

**Bio-enrichment of plant  
feedstuffs by  
*Aspergillus* spp. under  
solid-state  
fermentation for  
application to  
aquafeeds**

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Mestrado em Recursos Biológicos Aquáticos

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**Orientador**

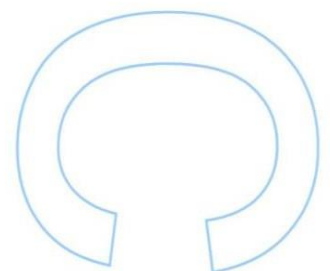
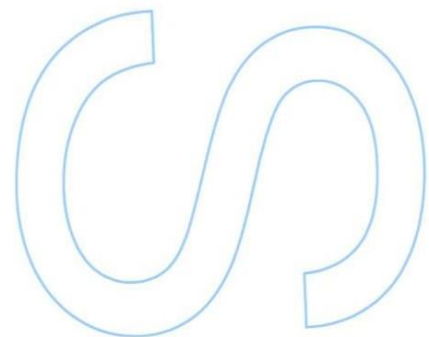
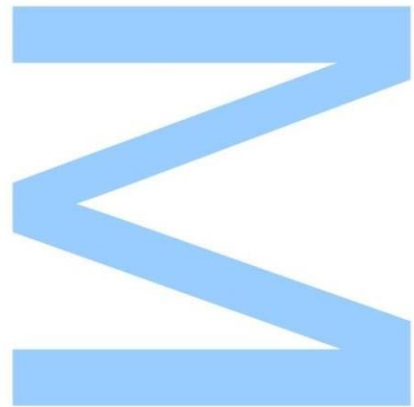
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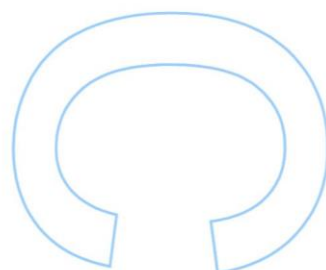
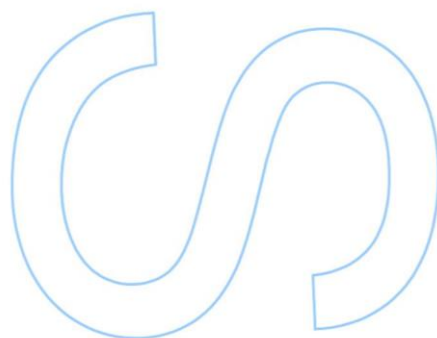
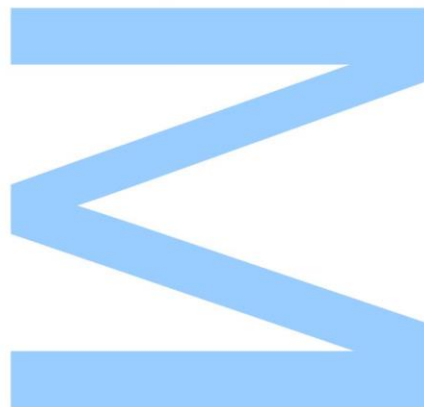
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Todas as correções determinadas  
pelo júri, e só essas, foram efetuadas.  
O Presidente do Júri,

Porto, \_\_\_\_/\_\_\_\_/\_\_\_\_



*Just pick a goal, a goal you truly want to achieve, and take a clear-eyed look at your weakness- not only you'll feel less confident, but so you can determine exactly what you need to work on. Then get to work. Celebrate small successes. Analyze your weaknesses. As you gain skill, you'll also gain a feeling of genuine confidence, one that can never be taken away – because you've earned it.*

Jeff Haden (2018). "The Motivation Myth"

### **Sworn Statement**

I, Ana Lúcia Silva Vieira, enrolled in the Master Degree Biological Aquatic Resources at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this dissertation reflects perspectives, research work and my own interpretations at the time of its submission. By submitting this dissertation, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution. I further declare that all references to other authors fully comply with the rules of attribution and are referenced in the text by citation and identified in the bibliographic references section. This dissertation does not include any content whose reproduction is protected by copyright laws. I am aware that the practice of plagiarism and self-plagiarism constitute a form of academic offense.

Ana Lúcia Silva Vieira

12-12-2022

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## Abstract

Aquaculture has been the fastest-growing industry in recent decades. Fishmeal, once the main ingredient in aquafeeds, has become unsustainable due to its high cost, demand, and fish exploitation. Nowadays, fishmeal is essentially produced from fishery by-products but remains the most expensive ingredient in fish feeds. Nonetheless, fishmeal has an optimal amino acid profile for aquaculture fish species, excellent palatability, and lacks anti-nutritional factors such as dietary fiber. The incorporation of fishmeal in aquafeeds is still required, particularly for some early growth stages of carnivorous fish. Still, it is gradually coming to be seen as a prime ingredient used in specific cases, rather than a common ingredient used indiscriminately. Plant feedstuffs are important ingredients for replacing fishmeal, but they present some disadvantages. For instance, they have low palatability, digestibility, and anti-nutritional factors, that reduce nutrient absorption and, consequently, growth performance and feed efficiency of the fish. Therefore, new technologies or nutritional approaches have been developed to reduce these plant feedstuff constraints, guaranteeing adequate and efficient inclusion in aquafeeds, especially for carnivores. Solid-state Fermentation (SSF) of plant feedstuffs by fungi may help mitigate the aforementioned disadvantages, increase nutrient content and availability, and add fiber-degrading enzymes and phenolic compounds. In addition, fermented substrates may exhibit antioxidant and immunomodulatory properties due to the enrichment of antioxidants and  $\beta$ -glucans, mannans, and chitin present in the fungal cell wall.

In this study, SSF was applied to enhance the nutritional properties of plant-based aquafeed ingredients. A holistic approach was then used to assess the effect of these diets on fish performance, feed and nutrient utilization efficiency, whole-body composition, digestive enzyme activity, plasma metabolites, liver, and gut oxidative stress status, liver, intestine, and muscle LPO, evaluate inflammatory and metabolic gene expression, and the modulation of the gut microbiota.

Chapter 2 shows the results of the SSF of a plant-feedstuff mixture (rapeseed meal, soybean meal, rice bran, and sunflower seed, each 25%) with 3 fungi. This mixture was fermented with 3 different fungi, namely *Aspergillus ibericus* MUM 03.49; *Aspergillus niger* CECT 2088; *Aspergillus niger* CECT 2915, aiming to maximize the proximal composition, namely protein content, enzyme activity, and *in vitro* protein digestibility of this mixture. Among the three fungi, *Aspergillus niger* 2088 CECT was selected as the most suitable fungus to up-grade the nutritional

value of the plant-feedstuff mixture. This fungus was used to ferment the plant-ingredient mixture to be used in the following research.

In Chapter 3, the results of the growth trial are presented in which the dietary inclusion of the fermented mixture was tested using European sea bass as a biological model. Four isoproteic (42%) and isolipidic (18%) experimental diets were formulated, including 20% and 40% of plant-feedstuff mixture, unfermented and fermented by SSF (diets 20 Mix, 40 Mix, 20 SSF, and 40 SSF, respectively), with the filamentous fungus *A. niger* CECT 2088 for 7 days, as described in Chapter 2. These four diets were tested in a growth trial with European sea bass juveniles (*Dicentrarchus labrax*, L.; initial body weight of 10 g), in a recirculating water system. All diets were fed in triplicate groups for 50 days. At the end of the growth trial, digestive enzymes, plasma metabolites, liver, and gut oxidative stress, liver, gut, and muscle LPO, inflammatory and metabolic gene expression, and gut microbiota modulation were assessed.

In diets containing 40% of Mix, SSF decreased growth performance, feed intake, efficiency, and protein retention. In fish fed the 20% Mix diets, SSF resulted in similar growth performance, lower feed intake but higher feed efficiency, and protein retention. Overall growth performance and feed conversion efficiency, were higher in fish fed the 20% Mix than those fed the 40% Mix, regardless of SSF. Whole-body dry matter and protein increased with SSF. Digestive enzyme activity was also affected by dietary SSF and by the inclusion level. Oxidative stress enzymes were affected by both SSF and the inclusion level, but LPO was not affected in all three tissues. In terms of inflammatory gene expression, SSF appears to have an immunostimulatory effect, as it decreases TGF- $\beta$  gene expression, and, although not significantly, increases IL-8, IL-10, and TNF- $\alpha$  gene expression. Cathepsin D, responsible for the hydrolysis of intracellular proteins and peptides to maintain cell homeostasis, was also increased by SSF. The gut microbiota was modulated by the dietary treatments. SSF diets promoted higher microbial diversity and the presence of *Bacillus* spp. with antifungal activity.

Overall, this study concludes that SSF of a plant-feedstuff mixture by *A. niger* CECT 2088 SSF resulted in enzyme production and increased *in vitro* protein digestibility. Additionally, dietary inclusion of a plant-feedstuff mixture fermented by *A. niger* CECT 2088 can have beneficial effects, on feed utilization efficiency, when included at 20% of the diet, while higher inclusion level decrease growth performance and feed efficiency of the fish. In addition, due to its immunostimulatory properties, and by increasing the presence of some *Bacillus* spp., considered probiotics, SSF reduced the incidence of diseases in aquaculture. Future studies should test

lower inclusion levels of this fermented mix and conduct further studies on the immunostimulatory and probiotic properties of this mixture.

**Keywords:** Aquaculture, Plant-feedstuffs, Solid-State Fermentation, *Aspergillus* spp., enzymes production, Growth performance, Oxidative stress, Inflammatory status, Gut microbiota modulation.

## Resumo

A aquacultura tem-se apresentado como uma indústria crescente nos últimos anos, face ao crescimento da população humana. Ingredientes como a farinha de peixe, outrora incorporados em grande quantidade em dietas para aquacultura, são atualmente considerados insustentáveis, devido ao seu elevado custo, elevada demanda e escassez, devido à sobreexploração pesqueira. Porém, este ingrediente é rico em aminoácidos e ácidos gordos essenciais, não possui fatores anti-nutricionais e fibra e apresenta boa palatibilidade em dietas para peixes, especialmente para espécies carnívoras, como o robalo. Assim, de forma a reduzir o nível de inclusão da farinha de peixe em dietas para peixes, é necessária a inclusão de ingredientes de origem vegetal. Porém, estas alternativas possuem baixa palatibilidade, baixa digestibilidade e apresentam fatores antinutricionais, que impossibilitam a absorção completa dos nutrientes e, por conseguinte, diminuem o crescimento, a eficiência alimentar e a performance dos peixes. Neste sentido, surge a necessidade de serem desenvolvidas estratégias para mitigar estes efeitos menos positivos dos ingredientes de origem vegetal. A Fermentação em Estado Sólido (SSF), sendo um processo com baixa pegada ambiental e de baixo custo, valoriza nutricionalmente ingredientes de origem vegetal e sub-produtos agrícolas, por exemplo, através da produção de enzimas que degradam a fibra, as carboidrases, e pela libertação de antioxidantes da parede celular dos ingredientes vegetais. Além disso, os fungos utilizados nesta fermentação poderão apresentar propriedades imuno-modulatórias, devido à presença de  $\beta$ -glucanos, mananos e quitina na sua parede celular.

Neste estudo, a SSF foi usada com o objetivo de melhorar as propriedades nutricionais destes ingredientes. Posteriormente, foi feita uma análise holística, de modo a se compreender qual o efeito destas dietas fermentadas na saúde dos peixes. Especificamente, foi avaliado o crescimento dos peixes, a eficiência alimentar e de retenção proteica, a composição corporal dos peixes, os metabolitos plasmáticos, a atividade das enzimas digestivas, o estado oxidativo no fígado e no intestino, a peroxidação lipídica no fígado, no intestino e no músculo, a expressão génica de genes associados a processos inflamatórios e metabólicos e ainda a modulação da microbiota intestinal dos peixes alimentados com estas dietas.

No capítulo 2 são apresentados os resultados de três fermentações de uma mistura de ingredientes vegetais, composta por farinha de colza, farinha de soja, farelo de arroz e sementes de girassol (25% de cada ingrediente). Esta mistura foi fermentada com três espécies de fungos diferentes: *Aspergillus ibericus* MUM 03.49; *Aspergillus niger* CECT 2088; *Aspergillus niger* CECT

2915, com o objetivo de otimizar a composição proximal, nomeadamente o teor proteico, a atividade enzimática e a digestibilidade da proteína *in vitro*. Entre estes três fungos, o *Aspergillus niger* CECT 2088 foi escolhido como o fungo que mais enriqueceu nutricionalmente a mistura.

No capítulo 3 são apresentados os resultados do ensaio de crescimento com robalo, que testou a inclusão da mistura fermentada. Foram formuladas quatro dietas experimentais isoproteicas (42%) e isolipídicas (18%), com níveis elevados de ingredientes vegetais (20% e 40%) com a mistura não fermentada e fermentada por SSF (20 Mix, 40 Mix, 20 SSF e 40 SSF, respetivamente), com o fungo *A. niger* CECT 2088 durante 7 dias, descrita no capítulo 2. Posteriormente, estas dietas foram testadas num ensaio de crescimento com robalos juvenis (*Dicentrarchus labrax*), com um peso inicial de 10 gramas. Nas dietas foram fornecidas aos peixes em triplicado durante 50 dias, num sistema de recirculação de água. As enzimas digestivas, os metabolitos plasmáticos, o estado oxidativo no fígado e no intestino, a peroxidação lipídica no fígado, no intestino e no músculo, a expressão génica de genes associados a processos inflamatórios e metabólicos e ainda a modulação da microbiota intestinal foram avaliados no final do ensaio de crescimento.

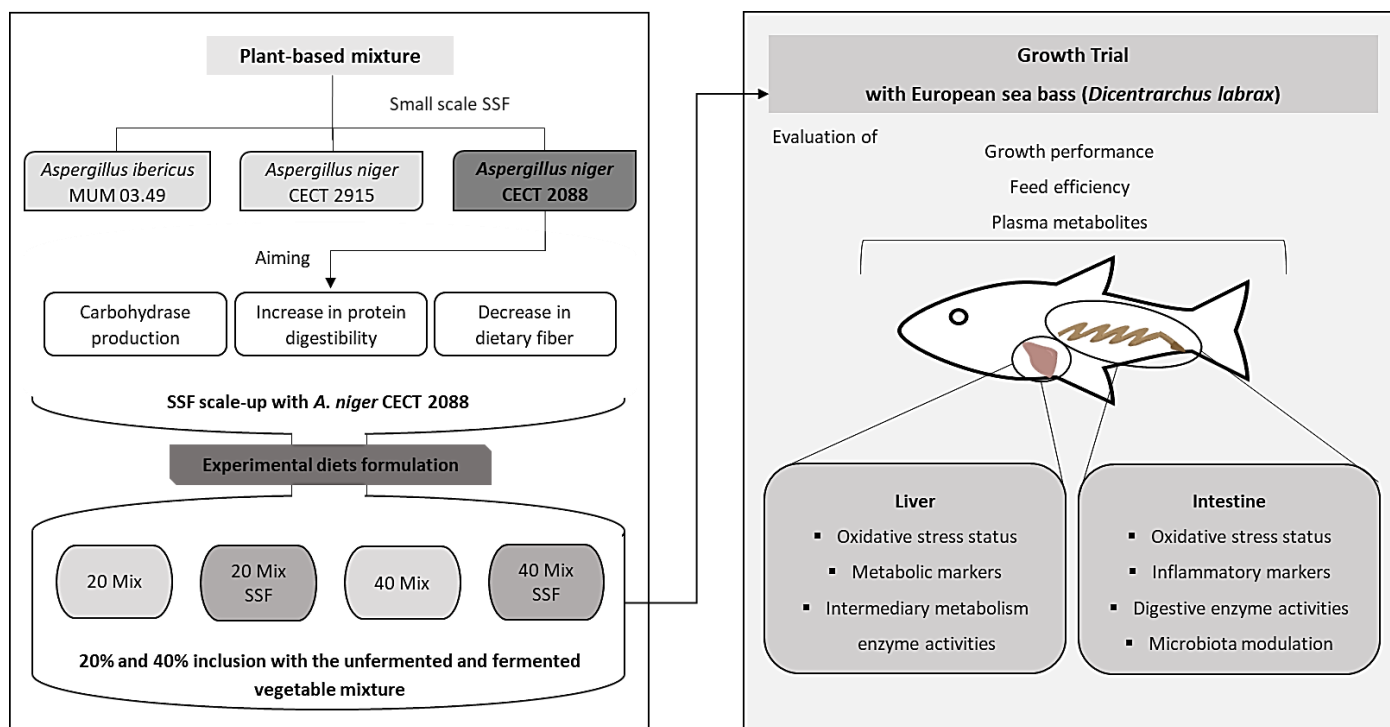
As dietas que incluíram 40% da mistura, a fermentação reduziu o crescimento, a ingestão a eficiência alimentar e a retenção proteica dos peixes, enquanto que nas dietas que incluíram 20% da mistura, a fermentação não afetou o crescimento, diminuiu a ingestão, mas melhorou a eficiência alimentar e a retenção proteica dos peixes. Após o ensaio, o teor de matéria seca e proteína corporal aumentou com a inclusão da mistura fermentada. Do mesmo modo, a atividade das enzimas digestivas também foi alterada com a fermentação e com o nível de inclusão da mistura nas dietas. Em suma, foi concluído que a dieta 20SSF foi vantajosa para os peixes, já que permite um uso da dieta mais eficiente, comparando com a respetiva dieta não fermentada. Ainda assim, em futuros estudos deveriam ser testados níveis de inclusão desta mistura fermentada mais baixos. As enzimas intervenientes no stress oxidativo foram afetadas pelos tratamentos das dietas, mas a peroxidação lipídica não foi afetada nos três tecidos testados. Relativamente à expressão de genes inflamatórios, a SSF aparenta ter propriedades imuno-estimulantes, na medida em que a expressão génica do TGF- $\beta$  diminuiu e, embora não significativamente, a expressão dos genes IL-8, IL-10 e TNF- $\alpha$  aumentou. Do mesmo modo, a expressão da catepsina D, responsável pela hidrólise proteica intracelular, mantendo a homeostasia celular, aumentou com a SSF. A microbiota intestinal foi modulada pelos tratamentos nas dietas, já que se verificou um aumento da diversidade e abundância

microbiana, bem como a presença de várias espécies do género *Bacillus* com atividade anti-fúngica.

Concluindo, a SSF com o fungo *A. niger* CECT 2088 aumenta a produção de enzimas e a digestibilidade da proteína *in vitro*. Além disso, a inclusão da mistura fermentada com este fungo em dietas para aquacultura apresenta vantagens no crescimento dos peixes, quando incluída em níveis ponderados (20%), enquanto que, em níveis mais elevados de inclusão (40%) reduzem o crescimento e a eficiência alimentar dos peixes. Além disso, a SSF poderá reduzir a incidência de doenças em aquacultura, apresentando propriedades imuno-estimulantes e por estimular a presença de algumas bactérias do género *Bacillus* no intestino do peixe, com propriedades probióticas. Futuramente, deveriam ser testados níveis mais reduzidos desta mistura fermentada, bem como estudar as suas propriedades imuno-estimulantes e probióticas em robalo.

**Palavras-chave:** Aquacultura, Ingredientes de Origem Vegetal, Fermentação em Estado Sólido, *Aspergillus* spp., Produção de enzimas, Crescimento, Stress oxidativo, Estado Inflamatório, Modulação da microbiota intestinal

## Graphical Abstract



## Scientific Divulagation

### Parts of the present work are presented at the following events:

Vieira, L., Filipe, D., Belo, I., Oliva-Teles, A., Peres, H., 2022. "Nutritional value of fermented plant-feedstuff mixture with *Aspergillus niger* for European sea bass (*Dicentrarchus labrax*)". Presented as an **oral communication** at the 15th edition of the Youth Research Meeting (IJUP), which took place on May 4-6, 2022, in Porto, Portugal.

Vieira, L., Filipe, D., Magalhães, R., Belo, I., Oliva-Teles, A., Peres, H., 2022. "Nutritional evaluation of fermented plant-feedstuff mixture with *Aspergillus niger* for European sea bass (*Dicentrarchus labrax*)". Presented as an **oral communication** at the first annual Ocean3R meeting, which took place on June 1, 2022, in Porto, Portugal.

Vieira, L., Filipe, D., Magalhães, R., Soares, F., Belo, I., Oliva-Teles, A., Peres, H., 2022. "Solid-State fermented plant-based diets for European sea bass (*Dicentrarchus labrax*): complementing trial interpretation with a nutrient-based model". Presented as a **poster** at the Aquaculture Europe 2022, which took place on September 27-30, 2022, in Rimini, Italy.

