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Assessing antioxidant and antimicrobial potential of brewery, winery and olive-oil byproducts extracts produced through solid state fermentation in European sea bass

João Manuel Félix Silva

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Dissertação de Mestrado apresentada à
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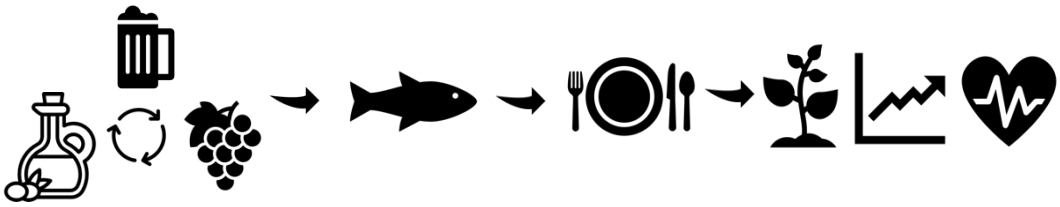

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João Manuel Félix Silva

Mestrado em Tecnologia e Ciência Alimentar

Departamento de Química e Bioquímica

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

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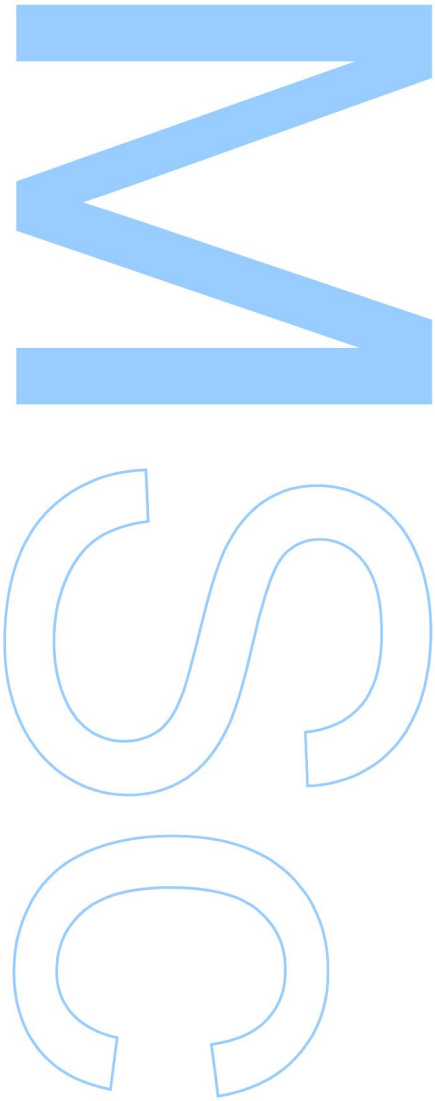

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Resumo

Com vista a colmatar a incessante procura de peixe na sociedade moderna, o desenvolvimento sustentável da aquacultura é mandatário. Para garantir a qualidade total e a segurança do consumidor dos peixes fornecidos pela aquacultura, estratégias nutricionais alternativas para o combate de doenças bacterianas são necessárias, evitando o uso de fármacos sintéticos.

Os subprodutos da agroindústria, como os das indústrias vitivinícola e da produção de azeite, constituem uma fonte de baixo custo de compostos bioativos. A fermentação em estado sólido é um processo biotecnológico que pode ser aplicado aos subprodutos para aumentar a disponibilidade dos compostos bioativos.

O objetivo deste estudo foi desenvolver um extrato enriquecido em compostos bioativos, inovador e produzido a partir de um processo de fermentação em estado sólido, com a aplicação do fungo *Aspergillus ibericus,* de subprodutos de agroindústrias locais e avaliar o seu potencial antimicrobiano contra patogénicos de peixe.

Para o efeito, fermentou-se com *A. ibericus* MUM 03.49 uma mistura de bagaço de uva, rebentos de videira e bagaço de azeitona, previamente otimizada pelo seu potencial antioxidante O potencial antimicrobiano destes extratos aquosos contra patogénicos de peixes de alta incidência na aquacultura: *Aeromonas bestiarium*, *Aeromonas bivalvium*, *Aeromonas hydrophila*, *Aeromonas salmonicida* and *Aeromonas veronii*; *Edwardsiella tarda*; *Photobacterium damselae subs. damselae* and *Photobacterium damselae subs. piscicida*; *Sigella sonnei*; *Tenacibaculum maritimum*; *Vibrio anguillarum*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*; *Streptococcus agalactiae*, *Streptococcus iniae* and *Streptococcus parauberis*, foi avaliado em ensaios de difusão em placa de petri.

A somar, um bioensaio in vivo em robalo europeu (*Dicentrarcus labrax*) teve lugar para determinar o potencial antimicrobiano contra *Photobacterium damselae* subs*. Piscicida*, assim como o efeito imuno-estimulante da incorporação dos extratos em formulações para aquacultura. O extrato da mistura otimizada não fermentada foi eficaz contra as espécies bacterianas dos gêneros *Aeromonas*, *Edwardsiella*, *Photobacterium*, *Vibrio* e *Streptococcus*, enquanto a mistura otimizada fermentada inibiu *Streptococcus iniae* e *Photobacterium damselae* subsp*. piscicida*. No decorrer do bioensaio, os robalos foram alimentados com 4 formulações diferentes durante 10 semanas, que incluíam 0 (controlo), 0.4% e 0.8% da mistura fermentada otimizada e 0.4% da mistura otimizada não fermentada.

Os peixes foram mantidos num sistema de recirculação termorregulado, e foram desafiados com uma solução de *Photobacterium damelae* subs*. piscisida* quimicamente inativada. Posteriormente, análises hematológicas e imunológicas ocorreram para determinar de forma geral a influencia dos diferentes tratamentos na saúde dos robalos.
A mistura otimizada não fermentada foi efetiva contra o desenvolvimento bacteriano da bactérias dos géneros *Aeromonas*, *Edwardsiella*, *Photobacterium*, *Vibrio* e *Streptococcus*, enquanto que a mistura fermentada otimizada foi efetiva na inibição das estirpes *Streptococcus iniae* e *Photobacterium damselae* subs*. piscicida.*
O bioensaio in vivo em robalo revelou que a incorporação do o extrato da mistura otimizada não fermentada a 0.4% resultou num aumento na contagem total de glóbulos brancos.
Pela análise da expressão dos genes imuno-modulatórios concluímos que a mistura otimizada fermentada 0.4% despoletou um aumento na expressão de citocinas proinflamatórias Casp-3 e IL-6. E por fim, a incorporação de extrato fermentado a um nível de 0,8% de incorporação diminuiu a expressão do gene Cox-2.
Em suma este trabalho sugere que extratos de subprodutos da indústria do vinho e do azeite fermentados e não fermentados foram capazes de não só demonstrar um potencial antibacteriano contra um largo espectro de microrganismos patogénicos de peixe como ainda revelarem um potencial efeito imuno-estimulante quando incorporados em dietas de peixe.

Palavras-chave: Aquacultura sustentável, ingredientes funcionais, subprodutos, fermentação no estado sólido, economia circular.

Abstract

In order to ensure the future global fish provision, sustainable development of aquaculture is mandatory. To guarantee the total quality and consumer safety of fish provided by aquaculture, alternative nutritional strategies for bacterial disease management are required, preventing the use of synthetic drugs and antimicrobial resistance development.

Agroindustry byproducts, like those from the winery and olive oil industries, constitute a low-cost source of add-value bioactive compounds.

Solid-state fermentation is a biotechnological process that may be applied to increase the availability of their bioactive compounds.

This study aimed to develop an innovative bioactive-enriched extract obtained from the solid-state fermentation with *Aspergillus ibericus* of agroindustry by-products and assay its antimicrobial potential against high incidence fish pathogens.

For that purpose, brewers spent grains and an optimized mixture of exhausted grape marc, vine trimming shoots and exhausted olive pomace that maximizes the antioxidant potential were fermented with *A. ibericus* MUM 03.49. The antimicrobial potential of these aqueous extracts against *Aeromonas bestiarium*, *Aeromonas bivalvium*, *Aeromonas hydrophila*, *Aeromonas salmonicida* and *Aeromonas veronii*; *Edwardsiella tarda*; *Photobacterium damselae* subs*. damselae* and *Photobacterium damselae* subs. *piscicida*; *Sigella sonnei*; *Tenacibaculum maritimum*; *Vibrio anguillarum*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*; *Streptococcus agalactiae*, *Streptococcus iniae* and *Streptococcus parauberis*, was addressed by well-diffusion assays. Moreover, an in vivo trial in European Sea bass (*Dicentrarchus labrax*) was conducted to assay the antimicrobial potential against *Photobacterium damselae* subs. *piscicida* and immunostimulant potential of dietary extracts incorporation in aquafeeds. European sea bass juveniles were fed with 4 different diets, including 0 (control), 0.4%, and 0.8% of the optimized fermented mixture extract and 0.4% of the optimized unfermented mixture extract), for 10 weeks. Fish were maintained in a thermoregulated water recirculation system, and then challenged by injection of a chemical inactivated *Photobacterium damselae* subs*. piscicida* solution. Posteriorly, hematological and immunological

analyses were conducted to compare the over-all fish health status after the dietary treatment.

The unfermented optimized mixture extract was effective against bacterial species from *Aeromonas*, *Edwardsiella*, *Photobacterium*, *Vibrio*, and *Streptococcus* genera, while the fermented optimized mixture inhibited *Streptococcus iniae* and *Photobacterium damselae* subsp. *piscicida* bacterial growth.

