

Novel grape functional extracts to prevent bacterial infections in European seabass aquaculture

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Relatório de estágio

Mestrado em Biologia e Gestão da Qualidade da Água

Departamento de Biologia

2023

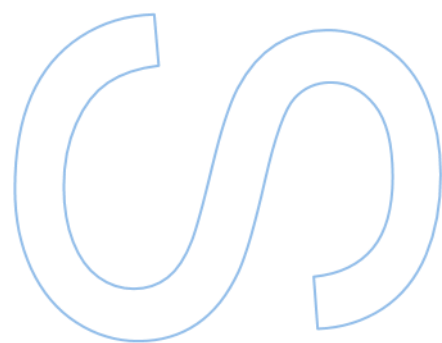
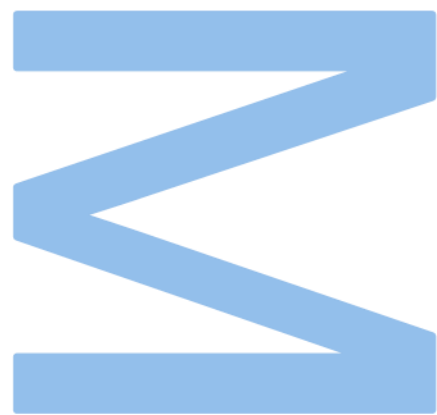
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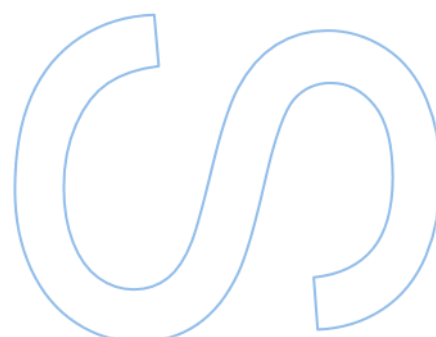
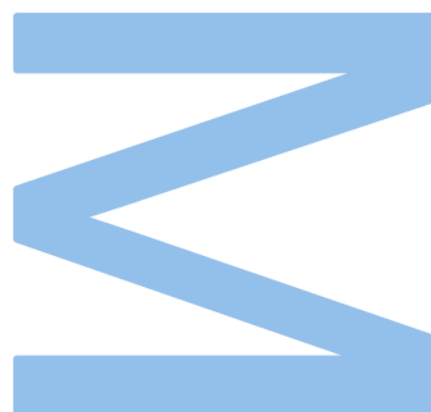
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This internship report was performed under the European project NeoGiANT financed by the European Union's Horizon 2020 Research and Innovation program under grant agreement NO 101036768.



Acknowledgements

First of all, I would like to thank the Faculty of Sciences of the University of Porto (FCUP) for the opportunity to pursue a master's degree in Biology and Water Quality Management. Also, I would like to thank the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) for welcoming me and the opportunity to work in their facilities.

Special thanks:

To my supervisor, Dr. Cláudia Serra for the great opportunity that she gave me by accepting me as her master's student and for all the help and support.

To my co-supervisors, Dr. Rafaela Santos and Dr. Rui Magalhães for their invaluable guidance, availability, and patience. For sharing their experience and knowledge which were essential for the successful completion of this report.

To Professor José Américo Sousa for sharing his knowledge in the field of fish diseases and for mentioning Dr. Cláudia Serra as a potential supervisor.

To Ricardo and Lúcia for always being ready to help me when I needed it.

To my friends Pedro A., Inês, António, Henrique, Cheyenne and Pedro L. for the friendship, motivation, and for all the moments we had together.

To my family:

Firstly, to my parents, Maria da Luz e Paulo Sousa, for all their love, support, patience and encouragement to pursue my dreams.

To my brother, Tiago Monteiro, for his support and help when I needed the most.

To my boyfriend, André Almeida, for being there for me when I was lost, and for all the love, support and patience throughout this journey.

The accomplishment of this internship report would not be possible without all of you.

Resumo

A população mundial tem vindo a aumentar ao longo dos anos provocando um aumento da procura de alimento, particularmente fontes proteicas como o peixe. Devido a este aumento, a aquacultura tem-se tornado cada vez mais intensiva o que levou ao aparecimento de inúmeras doenças nos peixes como, por exemplo, doenças bacterianas. O método utilizado durante muitos anos para prevenir e tratar as infeções bacterianas foi a utilização de grandes quantidades de antibióticos, agravando a problemática da resistência aos antimicrobianos entre os agentes patogénicos de peixes. Nos últimos anos, têm emergido novas medidas de prevenção de doenças bacterianas e uma das alternativas promissoras é a utilização de extratos funcionais.

Sabendo das propriedades bioativas dos extratos funcionais o objetivo deste estudo era descobrir se extratos de bagaço de uva branca podem ser utilizados em rações de aquacultura para robalo Europeu (*Dicentrarchus labrax*) com o intuito de prevenir o aparecimento de doenças bacterianas. Para tal, a atividade antimicrobiana dos extratos água100%/acetona volatilizada (AC), etanol/água (50%/50%) (ET) e propileno glicol/água (50%/50%) (PG) foi inicialmente avaliada, *in vitro*, contra diferentes espécies bacterianas patogénicas para peixes (*A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *P. aeruginosa*, *S. aureus*, *E. tarda*, *S. sonnei*, *Ph. damsela* subsp. *damsela*, *Ph. damsela* subsp. *piscicida*, *Y. ruckeri*, *T. maritimum*, *S. iniae*, *S. parauberis* e *S. agalactiae*) usando o método por difusão em agar. Verificou-se que os extratos de bagaço de uva apresentam atividade antimicrobiana contra as bactérias *V. harveyi*, *V. vulnificus*, *Ph. damsela* subsp. *damsela*, *Ph. damsela* subsp. *piscicida*, *T. maritimum* e *S. agalactiae*. De seguida, os extratos AC, ET e PG foram incorporados em dietas para robalo em três concentrações diferentes de polifenóis (45, 200 e 450 ppm), perfazendo um total de 10 dietas experimentais: uma dieta controlo (semelhante a uma ração comercial) e nove dietas suplementadas. Estas foram usadas para alimentar juvenis de robalo, com 23,3g de peso médio inicial, durante 42 dias. Após esse período, e com o intuito de avaliar se as dietas experimentais melhoram a sobrevivência dos animais quando sujeitos a desafios bacterianos, os robalos foram infetados com 100 µL de 3×10^8 CFUs de *Photobacterium damsela* subsp. *piscicida* mL⁻¹ e, passados 7 dias foram novamente infetados com 100 µL de 1×10^8 CFUs de *Vibrio anguillarum* mL⁻¹. No ensaio *in vivo* não se observaram diferenças significativas entre as dietas suplementadas com extratos e a dieta de controlo no crescimento e na utilização de

alimento. No desafio bacteriano, os extratos de subprodutos de uva não conseguiram melhorar significativamente a sobrevivência dos peixes contra a infeção conjunta de *Ph. damsela* subsp. *piscicida* e *V. anguillarum* apesar de se terem observado valores de sobrevivência mais elevados em animais alimentados com as dietas PG suplementadas com 45 e 200 ppm de polifenóis.

Concluindo, os extratos AC, ET e PG testados neste estudo nas concentrações de 45, 200 e 450 ppm de polifenóis, não tiveram efeito negativo na performance dos animais, não afetando o crescimento, a ingestão e utilização do alimento, mas também não foram capazes de melhorar a taxa de sobrevivência dos juvenis de robalo quando infetados com *Ph. damsela* subsp. *piscicida* e *V. anguillarum*. Apesar dos extratos funcionais de uva testados apresentarem propriedades antimicrobianas capazes de inibir o crescimento dos organismos patogénicos alvo, é necessário um estudo mais aprofundado das concentrações a utilizar em rações assim como aferir a sobrevivência dos animais quando desafiados com outras espécies bacterianas.

Palavras-chave: Aquacultura, Robalo Europeu (*Dicentrarchus labrax*), Subprodutos de uva, Extratos funcionais, Polifenóis, Doenças bacterianas

Abstract

The world's population has been increasing over the years, leading to a higher search for food, particularly protein sources like fish. Due to this increase, aquaculture has become more intensive, resulting in the emergence of numerous diseases in fish such as bacterial diseases. For many years, the method used to prevent and treat bacterial infections was the use of large quantities of antibiotics, aggravating the problematics of antimicrobial resistance among fish pathogens. In recent years, new measures to prevent bacterial diseases have emerged, and one of the promising alternatives is the use of functional extracts.

