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Valorization of Brewer's Spent Grain through Solid-State Fermentation as a Novel Aquafeed Ingredient

Tássia Tamires Estevão Rodrigues

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Tássia Tamires Estevão Rodrigues
Thesis carried out as part of the Biology Doctoral Program
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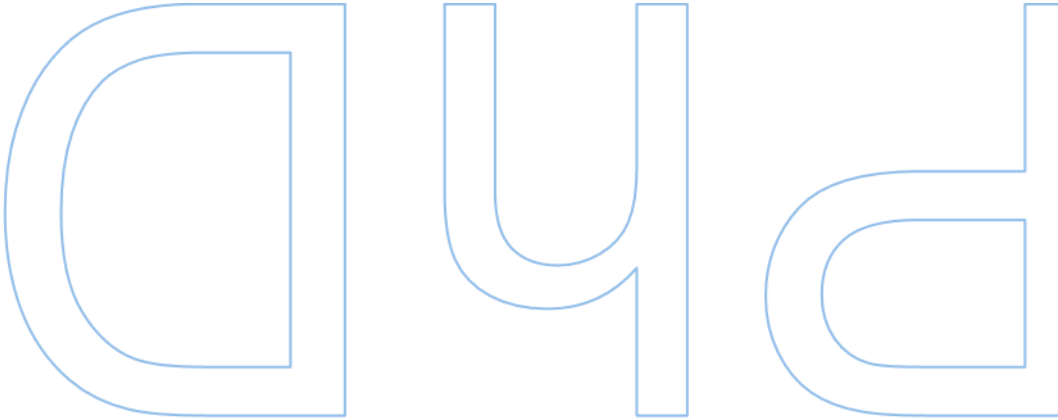

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To my beloved son, João

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Resumo

A substituição de ingredientes tradicionais agrícolas por subprodutos da agroindústria na dieta de peixes carnívoros representa uma importante estratégia para promover a sustentabilidade na aquicultura. Além disso, a dieta é responsável por aproximadamente 50-70% dos custos de produção na aquacultura. Ao utilizar subprodutos como ingredientes alternativos é possível reduzir a dependência de recursos alimentares convencionais, como a soja, o trigo e o milho além de valorizar resíduos da agroindústria, contribuindo para a economia circular e a redução do impacto ambiental.
A dreche é o subproduto mais abundante da indústria cervejeira. Suas características nutricionais como o teor em minerais e vitaminas, elevado nível de fibra e níveis consideráveis de proteína tornam a dreche um ingrediente interessante na alimentação animal, com utilização mais frequente na alimentação de gado.
No entanto, para a dieta de peixes, a dreche contém compostos que podem dificultar a digestão e a absorção pelos peixes, como altos níveis de fibra, celulose, hemicelulose e lignina. A aplicação da fermentação em estado sólido surge como uma solução eficaz para a valorização deste produto, pois esse processo biotecnológico utiliza microrganismos para quebrar as paredes celulares vegetais, tornando os nutrientes mais disponíveis para a absorção pelos peixes. Assim, a combinação da utilização de subprodutos da agroindústria com a fermentação sólida não só melhora a digestibilidade e o aproveitamento dos nutrientes, mas também contribui para a sustentabilidade e a eficiência alimentar na aquacultura.
Diante disso, o objetivo desta pesquisa foi testar a eficiência da dreche como ingrediente alternativo a ingredientes tradicionais agrícolas na dieta do robalo Europeu (*Dicentrarchus labrax*) e da corvina (*Argyrosomus regius*), espécies com grande importância económica e cultural na aquacultura europeia. Para isso foi utilizada uma abordagem multidisciplinar, considerando o desempenho zootécnico, composição da carcaça, digestibilidade de nutrientes, atividade de enzimas relacionadas com o estado oxidativo, digestão e metabolismo intermediário, bem como parâmetros bioquímicos plasmático e integridade da morfologia intestinal de ambas as espécies.
A eficiência da dreche como ingrediente alternativo na dieta de juvenis de robalo europeu está descrito no **Capítulo 2**. A dreche utilizada era composta por cevada, malte Pilsen e milho Gritz e foi obtida após a filtração da matéria-prima, e foi fornecida pela Unicer-Bebidas de Portugal, SA (Matosinhos, Portugal). O micro-organismo utilizado na

fermentação foi o *Aspergillus ibericus* MUM 03.49, fornecido pela Micoteca da Universidade do Minho (Braga, Portugal). Antes da fermentação a dreche foi esterilizada (121 ◦C, 15 min) e o teor de humidade ajustado para 75% p/p (base húmida) com água destilada estéril. A dreche foi inoculada com 2 mL de solução de esporos a uma concentração de 10^6 células mL^{-1} em bandejas contendo 400g de dreche. As bandejas foram incubadas a 25 ◦C por 7 dias e agitadas manualmente todos os dias. No final do processo a dreche fermentada (BSG-SSF) foi armazenada a 4 ◦C até que as dietas teste fossem produzidas. Para fins analíticos, amostras de 3 bandejas diferentes foram coletadas aleatoriamente para caracterizar a BSG-SSF. Foram formuladas 5 dietas (18% de lipídios brutos e 45% de proteína bruta), sendo uma dieta controlo, duas dietas contendo dreche não fermentada (10BSG e 20BSG) e outras duas dietas contendo dreche fermentada (10BSG-SSF e 20BSG-SSF). A dreche foi incluída nas dietas em substituição de um mistura de ingredientes vegetais (farinha de trigo, soja, colza e girassol). A fermentação demonstrou afetar a composição nutricional da dreche, incluindo um aumento de 21% no conteúdo de proteína e reduções no conteúdo de lipídios (49%), celulose (30%), hemicelulose (34%) e lignina (7.3%). A atividade antioxidante e os compostos fenólicos totais eram residuais antes da fermentação em estado sólido, mas aumentaram significativamente após a mesma. A incorporação dietética de 20% de BSG-SSF aumentou a digestibilidade da matéria seca, proteína, isoleucina, glutamato, lipídio e energia em comparação com a dieta 20BSG. Além disso, a fermentação, também, modulou a atividade das enzimas digestivas, reduzindo as atividades da protease total e da tripsina em peixes alimentados com a dieta 10BSG-SSF em comparação com a dieta controlo. Peixes alimentados com a dieta 20BSG apresentaram alterações negativas na histomorfologia intestinal em comparação com aqueles alimentados com a dieta controlo, e o BSG-SSF pareceu mitigar esses efeitos. Os resultados da performance de crescimento, composição da carcaça, metabolismo intermediário, atividade enzimática antioxidante e metabolismo intermediário são apresentados no **Capítulo 3**. As dietas foram fornecidas a grupos triplicados de juvenis de robalo Europeu (peso inicial de 49 g) por 68 dias. No final do ensaio, verificou-se que a inclusão dietética de 10% e 20% de dreche reduziu o crescimento e a eficiência alimentar dos animais. Em comparação, a inclusão dietética de 20% de BSG-SSF promoveu um crescimento e eficiência alimentar semelhantes aos do grupo controlo. Além disso, a inclusão dietética de 10% de BSG-SSF melhorou o crescimento e a eficiência alimentar em comparação com o controlo. O conteúdo de proteína nos animais não foi afetado pela composição da dieta. Contudo, independente do processo de fermentação, os conteúdos de lipídios e energia diminuíram com o aumento dos

níveis de dreche nas dietas. O aumento dos níveis dietéticos da dreche reduziu os níveis plasmáticos de glicose e fosfolipídios e as atividades hepáticas da glucoquinase e da enzima málica, independentemente do processo de fermentação. Independentemente do nível de inclusão dietética, a BSG-SSF aumentou os níveis plasmáticos de triglicerídeos e diminuiu as atividades da transaminase hepática, mas não afetou a atividade da enzima hepática chave β-oxidação (3-hidroxiacil-CoA desidrogenase) ou lipogénese (síntese de ácidos gordos). Tanto no fígado quanto no intestino, a inclusão dietética de BSG-SSF diminuiu a atividade da enzima antioxidante (glutationa redutase e glutationa peroxidase) e a peroxidação lipídica.

No **Capitulo 4**, foi avaliada a utilização da dreche como ingrediente alternativo na dieta da corvina. Foram avaliados a performance de crescimento, a composição da carcaça, o metabolismo plasmático, a atividade das enzimas digestivas, do stresse oxidativo e do metabolismo intermediário. Devido ao seu nível trófico elevado, a dreche fermentada e não fermentada foi incluída em níveis menores em comparação com os testes realizados com o robalo Europeu. Três dietas foram formuladas: uma dieta controlo e duas dietas contendo 10% de dreche fermentado (10BSG-SSF) ou não fermentado (10BSG). A inclusão da dreche na dieta fez-se pela substituição de 14% da mistura de ingredientes vegetais. As dietas foram testadas em triplicado em peixes com peso inicial médio de 83 g. No final do ensaio, o peso final, ganho de peso, consumo de ração e eficiência de utilização do alimento foram semelhantes nos peixes alimentados com as dietas controlo e 10BSG-SSF. Já na dieta 10BSG o desempenho de crescimento foi menor que a dieta controlo. Não foram detetadas diferenças entre grupos na atividade de enzimas hepáticas do metabolismo intermediário ou em enzimas digestivas intestinais. Peixes alimentados com a dieta dreche não fermentada exibiram um aumento da lâmina própria no intestino anterior e vacuolização supranuclear no intestino distal em comparação ao controlo. Já os peixes alimentados com a dieta contendo dreche fermentada lâmina própria no intestino anterior e vacuolização supranuclear no intestino distal foram semelhantes ao da dieta controlo.

Em conclusão, de um modo geral, os resultados deste trabalho indicam que a fermentação da dreche com o fungo *Aspergillus ibericus* demonstrou melhorar o perfil nutricional, o aumento da atividade antioxidante e de compostos fenólicos após o processo de fermentação. Além disso, a diminuição de fatores antinutricionais, como celulose e hemicelulose, e o aumento do teor de proteína pontuam como fatores positivos na transformação do perfil nutricional da dreche após o processo de fermentação. De facto, a fermentação é uma técnica promissora para melhorar a qualidade nutricional de subprodutos agroindustriais de baixo valor, aumentando o seu

uso potencial como ingredientes para ração de animais aquáticos e contribuindo para reduzir a pegada climática e ambiental da produção aquícola. Além disso, para juvenis de robalo Europeu, a inclusão da dreche fermentada demonstra não comprometer a digestibilidade da dieta, em contraste com a dreche não fermentada, que compromete tanto a digestibilidade, como a morfologia da histologia do intestino, tanto de juvenis de robalo Europeu, como da corvina. O desempenho de crescimento para ambas as espécies, no maior nível de inclusão de dreche fermentada testado, igualou o desempenho de uma dieta controlo. Além disso, as atividades das enzimas digestivas demonstram que os peixes alimentados com as dietas fermentadas apresentam uma menor atividade enzimática da protease e da tripsina. Porém, enquanto a inclusão nas dietas, contendo dreche não fermentada, levou a um deficit da defesa antioxidante e da atividade das enzimas do metabolismo intermediário, a dreche fermentada teve efeito positivo no estado oxidativo e na atividade das enzimas do metabolismo intermediário. Embora a inclusão da dreche fermentada com *A. ibericus* na dieta de peixes carnívoros contribua para reduzir a pegada de carbono da produção aquícola, sem comprometer as suas integridades fisiológicas e nutricionais, como demonstra este estudo, podem encontrar-se entraves na sua utilização na industria de fabrico de ração para peixes, devido à variabilidade na composição da dreche entre diferentes lotes, o que pode levar a diferentes resultados finais na fermentação. Será, por isso, necessário investir na padronização e certificação do produto.

Palavras-chave: fermentação em estado sólido, dreche, subproduto agroindustrial, economia circular, nutrição de peixes, aquacultura.

Abstract

Replacing traditional agricultural ingredients with agro-industrial by-products in the diet of carnivorous fish represents a transformative strategy for enhancing aquaculture's environmental, economic, and social sustainability. The efficient valorization of by-products enables their conversion into valuable feed alternatives, reducing feed production costs, supporting a circular economy, and contributing to a more resilient food system essential for feeding the world's growing population. Brewer's spent grain (BSG), a significant by-product of the brewing industry, shows potential as an aquafeed ingredient. However, its low protein content and high levels of non-starch polysaccharides (NSP), which negatively impact fish growth and health, limit its applicability.

Solid-state fermentation (SSF) is an eco-friendly and cost-effective biotechnological process capable of hydrolyzing NSP, increasing protein content, and enriching by-products with bioactive compounds such as antioxidants and enzymes. It was hypothesized that applying SSF to BSG could enhance its nutritional value, increasing its feasibility as an alternative feed ingredient.

This research aimed to assess the potential of SSF-treated BSG with *Aspergillus ibericus* as a sustainable alternative to traditional agricultural ingredients in diets for European sea bass (*Dicentrarchus labrax*) and meagre (*Argyrosomus regius*), species of high economic and cultural significance in European aquaculture. By using a multidisciplinary approach, the study evaluated the effects of dietary BSG and SSF-BSG on zootechnical performance, carcass composition, nutrient digestibility, intermediary metabolism, intestinal and hepatic oxidative stress, plasma metabolites, and intestinal morphology in both species.

Chapter 2 describes the fermentation process of BSG with *Aspergillus ibericus* MUM 03.49. The fermentation increased protein content (21%) and decreased the lipid (49%), cellulose (30%), hemicellulose (34%), and lignin (7.3%) contents. Antioxidant activity and total phenolic content, residual in the unfermented BSG, increased after fermentation. Chapter 2 also reports a digestibility trial in juvenile European sea bass to evaluate nutrient and energy digestibility, digestive enzyme activity, and intestinal histomorphology of BSG and SSF-BSG. Five diets were tested, including a control diet (without BSG), two with 10% and 20% BSG, and two with 10% and 20% SSF-BSG. At 20% inclusion, SSF-BSG improved the digestibility of protein, lipids, energy, and certain amino acids compared to BSG diets. Further, SSF-BSG modulated digestive enzyme

activity by reducing protease and trypsin activities and mitigated the adverse histomorphological changes induced by BSG.

Chapter 3 reports a growth trial evaluating the impact of dietary inclusion of 10 and 20% BSG and SSF-BSG on growth performance, feed utilization, plasma metabolite profile, intermediary metabolism, and liver and intestine oxidative status in juvenile European sea bass (initial body weight of 49 g) fed over 10 weeks. Dietary inclusion of BSG (10% and 20%) reduced growth and feed efficiency. In comparison, the 20% SSF-BSG diet promoted growth and feed efficiency similar to that of the control group, and the 10% SSF-BSG diet promoted growth and feed efficiency higher than the control diet. Whole-body protein content was unaffected, but lipid and energy contents decreased with increasing dietary BSG levels, regardless of fermentation. Plasma glucose and phospholipid levels and hepatic activities of glucokinase and malic enzymes decreased with increasing BSG levels, irrespective of fermentation. Dietary SSF-BSG incorporation increased plasma triglyceride levels and decreased hepatic transaminase activities but did not affect hepatic key enzyme activity of β-oxidation (Hydroxyacyl Co-A dehydrogenase) or lipogenesis (malic and fatty acid synthase). In both the liver and intestine, dietary inclusion of BSG-SSF decreased antioxidant enzyme activity and lipid peroxidation.

Chapter 4 describes a growth trial testing diets including 10% of BSG or SSF-BSG in meagre (initial body weight 80g). Growth performance, feed efficiency, intestinal digestive enzymes, and metabolic and antioxidant responses in fish fed the diet with BSG-SSF were comparable to the control diet. In contrast, BSG caused adverse changes in intestinal morphology. SSF-BSG maintained intestinal health without significant effects on intermediary metabolism or digestive enzyme activities.

Overall, the results of this research indicate that SSF is a promising technique for improving the nutritional quality of low-value agro-industrial by-products, such as BSG. SSF-BSG has increased protein content, enhanced antioxidant activity, and reduced antinutritional factors, significantly improving its suitability as a feed ingredient. In juvenile European sea bass, dietary inclusion of SSF-BSG improved nutrient and energy digestibility without compromising intestinal morphology, whereas BSG adversely affected both. Growth performance at the highest levels of SSF-BSG inclusion matched that of the control diet in European sea bass and meagre.

Moreover, SSF-BSG modulated enzymatic activities, reducing digestive enzyme activity, decreasing oxidative stress, and exacerbating intermediary metabolism deficits. Despite its potential to lower the environmental footprint of aquaculture and promote circular economy principles, the use of fermented BSG by the fish feed industry may face

challenges related to batch variability, standardization, and certification processes. These effects were more pronounced in sea bass than in meagre, possibly due to differences in initial body weight and the fact that only the lowest inclusion level was tested in meagre.

These findings highlight the potential of SSF-BSG as a sustainable, nutritionally enhanced ingredient for carnivorous fish aquafeeds, maintaining intestinal health and performance while addressing the environmental and economic challenges of modern aquaculture. Further studies will be necessary to evaluate the economic feasibility of SSF-BSG as an alternative ingredient for both species, as well as a detailed life cycle assessment to quantify the environmental benefits of using SSF-BSG to replace conventional ingredients like soybean, wheat, and corn-based feedstuffs. The variability in BSG composition between industries will also need to be addressed, as well as standardization and product certification.

Keywords: solid-state fermentation, BSG, agro-industrial by-product, circular economy, fish nutrition, aquaculture.

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Figure List

Abbreviation List

AA – Amino Acid
AAC – Atomic Absorption Coefficient
ABW – Average Body Weight
ADC – Apparent Digestibility Coefficient
ADCP – Protein Apparent Digestibility Coefficients
AIPCE – Association of Fish Processors and Traders in Europe
ALAT – Alanine Aminotransferase
ANOVA – Analysis of Variance
AOAC – Association of Official Analytical Chemists
ARA – Arachidonic Acid
ASAT – Aspartate Aminotransferase
ATCC – American Type Culture Collection
ATP – Adenosine Triphosphate
AZTI – Marine and Food Research Institute (Spain)
BSG – Brewer's Spent Grain
CAT – Catalase
CE – Circular economy
CHO – Cholesterol
CIIMAR – Interdisciplinary Centre of Marine and Environmental Research
CL – Crude Lipid
CP – Crude Protein
CPT-1 – Carnitine Palmitoyltransferase-1
DGI - Daily Growth Index
DHA – Docosahexaenoic Acid
DM – Dry Matter
DW – Dry Weight
DNA – Deoxyribonucleic Acid
EAA – Essential Amino Acids
ECR – Economic Conversion Ratio
EFA – Essential Fatty Acids
EPI – Economic Profit Index
EPA – Eicosapentaenoic Acid
EU – European Union
FA – Fatty Acid
FAO – Food and Agriculture Organization
FBW – Final Body Weight
FCR – Feed Conversion Ratio
FCUP – Faculty of Sciences of the University of Porto
FCT – Foundation for Science and Technology
FE – Feed Efficiency
FI – Feed Intake
FM – Fish Meal
FO – Fish Oil
G6PDH – Glucose-6-Phosphate Dehydrogenase
GLU – Glucose
GPx – Glutathione Peroxidase
GR – Glutathione Reductase
IBW – Initial Body Weight
ISO – International Organization for Standardization
LA – Lauric Acid
LCFA – Long-Chain Fatty Acids
LC-PUFA – Long-Chain Polyunsaturated Fatty Acids
LDL – Low-Density Lipoprotein

M – Million
MDA – Malondialdehyde
MUFA – Monounsaturated Fatty Acid
N – Nitrogen
NFE – Nitrogen-Free Extract
NSP – Non-starch polysaccharides
NUTRIMU – Fish Nutrition and Welfare Research Group
PAP – Processed Animal Proteins
PDF – Partially Defatted
PER – Protein Efficiency Ratio
PUFA – Polyunsaturated Fatty Acid
RAS – Recirculating Aquaculture Systems
ROS – Reactive oxygen species
SEM – Scanning electron microscopy
SFA – Saturated Fatty Acids
SGR – Specific Growth Rate
SOD – Superoxide Dismutase
SSF – Solid-State Fermentation
TG – Triglycerides
TL – Trophic Level
TP – Total Protein
UI – International Unit
UN – United Nations
VO – Vegetable Oil
WG – Weight Gain

Chapter 1:

General Introduction

1 Aquaculture Production Insights

1.1 Global Aquaculture Production

With the world population surpassing 8 billion people last year (FAOSTAT, 2024), the demand for food, especially for animal protein sources, has also been under pressure to intensify its production, requiring sustainable solutions to increase output without compromising natural resources. The Food and Agriculture Organization of the United Nations (FAO), in turn, has established as its primary goal the expansion of sustainable aquaculture production by improving efficiency in production systems and promoting access to technological and financial resources, ensuring that ethically and sustainably increased production keeps pace with population growth (FAO, 2024a). According to FAO data, aquaculture has been one of the fastest-growing agricultural activities in recent years, with an average growth rate of 4.9% over the last 24 years. In 1980, aquaculture ceased to be an insignificant alternative source of fish, accounting for 9.1% of global fish production. In 2021, aquaculture surpassed fishing in absolute terms, accounting for 51% of global fish production. In 2023, global aquaculture production was 130.9 million tons (**Figure 1**). Of this production, 87% (113 million tons) was directed to human consumption, with fish production accounting for 72% of aquaculture production (94.4 million tons, excluding algae) (FAO, 2024a).

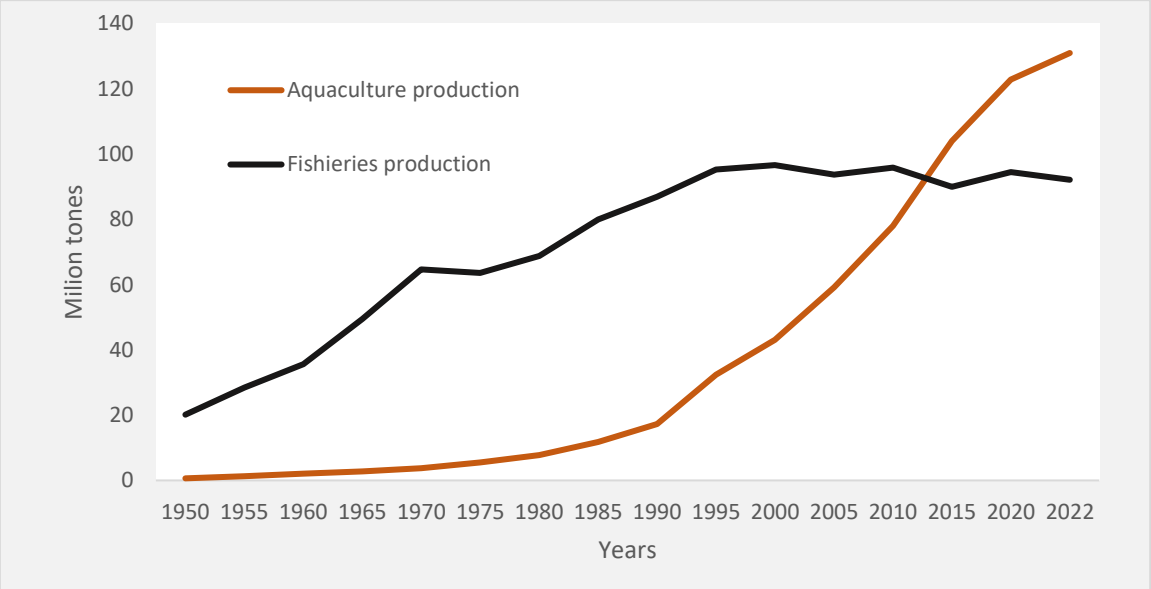

Figure 1: Aquaculture production and capture fisheries (1950-2022). Source: Fishstat (FAO 2024)

From 1980 to 2024, aquaculture increased its production by 121.2 million tons, while fishing has stagnated at approximately 90 million tons since 1996 (FAO, 2000).

Although the growth of aquaculture is associated with factors such as the increasing human population, the consequent need for food protein, rising income levels, and changes in eating habits (FAO, 2024a; Naylor et al., 2021), the stagnation of fisheries is one of the most significant drivers (Bjørndal et al., 2024). The scarcity of natural resources poses challenges not only to biodiversity but also to food security (Sunderland, 2011). In this scenario, aquaculture has emerged as a strategic response to the growing global demand for fish, offering a sustainable, environmental, and economic complement to fisheries (Garlock et al., 2024; Martinez-Porchas & Martinez-Cordova, 2012). Efficient fish production in controlled farming systems reduces pressure on marine environments and promotes the rational use of natural resources (Naylor et al., 2021, 2000). Thus, aquaculture is essentially the primary reason fish production has continued to grow and meet global demand (Garlock et al., 2020).

The most significant growth in aquaculture activity is observed in Asian and developing countries (Cojocaru et al., 2022; Belton et al., 2018; Thilsted et al., 2016). Among the most significant fish producers, Asian countries dominate, with China accounting for 60% of the world's aquaculture production (FAO, 2024). This large production is directly proportional to the annual fish consumption, reaching 47.4 kg per capita in this country, significantly exceeding the world average of 20.1 kg (FAO, 2024b).

In addition to meeting the growing global demand for food, aquaculture contributes significantly to improving human nutrition by providing a highly valuable source of nutrients. Fish is a high-quality animal protein source due to its good digestibility and adequate amino acid profile. Fish also provides essential minerals, including iodine, zinc, and iron, as well as vitamins A and D. Additionally, fish is low in saturated fat and rich in long-chain polyunsaturated fatty acids (LC-PUFA), particularly eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), which are essential to support human health (Moutinho et al., 2023; Maulu et al., 2021).

1.2 Aquaculture Production in the European Union

Aquaculture plays a pivotal role in both the economy and environmental sustainability of the European Union (EU) (Bostock et al., 2016). According to the latest data from FAO (2024), the sector's production reached 1.2 million tons. This production is primarily driven by mollusk farming, accounting for more than 50% of the total volume, followed by marine and freshwater fish, representing 21% and 28%, respectively. The leading producers in the EU, which together account for 64% of total production, include France—renowned for its mussel and oyster farming—Spain and Italy, both with

significant mollusk and fish farming industries, and Greece, the largest producer of marine fish (FAO, 2024a).

Production in the EU has increased by only 6% since 2007 (STECF, 2020), totalling 1.2 million tonnes of aquatic organisms, worth 4.8 billion euros, a growth lower than observed worldwide. Therefore, domestic production remains insufficient to meet the per capita consumption of 23.0 kg in 2023 (AIPCE, 2024). EU fishery products imports account for 5.44 million tonnes, with a total value of around 33.2 billion dollars in 2022, excluding intra-European Union trade (EuroStat, 2024).

Although over 70 species are produced in the EU (FAO, 2024a), overall production is dominated by a few finfish (particularly trout, seabream, seabass, carp, tuna, and salmon) and mollusks (particularly mussels, oysters, and clams). The most recent data indicates that rainbow trout (*Oncorhynchus mykiss*) accounts for 18% of EU production, followed by sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*), which together contribute 20%. Mussels (*Mytilus galloprovincialis* and *Mytilus edulis*) represent about 14%, carp (*Cyprinus carpio*) 14%, and oysters (*Magallana gigas*) 10% of total production (FAO, 2024a). The importance of these species is not limited to production but also to demand by the European market, where they are highly appreciated (Bjørndal et al., 2019).

These species are predominantly farmed in floating cages in the sea, although some are also produced in water recirculation systems and coastal earthen ponds (Ahmed & Turchini, 2021; Badiola et al., 2012). Furthermore, investments in genetic improvements, disease control, nutritional requirements, and the use of more sustainable alternative ingredients in aquafeeds have increased productive efficiency, reducing costs and environmental impacts (Colombo et al., 2022; Glencross, 2020), making these species more attractive for aquaculture. However, production costs of carnivorous species remain high due to their high protein requirements (Oliva-Teles et al., 2024).

In the EU, efforts to promote sustainable aquaculture have been ongoing, with varying degrees of intensity (Puszkarski & Śniadach, 2022). A significant step was taken in 2013 when the European Commission issued a joint Communication titled "Strategic Guidelines for the Development of Aquaculture in the EU" (EC,2011). This document links aquaculture to the EU's "blue growth" strategy, part of the Integrated Maritime Policy. The policy assumes that all issues related to Europe's oceans and seas are interconnected, and only coordinated action can ensure that the planned results are achieved.

In 2014, the European Maritime and Fisheries Fund (EC, 2014) was established with an investment of 4.3 billion euros, financing projects from 2014 to 2020. The fund's goal

was to continue promoting environmental sustainability and innovation in aquaculture, in line with the Common Fisheries Policy. For aquaculture, it aimed to support small and medium-sized aquaculture companies, support innovation, research, technological modernization, and the promotion of environmentally responsible practices (Puszkarski & Śniadach, 2022).

In 2019, the European Green Deal was launched as a comprehensive response to climate and environmental challenges, directly aligned with the UN 2030 Agenda and its Sustainable Development Goals (EC, 2015). This pact aimed to transform the EU economy toward sustainability, aiming for climate neutrality by 2050. The Green Deal covers several areas, notably the "Farm to Fork" strategy, which focuses on producing sustainable food and includes aquaculture in ecological chains. Through policy initiatives, legislation, and €1 trillion in funding, the Green Deal seeks to implement the 2030 Agenda objectives, reducing the EU's environmental and climate footprint while promoting a competitive and socially fair economy.

In 2021, the European Maritime, Fisheries and Aquaculture Fund was created, with an investment of 6.1 billion euros, financing projects between 2021 and 2027 (EC, 2021). This fund is focused on encouraging the development of aquaculture practices that minimize environmental impact, such as using water recirculation technologies and reducing carbon emissions in the sector. In addition, the fund supports projects aimed at reducing the use of traditional feed and developing more environmentally friendly alternatives, such as using plant-based or valorized by-product ingredients in aquafeeds. The EU Cohesion Policy also allocates significant resources to the development of aquaculture, with a focus on improving the sector's infrastructure and strengthening the blue economy (Guillen et al., 2019). This includes investments in sustainable practices, technological innovation, and the creation of conditions that promote the growth of aquaculture in coastal and marine regions (Guillen et al., 2019; Puszkarski & Śniadach, 2022). These resources are essential to support the transition to greener and more competitive production models aligned with the EU's environmental objectives. However, several challenges hinder the growth of aquaculture in the EU, including resistance to the adoption of new technologies due to high upfront costs, the scarcity of suitable farming spaces, pressure on marine and coastal ecosystems, and regulatory complexity (Puszkarski & Śniadach, 2022; Avdelas et al., 2021; Cavallo et al., 2021). To address these challenges, strategies aiming to increase the demand for aquaculture products in the EU should involve the definition, analysis, and discussion of their objectives, strategies, and policies at all relevant levels (Ertör & Ortega-Cerdà, 2017). In other words, improving collaboration between governments, industry, and academia will create

a more favorable environment for adopting sustainable practices and supporting sector growth. This collaborative approach will help ensure that European aquaculture achieves participatory, fair, and sustainable development.

2 Aquaculture Production Vs. Circular Economy

In recent decades, the aquaculture sector has been growing exponentially globally. Beyond being one of the largest suppliers of fish for human consumption (FAO, 2024b), it is considered by some to be essential for providing essential animal protein and essential fatty acids (Willett et al., 2019). However, its rapid growth has attracted some widespread criticism for its environmental and social impacts (Krause et al., 2020; Osmundsen & Olsen, 2017; Bacher, 2015; Whitmarsh & Wattage, 2006; Barrett et al., 2002).