In vivo European sea bass chemically inactivated bacterial challenge, the 0.4% optimized unfermented mixture extract triggered an increment in white blood cell count. For 0.4% optimized fermented mixture, we have a registered an increase in Casp-3 and IL-6 mRNA expression. Plus, 0.8% optimized fermented mixture was found to have a decreased Cox-2 gene expression.

This study ultimately suggest that wine and olive by-product's fermented and unfermented extracts dietary supplementation may have an immunostimulant role in European sea bass, and manifest antimicrobial activity against a wide spectrum of fish pathogens.

Keywords: Sustainable aquaculture, functional ingredients, agroindustry by-products, solid state fermentation, circular economy

Scientific divulgation

Oral communication

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Table of contents

Tables list

Figures list

Abbreviations list

AMR - Antimicrobial resistance

BHI - Brain Heart Infusion medium

BSG - Brewer spent grain

COP - Crude olive pomace

EGM - Exhausted grape marc

EOP - Exhausted olive pomace

FM - Fish meal

FO - Fish oil

GM - Grape marc

GRAS - Generally regarded as safe

Hb - Hemoglobin

Ht - Hematocrit

IP - Intraperitoneal

IP - intraperitoneal

IPMA - Instituto Português do Mar e da Atmosfera

MCH - Mean corpuscular hemoglobin

MCHC - Mean corpuscular hemoglobin concentration

MCV - Mean corpuscular volume

OP - Olive pomace

PBS - Phosphate-buffered saline solution

PhDP- *Photobacterium damselae* subs*. piscicida*

RBC- Red blood cells

SSF - Solid state fermentation

VTS - Vine trimming shoots

WBC - White blood cells

WO - Antioxidant activity optimized matrix (34% EOP : 30% EGM : 36% VTS)

Introduction

Aquaculture

Nowadays, aquaculture is one of the fastest-growing animal food industries. It is an ancient activity that involves farming of aquatic organisms, inland or outland, with the possibility to manipulate production parameters, for a more intensive, profitable, controlled, and sustainable fish, mollusks, and crustaceans production (Lucas 2015).
In 2018, as represented in Table 1, a total of 156 million tonnes of fish were consumed worldwide, equivalent to an estimated annual supply of 20.5 kg per capita. More than 50 percent of the fish directly used for human consumption was supplied by aquaculture. The amount of captured fish has been relatively stable, around 90 million tonnes (live weight), since 1986. Aquaculture is now responsible for sustaining the increasing world fish demand, since the sustainable amount of captured fish has been attained a long time ago. In 2018, 38 percent of total fisheries and aquaculture production were traded internationally with a first-sale value estimated at USD 164 billion (Food and Agriculture Organization of the United Nations 2020).
To follow up on the incessant demand of fish by modern society, optimized aquaculture strategies are required to increase the total quantity and quality of fish produced.
Two of the major issues that aquaculture has to face nowadays are the development of sustainable alternatives for the heavy dependence on the fish meal (FM) and fish oil (FO) (Iii, Barrows et al. 2007, Naylor, Hardy et al. 2009), and the continuous use of chemical, including antibiotics, to prevent the occurrence of bacterial infections (Preena, Swaminathan et al. 2020).

Table 1. World fisheries and aquaculture annual production and utilization in million tonnes from 1986 to 2018 adapted from (Food and Agriculture Organization of the United Nations 2020).

	1986–1995	1996–2005	2006–2015	2016	2017	2018
	Average per year					
	(million tonnes, live weight)					
Production						
Capture						
Inland	6.4	8.3	10.6	11.4	11.9	12.0
Marine	80.5	83.0	79.3	78.3	81.2	84.4
Total capture	**86.9**	**91.4**	**89.8**	**89.6**	**93.1**	**96.4**
Aquaculture						
Inland	8.6	19.8	36.8	48.0	49.6	51.3
Marine	6.3	14.4	22.8	28.5	30.0	30.8
Total aquaculture	**14.9**	**34.2**	**59.7**	**76.5**	**79.5**	**82.1**
Total world fisheries and aquaculture	**101.8**	**125.6**	**149.5**	**166.1**	**172.7**	**178.5**
Utilization[2]						
Human consumption	71.8	98.5	129.2	148.2	152.9	156.4
Non-food uses	29.9	27.1	20.3	17.9	19.7	22.2
Population (*billions*)[3]	5.4	6.2	7.0	7.5	7.5	7.6
Per capita apparent consumption (*kg*)	13.4	15.9	18.4	19.9	20.3	20.5
Trade						
Fish exports – in quantity	34.9	46.7	56.7	59.5	64.9	67.1
Share of exports in total production	*34.3%*	*37.2%*	*37.9%*	*35.8%*	*37.6%*	*37.6%*
Fish exports – in value (*USD billions*)	37.0	59.6	117.1	142.6	156.0	164.1

[1] Excludes aquatic mammals, crocodiles, alligators and caimans, seaweeds and other aquatic plants. Totals may not match due to rounding.
[2] Utilization data for 2014–2018 are provisional estimates.
[3] Source of population figures: UN DESA, 2019.

Vegetable Lipid and Protein sources in Aquaculture

FM and FO are extensively used as the main source of protein and lipids respectively, for carnivorous aquaculture fish (Oliva-Teles 2015). However, despite the excellent nutritional profile and palatability of these ingredients, FM and FO present several socio-economic, ethical, and environmental constraints. To find an alternative and eco-friendly protein and lipid sources is required for sustainable development of carnivorous fish aquaculture, stepping towards the reduction of the wild fisheries inputs to farmed fish outputs ratio, “fish in:fish out” (Iii, Barrows et al. 2007, Naylor, Hardy et al. 2009).

Although the use of plants and plants extracts as therapeutic and nutritional agents in aquaculture has exploded recently, medicinal plants have been used by rural fish farmers in order to improve water quality, reduce fish stress, increase fish resistance to pathogens and treat fish diseases (Caruso 2013). Vegetable proteins highly reduce the carbon footprint of aquafeeds formulation, stepping towards the aquaculture´s sustainable management (Naylor, Hardy et al. 2009). Presently, the most used vegetable

ingredients for aquafeed formulation are soybeans, corn, rapeseed, wheat, barley, cottonseed, and peas/ lupins (Hardy 2010). Plentiful scientific reports support the use of plants in aquaculture and their potential to produce various effects such as stress reducer, growth promotion, appetite stimulation, immunostimulant and to have antipathogen properties in fish and shrimp aquaculture due to their varied active principles such as alkaloids, terpenoids, tannins, saponins and flavonoids (Citarasu 2010, Chakraborty, Horn et al. 2013). Specifically, olive pomace (OP) has proven to have anti-atherogenic properties in *in vitro* studies in gilthead sea bream, and a reduction in the damage in steatosis group in goldfish (*Carassius auratus*) (Nasopoulou, Gogaki et al. 2013, Alesci, Cicero et al. 2014). Grape seeds and exhausted grape marc (EGM) phenolic compounds have shown to be effective in decreasing pro-inflammatory intestinal cytokines and improvement in growth performance in European sea bass (Magrone, Fontana et al. 2016)

Besides conventional vegetable ingredients, processed agroindustry by-products can act as an eco-friendly, low-cost, and consumer-friendly alternative to FM and FO. Contributing both to zero waste and circular economy principles, and aquaculture sustainable development.

Aquaculture pathogens and antimicrobial resistance

Antimicrobial resistance (AMR) is also a growing concern in the aquaculture sector. The indiscriminate use of antimicrobial agents in aquaculture lead to the acceleration of selective pressure resulting in the emergence of AMR (Furushita, Shiba et al. 2003).
Approximately 73% of the major fish-producing countries reported to use chemically synthesized antibiotics such as oxytetracycline, florfenicol, and sulphadiazine moreover 55% applied erythromycin, amoxicillin, sulfadimethoxine, and enrofloxacin (Lulijwa, Rupia et al. 2020).
The aquaculture application of these antibiotics target several bacterial diseases frequently encountered in aquaculture, which affect successful production. Bacterial infections are mainly derived from gram-negative pathogens such as bacteria from the *Aeromonas*, *Vibrio*, *Edwardsiella*, *Flavobacterium*, *Pseudomonas*, *Photobacterium*, *Shigella,* and *Yersinia* genera; rarely by gram-positive ones such as *Streptococcus* and *Staphylococcus* (Lulijwa, Rupia et al. 2020). **Table 2** compiles the most important bacterial diseases in global aquaculture according to the Food and Agriculture

Organization of the United Nations (Toranzo, Magariños et al. 2005). In this work we are going to focus on *Aeromonas, Edwardsiella, Photobacterium, Shigella, Vibrio* and *Streptococcus* genera, which are the high incidence marine fish pathogens, and assay the antimicrobial potential of SSF and unfermented BSG and wine and olive byproducts extracts against these fish pathogens.

