TANGUY RABILLÉ

Development of sustainable weaning feeds for gilthead sea bream (*Sparus aurata*): the potential of fungi meal from *Paecilomyces variotii* as protein source to replace fishmeal

Faculdade de Ciências e Tecnologia

2024

TANGUY RABILLÉ

Development of sustainable weaning feeds for gilthead sea bream (*Sparus aurata*): the potential of fungi meal from *Paecilomyces variotii* as protein source to replace fishmeal

Mestrado em aquacultura e pescas

(Especialidade em aquacultura)

Trabalho efetuado sob a orientação de:

Dr Marta Carvalho (Universidad de Las Palmas de Gran Canaria)

Dr Cláudia Aragão (Centre of Marine Sciences, Universidade do Algarve)

Faculdade de Ciências e Tecnologia

2024

DEVELOPMENT OF SUSTAINABLE WEANING FEEDS FOR GILTHEAD SEA BREAM (*SPARUS AURATA*): THE POTENTIAL OF FUNGI MEAL FROM *PAECILOMYCES VARIOTII* AS PROTEIN SOURCE TO REPLACE FISHMEAL

Declaração de autoria

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Tanguy Rabillé

Acknowledgements

I would like to express my gratitude to Dr Daniel Montero for his assistance during the project. I would especially thank my internal supervisor Dr Cláudia Aragão. I have learned a lot thanks to her feedback, support and guidance during the writing part and I recognize that I might have used a bit too much of her patience sometimes. I would also like to give a warm thank you to Dr Marta Carvalho, who helped me a lot throughout the year and was always available to me. I would like to thank all the people who helped me during the sampling and analysis. So, thank you, Samira, Adnane, Luis, Victor, Ruben and Carmen, I am really grateful for what you did. Finally, I would like to thank Dr Elsa Cabrita for her patience and for her inspiring lessons.

The project leading to these results has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 871108 (AQUAEXCEL3.0). The work was performed at ULPGC-FITU (Feed Ingredients and Additives Testing Unit) research infrastructure, under grant agreement PID is 27403 (TNA programme). This output reflects only the author's view, and the European Union cannot be held responsible for any use that may be made of the information contained therein.

Abstract

In recent years, intensive efforts have been made by aquaculture research and industry to identify novel alternatives to fishmeal, aiming to create more efficient and sustainable feeds in response to the sector's ongoing expansion. Fishmeal, a traditional source of high-quality protein in aquaculture, has become unsustainable in modern aquaculture due to the overexploitation of wild fish stocks, thus alternative protein sources are needed. This study explores the potential of sustainable weaning feeds for gilthead sea bream (*Sparus aurata*) larvae by evaluating the fungal meal, *Paecilomyces variotii* (PEKILO®) as a protein source to replace fishmeal. The research involved formulating diets with increasing levels of *P.variotii* (5, 10, and 15% inclusion) and evaluating their impact on larval growth performance, survival, stress resistance, and disease resistance against *Vibrio anguillarum*. The experiment was conducted under controlled conditions with larvae (28 dph) fed over 18 days. Results indicated that *P.variotii* could effectively replace fishmeal up to 15% without negatively affecting growth performance and survival. Additionally, larvae fed with higher levels of *P.variotii* exhibited no significant amelioration regarding stress tolerance and pathogen resistance. Increasing levels of *P.variotii* did not significantly affect whole-larvae proximate composition but did affect their fatty acid content. In fish fed the *P.variotti* diets, n-9 fatty acids were significantly lower, while DHA and n-6 fatty acids were significantly higher. Moreover, the inclusion of *P.variotii* in larval diets led to no significant changes in gene expression related to immune function. This study demonstrates the potential of *Paecilomyces variotii* fungal meal as a sustainable alternative to fishmeal in the larval diets for gilthead sea bream, offering a promising solution for improving the sustainability of aquaculture.

Keywords:
Aquaculture, filamentous fungi, *Paecilomyces variotii*, *Sparus aurata*, sustainable feeds

Resumo

O crescimento contínuo do sector da aquacultura tem levado a esforços intensos por parte da indústria e dos investigadores no sentido de identificar novas alternativas à farinha de peixe para obter alimentos mais eficazes e sustentáveis para peixes. Este estudo investigou o desenvolvimento de rações de desmame sustentáveis para larvas de dourada (*Sparus aurata*), centrando-se na utilização da farinha de fungos *Paecilomyces variotii* (PEKILO®) como potencial fonte de proteína para substituir a farinha de peixe, um ingrediente fundamental mas não sustentável nas dietas de aquacultura. A farinha de peixe é conhecida pela sua proteína de alta qualidade, aminoácidos essenciais e ácidos gordos n-3, o que a torna um ingrediente ideal, especialmente durante a fase larvar dos peixes. No entanto, a sua produção é insustentável do ponto de vista ambiental e económico. Este estudo procurou contribuir para a resolução deste problema, avaliando se a farinha fúngica de *P. variotii* pode ser uma alternativa viável nas rações para peixes marinhos, particularmente para as larvas de dourada, onde as necessidades nutricionais são elevadas. A investigação visou avaliar indicadores-chave de desempenho, incluindo crescimento, taxas de sobrevivência, tolerância ao stress, respostas imunitárias e resistência a agentes patogénicos em larvas de dourada alimentadas com diferentes níveis de *P.variotii*. Foram formuladas quatro dietas experimentais: uma dieta de controlo (CTRL), com farinha de peixe como fonte primária de proteína, e três dietas experimentais com 5, 10 e 15% de inclusão de farinha de fungos (substituindo até 100% da farinha de peixe). As dietas eram isonitrogénicas, isolipídicas e isoenergéticas para garantir um conteúdo nutricional comparável. A inclusão de *P.variotii* foi concebida para avaliar se a qualidade proteica, as propriedades promotoras de crescimento e os efeitos imunomoduladores da farinha fúngica poderiam corresponder aos da farinha de peixe. As larvas de dourada foram criadas num ambiente controlado e alimentadas com as dietas experimentais a partir dos 28 dias pós-eclosão (dph) durante 18 dias. Os peixes foram criados em condições controladas e monitorizados diariamente. A alimentação foi dada a cada 45 minutos, das 8:00 às 20:00 horas, durante toda a experiência. No final do período de alimentação, as larvas foram submetidas a um teste de stress (exposição ao ar durante 2 minutos) e a um desafio patogénico com *Vibrio anguillarum* para avaliar a resposta ao stress e a resistência à doença, respetivamente. Os resultados demonstraram que a farinha de fungos *P. variotii* pode substituir eficazmente a farinha de peixe até 15% de inclusão sem afetar negativamente o crescimento ou a sobrevivência. As larvas alimentadas com as dietas de 5, 10 e 15% de farinha de fungos apresentaram taxas de crescimento semelhantes às da dieta controlo, indicando que a qualidade proteica da farinha de fungos foi suficiente para suportar um crescimento normal. Esta descoberta é significativa, uma vez que o desempenho do crescimento é um fator crítico na aquacultura, particularmente durante a fase larvar, quando os peixes têm elevadas exigências nutricionais. A composição proximal e os perfis de ácidos gordos das larvas também foram analisados para avaliar se a inclusão de farinhas de fungos tinha impacto na qualidade nutricional das larvas. Os resultados não mostraram diferenças significativas no conteúdo de proteínas, lípidos ou cinzas entre o controlo e as larvas alimentadas com farinha de fungos, indicando que o *P.variotii* tem um desempenho equivalente ao da farinha de peixe. Os perfis de ácidos gordos foram afetados, com uma diminuição significativa dos ácidos gordos n-9, bem como um aumento significativo dos ácidos gordos n-6 e DHA. Em termos de tolerância ao stress, as larvas alimentadas com 10% de *P.variotii* apresentaram uma taxa de sobrevivência mais baixa, enquanto os peixes alimentados com 5 e 15% apresentaram uma taxa de sobrevivência semelhante à do controlo. A resistência ao stress é uma caraterística valiosa em aquacultura, uma vez que as larvas são frequentemente expostas a fatores de stress ambiental que podem ter impacto na sobrevivência e na saúde a longo prazo. Após o desafio patogénico com *V.anguillarum*, a taxa de sobrevivência não foi significativamente diferente entre os tratamentos, o que não confirmou

os potenciais efeitos imunomoduladores de *P.variotii,* pelo menos nas doses testadas. A presença de compostos bioativos como os β-glucanos em *P.variotii* pode contribuir para uma melhor resposta imunitária dos peixes alimentados, uma vez que os β-glucanos são conhecidos por estimular o sistema imunitário inato dos peixes, promovendo uma maior resistência aos agentes patogénicos. A análise da expressão genética reforçou os resultados imunomoduladores anteriores, com as larvas alimentadas com as dietas de *P.variotii* a não apresentarem diferenças significativas na expressão de genes relacionados com o sistema imunitário. Estes genes estão envolvidos em processos imunitários fundamentais, incluindo o reconhecimento de agentes patogénicos, a inflamação e a atividade antimicrobiana. A expressão relativa destes genes sugere que *P.variotii* nas doses testadas não melhorou o sistema imunitário como esperado. A capacidade de *P.variotii* para potenciar o crescimento, aumentar a resistência ao stress e melhorar a função imunitária em larvas de dourada realça o seu potencial como ingrediente funcional em dietas de aquacultura. Além disso, o uso de fontes de proteína microbiana como *P.variotii* contribui para reduzir a dependência da indústria da aquacultura de peixes capturados na natureza para a produção de ração, alinhando-se com os esforços globais para melhorar a sustentabilidade dos sistemas alimentares. Em conclusão, este estudo demonstra a viabilidade da utilização da farinha fúngica de *Paecilomyces variotii* como alternativa sustentável à farinha de peixe na alimentação de larvas de dourada. A farinha fúngica suportou taxas de crescimento e de sobrevivência comparáveis às alcançadas com a farinha de peixe, tendo simultaneamente a capacidade de melhorar a resistência dos peixes ao stress e aos agentes patogénicos. Estes resultados sugerem que *P.variotii* pode desempenhar um papel fundamental na redução da dependência da farinha de peixe na aquacultura, contribuindo para sistemas de produção mais sustentáveis e resilientes. A investigação futura deve centrar-se na otimização dos níveis de inclusão, no equilíbrio da composição de ácidos gordos, na avaliação dos efeitos a longo prazo na saúde dos peixes e na avaliação do potencial das farinhas fúngicas para serem utilizadas nas dietas de outras espécies de aquacultura.

Termos chave :
Alimentos sustentáveis, aquacultura, fungos filamentosos, *Paecilomyces variotii*, *Sparus aurata*

Table of Contents

Table index

Figure index

List of Abbreviations

ARA	Arachidonic Acid
cd4	*cd4-full*
cd8α	*CD8 alpha chain precursor*
CP	Crude Protein
CTRL	Control
β-actin	*Cytoplasmic β-actin 1*
DHA	Docosahexaenoic Acid
EFAs	Essential Fatty Acids
ef1α	Elongation factor 1 α
EPA	Eicosapentaenoic Acid
FAO	The Food and Agriculture Organization (FAO)
FF	Filamentous fungi
FCR	Feed conversion ratio
FM	Fishmeal
il-1β	*Interleukin 1-beta*
LC-PUFAs	Long-Chain Polyunsaturated Fatty Acids
MOS	Mannan-Oligosaccharides
MUFA	Monounsaturated Fatty Acids
P5	5% inclusion of P.variotii (33% of FM replacement)
P10	10% inclusion of P.variotii (66% of FM replacement)
P15	15% inclusion of P.variotii (100% FM replacement)
SFA	Saturated Fatty Acids
SGR	Specific Growth Rate
SmF	Submerged Fermentation
SSF	Solid-State Fermentation
tnf-α	*tumour necrosis factor-alpha*
Tm	Annealing Temperature

1. Introduction

1.1 Status of global aquaculture

Aquaculture has experienced significant growth in recent decades to meet the growing global demand for aquatic products. The Food and Agriculture Organisation (FAO) reported that aquaculture production has expanded in the past seven decades from 19 million tonnes in 1950 to 177.8 million tonnes in 2020. Moreover, global aquatic food consumption increased in 60 years, from an average per capita of 9.9 kg in 1960 to 20.7 kg in 2022 (FAO 2022, 2024). With the increasing demand for aquatic products and the struggle for fisheries to keep pace due to the decrease in wild fish stocks and the stagnation of production (Gephart *et al.* 2017), the aquaculture industry is facing an increasing demand that leads to the creation of many opportunities for the sector.

Furthermore, in 2022, global production of aquatic animals hit a record 185 million tonnes (live weight equivalent), marking a 4% increase from 2020. Aquaculture contributed an estimated 94 million tonnes, accounting for 51% of the total, overtaking capture fisheries for the first time (FAO 2024). Of the 2022 overall aquatic production, over 164.6 million tonnes were destined for human consumption while the remaining 20.8 million tonnes were used mainly to produce fishmeal and fish oil (FAO 2024).

1.2 Dietary importance of fishmeal and fish oil

In recent decades, the overexploitation of wild fish stocks has reduced the availability and driven up the cost of fishmeal (FM). Additionally, increasing awareness of how reliance on forage fisheries impacts the sustainability of aquaculture has encouraged the replacement of FM derived from wild fisheries in fish feeds (FAO 2024).

Fishmeal is the crude flour obtained after drying and grinding fish material. Additions of FM in aquafeeds result in increased feed efficiency, food palatability, nutrient uptake, digestion and absorption (Hodar *et al.* 2020). Indeed, FM is an excellent source of high-quality protein, amino acids, vitamins, minerals and omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These nutrients are vital for the rapid growth and

development of fish larvae, whose nutritional needs are particularly high due to their fast metabolic rates. The presence of these essential nutrients in fishmeal helps ensure that the larvae have the necessary building blocks for proper tissue development, immune function and overall health (Oliva-Teles *et al.* 2015, Hodar *et al.* 2020).

Fish oil is an excellent lipid source in aquafeeds, especially in terms of long-chain omega-3 fatty acids, particularly EPA and DHA. Indeed, these fatty acids are essential for the proper development of fish larvae, especially in terms of brain and eye development. DHA, in particular, is vital for the formation of neural tissues and retinal cells, which are essential for the sensory and cognitive functions of young fish (Izquierdo 1996). Adequate intake of these fatty acids during the larval stage ensures that fish develop the necessary physiological systems for survival and growth (Bell 2008).

Efforts are still needed to undertake an in-depth assessment of the consequences of feeding very low FM and fish oil diets to marine species (less than 10% inclusion levels), and additional knowledge is welcome to overcome the nutritional disorders that can lead to reduced growth and survival. Most of the symptoms that come from nutrient deficiencies from replacing FM and FO in aquafeeds disappear with the supplementation with essential fatty acids and phospholipids as seen in gilthead sea bream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*) (Olsen *et al.* 2003, Benedito-Palos *et al.* 2008).

Furthermore, it has been recognised that FM and FO in the diets of gilthead sea bream larvae can be problematic due to inherent variability in fishmeal composition due to species, season, geographic origin and processing variation in quality of the wild caught fish (Opstvedt *et al.* 2003, Bragadóttir *et al.* 2004). To address this, a significant portion of aquaculture research over the past two decades has focused on substituting FM with more consistently available, cost-effective, and sustainable raw materials. Other possibilities such as plant-based proteins could be more suitable for modern aquaculture but have sustainability and nutritional composition issues (Fry *et al.* 2016).

1.3 Plant-based proteins

The use of plant proteins as a replacement for FM in aquaculture has gained significant interest due to several economic, and nutritional advantages. Plant proteins are in certain cases more sustainable and environmentally friendly than FM. Plant proteins are derived from renewable crops, which can be cultivated sustainably (Naylor *et al.* 2009). Moreover, plant

proteins, such as soy, pea, and wheat gluten, tend to be more cost-effective than FM. The lower cost of production and greater availability of plant-based ingredients contribute to reduced feed costs in aquaculture, making them an economically viable alternative (Hardy 2010). In addition, using plant proteins minimises the dependency on marine resources, helping to alleviate the pressure on fish stocks and contribute to more balanced ecosystems. This shift also aligns with global efforts to promote sustainable fishing practices and reduce the carbon footprint of aquaculture (Tacon *et al.* 2008).

Plant-based proteins can indeed be a problem in fish feeding, especially at the early stages of development like in larvae that require a highly nutritional protein. Compared to FM, plant meal lacks essential amino acids such as lysine, methionine, and tryptophan, which are critical for the growth and development of fish larvae (Kaushik *et al.* 2010). However, this problem can be avoided by proper formulation and supplementation with essential amino acids. Additionally, plant proteins generally have low levels of essential long-chain omega-3 fatty acids, such as EPA and DHA that are essential nutrients for larvae. Those deficiencies can lead to suboptimal larval growth, poor muscle development, reduced immune functions and overall lower survival rates if the diet is not properly supplemented (Hardy 2010, Tocher 2015). Depending on the feed species and the plant-based meal, some nutritional limitations can occur. Indeed, many plant-based proteins contain anti-nutritional factors such as protease inhibitors, lectins, and phytic acid (Gatlin III *et al.* 2007). For example, soybean meal contains anti-nutritional factors (Krogdahl *et al.* 2010) and induces enteritis in the distal intestine of the fish, which affects its health and growth significantly (Baeverfjord *et al.* 1996, Urán *et al.* 2009, Hardy 2010). These compounds can interfere with the digestion and absorption of nutrients in fish larvae, leading to reduced feed efficiency and growth. For example, trypsin inhibitors in soy protein can impair protein digestion, while phytic acid can reduce the availability of essential minerals like phosphorus, calcium, and zinc (Gatlin III *et al.* 2007, Hardy 2010). Moreover, many plant proteins are associated with higher fiber content and other components that are less digestible for fish larvae. This lower digestibility can result in reduced nutrient absorption, leading to slower growth rates and increased feed conversion ratios (FCR) (Glencross *et al.* 2007). However, recent advances in feed technology have improved the digestibility and nutritional value of plant proteins, making them suitable for a wide range of aquaculture species (Hossain *et al.* 2024).

Nevertheless, plant-based protein sources may harm seriously the environment through deforestation or large-scale monoculture. For example, in Brazil, the continuous intensification of soybean production led to an increase in greenhouse gas emissions (Øverland *et al.* 2013,

Øverland *et al.* 2017). In this context, the search for sustainable and effective alternatives to FM has become a crucial area of research in modern aquaculture. Moreover, the ongoing war between Russia and Ukraine has severely impacted global food and feed prices. Russia and Ukraine were both the main producers of wheat and other vegetables. As a result, plant-based protein feed lost interest, and to overcome this problem the inclusion of microbial ingredients in diets has emerged as a promising strategy to improve the performance and health of fish.