The environmental impacts of aquaculture could be minimized or even negated through appropriate environmental management (Read & Fernandes, 2003). Applying methodologies to assess these impacts is essential for fostering more sustainable practices within the industry (Bohnes et al., 2019; Papatryphon et al., 2004). Some of these methodologies include carbon footprint analysis (Li et al., 2024; Chang et al., 2017), water footprint analysis (Q. Jiang et al., 2022; Mekonnen & Gerbens-Leenes, 2020), nutrient cycling analysis (Huang et al., 2024; Rafiee & Saad, 2005; Schneider et al., 2005), ecotoxicology analysis (Lin et al., 2023; Arias-Andrés et al., 2014), land use pattern analysis (Davis et al., 2021), and by-product life cycle analysis (ISO 14040 and ISO 14044). These methodologies generally follow the same core tools:

***1. Definition of objective and scope*:** Determine the purpose of the analysis by comparing production technologies to determine which has the least impact, mapping environmental impacts caused by the production system used, improve the production cycle by identifying environmental bottlenecks from feed production to the processing and transportation phase, and support decisions with data that that can guide policy or help companies adopt more sustainable practices.

2. Inventory analysis: This step requires collecting and organizing accurate data on the materials and energy flows at each stage of the production system, including information on agricultural or industrial inputs for feed production and data on transportation and disposal.

3. Functional Unit: Identify the functional unit to standardize comparisons between the production systems based on factors like the quantity of the final product, nutritional value (measured in kg of edible protein produced), or temporal dimension (measured by the amount produced per unit of time).

4. Life Cycle Assessment: The data collected (inventory phase) is interpreted and linked to specific environmental impact categories: a) Climate change: emission of greenhouse gases (carbon dioxide, methane, and nitrous oxide) at different stages of the aquaculture system. b) Eutrophication: accumulation of nutrients (nitrogen and phosphorus) in water bodies, contributing to excessive algal blooms and water quality degradation. c) Acidification: increase in acidity in soils and water bodies. d) Ecotoxicity: impact of toxic substances on the environment, particularly on organisms in aquatic and terrestrial ecosystems. e) Energy consumption and water resources: energy and water use throughout the system's life cycle.

5. Interpretation of results: Aims to transform the data into practical insights for decision-making. The life cycle impact analysis results are critically evaluated to identify areas for improvement and strategies to reduce environmental impacts.

These methodologies, which combine quantitative and qualitative approaches, are essential for more informed decision-making and aligning aquaculture production with natural resource conservation and sustainable development principles (Stevens et al., 2018; Troell et al., 2014).

In aquaculture, the most significant environmental impacts are related to the disposal of effluents and diet manufacture (Martinez-Porchas & Martinez-Cordova, 2012b; Naylor et al., 2000). Aquaculture effluents can significantly impact aquatic ecosystems due to the high concentration of organic matter and nutrients, such as nitrogen and phosphorus (Ahmad et al., 2021). These compounds contribute to eutrophication, a process in which excessive nutrient enrichment leads to uncontrolled growth of algae and phytoplankton (Kang et al., 2021; Jiang et al., 2012), reducing dissolved oxygen levels, harming aquatic organisms and compromising local biodiversity (Jana' & Sarkar2, 2005). It is essential to implement sustainable management practices to mitigate these impacts, such as the use of balanced feeds, natural effluent treatment systems, and water recirculation techniques (Ahmad et al., 2021; Jana' & Sarkar2, 2005; Subasinghe et al., 2009). These measures help reduce effluents' pollutant load and promote more sustainable aquaculture (Ahmad et al., 2021; Subasinghe et al., 2009; Jana' & Sarkar, 2005). Optimal feed management includes using well-balanced feeds that meet the nutritional and energy requirements of the species while also adopting cost-effective feeding regimes (Oliva-Teles et al., 2015). It is important to note that animals absorb only a fraction of the feed provided (Fry et al., 2018). Much of the nitrogen and carbon are excreted through the branchia and dissolved in the water column, while much of the phosphorus is released as particulate matter that settles into the seabed (Duan & Firdaus, 2024; Khanjani & Sharifinia, 2020). White (1992) highlighted that poor feed quality and suboptimum feeding strategies substantially

influence the environmental impact of open-water and coastal aquaculture systems. Furthermore, fishmeal and plant-based ingredients have a carbon footprint that intensifies the environmental effect (Li et al., 2024; Gokulakrishnan et al., 2023). Carbon footprint calculation considers all relevant sources and sinks within a given spatial and temporal boundary (Gokulakrishnan et al., 2023; Chang et al., 2017; Jiang et al., 2012). Fishmeal in aquafeeds comes from fishing for small pelagic fish such as sardines, anchovies, and mackerel (Tacon et al., 2022). In addition, by-products of the fishing industry, such as viscera and carcasses, are increasingly used (Bechtel, 2006). Much of this fishing comes from Latin American countries such as Chile and Peru (Olsen & Hasan, 2012). As a result, aquaculture also exerts an effort on natural fish stocks, exacerbated by the growing demand for animal protein worldwide. The use of alternative protein sources is essential to minimizes the environmental impact of dependence on fishmeal, including vegetable proteins (Hussain et al., 2024), plant by-products (Estévez et al., 2022; Fernandes et al., 2022), insect-based proteins, animal protein sources (Moutinho et al., 2023, 2017), and microorganisms (Agboola et al., 2022).

Plant-based ingredients, particularly soybeans, wheat, corn, and rice, constitute the most extensive ingredients used in aquafeeds (Glencross et al., 2023). However, their use faces challenges related to competitiveness with other production chains, particularly human food and terrestrial animal nutrition (Sandström et al., 2022). Crops such as soybeans, wheat, and corn are also staple foods for human consumption, which can generate economic and ethical pressure due to direct competition for these commodities (Sandström et al., 2022). In 2023, 15.7 million tons of corn, 57.8 million tons of wheat, and 1.97 million tons of soybeans were imported into the EU (European Union, 2024). These imports primarily come from Latin American countries, such as Brazil, the EU's main supplier. Cereals, such as wheat and corn, come mainly from Ukraine and the United States, with varying domestic production conditions and demand (European Union, 2024). The large-scale use of plant-based ingredients from different continents increases the consumer market's carbon footprint and transportation costs, increasing aquaculture costs (Troell et al., 2014). Thus, the challenge of meeting the demand for fish lies in increasing aquaculture production sustainably while minimizing its economic and environmental impacts (Regueiro et al., 2022).

The circular economy (CE) concept arises from the need to enhance production systems sustainability by maximizing resource use and implementing a holistic approach. The CE model promotes keeping products, materials, and resources in use for as long as possible, emphasizing the reuse, recycling, and recovery of materials throughout their life cycle. By adopting practices that minimize waste and promote efficiency, a circular

economy reduces environmental impact and fosters innovation and the creation of economic value, contributing to a more sustainable and resilient future (Masi et al., 2024). The potential benefits of CE in aquaculture can be categorized into two main strategies: **closed-loop circularity** and **open-loop circularity** (Masi et al., 2024).

1. "Closed-loop" circularity strategies: all the materials waste and by-products are recycled and reintegrated into the production system from which they originated. In other words, waste and by-products generated during aquaculture production are processed and reused as raw material, promoting self-sufficiency and reducing the need for external resources (Bulc et al., 2011). One example of a closed-loop system in aquaculture is a biofloc culture system, where the microbial community present in the system acts in the transformation of toxic nitrogen compounds (such as ammonia) into less harmful forms or microbial biomass, which can be reused as feed for the fish (Khanjani & Sharifinia, 2020; Hargreaves, 2013). This process helps maintain water quality and reduces the need for external chemicals, making it a more sustainable and resource-efficient aquaculture approach. Furthermore, aquaculture waste can be directed to anaerobic digestion processes, generating biogas (renewable energy) and organic fertilizers that return to the biosphere, promoting circularity and avoiding waste (Khanjani & Sharifinia, 2020; Hargreaves, 2013; Bulc et al., 2011).

2. "Open-loop" circularity strategies: Materials and waste are recycled or reused in different production processes outside the original chain. In this case, both by-products from aquaculture and other types of enterprises can be used as feed ingredients, contributing to the circular economy in a broader context (Fernandes et al., 2022; Stevens et al., 2018; Wong et al., 2016).

The Ellen MacArthur Foundation's Butterfly Diagram (Masi et al., 2024) illustrates that the circularity strategies applicable to biological flows vary regarding the degree of closure (**Figure 2**). These strategies can range from more closed systems, such as cascade processes, representing a more intense form of closed-loop cycles, to more open systems, involving interactions with other economic sectors, such as energy or fertilizer production. In the latter case, these connections tend to be more open (open-loop), amplifying the impact and intersectoral collaboration (Masi et al., 2024). The Butterfly Diagram represents the flows of materials in technical and biological cycles. This model is particularly relevant in integrated aquaculture systems, where residues and by-products are reused, minimizing waste and promoting sustainability (Masi et al., 2024).

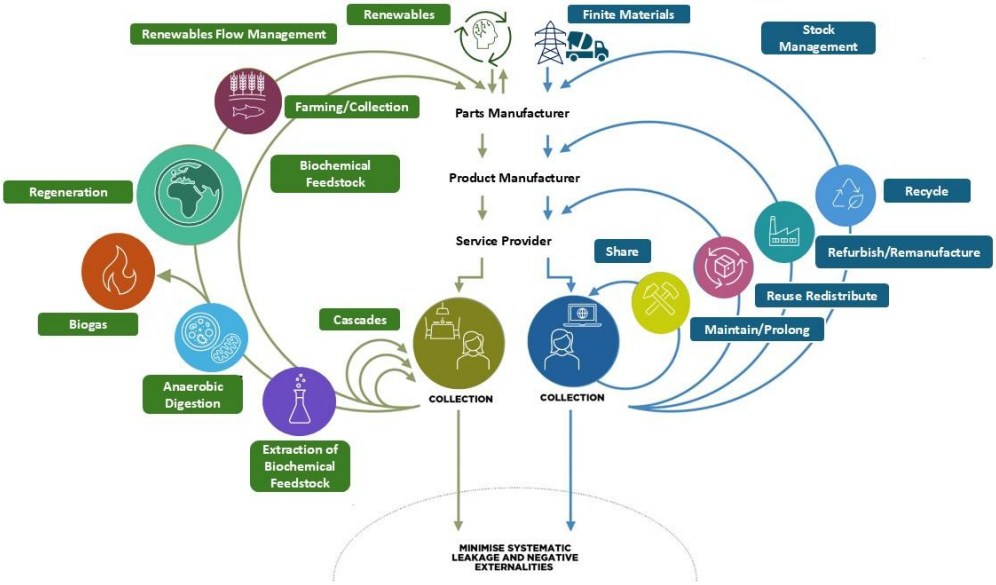

Figure 2: Ellen MacArthur Foundation's Butterfly Diagram. Source: Adapted from Circular Economy Systems Diagram (EMF, 2019).

In the technical flow, non-consumable materials such as cultivation structures and plastic packaging are optimized, aiming at their most significant use within the production system. Super-intensive aquaculture technologies, such as water recirculation systems (RAS), are designed to be durable and efficient, with a focus on ensuring that the systems operate optimally, with the least amount of maintenance possible and the most extended lifespan (Ahmed & Turchini, 2021; Badiola et al., 2012). In addition, RAS promotes water recycling, nutrient reuse, waste reduction, and integration with other food production systems, generating a more efficient, sustainable system with a lower environmental impact (Ahmed & Turchini, 2021; Badiola et al., 2012). The reuse and/or recycling of materials such as paper, piping, and metals are also part of the technical flow of the butterfly graph. Packaging or cultivation structures (cages and tanks) can be reused, repaired, or recycled. For example, after proper maintenance, reusing nets or farming systems prevents these materials from becoming waste and maintains their economic value in the production chain.

Within the biological flow diagram, by-products such as agro-industrial waste or fish processing leftovers can be used as ingredients for aquafeeds, forming a cascade of reusable nutrients (Zhang et al., 2023; Kokou & Fountoulaki, 2018; Wong et al., 2016). Several studies have demonstrated the effectiveness of using agro-industrial by-products, both as raw or technologically transformed forms, in the diets of commercially farmed fish (Estévez et al., 2022; Fernandes et al., 2022; Nazzaro et al., 2021; San

Martin et al., 2020). However, to align with the circular economy principles, with an emphasis on waste recovery, the use of by-products with lower economic and protein value may require technological treatments to increase their suitability for inclusion in animal feeds, including aquafeeds. By-product transformation processes include, for example, chemical, physical, and biological processes. Physical processes include grinding and sieving, extrusion, and steaming (Ahmed et al., 2010), which alter the physical properties of by-products, making them more suitable for feed formulations. Chemical processes involve chemical hydrolysis (Fernandes et al., 2022; San Martin et al., 2020), toxin neutralization (Maksimenko et al., 2024; Thazeem et al., 2018), and saponification (Lian et al., 2005) that modify the chemical composition and eliminate undesirable compounds. Biological processes, in turn, use microorganisms or enzymes to modify the by-products. They can be fungal fermentation (Estevão-Rodrigues et al., 2024; Karimi et al., 2018) or bacterial fermentation (Das & Ghosh, 2015) and enzymatic hydrolysis (Vázquez et al., 2019), increasing the bioavailability of nutrients. Additionally, the combination of techniques, such as the application of chemical and/or enzymatic hydrolysis followed by fermentation, can be a highly effective approach to enhance the benefits of processing (Wang et al., 2024; Fernandes et al., 2022).

Aquaculture, therefore, has the potential to align with the CE principles, promoting sustainable practices that maximize resource reuse, minimize waste, and reduce environmental impact. The CE guidelines focus on these principles, aiming to reduce waste disposal, valorize by-products from primary industries, ensure the renewal of resources, and create new value chains with economic benefits. The combination of research, technological development, and the implementation of supporting technologies facilitates a transition towards more sustainable aquaculture practices that align with long-term ecological and economic goals.

However, while direct and indirect CE measures contribute to achieving strategic biodiversity and climate/energy objectives, circular strategies alone cannot be considered a universal solution to biodiversity-related challenges (ETC, 2023). It is crucial to give explicit attention to sourcing that favors biodiversity conservation to ensure a positive impact of CE strategies on biodiversity (ETC, 2023). This principle is often overlooked or assumed in CE policies. It goes beyond mitigating negative impacts, aiming instead to promote positive outcomes for biodiversity (ETC, 2023).

3 Solid State Fermentation

Aligned with the principles of the CE and the focus on resource valorization, reintroducing agro-industrial by-products with low economic and nutritional value into the food chain

often requires technological treatments to enhance their suitability for use in animal feed, including aquafeed.

Solid State Fermentation (SSF) has emerged as a promising biological process for converting agro-industrial by-products into value-added products. This ancient technique uses solid substrates as a source of nutrients for the growth of specific microorganisms without free water (**Figure 3**) (Hölker & Lenz, 2005). Microorganisms grow directly in the solid matrix in controlled temperature and humidity environments, favoring the production of a wide range of compounds, including bioactive compounds (El-Bakry et al., 2015; Hölker & Lenz, 2005; Durand, 2003). During the SSF process, the substrate provides carbon, nitrogen, and other components, supporting microbial cell growth(Hölker & Lenz, 2005). Conditions closer to natural habitats facilitate microbial development; however, adequate aeration is crucial to avoid substrate agglomeration and ensure uniform growth (Hölker & Lenz, 2005).

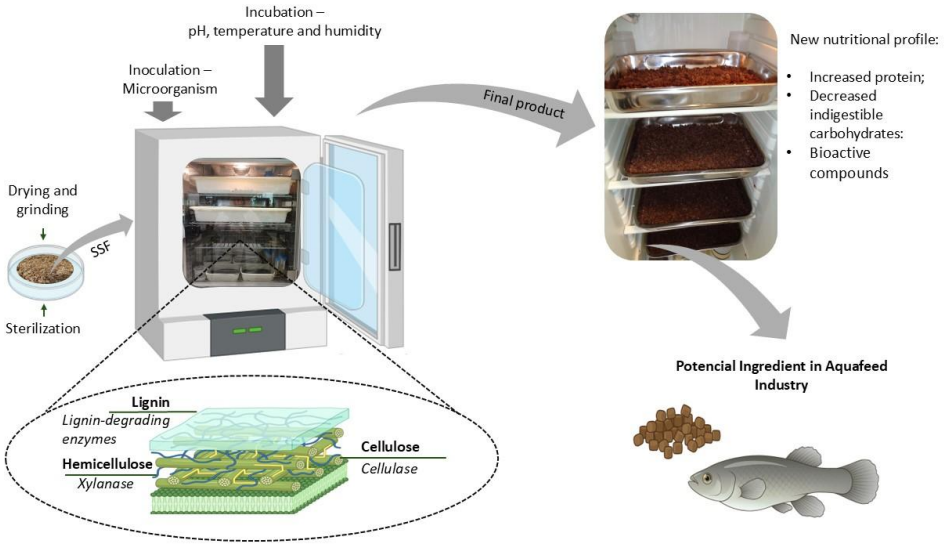

Figure 3: Transformation of agro-industrial by-products by solid-state fermentation into potential aquaculture feeds.

SSF is considered an environmentally friendly technique due to its low water usage, minimal effluent generation, energy efficiency, cost-effectiveness, and high adaptability in different environmental conditions(Hahryari & Niknezhad, 2022; Hölker & Lenz, 2005), making it advantageous compared to the submerged fermentation technique (Teigiserova et al., 2021; Durand, 2003).

Furthermore, SSF can convert agro-industrial by-products with lignocellulosic profiles, such as bagasse, bran, and husks, which are valuable nutrient sources but are primarily

used in ruminant nutrition. SSF can convert these by-products into high-value products, contributing to the circular economy by minimizing waste generation and promoting sustainable practices (López-Gómez et al., 2020; Leite et al., 2019; Berbel & Posadillo, 2018; Hölker & Lenz, 2005).

However, the heterogeneity of agro-industrial by-products poses challenges for scaling the process for commercial feed applications. Variability in raw material composition, chemical structure differences, and component proportion variation depend on the origin and plant part used (Strong et al., 2022; Leite et al., 2019; Hölker & Lenz, 2005). Therefore, each by-product must be carefully evaluated to ensure the efficiency and consistency of SSF processes (Strong et al., 2022; Leite et al., 2019; Hölker & Lenz, 2005).

The heterogeneity of substrates used in SSF remains a significant barrier to commercialization. Addressing this requires optimizing substrate selection, improving process control, and exploring innovative applications for SSF products. Research efforts should focus on enhancing the scalability of SSF to meet industrial demands while maintaining environmental and economic benefits.

Several microorganisms are used in SSF, such as bacteria, yeasts, and fungi (Hölker & Lenz, 2005). Filamentous fungi, in particular, possess advantageous characteristics, such as rapid growth under low humidity, efficient production of extracellular enzymes, growth in complex substrates, and resistance to pH and temperature variations. Their filamentous structure, with hyphal growth, facilitates substrate penetration, making them particularly suitable for SSF (Durand, 2003). Filamentous fungi are rich sources of bioactive metabolites, such as organic acids, vitamins, prebiotics, and enzymes, which can be incorporated into functional feeds to promote animal health (Wang et al., 2023; Strong et al., 2022). Fungi such as *Aspergillus* and *Trichoderma* are known for producing extracellular enzymes, antioxidants, antimicrobials, and immunomodulatory compounds, which improve animal health and feed bioavailability (Leite et al., 2019; Hölker & Lenz, 2005).

Fungi such as *Trichoderma spp., Aspergillus spp.* and *Phanerochaete spp., particularly Trichoderma reesei*, *Aspergillus niger*, and *Phanerochaete chrysosporium,* produce lignocellulolytic enzymes, such as cellulase, xylanase, and ligninase, which are essential for the degradation of lignocellulosic plant materials (López-Gómez et al., 2020). These fungi have already been shown to effectively degrade agro-industrial by-products such as fruit pulp, sugarcane bagasse, wheat residues, and soybean bagasse, producing enzymes, organic acids, antioxidants, and other products of industrial interest. Lignocellulolytic enzymes have significant industrial applications, such as biomass

biodegradation, biofuel production, and wastewater treatment (López-Gómez et al., 2020). SSF thus contributes to waste reduction in the feed industry and promotes more sustainable practices (López-Gómez et al., 2020; Leite et al., 2019).

In aquafeed production, SSF is emerging as a strategy to improve the nutritional profile of plant-based ingredients, particularly agro-industrial by-products (Amaral et al., 2023; Vieira et al., 2023; Fernandes et al., 2022; Dawood & Koshio, 2020). These by-products, typically low in protein and high in fiber, are unsuitable for carnivorous species, which have high dietary protein requirements and limited ability to digest non-starch polysaccharides (NSPs). High NSP-based diets have been associated with reduced nutrient digestibility and damage to the intestinal health of the fish (Magalhães et al., 2016; Oliva-Teles et al., 2015). The SSF process enhances the nutritional value of these by-products by degrading lignocellulosic components, improving digestibility, and increasing protein, amino acid, lipid, and micronutrient content while minimizing water usage during this process (Dias, 2016; Leite et al., 2016). Additionally, the SSF process leads to the accumulation of yeast biomass, rich in β-glucans, and the secretion of bioactive compounds, such as exoenzymes and phenolic compounds, which remain in the final product (Dias, 2016; Leite et al., 2016). These bioactive molecules may promote fish health, making SSF-treated ingredients functional feedstuffs with potential health benefits for humans if accumulated in fish fillets (Afonso et al., 2015; Ramalho Ribeiro et al., 2015). Moreover, SSF of by-products can improve the nutritional profile of the final mixture by increasing protein content while reducing fiber, lipid levels, and anti-nutritional factors such as tannins, phytic acid, saponins, and alkaloids (Manna et al., (2022).

SSF-treated agro-industrial by-products can replace traditional agricultural ingredients such as soybean, corn, and wheat in aquafeed production. These conventional ingredients are often expensive, have a high carbon footprint, and compete with human food with sustainable and undervalued alternatives. By contrast, SSF transforms low-cost, locally produced agro-industrial by-products (Estevão-Rodrigues et al., 2024; Das et al., 2022; Manna et al., 2022; Ismail et al., 2021).

Furthermore, SSF not only improves the nutritional profile of agro-industrial by-products but also enhances their digestibility (Estevão-Rodrigues et al., 2024; Das et al., 2022; Dawood & Koshio, 2020; Filipe et al., 2020; Mandal & Ghosh, 2019). SSF increases the availability of essential amino acids (EAA) and enriches the feed with bioactive compounds and enzymes (Fernandes et al., 2021; Dawood & Koshio, 2020). SSF-treated plant-based ingredients and agro-industrial by-products thus enhance their nutritional value, allowing their use as alternative feed ingredients in aquafeeds generally without compromising the growth performance, digestibility, nutritional status, and

immune health of the aquaculture fish species, particularly carnivorous species. A summary of benefits for aquaculture animals of SSF of Plant-Based Ingredients and agro-industrial by-products is presented in **Table 1. 1**.

Table 1. 1: Nutritional Effects of Solid-State Fermentation (SSF) on Plant-Based Ingredients and Agro-Industrial By-Products in Aquaculture Feeds.

microorganisms; Fermentation conditions	**Fermented substrate and replaced dietary ingredient**	**Fish species; Trophic level**	**Trial conditions**	**Results**	**Reference**
***Bacillus subtilis* sub sp. *subtilis* (JX292128) Temp.: 25-50°C; pH: 3-8; period: 48h**	Sesame oil cake ↔ Fishmeal	*Labeo rohita;* TL: 2.2 ±0.12	Growth and digestibility trial; IBW: 3.1g; 120 days feeding; Diets: 29% Prot.; 7.6% Lip	40% SSF increases weight gain, feed conversion rate, protein efficiency index, apparent net protein utilization, digestive enzyme activities, carcass composition, and digestibility of proteins, lipids, and phosphorus.	(Das & Ghosh, 2015)
***Saccharomyces cerevisiae.* Temp.: 40°C; period: 48h**	Soybean meal ↔ Fishmeal	*Oreochromis niloticus;* TL: 2.0 ±0.0	Growth and digestibility trial; IBW: 10g; 90 days feeding; Diets: 40% Prot.; 7.5% Lip	25 and 50% SSF improved the digestibility of proteins, lipids and energy. It increases the body composition of protein and ash and improves hematological parameters such as ALT and AST.	(Hassaan et al., 2015)
***Saccharomyces cerevisiae.* Temp.: 40°C; period: 48h**	Soybean meal ↔ Fishmeal	*Fenneropenaeus indicus*	Growth trial; IBW: 0.2g; 90 days feeding; Diets: 30% Prot.; 9.5% Lip	50% SSF does not compromise final body weight, weight gain, feed conversion rate, body composition of dry matter, lipids and proteins.	(Sharawy et al., 2016)
Pseudoalteromonas . Sp D11, Bacillus subtilis A142, Saccharomyces cerevisiae Y23 and Lactobacillus plantarum L54	Diet SSF↔ Diet	*Apostichopus japonicus*; TL: 2.0 ±0.00	Growth and digestibility trial; IBW: 62g; 30 days feeding; Diets: 18% Prot.	SSF diet increased weight gain, final weight gain, specific growth ratio, enzymatic activity of protease, alginase, amylase and cellulase.	(Wang et al., 2017)

Temp.: 26°C; period: 5 days					
***Aspergillus niger* (ATCC 6275) Temp.: 35°C; period: 3 days**	Groundnut oil cake ↔ Fishmeal	*Penaeus vannamei*	Growth trial; IBW: 3.0g; 45 days feeding; Diets: 37% Prot.	72.5% SSF increased feed conversion rate, feed and protein efficiency and hemolymph indices.	(Jannathulla et al., 2018)
***Pichia kudriavzevii.* Temp: 32°C, pH: 5.5, period: 15 days**	*Pistia* leaf meal ↔ Fishmeal	*Labeo rohita;* TL: 2.2 ±0.12	Growth trial; IBW: 3.3g; 54 days feeding; Diets: 42 Prot.; 11% Lip	20% SSF improved protein intake and lipid digestibility. The results were similar to those of the control diet in dry matter intake, lipase, amylase and protease enzyme activity.	(Mandal & Ghosh, 2019)
***Aspergillus niger* (ATCC 6275) Temp.: 35-37°C; pH: 6.7-7; period: 3 days**	Groundnut oil cake, rapeseed meal and sunflower oil cake ↔ Fishmeal	*Penaeus vannamei*	Growth and digestibility trial; IBW: 3.4g; 45 days feeding; Diets: 37% Prot.; 7% Lip.	70.8% SSF did not affect weight gain and diet digestibility. Increasing SSF as a substitute for fishmeal led to a decrease in protease and amylase activity, body lipid composition, total hemolymph protein, cholesterol and triglycerides.	(Dayal et al., 2020)
***Aspergillus oryzae* Temp.: 30°C; period: 2 days**	Olive cake ↔ Corn gluten meal and wheat bran	*Oreochromis niloticus;* TL: 2.0 ±0.0	Growth trial; IBW: 16.7g; 90 days feeding; Diets: 30% Prot.; 19 kJ g −1, energy	10% SSF increased weight gain, final weight gain, feed intake, mean high-density lipoprotein cholesterol and serum lysozyme level.	(Ismail et al., 2021)
***Sachharomyces cerevisiae* and *Bacillus subtilis.* Temp.: 37°C; period: 48h**	*Bassia latifolia* oil cake ↔ Soybean meal	*Labeo rohita;* TL: 2.2 ±0.12	Growth and digestibility trial; IBW: 3.1g; 120 days feeding; Diets: 29% Prot.; 7.6% Lip	20% SSF increases final body weight, weight gain and intake, digestibility (proteins, lipids and energy). It does not influence the immunological glycemic	

				parameters of bacterial agglutination, lysozyme and haemoglutination activity.	
***Aspergillus ibericus* (MUM 03.49) Temp.: 25°C; period: 7 days**	*Ulva rigida* ↔ Fishmeal	*Dicentrarchus labrax;* TL: 3.5 ±0.50	Growth trial; IBW: 106.9g; 64 days feeding; Diets: 47% Prot.; 18% Lip.	5% SSF increased feed efficiency and protein efficiency ratio, and total alkaline protease activity levels were equal to those of the control diet.	(Fernandes, et al., 2022)
***Saccharomyces cerevisiae, Candida utilis, and Yarrowia lipolytica* Temp.: 30°C; period: 9 hours**	food waste ↔ % Conventional diet	*Procambarus clarkil;* TL: 2.8 ±0.01	Growth and digestibility trial; IBW: 62g; 45 days feeding; Diets: 18% Prot.	30% SSF increased weight gain rate, special growth rate and feed conversion ratio. It increased the enzymatic activity of alkaline phosphatase, amylase, trypsin, lysozyme and superoxide dismutase.	(Li et al., 2023)
***Aspergillus niger* CECT 2088 Temp.: 25°C; period: 7 days**	A mixture of plant ingredients (rapeseed, soybean, rice bran, and sunflower seed meal mixture; equal parts) ↔ Non-fermented mixture	*Dicentrarchus labrax;* TL: 3.5 ±0.50	Growth trial; IBW: 20.25g; 42 days feeding; Diets: 42% Prot.; 18% Lip.	20% SSF was not able to mitigate the impact of environmental stress caused by salinity and temperature oxidation on physiological performance.	(Amaral et al., 2023)
***Aspergillus niger* CECT 2088 Temp.: 25°C; period: 7 days**	A mixture of plant ingredients (rapeseed, soybean, rice bran, and sunflower seed meal mixture; equal parts) ↔ Non-fermented mixture	*Dicentrarchus labrax;* TL: 3.5 ±0.50	Digestibility trial; IBW: 10g; 14 days feeding; Diets: 42% Prot.; 18% Lip.	20% SSF showed performance equivalent to that of a control diet in terms of growth, feed digestibility, digestive enzyme activity and plasma metabolites.	(Vieira et al., 2023)
***Aspergillus ibericus* (MUM 03.49)**	Brewer's spent grain ↔ Plant feedstuff mixture	*Dicentrarchus labrax;* TL: 3.5 ±0.50	Digestibility trial; IBW: 92g; 60 days feeding; Diets: 45% Prot.; 18% Lip.	20% SSF increased the digestibility of dry matter, protein, isoleucine, glutamate, lipid and energy. 10% SSF	(Estevão-Rodrigues et al., 2024)

Temp.: 25°C; period: 7 days				modulated digestive enzyme activity, reducing total protease and trypsin activities.	
***Aspergillus niger* (CECT 2915) and *Aspergillus ibericus* (MUM 03.49) Temp.: 25°C; period: 7 days**	By-products *Gelidium* sp. and sunflower cake ↔ Plant feedstuff mixture	*Dicentrarchus labrax;* TL: 3.5 ±0.50	Growth trial; IBW: 35g; 63 days feeding; Diets: 44% Prot.; 16% Lip.	10% SSF by *A. niger* negatively affected overall growth, feed intake, and utilization. Plasma metabolite levels, hepatic antioxidant enzyme activity, and lipid peroxidation were similar between diets. 10% SSF by *A. ibericus* increased feed utilization efficiency by 25%.	(Ferreira et al., 2024)

4 Brewers Spent Grain – BSG

The generation of waste and by-products is inherent to any productive sector (Georganas et al., 2023; Reguengo et al., 2022; Gómez-García et al., 2021). The growing ecological awareness that emerged at the end of the 20th century highlighted the significant challenge humanity faces: balancing economic growth, social equality, and environmental sustainability in the production of goods and services(Reguengo et al., 2022; Gómez-García et al., 2021). The agro-industrial and food sectors produce large amounts of liquid and solid wastes (Reguengo et al., 2022; Fărcaș et al., 2021; Carley & Yahng, 2018). These residues can present serious problems of final disposal and pollutant potential, in addition to often representing losses of biomass and high-value nutrients (Georganas et al., 2023; Gómez-García et al., 2021; Carley & Yahng, 2018). Unlike what happened in the past, when by-products were disposed of in landfills or used without treatment for animal feed or fertilizer, current practices emphasize minimizing, recovering, and converting by-products and waste in agro-industrial chains (Laufenberg & Schulze, 2009).

The beer industry is one of the largest global economic sectors, with China leading world production, followed by the EU. The EU industry collectively produces 34.6 billion hectoliters of beer annually (EBT, 2024). Portugal contributes 7.5 billion hectoliters of this total (EBT, 2024). However, beer production brings significant environmental challenges due to the generation of millions of tons of by-products, which raises ecological concerns (Fărcaș et al., 2021; Carley & Yahng, 2018).