Given the bioactive properties of functional extracts, the goal of this study was to determine whether extracts from white grape marc (or pomace) could be used in European seabass (*Dicentrarchus labrax*) aquaculture feeds in order to prevent the emergence of bacterial diseases. To do this, the antimicrobial activity of water100%/volatilised acetone (AC), ethanol/water (50%/50%) (ET), and propylene glycol/water (50%/50%) (PG) extracts was evaluated, *in vitro*, against different bacterial species pathogenic for fish (*A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *P. aeruginosa*, *S. aureus*, *E. tarda*, *S. sonnei*, *Ph. damsela* subsp. *damsela*, *Ph. damsela* subsp. *piscicida*, *Y. ruckeri*, *T. maritimum*, *S. iniae*, *S. parauberis* and *S. agalactiae*) using the well diffusion assay method. Grape marc extracts exhibited antimicrobial activity against the bacteria *V. harveyi*, *V. vulnificus*, *Ph. damsela* subsp. *damsela*, *Ph. damsela* subsp. *piscicida*, *T. maritimum*, and *S. agalactiae*. Subsequently, AC, ET and PG extracts were incorporated into seabass diets at three different concentrations of polyphenols (45, 200 and 450 ppm), making a total of 10 experimental diets: a control diet (similar to a commercial feed) and nine supplemented diets. These were used to feed seabass juveniles with an average initial weight of 23.3g for 42 days. After this period, and to evaluate whether experimental diets improve the survival of the animals when subjected to bacterial challenges, fish were infected with 100 µL of 3×10^8 CFUs of *Photobacterium damsela* subsp. *piscicida* mL⁻¹ and, after 7 days they were infected again with 100 µL of 1×10^8 CFUs of *Vibrio anguillarum* mL⁻¹. In the *in vivo* trial, no significant differences were observed between the diets supplemented with extracts and the control diet in terms of growth and feed utilization. In the bacterial challenge, grape by-product extracts did not significantly improve fish survival against the joint infection of *Ph. damsela*

subsp. *piscicida* and *V. anguillarum*, although higher survival rates were observed in animals fed PG diets supplemented with 45 and 200 ppm of polyphenols.

In conclusion, the AC, ET and PG extracts tested in this study at concentrations of 45, 200 and 450 ppm of polyphenols, did not have a negative effect on animal performance, not affecting growth, feed intake, or utilization, but were also unable to improve the survival rate of seabass juveniles when infected with *Ph. damsela* subsp. *piscicida* and *V. anguillarum*. Although functional grape extracts have antimicrobial properties capable of inhibiting the growth of target pathogenic organisms, further studies are needed to determine the ideal concentrations to be used in feeds and to assess the survival of animals when challenged with other bacterial species.

Keywords: Aquaculture, European seabass (*Dicentrarchus labrax*), Grape by-products, Functional extracts, Polyphenols, Bacterial diseases

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List of Abbreviations

AC: Grape marc extract water100%/volatilised acetone
BHI: Brain Heart Infusion medium
CFUs: Colony Forming Units
CIIMAR: Interdisciplinary Centre of Marine and Environmental Research
CTR: Control diet
DGI: Daily Growth Index
dpi: Days post-infection
ET: Grape marc extract ethanol/water (50%/50%)
EU: European Union
EUMOFA: European Market Observatory for Fisheries and Aquaculture
EUR: Euro
FAO: Food and Agriculture Organization of the United Nations
FE: Feed Efficiency
FI: Feed Intake
g: Grams
h: Hours
hpi: Hours post-infection
kg: Kilograms
L: Litres
mg: Milligrams
mm: Millimetres
mL: Millilitres
MT: Million Tonnes
OD: Optical Density
PBS: Phosphate-buffered saline
ppm: Parts per million
PG: Grape marc extract propylene glycol/water (50%/50%)
Rpm: Rotation per minute
WHO: World Health Organization
µL: Microlitres

1. Introduction

1.1. Aquaculture

Aquaculture is the production of freshwater or saltwater organisms, such as crustaceans, fish, molluscs, and plants (EUMOFA 2023a). This sector has been recognised for its essential contribution to global food production, security and human population nutrition in the twenty-first century. Aquaculture holds great significance due to the increasing human population (1.6% per year from 1961 to 2019) and capture fisheries alone cannot meet the high demand for seafood (Figure 1).

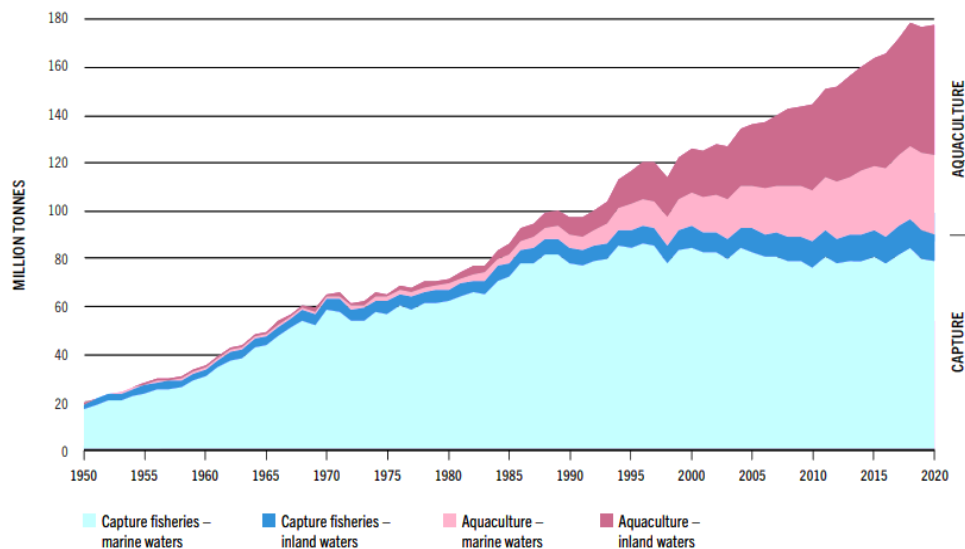


Figure 1. World Capture Fisheries and Aquaculture Production. (Note: Aquatic mammals, reptiles and algae are not included in this data). Source: FAO (2022)

The global consumption of aquatic foods grew about 3.0% each year from 1961 to 2019. The per capita consumption of aquatic animal foods grew from 9.0 kg in 1961 to 20.2 kg in 2020 (Figure 2). In 2020, aquaculture contributed 88 million tonnes (MT) to the global production of aquatic animals. Additionally, in the same year, this industry represented 56% of the available aquatic animal food production for human consumption (FAO 2022).

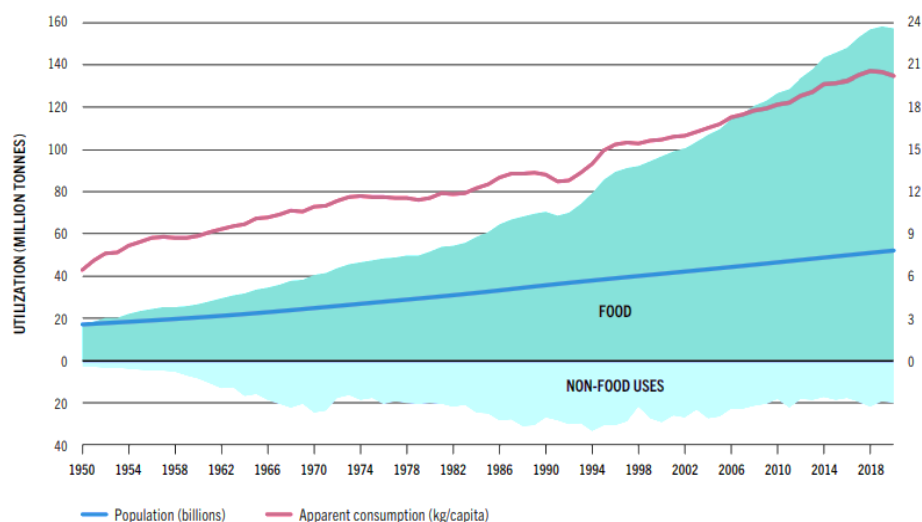


Figure 2. World Fisheries and Aquaculture Production: Utilization and Apparent Consumption. (Note: Aquatic mammals, reptiles and algae are not included in this data). Source: FAO (2022)

In the European Union (EU), fish is an important food resource and in 2021 its average consumption per capita was 24.16 kg showing a 0.5% increase from 2020. However, over 70% of this consumption is provided from imported products (EUMOFA 2022; European Commission 2021). In 2020, EU fisheries and aquaculture production was approximately 5 MT, making the European Union the sixth producer worldwide. EU aquaculture represented 33% of the European production (EUMOFA 2022). The majority of EU production is concentrated in four Mediterranean countries (Spain, France, Italy and Greece). Trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), seabream (*Sparus aurata*), European seabass, turbot (*Scophthalmus maximus*) and sole (*Solea solea*) are the most produced fish in the EU. In Portugal, some species, such as turbot, seabream and European seabass present a high economic value and their production is increasing. In particular, the seabass price in 2021 was 11% higher than in 2020, being 7.42 EUR kg⁻¹ (EUMOFA 2022; EUMOFA 2023a; European Commission 2021).

1.1.1. European seabass (*Dicentrarchus labrax*, Linnaeus, 1758)

European seabass belongs to the Actinopterygii class, Perciformes order and Moronidae family. This marine species presents a silvery grey elongated body with blue reflections, has two separate dorsal fins (one with 8-10 spines and the other with 12-13 soft rays), an anal fin with 3 spines and 10-12 soft rays, a lateral line with 62-74 scales and a caudal fin slightly forked (FAO 2023a; Fishbase 2023).

Seabass are euryhaline (3-35‰) and eurythermal (5-28°C) and inhabit coastal waters, estuaries, lagoons and occasionally rivers. This species is carnivorous, and it's found in the eastern Atlantic (Norway to Morocco, the Canary Islands and Senegal), the Mediterranean and the Black Sea (FAO 2023a; FAO 2023b; Fishbase 2023).

European seabass is one of the most important fish cultured in Mediterranean areas and was the first marine non-salmonid species to be cultured in Europe (FAO 2023b). In 2016 the EU member states produced 82.000 tonnes of seabass that were valued at 555 million US dollars (Llorente *et al.* 2020). The seabass production in Europe has grown strongly in the last decade reaching about 81.369 tonnes in 2020 in the EU Member States. The main producers are Turkey, Greece and Spain (FAO 2023b; EUMOFA 2023b).

