# Potential of solid-state fermented wineries and olive oil by-products as functional ingredients for European seabass feeds

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Mestrado em Recursos Biológicos Aquáticos
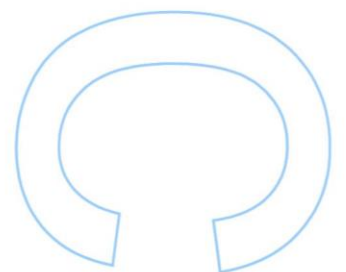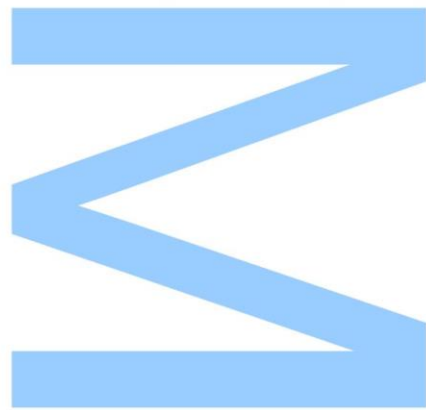
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2020

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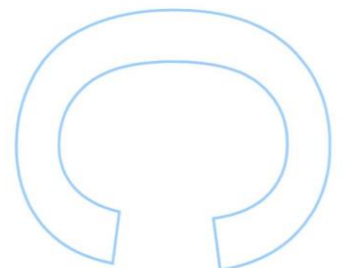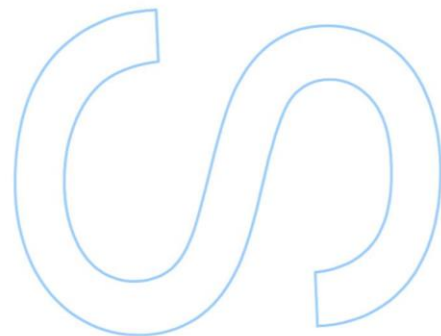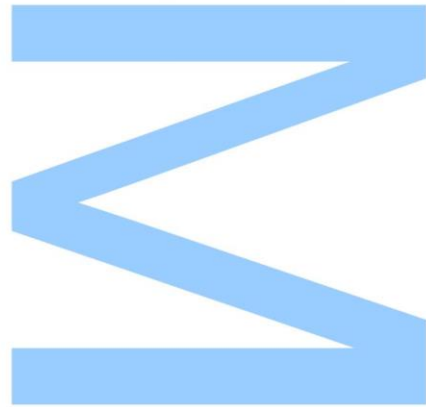UNIVERSIDADE DO PORTO

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

O Presidente do Júri,

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"Science never solves a problem without creating at least ten more." - George Bernard Shaw

# Acknowledgments

Going through a Master's Degree and doing this research project was not an easy task, and I could not have done it without the help of many people that were always beside me, and for that, I am truly grateful and it is for them that I give my biggest thanks.

First, to Dr Helena Peres, my biggest thank you, for all of the guidance and support, availability, and doing her best in spreading her knowledge in the most kind of ways.

To Dr Carolina Castro, for always being there supporting and teaching all the tasks done throughout the year, as well as helping so much in the research done for the writing of this dissertation.

Thank you to Dr Paula Enes, Dr Ana Couto, Dr Filipe Coutinho for all the help they provided in the experimental part of the work, their help was essential to overcome many difficulties that happened.

A special thank you to Helena Fernandes, Nicole Pires, Sara Moutinho, and Diogo Filipe, all of them had their individual projects, but they never refused to lend me a hand and help with everything I needed, many hours were passed alongside these colleagues and I am very grateful for the amazing kindness and guidance.

A massive thank you to all of the NUTRIMU group and group leader Dr. Aires Oliva Teles for supporting my work.

To Isa Quinzico, my biggest thank you, I could not have asked for a better partner from the beginning till the very end, I am very grateful for having her at my side and despite all that went wrong and all of the adventures that we had, we persevered and finally made it to the end. Also would like to thank my other colleagues, Carla Sousa, Carmo Cunha, and Maria Serrano, which passed through the same as me and were always willing to help with everything they could.

Finally, to all of my friends that supported me during all of this year and the years before, I love you all, your persistence and lecturing were essential to always put me on the correct path and pushed me to finalize this dissertation. To my family, thank you so much, my Mom and Dad accompanied me in all of my academic journey, and their love, support, and values to always pursue my dreams, led me to this moment. To my big sister, thank you for clarifying the dumbest questions and being always concerned with how my work was going. To all the people that I did not mention but passed through my life and left a mark on me, a big thanks, you all shaped me into the person I am today.

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# Resumo

A aquacultura é a indústria de produção alimentar com o crescimento mais rápido no mundo, mas tem sido desafiada por várias restrições, como a crescente preocupação com a sustentabilidade dos recursos primários e o objetivo de contribuir para uma Economia Circular Azul. A substituição parcial da farinha de peixe (FM) por ingredientes vegetais, muito mais ecológicos, está agora bem estabelecida para diferentes espécies carnívoras. No entanto, para atingir altos níveis de reposição e aumentar o uso de rações vegetais com baixo teor de proteína, para substituir rações vegetais convencionais, como farinhas de soja, milho e trigo, é necessário maior esforço de pesquisa. A presença de fatores antinutricionais e de polissacarídeos não amiláceos (NSP) nestas fontes vegetais é um obstáculo a ser superado, para que o crescimento e o valor nutricional dos peixes cultivados não sejam afetados negativamente. Recentemente, muitos estudos têm-se concentrado em aditivos para rações com compostos bioativos, como enzimas, prebióticos e probióticos, para combater estas desvantagens antinutricionais. Para além disso, a busca por novos antioxidantes naturais para serem usados na alimentação em aquacultura também tem sido uma prioridade devido à recente proibição, pela comissão da EU em 2017, do uso de alguns dos antioxidantes mais usados, como a etoxiquina.

A fermentação em estado sólido (SSF) é um processo ecológico, que utiliza pouca água e energia com fungos ou bactérias que produzem enzimas exógenas e libertam antioxidantes com diversas aplicações biotecnológicas. Esta fermentação pode ser realizada utilizando resíduos/subprodutos de agroindústrias como substrato, contribuindo para a economia circular. Em Portugal, a indústria do vinho e do azeite tem um grande impacto na economia. A produção portuguesa de vinho e azeite é bastante elevada, portanto aproveitar a elevada produção de resíduos/subprodutos derivados, reciclando, valorizando e transformando-os em produtos de alto valor acrescentado para incorporação em rações usadas na aquacultura, pode ser muito vantajoso.

Para este trabalho, uma mistura de bagaço de azeitona (OP) e bagaço de uva (GP), previamente otimizado para maximizar a produção de enzimas e antioxidantes, foi utilizada para produzir dois extratos funcionais, com ou sem SSF desses subprodutos. Estes extratos foram usados como ingredientes funcionais para alimentos aquáticos. Um ensaio de crescimento foi realizado com robalo europeu para avaliar a inclusão destes extratos na dieta; cinco dietas experimentais foram formuladas para serem isoproteicas (50% de proteína bruta) e isolipídicas (18% de lipídios brutos), com 25% de farinha de peixe. As dietas teste foram formuladas de forma semelhante à dieta controlo,

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mas adicionou-se o extrato obtido da mistura fermentada a 0,4% e 0,8%, e o extrato obtido da mistura não fermentada a 0,4%.

Parâmetros zootécnicos, composição de corpo inteiro, atividade de enzimas digestivas de enzimas endógenas e peroxidação de lípidos musculares ao longo da validade foram avaliados neste trabalho. O ganho de peso, o índice de crescimento diário, a eficiência alimentar, o consumo de ração e a taxa de eficiência proteica não foram afetados pela suplementação alimentar com nenhum dos extratos testados. A composição de corpo inteiro, índices somáticos e enzimas digestivas também não foram afetados. Por outro lado, os níveis de peroxidação lipídica registados no tecido muscular foram menores nos peixes alimentados com as dietas experimentais enriquecidas com os extratos do que com a controlo, o menor valor registado corresponde à dieta suplementada com o extrato não fermentado, tanto após uma noite como após 3 dias de refrigeração.

Em conclusão, a inclusão dietética de subprodutos vinícolas e do azeite, principalmente que não foram submetidos ao processo SSF, tem uma forte componente antioxidante que ajuda a reduzir a peroxidação de lípidos musculares do robalo europeu, sem afetar negativamente o crescimento, a eficiência alimentar, a composição corporal total ou a atividade das enzimas digestivas. Mais estudos são necessários para avaliar melhor o potencial antioxidante destes novos ingredientes funcionais produzidos a partir de vinícolas e subprodutos do azeite.

Palavras-chave: resíduos da agroindústria; fermentação em estado sólido; economia circular; Robalo europeu; antioxidantes

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# Abstract

Aquaculture is the fastest growing food production industry in the world but has been challenged by several constraints, including sustainability issues and compliance with the Blue Circular Economy. The partial replacement of fish meal (FM) with plant-based ingredients, more environmentally friendly, is now well-established for several carnivorous fish species. Nevertheless, to attain high replacement levels and to increase the use of low-protein plant feedstuffs, to replace conventional plant feedstuffs, like soybean, corn, and wheat meals, further research effort is needed. The presence of antinutritional factors and non-starch polysaccharides (NSP) in these plant-based sources is an obstacle to overcome so that the growth and nutritional value of farmed fish are not negatively affected. Recently, many studies have focused on the use of bioactive feed additives, as enzymes, prebiotics, and probiotics, to counter these antinutritional drawbacks. Moreover, the search for novel and natural antioxidants to be used in aquafeeds is now a priority due to the recent ban, by the Eu commission in 2017, on the use of some of the most common antioxidants used, as ethoxyquin.

The solid-state fermentation (SSF) is an eco-friendly process, using little water and energy with fungi or bacteria that produce exogenous enzymes and release antioxidants with many biotechnological applications. This fermentation can be performed using agro-industry wastes/by-products as a substrate, contributing to the circular economy. In Portugal, the wine and olive oil industries have a big impact on the economy. The Portuguese wine and olive oil production is quite high, so taking advantage of the high production of derived wastes/by-products, recycling, adding value, and transforming them into high added value products to be incorporated aquafeeds can be very advantageous.

For this work, a mixture of olive pomace (OP) and grape marc (GP), previously optimized to maximize the production of enzymes and antioxidants, was used to produce two functional extracts, with or without SSF of these by-products. These extracts were used as functional ingredients to aquafeeds. A growth trial was performed with European seabass to assess the effect of the dietary inclusion of these extracts; five experimental diets were formulated to be isoproteic (50% crude protein) and isolipidic (17% crude lipid), with 25% of fisheries meal. Test diets were formulated similarly to the control diet, but adding the extract obtained from the fermented mixture at 0.4% and 0.8%, and the extract obtained from the unfermented mixture at 0.4%.

Zootechnical parameters, whole-body composition, digestive enzyme activity of endogenous enzymes, and muscle lipid peroxidation throughout the self-life were

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assessed in this work. Weight gain, daily growth index, feed efficiency, feed intake, and protein efficiency ratio were not affected by the dietary supplementation with any of the extracts tested. Whole-body composition, somatic indices, and digestive enzymes were also not affected. On the other hand, lipid peroxidation levels registered in the muscle tissue were lower in fish fed the experimental diets enriched with the extracts than with the control, and the lowest value was registered with the diet supplemented with the unfermented extract, both after one night and 3 days in the refrigerator.

In conclusion, dietary inclusion of extracts obtained from wineries and olive mill by-products, mainly that not submitted to the SSF process, have a strong antioxidant component that helps to reduce the muscle lipid peroxidation of European seabass, without negatively affecting growth, feed efficiency, whole-body composition, or digestive enzymes activity. Further research is needed to better evaluate the antioxidant potential of these novel functional ingredients produced from wineries and olive mill by-products.

**Keywords:** agro-industry wastes; solid-state fermentation; circular economy; European seabass; antioxidants

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# Abbreviations

**ABW** – Average Body Weight

**BSA** – Bovine Serum Albumin

**CMC** – Carboxymethylcellulose

**COP** – Crude Olive Pomace

**DGI** – Daily Growth Index

**DNS** – 3,5-Dinitrosalicylic Acid

**DPPH** – 2,2-Diphenyl-1-picrylhydrazyl

**EGM** – Exhausted Grape Marc

**EOP** – Exhausted Olive Pomace

**EU** – European Union

**FAO** – United Nations Food and Agriculture Organization

**FBW** – Final Body Weight

**FCR** – Feed Conversion Ratio

**FE** – Feed Efficiency

**FEAMP** – European Maritime and Fisheries Fund

**FI** – Feed Intake

**FM** – Fish Meal

**FO** – Fish Oil

**FWO** – Fermented Mixture

**GM** – Grape Marc

**GRAS** – Generally Regarded As Safe

**HIS** – Hepatosomatic Index

**IBW** – Initial Body Weight

**IU** – International Unit

**LD50** – Median Lethal Dose

**LPO** – Lipid Peroxidation

**MDA** – Malondialdehyde

**NSP** – Non-starch Polysaccharides

**OP** – Olive Pomace

**PER** – Protein Efficiency Ratio

**PNG** – p-Nitrophenyl-β-D-glucopyranoside

**PUFA** – Polyunsaturated Fatty Acids

**ROS** – Reactive Oxygen Substances

**SEM** – Standard Error of the Mean

**SGR** – Specific Growth Rate

**SSF** – Solid-state Fermentation

**TBA** – Thiobarbituric Acid

**TBRAS** – Thiobarbituric Acid Reactive Substances

**UWO** – Unfermented Mixture

**VSI** – Visceral Index

**VTS** – Vine Shoot Trimmings

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# 1.Introduction

## 1.1 Aquaculture

Aquaculture is a very important and growing industry all around the globe. With the capture fisheries production at a stable point (Figure 1) and the continuing growth of the human population, aquaculture is the only sustainable alternative to guarantee the supply of fish for human consumption (FAO, 2018). In 2014, aquaculture contributed to the total supply of fish for human consumption overtook, for the first time, that of capture fisheries. Thirty-five countries, representing 45 % of the world's population, also produced more farmed than wild-caught fish, e.g. China, India, Viet Nam, Bangladesh, and Egypt (FAO, 2016).

