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Actinomycetota-Mediated Solid-State Fermentation of *Gelidium* sp. By-Products: Evaluation of Digestibility in European Seabass

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Abstract

Aquaculture significantly supports global food security by providing a reliable and sustainable source of protein, and it contributes to circular economy goals by optimizing resource use and minimizing waste. Through efficient production practices, aquaculture helps enhance food availability while promoting sustainability. This research aimed to evaluate the benefits of employing circular economy concepts with regard to aquaculture, particularly the application of industrial by-products from macroalgae to form a sustainable aquafeed. The study assessed the suitability of *Gelidium* by-product (GBP) as a feed ingredient and investigated the concept of solid-state fermentation of GBP with Actinomycetota strains obtained from macroalgae, specifically, the relevance of solid-state fermentation to reduce crude fiber content and improve digestibility of GBPs by European seabass. Digestibility measurements were conducted over two randomized periods, using five different diets: a control diet witout the addition of GBP, and diets with 5% (G5), 10% (G10) and 15% (G15) of GBP, and 15% of NaOH-treated GBP (G15 NaOH), with feces collected daily for analysis. Chemical analyses included the determination of dry matter, ash, protein, lipid, neutral detergent fiber (NDF), and acid detergent fiber (ADF) contents. As GBP content in the feed increased, the protein digestibility coefficient decreased, with the G15 diet showing the lowest digestibility (85.6%). However, the G15 NaOH diet, in which the GBP was pre-treated with NaOH, achieved a higher protein digestibility of 91.4%. The lipid digestibility coefficient remained consistent across all treatments. SSF was performed on GBP using three selected Actinomycetota strains which previously isolated from macroalgae—*Cellulosimicrobium funkei* (R177), *Streptomyces violascens* (F26), and *Rhodococcus chondri* (R104)—to reduce fiber content and enhance nutrient availability. The antimicrobial activity of the fermented GBP was also assessed against ten reference microorganisms. GBP fermented by *CeIIuIosimicrobium funkei* (R177) reduced neutraI detergent fiber (NDF) from 59.1% to 55.0% and significantly Iowered acid detergent fiber (ADF). *Streptomyces vioIascens* (F26) further reduced NDF to 53.5%, whiIe *Rhodococcus chondri* (R104) achieved moderate reductions. These resuIts demonstrate SSF's potentiaI to enhance GBP digestibiIity and nutrient avaiIability in fish diets. The present study concludes that solid-state fermentation can be used to reduce fiber and improve the digestibility of GBP in fish diets.

Keywords: *Gelidium* by-products, solid-state fermentation, actinomycetota, european seabass, digestibiIity, fiber

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List of Abbreviations

EU	European Union
OD	Optical density
SSF	Solid-state fermentation
GBP	*Gelidium* by-products
MHA	Mueller-Hinton agar
SDA	Sabouraud Dextrose agar
TSA	Tryptic Soy agar
MA	Marine agar
TSYA	Tryptic Soy Yeast agar
ADC	Apparent digestibility coefficients
NDF	Neutral detergent fiber
ADF	Acid detergent fiber
DMSO	Dimethyl sulfoxide

1. Introduction

1.1 The Fundamental Role of Aquaculture in the Circular Economy

Aquaculture, when placed within the context of the circular economy, becomes a key driver of development, reduction of poverty, and food security on a planetary scale (Virapat, 2022; Jones et al., 2014; Subasinghe et al., 2009). The aquaculture industry's consistent growth, driven by the increased global demand for seafood, underscores the need for sustainable resource management methods and waste reduction (Colombo et al., 2022). Based on knowledge and strategy, Chary (2023) outlines several key areas of engagement to enhance the circularity of aquaculture: increasing the production and demand of aquaculture species, funding research breakthroughs, and developing outreach programs to raise consumers' awareness of environmental-friendly seafood. A critical aspect of these priorities is using nutrient-rich by-products in aquatic systems and improving production output (Chary, 2023).

The European Union (EU) has considered the potential of aquaculture and recommended its use in a conservation-friendly manner compatible with the standard fisheries policy (Cappell & Huntington, 2023). In 2021, the EU aquaculture sector generated substantial economic value, with 1.1 million tons of aquatic life worth €4.2 billion (Eurostat, 2023).

In the context of the aquaculture business area, the principles of the circular economy are evident, aiming to minimize waste production and improve productivity (Skretting, 2022). By-products and waste from wild fish and aquaculture can be converted into valuable nutrients, demonstrating that aquaculture operations have the potential to create positive change. (Skretting, 2022). For instance, omnivorous species can contribute to nutrients cycling in the circular food system by converting plant by-products into sea-food protein, with approximately 35% of the fish meal fed to the farmed fish in aquaculture derived from the fish processing by-products (Chary, 2023; Skretting, 2022). Detritivorous species, like sea cucumbers and sea urchins, show promise to occupy the niche of using low-quality organic compound due to their unique feeding habits and ecological roles. These detritivorous species can feed on organic matter that is often considered waste, such as fish feces and other decomposing materials. By doing so, they contribute to nutrient cycling within their ecosystems and can convert these low-quality organic inputs into valuable nutrients, thus closing the loop within aquaculture (Chary, 2023).

Figure 1, which depicts the changes in ingredient classes in compound feeds for omnivorous and carnivorous fish in aquaculture, is relevant to the future development of aquafeed and the integration of circular economy principles into aquafeed formulation (Chary, 2023). Figure 1 highlights changes in feedstuff

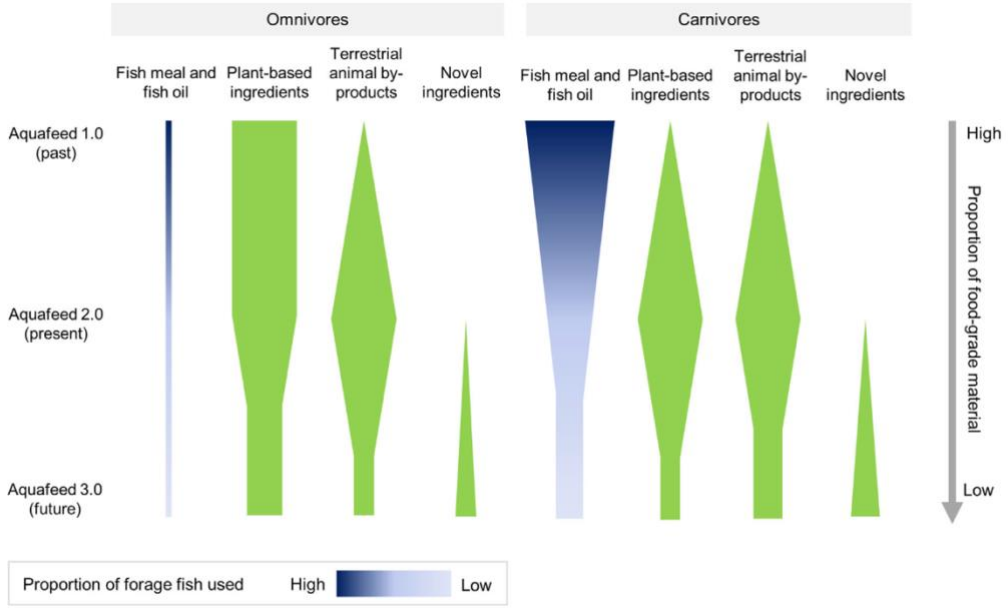

Figure 1. Diagram showing the evolution of ingredient types in compound feed for omnivorous and carnivorous aquaculture species (Chary et al., 2023).

sourcing from a circular economy and anticipated adjustments in the ratio of the different feed ingredients to make feeds more sustainable (Chary, 2023). The world of aquaculture must improve feed-sustainability by applying circular economy principles to aquafeed production, ensuring long-term global protein efficiency (Skretting, 2022).

1.2 The Challenges of Circular Economy in Aquaculture

Implementing circular economic concepts in aquaculture presents benefits and drawbacks. To effectively implement a circular model in aquaculture, it is crucial to evaluate the environmental impact of processes and inputs, despite the challenges posed by various methods and approaches (Regueiro et al., 2021). Most metrics for circularity are based on the technical cycles of products or physical materials derived from non-renewable sources. These metrics focus on tracking the life cycle of non-renewable materials, such as metals, plastics, and other resources, and promote resource efficiency, waste reduction, as well as sustainable practices. While these traditional metrics have been valuable in assessing the circularity of industrial processes and material flows, their applicability to sectors like aquaculture, especially within integrated multi-trophic systems, may be limited due to the biological, ecological, and renewable resource-dependent nature of aquaculture operations (Checa, 2024). Sustainable aquaculture feed production requires that feed resources are produced and sourced within a circular bioeconomy framework to enhance a robust feed chain (Colombo et al., 2022).

Transitioning to a circular bioeconomy in aquaculture would imply the conceptual and design of appropriate production systems, reusing wastes and by-products, sectoral impacts, and exploring new recycling options (Verreth et al., 2023). A circular economy relationship can be developed by utilizing aquaculture waste and resources, promoting profitability and sustainability (Peceño et al., 2022). Key areas for enhancing circularity in aquaculture are identified, including core species production, waste reduction, nutrient recycling, modifications to aquafeed formulation practices, consumer education, and research priorities. (Chary, 2023).

The potential for sustainable development should be unlocked through innovations, such as bio-based ropes enabling circular and low-impact mussel and seaweed aquaculture in the EU aquaculture sector (Arantzamendi et al., 2023). The potential of microalgae as a sustainable ingredient lies in its ability to aid in food security and support the practices of a circular economy (Yarnold et al., 2019; Ahmad et al., 2022).