Table 2. Most important fish diseases affecting global aquaculture. Adapted from (Haenen 2017)

Gram-negative bacteria	Gram-positive bacteria
Vibriosis *(Vibrio. anguillarum, V. harveyi clade, V. Parahaemolyticus, V. salmonicida, V. vulnificus, Photobacterium damselae)*	Mycobacteriosis *(Mycobacterium fortuitum, M. marinum, Norcardia asteroides, N. crassostreae, N. seriolae)*
Aeromonasis *(Aeromonas. hydrophila, A. sobria, A. veronii, A. jandaei, A. salmonicida)*	Streptococcocsis *(Streptococcus agalactiae, S. iniae, Lactococcus garvieae, Aerococcus viridans)*
Edwardsiellosis *(Edwardsiella anguillarum, E. ictalurid, E. piscicida, E.tarda, Yersinia ruckeri)*	Renibacteriosis *(Renibacterium salmoninarum)*
Pseudomonasis *(Pseudomonas anguilliseptica, P. fluorescens)*	Infection with Anaerobic Bacteria *(Clostridium botulinum, Enterobacterium catenabacterium)*
Flavobacteriosis *(Flavobacterium . branchiophilum, F. columnare, F. psychrophilum, Tenacibaculum maritimum)*	
Infection with Intracellular Bacteria *(Piscirickettsia salmonis, Hepatobacter penaei, Francisella noatunensis, Chlamydia spp.)*	

Agroindustry byproducts

The valorization of agroindustry by-products is one of the directives present in the Circular Economy Action Plan, European Commission, March 2020, one of the main building blocks of the European Green Deal 2020 agenda. Give a second life to these once wasted matrixes has economic and environmental benefits for the agroindustry sector.

Reuse is the best way to reduce, not only energetic and carbon emissions responsibilities could be avoided by the biodegradation or incineration of these by-products from agroindustry processers, but also they could profit from selling them. Brewery, wine, and olive oil production constitute three of the most demarked agroindustry's in Portugal northern region, by so this work is developed in order to access the economical, nutricional and medicinal potential of these three extracts incorporation in alternative aquafeeds formulations.

Brewers' Spent Grain

Brewer Spent Grain (BSG) is by far the most abundant byproduct of the brewing industry. BSG corresponds to the insoluble part of the grains dispersed in wort following the mashing process. Wort is separated from BSG through a filtration process and then fermented into beer (Mussatto 2014). Approximately 39 million tons of BSG are produced annually and for every 100 liters of beer brewed, 20kg of BSG are produced (Lynch, Steffen et al. 2016).

BSG are the husks of barley malt grain with the pericarp and seed coat layer of these grains (Mussatto 2014). BSG is not a stable matrix, its composition varies with factures such as the mix of grains used, harvest time, and brewery conditions. Despite the variations reported, BSG is mainly constituted by fiber (30-50%) and protein (19-30%). Total essential amino acids represent 30% of the total protein, including lysine, phenylalanine, isoleucine, threonine, and tryptophan, being lysine the most abundant (14% total protein). Comparativel to other cereal matrixes, which are generally deficient in lysine, this high lysine content of BSG is of high nutritional significance (Kaur and Saxena 2004, Lynch, Steffen et al. 2016).

BSG has a high fibrous content (mainly arabinoxylan and β-glucan). In humans, β-glucan has proven to reduce LDL-cholesterol, lowering the risk of cardiovascular disease (Ho, Sievenpiper et al. 2016). In fish, β-glucan, oral administration at low concentration resulted in an immunostimulatory effect (Vetvicka, Vannucci et al. 2013). BSG is also a source of mineral elements like silicon, phosphorus, calcium, magnesium, sulfur, potassium, iron, and zinc (Lynch, Steffen et al. 2016), and phenolic compounds, like hydroxycinnamic acid (Ho, Sievenpiper et al. 2016).

Olive Pomace

The European Union will be responsible for 64% of the olive oil produced worldwide in 2020, approximately 3,14 million tones. The major world producers are located around the Mediterranean region, namely, Spain, Italy, Greece, and Portugal (IOOC, 2019). Portugal is expected to be the third higher producer by 2030 (Dawson 2020).

Olive pomace (OP) is the most abundant by-product of olive oil industry processing. This organic value-added matrix is constituted by the remaining skins, pulp, fragmented olives, and residual olive oil and mainly composed of polysaccharides, protein, and

unsaturated fatty acids like oleic acid and other C2-C7 fatty acids (Mateo and Maicas 2015). The solid matrix obtained directly from olive oil extraction is classified as crude olive pomace (COP) and has a high amount of residual lipids. A second extraction with organic solvents of residual olive oil from COP results in exhausted olive pomace (EOP) (Molina-Alcaide and Yáñez-Ruiz 2008) (Bárbara, Fernando et al. 2012) (2014). The waste of these residues represents an environmental hazard due to its organic load and the combustion processes that release toxic gases into the atmosphere (Niaounakis and Halvadakis 2006). OP is a sustainable and low-cost source of a variety of polyphenols from different classes like phenolic acids, alcohols, lignans, and flavones. In this work, we are going to use COP since it has a higher olive oil content than EOP, which makes the COP a more unstable matrix (Dal Bosco, Mourvaki et al. 2012, Terramoccia, Bartocci et al. 2013). **Table 3** compiles the proximate composition of COP and EOP.

Table 3. Proximate composition (dry matter basis) of crude and exhausted olive pomace (adapted from (Leite, Salgado et al. 2016).

Parameter	COP	EOP
Total solids (%)	26.5 ± 0.4	90.1 ± 0.1
Ash (%)	6.6 ± 0.5	3.4 ± 0.2
Lignin (%)	43.2 ± 0.5	41.62 ± 0.04
Hemicellulose (%)	22.3 ± 0.8	24.1 ± 0.2
Cellulose (%)	12.5 ± 0.9	11 ± 2
Lipids (%)	16.7 ± 0.09	4 ± 2
Proteins (mg/g)	4 ± 1	2.6 ± 0.3
Reducing Sugars (mg/g)	96 ± 6	42 ± 2
Phenols (mg/g)	8.4 ± 0.3	8.9 ± 0.2
N (%)	0.6 ± 0.1	1.27 ± 0.07
C (%)	49.7 ± 0.7	46 ± 1
Ca (g/kg)	1.16 ± 0.04	1.8 ± 0,2
K (g/kg)	17 ± 1	14.2 ± 0,7
Mg (mg/kg)	474 ± 22	473 ± 57
Zn (mg/kg)	12 ± 0	10.5 ± 0.7
Cu (mg/kg)	11.5 ± 0.7	11 ± 1
Fe (mg/kg)	42 ± 2	147 ± 33
Mn (mg/kg)	8.6 ± 0.1	10.2 ± 0.4
Cr (g/kg)	<22	<22
Ni (mg/kg)	<22	<22
Pb (mg/kg)	<22	<22
Na (mg/kg)	373 ± 35	92 ± 5

Winery by-products

Portugal is the world´s 11th wine producer with 7 million hectoliters per year (International Organization of Vine and Wine 2019). The most relevant solid by-products derived from the winery are grape stalk, grape leaves, grape pomace, grape marc (GM), and vine trimming shots (VTS) (Oliveira, Salvador et al. 2013). Grape marc is essentially grape peels (65%) and seeds, that result from the pressing process in winemaking (Mateo and Maicas 2015). Most, the liquid resultant form grapes physical processing, constitutes only by 70 % of the grapes total volume while 30 % will result in grape marc (Devesa-Rey, Vecino et al. 2011). European regulations prohibit the discard of GM directly to the environment without being processed in alcohol distilleries and finally converted into

exhausted grape marc (EGM). The main applications for EGM nowadays are the use as fertilizers and charcoal (Devesa-Rey, Vecino et al. 2011).

VTS are the lignocellulosic materials that result from the pruning of the vine, usually, it is burned only to clean the wine producing fields, making it one of the major contributors to the wine footprint (Devesa-Rey, Vecino et al. 2011). VTS is mainly constituted by cellulose, hemicellulose, and lignin. The minor fraction of VTS is constituted by phenolic and volatile compounds with greater importance for the industry, such as stilbenes and other molecules with anti-oxidant potential like catechin, ellagic acid trans-resveratrol, and ferulic acid (Cebrián, Sánchez-Gómez et al. 2017).

EGM is usually generated in a distillery after washing to extract the remaining alcohol and tartrates. As VTS, EGM is mainly a hemicellulose matrix that can act as a source of xylose and glucose with a very high content of phenolic compounds with antioxidant properties such as tannins, anthocyanins, stilbenes, and flavanols (Devesa-Rey, Vecino et al. 2011, Kennedy JA (2002)). **Table 4** compiles the proximate composition of EGM and VTS.

Table 4. Proximate composition (dry matter basis) of VTS and EGM, adapted from (Salgado, Abrunhosa et al. 2013).

Parameter	Vine trimming shoots	Exhausted grape marc
Klaxon lignin (%)	37.34 ± 0.02	57.67 ± 0.01
Hemicellulose (%)	9.73 ±0.01	5.84 ± 0.01
Cellulose (%)	29.56 ± 0.03	14.37 ± 0.01
Lipids (mg/g)	29.6 ± 0.00	21.3 ± 0.00
Reducing Sugars (mg/g)	55.35 ± 0.05	3.00 ± 0.01
Phenols (mg/g)	1.25 ± 0.04	0.19 ± 0.01
C (g/kg)	453.56 ± 2.21	482.37 ± 16.07
N (g/kg)	5.62 ± 0.71	16.97 ± 6.26

Solid-State Fermentation

Solid-state fermentation (SSF) is defined as the fermentation of a solid dry matrix (inert support) in a system with the absence, or near absence, of free water. The substrate has to have the required nutritional conditions and enough moisture to grantee the microorganism growth (Pandey 2003). The advantages of this technique, when

compared with traditional fermentation, is the reduced amount of water required, the large volume of organic matter processed and overall lower energy expenditure (Salgado, Abrunhosa et al. 2014, Moccia, Flores-Gallegos et al. 2019).

Different species and strains of microorganisms could be recruited for the SSF process, for different applications such as bioremediation, production of biofuels, and production or extraction of antibiotics, organic acids, enzymes, biopesticides, bioherbicides, biosurfactants, aromatic and bioactive compounds with potential application in food and agroindustry (Salgado, Abrunhosa et al. 2014).