For European seabass farming to be effective this species presents certain nutrition needs, such as crude protein levels between 42-48% of the diet, lipid levels between 18-20%, 1% of w3 polyunsaturated fatty acids (PUFA), vitamin C ~50 mg kg⁻¹ and vitamin E at concentrations of 500 mg kg⁻¹ (FAO 2023c).

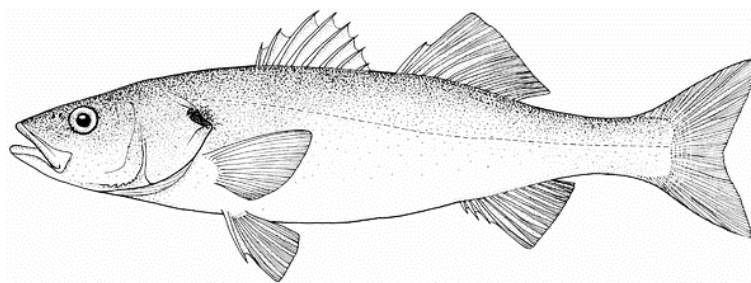


Figure 3. European seabass (*Dicentrarchus labrax*, L. 1758). Source: FAO (2022)

1.2. Diseases in aquaculture

Disease outbreaks are a top concern for the aquaculture industry because they can cause high mortality rates, leading to a decrease in production and consequent significant economic losses (Aziz and Abdullah, 2021; Irshath *et al.* 2023). The increasing demand for seafood for human consumption led to the intensification of aquaculture production. Species that are cultured intensively, like seabass, experience significant amounts of stress that weaken their immune system, being more sensitive to diseases caused by bacteria, viruses, fungi or parasites (FAO 2023b; Frans *et al.* 2008; Irshath *et al.* 2023).

Bacterial pathogens are the most frequent cause of infections in aquaculture. The most common and devastating bacterial infections responsible for economic losses are vibriosis, furunculosis, streptococcosis, photobacteriosis and tenacibaculosis (Irshath *et al.* 2023). Additionally, there is increasing evidence of zoonotic microbes (microorganisms that can be transmitted to humans from animals) among the major fish diseases, affecting public health. This transmission can occur via ingestion of contaminated seafood or water or through topical contact. Some fish pathogens already reported as zoonotic organisms, include *V. vulnificus*, *V. parahaemolyticus*, *A. hydrophila*, *S. iniae* and *E. tarda* (Meyer, 1991; Weir *et al.* 2012; WHO 2020).

1.2.1. Vibriosis

Vibriosis refers to a systemic infection caused by *Vibrio* species. *Vibrio* spp. is a group of gram-negative, rod-shaped bacteria common in marine and estuarine ecosystems. These species prefer warm and brackish water and can be found in the water column or associated with a host (Baker-Austin *et al.* 2018; Novriadi, 2016). Some species that cause vibriosis are *Vibrio anguillarum*, *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* (Austin, 2005; Novriadi, 2016).

This infection is described in numerous fish species like European seabass, seabream, Atlantic salmon, rainbow trout and turbot (Toranzo *et al.* 2005). Signs of vibriosis include lethargy, loss of appetite, organ necrosis, exophthalmia and skin lesions (Novriadi, 2016; Sanches-Fernandes *et al.* 2022). The primary routes of infection occur via the mouth, gills, and skin lesions (Mohamad *et al.* 2019).

1.2.2. Furunculosis

Furunculosis is a bacterial disease caused by *Aeromonas salmonicida*, one of the oldest known fish pathogens that occurs worldwide (Buller *et al.* 2021; Irshath *et al.* 2023). This pathogen is a gram-negative and non-motile bacterium that infects fish from fresh, brackish and seawater (Boily *et al.* 2019). In addition to *A. salmonicida* other *Aeromonas* species, such as *A. hydrophila* and *A. veronii*, are dangerous fish pathogens resulting in economic losses for the aquaculture industry (De Silva *et al.* 2021; Irshath *et al.* 2023).

Infections caused by *Aeromonas* species are common in salmonids but affect several non-salmonid fish, such as eels (*Anguilla* spp.), sea lamprey (*Petromyzon marinus*) and seabass (Buller *et al.* 2021). Although the clinical signs differ with the pathogenic species the main signs are furuncles, haemorrhage, internal lesions and ulcers. These ulcers are formed upon rupture of the furuncles and release a significant number of bacteria into the environment (Aziz and Abdullah, 2021; Boily *et al.* 2019; Buller *et al.* 2021). The primary routes of infection occur via the gills, skin, and intestinal tract (Buller *et al.* 2021).

1.2.3. Streptococcosis

Streptococcosis is a disease caused by *Streptococcus* spp., which is a group of gram-positive bacteria (Doan *et al.* 2022; Toranzo *et al.* 2005). The etiological agents of this disease can survive in water and sediment for long periods. This disease is primarily caused by *S. agalactiae*, and *S. iniae* and common in warm water (Aziz and Abdullah, 2021; Toranzo *et al.* 2005). Streptococcosis is a serious infectious disease in both freshwater and marine aquaculture causing significant economic losses and is also a zoonotic disease affecting food safety for human consumption (Doan *et al.* 2022).

This infection was first reported in rainbow trout but now is described in numerous fish species, such as yellowtail (*Seriola dorsalis*), eel, turbot, and Atlantic salmon (Irshath *et al.* 2023). Some of the clinical signs of streptococcosis are skin discolouration, exophthalmia, corneal opacity, abdominal distention, and abnormal swimming (Doan *et al.* 2022).

1.2.4. Photobacteriosis

Photobacteriosis is caused by a gram-negative and halophilic bacterium called *Photobacterium damsela* subsp. *piscicida*. This bacterial species can survive outside the host and be detected in temperatures between 14 and 29°C and a wide range of salinities (Aziz and Abdullah, 2021).

Photobacteriosis affects several fish species around the world and in Mediterranean countries impacting European seabass, gilthead seabream and sole. This infection is also known as pseudotuberculosis because it is characterized by the presence of white nodules in the spleen and kidney (Aziz and Abdullah, 2021; Toranzo *et al.* 2005). Besides the granulomatous nodules, photobacteriosis clinical signs are lethargy, loss of equilibrium, dark skin, haemorrhages in the head and gills and necrosis in the liver, spleen and kidney (Aziz and Abdullah, 2021; Santos *et al.* 2022).

In addition to *Ph. damsela* subsp. *piscicida*, there is another emerging pathogen in marine aquaculture called *Photobacterium damsela* subsp. *damsela*. This bacterial species was found in different types of fish worldwide, such as European seabass, rainbow trout, seabream, yellowtail, and turbot. Signs of this disease are ulcers and haemorrhage in the mouth, eyes and muscles (Hassanzadeh *et al.* 2015; Rivas *et al.* 2013).

1.2.5. Tenacibaculosis

Tenacibaculosis or marine flexibacteriosis is a bacterial disease caused by a filamentous, gram-negative pathogen called *Tenacibaculum maritimum*. This bacterium is exclusively found in marine environments, where it attaches itself to the surface of other organisms (Mabrok *et al.* 2023; Toranzo *et al.* 2005).

Tenacibaculosis affects a large number of marine fish species like seabass, turbot, sole and gilthead seabream (Irshath *et al.* 2023; Toranzo *et al.* 2005). This infection is common in higher temperatures (15-34°C) (Mabrok *et al.* 2023; Toranzo *et al.* 2005). Clinical signs of this disease are ulcers, necrosis, eroded mouth, fin, and tail rot (Irshath *et al.* 2023). The severity of the infection is influenced by stress, skin surface condition and fish sanitary status because it can be a secondary infection after parasitic infestations (Mabrok *et al.* 2023).

1.3. Disease control strategies

Antibiotics have been used as prophylactic and therapeutic agents to fight bacterial infections in aquaculture for many years contributing to the spread of antibiotic-resistant bacteria (Chen *et al.* 2020; Okeke *et al.* 2022; Pérez-Sánchez *et al.* 2018). Several fish pathogens developed resistance to the antibiotics, namely *Vibrio* spp., *Aeromonas* spp., *Photobacterium* spp., *Streptococcus* spp. and *Tenacibaculum maritimum* (Defoirdt *et al.* 2011; Haenni *et al.* 2018; Petchimuthu *et al.* 2022; Thyssen and Ollevier, 2001).

Nowadays, the usage of antibiotics as a preventive measure is prohibited in the EU, but the diseases outbreaks caused by antibiotic-resistant bacteria keep increasing. Therefore, this reinforces the need to establish new measures to prevent fish diseases (Chen *et al.* 2020; Pérez-Sánchez *et al.* 2018). Over the years, several alternatives to antibiotics have been studied, namely vaccines, probiotics, prebiotics and bacteriophages (Bondad-Reantaso *et al.* 2023; Cabello *et al.* 2016; Pérez-Sánchez *et al.* 2018). Another promising strategy to prevent fish bacterial infections is the use of functional extracts as they have antimicrobial activity (Beltrán *et al.* 2022).