1.4 Microbial ingredients as novel ingredients for aquafeeds

Microbial ingredients are a category of ingredients that includes yeast, fungi, algae and bacteria (Nasseri *et al.* 2011, Øverland *et al.* 2017). These microorganisms grow very rapidly and produce a high yield. For example, it has been estimated that a gain of 1 kg of protein can be obtained in one day's growth from a 500 kg steer, while 500 kg of yeast would produce several tons of protein in one day (Gamboa-Delgado *et al.* 2018). Microorganisms have high protein content, are highly efficient in converting a variety of substrates, which leads to rapid biomass production and can be classified according to the carbon and the energy sources that they can use. Indeed, this microbial diversity has allowed their production through a wide range of available energy sources (solar or artificial light) and carbon substrates from analytical grade monosaccharides to agriculture and food industry wastes, and methane (Gamboa-Delgado *et al.* 2018).

Moreover, modern microbial production methods can be seasonal independent and can operate continuously, which permits a yearly supply of protein. Industries succeeded in maintaining a consistent microbial production from microalgae, yeasts and bacteria; some of these production methods are patented and generate bacterial biomass also from alternative substrates (Glencross *et al.* 2014, Goodall *et al.* 2016).

Furthermore, microbial-derived products in animal nutrition are supplied in the feeds as protein, specific amino acids, vitamins, and pigments. Their role as palatability agents and immune response enhancers has also been demonstrated (Gamboa-Delgado *et al.* 2018). Indeed, this field has recently been at the centre of interest in animal feeds since the governmental restrictions and elimination of prophylactic growth-promoting antibiotics in animal feeds within the European Union and the United States. Since then, solutions like alternative products such as microbial ingredients-based supplements have emerged to support animal health and growth performance (Agboola *et al.* 2021).

1.4.1 Microalgae

Over the past 50 years, microalgae have been extensively studied for their potential to produce oils, proteins, carbohydrates, pigments, and other valuable products for a range of applications. Microalgae encompass a wide variety of organisms with differing traits. Some species produce biogenic toxins, such as purines, while others contain non-biogenic toxins like heavy metals (Ansari *et al.* 2021).

Certain microalgal species are frequently utilized in aquaculture systems because they are easy to cultivate and offer a well-balanced nutritional profile, among other advantages. For example, dried *Spirulina* sp. and *Chlorella* sp. are used in various ways, such as food supplements, cosmetics, or even in the pharmaceutic industry. Live microalgae are also a key element in zooplankton production which is a key feed at the larval stage of many fish and crustacean species in aquaculture.

Microalgae have a high nutritional value. Indeed, they provide antioxidants, pigments and other bioactive compounds, which make them a good feed source for zooplankton, commonly found in aquatic systems and used as primary food for newly hatched larvae (Tibaldi *et al.* 2015). Microalgae in the late log phase of growth typically contain 10-20% lipids, 20-40% carbohydrates, and 40-60% proteins (Moheimani *et al.* 2012), but they also have vitamins, pigments, antioxidants and metal traces (Ho *et al.* 2012, Pancha *et al.* 2015). Unlike many plant-based protein sources, microalgae are a rich source of omega-3 fatty acids, particularly EPA and DHA, which are crucial for the health and growth of aquatic organisms and are typically derived from FM (Carneiro *et al.* 2020). This profile makes it a suitable alternative to FM in aquafeeds. However, the biochemical composition of microalgae biomass is highly dependent on the specific species, as well as the cultivation conditions and strategies used. Different microalgae species can exhibit considerable variation in their content of lipids, proteins, carbohydrates, and other bioactive compounds. Additionally, factors such as light intensity, nutrient availability, and growth phase can influence these nutritional differences (Ansari *et al.* 2021). In addition to their favourable biochemical composition, several other factors such as shape, size, palatability, digestibility, and cell wall composition make microalgae an excellent feed ingredient for fish. The use of microalgae for aquaculture feeds can have multiple commercial and environmental advantages. In fact, studies have shown that feeds supplemented with microalgae had positive effects on fish morphometric characteristics

(size, weight) and nutritional value (Rossi *et al.* 2012, Abdulrahman 2014) (Babuskin *et al.* 2014, Chen *et al.* 2015). However, completely replacing FM in aquafeeds with microalgae has shown some adverse effects. In fact, Badwy *et al.* (2008) have shown that above 50% replacement of FM in Nile tilapia diets, fiber content increases, which in term decreased the feed digestibility.

Unfortunately, cultivating microalgae can be expensive due to the need for controlled environments, specialised equipment, and high-energy inputs. Achieving the large-scale production necessary to meet the demands of the aquaculture industry can be difficult. The variability in the nutritional content of microalgae can make it challenging to ensure consistent quality and nutritional profiles in aquafeeds. Furthermore, not all fish find microalgae palatable (Richmond 2004). Ensuring that microalgae are accepted by different species of fish can be a challenge, which may require additional processing or blending with other ingredients to improve taste and acceptance (Richmond 2004).

Finally, microalgae are also in demand in industries such as biofuels, nutraceuticals, and pharmaceuticals. This competition can drive up prices and limit availability for aquafeeds. This makes microalgae less competitive compared to traditional feed ingredients like FM and soybeans.

1.4.2 Bacteria

Sources of bacterial biomass have been recently evaluated in aquafeeds (Jones *et al.* 2020). In fact, bacteria are a great source of protein with a crude protein (CP) content of over 80% (Jones *et al.* 2020). Bacteria multiply rapidly and are easy to maintain, making bacterial proteins more sustainable and environmentally friendly alternatives to FM. In contrast, bacterial proteins can be produced using renewable resources, such as waste streams from agriculture and industry, or even from carbon dioxide, which minimizes their environmental footprint (Øverland *et al.* 2017). The production of bacterial proteins can be highly controlled, ensuring consistent quality and nutritional composition. This is a significant advantage over FM, where the nutritional content can vary depending on the species of fish used and the processing methods. Consistent feed quality is crucial for the optimal growth and health of farmed fish, especially in larvae rearing which needs an important and high-quality feed (Jones *et al.* 2020). Moreover, bacterial proteins offer a high protein content and a balanced amino acid profile, which is crucial for the rapid growth and development of fish larvae. Unlike many

plant-based proteins, bacterial proteins typically contain all the essential amino acids in proportions similar to FM, ensuring that larvae receive the necessary nutrients for optimal growth (Oliva-Teles *et al.* 2015). Additionally, bacterial proteins are highly digestible, meaning that fish larvae can efficiently absorb and utilize the nutrients provided, leading to improved growth rates and feed conversion efficiency (Seong *et al.* 2018). Some bacterial proteins also offer additional benefits, such as enhancing gut health and boosting the immune system of fish larvae, further supporting their survival and overall health (Ringø *et al.* 2012). Delamare-Debouteville *et al.* (2019) tested a purple phototrophic bacteria (PPB) yielding biomass from wastewater, being able to replace up to 66 % of the FM in Asian sea bass (*Lates calcarifer*) diets. Additionally, Øverland *et al.* (2010) studied the effect of a bacterial meal derived from *Methylococcus capsulatus* on pigs, broiler chickens, mink (*Mustela vison*), fox (*Alopex lagopus*), Atlantic salmon (*Salmo salar*), rainbow trout, and Atlantic halibut (*Hippoglossus hippoglossus*). Based on criteria of amino acid composition, digestibility, and animal performance and health, it was concluded to be a promising bacteria meal.

1.4.3 Yeasts

Yeasts contain a great amount of protein with 40 to 55%, as well as bioactive components that are beneficial for fish growth and development (Øverland et al. 2013, Rawling et al. 2019). Furthermore, research has reported benefits from nutritional supplements and functional supplement feeds regarding the gut health of fish, as well as their role in the immune response (Torrecillas et al. 2012).Indeed, the main bioactive components present in yeast are mannan-oligosaccharides (MOS), β-glucan and chitin, usually present in the cell wall, representing 26-32% of the total dry weight of the cell (Klis et al. 2002, Schiavone et al. 2014). The yeast cell wall contains about 85-90% polysaccharides, glucans and mannans with a small amount of chitin (Nguyen et al. 1998, Schiavone et al. 2014). It has been shown that β-glucans have a significant effect on the immune responses and survival of the host after a pathogen exposition in fish, including European seabass (*Dicentrarchus labrax*) (Bonaldo et al. 2007), rainbow trout (Guselle et al. 2007) and Atlantic salmon (Bridle et al. 2005). β-glucan binds to dectin-1 receptors that are present on the surface of several immune cells such as neutrophils, eosinophils, macrophages, monocytes, dendritic cells and T cells (Volman et al. 2008), which will activate NF-κB through intracellular signalling, which leads to cytokine production, phagocytosis and respiratory burst (Volman et al. 2008). Mannan-oligosaccharides are an

important compound in feeds. In fact, Torrecillas et al. (2014), Agboola (2022) have shown that MOS positively influences the health and growth performance of fish through its ability to bind enteropathogenic bacteria which prevents the host from colonization but it has no effects on growth rate, feed intake or the immune system (Agboola et al. 2021).

Moreover, only a few studies have considered yeast as a macro protein ingredient in aquafeeds. Of the few available, most studied S. cerevisiae, due to its high availability as a by-product from various industry processes such as alcohol, bio-ethanol or beer production (Ferreira et al. 2010). A majority of the studies on aquatic organisms have shown that S. cerevisiae could partially replace FM or soy protein without having a negative effect on the growth performance. In fact, great results have been obtained in Nile tilapia (*Oreochromis niloticus*) (Abass et al. 2018), catfish (*Clarias gariepinus*) (Essa et al. 2011), goldfish (*Carassius auratus*) (Gumus et al. 2016), lake trout (*Salvelinus namaycush*) (Rumsey et al. 1990), rainbow trout (Siwicki et al. 2004, Guselle et al. 2007, Huyben et al. 2017, Vidakovic et al. 2020), Arctic charr (*Salvelinus alpinus*) (Vidakovic et al. 2016), Atlantic salmon (Robertsen et al. 1990, Bridle et al. 2005, Øverland et al. 2013), shrimp (*Litopenaeus vannamei*) (Guo et al. 2019), European seabass (Oliva-Teles et al. 2001), pink snapper (*Pagrus auratus*) (Cook et al. 2003) and gilthead sea bream (Cuesta et al. 2007, Fronte et al. 2019).

Despite the studies documenting the nutritional value of yeast on various aquatic organisms, incorporating yeast in commercial aquafeed diets faces many problems. Indeed, yeasts often have an imbalanced amino acid profile compared to FM. Furthermore, yeast cell walls contain a complex network of polysaccharides that could make digestion difficult (Murray *et al.* 1986, Yamada *et al.* 2005) and potentially reducing the availability of nutrients. However, this could be solved with exogenous enzymes that are commercially available to digest specific cell wall components, such as mannanase, glucanase, chitinase and glucosidase. Rimoldi *et al.* (2020) have tested different levels of inclusion of autolysed dried yeast in feeds for gilthead sea bream and results have shown an increase in beneficial bacteria within the gut microbiome. These fish also showed an increased and greater gut microbial diversity, which is generally linked to a healthier and more resilient gut ecosystem (Rimoldi *et al.* 2020, Estévez *et al.* 2021). Growth and feed utilisation, immune and health parameters have also been positively affected in fish fed yeast-supplemented diets (Rimoldi *et al.* 2020) or at least performance indicators presented equivalent results to the control (Estévez *et al.* 2021).

However, yeast-based feeds are often promoted as more sustainable but large-scale yeast production still has environmental impacts, including the use of substrates (e.g., sugars) and energy. These factors need to be balanced against the environmental benefits of other

ingredients. There also might be regulatory hurdles in some regions regarding the approval of new feed ingredients like in Europe with the European Commission. Additionally, consumer acceptance of farmed fish fed with yeast-based diets can vary, potentially affecting marketability.

1.4.4 Fungi

Fungi represent one of the most extensive groups of eukaryotic organisms, forming a distinct kingdom separate from plants, protists, animals, and bacteria. Estimates suggest approximately 2.2 to 3.8 million fungal species, although only 120,000 of these species have been described and identified to date (Hawksworth *et al.* 2017). Fungi are categorised into four distinct groups: *Chytridiomycota*, *Zygomycota*, *Ascomycota*, and *Basidiomycota* (Lennartsson 2012).

Filamentous fungi (FF), known as "Dikarya" are branched, multicellular organisms that form elongated, thread-like structures called hyphae (Nalage *et al.* 2016). Furthermore, FF can be grown using both submerged fermentation (SmF) and solid-state fermentation (SSF) methods. SmF involves the cultivation of microorganisms in a liquid nutrient medium. This method is widely used in industrial processes for the production of enzymes, antibiotics, and other metabolites (Ramesh *et al.* 2022). In SmF, the microorganisms, such as bacteria or fungi, are submerged in the liquid, which provides the necessary nutrients and conditions for growth and product formation. The liquid medium allows for easier control of environmental factors such as pH, temperature, and oxygen levels. This method suits microorganisms that thrive in high-moisture environments (Fazenda *et al.* 2008). SSF is a process where microorganisms grow on solid materials without free-flowing water. The substrates used in SSF, such as agricultural residues or food industry by-products, provide both nutrients and physical support for the microorganisms (Pandey 2003). SSF is particularly advantageous for the production of certain enzymes, organic acids, and bioactive compounds that are often not efficiently produced in liquid cultures. This method mimics the natural habitats of many fungi and other microorganisms, which can lead to higher yields of desired products. SSF is also considered more environmentally friendly due to lower water usage and simpler waste management (Pandey *et al.* 2008). One of the main problems in FF cultivation is the cost of defined synthetic media such as glucose or sucrose, but this high cost has driven scientists' efforts to develop

new media formulations using affordable by-products and waste materials that can adequately support and meet the nutritional needs for microbial growth (Andualem *et al.* 2013). Taking in account those previous criteria a substrate for fungal cultivation should contain enough macro and micro-nutrients, be cheap, and be easily degradable and available (Demirbas 2009).

Filamentous fungi have emerged as a viable alternative source of MI for aquafeeds. Like yeast, FF are resilient organisms capable of thriving in diverse growth conditions, including lower pH levels than bacteria (pH=4.7) by degrading carbohydrates, proteins and lipids into fatty acids, sugars and amino acids using enzymes (Ferreira *et al.* 2013). Their ability to grow on a wide variety of substrates and utilise low concentrations of these substrates makes them ideal for use in biorefineries processing different waste streams (Bajpai 2017), such as agriculture (soybean, grains, legumes, rice, potato, corn, wheat etc.) and forestry residues (Taherzadeh *et al.* 2003, Singh *et al.* 2005, Shuvaeva *et al.* 2010, Lennartsson 2012), stillage from the bioethanol production industry (Taherzadeh *et al.* 2003), spent sulphite liquor from the paper and pulp industry (Asadollahzadeh *et al.* 2018), molasses from sugar industry, lignocellulosic residues (Sues *et al.* 2005) and starch-based effluents (Jasti *et al.* 2008).

Karimi *et al.* (2019) have grown FF from ethanol production waste and its fungal biomass has shown a balanced amino acid profile, a lipid level from 3.5 to 7% and a high protein content of 44.7 to 55.6%. Analyses have also shown a significant level of beneficial minerals such as magnesium, potassium, calcium and traces of zinc and iron (Karimi *et al.* 2019). Furthermore, several species of FF are being used as fermented food for human consumption, like in Japanese gastronomy for the production of shoyu, sake and miso (*Aspergillus oryzae*) (Terabayashi *et al.* 2010) or even in Indonesian gastronomy for the production of soybean-based cake (*Neurospora intermedia*) (Singh *et al.* 2005). Fungal biomass is rich in proteins, fats, amino acids, and carbohydrates such as chitosan and chitin, making it a viable option for supplementing animal feed (Stahl *et al.* 1996).

The protein content of fungal biomass varies depending on the species and cultivation conditions, typically ranging from 40 to 50% in zygomycetes and ascomycetes (Archer *et al.* 2008). Its protein content is also influenced by the harvesting, dewatering, drying approach (Dufossé *et al.* 2014) and the cultivation medium where the nitrogen content acts directly on the fungal protein yield (Nitayavardhana *et al.* 2013). Analysis of the amino acid composition of fungi meal has revealed that it is comparable to FM except in terms of methionine where fungi meal may need some supplementation (Karimi *et al.* 2019). The previous statement cannot be generalized due to species differences and further studies might be appropriate to determine the potential of a fungal biomass as an aquafeed ingredient.

Several fungal species have previously been examined for their amino acid production capacity like the *R. oligosporus* in the zygomycetes group which cultivated on thin stillage presented significant concentrations of arginine, aspartic acid, cysteine, phenylalanine, glutamic acid, histidine, isoleucine, leucine, lysine, proline, serine, tyrosine, and valine (Van Leeuwen *et al.* 2013). In that study amino acid amounts were similar (methionine was higher) than those present in soybean meal. Furthermore, the protein level reported in *R. oligosporus* was up to 50% with a balanced amino acid profile similar to fishmeal and soybean meal (Nitayavardhana *et al.* 2013). In addition to its protein content, the fungal biomass contains 20 to 25% of lipids (Pedneault *et al.* 2008). The lipid content is still dependent on the species and its cultivation method. In FF, fatty acids are often structured as membrane phospholipids and storage triacylglycerol including palmitic and stearic acids, and other unsaturated fatty acids such as linoleic, oleic, palmitoleic, and linolenic acids (Stahl *et al.* 1996).

In past decades, antibiotics and other medicines have been overly used in aquaculture and have promoted the growth and selection of different pathogens, which causes bacteria strains to develop resistance (Kesarcodi-Watson *et al.* 2008, Ringø *et al.* 2012). Techniques such as vaccination could prevent fish diseases. Still, it is laborious and expensive and in the case of hatchery, those methods have proven to be not as efficient as expected (Staykov *et al.* 2007). To find alternative preventive and curative treatment techniques several immunostimulant compounds have been identified such as chitosan, chitin, mannan oligosaccharide and β-glucan (Gopalakannan *et al.* 2006). Those previous compounds have demonstrated their effects as immunostimulants (Awad *et al.* 2010, Holdt *et al.* 2011, Jung-Schroers *et al.* 2016). The fungal cell wall composition differs between species, it is a complex structure composed of 80-90% of polysaccharides and constitutes about 15-30% of the total cell mass (Nimrichter *et al.* 2005). The dietary inclusion of immunostimulants like β-glucans, mannan oligosaccharides, chitin, and chitosan in diets has been widely recognized for boosting immune system functions, improving stress responses, and increasing disease resistance in both marine and freshwater fish species (Ringø *et al.* 2010).

Filamentous fungi are also well known for pigment production, they produce a wide range of pigments depending on the species such as melanins, flavins, carotenoids, quinones and phenazines (Velmurugan *et al.* 2010). Pigments have important biological roles thanks to their functions as anti-oxidative, anti-carcinogenic, immunostimulant, free radical killing and protection against viruses and bacteria (Steglich 1981, Velíšek *et al.* 2011). Nowadays the flesh colouration of the fish is an important criterion for the consumers. Carotenoids play an important role in this colouration and it is uniquely dependent on the pigment input of the diet

because fish cannot produce this pigment (Torrissen *et al.* 1990). Aside from the colouration effect, pigments act positively on broodstock performance (Sawanboonchun *et al.* 2008), growth performance (Storebakken *et al.* 1996), disease resistance (Tachibana *et al.* 1997) and immune functions (Amar *et al.* 2004). The use of filamentous fungal biomass as a FM substitute and nutritional supplement in fish feed offers a promising solution to some challenges faced by the aquaculture industry. Fungal biomass is rich in protein, fatty acids, pigments, and immunostimulants, enhancing the nutritional quality of fish feed. However, since the composition and concentration of these components can vary significantly across different fungal strains, further research is needed to standardize the use of fungal biomass in fish feed formulations.