The ideal microorganism to be applied in SSF are those that can grow on cheap substrates, that are susceptible to genetic manipulation and biosafe. Since the SSF has a low level of free water, actinomyces and fungi are the most used due to their ability to propagate in these conditions (Fleuri, Kawaguti et al. 2013).

In this work, we are going to assess the potential of solid state fermented Portuguese agroindustry by-products by *Aspergillus ibericus spp.* MUM 03.49, a GRAS (generally regarded as safe) fungi previously isolated from wine grapes by Serra et al. (2016) (Serra, Cabanes et al. 2006), to be incorporated in European sea bass aquafeeds as functional ingredients.

Objective

The present study aims to assay the potential of *Aspergillus ibericus* solid-state fermented extracts of brewery, winery, and olive oil industry byproducts incorporation as functional ingredients in aquafeeds formulations for European sea bass. This analysis will compile well-diffusion assays to access in vitro antimicrobial potential of the byproducts extracts against high incidence fish pathogens, and an in vivo trial, to access the influence of dietary supplementation with solid-state fermented extracts in immunological overall status and in vivo antimicrobial potential in European sea bass.

Material and Methods

Agriculture by-products

Portuguese agroindustry byproducts that were the object of study are compiled in **Table 2**. BSG was kindly provided by Unicer, Bebidas de Portugal SGPS SA (Matosinhos, Portugal). This matrix was collected after the filtration of must, and results from a mixture of Pilsen malt, barley, and corn grits.

EOP, prevenient from the extraction of residual olive oil from the olive pomace, EGM, the solid matrix left from grape marc distillation, and VTS, vine shoots resultant from wine pruning were collected from wineries and olive oil industries in the north of Portugal during the 2017 season.

A mixture of the three agroindustry byproducts (WO), 30%EGM, 36%VTS, and 34%EOP was previously optimized by a simplex-centroid mixture design to maximize production of bioactive compounds (Filipe 2019), was also used in this study.

Table 2. Portuguese agroindustry byproducts matrixes that will be the object of study.

BSG	Brewer´s Spent Grains
EOP	Exhausted Olive Pomace
EGM	Exhausted Grape Marc
VTS	Vine Trimming Shoots
WO	Antioxidant activity optimized matrix (34% EOP : 30% EGM : 36% VTS)

Solid-State Fermentation (SSF)

For the BSG and WO SSF, *Aspergillus ibericus* from Micoteca da Universidade do Minho (MUM: reference: *Aspergillus ibericus* MUM 03.49) preserved in glycerol at -80 ºC was cultured in malt extract agar plates (MEA: 20 $g.L^{-1}$ malt extract, 1 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ glucose and 20 $g.L^{-1}$ agar).

SSF was carried out in 500 ml plugged Erlenmeyer flasks, with 10 g of substrate with 75% humidity and a 15 carbon: nitrogen ratio normalized with urea. Flasks with substrate were sterilized at 121ºC for 15 minutes and inoculated with *Aspergillus ibericus*. Inoculation was made using a spore solution (0.1%peptone and 0.01% Tween-80). Fungal concentration was adjusted to 10^6 spores/ml in a final volume of 2 ml. To the

control flasks, 2 ml of the peptone and Tween-80 solution was added. SSF occurred at 25º C for 7 days for SSF to occur.

Aqueous Extraction

After SSF of the extracts, produced enzymes and antioxidant compounds were aqueous extracted. For that, the fermented product was washed with distilled and sterilized water (solid/water of 1:5 w/v) for 30 minutes, with constant agitation, at room temperature. Solid was then filtered through a fine-mesh net and centrifuged at 11200 g for 10 minutes at 4ºC. The resulting SSFed extracts from this filtration process were stored at -20ºC.

Lyophilization of SSFed extract

In a glass square glass container (15x15 cm base) were placed 200 ml of solid state fermented extract and frozen at -80ºC. Then, lyophilization was carried out at -51± 1ºC and a vacuum pressure between 30 and 50 mTorr until visually confirmed widespread lyophilization. Lyophilized extract was stored at 4ºC in absence of light.

Total Phenolic content determination

Total phenolic content was determined for each aqueous extract in order to normalize posterior antimicrobial activity well-diffusion assays.
The total phenolic concentration was determined by the Folin-Ciocalteau method (Commission Regulation (EEC) No. 2676/90), using a standard curve of gallic acid solution in methanol/water, 50/50, v/v. For the analysis, 20 µL of sample standard, 100 µL of Folin-Ciocalteau previously diluted (1:10 H_2O), and 80 µL of Na_2CO_3 75 $\mu g.mL^{-1}$ were left to react in absence of light for 30 min at 40ºC and the absorbance was read at 750 nm in a Multiscan GO microplate reader (model 5111 9200; Thermo Scientific, Nanjing, China).

Microbiology

Well diffusion assays

Antimicrobial potential of WO and BSG fermented and unfermented (WO_F, WO_U, BSG_F and BSG_U) extracts were assayed by well diffusion assays. Each microorganism was tested in previously optimized growth conditions, described in Table 3.

Aeromonas bestiarium, Aeromonas bivalvium, Aeromonas hydrophila, Aeromonas salmonicida and *Aeromonas veronii; Edwardsiella tarda; Photobacterium damselae* subs. *damselae* and *Photobacterium damselae* subs. *piscicida; Sigella sonnei; Tenacibaculum maritimum; Vibrio anguillarum, Vibrio harveyi, Vibrio parahaemolyticus,* and *Vibrio vulnificus; Streptococcus agalactiae, Streptococcus iniae* and *Streptococcus parauberis* previously isolated colonies were was inoculated in 5 ml of bacteria growth medium, Bacto™ Brain Heart Infusion (BHI), Sparks, USA or Bacto™ Marine Broth, Sparks, USA for 24 or 48 hours at 25 or 37º with constant agitation at 120 rpm.

Inoculum concentration was adjusted to an optical density (λ=600nm) of 0.05 and swabbed in a 10 cm diameter Petri dish with approximately 20 ml of growth medium and 1,6% (m/m) agar. Six wells of 70 mm diameter were spread on the Petri dishes using a sterilized 1000µL micropipette tip, 100µL of 200 $mg.ml^{-1}$ WO_F, 208 $mg.ml^{-1}$ WO_U, 184 $mg.ml^{-1}$ BSG_F and 173 $mg.ml^{-1}$ BSG_U extracts filtered extract (0.2 µm) solution was added to the wells. The bacteria have grown for more 24 or 48 hours at 25 or 37ºC in contact with the extract's solution.

All tests were performed in triplicate, except for bacteria from the *Vibrio* genera which were performed in duplicate. For negative control, 100 µL of sterilized water was used. The results were visually analyzed and registered by images taken in a ChemiDoc™ Gel Imaging System (BioRad).

Table 3. Bacterial strains and growth conditions.

Bacterial Species	Medium	Time/h	Temp./ºC	Strain	Source
Aeromonas bestiarum	Brain Heart Infusion	24	25	Fish isolate	NUTRIMU collection
Aeromonas bivalvium	Brain Heart Infusion	24	25	Fish isolate	NUTRIMU collection
Aeromonas hydrophila	Brain Heart Infusion	24	25	Fish isolate	BCCM/LMG
Aeromonas salmonicida	Brain Heart Infusion	24	25	LMG 3780	BCCM/LMG
Aeromonas veronii	Brain Heart Infusion	24	25	Fish isolate	NUTRIMU collection
Edwardsiella tarda	Brain Heart Infusion	48	37	LMG 2793	BCCM/LMG
Photobacterium damselae subs. Damselae	Brain Heart Infusion	24	25	LMG 7892	BCCM/LMG
Photobacterium damselae subs. Piscicida	Brain Heart Infusion	48	25	*Lg*$_{h41/01}$	*
Shigella Sonnei	Brain Heart Infusion	24	37	LMG 10473	BCCM/LMG
Tenacibaculum maritimum	Marine Broth	48	25	LMG 11612	BCCM/LMG
Vibrio anguillarum	Brain Heart Infusion	24	25	DSM 21597	DSMZ
Vibrio harveyi	Brain Heart Infusion	24	25	Fish isolate	NUTRIMU collection
Vibrio parahaemolyticus	Brain Heart Infusion	24	25	LMG 2850	BCCM/LMG
Vibrio vulnificus	Brain Heart Infusion	24	25	LMG 13545	BCCM/LMG
Streptococcus agalactiae	Brain Heart Infusion	48	37	LMG 15977	BCCM/LMG
Streptococcus iniae	Brain Heart Infusion	24	37	LMG 14520	BCCM/LMG
Streptococcus parauberis	Brain Heart Infusion	24	37	LMG 12174	F. Tavares

Bacterial strains were obtained from bacterial collections (BCCM/LMG, Belgian Coordinated Collections of Microorganisms, Laboratory of Microbiology, Department of Biochemistry and Microbiology, Faculty of Sciences of Ghent University, Ghent, Belgium; DSMZ, DSM Collection, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; CECT, Spanish Type Culture Collection, Valencia, Spain), from our laboratory stocks (NUTRIMU collection) or kindly supplied by F. Tavares (Centro de Investigação em Biodiversidade e Recursos Genéticos) and M.A. Morinigo (Universidad Málaga).

*Diaz-Rosales, P., Chabrillon, M., Morinigo, M.A. & Balebona, M.C. (2003). Survival against exogenous hydrogen peroxide of *Photobacterium damselae* subsp. *piscicida* under different culture conditions. *J Fish Dis,* 26**:** 305-8. https://doi.org/10.1046/j.1365-2761.2003.00455.x.

Inactivated *Photobacterium damselae* subs. piscicida.

