).

## Task III. Growth trial

### Experimental Diets

Five diets were formulated to be isoproteic (45% crude protein) and isolipidic (18% crude fat). A control diet was formulated to include fish meal and a blend of plant feedstuffs as protein sources and fish oil as the main lipid source. Four other diets were formulated similar to the control diet but replacing the content of soybean and wheat meal by 10 or 20 % of DDGS and SSF-DDGS. 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) trough 3 mm die. Pellets were sleeved and stored after dried in an oven for 48h at 35 $^{o}$C. The proximate composition of ingredients tested in this trial is present above in table 3.

**Table 3** -Diet formulation and proximate composition (% dry weight) of the experimental diets used in the growth trial.

| | Diets | | | | |
|---|---|---|---|---|---|
| | **Control** | **10DDGS** | **10SSFDDGS** | **20DDGS** | **20SSFDDGS** |
| **Ingredients (% dry weight)** | | | | | |
| DDGS[6] | — | 10 | — | 20 | — |
| SSF-DDGS[7] | — | — | 10 | — | 20 |
| Fish meal[1] | 20 | 20 | 20 | 20 | 20 |
| Corn gluten[2] | 17.5 | 17.5 | 17.5 | 17.5 | 17.5 |
| Wheat gluten[3] | 5 | 5 | 5 | 5 | 5 |
| Soybean meal[4] | 20.1 | 16.3 | 15.3 | 12.6 | 10.5 |
| Wheat meal[5] | 15.3 | 10.1 | 11.1 | 4.8 | 7.0 |
| Fish oil | 14.6 | 14.0 | 14.0 | 13.4 | 13.4 |
| Vitamin premix[8] | 1 | 1 | 1 | 1 | 1 |
| Choline chloride (50%) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Mineral premix[9] | 1 | 1 | 1 | 1 | 1 |
| Binder[10] | 1 | 1 | 1 | 1 | 1 |
| Agar | 1 | 1 | 1 | 1 | 1 |
| Taurine | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| $NaH_2PO_4$ | 2.5 | 2.1 | 2.1 | 1.7 | 1.7 |
| **Proximate composition (% dry weight)** | | | | | |
| Dry matter (%) | 92.8 | 94.3 | 94.8 | 90 | 89 |
| Crude protein | 36.9 | 41.4 | 37 | 41.5 | 42.9 |
| Crude lipids | 15.6 | 15.6 | 15.4 | 15.2 | 16 |
| Ash | 7.2 | 5.7 | 5.2 | 10 | 11 |

[1]Fish meal (CP: 77.5%; CL: 9,5%); Pesquera Diamante, Peru,
[2]Corn gluten (CP: 72.1%; CL:2.1%); Sorgal, S.A. Ovar, Portugal;
[3]Wheat gluten (CP: 84.4%; CL: 1.8%), Sorgal, S.A. Ovar, Portugal;
[4]Non GOM Soybean meal (CP: 51.1; CL:2.6%); Sorgal, S.A. Ovar, Portugal;
[5]Wheat meal (CP: 13.4%; CL: 3.2%); Sorgal, S.A. Ovar, Portugal;
[6]Dried distillers grains with solubles[6] (CP: 22.6%; CL: 3.4%); Sorgal, S.A. Ovar, Portugal
[7]Solid state fermented dried distillers grains with solubles[7] (CP: 30.1%; CL: 4.3%); Sorgal, S.A. Ovar, Portugal
[8] 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; mascorbyl monophosphate, 50; inositol, 400.
[9] Minerals (mg $kg^{-1}$ diet): cobalt sulfate, 1.91; copper sulfate, 19.6; iron sulfate, 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).
[10] Binder (Aquacube. Agil, England).

### Animals, experimental conditions, and sampling

A growth trial was performed with European sea bass (*Dicentrarchus labrax*) at the Marine Zoology Station, University of Porto.

The trial was performed in a thermo-regulated recirculation water system equipped with 15 fiberglass tanks of 60L water capacity and supplied with a continuous flow of filtered seawater. During the trial, water temperature was maintained constant at 22±1 ºC, dissolved oxygen was kept above 90% of saturation, salinity averaged 34±1‰; nitrogenous compounds were kept below 0.02 mg $l^{-1}$, and photoperiod was controlled to 12 h light and 12 h dark. Temperature, dissolved oxygen, salinity and nitrogenous compounds were monitored during the entire trial,

Two hundred and fifty fish were acclimatized to the experimental facilities and water temperature for two weeks before the beginning of the feeding trial. During this period fish were fed a commercial diet once a day, 5 times per week. Thereafter, 15 homogenous groups of 15 European sea bass juveniles, with an average initial body weight of 30 g, were constituted, and each experimental diet was randomly assigned to triplicates of these groups. Fish were fed by hand twice a day (at 9 a.m. and 4 p.m.) 6 days a week, until visual apparent satiety (established after the first three pellets reach the bottom of the tank and are ignored by the fish). Utmost care was taken to avoid feed waste and to assure that all feed supplied was consumed. The trial lasted 12 weeks and at the end of the trial fish were bulk weighed following one day of feed deprivation. At the beginning of the trial, six fish from the initial stock, and at the end of the trial, five fish from each tank were randomly sampled and pooled for whole-body composition analyses. Wet weight and liver weight were recorded for determination of hepatosomatic index. Due to the time limitation, this data are not presented in this thesis.

## Chemical analyses performed in ingredients, diets, and faeces

### Sample preparation for SSF

Prior to analysis, dietary ingredients, including non-fermented and fermented DDGS, diets and faeces were finely ground to obtain a homogenous sample.

Chemical analyses of both DDGS (before and after SSF) were performed according to standard methods (Chemists and Horwitz, 1980) as follows: dry matter, by drying the samples at 105 ºC until constant weight; ash by incineration in a muffle furnace at 450

ºC for 16h. Crude protein content (N x 6.25) by the Kjeldahl method succeeding acid digestion, using Kjeltec digestor and distillation units (Tecator Systems, Hõganäs, Sweden; model 1015 and 1026, respectively); lipid content by extraction with petroleum ether using a Soxtec system (Tecator Systems, Hõganäs, Sweden; extraction unit model 1043 and service unit model 1046); chromic oxide by acid digestion according to Furukawa (1966); total phosphorus by colorimetric method according to Fiske and Subbarow (1925). Gross energy by direct combustion of samples in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261), and starch content according to Beutler (1984).

Cellulose, hemicellulose, and Klason lignin of unfermented DDGS and SSF-DDGS, experimental diets and faeces were analysed by quantitative acid hydrolysis in a two-stage acid treatment (the first stage with 72 wt% sulfuric acid at 30º C for 1 hour and the second stage after dilution of medium to 4 wt% sulfuric acid at 121º C for 1 hour). Posteriorly each resulting content was filtered through a Gooch container with known weight.