In beer production, cereals go through a milling stage and are mixed with water to form a homogeneous paste. This mixture is gradually heated until it reaches 78 °C, the ideal temperature for the natural enzymes presented in the grain to hydrolyze the endosperm, breaking down complex carbohydrates into fermentable sugars and releasing important nutrients for fermentation (Mussatto et al., 2006). The liquid portion, known as wort, is separated from the brewer's spent grain (BSG) through filtration and washing. During filtration, the mixture of wort and BSG passes through a bed of crushed malt or other filtering material, which allows the liquid to drain, retaining the solid material (**Figure 4**) (Buffington, 2014). To maximize the extraction of residual sugars, the solid BSG is subjected to successive washing and draining. The washing water containing the extracted sugars is then added to the wort, improving sugar recovery efficiency(Agrawal et al., 2023). The wort is subsequently transferred to the next stage of beer production: boiling. At this stage, hops and other ingredients are added before fermentation.

Meanwhile, the BSG, a by-product of the process, is dried and stored for potential further use (Agrawal et al., 2023).

Figure 4: Brewer's spent grain from Unicer (Matosinhos, Portugal) after the drying process. Source: Personal collection.

The beer production process consumes large volumes of water, generating effluents rich in organic matter, suspended solids, and nutrients, which require treatment before being discarded (Carley & Yahng, 2018). However, the largest residue generated by this industry is solids, which include sediments formed during the wort boiling and cooling processes, containing hop remains, coagulated proteins, and fine particles (Carley & Yahng, 2018). The most abundant by-product generated by the beer industry is BSG, representing approximately 85% of the total solid waste produced (Mussatto, 2009). It corresponds to approximately 31% of the original weight of the cereals used and generates, on average, 20 kilograms of waste for every 100 liters of beer produced (Mussatto, 2009). The second most abundant waste is residual yeast, composed of spent yeast cells that can no longer be reused in fermentation processes (Carley & Yahng, 2018).

BSG comprises 12–33% cellulose and 19–42% hemicellulose (Lynch et al., 2016), with up to 40% arabinoxylan in dry matter (Lynch et al., 2016). In addition, BSG contains approximately 45–49% carbon, reflecting its composition rich in organic compounds (Lynch et al., 2016). Currently, the use of BSG as a feedstock is limited, mainly as animal feed, landfill (Westendorf & Wohlt, 2002), and, more recently, bioethanol (Bianco et al.,

2020). Regarding animal feed, BSG is a good ingredient for ruminant feed (Gupta et al., 2010). Besides its role as an animal feed by-product, some components of BSG are also considered precursors for food-grade chemicals or energy sources in microbial fermentations (Gupta et al., 2010). Energy recovery from BSG has been considered feasible through thermochemical and biochemical processes, but most studies have focused mainly on bioethanol rather than other uses (Chattaraj et al., 2024; Gómez et al., 2010). The released sugars can be microbially converted into several products, such as organic acids, ethanol, glycerol, food additives, and butanol (Puligundla & Mok, 2021; Mussatto & Teixeira, 2010). In particular, lactic acid has applications in food, fermentation, pharmaceuticals, and chemicals (Naibaho et al., 2024).

In aquafeed, studies on using BSG have gained attention as a sustainable alternative to replace traditional ingredients, offering the potential to reduce costs and add value to the beer industry by-products (Karlsen & Skov, 2022; Jayant et al., 2018). Studies indicate that for striped catfish (*Pangasianodon hypophthalmus*) fingerlings, replacing 50% of soybean meal with raw BSG, in addition to not compromising growth performance compared to a control diet, reduces feed costs by 27.6% (Jayant et al., 2018). In contrast, for Nile tilapia (*Oreochromis niloticus*) and channel catfish (*Ictalurus punctatus*), it has been shown that replacing 27% of soybean meal and wheat bran with BSG supplemented with 0.02% of a commercial enzyme additive (Allzyme™) resulted in negative impacts on growth performance and body composition (Tidwell et al., 2023). Although omnivorous species have higher dietary flexibility than carnivorous species, which makes them more tolerant to the inclusion of plant-based ingredients in their diets, high lignocellulosic ingredients may reduce the efficiency of nutrient absorption (Erasmus, 2009).

For species with carnivorous feeding habits, such as rainbow trout and gilthead sea bream, dietary inclusion of 20-30% BSG after prior enzymatic hydrolysis in place of fishmeal resulted in growth performances similar to that of a control diet and did not negatively interfere with the digestibility (Nazzaro et al., 2021). Furthermore, BSG extracts processed by SSF incorporated into the diet of European sea bass improved diet digestibility, growth performance, and antioxidant defenses (Fernandes et al., 2022; Fernandes et al., 2021). Thus, the use of BSG in fish diets is directly related to the application of bioprocesses that aim to reduce the presence of antinutritional factors, increase the bioavailability of nutrients, and improve the digestibility of BSG (Estevão-Rodrigues et al., 2024; Fernandes, 2022; Nazzaro et al., 2021; San Martin et al., 2020).

5 European sea bass (*Dicentrarchus labrax* Linnaeus 1758, Moronidae)

5.1 Biology of the Species

European sea bass (*Dicentrarchus labrax*) is a euryhaline and eurythermal species found in the Northeast Atlantic, Mediterranean, and Black Seas coasts (Vandeputte et al., 2019; Kousoulaki et al., 2015) (**Figure 5**). Although primarily marine, after spawning in winter in deeper coastal waters, the larvae are transported by currents to estuarine areas and lagoons, which serve as nursery areas (Pontual et al., 2023). These areas offer favorable conditions, such as higher temperatures, abundant food availability, and protection from predators, essential for the growth and survival of the juveniles (Pontual et al., 2023). Juveniles remain in these habitats until they reach a size that allows them to return to coastal and marine waters, usually around the first or second year of life (Pontual et al., 2023).

Figure 5: European sea bass (*Dicentrarchus labrax*). Source: Personal collection

Adults can reach up to 1 m in length and weigh up to 12 kg in their natural environment, although smaller specimens are more common (Goossens et al., 2024). They have an elongated, fusiform body with a silvery coloration on the belly and grey or greenish on the back (Pickett & Pawson, 1994). The terminal mouth and slightly prominent lower jaw are adaptations for capturing prey in the water column, aligning with their carnivorous diet of small fish, crustaceans, and mollusks (Pickett & Pawson, 1994). Distinctive features include separate dorsal fins, the first being spiny and the second with soft rays (Pickett & Pawson, 1994). Their physiological adaptability to a wide range of salinities (0-40 ppt salinity) and temperature (2 – 32 °C) grants them significant ecological plasticity (Pontual et al., 2023; Vandeputte et al., 2019). Typically, they inhabit waters with

temperatures between 10 °C and 25 °C and depths of up to 100 meters, with preferences for shallower areas (Pontual et al., 2023; Vandeputte et al., 2019).

5.2 Aquaculture Production

There are historical records of extensive European sea bass (*Dicentrarchus labrax*) farming dating back to the Roman era, around the 1st century BC, in coastal ponds and lagoons across the Mediterranean region.

During the Middle Ages, these practices persisted and became well established in Valli (Italy), Esteros (Spain), and Salinas (France). The extensive farming system remained largely unchanged for centuries until it underwent modernization in the 20th century, coinciding with the development of intensive aquaculture, particularly from the 1970s onwards (Kousoulaki et al., 2015). Initially, production methods involved capture and fattening in farms, but advances in captive breeding and the development of specific diets allowed the expansion of its production. Since then, European sea bass has become one of European aquaculture's main marine fish species. Indeed, today, European sea bass production is one of the pillars of Mediterranean aquaculture, standing out as one of the most produced and highly valued species (Vandeputte et al., 2019; Kousoulaki et al., 2015). The leading producers include Greece, Turkey, Spain, Italy, and France, benefiting from favorable environmental conditions, including temperate waters and a long tradition in marine aquaculture (FAO, 2024a). European sea bass is the fourth most produced species in Europe. In 2022, its production reached 23.7 tonnes, valued at 1.57 billion euros, with Turkey and Greece contributing around 69% of this output (EuroStat, 2024) (**Figures 6 and 7**).

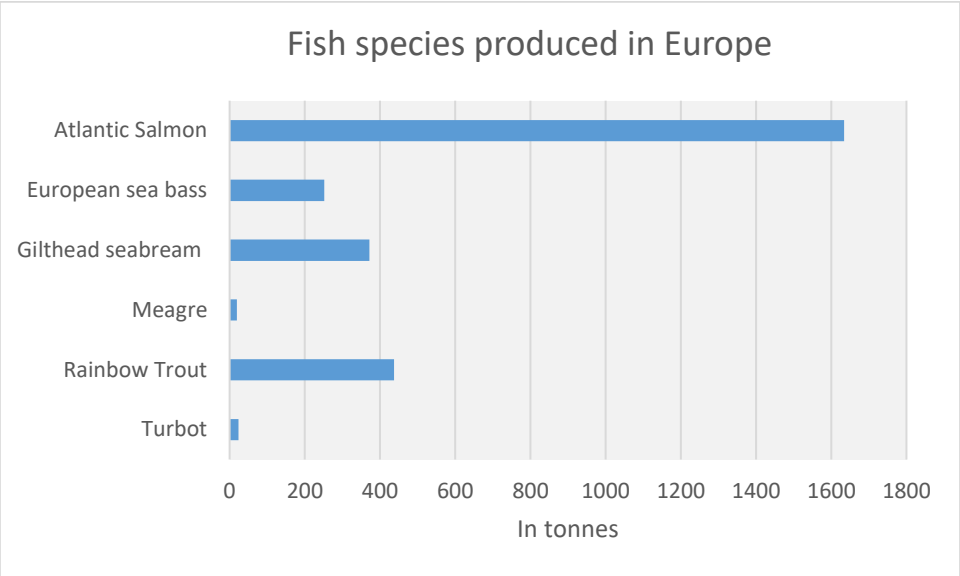

Figure 6: Total aquaculture production of farmed fish species in Europe in 2022, adapted from (EuroStat, 2024).

In 2022, Atlantic salmon and rainbow trout dominated European aquaculture production by volume. Despite lower production volumes, European sea bass accounted for a 10.1% market share, accounting for €1.57 billion in 2022 (EuroStat, 2024). This difference reflects market strategies and aquaculture systems adapted to species characteristics and market preferences.

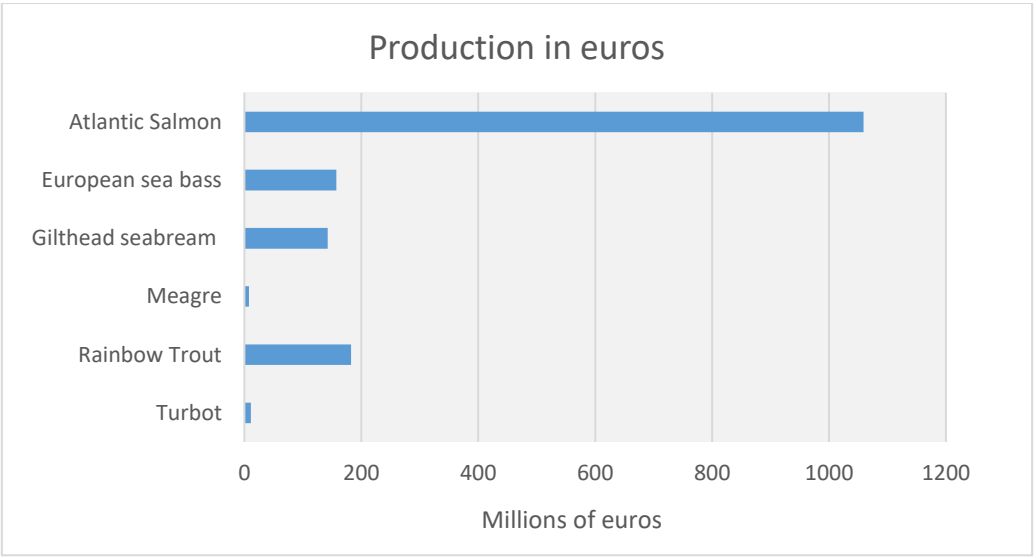

Figure 7: Total aquaculture production in euros of farmed fish species in Europe in 2022, adapted from (EuroStat, 2024).

European sea bass is associated with a refined flavor, firm texture, and high culinary versatility, positioning it as a premium fish in international markets (Pérez-Lloréns et al., 2021). In addition, its adaptability to intensive and semi-intensive farming systems, combined with a known and well-studied life cycle, favors its large-scale production (Vandeputte et al., 2019). The species also play an important role in strengthening coastal economies and creating jobs in producing regions (FAO, 2024a).

5.3 Nutritional requirements

As a carnivorous fish occupying a trophic position around 3.5±0.5 (Fishbase), European sea bass requires a high dietary protein content (Kousoulaki et al., 2015). Although primary studies have determined the protein requirements of European sea bass at 52-60% (Alliot et al., 1973; Metailler et al., 1981), more recent studies estimated requirement at around 43% to 48%, being higher in the early growth phases (Peres & Oliva-Teles, 1999; Dias et al., 1998). Optimum dietary protein requirements do not appear to be affected by water temperature (Peres & Oliva-Teles, 1999). The optimum dietary digestible protein-to-digestible energy ratio (DP/DE) for achieving maximum growth and feed utilization was initially estimated at approximately 24-27 mg/kJ (García Gallego et al., 1994; Pérez et al., 1997). However, subsequent studies proposed a lower DP/DE ratio of around 21–22 mg/kJ (Dias et al., 1998; Lanari et al., 1999; Peres and Oliva-Teles, 1999).

Although protein requirements are often considered when determining nutrient requirements, fish do not have specific protein requirements; they require amino acids (Oliva-Teles et al., 2024). European sea bass requires 10 EAA, as do other species (Oliva-Teles et al., 2024). Requirements have been estimated by dose-response studies using semi-purified diets, including lysine (4.8% protein) (Tibaldi et al., 1994); methionine and cysteine (4.0% protein) (Tibaldi et al., 1994), arginine (2.0–4.6% protein) (Azeredo et al., 2015); threonine (2.6–3.0% protein) (Tibaldi & Tulli, 1999); tryptophan (0.5–0.7% protein) (Tibaldi et al., 1994). Additionally, the requirements for all EAA were determined using the ideal protein method to be (as % protein): methionine + cystine, 4.4; arginine, 4.1; lysine, 4.8; threonine, 2.7; tryptophan, 0.6; histidine, 1.6; isoleucine, 2.6; leucine, 4.3; phenylalanine + tyrosine, 2.6 (Kaushik et al., 1998).

Fish preferentially use protein over lipids or carbohydrates as an energy source. Therefore, improving the utilization of protein for tissue synthesis rather than for energy purposes is important from a nutritional, environmental, and economic point of view (Peres & Oliva-Teles, 1999). Earlier studies reported that optimal dietary lipid levels range between 12-20% (Lanari et al., 1999; Peres & Oliva-Teles, 1999). Diets containing

12% to 24% of lipid levels have not been shown to enhance growth or feed utilization efficiency in European sea bass but were associated with increased energy utilization and lipid deposition in body tissues (Peres & Oliva-Teles, 1999). However, lipid levels of around 30% were reported to reduce protein and energy retention (Peres & Oliva-Teles, 1999). As a marine species, the European sea bass has a limited capacity to endogenously synthesize long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (ARA), from their precursors (C18-PUFA). This limitation is due to the reduced activity of specific elongases and desaturases (Tocher, 2015). Consequently, European seabass has a dietary requirement for EPA and DHA, estimated at approximately 0.7% of the diet, with an optimal DHA: EPA ratio of 1.5:1 (Skalli & Robin, 2004). ARA levels below 0.2% of the diet were reported to be detrimental to fish growth (Skalli & Robin, 2004; Torrecillas et al., 2018) but not survival; higher ARA levels (up to 6% of total fatty acids) improved tissue fatty acid composition (Torrecillas et al., 2018).

Carbohydrates are not an essential nutrient for European sea bass but serve as an energy source. However, European sea bass cannot digest non-starch polysaccharides (Enes et al., 2011). The detrimental effects of excessive dietary incorporation of complex polysaccharides on nutrient digestibility in the diet of European sea bass are well documented (Fountoulaki et al., 2022; Magalhães et al., 2015; Enes et al., 2011; Tibaldi et al., 2006).

European sea bass also has a limited capacity to digest starch, particularly its raw form (Kamalam et al., 2017). Digestibility depends on the inclusion level and water temperature, increasing with higher temperatures but decreasing as the inclusion level rises (Moreira et al., 2008). Moreover, the botanical origin of carbohydrates also affects digestibility (Enes et al., 2011; Moreira et al., 2008). Studies have shown that diets containing 25% gelatinized starch reduce feed intake and growth in European sea bass, indicating that dietary digestible carbohydrates should not exceed 20% (Enes et al., 2011; Moreira et al., 2008).

Minerals are fundamental in several biological processes, such as growth, bone development, enzymatic functions, and electrolyte balance. However, information on the mineral requirements of European sea bass is limited to phosphorus. Phosphorus is essential for metabolic processes such as protein and lipid synthesis, bone formation, and ATP production, the primary source of cellular energy. An optimum dietary phosphorus level of 0.65% diet was estimated to be required to optimize the growth of European sea bass (Oliva-Teles & Pimentel-Rodrigues, 2004). Dietary phosphorus in

phytate form is not digested by European sea bass due to the absence of the enzyme phytase (Oliva-Teles et al., 1998).

The inclusion of vitamins in the diet of European sea bass can also reflect on animal health and growth performance. As for minerals, available information on vitamin requirements is limited. The dietary requirement for vitamin C (ascorbic acid) for European sea bass has been estimated to be 5 mg/kg of diet to optimize growth. To achieve a high concentration of ascorbic acid in muscle tissue, including 31 mg/kg in the diet is necessary. The recommended level increases to 121 mg/kg in the diet to maximize ascorbic acid storage in the liver. These values reflect the importance of vitamin C in growth and supporting crucial metabolic and antioxidant functions in fish tissues (Fournier et al., 2000). Darias et al. (2010) observed that dietary inclusion of vitamin D (up to 19.2 IU/ g diet) was required to support optimal morphological development of European sea bass larvae. For vitamin A, a dietary level of 31 mg/ kg diet improved the growth of European sea bass larvae and decreased malformation development (Villeneuve et al., 2005). Furthermore, vitamin A levels between 5 and 10 mg/kg were more effective in improving growth performance (Villeneuve et al., 2005), while higher levels, up to 25 mg/kg diet, enhanced the survival rates for European sea bass (Mazurais et al., 2007).

6 Meagre (*Argyrosomus regius* Asso, 1801)

6.1 Biology of the Species

Meagre (*Argyrosomus regius*) is a marine fish species widely distributed in temperate and subtropical waters of the Eastern Atlantic, ranging from the Bay of Biscay to Senegal, including the Mediterranean Sea and, occasionally, the Black Sea (Quero & Vayne, 1985; Champagnat et al., 1979). Meagre inhabits coastal and estuarine areas, is found at depths ranging from 15 to 300 meters, and prefers sandy or muddy substrates. (Kružić et al., 2016; Quero & Vayne, 1985).

Meagre has an elongated and slightly compressed body with a bright silvery coloration and a clearly visible lateral line that extends to the caudal peduncle (**Figure 8**). Adults can reach up to 2 meters in length and weigh about 50 kg, although smaller specimens are more common (Quero & Vayne, 1985). The lower jaw is slightly projected, a common characteristic in predators that feed on marine and estuarine substrates (Quero & Vayne, 1985).

Figure 8: Meagre (*Argyrosomus regius*). Source: Internal collection of the Instituto Português do Mar e da Atmosfera

Meagre a is a benthopelagic species with predominantly carnivorous feeding habits, feeding on crustaceans, cephalopods, and other fish (Almeida et al., 2022; Quero & Vayne, 1985; Champagnat et al., 1979). It has broad thermal preferences, with ideal growth at temperatures close to 26°C (Quero & Vayne, 1985). Spawning occurs in brackish or marine waters during the warmer months, with adults migrating to specific areas for reproduction (Champagnat et al., 1979). Juveniles live in estuaries until adulthood (Quero & Vayne, 1985).

6.2 Aquaculture Production

Meagre aquaculture production began in 1990 in southern France (Camargue, Cannes, and Corsica) and Italy (La Spezia and Orbetello) (GFCM, 2009). Meagre is produced predominantly on the Mediterranean coast in Greece, Spain, Croatia, and Portugal. The largest producers in the European Union are Spain, France, and Greece (FAO, 2024a). Land-based tanks and sea cages are the most widely used farming systems (EUMOFA, 2022).

Meagre production has grown significantly in Europe, especially in Spain, Portugal, France, Italy, and Greece. In fact, in less than 10 years, meagre production has increased by 722% in volume and 867% in value (EuroStat, 2024) (**Table 1. 2**). Approximately 80% of the meagre consumed in Europe comes from aquaculture (FAO, 2024).

Table 1. 2: Meagre farm production in Europe, according to data published by Eurostat (2024).

	2013	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**	**2021**	**2022**
Volume (tons)	144	589	587	696	621	831	1176	1682	1125	1188

Value (millions €)	8.3	31.0	31.1	40.2	32.9	45.2	59.4	79.2	58.6	79.8

Characterized by fast growth, high-quality meat rich in LC-PUFA (Pfalzgraff et al., 2023), and well-established farming methods, meagre has a high potential to diversify European aquaculture (Konstantinidis et al., 2021). However, despite significant advances in aquaculture technology development, compared to other fish species, meagre production in Europe remains limited (FAO, 2024a). Some challenges include low penetration in the consumer market, negligible diversification of farming localization, high production costs associated with high nutritional requirements, and technical difficulties since meagre production in captivity is still a relatively new process compared to the cultivation of other exploited fish species (EUMOFA, 2022; Kružić et al., 2016). In addition, insufficient marketing strategies and labeling initiatives have not yet been widely implemented (Newton et al., 2008). Overcoming these limitations depends on investments in applied research, more significant efforts to promote the product and expansion of the production infrastructure, consolidating meagre as an economically viable and sustainable species for European aquaculture (Konstantinidis et al., 2021; EUMOFA, 2022).

6.3 Nutritional requirements

Although commercial diets specifically designed for meagre are currently available, diets formulated for sea bream and European sea bass have also been used, given the approximated nutritional requirements of these species (Chatzifotis et al., 2012; Duncan et al., 2012). Initially, meagre diets were predominantly based on fishmeal and fish oil (FAO, 2010). However, with the increasing emphasis on aquaculture sustainability, alternative ingredients such as plant proteins and non-marine lipids have been explored to reduce reliance on traditional ingredients (Carvalho et al., 2019). Meagre has an efficient feed utilization, and diets are designed to optimize protein and energy retention (Carvalho et al., 2019; Pfalzgraff et al., 2023)

As a carnivorous fish with a high trophic level 4 (FishBase), meagre has a high need for dietary protein, with an optimal level between 43-47% (Jauralde et al., 2021; Fountoulaki et al., 2017). However, studies on EAA requirements are limited. Methionine requirement was estimated to be 0.1-1.5% diet (Moura et al., 2018), histidine 3% diet (Saavedra et al., 2020), and tryptophan 0.5-0.8% diet (Teixeira et al., 2023; Gonzalez-Silvera et al., 2018). High dietary tryptophan levels, up to 1% of the diet, have been reported to

decrease growth and feed utilization (Teixeira et al., 2023). A dietary phenylalanine level of 5% was shown to be an effective strategy to enhance stress response (Salamanca et al., 2021). Although not an amino acid, taurine is essential for lipid metabolism, calcium regulation, immune function, and protection against stress, and the optimum taurine level for meagre is 0.5-1% diet (Moura et al., 2018).

The optimum dietary lipid levels for meagre are between 12-17% (Matulić et al., 2024). The essential fatty acids (EPA + DHA + ARA) requirement has been estimated to be 0.7-2% of the diet (Pfalzgraff et al., 2023; Carvalho et al., 2019). It is recommended to include in the diets 1-2% EPA and DHA (Pfalzgraff et al., 2023) and 0.8% ARA (Khalil et al., 2018) to maximize growth performance and liver health,

Meagre, has low efficiency in utilizing dietary carbohydrates (Adebowale et al., 2019; Castro, Corraze, et al., 2015). Dietary carbohydrate levels above 20% lead to increased hepatic lipogenesis, lipid accumulation in the liver, and alterations in the intestinal microbiota (Zheng et al., 2023). However, it was reported that juvenile meagre fed a diet containing 23.5% carbohydrates did not experience compromised growth performance or feed utilization, suggesting that at this level, carbohydrates can be effectively used when balanced with adequate lipids and protein sources and levels (Chatzifotis et al., 2010). (Oliva-Teles et al., 2015a; Peres & Oliva-Teles, 1999). Since fish cannot digest complex and fibrous carbohydrates, such as cellulose and hemicellulose, the amount of crude fiber in meagre diets should be less than 7% of the diet to limit the amount of undigested material entering the culture system (Kamalam et al., 2017).

Studies indicate that DL-α-tocopherol, or vitamin E, when included in diets at 300 and 450 mg/kg (Ruiz et al., 2019; Lozano et al., 2017), ascorbic acid at 50 to 210 mg/kg, and menaquinone at 23 mg/kg improved meagre growth performance, filet quality, antioxidant defense, body composition, and liver health (Ruiz et al., 2019). Other studies suggest that supplementing with 1200 to 3200mg/kg of ascorbic acid, in combination with minerals such as manganese (40 mg/kg), zinc (200 mg/kg), and selenium (1.5 mg/kg), helped to reduce lipid peroxidation, and indicated that deficiency of antioxidant nutrients, especially vitamin C, is strongly related to the development of systemic granulomatosis (Ruiz et al., 2021). Diets rich in fishmeal (60%) and with adequate levels of phosphorus (15 g/kg) can also mitigate the occurrence of systemic granulomatosis (Tsertou et al., 2020).

7 Objectives

To increase aquaculture circularity, it is of utmost importance to explore alternative ingredients to traditional and imported agricultural products, such as soybean meal, by incorporating locally available, low-economic value agro-industrial by-products. However, these by-products' low protein and high fiber content limit their dietary inclusion, particularly in carnivorous fish species like European seabass and meagre. Therefore, the technological improvement of agro-industrial by-products using green technologies may prove an important strategy for valorization as novel aquafeed ingredients within a circular economy approach. Within this context, SSF technology appears as an innovative strategy for upgrading the nutritional value of agro-industrial by-products. SSF is an eco-friendly process with low energy and water consumption that, through the growth of specific microorganisms (bacteria or fungi), may convert agro-industrial by-products into new ingredients enriched with microbial biomass and bioactive compounds produced during the fungi growth. Brewer's spent grain (BSG) is the most abundant by-product generated in the beer industry. It is estimated that for every 100 liters of beer produced, approximately 20 kg of BSG are generated, meaning that approximately 69.2 million tons of BSG were produced in 2023 (EBT, 2024). SSF of BSG may increase its nutritional value by enriching it with nutrients and functional properties that promote fish growth performance and feed utilization.

Thus, this thesis aimed to apply SSF with *Aspergillus ibericus* to BSG to develop novel low-carbon footprint ingredients to be used as alternatives to traditional agricultural ingredients for aquafeeds, using European sea bass and meagre as biological models. These species were chosen for being carnivorous and relevant species in Mediterranean aquaculture.

In chapter two, a characterization of non-fermented and fermented BSG in terms of protein, lipids, energy, cellulose, hemicellulose, and lignin was carried out. In addition, the enzymatic activity of cellulase and hemicellulase and total phenolics and antioxidant compounds were determined. The study then evaluated the effectiveness of replacing 10% and 20% of a vegetable ingredients mix in a control practical diet with non-fermented and fermented BSG. The effects of the diets on the apparent digestibility of nutrients and energy, digestive enzyme activity, and intestinal histology of juvenile European sea bass were tested.

In chapter three, the impact of the same diets was evaluated in terms of growth performance, feed utilization efficiency, body composition, plasma metabolites, hepatic activities of enzymes of intermediary metabolism, and intestinal and hepatic digestive and antioxidant enzymes activity and lipid peroxidation of juvenile European sea bass.

In chapter four, the effectiveness of incorporating 10% of fermented and non-fermented BSG in meagre diets was evaluated. The study assessed growth performance, carcass composition, plasma metabolites, the activity of digestive enzymes, the hepatic activity of enzymes of intermediary metabolism, hepatic and intestinal antioxidant enzymes activity, and lipid peroxidation.

Finally, chapter five provides a general discussion of the results obtained, a conclusion on the relevance of fermented BSG as a new strategy for ingredient valorization for aquafeeds, and future research perspectives.

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Chapter 2:

Effect of Solid-State Fermentation of Brewer's Spent Grain on Digestibility and Digestive Function of European seabass (*Dicentrarchus labrax*) Juveniles

Estevão-Rodrigues, T., Fernandes, H., Moutinho, S., Filipe, D., Fontinha, F., Magalhães, R., Couto, A., Ferreira, M., Gamboa, M., Castro, C., Belo, I., Salgado, J., Oliva-teles, A., Peres, H.

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Effect of Solid-State Fermentation of Brewer's Spent Grain on Digestibility and Digestive Function of European Seabass (*Dicentrarchus labrax)* Juveniles

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Abstract: This study assessed the effects of dietary inclusion of solid-state fermented (SSF) brewer's spent grain (BSG) with *Aspergillus ibericus* on nutrient and energy digestibility, digestive enzyme activity, and intestinal histomorphology of juvenile European seabass (*Dicentrarchus labrax*). Five diets (18 % crude lipids and 45 % crude protein) were formulated, including a control diet (without BSG), two diets with 10 % and 20 % unfermented BSG (10BSG and 20BSG), and two other diets with 10 % and 20 % fermented BSG (10BSG-SSF and 20BSG-SSF). SSF affected the BSG's nutritional composition, including a 21 % increase in protein content and reductions in lipid (49 %), cellulose (30 %), hemicellulose (34 %), and lignin (7.3 %) content. Antioxidant activity and total phenolic compounds were minimal before SSF but significantly increased after SSF. Dietary incorporation of 20 % BSG-SSF increased the digestibility of dry matter ($p<0.01$), protein ($p=0.03$), isoleucine ($p=0.03$), glutamate ($p=0.02$), lipids ($p=0.04$) and energy ($p=0.04$) compared to the 20BSG diet. Moreover, SSF also modulated digestive enzyme activity, reducing total protease ($p=0.03$) and trypsin ($p=0.01$) activities in fish fed the 10BSG-SSF diet compared to the control diet. Fish fed the 20BSG diet showed changes in intestinal histomorphology compared to the control diet, and the SSF appeared to mitigate these effects.

Overall, these results indicate that SSF is a promising technique for enhancing the nutritional quality of low-value agro-industrial by-products, such as BSG, increasing their potential use as feed ingredients and contributing to reducing the climate and environmental footprint of aquaculture production.