For the European sea bass chemically inactivated bacterial challenge, a chemically inactivated *Photobacterium damselae* subs. *piscicida* (PhDP) solution was prepared. An isolated colony of PhDP was inoculated in 2L of BHI medium and grown for 48 hours. Then, formaldehyde was added to the growth medium (39% w:v, VWR chemicals, Prolab, EU) to a 2% v/v solution and left over-night at room temperature to chemically inactivate the bacteria. Then the inactivated bacterial culture was centrifuged at 6500 g for 30 min at 4ºC, resuspended and washed 3 times with phosphate-buffered saline solution (PBS) solution, and centrifuged in the same conditions. Finally, inactivated culture was resuspended to a final concentration of 10^8 CFU/ml in PBS and kept at -80ºC until the European sea bass challenge.

In vivo European sea bass feeding trial

Aquafeed formulation

To assess the potential of WO extract as functional ingredients for European sea bass (*Dicentrarchus labrax*) aquafeeds. A total of four diets were formulated, as described in **Table 4**. A non-supplemented diet was used as control; test diets were made equally to control diet but including 0.4 and 0.8% of fermented WO (diets WO_F 0.4 and WO_F 0.8 respectively) or 0.2% of unfermented WO, adjusted to have the same antioxidant level of diet WO_F0.4 (diet WO_U 0.4). Dietary ingredients were pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA), through a 3 mm die. Pellets were dried in a drying oven for 48 h at 60ºC and stored at -20°C temperature in plastic containers until used. The diets were tested in triplicate in European sea bass.

Table 4. Composition (% dry matter) and proximate analyses of the experimental diets.

Diet	CTRL	WO_F 0.4	WO_F 0.8	WO_U 0.4
Ingredients (% dry matter)				
Fish meal [1]	20	20	20	20
CPSP[2]	5	5	5	5
Wheat gluten[3]	12.1	12.1	12.1	12.1
Soy protein concentrate [4]	11.4	11.4	11.4	11.4
Sunflower meal[5]	8.5	8.5	8.5	8.5
Rice bran[6]	10	10	10	10
Rapeseed meal[7]	8	8	8	8
Whole-wheat meal[8]	5.4	5.4	5.4	5.4
Hemoglobin AP310[9]	3	3	3	3
Hydrolyzed shrimp[10]	1.2	1.2	1.2	1.2
Fish oil[11]	11.2	11.2	11..2	11.2
Vitamin premix[12]	1	1	1	1
Mineral premix[13]	1	1	1	1
Choline chloride (50%)[14]	0.5	0.5	0.5	0.5
Betaine[15]	0.2	0.2	0.2	0.2
Binder [16]	1	1	1	1
Taurine [17]	0.5	0.5	0.5	0.5
WO_F extract		0.34	0.68	
WO_U extract				0.19
Proximate Composition (dry matter basis)				
Dry matter (%)	95.8	94.8	95.6	94.4
Crude protein	50.3	50.1	50.0	49.6
Crude lipid	17.2	16.3	16.2	16.6
Ash	7.5	7.3	7.7	7.6
NFE[18]	25.0	26.3	26.1	26.2

[1]Pesquera Centinela, Steam Dried LT, Chile (CP: 79.7; CL: 10,5). Sorgal, S.A. Ovar, Portugal.
[2] Soluble fish-protein concentrate (CP: 80.2%; CL: 15.40%). Sopropeche, France
[3] Wheat gluten (CP: 80%; CL: 1.74%), Sorgal, S.A. Ovar, Portugal.
[4] Soy protein concentrate (CP: 48.57%; CL: 2.52%), Sorgal, S.A. Ovar, Portugal.
[5] Sunflower (CP: 40.4%; CL: 1.0%), Sorgal, S.A. Ovar, Portugal.
[6] Rice bran (CP: 14.20%; CL: 13.20%), Sorgal, S.A. Ovar, Portugal.
[7] Rapeseed (CP: 41.14%; CL: 5.80%), Sorgal, S.A. Ovar, Portugal.
[8] Whole-wheat (CP: 12.23%; CL: 3.19%), Sorgal, S.A. Ovar, Portugal.
[9] Hemoglobin powder AP310P; APC Europe S.A.
[10] Hydrolyzed shrimp (CP: 69.8%; CL: 12.1%), Sorgal, S.A. Ovar, Portugal.
[11] Fish oil, Sorgal, S.A. Ovar, Portugal.
[12]Vitamin premix (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[13]Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc

oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).
[14]Choline chloride (50%), Sorgal, S.A. Ovar, Portugal.
[15] Betaine, Sorgal, S.A. Ovar, Portugal.
[16] Binder, Aquacube. Agil, UK.
[17]Taurine, Sorgal, S.A. Ovar, Portugal.
[18]NFE (Nitrogen free extract) = 100 − (crude protein + crude lipid + ash)

Fish trials and experimental design

European sea bass juveniles were provided by Instituto Português do Mar e da Atmosfera (IPMA) pilot aquaculture facility at Olhão (Algarve, Portugal) in early 2019 and transported to Marine Zoological Station (Foz do Douro, Portugal), where they were acclimated to the experimental conditions for 4 weeks while fed with a commercial diet suitable for European sea bass juveniles (AquaGold, Aquasoja; Sorgal, S.A., Portugal) in seawater.

Groups of 18 juveniles were established, with an average individual body weight of 23.0 ±1 g, and randomly distributed in 18 fiberglass tanks of 100 L water capacity in a thermoregulated recirculation water system. Growth trial was conducted for 66 days and according to the European Union Directive (2010/63/EU) on the protection of animals for scienti*fic* purposes. During this period, fish were hand-fed twice a day, six days a week until apparent satisfaction. Water temperature was stabilized at 23± 1 ºC, salinity kept at 35± 1 ‰, nitrogenous compounds below 0.02 mg.L^{-1}, dissolved oxygen was kept near saturation, and a photoperiod of 12h light: 12h dark regime provided by artificial light.

Chemically inactivated *Photobacterium damselae* subs. *piscicida* challenge

After 66 days, 8 fish per tank were randomly selected and separated for being challenged, which result in 24 fish per treatment, fish were anesthetized by immersion in 2-phenoxyethanol (1500 ppm; Sigma) and intraperitoneal (IP) injected with either 2 ml of $1x10^8$ inactivated CFUs of *PhDP* /ml PBS solution or strictly PBS control solution. Then, fish were reallocated in duplicate tanks in the same recirculated seawater system according to dietary treatments and stimuli.

Sampling

Six fish from each tank were randomly sampled at 4 and 24 h after IP injection. Blood was collected from the caudal vein using heparinized syringes and placed in heparinized tubes for hematocrit (Ht), hemoglobin (Hb), and hematological profile determination. The

remaining blood was centrifuged (10000 g for 10 min at room temperature) and plasma collected and stored at -20ºC until analysis of innate humoral parameters. After blood collection, fish were euthanized with a sharp blow in the head. Distal intestines were collected and preserved in individual Eppendorf's containing RNA later and frozen at -80ºC until gene expression analysis.

Hematological Profile

Fresh blood collected from the caudal vein using heparinized syringes was used for Ht and Hb determination. Ht value was determined by microcentrifugation (10000 g for 10 min, at room temperature) and Hb was determined using Drabkin's solution (Spinreact, ref. 1001230; Girona, Spain). Hematological profile also entailed total white (WBC) and red blood (RBC) cells count. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated from WBC, RBC, HT, and Hb values, using the following formulas:

-MCV (μm^3) =(Ht/RBC) x 10

-MCH (pg $cell^{-1}$)= (Hb/RBC) x10

-MCHC (g 100 ml^{-1})= (Hb/Ht) x100

Gene expression analysis

Analyses of mRNA levels were performed on distal intestine samples (n=6 per treatment). Total RNA was extracted using a commercial kit (Bio-RAD Direct-zol™ RNA Miniprep) accordingly to the manufacture recommendations, eluted in 50 µL RNase/DNase free water, and stored at -80ºC.

RNA concentration purity and integrity of each sample were assayed by spectrophotometry (µDrop™ plate, ThermoScientific). For each total RNA sample, the concentration was measured in duplicate on 1.2 µL aliquots. For synthesis of the first-strand cDNA, a commercial kit was used (the NZY First-Strand cDNA Synthesis Kit [nzytech. MB12501]. Resultant cDNAs were checked by conventional PCR and stored at -20ºC.

Gene expression levels were determined by real-time quantitative PCR analysis (CFX Connect™ Real-Time System, Bio-Rad, California, USA). Analysis was carried out with 0.4 µL diluted cDNA (1:8), 0,2 µL of 10µM primer solution, 5 µL SsoAdvanced Universal

SYBR® Green supermix (Bio-Rad), and DNase/RNase-free water until a total volume of 10 µL. Primers, found in the literature are compiled in Table 8.

Table 5. List of primers used for the mRNA expression analysis, with the corresponding annealing temperature and primer efficiencies.