1.4.5 Paecilomyces variotii

Paecilomyces variotii is a filamentous fungus, it can be a potentially cost-effective and sustainable option for aquafeed production due to its high availability (Mensah *et al.* 2024). *P. variotii* has a high crude protein content (60-70%) and contains bioactive compounds such as β-glucans (10-15%), mannans and nucleic acids which may bring health benefits to fish (Hooft *et al.* 2024). This microbial source has shown potential as an alternative protein source for various aquatic organisms with promising results, for instance, in salmonids. Recent studies aimed to evaluate increasing levels of *P. variotii* as a fishmeal replacer on growth performance, nutrient digestibility and utilization, as well as on the expression of immune-related biomarkers in the gut of Atlantic salmon at juvenile stage. Those studies have shown that as the inclusion level of *P. variotii* increases it exhibits immunomodulatory effects in the distal intestine of Atlantic salmon and that it could replace up to 20% of the crude protein of the diet for improved feed conversion ratio and nutrient utilization efficiency (Javed, 2023). Furthermore, a recent study from (Dahlberg 2019) on rainbow trout has shown a high digestibility of about 85%, indicating that it could be highly nutritious for aquatic species. In salmon diets, the inclusion of *P. variotii* has demonstrated a significant increase in growth performance as well as a better feed conversion ratio (Hooft *et al.* 2024). Moreover, previous studies have indicated the ability of this microbial source to enhance immune response and pathogen resistance in fish and crustacean larvae (Eroldoğan *et al.* 2023). Indeed, β-glucans, mannan oligosaccharides and other nucleic acids in diets have been widely recognised for improving immune system capacities, stress responses, and disease resistance in both marine and freshwater fish species

(Ringø *et al.* 2010), which stimulate both innate and adaptive immune responses, including T-cell activation and enhanced antimicrobial activity for a better pathogens resistance. In addition, Javed (2023) study has demonstrated that, in Atlantic salmon, dietary *P.variotii* inclusion led to immunomodulatory effects in the distal intestine.

1.5 PEKILO®

1.5.1 History

PEKILO® is a single-cell protein product derived from the cultivation of the fungus *Paecilomyces variotii*. The concept originated in Finland during the 1970s, driven by the need to find alternative and sustainable protein sources for animal feed. The Finnish company Cultor Oy conducted the development, which sought to utilise industrial by-products and waste streams more effectively. The innovative process involved cultivating the fungus on the waste streams from the pulp and paper industry, such as spent sulfite liquor, which provided a nutrient-rich substrate for fungal growth. The resulting biomass, rich in protein, was then processed and dried to create PEKILO® (Javed 2023, Hooft *et al.* 2024).

PEKILO® was notable not only for its high protein content comparable to other protein sources like soy or FM but also for its balanced amino acid profile (Table 1.1), making it an excellent feed ingredient for livestock, particularly pigs and poultry. This innovation was a significant advancement in the field of biotechnology and environmental management, as it offered a solution for recycling industrial waste while producing a valuable product.

With the increasing global focus on sustainability, climate change, and the need for efficient food production systems, there has been a renewed interest in MI like PEKILO®. The environmental benefits, such as reduced land use and lower greenhouse gas emissions compared to conventional protein production methods, have made it an attractive option. As the world grapples with the challenges of feeding a growing population while minimizing environmental impact, the legacy and principles behind PEKILO® are being revisited, potentially offering a sustainable solution for the future of food and feed industries.

In 1970, the PEKILO® process was patented and the PEKILO® trademark as well. Following, in 1971, the first continuous fermenter of about 1000 L was operated and a pilot test of a 15 000 L fermenter that could produce 30 kg of PEKILO® per hour and could run 3000 hours successively was tested. These tests have finally led to the establishment of two

industrial-scale PEKILO® plants that could produce up to 10000 tons of PEKILO® per year. It was in the year 1991 that the PEKILO® process had to stop because of the low demand and the discontinuous sulfite pulping. In the year 2017, PEKILO® was revived with the increased demand of alternatives to soymeal and FM and the new coming of a new biorefinery (EniferBio 2022).

Table 1.1: PEKILO® amino acid composition.

Amino acid (g/100g)	
Asp	4.55
Thr	2.35
Ser	2.36
Glu	7.09
Pro	1.86
Gly	2.62
Ala	4.64
Val	2.77
Met	0.75
Ile	2.26
Leu	3.67
Tyr	1.90
Phe	2.45
His	1.19
Lys	3.69
Arg	3.55
Cys	0.59
Trp	0.68
Tau	<100 ppm

Asp: aspartic acid; Thr: threonine; Ser: serine; Glu: glutamic acid; Pro: proline; Gly: glycine; Ala: alanine; Val: valine; Met: methionine; Ile: isoleucine; Leu: leucine; Tyr: tyrosine; Phe: phenylalanine; His: histidine; Lys: lysine; Arg: arginine; Cys: cysteine; Trp: tryptophan; Tau: taurine.

1.5.2 Production process

The modern PEKILO® process has evolved to utilise a broader range of by-products from biorefineries, including sulfite stillage from ethanol production. The primary carbon sources in this stillage include xylose, acetic acid, arabinose, and small amounts of glycerol, which originate from the yeast ethanol production process (Javed 2023). Unlike traditional

methods that relied on spent liquors from sulfite pulping, the modern approach leverages these varied substrates to cultivate *Paecilomyces variotii*. In the updated process, which is a submerged fermentation, the stillage is first clarified and sterilized before being fed continuously into a fermenter (Figure 1.1). Here, the solution is mixed and aerated under controlled pH and temperature conditions to promote optimal fungal growth. One of the key advantages of using *P. variotii* is that its fibrous structure facilitates easy separation, negating the need for specialised separators typically required in other MI processes (Toledo Marante *et al.* 2012). The biomass is continuously removed from the fermenter along with the broth. It is then filtered, washed, and dewatered through mechanical pressing, which significantly reduces energy consumption compared to traditional drying methods. The dewatering process achieves a dry solid content of 30-35%, minimizing the amount of water that needs to be evaporated later (Javed 2023). The separation of the fungus mycelium is facilitated by a drum filter, which is efficient and cost-effective, it is then dried through hot air to reach the final step of the formulation making place to the PEKILO® mycoprotein. This approach not only conserves water but also reduces both investment and operational costs, making the PEKILO® process economically competitive and environmentally sustainable compared to other MI.

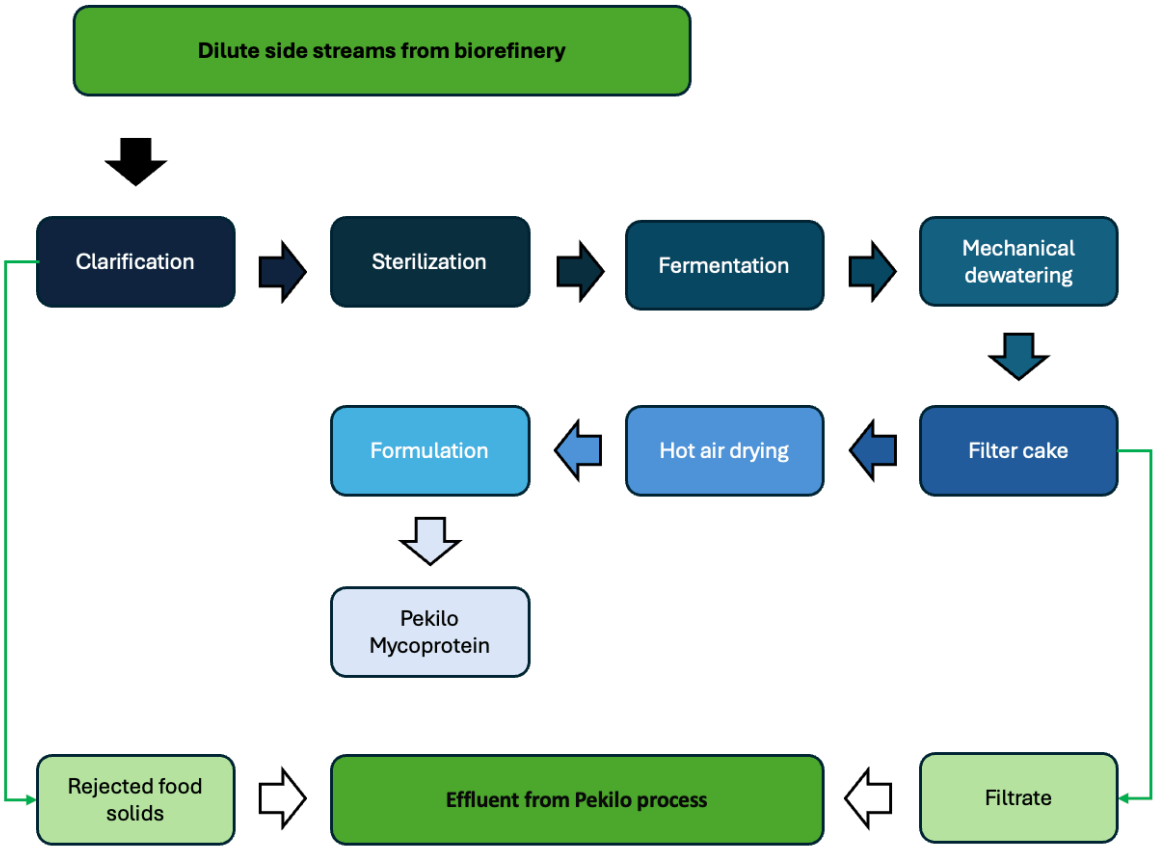


Figure 1.1: PEKILO® process by EniferBio (Javed 2023).

1.6 Status of gilthead sea bream aquaculture

Gilthead sea bream is one of the key Mediterranean aquaculture species that has traditionally been cultured in coastal lagoons and brackish/saltwater ponds, especially in the northern Adriatic valli in Italy and the Egyptian hosha (Svåsand *et al.* 2007). In the late 1970s, the increasing demand for fry for aquaculture enhanced the development of breeding techniques, and in the 1980s a reliable breeding program was established (Moretti *et al.* 2005). Nowadays, the worldwide source for gilthead sea bream production is aquaculture (Figure 1.2) with an average density of 20-100 kg/m^3 and an FCR of 1.5 to 2 (Svåsand *et al.* 2007). Aquaculture represented 101875 tonnes of live weight while fisheries represented 3888 tonnes of live weight in 2021. Gilthead sea bream functional and biological functions have been studied for decades accumulating knowledge throughout multiple research and leading to a significant improvement in their reproductive success, survival, and growth (Mhalhel *et al.* 2023).

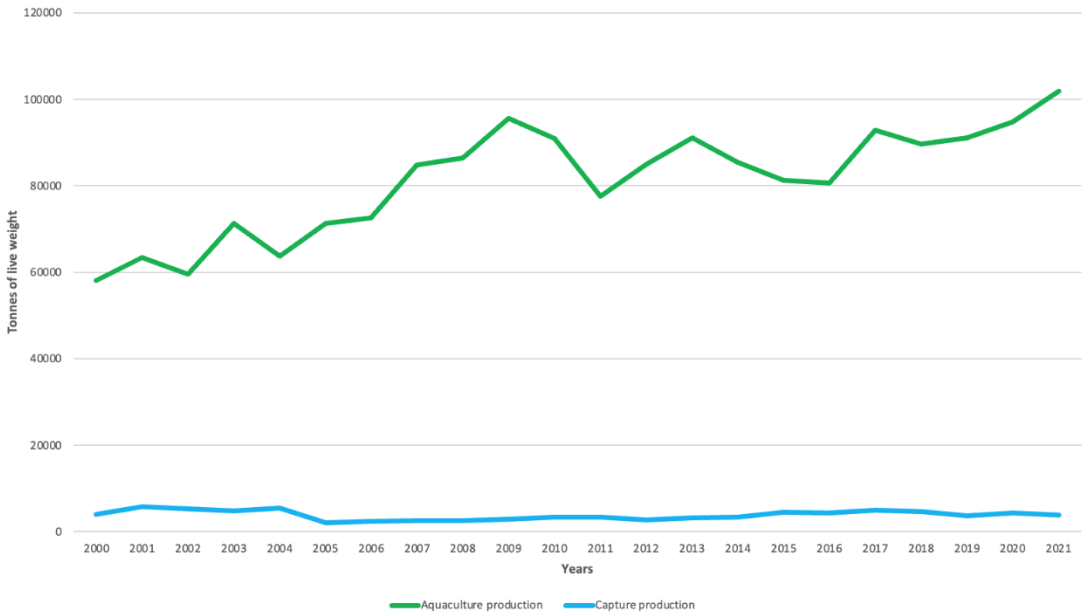


Figure 1.2: European fisheries capture and aquaculture production of gilthead sea bream (FAO, 2022)

1.6.1 Gilthead sea bream larviculture

Successful larviculture, the rearing of fish during their early life stages from hatchlings to juveniles, is critical for the sustainable production of sea bream. However, gilthead sea bream larviculture presents significant challenges related to nutrition, environmental conditions, and the management of stressors during early development.

The optimal rearing conditions for sea bream larvae include water temperatures between 18 and 22°C, salinity levels around 35 ppt, and stable photoperiod regimes that facilitate feeding and growth (Divanach *et al.* 2000). Larviculture is a critical stage of gilthead sea bream production in aquaculture, but it presents several challenges. One of the primary issues is the sensitivity of larvae to environmental fluctuations, including water quality, temperature, and nutritional imbalances (Izquierdo 2004). Ensuring proper nutrition, particularly regarding essential fatty acids, vitamins, and digestible proteins, is vital for their development. Nutritional deficiencies often result in skeletal deformities, swim bladder dysfunction, and reduced survival rates, making it a key area of focus in larval rearing research (Hamre *et al.* 2013).

The use of live feeds such as rotifers and artemia remains a standard for the gilthead sea bream larval industry rearing. However, the inconsistent nutritional composition of live

feeds and their susceptibility to contamination present significant challenges. Enrichment of live feeds with essential nutrients, such as DHA and EPA, has helped mitigate these issues, but there is growing interest in developing high-quality, cost-effective formulated diets that could replace or supplement live feeds (Hamre *et al.* 2013).

Finally, molecular tools are increasingly being employed to better understand the physiological responses of larvae to dietary and environmental stressors. Gene expression studies are providing insights into how larvae respond to these challenges, opening up new opportunities for targeted interventions designed to enhance survival rates and growth performance (Sajina *et al.* 2022).

1.6.2 Gilthead sea bream larvae nutritional requirements

Gilthead sea bream is a valuable species in aquaculture, prized for its market appeal and robust growth potential. Meeting the nutritional requirements of these fish is critical for their health and optimal development, which can vary based on their life stages.

Nutrition plays a pivotal role in the successful rearing of gilthead sea bream larvae. The early life stages are characterized by rapid growth and the formation of critical organs, such as the digestive system, bones, and nervous tissue. Therefore, larvae require well-balanced diets rich in proteins, lipids, vitamins, and minerals to support these developmental processes.

Protein is a fundamental component of the diet, essential for growth and tissue repair. The early stages of growth involve high rates of protein synthesis, making it necessary to provide sufficient dietary protein. The recommended dietary protein content for larvae is 50-60% protein (Conceição *et al.* 2010). The quality of protein, in addition to its quantity, is also critical for larval development. The protein source must provide essential amino acids in the correct proportions to support muscle growth and overall development. Carnivorous fish, such as gilthead sea bream, predominantly rely on protein over lipids or carbohydrates for energy, which is why diets have to be formulated with the optimum essential amino acid profile to ensure a better amino acid utilization during growth, thus reducing the nitrogen excretion resultant from catabolism (Peres *et al.* 1999). A deficiency or imbalance in these amino acids can lead to impaired growth, reduced survival rates, and increased susceptibility to diseases (Gaber *et al.* 2016).

For gilthead sea bream larvae, dietary lipids are an essential source of energy, as well as essential fatty acids (EFAs), which are crucial for growth, development, and maintaining

overall health. The recommended lipid levels for larvae are generally in the range of 10 to 15% of the diet (Grisez *et al.* 1997, Turchini *et al.* 2010). This lipid content provides the necessary energy while also supplying EFAs, particularly long-chain polyunsaturated fatty acids (LC-PUFAs) such as DHA and EPA, which are important for energy provision, cell membrane integrity, and the development of the central nervous system in larvae (Izquierdo 1996). DHA, in particular, plays a crucial role in brain development, visual acuity, and the formation of cellular membranes. Maintaining an appropriate lipid level is critical not only for energy but also for optimizing the protein-sparing effect, where proteins are utilized more efficiently for growth rather than being used for energy, thus enhancing the overall growth and health of the fish (Izquierdo 1996). Finally, deficiencies in these EFAs can lead to developmental abnormalities, including impaired vision, poor neural development, and reduced survival (Hamre *et al.* 2013).

Vitamins and minerals play a crucial role in supporting various physiological functions, such as bone development, metabolic processes, and immune responses. Specifically, vitamins like A, D, E, and C are vital for maintaining vision, regulating calcium metabolism, providing antioxidant protection, and enhancing immune function. Meanwhile, essential minerals like calcium, phosphorus, magnesium, and iron are fundamental for proper skeletal development, enzymatic activity, and oxygen transport. A deficiency or imbalance in these micronutrients can result in significant health issues, including skeletal deformities, weakened immune defences, and reduced viability during the larval stage (Izquierdo 2004). Early nutritional interventions can have lasting impacts on the growth, health, and feed efficiency of fish. Proper nutrition during the larval stage can program metabolic pathways, improve feed conversion ratios, and enhance overall fish performance throughout their lifecycle (Izquierdo 2004).

Formulated diets specifically designed for sea bream larvae are currently being developed, with research aiming to fine-tune nutrient composition to precisely meet larval needs. Progress in feed formulation technology and ingredient processing has led to the creation of more digestible and nutritionally balanced diets, which hold the potential to eventually replace live feeds (Basford 2021).

1.7 Objective

The objective of this thesis is to explore the potential of using a MI product, composed of fungi meal from *Paecilomyces variotii* (PEKILO®), as a fishmeal replacer in the weaning diets of gilthead sea bream. Specifically, this study aims to evaluate the effect of *P.variotii* inclusion on key larval performance indicators, such as growth, survival, stress resistance by air exposure, pathogen resistance to *Vibrio anguillarum* infection, and body composition, alongside immune-related genomic parameters. The findings may serve as a foundation for further research on microbial sources of protein in feeding larvae of other aquatic organisms and the broader replacement of FM in larvae diets.