The filtrate was analysed by High-Performance Liquid Chromatography (HPLC) system for measure cellulose and hemicellulose. Using a Jasco830-IR intelligent refractive-index detector and a Varian MetaCarb 87H column. The increase weigh of the Gooch container matches to Klason lignin.

Free reducing, sugars, total phenols and proteins in solid residues (after SSF with the different microorganisms and without fermentation) were measured after extraction with water 1:4 (w/v). Reducing sugars were determined by the dinitrosalicylic acid method. Protein was determined by Bradford (1976) method. Total phenols were determined by the Folin-Ciocalteau method using caffeic acid as a standard.

### Enzyme activity assays in fermented-DDGS

Cellulase (endo-1, 4-ß-glucanase) activity was determined with the enzymatic kit Azo-CM-Cellulose S-ACMC 04/07 (Megazyme International, Ireland). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of glucose reducing sugar equivalents from CMCellulose in 1 min at 40 °C and pH 4.5. Xylanase (endo-1, 4-ß-xylanase) activity was determined with the enzymatic kit Azo wheat arabinoxylan AWX 10/ 2002 (Megazyme International, Ireland). One unit of enzyme

activity was defined as the amount of enzyme required to release 1 µmol of xylose reducing sugar equivalents from wheat arabinoxylan in 1 min at 40 °C and pH 4.5.

## Digestive Enzymes Activities

For the digestive enzymatic activity measurement, each intestine portion was homogenized in ice, using an ultrapure water buffer (pH 8.0) and centrifuged (23,000 x g: 30 min: 4 ºC). The supernatant was collected, divided into several aliquots and stored at -80 ºC, until analyses.

Total protease activity was measured by the casein hydrolysis method according to Hidalgo et al. (1999). The reaction mixture containing casein (1% w/v; 0.125 mL) and homogenate supernatant (0.05 mL) was incubated for 1 hour at 37 ºC and stopped by adding 0.6 trichloroacetic acid (8% w/v) solution. After being kept for 1 h at 2 ºC, 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. A calibration curve was established using tyrosine solution. One unit of enzyme activity was defined as the amount of enzyme needed to catalyse the formation of 1.0 µmol of tyrosine per min.

α-Amylase (EC 3. 2. 1. 1) and lipase (EC 3. 1. 1. 3) activities were measured using commercial kits from Spinreact (ref. 41201 and 1001275, respectively), following the manufacturer methodology. α-Amylase activity was measured at 405 nm by the rate of 2-chloro-4-nitrophenol formation at 37 ºC. Lipase activity was measured at 580 nm by the rate of methyl resorufin formation was quantified photometrically at 37 º C. Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) activities were determined according to Faulk et al. (2007). Trypsin activity was measured using Nα-benzoyl-L-arginine 4-nitronilide hydrochloride (1mM BAPNA) as substrate combined with trypsin assay buffer (50 mM Trizma, 20mM $CaCl_2$, pH8.2). Production of p-nitroaniline was monitored at 37 ºC and read at 410 nm. Chymotrypsin activity was measured using N-benzoyl-L-tyrosine ethyl éster (0.566 mM BTEE) as substrate combined with chymotrypsin assay buffer (0.1 M Trizma, 25 mM $CaCl_2$, pH7.8). Hydrolysis of BTEE was measured at 256 nm at 37 ºC.

All enzyme activities were determined using a Power Wavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA).

### Specific digestive enzymatic activity

All enzyme activities were expressed per mg of soluble protein (specific activity). Protein concentration was determined by the method described by Bradford (1976) using bovine serum albumin solution as standard. One unit (U) of enzyme activity was defined as µmol of product generated per minute at assay temperature.

## Statistical analyses

Before analysis, all data obtained were checked for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test). If necessary, variables were normalized by log transformation or arcsin square root transformation, for data expressed as decimal or percentage, respectively. All statistical analysis was performed using SPSS 24.0 software package for windows. Data are presented as means and pooled standard error of the mean (SEM).

ADC's of diets and ingredients were analysed by one-way analysis of variance (ANOVA). Digestive enzyme activity was analysed using a two-way ANOVA and if the interaction was significant, a one-way ANOVA was performed for each intestine section. For growth performance data statistical effects of DDGS level and SSF in test diets, excluding the control diet, were evaluated using a two-way ANOVA. If two-way ANOVA interaction was significant, a one-way ANOVA was performed for each DDGS level. The Dunnett test was used to compare control diet against each test diet. When p-values were significant ($P<0.05$), means were compared with Tukey´s HSD test (Tukey, 1949).

# Results

## Solid state Fermentation

Three filamentous fungi from MUM collection (Micoteca of University of Minho) were evaluated *Aspergillus ibericus*, *Aspergillus uvarum,* and *Aspergillus niger*. Three fungi were selected to perform SSF using as solid substrate DDGS. The chemical composition of DDGS before and after SSF is present in Table 4. The nitrogen and hemicellulose content increased in fermentations using *A. ibericus* and *A. uvarum* and decreased with *A. niger*. Irrespectively the fungi, cellulose, neutral detergent fibre (NDF) and acid detergent fibre (ADF) content of DDGS decreased after fermentations. Similarly, lignin also decreased after fermentation with *A. ibericus* and *A. uvarum*, whereas with *A. niger* was not affected. Ash content also increased in all the fermentations.

**Table 4**- Chemical composition (g/kg DM) and enzymatic activity (U/g) of DDGS unfermented and after SSF with *Aspergillus ibericus, Aspergillus uvarum,* and *Aspergillus niger.*

| | | Fungi | | |
|---|---|---|---|---|
| | **Unfermented** | ***A. ibericus*** | ***A. uvarum*** | ***A. niger*** |
| Nitrogen | 42.7 | 47.4 | 49.8 | 35.3 |
| Hemicellulose | 229.3 | 267.4 | 246.3 | 209.1 |
| Cellulose | 405.2 | 243.5 | 208.3 | 212.0 |
| Lignin | 176.4 | 119.4 | 139.2 | 171.4 |
| NDF | 810.9 | 630.3 | 593.8 | 592.5 |
| ADF | 581.6 | 362.9 | 347.5 | 383.4 |
| Ash | 38.1 | 52.8 | 42.4 | 54.9 |
| Enzymatic activity | | | | |
| Cellulase | — | 43.4 | 15.3 | 27.4 |
| Xylanase | — | 68.0 | 152.1 | 15.3 |

Total protein, reducing sugars and total phenols of DDGS before and after SSF in presented in table 5. All the tested parameters increased after SSF, regardless the fungi used, compared to the unfermented DDGS. The initial protein content of DDGS was very low (0.27 mg/g of dry waste), *A. ibericus* and *A. uvarum* showed similar results in increasing protein content of DDGS (0.30 and 0.31 mg/g of dry matter, respectively) while *A. niger* decreased the protein content of DDGS (0.22 mg/g of dry matter). For total phenols content of DDGS (1.5±0.03 mg/g of dry matter) increased more than threefold

after fermentation with *A. ibericus* and *A. uvarum* (4.9±0.02 and 5.0±0.49 mg/g of dry waste, respectively) while with *A. niger* increased more than sixfold (9.6±0.02 mg/g of dry waste). Similarly, reducing sugars were also increased after fermentation of DDGS from (9.5±0.5 mg/g dry matter) to 31.9 - 38.1 mg/g dry matter with *A. ibericus* and *A. niger* and to 28.4 mg/d dry matter with *A. uvarum* (28.4±1.8 mg/g of dry waste).