Animal welfare should be improved by upgrading by-products through different species, reducing competition for feed resources between livestock and aquaculture (Riel et al., 2023). Successful aquaculture enterprises demonstrate that the solution lies in uniting social economy principles with circular and green economy strategies (Barna et al., 2023). For instance, producing *Artemia,* commonly known as brine shrimp, as a protein substitute from agricultural waste can contribute to a circular economy and tackle protein scarcity (Ogburn et al., 2023).

Environmental sustainability challenges for aquaculture include nutrient load management and sustainability of the practice (Fadum, 2024). It has become apparent that lack of knowledge, financial constraints, and not accounting for operational expenses are significant limitations requiring capacity building or financial support for aquaculture operators (Berio & Salugsugan, 2022). Effective knowledge exchange in sustainable aquaculture is mandatory, as knowledge isolation can inhibit the most effective information and best practices (Jones et al., 2014).

1.3 Potential of Macroalgae and its By-Products as a Valuable Resource

Macroalgae, commonly referred to as seaweeds, have multiple industrial applications and are a prized commodity (Rahikainen et al., 2021). They can be used as raw feedstock for biorefineries to manufacture biomaterials and bioenergy, representing a sustainable source in various industries (Arias et al., 2023; Kostas et al., 2021). Besides its nutritional value, macroalgae is cost-effective, easy to cultivate, and thrives well in harsh environments, making it a multi-functional resource capable of meeting global demand (Adarshan, 2023).

They are utilized in pharmaceuticals, nutraceuticals, cosmetics, and energy industries, showing their versatility and importance (Adarshan, 2023).

The potential of macroalgae as a renewable energy source has recently gained attention due to their ability to reduce carbon dioxide emissions and their application as a biofuel feedstock (Fernandes et al., 2019). Production methods range from tank/tumble culture, sea floor planting and attachment on artificial substrates. The chemical composition of macroalgae depends on environmental conditions and the genetic differences among the species (Jiménez-Peñalver et al., 2016). The macroalgae comprise proteins, lipids, carbohydrates, vitamins, and minerals, making them essential for various uses (Jiménez-Peñalver et al., 2016). Marine algae-based aquaculture offers solutions for future nutritional gaps and environmental sustainability, which aligns with the circular economy principles (Greer et al., 2022).

Using macroalgae and its by-products as potential raw ingredients for aquaculture feed production could serve as sustainable alternatives for fish meal and soybean, the traditional feedstuffs. Incorporating macroalgae in aquafeeds may contribute to improving feed efficiency and fish health and reduce environmental impacts, gaining interest in sustainable aquaculture (larsen et al., 2022). The future of fed aquaculture depends on replacing traditional feed ingredients derived from wild-caught fish with alternative, more sustainable sources. These alternatives could include macroalgae and single-celled organisms, which are produced through agriculture or aquaculture (Herath, 2022). By incorporating these plant-based and microbial ingredients into aquaculture feeds, it is possible to maintain the key health benefits for the farmed fish while reducing the reliance on wild fisheries (Herath, 2022).

Direct implementation of macroalgae, a component of sustainable aquaculture as well, into aquafeed could narrow the deficit between the quality requirement of feed and the dependence of industry on conventional protein sources of fish meal, leading to a more environmentally friendly and sustainable production methodology of aquafeed (Ghamkar & Hicks, 2021).

Besides their nutritional importance, macroalgae represent a viable alternative to overcoming the feed-food competition in aquaculture systems (Naiel et al., 2021). Integrating macroalgae into aquafeed formulations allows aquaculture to avoid competing with livestock for feedstuffs, thereby supporting aquaculture's more sustainable resource utilization practices (Naiel et al., 2021). The successful incorporation of macroalgae by-products into aquafeed could support this effort while also adding positively to a circular economy, broader valorization of organic waste, and general improvement of the sustainability footprint of aquafeed production (Hamid, 2021). In addition to plant-based replacements, macroalgae have been used as an alternative to fishmeal,

particularly to omnivorous species, contributing feed source variety in promoting sustainability in aquaculture (Bu, 2023). On the other hand, carnivorous fish, due to their limited ability to digest complex carbohydrates, may face challenges in utilizing macroalgae efficiently. However, with the right technological solutions, we can help them overcome these challenges. One key technological process to enhance the nutritional value of macroalgae involves the extraction of high-value biologically active oligosaccharides alongside protein with essential amino acids (Sadhukhan et al., 2019). The valorization of seaweed carbohydrates through autohydrolysis has been identified as a selective and sustainable pretreatment method, highlighting the potential for enhancing the nutritional value of macroalgae (Gomes-Dias et al., 2020).

Several studies have indicated the biochemical potentials of macroalgae as an ingredient for aquafeed of different fish species (Hua et al., 2019). Innovative bioprocessing, such as using *Ulva rigida* to enhance its nutritional quality for aquafeed formulation, indicates its diverse nature and scope for formulating aquafeed (Fernandes et al., 2019). Macroalgae is currently being integrated into aquafeed, aligning with circular economy principles in aquaculture. The fermentation of macroalgae allows for their high nutrient content to be brought into the feed, contributing to the aquaculture sector's circular economy (Ang et al., 2021).

1.4 Red macroalgae, *Gelidium* sp.

Gelidium sp., a group of marine macroalgae belonging to the phylum Rhodophyta, is commonly known as red algae (Carpena et al., 2022). These algae are studied and used for environmental importance in diverse fields (Matias et al., 2022; Mouga et al., 2022; Park et al., 2012); Kim et al., 2012). The red color of *Gelidium* sp. is attributed to phycoerythrin, a photosynthetic pigment responsible for the excellent light absorption required for photosynthesis (Ganesan et al., 2022; Cotas et al., 2020). Some of the red algae, like *Gelidium crinale*, are widely distributed in tropical and temperate waters (Boo et al., 2023). It is challenging to identify smaller to medium-sized *Gelidium* species, due to their high degree of morphological variation (Kim & Boo, 2012).

Agar is a key polysaccharide component extracted from the cell walls of red algae in the *Gelidium* genus. This versatile compound has numerous industrial applications (Fu & Kim, 2010). Agarases are a group of enzymes that can degrade agar, and they are essential for producing agar-derived oligosaccharides with various bioactive properties. These oligosaccharides are utilized in the food, cosmetics, and pharmaceutical industries (Fu & Kim, 2010). A study conducted by Paiva et al. (2017) on selected Azorean macroalgae, like *Gelidium microdon*, evaluated their nutritional and functional bioactivity value. The study revealed

that *Gelidium microdon* and other macroalgae contained highly digestible proteins, particularly rich in aspartic and glutamic amino acids, contributing significantly to the total amino acid content (Paiva et al., 2017). Also, *Gelidium microdon* contains high dietary fiber (33.7%), protein (15.7% to 23.4%), and ash (10.7% to 20.7%) content (Paiva et al., 2017). In another study, Cavaco et al. (2021) analyzed the seasonal nutrition value of *Gelidium corneum* in central Portugal. This study showed high protein (up to 16.3 ± 0.3%) and carbohydrate (up to 39.5 ± 3.3%) contents of the *Gelidium corneum* biomass, with lower levels of lipids (up to 2.8 ± 0.3%), mainly in the summer (Cavaco et al., 2021). These results underscore the dietary significance and the potential nutraceutical properties of *Gelidium* seaweeds for a healthy diet ecosystem.

1.4.1 *Gelidium* by-products

Gelidium by-products (GBP) are residual materials that remain after extracting valuable compounds like agar and agarose from species of the agar-producing red algal genus *Gelidium* (Lebbar et al., 2018). The GBP consists of 30-40% carbohydrate, providing a carbon backbone for several applications (Bondar et al., 2022). GBP has the potential as a sustainable carbon platform for the coproduction of valuable compounds like poly-3-hydroxybutyrate and gluconic acid (Bondar et al., 2022). Additionally, the protein fraction from red seaweed industrial solid residue after agar extraction has high antioxidant activity and could be valorized, which opens the possibility of producing new by-products from *Gelidium* (Trigueros et al., 2021). This GBP processing can be subjected to many cascade valorization strategies to exploit their potential in several industries (McReynolds et al., 2023). The by-products can be utilized to produce biofuels, biobased chemicals, biochar, and other sustainable materials to support the circular economy and resource efficiency (McReynolds et al., 2023; Tayibi et al., 2019).

The study by Tůma et al. (2020) highlights the nutritional content of GBP and its potential as a sustainable and nutrient-rich resource. As shown in Table 1, the presence of proteins, lipids, carbohydrates, vitamins, and minerals in *Gelidium* residues makes them a valuable source of organic compounds with various economic applications (Tůma et al., 2020).

Table 1. The chemical composition of *Gelidium sesquipedale* residues after agar extraction (Adapted from Tůma et al., 2020).