Vieira, L., Filipe, D., Magalhães, R., Fontinha, F., Martins, N., Serra, C. R., Oliva-Teles, A., Belo, I., Peres, H., 2022. "Solid-state fermented plant-feedstuff mixture with *Aspergillus niger* for European sea bass (*Dicentrarchus labrax*) juveniles effects on oxidative stress, immune system, and gut microbiota". Presented as a **poster** at the Aquaculture Europe 2022, which took place on September 27-30, 2022, in Rimini, Italy.

Vieira, L., Filipe, D., Magalhães, R., Ferreira, M., Oliva-Teles, A., Belo, I., Peres, H., 2022. "Nutritional evaluation of diets including a solid-state fermented plant-feedstuff mixture with *Aspergillus niger* for European sea bass (*Dicentrarchus labrax*) juveniles". Presented as an **oral communication** at the Aquaculture Europe 2022, which took place on September 27-30, 2022, in Rimini, Italy.

Vieira, L., Filipe, D., Magalhães, R., Ferreira, M., Oliva-Teles, A., Belo, I., Peres, H., 2022. "Bio-enrichment of plant feedstuffs by *Aspergillus* spp. under solid-state fermentation for application to aquafeeds". Presented as an **oral communication** at the 6<sup>th</sup> Aquaimprove, which took place on October 19, 2022, in Porto, Portugal.

### Scientific divulgation within the Ocean3R project

Within this work and the Ocean3R project, an online scientific divulgation composed of ten e-posts regarding the importance of scientific investigation in aquaculture took place on Ocean3R's website and CIIMAR's Facebook, Instagram, and LinkedIn.

### Awards

Winner of the SPAROS (Lda.) FEEDNETICS™ Prize for Aquaculture Nutrition Research Students 2022 for Aquaculture Europe 2022, with the abstract "Solid-State fermented plant-based diets for European sea bass (*Dicentrarchus labrax*): complementing trial interpretation with a nutrient-based model." The SPAROS grant included a flight ticket, accommodation, and the conference registration fee.

## Index

|                                                                                                                                                                                                                               |      |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| Abstract                                                                                                                                                                                                                      | I    |
| Resumo                                                                                                                                                                                                                        | IV   |
| Graphical Abstract                                                                                                                                                                                                            | VII  |
| Scientific Divulcation                                                                                                                                                                                                        | VIII |
| Awards                                                                                                                                                                                                                        | IX   |
| Figure List                                                                                                                                                                                                                   | XI   |
| Abbreviations List                                                                                                                                                                                                            | XII  |
| Chapter 1 - General Introduction                                                                                                                                                                                              | 1    |
| 1.1. Aquaculture Production                                                                                                                                                                                                   | 2    |
| 1.2. European sea bass ( <i>Dicentrarchus labrax</i> , Linnaeus, 1758)                                                                                                                                                        | 5    |
| 1.2.1. Biology of the species                                                                                                                                                                                                 | 5    |
| 1.2.2. Nutritional requirements                                                                                                                                                                                               | 6    |
| 1.3. Fishmeal and alternative ingredients for European sea bass diets                                                                                                                                                         | 8    |
| 1.4. Solid-State Fermentation                                                                                                                                                                                                 | 11   |
| 1.4.1. <i>Aspergillus</i> spp.                                                                                                                                                                                                | 14   |
| Aims                                                                                                                                                                                                                          | 16   |
| Chapter 2 - Enrichment of a vegetable protein mixture's nutritional value for use in animal feed through SSF                                                                                                                  | 17   |
| Chapter 3 - Effect on growth performance, well-being, and gut microbiota of European sea bass ( <i>Dicentrarchus labrax</i> ) juveniles fed with diets containing a SSF plant-feedstuff mixture with <i>Aspergillus niger</i> | 30   |
| Chapter 4 – General conclusions                                                                                                                                                                                               | 72   |
| References                                                                                                                                                                                                                    | 74   |

## Figure List

Fig. 1 – Word captures fisheries and aquaculture production from 1950 until 2020. Retrieved from FAO (2022).

Fig. 2 – Word aquaculture production from 1992 to 2020. Retrieved from FAO (2022).

Fig. 3 - European Sea Bass (*Dicentrarchus labrax*). Retrieved from Scandinavian Fishing Year Book.

Fig. 4 - Schematic representation of the plant cell wall structure of lignocellulolytic materials. Retrieved from Brethauer, Shahab, and Studer (2020).

## Abbreviations List

ARA – Arachidonic Acid

A<sub>w</sub> – water activity

BGS - Brewer's spent grain

DDGS - Distiller's dried grains with soluble

DHA - Docosahexaenoic Acid

EPA - Eicosapentaenoic Acid

EU – European Union

EUR – Euros

FAO - United Nations Food and Agriculture Organization

LC-PUFA – Long-chain polyunsaturated fatty acids

NSP – Non-starch polysaccharides

ROS - Reactive Oxygen Species

SmF – Submerged Fermentation

SSF – Solid State Fermentation

## Chapter 1 - General Introduction

### 1.1. Aquaculture Production

The world population is expected to grow more than 25% by 2050, representing an increase from 7.5 billion to 9.4 billion people (DNV, 2021; FAO, 2009b). Seafood is considered an essential part of the global food consumption patterns (Béné, 2006) due to its high and unique nutritive characteristics, such as good quality protein, lipids (rich in omega-3 highly unsaturated fatty acids), micronutrients (Béné et al., 2015), and because it represents the main source of food, especially in developing countries (Klinger & Naylor, 2012). It is estimated that 950 million people rely on fish as their primary protein source (Pradeepkiran, 2019). Consequently, global demand for seafood and seafood production is rising since the world population grows and consumes more fish (Francis et al., 2001; Klinger & Naylor, 2012), and it is expected to continue to rise.

For years, fisheries were the main source of fish for human consumption (Martins, 2017). Global fish consumption increased by 3.1% from 1961 to 2017 (FAO, 2020), motivating fisheries to increase, to fulfill the population's needs. Nevertheless, due to its continuous unsustainable exploitation which has led to the decline of wild fish stocks, global fisheries have stagnated since the late-1980s. The aquaculture industry, however, started to grow continuously in response to the population increase and fish demand. More recently, the development of new technologies allowed a greater evolution and more sustainable production (DNV, 2021).

Aquaculture is defined as the farming of aquatic organisms with human intervention in the process to enhance production, such as feeding, stocking, etc., with ownership of the stock (Edwards & Demaine, 1998). It is carried out under more or less controlled systems in an extensive or intensive production. Consequently, some not well-planned intensive productions can cause some environmental impacts, affecting local ecosystems, for instance (Bohnes et al., 2022). Therefore, one of aquaculture's biggest challenges is reaching sustainable production. To face that, some methods to improve aquaculture management are being tested, new sources of fish feed are being developed to improve fish growth and control disease outbreaks, and more sustainable productions, such as IMTA, are being assessed to reduce the environmental footprint. Nonetheless, scientific investigation plays a vital role in these achievements, since it is expected that from 2018 to 2050, marine aquaculture production will increase from 30 to 74 million tons per year (DNV, 2021). Nowadays, most of the fish products consumed are from

aquaculture. This production is expected that this consumption will increase by 24 million tons from 2020 to 2030 (FAO, 2022) (Fig. 1).

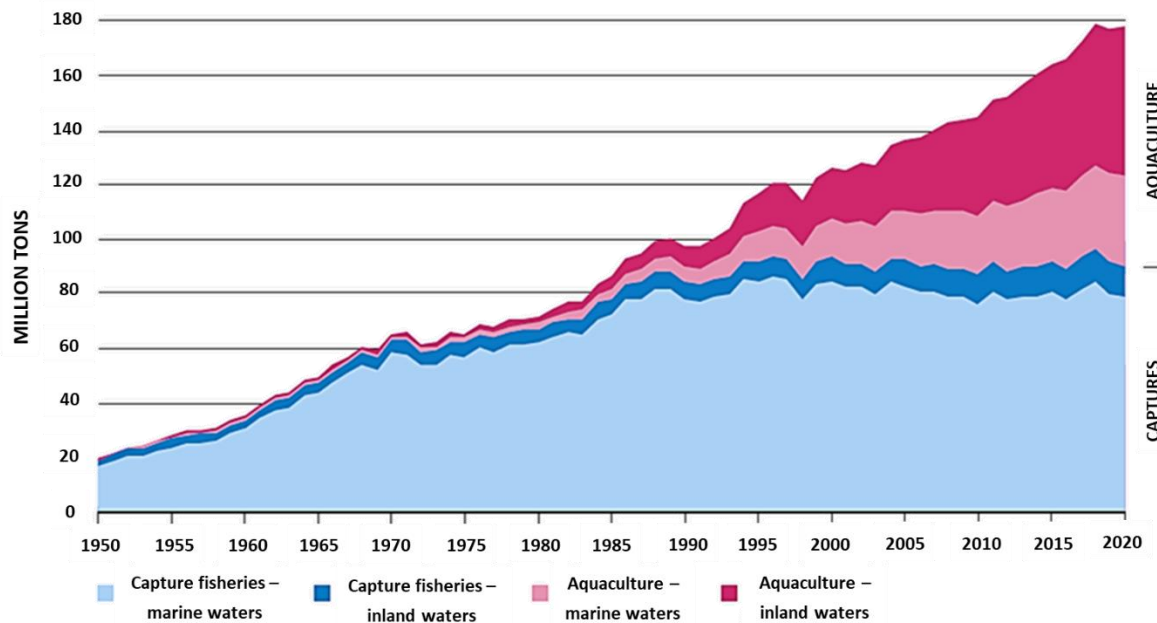


Fig. 1 - World captures and aquaculture production from 1950 to 2020. Retrieved from FAO (2022).

In the past few years, aquaculture has been increasing exponentially. In 2018, aquaculture production reached 114.5 million tons (FAO, 2020), and in 2020, 112 million tons. Similarly, world exports of aquatic products, excluding algae, peaked in 2018, recording a total of 67 million tons, followed by a decrease in 2020 of 60 million tonnes. This slight decrease in production is due to several factors, mainly the COVID-19 pandemic, the decrease in China's production, due to some policy changes regarding environmental protection, and anchoveta catch reduction (FAO, 2022). Nonetheless, it is expected to continue growing (Tacon et al., 2011), following the same trends up to 2018.

In 2018, when aquaculture recorded the highest production and profits, China was the major fish producer, accounting for 35% of global fish production, and in 2020 recorded the same production statistics. Excluding China, production was headed by Asia (34% in 2018 vs. 35% in 2020), followed by the Americas (14% in 2018 and 12% in 2020), Europe (10% in 2018 and 2020), Africa (7% in 2018 and 2020), and Oceania (1% in 2018 and 2020). The total production is dominated by aquatic animals' (82.1 in 2018 vs. 35.1 million tons in 2020), followed by marine algae (32.4 in 2018 vs. 36 million tons in 2020) and ornamental seashells and pearls (26 000 tons) (Fig. 2). Concerning aquatic animals, farming was led by finfish (54.3 in 2018 and

57.7 million tons in 2020), followed by mollusks (17.7 million tons in 2018 and 2020), crustaceans (9.4 in 2018 vs 11.2 million tons in 2020), marine invertebrates (435 400 in 2018 and 525 000 tons in 2020), aquatic turtles and frogs (370 000 and 131 300 tons, respectively, in 2018) (FAO, 2020, 2022).

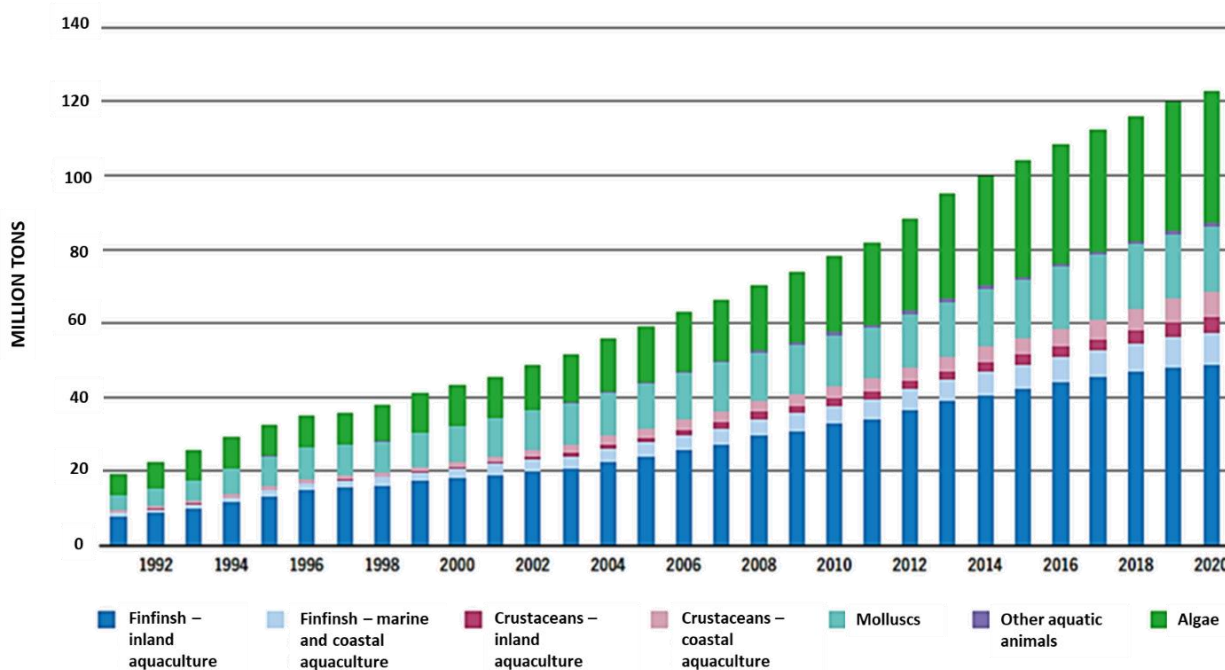


Fig. 2 - World aquaculture production from 1992 to 2020. Retrieved from FAO (2022).

In Europe, aquaculture production covers only 10% of the seafood consumed in the European Union (EU) and less than 2% of the world's production. 70% of the aquaculture production in the EU is in Spain, France, Italy, and Greece. The main species produced in the EU are mussels, trout, sea bream, oysters, seabass, carp, and clams (EuropeanCommission, 2021a). Up to 2050, European finfish production will continue to grow, from 1.8 to 3.9 million tons per year. In 2020, Portugal recorded the highest fish consumption in the EU, around 60.9 kg/capita/year, being the European average of 23.82 kg/capita/year, and in 2019 had the highest per capita disbursement (EUR 371), more than the triple of the EU average (EUR 110) (EuropeanCommission, 2020). In 2020, Portugal registered a production of 3 000 tons of turbot (*Psetta maxima*), 2 000 tons of sea bream (*Sparus aurata*), 600 tons of rainbow trout (*Oncorhynchus mykiss*), 500 tons of sea bass (*Dicentrarchus labrax*), and 160 tons of sole (*Solea senegalensis*), totalizing a production of 6 320 tons of marine fish (FEAP, 2021).

## 1.2. European sea bass (*Dicentrarchus labrax*, Linnaeus, 1758)

### 1.2.1. Biology of the species

European sea bass (*Dicentrarchus labrax*) (Fig. 3) belongs to the Moronidae family and is a coastal marine teleost that lives in shallow waters (<100 meters) that can also occur in estuaries and brackish lagoons (FAO, 2009a). It is a euryhaline (0-40‰) and eurythermal (2-32 °C) species and shows oceanodromous behavior. It is distributed through the coasts of the Mediterranean Sea, the Black Sea, and the eastern Atlantic Ocean from Norway to Morocco, the Canary Islands, and Senegal (Volckaert et al., 2008). This wide geographical distribution is due to its physiological adaptations, mainly its tolerance to a wide range of salinity and temperature and its ability to regulate osmotic stress (Pérez-Ruzafa & Marcos, 2014; Vandeputte et al., 2019).

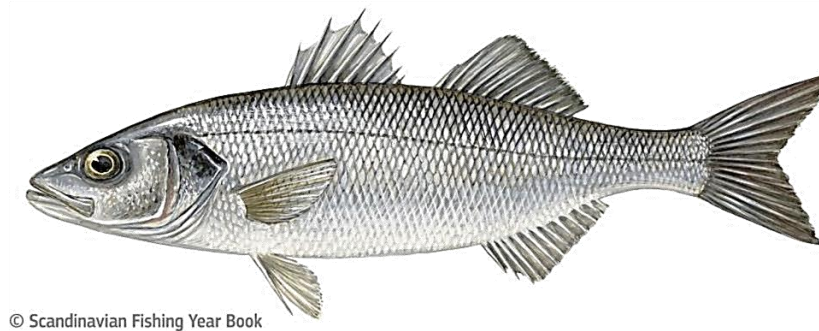


Fig. 3 - European Sea Bass (*Dicentrarchus labrax*). Retrieved from: Scandinavian Fishing Year Book.

*D. labrax* is a carnivorous top predator, feeding plankton at the larval stages and fish (up to 36%) and crustaceans (48%) at the juvenile and adult stages. Analysis of gut content shows that, in the wild, polychaetes, seagrasses, seaweeds, aquatic insects, and snails also enter this species' alimentation, although in lesser amounts (Pérez-Ruzafa & Marcos, 2014; Vandeputte et al., 2019).

This species is well domesticated, has a high commercial value, and is among the most exploited fish species on the European coast aquaculture, mainly in the Mediterranean Sea (FAO, 2009a; Pérez-Ruzafa & Marcos, 2014), totalizing an adult production of 189 023 tons and 540 784 thousand units of juveniles in 2020 (FEAP, 2021). With declining fisheries, aquaculture of this species is extremely important to provide a high-quality product to the population (Vandeputte et al., 2019; Vazquez & Munoz-Cueto, 2014).

In Europe, Portugal is the eighth-producing country of E. sea bass (FEAP, 2021), and it is one of the most appreciated and consumed fishes, with a high market value. Nonetheless,

sustainable intensive production of sea bass depends on formulating more economical aquafeeds with a low carbon footprint. To achieve that, the inclusion of new ingredients is studied, and the adequacy of these diets to the nutritional requirements of the species and development stage is of utmost importance to guarantee maximum growth performance and feed efficiency (FAO, 2009a; Vandeputte et al., 2019), stress tolerance, and health of fish (Trichet, 2010).

### 1.2.2. Nutritional requirements

Every species has specific nutritional requirements, and the administrated diets must meet the minimum levels of these requirements (Allen & Steeby, 2011) to avoid certain deficiencies that will negatively affect fish performance and make them more vulnerable to diseases (Oliva-Teles, 2012).

Protein is one of the most expensive nutrients in formulated aquafeeds (da Silva et al., 2014). For European sea bass, the optimal dietary protein level is circa 55% for juveniles (< 20 g), and around 45 for adults (> 200 g) (NRC, 2011). Nonetheless, some studies also report that juveniles with 6 g seemed satisfied with diets containing 48% of protein (Peres & Oliva-Teles, 1999). However, fish do not have protein, but amino acid requirements and the requirement of some of the most limiting essential amino acids are compiled in NRC (2011). Sea bass requires ten essential amino acids that are not synthesized endogenously, these being lysine, arginine, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, and valine; seven nonessential amino acids, such as alanine, asparagine, aspartate, glutamate, glycine, and serine, two semi-essential, cysteine and tyrosine and some considered as “conditionally” essential amino acids, as taurine, glutamine, hydroxyproline, and proline (Li et al., 2008; Martins, 2017; Oliva-Teles, 2000). Some marine fish lack or have a low capacity to synthesize taurine so its inclusion may be mandatory for growth performance improvement (Martins et al., 2018). For sea bass, taurine is considered a conditionally essential amino acid as its deficiency has been reported to reduce growth performance and feed efficiency (Takagi et al., 2011). Moreover, for some other species, such as red sea bream (*Pagrus major*) (Takagi et al., 2011), and yellowtail (*Seriola quinqueradiata*) (Takagi et al., 2005), the development of green liver syndrome is observed.

Lipids are another essential component in aquafeeds as a source of fatty acids, liposoluble vitamins, pigments, and energy. Besides being the main conventional energy source in sea bass

diets, lipids are also the source of fatty acids, including long-chain polyunsaturated fatty acids (LC-PUFAs), essential fatty acids for this species. Indeed, like the majority of marine fish, sea bass lacks the enzymes to synthesize LC-PUFAs, mainly Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA), and Arachidonic acid (ARA) (NRC, 2011; Oliva-Teles, 2000). Nowadays, E. sea bass dietary lipid requirements range between 18% and 20% (FAO, 2021). Initially, a requirement of 1% of fatty acid was proposed for sea bass (Coutteau et al., 1996). Further, using an EPA/DHA (1:1.5) ratio, it was suggested a minimum dietary requirement of 0.7% (Skalli & Robin, 2004). Nonetheless, these requirements may vary with fish size, or dietary lipid levels (Magalhães, 2022).