**Table 5**- Protein, reducing sugars and total phenols content (mg/g dry waste) of DDGS unfermented and after SSF fermentation with *Aspergillus ibericus, Aspergillus uvarum,* and *Aspergillus niger.*

| | | **Fungi** | | |
|---|---|---|---|---|
| | **Unfermented** | ***A. ibericus*** | ***A. uvarum*** | ***A. niger*** |
| Protein | 0.27 | 0.3 | 0.31 | 0.22 |
| Reducing sugars | 5.2 | 31.9 | 22.7 | 38.1 |
| Total phenols | 1.5 | 4.9 | 5.1 | 9.6 |

The cellulase and xylanase activity in DDGS after SSF by the three *Aspergillus* sp. fungi are presented in Figure 7. The fermentation with *A. ibericus* promoted the highest cellulase activity (43.4±4.6 U/g of solid substrate) while the *A. uvarum* promoted the highest xylanase activity (152.1±42.3 U/g of solid substrate) among the tested fungi species.

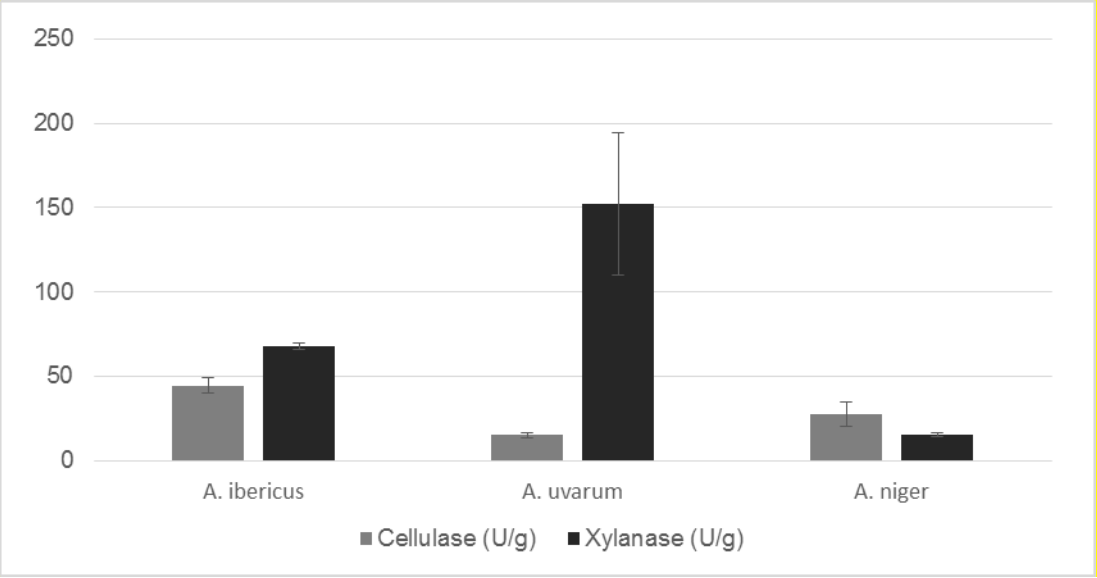



**Figure 7**- Enzymatic activity of DDGS after SSF fermentation with *Aspergillus ibericus*, *Aspergillus uvarum, and Aspergillus niger*

Based on the upgrading nutritional content and enzymatic activity, *A. ibericus* was selected for SSF in a larger scale to produce SSF-DDGS to be used as a feed ingredient in seabass digestibility and growth trial. Besides the upgrading of the nutritional value of DDGS, the chosen strain of *A. ibericus* was recently discovered and it potential to be used in SSF process has been little studies.

## Diets and ingredient digestibility

The ADC of the experimental diets are presented in Table 6. The ADC of dry matter, organic matter, lipids and starch of reference diet was higher for reference diet than those of test diets. Protein digestibility of SSF-DDGS diet (93.9%) was higher than that of the reference diet (91.9%). Digestibility of lipid and energy digestibility were higher (96.7% and 92.6%, respectively) in SSF-DDGS diet than in the DDGS diet (93.6% and 87.3%, respectively). Phosphorus digestibility was similar among test diets.

**Table 6**- Apparent digestibility coefficients (ADCS %) of the experimental diets[1]

| | Diets | | | |
|---|---|---|---|---|
| | **Reference** | **DDGS** | **SSF-DDGS** | **SEM** |
| Dry matter | 83.7$^{b}$ | 75.5$^{a}$ | 74.5$^{a}$ | 0.7 |
| Organic matter | 85.1$^{b}$ | 75.8$^{a}$ | 74.4$^{a}$ | 0.7 |
| Protein | 91.9$^{a}$ | 92.7$^{ab}$ | 93.9$^{b}$ | 0.3 |
| Lipids | 98.9$^{c}$ | 93.6$^{a}$ | 96.7$^{b}$ | 0.7 |
| Starch | 88.1$^{b}$ | 81.1$^{a}$ | 81.3$^{a}$ | 0.2 |
| Energy | 93.9$^{b}$ | 87.3$^{a}$ | 92.6$^{b}$ | 1.2 |
| Phosphorus | — | 90.7$^{a}$ | 91.2$^{a}$ | 1.0 |

[1] Means (n = 3) with different superscript letters are significantly different ($p<0.05$). SEM: pooled standard error of the mean.

Apparent digestibility coefficients of the ingredients are presented in Table 7. SSF of DDGS led to a significant increase of digestibility of protein, lipids, and energy while dry matter and organic matter ADC were not affected.

**Table 7**- Apparent digestibility coefficients (ADC %) of DDGS and SSF-DDGS[1].

| | Diets | | |
|---|---|---|---|
| | **DDGS** | **SSF-DDGS** | **SEM** |
| Dry matter | 56.5 | 51.9 | 2.3 |
| Organic matter | 54.0 | 58.7 | 2.3 |
| Protein | 92.4[a] | 98.3[b] | 1.5 |
| Lipids | 87.9[a] | 98.3[b] | 2.4 |
| Energy | 71.7[a] | 89.6[b] | 4.1 |

[1] Means (n = 3) with different superscript letters are significantly different ($p<0.05$). SEM: pooled standard error of the mean.

## Digestive enzymes

The specific activity of protease, amylase, lipase, trypsin, and chymotrypsin in anterior and distal intestine sections of sea bass juveniles fed the experimental diets are present in Table 8.

Irrespectively of dietary treatment, digestive enzymes activity was higher in distal intestine than in the anterior intestine, except for amylase and chymotrypsin.