Keywords: agro-industrial by-products, circular economy, digestibility, histomorphology, treatment solid-state fermentation

1 Introduction

The world population is expected to surpass nine billion people by 2050, which will demand a 70 % increase in food production systems (FAO, 2022). To meet this demand, aquaculture production must also increase, aligning with the sustainable food production practices and food security objectives outlined in the United Nations' Sustainable Development Goals (FAO, 2022; Charles et al., 2010). For this purpose, aquaculture production needs to reduce the traditional ingredients in aquafeeds, such as fisheries and agricultural feedstuffs, which compete directly with human consumption, are expensive, and have a high carbon footprint (Colombo et al., 2022). In this context, low-cost agro-industrial by-products can be viable alternatives due to their abundant availability, minimal use in human consumption, and low carbon footprint (Berbel and Posadillo, 2018). Furthermore, these biomasses are often discarded, directly causing adverse environmental impacts. Therefore, the valorization of agro-industrial by-products as feedstuffs would simultaneously benefit the aquaculture industry and ecosystem integrity based on an eco-friendly and circular economic framework (Fernandes et al., 2021; Filipe et al., 2020; Diogenes, et al., 2018; Obirikorang et al., 2015). Brewer's spent grain (BSG) is the main by-product of the brewing industry, accounting for approximately 80 %–85 % of the total solid waste produced (Mussatto, 2014; Mussatto et al., 2006). In 2021, the EU produced approximately 32 billion liters of beer, generating over 6 billion tons of BSG (Eurostat, 2021). In addition to direct discarding, BSG is often used as feed for ruminants, but its further utilization is limited by its high moisture and sugar content, which hinders its transport and storage (Lao et al., 2020; Westendorf and Wohlt, 2002). Nonetheless, the valorization of BSG is particularly compelling, showing the potential to yield a diverse array of compounds, such as enzymes (Liguori et al., 2015), phenolic compounds (Leite et al., 2019), lactic acid (Mussatto, 2014), and ethanol (Parchami et al., 2022). Despite its potential, the exploration of BSG nutritional improvement and its application in animal feeds, including aquatic feeds, has been limited. In aquaculture, studies using BSG as a substitute for fish meal or agricultural feedstuffs have only been conducted in omnivorous species, such as carp (*Cyprinus carpio* L.) (Morgan et al., 2013) and Nile tilapia (*Oreochromis niloticus*) (Tidwell et al., 2023). The high fiber (up to 50 % DM) and moderate protein content (between 20 % and 30 % DM) limit BSG inclusion in diets for carnivorous fish (Nazzaro et al., 2021; San Martin et al., 2020). Similar to other animals, fish lack intestinal enzymes capable of degrading non-starch polysaccharides (NSP), hindering the dietary inclusion of high levels of fiber-rich ingredients because they may negatively affect

growth, health, nutrient digestion, and metabolism (Dawood and Koshio, 2020; Glencross et al., 2020; Kokou and Fountoulaki, 2018; Oliva-Teles et al., 2015; Sinha et al., 2011). Biotechnological approaches can be used to improve the nutritional value of BSG and increase its potential for use in aquafeeds. However, including bioprocessed BSG in aquafeeds has yielded mixed results. For example, dietary inclusion of enzymatically hydrolyzed BSG decreased lipids and protein weight gain and digestibility in rainbow trout (*Oncorhynchus mykiss*) (Estévez et al., 2022). Similarly, in sea bream (*Sparus aurata*), dietary replacement of fish meal with 20 % dry or hydrolyzed BSG reduced weight gain and feed efficiency (Estévez et al., 2021). In contrast, including up to 20–30 % enzymatically treated BSG in diets for rainbow trout (*Oncorhynchus mykiss*) did not affect protein digestibility, growth, and feed efficiency (Nazzaro et al., 2021). Innovative microbial fermentation techniques are gaining attention as strategies for increasing the nutritional value of agroindustrial by-products with minimal environmental impact. Solid-state fermentation (SSF) involves the growth of selected microorganisms in a solid matrix that functions as a nutritional and physical support (Bhargav et al., 2008). SSF requires low energy and water volumes and usually uses low-cost and abundant by-products as fermentative substrates, decreasing operating costs and environmental impact and avoiding the formation of toxic compounds (Soccol et al., 2017). Fungi, in particular, are commonly used in SSF due to their ability to produce a wide range of enzymes that degrade the complex cell wall polysaccharides of agro-industrial by-products, such as BSG (Bhargav et al., 2008). The efficiency of SSF of BSG by different fungi, e.g. *Mortierella alpina*; *Rhizopus oryzae*, *Aspergillus niger* and *Aspergillus ibericus*, to enhance its nutritional value and produce fungal enzymes has been demonstrated through the degradation of the lignocellulosic matrix and the production of highly active lignocellulosic enzymes, particularly with the *Aspergillus ibericus* (Jacobs et al., 2009; Leite et al., 2019; Chin et al., 2022). While solid-state fermentation (SSF) has been employed to yield diverse bioactive compounds from agro-industrial by-products (Fernandes et al., 2021; Salgado et al., 2015; Filipe et al., 2020), there is currently a lack of studies assessing the effectiveness of SSF in enhancing the BSG nutritional value for fish nutrition. Therefore, this study aimed to evaluate 1) the nutritional changes in BSG after SSF with *Aspergillus ibericus* and 2) compare the effects of dietary inclusion of non-fermented and SSFe d BSG on diet digestibility, intestinal function, and intestinal histomorphology of European seabass (*Dicentrarchus labrax*) juveniles.

2 Materials and Methods

2.1 Solid-state fermentation

The brewer's spent grain (BSG) was supplied by Unicer-Bebidas de Portugal, SA (Matosinhos, Portugal). BSG was composed of barley, Pilsen malt, and corn Gritz and was obtained after the raw material filtration process. *Aspergillus ibericus* MUM 03.49 was used in the solid-state fermentation (SSF) of BSG and was supplied by Micoteca of the University of Minho (Braga, Portugal). The details of the optimization of the SSF of BSG are described in Fernandes et al. (2022). Briefly, prior to SSF, BSG was sterilized (121 ◦C, 15 min), and the moisture content was adjusted to 75 % w/w (wet basis) with sterile distilled water. Then, 400 g of BSG (dry weight) was placed in each previously sterilized (121 ◦C, 15 min) fermentation tray and inoculated with 2 mL of spore solution at a concentration of 10^6 cells mL− [1]. The trays were incubated at 25 ◦C for 7 days and manually stirred every day. At the end of the SSF process, the fermented BSG (BSG-SSF) was pooled and stored at 4 ◦C until the test diets were produced. For analytical purposes, samples from 3 different trays were randomly collected to characterize the BSG-SSF (**Table 2.1**).

Table 2. 1. Composition of brewer's spent grain before (non-fermented BSG) and after seven days of solid-state fermentation with *Aspergillus ibericus* MUM 03.49 (BSG-SSF).

Dry matter basis	**Non-Fermented BSG**	**BSG-SSF**
Crude Protein (%)	26.7 ± 0.5	32.3 ± 0.2
Crude Lipids (%)	5.7 ± 0.04	2.8 ± 0.0
Klason lignin (%)	15.0 ± 0.4	13.9 ± 0.2
Cellulose (%)	21.1 ± 1.0	14.8 ± 0.1
Hemicellulose (%)	23.7 ± 1.4	15.7 ± 0.1
DPPH (µmol trolox equivalents g^{-1})	0.6±0.0	64.2 ± 5.2
Total phenolics (mg gallic acid equivalents g^{-1})	0.6 ± 0.1	11.8 ± 0.2
Cellulase (U g^{-1})	—	925.1 ± 46.3
Xylanase (U g^{-1})	—	67.7 ± 24.1

Values are presented as mean (n=3) ± standard deviation.

2.2 Experimental diets

A control diet (45 % crude protein, 18 % crude lipids) was formulated to contain 15 % fish meal and 65.8 % plant feedstuffs. Four other diets were formulated similar to the control diet but partially replacing the plant feedstuff mixture with 10 % or 20 % of unfermented (10BSG and 20BSG diets) or BSG-SSF (10BSG-SSF and 20BSG-SSF diets) products. Chromium oxide was used as a digestibility marker and added to the

diets at 1 %. The ingredients were finely ground, thoroughly mixed, and pelleted using a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA) through a 2 mm die. The diets were placed on trays and dried in an oven at 60 ◦C for 24 h. The ingredients and proximal composition of the experimental diets are presented in **Table 2.2**, and the amino acid composition is shown in **Table 2.2**.

Table 2. 2. Ingredients and proximal compositions of experimental diets.

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF
Ingredient (% dry matter)					
BSG[1]	—	10	—	20	—
BSG-SSF[2]	—	—	10	—	20
Fish meal[3]	15	15	15	15	15
Pea protein concentrate[4]	10	10	10	10	10
Corn gluten[5]	12.5	12.5	12.5	12.5	12.5
Soybean meal[6]	17.6	15	15	10	10
Wheat gluten[7]	11.7	4.6	4.6	—	—
Rapeseed meal[8]	7.5	7.0	7.0	5.0	5.0
Sunflower meal[9]	5.6	5.3	5.3	3.8	3.8
Hemoglobin[10]	—	—	—	2.2	2.2
Hydrolyzed shrimp[11]	1.2	1.2	1.2	1.2	1.2
Fish oil[12]	14	14.4	14.4	14.9	14.9
Vitamin premix[13]	1	1	1	1	1
Choline chloride (50%)	0.5	0.5	0.5	0.5	0.5
Mineral premix[14]	1	1	1	1	1
Binder[15]	1	1	1	1	1
Dicalcium phosphate	—	0.1	0.1	0.5	0.5
Methionine[16]	0.1	0.1	0.1	0.1	0.1
Taurine[16]	0.3	0.3	0.3	0.3	0.3
Chromium oxide	1	1	1	1	1
Proximate composition (% dry matter)					
Dry matter (DM, %)	88.5	87.3	87.7	88.2	87.3
Crude protein (CP)	45.3	45.6	46.9	45.9	46.3
Crude lipid (CL)	18.0	18.1	17.9	18.3	18.7
Gross energy (kJ g^{-1} DM)	23.1	23.2	23.7	23.2	23.7
Ash	6.33	6.84	6.49	6.00	6.30
Cellulose	4.21	6.20	4.71	7.07	5.35
Hemicellulose	2.54	2.43	1.85	2.11	1.79
Klason lignin	12.2	10.0	10.1	12.8	12.6
Essential amino acids (% dry matter)					
Lysine	2.17	1.94	2.26	2.15	2.14
Arginine	1.63	1.67	1.62	1.68	1.59

Histidine	0.71	0.74	0.89	0.78	0.82
Isoleucine	1.30	1.29	1.30	1.28	1.32
Leucine	5.24	5.53	5.66	5.91	5.63
Valine	1.33	1.34	1.45	1.44	1.50
Methionine	1.20	0.97	0.95	1.24	1.39
Phenylalanine	2.07	2.00	1.97	2.25	2.05
Threonine	1.14	1.16	1.34	1.14	1.43
Non-essential amino acids					
Tyrosine	0.78	0.83	0.83	0.89	0.85
Aspartic Acid	2.77	2.76	2.75	2.51	2.69
Glutamic Acid	3.91	4.01	3.96	4.15	3.71
Serine	4.42	5.07	4.79	4.58	4.63
Glycine	5.53	5.39	5.37	5.37	5.45
Alanine	4.93	5.22	4.91	5.62	5.30
Proline	5.06	5.31	5.37	5.02	5.27
Tyrosine	0.78	0.83	0.83	0.89	0.85

[1]Brewer's spent grain, Unicer-Bebidas de Portugal, SA. Matosinhos, Portugal; composition detailed in **Table1**

[2]Fermented brewer's spent grain; composition detailed in Table 1.

[3] Pesquera Centinela, Steam Dried LT, Chile (CP: 66.8; CL: 9,6). Sorgal, S.A. Ovar, Portugal.

[4]Pea protein concentrate (CP:45.1%, CL: 2.84%)

[5] Corn gluten (CP:80.1% ; CL:4.07%). Sorgal, S.A. Ovar, Portugal

[6] Soybean meal 48 (CP: 44.9%; CL: 1.4%), Sorgal, S.A. Ovar, Portugal.

[7]Wheat gluten (CP: 74.7%; CL: 1.74%), Sorgal, S.A. Ovar, Portugal.

[8]Rapeseed (CP: 34.6%; CL: 2.6%), Sorgal, S.A. Ovar, Portugal.

[9] Sunflower (CP: 28.5%; CL: 1.48%), Sorgal, S.A. Ovar, Portugal

[10] Hemoglobin powder AP310P; APC Europe S.A.

[11] Hydrolyzed shrimp (CP: 69.8%; CL: 12.1%). Sorgal, S.A. Ovar, Portugal.

[12] Fish oil Sorgal, S.A. Ovar, Portugal.

[13]Vitamin premix (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

[14] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).

[15] Aquacube. Agil, UK.

[16] Feed grade amino acids, Sorgal, S.A. Ovar, Portugal.

2.3 Digestibility trial

The digestibility trial was approved by the Ethics Committee of CIIMAR and the National Competent Authority (Direção Geral de Alimentação e Veterinária - DGAV) (reference ORBEA_CIIMAR_27_2019). (reference ORBEA_CIIMAR_27_2019). All procedures were performed by accredited scientists in compliance with the European Union (Directive 2010/63/EU) and Portuguese (Decreto Lei n◦ 113/2013) guidelines on protecting animals for scientific purposes. Juveniles of European seabass were purchased from commercial aquaculture (Maresa, Huelva, Spain). Fish were transported to the experimental facilities at the Interdisciplinary Center of Marine and Environmental Research (CIIMAR, Matosinhos, Portugal) and quarantined for two weeks, during which they were fed a commercial diet (Neo Gold Blue seabream/seabass diet (48 % protein and 17 % lipids; Sorgal, S.A., Portugal). After this period, the fish were transferred to the experimental system and acclimatized for 2 weeks. The experimental system consisted of a thermoregulated water recirculation system with 12 fiberglass tanks of 60 L capacity, equipped with a feces-settling column connected to the outlet of each tank. During the trial, the water flow in each tank was maintained at 5 L min^{-1}, the average water temperature was 22 ± 1◦C, salinity was 35 ± 1 ‰, nitrogenous compounds were maintained below 0.02 mg/L, and dissolved oxygen was above 90 %. The photoperiod was adjusted to a 12/12 h light/dark cycle. A completely randomized block design was used to test each diet in duplicate in two sequential periods (blocks) to obtain four replicates per treatment. The same group of fish (initial average body weight of 92 g) was used in each block and was randomized between blocks. The fish were adapted to the experimental diets for 7 days in each period before starting fecal collection. Fish were manually fed twice daily, at 9:00 a.m. and 4:00 p.m., until apparent visual satiety. Feces were collected for 22 consecutive days before the first daily feeding, centrifuged (3000 × g, 10 min), pooled for each tank, and stored at − 20 ◦C until analysis. Forty-five minutes after the afternoon meal, the feces-settling columns and pipes were thoroughly cleaned to eliminate any residual feces or uneaten feed. The second block period was extended to 60 days of feeding for intestinal sampling purposes. Six hours after the morning meal, 4 fish per tank (8 per treatment) were randomly sampled and killed by an overdose of anesthesia (10 mL L^{-1}; ethylene glycol monophenyl ether), followed by cervical dislocation. The intestine was excised, freed of perivisceral fat and connective tissues, and divided into two regions: the anterior intestine (from the pyloric cecum to the distal intestine) and the distal intestine (visually distinguished by increased diameter, thicker mucosa, and darker mucosa). For histological analyses, tissue samples (approximately 0.5 cm) from each region were collected, washed in phosphate-buffered saline (PBS),

fixed in phosphate-buffered formalin (4 % w/v; pH 7.4) for 24 h, and then transferred to 70 % v/v ethanol for storage until processing. The remaining tissue from both regions was pooled per fish and stored at − 80 ◦C until the digestive enzyme activity was analyzed. The apparent digestibility coefficients (ADC) of dry matter, protein, lipids, and energy in the experimental diets were calculated as follows:

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

2.4 Chemical analysis

2.4.1 Proximal composition

The proximal composition of BSG, before and after SSF, dietary ingredients, diets, and feces were analyzed as follows: dry matter, by drying the samples at 105 ◦C until constant weight; ash, by incineration in a muffle furnace at 450 ◦C for 16 h; protein (N × 6.25) by the Kjeldahl method following acid digestion, using Kjeltec digestion and distillation units (Tecator Systems, Hogan ¨ as, ¨ Sweden; models 1015 and 1026, respectively); lipids were extracted with petroleum ether using a Soxtec system (Tecator Systems, Hogan ¨ ¨ as, Sweden; extraction unit model 1043 and service unit model 1046) for ingredients and diets, or following a gravimetric method (Folch et al., 1957) for feces because of the limited amount of sample available; gross energy, by direct combustion in an adiabatic bomb calorimeter (PARR Instruments, Moline, Il, USA; PARR model 1261). Cellulose, hemicellulose, and lignin contents were determined as described by Filipe et al. (2020). Dietary amino acid quantification was performed by high-performance liquid chromatography (HPLC), according to Cocuron et al. (2017).

2.4.2 Phenolic compounds and antioxidant activity

To quantify the phenolic compounds and antioxidant activity of BSG-SSF, an aqueous extraction was performed using distilled water at a solid/liquid ratio of 1:5 (w/v). The mixture was agitated at 150 rpm for 30 min at room temperature, filtered through a fine mesh, sieved, and centrifuged at (15,000 g for 10 min at 4◦C). The resulting supernatant was filtered through a syringe filter (0.45 µm, ø25 mm), and the extract was stored at − 20 ◦C until analysis. The Folin-Ciocalteu method was used to determine the total phenolic compounds, following Filipe et al. (2020). Briefly, 100 µL of the diluted sample (1:2) (or 100 µL of distilled water as a blank), 2 mL of 15 % Na2CO3, 500 µL of Folin–Ciocalteu reagent, and 7.4 mL of distilled water were mixed in a glass tube and incubated at 50 ◦C

for 5 min. After cooling, the mixtures were mixed, and the absorbance was read at 740 nm using a calibration curve with caffeic acid (CA) at concentrations ranging from 0 to 2 g L^{-1}. The total phenolic compounds were expressed in milligrams of gallic acid equivalents per gram of dry solids. Antioxidant activity was measured using the 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH) as described by Blois (1958) and adapted by Filipe et al. (2020). Briefly, 200 µL of several dilutions of each sample were added to a microplate and mixed with 100 µL of DPPH. Methanol was used as a blank. The reaction was performed in the dark for 30 min, and the absorbance was read at 517 nm. A calibration curve was prepared using the standard 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox). The results are expressed in µmol of Trolox equivalents (TE) per gram of dry solid substrate (µmol TE g^{-1}).

2.4.3 Cellulase or xylanase activity

To measure xylanase (endo-1,4-β-xylanase) and cellulase (endo-1,4-β-glucanase) activities in BSG-SSF, carboxymethylcellulose 2 % w/v (CMC) and xylan 1 % w/v in 0.05 N sodium citrate buffer (pH 4.8) were used as substrates, respectively. The enzyme activity was measured as described by Filipe et al. (2020). One unit of cellulase or xylanase activity was defined as the amount of enzyme required to release 1 µmol of glucose or xylose per minute under assay conditions.

2.4.4 Digestive enzyme activity

The whole intestine was homogenized (1:4 w/v) using an ice-cold buffer solution (100 mM Tris-HCl, 0.1 mM EDTA, and 0.1 % (v/ v) Triton X-100, pH 7.8) and centrifuged (30,000 × g for 30 min at 4 ◦C). The resulting supernatants were collected, divided into aliquots, and stored at − 80 ◦C until analysis. The activities of alpha-amylase (EC 3.2.1.1), trypsin (EC 3.4.21.4), and lipase (EC 3.1.1.3) were determined as described by Magalhaes˜ et al. (2015). Total alkaline protease (TAP) activity was measured according to Walter (1984) and adapted by Hidalgo et al. (1999), using casein as a substrate (1 % w/v) at pH 8, using 0.1 M tris HCl as a buffer. The reaction was stopped with 8 % tri chloroacetic acid (w/v), and the absorbance was read at 280 nm against the blank. All enzyme activities were determined at 37 ◦C using a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China).

2.4.5 Histological processing

The anterior and posterior intestine samples were processed following routine procedures and stained with hematoxylin and eosin. The histomorphology of the two intestinal sections was evaluated using a double-blind semiquantitative scoring system ranging from 1 to 5, according to the criteria suggested by Krogdahl et al. (2003): 1) widening and shortening of intestinal folds, (2) number of goblet cells, (3) widening of the lamina propria, (4) size and regularity of supranuclear vacuoles, (5) changes in enterocytes nucleus position. Images were acquired using the Zen software (Blue edition; Zeiss, Jena, Germany).

2.5 Statistical analysis

Data are presented as mean and pooled standard error of the mean (SEM). Data were checked for normality and homogeneity of variance and normalized when necessary. Non-orthogonal contrasts were performed to compare each test diet with the control diet, non-fermented BSG versus BSG-SSF diets, dietary incorporation level (10 versus 20), and interactions of incorporation level versus BSG processing. Non-parametric Kruskal-Wallis tests followed by pairwise comparisons with Bonferroni adjusted p-values were used for histological data because they did not follow normality and homogeneity of variance principles. To test the effect of dietary inclusion level (10–20 %) and fermentation process (fermented and non-fermented) on histological data, the Kruskal-Wallis test was used, excluding the control diet. All statistical analyses were performed using IBM SPSS Statistics software version 26 (IBM, NY, USA).

3 Results

The composition and enzymatic activity of BSG before and after 7 days of SSF with *Aspergillus ibericus* are presented in **Table 2. 1.** SSF increased the crude protein content of BSG by 21 %, from 26.7 % to 32.3 %, but decreased the lipid content by 49 %, from 5.7 % to 2.8 %. Furthermore, SSF reduced lignin, cellulose, and hemicellulose contents by 7.3 %, 30 %, and 34 %, respectively, compared to unfermented BSG. Antioxidant activity and total phenolic compounds were nearly zero in BSG, and after SSF, increased significantly to 64 μmol Trolox equivalents g− 1 and 12 mg gallic acid equivalents g− 1 , respectively. Xylanase (68 U g− 1 DM) and cellulase (925 U g− 1 DM) activities were also detected after SSF, while no activity was observed in the unfermented product.

The ADC of proteins, lipids, energy, and amino acids are shown in **Table 2. 3**. Dietary inclusion of 10% BSG reduced the ADC of energy ($p=0.04$), whereas dietary inclusion of 20% BSG decreased the ADC of dry matter ($p<0.01$), protein ($p=0.01$), lipids ($p=0.04$), and energy ($p<0.01$). However, irrespective of the dietary inclusion level, BSG-SSF restored ADC to values similar to those observed in the control group. Regardless of the

dietary inclusion level, the ADC of dry matter, lipids, and energy of BSG-SSF-based diets were higher than those of BSG-based diets. Regardless of the treatment, the ADC of dry matter, protein, and energy of the diets with 20% incorporation of BSG products were lower than those of diets with 10% BSG products. Compared to the unfermented product, the ADC of dry matter ($p<0.01$), protein ($p=0.03$), lipids ($p=0.04$), and energy ($p=0.04$) were higher in the diets containing 20% BSG-SSF, but no differences between groups were observed at the 10% incorporation level. Amino acid digestibility was not significantly affected by the ingredient source or dietary incorporation level. Nonetheless, the ADC for isoleucine ($p=0.03$) and glutamic ($p=0.02$) acid were higher in the 20BSG-SSF diet than in the 20BSG diet. The ADC of aspartic acid was lower in the BSG-SSF diets than in the BSG diets ($p=0.04$). The ADC of proline was higher in the 20BSG diet than that in the control ($p=0.04$), and the ADC of glutamate ($p=0.02$) and Isoleucine ($p=0.03$) was higher in the 20BSG diet than in the 20BSG-SSF diet.

Table 2. 3. Apparent digestibility coefficients (%) of European seabass juveniles fed the experimental diets.

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF	SEM
Dry Matter	62.8	60.7	60.6	51.8	63.7	1.1
Organic matter	68.9	65.7	66.1	60.9	68.3	1.1
Protein	88.4	88.4	88.4	85.8	88.0	0.4
Lipids	94.2	93.6	93.7	91.0	94.5	0.4
Energy	71.4	67.7	69.1	64.2	68.3	0.7
Essential amino acids						
Lysine, Lys	90.0	89.9	90.8	90.0	90.9	0.4
Arginine, Arg	86.8	89.4	89.8	87.0	86.3	0.6
Histidine, His	89.2	88.2	90.2	88.7	88.6	0.4
Isoleucine, Ile	87.6	85.1	88.3	88.7	84.2	0.7
Leucine, Leu	90.5	92.8	87.1	91.5	90.8	0.7
Valine, Val	83.3	85.4	85.7	82.1	85.0	0.7
Methionine, Met	86.9	88.4	88.2	91.9	94.0	0.7

Phenylalanine, Phe	86.2	85.7	84.9	88.5	87.7	05
Threonine, Thr	87.3	86.7	88.5	87.0	88.1	06
Non-essential amino acids						
Tyrosine, Tyr	89.3	90.8	86.5	86.7	86.8	0.7
Aspartic acid, Asp	86.6	85.4	84.1	92.9	86.6	1.1
Glutamic acid, Glu	84.4	84.9	82.8	82.1	87.8	0.7
Serine, Ser	87.7	88.7	83.7	87.7	88.8	0.6
Glycine, Gly	87.6	87.9	85.0	88.9	88.4	0.6
Alanine, Ala	86.7	86.7	84.2	90.2	87.5	0.9
Proline, Pro	90.3	87.6	85.8	87.8	88.6	1.0

Non-orthogonal contrast

	Dry Matter	**Protein**	**Lipids**	**Energy**	**Lys**	**Ile**	**Leu**	**Asp**	**Glu**	**Pro**
Control vs 10BSG	0.24	0.97	0.59	**0.04***	0.95	0.17	0.19	0.67	0.78	0.19
Control vs 10BSG-SSF	0.26	0.99	0.63	0.18	0.57	0.07	0.87	0.98	0.09	0.39
Control vs 20BSG	**<0.01***	**0.01***	**0.04***	**<0.01***	0.60	0.68	0.06	0.40	0.37	**0.04***
Control vs 20BSG-SSF	0.63	0.65	0.81	0.08	0.97	0.50	0.54	**0.04***	0.21	0.23
BSG vs BSG-SSF	**0.03***	0.07	**0.04***	**0.04***	0.92	0.83	0.29	**0.03***	0.42	0.29
Level (10 vs 20)	**<0.01***	**0.03***	0.15	**0.03***	0.43	0.55	0.02*	**0.09**	0.15	0.72
10BSG vs 10BSG-SSF	0.96	0.96	0.95	0.42	0.92	0.06	0.45	**0.02***	0.13	0.91
20BSG vs 20BSG-SSF	**<0.01***	**0.03***	**0.04***	**0.04***	0.95	**0.0.3***	**0.04***	0.39	**0.02***	0.18
Interaction (Level, SSF)	**<0.01***	0.13	0.13	0.25	0.98	**<0.01***	0.05	0.23	**0.01***	0.38

Values are presented as mean (n = 4) and pooled standard error of the mean (SEM). * Denotes significant differences (non-orthogonal contrast analysis) of control group versus each test group (10BSG, 10BSG-

SSF, 20BSG, 20BSG-SSF); BSG versus BSG-SSF diets; BSF level 10% versus SF level 20%; 10BSG versus 10BSG-SSF; 20BSG versus 20BSG-SSF; and interaction (level and SSF).

The intestinal enzyme activities of European seabass fed the experimental diets are presented in **Table 2. 4**. Compared to the unfermented product, enzyme activity was not affected by SSF. However, fish fed the 10BSG-SSF diet had lower total protease (p=0.03) and trypsin activities (p=0.01) than those fed the control diet. Irrespective of the treatment, amylase (p=0.02) activity was higher in fish fed the 20 % diets than in those fed the 10 % diets.

Table 2. 4. Intestine enzyme activity (mU mg^{-1} protein) of European seabass juveniles fed the experimental diets.

	Control	**10BSG**	**10BSG-SSF**	**20BSG**	**20BSG-SSF**	**SEM**
Total proteases	5.0	3.7	3.5	3.8	4.3	0.2
Trypsin	135.9	108.3	77.01	124.6	113.6	7.4
Amylase	122.7	106.3	87.6	115.4	159.4	8.4
Lipase	7.0	6.3	5.4	6.0	7.0	0.4

Non-orthogonal contrasts

	Total proteases	**Trypsin**	**Amylase**	**Lipase**
Control vs 10BSG	0.26	0.23	0.47	0.55
Control vs 10BSG-SSF	**0.03***	**0.01***	0.13	0.18
Control vs 20BSG	0.06	0.61	0.75	0.37
Control vs 20BSG-SSF	0.63	0.31	0.12	0.97
BSG vs BSG-SSF	0.84	0.18	0.44	0.94
Level (10 vs 20)	0.46	0.09	**0.02***	0.46
10BSG vs 10BSG-SSF	0.26	0.16	0.42	0.46
20BSG vs 20BSG-SSF	0.16	0.62	0.06	0.40
Interaction (Level, SSF)	0.07	0.52	0.06	0.26

Values are presented as mean (n = 8) and pooled standard error of the mean (SEM). *Denotes significant differences (non-orthogonal contrast analysis) of control group versus each test group (10BSG, 10BSG-SSF, 20BSG, 20BSG-SSF); BSG versus BSG-SSF diets; BSF level 10% versus SF level 20%; 10BSG versus 10BSG-SSF; 20BSG versus 20BSG-SSF; and interaction (level and SSF).

Histological assessment of the anterior and distal intestines of European seabass fed the experimental diets is presented in **Table 2.5**. In the anterior intestine, fold height and lamina propria width were not affected by dietary treatments. The number of goblet cells was lower in fish fed the experimental diets than in the control. Compared to the control diet, the position of enterocytes was the same in fish fed the 10BSG, 10BSG-SSF, and 20BSG-SSF diets than the control diet. Fish fed the 20BSG (p=0.05) diet had higher scores in the position of the enterocyte nuclei than fish fed the control diet. In the distal intestine, only supranuclear vacuolation (p=0.02) was affected by diet composition, and it was higher in fish fed the 20BSG diet than in those fed the control diet (**Fig. 1**). In the anterior intestine, no differences between the experimental groups were observed in the mean score. However, the mean score was higher in fish fed the 20 % diets than in the control, irrespective of treatment. In the posterior intestine, no differences in the mean scores were observed between the groups.

Table 2. 5. Score-based evaluation of the anterior and distal intestine histology of European seabass fed the experimental diets[1].