Carnivorous fish do not utilize carbohydrates efficiently. Despite fish possessing  $\alpha$ -amylase, fish lack the enzymes to degrade other complex carbohydrates, like hemicellulose and cellulose. Moreover, fish also poorly regulate postprandial hyperglycemia (Enes et al., 2009). However, as it constitutes a cheap energy source and spares the utilization of protein and lipids for energy purposes, incorporating carbohydrates in aquafeed is recommended (Martins, 2017). Nevertheless, carbohydrate pre-treatments, such as extrusion or gelatinization, are known to improve carbohydrate utilization (Dias et al., 1998; Kaushik, 2002) and digestibility (Peres & Oliva-Teles, 2002). Peres and Oliva-Teles (2002) confirmed that the inclusion of gelatinized starch in diets improved fish performance and that a mixture of 12.5% of raw starch plus 12.5% of gelatinized starch was more advantageous than 25% of inclusion of raw or gelatinized starch. Indeed, Lanari et al. (1999) reported that the dietary incorporation of gelatinized ingredients should not exceed 20%.

Available data on vitamin requirements are scarce for European sea bass. According to Kaushik (2002) and NRC (2011), there is evidence that the dietary allowances for *D. labrax* do not differ from those defined for the salmonids. Among all vitamins, vitamins A, C, and E are the most well-studied. Vitamin A is important for skeletal development and is recommended the inclusion of 35 mg kg<sup>-1</sup> (Villeneuve et al., 2005). Vitamin C is involved in skeletal deformities in fish, so it is recommended the inclusion of at least 50 mg kg<sup>-1</sup> (Kaushik, 2002). A supply of 500 mg kg<sup>-1</sup> for vitamin E has been recommended to prevent peroxidative damage against reactive oxygen species (ROS) (Messenger et al., 1992).

Mineral requirements are not well defined for sea bass, due to a lack of data (Kaushik, 2002), except for phosphorus, with an estimated requirement of 0.65% for European sea bass (Oliva-Teles & Pimentel-Rodrigues, 2004).

### 1.3. Fishmeal and alternative ingredients for European sea bass diets

Fishmeal is the most complete ingredient in aquafeeds and feed nutrition. It contains a balanced profile of essential amino acids, fatty acids, minerals, and vitamins ideal for the majority of fish species. It is also highly digestible and palatable and has no anti-nutritional factors for fish (Abowei & Ekubo, 2013; Hardy, 2010; Oliva-Teles & Gonçalves, 2001; Tacon & Metian, 2015). Along with fishmeal, fish oil is the major source of LC-PUFA (EPA and DHA) (FAO, 2020). Over the last decade, most of the fishmeal production came from wild fisheries, mainly through the processing of the whole fish, mainly small marine pelagic fish, such as anchovies, mackerels, or sardines (Jannathulla et al., 2019), culminating in the overexploitation of marine fish stocks (Péron et al., 2010). Currently, most of the fishmeal production comes from by-catch fisheries or from fisheries and aquaculture trimmings and by-products, which were commonly discarded. However, fishmeal production has been decreasing over the years, due to its unsustainability and the impact of climate change and meteorological phenomena, such as *El Niño*, on the oceanic net production and consequent fish abundance (Jannathulla et al., 2019). Overall, fishmeal is available in limited quantities, and sustainable alternatives are needed (Schalekamp et al., 2016).

Sustainable aquaculture production of *D. labrax* depends on reducing dietary fishmeal incorporation. As aquafeed represents over 50% of production costs (Allen & Steeby, 2011), and due to the need to reduce the environmental footprint and approach to sustainable aquaculture, as proposed by the European Green Deal (EuropeanCommission, 2021b), the amount of fishmeal should be reduced as much as possible without compromising fish growth and well-being, and replaced with alternative protein sources (Allen & Steeby, 2011). However, for marine finfishes, 20% to 65% of fishmeal, especially to support larval and fry survival, continues to be used in aquafeed (Naylor et al., 2009; Tacon et al., 2011), since a total replacement reduces growth performance, feed efficiency (Lin et al., 2022), and voluntary feed intake (Seong et al., 2020). Therefore, this continuous demand for fishmeal leads to an increase in the high price of aquafeeds (Naylor et al., 2009).

Partial replacement of fishmeal may be accomplished through the incorporation of alternative protein sources. For instance, animal by-products, such as feathers, blood, and poultry meals represent a cost-effective solution with good palatability and a complete amino acid profile (Naylor et al., 2009). However, these ingredients have high ash and saturated lipid

levels, which may increase phosphorus loss and environmental waste, and affect the nutritional quality of fish fillets (Oliva-Teles et al., 2015). Insects are another alternative protein source, as they have a good profile of amino acids, are rich in fatty acids, and are a good source of vitamins and minerals (Henry et al., 2015). Single-cell proteins, such as yeasts, can also be used in aquafeeds due to their high protein content and palatability (Oliva-Teles & Gonçalves, 2001). Micro and macroalgae are also reliable substitutes, with balanced amino acid and fatty acid profiles (Silva et al., 2015). Some agro-industrial by-products are also good alternatives, such as distiller's dried grains with soluble (DDGS), cereal by-products from the biofuel industry (Ray et al., 2022), or Brewer's spent grain (BSG), even though both contain high levels of fiber (Fernandes et al., 2022). However, plant feedstuffs are the most abundant alternative protein sources in aquafeeds (Gatlin et al., 2007; Tacon et al., 2011).

Although sea bass, as a carnivorous fish, has a limited capacity to use vegetable protein (Magalhães et al., 2016), a partial replacement of fishmeal with alternative plant-based ingredients has already been attained (Vandeputte et al., 2019). Plant feedstuffs such as oilseeds, legumes, plant concentrates, and cereals are often used as alternative plant-protein in aquafeeds (Oliva-Teles et al., 2015). These ingredients are well accepted by the industry due to their wide availability, sustainability, and reduced costs (Abowei & Ekubo, 2013; Castillo & Gatlin, 2015; Samuel & Shapawi, 2016). Particularly, ingredients such as sunflower and rapeseed meals do not represent a competitive threat regarding human alimentation and are locally produced. Beyond its sustainability, its inclusion in aquafeeds increases the local economy and lowers the carbon footprint associated with transportation. Soybean meal is an economical option and the leading crop that is globally produced. It represents a high-quality source of crude vegetable protein and is usually one of the main fishmeal ingredient alternatives (Gatlin et al., 2007). Rice bran is an abundant by-product of rice milling and contains interesting bioactive compounds, such as phenolic compounds with antioxidant activity, namely gallic, vanillic, or ferulic acids. Hence, it can be applied in aquafeeds as a functional ingredient since it can reveal anti-oxidant and anti-inflammatory properties when submitted to some treatments, such as Solid-State Fermentation (Dey et al., 2016; Janarny & Gunathilake, 2020).

However, dietary plant feedstuffs present some constraints, particularly for carnivorous fish. Although the carbohydrate component of plant-based ingredients provides a cheap source of energy for fish, it is poorly digested compared to protein and lipids (Sinha et al., 2011) and contributes to higher levels of nutrient excretion and waste (Naylor et al., 2009). Furthermore, fishmeal replacement with plant feedstuff may also affect the gut health and microbiota, which

may affect the fish's health (Oliva-Teles et al., 2015). The majority of the plant ingredients show poor digestibility, low palatability, high fiber content, amino acid limitations, and anti-nutritional factors, like non-starch polysaccharides, phytates, saponins, tannins, and protease inhibitors, which may decrease nutrient utilization and fish performance (Castillo & Gatlin, 2015; Conceição et al., 2018; Hardy, 2010; Marcotuli et al., 2020; Sinha et al., 2011; Supriyati et al., 2015).

Non-starch polysaccharides (NSP) can make up to 90% of the plant cell wall and lignocellulosic materials (Selvendran & Du Pont, 1980). NSPs are constituted by cellulose, hemicellulose, lignin, pectin, etc. (Fig. 4), and are poorly digested by carnivorous fish since they do not have the endogenous enzymes to degrade them, such as  $\beta$ -glucanases, xylanases, or cellulases (Maas et al., 2019; Sinha et al., 2011), or they are very scarce (Caprita et al., 2010; Castillo & Gatlin, 2015). Therefore, these NSPs remain indigestible, cannot be used as an energy source, and may interfere with the uptake of other nutrients like proteins and lipids since high levels of fiber lower nutrient absorption (Khajali & Slominski, 2012). Additionally, NSPs can induce modifications in digesta viscosity, gut morphology, and gut microbiota composition and may induce physiological and inflammatory disorders (Anwar et al., 2020; Hardy, 2010; Kumar et al., 2012; Maas et al., 2020; Sinha et al., 2011). Hence, among other disadvantages, NSPs can reduce nutrient digestibility and utilization and so may impair fish growth performance and health (Maas et al., 2019). Moreover, fish's reduced NSPs digestion increases waste production, and thus, the environmental footprint (Maas et al., 2020).

There are several strategies to improve nutrient bioavailability, utilization, and digestibility and to reduce or eliminate the anti-nutritional factors of plant-feedstuffs, such as extrusion pelleting (Hardy, 2010), hydrothermal processes (Castillo & Gatlin, 2015), heat processing, solvent extraction (Oliva-Teles et al., 2015), or probiotics supplementation (Lee et al., 2010; Maas et al., 2021; Wealleans et al., 2017). Also, supplementation with exogenous enzymes, such as phytases, xylanases, and cellulases (Maas et al., 2020; Magalhães et al., 2016; Oliva-Teles et al., 2015), which are produced by fermentation (Dai et al., 2017; Fernandes et al., 2019; Qazi et al., 2011), is a valuable solution to increase these compounds' nutritional value and reduce NSPs.

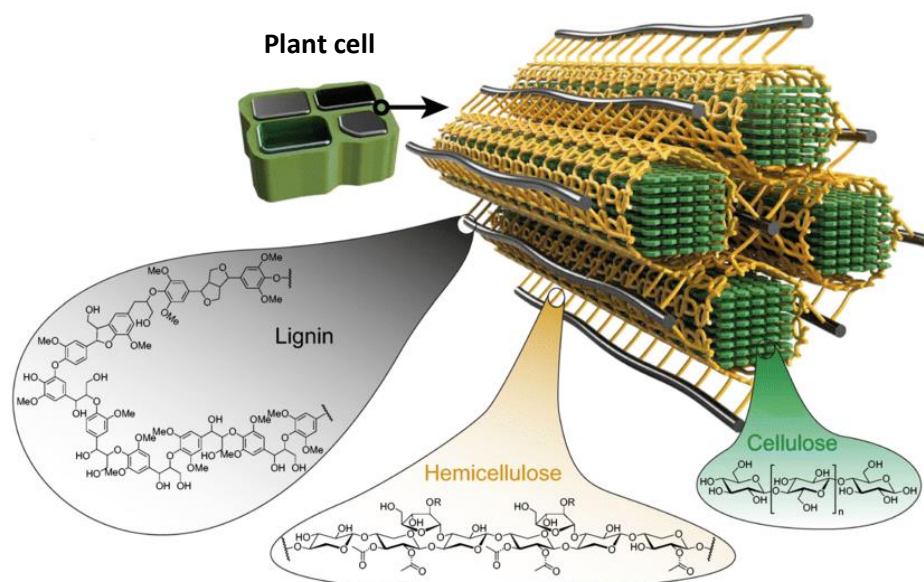


Fig. 4 – Schematic representation of the plant cell wall structure of lignocellulosic materials. Retrieved from Brethauer et al. (2020).

## 1.4. Solid-State Fermentation

Solid-State Fermentation (SSF) is a cost-effective and eco-friendly biotechnological process for large-scale microbial bioconversion and biodegradation (López-Gómez et al., 2020; Manan & Webb, 2017; Soccol et al., 2017). It uses solid biomass as a substrate for microorganism growth in the absence of free-running water, mimicking the natural growth conditions of some microorganisms (Kumar et al., 2003; Leite et al., 2021; Soccol et al., 2017), with minimal or no pre-treatment of the substrates (Yazid et al., 2017). It differs from submerged fermentation (SmF) as it uses less water and, therefore, has a lower risk of bacterial contamination (Leite et al., 2016). For these reasons, the sterilization process may be avoided in SSF, reducing energy consumption, process cost, and environmental impact. Also, in SSF, higher titers of enzyme activity are achieved with higher stability, as the enzyme degradation by proteases is also reduced compared to SmF (Leite et al., 2021). Moreover, SmF commonly uses soluble substrates, such as sugars, while SSF uses insoluble substrates, such as lignocellulosic substrates (Fernandes et al., 2022; Raimbault, 1998).

The most common substrates for SSF are agro-industrial by-products and plant crops (Olukomaiya et al., 2020; Pandey, 2003) due to their great abundance (Verduzco-Oliva & Gutierrez-Urbe, 2020), thus promoting the Circular Economy Action Plan proposed by the European Commission (EuropeanCommission, 2019), and the global goals promoted by the

United Nations regarding the Decade of Ocean Science for Sustainable Development. These substrates have a lignocellulosic nature, constituted by cellulose, hemicellulose, lignin, and other fibers (Soccol et al., 2017), and have the optimal characterization to produce multiple compounds, such as lignocellulosic enzymes (Leite et al., 2021). Several studies described some by-products and plant-feedstuffs as good substrates for SSF, such as Brewer's spent grain (Fernandes et al., 2022), rice bran (Janarny & Gunathilake, 2020), olive pomace (Filipe et al., 2020), or oilseed cakes (Sousa et al., 2021).

In general, yeast and fungi are the most suitable microorganisms for SSF due to their lower water activity requirements, typically around 0.5-6 a<sub>w</sub> (López-Gómez et al., 2020; Thomas et al., 2013), and their ability to grow in SSF conditions (Janarny & Gunathilake, 2020). Filamentous fungi are the preferred microorganisms due to their hyphal way of growth, which allows them to grow in solid substrates (De la Cruz Quiroz et al., 2015) and produce numerous extracellular enzymes (Vishwanatha et al., 2010). However, some genera of bacteria can also be successfully used in SSF, such as *Bacillus* and *Lactobacillus* (Singhania et al., 2009). All of these microorganisms produce important compounds, such as enzymes, organic acids, antibiotics, single-cell proteins, polyunsaturated fatty acids, and antioxidants and phenolic compounds become more bioavailable (Bhargav et al., 2008; Filipe et al., 2020; Yang et al., 2021; Yazid et al., 2017).

During SSF, fungal growth may contribute to the increase of the crude protein content of the substrate due to the increase of fungal biomass. Moreover, the substrate's protein content becomes more bioavailable due to extracellular fungal digestion through the action of extracellular enzymes. Among the extracellular enzymes, some proteases may be secreted and digest protein into small peptides (Olukomaiya et al., 2019). Particularly, as was shown by some studies, after SSF with *Lactobacillus reuteri* and *Saccharomyces cerevisiae*, crude protein of fish by-products and lemon peel increased by up to 40%, compared to the initial substrate (Tropea et al., 2021). Hence, SSF enhances the nutritional quality of the fermented substrate due to the production of mycoprotein-enriched biomass and some bioactive compounds.

Lipases, amylases, laccases, proteases, phytases, hemicellulases, cellulases, and xylanases are some of the produced enzymes, being the last two ones the most abundantly produced (Olukomaiya et al., 2019; Soccol et al., 2017). Cellulases are a class of enzymes constituted by endoglucanases, exoglucanases, and  $\beta$ -glucosidases (Soccol et al., 2017) and, together with the previous enzymes, are responsible for the partial hydrolysis of lignocellulosic materials, mainly

the plant cell walls (Ibarruri et al., 2021; Sinha et al., 2011). Along with the preceding enzymes, laccases and peroxidases form an enzyme consortium that degrades the lignin present in cellulosic compounds that inhibits access to cellulases and hemicellulases to cellulose and hemicellulose (Arredondo-Santoyo et al., 2020; Steudler et al., 2019). Phytases may also be produced by SSF and catalyze the release of phosphate from phytate, making it available for fish (Singh et al., 2008), and reducing phosphorus waste (Saithi & Tongta, 2016). At the end of SSF, these enzymes remain in the fermented substrate and can be included in aquafeeds as exoenzymes. In addition, most commercial exoenzymes, widely used in aquafeeds, are prevent from SSF (Verduzco-Oliva & Gutierrez-Urbe, 2020).

Fiber-degrading enzymes reduce dietary fiber content, as described by Shi et al. (2016), and Hu et al. (2016) with SSF using *Aspergillus niger*, producing numerous monosaccharides that are more easily used by fish's intestinal flora (Yang et al., 2021). Additionally, they improve the nutritional quality of the feed, feed digestion, fish's performance (Sinha et al., 2011), intestinal morphology, and absorption (Davies et al., 2021; Olukomaiya et al., 2019), by allowing the intestinal microbiota and endogenous fish enzymes to better access the remaining nutrients (Arredondo-Santoyo et al., 2020; Das et al., 2021).

As a consequence, overall nutrient digestibility is enhanced by carbohydrase activity by the NSPs hydrolysis, as was studied in SSF of BSG by *A. ibericus* (Fernandes et al., 2021). Additionally, during SSF, numerous proteases are produced as fungal metabolites, which will degrade dietary proteins and make them more easily accessed by other protein-degrading enzymes, as described by Shi et al. (2016) with *Aspergillus niger*'s SSF. Hence, protein digestibility increases (Olukomaiya et al., 2019), as was observed in fermented pea protein by *A. oryzae* and *A. niger*, as an *in vitro* protein digestibility augmented by 6.1% and 4.5% (Kumitch et al., 2020). Furthermore, SSF may alter the fragrance and palatability of substrates, which may stimulate fish's appetite and improve its performance (Das et al., 2021; Yang et al., 2021).

During fish's metabolism, reactive oxygen species (ROS) are produced as a natural response to oxygen metabolism, inducing oxidative stress (Dey et al., 2016). Antioxidants are molecules that reduce oxidative stress with their free radical scavenging properties (Dey & Kuhad, 2014). In particular, phenolic compounds are found in the cell wall matrix, imprisoned in phenol rings, linked with hydroxyl groups, lipids, and other compounds, and are not bioavailable. Consequently, they are only liberated by a hydrolysis process, such as SSF fungal enzymatic hydrolysis, carried out by beta-glucosidase, for example (Ajila et al., 2012; Shahidi & Yeo, 2016).

Some phenolics have been found to have antimicrobial, antiviral, and anti-inflammatory properties. For instance, flavonols and tannins appear to suppress some microbial virulence factors, such as inhibition of biofilm formation or neutralization of some bacterial toxins (Daglia, 2012; Laddomada et al., 2015). In other studies, SSF rice koji by *A. candidus* increased antioxidant activity and revealed an inhibitory effect on linoleic acid peroxidation, which indicates lower oxidative stress (Yen et al., 2003). Also, oats SSF with *Cordyceps militaris* augmented phenolic acid contents and antioxidant capacity and, consequently, successfully increased DNA damage protection (Xiao et al., 2015). Other reports revealed that SSF rapeseed with *A. oryzae* significantly enhanced immune responses and bactericidal activity in red sea bream (Dossou et al., 2018) and decreased oxidative stress (Dossou et al., 2018).

However, SFF faces some challenges in its process. For instance, due to the complexity of strains of microorganisms, contaminations may occur, drug-resistant genes may be transferred, and toxic metabolites may be synthesized (Ezekiel et al., 2019). In addition, physicochemical parameters of the fermentation may interfere with the final product, such as temperature, humidity, pH, aeration, particle size (Leite et al., 2021; Rodriguez-Leon et al., 2008), and acetic acid production by the microorganisms, which may decrease the palatability and appetite (Yang et al., 2021). Occasionally, SSF can lead to a decrease in nutrient content, such as vitamins and amino acids (Singhania et al., 2009). Additionally, some fungi may produce mycotoxins under certain conditions and may be harmful to fish. Also, fungal spores are produced as a natural step of the fungal life cycle and may have controversial effects on fish nutrition and immunology, which will be explained in detail further in this introduction.

#### 1.4.1. *Aspergillus* spp.

*Aspergillus* spp. are described as great producers of exogenous enzymes that have various applications, such as in the food, pharmaceutical or biotechnology industries (Leite et al., 2021; Gholami-Shabani et al., 2022). Particularly, *A. niger* (Leite et al., 2021; Pothiraj et al., 2010; Sousa et al., 2021) and *A. ibericus* (Sousa et al., 2018) have been described as two of the most important producers of lignocellulolytic enzymes. *Aspergillus* spp. belong to the Ascomycota filamentous fungus, which are among the most abundant fungi in the world.