Gene	Primer	Primer Sequence	Annealing Temperature	Efficiencies	Reference
18S rRNA	18S-DI-F2	AGGGTGTTGGCAGACGTTAC	65º	1.91	Sepulcre et al., 2007
	18S-DI-R2	CTTCTGCCTGTTGAGGAACC			
Elongation factor 1α	EF1α-DI-F	GCTTCGAGGAAATCACCAAG	60º	1.97	Sepulcre et al., 2007
	EF1α-DI-R	CAACCTTCCATCCCTTGAAC			
Tumor necrosis factor α	TNFα-DI-F2	AGCCACAGGATCTGGAGCTA	60º	2,00	Sepulcre et al., 2007
	TNFα-DI-R2	GTCCGCTTCTGTAGCTGTCC			
Interleukin-1β	IL1β-DI-F1	ATCTGGAGGTGGTGGACAAA	60º	2.01	Sepulcre et al., 2007
	IL1β-DI-R1	AGGGTGCTGATGTTCAAACC			
Caspase 3	Casp3-DI-F	CTGATTTGGATCCAGGCATT	65º	1.96	Sepulcre et al., 2007
	Casp3-DI-R	CGGTCGTAGTGTTCCTCCAT			
Caspase 9	Casp9-DI-F	GGCAGGACTCGACGAGATAG	65º	2,04	Sepulcre et al., 2007
	Casp9-DI-R	CTCGCTCTGAGGAGCAAACT			
Ciclo-oxigenase 2	COX2-DI-F1	AGCACTTCACCCACCAGTTC	60º	1.98	Sepulcre et al., 2007; Román et al., 2013
	COX2-DI-R1	AAGCTTGCCATCCTTGAAGA			
Interleukin 6	IL6-DI-F	ACTTCCAAAACATGCCCTGA	60º	1,99	Sepulcre et al., 2007; Román et al., 2013
	IL6-DI-R	CCGCTGGTCAGTCTAAGGAG			
Interleukin 8	IL8-DI-F	GTCTGAGAAGCCTGGGAGTG	60º	1.91	Sepulcre et al., 2007
	IL8-DI-R	GCAATGGGAGTTAGCAGGAA			
Melanocortin Receptor 2	MC2R-DI-F	TTGCAGTGGACCGTTACATC	52º	1.97	Agullero et al., 2013
	MC2R-DI-R	GGCAACGAAGCAGATCATGA			

Primer efficiency was validated with serial two-fold dilutions of cDNA and calculated from the regression line slope of the quantification cycle vs. relative cDNA concentration (Pfaffl 2001). Primers efficiency were accepted between 83 and 115% (slope -3.6 and -3.0, 1.1 $>r^2>0.98$). Annealing temperature was determined through a temperature gradient. Incubation temperature was 95ºC for 30s for iTaq^{TM} DNA polymerase

activation. A total of forty steps were performed, with denaturing temperature of 95ºC for 15 sec, and 30 s at 52º/60º or 65º C for annealing. A final extension step at 60º for 30 sec was carried out. The results were normalized using two previously validated house-keeping genes, 18S ribosomal RNA (18s) and elongation factor 1α (ef1α) genes. The expression levels are given as mean normalized values ± standard error (SE) corresponding to the ratio between Cq value of the target gene transcripts and the geometric mean of Cq values of reference genes ef1α and 18s, according to (Vandesompele et al., 2002)).

Statistical analysis

Gene expression data are presented as fold change levels (means ± SD), calculated by dividing each parameter value from fish IP injected with chemically inactivated Phdp by the mean value from control fish, IP injected with PBS. Data were analyzed by a two-way ANOVA, with time and diet as factors. When significant interaction between factors was found, one-way ANOVA was performed for each factor. Both procedures were followed by Tukey post hoc test to identify differences in the experimental treatments. For all data, the probability level for rejection of the null hypothesis was 0.05. Previously to ANOVA, data were tested for normality and homogeneity (Shapiro-Wilk and Leven' s tests, respectively) and when necessary transformed to achieve ANOVA assumptions. All statistical analyses were performed using the computer package SPSS 26 software package (SPSS® Inc.) for windows.

Results

Antimicrobial assays

Total phenolic content of WO_F, WO_U, BSG_F, and BSG_U determined by Folin-Ciocalteau method and expressed in mg gallic-acid. mg^{-1} is compiled in **Table 6**. The BSG extracts used in the bioanalysis had a slightly superior total phenolic content when compared with the WO extracts. The unfermented extracts tended to have higher phenolic content than the fermented ones for the same matrix.

Table 6. Total phenolic content of WO_F, WO_U, BSG_F, and BSG_U extracts.

Extract	Total Phenolic Content (mg gallic-acid. mg^{-1})	Concentration of the lyophilized extract ($mg.ml^{-1}$)
WO_F	52.2	208
WO_U	54.7	200
BSG_F	60.3	184
BSG_U	65.0	173

Total phenolic content of WO_F, WO_U, BSG_F, and BSG_U extracts was determined by the Folin-Ciocalteau method, using a standard curve of gallic acid solution in methanol/water, 50/50, v/v. The total phenolic content values are expressed in means of 3 independent dilutions for each extract in gallic-acid $mg.mg^{-1}$. Plus, the concentration of the lyophilized extract to be used to normalize the posterior antimicrobial bioassays is also described in mg of solid per ml of sterilized water.

Well-Diffusion Assays

Antimicrobial potential of WO_F, WO_U, BSG_F, and BSG_U extracts against bacteria from *Aeromonas*, *Edwardsiella*, *Photobacterium*, *Shigella*, *Tenacibaculum*, *Vibrio,* and *Streptococcus* genus are visually compiled from Figure 1 to 7. In Table 10 are listed the measured inhibition halos from 3 independent bioassays, except for bacteria from the Vibrio genera which are prevenient from 2 independent bioassays, represented in mean ± SD form.

BSG_F and BSG_U extract solutions were ineffective against all 17 pathogens in the respective concentrations tested. On the other end, WO_U extract was effective against *Aeromonas bestiarium, Aeromonas hydrophila, and Aeromonas bivalvium; Edwardsiella tarda; Photobacterium damselae* subs. *damselae* and PhDP*; Tenacibaculum maritimum; Vibrio anguillarum, Vibrio harveyi, Vibrio parahaemolyticus,* and *Vibrio vulnificus and Streptococcus agalactiae.* WO_F extract has shown antimicrobial potential against *Streptococcus iniae, and* PhDP*.*

The highest inhibition halos were found for *Photobacterium damselae* subs. *piscicida,* Figure 3, both for WO_F and WO_U extracts (1,17 ± 0,06 cm and 1,27 ± 0,06 respectively). By so, the potential of both extract, WO_F, and WO_U extracts, were chosen to test their immunomodulation effect in European sea bass challenged by PhDP chemically inactivated bacterial challenge after being fed with different levels of incorporation of WO fermented and unfermented extracts for 10 weeks.

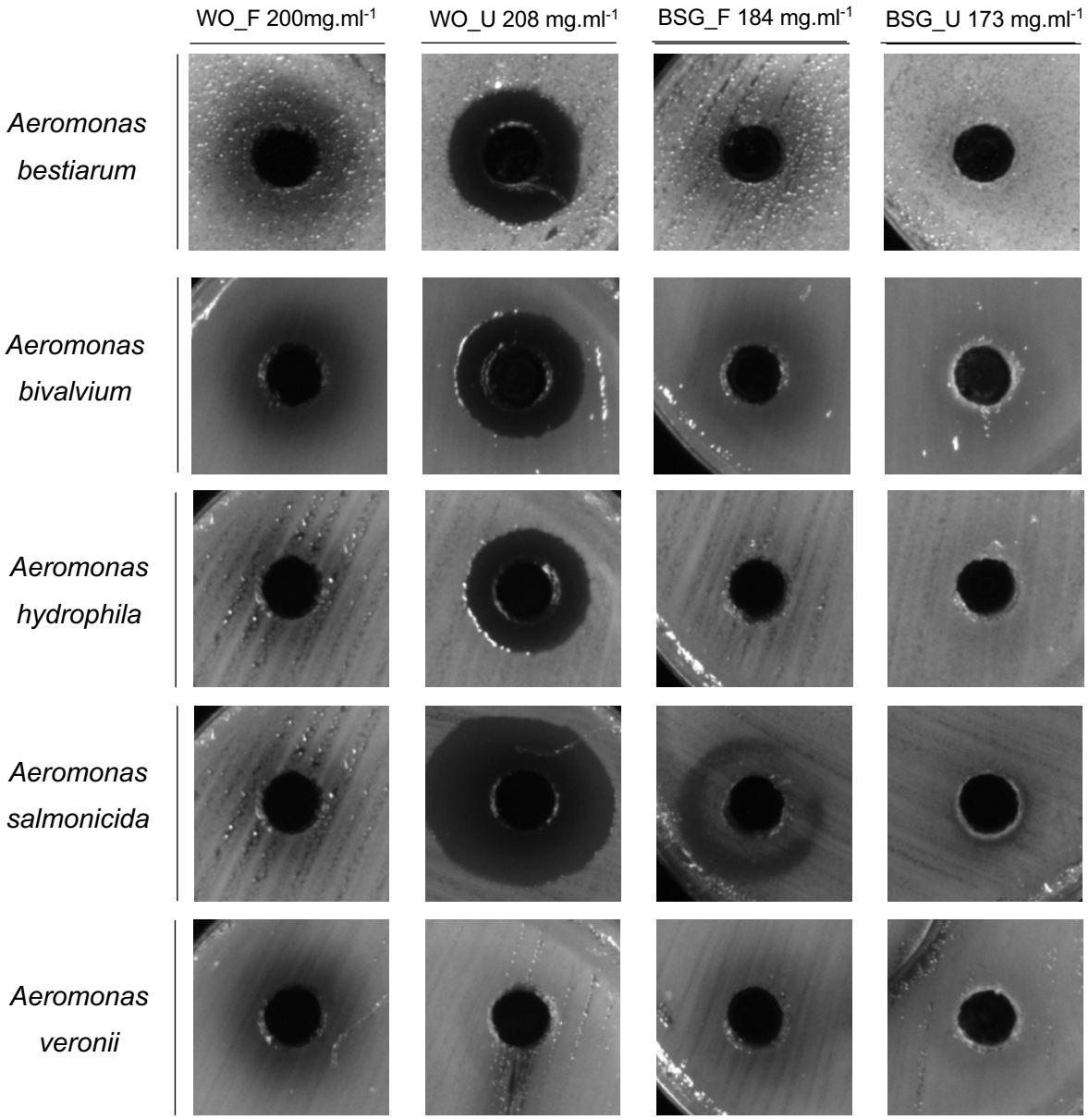

Figure 1. Well diffusion assays of 100 µL of 200 mg.ml^{-1} WO_F, 208 mg.ml^{-1} WO_U, 184 mg.ml^{-1} BSG_F and 173 mg.ml^{-1} BSG_U extracts in BHI plates. *Aeromonas bestiarium, Aeromonas bivalvium, Aeromonas hydrophila, Aeromonas salmonicida and Aeromonas veronii* isolated colonies were inoculated in 5ml BHI medium, grown for 24 hours at 25ºC at constant agitation of 120rpm. The inoculum was adjusted to an optical density of 0,05, spread by swab technique and grown for 24h at 25ºC in contact with the water diluted aqueous extracts.