Total protease activity was not different between groups, whereas trypsin and chymotrypsin had higher activity with the test diets than with the reference diet. Similarly, lipase activity was higher in fish fed the test diets than with the reference diet, except in the anterior intestine of fish fed the DDGS diet. Amylase activity was higher in fish feed the DDGS diet than with the other diets.

**Table 8**- Specific activity of protease, amylase, lipase, trypsin, and chymotrypsin (mU mg protein$^{-1}$) in anterior and distal intestine sections.

| | Diets | | | |
|---|---|---|---|---|
| | **Reference** | **DDGS** | **SSF-DDGS** | **SEM** |
| **Proteases** | | | | |
| Anterior intestine | 467.4 | 487.4 | 422.2 | 13.7 |
| Distal intestine | 342.6 | 302.9 | 403.9 | 21.1 |
| **Trypsin** | | | | |
| Anterior intestine | 5.4 | 10.5 | 12.8 | 0.9 |
| Distal intestine | 10.9 | 17.0 | 16.4 | 1.2 |
| **Chymotrypsin** | | | | |
| Anterior intestine | 694.3 | 1192.8 | 1283.4 | 70.7 |
| Distal intestine | 976.2 | 1469.8 | 1336.3 | 71.2 |
| **Lipase** | | | | |
| Anterior intestine | 365.9$^{a}$ | 497.0$^{ab}$ | 541.2$^{b}$ | 0.5 |
| Distal intestine | 478.4$^{a}$ | 864.6$^{b}$ | 694.6$^{b}$ | 0.5 |
| **Amylase** | | | | |
| Anterior intestine | 6.2 | 7.1 | 6.5 | 30.3 |
| Distal intestine | 6.7 | 9.8 | 6.5 | 44.3 |

**Two-Way ANOVA[1]**

| | **Diet** | **Intestine section** | **Interaction** | **Diets** | | |
|---|---|---|---|---|---|---|
| | | | | **Ref.** | **DDGS** | **SSF-DDGS** |
| Proteases | ns | *** | * | — | — | — |
| Trypsin | *** | ** | ns | a | b | b |
| Chymotrypsin | *** | ns | ns | a | b | b |
| Lipases | *** | *** | * | — | — | — |
| Amylase | * | ns | ns | ab | c | b |

Values presented as means (n = 6) and pooled standard error of the mean (SEM).
[1]Two-way ANOVA: ns: non-significant (P>0.05); *P<0.05; **P<0.01; ***P<0.001. If two-way ANOVA interaction was significant, a one-way ANOVA was performed for each intestine section (P < 0.05).

## Growth Trial

Data on growth performance and feed utilization of European sea bass feed the experimental diets are presented in table 9. No pathological signs were observed during the growth trial, and mortality was very low and unaffected by the dietary treatment. Growth performance, expressed as final body weight, weight gain or daily growth index was affected by dietary treatment. Compared to the control diet, those incorporating 20% of fermented or non-fermented DDGS lead to a significantly lower final body weight,

weight gain, and daily growth index, while no differences were observed for feed intake and feed or protein utilization. Comparing the effect of fermented versus non-fermented DDGS; the inclusion of 10% SSF-DDGS promoted higher weight gain and daily growth index that 10% DDGS diets. However, no effect was observed for feed intake and feed utilization. Protein efficiency ratio has also lower in the diets 20DDGS and 20SSF-DDGS diets compared to the SSF-DDGS diet.

**Table 9**- Growth performance and feed utilization efficiency of European sea bass fed the experimental diets[1]

| | Diets | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **10DDGS** | **10SSF-DDGS** | **20DDGS** | **20SSF-DDGS** | **SEM** |
| Initial body weight (g) | 29.8 | 29.6 | 29.5 | 29.8 | 29.7 | 0.01 |
| Final body weight (g) | 82.1 | 80.5¥ | 86.4¥ | 70.7‡ | 63.4‡ | 2.4 |
| Weight gain (g/kg/d) | 10.5 | 10.4¥ | 11.0¥ | 9.1‡ | 8.1‡ | 0.3 |
| Weight gain (%IBW) | 175.5 | 172.3¥ | 192.2¥ | 137.3‡ | 113.4‡ | 8.2 |
| Daily growth index | 1.40 | 1.38¥ | 1.49¥ | 1.16‡ | 1.00‡ | 0.05 |
| Ing (gMS/kg/d) | 10.5 | 10.4 | 11.0 | 9.1 | 8.1 | 0.3 |
| Feed efficiency | 0.79 | 0.78 | 0.83 | 0.67 | 0.73 | 0.03 |
| Protein efficiency ratio | 2.2 | 1.9 | 2.2 | 1.6‡ | 1.7‡ | 0.08 |
| Mortality (%) | 2.2 | 2.2 | 4.4 | 6.7 | 2.2 | 1.4 |

**Two-Way ANOVA[1]**

| | | | | Diets | | | |
|---|---|---|---|---|---|---|---|
| | **DDGS level** | **SSF** | **Interaction** | **10DDGS** | **10SSF-DDGS** | **20DDGS** | **20SSF-DDGS** |
| Initial body weight (g) | ns | ns | ns | — | — | — | — |
| Final body weight (g) | *** | ns | * | | | | |
| Weight gain (g/kg/d) | *** | ns | * | | | | |
| Weight gain (%IBW) | *** | ns | ** | | | | |
| Daily growth index | *** | ns | * | | | | |
| Ing (gMS/kg/d) | ns | ns | ns | — | — | — | — |
| Feed efficiency | ns | ns | ns | — | — | — | — |
| Protein efficiency ratio | * | ns | ns | ab | b | a | a |

Values presented as means (n = 3) and pooled standard error of the mean (SEM).
In the same row ‡ denotes significant differences against control diet ((P < 0.05; Dunnett test).
[1]Two-way ANOVA analysis excluding the control diet: ns: non-significant (P>0.05); *P<0.05; **P<0.01; ***P<0.001. If two-way ANOVA interaction was significant, a one-way ANOVA was performed for each DDGS level; ¥ denotes differences between 10%DDGS diets (*T*-test; P < 0.05).

# Discussion

## Solid state fermentation

Solid state fermentation has been applied in the production of metabolites, such as enzymes, antibiotics, and other value-added products, as organic and antioxidants compounds. Moreover, SSF holds tremendous potential for the production of new added-value feed ingredients, in those processes where the crude fermented products may be used directly in animal feeding (Tengerdy, 1998)

In SSF, the selection of a suitable substrate for the solid fermentation process is an important issue. The use of surplus/waste as a substrate for SSF allows the reduction of the final product cost and the implementation of a closed, sustainable, and environmentally friendly product chain (Fleuri et al., 2013). DDGS, a surplus from the ethanol industry, has proven to be a suitable substrate for SSF (Hoskins and Lyons, 2009). The two limiting factors of DDGS as SSF substrate are the possible lack of readily available nitrogen and the porosity of the material (Yang et al., 2012). Indeed, the porosity of the material plays an important role in SSF due to the aerobic nature of the SSF microorganisms, ensuring oxygen for its metabolism.