Their wide distribution in soil and air is a consequence of their wide dispersal through their asexual spores, the conidia, continuously produced during their life cycle (Krijgsheld et al., 2013). An important feature of the spores and the mycelium of *Aspergillus* species for potential

applications in animal nutrition is their cell wall, which is mainly composed of layers of structural polysaccharides that constitute up to 50% to 80% of the cell wall, and are subdivided into glucans, chitin, and mannans (Isaza-Pérez et al., 2020).

Glucans are the most abundant polysaccharides in the fungal cell wall and account for 50% to 60% of its dry weight, followed by mannans, ranging from 35% to 40%, and chitin with 10% to 20% (Arana et al., 2009; Garcia-Rubio et al., 2020). These compounds are thought to have immunogenic properties for fish and “pathogen-associated molecular patterns” (PAMPs), as a result of their capacity to stimulate phagocytic activity (Erwig & Gow, 2016). For instance,  $\beta$ -glucans can stimulate cellular and humoral responses during infections (Erwig & Gow, 2016). In mammals,  $\alpha$ -mannans lead to some proinflammatory gene expression, such as TNF- $\alpha$ , and host antifungal activity (Loures et al., 2015). Other authors suggest that chitin may also have proinflammatory properties, for example, by inducing the expression of interleukin-10 in mouse macrophages (Wagener et al., 2014).

## Aims

Considering the challenges aquaculture faces towards achieving carbon-free production, using vegetable ingredients and agriculture by-products is an excellent option to reduce aquafeeds' carbon footprint and promote a circular economy. Additionally, Solid-state Fermentation, being an eco-friendly process, is a promising approach to help mitigate the nutritional constraints given by plant feedstuffs to fish.

Consequently, this study's aims were to:

1. Evaluate the effects of SSF in a plant feedstuffs mixture and the production of bioactive compounds by three different *Aspergillus* spp. strains.
2. Access the effect of dietary inclusion of the fermented mixture of plant feedstuffs chosen in chapter 1, on growth performance, feed utilization efficiency, protein retention, and digestive enzyme activity of European sea bass (*Dicentrarchus labrax*) juveniles.
3. Determine the effect of dietary inclusion of a fermented mixture of plant feedstuffs on plasma metabolites, oxidative stress status, inflammatory and protein degradation gene expression, and microbiota modulation of European sea bass (*Dicentrarchus labrax*) juveniles.

## Chapter 2 - Enrichment of a plant feedstuff mixture's nutritional value for use in non-ruminant animal feed through SSF

## Enrichment of a plant feedstuff mixture's nutritional value for use in non-ruminant animal feed through SSF

### 1. Materials and methods

#### 1.1. Solid-State Fermentation

Firstly, a plant feedstuff mixture composed of 25% (w/w) each rapeseed meal, soybean meal, rice bran, and sunflower seed meal was adopted based on the inclusion profile of plant ingredients used in a commercial diet for European sea bass (personal communication from Sorgal S.A., Portugal). Then, this mixture was fermented in triplicate with 3 fungi strains (*Aspergillus ibericus* MUM 03.49; *Aspergillus niger* CECT 2088; *Aspergillus niger* CECT 2915) to obtain mycoprotein-enriched biomass with enzymatic activity. An inoculum of each fungus was prepared with a peptone solution (0.1% peptone and 0.01% Tween-80) previously sterilized by autoclave (121 °C for 15 minutes) containing 10<sup>6</sup> spores/ml, 2 ml of this spore solution was inoculated into 10g of substrate mixture at 75% humidity prepared in 500 ml Erlenmeyer's flasks sterilized by autoclave (121 °C for 15 minutes) and fermented for 7 days at 25 °C.

Proximate composition, enzymatic activity, total phenols, total antioxidants, ferric reducing power, iron chelating activity, flavonoids, orthodiphenols, superoxide dismutase, and *in vitro* protein digestibility were determined before and after the SSF with each fungus.

#### 1.2. *In vitro* protein digestibility

*In vitro* protein digestibility was measured according to Morales et al. (2018). Briefly, an *in vitro* two-stage digestion of each fermented substrate was made, acidic digestion with pepsin and alkaline digestion with pancreatin. The total protein content of the substrate before and after digestion was determined by the Kjeldahl method. The protein digestibility was calculated as the difference between the protein content before and after digestion in each fermented substrate.

#### 1.3. Enzymatic activity

At the end of fermentation, an aqueous extraction of the fermented substrate was done to evaluate bioactive compounds, and water was added to the substrate in a 1:5 ratio (w/v) and left with constant stirring for 30 minutes. The substrate was then filtered through a fine-mesh net and centrifuged at 11200 g for 10 minutes at 4 °C. The supernatant was then filtered by

vacuum through filter paper (pore size of 11  $\mu\text{m}$ ). The resulting extract was then stored at  $-20^{\circ}\text{C}$  until analysis.

Fermented mixture activity of xylanase and cellulase was determined by measuring the release of xylan and carboxymethyl cellulose (CMC) after enzymatic hydrolysis using the 3,5-Dinitrosalicylic acid (DNS) method as described in (Leite et al., 2019),  $\beta$ -glucosidase was analyzed with p-Nitrophenyl- $\beta$ -D-glucopyranoside (PNG) as substrate as described by (Leite et al., 2016), protease activity was determined using Azocasein as a substrate (Sousa et al., 2021) and lipase activity was measured using p-nitrophenyl butyrate as a substrate (Gomes et al., 2011).

#### **1.4. Antioxidant activity**

Total phenols were determined using the Folin-Ciocalteu method (Commission Regulation (EEC) No. 2676/90). Total flavonoids were measured with a method described in (Gouveia & Castilho, 2011), and total ortho-diphenols according to the sodium molybdate assay seen in (Domínguez-Perles et al., 2014). Total Antioxidant activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay as described by (Dulf et al., 2015) and 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation assay like in (Miller et al., 1993). Other parameters measured include antioxidant potential by the ferric reduction antioxidant power (FRAP) assay as seen in (Fernandes et al., 2016), Iron (II) chelating activity (ICA) as (Oliveira et al., 2018), and superoxide dismutase activity (SOD) was measured as described in (Oliveira et al., 2018).

#### **1.5. Proximal composition of the fermented product**

Firstly, in order to analyze cellulose and hemicellulose levels by screening free sugars released during an quantitative enzymatic hydrolysis, starch was removed utilizing a two-step process, the first step involved an extraction process, where 120 mL of an 80% ethanol solution was added to 12 g of the sample. This mixture was then put in an ultra-sound bath for 15 minutes, centrifuged at 1000  $g$  (10 minutes at  $4^{\circ}\text{C}$ ), and the supernatant was recovered to a metallic cup with a known weight. The ultra-sound bath treatment, the centrifuge process, and the supernatant recovery were repeated 5 times and recovered into different metallic cups. These were then dried in an oven at  $105^{\circ}\text{C}$  overnight as well as the solid that remained at the end of the treatment, which was weighed. The second step involved the removal of the starch. 2 g of solid that was recovered from the previous step was weighed, then 20 mL of 0.1 M acetate

buffer (pH of 5) and 160  $\mu$ L of thermostable  $\alpha$ -amylase were added. Then an incubation period occurred at 100 °C for one hour, stirring regularly every 10 minutes. After cooling, 270  $\mu$ L of highly pure amyloglucosidase (70 U/g) was added, and the samples were incubated at 60 °C for 4 hours with agitation. The blank was done by adding buffer instead of enzymes. Then, 2 mL of the sample was recovered and centrifuged at 11200 *g* for 5 minutes, the sample is then filtered with a 0.45  $\mu$ m filter into a 1.5 mL HPLC vial and stored for analysis. To the remainder mixture, 80 mL of absolute ethanol was added, this was then stirred at 4 °C for one hour. After this step the mixture was then centrifuged at 11200 *g* for 5 minutes, the supernatant was discarded and the remaining residue was washed by resuspension with 80 mL of an 80% ethanol solution and centrifuged again at 11200 *g* for 5 minutes, after discarding the supernatant, another washing process was done with 25 mL of an acetone solution and another centrifugation process at 11200 *g* for 5 minutes was done, these last 2 washing steps were repeated 2 times. Finally, the remaining solids were left at 60 °C in an oven overnight.

Cellulose, hemicellulose, and Klason lignin were determined in these solids using quantitative acid hydrolysis (QAH) in an acid treatment with two stages, and HPLC with a Jasco830-IR intelligent refractive-index detector and a Varian MetaCarb 87H column were used in this process. Total nitrogen content was determined by the Kjeldahl method (Kjeltec system; digester model 1015 and distillation models 1026; Tecator Systems, Höganäs, Sweden). Total lipids were determined by extraction with petroleum ether utilizing a Soxtec system (SoxTec extraction system; Tecator systems; extraction unit model 1043 and service unit model 1046). Gross energy was determined by direct combustion with an adiabatic calorimeter bomb (PARR Instruments; Moline, IL, USA; PARR model 1261).

### 1.6. Statistical analysis

The bioactive compound's activity and composition of the fermented substrate produced by the *A. ibericus*, *A. niger* 2088, and *A. niger* 2915 were analyzed for significant differences with a one-way ANOVA. For all data, the probability level of 0.05 was used for the rejection of the null hypothesis. All statistical analysis was performed using SPSS 27.0 software package for Windows (IBM, USA).

## 2. Results and discussion

### Total protein

Animal feed's high-quality protein is of utmost importance for animals' growth. In this study, SSF significantly increased the total protein of the plant feedstuff mixture among all fungi, from 22% in *A. niger* 2088 to 32% in *A. ibericus* (Table 1). The fungus is a source of crude protein, and the increase in total protein content during the SSF may due to microbial growth, although it may vary depending on the fungi and substrate utilized, as an increase in protein is not always achieved (Sousa et al., 2022).

Similarly, different studies have reported a protein content increase in fermented substrates. For example, fermented lemon peel with *Lactobacillus reuteri* and *Saccharomyces cerevisiae* reached a protein increase of 76% compared to the unfermented substrate (Tropea et al., 2021). In washed and unwashed macroalgae *Ulva rigida*, SSF with *A. ibericus* increased total protein content by 8% and 21%, respectively (Fernandes et al., 2019). In another study, a fungi and bacteria consortium using *A. niger*, *Candida utilis*, and *Bacillus subtilis* increased the total protein of *Moringa oleifera* leaf meal by 30% (Shi et al., 2020). Using soybean, rapeseed, and sunflower oilseed cakes, SSF with *A. ibericus*, *A. uvarum*, and *A. niger* significantly improved protein content, mainly in brewer's spent grain (BSG), by 38.5% with *A. ibericus* (Sousa et al., 2018). Contrarily, other authors have also observed a decrease in the total protein content of fermented substrate relative to the unfermented. SSF of oilseed cakes with *R. oryzae* and *A. ibericus* significantly reduced crude protein content. This may occur when fungi use substrate nitrogen to ensure their growth (Sousa et al., 2021).

Soluble protein and energy levels were not statistically affected by SSF, and this may be due to the use by the fungi of energy and soluble protein to grow, thus not allowing for an increase to be seen (Olukomaiya et al., 2019). Depending on the fungus species, fermentation conditions and substrate utilized, soluble protein may increase or decrease. For example, SSF with *Bacillus subtilis* was able to increase the soluble protein of soybean meal from 6.26% to 22.78% (Dai et al., 2017).

### Protein digestibility

SSF may improve protein digestibility due to the production of microbial proteases, which hydrolyze substrate protein into small monomers, increasing protein digestibility. Moreover,

protein digestibility may also be indirectly enhanced through the reduction of anti-nutritional compounds, as trypsin inhibitors (Gao et al., 2013), and the lignocellulosic matrix of the substrate (Olukomaiya et al., 2019; Ranjan et al., 2019). In the present study, protein digestibility significantly increased with SSF, mainly in the two *A. niger* strains, reaching 50% and 58% in *A. niger* CECT 2088 and *A. niger* CECT 2915, respectively. Some earlier studies report increased protein digestibility of different substrates after fermentation with *A. niger*. For instance, Kumitch et al. (2020) concluded that fermented pea protein by *Aspergillus oryzae* and *A. niger* increased *in vitro* protein digestibility by 6.1% and 4.5%, respectively. In another study, SSF with *A. niger* increased the protein digestibility of *M. oleifera* leaf meal by 35% (Wang et al., 2018). A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed that SSF of soybean by *S. cerevisiae* increased protein digestibility, by fungal digestion of the protein demonstrated with the appearance of oligopeptides with molecular weight lower than 14 kDa in fermented soybean (Zahir et al., 2020). Contrarily, other authors have also observed a decrease in protein digestibility. For example, *in vitro* protein digestibility of de-oiled rice bran fermented with *Rhizopus oryzae* decreased by 17% (Ranjan et al., 2019). Simulating an *in vivo* digestion in an *in vitro* approach is a highly complex process, due to the difficulty in simulating real digestive conditions (Moyano et al., 2015). Hence, these contradictory results may be due to differences in the methods and in the matrix used since the latter study determined *in vitro* digestibility through the exposure of the substrate to a basic and acidic pH. In the present study, the mixture was submitted to an acidic and basic pH, respectively, and two gastric enzymes were also added, which might be more coherent to an *in vivo* fish digestion.

### Exogenous enzymes

Cellulose and hemicellulose are the main components of the plant cell wall (Soccol et al., 2017), the presence of these compounds in the fermentation substrate induce the production of fungal exogenous carbohydrase's (Kaur et al., 2020). Therefore, as the fermented plant-feedstuff mixture in this study contained high fiber content, fiber degrading enzymes production might be equally high, to decrease fiber content. *Aspergillus* species are well-established fungi in the industry as exoenzyme enzyme producers, particularly carbohydrases (Leite et al., 2021). In the present study, SSF decreased cellulose content, while hemicellulose content reduction was not statistically significant. The decrease in fiber content is related to the fungi's exoenzyme production during the fermentation process, namely cellulase, xylanase, and beta-glucosidase. The results in this work show that *A. niger* 2088 was the highest cellulase and xylanase producer,

even though it was not statistically different from the other two fungi. Beta-glucosidase also reached a high production of 48.4 U/g with *A. ibericus* and 32.8 U/g with *A. niger* 2088. Similar to our findings, in SSF of BSG with *A. niger*, xylanase production was higher than cellulase (Sousa et al., 2018), and using *A. ibericus* in BSG, xylanase was also circa 7-fold higher than cellulase production (Fernandes et al., 2022). In fermented oilseed cakes SSF with *A. niger* xylanase production was 6-fold higher than cellulase (Sousa et al., 2021). This high production of lignocellulolytic enzymes can also be seen in fermented *M. oleifera*, where *A. niger* produced 564 U/g of xylanase (Wang et al., 2018). As cellulase is usually the most produced carbohydrase, due to the majority of the substrates richness in cellulose (Fernandes et al., 2019), the higher xylanase activity reported in these studies may be due to hemicellulose being present in the outer layer of the plant cell wall, and, as aforementioned, it's presence will firstly stimulate the production of xylanases and only then exposing cellulose for higher cellulase production.

*Aspergillus* fungi are also known for their proteolytic activities (Mukhtar & Haq, 2009). In this study, protease production was highest in *A. niger* 2088, with 5.1 U/g, which are within the range reported for SSF of other lignocellulosic substracts. *A. niger* IHG<sub>9</sub> fermented lead to the protease production of 5.2. U/g sunflower meal, 3.2 U/g wheat bran, 4.8 U/g soybean meal, 4.0 U/g cottonseed meal, and by 3.3 U/g rapeseed meal (Mukhtar and Haq, 2009). Also, the SSF of soybean, rapeseed, and sunflower oilseed cakes, reached the maximum protease activity with *A. niger* by 157 U/g (Sousa et al., 2021). In SSF with *Penicillium camemberti*, rapeseed cake protease production reached 380 U/mg (Boratyński et al., 2018). In the present work, as aforementioned, protease production could have contributed to a higher protein digestibility, through the hydrolysis of proteins into peptides and small monomers.

Fungi are widely regarded as the best lipase sources for industrial applications, using agroindustrial wastes or oil by-products, including rice bran (Kumar & Ray, 2015), which is present in the plant-feedstuff mixture used in this work. However, to maximize lipase production, substrate supplementation may be required, such as ammonium chloride or peptone, as nitrogen and carbon sources, respectively (Ramos-Sánchez et al., 2015). In the present work, lipase production by the three fungi species was similar, even though it tended to be higher with *A. niger* 2088, reaching 3.3 U/g of dry substrate. Moreover, in SSF rapeseed cake with *P. camemberti*, a 2% supplementation of lactose and calcium chloride in optimal conditions increased lipase production from 22 U/mg to 247 U/mg (Boratyński et al., 2018).

### Antioxidant activity

In the literature, fermentation has been shown to enhance phenolic compounds and their antioxidant activity (Dulf et al., 2016; Hur et al., 2014). Indeed, phenolic compounds are present in lignin, reducing their bioavailability (Kleinert & Barth, 2008). During the SSF, the produced fungal carbohydrase's, such as beta-glucosidase, catalyze the hydrolysis of phenolic glycosidic linkages, open the phenol rings and release free phenolic compounds (Ajila et al., 2012). However, depending on the fermentation conditions, the degradation of phenolic compounds may also occur, resulting in a decrease in antioxidant activity (Hur et al., 2014). Peroxidases and laccases are fungal enzymes known for their oxidant properties (Leite et al., 2021). However, both enzymes produce reactive oxygen species (ROS) that catalyze the oxidation of phenols and antioxidants, resulting in the formation of polymers (Nagarajan et al., 2020). This may lead to the lignification of the free phenols and antioxidants and reduction of the antioxidant properties of the substrate (Vazquez-Duhalt et al., 1994).

In the present work, SSF did not affect total phenols and flavonoids content and, inclusively, reduced the ortho-diphenols content and total antioxidant activity, although it was not significant. SSF can potentially decrease or increase antioxidant activity, depending on the fermentation conditions. For example, SSF with *A. oryzae* and *A. niger* increased total phenolic content in pea protein-enriched flour by 31% and 26%, respectively (Kumitch et al., 2020), as did in by-products of plum fruit, plum pomace, and plum brandy distillery wastes, fermented with *R. oligosporus* and *A. niger* (Dulf et al., 2016). However, a reduction in total phenolic compounds was reported by other authors in EOP (exhausted olive pomace) SSF with *A. ibericus*, *A. uvarum*, or *A. niger* (Sousa et al., 2018). The reduction of antioxidant activity in this work may be related to fermentation time. As was described by Filipe et al. (2020), fungal enzyme production during SSF with *A. ibericus* started after 2 days of the fermentation, and phenols and antioxidants reach their highest activities between the second and third day of the fermentation, followed by a decrease of their contents. Hence, the low antioxidant activity observed in this study may result from the production of peroxidases and laccases, during the 7 days of fermentation.

### 3. Conclusion

Solid-state fermentation of a mixture of rapeseed meal, soybean meal, rice bran, and sunflower seed meal with *A. ibericus* MUM 03.49; *A. niger* CECT 2088; *A. niger* CECT 2915 increased protein content and digestibility, decreased fiber content, and added highly active

carbohydrases, mainly cellulase and xylanase. *A. niger* CECT 2088 led to a higher yield in carbohydrases and fiber reduction compared to *A. niger* CECT 2915 and *A. ibericus*. Overall, the result highlight the potential of SSF with a mixture of rapeseed meal, soybean meal, rice bran, and sunflower seed meal to improve its nutritional value as a novel feedstuff for animal nutrition.