Parameter	Value	Unit
Dry matter (DM)	94.0± 0.8	%
Moisture	6.1 ± 0.8	%
Total fiber	76.3	% dry matter
Proteins	0.7	% dry matter
Lipids	3.4	% dry matter
Ash	16.4 ± 1.2	% dry matter
Carbohydrate	45.0 ± 2.0	% dry matter
Agar	7.3	% dry matter
Cellulose	37.1 ± 0.0	% dry matter
Starch	0.8 ± 0.3	% dry matter
Minerals		
Phosphorous	7.2	g/kg dry matter
Sodium	1.7	g/kg dry matter
Potassium	1.9	g/kg dry matter
Calcium	31.7	g/kg dry matter
Iron	1248.2	mg/kg dry matter
Copper	25.4	mg/kg dry matter
Zinc	243.3	mg/kg dry matter
Chromium	23.3	mg/kg dry matter
Lead	5.8	mg/kg dry matter

1.5 Marine Actinomycetota

Marine Actinomycetota is a group of diverse bacteria that live in a number of marine habitats including coastal and intertidal regions, marine sediments, seaweeds, fish, shrimps, mollusks, and mangroves (Mahmoud & Kalendar, 2016; Kasanah & Triyanto, 2019). These bacteria are characterized by a high diversity of morphological, physiological, and biochemical features that facilitate their survival in marine habitats (Jose & Jha, 2017). Actinomycetota are recognized as unique metabolic factories of bioactive compounds with diverse biological activities (Dhakal et al., 2017). The existence of indigenous marine Actinomycetota, such as *Rhodococcus marinonascens*, indicates that these microorganisms are well adapted inhabitants of marine environments and they can be regarded a promising reservoirs of new bioactive molecules (Siro et al., 2022). Secondary metabolites produced by marine Actinomycetota exhibit diverse chemical structures that correlate with their unique marine living conditions

(Ulfah et al., 2021). Research has extensively documented the bioactive potential of marine Actinomycetota showing their antimicrobial, anticancer, antifungal, antiviral, and anti-inflammatory activities (Girão et al., 2019; Ribeiro et al., 2020). Thus, marine Actinomycetota provide a wide array of bioactive compounds that can be explored for various applications in medicine, biotechnology, and environmental science (Ebrahimi et al., 2023), underlining the significant biotechnological value of these microorganisms.

1.5.1 *Cellulosimicrobium* Genus

The *Cellulosimicrobium* genus comprise Gram-positive, aerobic, non-spore-forming actinobacteria that have been isolated from various marine environments, including marine sediments, marine sand, and macroalgae (Girão et al., 2024; Oh et al., 2018). This genus is promising in terms of producing novel natural products; thus, these bacteria are an exciting subject of biotechnological interest (Girão et al., 2024; Hamada et al., 2016; Oh et al., 2018). For instance, Girão et al., (2024) have successfully isolated two novel linear peptides, cellulamides A and B, from the culture of the macroalgae-associated *Cellulosimicrobium funkei* strain CT-R177, obtained from the Cholorophyta macroalga *Codium tomentosum*, collected from the northern Portuguese coast. Similarly, Song and Wei (2010) demonstrated that marine-derived *Cellulosimicrobium* species can produce enzymes through fermentation processes, capable of degrading lignocellulosic materials (Song & Wei, 2010). Their study reported the production and characterization of cellulases and xylanases by *Cellulosimicrobium cellulans* grown in pretreated and extracted bagasse and minimal nutrient medium M9 (Song & Wei, 2010). Furthermore, *Cellulosimicrobium cellulans* has been identified as a major source of yeast-lytic enzymes like endo-β-1,3-glucanases, proteases, and mannanases, which are crucial for cellulose degradation (Özcan et al., 2013). Additionally, Kim et al. (2012) reported the production of a mixture of cellulases and α-galactosidases through SSF using cellulolytic bacteria, including *Cellulosimicrobium* sp., highlighting the enzyme production potential of marine-derived *Cellulosimicrobium* strains.

1.5.2 *Streptomyces* Genus

The genus *Streptomyces* comprises a group of filamentous, spore-producing Gram-positive bacteria that are widely distributed in nature, with a significant presence in the marine environment (Khadayat et al., 2020; Lee et al., 2020). This genus is well known for producing a wide array of bioactive secondary

metabolites, including antibiotics, antifungals, and compounds with cytotoxic activities (Chen et al., 2016; Du et al., 2018; Ward & Allenby, 2018).

In the marine environment, *Streptomyces* strains have been isolated from a variety of sources, such as seawater, sediments, and macroalgae (Romanenko et al., 2008; Gupta et al., 2014; Ismail et al., 2018). These marine-derived Streptomyces have important roles in the degradation of complex organic compounds such as polysaccharides, proteins, and lipids (Hong & Cho, 2013; Chen et al., 2016; Liu et al., 2017), synthesizing a variety of cellulases, proteases and lipases with industrial significance (Kurtböke, 2017). Some of these enzymes may have a vital function in terms of processing and modifying various feed ingredients used in aquaculture feeds. For instance, cellulases produced by *Streptomyces* can break down cellulose, a major component of plant-based feed ingredients, improving their digestibility and nutritional value for aquatic organisms (Vandamme et al., 2005). Likewise, proteases and lipases from *Streptomyces* can improve the digestibility of protein and lipid sources in the aquaculture feed and consequently increase the feed conversion rate in the performance of the aquaculture species (Al-Dhabi et al., 2019).

1.5.3 *Rhodococcus* Genus

Rhodococcus species are widely recognized for their ecological significance, thriving in various natural environments such as soil, water, and marine sediments (Hernández et al., 2008). These aerobic, non-sporulating bacteria are found in diverse habitats, including tropical and arctic soils as well as marine and deep-sea sediments (Urbano et al., 2014). A recent study by Girão et al. (2024) emphasizes the presence of this genus in marine ecosystems, specifically in macroalgae.

One of the most notable features of *Rhodococcus* spp. is their ability to degrade a variety of plant biomass and lignocellulosic materials (Cappelletti et al., 2020). This property suggests that these bacteria have potential to degrade GBP, which are expected to be rich in cellulose and other complex carbohydrates. The degradation of fibrous materials by *Rhodococcus* could lead to the production of valuable compounds, including biosurfactants, carotenoids, and lipids (Cappelletti et al., 2020). *Rhodococcus* species produce specific enzymes that facilitate the degradation of lignocellulosic biomass, such as lignin-degrading enzymes, including lignin peroxidases, manganese peroxidases, and laccases. These enzymes play a crucial role in breaking down lignin and other complex aromatic compounds present in plant fibers (Cappelletti et al., 2020). Additionally, *Rhodococcus* has been shown to possess cellulases and xylanases, which are vital for the degradation of lignocellulosic materials. The presence of these enzymes in *Rhodococcus* strains has been confirmed in several studies (Saini et al., 2016; Vu, 2023; Okeke & Lu, 2010). Moreover, the ability of *Rhodococcus* to produce

xylanases has been well-documented, demonstrating their capacity to degrade xylan, a key component of hemicellulose (Okeke & Iu, 2010). Xylanases are essential for breaking down hemicellulose into xylose and other sugars, thereby enhancing the overall efficiency of biomass conversion processes (Okeke & Iu, 2010).

1.6 Solid-State Fermentation

Solid-state fermentation (SSF) is a process where microbial cultures develop on moist solid substrates with limited water content, in the absence or near absence of free water (Dobrev et al., 2018). SSF has also been acknowledged to enhance the functional properties of different feedstuffs, resulting in improved properties for a wide range of applications, including bread quality (Chandrasekar et al., 2022). Figure 2 presents a general overview of the SSF process of converting organic waste to bio-products.

SSF has advantages, such as the hydrolysis of complex polysaccharides to disaccharides and monosaccharides (Vaitkevičienė et al., 2022). SSF has been used to produce enzymes by utilizing cellulosic by-products as substrates (Shittu et al., 2009). This process can also help upgrade the nutritional value of certain food and for aflatoxin production (Chahal et al., 1983). Furthermore, it has also been used to reduce the phytate content in whole-grain cereals, providing a means of upgrading grains through fermentation (Nambi, 2017). This is a simple, cost-effective and widely used process with high yield and effective product recovery (G & Sukumar, 2020). The biotransformation of organic wastes into bio-products by microorganisms has been a very promising research field using the technique of SSF (Yazid et al., 2017). SSF is currently viewed as a very attractive technology to develop in order to obtain higher added-value compounds in modern fermentation processes (Manan & Webb, 2017).

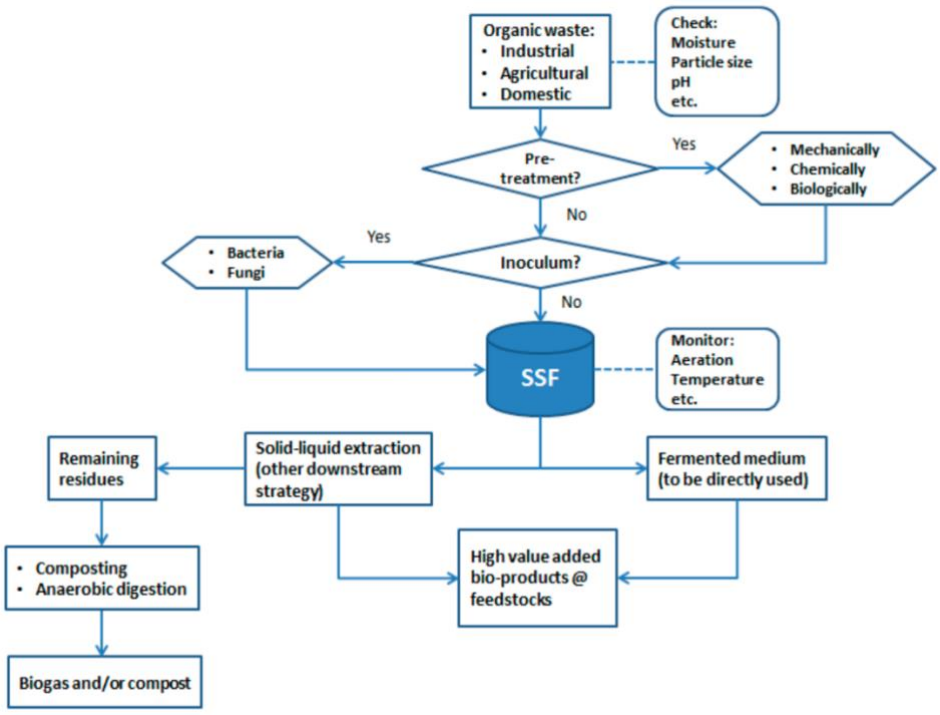

Figure 2. Flowchart of organic waste valorization into bio-products via SSF (SSF) (Yazid et al., 2017).