1.4. Functional extracts in aquaculture

Functional extracts are compounds capable of eliciting physiological, immunological, or metabolic responses within a living organism and can be obtained from plants, algae, agro-industrial waste, and microbes, among others (Banik *et al.* 2022). In aquaculture, functional extracts can be used to increase the feed's nutritional properties, stimulate appetite and increase the fish's immune and health status due to their antimicrobial and antioxidant properties (Filipe *et al.* 2023; Reverter *et al.* 2014).

The use of industrial by-products can promote waste recycling and decrease the utilization in aquafeeds of other ingredients that can be directed for human food (Caipang *et al.* 2019). The wine industry produces large amounts of by-products, mainly grape by-products with a valuable composition in phytochemicals with health-promoting properties (Gouveinhas *et al.* 2018).

Phytochemicals are synthesized by plants' secondary metabolism and are considered safer than synthetic drugs (Beltrán *et al.* 2022). The most significant phytochemicals are phenolic compounds, of which polyphenols are one of the most important. Phenolic compounds are ideal components to include in aquafeeds because

they present biological activities, such as immunostimulant, anti-inflammatory, antioxidant, and antimicrobial properties. One source of these compounds is grape by-products (grape marc), namely skins, pulp, seeds, stems and leaves that come from wine production (Beltrán *et al.* 2022; Leal *et al.* 2020). The most abundant polyphenols in grape by-products are flavonoids, specifically anthocyanidins, flavonols, flavan-3-ols, and chalcones (Silva *et al.* 2021). Several studies have already been conducted utilizing grape by-products in aquacultured species (Arciuli *et al.* 2017; Martínez-Antequera *et al.* 2023; Souza *et al.* 2019). Arciuli *et al.* (2017) observed that the immune response of seabass was better when fed the diet supplemented with 200 mg kg⁻¹ of grape seed extract. In the case of Martínez-Antequera *et al.* (2023), the results showed that phenolic compounds of two wine by-products (grape marc and wine lees) have a protective effect against induced stress produced by hypoxia when added (100 g kg⁻¹ of grape marc or wine lees) to the diet of golden grey mullet (*Liza aurata*). Additionally, Souza *et al.* (2019) concluded that grape marc flour can be used to prevent liver damage induced by the bacterial pathogen *P. aeruginosa* when added to grass carp (*Ctenopharyngodon idella*) diet at 300 mg kg⁻¹.

1.5. Objectives

Taking into consideration the urgent need to find new sustainable alternatives to fight aquaculture bacterial diseases, this study aimed to assess the potential of three white grape marc extracts to inhibit important bacterial fish pathogens and evaluate their *in vitro* potential against several bacteria pathogens, and the effect of their dietary inclusion at three concentrations on European seabass growth and disease resistance. To accomplish this, the first goal of this project was to characterize *in vitro* the antimicrobial profile of the functional extracts against a broad range of fish pathogens, and the second goal was to evaluate *in vivo* if these extracts and concentrations improve European seabass growth and protect it against an infection challenge.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

The fish bacterial pathogens used in this study are listed in Table 1. All strains were grown at 140 rpm in Brain Heart Infusion (BHI) for 24h (except *Tenacibaculum maritimum* which was grown in Marine medium (MA) for 48h) at 28°C or 37°C (for *Edwardsiella tarda*, *Pseudomonas aeruginosa*, *Shigella sonnei*, *Staphylococcus aureus*, *Streptococcus iniae*, *Streptococcus parauberis* and *Streptococcus agalactiae*).

Table 1. Bacterial pathogens used in this study

Bacterial strains	Strain
<i>Aeromonas hydrophila</i>	LMG 2844
<i>Aeromonas salmonicida</i>	LMG 3780
<i>Aeromonas veronii</i>	NUTRIMU Isolate
<i>Aeromonas bivalvium</i>	NUTRIMU Isolate
<i>Edwardsiella tarda</i>	LMG 2793
<i>Photobacterium damsela</i> subsp. <i>damsela</i>	LMG 7892
<i>Photobacterium damsela</i> subsp. <i>piscicida</i>	Lg 41/01
<i>Pseudomonas aeruginosa</i>	CECT 108
<i>Shigella sonnei</i>	LMG 10473
<i>Staphylococcus aureus</i>	LMG 8224
<i>Streptococcus iniae</i>	LMG 14520
<i>Streptococcus agalactiae</i>	LMG 15977
<i>Streptococcus parauberis</i>	LMG 12174
<i>Tenacibaculum maritimum</i>	LMG 11612
<i>Vibrio anguillarum</i>	DSMZ 21597
<i>Vibrio harveyi</i>	NUTRIMU Isolate
<i>Vibrio parahaemolyticus</i>	LMG 2850
<i>Vibrio vulnificus</i>	LMG 13545
<i>Yersinia ruckeri</i>	DSM 18506

Strains were purchased from bacterial collections, namely, CECT (Spanish Type Culture Collection), DSM (German Collection of Microorganisms), DSMZ (German Collection of Microorganisms and Cell Cultures GmbH), and LMG (Laboratory of Microbiology of Ghent University), obtained from the Nutrimu group culture collection, or kindly provided by Díaz-Rosales, P. *et al.* (2003).

2.2. Grape by-products functional extracts

Three different grape functional extracts were used in this study, namely: (i) Grape marc extract water100%/volatilised acetone (AC); (ii) Grape marc extract ethanol/water (50%/50%) (ET) and (iii) Grape marc extract propylene glycol/water (50%/50%) (PG). These extracts were obtained from grape by-products using different solvents to extract the bioactive compounds and further dissolved in water, ethanol and propylene glycol, respectively.



Figure 4. Grape functional extracts used in this study. (I) Grape marc extract water100%/volatilised acetone (AC); (II) Grape marc extract ethanol/water (50%/50%) (ET); (III) Grape marc extract propylene glycol/water (50%/50%) (PG)

2.3. Antimicrobial activity

The antimicrobial activity of the functional extracts was evaluated using a well diffusion assay, as described by Santos *et al.* (2021).

The bacterial pathogens ($OD_{600} \sim 0.1$) were spread on BHI or MA plates with a cotton swab in 3 different directions to ensure uniform coverage. Then, 9 mm diameter wells were made and filled with 100 μ L of each extract and solvent (negative control) previously sterilized by filtration with a 0.22 μ M cellulose acetate filter. Plates were incubated for 24 or 48 hours at 28°C or 37°C. Zones of growth inhibition around the wells were measured (mm) and considered a positive result for antimicrobial activity. All plates were photographed in the Gel-doc™ XR⁺ system using the Image Lab™ software (Bio-Rad, EU).

2.4. Diets composition

Ten diets were formulated to be isoproteic (45%) and isolipidic (18%). One was used as a control diet and the other nine were supplemented with AC, ET and PG extracts at 3 different polyphenol concentrations (45, 200 and 450 ppm). All diets were supplemented with methionine, taurine and dicalcium phosphate to avoid essential amino acid and phosphorus deficiencies. All diet ingredients were thoroughly mixed and dry-pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA). The diets were stored in airtight bags at -20°C until further use. The diets composition is presented in Table 2.

Table 2. Ingredients and proximate composition of the experimental diets

	Diets									
	CTR	AC45	AC200	AC450	ET45	ET200	ET450	PG45	PG200	PG450
Ingredients (% dry weight)										
Fish meal ^a	16.48	16.48	16.48	16.48	16.48	16.48	16.48	16.48	16.48	16.48
Salmon hydrolysate ^b	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Soybean meal ^c	22.73	22.73	22.73	22.73	22.73	22.73	22.73	22.73	22.73	22.73
Corn gluten meal ^d	19.44	19.44	19.44	19.44	19.44	19.44	19.44	19.44	19.44	19.44
Wheat gluten meal ^e	10.65	10.65	10.65	10.65	10.65	10.65	10.65	10.65	10.65	10.65
Rapeseed meal ^f	5.62	5.62	5.62	5.62	5.62	5.62	5.62	5.62	5.62	5.62
Wheat meal ^g	11.74	11.74	11.74	11.74	11.74	11.74	11.74	11.74	11.74	11.74
Fish oil	7.98	7.98	7.98	7.98	7.98	7.98	7.98	7.98	7.98	7.98
Colza oil	7.98	7.98	7.98	7.98	7.98	7.98	7.98	7.98	7.98	7.98
Vitamin premix ^h	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mineral premix ⁱ	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Binder ^j	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Dicalcium phosphate	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66
Methionine	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
Taurine	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Extract incorporation per kg diet										
AC (mL)	3	13	30							
ET (mL)					10	44	100			
PG (mL)								10	44	100
Proximate analysis (% dry weight)										
Dry matter	88.95	91.33	90.94	90.64	91.71	89.73	87.77	88.26	90.09	90.87
Crude protein	44.8	45.7	45.2	45.7	45.8	45.9	45.4	45.3	45.8	45.7
Crude lipids	18.13	18.89	18.47	18.68	18.61	18.35	18.79	18.58	18.41	18.43
Ash	8.56	8.04	7.99	7.96	8.57	8.34	8.01	7.88	7.61	7.76

^a Pesquera Centinela, Steam Dried LT, Chile (CP: 61.1 %; CL: 9.8 %). Sorgal, S.A. Ovar, Portugal.

^b Sorgal, S.A. Ovar, Portugal (CP: 67 %; CL: 11 %).

^c Non-GMO; Cargill France SAS, St. Germain-en-Laye, France (CP: 43.5 %; CL: 2.0 %)

^d Sorgal, S.A Ovar, Portugal (CP: 75.4 %; CL: 2.8%).

^e Sorgal, S.A Ovar, Portugal (CP: 54.9 % CL: 2.2 %).