2. Material and methods

Animal manipulation during these experiments complied with the guidelines of the European Union Directive (2010/63/EU) and Spanish legislation (RD 53/2013) for animal experiments. Experiments were performed at ECOAQUA Institute of University of Las Palmas de Gran Canaria (Canary Island, Spain). Discomfort, stress and pain to the experimental animals were avoided, as much as possible, during the experiment. The project was submitted in advance to the ethics review committee at the University of Las Palmas de Gran Canaria.

2.1 Experimental diets

Four isonitrogenous, isolipidic, and isoenergetic experimental microdiets were prepared to evaluate the nutritional value of *P. variotii* in gilthead sea bream feeds (Table 2.1). The control diet (CTRL) contained a balanced mixture of marine proteins (fishmeal and squid meal). In the P5, P10 and P15 diets, marine raw materials were partially replaced by the inclusion of 5, 10 and 15% of a single-cell protein derived from the filamentous fungus (*Paecilomyces variotii*; PEKILO®, Norway). Each experimental microdiet was assessed in triplicate (n = 3) and prepared in ECOAQUA, Instituto Universitario de Acuicultura Sostenible y Ecosistemas Marinos. All the ingredients were ground (Braun, Suderm, Germany) and sieved (Filtra, Barcelona) < 125 μm before microdiet preparation (Eryalçin *et al.* 2017). Moreover, water-soluble components were mixed following an increasing order of ingredient content

(Figure 2.1) and then the lipid source and fat-soluble vitamins were added. Finally, distilled water with dissolved gelatin was added. The paste was further mixed by hand until a stiff dough was obtained and was then passed through a mincer (Severin, Suderm, Germany). The obtained strands were dried in the oven (Ako, Barcelona, Spain) at 37 °C for 24 h. After drying, the strands were ground and sieved again into two different particle sizes, < 250 μm and 250–500 μm, and then stored at – 80 °C until use (Figure 2.1).

Experimental diets were analysed at Servicio de Análisis para Acuicultura y Biotecnología de Alta Especialización (SAABAE) for biochemical composition: ash, crude protein, crude lipids, fatty acids (Table 2.1, Table 2.2).

Table 2.1: Formulation and proximate composition of the experimental diets.

	Experimental diets			
Ingredients (%)	**CTRL**	**P5**	**P10**	**P15**
Squid meal[1]	63.00	63.00	63.00	63.00
Fishmeal[2]	15.00	10.00	5.00	0.00
P.variotii[3]	0.00	5.00	10.00	15.00
Krill oil[4]	6.10	6.10	6.10	6.10
Gelatin[5]	3.00	3.00	3.00	3.00
Mineral premix[6]	4.50	4.50	4.50	4.50
Vitamin premix[7]	5.40	5.40	5.40	5.40
Attractants[8]	3.00	3.00	3.00	3.00
Hydroxy-selenomethionine	0.04	0.04	0.04	0.04
Proximate composition (% DM)				
Protein	64.79	63.96	62.14	62.24
Lipids	10.15	11.08	10.34	10.01
Ash	9.18	8.31	7.36	6.53

CTRL: control diet with 15% of fishmeal; P5: 5% inclusion of *P.variotii* (33% of FM replacement); P10: 10% inclusion of *P.variotii* (66% of FM replacement); P15: 15% inclusion of *P.variotii* (100% FM replacement).

[1] FF SKAGEN A/S (Denmark).

[2] Bacarel.

[3] PEKILO®, EniferBio

[4] Sel-Plex® 2000, 2000 mg Se/kg, yeast derived selenium; Alltech.

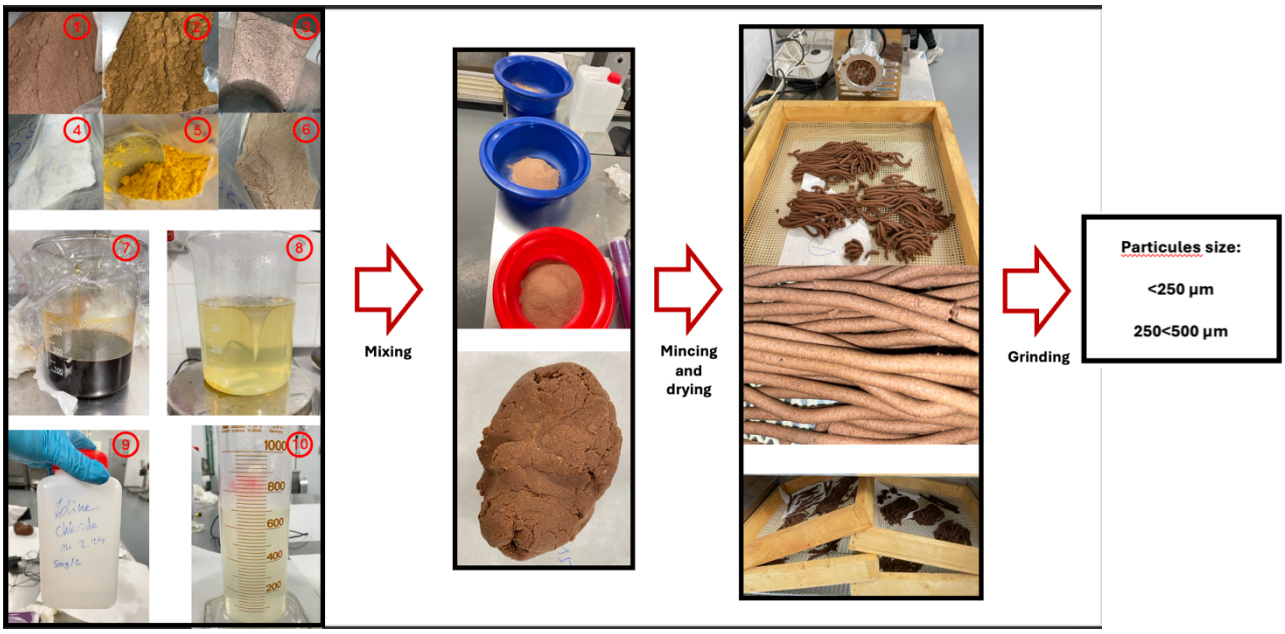
[5] Panreac.

[6] Mineral premix (mg/100 g) supplied for 100 g of diets: NaCl, 215.133 mg; $MgSO_4.7H_2O$, 677.545 mg; $NaH_2PO_4.H_2O$, 381.453 mg; K_2HPO_4, 758.949 mg; $Ca(H_2PO_4).2H_2O$, 671.610 mg; $FeC_6H_5O_7$, 146.884 mg; $C_3H_5O_3.1/2Ca$, 1617.21 mg; $Al_2(SO_4)_3.6H_2O$, 0.693 mg, $ZnSO_4.7H_2O$, 10.1 mg; $CuSO_4.5H2O$, 2.4 mg; $MnSO_4.H_2O$, 27.7 mg; KI, 0.742 mg; $CoSO_4.7H_2O$, 10.706 mg.

[7] Vitamin premix (mg/100 g) supplied for 100 g of diets: Cyanocobalamin, 0.03 mg; Astaxanthin, 5.0 mg; Folic acid, 5.44 mg; Pyridoxine-HCl, 17.28 mg; Thiamine-HCl, 21.77 mg; Riboflavin, 72.53 mg; Calcium pantothenate,

101.59 mg; 4-Aminobenzoic acid, 145 mg; Nicotinic acid, 290.16 mg; Inositol, 1450.90 mg; Retinoic acid, 0.24 mg; Cholecalciferol, 0.0063 mg; Menadione, 7.0 mg; α-Tocopherol acetate, 150.0 mg.

[8] Attractants premix (mg/100 g) supplied for 100 g of diets: Inosine 5-monophosphate, 500 mg; Betaine, 660 mg; L-Serine, 170 mg; L-Tyrosine, 170 mg; Phenylalanine, 250 mg; DL-Alanine, 500 mg; L-Aspartic acid sodium salt, 330 mg; L-Valine, 250 mg; Glycine, 170 mg.



[1] Squid meal

[2] Fishmeal

[3] Attractants premix

[4] Mineral premix

[5] Vitamin premix

[6] *P.variotii*, PEKILO®

[7] Krill oil: Sel-Plex® 2000

[8] Gelatin

[9] Chlorine-chloride

[10] Vitamin C

Figure 2.1: Experimental diet preparation

Table 2.2: Fatty acid composition of experimental diets (% of total identified fatty acids)

	Experimental diets			
Fatty acid (%)	**CTRL**	**P5**	**P10**	**P15**
14:0	8.10	8.38	8.40	8.54
14:1n-7	0.12	0.12	0.13	0.12
14:1n-5	0.24	0.24	0.24	0.25
15:0	0.56	0.50	0.47	0.45
15:1n-5	0.05	0.06	0.05	0.06
16:0ISO	0.11	0.11	0.10	0.10
16:0	25.48	24.31	24.38	24.50
16:1n-7	5.10	5.31	5.10	5.10
16:1n-5	0.29	0.26	0.26	0.25
16:2n-6	0.02	0.04	0.01	0.00
16:2n-4	0.44	0.51	0.47	0.47
17:0	0.14	0.16	0.18	0.17
16:3n-4	0.25	0.22	0.22	0.18
16:3n-3	0.31	0.28	0.29	0.30
16:3n-1	0.12	0.09	0.09	0.08
16:4n-3	0.56	0.67	0.68	0.71
16:4n-1	0.02	0.05	0.04	0.02
18:0	5.40	4.52	4.35	4.14
18:1n-9	12.14	12.22	11.87	11.09
18:1n-7	4.55	4.71	4.52	4.49
18:1n-5	0.19	0.18	0.18	0.17
18:2n-9	0.06	0.07	0.07	0.08
18:2n-6	2.88	4.58	6.04	7.46
18:2n-4	0.02	0.07	0.06	0.05
18:3n-6	0.09	0.16	0.14	0.13
18:3n-4	0.07	0.06	0.05	0.06
18:3n-3	0.77	0.84	0.78	0.74
18:3n-1	0.00	0.03	0.02	0.00
18:4n-3	1.72	2.05	2.04	2.07
18:4n-1	0.04	0.04	0.07	0.05
20:0	0.20	0.17	0.15	0.13
20:1n-9	0.14	0.20	0.16	0.14
20:1n-7	4.55	3.67	3.58	3.54
20:1n-5	0.31	0.32	0.29	0.27
20:2n-9	0.03	0.02	0.03	0.03
20:2n-6	0.28	0.22	0.23	0.22
20:3n-9	0.03	0.05	0.04	0.03

20:3n-6	0.05	0.08	0.05	0.04
20:4n-6 (ARA)	1.09	0.94	0.88	0.84
20:3n-3	0.49	0.42	0.42	0.44
20:4n-3	0.25	0.25	0.24	0.23
20:5n-3 (EPA)	10.29	11.45	11.55	11.65
22:1n-11	0.42	0.86	0.85	0.85
22:1n-9	0.87	0.14	0.14	0.13
22:4n-6	0.16	0.15	0.13	0.11
22:5n-6	0.27	0.22	0.18	0.14
22:5n-3	0.47	0.47	0.41	0.36
22:6n-3 (DHA)	10.26	9.51	9.37	9.02
SFA	39.85	37.99	37.85	37.85
MUFA	60.01	61.85	61.97	61.98
n-3	25.12	25.95	25.77	25.50
n-6	4.83	6.39	7.67	8.94
n-9	13.28	12.70	12.32	11.50
EFAs	21.64	21.91	21.80	21.50

CTRL: control diet with 15% of fishmeal; P5: 5% inclusion of *P.variotii* (33% of FM replacement); P10: 10% inclusion of *P.variotii* (66% of FM replacement); P15: 15% inclusion of *P.variotii* (100% FM replacement).
ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; EFAs: essential fatty acids (ARA, EPA, DHA).

2.2 Fish and experimental conditions

Larvae were obtained through natural spawn from the gilthead sea bream broodstock present at Grupo de Investigación en Acuicultura (GIA) from Unievrsidade de Las Palmas de Gran Canaria (ULPGC). A total of 18300 larvae were necessary. From hatching to the trial phase, larvae were reared in 2000 L tanks at Parque Cientifico Tecnologico Marino, Taliarte, Gran Canaria. At first, larvae were fed each day with rotifers (*Brachinous plicatilis*) enriched with Ori-Green (Skretting, France), 10 rotifers/mL and green water technique, both pumped from 9:00 am to 7:00 pm (April to May period). The trial started when larvae reached 28 days post-hatch (dph). Larvae were assigned to 12 fiberglass cylinder tanks with conical bottoms (170 L), at 8894 larvae/m^3 density (Figure 2.2). Tanks were supplied with flow-through filtered seawater (37 mg/L salinity) at an increasing debit from 1 L/min at the beginning of the experiment to 2 L/min at the end of the experiment and continuous aeration (125 mL/min) during the entire experiment aiming for 6–8 g/L dissolved O_2. Water conditions such as dissolved oxygen and temperature were daily monitored during the entire trial, the average

temperature was 22.2 ± 0.8 ºC and the dissolved oxygen was 6.57 ± 0.1 g/L in every tank. Larvae were hand-fed every 45 min from 8:00 am to 8:00 pm for 18 days until 47 dph. Fish were fed the five first days with a total of 3 g/tank/day of experimental diets (2 g of size 250-500 µm and 1 g of size <250 µm), followed by 3.5g/tank/day for 3 days (2 g of size 250-500 µm and 1g of size <250 µm), 3.5g/tank/day of size 250-500 µm for 6 days, finishing with 4 g/tank/day of size 250-500 µm. Photoperiod was artificially maintained with a 12h light and 12h dark regime from 8:00 am to 8:00 pm. Larvae mortality was daily registered by individually counting the dead larvae. At the end of the trial, the survival rate in each tank was calculated.

CTRL: control diet with 15% of fishmeal; P5: 5% inclusion of *P.variotii* (33% of FM replacement); P10: 10% inclusion of *P.variotii* (66% of FM replacement); P15: 15% inclusion of *P.variotii* (100% FM replacement).

Figure 2.2: Treatment arrangement, overview of the experiment room and closer picture of a tank.

2.3 Stress test

At the end of the feeding trial, larvae from each tank were submitted to a stress test. Thirty larvae per tank were submitted to an air exposure test by scooping them with a net out of the water for 2 min. After 2 min, fish were placed in a bucket (Figure 2.3) supplied with seawater and aeration to further determine survival at 24 h post-stress (Carvalho *et al.* 2022).

Figure 2.3: Tank set-up after stress exposure test.

2.4 Pathogen challenge

Thirty specimens of the remaining larvae from each tank were subjected to a pathogen resistance test against *Vibrio anguillarum* (Figure 2.4), with a sublethal dose of the pathogen (LD70), as described by Hanif *et al.* (2005). The experiment was performed in triplicate, mortality was recorded for 48 h, and the relative percentage of survival (RPS) was calculated at the end of the challenge.

Figure 2.4: Pathogen challenge test set-up

2.5 Growth determination

To determine growth, samplings were done during the experiment, at the beginning (28 dph) and at the end of the trial (47 dph). Growth performance was evaluated by measuring the total length of larvae at both sampling points (n = 30 per tank) under a profile projector, Leica M50 (Leica microsystems, Germany) using the Leica LAS EZ (Leica, Microsystems, Germany) software and dry weight was measured by drying larvae in an oven at 105 °C until constant weight. Biomass was calculated by multiplying the larvae's dry weight by the number of remaining live larvae. At the end of the trial, alive larvae were counted to calculate the final survival rate. Finally, the remaining larvae were euthanised using ice-cold water, washed with fresh water and stored at -80 °C for further analysis.

2.6 Proximate composition and fatty acid profiles

All the remaining larvae after all the other samples were done were used for proximate composition and fatty acid profile. The whole larvae and feed samples were finely ground until homogeneity of samples. To determine the proximate composition, feed and whole larvae body samples were weighed and moisture, ash and crude protein contents were analysed according to AOAC *et al.* (1931). Total lipids were also extracted and analysed using chloroform:methanol solution (2:1) according to Folch *et al.* (1957). Following lipid extraction, fatty acids methyl esters were obtained by transmethylation of the lipids and were then separated by gas-liquid chromatography (GLC) and quantified by Flame Ionisation Detection (FID) (Izquierdo 1989).

2.7 Relative gene expression

From each tank, 30 larvae were randomly sampled at 47 dph before pathogen exposure (0 h) and at 49 dph after 48 h of pathogen exposure (48 h), and washed with DEPC water to be stored in RNALater® (Sigma, Madrid, Spain) at –80 ºC until analysis. Total RNA was extracted and purified from whole larvae samples while keeping them on ice. Samples were homogenised using Tissuelyser II (Qiagen) with Tri Reagent (Sigma-Aldrich, Sant Louis, MO, USA). RNA was separated by adding 250 µL of chloroform and centrifuged at 12000 x g, for 30 min at 4 ºC. Following centrifugation, the upper phase was isolated and mixed with EtOH 70% and passed through a RNeasy spin column (kit-RNeasy) and centrifugated at 8000 x g for 15 s. RNA purification was done by adding RW1 and RPE buffers with centrifugation between each addition of solutions. Pure RNA was retained and bonded to the column's membrane and to finally draw the final purified RNA, 35 µL of RNase-free water was added. Every step was done following the established protocol described by the manufacturer. Moreover, quantification of the RNA was determined using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA synthesis was done using the iScript cDNA Synthesis kit (Bio-rad, Hercules, CA, USA). The *mRNA* levels of *interleukin 1-beta (il-1β)*, *cd4-full (cd4)*, *CD8 alpha chain precursor (cd8α)* and *tumour necrosis factor-alpha (tnf-α)* were determined by RT-PCR (iQ5 Multicolour Real-Time PCR detection system, Bio-Rad), with *cytoplasmic β-actin 1 (β-actin)* and *elongation factor 1 (ef1α)* used as housekeeping genes

for 0 h samples and 48 h samples respectively. Every primer sequence with its respective concentrations and annealing temperatures is presented in Table 2.3. Regarding RT-PCR, each well-contained a mix of 10 µL of Brillant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA), 1 µL of each primer at a concentration of 10 mM, 3 µL of cDNA at dilution 1:10 and 5 µL of RNA-free water (Omega Bio-tek, Inc). Control blanks for RT-PCR were as mentioned previously, except cDNA dose was replaced by 3 µL of RNA-free water (Omega Bio-tek, Inc). RT-PCR conditions were as follows: a first step of 3 min at 95 ºC followed by 40 cycles of 10 s at 95 ºC, 30 s at the respective annealing temperature (Tm, Table 2.3), 30 s at 72 ºC, 1 min at 95 ºC, and a final 10 s from 55 ºC to 95 ºC. The resulting data was used to calculate the relative gene expression according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 2.3: Primers sequences, annealing temperatures and GenBank accession number of genes assessed on whole gilthead sea bream larvae fed the experimental diets.