Moreover, in order to maximize the SSF process, it is also important to screen suitable microorganism and select the most suitable one for the substrate used in the solid fermentation process (Yang et al., 2012).For the fermentation of DDGS fungi seems to be the most adapted as their hyphae can grow on particle surfaces and penetrate the interparticle spaces and, thereby, colonize solid substrate (Lio and Wang, 2012). Three different fungi *A. ibericus*, *A. niger*, and *A. uvarum* were chosen for the SSF of DDGS based on its capability to produce enzymes and improve the nutritional value of other agro-industrial by-products with physical characteristics similar to DDGS (Salgado et al., 2014a; Salgado et al., 2016).

The current results showed that SSF with *A. ibericus* and *A. uvarum* increased crude protein content of DDGS, while the opposite was true for the *A. niger*. SSF with *A. ibericus* and *A. uvarum* increased crude protein content up to 11% while with *A.niger* it was decreased about

19%, highlighting the importance of the adequacy of the microorganism versus fermentation product. This increment in DDGS protein level is considerably higher than that obtains trough the fermentation of DDGS by *Aspergillus oryzae*, *Trichoderma* reesei, and *Phanerochaete chrysosporium* that increased the protein level up to 1.3-4-2%, (Lio and Wang, 2012). Higher values were, however, obtained by Zhang et al. (2013) who reported an increase of 25.2% of DDGS crude protein after SSF using a combination of *Trichoderma viride* and *Rhodopseudomonas palustris*. The different efficiency of SSF to upgrading the nutritional value of DDGS may be dependent on the feedstock and composition, process methods and parameters, and fermentation yeast (Liu, 2011). Similarly, the increase of crude protein level through the SSF has been reported for other fermentation substrates like maize stalk (Darwish et al., 2012), soybean meal (Hassaan et al., 2015; Lio and Wang, 2012), vegetable waste (Rajesh and Raj, 2010), straw (from rice, wheat, barley, soybean, canola, and pea), or rice husk (Nasehi et al., 2016). For groundnut oil cake it was reported that SSF increased protein, lysine and methionine levels (Ghosh and Mandal, 2015).

The increase in DDGS protein content could possibly be attributed to fungal biomass as reported by Chen et al. (1995) as well as to the segregation of some amino acids (Belewu and Sam, 2010; Hong et al., 2004). Indeed, SSF has also been reported to increase the content of some free amino acids during the process of fermentation (Belewu and Sam, 2010; Ghosh and Mandal, 2015). For soybean meal, the SSF process increased total essential amino acids level up to 13.5% and non-essential amino acids up to 18.4% (Hassaan et al., 2015).

In this study, SSF reduced substantially the cellulose content of DDGS, totalizing a 40-49% of reduction. DDGS contains relatively high concentration of fibre and previous studies have demonstrated that SSF may be used to reduce the fibre fraction of DDGS, improving its feasibility to be used as feedstuff for monogastric animals (Lio and Wang, 2012; Yang et al., 2012). Also, for other feed ingredients like maize stalk (Darwish et al., 2012) or groundnut oil cake (Mandal and Ghosh, 2013), crude fibre content also decreased through the SSF process.

In the present study, the reduction of DDGS cellulose content corresponded well to the activity of cellulase as well as to the levels of reducing sugars measured in SSF-DDGS. So, the decrease in cellulose could be attributed to the action of produced enzymes that degrade cellulose to reducing sugars as they grow on the substrate (Egwim and Onwuchekwa, 2016;

Tengku Norsalwani et al., 2012). Normally, the accessibility of enzymes to cellulose of lignocellulosic materials is limited (Arantes and Saddler, 2010). For this reason, the enzymatic hydrolysis is hampered and the materials should be pre-treated. In this case, DDGS is a by-product that has been undergone in the industrial process a several treatments as milled, thermic treatments that could have acted as pre-treatment and to facilitate the cellulose degradation by enzymes. Cellulases can break down the cellulose molecule into monosaccharides or shorter polysaccharides. Thus, based on the detected activity of cellulases in SSF-DDGS, a decrease in cellulose content in DDGS would be expected with the synthesis of cellulase after SSF. It was been reported that DDGS may be a good SSF substrate for enzyme production, namely cellulase and xylanase since it contains a moderate amount of nitrogen (Haltrich et al., 1996; Iyayi and Losel, 2001). Indeed, nitrogen source and availability in SSF substrate is an important factor for microbial growth and enzyme production (Yang et al., 2012).

Xylan is the major component of hemicellulose, and xylanase breaks down hemicellulose into xylose or xylooligosaccharides. In the present study, even though SSF lead to the segregation of xylanase, no decreased of hemicellulose was detected, which may be due to the presence of xylanase inhibitors (Sansen et al., 2004). Lio and Wang (2012) showed that fibre content in DDGS after SSF decreased substantially due to the effect of cellulase and xylanases, although xylanase activity was significantly higher than cellulase activity and hemicellulose content decreased and cellulose content increased after SSF. Yang et al. (2012) also reported a relationship between cellulase and xylanase activity and the decrease in fibre content of DDGS after SSF. Differences in enzyme activity of SSF-DDGS may be related with different composition of DDGS as process conditions

Among the fungi, *A. ibericus* seems to lead to the higher production of both cellulase and xylanase. Recent investigations in SSF using *Aspergillus* sp. shows that *A. ibericus* have found to be the best producer of lipase in different agro-industrial by-products substrates (Oliveira et al., 2016; Salgado et al., 2014a).

Based on the upgrading of DDGS protein content and enzymes activities observed, SSF by *A.ibericus* was choose for the production of large quantities of fermented DDGS to use in fish trials.

## Digestive Trial

The protein digestibility coefficient is a key factor for the evaluation of the diet quality, measured as its potential to promote synthesis of new tissue (Kumar et al., 2011). In general, protein digestibility of diets decreased as FM is replaced with DDGS.

In the present study, in general, DDGS digestibility of protein and lipid were high. DDGS protein digestibility (92%) was similar to that of FM (92%), the only protein source in the reference diet. However, dry matter (57%), organic matter (54%) and energy (72%) digestibility of DDGS were lower than that reported for FM or soybean meal (Gomes da Silva and Oliva-Teles, 1998). Differences in digestibility of dry matter, organic matter, and energy between DDGS and soybean meal may be due to differences in fiber content, as lower digestibility values has been correlated to higher fiber content plant feedstuffs. In the present study, DDGS neutral detergent fiber averaged 810.9 g/kg and 581.6 acid detergent fiber, values must lower than that of soybean meal. For carnivorous fish, as European sea bass, non-starch polysaccharides (NSP) are not digested, being associated with the reduction of digestibility of dry matter, organic matter and energy (Francis et al., 2001; Thompson et al., 2008).