In 2016, total fish production reached a peak of 171 million tonnes, of which 88 % are used directly in human consumption. Moreover, 50 % of the consumed fish derived from aquaculture. A new record of 20.3 kg per capita of consumption was reached, in 2016, and, in 2015, 17 % of the animal protein consumed by the global population was from fish only (FAO, 2018).
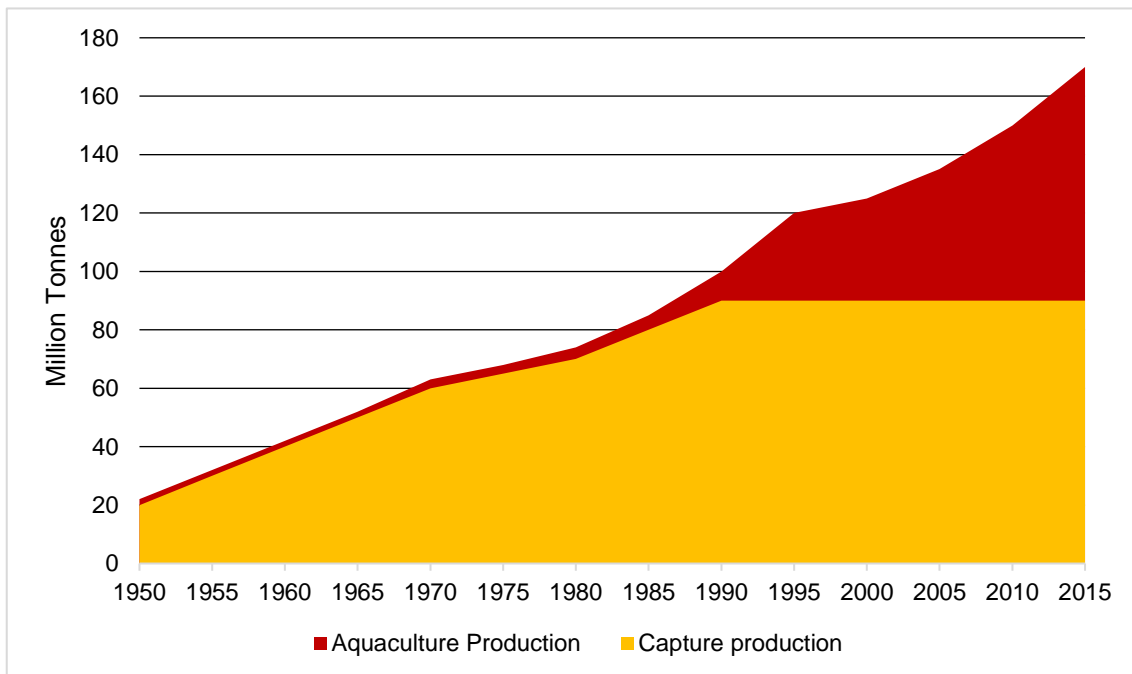


**Figure 1: World capture fisheries and aquaculture production. (Excludes aquatic mammals, crocodiles, alligators and caimans, seaweeds, and other aquatic plants; the numbers presented approximate the ones indicated by FAO for illustration purposes). Adapted from (FAO, 2018).**

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Although aquaculture is presented as a solution to overfishing, it is also one of the reasons for the declining of wild fish populations through the capture of this wild-fish to produce fish meal (FM) and fish oil (FO) necessary to produce salmon and other carnivore species (Ahmed et al., 2019). Indeed, aquaculture fish production is an industry in fast expansion and so it is of utmost importance that sustainable aquaculture practices are executed. Even though aquaculture has been recognized for its importance to cope with the world seafood demand, aquaculture has been related to several environmental issues. Habitat destruction, water pollution and eutrophication, disease outbreaks, and the introduction of invasive species are worrying problems that must be considered (Ahmed et al., 2019; Naylor et al., 2000). European Union, through the European Maritime and Fisheries Fund (FEAMP; 2014/2020), wants aquaculture to grow sustainably and efficiently in the future. To attain this objective in Portugal, sustainable aquaculture practices have to be adopted, including further research to develop new and low-impact aquaculture practices and new seafood products with higher value through different methods that can fulfill these objectives (Valente et al., 2015).

## 1.2 Aquaculture in Portugal

Portugal has the highest fish consumption per capita in the European Union (EU). From 2015 to 2016, fish consumption increased by about 3 %, attaining 57 kg per person per year, while in the EU the average per capita fish consumption was 24.35 kg, in the same year (EUMOFA, 2018). Nevertheless, most of the consumed fish is obtained through their capture and aquaculture still has a small impact on the total Portuguese fish consumption. In fact, Portuguese aquaculture production represents 0.7 % of the total EU aquaculture production (EUMOFA, 2018) (Figure 2) and reached 12 549 tonnes (81.3 million Euro) in 2017 (INE, 2018).

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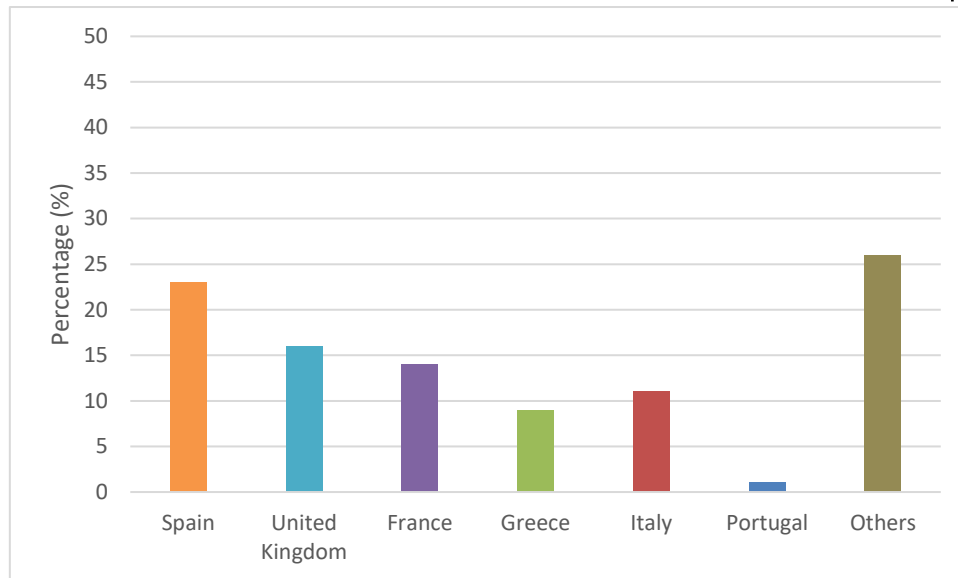
**Figure 2: EU aquaculture production overview in 2017, represented by percentage total output of aquatic organisms. Adapted from (Eurostat, 2019).**

Aquaculture production is dominated by marine species, representing 94.4 % of the total production, and of those, 37.5 % is due to marine fish production (INE, 2018). In terms of total Portuguese aquaculture, trout is the only freshwater species produced, especially in the north and centre regions. Marine aquaculture production includes shellfish like clams, mussels, and oysters; and marine fish produced are turbot, gilthead seabream, European seabass, and sole. In 2017, it was observed a decrease in gilthead seabream (-13.2 %) while the production of turbot European seabass increased around 17.4 % and 64.2 %, respectively (INE, 2018).

## 1.3 European seabass (*Dicentrarchus labrax* (Linnaeus, 1758)

European seabass (Figure 3) is a marine teleost species, belonging to the Moronidae family. It has a geographical distribution in the Eastern Atlantic from Norway to Morocco, the Canary Islands, and Senegal. Also present in the Mediterranean and Black Sea. They are benthopelagic, inhabiting shallow waters as well as various kinds of bottoms of coastal areas up to 100-150 m depth and are also eurythermic and euryhaline species, being recurrent in coastal inshore waters, estuaries, brackish water lagoons, and, occasionally, in rivers (Froese et al., 2019)

European sea bass is a piscivorous species typically feeding on small fish, prawns, crabs, and cuttlefish (FAO, 2019).

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European sea bass is a gonochoric species and sexual maturity generally occurs at an age of 2 to 4 years in the Mediterranean populations, while in the Atlantic populations sexual maturity occurs later (males between 4-7 years of age, and 30 to 40 cm of length; and females between 5-8 years of age, with 36-46 cm of length). Spawning season occurs just once a year, mostly in winter for the Mediterranean population (December to March), extending up to June for the Atlantic populations (Froese et al., 2019).



**Figure 3: European seabass (*Dicentrarchus labrax*; Linnaeus, 1758).**

Under farm conditions, they reach commercial size (300-400 g) in about 24 to 36 months. Their production is also considered a success on Mediterranean aquacultures (FAO, 2019).

To obtain satisfactory growth rates, protein requirements are relatively high (>40 %), considering that this is a carnivorous species. In the last decades, research has focused efforts to develop new feed formulations with reduced protein levels. Optimum dietary protein level averaged 50 % (Oliva-Teles, 2000). Peres and Oliva-Teles (1999a) established that the dietary protein requirement for growth was satisfied with a diet containing 48 % protein and was not affected by the water temperature. Nevertheless, through the optimization of the dietary digestible protein/digestible energy, the optimum dietary protein levels may be reduced. For example, Dias et al. (1998) obtained optimal growth rates with a dietary protein level of 43 %, corresponding to 40 % of digestible protein, and a digestible protein to digestible energy ratio of 19–20 mg/kJ. Perez et al. (1997) determined the optimum dietary level of 45 %, in a diet with 22.3 mg/kJ of crude protein to crude energy. Kousoulaki et al. (2015) review the bibliographic data and concluded that dietary protein levels of up to 56 % increase significantly the protein efficiency ratio. These differences are possibly related to diet formulation, including the digestible energy level and level and profile of essential amino acids, fish size, and dietary digestible energy level.

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Concerning optimum dietary lipid levels, various studies suggest that levels above 12 % do not have any beneficial effects on growth performance (Peres et al., 1999a; Perez et al., 1997), but protein efficiency ratio (PER) and energy retention increase with the increase of dietary lipid level from 12 to 24 % have been reported (Peres et al., 1999a; Perez et al., 1997). Dias et al. (1998) reported that dietary lipid levels up to 18-19 % enhanced growth performances and feed efficiency. Regarding the dietary replacement of fish oil by soybean oil, it was observed that the incorporation of 6-10% had no negative effects on growth rate, feed efficiency, and body composition (Kousoulaki et al., 2015).

Carbohydrates are not an essential nutrient, but their inclusion in diets can spare the use of proteins and lipids for energy purposes (Wilson, 1994). There are no specific requirements for dietary carbohydrates levels, nevertheless, it is recommended to be limited to 20 %. European seabass has a limited capacity to digest and metabolize carbohydrates (Enes et al., 2011). Furthermore, the utilization of gelatinized starch over glucose significantly increases feed efficiency, and the digestive utilization of diets (Enes et al., 2011). The type and amount of carbohydrates used, water temperature, and feeding frequency should also be considered when formulating diets for European seabass (Kousoulaki et al., 2015).

Since the data on minerals and vitamins is very scarce, requirement levels for salmonids (NRC, 2011) are used in experimental diets with European seabass, which are the following: 50 mg/kg diet for ascorbic acid, 2500 IU/kg diet for vitamin A, 2400 IU/kg diet for vitamin $D_3$, and 0.65% of dietary levels of phosphorous (Kaushik et al., 1998; Oliva-Teles et al., 2004).

## 1.4 The fish meal and fish oil problematic

Intensive aquaculture production depends on the provision of adequate diets to guarantee maximum production. In 2017, global feed usage in aquaculture was estimated to be 51 229 thousand tons (Tacon, 2019). For the majority of fish species produced under intensive aquaculture systems, FM and FO are still the preferred ingredients used in their diets due to their optimal nutritional values and palatability (Tacon et al., 2015). Even though during the last decades, the use of FM and FO have been reduced, its inclusion still is a common practice (Figure 4). However, the incorporation of these ingredients contradicts the primary directives for sustainable aquaculture, due to the environmental, economic, and social impact of their use (Gatlin

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et al., 2007; Sinha et al., 2011). In marine carnivorous fish, it is especially important to optimize this substitution, where the incorporation levels of FM and FO are still relatively high. Indeed, partially replacing FM and FO was already achieved, but total replacement leads to problems in growth and feed intake (Kaushik et al., 2004; Kousoulaki et al., 2015).
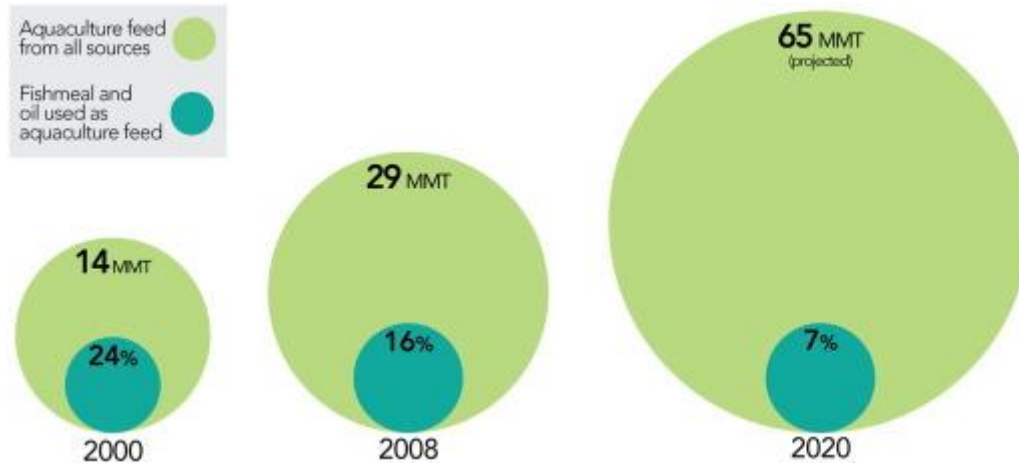


**Figure 4: Global aquafeed production (values for 2000, 2008 and projected for 2020), and inclusion levels of fishmeal (Fry et al., 2016).**

## 1.5 The potential of vegetable-based diets

The use of plant ingredients in substitution of FM and FO seems to be an economically sustainable alternative, as they are more available at a lower price. However, these ingredients present several constraints to its incorporation in aquafeeds, namely the presence of anti-nutritional factors and deficiencies in some essential amino acids, fatty acids, and minerals. The judicious feed formulation may overcome some of them, including restoring the ideal essential amino acid and fatty acid profile. However, the carbohydrate component present in these ingredients is a challenge because, despite being a cheap energy source for fish, it is poorly utilised by fish (Enes et al., 2011; Sinha et al., 2011). In fact, non-starch polysaccharides (NSP) are highly abundant in common plant ingredients, like soybean meal, but are not digested by carnivorous fish (Allan et al., 2000; Naylor et al., 2009; Sinha et al., 2011).

To overcome these constraints, research has been devoting efforts to develop strategies to ensure higher inclusion levels of plant ingredients in fish diets. The use of attractants to improve palatability has shown an increase in feed efficiency, weight gain, and feed intake on European seabass (Dias et al., 1997). Genetic manipulation of grains

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and oilseeds to improve the amino-acid profile and to reduce antinutritional compounds is another strategy (Naylor et al., 2009). Pre-treatments, like extrusion, for a more efficient digestibility, reducing the antinutritional factors content have also demonstrated an improvement in the bioavailability of protein and energy on several plant feedstuffs (Glencross et al., 2012).

The use of the dietary supplement with exogenous enzymes to reduce the negative effects of antinutritional compounds and improve nutrient digestibility has also been studied and implemented. Fish do not hydrolyse the phytic acid and so, dietary supplementation with phytase has been proved to be an effective strategy to increase the bioavailability of dietary phosphorus, reducing the phosphorus release to the environment (Debnath et al., 2005). Similarly, fish also do not hydrolyse the β-glycosidic bonds of NSP, and dietary supplementation with carbohydrases has shown to improve energy yielding nutrients digestibility, through a direct action of the digestibility of the complex carbohydrates or indirectly by affecting the physical characteristics of the digesta (Castillo et al., 2015; NRC, 2011). These carbohydrases hydrolyse polymeric carbohydrates and the two most prominent in the market right now are glucanase and xylanase (Adeola et al., 2011).

Another method in fish nutrition for the repair or prevention of adverse effects on feeding farmed fish with alternative ingredients is using prebiotics and probiotics (Dawood et al., 2016; Guerreiro et al., 2017). Through the gut modulation effect, prebiotics and probiotics may enhance growth performance, feed efficiency, immune status, disease resistance, and stress responses (Guerreiro et al., 2017).