5′–3′ primer sequence				
Gene	**Forward**	**Reverse**	**Tm (°C)**	**GenBank accesion number**
il-1β	AGCGACATGGCACGATTTC	GCACTCTCCTGGCACATATCC	62	AJ277166.2
tnf-α	CTCACACCTCTCAGCCACAG	TTCCGTCTCCAGTTTGTCG	62	AJ413189.2
cd8	CTCGACTGGTCGGAGTTAA	TCCATCAGCGGCTGCTCGT	62	AJ878605
cd4	TCCTCCTCCTCGTCCTCGTT	GGTGTCTCATCTTCCGCTGTCT	60	AM489485
β-actin	TCTGTCTGGATCGGAGGCTC	AAGCATTTGCGGTGGACG	60	KY388508.1.
ef1α	CATGGTTGTGGAGCCCTTCT	TCCTGCACGACCATTCATTTC	58.1	AF184170

Tm, annealing temperature; *il-1β*, interleukin 1-beta; *tnf-α*, tumour necrosis factor alpha; *cd8*, cd8 alpha chain precursor; *cd4*, cd4-full; *β-actin*, beta-actin; *ef1α*, elongation factor 1-alpha.
β-actin and ef1α cDNA were used as an internal control for 0 h and 48 h respectively.
Gen Bank: https://www.ncbi.nlm.nih.gov

2.8 Calculations

- Biomass (g): DW × RL,

where, DW = dry weight in g; RL = number of remaining larvae.

- Body weight gain (dry weight, g): FBW− IBW,

where, FBW = final body weight in g/fish; IBW = initial body weight in g/fish.

- Total length gain (mm): FBL− IBL,

where, FBL = final body length in mm/fish; IBL = initial body length in mm/fish.

- Specific growth rate (SGR, % body weight/day): [((ln (FBW) – ln (IBW))/experimental period (days)] × 100.

2.9 Statistical analysis

Means and standard deviation were calculated for each parameter. All data were tested for normality and homogeneity of variances with Levene's test. Data were analyzed through a one-way analysis of variance and by using IBM SPSS Statistics software (29.0 version; SPSS Inc., Chicago, IL, USA) followed by Tukey's multiple comparison test to determine differences between treatment means. The significance was set at $P < 0.05$.

3. Results

3.1. Larval performance

During the experiment, observations have shown that larvae from each treatment accepted well the experimental diets. Growth performance data are presented in Table 3.1. There were no significant differences in final body weight, final body length, body weight gain, total length gain, SGR, biomass, survival, survival after air exposure and survival after pathogen exposure among larvae fed different levels of *P.variotii* (5, 10 and 15%) (Table 3.1). Despite the lack of significance, P10 has shown lower average values of final body weight,

final body length, body weight gain, total length gain, SGR, biomass, survival after air exposure and survival after pathogen exposure compared to the other treatments.

Table 3.1: Growth performance of gilthead sea bream larvae (47 dph) fed diets with different levels of dietary P.variotii for 18 days.

	Experimental diets			
	CTRL	**P5**	**P10**	**P15**
Initial dry weight (mg)	0.45 ± 0.05	0.45 ± 0.06	0.45 ± 0.07	0.45 ± 0.08
Initial total length (mm)	8.34 ± 1.00	8.34 ± 1.01	8.34 ± 1.02	8.34 ± 1.03
Final body weight (g)	2.76 ± 0.20	2.47 ± 0.53	2.15 ± 0.32	2.62 ± 0.56
Final body length (mm)	13.35 ± 1.32	12.87 ± 1.62	12.42 ± 1.44	13.07 ± 1.61
Body weight gain (dry weight, mg)	2.76 ± 0.18	2.47 ± 0.39	2.15 ± 0.28	2.62 ± 0.30
Total length gain (mm)	5.01 ± 1.32	4.54 ± 1.47	4.08 ± 1.43	4.74 ± 1.54
SGR (% body weight day-1)	10.08 ± 0.22	9.39 ± 1.01	8.68 ± 0.44	9.71 ± 1.12
Biomass (g)	2.26 ± 0.25	1.85 ± 0.50	1.61 ± 0.03	1.75 ± 0.54
Survival (%)	54.7 ± 8.4	49.7 ± 6.4	50.1 ± 3.2	43.9 ± 5.5
Survival after air exposure(%)	88.54 ± 7.24^{ab}	97.7 ± 3.98^{a}	76.51 ± 6.60^{b}	96.67 ± 5.77^{a}
Survival after pathogen exposure(%)	79.98 ± 23.08	80.18 ± 8.09	73.79 ± 8.09	70.86 ± 15.56

CTRL: control diet with 15% of fishmeal; P5: 5% inclusion of *P.variotii* (33% of FM replacement); P10: 10% inclusion of *P.variotii* (66% of FM replacement); P15: 15% inclusion of *P.variotii* (100% FM replacement). Values (mean ± SD). The absence of letters in the same row indicates no significant differences ($p < 0.05$).

3.2. Proximate composition and fatty acid profiles

After 18 days of the feeding experiment, whole-body compositions were assessed, and despite the lack of significant differences between treatments a tendency to a slight increase in ash content could be noted in fish fed with increasing *P.variotii* inclusion (Table 3.2).

Table 3.2: Final proximate composition of whole-body of gilthead sea bream larvae fed the experimental diets for 18 days.

	Experimental diets			
Whole body DW (%)	**CTRL**	**P5**	**P10**	**P15**
Ash	1.87 ± 0.29	1.97 ± 0.20	2.03 ± 0.18	2.08 ± 0.36
Total lipid	2.62 ± 0.42	2.31 ± 0.26	2.46 ± 0.27	2.56 ± 0.47
Crude protein	11.53 ± 2.10	11.61 ± 1.54	12.02 ± 1.52	11.22 ± 0.74
Moisture	82.54 ± 0.41	84.27 ± 1.82	84.23 ± 1.78	84.3 ± 3.38

DW = dry weight. CTRL: control diet with 15% of fishmeal; P5: 5% inclusion of *P.variotii* (33% of FM replacement); P10: 10% inclusion of *P.variotii* (66% of FM replacement); P15: 15% inclusion of *P.variotii* (100% FM replacement).

Values (mean ± SD). The absence of letters in the same row indicates no significant differences ($p < 0.05$).

In terms of fatty acids analysis, the increase in dietary *P.variotii* significantly ($p < 0.05$) decreased the larvae whole-body content in n-9 fatty acids, particularly, oleic acid (18:1n-9), linoleic acid (18:2n-9) and 20:1n-9. On the contrary, DHA and n-6 fatty acids, particularly, linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) have significantly ($p < 0.05$) increased by the increase in dietary *P.variotii* levels. Moreover, the increase in dietary *P.variotii* inclusion significantly ($p < 0.05$) decreased 14:0, 14:1n-7, 15:0, 16:0, 16:1n-7, 16:3n-4, 18:1n-5 and 20:1n-7.

Table 3.3: Fatty acids composition of whole gilthead sea bream larvae fed the experimental diets for 18 days (% of total identified fatty).

	Experimental diets			
Fatty acid (%)	**CTRL**	**P5**	**P10**	**P15**
14:0	2.75 ± 0.12[a]	2.68 ± 0.19[ab]	2.37 ± 0.12[b]	2.66 ± 0.05[ab]
14:1n-7	0.03 ± 0.00[a]	0.02 ± 0.00[ab]	0.02 ± 0.00[b]	0.03 ± 0.00[ab]
14:1n-5	0.13 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.14 ± 0.01
15:0	0.44 ± 0.02[a]	0.40 ± 0.02[ab]	0.39 ± 0.03[b]	0.41 ± 0.02[ab]
15:1n-5	0.04 ± 0.01	0.027 ± 0.00	0.03 ± 0.00	0.03 ± 0.01
16:0ISO	0.01 ± 0.01	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
16:0	24.57 ± 0.87[a]	22.83 ± 0.45[ab]	22.51 ± 0.62[b]	23.27 ± 0.75[ab]
16:1n-7	3.19 ± 0.10[a]	2.98 ± 0.24[ab]	2.70 ± 0.19[b]	2.80 ± 0.05[ab]
16:1n-5	0.35 ± 0.01	0.34 ± 0.01	0.35 ± 0.01	0.32 ± 0.02
16:2n-6	0.11 ± 0.00	0.10 ± 0.02	0.10 ± 0.01	0.09 ± 0.01
16:2n-4	0.49 ± 0.06	0.38 ± 0.05	0.49 ± 0.06	0.40 ± 0.13
17:0	0.07 ± 0.00	0.07 ± 0.01	0.07 ± 0.00	0.07 ± 0.00
16:3n-4	0.34 ± 0.01[a]	0.29 ± 0.01[b]	0.29 ± 0.01[b]	0.27 ± 0.01[b]
16:3n-3	0.29 ± 0.04	0.28 ± 0.02	0.30 ± 0.05	0.27 ± 0.05
16:3n-1	0.52 ± 0.02	0.55 ± 0.01	0.53 ± 0.01	0.55 ± 0.01
16:4n-3	0.44 ± 0.06	0.49 ± 0.13	0.59 ± 0.05	0.61 ± 0.05
16:4n-1	0.17 ± 0.01	0.19 ± 0.02	0.19 ± 0.01	0.19 ± 0.01
18:0	7.97 ± 0.34	7.74 ± 0.09	8.05 ± 0.15	7.93 ± 0.27
18:1n-9	13.29 ± 0.20[a]	12.67 ± 0.52[ab]	12.31 ± 0.27[ab]	12.17 ± 0.05[b]
18:1n-7	4.23 ± 0.15	4.37 ± 0.14	4.18 ± 0.11	4.29 ± 0.10
18:1n-5	0.18 ± 0.00[a]	0.18 ± 0.01[ab]	0.16 ± 0.00[b]	0.17 ± 0.00[ab]
18:2n-9	0.38 ± 0.06[a]	0.22 ± 0.06[b]	0.19 ± 0.03[b]	0.18 ± 0.02[b]
18:2n-6	2.83 ± 0.11[a]	4.02 ± 0.51[ab]	4.15 ± 0.64[ab]	5.62 ± 0.10[b]
18:2n-4	0.12 ± 0.00	0.11 ± 0.01	0.10 ± 0.00	0.10 ± 0.00
18:3n-6	0.30 ± 0.02	0.31 ± 0.03	0.27 ± 0.03	0.29 ± 0.01
18:3n-4	0.05 ± 0.01	0.05 ± 0.02	0.04 ± 0.01	0.04 ± 0.01
18:3n-3	0.90 ± 0.05	0.87 ± 0.05	0.85 ± 0.04	0.83 ± 0.01
18:3n-1	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01
18:4n-3	0.74 ± 0.05	0.71 ± 0.07	0.62 ± 0.01	0.65 ± 0.06
18:4n-1	0.48 ± 0.11	0.47 ± 0.12	0.58 ± 0.08	0.59 ± 0.11
20:0	0.29 ± 0.01	0.27 ± 0.00	0.29 ± 0.01	0.27 ± 0.01
20:1n-9	0.13 ± 0.00[a]	0.11 ± 0.02[ab]	0.10 ± 0.02[ab]	0.09 ± 0.00[b]
20:1n-7	2.20 ± 0.08[a]	1.84 ± 0.11[b]	1.67 ± 0.07[b]	1.75 ± 0.07[b]
20:1n-5	0.23 ± 0.01	0.23 ± 0.02	0.21 ± 0.02	0.24 ± 0.04
20:2n-9	0.07 ± 0.01	0.05 ± 0.02	0.05 ± 0.01	0.04 ± 0.00
20:2n-6	0.72 ± 0.10	0.71 ± 0.14	0.85 ± 0.08	0.86 ± 0.12
20:3n-9	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.00

20:3n-6	0.13 ± 0.01	0.15 ± 0.02	0.16 ± 0.03	0.15 ± 0.01
20:4n-6 (ARA)	2.24 ± 0.08^{a}	2.37 ± 0.16^{ab}	2.60 ± 0.16^{b}	2.30 ± 0.14^{ab}
20:3n-3	0.45 ± 0.01	0.42 ± 0.01	0.44 ± 0.02	0.45 ± 0.03
20:4n-3	0.40 ± 0.10	0.39 ± 0.12	0.55 ± 0.15	0.51 ± 0.10
20:5n-3 (EPA)	9.06 ± 0.58	9.42 ± 0.26	8.76 ± 0.15	8.83 ± 0.45
22:1n-11	0.77 ± 0.04	0.64 ± 0.09	0.64 ± 0.07	0.74 ± 0.23
22:1n-9	0.11 ± 0.00	0.11 ± 0.01	0.10 ± 0.00	0.11 ± 0.01
22:4n-6	0.18 ± 0.00	0.17 ± 0.00	0.20 ± 0.02	0.16 ± 0.01
22:5n-6	0.69 ± 0.06	0.71 ± 0.04	0.81 ± 0.08	0.68 ± 0.05
22:5n-3	0.93 ± 0.05	1.08 ± 0.08	1.11 ± 0.07	1.03 ± 0.07
22:6n-3 (DHA)	15.87 ± 1.22^{a}	17.73 ± 0.79^{ab}	18.74 ± 1.05^{b}	16.72 ± 1.11^{ab}
SFA	36.13 ± 1.34	34.02 ± 0.63	33.72 ± 0.78	34.64 ± 1.03
MUFA	63.80 ± 1.34	65.91 ± 0.63	66.21 ± 0.78	65.29 ± 1.03
n-3	29.08 ± 1.90	31.37 ± 1.08	31.95 ± 1.01	29.88 ± 1.50
n-6	7.20 ± 0.26^{a}	8.54 ± 0.80^{b}	9.14 ± 0.56^{bc}	10.15 ± 0.05^{c}
n-9	14.00 ± 0.19^{a}	13.17 ± 0.59^{ab}	12.77 ± 0.29^{b}	12.61 ± 0.04^{b}
EFAs	27.17 ± 1.80	29.52 ± 1.00	30.10 ± 1.17	27.84 ± 1.49

CTRL: control diet with 15% of fishmeal; P5: 5% inclusion of *P.variotii* (33% of FM replacement); P10: 10% inclusion of *P.variotii* (66% of FM replacement); P15: 15% inclusion of *P.variotii* (100% FM replacement).
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ARA: arachidonic acid; EFAs: essential fatty acids (ARA, EPA, DHA).
Values (mean ± SD) with different superscript letters in the same row are significantly different ($p < 0.05$).

3.3 Health-related gene expression

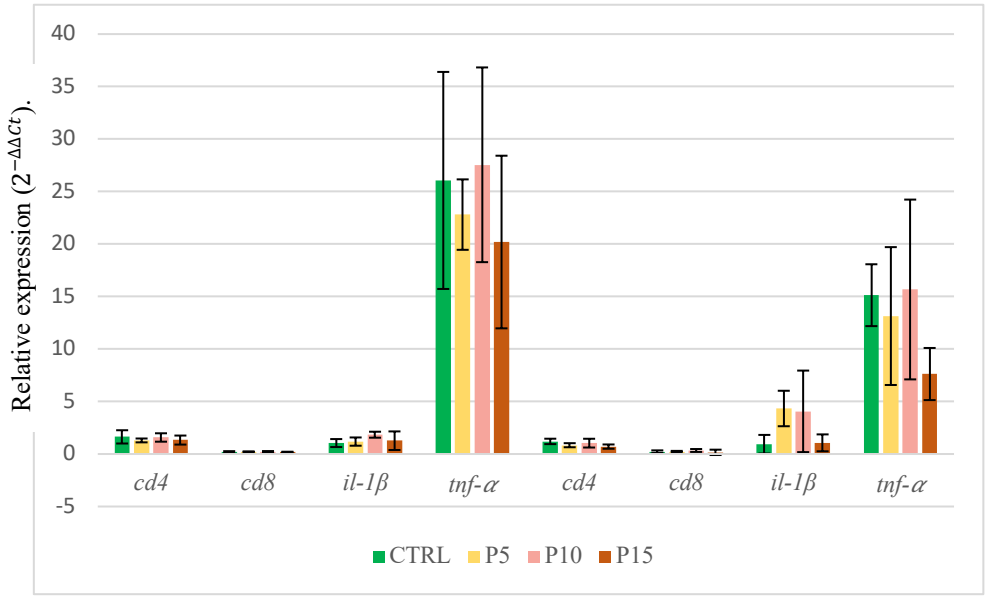
After 18 days of feeding the experimental diets, there were no significant differences in the basal relative expression of immune-related genes among larvae fed the different experimental diets (Table 3.4). There were also no significant differences between the same genes after 48 h of exposure to *V.anguillarum* (48 h, Table 3.4).

Furthermore, although not significant, *il-1β* relative expression rose after 48 h of pathogen exposure for the larvae fed increasing levels of *P.variotii*. On the contrary, *tnf-α* and *cd4* relative expression decreased after 48 h of pathogen exposure among fish fed the different experimental microdiets (Figure 3.1).

Table 3.4: Expression of immune-related genes in the whole body of gilthead sea bream larvae (47 dph) fed different levels of dietary P.variotii for 18 days.

	Experimental diets			
	CTRL	**P5**	**P10**	**P15**
0h				
il-1β	1.04 ± 0.38	1.18 ± 0.39	1.84 ± 0.28	1.27 ± 0.89
cd4	1.63 ± 0.63	1.29 ± 0.18	1.58 ± 0.40	1.33 ± 0.43
cd8	0.18 ± 0.09	0.20 ± 0.05	0.22 ± 0.07	0.18 ± 0.04
tnf-α	26.06 ± 10.34	22.81 ± 3.36	27.55 ± 9.27	20.19 ± 8.22
48h				
il-1β	0.93 ± 0.89	4.34 ± 1.69	4.06 ± 3.88	1.06 ± 0.81
cd4	1.20 ± 0.26	0.84 ± 0.19	1.04 ± 0.42	0.71 ± 0.20
cd8	0.23 ± 0.12	0.23 ± 0.07	0.34 ± 0.25	0.16 ± 0.14
tnf-α	15.13 ± 2.95	13.14 ± 6.57	15.67 ± 8.56	7.62 ± 2.48

CTRL: control diet with 15% of fishmeal; P5: 5% inclusion of *P.variotii* (33% of FM replacement); P10: 10% inclusion of *P.variotii* (66% of FM replacement); P15: 15% inclusion of *P.variotii* (100% FM replacement). Values (mean ± SD). The absence of letters in the same row indicates no significant differences ($p < 0.05$).



CTRL: control diet with 15% of fishmeal; P5: 5% inclusion of *P.variotii* (33% of FM replacement); P10: 10% inclusion of *P.variotii* (66% of FM replacement); P15: 15% inclusion of *P.variotii* (100% FM replacement). Values (mean ± SD). The absence of letters in the same column indicates no significant differences ($p < 0.05$).

Figure 2.1: Relative expression of fatty acid metabolism-related genes of gilthead sea bream larvae fed increasing levels of dietary P.variotii for 18 days.