**Table 1** – Composition of plant feedstuff mixture (soybean meal, rice bran, rapeseed meal, sunflower meal) unfermented and fermented with *A. niger* 2088, *A. niger* 2915, and *A. ibericus* 03.49. Mean ( $\pm$ SD). Values with different superscript letters denote significant differences ( $p < 0.05$ ).

|                                                    | <i>Aspergillus niger</i><br>CECT 2088 |     | <i>Aspergillus niger</i><br>CECT 2915 |     | <i>Aspergillus ibericus</i><br>MUM 03.49 |     | Unfermented       |     |
|----------------------------------------------------|---------------------------------------|-----|---------------------------------------|-----|------------------------------------------|-----|-------------------|-----|
|                                                    | MD                                    | SD  | MD                                    | SD  | MD                                       | SD  | MD                | SD  |
| <b>Proximal composition (% dry matter)</b>         |                                       |     |                                       |     |                                          |     |                   |     |
| Total protein                                      | 38.3 <sup>ab</sup>                    | 2.0 | 39.2 <sup>a</sup>                     | 0.3 | 44.1 <sup>a</sup>                        | 2.0 | 30.1 <sup>b</sup> | 1.1 |
| Soluble protein (mg/g)                             | 29.3                                  | 0.6 | 18.6                                  | 0.4 | 25.4                                     | 1.3 | 31.7              | 1.7 |
| Energy (kJ/g)                                      | 18.1                                  | 0.3 | 18.0                                  | 0.3 | 17.2                                     | 0.3 | 20.5              | 2.5 |
| Protein digestibility                              | 72.6 <sup>ab</sup>                    | 2.5 | 86.6 <sup>a</sup>                     | 7.0 | 47.6 <sup>bc</sup>                       | 3.0 | 36.6 <sup>c</sup> | 6.1 |
| Cellulose                                          | 9.2 <sup>b</sup>                      | 0.5 | 8.1 <sup>b</sup>                      | 0.9 | 9.3 <sup>b</sup>                         | 2.3 | 15.0 <sup>a</sup> | 0.5 |
| Hemicellulose                                      | 6.4                                   | 0.7 | 6.2                                   | 0.6 | 6.5                                      | 2.2 | 9.7               | 1.2 |
| <b>Enzymatic activity (U/g dry substrate)</b>      |                                       |     |                                       |     |                                          |     |                   |     |
| Cellulase                                          | 10.1                                  | 2.1 | 5.1                                   | 1.0 | 7.5                                      | 2.0 |                   |     |
| Xylanase                                           | 51.4                                  | 8.2 | 25.0                                  | 9.9 | 18.0                                     | 0.4 |                   |     |
| Beta-glucosidase                                   | 32.8 <sup>b</sup>                     | 0.1 | 5.4 <sup>c</sup>                      | 0.0 | 48.4 <sup>a</sup>                        | 1.4 |                   |     |
| Protease                                           | 5.1 <sup>a</sup>                      | 0.1 | 3.3 <sup>c</sup>                      | 0.1 | 4.0 <sup>b</sup>                         | 0.3 |                   |     |
| Lipase                                             | 3.3                                   | 1.6 | 0.7                                   | 0.7 | 0.3                                      | 0.1 |                   |     |
| <b>Antioxidant activity (per g of dry matter)</b>  |                                       |     |                                       |     |                                          |     |                   |     |
| Total phenols (mg of gallic acid equivalents/g)    | 0.4                                   | 0.0 | 0.3                                   | 0.0 | 0.7                                      | 0.1 | 0.7               | 0.1 |
| Ortho-diphenols (mg of gallic acid equivalents /g) | 0.1 <sup>c</sup>                      | 0.0 | 0.0 <sup>c</sup>                      | 0.0 | 2.4 <sup>b</sup>                         | 0.1 | 9.6 <sup>a</sup>  | 0.1 |
| Flavonoids (mg of quercetin equivalents/g)         | 0.9                                   | 0.3 | 0.1                                   | 0.0 | 0.7                                      | 0.1 | 0.6               | 0.0 |
| DPPH (μmol trolox equivalents/g)                   | 0.1 <sup>c</sup>                      | 0.1 | 0.5 <sup>cb</sup>                     | 0.4 | 1.8 <sup>b</sup>                         | 0.1 | 5.1 <sup>a</sup>  | 0.2 |
| ABTS (μmol trolox equivalents/g)                   | 44.0 <sup>b</sup>                     | 2.5 | 34.0 <sup>b</sup>                     | 3.6 | 29.2 <sup>b</sup>                        | 1.9 | 86.3 <sup>a</sup> | 4.6 |
| FRAP (mM of Fe2+ equivalents/g)                    | 0.1 <sup>b</sup>                      | 0.0 | 0.1 <sup>c</sup>                      | 0.0 | 0.1 <sup>b</sup>                         | 0.0 | 0.1 <sup>a</sup>  | 0.0 |
| SOD (μmol ascorbic acid equivalents/g)             | 0.3 <sup>ab</sup>                     | 0.0 | 0.1 <sup>b</sup>                      | 0.1 | 0.3 <sup>a</sup>                         | 0.0 | 0.4 <sup>a</sup>  | 0.0 |

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Chapter 3 - Effect on growth performance, well-being, and gut  
microbiota of European sea bass (*Dicentrarchus labrax*) juveniles  
fed with diets containing a SSF plant-feedstuff mixture with  
*Aspergillus niger*

# Effect on growth performance, well-being, and gut microbiota of European sea bass (*Dicentrarchus labrax*) juveniles fed with diets containing a SSF plant-feedstuff mixture with *Aspergillus niger*

## 1. Materials and methods

This study was approved by the ORBEA Animal Welfare Committee of CIIMAR (ORBEA; reference ORBEA\_CIIMAR\_27\_2019) according to the European Union directive 2010/63/EU and the Portuguese Law (DL 113/2013). All procedures were performed by certified scientists (Functions a, b, c & d defined in article 23 of European Union Directive 2010/63), in compliance with the Federation of Laboratory Animal Science Association (FELASA) recommendations and the EU Directive (2010/63/EU) on the protection of animals for scientific purposes.

### 1.1. Solid-State Fermentation scale-up

In Chapter 2, *A. niger* CECT 2088 produced higher cellulase and xylanase contents than the other two fungi. Additionally, total protein, protein digestibility, and fiber reduction increased with *A. niger* CECT 2088 SSF. Therefore, this fungus was chosen to perform the scale-up SSF to include in the experimental diets. The fermentation was done as described in Chapter 2, but using fermentation trays. The plant-feedstuffs mixture (400 g) was inoculated with 80 ml of spore solution containing  $10^6$  spores/ml with the same fermentation conditions.

### 1.2. Experimental diets

Four isoproteic and isolipidic diets (42% crude protein and 18% crude lipids) were formulated to include the non-fermented and fermented plant feedstuff mixture at 20% and 40% (diets 20 Mix, 40 Mix, 20 SSF, and 40 SSF, respectively). Dietary ingredients were ground, mixed, and pelleted utilizing a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA), through a 3 mm die. After pelleting, diets were dried (60 °C, 48h) and kept at -20 °C until use. The ingredients and proximate composition of the experimental diets are presented in Table 1.

### 1.3. Growth trial and experimental conditions

The growth trial was performed at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto, Portugal, with European sea bass (*Dicentrarchus labrax*) juveniles purchased from a certified hatchery in Sonrionansa S.L. (Cantabria, Spain). Fish were transported to the experimental facilities, kept in quarantine in a recirculation aquaculture system (RAS) with a 2000 L tank for 15 days, and fed a commercial diet (NEO diet, Aquasoja; Sorgal, S.A., Portugal).

Fish were then moved to the experimental facility, where they were acclimatized for 2 months. The experiment was performed in a thermo-regulated recirculation water system (RAS) equipped with 12 tanks with 300 L of water capacity. During the trial, water flow was established at about 5 L min<sup>-1</sup> per tank, the water temperature was set to 24 °C, salinity averaged 35‰, and dissolved oxygen averaged 8 g/L. Fish were maintained on a 12h light: 12 dark photoperiod.

After the quarantine period, 12 homogeneous groups of 20 seabass (initial body weight of 10 g) were transferred to the tanks. The diets were randomly distributed to triplicate groups, and fish were hand-fed twice a day, six days a week, until apparent satiation. Feed intake was determined weekly. The trial lasted 50 days, and an intermediate weighing was carried out four weeks after the beginning of the experiment. During the test, dead fish were removed daily, and their weight was recorded.

### 1.4. Sampling

Five fish from the initial population were sampled for whole-body composition analysis. At the end of the growth trial, fish were bulk-weighted following one day of feed deprivation to determine growth parameters. Three fish from each tank were randomly sampled for whole-body composition analysis, two more had their pooled intestine mucosa and digesta collected for microbiota analysis. Another three fish were randomly sampled after 4h of the first meal. Blood was collected from the caudal vein with heparinized syringes, centrifuged at 10000 rpm for 10 minutes and the plasma was stored at -80 °C until analysis. Then, fish were killed, and liver, intestine, and muscle were collected for posterior analysis and stored at -80 °C. Liver and intestine samples were also collected from three fish for RT-PCR analysis, kept in RNA later and stored at -80 °C. For whole-body composition analysis, fish were dried at 100 °C and then finely ground and stored in a desiccator until use.

### 1.5. Carbohydrase activity determination

At the end of scale-up fermentation, an aqueous extraction was done following the same conditions as in the Chapter 2, to determine cellulase (endo-1,4- $\beta$ -glucanase), xylanase (endo-1,4- $\beta$ -xylanase), and  $\beta$ -glucosidase activities (Table 1).

### 1.6. Antioxidant activity determination

To determine the antioxidant activity of the fermented product in the experimental diets, a double extraction with water/methanol (50:50 ratio) and with acetone/water (70:30 ratio) was performed. Then, with constant stirring at room temperature for 30 min, samples were filtrated and centrifugated. Then, total antioxidant activity (DPPH) and total phenolic compounds were accessed as described in Chapter 2 (Table 1).

### 1.7. Bromatological analysis

The chemical composition of the ingredients, fermentation products, diets, and whole fish was determined following standard procedures of the Association of Official Analytical Chemists (AOAC International, 2016). Dry matter, total protein, total lipids, ash, and gross energy were analyzed as described in Chapter 2 (Table 1).

### 1.8. Digestive enzyme activity determination

Intestine samples were homogenized (1:6 w/v) in ice-cold buffer (100 mM Tris-HCl, 0.1% mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8). Samples were then centrifuged at 30,000 $\times$ g for 30 min at 4 °C and the supernatants were stored at -80 °C until analysis. Samples were measured at 37 °C in a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China).  $\alpha$ -Amylase (EC 3.2.1.1) and lipase (EC 3.1.1.3) were determined using commercial kits by (Spinreact ref. 1001274 and 41201, respectively) adapted to European sea bass, as described by Magalhães et al. (2016). Total alkaline proteases were determined according to Fernandes et al. (2022).

### 1.9. Plasma metabolites determination

Analyses of plasma glucose, cholesterol, triglycerides, and total protein were performed using commercial kits from SpinReact, S.A. (Gerona, Spain), ref.: 1001191, ref.: 1001091, ref.:

41031, ref.:1001291 respectively. All plasmatic parameters were analyzed in a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China).

### 1.10. Oxidative and metabolic enzyme activity determination

Intestine, muscle, and liver samples were homogenized (1:6 w/v for liver and intestine; 1:4 w/v for muscle) in ice-cold buffer (100 mM Tris-HCl, 0.1% mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8). Samples were then centrifuged at 30,000 *g* for 30 min at 4 °C, and the supernatants were stored at –80 °C until analysis.

Oxidative stress enzymes were determined in the liver and intestine as follows: catalase (CAT, EC 1.11.1.6), as in Aebi (1984); glutathione peroxidase (GPX, EC 1.11.1.9), as seen in Flohé and Günzler (1984); glutathione reductase (GR, EC 1.6.4.2) and Glucose-6-phosphate-dehydrogenase (G6PDH, EC 1.1.1.49), following Morales et al. (1990) and superoxide dismutase (SOD, EC 1.15.1.1), as described in McCord and Fridovich (1969).

Aspartate (ASAT/GOT, EC 2.6.1.1) and alanine aminotransferase (ALAT/GPT, EC 2.6.1.2) activities in the liver were accessed with commercial kits (Spinreact, ASAT/GOT; 41,273; ALAT/GPT; 41,283). Glutamate dehydrogenase (GDH, EC 1.4.1.2) activity was calculated according to Morales et al. (1990).

Enzymatic activity was expressed as units (CAT, SOD,  $\alpha$ -Amylase, lipase, protease) or milliunits (GPX, G6PDH, GR, ALAT, ASAT, and GDH). A unit (U) of enzymatic activity was described as the amount of enzyme necessary to release 1  $\mu$ mol of substrate per min in the experimental conditions, except for SOD, as one unit of enzymatic activity was defined as the quantity of enzyme needed to result in 50% inhibition of ferricytochrome C. All enzyme activities were expressed per mg of soluble protein. Soluble protein was calculated according to Bradford (1976) with a protein assay kit (Biorad, Ref. 5000006; Algés, Portugal) using bovine serum albumin as standard.

### 1.11. Lipid Peroxidation

Liver, intestine, and muscle lipid peroxidation were measured as the content of reacting substances of thiobarbituric acid and expressed as nmol MDA  $g^{-1}$  tissue, as described by Buege and Aust (1978). Briefly, 100  $\mu$ l of homogenate was mixed with 500  $\mu$ l of a solution containing 15 % (w/v) trichloroacetic acid (TCA), 0,375 % (w/v) thiobarbituric acid (TBA), 80 % (v/v) HCl 0,25 N and 0,01 % (w/v) butylated hydroxytoluene. Samples were then heated at 100 °C for 15 min,

cooled, and centrifuged (1500 g x 10 min). The supernatant was recovered and absorbance was read at 535 nm.

### 1.12. Gene expression

Total RNA from intestine and liver were isolated using TRIzol reagent (Direct-zol™ RNA Miniprep, Zymo Research) according to supplier recommendations. The purified RNA was eluted in 50 µl of DNase/RNase/Protease-free water and treated with DNase I (Direct-zol™ RNA Miniprep, Zymo Research) to avoid contamination by DNA. To access RNA quality, a spectrophotometrically µDrop™ plate (ThermoScientific) was used, and quantity was screened by electrophoresis on 1% agarose gel. 0.5 µg/µl of RNA was then converted into cDNA with a NZY First-Strand cDNA Synthesis Kit (NZYTech. MB12501, Lisbon, Portugal).

Real-time quantitative PCR (CFX Connect™ Real-Time System, Bio-Rad, California, USA) was used to analyze gene expression. Primers were gathered from the literature (Table 2) and the quality of primer pairs was measured with the Beacon Designer Program. Primer efficiency was analyzed with the method proposed by Pfaffl (2001), using five serial threefold dilutions of cDNA.

### 1.13. Microbiota composition

Digesta and mucosa were submitted to a DNA extraction, polymorphism analyses of 16S rRNA genes by denaturing gradient gel electrophoresis (DGGE), band excision, and re-amplification were performed as described by Castro et al. (2019). Briefly, PCR products were loaded onto a polyacrylamide gel at 8% with a denaturing gradient of 40 to 60% 7 M urea/40% formamide. Verified bands were excised, amplified, and then sequenced to identify microbiota operational taxonomic units (OTUs). To identify close known species, a phylogenetic analysis was done as described by Castro et al. (2019).

### 1.14. Statistical analysis

All data were checked for normality and homogeneity of variances and normalized when applicable. Data analysis was accessed using a Two-way ANOVA with SSF and the inclusion level as independent variables. If an interaction was significant, a t-test was performed for each factor. A p-value <0.05 was used as the significance level for all statistical analyses. Statistical analyses were performed with IBM SPSS Statistics software version 26 (IBM, New York, USA).

## 2. Results

During the trial, non-fermented diets (20 Mix and 40 Mix) were well accepted by fish, while fermented diets (20 SSF and 40 SSF) were not. Nevertheless, no signs of stress or disease were noticed during the trial. Mortality was low but higher in those fed the SSF including diets (Table 3).

Irrespective of SSF, the growth performance of fish fed with the 40 Mix diets was lower than with the 20 Mix diets. Regarding the SSF effect, the growth performance of fish fed with 40 SSF was lower than that of the respective unfermented diet. Feed intake decreased with SSF and was lower in fish fed the 40 Mix and 40 SSF diets compared to the 20% inclusion level diets. Feed efficiency increased when fish were fed the 20 SSF diet. However, both feed efficiency and protein efficiency ratio (PER) decreased with the fermented inclusion in the 40 SSF diet. Concerning the inclusion effect, FE and PER were higher in fish fed the 20 Mix diets than in the 40 Mix diets. Nitrogen intake (NI) decreased when fish were fed the 40 SSF diet, compared to the 20 SSF diet. Regarding the inclusion effect, fish fed the 40 Mix diets had higher NI than fish fed the 20% respective ones. Nitrogen retention (NR) decreased within the 40% inclusion level when fish were fed the 40 SSF diet. Irrespective of the fermentation, the NR of fish fed with the 20 SSF diet was higher than the 40 SSF diet.

Whole-body ash and lipid contents were unaffected by dietary SSF. Regarding the inclusion level, whole-body dry matter content increased when fish were fed the 20% Mix diet, rather than at 40%. Irrespective of the inclusion level, fish fed the 20 Mix diet had higher dry matter than 20 Mix SSF. Dietary SSF increased whole-body protein in the 20 Mix SSF diet and decreased in the 40 Mix SSF diet. Gross energy content was affected by the inclusion level, being higher when fish were fed the 20% inclusion diets (Table 4).

Amylase activity was lower in fish fed the 40 SSF diet than the 40 Mix diet, while no differences were observed for the 20 Mix diets. Lipase activity was higher in fish fed the 40 Mix than in those fed the 20 Mix diets, while SSF increased lipase activity, but only in the 20 Mix diets. Total alkaline proteases decreased when fish were fed with 20% inclusion level diets, irrespective of SSF. Regarding the SSF effect, fish fed with non-fermented diets had higher activity of proteases (Table 5).

Plasma total protein, cholesterol, and triglyceride levels were unaffected by dietary treatments (Table 6). Within the 40 Mix diets, glucose content increased with SSF.

In the intestine, catalase (CAT), glutathione reductase (GR), and glucose 6-phosphate dehydrogenase (G6PDH) activities were unaffected by the plant feedstuffs mix inclusion level nor by SSF (Table 7). Glutathione peroxidase (GPX) was higher in fish fed the 40 Mix diets than in the 20 Mix diets. SSF increased superoxide dismutase (SOD) activity in fish fed the 20 SSF diet, while the opposite was observed for fish fed the 40 SSF diets. Concerning the inclusion effect, SOD activity of fish fed the 40 SSF was lower than 20 SSF.

Liver glutamate dehydrogenase (GDH) and aspartate aminotransferase (ASAT) activities were not affected by the diet composition (Table 7). Alanine aminotransferase (ALAT) activity was higher in fish fed the 20 Mix than the 40 Mix plant feedstuffs diets. Liver CAT activity was higher in fish fed the 40 Mix than the 20 Mix plant feedstuffs mix diets, while the opposite was observed for GR and G6PDH. GPX and G6PDH activities were lower in the fermented diets than in the un-fermented ones.

Hepatic SOD activity was higher in fish fed the 40 SSF diet than the 20 SSF diet. Concerning the SSF effect, SOD activity increased when fish were fed the 20 SSF compared to fish fed the 20 Mix diet. The intestine, liver, and muscle lipid peroxidation (LPO) were not affected by dietary treatments.

Gut caspase 3 (casp 3), interleukin 8 (IL-8), interleukin 10 (IL-10), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), and liver insulin-like growth factor-I (IGF-I) gene expression were unaffected by dietary treatments (Fig. 1a, b, c, e, and g). Intestine transforming growth factor  $\beta$  (TGF- $\beta$ ) (Fig. 1d) gene expression was lower, and liver cathepsin D (Cat D) (Fig. 1f) gene expression was higher in the fermented diets.

Bray-Curtis dendrogram obtained from the DGGE fingerprints analysis (Fig. 2) showed an illustrative grouping tendency between the majority of the non-fermented diets (20 Mix and 40 Mix) and the fermented diets (20 SSF and 40 SSF).

PCR-DGGE fingerprints of fish fed the experimental diets showed that SSF increased the average intestinal number of operational taxonomic units (OTUs), microbial richness, and diversity indices (Table 8). This was confirmed by analysing the sequenced gel bands that showed the appearance of numerous *Bacillus* spp. (bands 1, 2, 3, 4, 7, 8, and 9) and a *Clostridium* sp. strain (band 6) in fish fed with the SSF diets. The inclusion level increased microbial evenness, diversity, and SIMPER similarity indices when fish were fed the 40 Mix diets (Table 9).

### 3. Discussion

Solid-state fermentation has been described as a tool for agroindustrial by-products, and low-carbon footprint plant feedstuffs nutritional valorization (Fernandes et al., 2021; Kumitch et al., 2020; Stephen et al., 2022). *A. niger* SSF is well described within the biotechnology industry (Sousa et al., 2018; Wang et al., 2018) and is referred to as a suitable fungus to include in animal feeds (Dawood & Koshio, 2020; Chuang et al., 2020; Kong et al., 2021). Besides, *A. niger* SSF is used to produce commercial enzymes used in aquafeeds, such as Synergen™ (Anwar et al., 2020; Diógenes et al., 2018; Magalhães et al., 2018), or Allzyme™ (El-Sayed et al., 2014; Moura et al., 2012), produced by Alltech.

Several studies have demonstrated that SSF of different substrates leads to the production of add-value functional extract or enriched fermented substrates with applicability in aquaculture. For example, SSF of brewery by-products leads to the production of an extract with high enzymatic activity, including carbohydrases (Fernandes et al., 2022), *in vitro* and *in vivo* increased digestibility (Fernandes et al., 2021). Also, the addition of a commercial carbohydrase produced by *A. niger* SSF increased rainbow trout (*Oncorhynchus mykiss*) growth performance (Davies et al., 2021). Other authors described that SSF may enhance feed palatability, as observed in rohu juveniles (*Labeo rohita*) fed with a SSF fish feed with *S. cerevisiae* (Das et al., 2021). Further, SSF of de-oiled sunflower seed meal with *S. cerevisiae* and *B. subtilis* was found to decrease fiber content after fermentation (Hassaan et al., 2018), and after SSF of soybean meal and sunflower oil cake, anti-nutritional factors, such as saponins, phytic acid, and trypsin inhibitor decreased (Jannathulla et al., 2018).