Dietary supplementation with natural bioactive rich additives is another strategy that has recently received more attention. Bioactive compounds are present in plant-based ingredients that have antioxidant, anti-inflammatory, and anticarcinogenic properties, with possible effects on the promotion of growth, feed efficiency, and resistance to stress and diseases of aquaculture fish (Citarasu, 2012). These compounds offer a natural alternative to synthetic compounds, such as antibiotics, reducing the human risk of consumption products from antibiotic-treated animals (Citarasu, 2012). Moreover, the use of synthetic antioxidants in feed formulation results in the presence of these compounds in the fish fillet that, even after two weeks of starvation before slaughter reducing the maximum levels of antioxidants, are found in farmed fish (Hamre et al., 2010). Bioactive compounds can have different origins, with medicinal plants being the most well studied and common one because of their low cost, availability, and composition on secondary metabolites, but recently there has been an

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effort in producing rich bioactive compounds extracts from agroindustry wastes (Bulfon et al., 2015; Citarasu, 2012; Novelli et al., 2017).

## 1.6 Agroindustry wastes

Great quantities of waste are generated by agroindustrial operations all around the world. This waste has massive potential in its reutilization, to produce other compounds that are more valuable so that other industries can benefit from using them, like the aquafeed industry. Moreover, the waste producers benefit from their reuse considering that they will not have to dispose or incinerate them, which can be costly (Harris et al., 2018). The process of recycling these by-products for several other sectors is crucial for a working Circular Economy as it is also one of the objectives of the EU's circular economy package (Commission, 2019). In Portugal, olive oil and wine production are two of the most relevant agro-food industries. Just in 2018, wine production reached 5.2 hectolitres and olive oil 1.1 hectolitres (INE, 2019).

The main by-products from the olive oil production, include olive pomace (OP), a wet solid waste generated from the two-phase extraction system of olive oil, mainly constituted by olive pulp, skin, and stone, which is categorised in crude olive pomace (COP) and exhausted olive pomace (EOP) depending on the percentage of residual oil in the final composition (Molina-Alcaide et al., 2008). These by-products contain compounds nutritionally relevant, such as sugar, fatty acids, polyalcohols, and polyphenols (Molina-Alcaide et al., 2008; Roig et al., 2006). Their composition is presented in Table 1 and Figure 5 represented the processes of olive oil production with the corresponding derived wastes.

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**Table 1: Proximate composition (dry matter basis) of crude olive pomace (COP) and exhausted olive pomace (EOP). Adapted from (Leite et al., 2016).**

| Compound | COP | EOP |
|---|---|---|
| Humidity (%) | 73.5 ± 0.4 | 9.9 ± 0.1 |
| Total solids (%) | 26.5 ± 0.4 | 90.1 ± 0.1 |
| Ash (%) | 6.6 ± 0.5 | 3.4 ± 0.2 |
| Lignin (%) | 43.2 ± 0.5 | 41.62 ± 0.04 |
| Hemicellulose (%) | 22.3 ± 0.8 | 24.1 ± 0.2 |
| Cellulose (%) | 12.5 ± 0.9 | 11 ± 2 |
| Lipids (%) | 16.7 ± 0.09 | 4 ± 2 |
| Proteins (mg/g) | 4 ± 1 | 2.6 ± 0.3 |
| Reducing Sugars (mg/g) | 96 ± 6 | 42 ± 2 |
| Phenols (mg/g) | 8.4 ± 0.3 | 8.9 ± 0.2 |
| N (%) | 0.6 ± 0.1 | 1.27 ± 0.07 |
| C (%) | 49.7 ± 0.7 | 46 ± 1 |
| Ca (g/kg) | 1.16 ± 0.04 | 1.8 ± 0.2 |
| K (g/kg) | 17 ± 1 | 14.2 ± 0.7 |
| Mg (mg/kg) | 474 ± 22 | 473 ± 57 |
| Zn (mg/kg) | 12 ± 0 | 10.5 ± 0.7 |
| Cu (mg/kg) | 11.5 ± 0.7 | 11 ± 1 |
| Fe (mg/kg) | 42 ± 2 | 147 ± 33 |
| Mn (mg/kg) | 8.6 ± 0.1 | 10.2 ± 0.4 |
| Cr (g/kg) | <22 | <22 |
| Ni (mg/kg) | <22 | <22 |
| Pb (mg/kg) | <22 | <22 |
| Na (mg/kg) | 373 ± 35 | 92 ± 5 |

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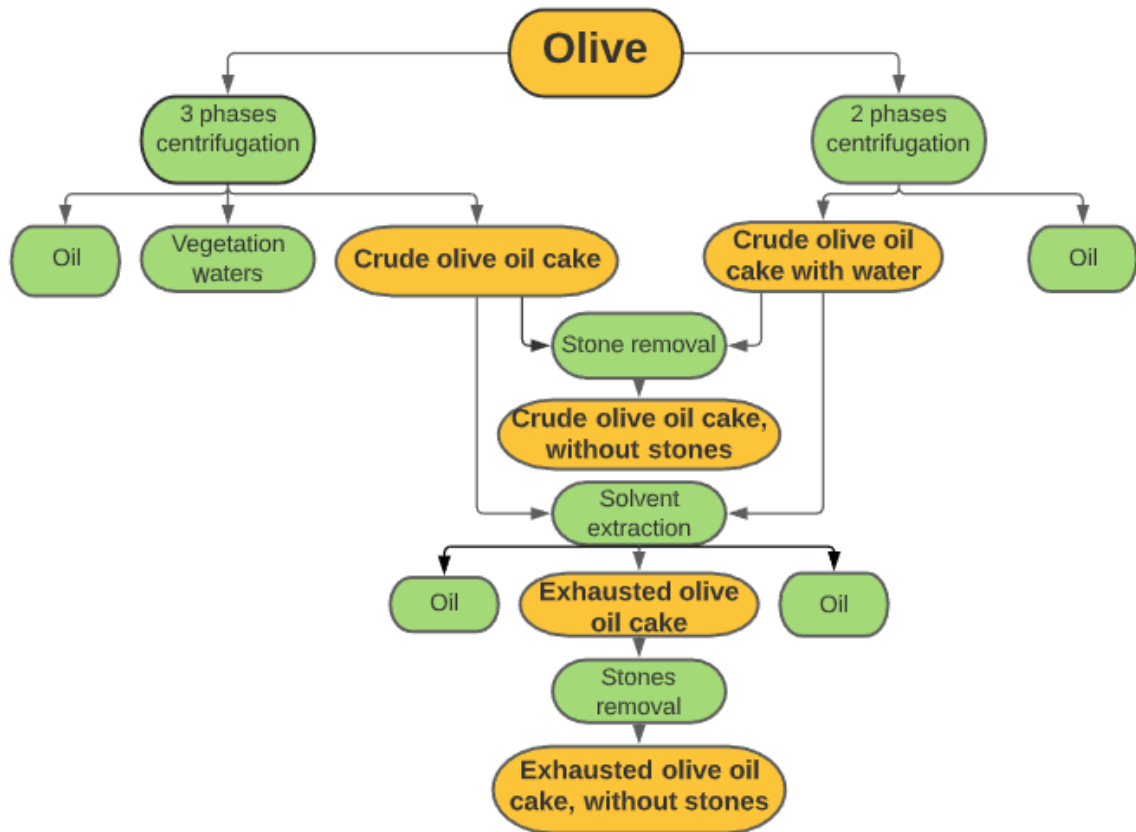


**Figure 5: Diagram of the production of olive oil and the by-products generated (crude olive oil cake – COP; exhausted olive oil cake – EOP). Adapted from (Cabrera et al., 2015).**

From the winery industries, the by-products generated and important for this work are vine shoot trimmings (highly abundant with a low economic value), produced with the pruning of the vine, and exhausted grape marc (EGM), grape marc obtained through the crushed grapes of a pressing process and constituted by grape peels and seeds that were processed in alcohol distilleries, which have phenolic and antioxidant compounds (Bucić-Kojić et al., 2013; Devesa-Rey et al., 2011; Furiga et al., 2009; Mateo et al., 2015). Their composition is presented in Table 2 and Figure 6 it is presented the wineries production processes with the corresponding derived wastes used in this work.

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**Table 2: Proximate composition (dry matter basis) of vine shoot trimmings (VTS) and exhausted grape marc (EGM). Adapted from (Salgado et al., 2013).**

| Compound | VTS | EGM |
|---|---|---|
| C (g/kg) | 453.56±2.21 | 482.37±16.07 |
| N (g/kg) | 5.62±0.71 | 16.97±6.26 |
| C/N | 80.76 | 28.42 |
| Cellulose (%) | 29.56±0.03 | 14.37±0.01 |
| Hemicelluloses (%) | 9.73±0.01 | 5.84±0.01 |
| Klason lignin (%) | 37.34±0.02 | 57.67±0.01 |
| Reducing sugars (mg/g) | 55.35±0.05 | 3.00±0.01 |
| Protein (mg/g) | 1.27±0.03 | 1.30±0.00 |
| Total phenols (mg/g) | 1.25±0.04 | 0.19±0.01 |
| Lipids (mg/g) | 29.6±0.00 | 21.3±0.00 |
| Moisture (%) | 6.08±0.09 | 11.03±0.12 |
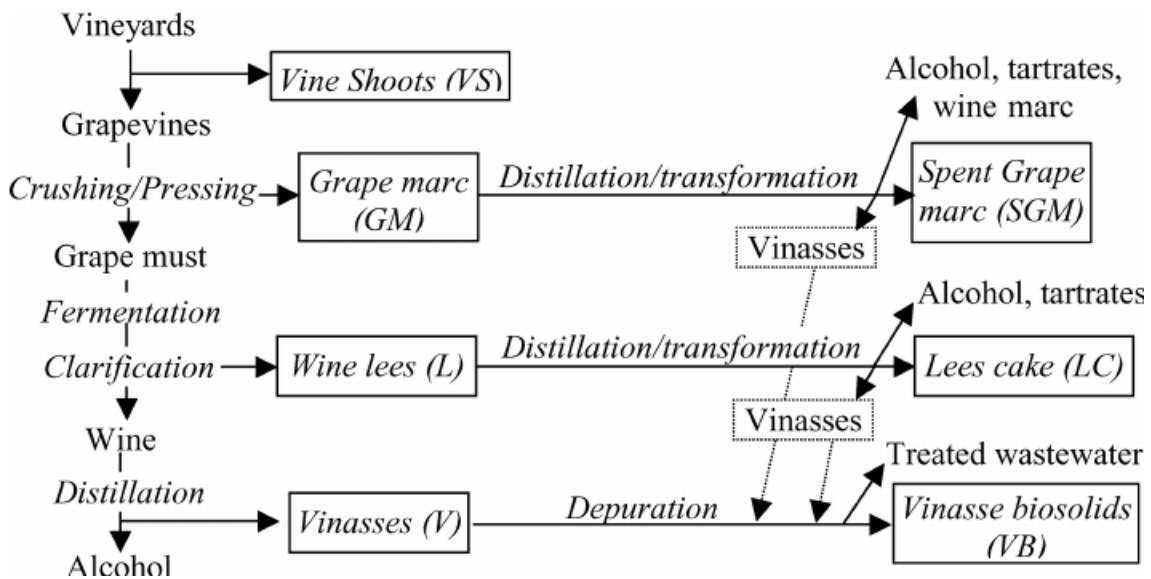


**Figure 6: Diagram of wine production and the by-products generated (vine shoot trimmings – VST; exhausted grape marc – EGM). Adapted from (Nogales et al., 2005).**

The direct use of main by-products from the winery and olive industries as feed ingredients is limited to ruminants. For these, the dietary incorporation is also limited to

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about 30% due to its low nutritional value and its antinutritional factors (Zept et al., 2011). To take advantage of the several bioactive compounds presented in these by-products, biotechnological processes must be developed to potentiate its use. Indeed, winery and olive mill wastes are plant-based by-products, with high levels of lignin, cellulose, and hemicellulose components. This cellulosic matrix reduces and limit access to the bioactive compounds and needs to be destroyed to make these compounds more available (Murad et al., 2010). Solid-state fermentation may be used for this purpose.

## 1.7 Solid-state fermentation

Solid-state fermentation (SSF) is a fermentation process that occurs in the absence or near absence of free water to produce biotechnological products (Pandey, 2003). It can use agro-industrial wastes as substrates that are nutrient-rich and have low water availability and reduces the final product cost and represents the implementation of a closed, sustainable, and environmentally friendly product chain (Fleuri et al., 2013). It enables enzyme production from the growth of microorganisms, like bacteria or fungi (Moura et al., 2012). During the fermentation process, fungi growth enriches the nutritional value of the substrate and produce valuable compounds such as enzymes, organic acids, single-cell proteins, polyunsaturated fatty acids, biopesticides, biofuel, antibiotics, and aromas, with a great interest for the biotechnology industry (Graminha et al., 2008). Therefore, SSF represents a way of adding value to neglected wastes and also minimizing the impact of their disposal by agroindustrial operations (Singhania et al., 2008).

Fungi have demonstrated to be very efficient, secreting metabolites and enzymes with a high growth rate, being *Aspergillus sp.* one of the several species with more interest(Novelli et al., 2015). Furthermore, this species is considered a GRAS (Generally Regarded As Safe) fungi, being proved to not produce any alarming toxins (Serra et al., 2006).

The final product of SSF can be totally incorporated in animal diets, as an ingredient, or the produced bioactive compounds can be extracted from it and then supplemented in the diets. For fish, Moura et al. (2012) have demonstrated that the inclusion in a plant-based diet of an enzyme complex obtained by SSF (Allzyme® SSF, Alltech) improved the growth performance of Nile Tilapia, due to the increased bioavailability of the dietary nutrients through the action of these enzymes.