In general, the present result of digestibility coefficients of DDGS correlates well with those determined previously for this species by Magalhães et al. (2015). For other species, protein digestibility was also similar. For rainbow trout, protein digestibility of DDGS based diets was estimated to be similar to FM, if DDGS is incorporated at low levels. Stone et al. (2005) observed for rainbow trout that even tough protein digestibility was high (>90%), it was reduced by the increase of dietary replacement of FM by DDGS, from 0, 250, 500, or 750 g/kg FM protein. However, other authors reported that protein digestibility of 20% DDGS based diet could be higher than that of 10% DDGS or FM based diets if supplemented with limiting essential amino acids (Barnes et al., 2012).

The inclusion of vegetable oils in carnivorous fish diets can negatively affect digestive and absorptive processes due to functional modifications at the intestine level induced by lipids (Santigosa et al., 2011). In present study, digestibility of lipids in DDGS was lower than that of FM, the only lipid source in reference diet, but was similar to those reported for European sea bass (87-89%) (Magalhães et al., 2015) and for rainbow trout (79-89%) (Cheng and Hardy, 2004).

DDGS digestibility of dry matter, organic matter, and energy were moderate to low, as similarly reported by Magalhães et al. (2015) for the same species. The high DDGS fiber content (58% ADF and 81% NDF) is probably the responsible of these results, as previously stated by (Magalhães et al., 2015)

SSF is a process with the capability to produce microbial enzymes, increase protein content and reduce fibre content of agro-industrial residues, where, microorganisms grow on solid substrates in a limited amount of free water sufficient to support the growth of cells. During fermentation is expected some modifications in the substrate like an increase in the nutrient content through microbial synthesis (Wee, 1991), inactivation of anti-nutritional factors (Reddy and Pierson, 1994), and addition of exoenzymes (Salgado et al., 2014b).

The SSF of DDGS increased the protein content in about 11%, reduced cellulose, lignin, ADF and NDF in about 34, 32, 38 and 22%, respectively, and added exogenous enzymes like cellulase and xylanase. This upgrading of DDGS composition induced considerable changed in digestibility of this ingredient in European sea bass. Comparatively to the unfermented DDGS, SSF increased DDGS digestibility of protein, lipids, and energy in about 6, 12 and 25%. To our knowledge, this is the first study aiming the evaluation of the potential of SSF by *A. ibericus* to improve digestibility of DDGS in European sea bass.

For terrestrial animals, SSF has shown to improved digestibility of the ingredient. Studies in chickens show that protein digestibility of fermented rapeseed meal did not differ from birds fed the control, but ADC of energy and phosphorus here higher for fermented rapeseed meal than for unfermented rapeseed meal (Chiang et al., 2010). Trials in pigs also showed that the inclusion of SSF soybean meal had higher protein digestibility than the unfermented soybean meal (Chen et al., 2010).

The improvement of digestibility of SSF plant feedstuffs may be related to several chemical or physical alterations that occur during the fermentation, including reduction/ degradation of anti-nutritional factors, reduction of molecular weight of proteins, added exo-enzymes (as lipases and carbohydrases), reduction and improvement of breakdown of fiber into lower molecular weight carbohydrates. As it was aforementioned, in the present study SSF of DDDGS increase protein content, reduced fiber content and added cellulase and xylanase to DDGS. So, the observed increase of protein, lipid, and energy digestibility resulted from the combination of these factors, which together make the DDGS more digestible than

before the SSF. Moreover, the reduction of complex polysaccharides and the secretion of exo-enzymes may have reduced the intestinal viscosity and increased the exposure the digesta to endogenous digestive enzymes and so increased the digestibility SSF-DDGS. Indeed, dietary non-starch polysaccharides (NSP) has been known to induce changes in gut transit, physiology, and morphology in fish, reducing considerably the digestibility (Sinha et al., 2011). Some of these NSP are present as part of the cell wall, thus shielding substrates from contact with the digestive enzymes and increasing digesta viscosity which interferes with digestion and absorption due to their chemical nature (Castillo and Gatlin, 2015).

Cellulase and xylanase that were synthetized during the fermentation of DDGS are some of the exogenous enzymes that have been tested to supply plant feedstuff based diets in aquafeeds and was revised by Castillo and Gatlin (2015). Dietary supplementation with exogenous carbohydrases can hydrolyze complex NSP present in plant feedstuffs that non-ruminant animals are incapable of hydrolyzing with their endogenous pool of digestive enzymes (Castillo and Gatlin, 2015). Dietary supplementation with carbohydrases did not affect only the digestibility of the carbohydrate/energy fraction but affect the overall digestive utilization of diet (Magalhães et al., 2016; (Xavier et al., 2012; Zhou et al., 2013). In the present study, the segregation of other enzymes, as lipase and proteases, into the SSF-DDGS substrate has also to be equated, which may also have contributed to the increase of digestibility efficiency. Indeed, *A. ibericus* has been reported has a well suitable fungus for lipase production by SSF (Salgado et al., 2014a), which may also contribute to the differences observed between unfermented DDGS and SSF-DDGS for lipids and energy digestibility.

In present study, phosphorus digestibility was high (91%) and not affected by the SSF. Even tough, nearly 60 to 70% of phosphorus in cereals is bound to phytate, which is poorly available for fish (Oliva-Teles et al., 1998), the amount of phytate-phosphorus in corn-DDGS is lower than that of corn. DDGS is a by-product of ethanol production, and during this fermentation the phytate-phosphorus is partially degraded, increasing phosphorus availability (Widyaratne and Zijlstra, 2007). Thereby, due to the low levels of phytate-phosphorus in the unfermented DDGS, the subsequent SSF of DDGS may had little effect on the bioavailability of phosphorus, justifying both high phosphorus ADC. However, for ingredients with a high amount of phytate-phosphorus, SSF may improve is bioavailability. Studies have showed that marine yeasts isolated from the gut of sea cucumber (*Holothuria*

*scabra*) and marine fish (*Hexagrammos otakii*) had the ability to produce large amount of extracellular phytase, which might play an important role in phytate degradation within marine animals gut (Li et al., 2008a; Li et al., 2008b). Moreover, studies in diets for olive flounders showed that microbial fermentation of soybean had increased phosphorus availability (Kim et al., 2010).

To the best of our knowledge, little research has been conducted on the effect of SSF on the feedstuffs digestibility in fish. Research has been centered on the study of the effect of increased dietary level on SSF feedstuff. For Nile tilapia fingerlings, it was reported that protein digestibility of 75% of fermented de-oiled *Jatropha curcas* seed meal based diet was lower than values obtained for 25% of fermented de-oiled *Jatropha curcas* seed meal or control diet (Hassaan et al., 2016). Also for soybean meal, it was observed a reduction of protein digestibility when SSF soybean meal was incorporated in the diet at higher incorporation rate than 25% (Hassaan et al., 2015). For black sea bream, partial replacement of FM by SSF soybean meal did not negatively affect protein and lipids (Azarm and Lee, 2014). However, previously, Zhou et al. (2011) showed that protein digestibility of a 10% SSF soybean meal diet was similar to the control diet, decreasing with a higher replacement level of FM by SSF soybean meal. Also in black sea bream, the replacement of FM by fermented cottonseed meal negatively affected dry matter, protein, and lipid digestibility (Sun et al., 2015). For juvenile *Myxocyprinus asiaticus*, Yuan et al. (2013) showed a progressive decline in the ADC value of protein with increasing replacement levels of FM by SSF soybean meal in diets.