SSF of feedstuffs for aquafeeds is a promising approach to improve diet quality and the overall physiological health of farmed fish (Das et al., 2021). Feeding fermented products in aquafeed has shown promising results in improving non-specific immune responses and disease resistance in farmed fish, which could be a useful replacement to warrant reductions in the use of antibiotics in the aquaculture industry (Kim et al., 2009). For instance, in Rohu (*labeo rohita*), fish feed containing sesame oil cake and mahu oil cake, solid fermented by *Saccaromyces cerevisiae,* has been shown to improve growth performance, digestive enzyme activities, and innate immunity aspects (Das et al., 2021). Moreover, SSF has been observed to improve the growth performance of catfish fed pelletized fish feeds with Black Soldier Fly larvae meal, suggesting that SSF can be used to enhance feed utilization and growth in aquaculture species (Hariyono et al., 2022). The potential of SSF to improve the nutritive value of agricultural residues, such as sorghum straw, by protein enrichment of *Aspergillus oryzae* cultivated in solid-state processes was also described (Bathe et al., 2012). This highlights the potential of SSF to enhance the nutritional profile of feed raw materials, which is critical for developing cost-effective and nutritionally balanced aquafeeds. This demonstrates that SSF can enhance the nutritional value of feed ingredients and help aquafeeds to be sustainable in the future. A significant area of interest is the valorization of macroalgae through fermentation technology for the aquafeed industry, which presents significant potential for improvement of

digestibility, growth rate, feed efficiency, and body composition of aquaculture animals as well (Ang et al., 2021). Indeed, sequential bioprocessing through SSF of seaweed (*Ulva rigida*) showed the production of lignocellulolytic enzymes and improved its nutritional quality for aquaculture feed (Fernandes et al., 2019).

Optimizing the conditions for SSF is crucial to achieving the desired outcomes. Several key factors influence the SSF process, including temperature, pH, moisture level, solid-to-liquid ration, fermentation duration, and water activity of the substrate, as microbes rely on this parameter to thrive (Bai et al., 2019; Robinson & Nigam, 2008; Nigam & Singh, 1994). Maintaining an appropriate temperature and pH, typically around 30^{o}C and 7, respectively, is vital for supporting microbial activity and growth during SSF (Bai et al., 2019). The optimal fermentation duration can vary with some studies reporting 168 hours as an ideal timeframe for specific SSF processes (Bai et al., 2019).

SSF utilizing actinobacteria has gained significant attention in recent years as a promising biotechnological approach for various applications. Several studies have demonstrated the potential of actinobacteria-driven SSF for the production of enzymes, antibiotics, and other valuable compounds (Sharma et al., 2014; Tiwari et al., 2018). One notable application of actinobacteria in SSF is the production of enzymes, such as cellulases and xylanases, which are crucial for the degradation of lignocellulosic biomass (Brijwani et al., 2010). *Streptomyces* species, in particular, have been extensively studied for their ability to produce these enzymes under SSF conditions using agricultural residues as substrates (Nascimento et al., 2009; Sohail et al., 2009). The optimization of SSF parameters such as temperature, pH, and moisture content, has led to significant improvements in enzyme yields (Bai et al., 2019; Mitra et al., 2020). In addition to enzyme production, actinobacteria-based SSF has also been employed for the synthesis of antibiotics and other bioactive compounds. Researchers have successfully utilized SSF to enhance the production of antibiotics like tyrosine, neomycin, and streptonigrin by optimizing the fermentation conditions and media composition (Tiwari et al., 2018; Zhu et al., 2014).

1.7 *Dicentrarchus labrax* (linnaeus, 1758)

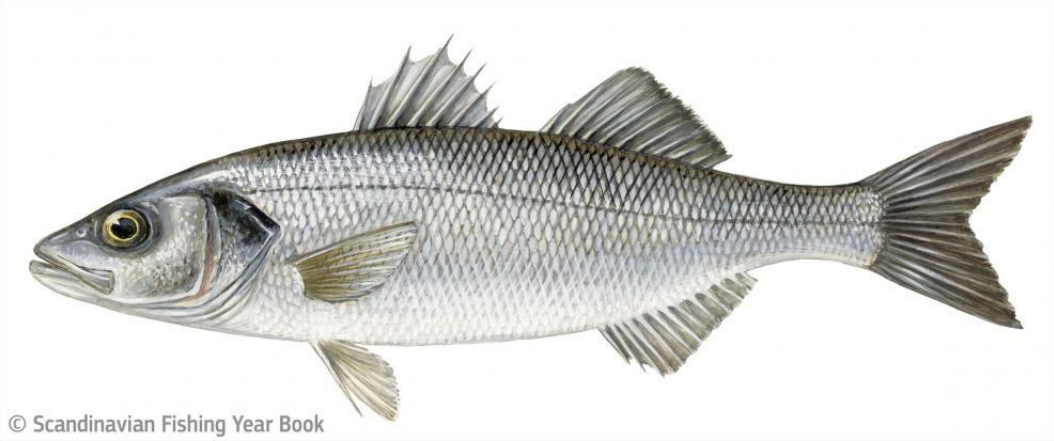

Figure 3. *Dicentrarchus labrax* (European Commision, n.d.).

The European seabass, Figure 3, is a commercially important marine teleost fish species found in the coastal waters of the Mediterranean Sea and eastern Atlantic Ocean (Pickett & Pawson, 1994). Seabass is a predatory fish that plays a key role in coastal ecosystems (Pickett & Pawson, 1994). The seabass exhibits distinct habitat preferences and migratory behaviors throughout its life cycle, allowing it to exploit different ecological niches (Pickett & Pawson, 1994). In its embryonic and larval stages, European seabass resides in the marine zone, and as a juvenile, it moves to coastal waters, estuaries, and lagoons where there is sufficient plankton for feeding (Kaitetzidou et al., 2015). The importance of these habitats is demonstrated by the fact that the distribution of juvenile seabass nursery areas on the shoreline is directly related to the inshore nursery grounds. Recruitment at different inshore nursery locations is crucial in recruiting juvenile seabass to adult standing stocks (Cobain et al., 2019). Recent studies by Dambrine et al. (2021) have pointed to offshore areas as necessary for seabass spawning and have highlighted the importance of specific marine environments for the reproductive success of the species.

The European seabass is a euryhaline species, tolerating salinity levels from 3 to 33 ppt (Young & Shaikhi, 2022). It commonly spawns at the mouth of estuaries in salinities ranging from 28 to 34 ppt. Temperature is another important abiotic factor influencing the habitat and behaviour of European seabass. This species shows ectothermal behaviour with a temperature spectrum between 5 to 28^{o}C (Young & Shaikhi, 2022). For example, it has been suggested that ocean warming could benefit some populations of European seabass, such as those found in the Mediterranean, while harming others (Howald et al., 2019).

1.7.1 Aquaculture production

The European seabass (*Dicentrarchus labrax)* is one of the most important fish species for Mediterranean aquaculture, with total production reaching 305,000 tonnes in 2021, of which 98% came from aquaculture and 2% from fisheries (EUMOFA, 2021; Peñaloza et al., 2021). Turkey is the largest producer, accounting for 52.2% of global seabass output, followed by Greece (15.7%) and Egypt (11.5%) (Compassion in Food Business, 2021).

The production of European seabass has become increasingly important for the aquaculture industry in Mediterranean countries. Over the past two decades, the contribution of Mediterranean nations to the global aquaculture output of European seabass has steadily increased (Smyrli et al., 2017). This growth can be attributed to the adoption of diverse aquaculture systems for seabass production, each presenting unique strengths and challenges regarding production capacity and environmental sustainability (Georgopoulou et al., 2021). Historically, seabass farming was conducted on a small scale in coastal lagoons using wild-caught juveniles, but the industry has since shifted towards more intensive, hatchery-based production systems (ASC, 2022).

The production cycle of European seabass can be broadly categorized into extensive and intensive systems. Figure 4 illustrates the production cycle of European seabass in extensive systems such as estuaries or ponds, where seabasses are cultured in natural water bodies (Food and Agriculture Organization of the United Nations, 2024). On the other hand, Figure 5 provides an example of the type of production in intensive systems, where seabasses are reared in controlled, land-based facilities (Food and Agriculture Organization of the United Nations, 2024). The diversity of aquaculture approaches employed for this species highlights the versatility and economic importance of the European seabass within the Mediterranean region.