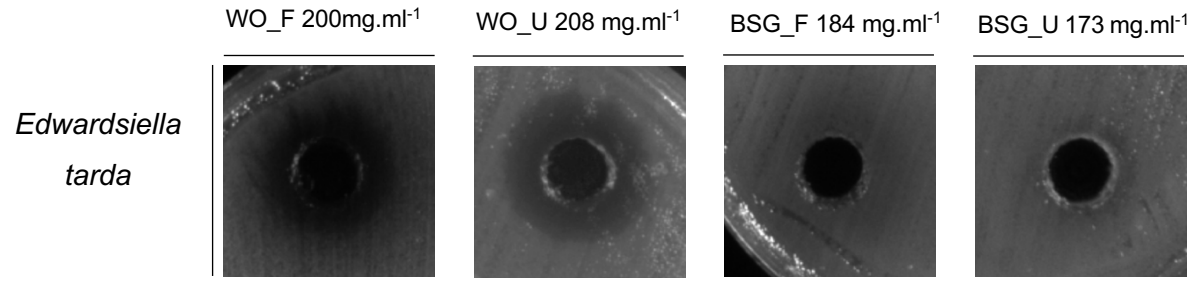

Figure 2. Well diffusion assays of 100 µL of 200 mg.ml^{-1} WO_F extract, 208 mg.ml^{-1} WO_U extract, 184 mg.ml^{-1} BSG_F and 173 mg.ml^{-1} BSG_U extract against *Edwardsiella tarda* in BHI plate. *Edwardsiella tarda* isolated colony was inoculated in 5ml BHI medium, grown for 48 hours at 37º with constant agitation at 120 rpm. The inoculum was adjusted to an optical density of 0,05, spread by swab and grown for 48h at 37º in contact with the water diluted aqueous extracts.

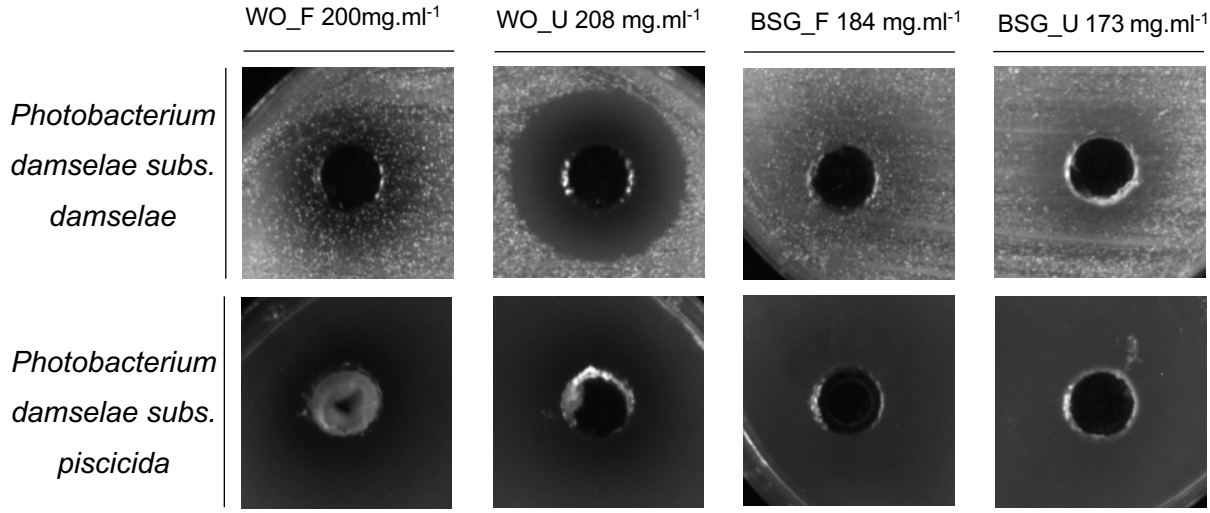

Figure 3. Well diffusion assays of 100 µL of 200 mg.ml^{-1} WO_F, 208 mg.ml^{-1} WO_U, 184 mg.ml^{-1} BSG_F and 173 mg.ml^{-1} BSG_U extracts against *Photobacterium damselae subs.damselea and Photobacerium damselae subs piscicida* in BHI. Both bacteria were separately inoculated in 5 ml BHI medium, grown for 24 and 48h respectively at 25 º C at 120rpm. The inoculum was adjusted to an optical density of 0,05 spread by swab and grown for 24 and 48h at 25º respectively in contact with the water diluted extracts.

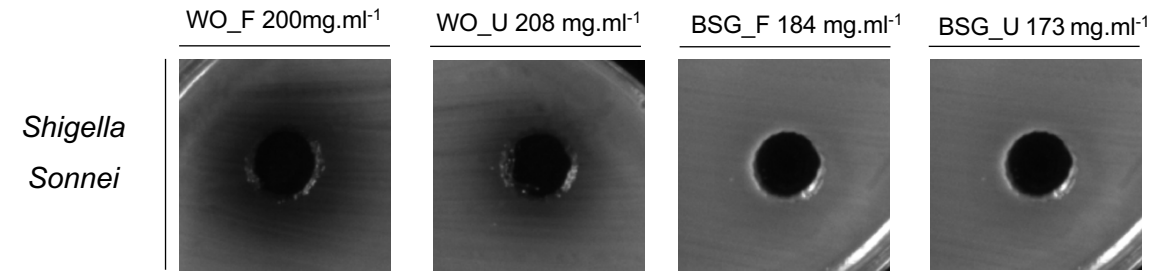

Figure 4. Well diffusion assays of 100 µL of 200 mg.ml^{-1} WO_F extract, 208 mg.ml^{-1} WO_U extract, 184 mg.ml^{-1} BSG_F and 173 mg.ml^{-1} BSG_U extract against *Shigella Sonnei* in BHI plates. *Shigella Sonnei* isolated colony was inoculated in 5ml BHI medium, grown for 24 hours at 37º with constant agitation at 120rpm. The inoculum was adjusted to an optical density of 0,05, spread by swab and grown for 24h at 37º in contact with the water diluted aqueous extracts.

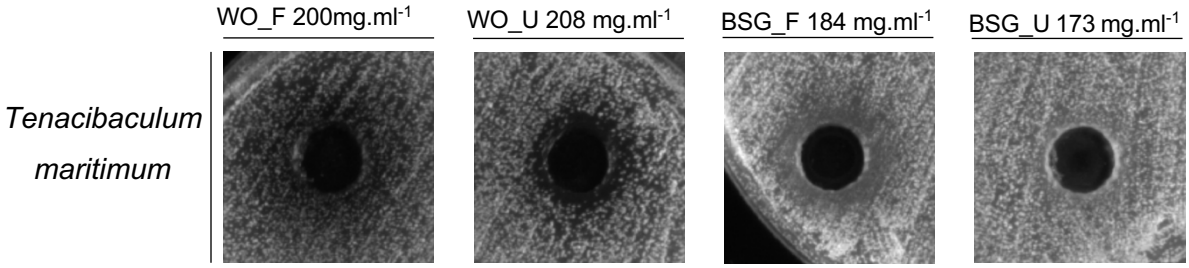

Figure 5. Well diffusion assays of 100 µL of 200 mg.ml^{-1} WO_F extract, 208 mg.ml^{-1} WO_U extract, 184 mg.ml^{-1} BSG_F and 173 mg.ml^{-1} BSG_U extract against *Tenacibaculum maritimum* in Marine Broth plates. *Tenacibaculum maritimum* isolated colony was inoculated in 5ml Marine Broth medium, grown for 24 hours at 25ºC with constant agitation at 120 rpm. The inoculum was adjusted to an optical density of 0,05, spread by swab and grown for 48h at 25ºC in contact with the water diluted aqueous extracts.