In the present study, although SSF had reduced the fibre content of DDGS, digestibility of dry and organic matter did not show significant differences between DDGS and SSF-DDGS. These results are similar to those obtained by Azarm and Lee (2014) using fermented soybean meals in diets for black sea bream, reporting that, although fibre content has reduced by SFF the digestibility of dry matter decreased with the increase of dietary fermented soybean meal level. It is known that fish, especially carnivorous species, poorly digest complex carbohydrates; starches can be digested but the majority of NSP are no digestible for monogastric animals, including fish (Enes et al., 2011). The high amount of NSP present in corn DDGS may have contributed to the low digestibility of energy through mechanisms involving a binding action with bile salts combined with changes in digesta viscosity and transit rate (Francis et al., 2001). Indeed, in general, the addition of NSP to the

diet reduced dry matter and organic matter digestibility but not protein digestibility (Castillo and Gatlin, 2015; Glencross et al., 2012). Dietary NSP may also affect lipid digestibility as it interferes with the micelle formation in the gastrointestinal tract (Enes et al., 2011). However, the effect of NSP on digestibility clearly depend on the level and type of polysaccharide. For example, in red drum, it was detected that an adequate amount of NSP in the diet may acted as prebiotic enhancing nutrient and energy digestibility (Burr et al., 2008).

Several factors may affect the digestive enzyme production in fish, such as feeding habits, food preferences, diets formulations and anti-nutritional factors (ANF) (Pavasovic et al., 2007). So, the study of the modulation of the digestive enzymatic activity through the dietary FM replacement by plant protein feedstuffs is crucial to evaluate the interaction between diet and digestive function.

Bibliographic comparison of digestive enzymatic activity is not easy due to diverse protocols used (Hidalgo et al., 1999) and different distributions patterns of digestive enzymes between species (Corrêa et al., 2007). In fish, nutrient absorption is known to take place in the anterior intestine and, to a lower extent, in the distal intestine (Gai et al., 2012). In this study, results showed higher lipase, amylase, trypsin and chymotrypsin activity in the distal intestine. This uncommon elevated digestive enzyme activity observed in the distal intestine may be due to a possible drag of secreted mucous to this part of the digestive tract, as previously observed by Magalhães et al. (2015) for European sea bass and meagre and by Gai et al. (2012) for rainbow trout.

Overall, digestive protease, trypsin, chymotrypsin and lipase activity were not affected by the dietary inclusion of unfermented or fermented DDGS. The exception was amylase activity that was higher for DDGS than for SSF-DDGS. However, compared to the FM reference diet, the dietary inclusion of DDGS or SSF-DDGS significantly increased the activity of trypsin, chymotrypsin, and lipase.

Total protease activity was high, reflecting the carnivorous feeding habits of European sea bass. Strong protease activity may be predisposed by diet composition (Pérez-Jiménez et al., 2009; Santigosa et al., 2008), however, this was not the case in this study as dietary protein content of reference, DDGS, and SSF-DDGS diets differed considerably. Protease activity was previously determined in European sea bass fed DDGS based diets and, as in

the current study, no differences relatively to the fish meal based reference diet were also noticed (Magalhães et al., 2015).

Dietary inclusion of DDGS or SSF-DDGS increased trypsin and chymotrypsin activity. However, a decline of the activity of these enzymes has been observed with the dietary replacement of FM by plant protein sources, due to these proteases being highly sensitive to plant ANF (Hassaan et al., 2016; Saha and Ghosh, 2013). In present study due to the fermented nature of DDGS and double fermented nature of SSF-DDGS, that amount of ANF is residual, justifying a higher activity of these enzymes. Similar results were also obtained by Azarm and Lee (2014) with diets containing different inclusion rates of fermented soybean meal.

Lipase specificity is known to change in function of the unsaturation degree and of the chain length of dietary fatty acids (Castro et al., 2016). In the present study, fish fed the DDGS or SSF-DDGS diet had higher lipase activity that those fed the FM reference diet. Fish seem to adapt their digestive processes to improve lipid digestibility, including the modulation of the digestive enzyme activity (Bogevik et al., 2009; Fountoulaki et al., 2005). In the present study, the lower lipid level of test diets may justify the higher lipase activity in order to maximize the lipid absorption. Besides, the hypothesis of other exogenous enzymes, besides cellulase and xylanase, have been segregated cannot be ruled out. Some studies of SSF using *A. ibericus* showed that this microorganism is a very good producer of lipase (Oliveira et al., 2016; Salgado et al., 2014a). Salgado et al. (2014a) also registered protease activity after SSF using *A. niger*.

Amylase activity was also in accordance with the feeding habits of European sea bass. Amylase activity is usually affected by diet composition, with higher activity being observed in fish fed higher carbohydrate levels (Corrêa et al., 2007; Fountoulaki et al., 2005; Keshavanath et al., 2002; Pérez-Jiménez et al., 2009). Higher amylase activity observed in SSF-DDGS than that of DDGS diet may be related to the reduction of digesta viscosity and a higher digesta exposure to digestion enzymes (Adeola and Bedford, 2004). Actually, studies in other fish species showed that dietary NSP increases water content of digesta, forming a gum-like mass that increases digesta viscosity, obstructing the activity of digestive enzymes and so reducing diet utilization (Sinha et al., 2011). Also, changes in digesta viscosity and transit time reflected reduced nutrient and energy bioavailability for soybean meal based diets in European sea bass (Tibaldi et al., 2006). Also, the digestion of plant

feedstuff cell walls by NSPase releases cell nutrients that may stimulate exocrine pancreatic secretion (Li et al., 2009).

In the present study, exogenous enzymes segregated by fungi during the SSF process may also influence the digestibility. For example, cellulase increased proteases, amylase, and lipase activities of rohu and grass carp (Xavier et al., 2012; Zhou et al., 2013); dietary xylanase increased trypsin, chymotrypsin, amylase, and lipase activities of Jian carp (Jiang et al., 2014). Cellulase activity has been observed in several fish species indicating that fish may be able to utilize cellulose and similar fibrous carbohydrates (Chakrabarti et al., 1995). However, this activity may be a direct result of the ingestion of cellulases and/or cellulolytic bacteria contained within the food (Lindsay and Harris, 1980). However, other authors reported that exo-enzymes had little effect on the activity of endogenous digestive enzymes (Castillo and Gatlin, 2015; Magalhães et al., 2016).