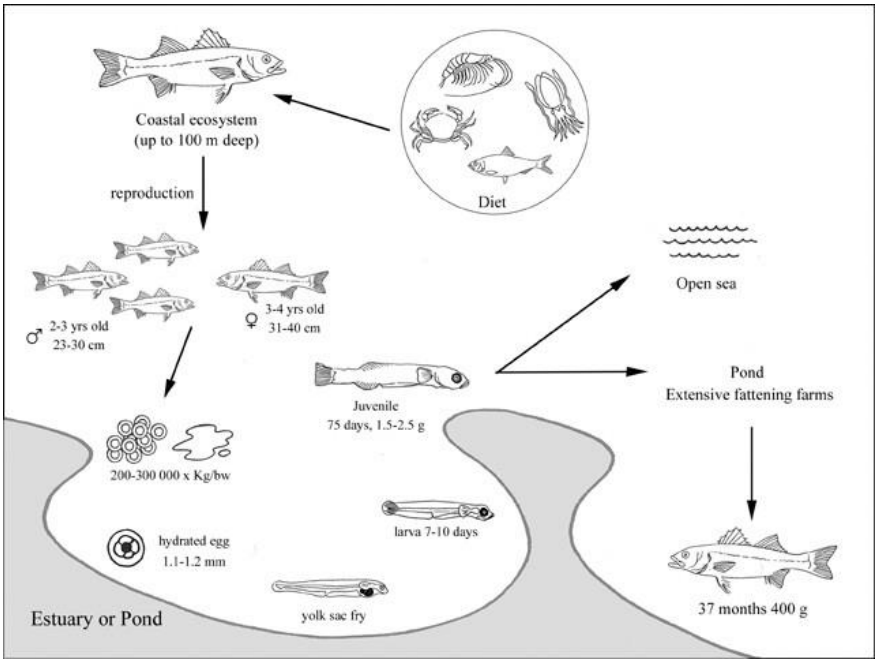

Figure 4. Production of European seabass in extensive system (Food and Agriculture Organization of the United Nations, 2024).

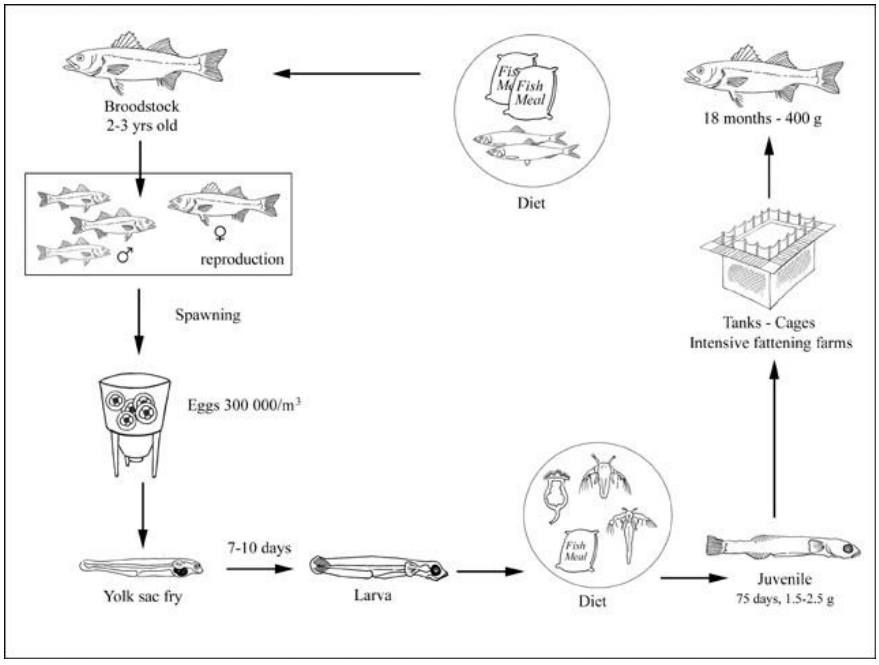

Figure 5. Production of European seabass in intensive system (Food and Agriculture Organization of the United Nations, 2024).

1.7.2 Nutritional Requirements of European Seabass

In aquaculture, providing an optimized diet is crucial to optimize growth, health, and welfare of the species (Kousoulaki et al., 2015). European seabass is a carnivorous fish species with a high protein requirement, particularly from animal protein sources (Sallam et al., 2021). Studies have indicated that the dietary protein requirement for growth in European seabass is estimated to be around 45-48% of the diet, with protein being efficiently utilized, especially at lower water temperatures (Perés & Oliva-Teles, 2005). Dietary interventions with specific aminoacids like tryptohphan and methionine have been explored to modulate immune responses and inflammatory reactions in European seabass (Machado et al., 2022). Table 2 summarizes the essential amino acid requirements of European seabass.

Table 2. Essential amino acid requirements for European seabass (Adapted from Kousoulaki et al., 2015).

Essential amino acids (EAA)	Requirement (g kg^{-1} of crude protein)	Fish size (g)
lysine	44	0.9
Methionine + Cysteine	18-19	13.4
Threonine	23-26	7.5
Arginine	39	2
Tryptophane	5-7	fingerling

1.8 Objectives of the study

This research thesis aims to advance sustainable aquaculture by evaluating the potential of GBP as an ingredient in aquafeed and exploring treatments to enhance its bioavailability. The study focuses on the SSF of GBP by Actinomycetota isolated from macroalgae, with the goal of incorporating these industrial by-products, typically discarded, into nutritious, low-fiber fermented macroalgae, aligning with the principles of circular economy. To achieve this, the research is structured around two central aims and employs a holistic approach that integrates both *in vitro* and *in vivo* methods. Unfermented GBP was evaluated for nutrient digestibility by the European seabass, a core species in Mediterranean aquaculture, and in the second part GBP was subjected to solid-state fermentation using three Actinomycetota strains in order to improve its nutritional value. Ultimately, the research aims to create opportunities for novel approaches in

aquaculture, establishing sustainable solutions and promoting circular economy principles in utilizing marine resources.

2. Materials and Methods

2.1 Experimental diets

The GBP, sourced from Iberagar Sociedade Luso-Espanhola de Coloides Marinhos SA, was initialy ground into a finer grain size. A portion of GBP was subjected to solid alkaline treatment with a 1N NaOH solution at a solid-to-ratio of 4:3, and then autoclaved at 121°C for 30 minutes. The digestibility of untreated and solid alkaline treated GBP was determined in the digestibility trial. For that purpose, five diets were formulated to evaluate the effects of varying levels of GBP supplementation, both without and with NaOH treatment, on the nutritional performance of the fish. In the formulation of experimental diets, a base of fish meal, CPSP, corn gluten, and pea protein concentrate was used as the primary protein sources. Variations among the diets were achieved by adjusting the levels of soybean meal, wheat gluten, and incorporating GBP. The control diet contained no GBP and had higher levels of soybean meal and wheat gluten. The G5 diet included 5% GBP, which replaced 10% of the wheat and soybean meal. The G10 diet incorporated 10% GBP, replacing 20% of these ingredients, while the G15 diet contained 15% GBP, substituting 25% of the wheat and soybean meal. Additionally, the G15NaOH diet featured 15% GBP that had been pre-treated with NaOH, replacing a similar proportion of wheat and soybean meal.

All diets were enriched with fish oil, vitamin and mineral premixes, choline chloride, shrimp hydrolysate, a binder, and chromium oxide (Cr_2O_3) for digestibility tracking. Methionine levels were adjusted according to diet composition to ensure optimal amino acid balance.

All dietary ingredients were finely ground, and proximate composition was analyzed before the feed formulation. Following the feed formulation, ingredients were well mixed in a Hobart mixer, and the mixture was then dry pelleted without steam using a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA). Pellets were then dried in an oven at 50°C for 48 hours and stored in the refrigerator until used. The ingredients and proximate composition of the experimental diets are presented in Table 3.

Table 3. Ingredient composition of the experimental diets.

	Control	G5	G10	G15	G15NaOH
Ingredients (g/kg DM)					
Fish meal[a]	421.3	421.3	421.3	421.3	421.3
CPSP[b]	142.0	142.0	142.0	142.0	142.0
Corn gluten[c]	459.7	459.7	459.7	459.7	459.7
Wheat gluten[d]	25.2	6.7	0.0	0.0	0.0
Soybean meal[e]	493.2	493.2	475.7	448.5	448.5
Pea Protein Concentration[f]	277.8	277.8	277.8	277.8	277.8
Wheat meal[g]	485.8	361.1	241.5	124.9	124.9
GBP[h]	0.0	142.0	284.1	426.1	462.4
Fish oil[i]	299.7	303.0	306.3	309.5	309.5
Vitamin premix[j]	25.0	25.0	25.0	25.0	25.0
Mineral premix[j]	25.0	25.0	25.0	25.0	25.0
Choline chloride (50%)	12.5	12.5	12.5	12.5	12.5
Shrimp hydrolisate[k]	30.0	30.0	30.0	30.0	30.0
Binder[l]	25.0	25.0	25.0	25.0	25.0
Methionine	1.4	1.9	2.3	2.7	2.7
Chromium	12.5	12.5	12.5	12.5	12.5
Proximate analysis (% DM)					
Dry matter (%)	94.7	94.7	95.6	95.4	95.0
Crude protein	43.3	43.4	41.6	41.6	39.2
Crude lipid	15.8	15.0	15.7	14.4	14.3
Ash	8.3	12.5	17.4	23.1	24.2
NDF	13.4	14.4	16.4	18.7	17.9
Inorganic ADF	3.0	4.4	6.8	9.1	7.1
Organic ADF	1.9	4.0	4.8	5.3	3.9

[a] Pesquera Centinela, Steam Dried IT, Chile. Sorgal, S.A. Ovar, Portugal (CP: 70.3 %; CI: 12 %)
[b] Sopropeche G, France (CP: 80.2%; CI: 15.4%)
[c] Sorgal, S.A Ovar, Portugal (CP: 62.8 % CI:1.2%)
[d] Sorgal, S.A Ovar, Portugal (CP: 73.5 % CI: 1.7 %)
[e] Non-GMO; Cargill France SAS, St. Germain-en-laye, France (CP: 56.4 %; CI:1.9 %)
[f] Sorgal, S.A Ovar, Portugal (CP: 54.9 %; CI: 3.3%).
[g] Sorgal, S.A Ovar, Portugal (CP: 13.8 %; CI: 3.2 %)
[h] Iberagar Sociedade Iuso-Espanhola de Coloides Marinhos SA (CP: 22.5 %; CI: 0.4%)
[i] Vitamins (mg kg-1 diet): retinol, 18000 (IU kg-1 diet); calciferol, 2000 (IU kg-1 diet); alfa tocoferol, 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.
[j] 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
[k] Sorgal, S.A Ovar, Portugal
[l] Aquacube. Agil, UK

2.2 Digestibility trial

This trial was approved by the CIIMAR animal welfare body (ORBEA-CIIMAR; reference ORBEA_CIIMAR_27_2019) and subsequently approved by the Direção Geral de Alimentação e Veterinária (DGAV). All researchers involved in this trial followed the guidelines of the European Union (directive 2010/63/EU) and Portuguese law (Decreto lei no. 113/2013, de 7 de Agosto) regarding the protection of animals used for scientific purposes and was conducted by accredited researchers in accordance with FELASA category C recommendations. The experiment occurred at CIIMAR and utilized juvenile European seabass (*Dicentrarchus labrax*).