In the present study, the inclusion of SSF plant feedstuff mixture induced the production of cellulase and  $\beta$ -glucosidase, which were detected in the experimental diets. However, even though xylanase activity was detected in the fermented mixture, its activity was not detected in the experimental diets, possibly due to its lower thermostability (Kaur et al., 2021) and consequent loss during the diets manufacturing and drying processes. Fungal carbohydrase production and, concomitantly, the substrate lignocellulolytic matrix degradation through SSF had been reported for several plant feedstuff. SSF of rapeseed and soybean oil cakes with *R. oryzae*, *A. ibericus*, and *A. niger* led to the reduction of acid detergent fiber (ADF) of circa 67% and 69%, while neutral detergent fiber (NDF) decreased up to 63% and 53%, respectively, attributed to the production of carbohydrases (Sousa et al., 2021).

The inclusion of the fermented plant feedstuff mixture in 20% and 40% did not affect total phenols content, but total antioxidants slightly decreased. The fermentation conditions of SSF of the plant feedstuffs were optimized to maximize the production of carbohydrases, so part of the antioxidants may have been lost during the fermentation process. Indeed, depending on the substrate composition, SSF may increase or decrease the content of phenolic compounds and antioxidant activity (Dulf et al., 2016). For instance, pea protein-enriched flour SSF with *A. oryzae* and *A. niger* increased total phenolic activity up to 30.9% and 26.1%, respectively, after 6 hours of fermentation (Kumitch et al., 2020). However, SSF of wheat and oat bran with *S. cerevisiae* decreased total phenolic and antioxidant activity after 3 and 4 days for wheat and oat bran, respectively (Călinoiu et al., 2019). The reduction of the antioxidant activity during SSF may be due to the production of some enzymes, such as laccases and peroxidases (Leite et al., 2021), that catalyze antioxidants and phenols oxidation (Nagarajan et al., 2020). Furthermore, the antioxidant content of fermented substrates is related to the fermentation time. The kinetic production/release of the secondary compounds during SSF by *A. ibericus* attained the highest production between the second and the third day of fermentation, followed by a decrease, while enzyme activity production starts two days after the fermentation (Filipe et al., 2020).

In this work, the 20 SSF diet did not affect growth performance in European sea bass juveniles, but when fish were fed the 40 SSF diet, growth performance decreased, suggesting that the success of dietary inclusion of fermented ingredients may be related to the dietary inclusion levels. For different fish species, the dietary incorporation of fermented ingredients improved growth performance. For example, the SSF of corn husk, rice bran, palm, rapeseed, and soybean meal by a mixture of *Lactobacillus* spp., yeast, and *Bacillus* spp., and the dietary inclusion of 3%, 5%, and 8% increased gibel carp (*Carassius auratus gibelio* var. CAS V) juveniles growth performance when 5% of the fermented mixture was included (Cao et al., 2021), while the inclusion of 8% had no positive effects. For turbot (*Scophthalmus maximus* L.), the inclusion of a fermented soybean meal with *A. awamori* did not affect growth performance compared to the non-fermented soybean meal-based diets (Li et al., 2019). For rainbow trout and whiteleg shrimp (*Litopenaeus vannamei*), the inclusion of 30% of fermented soybean and 50% of fermented corn gluten by *B. subtilis* U304 decreased growth performance combatively to the control un-fermented diet (Moniruzzaman et al., 2018; 2020).

The reduced growth performance described in this study, particularly in fish fed the 40 SSF diet, may be related to the lower voluntary feed intake. Although SSF of plant feedstuffs has been reported as an enhancer of animal feed palatability, stimulating animal appetite (Yang et

al., 2021), other authors have reported that low pH and high concentrations of some metabolites produced by the fungi may decrease feed palatability and, consequently, feed intake (Brooks, 2008). The reduced growth performance may also be attributed, at least in part, to the presence of fungal spores in experimental diets. The fermented plant-feedstuff mixture used in the present study comprises degraded plant feedstuffs, the fungus itself, the fungi metabolic compounds, and fungal spores. Spores are naturally produced during sporulating fungi life cycles, such as *Aspergillus*, as a part of asexual reproduction (Krijgsheld et al., 2013). Fungal spores cell walls are composed of glucans, glycoproteins, melanin, and chitin, which may represent up to 20% of the cell wall (Garcia-Rubio et al., 2020). In the fish gut, chitin disruption is attributed to chitinolytic enzymes (Fines & Holt, 2010). However, carnivorous fish, such as European sea bass, are not able to produce these enzymes, or their production is inefficient (Guerreiro et al., 2020). Consequently, fungal spores are undigestible and may have interfered with another nutrient uptake, decreasing overall seabass growth performance. Moreover, some fungi species, including *A. niger*, produce some secondary metabolites, such as hydrolytic and proteolytic enzymes, adhesins, and kinases (Sarkar et al., 2021). Despite *A. niger* being reported as nonpathogenic, and a Generally Regarded As Safe (GRAS) fungi by FDA (US Food and Drug Administration; Meyer & Schmidhalter, 2012), in the present study, the production of these metabolites may have caused some metabolic interferences that may impair sea bass growth (Ráduly et al., 2020). Although no signs of external or internal disease were detected, more studies are needed to determine the effect of these metabolites on sea bass.

In this study, the dietary incorporation of 20% of the fermented mixture increased FE and PER, while the inclusion of 40% reduced both, probably due to the lower growth rate of fish fed the 40 SSF diet. Similarly, in gibel carp juveniles fed with diets including up to 5% of SSF feedstuff mixture by *Lactobacillus* spp., *Bacillus* spp., and yeast increased, FE was increased, while the incorporation levels add no effect on FE (Cao et al., 2021). In rohu juveniles, the dietary inclusion of SSF *M. oleifera* leaf meal increased FE and PER compared to the un-fermented leaf meal (Manna et al., 2022). Further, in rohu fed with fermented diets by *S. cerevisiae*, FE increased (Das et al., 2021). Contrarily, the dietary inclusion of 40% fermented soybean meal or 50% fermented corn gluten by *B. subtilis* U304 significantly decreased FE in rainbow trout (Moniruzzman et al., 2018) as well as in whiteleg shrimp (Moniruzzman et al., 2020).

In the present work, European sea bass juveniles whole-body protein increased in fish fed the 20 SSF diet but decreased in the 40 SSF one. The increase in whole-body total protein in fish fed the 20 SSF diet is mainly attributed to the reduced whole-body lipid content were fed the 20

SSF diet is mainly attributed to the reduced whole-body lipid content. Similar results were found when rohu juveniles were fed diets including 15% and 30% of a SSF *M. oleifera* leaf meal by *A. niger*, where an increase of whole-body protein was observed. However, whole-body lipid content decreased when fish were fed these diets (Manna et al., 2022). For other species, the dietary inclusion of fermented plant feedstuffs did not affect the protein, lipid, ash, and moisture contents of whole-body gibel carp (Cao et al., 2021) and rainbow trout (Moniruzzaman et al., 2018).

Diet composition strongly modulates digestive enzymatic activity (Magalhães et al., 2015). In this work, amylase activity decreased when the SSF mixture was included at 40% (40 SSF diet) but not when included at 20% (20 SSF diet), while the opposite trend was observed for the lipase activity. Diets including 40% of plant feedstuffs mix had higher amylase activity than the diets including 20% probably related to the presence of more amylaceous ingredients. In the 40 Mix diets, the decrease of amylase activity in the 40 SSF diet may be due to the starch hydrolise during the fermentation process. In rohu juveniles fed diets including 15% and 30% of a SSF *M. oleifera* leaf meal, lipase activity was unaffected, while amylase activity increased, compared to the non-fermented diets (Manna et al., 2022). Further, in rohu fed with fermented diets by *S. cerevisiae*, amylase and protease activities increased (Das et al., 2021). During SSF, fungi produce exoenzymes, including cellulase,  $\beta$ -glucosidase, xylanase, amylase, lipase, and proteases, that remained in the mixture and were introduced to the SSF-experimental diets as exoenzymes. Therefore, in the present study, the enzymes produced during the SSF and present in the diet, including the fermented plant-feedstuffs ingredients, may have contributed to the modulation of the digestive activity. Indeed, it has been reported that dietary supplementation with exogenous enzymes may affect digestive enzyme activity. For instance, lipase and amylase activities increased with dietary supplementation with xylanase in Jian carp (*Cyprinus carpio* var. Jian) (Jiang et al., 2014), and with carbohydrases supplementation in turbot (Diógenes et al., 2018), and white sea bream (*Diplodus sargus*) (Magalhães et al., 2016). However, in rainbow trout juveniles fed a supplemented diet with SSF-produced enzymes, lipase and amylase activity was unaffected (Davies et al., 2021).

In the present study, total alkaline protease activity decreased with the inclusion of fermented plant feedstuffs mixture, irrespective of the inclusion level, which may be related to increased protein digestion during the SSF process. Indeed, *in vitro* digestibility of this plant feedstuffs mixture increased after the SSF (Chapter 2). Similar results were also observed in European sea bass, where the inclusion of 5% of SSF *Ulva rigida* did not affect amylase but

decreased lipase and protease activity (Fernandes et al., 2022). In this case, the author described that the lower protease activity might be related to the reduction of complex polysaccharides in the diet and higher access to the remaining nutrients. The present results also support this assumption due to the presence of cellulase and  $\beta$ -glucosidase in the fermented diets. Contrarily, in turbot fed with diets containing a supplementation of commercial carbohydrases, protease activity was unaffected (Diógenes et al., 2018).

Currently, one of the major constraints in aquaculture is fish diseases, which limit the productivity of this industry. SSF has been gaining attention due to its cheap and eco-friendly process and adding value to agroindustrial by-products or plant crops by increasing their nutritional value and enrichment with functional compounds. The functional compounds present in the fermented products may have antioxidant and immunostimulant properties, which help decrease oxidative stress and improve the innate immune system. Thus, SSF emerges as a potential tool to reduce aquafeed environmental footprint while helping to increase aquaculture species well-being and decrease disease incidence.

In the present work, plasma cholesterol, triglycerides, and total protein were unaffected by the dietary composition, while glucose increased in the 40 SSF diet, probably due to the increased availability of carbohydrates after the fermentation process. Indeed, several authors have reported increased plasma glucose levels with dietary supplementation with digestible carbohydrates (Enes et al., 2011). Also, dietary supplementation with exogenous carbohydrases may increase plasma glucose levels. For example, Rainbow trout juveniles fed a plant-based diet supplemented with a commercial carbohydrase showed higher plasma glucose than the unsupplemented diet (Davies et al., 2021). Moreover, in large yellow Croaker juveniles (*Larimichthys crocea*), the dietary inclusion of xylanase did not affect serum glucose, total protein, and cholesterol contents, while triglyceride levels increased (Luo et al., 2020). In rainbow trout fed diets including fermented soybean and corn gluten fermented meal by *Bacillus subtilis* U304, plasma glucose decreased when 30% of the fermented mixture was included and, similarly to the present study, triglycerides and total protein were not affected by SSF (Moniruzzaman et al., 2018). Also, in European sea bass fed a plant-based diet supplemented with a fermented extract rich in carbohydrases and obtained from the SSF of brewer's spent grain (BSG) with *Aspergillus ibericus*, plasma cholesterol, triglycerides, and total protein was not influenced by dietary BSG SSF (Fernandes et al., 2022). Further, *L. vannamei* fed with graded levels of fermented rapeseed meal, and soybean meal by *A. niger*, total protein, cholesterol, and

triglycerides decreased, while serum glucose increased with a graded increase in the fermented inclusion (5%, 7.5%, and 10%) (Jannathulla et al., 2018; Jannathulla et al., 2019).

During fish metabolism, reactive oxygen species (ROS) are produced continuously under physiological processes and play vital roles in fish metabolism. However, environmental stress may trigger some metabolic imbalances, leading to oxidative stress conditions, where ROS are abundantly produced, exceeding the endogenous capacity of the organism to neutralize, inducing DNA damage, protein denaturation, or lipid peroxidation (Hoseinifar et al., 2021). The endogenous antioxidant defense system of organisms, including fish, comprises non-enzymatic antioxidants, and enzymatic antioxidants, such as SOD, GPX, GR, and CAT (Mishra et al., 2015).

SOD is the first enzyme involved in antioxidant defense, by disrupting the superoxide anion in  $H_2O_2$  and oxygen (Feng et al., 2011). In this study, the dietary inclusion of SSF plant feedstuffs mix increased intestine SOD activity when fish were fed the 20 SSF diet, while GPX, CAT, and GR were not affected by SSF. Moreover, the intestine LPO was not affected, indicating that oxidative lipid damage was not observed in the present study. Neither the plant feedstuff mix nor the SSF affected the dietary phenolic compound level, but the total antioxidant activity decreased. Previously, in European sea bass fed a diet supplemented with an extract obtained from SSF of BSG with *A. ibericus*, it was observed that intestine SOD, CAT, GPX, GR, and G6PDH activities increased (Fernandes et al., 2022). In the present study, the whole fermented substrate was used, while in the study of Fernandes et al. (2022), only the extract was used. The whole fermentation product included fungal spores. These spores are not digested by carnivorous fish, due to their chitinous cell wall and the fish's lack of chitinolytic enzymes (Guerreiro et al., 2020). To our knowledge, no studies regarding the effect of dietary fungal spores on intestinal oxidative stress have been published.

Usually, oxidative stress is higher in the intestine, than in the liver due to the higher cellular turnover of the intestine, which, consequently, induces higher ROS production (Castro et al., 2016). However, in the present study, CAT and G6PDH activities were higher in the liver than in the intestine. Higher liver G6PDH was also reported in European sea bass (Fernandes et al., 2022) and sea bream (Castro et al., 2016; Magalhães et al., 2020). G6PDH, apart from being responsible for glutathione reduction and cellular redox balance maintenance, is also the first limiting enzyme in the pentoses phosphate pathway required for fatty acid biosynthesis (Fernandes et al., 2022), and nucleic acid metabolism (Kim et al., 2012). Further, G6PDH acts in glucose-6-phosphate, a product of glycolysis (Stanton, 2012). In this study, liver G6PDH decreased with the

incorporation of the SSF plant feedstuff mix in both inclusion levels (20 SSF and 40 SSF). However, serum glucose was only increased in the 40 SSF diet, so the decrease in G6PDH in both fermented diets may not be fully explained by dietary digestible carbohydrates content. Additionally, decreased G6PDH may compromise fish oxidative stress status (Zhang & Wang, 2007), reducing NADPH production. Indeed, GPX activity also decreased in fermented diets. Similarly, in *Labeo rohita* fed with SSF *M. oleifera* leaf extract by *A. niger*, both liver SOD and CAT activities decreased (Manna et al., 2022).

ALAT activity was higher in fish fed the 20 Mix diets, while ASAT and GDH activities were unaffected by dietary treatments. These enzymes are involved in amino acid catabolism pathways, and ALAT lower activity in the 40 Mix diets may be related to the higher incorporation of carbohydrates and, consequently, lower usage of dietary protein for energy purposes (Manna et al., 2022). Higher catabolic enzyme activity has been reported by other authors when fish are fed with more proteic diets or when protein is more available for fish, in amino acids (Gaye-Siessegger et al., 2006). Similar to the present results, in *Labeo rohita* fed a SSF *M. oleifera* leaf extract by *A. niger*, ASAT and ALAT activity increased (Manna et al., 2022).

In this study, liver, intestine, and muscle LPO was unaffected by the dietary treatments. Similarly to these results, diets including rice bran fermented with *S. cerevisiae* did not affect ROS production and LPO in *Brachionus plicatilis*, compared to a non-fermented rice bran-based diet (Monteiro et al., 2020). Nonetheless, other studies report a decrease in intestine LPO when E. sea bass were fed a supplemented diet with an extract obtained from SSF of BSG with *A. ibericus*. Interesting results were found regarding muscle LPO, showing that this SSF of BSG extract decreased MDA concentration and, therefore, may help to increase the shelf-life time (Fernandes et al., 2022). Nevertheless, in this study the BSG extract was rich in total antioxidants, which led to the decrease of the muscle LPO while in the present study, SSF decreased the antioxidant activity. Additionally, in zebrafish embryos, a rice bran SSF with *S. cerevisiae*, *B. subtilis*, and *L. plantarum*, decreased ROS production, and LPO (Liu et al., 2022).

Oxidative stress is intimately related to fish inflammatory status, since some oxidative stress conditions may trigger immune responses (Lugrin et al., 2014). As aforementioned, the diets containing the SSF mixture contained fungal spores. The cell wall of these spores is rich in chitin,  $\beta$ -glucans and mannans, which have immunomodulatory properties (Garcia-Rubio et al., 2020; Isaza-Pérez et al., 2020). Specifically,  $\beta$ -glucans and mannans are identified by pattern recognition receptors (PRR) of phagocytes, which have a major role in stimulating the

production of pro-inflammatory cytokines and in the activation of the innate immune response (Erwig & Gow, 2016).

In this work, pro-inflammatory cytokines casp 3, IL-8, and TNF- $\alpha$ , anti-inflammatory IL-10, and the somatic growth indicator IGF-I gene expression were unaffected by dietary treatments. Nonetheless, there is a tendency to increase these gene expressions when fish were fed diets including the SSF plant feedstuff mixture. Indeed, the inclusion of fermented ingredients in aquafeeds may upregulate pro-inflammatory gene expression and downregulate anti-inflammatory gene expression, as described by Zhang et al. (2021). Indeed, in European sea bass, a mannan supplemented diet upregulated casp 3 gene expression, while IL-8, IL-10, and TNF- $\alpha$  gene expression were unaffected (Torrecillas et al., 2015). Nile Tilapia fed with a  $\beta$ -glucan supplemented diet, while IL-8 gene expression was upregulated, casp 3 was not affected, compared to a control diet (Dawood et al., 2020), and when fed diets containing 1.5% of  $\beta$ -glucans supplementation, IL-10, TNF- $\alpha$ , and casp 3 liver gene expression was upregulated, compared to a control diet (Lu et al., 2019). Similarly, IGF-I expression increased in gibel carp fed with a fermented plant feedstuff mixture, composed of rapeseed meal, sprayed corn husk, rice bran, palm meal, and soybean meal (Cao et al., 2021). Further, in Pearl gentian grouper fed with diets including SSF soybean meal, IL8, and TNF- $\alpha$  gene expression was upregulated, while IL-10 gene expression was downregulated (Zhang et al., 2021). Contrary to the present study, in turbot fed with diets containing 45% and 60% of SSF soybean meal in substitution to fishmeal, pro-inflammatory TNF- $\alpha$  gene expression was downregulated, while the substitution of fishmeal by raw soybean meal upregulated TNF- $\alpha$  gene expression (Li et al., 2019).

In the present study, gut anti-inflammatory TGF- $\beta$  gene expression was lower in fermented diets. Contrary to our results, the inclusion of a SSF soybean meal by *A. awamori* in aquafeeds for turbot increased TGF- $\beta$  gene expression (Li et al., 2019). Further, chitooligosaccharides supplementation upregulated TGF- $\beta$ , and down-regulated TNF- $\alpha$  expression, compared to a control diet, in hybrid tilapia (*O. niloticus* x *O. aureus*) (Qin et al., 2014). Similar to our results, the inclusion of SSF soybean meal in aquafeeds decreased TGF- $\beta$  in Pearl gentian grouper (Zhang et al., 2021). Also, a mannan oligosaccharide supplementation down-regulated TGF- $\beta$  gene expression in E. sea bass (Torrecillas et al., 2015). The down-regulation of TGF- $\beta$  expression may be related to a compensatory mechanism regarding the non-significant increase in the pro-inflammatory gene expression (Torrecillas et al., 2015). Nonetheless, more studies are needed to understand these pathways.

Liver cathepsin D (Cat D) expression was upregulated when fish were fed the diets including the SSF plant mixture. Cathepsins are lysosomal proteases (Esteban, 2012), that may be involved in amino acid catabolism, and apoptosis pathways (Moreno et al., 2020). Contrary to the present study, in *Macrobrachium rosenbergii* fed with diets supplemented with  $\beta$ -glucans, Cat gene expression was not affected, while diets supplemented with *S. cerevisiae* upregulated Cat expression (Han et al., 2021). In this study, the authors refer that chitin and mannans may have a more prominent immunostimulatory effect, than  $\beta$ -glucans to Cat D expression. Interestingly, cathepsins are synthesized as inactive proenzymes (Chwieralski et al., 2006). During Cat D maturation, molecules of mannose-6-phosphate are recruited as part of the maturation process, and they are only active after this maturation (Szulc-Dąbrowska et al., 2020). Thus, *A. niger* cell wall mannans may be related to the recruitment of mannose molecules. Nevertheless, additional studies are needed to confirm this hypothesis and to investigate what would be the role of Cat D when fish were fed with diets including SSF ingredients.