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Figure 7 represents the general SSF process of a by-product, to the production of different products.
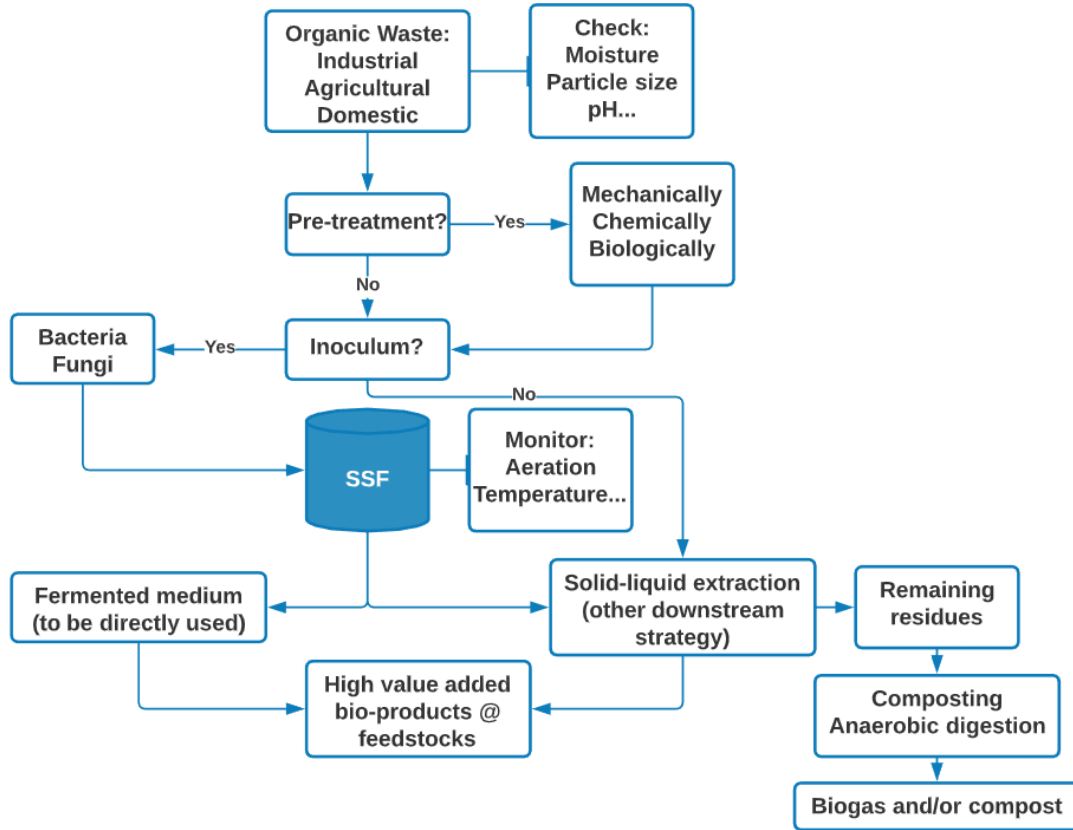


**Figure 7: Diagram of the Solid-state fermentation process. Adapted from (Yazid et al., 2017).**

## 1.8 Objectives

The aim of this study is to access the potential of the solid-state fermentation of grape marc (GM) and olive pomace (OP) mixture to produce a functional supplement for diets of European seabass (*Dicentrarchus labrax*), an important European aquaculture species. The prospective beneficial effects of the solid-state fermentation extract of GM and OP mixture will be evaluated in European seabass in terms of zootechnical performance, digestive enzymes, and lipid peroxidation of muscle during shelf-life.

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# 2. Materials and Methods

## 2.1 Solid-state fermentation and attainment of the extracts

An optimized mixture of olive mill wastes (COP and EOP) and winery wastes (EGM and VTS) was previously determined to maximize the production of bioactive compounds, namely enzymes and polyphenols (Filipe et al., 2019). The mixture comprises 0 % (w/w) COP, 30 % (w/w) EGM, 36 % (w/w) VTS, and 34 % (w/w) EOP and was fermented by *Aspergillus ibericus*. At the end of the fermentation period of 7 days, the produced enzymes and antioxidant compounds were extracted. For that, the fermented product was washed with distilled water (solid/water of 1:5 w/v) for 30 min, with constant stirring, at room temperature. Then, this mixture was filtered through a fine-mesh net and centrifuged at 11200 g for 10 minutes in 4 ºC. The supernatant was then filtered by vacuum through filter paper (pore size of 11 µm). The resulting SSF extract was then lyophilized to obtain a dry powder (Figure 8), allowing its incorporation in European seabass diets.

From the unfermented mixture of by-product, another extract was prepared by washing it with distilled water (solid/water of 1:5 w/v) and lyophilizing the obtained extract following the same procedure as done with the fermented mixture (Figure 8).
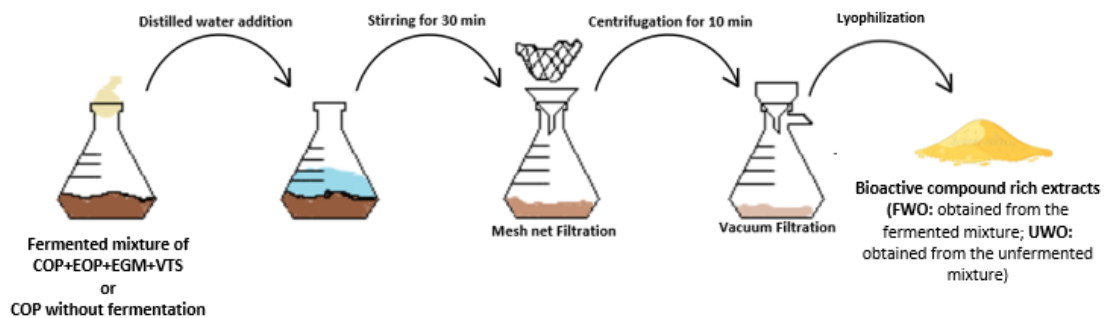


**Figure 8: Diagram representing the process to obtain the bioactive extract from a mixture of winery and olive by-products, submitted or not to a solid-state fermentation by *A. ibericus*. Adapted from (Filipe et al., 2019).**

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The composition of bioactive compounds present in the unfermented extract (UWO) and the fermented extract (FWO) was analysed before its incorporation into the diets (Table 3).

**Table 3: Bioactive compounds content of the lyophilized extracts, obtained before and after solid-state fermentation, of the optimal mixture with *A. Ibericus*.**

| | Extract | |
| --- | --- | --- |
| | **Unfermented mixture UWO** | **Fermented mixture FWO** |
| **Xylanase (U/g extract)** | - | 1864 |
| **Cellulase (U/g extract)** | - | 1165 |
| **Soluble protein (mg/g extract)** | 1.46 | 2.82 |
| **Total phenols (mg GAE/g extract)*** | 88.6 | 90.7 |
| **Total antioxidants (µmol TE/g extract)**** | 236.84 | 266.80 |

*GAE- gallic acid equivalents; **TE- trolox equivalents

## 2.2 Formulation of the diets

Four diets were formulated, attending to the specific optimum dietary levels of protein and lipid (50 % and 18 %, respectively) (Peres et al., 1999a; Peres et al., 1999b), amino acids (Peres et al., 2007), and total phosphorus (Oliva-Teles et al., 2004) estimated for European seabass.

A control diet was formulated, including 25 % of fisheries meals. Three other diets were formulated equally to the control diet, but incorporating the extract obtained from the fermented mixture (FWO) at 0.4 % or 0.8 % of the diet (diets FWO4 and FWO8, respectively) or the extract obtained from the unfermented COP at 0.4 % of the diet (diet UWO). The amount of FWO extract to be incorporated in diets FWO4 and FWO8 correspond to 4 U or 8 U of cellulase activity per gram of diet. The amount of unfermented COP extract to be incorporated in diets UWO equals the antioxidant activity of the extract 0.4 % of FWO added to the diet FWO4. The ingredients composition of the estimated enzymatic and antioxidant activity of the experimental diets are presented in Table 4.

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**Table 4: Composition of diets.**

| Diet | Control | FWO4 | FWO8 | UWO |
|---|---|---|---|---|
| Ingredients (% dry matter) | | | | |
| Fish meal[1] | 20 | 20 | 20 | 20 |
| CPSP[2] | 5 | 5 | 5 | 5 |
| Wheat gluten[3] | 12.1 | 12.1 | 12.1 | 12.1 |
| Soy protein concentrate[4] | 11.4 | 11.4 | 11.4 | 11.4 |
| Sunflower meal[5] | 8.5 | 8.5 | 8.5 | 8.5 |
| Rice bran[6] | 10 | 10 | 10 | 10 |
| Rapeseed meal[7] | 8 | 8 | 8 | 8 |
| Whole-wheat meal[8] | 5.4 | 5.4 | 5.4 | 5.4 |
| Hemoglobin AP310[9] | 3 | 3 | 3 | 3 |
| Hydrolysed shrimp[10] | 1.2 | 1.2 | 1.2 | 1.2 |
| Fish oil[11] | 11.2 | 11.2 | 11.2 | 11.2 |
| Vitamin premix[12] | 1 | 1 | 1 | 1 |
| Mineral premix[13] | 1 | 1 | 1 | 1 |
| Choline chloride (50%)[14] | 0.5 | 0.5 | 0.5 | 0.5 |
| Betaine[15] | 0.2 | 0.2 | 0.2 | 0.2 |
| Binder[16] | 1 | 1 | 1 | 1 |
| Taurine[17] | 0.5 | 0.5 | 0.5 | 0.5 |
| FWO extract | — | 0.34 | 0.68 | — |
| UWO extract | | —— | — | 0.19 |
| Proximate Composition (dry matter basis) | | | | |
| Dry matter (%) | 95.8 | 94.8 | 95.6 | 94.4 |
| Crude protein | 50.3 | 50.1 | 50.0 | 49.6 |
| Crude lipid | 17.2 | 16.3 | 16.2 | 16.6 |
| Ash | 7.5 | 7.3 | 7.7 | 7.6 |
| NFE[18] | 25.0 | 26.3 | 26.1 | 26.2 |
| Total phenols (mg GAE/g diet)[19] | 4.60 | 4.59 | 4.84 | 5.12 |
| Total antioxidants (µm TE/g diet)[20] | 48.1 | 126.8 | 119.8 | 91.1 |
| Xylanase (U/g diet) | n.d | n.d | n.d | n.d |
| Cellulase (U/g diet) | n.d | 1.16 | 6.6 | n.d |

[1]Pesquera Centinela, Steam Dried LT, Chile (CP: 79.7; CL: 10,5). Sorgal, S.A. Ovar, Portugal.
[2] Soluble fish-protein concentrate (CP: 80.2%; CL: 15.40%). Sopropeche, France
[3] Wheat gluten (CP: 80%; CL: 1.74%), Sorgal, S.A. Ovar, Portugal.
[4] Soy protein concentrate (CP: 48.57%; CL: 2.52%), Sorgal, S.A. Ovar, Portugal.
[5] Sunflower (CP: 40.4%; CL: 1.0%), Sorgal, S.A. Ovar, Portugal.
[6] Rice bran (CP: 14.20%; CL: 13.20%), Sorgal, S.A. Ovar, Portugal.
[7] Rapeseed (CP: 41.14%; CL: 5.80%), Sorgal, S.A. Ovar, Portugal.

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[8] Whole-wheat (CP: 12.23%; CL: 3.19%), Sorgal, S.A. Ovar, Portugal.
[9] Hemoglobin powder AP310P; APC Europe S.A.
[10] Hydrolyzed shrimp (CP: 69.8%; CL: 12.1%), Sorgal, S.A. Ovar, Portugal.
[11] Fish oil, Sorgal, S.A. Ovar, Portugal.
[12] Vitamin premix (mg kg$^{-1}$ diet): retinol, 18000 (IU kg$^{-1}$ diet); calciferol, 2000 (IU kg$^{-1}$ diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[13] Minerals (mg kg$^{-1}$ diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg$^{-1}$ diet); potassium chloride, 1.15 (g kg$^{-1}$ diet); sodium chloride, 0.4 (g kg$^{-1}$ diet).
[14] Choline chloride (50%), Sorgal, S.A. Ovar, Portugal.
[15] Betaine, Sorgal, S.A. Ovar, Portugal.
[16] Binder, Aquacube. Agil, UK.
[17] Taurine, Sorgal, S.A. Ovar, Portugal.
[18] NFE (Nitrogen free extract) = 100 − (crude protein + crude lipid + ash)
[19] GAE- gallic acid equivalents
[20] TE- trolox equivalents
n.d: not detected.

All dietary ingredients were finely ground, and proximate composition was analysed before the feed formulation. Following the feed formulation, ingredients were well mixed in a Hobart mixer and the mixture was then dry pelleted without steam using a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA). Pellets were sleeved and stored at -20 ºC after being dried in an oven for 48 h at 35 ºC (Figure 9).



**Figure 9: Experimental diets after being mixed, ground, and dry pelleted.**

## 2.3 Growth Trial

A growth trial was carried out at the Marine Zoology Station, at the University of Porto with European seabass juveniles obtained from a commercial hatchery. It was

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carried out in a thermoregulated water recirculation system equipped with 12 fiberglass tanks of 100L water-capacity (Figure 10). The tanks were supplied with filtered seawater through the use of 3 different filters, firstly a mechanic filter to retain the coarse particles and feces, secondly a biological filter that enabled the nitrification process, converting ammonia into nitrates and nitrites and, finally a second mechanical filter (sand filter) to retain the finer particles; at a water flow of about 3-5L/min per tank through the use of a pump. During the trial, the water temperature was maintained at 22-23 °C. Water temperature, dissolved oxygen, salinity, ammonia, and nitrites were measured daily during the entire trial, using an oxygen probe and PRODACtest kit (Figure 10). The photoperiod was established to be of 12 hours of light:12 hours of dark.

Before the beginning of the growth trial, the fish had a period of acclimatization to the experimental system for 15 days, being fed a commercial feed (Aquasoja, Sorgal, Portugal). At the beginning of the trial, 12 homogenous groups of 18 European seabass juveniles, with an average weight of 21.5 ± 1 g, were established and randomly distributed. Triplicate groups of these fish were fed one of the experimental diets, twice daily to visual satiety 6 days a week. The trial lasted 10 weeks.

At the end of the trial, fish were unfed for 1 day to empty gut content and bulk weighted. A total of 11 fish were collected from the initial stock of fish, as well as 2 fish from each tank at the end of the trial for whole-body composition analysis. The wet weight, viscera, and liver weight of these fish were recorded for the evaluation of visceral and hepatosomatic indices.

At the end of the trial, another 3 fish per tank (9 fish/diet) were randomly sampled, euthanized with a sharp blow to the head, and immediately eviscerated. The digestive tract was excised, adherent adipose and connective tissue were carefully removed. The intestine, directly after the stomach with the pyloric caeca, was fully removed and stored at -80 ºC until measurement of enzyme activity.

From the same fish, the right and left fillets were removed and the epaxial part of each fillet sectioned (i.e., the lateral dorsal part above the lateral line) for evaluation of the lipid peroxidation index after a night (T0) or after 3 days in the fridge at + 2/4 °C (T3), Time was defined according to the most common period of commercial shelf life for aquaculture fish trade in seafood markets.

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**Figure 10: Thermoregulated water recirculation system. a) – fiberglass tanks of 100L water-capacity; b) – biological filter; c) – PRODACtestkit.**

## 2.4 Chemical analysis

### 2.4.1 Extracts and Diets analysis

Both extracts, obtained from the unfermented mixture (UWO) and the fermented mixture (FWO) were analysed, including the analysis of xylanase, cellulase, total phenols, soluble protein, and antioxidants.

Xylanase, cellulase, total phenols, soluble protein were measured in the FWO extract while in the UWO the activity of the enzymes was not determined. The measurement of the enzymatic and antioxidant activity was used to define the inclusion levels of the extract in the diets.

For the analyses of non-starch polysaccharide enzyme activities, xylanase and cellulase activities were determined by measuring the release of reducing sugars after enzymatic hydrolysis using the 3,5-Dinitrosalicylic acid (DNS) method (Miller et al., 1959). For xylanase, the reaction mixture consisted of 250 µL of beechwood xylan substrate and 250 µL of diluted sample buffer or citrate buffer (0.05 N at 4.8 pH) for the blank. After an incubation period at 50 ºC for 15min, 500 µL of DNS was added and followed by a second incubation period at 100 ºC for 5 minutes. After being cooled at room temperature, 5 mL of distilled water was added, and the absorbance was read at 540 nm. For cellulase determination, carboxymethylcellulose (CMC; 2% in citrate buffer 0.05 N at a pH of 4.8) was used as a substrate, following the same procedure of xylanase determination, except for the first incubation that was performed at 50 ºC for 30 min. This was done in duplicate. An International Unit (IU) of enzymatic activity was stapled as the amount of enzyme needed to release 1 µmol of glucose per minute under standard assay conditions for cellulase assays; and 1 µmol of xylose per minute for xylanase assays. Results were expressed in units per gram of dry substrate used (U/g).