For the digestibility trial, a thermo-regulated water system with 10 fiberglass tanks of 60-liter capacity was utilized. These tanks were equipped with feces settling columns connected to outlets, designed based on the Guelph system as shown in Figure 6 (Cho et al., 1982). A total of 120 fish were allocated to the experimental setup and acclimatized to the tanks and water temperature over a two-week period. Following acclimatization, 10 groups of 10 European seabass, with an initial average body weight of 161 g, were randomly assigned to each tank. Digestibility measurements were conducted over two periods using a complete randomized block design.

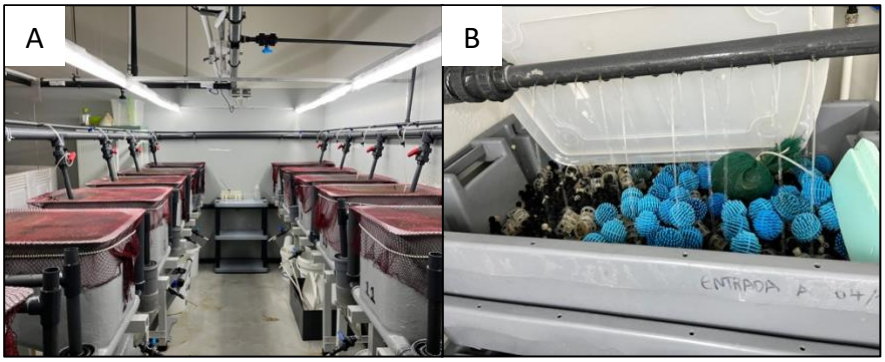

Figure 6. Thermoregulated water system. (A) Fiberglass tanks with 60l of water capacity each tank; (B) Biological filters.

In each period, the experimental diets were randomly allocated to duplicate groups of fish and the animals were hand-fed twice a day to apparent visual satiation at 9:30 hr and 16:30 hr. Fish were allowed to adapt to the experimental diets for 5 days, and then, feces accumulated in each settling column were pooled and collected daily before the morning meal for at least 15 days, depending on the total quantity of recovered feces (Magalhães et al., 2017). The diets were randomly reallocated to another duplicate group of fish, and the procedure was

repeated for another 15 days. The distribution of the experimental diet is stated in Table 4.

Table 4. Diet distribution for each period and each tank

Period	Tank									
	1	2	3	4	5	6	7	8	9	10
I	G5	G15NaOH	G10	Control	G15	G5	G10	G15	Control	G15NaOH
II	G15NaOH	G5	G15	G10	Control	G10	G5	G15NaOH	G15	Control

Daily, before the morning feeding, feces from each tank were directly collected into a 500 ml beaker and allowed to settle for 15 minutes. After sedimentation, the solid portion was transferred into 50 ml centrifuge tubes (two tubes per tank) and centrifuged for 10 minutes at 4000 g. The resulting feces pellets were pooled by each tank and stored at -20°C until analysis. The feces were dried at 70°C for 48 hours and then ground using mortar. Approximately 30 minutes after the afternoon feeding, the tanks, and settling columns were thoroughly cleaned, with 50% of the tank water being removed to ensure the elimination of any remaining feed or feces. Water temperature and salinity averaged 20°C and 32‰ throughout the trial, respectively.

The Apparent Digestibility Coefficients (ADC) for dry matter, protein, lipids, and energy of the diets were calculated using the following formula:

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

2.3 Chemical analyses

The proximate composition of diets and feces was analyzed using the following the standard procedures of the Association of Official Analytical Chemists (Official Methods of Analysis of AOAC International, 1997). Dry matter content was determined by drying the samples at 105°C until a constant weight was achieved. Ash was quantified by incinerating the samples in a muffle furnace at 450°C for 16 hours. Protein content was analyzed using the Kjeldahl method, which involves acid digestion, followed by distillation and titration using Kjeltec equipment (Tecator Systems, Höganäs, Sweden; models 1015 and 1026). Lipid was extracted from the samples using petroleum ether in a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046). lastly, NDF and ADF (organic and inorganic) contents were determined using Fibertherm (Fibretherm FT 12, Gerhardt). Procedures for NDF and ADF were carried out on fermented GBP samples as well. Chromium oxide content in the diets and feces was quantified using acid digestion (Bolin et al., 1952).

2.4 Actinomycetota culture

Three Actinomycetota strains (Table 5) were selected within the scope of this project. These microorganisms were previously isolated from the tissues of two macroalgae collected in the northern Portuguese coast (Girão et al., 2024) and preserved at -80 °C at CIIMAR. The Actinomycetota strains were streaked onto solid agar plates using Nutrient-poor Sediment Extract agar (NPS) and Actinomycete Isolation Agar (AIA) (Table 6) and incubated at 28 °C until colony growth. The time for incubation was set according to each strain requirements, typically ranging from 7 to 14 days.

Table 5. Selected Actinomycetota strains

Strain	**Culture Media**	**Species taxonomy[1]**
CC-R104	NPS	*Rhodococcus chondri*
CT-R177	AIA	*Cellulosimicrobium funkei*
CC-F26	AIA	*Streptomyces violascens*

[1]Taxonomic identification according to 16S rRNA gene sequencing as described by Girão et al., 2024.

Table 6. Formulation of the selective culture media used for Actinomycetota isolation

Culture Media	**Composition**
NPS[a]	1. Agar 17g 2. Marine sediment extract 100ml – obtained by washing beach sand with seawater 500ml
AIA[b]	1. Agar 17g 2. Sodium propionate 4g 3. K_2HPO_4 0.5g 4. Na_2CO_3 0.2g 5. $FeSO_4$ 0.2g 6. l-arginin 0.1g 7. $MgSO_4$ 0.2g

Note: All media were supplemented with cycloheximide (50 mg l^{-1}), nalidixic acid (50 mg l^{-1}) and nystatin (50 mg l^{-1}) (Sigma-Aldrich, MO, United States).

[a]Per litre of seawater.

[b]Per litre of 60:40 seawater/deionized water.

2.5 Solid-state Fermentation (SSF)

SSF was performed using 3 g of sterile GBP inoculated with 8 ml of an inoculum of the selected Actinomycetota strains, in 100 ml Erlenmeyer flasks. The three microbial inocula were prepared using 8 mL of sterile saline solution at 0.85% and a loopful of the corresponding bacterial strain. Each strain was tested in triplicate. A control without using any microorganism was also performed, in triplicate. The Erlenmeyer flasks were closed and incubated at 25 °C for 7 days.

2.6 Bioactivity assay

2.6.1 Crude extract preparation

After the 7 days of SSF, the biomass of fermented GBP was harvested and freeze-dried to preserve the integrity of organic compounds. Specifically, 1.2 g of the freeze-dried sample was used for crude extract preparation, as described by Girão et al. (2019). Briefly, the sample was subjected to organic extraction using 30 ml of a mixture of methanol and acetone 1:1 (v/v). The mixture was kept under agitation for 30 minutes. After extraction, the organic layer containing the crude extract was separated, and the solvents were evaporated using a rotary evaporator. The organic extracts obtained were then weighted and dissolved in dimethyl sulfoxide (≥99.9%, DMSO; Sigma-Aldrich, MO, United States) to prepare a stock solution at 1 mg ml^{-1}, which were used for the antimicrobial bioactivity assay.

2.6.2 Antimicrobial activity screening

Crude extracts derived from fermented and unfermented GBP were evaluated for their antimicrobial properties against ten reference microorganisms, as detailed in Table 7, using the agar-based disk diffusion method following as described by Girão et al. (2019). The bacterial strains (*Escherichia coli, Bacillus subtilis*, *Staphylococcus aureus*, and *Salmonella typhimurium*) were grown in Mueller-Hinton Agar (MHA); Sabouraud Dextrose Agar (SDA) was used to grow *Candida albicans*; Tryptic Soy Agar (TSA) was used for the growth of *Edwardsiella tarda*, A*eromonas hydrophila*, *Pseudomonas anguilliseptica*, *Yersinia ruckeri*, and *Listonella anguillarum*; Marine Agar (MA) for *Tenacibaculum maritimum*; and Tryptic Soy Yeast Extract Agar (TSYA) for *Lactococcus garvieae* (Liofilchem, Roseto d. Abruzzi, Italy). The assay was performed with the turbidity of each test organism adjusted within the 0.5 McFarland standard (OD_{625} = 0.08 - 0.13) in the corresponding culture media. These suspensions were used to inoculate agar plates, by evenly streaking the plates using a swab immersed in the suspension.