4. Discussion

Fishmeal, a protein-rich feed ingredient made from processed fish, has long been a staple in aquaculture diets. However, the growing demand for aquaculture products has led to significant environmental and economic concerns regarding the continued use of FM. A recent study done by Cashion *et al.* (2017) stapled the fact that the wild-caught fish used to make FM were food-graded fish that could be used for human consumption. In this sense, the replacement of FM as the main protein source in aquafeeds is critical to ensure the future of fish farming as the foundation for food security (Naylor *et al.* 2009, Troell *et al.* 2014). In recent years, microbial protein ingredients have raised a particular interest as they thrive on industry by-products, like brewing or waste treatment streams, that are cheap since they are meant to be discarded (Matassa *et al.* 2016, Øverland *et al.* 2017). In the present study, the potential of *Paecilomyces variotii* as a FM replacer in the diets at the larvae stage of the popular aquaculture Mediterranean species, gilthead sea bream, was demonstrated. In the following sections outcomes on fish general welfare and resistance are discussed for a potential commercially viable solution.

4.1 Growth performance

The replacement of the total CP content of the experimental diets with *P.variotii* ans squid meal did not significantly affect the survival rate in the experimental treatments compared to the control treatment. The lack of significant differences in growth performance parameters (final body weight, final body length, body weight gain, total length gain, and SGR) suggests that the experimental diets provided similar nutritional value to the control diet in supporting larval growth. This is consistent with previous findings where dietary modifications often resulted in negligible impacts on growth indicators in the early stages of fish development when baseline nutritional requirements were met (Hamre *et al.* 2013). In this study, larvae showed a lower survival rate than the gilthead sea bream larvae at the same age in Tseng *et al.* (2023) study. However, larvae in the current study presented a lower DHA level than in Tseng *et al.* (2023) study. This could explain the overall low survival rate of the larvae since DHA is an important compound in larvae metabolism (Rodriguez *et al.* 1994, Izquierdo *et al.* 2011). Indeed, larvae have higher EFAs requirements than juveniles (Izquierdo *et al.* 2011), especially DHA and EPA. Low levels of n-3 could lead to a low survival rate, reduced stress resistance,

decreased immune resistance, and altered feeding behaviour (Izquierdo 2005), which may explain the significantly lower survival after air exposure for larvae fed the experimental diets compared to the larvae fed the control diet.

Additionally, the values of the growth performance parameters from this studywere higher than in the study by Tseng *et al.* (2023) with 47 dph larvae fed with copper-supplemented diets.

The specific growth rate (SGR) of the fish fed the experimental diets was not significantly different from the control diet. However, previous studies for the same larvae age have shown SGR of about 10 to 15% (Mhalhel *et al.* 2023), while the SGR in this study was approximately 9.5% for all the experimental treatments.

Previous studies have already assessed the dietary inclusion of *P.variotii* as the main protein source in aquafeeds. A study in juveniles Atlantic salmon fed with increasing inclusion levels of *P.variotii* has found that it could replace up to 20% of the CP content with improved FCR, increased nutrient utilization, improved gut health as well as better immune-modulatory capacity in the distal intestine (Hooft *et al.* 2024). Similar key findings have been observed in the growth performance and gut health of freshwater-reared juveniles Atlantic salmon (Javed 2023). Also, in Nile tilapia (*Oreochromis niloticus*), similar growth performance as fish-fed conventional diets was observed (Alriksson *et al.* 2014). In the present study, *P.variotii* crude protein content was 62.51% and the growth performance of fish fed the experimental diets was not significantly different from the fish fed the control diet.

Other studies highlighted the potential for MIs to replace FM efficiently in gilthead sea bream feeds. Indeed, up to 66% of the total FM in diets could be replaced by a single-cell protein from *Methylococcus capsulatus* without interfering with the growth performance of the fed gilthead sea bream (Carvalho *et al.* 2023). In the same way, Marchi *et al.* (2023) showed that the inclusion of 20% MI derived from *Corinebacterium glutamicum* in sea bream feeds did not affect growth and feed efficiency. In a study by Oliva-Teles *et al.* (2006), the replacement of FM with brewers' yeast led to a significant improvement in the feed intake and growth performance. Finally, another experiment conducted on gilthead sea bream aiming to replace FM with a mixture of single-cell ingredients (66.7% of *Methylococcus capsulatus* and 33.3% *Saccharomyces cerevisiae*) for organic aquaculture showed increased growth performance as well as a better protein digestibility in the fed fish (Tampou *et al.* 2024).

No significant differences were observed regarding survival after pathogen exposure. Despite the lack of significance, there was a tendency for a decrease in larval survival after 48 hours of exposure to *Vibrio anguillarum* with increasing dietary levels of *P.variotii*. Since

P.variotii present a high amount of β-glucans, it was expected a better survival after pathogen exposure of the larvae fed the experimental diets. This effect was observed in Atlantic cod (*Gadus morhua*) fed yeast-derived mannan oligosaccharide and purified β-glucans and challenged with *Vibrio anguillarum*. These fish showed better growth of beneficial bacteria in gut microbiome, which resulted in a strengthened gut barrier and a better health of the fed fish (Torrecillas *et al.* 2014). The lack of significant differences in survival after pathogen exposure in the current study suggests that the dietary treatments did not markedly affect the immune competence of the larvae during the trial period. This finding is in line with studies showing that variations in diet composition often have limited short-term effects on immune parameters in early-stage larvae (Rønnestad *et al.* 2013).

4.2 Proximate and fatty acid composition

In the present study, *P.variotii* inclusion had no significant effect on the whole-body proximate composition of the fed larvae compared to the control diet. This suggests that the experimental diets, despite varying in formulation, did not significantly impact the nutritional composition of the larvae at this early developmental stage. One potential explanation for the absence of significant differences could be that gilthead sea bream larvae possess a high degree of dietary flexibility during their early life stages, allowing them to maintain homeostasis in terms of nutrient composition despite variations in diet. Previous studies have suggested that marine fish larvae, including gilthead sea bream, can often compensate for fluctuations in nutrient availability by adjusting their metabolic processes, but it might results in deformities and imbalanced growth (Rønnestad *et al.* 2013). This ability to adjust might be especially pronounced in the early stages of development when larvae are rapidly growing and require a steady supply of essential nutrients to sustain growth and organ development (Hamre *et al.* 2013).

P.variotii dietary inclusion in gilthead sea bream feeds significantly affected their fatty acid profile. Indeed, whole-larvae analysis showed a significant reduction in n-9 fatty acids with increased *P.variotii* inclusion. After analysis, oleic acid (18:1n-9), linoleic acid (18:2n-9) and gondoic acid (20:1n-9) were significantly lower in the whole-larvae compared to the control. Omega-9 fatty acids, and especially oleic acid, play several roles in the health and development of sea bream. Indeed, oleic acid serves as a significant energy source and lipid storage in the gilthead metabolism which assists muscle growth (Lenas *et al.* 2011). Those low

levels of n-9 fatty acids in whole larvae from experimental treatments can be explained by the low levels of fatty acids present in the experimental diets, which could directly affect the fish's fatty acid composition (Benitez 1989). This may explain the lower growth performance trend observed in fish fed the experimental diets. Similarly, Tseng *et al.* (2023) found that the low levels of dietary n-9 fatty acids induced lower growth in sea bream larvae.

On the contrary, with the increase in dietary *P.variotii* levels, DHA significantly increased in the whole larvae fatty acids analysis. Moreover, while comparing the DHA levels of the control and the experimental diets, a clear difference is observed, with the control reaching 10.26% while the P5, P10 and P15 diets reached 9.51, 9.37 and 9.02 % respectively. At the larval stage, low supply levels of n-3 fatty acids, especially DHA and EPA, can lead to altered feeding, decreased growth performance, reduced stress resistance, immune suppression as well as low survival (Izquierdo 2005, Izquierdo *et al.* 2011).

In this study, the levels of n-6 fatty acids were significantly higher in fish fed with increasing levels of *P.variotii* compared to the control diet. This suggests that the composition of the dietary lipids in the experimental diets significantly influenced the incorporation and metabolism of n-6 fatty acids in the larvae. It has been well-documented that n-3 and n-6 fatty acids can compete with n-9 fatty acids for incorporation into cell membranes and storage tissues due to shared metabolic pathways (Halver *et al.* 2003). Therefore, higher levels of n-3 or n-6 fatty acids could have reduced the incorporation of n-9 fatty acids into the body tissues, resulting in the observed lower levels of n-9 fatty acids in the larvae. Higher n-6 fatty acid levels in the whole larvae composition from the treatment groups reflect the higher content of these fatty acids in the experimental diets. When fish larvae are fed n-6 fatty acids enriched diets, their tissues tend to accumulate these fatty acids due to their limited ability to metabolically regulate the excess, especially at early developmental stages when lipid metabolism is still developing (Tocher 2010). Moreover, elevated n-6 fatty acid levels could have an impact on the physiological and developmental processes of the larvae. While n-3 and n-6 PUFAs are essential for growth and development in fish, an imbalance favouring n-6 fatty acids could influence key biological performance, such as inflammatory responses and immune functions (Bell *et al.* 2003). ARA is a precursor for the synthesis of pro-inflammatory eicosanoids, which can play critical roles in the immune and stress responses of the host (Bell *et al.* 1995). High levels of ARA have been reported (Tables 3.3) in fish fed increasing levels of *P.variotii*, therefore its significant high presence in experimental fed fish, there were no significant differences in survival after pathogen exposition between treatments to confirm its effect. However, higher n-6 fatty acids could have influenced the survival rate after air

exposure. Finally, while n-6 fatty acids are essential for fish, previous research has shown that an excessive intake relative to n-3 can alter the health and growth performance of the fed fish (Izquierdo *et al.* 1997). In this context, the increased n-6 fatty acid levels in the larvae-fed *P.variotii*-containing diets may need careful consideration in future dietary formulations to avoid long-term imbalances.

4.3 Immune-related gene expression

In this study, the effects of the dietary inclusion of *Paecilomyces variotii* on the immune response of gilthead sea bream larvae at 47 dph were evaluated before pathogen exposure (0h) and at 49 dph, after 48h of *Vibrio anguillarum* exposure (48h). Understanding the relationship between nutrition and immune response is critical for optimizing larval health and disease resistance in aquaculture.

In this study, increasing levels of *P.variotii* had no significant effect on the expression of the immune-related gene *il-1β* in gilthead sea bream larvae from 0h and 48h. *Il-1β*, a key pro-inflammatory cytokine, plays a crucial role in the early immune response to pathogen invasion. This lack of significant differences may indicate that the dietary inclusion of *P.variotii* does not have a direct effect on these specific immune markers, either at basal levels (0h) or in response to bacterial exposure (48h), it also indicates that replacement the FM replacement by P.variotii had no adverse effect on the immune response of the fed fish. However, *P.variotii* fed to post-smolts Atlantic salmon has been shown to induce immune responses, including the upregulation of pro-inflammatory cytokines like *il-1β* (Mensah et al. 2024), but these responses can vary depending on factors like dose, the presence of other pathogens, the overall health status of the fish, its developmental stage and the diet composition (Dawood *et al.* 2018).

Cd4 is a key marker of T-helper cells, which play a central role in coordinating adaptive immune responses, particularly in recognizing and responding to pathogens. However, the results showed no significant differences in *cd4* expression between the control and *P.variotii*-fed groups nor before (0h) and after pathogen exposure (48h). The absence of significant variation in *cd4* expression suggests that dietary supplementation with *P.variotii* at the tested levels did not positively or negatively influence the adaptive immune response of the larvae. This finding contrasts with Dawood *et al.* (2018) study, indicating that certain dietary

supplements, such as probiotics and microbial derivatives, can enhance immune functions by upregulating immune markers like *cd4*, particularly during pathogen exposure. Furthermore, previous studies on juveniles Atlantic salmon indicated that exposure to *P.variotii* could modulate immune responses in head kidney and spleen leukocytes, potentially enhancing the expression of pro-inflammatory cytokines and adaptive immune markers, including *cd4* (Mensah *et al.* 2024). Additionally, Reis *et al.* (2021) study on gilthead sea bream juveniles fed *Phaeodactylum tricornutum* demonstrated that despite the absence of FM, the fish displayed no significant changes in growth performance. However, the diets enriched with β-glucans derived from *Phaeodactylum tricornutum* showed an immunomodulatory effect, particularly in the downregulation of certain immune-related genes such as *cd4*. This effect was likely linked to an immune tolerance response, particularly in the intestinal tissues, after prolonged feeding of the β-glucan-enriched diet, thus contributing to an immune modulation that helps maintain gut health and reduces inflammatory responses (Reis *et al.* 2021). Finally, the lack of effect on *cd4* relative gene expression suggests that immune benefits from *P.variotii* may not involve enhanced T-helper cells responses, at least under the conditions of this experiment.

Cd8 is a gene associated with cytotoxic T-cells, which play a critical role in the adaptive immune response by targeting and eliminating infected or abnormal cells. The analysis of the relative expression of *cd8* in whole larvae samples revealed no significant differences in its relative expression between the experimental groups and the control diet, nor between 0h and 48h. Those results suggest that the dietary inclusion of *P.variotii* at the tested levels did not positively or negatively impact the cytotoxic T-cell-mediated immune response in gilthead sea bream larvae, at least regarding *cd8* expression. This finding contrasts with some studies where other microbial supplements have been shown to upregulate *cd8* expression, potentially enhancing the adaptive immune response and resistance to pathogens (Carballo *et al.* 2019). However, the lack of significant variation in *cd8* relative expression could indicate that *P.variotii* may not strongly influence the adaptive immune system or that its effects might be more prominent in other immune pathways (Dawood *et al.* 2018). Finally, the lack of effect on *cd8* relative gene expression suggests that immune benefits from *P.variotii* may not involve enhanced cytotoxic T-cell responses, at least under the conditions of this experiment.

Tumour necrosis factor alpha is a key pro-inflammatory cytokine involved in the early immune response to pathogens. It is an important mediator of immune regulation and plays a central role in initiating inflammation and the subsequent activation of various immune cells during infections. Despite the important decrease in *tnf-α* expression following pathogen exposure, no significant differences were observed between the control

and *P.variotii* treatments, nor were there statistically significant changes between 0h and 48h conditions. This result can indicate that while *tnf-α* expression naturally decreases after the pathogen challenge, potentially reflecting a resolution phase of the immune response, dietary supplementation with *P.variotii* did not alter positively or negatively the *tnf-α* mediated inflammatory response in gilthead sea bream larvae. This result is consistent with other studies where microbial dietary supplements, such as probiotics and fungi-derived components, did not necessarily lead to significant changes in *tnf-α* expression in fish, despite contributing to overall immune modulation (Dawood *et al.* 2018, Pogue *et al.* 2021). Finally, the absence of a strong effect on *tnf-α* expression might suggest that *P.variotii* could influence other immune pathways, rather than directly affecting pro-inflammatory cytokines like *tnf-α* (Mensah *et al.* 2024).

In this study on gilthead sea bream larvae fed diets supplemented with *Paecilomyces variotii*, the immune response was primarily assessed through *il-1β, cd4, cd8* and *tnf-α* expression and was not positively or negatively affected, but the role of other immune markers could provide a more comprehensive understanding of the effects of the diet. In this sense, it is possible that the benefits of *P. variotii* could manifest in other areas of immune function or under different experimental conditions. Further studies could explore different biomarkers or doses to fully capture the potential immune-modulatory benefits of *P.variotii*.

5. Conclusion

In summary, *P.variotii* has shown great potential to replace FM as the main protein source in gilthead sea bream diets. *P.variotii* could replace up to 15% of the crude protein content of the diet without adverse consequences on growth performance and survival. In addition, the fungal meal had little effect on the proximate composition of the whole larvae. Regarding fatty acid composition, *P.variotii* inclusion in larval diets can provide some nutritional benefits, particularly in boosting DHA levels. The observed reduction in n-9 fatty acids and increase in n-6 fatty acids highlight the need for careful consideration of fatty acid balance in future diet formulations to optimize growth and health outcomes. Interestingly, the lack of relative gene expression in this study has shown no positive or negative effects on the immune response of the fed fish and it also suggests that immune responses to dietary

components may be species or stage-specific, or that other immune pathways not measured in this study could be involved.

References

Abass, D. A., K. A. Obirikorang, B. B. Campion, R. E. Edziyie and P. V. Skov (2018). "Dietary supplementation of yeast (*Saccharomyces cerevisiae*) improves growth, stress tolerance, and disease resistance in juvenile Nile tilapia (*Oreochromis niloticus*)." Aquaculture International **26**(3): 843-855.

Abdulrahman, N. (2014). "Effect of replacing fishmeal with Spirulina spp. on carcass chemical composition of common carp *Cyprinus carpio L*." Iraqi Journal of Veterinary Sciences **28**(2): 67-70.

Agboola, J. O. (2022). "Can fish grow on trees? Nutritional and functional properties of yeasts in diets for Atlantic salmon (*Salmo salar*)."

Agboola, J. O., M. Øverland, A. Skrede and J. Ø. Hansen (2021). "Yeast as major protein-rich ingredient in aquafeeds: a review of the implications for aquaculture production." Reviews in Aquaculture **13**(2): 949-970.

Alriksson, B., A. Hörnberg, A. E. Gudnason, S. Knobloch, J. Arnason and R. Johannsson (2014). "Fish feed from wood." Cellulose Chemistry and Technology **48**(9-10): 843-848.

Amar, E. C., V. Kiron, S. Satoh and T. Watanabe (2004). "Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products." Fish & Shellfish Immunology **16**(4): 527-537.

Andualem, B. and A. Gessesse (2013). "Production of microbial medium from defatted brebra (*Milletia ferruginea*) seed flour to substitute commercial peptone agar." Asian Pacific Journal of Tropical Biomedicine **3**(10): 790-797.

Ansari, F. A., A. Guldhe, S. K. Gupta, I. Rawat and F. Bux (2021). "Improving the feasibility of aquaculture feed by using microalgae." Environmental Science and Pollution Research **28**(32): 43234-43257.

AOAC and A. o. O. A. Chemists (1931). Official methods of analysis of the Association of Official Analytical Chemists, Association of Official Analytical Chemists.

Archer, D. B., I. F. Connerton and D. A. MacKenzie (2008). Filamentous Fungi for Production of Food Additives and Processing Aids. Food Biotechnology. U. Stahl, U. E. B. Donalies and E. Nevoigt. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 99-147.

Asadollahzadeh, M., A. Ghasemian, A. Saraeian, H. Resalati and M. J. Taherzadeh (2018). "Production of fungal biomass protein by filamentous fungi cultivation on liquid waste streams from pulping process." BioResources **13**(3): 5013-5031.

Awad, E. and B. Austin (2010). "Use of lupin, *Lupinus perennis, mango, Mangifera indica*, and stinging nettle, *Urtica dioica*, as feed additives to prevent *Aeromonas hydrophila* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum)." Journal of Fish Diseases **33**(5): 413-420.

Babuskin, S., K. R. Krishnan, P. A. Saravana Babu, M. Sivarajan and M. Sukumar (2014). "Functional foods enriched with marine microalga *Nannochloropsis oculata* as a source of ω-3 fatty acids." Food Technology and Biotechnology **52**(3): 292-299.

Badwy, T. M., E. Ibrahim and M. Zeinhom (2008). Partial replacement of fishmeal with dried microalga (*Chlorella spp*. and *Scenedesmus spp.*) in Nile tilapia (*Oreochromis niloticus*) diets. 8th international symposium on tilapia in aquaculture.