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF	SEM
Anterior intestine						
Fold height	1.0	2.1	2.0	2.6	1.8	0.2
Goblet cells	1.2^{a}	$2.3^{\cdot b}$	2.8^{b}	2.6^{b}	2.5^{b}	0.2
Lamina propria[2]	2.2	2.1	2.3	3.0	2.1	0.1
Supranuclear vacuolization	1.2^{a}	$1.6^{a,b}$	1.2^{a}	2.2^{b}	$2.0^{a,b}$	0.1
Enterocyte nucleus position	1.2^{a}	$1.6^{a,b\blacklozenge}$	$1.2^{a\blacklozenge}$	2.2^{b}	$2.0^{a,b}$	0.1
Mean	1.5^{a}	$2.0^{a,b}$	$1.9^{a,b}$	2.5^{b}	2.1^{b}	0.2
Distal intestine						
Fold height	3.2	2.3	2.2	1.9	2.1	0.1
Goblet cells	2.0	3.0	3.5	2.2	2.7	0.3
Lamina propria[2]	2.0	2.6	2.2	2.3	3.1	0.2
Supranuclear vacuolization	2.0^{a}	$2.8^{a,b}$	$2.7^{a,b}$	4.7^{b}	3.1^{ab}	0.3

Enterocyte nucleus position	1.0	1.0	1.0	1.0	1.0	0.0
Mean	2.9	2.6	2.4	2.6	2.8	0.2

[1]Scores from 1 to 5, with 5 indicating major alterations.
[2]Width and cellularity of the lamina propria
Values are presented as mean scores (n = 8) and pooled standard error of the mean (SEM). Different lower-case letters represent statistical differences between dietary groups determined by comparing all Kruskal-Wallis pairs. Significance values were adjusted by Bonferroni correction for multiple tests. ♦ Denotes significant differences ($p<0.05$) between the BSG levels (10% versus 20%), and ¥ denotes significant differences between BSG processing methods (BSG versus BSG-SSF)

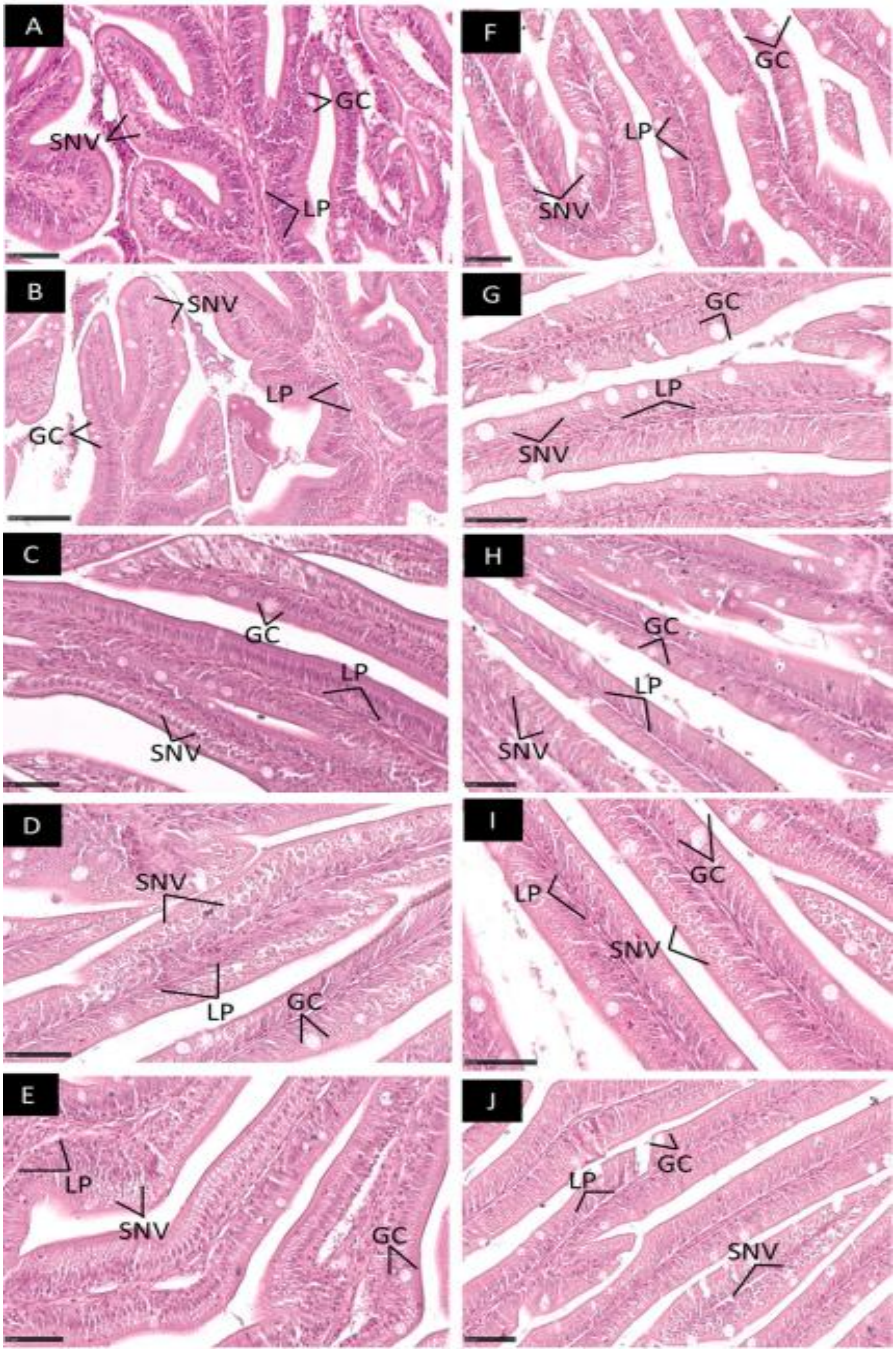

Fig. 1. Detailed of anterior (Figs. A to E) and distal (Figs. F to J) intestine of European seabass fed the control (A and F), 10BSG (B and G), 10BSGSSF (C and H), 20BSG (D and I), and 20BSG-SSF (E and J) for 68 days. Lamina propria: LP, goblets cells (GC); lamina propria: (LP) and supranuclear vacuoles (SNV). Scale bar: 25 mm; HeE staining.

4 Discussion

4.1 SSF of BSG with Aspergillus ibericus

Plant feedstuff cell walls are composed of highly complex carbohydrates that are not digested by monogastric animals, including fish, hindering plant feedstuff digestibility and nutrient absorption and negatively affecting fish growth, feed utilization, intestinal

function, and overall health status (Glencross et al., 2020; Kokou and Fountoulaki, 2018; Oliva-Teles et al., 2015). This is particularly relevant for carnivorous fish, for which only low-fiber and relatively high-protein plant feedstuffs, such as soybean, rapeseed, wheat gluten, and corn gluten, are used in the formulation of plant-based diets (Gatlin et al., 2007). Nonetheless, the sustainability and cost-effectiveness of aquafeeds may be compromised by using only traditional feedstuffs in the diet formulation. Therefore, technological approaches that reduce the complexity of plant carbohydrates and increase the nutrient bioavailability of underutilized feedstuffs using the circular economy concept may be of high practical value. This is the case for SSF, a relatively simple and low-technological process that hydrolyzes plant feedstuff fiber content and increases protein and other nutrient contents while also releasing enzymes, organic acids, vitamins, and other compounds that may increase the nutritional value of fermented feedstuffs (Leite et al., 2021).

In the current study, SSF of BSG by *A. ibericus* increased the protein content and decreased the cellulose, hemicellulose, lignin, and lipid contents of the fermented product. Previously, we have also observed that SSF with *A. ibericus* increased the protein content and decreased the lipid content of DDGS (Filipe et al., 2023) or of a mixture of plant feedstuffs (soybean, rapeseed, sunflower, and rice bran) (Amaral et al., 2023, Vieira et al., 2022). In other studies where BSG was fermented by *Rhizopus oligosporus* or *Rhizopus* sp., there was a decrease in lipid, fiber, sugars, and neutral detergent fiber, and an increase in the protein content of the fermented products (Cooray and Chen, 2018; Ibarruri et al., 2019). Moreover, SSF with BSG *Aspergillus oryzae* increased the protein content and decreased the lipid content of the fermented product (Ogunjobi et al., 2011). Differences in the nutrient composition of the BSG and SSF conditions, such as microorganism species, time, temperature, and bed height, led to differences in the final composition of the SSF products (Yang et al., 2021). The BSG nitrogen and carbon contents affect fungal growth, as nitrogen is used for fungal conidiation, and carbon is used as an energy source. Thus, the final composition of the SSF product depended on the total fungal biomass produced.

Filamentous fungi, particularly *Aspergillus* spp., are known to produce a wide range of enzymes, including carbohydrases, with higher enzymatic yields than those produced by yeast or bacteria (Ghorai et al., 2009). These enzymes are responsible for the degradation of the complex lignocellulosic matrix of the substrate. In the present study, high NSP content of BSG induced the production of cellulase and xylanase, with higher cellulase than xylanase activity. Similarly, SSF of different oilseed cakes by *A. ibericus* and *A. niger* led to higher cellulase and xylanase production than SSF of *Rhyzopus*

oryzae, which in turn led to higher production of β-glucosidase (Sousa et al., 2018). Enzymatic production differs among species of the genus *Aspergillus*. For example, in the SSF of DDGS with *A. ibericus*, *A. uvarum*, and A. carbonarius, higher cellulase production was obtained with *A. ibericus*, whereas higher xylanase production was obtained with *A. uvarum* (Filipe et al., 2023). Maximum enzymatic production also depends on the duration of fermentation. For instance, in the SSF of rice bran with *A. terreus*, xylanase production reached its maximum after 8 days, aminoglycosidase after 6 days, and β-glucosidase after 10 days, and decreased afterward (Liang et al., 2011).
Bioactive compounds, such as alkaloids and phenolic compounds entrapped in the substrate lignocellulosic matrix, are released during the SSF process (Martins et al., 2011; Devasagayam et al., 2004) during the SSF process. The release of phenolic compounds is particularly interesting owing to their potent antioxidant properties (Bhanja-Dey et al., 2016). In the present study, SSF of BSG increased the total phenolic content by more than 50 times. Similar results were obtained by the SFF of a mixture of ago-industrial by-products (exhausted grape marc, vine trimming shoots, and exhausted olive pomace) with *Aspergillus ibericus* (Filipe et al., 2020). An increase in total phenolic content was also observed in the SSF of dried distiller grains with solubles of A*spergillus ibericus*, *Aspergillus uvarum*, and *Aspergillus carbonarius* (Filipe et al., 2023). However, a reduction in phenolic compounds after SSF can also occur because of enzymes produced by fungi, such as laccases and peroxidases (Rodrıguez et al., 2004). For example, a reduction in phenolic compounds was observed in the SSF of olive pomace, exhausted grape marc, and vine shoot trimmings by *Aspergillus uvarum* (Salgado et al., 2015) or in the SSF of exhausted olive pomace by *Aspergillus Ibericus*, *Aspergillus uvarum*, and *Aspergillus niger* (Sousa et al., 2018).

4.2 Digestibility of unfermented and SSed BSG

BSG presents a potential and valuable source of nutrients for animal nutrition. However, its high fiber content (up to 50 %) may compromise nutrient availability (Mitri et al., 2022), limiting its utilization in aquafeeds, particularly for carnivorous fish species. In the present study, the replacement of a plant feedstuff mixture (soybean, rapeseed, sunflower, and wheat gluten meal) with BSG reduced the digestibility of dry matter, protein, lipids, and energy, and these effects linearly increased with the dietary replacement level. This is mainly related to the high fiber content of BSG. High dietary fiber levels reduce the digestibility of energy and may compromise the digestion of dietary nutrients by affecting the physical characteristics of the digesta and access of endogenous enzymes to the substrate (Adeola and Bedford, 2004). In European seabass, the detrimental effects of

excessive dietary incorporation of complex polysaccharides on nutrient digestibility have been well documented (Fountoulaki et al., 2022; Magalhães et al., 2015; Enes et al., 2011; Tibaldi et al., 2006). In contrast, in rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (*Sparus aurata*), dietary incorporation of 20 % BSG replacing fishmeal did not affect protein digestibility but decreased lipid digestibility (Nazzaro et al., 2021). However, in another study, the replacement of plant ingredients (soybean meal, wheat gluten, and wheat starch) with up to 15 % BSG did not affect protein and lipid digestibility in rainbow trout and gilthead seabream (Estevéz et al., 2022). This indicates that the adverse effects of dietary incorporation of BSG are related to species and dietary incorporation levels.

BSG is a highly heterogeneous lignocellulosic biomass, and differences in lignocellulosic composition may also have contributed to the observed results (Lynch et al., 2016). In the present study, the BSG included in the diets contained approximately 45 % NSP (24 % hemicellulose and 21 % cellulose) and 15 % lignin. Studies carried out by Nazzaro et al. (2021) and Estev´ez et al. (2022) indicated that the enzymatic hydrolysis of BSG reduced crude fiber content by 11.7 % compared to untreated BSG. Different carbohydrate fractions affect digestibility in divergent ways (Irvin et al., 2016). For example, starch is well tolerated (Oliva-Teles et al., 2015), while cellulose has little effect on protein digestibility but may decrease dry matter and energy digestibility (Glencross et al., 2020; Hansen and Storebakken, 2007; Kraugerud et al., 2007). In seabass, the dietary inclusion of up to 20 % insoluble dietary fiber (cellulose) had little impact on protein and dry matter digestibility (Dias et al., 1998), digesta characteristics, gut histology, and evacuation (Bonvini et al., 2018). However, 30 % sunflower meal (41 % NSP) decreased the dry matter but did not affect protein digestibility (Fountoulaki et al., 2022).

Previously, it was demonstrated that SSF of BSG with *A. ibericus* MUM 03.49 allowed the production of a highly active carbohydrases extract that can contribute to increasing dry matter and energy digestibility (Fernandes et al., 2021). In the present study, SSF of BSG increased the digestibility of dry matter and nutrients to a level similar to that of the control diet. These positive results can be attributed to the reduction in fiber content and the presence of fungal carbohydrases in the SSF product.

4.3 Digestive function and intestine histomorphology

Irrespective of the treatment, increased dietary inclusion of BSG reduced trypsin and amylase activity. These results align with the observed reduction in the ADC of protein and energy in diets.

The total proteases and trypsin activities were significantly lower in fish fed the 10BSG-SSF diet than in those fed the control diet. In absolute values, the activity of these enzymes was also lower in fish fed 20BSG-SSF than in the control, but the differences were not statistically significant. However, the reduction in proteolytic activity in the BSG-SSF groups was not reflected in differences in the ADC of the protein. During the SSF process, proteins may have been partially hydrolyzed by fungal proteases, and fungal proteases may have remained active in BSG-SSF. This may contribute to explaining the reduction in endogenous total proteases and trypsin activities. Previous studies have also observed that the dietary inclusion of SSF ingredients reduces endogenous digestive activity without compromising diet digestibility. For example, also in European seabass, the dietary inclusion of SSF *Ulva rigida* by *A. ibericus* (Fernandes et al., 2022) or SSF plant feedstuff mixture by *A. niger* (Vieira et al., 2022) decreased total intestinal proteases activity compared to the unfermented feedstuff. In Nile tilapia, digestive protease, and amylase activities also decreased when SSF guar and copra meal were included at the expense of fishmeal (Dileep et al., 2021).

The increase in dietary fiber can negatively affect the digestive process of fish (Raskovic et al., 2011); however, this was not observed in the present study. High dietary fiber levels may also negatively affect fish intestinal histomorphology, although European sea bass has been reported to have a relatively high fiber tolerance, and dietary fiber inclusion of up to 16 % does not affect gut morphology (Bonvini et al., 2018). In the present study, histomorphological alterations due to diet were primarily observed in the anterior intestine, and except for increased supranuclear vacuolization, no major differences were observed in the posterior intestine. The increase in goblet cells observed in the anterior intestine of fish fed diets containing fermented and non-fermented BSG may be associated with an increase in the fiber level of the diet, as the goblet cells protect the intestinal lining and help increase mucus production, facilitating fecal expulsion (Machado et al., 2013).

Goblet cells secrete mucin, which is crucial for the intestinal immune barrier in fish (Machado et al., 2013). While more goblet cells typically denote intestinal health, excessive numbers can thicken the mucus layer, hindering nutrient absorption (Kim and Khan, 2013). Studies show that high dietary fiber levels lead to mucus efflux from the intestine, possibly due to increased chyme volume physically scraping the mucus layer (Christelle et al., 2005). This suggests a link between high fiber and intestinal mucus efflux, requiring more goblet cells to maintain the mucus layer. Considering the harmful effects of dietary fiber on the fish intestinal epithelium, the increased number of goblet cells observed in the present study may indicate an attempt to diminish or repair the

mucosal damage imparted by the high dietary fiber content of BSG contacting diets, as previously observed in other fish species (Liu et al., 2022).

Even though nutrient absorption in the distal portion of the intestine is limited, the number of absorptive cells in this region indicates that the distal intestine of some fish plays a vital role in the uptake of intact proteins and other large-size molecules (Bakke-Mckellep et al., 2000). Additionally, the fiber level in the diet may have impaired selective permeability of the enterocytes' membrane in the distal part of the intestine. Thus, the increase in the size of supranuclear vacuolation of enterocytes may result from the indiscriminate absorption of dietary components that have no routes of entry into the body and, therefore, accumulate in vacuoles. Converging with current research, Couto et al. (2016) reported that meagre juveniles (*Argyrosomus regius*) fed diets containing high levels of carob germ seed meal also showed increased supranuclear vacuolation of intestinal enterocytes with concomitant accumulation of an amorphous hyaline substance of unknown nature, associated with a potential deleterious effect of tannins on the selectivity of cell membranes.

5 Conclusions

The present study showed that SSF of BSG by *A. ibericus* increased the protein content and decreased the cellulose and hemicellulose fractions of the fermented product. Including BSG in the diet reduced digestibility, whereas SSF-BSG did not. Moreover, SSF also decreased the deleterious effects of the dietary incorporation of BSG. Overall, present results indicate that SSF increases the nutritional value of BSG, a lignocellulosic-rich agro-industrial by-product, increasing the potential of its use in fish feeds and contributing to reducing the climate and environmental footprint of aquaculture production. Further studies, including a growth trial, are needed to evaluate the impact of these ingredients on growth performance and feed utilization.

CRediT authorship contribution statement

Tássia Estevão-Rodrigues: Investigation, Formal analysis, Writing - Original Draft. Helena Fernandes: Investigation, Writing - Review & Editing. Sara Moutinho: Investigation, Methodology. Diogo Filipe: Investigation. Filipa Fontinha: Investigation, Formal analysis. Rui Magalhães: Investigation. Ana Couto: Writing - Review & Editing, Resources. Marta Ferreira: Resources, Writing - Review & Editing. Margarida Gamboa: Investigation, Resources. Carolina Castro: Writing - Review & Editing. Isabel Belo: Writing - Review & Editing, Resources. José Salgado: Supervision, Writing - Review & Editing – Resources. Aires Oliva-Teles: Conceptualization, Writing - Review & Editing –

Resources. Helena Peres: Conceptualization, Supervision, Writing - Review & Editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 3:

Effect of Solid-Fermented Brewer's Spent Grain on Growth, Metabolism, and Oxidative Status of European Seabass (*Dicentrarchus labrax*)

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Effect of Solid-Fermented Brewer's Spent Grain on Growth, Metabolism, and Oxidative Status of European Seabass (*Dicentrarchus labrax*)

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Abstract: Replacing traditional agricultural ingredients with biotechnologically improved agro-industry by-products in fish diets promotes sustainable aquaculture, reduces production costs and carbon footprint, and promotes a circular economy. Brewer's spent grain (BSG) is one such by-product. Solid-state fermentation (SSF) of BSG with *Aspergillus ibericus* enhances its nutritional value and digestibility for European seabass. The present study further evaluates the potential of dietary inclusion of BSG-SSF on growth performance, feed utilization, plasma metabolite profile, intermediary metabolism, and oxidative status of European seabass juveniles compared to the unfermented product. A practical diet (45% protein; 18% lipids) was tested against diets incorporating 10 % or 20% of BSG or BSG-SSF, replacing plant-protein feedstuffs. Triplicate groups of European seabass juveniles (49 g initial weight) were fed for 10 weeks. Unfermented BSG (10% and 20%) reduced growth and feed efficiency. In comparison, the 20% BSG-SSF diet promoted growth and feed efficiency similar to the control group, while the 10% BSG-SSF diet surpassed the control diet. Whole-body protein content was unaffected, but lipid and energy contents decreased with increasing BSG levels, regardless of fermentation. Plasma glucose and phospholipid levels and hepatic activities of glucokinase and malic enzymes decreased with increasing BSG, irrespective of fermentation. BSG-SSF incorporation increased plasma triglyceride levels and decreased hepatic transaminase activities but did not affect hepatic key enzyme activity of β-oxidation or lipogenesis. It also reduced antioxidant enzyme activity and lipid peroxidation. In conclusion, BSG negatively impacted growth performance, while BSG-

SSF supported inclusion levels up to 20% without performance loss. Further, a 10% BSG -SSF outperformed the control diet.

Keywords: Alternative ingredients; Biocircularity; By-product; European seabass; Fermentation; Metabolism; Oxidative stress

1 Introduction

The growing global population is placing significant pressure on food production systems to meet the increasing demand for protein (FAO, 2022). Addressing this challenge requires adopting eco-friendly strategies, prioritizing resource circularity, and alleviating the pressure on the global food supply chain (van Zanten et al., 2023). In this context, the fed aquaculture industry must improve efficiency practices and implement sustainable measures to reduce reliance on traditional ingredients for aquafeed formulation (Colombo et al., 2022).

Over the past decades, substantial efforts have been directed to decrease the dependency on fishmeal and fish oil. Plant proteins have become a significant source of protein and lipids in aquafeed, even for carnivorous fish (Glencross et al., 2023). However, including high-quality plant ingredients and their derivatives in aquaculture diets presents sustainability challenges, such as deforestation, increased carbon footprint, higher costs, and competition with human food (Ahmed & Thompson, 2019). To address these concerns and align with European Union recommendations (AAC, 2023), a circularity approach focused on recycling biomass that does not compete with food sources is essential (Colombo et al., 2022).

Agro-industrial by-products present potential as alternative ingredients for fish feeds. Reintegrating these by-products into the food chain implies evaluating their feasibility and determining the most efficient processing treatment to improve their nutritional value (Chary et al., 2023). A primary challenge in incorporating these by-products into aquafeeds is their low protein content and, more critically, their high levels of non-starch polysaccharides (NSP), which can adversely impact fish growth and health (Sinha et al., 2011).

NSPs are the main component of plant cell walls and include various undigestible polysaccharides and lignin (Kraugerud & Svihus, 2011; Sinha et al., 2011). The polysaccharides include cellulose (linear β-(1-4)-D-glucose chains, insoluble and resistant to hydrolysis), hemicellulose (branched, shorter chains of diverse sugars), and lignin (an amorphous, hydrolysis-resistant structure), and pectins (Sinha et al., 2011). Fish lack the ability to digest NSP, and high dietary levels may impair digestion, absorption, and utilization of other nutrients (Sinha et al., 2011; Krogdahl et al., 2010).

However, appropriate processing treatments through physical, chemical, and biological methods can disrupt the complex structure of NSP into simpler compounds (Kraugerud & Svihus, 2011).

Solid-state fermentation (SSF) is a biotechnological process capable of hydrolyzing agro-industrial substracts. In SSF, the substrate serves as a solid support and a nutritional source for microorganisms, which secrete hydrolytic enzymes that disrupt the substrate's structure (El-Bakry et al., 2015). Enzymes such as hemicellulases and cellulases, generated during SSF, break NSP into monomers and low-molecular-weight oligosaccharides (Krishna, 2005). The type of enzymes produced depends on both the substrate and selected microorganism, with fungi being the preferred due to their adaptability to SSF conditions (Leite et al., 2021). Further, thermophilic fungi produce thermostable enzymes, which offer several advantages for various applications (Rigoldi et al., 2018). Overall, SSF transforms plant by-products into nutritionally enriched biomasses with higher protein content, reduced anti-nutritional factors such as NSP and phytate, and the addition of functional compounds, such as hydrolytic enzymes and antioxidants compounds (Leite et al., 2021).

Brewer's spent grain (BSG) is the primary by-product of the brewery industry, constituting 80% of total brewery waste. According to the Eurostat data from 2023, approximately 35 million liters of beer were produced in the EU in 2022, generating around 20 kg of BSG per 100 liters produced (Agrawal et al., 2023). BSG contains moderate protein, high fiber, minerals, and vitamin levels (Agrawal et al., 2023). However, its high fiber content limits its utilization in aquafeeds. BSG has been previously used as a substrate in SSF to produce modified biomass with increased protein content and reduced structural polysaccharides and to extract valuable compounds, such as enzymes and phenolic compounds with antioxidant capacity (Liguori et al., 2015).

It was previously shown that extract obtained from BSG after SSF has high potential as functional ingredients for European seabass (Fernandes et al., 2022). Moreover, SSF improved the nutritional profile of BSG, increasing protein content and reducing cellulose and hemicellulose levels, and improved the digestibility of dry matter, energy, protein, and amino acids in European seabass juveniles (Estevão-Rodrigues et al., 2024).

The present study further investigated the potential of dietary inclusion of non-fermented and solid-fermented BSG (BSG-SSF) on growth performance, feed utilization, and oxidative status in European seabass juveniles.

2 Materials and Methods

2.1 Ethical statement

This study was approved by the Animal Welfare Committee (ORBEA) of CIIMAR and the Portuguese National Authority for Animal Health (DGAV; reference ORBEA-CIIMAR-27-2019). The growth trial was performed by certified personnel in compliance with European Union Directive 2010/63/EU and Portuguese legislation (Decreto-Lei no. 113/2013).

2.2 Solid-state Fermentation (SSF)

Brewer's Spent Grain (BSG), comprising barley, Pilsen malt, and corn grits, was sourced from Unicer-Bebidas de Portugal, SA (Matosinhos, Portugal). *Aspergillus ibericus* MUM 03.49, provided by the University of Minho Micoteca (Braga, Portugal), was employed in the solid-state fermentation (SSF) of BSG.

SSF was performed in sterilized trays (121 ºC, 15 min) containing 400 g of sterilized BSG (moisture adjusted to 75% w/w) inoculated with 2 mL of *A. ibericus* MUM 03.49 spores solution (10^6 cells ml^{-1}). The trays were incubated at 25 ºC for 7 days with daily stirring. After fermentation, the BSG-SSF was pooled and stored at 4 ºC until diet production.

2.3 Experimental diets

Five approximately isoproteic and isolipidic diets (45% crude protein, 18% crude lipids) were formulated: a practical control diet including 15% fish meal and plant ingredients as protein sources, and 4 test diets including 10% or 20% of either BSG (diets 10BSG and 20BSG) or BSG-SSF (diets 10BSG-SSF and 20BSG-SSF). BSG and BSG-SSF replaced a mixture of soybean, rapeseed, sunflower, and wheat meal. Ingredients were ground, mixed thoroughly, and pelleted using a 2 mm die laboratory pellet mill (CPM: California Pellet Mill, Crawfordsville, IN, USA). Diets were then dried in an oven at 55 ºC for 24 h. Ingredients and proximate composition of experimental diets are presented in **Table 3. 1**.

Table 3. 1. Ingredients and proximal composition of the experimental diets

	Control	**10BSG**	**10BSG-SSF**	**20BSG**	**20BSG-SSF**
Ingredient (% dry matter)					
BSG[1]	—	10	—	20	—
BSG-SSF[2]	—	—	10	—	20
Fish meal	15	15	15	15	15

Pea protein concentrate	10	10	10	10	10
Corn gluten	12.5	12.5	12.5	12.5	12.5
Soybean meal	17.6	15	15	10	10
Rapeseed meal	7.5	7	7	5	5
Sunflower meal	6.5	6.2	6.2	4.8	4.8
Wheat meal	11.7	4.6	4.6	—	—
Hemoglobin	—	—	—	2.2	2.2
Fish oil	14.0	14.4	14.4	14.9	14.9
Dicalcium phosphate	—	0.1	0.1	0.5	0.5
Constant componests[3]	5.1	5.1	5.1	5.1	1.2
Proximate composition (% dry matter)					
Dry matter, DM	88.5	87.3	87.7	88.2	87.3
Crude protein	45.3	45.6	46.9	45.9	46.3
Crude lipids	18.0	18.1	17.9	18.3	18.7
Gross energy (kJ g^{-1} DM)	23.1	23.2	23.7	23.2	23.7
Ash	6.3	6.8	6.4	6.0	6.30
Cellulose	4.2	6.2	4.7	7.0	5.35
Hemicellulose	2.5	2.4	1.8	2.1	1.79
Klason lignin	12.2	10.0	10.1	12.8	12.6
Essential amino acids (EAA, % protein)					
Lysine, Lys	4.8	4.2	4.6	4.9	4.6
Arginine, Arg	3.6	3.6	3.6	3.5	3.4
Histidine, His	1.5	1.6	1.6	1.9	1.7
Isoleucine, Ile	2.9	2.8	2.7	2.8	2.8
Leucine, Leu	11.7	12.0	12.6	12.3	12.1
Valine, Val	2.9	2.9	3.1	3.1	3.2
Methionine, Met	2.6	2.1	2.6	2.0	3.0
Cysteine, Cys	1.2	1.2	1.1	1.1	1.1
Phenylalanine, Phe	4.6	4.3	4.8	4.2	4.4
Tyrosine, Tyr	1.7	1.8	1.9	1.8	1.8
Threonine, Thr	2.5	2.5	2.4	2.9	3.0
Sum EAA	40.5	39.3	41.4	40.9	41.5
Non-essential amino acids (NEAA, % protein)					
Aspartic acid, Asp	6.20	6.03	5.40	5.98	5.81
Glutamic acid, Glu	8.73	8.76	8.92	8.62	8.01
Serine, Ser	9.87	11.1	9.84	10.4	10.0
Glycine, Gly	12.4	11.8	11.5	11.7	11.8
Alanine, Ala	11.0	11.4	12.1	10.7	11.5
Proline, Pro	11.3	11.6	10.8	11.7	11.4
Sum NEAA	59.5	60.6	58.6	59.1	58.4

[1]Brewer's spent grain, Unicer-Bebidas de Portugal, SA. Matosinhos, Portugal. Crude protein: 26.7%, crude lipids: 5.7%, cellulose: 21.1%, hemicellulose: 23.7%, lignin: 15.0% (% dry matter). [2]Fermented

brewer's spent grain. Crude protein: 32.3%, crude lipids: 2.8%, cellulose: 14.8%, hemicellulose: 15.7%, lignin: 13.9% (% dry matter). [3]Constant components (% of the diet): vitamin premix, 1; mineral premix, 1; choline chloride, 0.5; shrimp hydrolysate, 1.2; binder, 1; methionine, 0.1; taurine, 0.3. Detailed composition of the ingredients, vitamins, and mineral premixes are presented in Estevão-Rodrigues et al. (Estevão-Rodrigues et al., 2024).

2.4 Growth Trial

European seabass (*Dicentrarchus labrax*) juveniles, sourced from commercial aquaculture (Maresa, Huelva, Spain), were transported by car in two closed and completely filled with oxygenated seawater (using an oxygen bottle). During transport, temperature, oxygen levels, and ammonia nitrogen were continuously monitored and controlled. Upon arrival at the Bioterium of Aquatic Organisms of CIIMAR (Matosinhos, Portugal), the fish were maintained in quarantine for 15 days in a 2000 L recirculating seawater system (RAS) and fed a commercial diet (NEOGOLD diet; 55% crude protein and 16% crude lipids, Aquasoja; Sorgal, S.A., Portugal). Then, fish were transferred to the experimental system consisting of a thermo-regulated RAS with 15 fiberglass tanks (300 L capacity) supplied by an aerated water flow. Throughout the trial, the water temperature was maintained at 22 ± 0.4 ºC, dissolved oxygen averaged 7.2 mg mL^{-1}, salinity was 27 ± 3‰, ammonia and nitrites levels were kept below 0.02 mg mL^{-1}, and the photoperiod was set to 12 hours light and 12 hours dark (12L:12D).

At the beginning of the trial, 15 groups of 16 fish (initial body weight: 49 g) were randomly assigned to the tanks, and experimental diets were tested in triplicate. Fish were hand-fed to visual satiety twice daily, 6 days a week, for 10 weeks. At the end of the trial, fish were bulk-weighed under light anesthesia (0.3 mL L^{-1} ethylene glycol monophenyl ether) after one day of feed deprivation. Five fish from the initial stock and 3 fish per tank at the end of the trial were euthanized by anesthetic overdose (10 mL L^{-1} ethylene glycol monophenyl ether), pooled, and stored at -20ºC for determination of the proximate composition analysis.

2.5 Fish sampling

Following the final weighing, the fish were fed for 3 additional days to mitigate stress resulting from handling. Subsequently, 4 hours after the morning meal, blood samples were collected from the caudal vein of 3 fish per tank using heparinized syringes. The collected blood was centrifugated (10,000 *g*, 10 min), and the resulting plasma was divided into aliquots and frozen at -80 °C until further analysis. The fish were then killed by decapitation, and the liver and whole intestine were dissected on an iced tray, immediately frozen in liquid nitrogen, and stored at -80 ºC for subsequent analysis. The

fish, liver, and viscera weights of these fish were recorded to determine the hepatosomatic (HSI) and visceral (VI) indices.

2.6 Proximate composition analysis

The proximate composition of dietary ingredients, diets, and whole-body fish was analyzed following standard AOAC methods. Dry matter was measured by drying samples at 105 °C until a constant weight was achieved. Ash content was determined through incineration in a muffle furnace at 450 °C for 16 hours. Protein content was analyzed using the Kjeldahl method (N × 6.25) with Kjeltec digestion and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026). Total lipid content was quantified via petroleum ether extraction using a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046). Energy content was determined by direct combustion in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261). Cellulose, hemicellulose, and lignin contents were analyzed following the method outlined by Vieira et al. (Vieira et al., 2023).