^f Sorgal, S.A. Ovar, Portugal (CP: 40 %; CL: 0.8 %).

^g Sorgal, S.A Ovar, Portugal (CP: 13.2 %; CL: 2.4 %).

^h Vitamins (mg/kg diet): retinol, 18,000 (IU/kg diet); calciferol, 2000 (IU/kg diet); alpha-tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

ⁱ Mineral (mg/kg diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g/kg diet); potassium chloride, 1.15 (g/kg diet); sodium chloride, 0.4 (g/kg diet).

^j Aquacube, Agil, UK.

2.5. Diets composition analysis

2.5.1. Crude protein (Kjeldahl method)

The protein content of diets was determined by the Kjeldahl method following acid digestion, using a Tecator digester and a Tecator distillation unit. Crude protein was calculated by multiplying the nitrogen content of the sample by the factor 6.25, assuming that the average nitrogen protein content is 16% (16gN/100 protein).

Digestion:

Approximately 0.5g of sample were added to the tubes of digestion. Then 1 Kjeldahl gum (catalyser) and 15 mL of concentrated sulfuric acid were added to the tubes. After this the tubes were placed in a Tecator digester, at 420°C, connected to a water hose, with the exhaustion system on. The sample digestion took 1h to complete and after this hour the tubes were cooled for 15 minutes.

Distillation and titration:

25 mL of boric acid and 10 drops of pH indicator were added to a 250 mL Erlenmeyer (this solution is orange). To make the distillation each tube and Erlenmeyer was placed in the Tecator distillation unit, for 3 minutes, where water and NaOH were automatically added (the solution becomes blue at the end of distillation). Then each Erlenmeyer was titrated with hydrochloric acid (HCl) (0.5 N) until the solution turned orange again and the titrated volume spent was registered.

2.5.2. Crude lipids (Soxhlet method)

The lipid content of diets was determined by the Soxhlet method, involving a continuous extraction with petroleum ether in a Soxhlet system for 4 hours. Approximately 1g of sample was added to the extraction cartridges and a piece of cotton was placed on top of the sample. Then the extraction cups were weighed and filled with 40 mL of petroleum ether. Every cup and cartridge was placed in the Soxhlet extractor. The samples were boiled for 1 hour in the petroleum ether, rinsed for 2 hours and the extracted lipids were completely collected in the extraction cups. After extraction, the solvent was evaporated in an oven for 30 min at 100°C and the extracted material was

weighed after cooling on a desiccator. Lipid content was estimated through the difference in weight of the cups before and after extraction.

2.5.3. Dry matter

The humidity content was determined by the total weight loss of the sample, expressed as a percentage of the original weight. Approximately 1g of the sample was placed in pre-dried, cooled and weighed porcelain crucibles. The crucibles were placed in an oven at 100°C for 24 hours. After 24h the crucibles were cooled on a desiccator for 30 min and weighed.

2.5.4. Ash content

The ash content is the inorganic residue obtained after incineration at 450°C. To determine the ash content the crucibles used to determine the humidity were placed in a muffle for 16h then cooled on a desiccator for 30 min and weighed.

2.6. Growth trial

All animal experiments were approved by the CIIMAR Ethical Committee for Managing Animal Welfare (ORBEA) in compliance with the European Union directive 2010/63/EU and the Portuguese Law (DL 113/2013).

The growth trial was performed at CIIMAR, Porto University, Portugal, with European seabass obtained from a commercial fish farm (Maresa, Marismas y Esteros, S.L., Huelva). Since the juveniles were already adapted to the experimental environment, being allocated in the stock tanks presented at the bioterium, no quarantine period was required.

This trial was conducted in 2 interconnected recirculating aquaculture systems (RAS) equipped with 18 and 21 fibreglass tanks of 90 L capacity, supplied with a continuous flow of seawater (salinity at $38 \pm 1 \text{ g L}^{-1}$), with oxygen levels close to saturation ($7\text{-}8 \text{ mg L}^{-1}$), thermoregulated to $24 \pm 1^\circ\text{C}$, $\text{pH} \approx 7$, with a photoperiod of 12:12h light:dark that is provided by artificial illumination. In these systems, the nitrogenous compounds were kept below 0.02 mg L^{-1} . At the beginning of the trial, 450 fish were evenly distributed

in 30 tanks with an average initial body weight of 23.3 g and the experimental diets were randomly assigned in triplicate groups (Table 3). The trial lasted 42 days and fish were fed by hand 6 times a week, 2 times a day, until apparent full satiation. Utmost care was taken to avoid feed losses. The containers of the diets were weighed, every week, to determine the intake of each tank. At the end of the trial, fish were slightly anaesthetized with 0.3 mL L⁻¹ of 2-phenoxyethanol and group-weighted.

Table 3. Diet distribution by the experimental system tanks and respective weights

Diet	Tank	10 fish (g)	5 fish (g)	Total (g)
CTR	5	234.8	115.3	350.1
	28	230.4	119.7	350.1
	15	234.4	115.8	350.2
AC 45	10	226.1	123.9	350.0
	17	231.8	116.7	348.5
	21	233.9	116.8	350.7
AC 200	9	231.8	118.7	350.5
	12	232.4	117.5	349.9
	20	233.7	116.4	350.1
AC 450	3	232.0	117.9	349.9
	6	230.5	119.3	349.8
	14	235.4	114.2	349.6
ET 45	2	232.3	117.7	350.0
	8	230.2	119.9	350.1
	18	234.5	115.6	350.1
ET 200	24	235.8	114.3	350.1
	29	234.8	114.8	349.6
	13	234.3	116.2	350.5
ET 450	26	231.0	118.3	349.3
	30	231.7	118.5	350.2
	16	236.5	113.7	350.2
PG 45	1	231.7	118.3	350.0
	25	236.2	114.4	350.6
	7	231.8	118.2	350.0
PG 200	23	237.6	112.3	349.9
	4	230.5	119.7	350.2
	19	236.5	113.7	350.2
PG 450	22	232.1	117.8	349.9
	27	231.7	118.8	350.5
	11	230.8	119.1	349.9

2.7. Sampling

At the end of the growth trial, 3 fish from each tank were randomly collected and euthanized by anaesthesia overdose with 2-phenoxyethanol (1 mL L^{-1}). Each fish's blood, liver, head kidney and intestine were sampled. The blood was collected from the caudal vein with heparinized syringes and centrifuged at $10.000 \times g$ for 10 minutes (Figure 5). The plasma obtained from blood centrifugation was stored in 1.5 mL Eppendorf tubes and frozen at -80°C for further analysis. Additionally, the liver was sampled to further analyse the fish's oxidative stress. The head kidney and distal intestine were collected, stored in RNAlater solution (1:10) for 24h at 4°C , and then frozen at -80°C until further analysis as described by Magalhães *et al.* (2021). To analyse the gut microbiota 3 fish per tank were sampled under aseptic conditions. Digesta samples were obtained by squeezing the intestinal content into a sterile tube. The intestine was collected and promptly frozen in liquid nitrogen and kept at -80°C until analysed (Figure 6) (Magalhães *et al.* 2022).



Figure 5. Sampling of European seabass blood



Figure 6. Sampling of European seabass intestine to gut microbiota analysis

2.8. Pre-challenges and infection models

Different fish pathogens, namely, *A. salmonicida*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *Ph. damsela* subsp. *piscicida* and *T. maritimum* were evaluated for their ability to cause infection and mortality in European seabass. For that, bacteria were inoculated in 25 mL of the corresponding medium and incubated at 28°C . After 24h or 48h (for *T. maritimum*), bacterial cells were harvested by centrifugation at

5000 x *g* for 15 min at room temperature, followed by 2 washes with 1x Phosphate Buffered Saline (PBS) (137 mM NaCl, 2.7 mM KCL, 10 mM Na₂HPO₄, 1.4 mM KH₂PO₄). Finally, the pellet was resuspended in 2.5 mL of 1x PBS and then diluted to reach different bacterial concentrations.

The fish infection trials were performed with European seabass obtained from 2 commercial fish farms (Atlantik Fish, Castro Marim, Algarve and Maresa, Marismas y Esteros, S.L., Huelva). Once in the experimental facilities fish were submitted to a quarantine period of 15 days before transfer to the experimental system. The trials were conducted in the same RAS system and the water quality parameters were maintained in the same conditions described in section 2.6. For the pre-challenges, 10 fish were injected with 100 µL of different bacterial concentrations of *A. salmonicida* (1x10⁸ - 1x10⁹ CFUs mL⁻¹), *V. anguillarum* (1x10⁷ - 1x10⁸ CFUs mL⁻¹), *V. harveyi* (1x10⁶ - 1x10⁸ CFUs mL⁻¹), *V. parahaemolyticus* (1x10⁷ - 1x10⁸ CFUs mL⁻¹), *V. vulnificus* (1x10⁶ - 1x10⁸ CFUs mL⁻¹) and *Ph. damsela* subsp. *piscicida* (1x10⁶ - 5x10⁸ CFUs mL⁻¹). In the case of *T. maritimum*, 10 fish were placed in tanks with 10L of water and exposed to different bacterial concentrations (3.4x10⁷ - 1x10⁸ CFUs mL⁻¹) by bath for 2h, 3.5h and 5h. After this period the fish were moved to tanks with 90L of water without the bacteria.