The replacement of fishmeal with plant feedstuffs is often followed by a modulation of the digestive physiology, for instance, gut microbiota (Moutinho et al., 2017). A balanced gut microbiota is of utmost importance in aquaculture fish for a better adaptation to environmental changing conditions and resistance to pathogens (Cerezuela et al., 2012).

In this study, the average number of operational taxonomic units (OTUs), microbial richness, and diversity increased with SSF, which may promote a potentially more resilient microbiota against pathogen outbreaks (Fontinha et al., 2021). Similar results were found in grass carp fed with a supplemented cellulase diet (Zhou et al., 2013) and in turbot when fed with DDGS based-diets (Diógenes et al., 2018). Moreover, a *Bacillus* sp. was identified in grass carp microbiota fed with a cellulase supplementation, suggesting that dietary cellulase increased microbial OTUs and density (Zhou et al., 2013). The inclusion of *A. niger* in diets for broilers significantly increased ileum microbiota *Bifidobacterium* and *Lactobacillus* OTUs and decreased *Escherichia coli* OTUs (Lin et al., 2020). Although these genera were not detected in our study, they sustain that *A. niger* supplementation in diets makes broilers more resilient to diseases since *Bifidobacterium* and *Lactobacillus* are known as probiotic genera and *E. coli* as a genus containing several pathogens. Indeed, other authors describe *A. niger* as a probiotic in common carp, since it can colonize gut microbiota, inhibit pathogens colonization, improve the immune system, and produce extracellular enzymes (Jasim et al., 2022).

Bacterial identification from fish fed the diets including the SSF plant mixture revealed that the most abundant genus in the detected bacteria is *Bacillus* spp., followed by *Clostridium* sp. Both genera belong to the Firmicutes phylum which, among Proteobacteria and Bacteroidetes, comprise 90% of fish intestinal microbiota (Egerton et al., 2018). *Bacillus* spp. are spore-forming bacteria naturally found in the fish gut and stimulate the host immune system, due to their probiotic properties, for instance, by upregulating some pro-inflammatory cytokines (Santos et al., 2022). Additionally, previous studies demonstrated *Bacillus* spp. potential to control several fish pathogens that belong to *Aeromonas* spp. (Gao et al., 2017), *Pseudomonas* spp. (Hamza et al., 2018), *Vibrio* spp. (Yatip et al., 2018), and *E. tarda* (Santos et al., 2021). Moreover, *Bacillus* spp. commonly produce NACs (Natural Antimicrobial Compounds), suitable to inhibit pathogen growth and proliferation (Santos et al., 2021). The majority of NACs are AMPs (Antimicrobial Peptides), mainly ribosomal peptides, which comprise enzymes, polyketides, and non-ribosomal peptides, that may present anti-fungal activity (Caulier et al., 2019). The produced enzymes include cellulases, glucanases, proteases, and chitinases, some of which inhibit fungal growth through their capacity to degrade fungal cell wall components (Caulier et al., 2019; Martínez-Culebras et al., 2021; Rangel et al., 2022; Xiao et al., 2009). Additionally, lipopeptides, such as iturins, fengycins, and surfactins, produced by some *Bacillus* are active against mycotoxin-producing fungi (Martínez-Culebras et al., 2021; Veras et al., 2016). Indeed, *B. subtilis* was able to degrade Aflatoxin B1, M1, and G1 (Gao et al., 2011). Specifically, within the *Bacillus* genus, we identified *B. thuringiensis*, *B. cereus*, *B. amyloliquefaciens*, and *B. velezensis* in the microbiota of fish fed diets containing the *A. niger* SSF mixture.

*B. thuringiensis* and *B. cereus* are often referred to as pathogenic species for fish and humans, due to their virulence factors (Ibrahim et al., 2010; Younes et al., 2020). They may present anti fungal activity, mainly by producing lipopeptides with anti fungal activity (Ajesh et al., 2013), or chitin (Martínez-Zavala et al., 2020), as described in the degradation of shrimp and crab powder by chitinases and chitosanases (Chang et al., 2007). Similarly, in some studies, *B. cereus* chitinase production inhibited some fungal growth, namely *F. oxysporum*, *R. solani*, and *C. gloeosporioides* (Thakur et al., 2021). Further, *B. cereus* chitinases were also found to inhibit conidia (fungal spores) germination of *F. verticillioides* (Morales-Ruiz et al., 2021). In addition, *B. thuringiensis* CECT 197 reduced mycotoxins Aflatoxin B1 (AFB1) and OTA after 48h of exposure to the mycotoxin (Illueca et al., 2021).

*B. amyloliquefaciens* and *B. velezensis* are known for their anti fungal activity due to their lipopeptides and polyketides production (Calvo et al., 2017), mainly surfactin and iturin (Kim et

al., 2020; Rabbee & Baek, 2020; Sansinenea & Ortiz, 2011; Younes et al., 2020), that stimulate the innate immune system by the production of numerous cytokines (Madan & Kishore, 2020). Indeed, when cultured, exhibits a large spectrum of activity against several fungi, namely *F. oxysporum*, *M. oryzae*, or *M. fructicola* (Torres et al., 2020), and *A. niger* (Zhao et al., 2014). Moreover, an in vitro assay described that *B. amyloliquefaciens* decreases Ochratoxin A (OTA) concentration after 48h of exposure to the mycotoxin (Illueca et al., 2021). Further, these bacteria are also associated with chitinolytic activity, as seen in the degradation of shrimp and crab powder marine wastes by chitinases (Wang et al., 2002). Additionally, *B. amyloliquefaciens* and *B. velezensis* are involved in the production of some commercial fungicides, such as Taegro® (Syngenta) (Calvo et al., 2017) and Serenade® (AgraQuest), respectively (Rabbee & Baek, 2020).

*Clostridium* spp. are spore-forming bacteria, commonly present in the soil and water, which comprise pathogenically but also beneficial species (Novoslavskij et al., 2016). Nevertheless, some *Clostridium* spp. may also have probiotic effects, such as *C. autoethanogenum*, which increased feed efficiency and protein retention in black sea bream (*Acanthopagrus schlegelii*) (Chen et al., 2020). In another study, the same species was referred to as a good substitute for fishmeal up to 30% of inclusion, without any adverse effects on Pacific white shrimp (Jiang et al., 2021), and largemouth bass (*Micropterus salmoides*) up to 49% of inclusion (Zhu et al., 2022). Further, *C. butyricum* may also present probiotic effects in *M. rosenbergii*, mainly by inhibiting *V. harveyi* growth in an *in vivo* challenge (Sumon et al., 2018).

In this study, we were not able to determine the *Clostridium* species, so we cannot infer if its effects were beneficial or detrimental. Nevertheless, no signs of external disease were found in the fish. Regarding *Bacillus*, as all of the identified species have been previously reported to present anti fungal activity, we may suggest that the fermented mixture served as a substrate for these microorganisms growth and that fungal spores were not well digested by fish, so these bacteria had beneficial effects for the fish, against *A. niger*. In future studies, it would be interesting to evaluate what compounds would have been produced by fish microbiota when fed diets containing the *A. niger* SSF mixture and the potential isolation of these antifungal NACs.

#### 4. Conclusion

In this work, SSF increased cellulase and  $\beta$ -glucosidase activity in the experimental diets, but xylanase was not detected. Total antioxidants decreased, and phenolic compound activity was not affected by SSF. SSF increased feed efficiency and protein retention of fish fed with the

20 SSF diet, but reduced growth performance, feed intake, efficiency, and protein retention of fish fed with the 40 SSF diet. The reduced growth performance of fish fed the 40 SSF diet is related to the lower voluntary feed intake.

Overall oxidative stress and LPO of the liver, intestine, and muscle were unaffected by the dietary inclusion level or SSF process of the plant-feedstuff mixture. SSF of plant feedstuffs appears to have some immunomodulatory properties in European sea bass, leading to a down regulation of TGF- $\beta$  expression. Moreover, the SSF of plant feedstuffs also had modulatory effects on intestinal microbiota by promoting a higher microbial diversity and the presence of *Bacillus* spp. with potential anti fungal properties, which may help fungal digestion in fish's gut.

The inclusion of moderate levels of the fermented mixture may be important and advantageous for fish and should be assessed further. Future studies should evaluate, the potential of extracts from this mixture SSF or the inclusion of non-sporulating fungi in SSF ingredients in aquafeeds. Further work should be done in order to clarify the mechanisms behind the modulation action of the fermented feedstuffs.

**Table 1** – Ingredient composition (% dry weight) and proximate analysis of the experimental diets.

|                                             | 20 Mix | 20 Mix SSF | 40 Mix | 40 Mix SSF |
|---------------------------------------------|--------|------------|--------|------------|
| <i>Feedstuff (% dry weight)</i>             |        |            |        |            |
| Fish meal <sup>1</sup>                      | 17.5   | 17.5       | 17.5   | 17.5       |
| CPSP <sup>2</sup>                           | 2.5    | 2.5        | 2.5    | 2.5        |
| Plant-feedstuff mix <sup>3</sup>            | 20     | 0          | 40     | 0          |
| SSF Plant-feedstuff mix <sup>4</sup>        | 0      | 20         | 0      | 40         |
| Wheat gluten meal <sup>5</sup>              | 7.5    | 7.5        | 7.5    | 7.5        |
| Corn gluten meal <sup>6</sup>               | 13.4   | 11.4       | 5.1    | 1.2        |
| Hemoglobin <sup>7</sup>                     | 5.0    | 5.0        | 5.0    | 5.0        |
| Wheat meal <sup>8</sup>                     | 14.2   | 15.5       | 4.0    | 6.6        |
| Fish oil                                    | 13.5   | 14.2       | 12.9   | 14.2       |
| Hydrolyzed Shrimp <sup>9</sup>              | 0.5    | 0.5        | 0.5    | 0.5        |
| Chromium oxide                              | 0.5    | 0.5        | 0.5    | 0.5        |
| Vitamin premix <sup>10</sup>                | 1.0    | 1.0        | 1.0    | 1.0        |
| Choline chloride                            | 0.5    | 0.5        | 0.5    | 0.5        |
| Minerals premix <sup>11</sup>               | 1.0    | 1.0        | 1.0    | 1.0        |
| Binder <sup>12</sup>                        | 1.0    | 1.0        | 1.0    | 1.0        |
| Dicalcium phosphate                         | 1.2    | 1.2        | 0.3    | 0.3        |
| Lysine <sup>13</sup>                        | 0.2    | 0.2        | 0.2    | 0.2        |
| Methionine <sup>13</sup>                    | 0.2    | 0.2        | 0.2    | 0.2        |
| Taurine <sup>13</sup>                       | 0.3    | 0.3        | 0.3    | 0.3        |
| <i>Proximate analysis (% dry weight)</i>    |        |            |        |            |
| Dry matter (%)                              | 96.8   | 90.8       | 96.3   | 90.4       |
| Crude protein                               | 42.3   | 41.4       | 41.0   | 41.4       |
| Crude lipids                                | 17.3   | 17.4       | 17.4   | 17.0       |
| Ash                                         | 8.1    | 9.3        | 9.4    | 9.8        |
| Gross Energy (MJ/kg)                        | 22.9   | 22.0       | 23.4   | 22.9       |
| <i>Bioactive compounds analysis</i>         |        |            |        |            |
| Cellulase (U/g)                             | nd     | 8.1        | nd     | 12.5       |
| β-glucosidase (U/g)                         | nd     | 3.8        | nd     | 2.3        |
| Xylanase (U/g)                              | nd     | nd         | nd     | nd         |
| DPPH (μmol trolox equivalents/g)            | 16.6   | 13.0       | 17.5   | 13.2       |
| Total phenols (mg galic acid equivalents/g) | 9.4    | 8.9        | 8.6    | 9.6        |

<sup>1</sup> Pesquera Centinela, Steam Dried LT, Chile (CP: 68.45%; CL: 12.56%). Sorgal, S.A. Ovar, Portugal.

<sup>2</sup> Soluble fish protein concentrate (CP: 80.2%; CL: 15.39%). Sopropêche, France.

<sup>3</sup> Plant-feedstuff mix (%DM) (CP: 33.0%; CL: 5.5%): 25% rapeseed meal (CP: 40.0%; CL: 5.8%); 25% soybean meal (CP: 51.9%; CL: 3.7%); 25% rice bran (CP: 14.2%; CL: 13.2%); 25% sunflower seed (CP: 40.2%; CL: 2.86%). Sorgal, S.A. Ovar, Portugal.

<sup>4</sup> SSF Plant-feedstuff mix (%DM) (CP: 38.03%; CL: 2.5%): 25% rapeseed meal; 25% soybean meal; 25% rice bran; 25% sunflower seed. Sorgal, S.A. Ovar, Portugal.

<sup>5</sup> Wheat gluten meal (CP: 80.5%; CL: 1.0%). Sorgal, S.A. Ovar, Portugal.

<sup>6</sup> Corn gluten meal (CP: 62.0%; CL: 2.8%). Sorgal, S.A. Ovar, Portugal.

<sup>7</sup> Hemoglobin (CP: 91.5%; CL: 0.4%). Sorgal, S.A. Ovar, Portugal.

<sup>8</sup> Wheat meal (CP: 14.33%; CL: 2.09%). Sorgal, S.A. Ovar, Portugal.

<sup>9</sup> Hydrolyzed Shrimp (CP: 69.8%; CL: 2.1%). Sorgal, S.A. Ovar, Portugal.

<sup>10</sup> Vitamin premix (mg kg<sup>-1</sup> diet): retinol, 18,000 (IU kg<sup>-1</sup> diet); calciferol, 2000 (IU kg<sup>-1</sup> 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.

<sup>11</sup> Mineral premix (mg kg<sup>-1</sup> 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<sup>-1</sup> diet); potassium chloride, 1.15 (g kg<sup>-1</sup> diet); sodium chloride, 0.4 (g kg<sup>-1</sup> diet).

<sup>12</sup> Aquacube, Agil, UK.

<sup>13</sup> Feed grade-amino acids. Sorgal, S.A. Ovar, Portugal.

**Table 2** – Forward and reverse primers used for RT-PCR.

| Gene                               | Acronym           | Efficiency | AT | Forward primer sequence (3'-5') | Reverse primer sequence (5'-3') | Accession nos. | Reference                                                   |
|------------------------------------|-------------------|------------|----|---------------------------------|---------------------------------|----------------|-------------------------------------------------------------|
| Elongation factor 1 $\alpha$       | <i>elf \alpha</i> | 100.9      | 60 | GCTTCGAGGAAATCACCAAG            | CAACCTTCCATCCCTTGAAC            | AJ866727       | (Sepulcre, Sarropoulou, Kotoulas, Meseguer, & Mulero, 2007) |
| 40s ribosomal protein              | <i>40 s</i>       | 94.5       | 55 | AGGGTGTGGCAGACGTTAC             | CTTCTGCCTGTTGAGGAACC            | AM490061       | (Sepulcre et al., 2007)                                     |
| Caspase 3                          | <i>casp 3</i>     | 96.7       | 60 | CTGATTTGGATCCAGGCATT            | CGGTCGTAGTGTTCCTCCAT            | DQ345773       | (Sepulcre et al., 2007)                                     |
| Interleukin 8                      | <i>il 8</i>       | 105.2      | 60 | GTCTGAGAAGCCTGGGAGTG            | GCAATGGGAGTTAGCAGGAA            | AM490063       | (Sepulcre et al., 2007)                                     |
| Interleukin 10                     | <i>il 10</i>      | 92.4       | 60 | CGACCAGCTCAAGAGTGATG            | AGAGGCTGCATGGTTTCTGT            | DQ821114       | (Sepulcre et al., 2007)                                     |
| Tumour necrosis factor $\alpha$    | <i>tnf \alpha</i> | 105.1      | 60 | AGCCACAGGATCTGGAGCTA            | GTCCGCTTCTGTAGCTGTCC            | DQ200910       | (Sepulcre et al., 2007)                                     |
| Transforming growth factor $\beta$ | <i>tgf \beta</i>  | 100.8      | 60 | GACCTGGGATGGAAGTGGAT            | CAGCTGCTCCACCTTGTTG             | AM421619.1     |                                                             |
| Insulin-like growth factor-I       | <i>igf-I</i>      | 98.1       | 54 | TACAGGCTATGGCCCAAT              | TTGGCAGGTGCACAGTACAT            | AJ579342       | (Carnevali et al., 2006)                                    |
| Cathepsin D                        | <i>cat D</i>      | 99.5       | 55 | CCTTCATCGCAGCCAAGTTT            | TATTTGGGGTCGGTTCCTCC            |                | (Moreno et al., 2020)                                       |

**Table 3** – Growth performance and feed utilization efficiency of European sea bass juveniles fed the experimental diets.

| Diets                                                          | 20 Mix | 20 Mix SSF | 40 Mix  | 40 Mix SSF | SEM  | Two-way ANOVA |           |                 |
|----------------------------------------------------------------|--------|------------|---------|------------|------|---------------|-----------|-----------------|
|                                                                |        |            |         |            |      | SSF           | Inclusion | SSF * Inclusion |
| Final body weight (g)                                          | 25.4 B | 23.8 B     | 21.6 Ab | 16.0 Aa    | 1.1  | **            | ***       | *               |
| Weight gain (g kg ABW <sup>-1</sup> day <sup>-1</sup> )        | 17.7 B | 16.7 B     | 15.0 Ab | 9.3 Aa     | 1.0  | **            | ***       | *               |
| Daily growth index                                             | 1.6 B  | 1.5 B      | 1.3 Ab  | 0.7 Aa     | 0.1  | **            | ***       | *               |
| Feed Intake (g kg ABW <sup>-1</sup> day <sup>-1</sup> )        | 19.1   | 15.5       | 17.4    | 12.2       | 0.8  | ***           | ***       | ns              |
| Feed efficiency                                                | 0.9 Ba | 1.1 b      | 0.9 Ab  | 0.8 a      | 0.04 | **            | ns        | *               |
| Protein efficiency ratio                                       | 2.2 B  | 2.6        | 2.1 Ab  | 1.8 a      | 0.1  | **            | ns        | *               |
| Nitrogen intake (g kg ABW <sup>-1</sup> day <sup>-1</sup> )    | 1.5 A  | 1.3        | 1.6 Ba  | 1.8 b      | 0.1  | **            | ns        | *               |
| Nitrogen retention (g kg ABW <sup>-1</sup> day <sup>-1</sup> ) | 0.3    | 0.4 B      | 0.3 b   | 0.2 Aa     | 0.02 | *             | ***       | **              |
| Mortality (%)                                                  | 0      | 5          | 1.7     | 5          | 1.0  | *             | ns        | ns              |

Values are presented as mean (n=3) and pooled standard error of the mean (SEM).

Two-way ANOVA: ns - non-significant; ( $p \leq 0.05$ ); \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

If the interaction was significant, SSF and inclusion level were analyzed by a t-test; means in the same line with different capital or small letters indicate significant differences ( $p < 0.05$ ) between inclusion level or SSF, respectively.

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

<sup>1</sup> Weight gain: [(Final weight – Initial weight) / Initial weight] x 100

<sup>2</sup> Feed efficiency: wet weight gain/dry feed intake.

<sup>3</sup> Daily growth index: ((FBW1/3 – IBW1/3)/time in days) x 100.

<sup>4</sup> Protein efficiency ratio: wet weight gain/crude protein intake.

<sup>5</sup> Nitrogen intake: (N intake x 1000) / (ABW x time in days).

<sup>6</sup> Nitrogen retention: (FBW x FBN) - (IBW x IBN) / (ABW x time in days); FBN and IBN: final and initial whole-body N content, respectively.