Baeverfjord, G. and A. Krogdahl (1996). "Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: a comparison with the intestines of fasted fish." Journal of Fish Diseases **19**(5): 375-387.

Bajpai, P. (2017). Single cell protein production from lignocellulosic biomass, Springer.

Basford, A. J. (2021). Alternative live feeds and live feed enhancements for marine larviculture, Southern Cross University.

Bell, J. (2008). Alternative lipid sources for fish feed and farmed fish quality. Improving Farmed Fish Quality and Safety, Elsevier**:** 275-299.

Bell, J. G., J. D. Castell, D. R. Tocher, F. M. MacDonald and J. R. Sargent (1995). "Effects of different dietary arachidonic acid: docosahexaenoic acid ratios on phospholipid fatty acid compositions and prostaglandin production in juvenile turbot (*Scophthalmus maximus*)." Fish Physiology and Biochemistry **14**: 139-151.

Bell, J. G. and J. R. Sargent (2003). "Arachidonic acid in aquaculture feeds: current status and future opportunities." Aquaculture **218**(1-4): 491-499.

Benedito-Palos, L., J. Gordon Bell, S. Kaushik, J. C. Navarro, J. Pérez-Sánchez and A. Sitjà-Bobadilla (2008). "High levels of vegetable oils in plant protein-rich diets fed to gilthead sea bream (*Sparus aurata* L.): growth performance, muscle fatty acid profiles and histological alterations of target tissues." British Journal of Nutrition **100**(5): 992-1003.

Benitez, L. V. (1989). "Amino acid and fatty acid profiles in aquaculture nutrition studies." Fish nutrition research in asia: 23-35.

Bonaldo, A., K. Thompson, A. Manfrin, A. Adams, E. Murano, A. L. Mordenti and P. P. Gatta (2007). "The influence of dietary ß-glucans on the adaptive and innate immune responses of European sea bass (*Dicentrarchus labrax*) vaccinated against vibriosis." Italian Journal of Animal Science **6**(2): 151-164.

Bragadóttir, M., H. Pálmadóttir and K. Kristbergsson (2004). "Composition and Chemical Changes during Storage of Fish Meal from Capelin (*Mallotus villosus*)." Journal of Agricultural and Food Chemistry **52**(6): 1572-1580.

Bridle, A., C. Carter, R. Morrison and B. Nowak (2005). "The effect of β-glucan administration on macrophage respiratory burst activity and Atlantic salmon, *Salmo salar L.*, challenged with amoebic gill disease–evidence of inherent resistance." Journal of Fish Diseases **28**(6): 347-356.

Carballo, C., P. I. S. Pinto, A. P. Mateus, C. Berbel, C. C. Guerreiro, J. F. Martinez-Blanch, F. M. Codoñer, L. Mantecon, D. M. Power and M. Manchado (2019). "Yeast β-glucans and microalgal extracts modulate the immune response and gut microbiome in Senegalese sole (*Solea senegalensis*)." Fish & Shellfish Immunology **92**: 31-39.

Carneiro, W. F., T. F. D. Castro, T. M. Orlando, F. Meurer, D. A. d. J. Paula, B. d. C. R. Virote, A. R. d. C. B. Vianna and L. D. S. Murgas (2020). "Replacing fish meal by *Chlorella sp*. meal: Effects on zebrafish growth, reproductive performance, biochemical parameters and digestive enzymes." Aquaculture **528**: 735612.

Carvalho, M., B. Marotta, H. Xu, P.-A. Geraert, S. Kaushik, D. Montero and M. Izquierdo (2022). "Complete replacement of fish oil by three microalgal products rich in n-3 long-chain polyunsaturated fatty acids in early weaning microdiets for gilthead sea bream (*Sparus aurata*)." Aquaculture **558**: 738354.

Carvalho, M., S. Torrecillas, D. Montero, A. Sanmartín, R. Fontanillas, A. Farías, K. Moutou, J. H. Velásquez and M. Izquierdo (2023). "Insect and single-cell protein meals as replacers of fish meal in low fish meal and fish oil diets for gilthead sea bream (*Sparus aurata)* juveniles." Aquaculture **566**: 739215.

Cashion, T., F. Le Manach, D. Zeller and D. Pauly (2017). "Most fish destined for fishmeal production are food-grade fish." Fish and Fisheries **18**(5): 837-844.

Chen, S.-M., K.-Y. Tseng and C.-H. Huang (2015). "Fatty acid composition, sarcoplasmic reticular lipid oxidation, and immunity of hard clam (*Meretrix lusoria*) fed different dietary microalgae." Fish & Shellfish Immunology **45**(1): 141-145.

Conceição, L. E., C. Aragão and I. Rønnestad (2010). "Protein metabolism and amino acid requirements in fish larvae." Avances en Nutrición Acuicola.

Cook, M. T., P. J. Hayball, W. Hutchinson, B. F. Nowak and J. D. Hayball (2003). "Administration of a commercial immunostimulant preparation, EcoActiva™ as a feed supplement enhances macrophage respiratory burst and the growth rate of snapper (*Pagrus auratus*, Sparidae (Bloch and Schneider)) in winter." Fish & Shellfish Immunology **14**(4): 333-345.

Cuesta, A., A. Rodríguez, I. Salinas, J. Meseguer and M. Á. Esteban (2007). "Early local and systemic innate immune responses in the teleost gilthead seabream after intraperitoneal injection of whole yeast cells." Fish & Shellfish Immunology **22**(3): 242-251.

Dahlberg, A. (2019). "Filamentous fungus Paecilomyces variotii in feed for Rainbow trout (*Oncorhynchus mykiss*)."

Dawood, M. A., S. Koshio and M. Á. Esteban (2018). "Beneficial roles of feed additives as immunostimulants in aquaculture: a review." Reviews in Aquaculture **10**(4): 950-974.

Delamare-Deboutteville, J., D. J. Batstone, M. Kawasaki, S. Stegman, M. Salini, S. Tabrett, R. Smullen, A. C. Barnes and T. Hülsen (2019). "Mixed culture purple phototrophic bacteria is an effective fishmeal replacement in aquaculture." Water Research X **4**: 100031.

Demirbas, A. (2009). "Political, economic and environmental impacts of biofuels: A review." Applied Energy **86**: S108-S117.

Divanach, P. and M. Kentouri (2000). "Hatchery techniques for specific diversification in Mediterranean finfish larviculture." Cahiers Options Méditerranéennes **47**: 75-87.

Dufossé, L., M. Fouillaud, Y. Caro, S. A. S. Mapari and N. Sutthiwong (2014). "Filamentous fungi are large-scale producers of pigments and colorants for the food industry." Current Opinion in Biotechnology **26**: 56-61.

EniferBio (2022). "The Original Mycoprotein." PEKILO® from https://enifer.com/about.

Eroldoğan, O. T., B. Glencross, L. Novoveska, S. P. Gaudêncio, B. Rinkevich, G. C. Varese, M. de Fátima Carvalho, D. Tasdemir, I. Safarik, S. L. Nielsen, C. Rebours, L. B. Lada, J. Robbens, E. Strode, B. Z. Haznedaroğlu, J. Kotta, E. Evliyaoğlu, J. Oliveira, M. Girão, M. I. Vasquez, I. Čabarkapa, S. Rakita, K. Klun and A. Rotter (2023). "From the sea to aquafeed: A perspective overview." Reviews in Aquaculture **15**(3): 1028-1057.

Eryalçin, K. M., S. Torrecillas, M. J. Caballero, C. M. Hernandez-Cruz, J. Sweetman and M. Izquierdo (2017). "Effects of dietary mannan oligosaccharides in early weaning diets on growth, survival, fatty acid composition and gut morphology of gilthead sea bream (*Sparus aurata*, L.) larvae." Aquaculture Research **48**(9): 5041-5052.

Essa, M. A., H. A. Mabrouk, R. A. Mohamed and F. R. Michael (2011). "Evaluating different additive levels of yeast, *Saccharomyces cerevisiae*, on the growth and production performances of a hybrid of two populations of Egyptian African catfish, *Clarias gariepinus*." Aquaculture **320**(1): 137-141.

Estévez, A., L. Padrell, B. Iñarra, M. Orive and D. S. Martin (2021). "Brewery by-products (yeast and spent grain) as protein sources in gilthead seabream (*Sparus aurata*) feeds." Aquaculture **543**: 736921.

FAO, F. a. A. O. o. t. U. N. (2024). "The state of world fisheries and aquaculture 2024." from https://openknowledge.fao.org/server/api/core/bitstreams/131ab804-f871-4562-bd0d-2457ebad0e47/content/cd0683en.html.

Fazenda, M. L., R. Seviour, B. McNeil and L. M. Harvey (2008). Submerged Culture Fermentation of "Higher Fungi": The Macrofungi. Advances in Applied Microbiology, Academic Press. **63:** 33-103.

Ferreira, I. M. P. L. V. O., O. Pinho, E. Vieira and J. G. Tavarela (2010). "Brewer's *Saccharomyces* yeast biomass: characteristics and potential applications." Trends in Food Science & Technology **21**(2): 77-84.

Ferreira, J. A., P. R. Lennartsson, L. Edebo and M. J. Taherzadeh (2013). "Zygomycetes-based biorefinery: Present status and future prospects." Bioresource Technology **135**: 523-532.

Folch, J., M. Lees and G. H. S. Stanley (1957). "A simple method for the isolation and purification of total lipids from animal tissues." Journal of Biological Chemistry **226**(1): 497-509.

Fronte, B., F. Abramo, F. Brambilla, M. De Zoysa and V. Miragliotta (2019). "Effect of hydrolysed fish protein and autolysed yeast as alternative nitrogen sources on gilthead sea bream (*Sparus aurata*) growth performances and gut morphology." Italian Journal of Animal Science **18**(1): 799-808.

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

Gaber, M. and M. Salem (2016). "Amino acid requirements of gilthead bream (*Sparus aurata*) juveniles." Sparus aurata.

Gamboa-Delgado, J. and J. M. Márquez-Reyes (2018). "Potential of microbial-derived nutrients for aquaculture development." Reviews in Aquaculture **10**(1): 224-246.

Gatlin III, D. M., F. T. Barrows, P. Brown, K. Dabrowski, T. G. Gaylord, R. W. Hardy, E. Herman, G. Hu, Å. Krogdahl and R. Nelson (2007). "Expanding the utilization of sustainable plant products in aquafeeds: a review." Aquaculture Research **38**(6): 551-579.

Gephart, J. A., L. Deutsch, M. L. Pace, M. Troell and D. A. Seekell (2017). "Shocks to fish production: Identification, trends, and consequences." Global Environmental Change **42**: 24-32.

Glencross, B., S. Irvin, S. Arnold, D. Blyth, N. Bourne and N. Preston (2014). "Effective use of microbial biomass products to facilitate the complete replacement of fishery resources in diets for the black tiger shrimp, *Penaeus monodon*." Aquaculture **431**: 12-19.

Glencross, B. D., M. Booth and G. L. Allan (2007). "A feed is only as good as its ingredients–a review of ingredient evaluation strategies for aquaculture feeds." Aquaculture Nutrition **13**(1): 17-34.

Goodall, J. D., N. M. Wade, D. J. Merritt, M. J. Sellars, K. Salee and G. J. Coman (2016). "The effects of adding microbial biomass to grow-out and maturation feeds on the reproductive performance of black tiger shrimp, *Penaeus monodon*." Aquaculture **450**: 206-212.

Gopalakannan, A. and V. Arul (2006). "Immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the immune system of *Cyprinus carpio* and control of *Aeromonas hydrophila* infection in ponds." Aquaculture **255**(1): 179-187.

Grisez, L., J. Reyniers, L. Verdonck, J. Swings and F. Ollevier (1997). "Dominant intestinal microflora of sea bream and sea bass larvae, from two hatcheries, during larval development." Aquaculture **155**(1): 387-399.

Gumus, E., B. Aydin and M. Kanyilmaz (2016). "Growth and feed utilization of goldfish (*Carassius auratus*) fed graded levels of brewers yeast (*Saccharomyces cerevisiae*)." Iranian Journal of Fisheries Sciences **15**(3): 1124-1133.

Guo, J., X. Qiu, G. Salze and D. A. Davis (2019). "Use of high-protein brewer's yeast products in practical diets for the Pacific white shrimp *Litopenaeus vannamei*." Aquaculture Nutrition **25**(3): 680-690.

Guselle, N., R. Markham and D. J. Speare (2007). "Timing of intraperitoneal administration of β-1, 3/1, 6 glucan to rainbow trout, *Oncorhynchus mykiss* (Walbaum), affects protection against the microsporidian *Loma salmonae*." Journal of Fish Diseases **30**(2): 111-116.

Halver, J. E. and R. W. Hardy (2003). Nutrient flow and retention. Fish nutrition, Elsevier**:** 755-770.

Hamre, K., M. Yufera, I. Rønnestad, C. Boglione, L. E. Conceição and M. Izquierdo (2013). "Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing." Reviews in Aquaculture **5**: S26-S58.

Hanif, A., V. Bakopoulos, I. Leonardos and G. J. Dimitriadis (2005). "The effect of sea bream (*Sparus aurata*) broodstock and larval vaccination on the susceptibility by Photobacterium damsela subsp. piscicida and on the humoral immune parameters." Fish & Shellfish Immunology **19**(4): 345-361.

Hardy, R. W. (2010). "Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal." Aquaculture Research **41**(5): 770-776.

Hawksworth, D. L. and R. Lücking (2017). "Fungal Diversity Revisited: 2.2 to 3.8 Million Species." Microbiology Spectrum **5**(4): 10.1128/microbiolspec.funk-0052-2016.

Ho, S.-H., C.-Y. Chen and J.-S. Chang (2012). "Effect of light intensity and nitrogen starvation on CO2 fixation and lipid/carbohydrate production of an indigenous microalga Scenedesmus obliquus CNW-N." Bioresource Technology **113**: 244-252.

Hodar, A., R. Vasava, D. Mahavadiya and N. Joshi (2020). "Fish meal and fish oil replacement for aqua feed formulation by using alternative sources: a review." Journal of Experimental Zoology India **23**(1).

Holdt, S. L. and S. Kraan (2011). "Bioactive compounds in seaweed: functional food applications and legislation." Journal of Applied Phycology **23**(3): 543-597.

Hooft, J. M., R. Montero, B. Morales-Lange, V. F. Blihovde, K. Purushothaman, C. M. Press, D. D. Mensah, J. O. Agboola, S. Javed, L. T. Mydland and M. Øverland (2024). "*Paecilomyces variotii* (PEKILO®) in novel feeds for Atlantic salmon: Effects on pellet quality, growth performance, gut health, and nutrient digestibility and utilization." Aquaculture **589**: 740905.

Hossain, M. S., B. C. Small, V. Kumar and R. Hardy (2024). "Utilization of functional feed additives to produce cost-effective, ecofriendly aquafeeds high in plant-based ingredients." Reviews in Aquaculture **16**(1): 121-153.

Huyben, D., A. Nyman, A. Vidaković, V. Passoth, R. Moccia, A. Kiessling, J. Dicksved and T. Lundh (2017). "Effects of dietary inclusion of the yeasts *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* on gut microbiota of rainbow trout." Aquaculture **473**: 528-537.

Izquierdo, M. (1989). Optimal EFA levels in Artemia to meet the EFA requirements of red seabream (*Pagrus major*). Proc. Third Int. Symp. on Feeding and Nutr. in Fish., Toba, Aug. 28-Sep. 1, Japan, 1989.

Izquierdo, M. (1996). "Essential fatty acid requirements of cultured marine fish larvae." Aquaculture Nutrition **2**(4): 183-191.

Izquierdo, M. (2004). "Nutritional requirements for finfish larvae."

Izquierdo, M. (2005). "Essential fatty acid requirements in Mediterranean fish species." Cahiers Options Méditerranéennes.

Izquierdo, M. and H. Fernandez-Palacios (1997). "Nutritional requirements of marine fish larvae and broodstock." Cah. Options Mediterr **22**: 243-264.

Izquierdo, M. and W. Koven (2011). Lipids. Larval Fish Nutrition**:** 47-81.

Jasti, N., S. K. Khanal, A. L. Pometto III and J. van Leeuwen (2008). "Converting corn wet-milling effluent into high-value fungal biomass in a biofilm reactor." Biotechnology and Bioengineering **101**(6): 1223-1233.

Javed, S. (2023). Filamentous fungi (*Paecilomyces variotii*) as an alternative sustainable ingredient in diets for Atlantic salmon (*Salmo salar*): effects on pellet quality, growth performance, nutrient digestibility and utilization, and immune-related biomarkers in the distal intestine during the freshwater phase, Norwegian University of Life Sciences.

Jones, S. W., A. Karpol, S. Friedman, B. T. Maru and B. P. Tracy (2020). "Recent advances in single cell protein use as a feed ingredient in aquaculture." Current Opinion in Biotechnology **61**: 189-197.

Jung-Schroers, V., M. Adamek, A. Jung, S. Harris, Ö.-S. Dóza, A. Baumer and D. Steinhagen (2016). "Feeding of β-1,3/1,6-glucan increases the diversity of the intestinal microflora of carp (*Cyprinus carpio*)." Aquaculture Nutrition **22**(5): 1026-1039.

Karimi, S., N. Mahboobi Soofiani, T. Lundh, A. Mahboubi, A. Kiessling and M. J. Taherzadeh (2019). "Evaluation of Filamentous Fungal Biomass Cultivated on Vinasse as an Alternative Nutrient Source of Fish Feed: Protein, Lipid, and Mineral Composition." Fermentation **5**(4): 99.

Kaushik, S. J. and I. Seiliez (2010). "Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs." Aquaculture Research **41**(3): 322-332.

Kesarcodi-Watson, A., H. Kaspar, M. J. Lategan and L. Gibson (2008). "Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes." Aquaculture **274**(1): 1-14.

Klis, F. M., P. Mol, K. Hellingwerf and S. Brul (2002). "Dynamics of cell wall structure in *Saccharomyces cerevisiae*." FEMS Microbiology Reviews **26**(3): 239-256.

Krogdahl, Å., M. Penn, J. Thorsen, S. Refstie and A. M. Bakke (2010). "Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids." Aquaculture Research **41**(3): 333-344.

Lenas, D. S., D. J. Triantafillou, S. Chatziantoniou and C. Nathanailides (2011). "Fatty acid profile of wild and farmed gilthead sea bream (*Sparus aurata*)." Journal für Verbraucherschutz und Lebensmittelsicherheit **6**(4): 435-440.

Lennartsson, P. R. (2012). Zygomycetes and cellulose residuals: hydrolysis, cultivation and applications, Chalmers Tekniska Hogskola (Sweden).

Marchi, A., A. Bonaldo, D. Scicchitano, M. Candela, A. De Marco, S. Falciglia, M. Mazzoni, G. Lattanzio, P. Clavenzani, F. Dondi, P. P. Gatta and L. Parma (2023). "Feeding gilthead sea bream with increasing dietary bacterial single cell protein level: Implication on growth, plasma biochemistry, gut histology, and gut microbiota." Aquaculture **565**: 739132.