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The soluble proteins were determined using the Bradford method (Bradford, 1976). A calibration curve was constructed using bovine serum albumin (BSA) solutions with concentrations between 0 g/L and 1 g/L. One unit (U) of enzyme activity was defined as µmol of product generated per minute at assay temperature.

Total phenols were determined using the Folin-Ciocalteu method (Commission Regulation (EEC) No. 2676/90): 100 µL of the diluted sample (1:2) or distilled water for the blank were mixed with 2mL of $Na_2CO_3$ (15%), 500 µL of Folin-Ciocalteu reagent, and 7.4 mL of distilled water and incubated at 50 ºC for 5min. After cooling at room temperature, absorbance was read at 700 nm. This was done in duplicate. A calibration curve was performed with gallic acid in concentrations between 0 and 2 g of gallic acid /L.

Antioxidant activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (Brand-Williams et al., 1995). Three wells of the first row of a microplate were plated with 200 µL of the sample. Several dilutions (1:2; 1:4; 1:10; 1:20; 1:40; 1:100; 1:200) were then made by adding methanol. Blank was made using only methanol. Then to each well, 100 µL of DPPH was added except for the control for each sample that had the same dilutions of the samples, 100 µL methanol added in place of DPPH for the control. The microplate was incubated at room temperature, in a dark place for 30 minutes, and after, the absorbance was read at 517 nm. The calibration curved was done using known quantities of 6-hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid (Trolox). Results were expressed in millimoles of Trolox equivalent per kilogram of the dry solid substrate (mmol Trolox/kg).

Diets were also analysed for their enzyme content (cellulase and xylanase), as well as total phenols and antioxidants after extraction with water 1:4 (w/v).

## 2.4.2 Proximate composition of ingredients, diets, and whole-body fish

Prior to analysis, dietary ingredients and diets were ground until homogenization. Initial and final fish carcasses were dried at 100ºC until constant weight, then ground, well mixed, and stored in a desiccator until use (Figure 11). The analyses of each ingredient, each diet, and whole-body fish composition were executed according to the standard methods (AOAC, 2019) (Figure 12):

- Water content – sample drying in an oven at 105 ºC until constant weight;
- Ash – destruction of all organic matter through the incineration on a muffle at 450 ºC for 16h;

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- Crude protein (N × 6.25) - by the Kjeldahl method after acid digestion using Kjeltec digestion and distillation units;
- Crude lipid - by petroleum ether extraction using a Soxtec system.



**Figure 11: Dried carcasses that were ground and well mixed before further analyses.**



**Figure 12: Apparatus for proximate composition analyses. a), b) – Kjeldahl system for crude protein content; c) – crucibles for humidity and ash content; d) – Soxtec systems for crude lipid content.**

### 2.4.3 Analyses of the digestive enzyme activities and muscle lipid peroxidation

Before the analysis of digestive enzyme activities and muscle lipid peroxidation, intestine was homogenized in five volumes of ice-cold water and muscle in four volumes of ice-cold water. Homogenates were centrifuged at 30,000 g for 30 min at 4 °C and the resultant supernatants were kept in aliquots and stored at −80 °C for further digestive enzyme assays.

The activity of key digestive enzymes was assessed in the intestine. Intestinal samples were homogenized (1:4 w/v) in ice-cold buffer (100 mM Tris-HCl, 0.1 mM EDTA and 0.1% (v/v) TritonX-100, pH 7.8). Homogenates were centrifuged ( at 30,000g, 30

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min, 4 °C) and the resultant supernatants were aliquoted and stored at -80 °C until analysis. All enzyme activities were measured at 37°C in a Multiskan GO microplate reader (model 5111 9200; Thermo Scientific, Nanjing, China). The optimal substrate and protein concentrations for measurement of each enzyme activity were established by preliminary assays.

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 °C. Lipase activity was measured at 580 nm by the rate of methyl resorufin formation was quantified photometrically at 37 ° C.

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 °C and stopped by adding 0.6 trichloroacetic acid (8% w/v) solution. After being kept for 1 h at 2 °C, samples were centrifuged at 1800 g for 10 min and the supernatant absorbance was read at 280 nm against blanks. A control blank for each sample was prepared adding the supernatant from the homogenates after incubation. A calibration curve was established using a 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.

The soluble proteins were determined using the Bradford method described previously.

Fillets were used to measure the lipid levels of peroxidation after a night or 3 days of refrigerated storage. Lipid peroxidation level was determined according to the methodology described by Buege et al. (1978) using malondialdehyde (MDA) as a marker of lipid peroxidation. An aliquot of supernatant from the homogenate (100 μl) was mixed with 500 μl of a previously prepared solution containing 15 % (w/v) TCA, 0·375 % (w/v) thiobarbituric acid (TBA), 80 % (v/v) HCl 0·25 N and 0·01 % (w/v) butylated hydroxytoluene. The mixture was heated to 100°C for 15 min. After being cooled to room temperature and centrifuged at 1500 g for 10 min, the absorbance was measured at 535 nm in the supernatant. MDA concentration was expressed as nmol MDA per g of tissue, calculated from a calibration curve.

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## 2.5 Zootechnical performance

At the end of the growth trial, zootechnical performance parameters were evaluated as follows:

- Average Body Weight (g) (ABW):

$$ABW\ (g) = \frac{FBW - IBW}{2}$$

- Daily Growth Index (DGI):

$$DGI\ (\%) = \frac{\left(Final\ average\ body\ weight^{\frac{1}{3}} - Initial\ average\ body\ weight^{\frac{1}{3}}\right)}{number\ of\ days} \times 100$$

- Feed Intake (FI) (g kg ABW$^{-1}$ day$^{-1}$):

$$FI = \frac{Feed\ intake\ (g\ dry\ matter\ per\ fish) \times 1000}{ABW \times number\ of\ days}$$

- Feed Efficiency (FE) ratio:

$$FE = \frac{Wet\ weight\ gain}{Dry\ feed\ intake}$$

- Protein Efficiency Ratio (PER):

$$PER = \frac{Wet\ weight\ gained}{dry\ protein\ ingestion}$$

- Hepatosomatic index (HSI):

$$HSI\ (\%) = \frac{liver\ weight}{body\ weight} \times 100$$

- Visceral Index (VI):

$$VI\ (\%) = \frac{Viscera\ weight}{body\ weight} \times 100$$

- Lipid intake (g kg ABW$^{-1}$ day$^{-1}$):

$$Lipid\ intake = \frac{Lipid\ intake\ (g\ dry\ matter) \times 1000}{ABW \times number\ of\ days}$$

- Lipid retention (g kg ABW$^{-1}$ day$^{-1}$):

$$\frac{((FBW \times carcass\ lipid\ content) - (IBW \times carcass\ lipid\ content)) \times 1000}{ABW \times number\ of\ days}$$

- Nitrogen intake (g kg ABW$^{-1}$ day$^{-1}$):

$$\frac{Nitrogen\ intake\ (g\ dry\ matter)\ \times 1000}{ABW\ \times number\ of\ days}$$

- Nitrogen retention (g kg ABW$^{-1}$ day$^{-1}$):

$$\frac{(FBW \times carcass\ nitrogen\ content) - (IBW \times carcass\ nitrogen\ content) \times 1000}{ABW \times number\ of\ days}$$

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## 2.6 Statistical analysis

The statistical analysis was made utilizing the software SPSS for Windows (Version 25).

Normality (Shapiro-Wilk) and homogeneity tests (Levene) were first executed to validate the data. Growth performance, whole-body composition, and digestive enzyme activity data were subjugated to a one-way ANOVA. Muscle lipid peroxidation was analysed through a two-way ANOVA, considering the diet and the time as the independent variables. Differences between the averages from significate P values (P<0.05) were obtained using Tukey's multiple comparison test.

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# 3. Results

## 3.1 Growth performance and feed utilization

No signs of pathological complications were observed during the growth trial. Mortality was very low and unaffected by dietary treatment. No differences among dietary treatments were observed concerning the growth performance expressed as final body weight, weight gain, and daily growth index. Nevertheless, the control and FWO4 diets have the highest values of FBW, weight gain, and daily growth index. Feed intake and feed utilization expressed as, feed efficiency and protein efficiency, showed no differences with dietary treatment. Although feed intake values were higher in the control and FWO4 diets, FWO8 showed the highest feed and protein efficiency, increasing circa 6 % relative to the unsupplemented control diet (Table 5).

Nitrogen (N) intake and N retention were not affected by experimental diets, but when N retention, expressed as a percentage of N intake, tends to be higher in fish fed the FWO8 diet. Lipid intake and retention were also not affected by dietary treatment. L intake tends to be higher in the control diet, and L retention expressed as a percentage of lipid intake tends to be higher in fish fed the FWO8 than with the other diets (Table 5).

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**Table 5: Growth performance and feed utilization of European seabass fed the experimental diets[1].**

| Diet | Control | FWO4 | FWO8 | UWO | SEM |
|---|---|---|---|---|---|
| Initial body weight (IBW; g) | 23.0 | 23.0 | 23.0 | 22.9 | 0.02 |
| Final body weight (FBW; g) | 61.7 | 61.6 | 59.1 | 57.5 | 1.01 |
| Weight gain (g/kg ABW /day) | 13.9 | 13.8 | 13.3 | 13.0 | 0.22 |
| Daily growth index[2] | 1.68 | 1.68 | 1.59 | 1.54 | 0.03 |
| Feed intake (g/kg ABW /day) | 19.9 | 19.1 | 18.3 | 18.6 | 0.29 |
| Feed efficiency[3] | 0.70 | 0.72 | 0.73 | 0.70 | 0.01 |
| Protein efficiency ratio[4] | 1.38 | 1.44 | 1.46 | 1.41 | 0.02 |
| Mortality (%) | 0 | 0 | 0 | 1.85 | 0.46 |
| N intake (g/kg ABW /day)[5] | 1.60 | 1.53 | 1.45 | 1.48 | 0.09 |
| N retention (g/kg ABW/day)[6] | 0.42 | 0.40 | 0.40 | 0.37 | 0.03 |
| N retention (% NI)[7] | 26.1 | 25.8 | 27.7 | 25.2 | 1.88 |
| L intake (g/kg ABW /day)[8] | 3.41 | 2.41 | 2.39 | 2.45 | 0.28 |
| L retention (g/kg ABW/day)[9] | 2.64 | 2.41 | 2.39 | 2.45 | 0.22 |
| L retention (% LI)[10] | 77.6 | 77.3 | 81.4 | 80.0 | 6.82 |

[1] Values presented as means (n = 3) and pooled standard error of the mean (SEM). Lack of different superscript letters denotes no statistical differences (One-Way ANOVA, $p > 0.05$).

ABW: average body weight (initial body weight + final body weight) / 2.

[2] FE: wet weight gain/dry feed intake.

[3] DGI: $(FBW^{1/3} - IBW^{1/3})$ / (time in days) $\times$ 100.

[4] PER: wet weight gain/crude protein intake.

[5] NI: (N intake $\times$ 1000) / (ABW $\times$ time in days).

[6] NR (g/kg ABW/day): (FBW $\times$ FBN) $-$ (IBW $\times$ IBN) / (ABW $\times$ time in days).

[7] NR (%NI): (NR/NI) $\times$ 100.

[8] LI: (L intake $\times$ 1000) / (ABW $\times$ time in days).

[9] LR (g/kg ABW/day): (FBW $\times$ FBL) $-$ (IBW $\times$ IBL) / (ABW $\times$ time in days).

[10] LR (%LI) = (LR/LI) $\times$ 100.

(IBN and FBN: initial and final body nitrogen content; IBL and FBL: initial and final body lipid content)

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## 3.2 Whole-body composition

At the end of the trial, whole-body composition was not affected by dietary treatment, but the fish fed the control diet tend to have a higher body lipid content and a lower body ash content than those fed the other diets (Table 6).

No significant statistical differences were observed among experimental diets concerning the hepatosomatic and visceral indexes (Table 6).

**Table 6: Whole-body proximate composition (% wet weight), hepatosomatic and visceral indices (HSI and VI) of seabass fed the experimental diets for 10 weeks.[1]**

| Diet | Initial | Control | FWO4 | FWO8 | UWO | SEM |
|------|---------|---------|------|------|-----|-----|
| *Whole-body composition* | | | | | | |
| Dry matter (%) | 24.9 | 34.8 | 33.3 | 33.6 | 33.4 | 0.26 |
| Protein | 14.7 | 17.3 | 16.7 | 16.9 | 16.6 | 0.12 |
| Lipids | 6.4. | 13.1 | 11.5 | 12.1 | 12.6 | 0.26 |
| Ash | 3.1 | 4.2 | 4.4 | 4.7 | 4.0 | 0.08 |
| *Indices* | | | | | | |
| HSI[2] (%) | — | 1.5 | 1.4 | 1.4 | 1.6 | 0.07 |
| VSI[3] (%) | — | 10.6 | 10.5 | 10.0 | 10.6 | 0.32 |

[1] Values presented as means (n = 3) for whole body composition and (n = 6) for hepatosomatic index (HIS) and viscerosomatic index (VSI)) and pooled standard error of the mean (SEM). Lack of different superscript letters denotes no statistical differences (One-Way ANOVA, p>0.05).

[2] Hepatosomatic index = (liver weight/body weight) × 100

[3] Visceral index = (visceral weight/body weight) × 100.

## 3.3 Digestive enzyme activities

Digestive enzyme activity results were not affected by dietary treatment. Nevertheless, UWO diet showed higher activity of proteases and lipase than the other dietary treatments, but in terms of amylase activity, FWO4 was the highest (Table 7).

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**Table 7: Specific activities (mU/mg protein) of intestinal proteases, amylase, and lipase in the intestine of European seabass fed experimental diets[1].**

| Diet | Control | FWO4 | FWO8 | UWO | SEM |
|---|---|---|---|---|---|
| Proteases | 81.6 | 80.5 | 80.6 | 103.6 | 36.7 |
| Amylase | 37.2 | 46.5 | 39.5 | 42.6 | 17.4 |
| Lipase | 4.1 | 5.3 | 4.7 | 6.9 | 2.60 |

[1] Values presented as means (n = 9) and pooled standard error of the mean (SEM). Lack of different superscript letters denotes no statistical differences (One-Way ANOVA, p>0.05).

## 3.4 Lipid peroxidation

Levels of lipid peroxidation (LPO) in the fillet of European seabass were affected by both diet and time of storage. It was observed that LPO was higher after 3 days than after one night of refrigerated storage, throughout all dietary treatments. At T0, LPO tends to decrease with the presence of the SSF-extract compared to the control diet. Irrespective of the time of storage, fillet of fish fed the diet supplemented with the UWO extract showed lower LPO than those fed the other diets. (Table 8 and Figure 13).

**Table 8: Fillet lipid peroxidation (TBARS; nmols MDA/ g muscle) of European seabass fed the experimental diets after a night or 3 days of refrigerated storage.**

| Storage time | Diets | | | | SEM |
|---|---|---|---|---|---|
| | Control | FWO4 | FWO8 | UWO | |
| Time 0 days | 27.8 | 23.6 | 24.8 | 20.3 | 7.4 |
| Time 3 days | 81.6 | 82.1 | 83.0 | 60.0 | 28.8 |

**Two-way ANOVA**

| | Variation source | | | Diets | | | |
|---|---|---|---|---|---|---|---|
| | Diet | Time | Interaction | Control | FWO4 | FWO8 | UWO |
| TBARS | * | *** | ns | a | ab | ab | b |

SEM: pooled standard error of the mean

Two-way ANOVA: *p < 0.05; ** p < 0.01; *** p < 0.001; ns: non-significant.