Subsequently, sterile paper disks (6 mm in diameter, Whatman, UK) were placed on the surface of the inoculated agar plates. Each disk was then loaded with 15 µl of the respective Actinomycetota crude extract at 1 mg ml^{-1} in DMSO. Antimicrobial activity was determined by measuring the diameter of the inhibition halo formed around each disk. Positive controls consisted in Enrofloxacin (1 mg ml^{-1}; Sigma-Aldrich, MO, United States) for all bacterial strains and Nystatin (1 mg ml^{-1}; Sigma-Aldrich, MO, United States) for the yeast *Candida albicans*. DMSO was used as negative control for all the microorganisms.

Table 7. Reference strains used for antimicrobial assay

Strains	Culture Medium	Incubation Temperature (°C)	Incubation Time (h)	Positive Control
Escherichia coli	MHA	37	24	Enrofloxacin
Bacillus subtilis	MHA	37	24	Enrofloxacin
Staphylococcus aureus	MHA	37	24	Enrofloxacin
Salmonella typhimurium	MHA	37	24	Enrofloxacin
Candida albicans	SDA	37	24h	Nystatin
Edwardsiella tarda	TSA	28	24h	Enrofloxacin
Aeromonas hydrophila	TSA	28	24h	Enrofloxacin
Pseudomonas anguiliseptica	TSA	25	48-72	Enrofloxacin
Yersinia ruckeri	TSA	28	24-28	Enrofloxacin
Listonella anguillarum	TSA	28	48	Enrofloxacin
Tenacibaculum maritimum	MA	25	24-48	Enrofloxacin
Lactococcus garvieae	TSYA	28	24-48	Enrofloxacin

2.7 Statistical analysis

Statistical analysis of ADC was carried out by analysis of variance according to a randomized complete block design, with each fecal collection period as block. Fermented GBP samples were analyzed by one-way analysis of variance. Significant differences between means were determined by Tukey's multiple range test. All analyses were performed using the probability level of 0.05 for rejection of the null hypothesis. To fulfill the assumptions of ANOVA, including normality and homogeneity of variances, transformations were applied to the data when necessary. Specifically, the ADC for lipids was subjected to natural logarithm transformation, while the NDF values of all samples underwent inverse

transformation, and the organic ADF of all data were log-transformed. All statistical analyses were performed using SPSS 29.0 software package for Mac.

3. Results

3.1 Digestibility trial

Throughout the trial period, fish adapted rather fast to the experimental diets and there was only one recorded death. The results of the ADC for dry matter, protein and lipid are shown in the Table 8.

The ADC of dry matter demonstrated significant variations among the dietary treatments ($p < 0.001$). The control diet yielded the highest ADC for dry matter at 78%, while the G15 diet exhibit the lowest value at 61.8%. intermediate ADCs were observed in the G5, G10, and G15NaOH diets, with values of 69.0%, 66.6%, and 73.8%, respectively. Similarly, the ADC of protein was significantly influenced by the dietary treatments ($p < 0.001$). The control diet again exhibited the highest ADC for protein at 94.5%, whereas the G15 diet recorded the lowest at 85.6%. the G5, G10, and G15NaOH diest presented intermediate protein digestibility values of 90%.7%, 88.3%, and 91.4%, respectively.

In contrast, the ADC of lipid did not show statistically significant differences across the dietary tratments ($p = 0.059$), with values ranging from 94.5% in the G5 diets to 98.1% in the G15NaOH diet. However, this result shows that lipid digestion remains constant in response to treatment and inclusion of GBP.

Table 8. The ADCs of the experimental diets

	Control	G5	G10	G15	G15NaOH	SEM	p-Value
Dry Matter	78.0^{a}	69.0^{bc}	66.6^{bc}	61.8^{c}	73.8^{ab}	1.5	< 0.001
Protein	94.5^{a}	90.7^{b}	88.3^{bc}	85.6^{c}	91.4^{b}	0.8	< 0.001
Lipid	97.7	94.5	97.3	97.2	98.1	0.4	0.059

Data expressed as means (n = 3) and pooled standard error of the mean (SEM).
Different superscript letters within the same row denote significant differences between dietary treatments (Tukey's test, $p < 0.05$).

3.2 Solid-state Fermentation (SSF)

The nutirional composition of the GBP before and after the SFF with three Actinomycetota strains is presented in Table 9. The analysis focused on the NDF, ADF, and ash content of the GBP. The NDF content significantly differed among the treatments ($p< 0.001$), with the control GBP exhibiting the highest value at 59.1%. The fermented GBP showed reduced NDF levels, with strain CC-R177 reducing NDF content to 55.0%, strain CC-F26 reducing to 54.5%, and strain CC-R104 to 53.5%. Similarly, the inorganic ADF values revealed significant differences ($p < 0.001$), with the control treatment again presenting the highest content at 42.1%. The fermented GBP demonstrated lower inorganic ADF levels, particularly

the one fermented by strain CC-R104, which had the lowest content, 30.7%. The GBP fermented by strains R177 and F26 showed values of 35.6% and 40.1%, respectively. The organic ADF results also indicated significant differences ($p < 0.001$), with the control having the highest organic ADF at 32.0%. The fermented GBP showed lower organic ADF values, being obtained the values of 27.1%, 27.5%, and 28.6% for GBP fermented by strains CC-R177, CC- R104 and CC- F26, respectively.

Lastly, the ash content varied significantly across the treatments ($p < 0.001$). The control had the lowest ash content at 39.9%, while the fermented GBP exhibited increased ash levels, 44.2%, 47.8% and 50.7% for strains CC-R177, CC-R104 and CC- F26, respectively.

Table 9. Comparison NDF, Inorganic ADF, Organic ADF, and Ash values of each treatment

	Control	CC-R177	CC-F26	CC-R104	SEM	p-Value
NDF	59.1^{a}	55.0^{b}	53.5^{c}	54.5^{bc}	0.8	< 0.001
Inorganic ADF	42.1^{a}	35.6^{b}	30.7^{c}	40.1^{d}	1.7	< 0.001
Organic ADF	32.0^{a}	27.1^{b}	27.5^{b}	28.6^{b}	0.7	< 0.001
Ash	39.9^{a}	44.2^{b}	50.7^{c}	47.8^{d}	1.5	< 0.001

CC-R177: *Cellulosimicrobium funkei*; CC-F26: *Streptomyces violacens*; CC-R104: *Rhodococcus chondri.*

Data expressed as means (n = 3) and pooled standard error of the mean (SEM).

Different superscript letters within the same row denote significant differences between dietary treatments (Tukey's test, $p < 0.05$).

3.3 Antimicrobial activity

In the agar diffusion assay, no inhibition zones were observed around the disks loaded with the crude extracts tested, indicating that the crude extracts obtained from the selected Actinomycetota strains did not show antimicrobial activity against the screened reference microorganisms.

4. Discussion

The increasing demand for aquaculture necessitates the exploration of novel nutrient sources for aquafeeds. Traditional ingredients like fish meal and fish oil are becoming unsustainable due to over-exploitation and environmental concerns, prompting a shift towards alternative protein and lipid sources, such as insect meals, plant-based ingredients, and even food waste (Fawole et al., 2021; Ghamkhar & Hicks 2021; Colombo & Turchini, 2021). In this context, this study investigates the use of GBP as a potential ingredient in aquafeeds. GBP utilization embodies the principles of a circular economy by converting an underutilized by-product into a valuable resource for aquafeeds.

The inclusion of GBP in aquafeeds has been associated with a reduction in digestibility. This finding aligns with other studies examining the effects of various macroalgae on fish diets. For instance, Norambuena et al. (2015) reported that juvenile Atlantic salmon (*Salmo salar)* experienced decreased protein digestibility when fed with diets containing *Ulva* spp. at levels of 10%. Similarly, Paiva et al. (2017) found that the incorporation of *Gelidium microdon* at levels of 10% in the diets of European sea bass resulted in decreased digestibility. Another study reported that the addition of *Ulva* spp. at 15% in diets for Nile tilapia also led to reduced digestibility (Okeke-Ogbuafor, 2024).

In our study, similar results were found where highest inclusion of GBP without any treatment (G15) showed a significant decrease to 61.8% in dry matter and 85.6% in protein. This is consistent with other research that has suggested high dietary fibre contents have a negative impact on nutrient absorption in fish. Fiber, being a complex carbohydrate, is not digested well by fish, particularly by carnivorous species like European sea bass. The fiber has an impact on fish health and feed conversion ratios and further categorizes into NDF and ADF and the presence of ash (Calvert et al., 1985; Ravindran et al., 1984). NDF encompasses the total fibrous content, including cellulose, hemicellulose, and lignin, which contribute to the feed's bulk and can impact nutrient availability (Van Soest, 1994). High NDF diet can lead to higher gut fill and poor nutrient digestibility and that in turn reduces feed conversion (Malik et al., 2020; Tye et al., 2017; Hatungimana et al., 2020). The ADF fraction, on the other hand, represents more resilient and less digestible fiber fractions like cellulose and lignin; thus ADF is associated with lower total tract digestibility of nutrient (Chen et al., 2018).