2.7 Plasma Metabolites

Plasma glucose (REF. 1001191), cholesterol (REF. 1001090), triglycerides (REF. 1001312), and phospholipids (REF. 1001140) levels were determined using enzymatic colorimetric kits (Spinreact, Girona, Spain), following the manufacturer's protocols. The absorbances of all samples were read on a microplate reader (Multiskan GO Model5111 9200, Thermo Scientific, Nanjing, China).

2.8 Enzymatic activity

Enzymatic analyses were performed at 37 °C, and absorbance changes were monitored using a microplate reader (Multiskan GO Model 5111 9200, Thermo Scientific, Nanjing, China). Enzymatic activity was reported as milliunits (GPx, GR, and G6PDH) or units (CAT) per milligram of soluble protein, which was quantified using the Bradford method (Bradford, 1976).

2.8.1 Intermediary metabolism

The liver samples were homogenized as described by Estevão-Rodrigues et al. (2024). Aspartate (ASAT/GOT, EC 2.6.1.1) and alanine aminotransferase (ALAT/GPT, EC 2.6.1.2) activities were assessed using commercial kits (Spinreact, ASAT/GOT: 41,273; ALAT/GPT: 41,283) adapted for fish by Diógenes et al. (2019). Malic enzyme (ME, EC 1.1.1.40), fatty acid synthase (FAS, EC 2. 3.1.38), 3-hydroxyacyl-CoA dehydrogenase

(HOAD, EC 1.1.1.35), hexokinase (HK, EC 2.7.1.1), glucokinase (GK, EC 2.7.1.2), and pyruvate kinase (PK; EC 2.7.1.40) activities were measured as described by Diógenes et al. (2019).

2.8.2 Oxidative stress

Liver and whole intestine samples were homogenized as described by Estevão-Rodrigues et al. (2024). Antioxidant enzyme activity analyses were performed as described by Diógenes et al. (2019). Catalase (CAT; EC 1.11.1.6) activity was determined by the reduction in H_2O_2 concentration at 240 nm. Glutathione peroxidase (GPx; EC 1.11.1.9) and glutathione reductase (GR; EC 1.11.1.9) were analyzed by measuring NADPH oxidation at 340 nm, while glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was evaluated based on $NADP^+$ reduction at the same wavelength.

2.9 Lipid peroxidation

Lipid peroxidation was measured by the malondialdehyde (MDA) concentration at 535 nm absorbance, with results expressed as nmol MDA g^{-1} tissue (Diógenes et al., 2019). Homogenate supernatants were reacted with a solution containing trichloroacetic acid, thiobarbituric acid, and butylated hydroxytoluene, heated at 100 °C for 15 minutes, cooled to room temperature, and centrifuged. The absorbance of the supernatant was then measured at 535 nm. MDA levels were expressed as nmol MDA per g of wet tissue, calculated using a calibration curve.

2.10 Statistical Analysis

Data were assessed for normality and variance homogeneity using the Kolmogorov-Smirnov and Levene's tests, respectively. Normalization procedures were applied when needed. Specific non-orthogonal contrast analyses were used to compare each test diet versus the control, BSG versus BSG-SSF diets, dietary BSG inclusion levels (10% versus 20%), and the interaction between BSG and SSF levels. All statistical analyses were performed using *IBM SPSS Statistics* software version 26 (IBM, NY, USA).

3 Results

Fish readily accepted all diets, and the dietary treatments did not significantly affect voluntary feed intake (**Table 3. 2**). Mortality was low and not significantly influenced by the dietary treatments. No fish died in the control and 10BSG groups. In the 10BSG-SSF, 20BSG, and 20BSG-SSF groups, one, three, and two fish died, respectively.

Irrespective of incorporation levels, the BSG diets led to a significant reduction of final body weight (FBW), weight gain (WG), daily growth index (DGI), feed efficiency (FE), and protein efficiency ratio (PER) compared to the control diet. In contrast, dietary BSG-SSF inclusion did not significantly reduce growth and feed utilization, with the 10% BSG-SSF diet promoting a better performance than the control diet. Daily nitrogen per kilogram of body weight was not significantly affected by the BSG level but increased with fermentation, while the opposite was observed for energy retention.

Table 3. 2. Growth performance of European seabass fed the experimental diets

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF	SEM
Final body weight (g)	92.8	85.4	100.0	82.9	89.9	1.68
Weight gain (WG, g kg ABW^{-1} day^{-1})	9.14	8.03	10.1	7.42	8.71	0.26
Daily Growth Index (DGI)[1]	1.28	1.10	1.45	1.01	1.21	0.04
Feed intake (FI, g kg ABW^{-1} day^{-1}) [2]	12.8	13.1	12.6	12.0	11.5	0.24
Feed efficiency (FE)[3]	0.71	0.62	0.81	0.64	0.76	0.02
Protein efficiency ratio (PER)[4]	1.58	1.36	1.72	1.34	1.64	0.05
Nitrogen retention (NR, g kg ABW^{-1} day^{-1})[5]	0.24	0.22	0.26	0.21	0.24	0.01
Energy retention (ER, kJ kg ABW^{-1} day^{-1})[5]	26.8	28.9	34.1	27.4	26.9	1.16
Survival (%)	100	100	97.9	93.8	95.8	0.99

Non-orthogonal contrasts	**FBW**	**WG**	**DGI**	**FI**	**PER**	**FE**	**NR**	**ER**	**Survival**
Control vs 10BSG	**0.013***	**0.009***	**0.010***	0.862	**0.028***	**0.039***	0.337	0.387	0.496
Control vs 10BSG-SSF	**0.016***	**0.018***	**0.015***	0.851	0.124	**0.042***	0.229	**0.038***	0.060
Control vs 20BSG	**0.002***	**0.001***	**0.001***	0.512	**0.022***	0.088	0.159	0.718	0.188
Control vs 20BSG-SSF	0.267	0.241	0.245	0.053	0.453	0.261	0.766	0.495	0.423
BSG vs BSG-SSF	**0.000***	**0.000***	**0.000***	0.212	**0.000***	**0.000***	**0.008***	0.439	0.998
10% vs 20%	**0.005***	**0.002***	**0.002***	0.071	0.480	0.656	0.088	**0.011***	0.073
Interaction (BSG vs SSF)	0.055	0.130	0.105	0.440	0.602	0.278	0.272	0.228	0.341

Values are presented as means (n=3) and pooled standard error of the mean (SEM). *Denotes significant differences (non-orthogonal contrast analyses; $P<0.05$). IBW or FBW: initial or final body weight; ABW, average body weight: (IBW + FBW)/2. [1]DGI: ((final body $weight^{1/3}$ - initial body $weight^{1/3}$)/ number of days) x 100 [2]Feed Intake: ((total intakex1000)/ABW) × number of days)). [3]FE: (wet weight gain / dry feed intake) [4]PER: (wet weight gain/crude protein intake) [5]NR and ER (g or kJ kg ABW^{-1} day^{-1}): ((FBW × carcass N or energy content) - (IBW × carcass N or energy content)) / (ABW × number of days).

Dietary treatments had no statistically significant effect on the whole-body dry matter and protein content of European seabass (**Table 3. 3**). Lipid and energy contents were significantly higher in fish fed the 10% than in the 20% BSG diets, regardless of fermentation. Whole-body ash content was significantly higher in fish fed the 20% BSG diets, irrespective of fermentation and, regardless of inclusion level, in BSG-SSF than in BSG diets. The energy content was significantly higher in fish fed the 20% than in the

10% BSG diets, irrespective of fermentation. The HSI was significantly higher in fish fed the 10% than the 20% BSG diets, regardless of fermentation, and it was also significantly higher in fish fed the 10BSG-SSF diet than in the control and 10BSG diets. VI was not significantly affected by BSG incorporation level or fermentation but was higher in fish fed the 10BSG diet compared to the control diet.

Table 3. 3. Whole-body composition (% wet weight) of European seabass fed the experimental diets

	Initial	**Control**	**10BSG**	**10BSG-SSF**	**20BSG**	**20BSG-SSF**	**SEM**
Dry matter, DM	28.5	30.0	32.7	31.5	31.4	29.7	0.45
Protein	15.3	15.9	16.1	16.2	16.2	16.1	0.12
Lipids	6.82	9.85	12.4	11.3	10.9	8.76	0.46
Energy (kJ g^{-1})	6.47	7.51	8.30	8.28	7.90	7.32	0.15
Ash	3.77	3.87	3.71	4.06	4.16	4.42	0.07
HSI[1]	ND	0.96	0.99	1.23	0.96	1.09	0.02
VI[2]	ND	8.88	10.49	10.28	9.20	9.13	0.20

Non-orthogonal contrast	**DM**	**Protein**	**Lipids**	**Energy**	**Ash**	**HSI**	**VI**
Control vs 10BSG	0.326	0.707	0.071	0.075	0.295	0.831	**0.039***
Control vs 10BSG-SSF	0.091	0.565	0.296	0.105	0.356	**0.007***	0.123
Control vs 20BSG	0.282	0.641	0.411	0.382	0.124	0789	0.412
Control vs 20BSG-SSF	0.368	0.686	0.413	0.682	**0.008***	0.788	0.683
BSG vs BSG-SSF	0.187	0.921	0.087	0.301	**0.027***	0.068	0.495
10% vs 20%	0.154	0.953	**0.044***	**0.039***	**0.007***	**0.014***	0.088
Interaction (BSG vs SSF)	0.819	0.851	0.614	0.443	0.744	**0.038***	0.923
10BSG-SSF vs 10BSG	0.153	0.532	0.074	0.091	0.721	**0.001***	0.608
20BSG-SSF vs 20BSG	0.820	0.584	0.423	0.305	0.757	0.998	0.923

Values are presented as mean (n = 3) and pooled standard error of the mean (SEM). *Denotes significant differences (non-orthogonal contrast analyses; $P<0.05$). [1]Hepatosomatic index: (Fish weight/Liver weight) X 100 [2]Visceral index: (Fish weight/Visceral weight) X 100

Plasma cholesterol levels were not significantly affected by diet composition (**Table 3. 4**). Further, compared to the control, plasma metabolites were unaffected at a 10% BSG

inclusion level, whether unfermented or fermented, except for a significant reduction in phospholipid levels in the BSG-SSF group. At a 20% inclusion level, independently of fermentation, plasma glucose, phospholipid, and triglyceride (only in the BSG diet) levels decreased.

Table 3. 4. Plasmatic metabolites levels (mg dl^{-1}) of European seabass fed the experimental diets.

	Control	10BSG	10BSG-SSF	20BSG	20BSG-SSF	SEM
Glucose	135.5	120.8	131.1	105.5	109.4	3.08
Cholesterol	104.7	90.2	97.0	91.9	93.3	2.45
Triglycerides	799.3	690.0	874.3	562.0	686.8	34.35
Phospholipids	1146.7	904.9	1118.6	699.6	709.1	41.55

Non-orthogonal contrast	Glucose	Cholesterol	Triglycerides	Phospholipids
Control vs 10BSG	0.087	0.068	0.299	**0.024***
Control vs 10BSG-SSF	0.599	0.448	0.474	0.786
Control vs 20BSG	**0.001***	0.149	**0.028***	**0.000***
Control vs 20BSG-SSF	**0.004***	0.107	0.285	**0.000***
BSG vs BSG-SSF	0.245	0.538	**0.036***	0.155
10% vs 20%	**0.004***	0.753	**0.032***	**0.000***
Interaction (BSG vs SSF)	0.688	0.477	**0.038***	0.121
10BSG vs 10BSG-SSF	0.057	0.61	0.075	0.064
20BSG vs 20BSG-SSF	0.61	0.43	0.223	0.074

Values are presented as mean (n = 9) and pooled standard error of the mean (SEM). *Denotes significant differences (non-orthogonal contrast analyses; $P<0.05$).

The diet composition did not significantly affect the activity of GDH, HK, HOAD, and FAS (**Table 3. 5**). Fermentation significantly decreased the activities of ASAT, ALAT, and PK, regardless of the dietary BSG incorporation level. In contrast, independently of fermentation, GK and ME activities were significantly reduced at the higher BSG incorporation level. Compared to the control group, GK and ME activities were reduced in the 20% group, independently of fermentation, while ASAT and ALAT also decreased but only in the 20% BSG-SSF group.

Table 3. 5. Enzymatic activity (mU mg^{-1} protein) of European sea bass fed experimental diets.

	Control	**10BSG**	**10BSG-SSF**	**20BSG**	**20BSG-SSF**	**SEM**
Amino acid catabolism						
Glutamate dehydrogenase (GDH)	48.6	55.1	52.1	45.7	49.3	2.06
Aspartate aminotransferase (ASAT)	482.1	431.9	329.1	460.5	362.0	19.8
Alanine aminotransferase (ALAT)	319.9	247.2	228.0	335.4	199.3	15.6
Glycolysis						
Hexokinase (HK)	1.65	1.30	1.67	1.26	1.42	0.10
Glucokinase (GK)	5.41	4.69	5.17	4.04	3.90	0.20
Pyruvate kinase (PK)	9.72	9.71	8.85	9.47	7.91	0.25
β-Oxidation						
Hydroxyacyl Co-A dehydrogenase (HOAD)	14.76	14.18	13.55	14.78	13.74	0.45
Lipogenesis						
Malic enzyme (ME)	4.86	4.94	3.83	3.42	3.33	0.19
Fatty acid synthase (FAS)	0.81	0.83	1.84	0.92	1.30	0.17

Non-orthogonal contrast	*Amino acid catabolism*			*Glycolysis*			*β-Oxidation*	*Lipogenesis*	
	GDH	**ASAT**	**ALAT**	**HK**	**GK**	**PK**	**HOAD**	**ME**	**FAS**
Control vs 10BSG	0.339	0.406	0.126	0.301	0.231	0.984	0.697	0.876	0.975
Control vs 10BSG-SSF	0.602	**0.014***	**0.049***	0.947	0.692	0.147	0.417	0.052	0.066

Control vs 20BSG	0.676	0.720	0.733	0.241	**0.015***	0.744	0.991	**0.008***	0.852
Control vs 20BSG-SSF	0.911	0.051	**0.011***	0.479	**0.026***	**0.020***	0.494	**0.005***	0.371
BSG vs BSG-SSF	0.950	**0.022***	**0.022***	0.272	0.685	**0.016***	0.455	0.106	0.076
10% vs 20%	0.211	0.471	0.365	0.543	**0.027***	0.380	0.716	**0.009***	0.560
Interaction (BSG vs SSF)	0.493	0.959	0.079	0.647	0.459	**0.000***	0.852	0.170	0.422
10BSG vs 10BSG-SSF	0.066	0.80	0.060	0.35	0.67	0.153	0.14	0.68	0.28
20BSG vs 20BSG-SSF	0.07	0.87	0.31	0.12	0.75	**0.044***	0.16	0.91	0.12

Values are presented as means (n=9) and pooled standard error of the mean (SEM). *Denotes significant differences (non-orthogonal contrast analyses; $P<0.05$).

In the liver, diet composition did not significantly affect CAT activity, except for a decreased activity in the 20%BSG-SSF group compared to the control group (**Table 3. 6**). G6PDH activity decreased with increasing BSG levels, regardless of fermentation, while fermentation increased G6PDH activity, irrespective of BSG level. SSF significantly decreased GR activity independently of dietary BSF inclusion levels, which was lower in the 20%BSG-SSF group than in the control group. SSF also significantly decreased LPO levels, independently of dietary BSF inclusion level, which was lower in the 20%BSG-SSF group than in the control group.

Table 3. 6. Enzymatic activity of European sea bass fed experimental diets.

	Control	**10BSG**	**10BSG-SSF**	**20BSG**	**20BSG-SSF**	**SEM**
Liver						
Catalase (CAT, U mg^{-1} protein)	153.9	141.4	134.7	138.1	120.5	3.41
Glutathione reductase (GR, mU mg^{-1} protein)	2.20	2.14	1.86	2.61	1.14	0.16
Glucose 6-phosphate dehydrogenase (G6PDH, mU mg^{-1} protein)	171.0	128.9	153.5	90.4	111.7	6.14
Lipid peroxidation (LPO, nmol MDA g^{-1} tissue)	20.14	28.12	23.82	24.69	20.02	0.92
Intestine						
Catalase (CAT, U mg^{-1} protein)	232.6	200.6	144.2	170.0	140.2	10.90
Glutathione reductase (GR, mU mg^{-1} protein)	24.24	23.14	16.67	22.01	17.45	0.96
Glutathione peroxidase (GPx, mU mg^{-1} protein)	36.09	36.62	20.29	48.24	31.67	3.15
Glucose 6-phosphate dehydrogenase (G6PDH, mU mg^{-1} protein)	12.18	10.54	15.18	9.57	14.02	0.85
Lipid peroxidation (LPO, nmol MDA g^{-1} tissue)	14.18	10.89	9.71	19.07	14.17	0.66

Non-orthogonal contrast	*Liver*				*Intestine*				
	CAT	GR	G6PDH	LPO	CAT	GR	GPx	G6PDH	LPO
Control vs 10BSG	0.221	0.903	**0.006***	**0.005***	0.318	0.695	0.956	0.536	**0.032***
Control vs 10BSG-SSF	0.064	0.488	0.233	0.175	**0.008***	**0.010***	0.114	0.259	**0.004***
Control vs 20BSG	0.124	0.403	**0.000***	0.096	0.055	0.428	0.207	0.326	**0.002***

Control vs 20BSG-SSF	**0.002***	**0.034***	**0.000***	0.965	**0.006***	**0.019***	0.643	0.487	0.995
BSGF vs BSG-SSF	0.096	**0.014***	**0.030***	**0.022***	0.061	**0.008***	**0.022***	**0.019***	**0.006***
10% vs 20%	0.226	0.709	**0.000***	0.062	0.444	0.927	0.104	0.569	**0.000***
Interaction (BSG vs SSF)	0.448	0.089	0.868	0.923	0.556	0.633	0.986	0.958	0.084

Values are presented as means (n=9) and pooled standard error of the mean (SEM). *Denotes significant differences (non-orthogonal contrast analyses; $P<0.05$).

In the intestine, CAT and GR activities were lower in fish fed the BSG-SSF diets than in the control (**Table 3. 6**). Independently of dietary inclusion level, GR and GPX activities were also lower in fish fed the BSF-SSF than in the BSG diets. LPO levels were also lower in fish fed the BSG-SSF than the BSG diets, but it increased with the dietary inclusion level.

4 Discussion

Low-protein, plant-derived ingredients are nutritionally challenged for use in feed for carnivorous fish, such as European seabass (trophic level 3.5 ± 0.5, FishBase), due to their high fiber content (Petereit et al., 2022). Results of this study showed that dietary incorporation of BSG reduced the growth performance of European seabass juveniles independently of the incorporation level. Differently, in gilthead seabream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*), dietary incorporation levels of BSG up to 10–15% did not impair growth performance (Estévez et al., 2021, 2022). On the other hand, for omnivorous fish species, such as striped catfish (*Pangasianodon hypophthalmus*) and Nile tilapia (*Oreochromis niloticus*), dietary BSG levels as high as 50% have been successfully used without adversely affecting fish performance (Jayant et al., 2018; Zerai et al., 2008).

The lower palatability of the diets, and consequently a reduced feed intake, can not explain the decreased growth performance of European seabass juveniles, as diet composition did not affect feed intake. The higher cellulose content of the BSG diets contributes to explaining the lower energy available for growth, as feed intake was not adjusted to the dietary energy intake, as is generally observed in fish (Cho & Kaushik, 1990), including European seabass. Indeed, it was previously shown that European seabass fed a 20% cellulose-supplemented diet increased voluntary feed intake, matching the digestible energy intake to the non-supplemented diet (Dias et al., 1998).

Solid-state fermentation has emerged as a promising strategy to address the challenges of utilizing low-valued agro-industrial by-products as feedstuffs (Cao et al., 2021; Ghosh & Mandal, 2015; Hassaan et al., 2015). In the present study, BSG-SSF significantly enhanced growth performance and feed utilization of European seabass compared to the unfermented product and promoted a growth performance similar to the control diet with dietary incorporation of 20%. Moreover, dietary incorporation of 10% BSG-SSF inclusively promoted a higher growth performance and feed utilization than the control group. These results may be attributed to the improved nutritional profile and nutrient digestibility of BSG-SSF compared to BSG. Indeed, a previous study showed that SSF of BSG reduced cellulose and hemicellulose content and increased crude protein content and the apparent digestibility of dry matter, lipids, and energy (Estevão-Rodrigues et al., 2024). Additionally, the dietary methionine content of BSG-SSF was higher than that of BSG, which was reflected in a dietary methionine content similar to that of the control diet. Previously, in European seabass, it was also shown that dietary incorporation of 20% SSF plant feedstuff mixture (rapeseed, soybean sunflower, rice bran, 25% each) with *A. niger* increased feed utilization efficiency and energy digestibility without compromising growth (Vieira et al., 2023). Also, in rohu (*Labeo rohita*) fingerlings, the dietary inclusion of 40% solid-fermented groundnut oil cake with yeast (*Pichia kudriavzevii*) increased the growth and PER (Ghosh & Mandal, 2015). Similarly, in gibel carp (*Carassius auratus gibelio*), a low inclusion level (3%) of plant ingredients (sprayed corn husk, rapeseed meal, soybean meal, palm meal, and rice bran) fermented with a *Lactobacillus* spp. and *Bacillus* spp. enhanced growth performance (Cao et al., 2021). For Nile tilapia, up to 37% of fishmeal in the diet can be replaced with fermented soybean meal; however, higher replacement levels reduced growth and feed utilization efficiency (Hassaan et al., 2015).

Body composition is a valuable indicator of animal nutritional status, reflecting the balance between nutrients and energy intake, expenditure, and storage (Jobling, 2001). In the current study, the body composition of fish fed the experimental diets was similar to that of fish fed the control diet. Similarly, the inclusion of a 20% SSF plant-based ingredient mixture in the diet had no impact on the body composition of European seabass (Vieira et al., 2023). However, in the test diets, whole-body lipid and energy content and HSI decreased with the increased dietary inclusion of BSG and BSG-SSF. HSI was also higher in fish fed a 10% SSF-BSG diet compared to the 10% BSG diet. This seems related to the increased cellulose content in the diets, leading to lower available energy to be deposited as lipids in viscera and muscle. Similar increases in HSI have been reported in largemouth bass (*Micropterus salmoides*) and African catfish

(*Clarias gariepinus*) fed diets containing non-fermented and fermented ingredients (Enyidi & Etim, 2020; Li et al., 2020), while other studies reported the opposite effects, such as in Furong crucian carp (Wang et al., 2024), or found no effects, as observed in rainbow trout (Moniruzzaman et al., 2018).

Plasma metabolites are also important biomarkers of fish nutritional status. In the present study, plasma glucose levels were reduced in fish fed the 20% BSG and BSG-SSF diets, likely due to the lack of wheat meal, the main source of starch (glucose) in the experimental diets. However, fermentation of ingredients has been shown to enhance plasma glucose levels, as observed in European seabass (Vieira et al., 2023) and rainbow trout (Davies et al., 2021), suggesting that fermentation increases carbohydrate bioavailability. In the present study, fermentation of BSG did not affect plasma glucose levels despite reductions in dietary cellulose and hemicellulose content. While plasma cholesterol was unaffected by diet composition, plasma triglycerides, and phospholipids were lower in the BSG diets and decreased with dietary levels, which may be related to dietary fiber content. The impact of dietary fiber on plasma lipid parameters in European seabass remains inconsistent. While some studies reported no effect on plasma cholesterol and triglycerides (Bonvini et al., 2018), others observed reductions in both parameters (Zhong et al., 2020; Diógenes et al., 2019). Fermentation of BSG increased the plasma triglycerides, probably due to the increased lipid digestibility observed in BSG-SSF diets (Estevão-Rodrigues et al., 2024).

The dietary inclusion of BSG decreased the PER and N retention, which indicates increased amino acid catabolism. However, no changes in hepatic GDH activity, which is the primary amino acid deaminase, were observed. However, ALAT and ASAT activities were higher in fish fed the BSG diets than the BSG-SSF diets, suggesting an increased need for amino acid interconversion to compensate for eventual amino acid imbalances in the BSG diets. In contrast, some studies have reported increased (Manna et al., 2022) or unchanged (Samad et al., 2022; Vieira et al., 2023) hepatic transaminase activity in fish-fed diets containing fermented ingredients.

While hepatic β-Oxidation (HOAD) and lipogenesis (FAS) enzyme activities were unaffected by the diet composition, GK and PK activity decreased with the dietary BSG and BSG-SSF levels, which align with the reduced plasma glucose levels observed in fish fed these diets and, therefore, the lower available glucose for glycolysis (Gatesoupe et al., 2014). ME activity, a NADPH-generating enzyme, was not affected by fermentation but was reduced with increasing BSG levels, which may be attributed to the reduced energy availability in diets with higher BSG inclusion. In seabass, a positive relationship between dietary digestible energy levels and ME and FAS activity has been reported

(Dias et al., 2003). In seabass and *Labeo rohita*, a positive relationship was also observed between dietary incorporation of fermented ingredients ME and FAS activity, suggesting higher energy availability (Vieira et al., 2023; Ghosh & Mandal, 2015). However, in this study, FAS activity remained unaffected.

The antioxidant system of animals is composed of enzymatic and non-enzymatic mechanisms that interact to regulate the balance between the generation and elimination of reactive oxygen species (Regoli & Giuliani, 2014). Several endogenous and exogenous factors can disrupt this balance, increasing susceptibility to oxidative damage of lipids, proteins, and DNA (Chowdhury & Saikia, 2020). Among these, exogenous factors such as abiotic parameters and diet composition are highly relevant. In the present study, dietary incorporation of BSG did not affect hepatic LPO levels but increased intestinal LPO levels. However, in both tissues, regardless of the inclusion level, fermentation of BSG restored LPO levels, decreased GR and GPX activities (GPX only measured in the intestine), and increased G6PDH activity. Compared to the control group, hepatic LPO levels were unaffected by diet composition (except for fish fed the 10BSG diet), while LPO levels were lower in fish fed the BSG-SSF diets than the BSG diets. On the other hand, in the intestine, LPO levels were lower in fish fed the 10% BSG and BSG-SSF diets than in the control group, while LPO levels were also lower in fish fed the BSG-SSF diet than the BSG diets.

Fermentation of BSG has been reported to release polyphenolic and other antioxidant compounds, including those present in BSG (Fernandes et al., 2022) and other by-products (Ibarruri et al., 2019) enhancing the antioxidant potential of fermented ingredients and mitigating oxidative damage in species such as European seabass (Amaral et al., 2023), Coho Salmon (*Oncorhynchus kisutch*) (Zhang et al., 2023) and Nile tilapia (Hao et al., 2025). As observed in this study, another study on European seabass also reported decreased hepatic LPO levels in diets including SSF ingredients without affecting antioxidant enzyme activity (Ferreira et al., 2024), suggesting that the antioxidant benefits of SSF of BSG may not be attributed to enzymatic antioxidant pathways but rather to the improved nutritional profile of BSG, with a reduction in cellulose and hemicellulose content and an increase in antioxidant levels.

The increased intestinal LPO levels observed with BSG diets may be due to their higher fiber content, which affects the intestinal oxidative status without triggering a full antioxidant response, as seen in sea bream and rainbow trout (Deng et al., 2021; Diógenes et al., 2019). The impact of dietary fiber on intestinal oxidative status appears to depend on its type and inclusion level (Montagne et al., 2003). Oxidative damage associated with excessive dietary fiber may be mitigated by hydrolyzing the fiber, as

observed with the addition of exogenous enzymes to the diet in carp (Monier, 2020) and sea bream (Ayhan et al., 2008) or through pre-treatment processes like fermentation, as observed in the present study.

5 Conclusions

Dietary inclusion of BSG led to reduced growth, feed utilization efficiency, plasma glucose, triglycerides, and a decrease in hepatic glucokinase (GK) and malic enzyme (ME) activity. Solid-state fermentation (SSF) of BSG alleviated these limitations, improving growth and overall feed and protein utilization. While BSG impaired liver and intestine oxidative status, SSF-BSG countered this effect in both tissues.

Overall, the results of this study indicate that BSG-SSF can be included in up to 20% of diets for European seabass juveniles without affecting growth, feed utilization, and hepatic and intestine oxidative status, thus alleviating the negative results observed with the dietary inclusion of BSG. These results suggests that BSG-SSF could be a valuable alternative to more traditional feed ingredients, offering both environmental and economic benefits.

Further studies are needed to fine-tune the incorporation levels of BSG-SSF for different fish species and to explore its long-term effects on fish well-being and health, ultimately contributing to enhanced sustainability in the aquaculture industry.

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Chapter 4:

Solid-State Fermentation of Brewer's Spent Grain Impacts Meagre (Argyrosumus regius) Growth, Intermediary Metabolism, Digestive Enzymes, Oxidative Stress, and Intestinal Histology

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Solid-state fermentation of Brewer's Spent Grain Impacts Meagre (*Argyrosumus regius*) Growth, Intermediary Metabolism, Digestive Enzymes, Oxidative Stress, and Intestinal Histology

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Abstract: It was previously observed that solid-state fermentation (SSF) with *Aspergillus ibericus* increased brewer's spent grain (BSG) protein content (21%) while reducing lipid (49%), cellulose (30%), hemicellulose (34%), and lignin (7.3%) contents. This study aimed to evaluate the potential of fermented BSG (BSG-SSF) as a novel feed ingredient for meagre (*Argyrosomus regius*) juveniles by evaluating growth performance, digestive function, intermediary metabolism, oxidative status, and intestinal histomorphology. Three diets were formulated: a control diet without BSG (15% fish meal and 66% plant ingredients) and two diets incorporating 10% BSG or BSG-SSF, replacing 14% of the plant ingredients. The diets were tested in triplicate for 10 weeks with meagre averaging 83 g initial weight. The dietary inclusion of BSG reduced fish growth performance and feed and protein utilization efficiency, while feed intake was unaffected. However, including BSF-SSF restored these parameters to the control diet levels. At the end of the trial, no significant differences were observed in whole-body composition, plasma metabolite profile, and intermediary metabolism enzyme activity. Hepatic and intestinal antioxidant enzyme activity and lipid peroxidation also remained similar among diets. However, the dietary inclusion of BSG and BSG-SSF negatively impacted anterior and distal intestine histomorphology compared to the control. Moreover, the mean histomorphological score was similar between the BSF and BSG-SSF diets in the

anterior intestine, while in the distal intestine, BSG diet scored higher than the BSG-SSF diet. Overall, the present results indicate that BSG-SSF could be included in meagre juvenile diets by up to 10% without affecting fish growth performance or oxidative status. This contributes to promoting aquaculture sustainability by valorizing agro-industrial by-products. Further efforts need, however, to be made to mitigate the negative impacts on intestinal histomorphology induced by dietary BSG inclusion.

Keywords: agro-industrial by-products, biocircularity, fermentation, meagre, metabolism, sustainability.

1 Introduction

Meagre (*Argyrosomus regius*) is a fast-growing marine fish distributed across temperate and subtropical waters of the Eastern Atlantic (Quero & Vayne, 1985; Champagnat et al., 1979). Valued for its high market demand, exceptional nutritional quality, low fillet fat content, and rapid growth (reaching 1.2 kg in less than two years), meagre has become an important species for diversifying European aquaculture (Soares et al., 2018; Fountoulaki et al., 2017; Duncan et al., 2012). Advances in breeding, farming protocols, and feed formulations have significantly contributed to its domestication and increasing production, particularly along the Mediterranean coast in countries like Greece, Spain, Croatia, and Portugal (FAO, 2024; Duncan et al., 2012). Annual meagre production exceeds 11,500 tonnes (FAO, 2024).