TBARS, thiobarbituric acid reactive substances.

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# 4. Discussion

Aquafeed industry has been searching for alternative ways to improve feeds through the incorporation of, for example, functional ingredients with bioactive and antioxidant properties (Citarasu, 2012). The wastes derivative from the olive mill and winery industries that normally have no value must be disposed off. Therefore, they can be recycled and incorporated in aquafeeds contributing to the sustainability of resources and following the guidelines of the Circular Economy (Harris et al., 2018). There has been an effort in reducing the number of synthetic antioxidants and antibiotics used in fish feeds, by replacing them with natural and eco-friendly compounds that enhance the fish immunological status and general health (Bai et al., 2015). This work aimed to evaluate growth performance, feed utilization, digestive, and fillet lipid peroxidation during shelf life of European seabass fed diets supplemented with an extract obtained from olive mill and winery by-products submitted or not to a SSF process.

## 4.1 Growth performance and feed utilization assessment

In the present study, daily growth index, feed intake, and feed efficiency showed no significant statistical differences when the extracts were incorporated in the experimental diets. However, the values were similar to other authors using plant-based protein diets in farmed fish (Castro et al., 2015; Hussain et al., 2020; Vicente et al., 2019). Diets with high quantities of plant-based protein normally result in poorer feed efficiency and growth performance (Kousoulaki et al., 2015). The replacement of marine ingredients, even by highly digested plant protein sources resulted in a reduction of feed intake and so reduction of growth and feed utilization (Dias et al., 2003; Emre et al., 2008; Tibaldi et al., 2003; Valente et al., 2006). Some studies have been pointing out that the previous SSF process may increase the biological value of feedstuffs. For example, Hassaan et al. (2015) concluded that SSF of soybean meal with *S. cerevisiae* improved the nutritional value of this feedstuff. Even though this increased the nutritive value, the dietary incorporation of high levels (75%) of fermented soybean meal showed significantly lower growth performance in Nile tilapia (*Oreochromis niloticus*) compared to those fed with only 50% inclusion levels (Hassaan et al., 2015). Another study in Japanese flounder (*Paralichthys olivaceus*), reported that diets with fermented soybean meal and squid by-products did not negatively affect growth, feed utilization, and health of fish if substitution of FM protein did not surpass 36% (Kader, Koshio, et al., 2012). Furthermore, although no statistical differences were observed, these authors observed

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that feed efficiency and protein efficiency ratio was slightly higher in the two diets with the fermented soybean meal.

The potential of utilization of extract obtained from SSF of by-products in aquafeeds to improve growth, feed utilization, well-being, immune status, or diet/fillet quality has been little studied. Some studies have shown that the incorporation of SSF extracts with bioactive compounds can have positive benefits on feed efficiency. These studies involve mainly the supplementation of enzymes obtained from SSF to hydrolyse complex NSP. Dietary supplementation with exogenous enzymes, produced during SSF, increased nutrient availability in white seabream (*Diplodus sargus*) when fed with plant-based diets (Magalhaes et al., 2016). The application of these enzymatic extracts in turbot (*Psetta maxima*) feeds also improved feed digestibility (Diógenes et al., 2018). For Nile tilapia, the supplementation of diets with an enzymatic complex obtained from SSF increased growth performance and the availability of sucrose and monosaccharides in the chyme (Moura et al., 2012).

Heidarieh et al. (2013) also observed a significant improvement of feed conversion ratio and specific growth rate of rainbow trout (*Oncorhynchus mykiss*) when 0.1% and 1% of Aloe vera extract were included in the feeds.

Regarding feed intake, studies have reported that plant protein-rich diets may be related to a decrease in feed ingestion (Gomes et al., 1993). Nevertheless, in this study, feed intake was high and not affected by the dietary treatments, suggesting that the presence of these extracts in the experimental diets did not negatively influence feed palatability. Similarly, the dietary incorporation of other extracts obtained from SSF also had little impact on the voluntary feed intake (Diógenes et al., 2018; Moura et al., 2012).

## 4.2 Proximate whole-body composition

At the end of the growth trial, fish body composition did not vary significantly between dietary treatments, suggesting that the proximal composition of the fillet should also not have been affected by the SSF-extracts. The final composition of European seabass whole body is within the range previously reported by several authors (Peres et al., 1999b). For fish with an average body weight of 60g, the lipid carcass content of 12% was the amount expected (Kousoulaki et al., 2015). The results obtained agree with other studies, reporting that FM replacements of the levels used in this study do not affect fish composition, in European seabass (Tibaldi et al., 2006), red sea bream (Kader, Bulbul, et al., 2012), rainbow trout (Moniruzzaman et al., 2018).

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Hepatosomatic and visceral indexes were not affected by the inclusion of the SSF-extracts in the diets. The present data is in accordance with other studies carried out in European seabass (Castro et al., 2015), gilthead seabream (*Sparus aurata*) (Koukou et al., 2012), and rainbow trout (Moniruzzaman et al., 2018).

## 4.3 Digestive enzyme activity in the intestine

Fish digestive enzymes can be influenced by feeding habits, diets formulation, antinutritional factors, or even feed preferences (Pavasovic et al., 2007). The effect of SSF-extracts in aquafeeds and its interaction with the digestive function was also evaluated in this study.

Overall, digestive enzyme activity was not affected by dietary treatment. Total protease activity was high, reflecting the carnivorous feeding habits of European seabass, as previously reported by the same species (Perez-Jimenez et al., 2009). Diet composition can influence the predisposition of protease activity, as a partial or total replacement of FM by plant protein sources can induce small (gilthead seabream) or big (rainbow trout) decreases in protease activity, depending on the species studied (Santigosa et al., 2008). Digestive enzyme activity may also be affected by dietary supplementation with exogenous enzymes (Castillo et al., 2015). However, other studies including diets supplemented with exogenous enzymes obtained from SSF did not observe any effect of the endogenous digestive enzyme activities, even though the digestibility of the overall diets was improved (Magalhaes et al., 2016). Contrarily, in turbot, the addition of a SSF enzyme complex to the diet enhanced the activity of amylase, while the activity of proteases and lipase were not affected (Diógenes et al., 2018).

In the present study, the influence of exogenous enzymes, other than cellulase and xylanase, could be considered. Indeed, during the SSF of winery and olive mill wastes the production of other enzymes was observed (Salgado et al., 2014).

Amylase activity also corresponded to the normal values registered in European seabass due to their feeding habits (Magalhães et al., 2015). Amylase activity is also influenced by the diet composition, as different studies have demonstrated higher activities being proportional to the carbohydrates levels (Correa et al., 2007; Fountoulaki et al., 2005; Perez-Jimenez et al., 2009).

Lipase activity changes with the unsaturation degree and the chain length of dietary fatty acids and the data present in this study is in accordance with the literature

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(Castro et al., 2016). No differences were registered between the experimental diets and the UWO treatment showed the highest value. Even though not analysed in the SSF-extracts used in the present study, *Aspergillus ibericus* is also a very good producer of lipase (Oliveira et al., 2016; Salgado et al., 2014). However, no effect of the SSF extract on the endogenous lipase activity was observed.

The focus of the evaluation of the digestive intestinal enzymes of European seabass was to demonstrate if the SSF-extracts included in the diets could influence these enzymes' activity. The data presented in this work shows that the exogenous enzymes segregated by the fungi in the SSF did not affect the activity of the endogenous fish digestive enzymes activity, which is also in accordance with other authors that reported very little interaction between endogenous and exogenous/dietary enzymes (Castillo et al., 2015; Magalhaes et al., 2016). Further research needs to be carried out, to assess and reveal the influence of these exogenous enzymes in the digestive functions of European seabass and other fish species.

## 4.4 Muscle lipid peroxidation levels

The balance between the influence of reactive oxygen species (ROS) and the antioxidant activity can be influenced by diets, inducing lipid, protein, and DNA damage and protein oxidation. Antioxidant enzymes, such as glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase are extremely important for the catalysis of ROS. Besides enzymatic antioxidant defences, fish also have some non-enzymatic defences, including glutathione (GSH) and several vitamins (for example C and E) (Perez-Jimenez et al., 2012). Both systems, the enzymatic and the non-enzymatic systems, contribute to the decrease of oxidation, including lipid peroxidation (Diógenes et al., 2019; Vicente et al., 2019). Lipid peroxidation causes the production of undesirable chemical compounds, such as aldehydes, ketones, and organic acids, which lead to a decrease in shelf-life and nutritional value of fish (lipid-containing food product) (Lourenço et al., 2019).

Lourenço et al. (2019) reviewed the role of natural antioxidants from plant sources in the food industry and their effects and applications on preservation and packaging. The antioxidative defence mechanism can be boosted by the intake of exogenous antioxidants, such as ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), carotenoids, and polyphenols found in fruits, vegetables, beverages, and cereals. Phenolic compounds present in these plant-based sources are varied, such as, ferulic acid,

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vanillin, gallic acid, caffeic acid, tannins, and flavonoids that not only have antioxidant properties but also present antimicrobial and antifungal activities. These antioxidant compounds can also be found in olive pomace and grape pomace. Olive pomace is remarkably rich in polyphenols and other compounds, including hydroxytyrosol and tyrosol derivatives, iridoid precursors, seicoridoids and derivatives, flavonoids, tocopherols, and lignans (Tripoli et al., 2005). Grape pomace also has several antioxidants such as anthocyanins, catechins, flavanols, gallic acid, vanillic acid, syringic acid, protocatechuic acid, epicatechin, and trans-resveratrol (Brezoiu et al., 2019).

In the food industry, synthetic antioxidants are often used in place of natural ones due to their availability, low cost, and high performance, and stability. Butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, and tert-butyl hydroquinone are some of the most used (Lourenço et al., 2019). Nevertheless, studies have reported a relationship between the long-term intake of synthetic antioxidants and health issues (Botterweck et al., 2000). So, there is a trend in the substitution of natural antioxidants. Food grade antioxidants must be approved by regulatory bodies, classifying them as GRAS (generally regarded as safe), but these compounds must be within strict rules to be considered as such; odour, colour, flavour cannot be affected and $LD_{50}$ needs to be lower than 1000mg/kg body weight. Some natural antioxidants have lower antioxidant activities than their synthetic counterparts, but that does not mean they are not a valuable alternative, providing they are used under the regulatory limits (Lourenço et al., 2019). Recently, the EU commission has been taking action and regulating, even more, the use of several synthetic antioxidants that are raising suspicion, regarding their safety as additives in the food industry. Ethoxyquin is a synthetic antioxidant that inhibits the oxidation of highly unsaturated fatty acids in fish meal and fish silage. EU Commission has suspended the use of this compound as a feed additive for all animal species and categories, due to a lack of data to fully assess the safety of the substance, (Byrne, 2017; Hals et al., 2020). Also, in fish, some evidence exists of the immune modulator effect of this antioxidant on fish immune status. In tilapia, the effect of dietary inclusion of ethoxyquin, even at the approved levels of ethoxyquin in feeds, leads to immunosuppression (Yamashita et al., 2009). Some alternatives have been developed, for example, the one developed by Borregaard, containing formic acid/lignosulphonic acid and propyl gallate, that can substitute completely ethoxyquin in feeds, although it also is a synthetic compound (Hals et al., 2020).

The use of antioxidants to retard the oxidation and stabilized feed during shelf-life is very important, particularly for aquafeeds that are rich in fish meal and oil rich in highly long chained polyunsaturated fatty acids (PUFA). PUFA are highly susceptible to

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oxidation by oxygen, making fish meal and oil especially prone to oxidation. In the present study, even though the diet oxidation through the shelf-life was not evaluated, it was observed that both extracts, but in particular the unfermented extract, had a positive effect on the reduction of fillet LPO when comparing to the un-supplemented control diet. Therefore, it is possible to affirm that the antioxidants and phenolic compounds present in the SSF-extracts may have exerted a protective effect of muscle lipids against oxidation. The potential of wine and olive oil by-products to reduce oxidation in minced fish and frozen mackerel fillets was already studied (Sánchez-Alonso et al., 2007; Tang et al., 2001), but to the best of our knowledge, this is the first study evidencing the potential of using these by-products as a feed additive to reduce the fillet lipid oxidation. Fish fillet is very susceptive to oxidation, due to the high level of PUFA. Fillet lipid oxidation is an important food quality issue, and oxidation leads to quality losses, associated with unpalatable flavour and odour, shortening of shelf life, losses of nutritional values (e.g., loss of polyunsaturated fatty acids, PUFAs), and possible production of unhealthy molecules are some of the extensive consequences of lipid oxidation in foods (Secci et al., 2016).

Interestingly, lower fish fillet lipid oxidation was also registered in the unfermented mixture, confirming the presence of potent antioxidants in these by-products. The potential use of natural antioxidant extracts from different plants has been studied. For example, a study on common carp fingerlings with supplementation of polyphenols collected from cabbage in a canola-based meal reported a significant increase in antioxidant activity (Hussain et al., 2020). Other studies that included purified quercetin (polyphenol) from onion, extract of oregano (carvacrol and thymol) and orange peel fragment (contains carotenoids, phenolic compounds, and flavonoids with antioxidant capacity) in their experimental diets registered an increase in the levels of superoxide dismutase, catalase (antioxidant enzymes), and also plasma lysozyme on channel catfish and Nile tilapia (Shin et al., 2010; Vicente et al., 2019; Zheng et al., 2009). Another study on gilthead sea bream, also reported that superoxide dismutase activity increased in the liver and higher hepatic catalase activity was registered, using white tea and methionine (Perez-Jimenez et al., 2012).

The potential of olive oil and winery by-products to obtain functional ingredients for aquaculture have been little studied. The effect of OP and OP oil on growth performance, fatty acid composition, and cardioprotective properties of gilthead sea bream and sea bass showed increased biological activity against platelet aggregation (Nasopoulou et al., 2011). More recently, Sioriki et al. (2016) also reported that dietary OP inclusion counteracts the action of the platelet-activating factor enzyme. GM

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extracted polyphenols, such as flavanols are potent inhibitors of oxidation in frozen mackerel fish muscle, revealing to have a similar antioxidant capacity to that of propyl gallate (Pazos et al., 2005). Another recent study, used red grape extracts as an additive of experimental diets to test its effects on intestinal and spleen immune responses of seabass, reporting that proanthocyanidins and catechins present on the extract generate lower levels of intestinal proinflammatory cytokines and induces a higher protective spleen response, boosting the overall immunological status of the fish, which results in higher survivability and higher quality of meat (Magrone et al., 2016). Our results corroborate these, suggesting a high antioxidant potential, reducing muscle lipid oxidation during shelf-life. However, further investigation of the influence of fermented and non-fermented extracts have on the antioxidant responses is needed to better assess the effects and role of specific polyphenols present in OP and GM.

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# 5. Conclusion

The present study tested the potential of two extracts obtained from an optimized mixture of olive pomace (OP) and grape marc (GM) submitted or not to a solid-state fermentation process with *Aspergillus ibericus*, as a functional ingredient for European seabass feeds (FWO and UWO extract, respectively). The effect of these extracts was evaluated through the assessment of growth performance, whole-body composition, digestive enzymes, and filet lipid peroxidation during the shelf-life.