Matassa, S., N. Boon, I. Pikaar and W. Verstraete (2016). "Microbial protein: future sustainable food supply route with low environmental footprint." Microbial Biotechnology **9**(5): 568-575.

Mensah, D. D., B. Morales-Lange, M. Øverland, K. Baruah and L. T. Mydland (2024). "Differential expression of immune-related biomarkers in primary cultures from Atlantic salmon (*Salmo salar*) exposed to processed *Paecilomyces variotii* with or without inactivated *Moritella viscosa*." Fish & Shellfish Immunology **148**: 109506.

Mhalhel, K., M. Levanti, F. Abbate, R. Laurà, M. C. Guerrera, M. Aragona, C. Porcino, M. Briglia, A. Germanà and G. Montalbano (2023). "Review on Gilthead Seabream (*Sparus aurata*) Aquaculture: Life Cycle, Growth, Aquaculture Practices and Challenges." Journal of Marine Science and Engineering **11**(10): 2008.

Moheimani, N. R., M. A. Borowitzka, A. Isdepsky and S. F. Sing (2012). Standard methods for measuring growth of algae and their composition. Algae for biofuels and energy, Springer**:** 265-284.

Moretti, A., M. P. Fernandez-Criado and R. Betillart (2005). "Manual on hatchery production of Seabass and Gilthead Seabream: Volume 2." Food & Agriculture Org. **2**: 6-15.

Murray, A. P. and R. Marchant (1986). "Nitrogen utilization in rainbow trout fingerlings (*Salmo gairdneri Richardson*) fed mixed microbial biomass." Aquaculture **54**(4): 263-275.

Nalage, D., G. Khedkar, A. Kalyankar, A. Sarkate, S. Ghodke, V. Bedre and C. Khedkar (2016). "Single cell proteins." Encyclopedia of food and health **4**: 790-794.

Nasseri, A., S. Rasoul-Amini, M. Morowvat and Y. Ghasemi (2011). "Single cell protein: production and process." American Journal of food technology **6**(2): 103-116.

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

Nguyen, T. H., G. H. Fleet and P. L. Rogers (1998). "Composition of the cell walls of several yeast species." Applied Microbiology and Biotechnology **50**(2): 206-212.

Nimrichter, L., M. L. Rodrigues, E. G. Rodrigues and L. R. Travassos (2005). "The multitude of targets for the immune system and drug therapy in the fungal cell wall." Microbes and Infection **7**(4): 789-798.

Nitayavardhana, S., K. Issarapayup, P. Pavasant and S. K. Khanal (2013). "Production of protein-rich fungal biomass in an airlift bioreactor using vinasse as substrate." Bioresource Technology **133**: 301-306.

Oliva-Teles, A., P. Enes and H. Peres (2015). Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish. Feed and Feeding Practices in Aquaculture. D. A. Davis. Oxford, Woodhead Publishing**:** 203-233.

Oliva-Teles, A. and P. Gonçalves (2001). "Partial replacement of fishmeal by brewers yeast (*Saccaromyces cerevisae*) in diets for sea bass (*Dicentrarchus labrax*) juveniles." Aquaculture **202**(3): 269-278.

Oliva-Teles, A., M. Guedes, C. Vachot and S. Kaushik (2006). "The effect of nucleic acids on growth, ureagenesis and nitrogen excretion of gilthead sea bream *Sparus aurata* juveniles." Aquaculture **253**(1-4): 608-617.

Olsen, R. E., B. Tore Dragnes, R. Myklebust and E. Ringø (2003). "Effect of soybean oil and soybean lecithin on intestinal lipid composition and lipid droplet accumulation of rainbow trout, *Oncorhynchus mykiss* Walbaum." Fish Physiology and Biochemistry **29**(3): 181-192.

Opstvedt, J., E. Nygård, T. A. Samuelsen, G. Venturini, U. Luzzana and H. Mundheim (2003). "Effect on protein digestibility of different processing conditions in the production of fish meal and fish feed." Journal of the Science of Food and Agriculture **83**(8): 775-782.

Øverland, M., A. Karlsson, L. T. Mydland, O. H. Romarheim and A. Skrede (2013). "Evaluation of *Candida utilis*, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* yeasts as protein sources in diets for Atlantic salmon (*Salmo salar*)." Aquaculture **402-403**: 1-7.

Øverland, M. and A. Skrede (2017). "Yeast derived from lignocellulosic biomass as a sustainable feed resource for use in aquaculture." Journal of the Science of Food and Agriculture **97**(3): 733-742.

Øverland, M., A.-H. Tauson, K. Shearer and A. Skrede (2010). "Evaluation of methane-utilising bacteria products as feed ingredients for monogastric animals." Archives of Animal Nutrition **64**(3): 171-189.

Pancha, I., K. Chokshi, T. Ghosh, C. Paliwal, R. Maurya and S. Mishra (2015). "Bicarbonate supplementation enhanced biofuel production potential as well as nutritional stress mitigation in the microalgae *Scenedesmus sp.* ." Bioresource Technology **193**: 315-323.

Pandey, A. (2003). "Solid-state fermentation." Biochemical Engineering Journal **13**(2): 81-84.

Pandey, A., C. R. Soccol and C. Larroche (2008). Current developments in solid-state fermentation, Springer Science & Business Media.

Pedneault, K., P. Angers, A. Gosselin and R. J. Tweddell (2008). "Fatty acid profiles of polar and neutral lipids of ten species of higher basidiomycetes indigenous to eastern Canada." Mycological Research **112**(12): 1428-1434.

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

Pogue, R., E. J. Murphy, G. W. Fehrenbach, E. Rezoagli and N. J. Rowan (2021). "Exploiting immunomodulatory properties of β-glucans derived from natural products for improving health and sustainability in aquaculture-farmed organisms: Concise review of existing knowledge, innovation and future opportunities." Current Opinion in Environmental Science & Health **21**: 100248.

Ramesh, C., V. R. Prasastha, M. Venkatachalam and L. Dufossé (2022). "Natural Substrates and Culture Conditions to Produce Pigments from Potential Microbes in Submerged Fermentation." Fermentation **8**(9): 460.

Rawling, M. D., N. Pontefract, A. Rodiles, I. Anagnostara, E. Leclercq, M. Schiavone, M. Castex and D. L. Merrifield (2019). "The effect of feeding a novel multistrain yeast fraction on European seabass (*Dicentrachus labrax*) intestinal health and growth performance." Journal of the World Aquaculture Society **50**(6): 1108-1122.

Reis, B., L. Ramos-Pinto, J. A. Martos-Sitcha, M. Machado, R. Azeredo, S. Fernández-Boo, S. Engrola, C. Unamunzaga, J. Calduch-Giner and L. E. Conceição (2021). "Health status in gilthead seabream (*Sparus aurata*) juveniles fed diets devoid of fishmeal and supplemented with *Phaeodactylum tricornutum*." Journal of Applied Phycology **33**: 979-996.

Richmond, A. (2004). Handbook of microalgal culture: biotechnology and applied phycology, Wiley Online Library.

Rimoldi, S., E. Gini, J. F. A. Koch, F. Iannini, F. Brambilla and G. Terova (2020). "Effects of hydrolyzed fish protein and autolyzed yeast as substitutes of fishmeal in the gilthead sea bream (*Sparus aurata*) diet, on fish intestinal microbiome." BMC Veterinary Research **16**(1): 118.

Ringø, E., R. E. Olsen, T. Ø. Gifstad, R. A. Dalmo, H. Amlund, G.-I. Hemre and A. M. Bakke (2010). "Prebiotics in aquaculture: a review." Aquaculture Nutrition **16**(2): 117-136.

Ringø, E., R. E. Olsen, J. G. Vecino, S. Wadsworth and S. Song (2012). "Use of immunostimulants and nucleotides in aquaculture: a review." J Mar Sci Res Dev **2**(1): 104.

Robertsen, B., G. Rθrstad, R. Engstad and J. Raa (1990). "Enhancement of non-specific disease resistance in Atlantic salmon, *Salmo salar L*., by a glucan from *Saccharomyces cerevisiae* cell walls." Journal of Fish Diseases **13**(5): 391-400.

Rodriguez, C., J. A. Perez, M. S. Izquierdo, J. Mora, A. Lorenzo and H. Fernandez-Palacios (1994). "Essential fatty acid requirements of larval gilthead sea bream, *Sparus aurata* (L.)." Aquaculture Research **25**(3): 295-304.

Rønnestad, I., M. Yufera, B. Ueberschär, L. Ribeiro, Ø. Sæle and C. Boglione (2013). "Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research." Reviews in Aquaculture **5**: S59-S98.

Rossi, W. and D. A. Davis (2012). "Replacement of fishmeal with poultry by-product meal in the diet of Florida pompano *Trachinotus carolinus* L." Aquaculture **338-341**: 160-166.

Rumsey, G. L., S. G. Hughes and J. L. Kinsella (1990). "Use of Dietary Yeast *Saccharomyces cerevisiae* Nitrogen by Lake Trout." Journal of the World Aquaculture Society **21**(3): 205-209.

Sajina, K., S. Krupesha Sharma, T. Sumithra, G. Tamilmani, M. Saktivel, K. Anikuttan and A. Gopalakrishnan (2022). "Nutrigenomics tools to address the emerging issues in marine fish larviculture." Advances in Agricultural, Animal and Fisheries Sciences: 20.

Sawanboonchun, J., W. J. Roy, D. A. Robertson and J. G. Bell (2008). "The impact of dietary supplementation with astaxanthin on egg quality in Atlantic cod broodstock (*Gadus morhua*, L.)." Aquaculture **283**(1): 97-101.

Schiavone, M., A. Vax, C. Formosa, H. Martin-Yken, E. Dague and J. M. François (2014). "A combined chemical and enzymatic method to determine quantitatively the polysaccharide components in the cell wall of yeasts." FEMS Yeast Research **14**(6): 933-947.

Seong, M., S. Lee, S. Lee, Y. Song, J. Bae, K. Chang and S. C. Bai (2018). "The effects of different levels of dietary fermented plant-based protein concentrate on growth, hematology and non-specific immune responses in juvenile olive flounder, *Paralichthys olivaceus*." Aquaculture **483**: 196-202.

Shuvaeva, G. P. and M. G. Sysoeva (2010). "Xylanase of the micromycete Rhizopus var. microsporus 595: Preparation, structural and functional characteristics, and application." Applied Biochemistry and Microbiology **46**(6): 641-647.

Singh, O. V., N. Kapur and R. P. Singh (2005). "Evaluation of agro-food byproducts for gluconic acid production by *Aspergillus niger* " World Journal of Microbiology and Biotechnology **21**(4): 519-524.

Siwicki, A. K., K. Kazuń, E. Głąbski, E. Terech-Majewska, P. Baranowski and S. Trapkowska (2004). "The effect of beta-1.3/1.6-glucan in diets on the effectiveness of anti-Yersinia ruckeri vaccine-an experimental study in rainbow trout (*Oncorhynchus mykiss*)." Polish journal of food and nutrition sciences **13**(Suppl. 2): 59-61.

Stahl, P. D. and M. J. Klug (1996). "Characterization and differentiation of filamentous fungi based on Fatty Acid composition." Applied and Environmental Microbiology **62**(11): 4136-4146.

Staykov, Y., P. Spring, S. Denev and J. Sweetman (2007). "Effect of a mannan oligosaccharide on the growth performance and immune status of rainbow trout (*Oncorhynchus mykiss*)." Aquaculture International **15**(2): 153-161.

Steglich, W. (1981). "Biologically active compounds from higher fungi." Pure and Applied Chemistry **53**(6): 1233-1240.

Storebakken, T. and U. C. Goswami (1996). "Plasma carotenoid concentration indicates the availability of dietary astaxanthin for Atlantic salmon, *Salmo salar*." Aquaculture **146**(1): 147-153.

Sues, A., R. Millati, L. Edebo and M. J. Taherzadeh (2005). "Ethanol production from hexoses, pentoses, and dilute-acid hydrolyzate by *Mucor indicus*." FEMS Yeast Research **5**(6-7): 669-676.

Svåsand, T., D. Crosetti, E. García-Vázquez and E. Verspoor (2007). "Genetic impact of aduaculture activities on native populations." Genimpact final scientific report (EU contract n. RICA-CT-2005-022802).

Tachibana, K., M. Yagi, K. Hara, T. Mishima and M. Tsuchimoto (1997). "Effects of feeding of β-carotene-supplemented rotifers on survival and lymphocyte proliferation reaction of fish larvae (Japanese parrotfish (*Oplegnathus fasciatus*) and Spotted parrotfish (*Oplegnathus punctatus*): preliminary trials." Hydrobiologia **358**(1): 313-316.

Tacon, A. G. and M. Metian (2008). "Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects." Aquaculture **285**(1-4): 146-158.

Taherzadeh, M. J., M. Fox, H. Hjorth and L. Edebo (2003). "Production of mycelium biomass and ethanol from paper pulp sulfite liquor by *Rhizopus oryzae*." Bioresource Technology **88**(3): 167-177.

Tampou, A., S. Andreopoulou, A. Vasilaki, I. Nengas, P. Berillis, E. Gisbert, I. T. Karapanagiotidis, E. Antonopoulou and E. Mente (2024). "Growth performance of gilthead sea bream (*Sparus aurata*) fed a mixture of single cell ingredients for organic diets." Aquaculture Reports **36**: 102105.

Terabayashi, Y., M. Sano, N. Yamane, J. Marui, K. Tamano, J. Sagara, M. Dohmoto, K. Oda, E. Ohshima, K. Tachibana, Y. Higa, S. Ohashi, H. Koike and M. Machida (2010). "Identification and characterization of genes responsible for biosynthesis of kojic acid, an industrially important compound from *Aspergillus oryzae*." Fungal Genetics and Biology **47**(12): 953-961.

Tibaldi, E., G. Chini Zittelli, G. Parisi, M. Bruno, G. Giorgi, F. Tulli, S. Venturini, M. R. Tredici and B. M. Poli (2015). "Growth performance and quality traits of European sea bass (*D. labrax*) fed diets including increasing levels of freeze-dried Isochrysis sp. (T-ISO) biomass as a source of protein and n-3 long chain PUFA in partial substitution of fish derivatives." Aquaculture **440**: 60-68.

Tocher, D. R. (2010). "Fatty acid requirements in ontogeny of marine and freshwater fish." Aquaculture Research **41**(5): 717-732.

Tocher, D. R. (2015). "Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective." Aquaculture **449**: 94-107.

Toledo Marante, F. J., R. Mioso, J. Bermejo Barrera, J. E. González González, J. J. Santana Rodríguez and I. H. Bravo de Laguna (2012). "Structural characterization and metabolite profiling of the facultative marine fungus *Paecilomyces variotii*." Annals of Microbiology **62**(4): 1601-1607.

Torrecillas, S., A. Makol, M. J. Caballero, D. Montero, A. K. S. Dhanasiri, J. Sweetman and M. Izquierdo (2012). "Effects on mortality and stress response in European sea bass, *Dicentrarchus labrax* (L.), fed mannan oligosaccharides (MOS) after *Vibrio anguillarum* exposure." Journal of Fish Diseases **35**(8): 591-602.

Torrecillas, S., D. Montero and M. Izquierdo (2014). "Improved health and growth of fish fed mannan oligosaccharides: potential mode of action." Fish & Shellfish Immunology **36**(2): 525-544.

Torrissen, O. J., R. W. Hardy, K. D. Shearer, T. M. Scott and F. E. Stone (1990). "Effects of dietary canthaxanthin level and lipid level on apparent digestibility coefficients for canthaxanthin in rainbow trout (*Oncorhynchus mykiss*)." Aquaculture **88**(3): 351-362.

Troell, M., R. L. Naylor, M. Metian, M. Beveridge, P. H. Tyedmers, C. Folke, K. J. Arrow, S. Barrett, A.-S. Crépin, P. R. Ehrlich, Å. Gren, N. Kautsky, S. A. Levin, K. Nyborg, H. Österblom, S. Polasky, M. Scheffer, B. H. Walker, T. Xepapadeas and A. de Zeeuw (2014). "Does aquaculture add resilience to the global food system?" Proceedings of the National Academy of Sciences **111**(37): 13257-13263.

Tseng, Y., K. M. Eryalçın, U. Sivagurunathan, D. Domínguez, C. M. Hernández-Cruz, C. Boglione, A. J. P. Philip and M. Izquierdo (2023). "Effects of the dietary supplementation of copper on growth, oxidative stress, fatty acid profile and skeletal development in gilthead seabream (*Sparus aurata*) larvae." Aquaculture **568**: 739319.

Turchini, G. M., W.-K. Ng and D. R. Tocher (2010). Fish oil replacement and alternative lipid sources in aquaculture feeds, CRC Press.

Urán, P. A., J. W. Schrama, S. Jaafari, G. Baardsen, J. H. W. M. Rombout, W. Koppe and J. A. J. Verreth (2009). "Variation in commercial sources of soybean meal influences the severity of enteritis in Atlantic salmon (*Salmo salar* L.)." Aquaculture Nutrition **15**(5): 492-499.

Van Leeuwen, J., S. K. Khanal, A. L. Pometto, M. L. Rasmussen and D. Mitra (2013). Fungi cultivation on alcohol fermentation stillage for useful products and energy savings, Google Patents.

Velíšek, J. and K. Cejpek (2011). "Pigments of higher fungi-A review."

Velmurugan, P., S. Kamala-Kannan, V. Balachandar, P. Lakshmanaperumalsamy, J.-C. Chae and B.-T. Oh (2010). "Natural pigment extraction from five filamentous fungi for industrial applications and dyeing of leather." Carbohydrate Polymers **79**(2): 262-268.

Vidakovic, A., D. Huyben, H. Sundh, A. Nyman, J. Vielma, V. Passoth, A. Kiessling and T. Lundh (2020). "Growth performance, nutrient digestibility and intestinal morphology of rainbow trout (*Oncorhynchus mykiss*) fed graded levels of the yeasts *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus*." Aquaculture Nutrition **26**(2): 275-286.

Vidakovic, A., M. Langeland, H. Sundh, K. Sundell, M. Olstorpe, J. Vielma, A. Kiessling and T. Lundh (2016). "Evaluation of growth performance and intestinal barrier function in Arctic Charr (*Salvelinus alpinus*) fed yeast (*Saccharomyces cerevisiae*), fungi (*Rhizopus oryzae*) and blue mussel (*Mytilus edulis*)." Aquaculture Nutrition **22**(6): 1348-1360.

Volman, J. J., J. D. Ramakers and J. Plat (2008). "Dietary modulation of immune function by β-glucans." Physiology & behavior **94**(2): 276-284.

Yamada, E. A. and V. C. Sgarbieri (2005). "Yeast (*Saccharomyces cerevisiae*) Protein Concentrate: Preparation, Chemical Composition, and Nutritional and Functional Properties." Journal of Agricultural and Food Chemistry **53**(10): 3931-3936.