The digestibility trial results revealed significant variations in the apparent digestibility coefficients (ADCs) for dry matter, protein, and lipid across different dietary treatments (Table 8). ADCs are important for determining the efficiency of feed, specifically how well ingredients will be absorbed and used by fish. By measuring the ADCs of energy-yielding nutrients in the feed—such as starch,

sugars, fat, protein, and non-starchy polysaccharides—we can determine the effectiveness of the feed in providing digestible nutrients to the fish (Tibbetts et al., 2004). High ADC values for dry matter, protein and lipids are indicating better nutrient utilization which is a prerequisite to optimize growth and health in aquaculture species (Chu et al., 2014; Tibbetts et al., 2006). Conversely, an excessive fiber content added to the diets interferes with the digestion and nutrients absorption, such as protein, fat, and carbohydrate contents, leading to lower ADC values for these nutrients (Magalhães et al., 2015; Herdiyanti et al., 2018). These findings underscore the necessity of careful management of fiber-rich ingredients in aquafeed formulations, where fish feed should have the acceptable concentration of NDF and ADF that varies depending on the kind of fish and nutrients necessary for balancing its diet (Calvert et al., 1985).

Our results demonstrate a clear relationship between the inclusion level of GBP in the diet and the observed ADC values. As the inclusion level of GBP increase from G5 to G15, ADC values for dry matter and protein decreased relative to the control diet. This trend highlights the negative impact of higher GBP inclusion levels on nutrient digestibility. Specifically, the ADC values for dry matter and protein in the G15 diet were significantly lower compared to the control, indicating reduced nutrient utilization with higher GBP levels.

One effective strategy to enhance the utilization of ingredients with high levels of fiber in aquafeeds is to pre-hydrolyze these ingredients through various methods, including chemical processes such as alkaline hydrolysis, or biological processes like SSF (Shi et al., 2017; Amalia, 2024). Pre-hydrolysis can break down complex carbohydrates into simpler, more digestible forms, thereby improving nutrient absorption in fish (Amalia, 2024). The use of alkaline hydrolysis has been shown to effectively reduce the fiber content in feed ingredients, facilitating better nutrient availability (Deng et al., 2020). Notably, the G15NaOH diet, which underwent solid-alkaline hydrolysis treatment, demonstrated an improved ADC of 73.8%, 91.4%, and 98.1% in dry matter, protein, and lipid, respectively. This improvement aligns with the findings of Zaki et al. (1994), where alkali treatments were shown to effectively reduce non-digestible fiber in the diet, thereby enhancing nutrient digestibility in fish. Alkaline treatments are known for reducing the nondigestible fiber content of different natural fibers. These treatments are usually based on the use of an alkaline solution, for example sodium hydroxide (NaOH), that removes impurities such as lignin, hemicellulose and pectin from the fiber surfaces (Aphichartsuphapkhajorn et al., 2019; Jayamani et al., 2015; Xiao-ping et al., 2015). Removal of these components cut down fiber loading, especially hemicellulose and lignin, which improves purification in cellulose with better fiber-matrix attachment (Morais et al., 2017; Sankarathil, 2023; Nurazzi et al., 2019).

In addition to chemical methods like alkaline hydrolysis, SSF offers numerous advantages for enhancing the utilization of high-fiber ingredients in aquafeeds, primarily by hydrolyzing fiber and increasing the bioavailability of nutrients (Shi et al., 2017). This fermentation process involves the growth of microorganisms on solid substrates with limited moisture, promoting the breakdown of complex carbohydrates and the degradation of anti-nutritional factors that can hinder nutrient absorption (Altop et al., 2023; Altop et al., 2019). Research has shown that SSF of Distiller's Dried Grains with Solubles (DDGS) can improve digestibility for European seabass juveniles (Filipe et al., 2023). Similarly SSF has been studied in the context of reducing antinutritional factors in leaf meals for Nile tilapia, showcasing its role in enhancing nutrient availability in fish diets (Kasiga & Iochmann, 2014). Furthermore, the application of SSF has been linked to improved digestive function and gut health in fish, as it promotes the breakdown of fibrous materials and enhances the bioavailability of nutrients (Davies et al., 2021).

In this study, the results demonstrated significant reductions in both NDF and ADF components following SSF using different Actinomycetota strains. A total of three Actinomycetota strains were tested to mediate the reduction in fiber content of GBP in this study. Of the tested strains, *Cellulosimicrobium funkei* (CC-R177) showed ability to degrade fiber components resulting in substantial reductions of NDF and ADF values. Specifically, NDF decreased from 59.1% in the control to 55.0% with CC-R177, and ADF content, both inorganic and organic fractions, was significantly reduced as well. These reductions suggest that CC-R177 effectively breaks down complex polysaccharides, such as lignocellulosic components, enhancing GBP digestibility. This finding suggests that Actinomycetota-mediated SSF processes can indeed reduce fiber content in GBP. Based on the studies by Arisa et al. (2023) and Das et al. (2021), it can be inferred that the genus *Cellulosimicrobium*, known for its cellulolytic activity, could potentially help reduce fiber content in ingredients through fermentation processes. *Streptomyces violascens* (CC-F26), the second strain we used in this study, also demonstrated significant results, reducing NDF to 53.5%, indicating effective degradation of fibrous components. *Streptomyces* strains have been studied for its potential to produce lignin degrading enzymes, such as laccases, under SSF conditions, indicating their capability to degrade lignin and potentially fiber components (Blánquez et al., 2017; Orozco et al., 2008). In contrast, CC-F26 exhibited the highest ash content, 50.7%. This increase may be due to the release or modification of mineral components during fermentation by CC-F26. Future studies are needed to better understand these changes and their impact on the SSF process of GBP, as well as their implications for the overall nutritional profile. Study by Omodara (2024) suggested that higher ash contents were recorded in fermented seeds associated with decreased antinutrients, resulting in increased

bioavailability of minerals and, consequently, higher ash content. Similarly, Agustina et al. (2022) mentioned that changes in material composition during fermentation could lead to an increase in ash content due to decomposition of original compounds and the formation of new compounds. *Rhodococcus chondri* (CC-R104) exhibited a moderate reduction in fiber content, with NDF decreasing to 54.5% and inorganic ADF to 40.1%. The reduction in organic ADF was also significant, dropping to 28.6%. Despite these improvements, CC-R104 resulted in a relatively high ash content of 47.8%. The modest fiber content reductions observed with CC-R104 suggest that its fiber-degrading capability may have limited practical value. However, due to its higher ash content, further studies are necessary to determine how CC-R104 affects mineral availability and what implications this has for feed formulation.

When comparing the results of this study to previous studies on SSF, the observed reduction in fiber content are consistent with findings reported by other researchers using different substrates and microorganisms. The study conducted by Jannathulla et al. (2017) shows that *Aspergillus niger* can reduce the cellulose content in guar meal from 7.90% to 5.72%, hemicellulose from 10.70% to 3.93%, and lignin content from 1.11% to 0.44%. Similarly, the use of thermotolerant fungi like *Fomes* sp. in the SSF of corn stover, demonstrates the effectiveness of SSF in breaking down fibrous substrates (Méndez-Hernández et al., 2019). These comparisons suggest that while the choice of microorganism and substrate can influence the degree of fiber breakdown, SSF consistently enhances fiber digestibility across a range of materials. One advantage of this study was the screening of varius Actinomycetota strains, each contributing to different extents of fiber hydrolysis. Notably, while previous research predominantly focused on fungal strains, this study demonstrates that bacterial strains, namely Actinomycetota, can also effectively reduce fiber content in GBP, broadening the scope for SSF applications in aquafeeds.

Moreover, while the reduction in fiber content is promising, it is important to note that no antimicrobial activity was detected under the specific conditions of this study. This outcome suggests that, although the bacterial strains effectively hydrolyzed the fiber, they did not produce significant antimicrobial compounds. Future studies should investigate alternative approaches, such as testing different solvents for extraction of the SSF biomass, adjusting the substrate-to-solvent ratios, and optimizing the extraction time, to potentially enhance antimicrobial properties. With the significant reduction in fiber content achieved through SSF, determining the digestibility of GBP after fermentation becomes a priority. Reduced fiber levels are expected to increase the bioavailability of nutrients, thereby improving the overall nutritional value of GBP in aquafeeds. Evaluating digestibility will provide crucial insights into how these improvements translate

into enhanced nutrient absorption and feed efficiency, further supporting the potential use of GBP in sustainable aquaculture.

5. Conclusion

The present study evaluated GBP as an alternative to fish meal for European seabass and thus helping to potentially improve the sustainable production of aquaculture. In an effort to enhance the nutritional value of GBP, which is generally a waste by-product in industrial practice, the intent was to subject it to a SSF process with Actinomycetota sourced from macroalgae.

Overall, the use of SSF for treatment of GBP has the potential to enhance the digestibility of this ingredient up to acceptable level for its further application in aquaculture diets. Of all the microbial strains under study, *Cellulosimicrobium funkei* CC-R177 has been among the best performing one in terms of degradation of non-digestible fiber and enhanced nutritional value of GBP. This is in line with the enzymatic profile of the strain, and more so with the cellulase enzymes that the strain is endowed with, given their role in hydrolysing complex polysaccharides present in the GBP. Although *Streptomyces violascens* (CC-F26) was proficient in lowing fiber content significantly, it caused high levels of ash content implying the fact that this strain requires further amendments before it can be recommended for use. The species *Rhodococcus chondri* (CC-R104) has demonstrated potential for reducing fiber content while simultaneously increasing ash levels. This suggests that further research is needed to fully understand the impact of these changes and to optimize the use of this microorganism.

This study constitutes a good basis for future research since enhanced SSF processes yield advantages in the production of aquafeed formulations in terms of sustainability and efficiency. Altogether, this investigation can be regarded as valuable advances in the field of sustainable aquaculture and the more effective utilization of the seas.

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