It can be concluded that the zootechnical parameters, measured as weight gain, daily growth, feed intake, and feed efficiency were not affected by both extracts. The juvenile's growth was within the higher range observed in other studies with this species fed plant protein-rich based diets. voluntary feed intake was not affected by the dietary inclusion of FWO or UWO extracts, indicating that no palatability issues seem to have occurred.

At the end of the growth trial, whole-body composition, and somatic indices (HIS and VSI) were similar among dietary treatments.

Digestive enzyme activity of amylase, lipase, and total proteases were also analysed, and the dietary treatment did not affect their activities.

Fillet lipid peroxidation levels showed that the diet with unfermented extract (UWO) led to lower fillet LPO than the SSF-extract (FWO), during the shelf-life.

Overall, this study showed that wineries and olive mill by-products, with little economic and biological value, have a potential to decrease the lipid peroxidation of European seabass fillet, suggesting a potential increase of the antioxidant value, without negatively influencing growth and feed utilization, whole-body composition, and digestive enzyme activity.

The development of novel natural antioxidants for animal feed to substitute the synthetic antioxidants, generally used in aquafeeds, is of particular importance due to the recent authorisation suspension, by the EU commission, to use some synthetic antioxidants, as ethoxyquin, for all animal species, since 2017. So, the application potential of these extracts as a feed natural antioxidant, during the shelf-life period should also be studied. Furthermore, studies focusing on the immunologic state of fish using fermented extracts on the diets and organoleptic analysis of the muscle tissues are needed, so the potential of these extracts can be further researched.

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The reutilisation of wineries and olive mill by-products to produce antioxidant-rich ingredients is a step in the path to a more sustainable and environmentally friendly world and in the project of the EU to a Circular Economy.

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# References

Adeola, O., & Cowieson, A. J. (2011). Opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. *J Anim Sci, 89*(10), 3189-3218.

Ahmed, N., & Thompson, S. (2019). The blue dimensions of aquaculture: A global synthesis. *Science of The Total Environment, 652*, 851-861.

Allan, G. L., Parkinson, S., Booth, M. A., Stone, D. A. J., Rowland, S. J., Frances, J., & Warner.Smith, R. (2000). Replacement of fish meal in diets for Australian silver perch, Bidyanus bidyanus: I. Digestibility of alternative ingredients. *Aquaculture, 186*(3-4), 293-310.

AOAC. (2019). *Official methods of analysis of AOAC international - 21st Edition, 2019* (J. George W. Latimer Ed.  Vol. 1).

Bai, S. C., Katya, K., & Yun, H. (2015). Additives in aquafeed: An overview. In D. A. Davis (Ed.), *Feed and Feeding Practices in Aquaculture* (pp. 171-202). Oxford: Woodhead Publishing.

Botterweck, A., Verhagen, H., Goldbohm, R., Kleinjans, J., & Brandt, P. V. D. (2000). Intake of butylated hydroxyanisole and butylated hydroxytoluene and stomach cancer risk: results from analyses in the Netherlands Cohort Study. *Food Chemistry Toxicology, 38*, 599-605.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1-2), 248-254.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a Free Radical Method to Evaluate Antioxidant Activity. *Laboratoire de Chimie des Substances Naturelles, 28*, 25-30.

Brezoiu, A. M., Matei, C., Deaconu, M., Stanciuc, A. M., Trifan, A., Gaspar-Pintiliescu, A., & Berger, D. (2019). Polyphenols extract from grape pomace. Characterization and valorisation through encapsulation into mesoporous silica-type matrices. *Food and Chemical Toxicology, 133*.

Bucić-Kojić, A., Sovová, H., Planinić, M., & Tomas, S. (2013). Temperature-dependent kinetics of grape seed phenolic compounds extraction: Experiment and model. *Food Chemistry, 136*(3-4), 1136-1140.

Buege, J. A., & Aust, S. D. (1978). Microsomal lipid peroxidation. *Methods in Enzymology, 52*, 302-310.

Bulfon, C., Volpatti, D., & Galeotti, M. (2015). Current research on the use of plant-derived products in farmed fish. *Aquaculture Research, 46*(3), 513-551.

Byrne, J. (2017). Ethoxyquin authorization suspended in the EU – are there viable alternatives? Retrieved 10/11/2020 https://www.feednavigator.com/Article/2017/06/12/Ethoxyquin-authorization-suspended-in-the-EU-are-there-viable-alternatives#

Cabrera, A. G., Cordoba, U., Heuzé, V., & AFZ. (2015). Olive oil cake and by-products.  Retrieved 10/12/2019 https://www.feedipedia.org/node/32

Castillo, S., & Gatlin, D. M. (2015). Dietary supplementation of exogenous carbohydrase enzymes in fish nutrition: A review. *Aquaculture, 435*, 286-292.

Castro, C., Corraze, G., Perez-Jimenez, A., Larroquet, L., Cluzeaud, M., Panserat, S., & Oliva-Teles, A. (2015). Dietary carbohydrate and lipid source affect cholesterol metabolism of European sea bass (Dicentrarchus labrax) juveniles. *British Journal of Nutrition, 114*(8), 1143-1156.

Castro, C., Couto, A., Perez-Jimenez, A., Serra, C. R., Diaz-Rosales, P., Fernandes, R., Corraze, G., Panserat, S., & Oliva-Teles, A. (2016). Effects of fish oil replacement by vegetable oil

FCUP | 48
Potential of solid-state fermented wineries and olive oil by-products
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blend on digestive enzymes and tissue histomorphology of European sea bass
(Dicentrarchus labrax) juveniles. *Fish Physiology and Biochemistry, 42*(1), 203-217.

Citarasu, T. (2012). Natural antimicrobial compounds for use in aquaculture. In *Infectious disease in aquaculture*.

Commission, E. (2019). Circular Economy Strategy - Closing the loop - An ambitious EU Circular Economy Package. Retrieved 30/11/2019 https://ec.europa.eu/environment/circular-economy/

Correa, C. F., de Aguiar, L. H., Lundstedt, L. M., & Moraes, G. (2007). Responses of digestive enzymes of tambaqui (Colossoma macropomum) to dietary cornstarch changes and metabolic inferences. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology, 147*(4), 857-862.

Dawood, M. A. O., & Koshio, S. (2016). Recent advances in the role of probiotics and prebiotics in carp aquaculture: A review. *Aquaculture, 454*, 243-251.

Debnath, D., Sahu, N. P., Pal, A. K., Baruah, K., Yengkokpam, S., & Mukherjee, S. C. (2005). Present scenario and future prospects of phytase in aquafeed - Review. *Asian-Australasian Journal of Animal Sciences, 18*(12), 1800-1812.

Devesa-Rey, R., Vecino, X., Varela-Alende, J. L., Barral, M. T., Cruz, J. M., & Moldes, A. B. (2011). Valorization of winery waste vs. the costs of not recycling. *Waste Management, 31*, 2327-2335.

Dias, J., Alvarez, M. J., Diez, A., Arzel, J., Corraze, G., Bautista, J. M., & Kaushik, S. J. (1998). Regulation of hepatic lipogenesis by dietary protein/energy in juvenile European seabass (Dicentrarchus labrax). *Aquaculture, 161*, 169-186.

Dias, J., Arzel, J., Aguirre, P., Corraze, G., & Kaushik, S. (2003). Growth and hepatic acetyl coenzyme-A carboxylase activity are affected by dietary protein level in European seabass (Dicentrarchus labrax). *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 135*(1), 183-196.

Dias, J., Gomes, E. F., & Kaushik, S. J. (1997). Improvement of feed intake through supplementation with an attractant mix in European seabass fed plant-protein rich diets. *Aquatic Living Resources, 10*(6), 385-389.

Diógenes, A. F., Basto, A., Estevão-Rodrigues, T. T., Moutinho, S., Aires, T., Oliva-Teles, A., & Peres, H. (2019). Soybean meal replacement by corn distillers dried grains with solubles (DDGS) and exogenous non-starch polysaccharidases supplementation in diets for gilthead seabream (Sparus aurata) juveniles. *Aquaculture, 500*, 435-442.

Diógenes, A. F., Castro, C., Carvalho, M., Magalhães, R., Estevão-Rodrigues, T. T., Serra, C. R., Oliva-Teles, A., & Peres, H. (2018). Exogenous enzymes supplementation enhances diet digestibility and digestive function and affects intestinal microbiota of turbot ( Scophthalmus maximus ) juveniles fed distillers' dried grains with solubles (DDGS) based diets. *Aquaculture, 486*, 42-50.

Emre, Y., Sevgili, H., & Sanli, M. (2008). A preliminary study on the utilization of hazelnut meal as a substitute for fish meal in diets of European sea bass (Dicentrarchus labrax L.). *Aquaculture Research, 39*(3), 324-328.

Enes, P., Panserat, S., Kaushik, S., & Oliva-Teles, A. (2011). Dietary Carbohydrate Utilization by European Sea Bass (Dicentrarchus labrax L.) and Gilthead Sea Bream (Sparus aurata L.) Juveniles. *Reviews in Fisheries Science, 19*(3), 201-215.

EUMOFA. (2018). The EU fish market. *European Market Observatory for fisheries and aquaculture 2018 edition*, 115.

Eurostat. (2019). Statistics Explained - Fishery Statistics. Retrieved 15/04/2020 https://ec.europa.eu/eurostat/statisticsexplained/

FAO. (2016). *The State of World Fisheries and Aquaculture 2016 - Contributing to food security and nutrition for all*. Retrieved from Rome:

FCUP 49
Potential of solid-state fermented wineries and olive oil by-products
as functional ingredients for European seabass feeds

FAO. (2018). *The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals*. Retrieved from Rome:

FAO. (2019). European seabass - *Dicentrarchus labrax*. Retrieved 30/11/2019 http://www.fao.org/fishery/affris/species-profiles/european-seabass/european-seabass-home/en/

Filipe, D., Peres, H., Castro, C., & Seara, J. (2019). *Optimization of solid-state fermentation of winery and olive mill by-products to produce enzymatic and phenolic value added products - its application to aquafeed.* (Master). Faculty of Sciences of the University of Porto,

Fleuri, F. L., Kawaguti, H. Y., Vianello, F., Novelli, P. K., & Okino-Delgado, O. K. (2013). Exploration of Microorganisms Producing Bioactive Molecules of Industrial Interest by Solid State Fermentation. *Food Quality, Safety and Technology*.

Fountoulaki, E., Alexis, M. N., Nengas, I., & Venou, B. (2005). Effect of diet composition on nutrient digestibility and digestive enzyme levels of gilthead sea bream (Sparus aurata L.). *Aquaculture Research, 36*(13), 1243-1251.

Froese, R., & Pauly, D. (2019). FishBase. Retrieved 09/09/2020 https://www.fishbase.se/Summary/SpeciesSummary.php?ID=63&AT=european+seabass

Fry, J. P., Love, D. C., MacDonald, G. K., West, P. C., Engstrom, P. M., Nachman, K. E., & Lawrence, R. S. (2016). Environmental health impacts of feeding crops to farmed fish. *Environ Int, 91*, 201-214.

Furiga, A., Lonvaud-Funel, A., & Badet, C. (2009). In vitro study of antioxidant capacity and antibacterial activity on oral anaerobes of a grape seed extract. *Food Chemistry, 113*.

Gatlin, D. M., Barrows, F. T., Brown, P., Dabrowski, K., Gaylord, T. G., Hardy, R. W., Herman, E., Hu, G. S., Krogdahl, A., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, E. J., Stone, D., Wilson, R., & Wurtele, E. (2007). Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquaculture Research, 38*(6), 551-579.

Glencross, B., Blyth, D., Tabrett, S., Bourne, N., Irvin, S., Anderson, M., Fox-Smith, T., & Smullen, R. (2012). An assessment of cereal grains and other starch sources in diets for barramundi (Lates calcarifer) - implications for nutritional and functional qualities of extruded feeds. *Aquaculture Nutrition, 18*(4), 388-399.

Gomes, E. F., Corraze, G., & Kaushik, S. (1993). Effects of dietary incorporation of a co-extruded plant protein (rapeseed and peas) on growth, nutrient utilization and muscle fatty acid composition of rainbow trout (Oncorhynchus mykiss). *Aquaculture, 113*(4), 339-353.

Graminha, E. B. N., Gongalves, A. Z. L., Pirota, R., Balsalobre, M. A. A., Da Silva, R., & Gomes, E. (2008). Enzyme production by solid-state fermentation: Application to animal nutrition. *Animal Feed Science and Technology, 144*(1-2), 1-22.

Guerreiro, I., Serra, C. R., Oliva-Teles, A., & Enes, P. (2017). Short communication: gut microbiota of European sea bass (Dicentrarchus labrax) is modulated by short-chain fructooligosaccharides and xylooligosaccharides. *Aquaculture International, 26*(1), 279-288.

Hals, K., & Lindahl, S. H. (2020). Ethoxyquin ban in the EU: Are there viable alternatives? Retrieved 10/11/2020 https://aquafeed.co.uk/entrada/ethoxyquin-ban-in-the-eu-are-there-viable-alternatives-21749

Hamre, K., Kolas, K., & Sandnes, K. (2010). Protection of fish feed, made directly from marine raw materials, with natural antioxidants. *Food Chemistry, 119*, 270-278.

Harris, S., Staffas, L., Rydberg, T., & Eriksson, E. (2018). Renewable Materials in the Circular Economy. *IVL Swedish Environmental Research Institute*.

FCUP | 50

Potential of solid-state fermented wineries and olive oil by-products
as functional ingredients for European seabass feeds

Hassaan, M. S., Soltan, M. A., & Abdel-Moez, A. M. (2015). Nutritive value of soybean meal after solid state fermentation with Saccharomyces cerevisiae for Nile tilapia, Oreochromis niloticus. *Animal Feed Science and Technology, 201*, 89-98.

Heidarieh, M., Mirvaghefi, A. R., Akbari, M., Sheikhzadeh, N., Kamyabi-Moghaddam, Z., Askari, H., & Shahbazfar, A. A. (2013). Evaluations of Hilyses, fermented Saccharomyces cerevisiae, on rainbow trout (Oncorhynchus mykiss) growth performance, enzymatic activities and gastrointestinal structure. *Aquaculture Nutrition, 19*(3), 343-348.

Hidalgo, M. C., Urea, E., & Sanz, A. (1999). Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. *Aquaculture, 170*(3-4), 267-283.

Hussain, S. M., Gohar, H., Rasul, A., Shahzad, M. M., Akram, A. M., Tariq, M., Hussain, M., Ali, M., & Khalid, A. (2020). Effect of polyphenols supplemented canola meal based diet on growth performance, nutrient digestibility and antioxidant activity of common carp (Cyprinus carpio Linnaeus, 1758) fingerlings. *Indian J. Fish., 67*(1), 72-79.

INE. (2018). Estatísticas da Pesca.

INE. (2019).  Retrieved 30/11/2019
https://ine.pt/xportal/xmain?xpgid=ine_tema&xpid=INE&tema_cod=1510

Kader, M. A., Bulbul, M., Koshio, S., Ishikawa, M., Yokoyama, S., Nguyen, B. T., & Komilus, C. F. (2012). Effect of complete replacement of fishmeal by dehulled soybean meal with crude attractants supplementation in diets for red sea bream, Pagrus major. *Aquaculture, 350*, 109-116.