**Table 4** – Whole-body composition (% fresh weight), of European sea bass juveniles fed with the experimental diets.

| Diets                                           | Initial | 20 Mix  | 20 Mix SSF | 40 Mix | 40 Mix SSF | SEM | Two-way ANOVA |           |                 |
|-------------------------------------------------|---------|---------|------------|--------|------------|-----|---------------|-----------|-----------------|
|                                                 |         |         |            |        |            |     | SSF           | Inclusion | SSF * Inclusion |
| Whole body composition (% <i>fresh weight</i> ) |         |         |            |        |            |     |               |           |                 |
| Dry matter                                      | 29.6    | 32.1 Bb | 30.6 a     | 30.3 A | 30.5       | 0.3 | *             | *         | *               |
| Protein                                         | 7.6     | 17.5    | 18.1       | 17.1   | 16.9       | 0.3 | *             | ns        | ns              |
| Lipid                                           | 16.8    | 10.6    | 9.1        | 9.2    | 9.2        | 0.2 | ns            | ns        | ns              |
| Ash                                             | 6.5     | 8.0     | 8.1        | 8.7    | 8.1        | 0.2 | ns            | ns        | ns              |
| Gross energy (KJ/g)                             | 7.6     | 8.3     | 7.5        | 7.9    | 7.5        | 0.1 | ns            | *         | ns              |

Values are presented as mean (n=3) and pooled standard error of the mean (SEM).

Two-way ANOVA: ns - non-significant; ( $p \leq 0.05$ ); \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\* $p < 0.001$ .

If the interaction was significant, SSF and inclusion level were analyzed by a t-test; means in the same line with different capital or small letters indicate significant differences ( $p < 0.05$ ) between inclusion level or SSF, respectively.

**Table 5** – Digestive enzyme activity of European sea bass juveniles fed with the experimental diets.

| Diets                    | 20 Mix  | 20 Mix SSF | 40 Mix  | 40 Mix SSF | SEM  | Two-way ANOVA |           |                 |
|--------------------------|---------|------------|---------|------------|------|---------------|-----------|-----------------|
|                          |         |            |         |            |      | SSF           | Inclusion | SSF * Inclusion |
| Amylase                  | 43.0 a  | 51.5       | 67.9 Bb | 41.2 A     | 3.6  | ns            | ns        | *               |
| Lipase                   | 24.9 Aa | 42.6 B     | 38.8 b  | 43.2       | 1.9  | *             | **        | *               |
| Total alkaline proteases | 310.6   | 226.5      | 344.4   | 330.3      | 14.7 | *             | **        | ns              |

Values are presented as mean (n=3) and pooled standard error of the mean (SEM).

Two-way ANOVA: ns - non-significant; ( $p \leq 0.05$ ); \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

If the interaction was significant, SSF and inclusion level were analyzed by a t-test; means in the same line with different capital or small letters indicate significant differences ( $p < 0.05$ ) between inclusion level or SSF, respectively.

**Table 6** – Plasma metabolites quantification of European sea bass juveniles fed with the experimental diets.

| Diets                 | 20 Mix | 20 Mix SSF | 40 Mix | 40 Mix SSF | SEM   | Two-way ANOVA |           |                 |
|-----------------------|--------|------------|--------|------------|-------|---------------|-----------|-----------------|
|                       |        |            |        |            |       | SSF           | Inclusion | SSF * Inclusion |
| Cholesterol (mg/dl)   | 89.9   | 91.0       | 85.1   | 103.5      | 2.9   | ns            | ns        | ns              |
| Total protein (mg/dl) | 202.6  | 197.5      | 217.7  | 192.8      | 192.8 | ns            | ns        | ns              |
| Triglycerides (mg/dl) | 753.5  | 820.5      | 747.5  | 732.8      | 25.6  | ns            | ns        | ns              |
| Glucose (mg/dl)       | 84.6   | 79.9       | 78.9 a | 84.5 b     | 1.9   | ns            | ns        | *               |

Values are presented as mean (n=9). SEM: pooled standard error of the mean. ns - non-significant; ( $P \leq 0.05$ ); \* $P < 0.05$ .

If an interaction was significant, SSF effects were analyzed by one-way ANOVA for 20% and 40% inclusion; means in the same line with different capital letters indicate significant differences ( $P < 0.05$ ) between inclusion level and small letters for SSF, respectively.

**Table 7** – Oxidative stress intestinal and hepatic enzyme activity, liver metabolic enzyme activity, and intestinal, hepatic and muscle lipid peroxidation of European sea bass juveniles fed the experimental diets.

| Diets                                     | 20 Mix  | 20 Mix SSF | 40 Mix  | 40 Mix SSF | SEM   | SSF | Two-way ANOVA |                 |
|-------------------------------------------|---------|------------|---------|------------|-------|-----|---------------|-----------------|
|                                           |         |            |         |            |       |     | Inclusion     | SSF * Inclusion |
| <b><i>Intestine Oxidative stress</i></b>  |         |            |         |            |       |     |               |                 |
| CAT <sup>1</sup>                          | 569.7   | 509.8      | 570.1   | 672.4      | 23.0  | ns  | ns            | ns              |
| GPX <sup>2</sup>                          | 52.6    | 52.0       | 64.4    | 75.5       | 4.2   | ns  | *             | ns              |
| GR <sup>3</sup>                           | 51.7    | 47.8       | 50.5    | 54.9       | 1.8   | ns  | ns            | ns              |
| SOD <sup>4</sup>                          | 540.0 a | 675.0 Bb   | 679.0 b | 524.9 Aa   | 21.9  | ns  | ns            | **              |
| G6PDH <sup>5</sup>                        | 6.4     | 6.5        | 7.9     | 6.6        | 0.3   | ns  | ns            | ns              |
| LPO <sup>6</sup>                          | 20.5    | 22.7       | 22.9    | 25.6       | 0.8   | ns  | ns            | ns              |
| <b><i>Liver amino acid catabolism</i></b> |         |            |         |            |       |     |               |                 |
| GDH <sup>7</sup>                          | 427.6   | 435.8      | 417.1   | 363.8      | 23.1  | ns  | ns            | ns              |
| ALAT <sup>8</sup>                         | 620.9   | 533.5      | 484.1   | 480.5      | 22.2  | ns  | *             | ns              |
| ASAT <sup>9</sup>                         | 2425.4  | 2087.1     | 1866.4  | 2070.5     | 101.4 | ns  | ns            | ns              |
| <b><i>Liver Oxidative stress</i></b>      |         |            |         |            |       |     |               |                 |
| CAT                                       | 820.4   | 887.4      | 983.9   | 1076.8     | 39.6  | ns  | *             | ns              |
| GPX                                       | 14.8    | 11.2       | 13.3    | 12.8       | 0.5   | *   | ns            | ns              |
| GR                                        | 15.3    | 16.3       | 11.4    | 13.4       | 0.6   | ns  | **            | ns              |
| SOD                                       | 138.1 a | 272.5 Bb   | 162.8   | 132.6 A    | 18.5  | ns  | ns            | *               |
| G6PDH                                     | 604.5   | 371.1      | 430.3   | 320.2      | 27.1  | *** | *             | ns              |
| LPO                                       | 13.7    | 13.1       | 12.6    | 13.3       | 0.6   | ns  | ns            | ns              |
| <b><i>Muscle Lipid Peroxidation</i></b>   |         |            |         |            |       |     |               |                 |
| LPO                                       | 39.7    | 44.1       | 36.3    | 42.1       | 1.9   | ns  | ns            | ns              |

Values are presented as mean (n=9). SEM: pooled standard error of the mean. ns - non-significant; ( $P \leq 0.05$ ); \* $P < 0.05$ ; \*\* $P < 0.01$ .

If an interaction was significant, SSF effects were analyzed by one-way ANOVA for 20% and 40% inclusion; means in the same line with different capital letters indicate significant differences ( $P < 0.05$ ) between inclusion level and small letters for SSF, respectively.

<sup>1</sup> Catalase

<sup>2</sup> Glutathione peroxidase

<sup>3</sup> Glutathione reductase

<sup>4</sup> Superoxide dismutase

<sup>5</sup> Glucose 6-phosphate dehydrogenase

<sup>6</sup> Lipid peroxidation

<sup>7</sup> Glutamate dehydrogenase

<sup>8</sup> Alanine aminotransferase

<sup>9</sup> Aspartate aminotransferase

**Figure 1** – Quantitative expression of gut's (A) caspase 3, (B) interleukin 8, (C) tumor necrosis factor-alpha, (D) transforming growth factor beta, and (E) interleukin 10 and liver's (F) cathepsin D and (G) insulin-like growth factor-I of European sea bass juveniles fed the experimental diets.

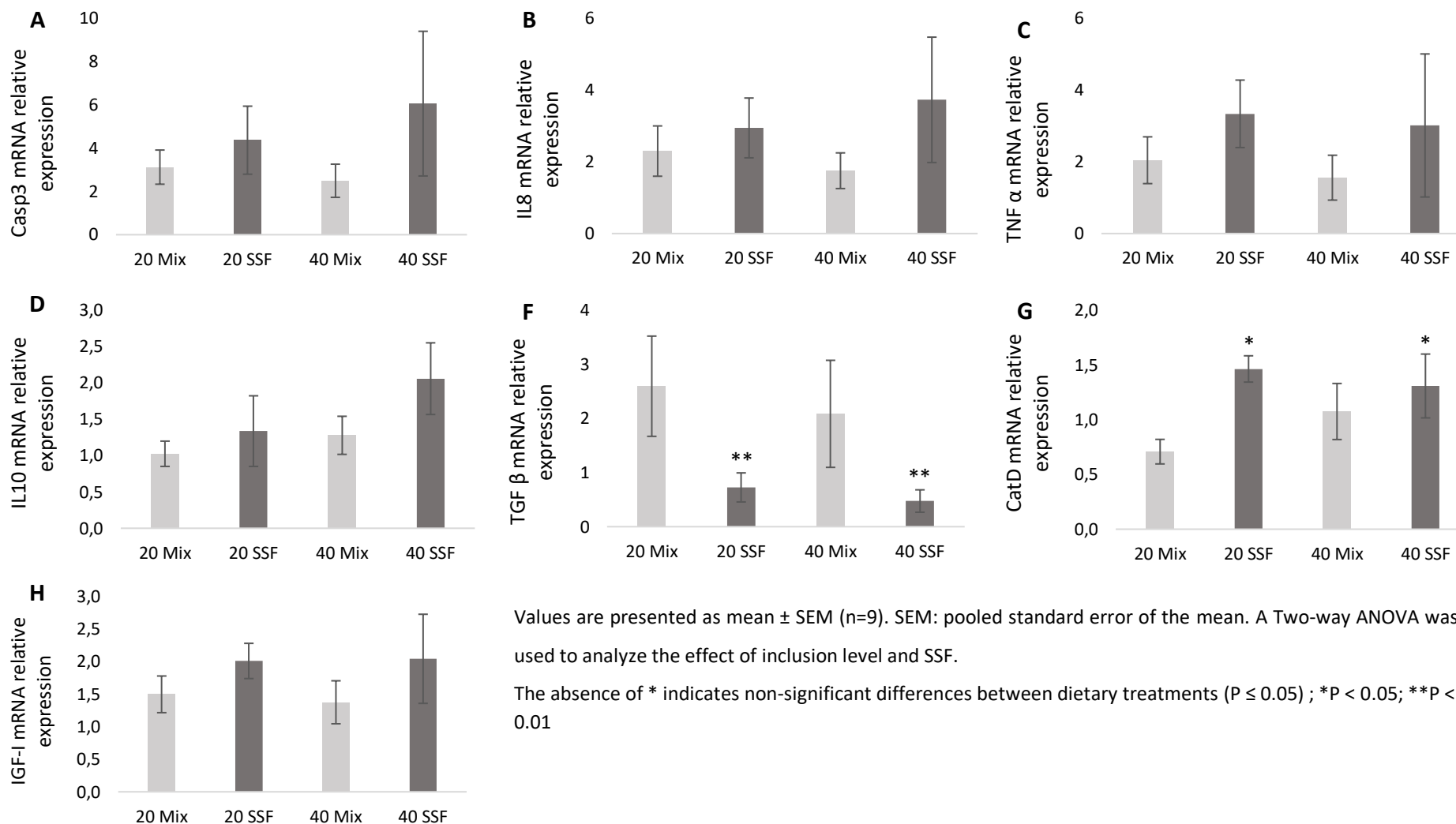


**Figure 2** – Dendrogram and DGGE fingerprints of the intestine pooled mucosa and digesta microbiota recovered from European sea bass juveniles fed the experimental diets.

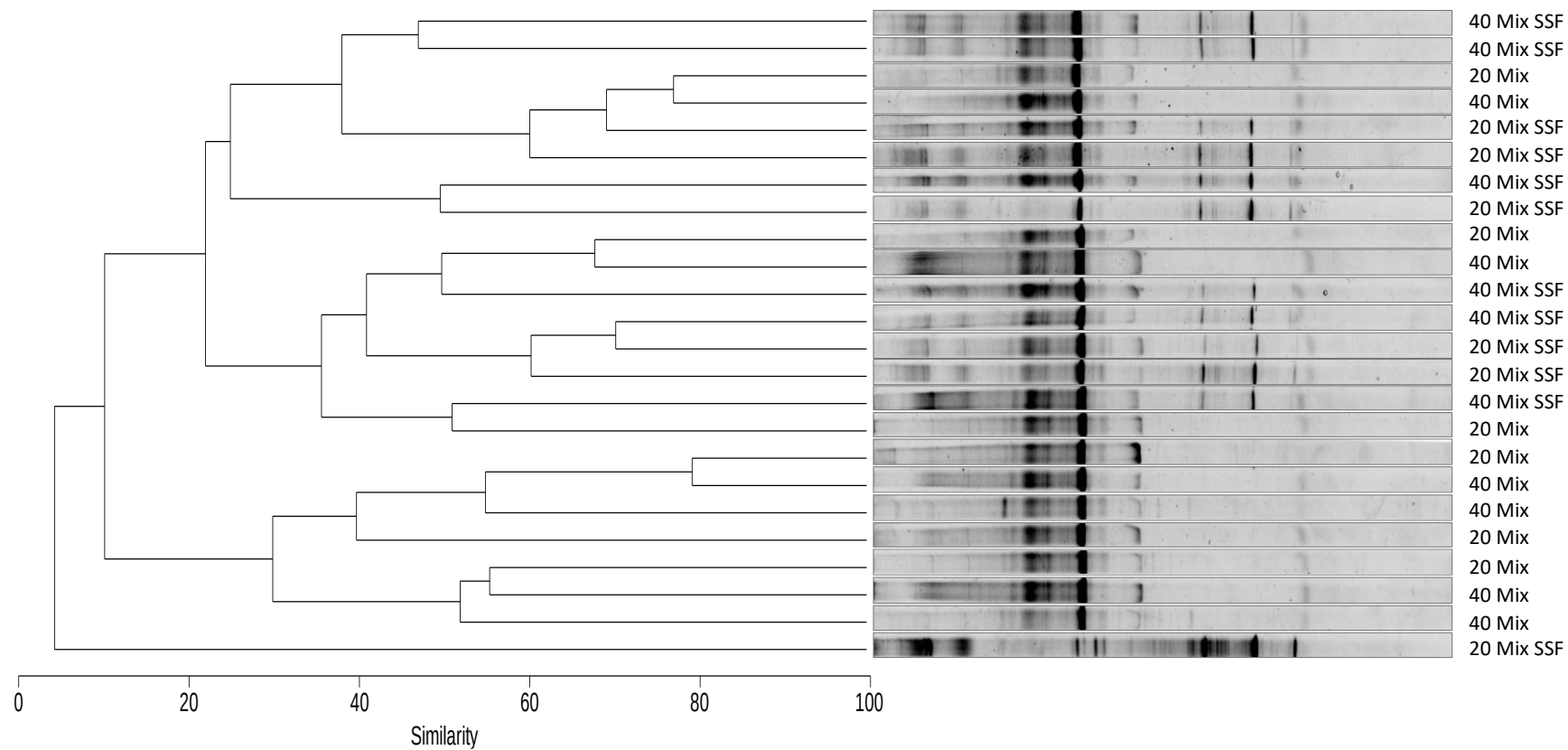


**Table 8** – Ecological parameters obtained from PCR-DGGE fingerprints of the European sea bass juveniles fed the experimental diets' microbiota.

| Diets                          | 20 Mix | 20 Mix SSF | 40 Mix | 40 Mix SSF | SEM  | Two-way ANOVA |           |                     |
|--------------------------------|--------|------------|--------|------------|------|---------------|-----------|---------------------|
|                                |        |            |        |            |      | Ferment       | Inclusion | Ferment * Inclusion |
| OTUs <sup>1</sup>              | 8.2    | 14.3       | 9.7    | 12.7       | 0.7  | **            | ns        | ns                  |
| Richness <sup>2</sup>          | 0.5    | 0.9        | 0.6    | 0.8        | 0.1  | **            | ns        | ns                  |
| Evenness <sup>3</sup>          | 1.7 Aa | 2.4 b      | 2.1 B  | 2.3        | 0.1  | ***           | ns        | **                  |
| Diversity <sup>4</sup>         | 0.8 Aa | 0.9 b      | 0.9 B  | 0.9        | 0.01 | ***           | *         | **                  |
| SIMPER Similarity <sup>5</sup> | 44.5   | 42.2       | 54.1   | 45.3       | 1.5  | ns            | *         | ns                  |

Values are presented as mean (n=6). SEM: pooled standard error of the mean. ns - non-significant; ( $P \leq 0.05$ ); \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

If an interaction was significant, SSF effects were analyzed by one-way ANOVA for 20% and 40% inclusion; means in the same line with different capital letters indicate significant differences ( $P < 0.05$ ) between inclusion level and small letters for SSF, respectively.

<sup>1</sup> OTUs: Average number of operational taxonomic units.

<sup>2</sup> Margalef richness index:  $d = (S - 1) / \log(N)$ .

<sup>3</sup> Simpson evenness index

<sup>4</sup> Shanon diversity index:  $H' = -\sum(\pi_i \ln(\pi_i))$ .

<sup>5</sup> SIMPER, similarity percentage within group replicates.

**Table 9** – Closest relatives (BLAST) to the sequenced PCR-DGGE gel bands of gut mucosa and digesta of European sea bass fed experimental diets.

| Band | Nearest neighbour                                                                          | Query cover (%) | Similarity (%) | Accession number |
|------|--------------------------------------------------------------------------------------------|-----------------|----------------|------------------|
| 1    | <i>Bacillus cereus</i> strain THt1-9 16S ribosomal RNA gene, partial sequence              | 96              | 99             | HQ333013.1       |
| 2    | <i>Bacillus thuringiensis</i> PAM58 gene for 16S ribosomal RNA, partial sequence           | 93              | 100            | LC716640.1       |
| 3    | <i>Bacillus amyloliquefaciens</i> strain E131 16S ribosomal RNA gene, partial sequence     | 96              | 100            | KY615347.1       |
| 4    | <i>Bacillus amyloliquefaciens</i> strain IUBT E2 16S ribosomal RNA gene, partial sequence  | 98              | 100            | MG237888.1       |
| 5    | Uncultured bacterium clone HR_1.2_16S_21 16S ribosomal RNA gene, partial sequence          | 76              | 83             | FJ875348.1       |
| 6    | <i>Clostridium</i> sp. strain H3-2 16S ribosomal RNA gene, partial sequence                | 70              | 100            | MN699050.1       |
| 7    | <i>Bacillus</i> sp. (in: Bacteria) strain ESA 657 16S ribosomal RNA gene, partial sequence | 98              | 99             | MT482572.1       |
| 8    | <i>Bacillus velezensis</i> strain ES2-4 chromosome, complete genome                        | 94              | 93             | CP097328.1       |
| 9    | <i>Bacillus</i> sp. (in: Bacteria) strain KA5 16S ribosomal RNA gene, partial sequence     | 94              | 98             | ON533508.1       |

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## Chapter 4 – General conclusions

The results presented in this thesis allow us to conclude that:

- SSF of the plant feedstuffs mixture increased cellulase and  $\beta$ -glucosidase activities. Antioxidant production decreased with SSF, and total phenols were unaffected. *In vitro* protein digestibility increased with SSF.
- SSF did not affect growth performance at the low inclusion level (20%) and negatively affected at the higher inclusion level (40%).
- SSF decreases feed intake, but lower inclusion levels increase feed efficiency.
- SSF did not affect amylase and lipase but decreased total alkaline protease activity.
- SSF did not affect plasma metabolites, except glucose, which increased in the 40 SSF diet.
- SSF did not affect liver, intestine, and muscle LPO, although it induced oxidative stress enzyme activity in the liver and intestine.
- SSF appears to have an immunomodulatory effect, mainly in increasing TGF- $\beta$  gene expression and modulating a non-significant increase in IL-8, IL-10, and TNF- $\alpha$ . It also increased cathepsin D gene expression.
- SSF increased gut microbiota diversity and abundance and promoted the presence of *Bacillus* spp. with antifungal activity.
- Further studies are needed to evaluate lower inclusion levels of this fermented mixture, to access the inclusion of an extract from this fermentation, or to better understand the effect of SSF on non-sporulating fungi.
- Further studies should also access the immunomodulatory and microbiota-modulating properties of  $\beta$ -glucans, mannans, and chitin and their benefic effects on fish in disease resistance.

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