Fish mortalities were followed by 7 days post-infection (dpi) and dead and moribund fish were daily removed or euthanized by anaesthesia overdose with 2-phenoxyethanol (1 mL L⁻¹).

2.9.Challenge

The challenge was performed with the fish used in the growth trial and fed with the experimental diets. This trial was conducted in the same RAS system and the water quality parameters were maintained in the same conditions described in section 2.6.

Fish were infected by intraperitoneal injection with 100 µL of 3x10⁸ CFUs mL⁻¹ *Ph. damsela* subsp. *piscicida*. After 7 days with no mortality, fish were infected again by intraperitoneal injection with 100 µL of 1x10⁸ CFUs mL⁻¹ *V. anguillarum*, an opportunistic bacterial species. Non-challenged fish, injected with 100 µL of 1x PBS were considered as negative control for the experiment. Fish mortalities were followed by 7 days post-infection with *V. anguillarum*. Dead and moribund fish were daily removed or euthanized with an overdose of 2-phenoxyethanol (1 mL L⁻¹). and clinical signs, weight

and days post-infection were registered. At the end of the trial, the remaining fish were euthanized with an overdose of anaesthetic (2-phenoxyethanol; 1 mL L⁻¹).

2.10. Statistical analysis

The growth trial results are expressed as the mean $\pm$ standard error of the mean (SEM). Normality and homogeneity of variances were assessed, and when required, data were log-transformed. Statistical analysis of the growth trial data was done by two-way analysis of variance (ANOVA) with the extract and concentration as fixed factors and non-orthogonal contrasts between the control and each test diet.

Statistical analysis of the challenge results was performed utilizing GraphPad Prism 8 software (GraphPad Software). The significance of the results was evaluated using the Kaplan-Meier method and analysed with the Log-rank (Mantel-Cox) test. Statistical significance was considered when $p > 0.05$.

3. Results

3.1. Well Diffusion Assays

In the first screening, 2 grape marc extracts - ethanol/water (50%/50%) and propylene glycol/water (50%/50%) were evaluated for their antimicrobial activity against a broad range of fish pathogens (Figure 7).

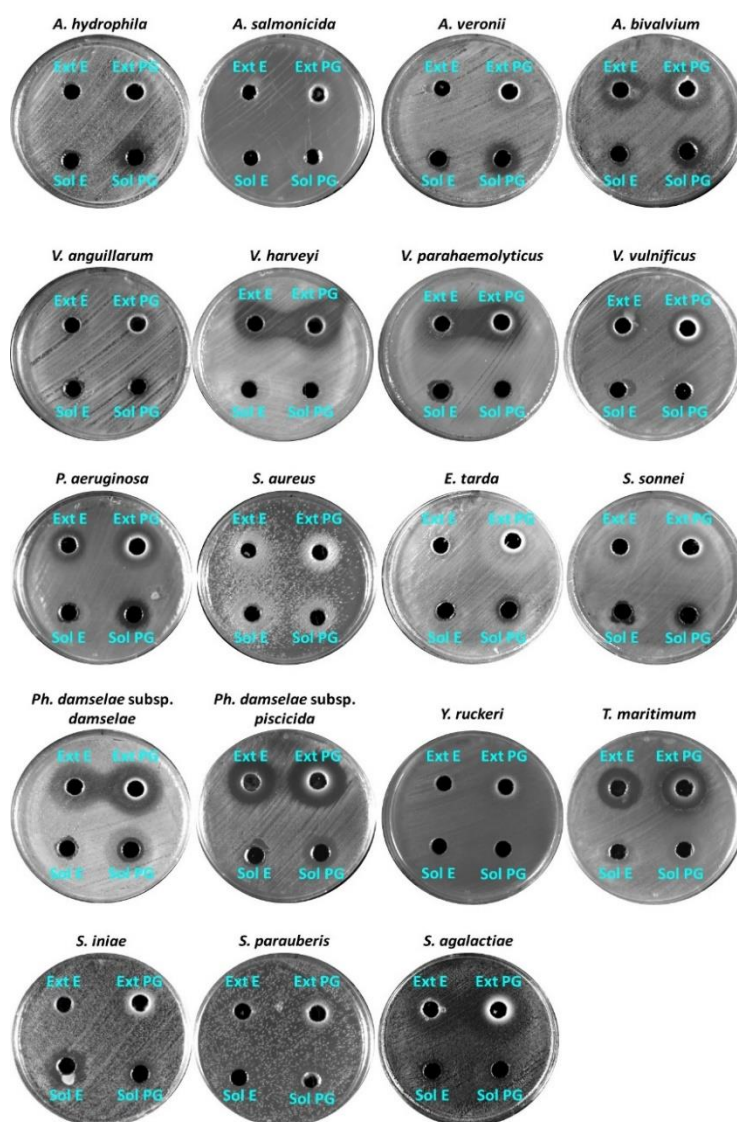


Figure 7. Growth inhibition halos for the bacterial strains *A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *P. aeruginosa*, *S. aureus*, *E. tarda*, *S. sonnei*, *Ph. damsela* subsp. *damsela*, *Ph. damsela* subsp. *piscicida*, *Y. ruckeri*, *T. maritimum*, *S. iniae*, *S. parauberis* and *S. agalactiae* around the wells filled with 100µL of the grape marc extract ethanol/water (50%/50%) (Ext E) and grape marc extract propylene glycol/water (50%/50%) (Ext PG); ethanol/water (50%/50%) (Sol E) and propylene glycol/water (50%/50%) (Sol PG). Photographs were taken in a Gel-doc™ XR+ system using the Image Lab™ software and are at the same scale.

The two extracts generated clear inhibitory halos against *V. vulnificus*, *Ph. damsela* subsp. *piscicida* and *T. maritimum*. Additionally, the extracts also interfered with the growth of *A. bivalvium*, *V. harveyi*, *V. parahaemolyticus*, *P. aeruginosa*, *Ph. damsela* subsp. *damsela* and *S. agalactiae*. The solvents ethanol/water (50%/50%) and propylene glycol/water (50%/50%) used as negative controls, slightly inhibited the growth of *A. hydrophila*, *A. veronii*, *A. bivalvium*, *V. vulnificus*, *P. aeruginosa*, *E. tarda*, *S. sonnei*, *Ph. damsela* subsp. *damsela*, *Ph. damsela* subsp. *piscicida* and *S. iniae*. Additionally, the solvents also demonstrated some inhibitory activity against *V. parahaemolyticus* and *T. maritimum*, respectively.

Based on the results of this first screening, the third grape marc extract - water100%/volatilised acetone was tested only against the bacterial strains that had their growth inhibited by the ethanol and propylene glycol extracts, namely *A. bivalvium*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *P. aeruginosa*, *Ph. damsela* subsp. *damsela*, *Ph. damsela* subsp. *piscicida*, *T. maritimum* and *S. agalactiae* (Figure 8).

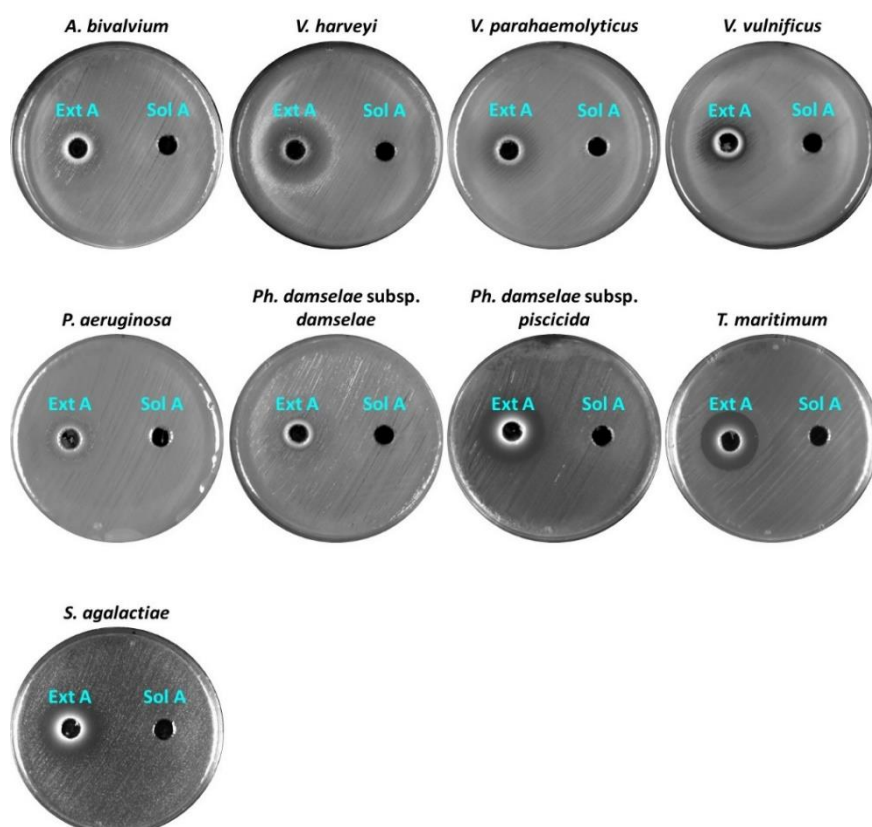


Figure 8. Growth inhibition halos for the bacterial pathogens *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *P. aeruginosa*, *Ph. damsela* subsp. *damsela*, *Ph. damsela* subsp. *piscicida*, *T. maritimum* and *S. agalactiae* around the wells filled with 100µL of the grape marc extract water100%/volatilised acetone (Ext A) and the solvent 100% water (Sol A). Photographs were taken in a Gel-doc™ XR+ system using the Image Lab™ software and are at the same scale.