Kader, M. A., Koshio, S., Ishikawa, M., Yokoyama, S., Bulbul, M., Nguyen, B. T., Gao, J., & Laining, A. (2012). Can fermented soybean meal and squid by-product blend be used as fishmeal replacements for Japanese flounder (Paralichthys olivaceus)? *Aquaculture Research, 43*(10), 1427-1438.

Kaushik, S. J., Coves, D., Dutto, G., & Blanc, D. (2004). Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, Dicentrarchus labrax. *Aquaculture, 230*(1-4), 391-404.

Kaushik, S. J., Gouillou-Coustans, M. F., & Cho, C. Y. (1998). Application of the recommendations on vitamin requirements of finfish by NRC (1993) to salmonids and sea bass using practical and purified diets. *Aquaculture, 161*(1-4), 463-474.

Koukou, F., Rigos, G., Henry, M. A., & Kentouri, M. (2012). Growth performance, feed utilization and non-specific immune response of gilthead sea bream (Sparus aurata L.) fed graded levels of a bioprocessed soybean meal. *Aquaculture, 364*, 74-81.

Kousoulaki, K., Sæther, B. S., Albrektsen, S., & Noble, C. (2015). Review on European sea bass (Dicentrarchus labrax, Linnaeus, 1758) nutrition and feed management: a practical guide for optimizing feed formulation and farming protocols. *Aquaculture Nutrition*, n/a-n/a.

Leite, P., Salgado, J. M., Venancio, A., Dominguez, J. M., & Belo, I. (2016). Ultrasounds pretreatment of olive pomace to improve xylanase and cellulase production by solid-state fermentation. *Bioresource Technology, 214*, 737-746.

Lourenço, S. C., Moldão-Martins, M., & Alves, V. D. (2019). Antioxidants of Natural Plant Origins: From Sources to Food Industry Applications. *Molecules, 24*.

Magalhães, R., Coutinho, F., Pousão-Ferreira, P., Aires, T., Oliva-Teles, A., & Peres, H. (2015). Corn distiller's dried grains with solubles: Apparent digestibility and digestive enzymes activities in European seabass (Dicentrarchus labrax) and meagre (Argyrosomus regius). *Aquaculture, 443*, 90-97.

Magalhaes, R., Lopes, T., Martins, N., Diaz-Rosales, P., Couto, A., Pousao-Ferreira, P., Oliva-Teles, A., & Peres, H. (2016). Carbohydrases supplementation increased nutrient utilization in white seabream (Diplodus sargus) juveniles fed high soybean meal diets. *Aquaculture, 463*, 43-50.

FCUP | 51
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as functional ingredients for European seabass feeds

Magrone, T., Fontana, S., Laforgia, F., Dragone, T., Jirillo, E., & Passantino, L. (2016). Administration of a Polyphenol-Enriched Feed to Farmed Sea Bass (Dicentrarchus labrax L.) Modulates Intestinal and Spleen Immune Responses. *Oxidative Medicine and Cellular Longevity, 2016*, 11.

Mateo, J., & Maicas, S. (2015). Valorization of winery and oil mill wastes by microbial technologies. *Food Research International, 73*.

Miller, R. B., Sinclair, A. C., & Hochachka, P. W. (1959). Diet, glycogen reserves and resistence to fatigue in hatchery rainbow trout. *Journal of Fisheries Research Board of Canada 16:321.*

Molina-Alcaide, E., & Yáñez-Ruiz, D. (2008). Potential use of olive by-products in ruminant feeding: A review. *Animal Feed Science & Technology, 147*.

Moniruzzaman, M., Bae, J. H., Won, S. H., Cho, S. J., Chang, K. H., & Bai, S. C. (2018). Evaluation of solid-state fermented protein concentrates as a fish meal replacer in the diets of juvenile rainbow trout, Oncorhynchus mykiss. *Aquaculture Nutrition, 24*(4), 1198-1212.

Moura, G. D. S., Lanna, E. A. T., Filer, K., Falkoski, D. L., Donzele, J. L., Oliveira, M. G. D. A., & Rezende, S. T. D. (2012). Effects of enzyme complex SSF (solid state fermentation) in pellet diets for *Nile tilapia*. *Revista Brasileira de Zootecnia, 41*(10), 2139-2143.

Murad, H. A., & Azzaz, H. H. (2010). Cellulase and Dairy Animal Feeding. *Biotechnology, 9*(3), 238-256.

Nasopoulou, C., Stamatakis, G., Demopoulos, C. A., & Zabetakis, I. (2011). Effects of olive pomace and olive pomace oil on growth performance, fatty acid composition and cardio protective properties of gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). *Food Chemistry, 129*(3), 1108-1113.

Naylor, R. L., Goldburg, R. J., Primavera, J. H., Kautsky, N., Beveridge, M. C. M., Clay, J., Folke, C., Lubchenco, J., Mooney, H., & Troell, M. (2000). Effect of aquaculture on world fish supplies. *Nature, 405*(6790), 1017-1024.

Naylor, R. L., Hardy, R. W., Bureau, D. P., Chiu, A., Elliott, M., Farrell, A. P., Forster, I., Gatlin, D. M., Goldburg, R. J., Hua, K., & Nichols, P. D. (2009). Feeding aquaculture in an era of finite resources. *Proceedings of the National Academy of Sciences, 106*(36), 15103-15110.

Nogales, R., Cifuentes, C., & Benitez, E. (2005). Vermicomposting of Winery Wastes: A Laboratory Study. *Journal of Environmental Science and Health Part B, 40*(4), 659-673.

Novelli, P. K., Barros, M. M., & Fleuri, F. L. (2015). Novel inexpensive fungi proteases: Production by solid state fermentation and characterization. *Food Chemistry, 198*(2016), 119-124.

Novelli, P. K., Barros, M. M., Pezzato, L. E., de Araujo, E. P., Botelho, R. D., & Fleuri, L. F. (2017). Enzymes produced by agro-industrial co-products enhance digestible values for Nile tilapia (Oreochromis niloticus): A significant animal feeding alternative. *Aquaculture, 481*, 1-7.

NRC. (2011). *Nutrient Requirements of Fish and Shrimp*: The National Academies Press.

Oliva-Teles, A. (2000). Recent advances in European sea bass and gilthead sea bream nutrition. *Aquaculture International, 8*(6), 477-492.

Oliva-Teles, A., & Pimentel-Rodrigues, A. (2004). Phosphorus requirement of European sea bass (Dicentrarchus labrax L.) juveniles. *Aquaculture Research, 35*(7), 636-642.

Oliveira, F., Moreira, C., Salgado, J. M., Abrunhosa, L., Venâncio, A., & Belo, I. (2016). Olive pomace valorization by Aspergillus species: lipase production using solid‐state fermentation. *Journal of the Science of Food and Agriculture*.

Pandey, A. (2003). Solid-state fermentation. *Biochemical Engineering Journal, 13*, 81-84.

Pavasovic, A., Anderson, A. J., Mather, P. B., & Richardson, N. A. (2007). Influence of dietary protein on digestive enzyme activity, growth and tail muscle composition in redclaw crayfish, Cherax quadricarinatus (von Martens). *Aquaculture Research, 38*, 644-652.

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Potential of solid-state fermented wineries and olive oil by-products
as functional ingredients for European seabass feeds

Pazos, M., Gallardo, J. M., Torres, J. L., & Medina, I. (2005). Activity of grape polyphenols as inhibitors of the oxidation of fish lipids and frozen fish muscle. *Food Chemistry, 92*(3), 547-557.

Peres, H., & Oliva-Teles, A. (2007). Effect of the dietary essential amino acid pattern on growth, feed utilization and nitrogen metabolism of European sea bass (Dicentrarchus labrax). *Aquaculture, 267*(1-4), 119-128.

Peres, M. H., & Oliva-Teles, A. (1999a). Effect of dietary lipid level on growth performance and feed utilization by European sea bass juveniles (Dicentrarchus labrax). *Aquaculture, 179*(1-4), 325-334.

Peres, M. H., & Oliva-Teles, A. (1999b). Influence of temperature on protein utilization in juvenile European seabass (Dicentrarchus labrax). *Aquaculture, 170*(3-4), 337-348.

Perez-Jimenez, A., Cardenete, G., Morales, A. E., Garcia-Alcazar, A., Abellan, E., & Hidalgo, M. C. (2009). Digestive enzymatic profile of Dentex dentex and response to different dietary formulations. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology, 154*(1), 157-164.

Perez-Jimenez, A., Peres, H., Rubio, V. C., & Oliva-Teles, A. (2012). The effect of dietary methionine and white tea on oxidative status of gilthead sea bream (Sparus aurata). *British Journal of Nutrition, 108*(7), 1202-1209.

Perez, L., Gonzalez, H., Jover, M., & Fernandez-Carmona, J. (1997). Growth of European sea bass fingerlings (Dicentrarchus labrax) fed extruded diets containing varying levels of protein, lipid and carbohydrate. *Aquaculture, 156*(3-4), 183-193.

Roig, A., Cayuela, M. L., & Sánchez-Monedero, M. A. (2006). An overview on olive mill wastes and their valorization methods. *Waste Management, 26*, 960-969.

Salgado, J. M., Abrunhosa, L., Venâncio, A., Domínguez, J. M., & Belo, I. (2013). Screening of winery and olive mill wastes for lignocellulolytic enzyme production from Aspergillus species by solid-state fermentation. *Biomass Conversion and Biorefinery, 4*, 201-209.

Salgado, J. M., L., A., Venâncio, A., Domínguez, J. M., & Belo, I. (2014). Screening of winery and olive mill wastes for lignocellulolytic enzyme production from *Aspergillus* species by solid-state fermentation. *Biomass Conversion and Biorefinery, 4*(3), 201-209.

Sánchez-Alonso, I., Jiménez-Escrig, A., Saura-Calixto, F., & Borderías, A. J. (2007). Effect of grape antioxidant dietary fibre on the prevention of lipid oxidation in minced fish: Evaluation by different methodologies. *Food Chemistry, 101*(1), 372-378.

Santigosa, E., Sanchez, J., Medale, F., Kaushik, S., Perez-Sanchez, J., & Gallardo, M. A. (2008). Modifications of digestive enzymes in trout (Oncorhynchus mykiss) and sea bream (Sparus aurata) in response to dietary fish meal replacement by plant protein sources. *Aquaculture, 282*(1-4), 68-74.

Secci, G., & Parisi, G. (2016). From farm to fork: lipid oxidation in fish products. A review. *Italian Journal of Animal Science, 15*(1), 124-136.

Serra, R., Cabañes, F. J., Perrone, G., Castellá, G., Venâncio, A., Mule, G., & Kozakiewicz, Z. (2006). *Aspergillus ibericus*: A new species of section Nigri isolated from grapes. *MYCOLOGIA, 98*(2), 295-306.

Shin, H. S., Yoo, J. H., Min, T. S., Lee, J., & Choi, C. Y. (2010). Effect of Quercetin on the Activity and mRNA Expression of Antioxidant Enzymes and Physiological Responses in Olive Flounder (Paralichthys olivaceus) Exposed to Cadmium. *Asian-Australasian Journal of Animal Sciences, 23*(6), 742-749.

Singhania, R. R., Patel, A. K., Soccol, C., & Pandey, A. (2008). Recent advances in solid-state fermentation. *Biochemical Engineering Journal, 44*(1), 13-18.

Sinha, A. K., Kumar, V., Makkar, H. P. S., De Boeck, G., & Becker, K. (2011). Non-starch polysaccharides and their role in fish nutrition – A review. *Food Chemistry, 127*(4), 1409-1426.

FCUP | 53
Potential of solid-state fermented wineries and olive oil by-products
as functional ingredients for European seabass feeds

Sioriki, E., Smith, T. K., Demopoulos, C. A., & Zabetakis, I. (2016). Structure and cardioprotective activities of polar lipids of olive pomace, olive pomace-enriched fish feed and olive pomace fed gilthead sea bream (*Sparus aurata*). *Food Research International, 83*, 143-151.

Tacon, A. G. J. (2019). Trends in Global Aquaculture and Aquafeed Production: 2000–2017. *Reviews in Fisheries Science & Aquaculture*, 1-14.

Tacon, A. G. J., & Metian, M. (2015). Feed Matters: Satisfying the Feed Demand of Aquaculture. *Reviews in Fisheries Science & Aquaculture, 23*(1), 1-10.

Tang, S., Sheehan, D., Buckley, D. J., Morrissey, P. A., & Kerry, J. P. (2001). Anti-oxidant activity of added tea catechins on lipid oxidation of raw minced red meat, poultry and fish muscle. *International Journal of Food Science & Technology, 36*(6), 685-692.

Tibaldi, E., Hakim, Y., Uni, Z., Tulli, F., de Francesco, M., Luzzana, U., & Harpaz, S. (2006). Effects of the partial substitution of dietary fish meal by differently processed soybean meals on growth performance, nutrient digestibility and activity of intestinal brush border enzymes in the European sea bass (Dicentrarchus labrax). *Aquaculture, 261*(1), 182-193.

Tibaldi, E., Tulli, F., Piccolo, G., & Guala, S. (2003). Wheat gluten as a partial substitute for fish meal protein in sea bass (D. labrax) diets. *Italian Journal of Animal Science, 4 (suppl. 2)*, 613-615.

Tripoli, E., Giammanco, M., Tabacchi, G., & Majo, D. D. (2005). The phenolic compounds of olive oil: Structure, biological activity and beneficial effects on human health. *Nutrition Research Reviews, 18*(1), 98-112.

Valente, L., Gouveia, A., Rema, P., Matos, J., Gomes, E., & Pinto, I. (2006). Evaluation of three seaweeds Gracilaria bursa-pastoris, Ulva rigida and Gracilaria cornea as dietary ingredients in European sea bass (Dicentrarchus labrax) juveniles. *Aquaculture, 252*(1), 85-91.

Valente, L. M. P., Dinis, M. T., & Gonçalves, F. H. (2015). Aquacultura em Portugal: presente e perspetivas futuras.

Vicente, I. S. T., Fleuri, F. L., Carvalho, P. L. P. F., Guimarães, M. G., Naliato, R. F., Müller, H. C., Sartori, M. M. P., Pezzato, L. E., & Barros, M. M. (2019). Orange peel fragment improves antioxidant capacity and haematological profile of Nile tilapia subjected to heat/dissolved oxygen‐induced stress. *Aquaculture Research, 50*, 80-92.

Wilson, R. P. (1994). Utilization of Dietary Carbohydrate by Fish. *Aquaculture, 124*(1-4), 67-80.

Yamashita, Y., Katagiri, T., Pirarat, N., Futami, K., Endo, M., & Maita, M. (2009). The synthetic antioxidant, ethoxyquin, adversely affects immunity in tilapia (Oreochromis niloticus). *Aquaculture Nutrition, 15*(2), 144-151.

Yazid, N. A., Barrena, R., Komilis, D., & Sánchez, A. (2017). Solid-State Fermentation as a Novel Paradigm for Organic Waste Valorization: A Review. *Sustainability, 9*(2), 224.

Zept, F., & Jin, B. (2011). Bioconversion of Grape Marc into Protein Rich Animal Feed by Microbial Fungi. *Chem. Eng. Process Tech, 1*, 2-7.

Zheng, Z. L., Tan, J. Y., Liu, H. Y., Zhou, X. H., Xiang, X., & Wang, K. Y. (2009). Evaluation of oregano essential oil (Origanum heracleoticum L.) on growth, antioxidant effect and resistance against Aeromonas hydrophila in channel catfish (Ictalurus punctatus). *Aquaculture, 292*(3), 214-218.