As a high-trophic-level species (4.3, according to Fishbase), meagre has a high dietary protein requirement, reaching up to 50% during early life stages (Kružić et al., 2016). Due to its high protein content, balanced amino acid profile, digestibility, and palatability, Fishmeal (FM) has traditionally been the primary protein source in carnivorous fish diets like meagre (Oliva-Teles et al., 2024). However, declining fish stocks, rising costs, and sustainability concerns have led to a reduction in FM inclusion in aquafeeds, including meagre diets (Macusi et al., 2023). Alternatives such as plant proteins, insects, microalgae, macroalgae, and animal by-products have been explored, but challenges such as reduced growth performance at high dietary inclusion levels persist (Matias et al., 2024; Couto et al., 2016; Ribeiro et al., 2015).

High-valued plant-based ingredients, such as soy protein concentrate, wheat, and corn gluten, are considered promising FM alternatives for meagre (Ribeiro et al., 2015). However, using plant proteins in aquafeeds raises environmental and economic sustainability concerns. Large-scale production often involves high water and land use, deforestation, and long-distance transportation, increasing the environmental footprint. Additionally, competition with human consumption can limit availability and increase

prices. Agricultural by-products, however, offer a sustainable alternative by reducing nutrient loss and minimizing environmental impact, besides contributing to biocircularity (van Zanten et al., 2023; Zhang et al., 2023). Brewer's spent grain (BSG), a by-product of the beer industry, is a promising candidate due to its availability and nutrient content (Pandey et al., 2023; Mitri et al., 2022; Mukasafari et al., 2018; Faccenda et al., 2017). While BSG has been successfully used in omnivorous fish diets like catla (*Catla catla*) and rohu (*Labeo rohita*) at inclusion levels up to 30% (Kaur & Saxena, 2004), its high fiber content (up to 50% dry matter) limits its use in carnivorous fish such as gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*), where inclusion levels above 20% reduce growth, feed efficiency, and digestibility (Estevão-Rodrigues et al., 2024; Estévez et al., 2022).

To enhance the use of BSG as an alternative ingredient for carnivorous species, technological treatments such as enzymatic hydrolysis and solid-state fermentation (SSF) have been explored (San Martin et al., 2020; Estevão-Rodrigues et al., 2024). SSF, in particular, has already been shown to improve BSG's nutritional profile (Ibarruri et al., 2019; Canedo et al., 2016). Indeed, SSF of BSG with *Aspergillus ibericus* increased protein content by 21% while reducing cellulose (30%), hemicellulose (34%), and lignin (7.3%) contents. Additionally, SSF added carbohydrase activity and increased the antioxidant levels of SSF-BSG. In European seabass, dietary incorporation of SSF-BSG with *A. ibericus* improved the animal's nutrient digestibility, growth performance, and oxidative status (Estevão-Rodrigues et al., 2025).

Building on these findings, the present study aimed to evaluate the potential of SSF-BSG as a novel feed ingredient for juvenile meagre (*Argyrosomus regius*). The research compared the effects on growth performance, digestive function, intermediary metabolism, oxidative status, and intestinal histomorphology of fish fed with diets including BSG, SSF-BSG and a BSG-free diet.

2 Material and Methods

2.1 Solid-state fermentation (SSF)

Brewer's spent grain (BSG), composed of barley, Pilsen malt, and Gritz corn, was provided by Unicer Bebidas de Portugal, S.A. (Matosinhos, Portugal). BSG was fermented with *Aspergillus ibericus* MUM 03.49 (Micoteca of the University of Minho, Braga, Portugal) at 25 °C for 7 days, as detailed in Estevão-Rodrigues et al. (2024). The fermented product, designated as BSG-SSF, was stored at 4 °C until diet manufacture. The nutritional composition, enzymatic activity, and antioxidant activity of BSG before and after SSF with *A. ibericus* are detailed in Estevão-Rodrigues et al. (2024).

2.2 Experimental diets

Three experimental diets (45% crude protein, 18% crude lipid) were formulated with 15% FM and 66% plant-based ingredients: a control, with no inclusion of BSG, and two other diets, including 10% BSG or BSG-SSF at the expense of plant ingredients (**Table 4. 1**). All ingredients were finely ground, mixed, and pelleted using a laboratory pellet mill (CPM: California Pellet Mill, Crawfordsville, IN, USA) through a 3.5 mm die and dried at 60 °C for 24 h.

Table 4 1. Ingredients and proximal composition of experimental diets

	Control	BSG	BSG-SSF
Ingredients (% dry matter)	—	10.0	—
BSG[1]	—	10.0	—
BSG-SSF[2]	—	—	10.0
Fish meal[3]	15.0	15.0	15.0
Pea protein concentrate[4]	10.0	10.0	10.0
Corn gluten[5]	12.5	12.5	12.5
Wheat gluten[6]	11.7	4.6	4.6
Plant meal mixture[7]	31.8	28.2	28.2
Hydrolyzed shrimp[8]	1.2	1.2	1.2
Fish oil	14	14.4	14.4
Vitamin premix[9]	1	1	1
Choline chloride (50%)	0.5	0.5	0.5
Mineral premix[10]	1	1	1
Binder[11]	1	1	1
Dicalcium phosphate	0	0.1	0.1
Methionine[12]	0.1	0.1	0.1
Taurine[12]	0.3	0.3	0.3
Proximate composition (% dry matter)			
Dry Matter (%)	88.5	87.3	87.7
Crude protein	45.3	45.6	46.9
Crude lipid	18.0	17.8	17.8
Gross energy (kJ g^{-1} DM)	23.1	23.2	23.7
Ash	6.33	6.84	6.49
Cellulose	4.21	6.20	4.71
Hemicellulose	2.54	2.43	1.85
Essential amino acids (% dry matter)			
Lysine, Lys	2.17	1.94	2.26
Arginine, Arg	1.63	1.67	1.62
Histidine, His	0.71	0.74	0.89
Isoleucine,Ile	1.30	1.29	1.30

Leucine,Leu	5.24	5.53	5.66
Valine, Val	1.33	1.34	1.45
Methionine, Met	1.20	0.97	0.95
Phenylalanine, Phe	2.07	2.00	1.97
Threonine, Thr	1.14	1.16	1.34
Non-essential amino acids (% dry matter)			
Tyrosine, Tyr	0.78	0.83	0.83
Aspartic acid, Asp	2.77	2.76	2.75
Glutamic acid, Glu	3.91	4.01	3.96
Serine, Ser	4.42	5.07	4.79
Glycine, Gli	5.53	5.39	5.37
Alanine, Ala	4.93	5.22	4.91
Proline, Pro	5.06	5.31	5.37

[1]All the ingredients were source by Sorgal, S.A. Ovar, Portugal. Brewer's spent grain was sourced by Unicer-Bebidas de Portugal, SA. Matosinhos, Portugal

[2]Fermented brewer's spent grain; detailed composition Estevão-Rodrigues et al (2024)(2024)

[3] Pesquera Centinela, Steam Dried LT, Chile (CP: 66.8; CL: 9,6).

[4]Pea protein concentrate (CP:45.1%, CL: 2.84%)

[5] Corn gluten (CP:80.1%; CL:4.07%).

[6] Wheat gluten (CP: 74.7%; CL: 1.74%).

[7]Plant ingredient mixture – Soybean meal : rapeseed meal : sunflower meal (2.5:1:1); Soybean meal (CP: 44.9%; CL: 1.4%), Rapeseed (CP: 34.6%; CL: 2.6%), Sunflower (CP: 28.5%; CL: 1.48%)

[8] Hydrolyzed shrimp (CP: 69.8%; CL: 12.1%). 8

[9]Vitamin premix (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

[10] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).

[11] Aquacube. Agil, UK.

[12] Feed grade amino acids, Sorgal, S.A. Ovar, Portugal

2.3 Growth Trial

The CIIMAR ORBEA Animal Welfare Committee (ORBEA) and the Portuguese National Authority for Animal Health (DGAV; reference ORBEA-CIIMAR-27-2019) approved the growth trial.

Meagre juveniles were provided by Instituto Português do Mar e da Atmosfera (IPMA; Olhão, Portugal) and transported to the Aquatic Organisms Bioterium at CIIMAR (Matosinhos, Portugal). The fish were transferred to the experimental system after the required quarantine period (15 days). The experimental system consisted of nine 300 L

fiberglass tanks equipped with a thermo-regulated recirculating water system (water flow rate of 10 L min^{-1}). Following a 30-day acclimatization to the rearing conditions, 9 meagre juveniles (initial body weight of 84 ± 1 g) were randomly distributed to each tank. Each experimental diet was randomly assigned to triplicate groups, and the fish were fed by hand to apparent satiety twice daily, six days a week, for 68 days. At the end of the trial, the fish were bulk-weighed under light anesthesia (0.3 mL L^1 ethylene glycol monophenyl ether) after 24 hours of feed deprivation. Throughout the trial, water quality parameters were monitored daily, including temperature (22 ± 0.5°C), salinity (27 ± 3‰), dissolved oxygen (above 7 mg L^{-1}), and pH (8 ± 0.5). Ammonia and nitrite levels were measured weekly (> 0.02 mg L^{-1}). The photoperiod was maintained at 12 hours light and 12 hours dark.

2.4 Sampling

For whole-body analysis, a sample of five fish from the initial stock and four fish per tank at the end of the growth trial were collected. The fish were euthanized by an overdose of anesthetic (10 mL L^{-1} ethylene glycol monophenyl ether), and whole-body, viscera, and liver weights were recorded to calculate the hepatosomatic and visceral indexes. To recover from the handling stress of the growth trial sampling, the remaining fish were fed for 3 more days. Four hours after the meal, blood from 3 fish per tank was sampled by punching the caudal vein with a heparinized syringe, centrifuged (5000 g, 10 min, 4 ºC) for plasma recovery that was divided into different aliquots and kept at -80 ºC for metabolite quantification. Fish were then killed by decapitation and dissected on an iced tray, and the liver and digestive tract were carefully removed and separated of adipose and adherent connective tissue. Liver and whole intestine samples were kept at −80 °C for enzymatic activity analyses. For histological analysis, two intestinal sections were collected: one from the anterior region (immediately after the pyloric caeca) and another from the distal region (just before the anus). The samples were rinsed with saline solution, fixed in 4% phosphate-buffered formalin (pH 7.4) for 24 hours, and preserved in 70% ethanol.

2.5 Ingredients, diets and whole body composition

Bromatological composition of ingredients, diets, and pooled whole fish was performed according to AOAC methods, including dry matter (determined after drying at 105 °C for 24 h); ash content (after incineration for 6 hours at 550 °C); crude protein (N × 6.25) following the Kjeldahl method (Kjeltec digestion and distillation units; Tecator Systems, Höganäs, Sweden; models 1015 and 1026); crude lipids (continuous extraction with

petroleum ether using a Soxtec extractor Model ST 2055 Soxtec™; FOSS, Hillerød, Denmark); gross energy (total combustion in a calorimeter, PARR model 1261; PARR Instruments, Moline, IL, USA). Cellulose, hemicellulose, and lignin contents were determined as described by Estevão-Rodrigues et al. (2025).

2.6 Plasma metabolites

Plasma triglycerides (REF. 1001312), glucose (REF. 1001191), and cholesterol (REF. 1001090) were measured using Spinreact colorimetric kits (Girona, Spain), with adaptation to a microplate reader (Multiskan GO Model5111 9200, Thermo Scientific, Nanjing, China).

2.7 Enzyme activities

Liver and whole intestine samples were homogenized in an ice-cold buffer solution (100mM Tris-HCl, EDTA, Triton buffer, pH 7.8) and centrifuged at 30,000 × g for 30 minutes at 4 °C. The supernatant was then divided into aliquots and frozen at −80 °C until further analysis. Enzymatic kinetic analysis was performed at 37 °C using a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China). Enzyme activity was expressed as milliunits or units per milligram of soluble protein, determined by the Bradford method (1976).

2.7.1 Digestive enzymes

The enzymatic activity of amylase (EC 3.2.1.1) and lipase (EC 3.1.1.3) was performed using commercial kits from Spinreact, S.A. (Girona, Spain), adapted to kinetic analysis, as described by Estevão-Rodrigues et al. (2025)(2025). For amylase, the production of 2-chloro-4-nitrophenol was monitored at 405 nm, and for lipase, methyl resorufin production was monitored at 580 nm. Trypsin (EC 3.4.21.4) activities were measured according to Faulk et al. (2007) following p-nitroaniline production was monitored at 410nm.

2.7.2 Intermediary metabolism enzymes

The enzymatic activities of aspartate (ASAT/GOT, EC 2.6.1.1) and alanine aminotransferase (ALAT/GPT, EC 2.6.1.2) were assayed using commercial kits (Spinreact, ASAT/GOT; 41,273; ALAT/GPT; 41,283) adapted to microplate reader. Malic enzyme (ME, EC 1.1.1.40), fatty acid synthase (FAS, EC 2.amely3.1.38), hexokinase (HK, EC 2.7.1.1), glucokinase (GK, EC 2.7.1.2), pyruvate kinase (PK; EC 2.7.1.40), and 3-hydroxyacyl-CoA dehydrogenase (HOAD, EC 1.1.1.35) activities were quantified as described by Estevão-Rodrigues et al. (2025) .

2.7.3 Antioxidant enzymes and lipid peroxidation

Catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPx; EC: 1.11.1.9), and glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activities were quantified as described by Estevão-Rodrigues et al. (2025). Lipid Peroxidation (LPO), measured as the level of malondialdehyde (MDA), as determined as described by Estevão-Rodrigues et al. (2025) and concentration expressed as nmol MDA g^{-1} tissue.

2.8 Intestinal morphology analysis

The samples were processed according to standard procedures. Histological images were captured using Zen software (Blue edition; Zeiss, Jena, Germany) and the morphological structures were evaluated as detailed in Estevão-Rodrigues et al. (2024).

2.9 Statistical analysis

Data normality and homogeneity of variances were verified by the Shapiro-Wilk and Levene's tests and submitted to one-way analysis of variance (ANOVA), followed by Tukey's test to determine statistically significant differences ($p < 0.05$) among dietary treatments. For intestinal morphology, due to the lack of normality and homogeneity of variances, Kruskal-Wallis tests were applied, followed by pairwise comparisons with Bonferroni-adjusted p-values. The software SPSS 25.0 for Windows (IBM® SPSS Statistics, New York, USA) was used for all analyses.

3 Results

In the present study, meagre readily accepted all diets, and no mortality was observed (**Table 4. 2**). Final body weight, weight gain, and daily growth index were significantly reduced with the dietary inclusion of BSG. However, incorporating BSG-SSF restored growth to those obtained with the control diet. Feed intake was higher in fish fed with the BSG diet than the BSG-SSF diet, and feed efficiency and protein efficiency ratios were lower in the BSG group than in the control and BSG-SSF groups. Nitrogen and retention (% intake) were also higher in fish fed the BSG-SSF diet those fed the BSG diet.

Table 4 2. Growth performance of meagre fed the experimental diets

	Control	BSG	BSG-SSF	SEM
Initial Body Weight (g)	83.1	84.4	84.5	0.51
Final Body Weight (g)	152.6^{b}	134.7^{a}	147.8^{b}	2.76
Weight Gain (g/kg ABW/day)	8.66^{b}	6.75^{a}	8.01^{b}	0.29
Daily Growth Index[1]	1.44^{b}	1.09^{a}	1.32^{b}	0.05

Feed Intake (g/kg ABW/day)	9.04[ab]	10.3[b]	7.80[a]	0.37
Feed Eficiency[2]	0.96[b]	0.66[a]	1.03[b]	0.06
Protein efficiency ratio[3]	2.12[b]	1.44[a]	2.20[b]	0.12
Nitrogen retention (% nitrogen intake)	31.2[a,b]	26.1[a]	37.1[b]	1.75
Energy retention (% energy intake)	25.1[a,b]	20.3[a]	28.8[b]	1.52
Survival (%)	100	100	100	0.00

Values are presented as mean (n = 3) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters are significantly different (One-way ANOVA; Tukey test; $p<0.05$).
ABW: average body weight
[1]Daily Growth Index: ((final body weight$^{1/3}$ - initial body weight$^{1/3}$)/ number of days) x 100
[2]Feed efficiency: wet weight gain / dry feed intake)
[3]Protein efficiency ratio: (wet weight gain/crude protein intake)
[4]NR and ER (% intake): ((FBW × carcass N or energy content) - (IBW × carcass N or energy content)) / (FI × N content in feed or energy content) X 100.
Survival: (100 x dead fish)/Initial density.

At the end of the trial, no differences were observed among dietary treatments for dry matter, protein, lipids, energy, and ash content (**Table 4. 3**). The hepatosomatic and visceral indexes were also not affected by the dietary treatments.

Table 4 3. Whole-body composition of meagre fed the experimental diets

	Initial	Control	BSG	BSG-SSF	SEM
Dry matter	23.3	23.2	24.5	23.9	0.36
Protein	14.4	14.6	15.8	15.5	0.18
Lipids	1.29	4.25	4.41	4.56	0.24
Energy (kJ g^{-1})	4.53	5.21	5.50	5.44	0.10
Ash	4.21	4.65	5.01	4.48	0.10
HSI (%)[1]	ND	0.94	0.94	0.79	0.08
VI (%)[2]	ND	5.01	5.17	4.11	0.36

Values are presented as means (n=3) and pooled standard error of the mean (SEM). An absence of different superscript letters indicates no significant differences (One-way ANOVA; $p \geq 0.05$).
ND: not determined.
[1]Hepatosomatic index: (fish weight/liver weight) X 100
[2]Visceral index: (fish weight/visceral weight) X 100

Regarding intestinal enzyme activity, trypsin activity was similar among dietary treatments. Amylase and lipase activities were lower in fish fed the control diet than in the BSG and BSG-SSF diets (**Table 4. 4**).

No significant differences among dietary treatments were observed in plasma glucose, cholesterol, and triglyceride levels. However, in absolute values, these parameters were higher in fish fed the control than in the BSG and BSG-SSF diets (**Table 4. 4**).

The activity of the amino acid catabolic enzymes, glycolysis, β-oxidation, and lipogenesis remained similar across treatments (**Table 4. 4**).

Table 4 4. Intestine enzyme activity, plasma metabolite levels, and hepatic intermediary metabolism enzymes activity of meagre fed the experimental diets

	Control	BSG	BSG-SSF	SEM
Intestine enzyme activity (mU mg protein^{-1})				
Trypsin	62.8	88.7	92.5	8.53
Amylase	26.5^{a}	41.2^{a,b}	51.7^{b}	3.07
Lipase	4.28^{a}	5.20^{a,b}	6.06^{b}	0.30
Plasma metabolite levels (mg dl^{-1})				
Glucose	64.7	62.7	62.7	1.78
Cholesterol	99.1	93.7	91.3	4.29
Triglycerides	539.7	481.8	392.7	41.1
Intermediary metabolism enzymes activity (mU mg protein^{-1})				
Amino acid catabolism				
Glutamate dehydrogenase, GDH	15.5	14.4	14.4	0.62
Aspartate aminotransferase, ASAT	2107.4	2003.1	1575.3	133.5
Alanine aminotransferase, ALAT	713.9	669.5	533.7	46.0
Glycolysis				
Hexokinase, HK	6.14	5.32	4.26	0.42
Glucokinase, GK	6.77	6.21	6.01	0.24
Pyruvate kinase, PK	28.2	25.5	25.9	0.87
β-Oxidation				
3-hydroxyacyl-CoA dehydrogenase, HOAD	16.0	17.7	18.2	1.27
Lipogenesis				
Malic enzyme, ME	8.82	8.04	7.72	0.48
Fatty acid synthase, FAS	0.07	0.07	0.08	0.01

Values are presented as mean (n=9) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters are significantly different (One-way ANOVA; Tukey test; $p<0.05$).

In both the liver and intestine, no differences between groups were observed in oxidative stress enzyme activity or lipid peroxidation levels (**Table 4. 5**).

Table 4. 5. Activities of oxidative stress enzymes (mU mg protein^{-1}) and lipid peroxidation levels (nmol MDA g^{-1}) in the intestine and liver of meagre fed the experimental diets.

	Control	BSG	BSG-SSF	SEM
Intestine				

Catalase, CAT (U mg^{-1} protein)	74.39	75.21	73.95	3.91
Glutathione reductase, GR	17.85	18.72	17.86	0.82
Glucose 6-phosphate dehydrogenase, G6PDH	28.68	32.14	31.49	5.90
Lipid peroxidation, LPO	15.76	15.75	15.75	0.74
Liver				
Catalase, CAT (U mg^{-1} protein)	272.4	315.1	236.8	18.6
Glutathione reductase, GR	6.96	5.74	6.41	0.40
Glucose 6-phosphate dehydrogenase, G6PDH	74.01	61.61	59.14	4.66
Lipid peroxidation, LPO	19.46	25.42	21.67	2.19

Values are presented as mean (n=9) and pooled standard error of the mean (SEM). An absence of different superscript letters indicates no significant differences (One-way ANOVA; $p \geq 0.05$).

No significant differences between groups were observed in the anterior intestine for fold height, goblet cells, enterocyte nucleus position, and supranuclear vacuolization (**Table 4. 6; Figure 1**). The lamina propria scored higher in fish fed with the BSG diet than the control, while the mean score for all parameters was higher in fish fed with the BSG and BSG-SSF diets than in the control diet.

Table 4. 6. Score-based evaluation of the anterior and distal intestine histology of meagre fed the experimental diets[1].

	Control	BSG	BSG-SSF	SEM
Anterior intestine				
Fold height	1.99	1.44	1.99	0.18
Goblet cells	1.66	1.99	1.77	0.18
Lamina propria[2]	2.22^{a}	3.33^{b}	2.99^{a,b}	0.18
Supranuclear vacuolization	1.66	1.22	2.11	0.18
Enterocyte nucleus position	1.11	1.99	1.99	0.18
Mean score	1.55^{a}	1.99^{b}	2.66^{b}	0.19
Distal intestine				
Fold height	2.88	3.00	2.44	0.26
Goblet cells	2.65	2.66	3.00	0.26
Lamina propria[2]	2.66	3.33	2.66	0.29
Supranuclear vacuolization	2.22^{a}	3.33^{b}	2.44^{a,b}	0.29
Enterocyte nucleus position	2.99^{a,b}	4.66^{b}	3.00^{a,b}	0.29
Mean score	2.55^{a}	3.22^{b}	2.66^{a,b}	0.19

Values are presented as mean scores (n=9) and pooled standard error of the mean (SEM). Different lower-case letters represent statistical differences between dietary groups, determined by comparing all Kruskal-Wallis pairs. Significance values were adjusted using the Bonferroni correction for multiple tests.
[1]Scores from 1 to 5, with 5 indicating significant alterations.
[2]Width and cellularity of the lamina propria.

In the distal intestine, fold height, goblet cells, and lamina propria scores were also unaffected by diet composition (**Table 4. 6; Figure 1**). The supranuclear vacuolization

and enterocyte nucleus position scores were highest in fish fed the BSG diet. Similarly, the mean score for all parameters was higher in fish fed the BSG diet compared to the control, whereas the mean score for the BSG-SSF diet did not differ significantly from the control.

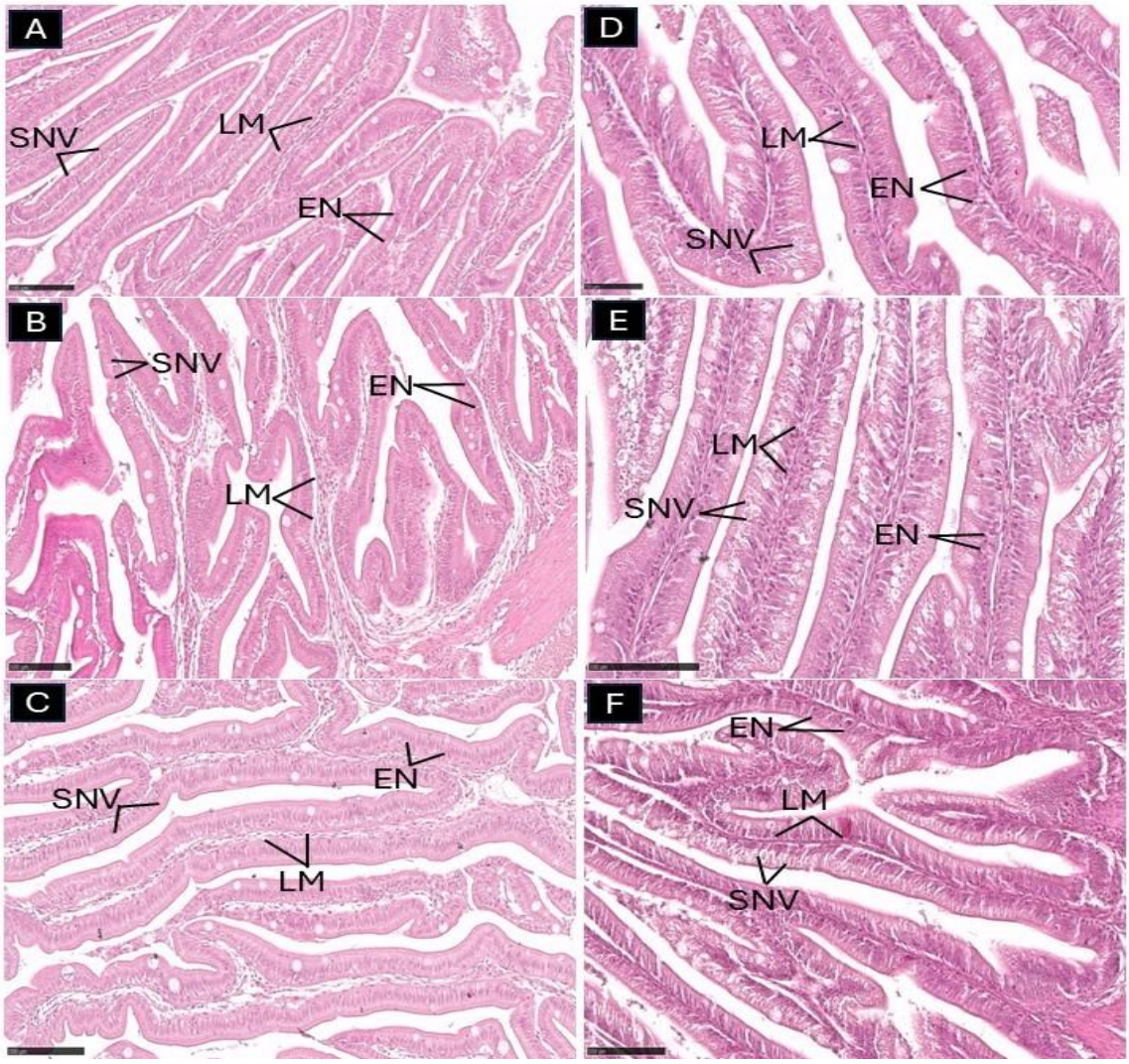

Figure 1: Detailed of anterior (Figs. A to C) and distal (Figs. D to F) intestine of meagre fed the control (A and D), BSG (B and E), BSG-SSF (C and F) diets. Enterocyte nucleus (EN), lamina propria (LP), and supranuclear vacuoles (SNV). Scale bar: 100 mm; HeE staining.

4 Discussion

Meagre is a high-trophic-level species with a high dietary protein to energy (29.6 to 22.4 g/MJ) requirments and a low tolerance for fibrous ingredients (Fountoulaki et al., 2017). High dietary inclusion levels of plant proteins have been associated with suboptimal growth performance, reduced feed efficiency, and gastrointestinal disorders, which often induce inflammatory responses (Ribeiro et al., 2015). Antinutritional factors in plant feedstuffs, particularly non-starch polysaccharides, have been reported to be one of the

highest challenges for meagre to cope with high plant-based ingredients (Couto et al., 2016; Ribeiro et al., 2015; Estévez et al., 2011). Consequently, the potential os dietary inclusion of low-nutritional value ingredients, e.g., low protein and high fiber plant-based ingredients, as agro-industrial by-products, in a meager diet will be mainly constrained by their high diet fiber content. However, to support sustainable aquaculture development based on the circularity principles, it is essential to valorize these underutilized feed resources, mainly those locally produced. Transitioning to aquafeeds with reduced reliance on traditional agricultural ingredients can provide environmental benefits (e.g., reduction of deforestation, consumption of arable land and water, and carbon footprint), reduce competition between aquaculture and other animal production sectors for raw ingredients, and yield economic advantages (Chary et al., 2023; van Zanten et al., 2023).

Solid-state fermentation (SSF) is a promising strategy to enhance the nutritional value of agro-industrial by-products due to its ability to degrade complex plant cell wall polysaccharides, increasing protein content and ingredient digestibility, besides adding valuable compounds, such as exoenzymes and antioxidant compounds (Yang et al., 2021). This approach has been applied to BSG. After SSF with *A. ibericus*, BSG registered a 21% increase in protein content and reductions in cellulose, hemicellulose, and lignin by 49%, 30%, and 7%, respectively. Additionally, BSG-SSF contained significant carbohydrase activity (xylanase and cellulase, 68 and 925 U/g dry BSG-SSF, respectively) and antioxidant activity (64 μmolTrolox equivalents/g and 12 mg gallic acid equivalents/g of dry BSG-SSF) (Estevão-Rodrigues et al., 2024).

In the present study, the dietary inclusion of 10% BSG, replacing a mixture of wheat gluten, soybean, rapeseed, and sunflower meals, reduced growth performance, feed efficiency, protein efficiency ratio, and nitrogen retention. These results underscore meagre's low tolerance for high-fiber ingredients. Similar findings were also reported in European seabass, another carnivorous fish species, where the dietary inclusion of 10 or 20% BSG compromised growth and dietary utilization (Estevão-Rodrigues et al., 2025, 2024). In contrast, moderate BSG inclusion (10–15%) did not compromise the growth performance in gilthead sea bream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*) (Estévez et al., 2022, 2021). On the contrary, for omnivorous species, such as striped catfish (*Pangasianodon hypophthalmus*) and Nile tilapia (*Oreochromis niloticus*), tolerate dietary BSG levels of up to 50% without negatively affecting zootechnical performance (Jayant et al., 2018; Zerai et al., 2008).

The growth reduction observed in the present study cannot be attributed to palatability issues, as voluntary feed intake was not affected by the dietary BSG inclusion. Instead,

the growth restriction likely resulted from reduced energy availability due to BSG's high fiber content, which is not digested by fish (Krogdahl et al., 2010). This is reflected in a significantly lower protein efficiency ratio and absolute lower values of nitrogen and energy retention (as a percentage of intake), indicating that part of the dietary protein was diverted for energy use instead of plastic purposes. Indeed, digestibility studies in meagre showed that ingredients with higher fiber content exhibit lower protein and energy digestibility. For instance, protein and energy digestibility values for wheat gluten, soy protein concentrate, and corn gluten are high (>90%) but moderate for soybean, rapeseed, and sunflower meals (78–84%). These differences are even more pronounced for energy digestibility, with values reaching 86% for soybean meal but decreasing to 59% and 46% for rapeseed and sunflower meals, respectively (Ribeiro et al., 2015)(Ribeiro et al., 2015). Considering that BSG has an even higher fiber content than sunflower meal, it is possible to infer that the observed growth reduction and decreased feed and protein utilization efficiency were due to a digestible energy limitation.