The grape marc extract water100%/volatilised acetone produced clearly inhibitory halos against *V. harveyi*, *Ph. damsela* subsp. *piscicida*, *T. maritimum* and *S. agalactiae*. And slightly inhibitory halos (<2mm) against *V. vulnificus* and *Ph. damsela* subsp. *damsela*. Finally, the solvent 100% water, as expected, did not inhibit any of the bacterial strains.

3.2. Growth trial

After 42 days, there were no differences between dietary treatments in all growth parameters assessed namely final body weight (FBW), weight gain (WG) and daily growth index (DGI). Nonetheless, fish duplicated their initial body weight and fish fed the ET200, ET450 and PG450 diets presented higher final body weight absolute values. Feed intake was similar in all tested diets with the lower value found in fish fed with AC45 diet. Dietary treatments did not affect feed efficiency and protein efficiency ratio (PER). No mortality was found in fish fed all the tested extracts with a survival rate of 100% in all diets (Table 4).

Table 4. Growth performance and feed utilization of European seabass

	AC45	AC200	AC450	ET45	ET200	ET450	PG45	PG200	PG450	CTR	SEM
Initial body weight (g)	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.3	0.01
Final body weight (g)	43.9	42.1	44.6	43.7	45.5	46.1	42.5	43.5	45.8	42.8	0.49
Weight gain (g kg ABW ⁻¹ day ⁻¹)	14.6	13.6	14.9	14.4	15.3	15.6	13.8	14.3	15.4	14.0	0.49
Daily growth index ^a	1.60	1.48	1.64	1.58	1.69	1.73	1.50	1.57	1.71	1.52	0.03
Feed intake (g kg ABW ⁻¹ day ⁻¹)	19.6	19.8	20.4	20.6	21.1	21.5	20.5	19.7	21.4	20.7	0.25
Feed efficiency ^b	0.74	0.69	0.73	0.70	0.72	0.73	0.67	0.73	0.72	0.68	0.01
Protein efficiency ratio ^c	1.63	1.53	1.60	1.53	1.57	1.60	1.48	1.58	1.57	1.52	0.02
Survival (%)	100	100	100	100	100	100	100	100	100	100	0.00

Values are presented as means (n = 3) and standard error of the mean (SEM). No statistical differences were found in the two-way ANOVA or the non-orthogonal contrast analysis.

ABW: Average Body Weight = (Initial Body Weight + Final Body Weight)/2).

^a DGI: Daily Growth Index = ((Final Body Weight^{1/3} – Initial Body Weight^{1/3})/Time in days) x 100.

^b FE: Feed Efficiency = Wet Weight Gain/Dry Feed Intake.

^c PER: Protein Efficiency Ratio = Wet Weight Gain/Crude Protein Intake.

3.3. Pre-challenges

The infection model of *A. salmonicida*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus* and *T. maritimum* couldn't be optimised for European seabass after several pre-challenges. Although seabass was exposed to several bacterial concentrations and infection routes (injection and bath in the case of *T. maritimum*) these 5 fish pathogens were not capable of causing at least 50% of mortality.

In the case of *Ph. damsela* subsp. *piscicida* 7 different concentrations were tested, and only 5×10^8 , 1×10^8 and 5×10^7 CFUs mL⁻¹ caused fish mortalities. Fish challenged with 5×10^8 CFUs mL⁻¹ presented 40% of mortality after 24h, 90% after 48h and 100% after 72h. The concentration 1×10^8 was tested two times. On the first test this concentration caused 100% mortality after 96h. On the second test, fish challenged with 1×10^8 showed only 20% mortality. And the concentration 5×10^7 CFUs mL⁻¹ only killed 3 fishes of a total of 10, 6 days post-infection.

To establish the *V. anguillarum* infection model, fish were exposed to 3 different concentrations - 1×10^8 , 5×10^7 and 1×10^7 CFUs mL⁻¹. Fish exposed to 1×10^8 CFUs mL⁻¹ started to show mortalities in the first hours post-infection, rapidly progressing reaching 100% after 24h. On the other hand, fish exposed to 5×10^7 CFUs mL⁻¹ showed 13% mortality 7 days post-infection.

Based on these results and the results of previous studies, the 3×10^8 CFUs of *Ph. damsela* subsp. *piscicida* (Unpublished data) and the 1×10^8 CFUs of *V. anguillarum* (Gonçalves *et al.* 2022) were selected to continue this work (challenge).

3.4. Challenge

European seabass juveniles were fed the experimental diets supplemented with the grape marc extracts before being challenged with *Ph. damselae* subsp. *piscicida* and *V. anguillarum*. There were no mortalities after the challenge with *Ph. damselae* subsp. *piscicida*, therefore 7 days after the challenge fish were infected with *V. anguillarum*. Although the diets supplemented with grape extracts failed to significantly improve fish survival when compared to the control group ($p > 0.05$) (Table 5), it was observed different survival rates.

Table 5. Survival rate of the fish fed with the diets supplemented with the grape marc extracts compared to the group control

	AC45	AC200	AC450	ET45	ET200	ET450	PG45	PG200	PG450
CTR	0.5696	0.6906	0.6261	0.3628	>0.9999	0.9356	0.6617	0.6241	0.7893
	ns	ns	ns	ns	ns	ns	ns	ns	ns

The survival results are illustrated in Figures 9, 10 and 11 which compare the survival rate of European seabass fed the diets supplemented with grape extracts with the control diet.

The survival rate of European seabass fed with grape marc extract water100%/volatilised acetone upon bacterial challenge is illustrated in Figure 9. One day post-infection (dpi), fish fed AC200 diet showed a 10% survival rate, while in fish fed AC45 and AC450 diets we only observed 7% and 8% survival rates, respectively. After 3 dpi the survival rate of fish fed with AC200 diet dropped to 7%. In the control group, fish mortalities reached 85% in the first 24 hours post-infection, increasing to 90% after 2 dpi.

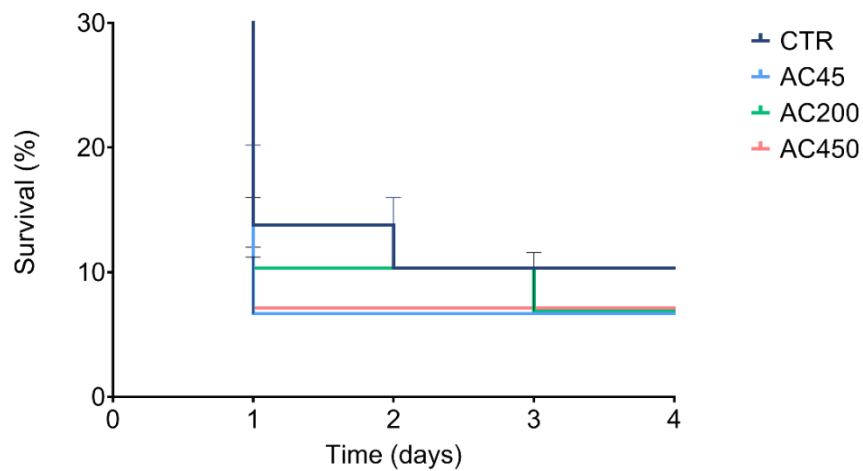


Figure 9. Survival rate of European seabass challenged with *V. anguillarum* after being fed the diets supplemented with grape marc extract water100%/volatilised acetone. Control group is composed of challenged fish fed with non-extracts diets. Statistical analysis was performed with GraphPad Prism 8 software.

The survival rate of European seabass fed with grape marc extract ethanol/water (50%/50%) upon bacterial challenge is illustrated in Figure 10. Mortalities in fish fed ET45 diet reached ~83% at 1 dpi rapidly progressing until day 3 post-infection, reaching 100% mortality. Fish fed diet ET450 showed 20% survival after 24 hpi progressing until day 4 with a final survival rate of 7%. Fish fed the control and ET200 diets showed the same survival rate and progression through time with 13% after 24 hpi and reaching a 10% survival rate from the second day onwards.

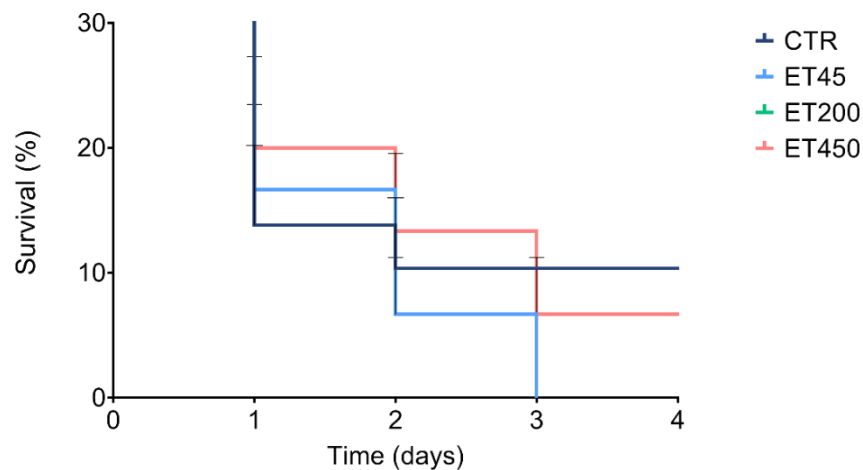


Figure 10. Survival rate of European seabass challenged with *V. anguillarum* after being fed the diets supplemented with grape marc extract ethanol/water (50%/50%). Control group is composed of challenged fish fed with non-extracts diets. Statistical analysis was performed with GraphPad Prism 8 software.