This increase of nutrient content and bioavailability can show that SSF may be a good process to enrich the nutritional and economic value of DDGS for use in aquafeeds formulation.

## Growth Trial

In general, poor growth performance is observed in fish fed diets containing high inclusion levels of plant protein sources due to low palatability, low protein, deficiency of some essential amino acids, less availability of phosphorus, and high content of ANF (Ye et al., 2011). Improvement and optimization of the nutritional composition of plant protein sources and the reduction of the potentially adverse effect is an essential step to enhance its use in fish diets, reducing the dependence on fisheries ingredients. Agro-industrial by-products like DDGS are attractive ingredients for use in aquaculture feeds because of their contents in protein, energy, and highly digestible phosphorus, and may reduce the diet cost compared to conventional plant protein ingredients. In this study, the potential of DDGS and SSF-DDGS to replace mixtures of typical plant protein ingredients were evaluated in diets for European sea bass by partially, while the FM levels were kept constant to avoid confounding effects of differences in FM levels.

Some studies using SSF products have been performed in fish nutrition. Studies in Nile tilapia showed that high inclusion (75%) of *Jatropha curcas* seed meal fermented with different microorganism and soybean meal fermented with yeast showed significantly lower growth performance compared to diets containing less fermented ingredient, while incorporation up to 50% had no significant difference in the growth performance or feed utilization (Hassaan et al., 2016; Hassaan et al., 2015). In rainbow trout, diets containing fermented soybean meal at 47.6% level attained similar growth performance to fish fed FM based diet (Yamamoto et al., 2010). In Japanese flounder (*Paralichthys olivaceus*) diets using a combination of fermented soybean meal and squid by-product revealed that 36% of FM protein could be substituted by these fermented products without any negative effects on growth, feed utilization and health/welfare of fish, however, higher replacement levels negatively affected growth performance although voluntary feed intake was not affected (Kader et al., 2012).

In the present study, the replacement of a mixture of plant protein by 10% of DDGS did not affect growth and feed utilization, while the increase up to 20% reduced growth and feed utilization. Considering the effect of SSF, the incorporation of 10% SSF-DDGS versus 10% DDGS increased significantly growth performance, measure as weight gain or daily growth index, but did not affect feed intake and feed efficiency. However, such beneficial effects of SSF were not observed when SSF-DDGS was incorporated at 20%. Based on the results obtained in the digestibility trial, the better performance of SSF-DDGS may be, at least part, attributed to the increased digestibility of the ingredient. Indeed, even though no significant differences were observed, feed utilization and protein efficiency ratio were increased up to 6.5 and 16%, respectively, with the replacement of DDGS by SSF-DDGS. These results may be related to the reduction of complex NSP during the SSF DDGS, as well as the presence of segregated exogenous enzymes to the SSF-DDGS that may have increased the nutrient availability (Magalhães et al., 2016).

In Nile tilapia, it was observed that the optimum dietary incorporation of DDGS is 10% and higher inclusion level reduced growth performance (Magdy et al. (2015). However, other authors reported higher inclusion level of DDGS. Welker et al. (2014) concluded that DDGS be included in the diet of juvenile hybrid tilapia at about 30% as a replacement of one-third protein from SBM-CM mixture without adverse effects. For hybrid catfish, DDGS may be incorporated up to 30% DDGS and in combination with other protein sources diet was not

deficient in lysine and supported good growth and feed utilization (Zhou et al., 2010). For rainbow trout, DDGS may be used to replace a mixture of plant ingredients, up to 25-30% without affecting growth performance (Overland et al., 2013). Also for rainbow trout, it was observed that DDGS could replace 15 and 22.5% of fish meal (Cheng and Hardy, 2004). Contrarily, other authors reported a reduction in growth rate and feed efficiency when 10% DDGS replaced fish meal, corn gluten meal, and wheat in diets for rainbow trout, even if supplemented with essential amino acids and phytase (Barnes et al., 2012).

Deficiencies in several essential amino acids may limit the use of DDGS as a protein source in fish diets. Azarm and Lee (2014) showed that up to 40% of FM in diets for juvenile black sea bream could be replaced by fermented soybean meal with supplementation of methionine, lysine, and taurine. Also, the reduction of growth and feed utilization observed in higher inclusions of the fermented product may be due to anti-nutritional factors introduced during the fermentation process (Zhou et al., 2011). Also, Lim et al. (2007) observed that 40% DDGS could be included in tilapia diets if supplemented with 0.4% lysine, whereas without lysine supplementation, fish grew poorer compared with the basal control diet.

In the present study, a mixture of soybean meal and wheat meal was replaced by DDGS, and so differences in complex NSP, nutrients content, and digestibility of soybean meal and DDGS may explain the differences between control diet and DDGS based diets. Moreover, poor growth performance of fish fed plant protein-rich diets may also be related to the reduction in the voluntary feed intake (Gomes et al., 1993). In the present study, no significant effect of DDGS incorporation in voluntary feed intake was observed, suggestion no palatability issues. The observed reduced effect of 20% DDGS inclusion rate in feed efficiency, may justify the poor growth of fish feed this diet.

# Conclusion

In the first phase of this experiment, three different fungi were tested to evaluate its potential to improve the nutritional quality of DDGS by SSF. All of them performed well, but fermentation by *A.ibericus* allowed a high increase in protein content, a high decrease in cellulose content, and the highest cellulase activity. Due to the high indigestible fibre content of DDGS products*, A.ibericus* was chosen to produce an added value ingredient to be tested in a digestibility and growth trial with European sea bass juveniles.

Compared to the unfermented DDGS, SSF greatly increases the digestibility of protein, lipid, and energy, even though digestibility of dry matter and organic matter were not affected. Also, digestive enzyme activity was not affected by the fermentation of DDGS, except amylase that was increased.

Dietary replacement of 10% soybean meal + wheat meal by DDGS in a 20% fishmeal diet significantly reduced growth performance, evaluated as weight gain and daily growth index, but feed intake and feed efficiency were not affected. The increase of the replacement level up to 20%, not only reduce growth performance but also impaired feed utilization. However, the replacement with 10% SSF-DDGS significantly increased growth performance and protein efficiency rate, when compared to the unfermented DDGS.

Overall, these results highlight the potential application of SSF to added value of low nutritional agro-industrial by-products, as DDGS, to be used as feed ingredients in carnivorous aquafeeds. Compared to the unfermented DDGS the increase of protein, decrease of fibre (cellulose), and the segregated exo-enzymes occurred during the SSF greatly increased its digestibility allowing a higher growth performance when incorporated in diets for European sea bass juveniles.

With the biological samples collected at the end of the digestibility and growth trials, further studies are being undertaken in order to evaluate the effect of SSF on the digestibility of the carbohydrate fraction as well as its effect on protein and energy metabolism and intestinal health and histomorphology.

Overall, together these results provide important information regarding the potential application of SSF of DDGS. Due to the “food-feed competition” and rising prices of soybean and other cereals, fermented DDGS may become a feasible alternative.

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