Dietary inclusion of BSG-SSF reversed all the adverse effects associated with the dietary inclusion of BSG, leading to growth performance and feed utilization efficiency comparable to the control diet. Similar effects were observed in European seabass, where dietary inclusion of up to 20% BSG negatively affected growth and feed utilization, where dietary replacing BSG with BSG-SSF led to growth and feed utilization similar (at 20% inclusion) or even higher (at 10% inclusion) than the control diet (Estevão-Rodrigues et al., 2025, 2024). Also, in European seabass, dietary inclusion of a plant feedstuff mixture (rapeseed, soybean, rice bran, and sunflower seed meals) fermented with *A. niger* did not affect growth but improved feed efficiency and digestibility compared to a non-fermented diet (Vieira et al., 2023). Similarly, the dietary inclusion of SSF of a mixture of *Gelidium* spp. by-product and sunflower meal (1:1, w/w) with *A. ibericus* did not affect the growth performance of European seabass but increased feed utilization efficiency compared to the control diet without *Gelidium* spp. by-products (Ferreira et al., 2024). However, feed intake and, consequently, growth was severely compromised when *A. niger*, instead of *A. ibericus*, was used to ferment *Gelidium* spp. by-products (Ferreira et al., 2024). In largemouth bass (*Micropterus salmoides*), the dietary inclusion of 30% soybean meal fermented with *Bacillus subtilis*, *Lactobacillus* sp., and yeast (1:1:2) supported growth performance similar to the control diet (He et al., 2020). In gibel carp (*Carassius auratus gibelio*), the dietary inclusion of 3% fermented plant-based ingredients using *Lactobacillus* spp. and *Bacillus* spp. improved growth performance (Cao et al., 2021). In *Labeo rohita* juveniles, the inclusion of SSF-treated *Moringa oleifera* leaf meal fermented with *A. niger* successfully replaced mustard oil cake without adverse

effects on the growth rate, feed, and protein utilization efficiency (Manna et al., 2022). In Nile tilapia, fermented soybean meal replaced up to 37% of dietary FM with no negative impact, although higher replacement levels led to reduced growth and feed utilization efficiency (Hassaan et al., 2015). SSF has also shown benefits for shrimp species. In *Penaeus vannamei*, up to 30% FM could be replaced with a mixture of soybean and sunflower meals fermented with *A. niger* (Jannathulla et al., 2018). Similarly, in Indian prawn (*Fenneropenaeus indicus*), up to 25% FM was successfully replaced by soybean meal fermented with *Saccharomyces cerevisiae* Sharaway et al. (2016).

Despite the observed differences in growth rates,the dietary treatments did not affect meager's whole-body composition. In European seabass, whole-body composition was also not affected by the dietary incorporation of BSG or BSG-SSF, except for increased ash content with a 10% and 20% BSG-SSF diet (Estevão-Rodrigues et al., 2025). Furthermore, European seabass whole-body composition was also unaffected by a diet including non-fermented or fermented *Gelidium* spp. by-products with *A. ibericus*. However, when *A. niger* was used for fermentation, body lipid and energy levels decreased (Ferreira et al., 2024). Contrarily, replacing 20% or 40% of non-fermented plant ingredient mixtures with the fermented mixture with *A. niger* increased European seabass body protein content (Vieira et al., 2023). In *Labeo rohita*, replacing up to 30% FM and rice bran with pistia leaf meal fermented with *P. kudriavzevi* did not affect body composition (Mandal & Ghosh, 2019).

Although not statistically significant, a trend toward reduced HSI and VI was observed with the inclusion of BSG-SSF. This reduction could suggest a shift in energy allocation strategies, favoring growth over energy storage. In meagre, while muscle lipid levels remained unchanged, liver and visceral fat increased with higher dietary lipid or energy levels, indicating that the liver and viscera are primary energy storage tissues (Fountoulaki et al., 2017). Conversely, in European seabass, an increase in HSI was observed in fish fed the BSG-SSF diet compared to those fed the BSG diet, likely reflecting the lower energy availability in fish fed the 10% BSG diet (Estevão-Rodrigues et al., 2025).

Digestive enzyme activity, plasma metabolites, and hepatic intermediary metabolism are key indicators of dietary modulation effect on digestion and post-prandial nutrient and energy metabolism (Martins et al., 2021; Castro et al., 2015). Previously, in meagre, digestive enzyme activities were followed over a 24-hour period after feeding, showing a moderate increase in trypsin activity and a significant increase in amylase activity four hours after feeding (Matias et al., 2023). In the present study, amylase and lipase activities were higher in fish fed with the BSG diets than in the control, and the difference

was statistically significant with the 10% BSG-SSF diet. Trypsin activity followed the same trend, but the differences were not statistically significant. Previously, it was reported that meager reduced digestive enzyme activity with high dietary inclusion of plant-based ingredients (Ribeiro et al., 2015; Estévez et al., 2011). The lower digestive enzyme activity observed in the present study in fish fed the control diet corroborates these observations. As in this study, lipase activities were unaffected (Estevão-Rodrigues et al., 2024). However, when European seabass was fed a fermented plant feedstuff mixture with *A. niger*, it was observed an increase in lipase activity, while trypsin and amylase activities remained stable (Vieira et al., 2023). In contrast, feeding a diet including *Ulva rigida* fermented with *A. ibericus* decreased lipase activity but did not affect trypsin or amylase activity (Fernandes et al., 2022). For Nile tilapia, replacing 50% FM with a 3:1 mixture of guar and copra meal fermented with *Saccharomyces yeast* decreased amylase activity (Dileep et al., 2021). The diversity of these results may be attributed to differences in feed formulation, such as the nature and quantity of each ingredient.

No differences among dietary groups were observed in plasma metabolite levels. Similar results were observed in European seabass with a dietary inclusion of 10% BSG and BSG-SSF, while with a 20% inclusion level, there was a reduction of plasma glucose, phospholipid, and triglycerides (2025), attributed to decreased feed utilization efficiency associated with the high BSG inclusion level. Conversely, dietary replacement of 20% plant ingredient mixture with the same mixture fermented by *A. ibericus* increased plasma glucose and triglyceride levels (Vieira et al., 2023). Similarly, in rainbow trout (*Oncorhynchus mykiss*), replacing 30% of dietary FM with soybean meal fermented with *Bacillus subtilis* decreased serum glucose and triglyceride levels (Moniruzzaman et al., 2018).

Also, no differences among dietary groups were observed in intermediary metabolism enzyme activities despite the differences in nitrogen and energy retention, particularly between the BSG and BSG-SSF groups. In European seabass, dietary inclusion of BSG-SSF reduced ASAT and ALAT activity but did not affect GDH activity, indicating a reduction in amino acid interconversion (Estevão-Rodrigues et al., 2025). As in the present study, β-oxidation and lipogenesis enzyme activities were unaffected, but glycolytic enzyme activity decreased in fish fed the BSG diet. Overall, these results indicate that at the dietary levels tested, dietary inclusion of BSG, particularly BSG-SSF, does not significantly alter fish's intermediary metabolism. Besides the effect dietary treatments, these results may also be related to the sampling time. As previously

mentioned, digestive enzyme activity peaks approximately four hours after the meal (Matias et al., 2023), suggesting that intermediary metabolism activity may occur later.

SSFmay enhanced antioxidant activity of lignocellulose ingredients by promoting the release of bound antioxidant compounds associated with cellulose, hemicellulose, and lipid components, thus increasing their bioactivity (Sarıtaş et al., 2024), as it was observed for BSG after SSF with *A. ibericus* (Estevão-Rodrigues et al., 2024). Enriched antioxidant ingredient may have an impact on the oxidative staus of fish (Taşbozan & Erbaş, 2023). In present study, the activities of hepatic and intestinal antioxidant enzymes and lipid peroxidation levels were unaffected by the dietary inclusion of BSG or BSG-SSF, indicating that 10% inclusion of BSG or BSG-SSF did not induce oxidative imbalance in the intestine or liver of meagre. However, in absolute values, the activity of antioxidant enzymes tended to be lower with the BSG-SSF diet compared to the BSG diet, which may indicate that fish fed the BSG-SSF diet were less exposed to ROS than those fed the BSG diet. In European seabass, intestine lipid peroxidation levels were reduced with the dietary inclusion of 10% BSG or BSG-SSF, but at 20% inclusion, lipid peroxidation was significantly higher than in the control group. In any case, lipid peroxidation levels were lower in fish fed the BSG-SSF diets than the BSG diets (Estevão-Rodrigues et al., 2025). Also in European seabass, dietary incorporation of *Gelidium* sp. fermented with *A. ibericus* did not affect hepatic antioxidant enzyme activity or lipid peroxidation (Ferreira et al., 2024). In contrast, European seabass exposed to oxidative stress induced by changes in temperature and salinity exhibited reduced hepatic lipid peroxidation when fed a fermented plant mixture with *A. niger* (Amaral et al., 2023). Similarly, in coho salmon and Nile tilapia, the use of fermented ingredients with *Bacillus pumilus* and *Bacillus velezensis*, respectively, reduced oxidative damage (Hao et al., 2025; Zhang et al., 2023).

The high fiber content of BSG can induce histomorphological changes in the intestine of carnivorous fish, potentially compromising intestinal mucosa integrity, triggering inflammatory responses, and reducing nutrient absorption efficiency (Gatlin et al., 2007) In meagre replacing 75% FM with plant ingredients has been shown to cause enteritis, while lower inclusion levels (50–60%, with 17.5% FM) only resulted in lipid droplet accumulation in enterocytes without significant changes to the intestinal epithelium (Ribeiro et al., 2015; Rodrigáñez et al., 2013). In the present study, including BSG in a low FM diet led to a few histomorphological alterations in both the anterior and distal intestines. These changes included increased width and cellularity of the lamina propria in the anterior intestine, as well as supranuclear vacuolization, altered enterocyte nucleus positioning, and elevated overall histomorphological alteration scores in both

intestinal regions. The increased width and cellularity of the lamina propria may indicate tissue proliferation or inflammatory cell infiltration, suggesting localized inflammation (Baeverfjord & Krogdahl, 1996). The increase in supranuclear vacuoles in enterocytes suggests lipid accumulation in enterocytes, potentially indicating lipid malabsorption (Deplano et al., 1989). These findings align with previous studies reporting increased supranuclear vacuolization in enterocytes with dietary inclusion of carob germ seed meal (Couto et al., 2016). However, low-fiber plant ingredients such as soy and pea protein concentrates did not induce similar histomorphological alterations (Ribeiro et al., 2015), suggesting that the observed effects are primarily attributable to the high fiber content of BSG.

Histomorphology mean scores alterations in the anterior intestine were higher in fish fed the BSG and BSG-SSF diets than the control. This effect can be primarily attributable to the high fiber content of BSG. In the posterior intestine, however, this negative effect was only observed in fish fed the BSG diet, suggesting that SSF contributed to mitigating the adverse effects of dietary fiber content.

In European seabass, adverse histomorphological changes in the anterior intestine were also observed, but only at a.dietary BSG or BSG-SSF inclusion of 20% (Estevão-Rodrigues et al., 2024). In the posterior intestine, however, no differences between groups were observed. As meagre is a species of a higher trophic level than European sea bass, this further confirms that fibrous material tolerance in carnivorous fish decreases with carnivory.

5 Conclusions

The results of the present study showed that the BSG is not well accepted by meagre even at a dietary inclusion level of 10% BSG. Treating BSG by SSF allowed overcoming these inconveniences, and a dietary inclusion level of 10% BSG-SSF promoted a growth performance, intestinal oxidative stress, and distal intestine histomorphology similar to that of the control. Future research should investigate the dietary inclusion of higher BSG-SSF levels and assess the long-term impacts on meagre well-being and production efficiency.

Overall, the present findings emphasize the potential of SSF as a promising strategy to improve the nutritional profile of agro-industrial by-products like BSG, contributing to the development of sustainable aquaculture diets.

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Chapter 5:

General conclusions, Final considerations, and Future prospects

General conclusions

Aquaculture production is projected to increase by 30% by 2030 to meet the growing demand for food from a rising human population (FAO, 2021). As aquaculture expands, there is a corresponding increase in the need for aquafeeds to feed intensive aquaculture production. In aquaculture, diet represents the most expensive production component (Hodar et al., 2020). In addition, the production of aquafeed, particularly for carnivorous species, remains highly reliant on ingredients with high environmental and economic impacts. These include marine-based products (fishmeal and fish oil) and plant-based ingredients (such as soybean meals and oil, rapeseed meals and oil, and corn and wheat meals and oil), which are mostly imported into Europe (Glencross et al., 2024; Gonzalez-Garcia et al., 2018). Thus, shifting to aquafeeds that are less dependent on these ingredients would have significant positive impacts on the ecosystem (e.g., reduction of overfishing, deforestation, carbon footprint, and use of arable land and water). Additionally, this shift could yield economic benefits (e.g., valorization of underutilized feed resources and the use of locally produced ingredients) and reduction of competition between aquaculture and other animal production sectors for raw ingredients (Lizardi-Jiménez & Hernández-Martínez, 2017).

Incorporating agro-industrial by-products as alternatives to traditional plant-based ingredients in aquafeeds represents an innovative and sustainable approach to aquaculture (Fernandes et al., 2022). This strategy adds value to by-products that would otherwise be underutilized or discarded and supports the circular economy (Masi et al., 2024; Chary et al., 2023). However, nutritional challenges remain for aquaculture species, particularly carnivorous fish. These by-products contain antinutritional factors, such as high levels of fiber, tannins, and protease inhibitors that reduce digestibility, compromising the overall welfare of fish (Oliva-Teles et al., 2024; Glencross, 2020; Kokou & Fountoulaki, 2018). Fungal SSF is emerging as a promising strategy to enhance the nutritional value of these by-products due to its ability to produce a variety of enzymes that degrade complex plant cell wall polysaccharides (Dawood & Koshio, 2020), such as those found in brewer's spent grain (Bhanja Dey et al., 2016).

In the current study, SSF of BSG by *A. ibericus* resulted in increased protein content and significant reductions in cellulose, hemicellulose, and lignin levels. Additionally, the fermentation process led to the production of enzymes such as cellulase and hemicellulase and the release of antioxidant compounds. These findings align with previous research where nutritional improvements of *A. ibericus* fermentation increased the nutritional value and made them more suitable for incorporation into aquafeeds. Examples include distiller's dried grains with solubles (Filipe et al., 2023), agar by-

product blends with sunflower cake (Ferreira et al., 2024), and *Ulva rigida* (Fernandes et al., 2022), where SSF with *A. ibericus* improved the nutritional profile by increasing protein content and reducing cellulose, hemicellulose, and lignin levels.

Filamentous fungi, particularly *Aspergillus* spp., are well-known for producing a wide range of enzymes, including carbohydrases, which have higher enzymatic yields compared to those produced by yeasts or bacteria (Baltussen et al., 2020; Contesini et al., 2010). Thus, the dietary incorporation of fermented products will also add enzymatic activity to the diet, which may be beneficial for the degradation of complex lignocellulosic matrices of plant ingredients, thereby improving dietary bioavailability.

This study evaluated the efficacy of SSF of BSG in European seabass and meagre. Both BSG and fermented BSG were studied alongside a control diet excluding BSG. Diets were formulated with a mixture of traditional plant ingredients, and BSG and fermented BSG were incorporated, replacing this plant ingredients mixture. For European seabass, two incorporation levels of BSG were tested (10% and 20%), while for meagre, only 10% dietary BSG was determined. This strategy was based on the high trophic level of meagre, estimated at 4.3 ± 0.75 (FishBase), indicating a highly carnivorous species with a lower tolerance for fibrous ingredients (Fountoulaki et al., 2017). Furthermore, in the present study, the digestibility of BSG and fermented BSG was evaluated only for European seabass due to the restriction of available meagre and the need to proceed with the thesis.

In European seabass, replacing a mixture of plant-based ingredients with increasing levels of BSG decreased the digestibility of dry matter, protein, lipids, and energy. These outcomes are attributed to BSG's high fiber content, reaching up to 50%, potentially compromising nutrient availability and limiting its use in aquatic feeds, especially for carnivorous fish species like European seabass (Mitri et al., 2022). The adverse effects of excessive dietary incorporation of complex polysaccharides on nutrient digestibility in European seabass have been documented (Fountoulakiet al., 2022; Magalhães et al., 2015; Enes et al., 2011; Tibaldi et al., 2006). High levels of dietary fiber can reduce energy digestibility and hinder the digestion of nutrients by altering the physical characteristics of the digesta and limiting the access of endogenous enzymes to substrates (Adeola & Bedford, 2004). Contrarily, incorporating BSG-SSF increased the digestibility of dry matter, protein, the amino acids proline and aspartic acid, lipids, and energy. These benefits are likely due to the lower fermented BSG fiber content and the presence of exogenous enzymes resulting from the fermentation process. The positive effects of dietary inclusion of exogenous carbohydrases on nutrient digestibility have been previously observed (Liang et al., 2022; Castillo & Gatlin, 2015). Specifically, the

beneficial impact of dietary incorporation of crude extracts obtained from SSF of BSG on digestibility in European seabass has been demonstrated, where the inclusion of 0.4% of this extract increased in vitro and in vivo digestibility of plant-based diets for European seabass (Fernandes et al., 2022; Fernandes et al., 2021).

The positive impact of SSF on the nutritional profile of BSG enhanced growth and feed utilization efficiency in both species. In European seabass, replacing BSG with BSG-SSF increased DGI and FE by approximately 32% and 20% at inclusion levels of 10% and 20%, respectively. For meagre, DGI increased by about 22%, and FE improved by approximately 40% with diets incorporating 10% BSG-SSF. In both species, BSG-SSF-based diets promoted a DGI and FE similar to the control diet without BSG. Additionally, dietary incorporation of 10% BSG-SSF in European seabass resulted in higher growth performance and feed utilization than in the control diet without BSG. These results can be attributed to the improved nutritional profile of BSG-SSF, particularly the reduced fiber content and the presence of exogenous enzymes introduced during the SSF process, as mentioned previously.

For meagre, dietary inclusion of BSG did not affect carcass composition or hepatosomatic and viscerosomatic indices. In European sea bass, whole-body lipid and energy content and HSI decreased with increasing dietary inclusion of BSG and BSG-SSF. HSI was also higher in fish fed a 10% SSF-BSG diet compared to the 10% BSG diet. This appears to be related to the increased cellulose content in the diets, leading to less energy available to be deposited as lipids in the viscera and muscles. Similar increases in HSI have been reported in sea bass (*Micropterus salmoides*) and African catfish (*Clarias gariepinus*) fed diets containing fermented and non-fermented ingredients (Enyidi & Etim, 2020; Li et al., 2020), while other studies have reported opposite effects, as in *Furong crucian* carp (Wang et al., 2024), or found no effects, as observed in rainbow trout (Moniruzzaman et al., 2018).

In European seabass, the dietary inclusion of BSG-SSF (statistically significant only at the 10% inclusion level) reduced total protease and trypsin activities compared to the control diet. However, this reduction in proteolytic activity in the BSG-SSF groups did not correspond to differences in protein digestibility. These findings may be attributed to the hydrolysis of proteins during the SSF process, where fungal proteases may have partially degraded BSG proteins and residual fungal proteases may remain active in BSG-SSF, potentially leading to decreased endogenous protease and trypsin activities. Previous studies have also observed that dietary inclusion of SSF ingredients reduces endogenous digestive activity without compromising dietary digestibility (Fernandes et al., 2022; Dileep et al., 2021).

On the other hand, for the European seabass fed the 10BSG-SSF diet, there was an increase in the enzymatic activities of amylase and lipase, which may indicate that although dietary BSG may reflect a more significant enzymatic effort for the hydrolysis of plant components, the increase in enzymatic activity in fermented diets suggests a synergistic interaction between the enzymes produced by fungi during SSF and the digestive enzymes of the fish itself. This synergy increases digestive efficiency and the metabolic use of nutrients. During the SSF process, *A. ibericus* may produce extracellular enzymes such as cellulases, xylanases, amylases, phytases, and proteases, which, together with endogenous enzymes, assist in the degradation of structural components of BSG. The BSG fermentation process has been shown to mitigate the intestinal histomorphology induced by BSG.

For meagre, amylase, and lipase activities were highest in the 10% BSG-SSF diet, the diet that registered the lowest feed intake. These results are likely attributed to the pre-digestion of BSG during the SSF process, which could have facilitated the access of endogenous digestive enzymes to the digesta, similar to the effects of exogenous enzyme supplementation (Castillo & Gatlin, 2015). Meagre has also been shown to have reduced digestive enzyme activity with high plant-based ingredient inclusion (Ribeiro et al., 2015; Estévez et al., 2011), potentially impairing nutrient absorption and reducing plasma metabolite levels and intermediary metabolism activity.

This study observed intestinal histomorphological changes mainly in the foregut for European sea bass and distal intestine for meagre. BSG diets induced more significant enterocyte degradation (nuclear position and vacuolization) in both species and increased supranuclear vacuolization and lamina propria size than BSG-SSF and control diets. These results suggested that BSG-SSF may alleviate the progression of mucosal layer inflammation caused by high dietary inclusion of BSG. Goblet cells protect the intestinal lining and help increase mucus production, facilitating fecal expulsion (Machado et al., 2013). Studies have shown that high levels of dietary fiber led to mucus efflux from the intestine, possibly due to increased chyme volume that physically scrapes the mucus layer (Piel et al., 2005). This suggests a link between high fiber content and intestinal mucus efflux, requiring more goblet cells to maintain the mucus layer. Furthermore, the level of dietary fiber may have impaired the selective permeability of the enterocyte membrane in the distal part of the intestine. Thus, the increase in the size of supranuclear vacuolization of enterocytes may result from the indiscriminate absorption of food components that have no entry pathways into the body and, therefore, accumulate in vacuoles. Converging with the current research, Couto et al. (2016) reported that meagre juveniles fed diets containing high levels of carob seed flour also

presented increased supranuclear vacuolization of intestinal enterocytes with concomitant accumulation of an amorphous hyaline substance of unknown nature, associated with a potential deleterious effect of tannins on cell membrane selectivity.

Plasma metabolites and the activity of key enzymes in intermediary metabolism are crucial biomarkers for assessing dietary nutrient utilization. Although no significant differences were identified in glucose levels in meagre, absolute values followed the same trend observed in European sea bass, where glucose levels decreased as BSG levels increased. For European sea bass, compared to the control, plasma metabolites were not affected at a 10% BSG inclusion level, whether fermented or not, except for a significant reduction in phospholipid levels in the BSG-SSF group. At a 20% inclusion level, plasma glucose, phospholipids, and triglycerides (only in the BSG diet) decreased regardless of fermentation. In absolute terms, this trend was also observed for meagre and the 10% BSG level, fermented and unfermented, and the control diet. The reduction in glucose is likely attributed to the replacement of wheat meal—a starchy ingredient—with BSG. Although plasma cholesterol was not affected by diet composition, plasma triglycerides and phospholipids were lower in BSG diets and decreased with dietary BSG incorporation levels, which may be related to dietary fiber content. The impact of dietary fiber on plasma lipid parameters in European sea bass remains inconsistent. Although some studies reported no effect on plasma cholesterol and triglycerides (Bonvini et al., 2018), others observed reductions in both parameters (Bonvini et al., 2018; Diógenes et al., 2018). BSG fermentation increased plasma triglycerides, likely due to the increased lipid digestibility observed in BSG-SSF diets.

In European sea bass, dietary inclusion of BSG did not affect the amino acid catabolism enzyme activities. However, ALAT and ASAT activities were reduced in fish-fed SSF-BSG diets, while GDH activity remained unaffected. This suggests a decreased need for amino acid interconversion, aligning with the observed improvements in protein digestibility and protein efficiency ratio. Although β-oxidation and lipogenesis enzyme activities were not affected by diet composition, GK and PK activity decreased with dietary levels of BSG and BSG-SSF. This corresponds with the reduced plasma glucose levels observed in fish fed these diets and, thus, the lower glucose available for glycolysis (Gatesoupe et al., 2014). The activity of ME, an NADPH-generating enzyme, was not affected by fermentation but was reduced with increasing BSG levels, possibly due to reduced energy availability in diets with higher BSG inclusion. A positive relationship was also observed in European sea bass between dietary incorporation of fermented ingredients and ME and FAS activity, suggesting increased energy availability, as previously observed (Vieira et al., 2023; Pelletier & Tyedmers, 2011).

For meagre, intermediary metabolism enzyme activities did not differ among dietary groups. These results were partly attributed to the sampling time, 4 hours post-feeding. As noted, digestive enzyme activity peaks around 4 hours after a meal (Matias et al., 2023), suggesting that intermediary metabolism activity may occur later.

Fermentation of BSG has been reported to release polyphenolic and other antioxidant compounds, enhancing the antioxidant potential of fermented BSG (Fernandes, 2022). Although dietary BSG did not affect hepatic LPO levels in European sea bass, it increased intestinal LPO levels. However, regardless of inclusion level, BSG-SSF restored intestinal LPO levels, decreased GR and GPX activities, and increased G6PDH activity. This reduction in intestinal LPO occurred without increased antioxidant enzyme activity or NADPH input, likely due to reduced exposure to reactive oxygen species (ROS), attributed to the increased antioxidant activity of BSG-SSF, which mitigates oxidative damage in the intestine. These findings align with previous studies on European seabass, which reveal that dietary ingredient composition manipulation affects oxidative stress, with the intestine being more prone to oxidative damage than the liver due to direct contact with the diet (Martins et al., 2024; Coutinho et al., 2017; Castro et al., 2015). The effect of BSG-SSF on oxidative stress in European seabass is likely due to reduced fiber content and the presence of functional compounds like exoenzymes and antioxidants. Similar positive modulation of intestinal oxidative stress has been demonstrated in European seabass (Amaral et al., 2023), Coho Salmon (*Oncorhynchus kisutch*) (Zhang et al., 2023) and Nile tilapia (Hao et al., 2025). In contrast, no changes in the oxidative status of the liver and intestine were observed in meagre, likely due to the more conservative dietary formulation and lower BSG inclusion levels.

Final considerations

The results of this study demonstrate that the inclusion of BSG in the diets of both juvenile European sea bass and meagre negatively impacted zootechnical performance and feed utilization. However, solid-state fermentation (SSF) of BSG using *Aspergillus ibericus* significantly enhanced its nutritional profile, making it a viable alternative to traditional plant-based ingredients in aquafeeds. Incorporating up to 20% fermented BSG in the diets of juvenile European sea bass and up to 10% in meagre (the highest inclusion levels tested) did not adversely affect growth performance, feed utilization, hepatic and intestinal oxidative status, or intestinal histomorphology. This alleviated the negative effects observed with the dietary inclusion of unfermented BSG.

The results underscore the potential of incorporating agro-industrial by-products, particularly BSG, as alternatives to conventional plant-based ingredients in aquafeeds. Furthermore, utilizing this by-product from the beer industry reduces dependence on

conventional plant resources, adds value to industrial waste, and promotes more sustainable aquaculture practices in line with circular economy principles.
Key findings from this study include:

- SSF of BSG increased protein content by 21%, and decreased lipid (51%), lignin (7%), cellulose (30%), and hemicellulose (34%) content by 49% and 7%, respectively and add high xylanase (67.7 U g^{-1}) and cellulase (925.1 U g^{-1}).
- For European seabass, replacing a plant ingredient mixture with 10% and 20% BSG decreased growth performance and increased feed and protein utilization efficiency
- For European seabass, replacing a plant ingredient mixture with 10% and 20% BSG-SSF increased decreased growth performance and increased feed and protein utilization efficiency to levels similar to the control diet. Including 10% BSG-SSF surpassed the zootechnical performance of the non-BSG control diet.
- For meagre, replacing a plant ingredient mixture with 10% BSG decreased growth performance and increased feed and protein utilization efficiency.
- For meagre, replacing a plant ingredient mixture with 10% BSG-SSF increased growth performance and feed and protein utilization efficiency to levels similar to the non-BSG control diet.
- For juvenile European sea bass, BSG fermentation increased the digestibility of dry matter, protein, certain amino acids, energy, and lipids, while BSG inclusion negatively affected the diet digestibility.
- For European sea bass juveniles, including 10% BSG SSF decreased the intestinal activity of total proteases and trypsin, which did not affect protein digestibility. Contrarily, for meagre juveniles, amylase, and lipase activities were higher in the 10% BSG-SSF diet than in the 10% BSG diet.
- For European sea bass juveniles, plasma glucose levels were reduced in the 20% BSG and BSG-SSF diets, likely due to the lack of wheat meal, the primary source of starch (glucose) in the experimental diets. For meagre, no differences in plasma metabolites were observed.
- For European sea bass juveniles, dietary BSG inclusion did affect hepatic LPO levels but increased intestinal LPO levels. BSG-SSF restored intestinal LPO in both tissues, reduced GR and GPX activities, and increased G6PDH activity.
- For meagre juveniles, dietary incorporation of BSG or BSG-SSF did not affect hepatic or intestinal oxidative status.

- For European seabass, ALAT and ASAT activity were higher in fish fed BSG diets than in fish fed BSG-SSF diets, suggesting a greater need for amino acid interconversion to compensate for potential amino acid imbalances in BSG diets. Hepatic β-oxidation and lipogenesis enzyme activities were not affected by diet composition, with GK and PK activity decreasing with dietary BSG and BSG-SSF levels, which aligns with the reduced plasma glucose levels observed in fish fed these diets.
- For meagre juveniles, dietary incorporation of BSG or BSG-SSF did not affect intermediary metabolism.
- For European sea bass and meagre juveniles, dietary incorporation of BSG negatively impacted intestinal lamina propria, enterocyte, and supranuclear vacuolation of intestinal morphology; however, the SSF of BSG effectively mitigated these effects, maintaining intestinal health comparable to that observed with a control diet.

Future perspectives

Based on current research, SSF of BSG with *Aspergillus ibericus* demonstrates significant potential as an alternative ingredient in aquafeeds, including for carnivorous species such as European seabass and meagre. The substantial impact of BSG-SSF on growth, feed utilization efficiency, intestinal histomorphology, and oxidative status of the intestine highlights its potential as a promising novel ingredient, even for highly carnivorous species, such as those tested in this thesis.

However, several key areas warrant further investigation. Identifying a broader range of enzymes produced during the SSF of BSG, such as amylase and proteases, is crucial to understanding the dynamics of the interaction between the exoenzymes generated by fermentation and the digestive endoenzymes of fish. This analysis would facilitate the evaluation of potential synergies between these enzymes, providing insights into whether they work together to enhance nutrient digestion and absorption, as well as optimizing the use of BSG as a dietary ingredient.

Although the inclusion of 10-20% BSG-SSF in the diets of carnivorous fish has shown effectiveness in improving growth performance and overall diet digestibility, conducting a specific digestibility assay for BSG-SSF would be essential for better understanding its functional role in aquafeed. Evaluation of the digestibility of the ingredient, BSG-SSF, would provide precise data on its nutrition and energy contribution to diet nutritional, allowing the dietary formulation based on the digestible basis rather than on dry matter base.

It is also necessary to identify the immunological responses caused by the dietary inclusion of BSG-SSF in the diet of carnivorous fish. Although during the SSF process bioactive products are produced that help strengthen immunity, it would be advantageous to further evaluate the potential immunomodulatory effects of this ingredient and the impact on animal health and performance. In addition, it is necessary to explore the modulatory effects of dietary inclusion of BSG-SSF on the intestinal microbiota, since changes in the intestinal microbiota provide valuable information on the health and general functionality of the intestine.

A controlled in vivo thermal and bacterial challenge would be a valuable approach to evaluate the immunological efficacy of the diet containing BSG-SSF and its influence on the fish immune system. In a scenario of pronounced climate change, a thermal challenge for fish offers crucial advantages to evaluate the resistance and adaptability of species to temperature variations. This type of study is essential to understand the thermal limits of species and how the inclusion of fermented BSG (BSG-SSF) can influence these limits. In addition, this methodology would allow for simulating stress and infection conditions similar to those faced in commercial aquaculture systems, providing accurate data on the ability of fish to respond to environmental variations and pathogens. Another point that challenges in a controlled environment would allow would be the identification of synergies between bioactive compounds generated during the fermentation process, such as beta-glucans, bioactive peptides, and antioxidant compounds, and the modulation of the immune system, providing insights into the mechanisms by which BSG-SSF promotes immunological resistance and growth performance.

In addition, it would be interesting to perform cost analyses, economic feasibility analyses, and assessments of reducing the carbon footprint associated with replacing traditional agricultural ingredients with BSG-SSF. These studies could demonstrate the benefits of this substitution, highlighting the positive impact on the sector and aiming to make aquaculture more sustainable. Such an approach would also promote the circular economy by valuing agro-industrial waste and integrating it efficiently into feed production, aligning economic and environmental sustainability.

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