The survival rate of European seabass fed with grape marc extract propylene glycol/water (50%/50%) upon bacterial challenge is illustrated in Figure 11. Mortalities in fish fed PG450 diet reached ~77% at 1 dpi, and rapidly progressed until day 3 post-infection, reaching 93% mortality. One day post-infection, fish fed PG45 and PG200 diets showed 20% and 21% survival rates, respectively. After 2 dpi the survival rate of fish fed with PG45 and PG200 diets dropped to 13% and 14%, respectively.

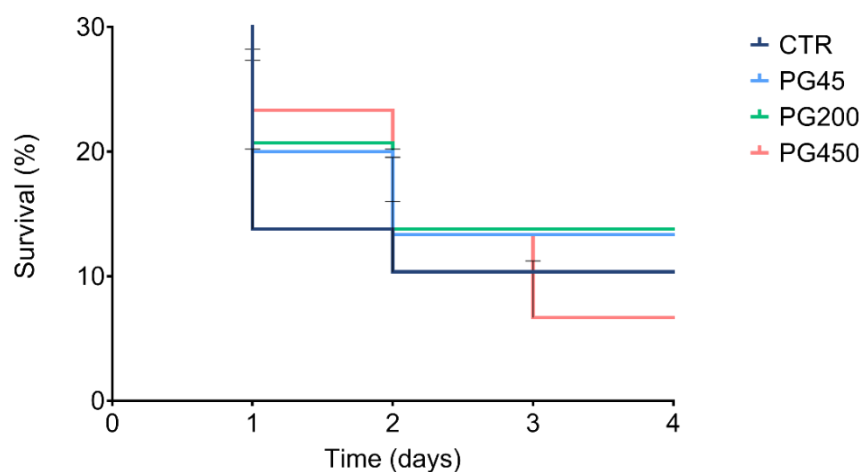


Figure 11. Survival rate of European seabass challenged with *V. anguillarum* after being fed the diets supplemented with grape marc extract propylene glycol/water (50%/50%). Control group is composed of challenged fish fed with non-extracts diets. Statistical analysis was performed with GraphPad Prism 8 software.

4. Discussion

Although the use of antibiotics as preventive measures and growth promoters is prohibited within EU countries, they have been used in worldwide aquaculture for many years contributing to the spread of antibiotic-resistant bacteria. (Chen *et al.* 2020; Okeke *et al.* 2022; Pérez-Sánchez *et al.* 2018). It is thus necessary and urgent to find sustainable, and safer alternatives to antibiotics (Beltrán *et al.* 2022; Dawood *et al.* 2022). Accordingly, this study aimed to discover if functional extracts of grape by-products can be used to prevent bacterial infections in European seabass production.

Antimicrobial activity

Grape by-products contain a high quantity of phytochemicals with immunostimulant, anti-inflammatory, antioxidant, and antimicrobial properties (Beltrán *et al.* 2022; Gouveinhas *et al.* 2018; Leal *et al.* 2020). These health-promoting properties are caused by the presence of phenolic compounds, specifically polyphenols (Dawood *et al.* 2022; Gouveinhas *et al.* 2018). Knowing this, the antimicrobial activity of the grape marc extracts (AC, ET, PG) was evaluated against several fish pathogens, namely *A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. bivalvium*, *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *P. aeruginosa*, *S. aureus*, *E. tarda*, *S. sonnei*, *Ph. damsela* subsp. *damsela*, *Ph. damsela* subsp. *piscicida*, *Y. ruckeri*, *T. maritimum*, *S. iniae*, *S. parauberis* and *S. agalactiae*. We chose these bacterial species because they cause high mortality rates and significant economic losses in aquaculture. The results (after 2 independent screenings) showed that these extracts can inhibit the growth of gram-negative and gram-positive bacteria, such as *V. harveyi*, *Ph. damsela* subsp. *piscicida*, *T. maritimum*, *S. agalactiae*, *V. vulnificus* and *Ph. damsela* subsp. *damsela*.

Although the extracts used in this study present antibacterial activity against gram-positive and gram-negative in similarity with other studies, the results showed that the grape marc extracts inhibited several gram-negative bacteria but only could inhibit 1 of the 4 gram-positive bacteria tested. In contrast, the majority of the studies state that grape extracts inhibit more effectively gram-positive bacteria, and that the resistance of gram-negatives is due to the presence of thick lipopolysaccharide (LPS) layer (Gouveinhas *et al.* 2018; Silva *et al.* 2018; Xu *et al.* 2015). The results of the present work can indicate that the grape marc extracts used (AC, ET and PG) present different

compounds that are able to overcome the LPS layer and enter the cytoplasm of gram-negative bacteria.

Growth trial

In this study, each extract was tested at 3 different concentrations of polyphenols (45, 200 and 450 ppm), which were chosen based on the concentrations used in other studies (Chien *et al.* 2023) and also taking into consideration the maximum volume of liquid extract that can be added per Kg of feed, which is 100 mL.

The growth trial results showed that the inclusion of grape by-product functional extracts in European seabass diets did not improve or decrease fish growth performance. Feed intake and efficiency were similar among dietary groups showing that the different extracts did not reduce feed palatability or fish capacity to utilize it for growth purposes.

Differently, proanthocyanidins extracted from grape seeds and added with graded levels (200, 400, 600 and 800 mg kg⁻¹) to Nile tilapia (*Oreochromis niloticus*) fingerlings diets improved growth performance (Zhai *et al.* 2014). Mousavi *et al.* 2021 also showed that a grape (*Vitis vinifera*) seed extract improved the growth performance of rainbow trout at dietary inclusion of 100 and 200 mg kg⁻¹.

In the case of Tarricone *et al.* (2023), the results showed that the specific growth rate of European seabass fed with a diet supplemented with 200 mg kg⁻¹ of red grape extract was considerably lower compared to the control group (without supplementation). Moreover, this concentration of red grape extract negatively affected the growth performance of European seabass (Tarricone *et al.* 2023) contrary to the highest concentration of white grape marc extracts 450 ppm used in the present study.

Challenge

In this study, we performed several pre-challenges to determine the median lethal dose (LD₅₀) and to select the best bacterial species to challenge the fish after being fed with the experimental diets.

To evaluate the *in vivo* efficacy of the grape marc extracts in preventing fish bacterial infections, fish were challenged with *Ph. damselae* subsp. *piscicida*, one of the bacterial species inhibited by the grape functional extracts in the *in vitro* trial. Unexpectedly and contrarily to what was expected following the results of the pre-challenges, after monitoring fish survival for 7 days, no mortalities could be observed in

fish challenged with *Ph. damselae* subsp. *piscicida*, independently of the diet provided. Possible technical errors when preparing the bacterial inoculum cannot be ruled out.

Since no mortalities were observed, a secondary infection was made with an opportunistic fish pathogen, *V. anguillarum*. Although this bacterial species was not inhibited by the extracts in the *in vitro* trial, *V. anguillarum* has been the most important seabass pathogen in the past 10 years and it's responsible for high mortality rates and severe economic losses in the aquaculture industry (Frans *et al.* 2011; Mougin *et al.* 2021). Additionally, this fish pathogen is resistant to several antibiotics, reinforcing the importance of discovering new alternatives to control this disease (Kapetanović *et al.* 2022). Also, it was interesting to assess if the functional extracts increased the fish's ability to cope with two simultaneous infections.

The results of the present study showed that the grape marc extracts did not significantly improve fish survival but fish-fed diets PG45 and PG200 presented higher survival rates than fish-fed PG450 and control diets. Even though this difference is not statistically significant, these results are helpful for future studies where PG extract can be tested at higher concentrations.

On the contrary, Mehrinakhi *et al.* (2021) showed that an extract of grape seeds increased the resistance of common carp when challenged with 1×10^8 CFUs of *A. hydrophila*, after being fed diets supplemented with 3 different concentrations of grape seed extract, 10, 20, and 30 g kg⁻¹, with all three dietary concentrations showed higher survival rates than the control group (Mehrinakhi *et al.* 2021). The fact that the incorporation level of grape marc extracts used in our study is much lower than the one used in Mehrinakhi, might explain the lack of results under our experimental conditions and opens the possibility to further explore these extracts at higher incorporations.

5. Conclusions

The *in vitro* trial showed that the white grape marc extracts (AC, ET and PG) can inhibit the growth of important aquaculture pathogens, either gram-negative or gram-positive bacteria, probably due to their high concentrations of phytochemicals, specifically polyphenols.

The results of the growth trial showed that these concentrations of grape functional extracts can be used in feeds of European seabass given that they do not negatively impact fish growth performance, feed utilization, and survival. Therefore, without any negative effects found in this study, higher concentrations should be tested to assess if enhanced growth performance and feed utilization of European seabass is possible, as has been observed in other species with different grape extracts.

The *in vivo* challenge trial showed that the diets supplemented with grape marc extracts did not present statistically higher survival rates when compared to the control group but a tendency to higher survival rates in fish-fed diets PG45 and PG200 was found. Although *V. anguillarum* is a very important bacteria in European seabass production, perhaps a more sensitive bacterial species to the white grape marc extracts antibacterial activity should be used in future *in vivo* challenges.

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