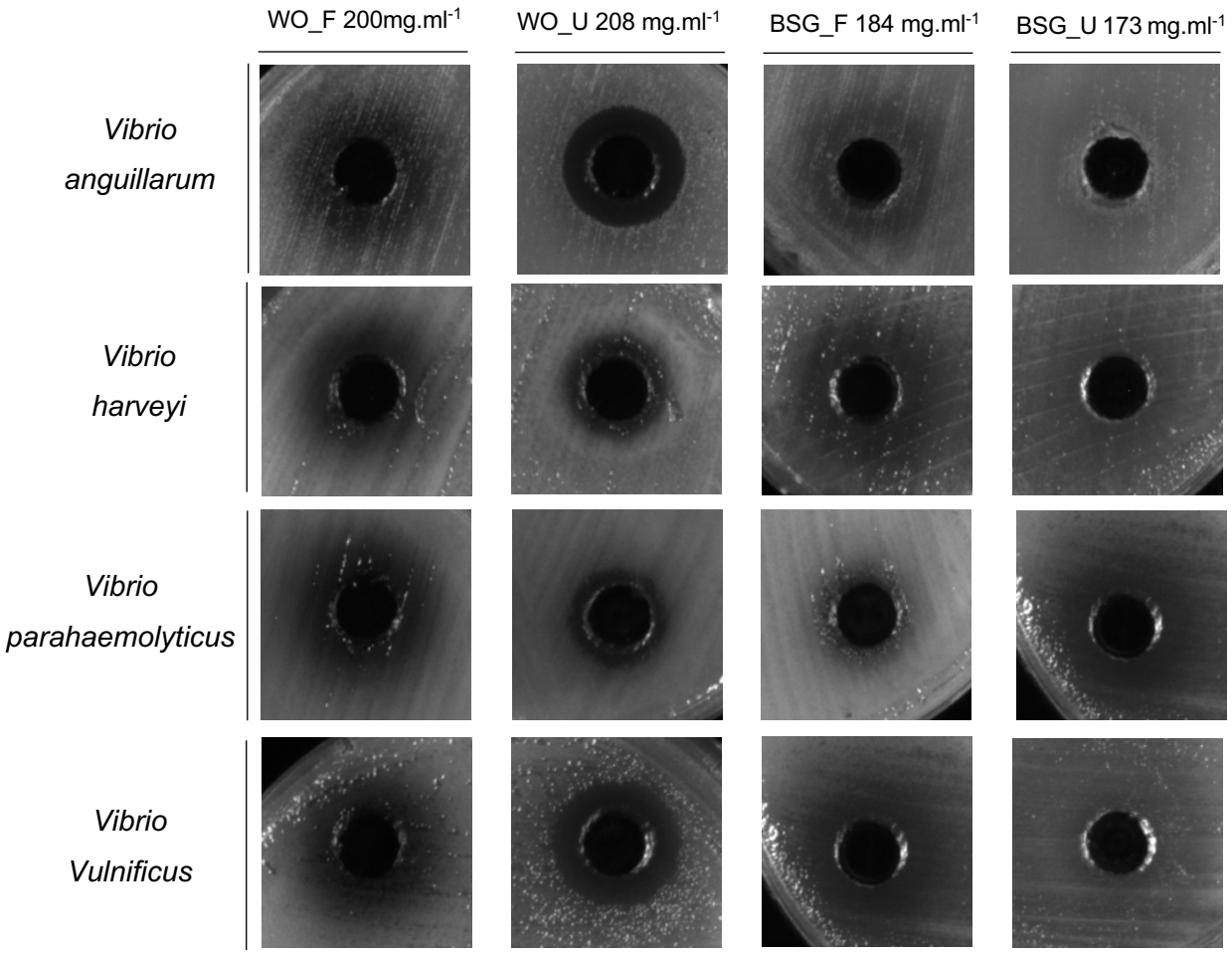

Figure 6. Well diffusion assays of 100 µL of 200 mg.ml^{-1} WO_F extract, 208 mg.ml^{-1} WO_U extract, 184 mg.ml^{-1} BSG_F and 173 mg.ml^{-1} BSG_U extract against *Vibrio anguillarum, Vibrio harveyi, Vibrio parahaemolyticus and Vibrio Vulnificus* in Brain Heart Infusion plates. One of each *Vibrio* isolated colony was inoculated in 5ml BHI medium, grown for 24 hours at 25ºC with constant agitation at 120rpm. The inoculum was adjusted to an optical density of 0,05, spread by swab and grown for 24h at 25ºC in contact with the water diluted aqueous extracts.

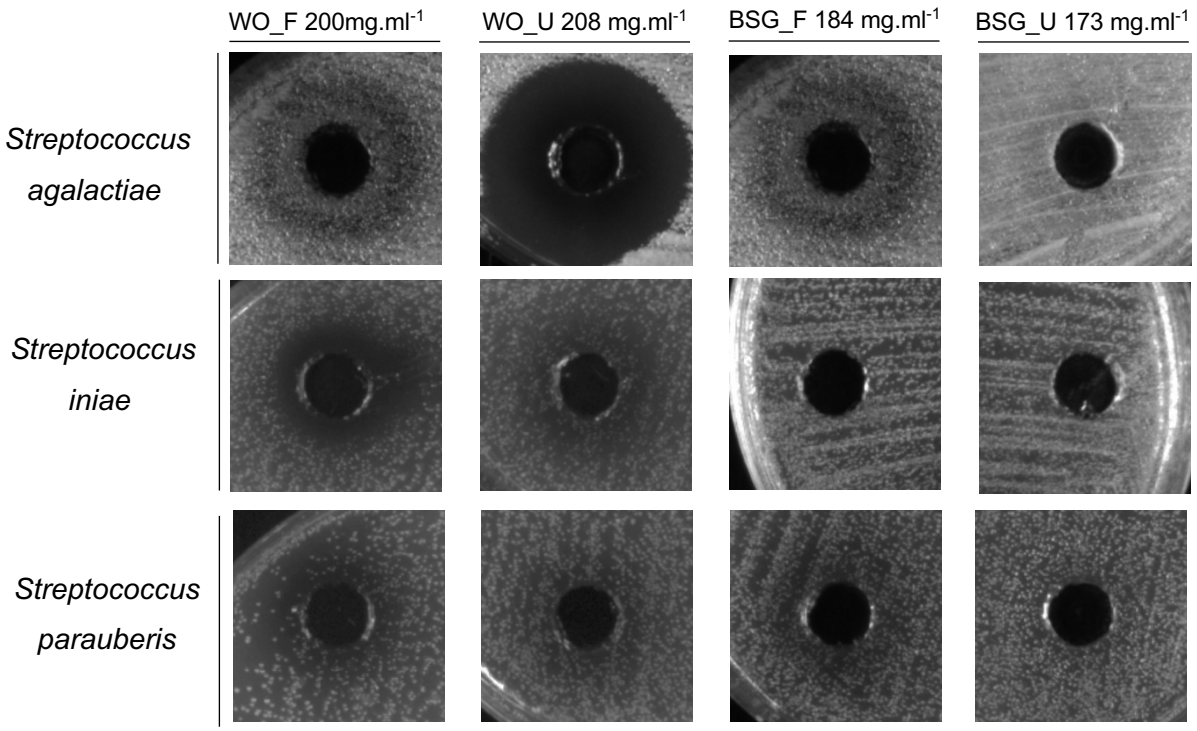

Figure 7. Well diffusion assays of 100 µL of 200 mg.ml^{-1} WO_F extract, 208 mg.ml^{-1} WO_U extract, 184 mg.ml^{-1} BSG_F and 173 mg.ml^{-1} BSG_U extract against *Streptococcus agalactiae, Streptococcus iniae, and Streptococcus parauberis.* in BHI. *One of each Streptococcus* isolated colony was inoculated in 5ml BHI medium, grown for 24 hours at 37ºC with constant agitation at 120rpm. The inoculum was adjusted to an optical density of 0,05 and spread by swab and grown for 24h at 37ºC in contact with the water diluted aqueous extracts.

Table 7. Antimicrobial activity summary of the WO_F, WO_U, BSG_F, and BSG_U extracts bioassays against 17 fish pathogens tested.

	Inhibition halo (cm)			
Bacteria	**WO_{mix}_F**	**WO_{mix}_U**	**BSG_F**	**BSG_U**
Aeromonas bestiarum	-	0.53 ± 0.1	-	-
Aeromonas bivalvium	-	0.47 ± 0.2	-	-
Aeromonas hydrophila	-	0.47± 0.1	-	-
Aeromonas salmonicida	-	1.01± 0.06	-	-
Aeromonas veronii	-	-	-	-
Edwardsiella tarda	-	0.23 ± 0.06	-	-
Photobacterium damselae subsp. damselae	-	0.47 ± 0.05	-	-
Photobacterium damselae subsp. piscicida	1.17 ± 0.06	1.27 ± 0.06	-	-
Shigella sonnei	-	-	-	-
Tenacibaculum maritimum	-	0.23 ± 0.06	-	-
Vibrio anguillarum	-	0.25 ± 0.07	-	-
Vibrio harveyi	-	0.20 ± 0.00	-	-
Vibrio parahaemolyticus	-	0.10 ± 0.00	-	-
Vibrio vulnificus	-	0.35 ± 0.07	-	-
Streptococcus agalactiae	-	0.75 ± 0.07	-	-
Streptococcus iniae	0.45 ± 0.07	-	-	-
Streptococcus parauberis	-	-	-	-

Inhibition halos are represented in cm^{-1} ± SD value of triplicate analysis for all the bacteria except for bacteria from the *Vibrio* genera where we have cm^{-1} ± SD value of duplicate analysis of the extracts potential.

Hematology

Table 8 compiles the hematological profile of European sea bass fed experimental diets incorporating WO_F and WO_U extract for 10 weeks and after 4 and 24 hours of being challenged with chemical inactivated PhDP IP injection. A significant increase in WBC count was observed in fish fed the WO_U 0.4 diet relative to the control diet, regardless of the time factor. A significant reduction in WBC level between hour 4 and 24 was registered in all the dietary treatments.

RBC count was highest in fish the WO_U 0.4 diet, regardless of the time. At 4 and 24 hour after IP injection, RBC level was higher in fish fed the WO_F 0.8 than those fed the WO_F0.4. Since no interactions were detected for the time factor, all 4 diets have had an increment of RBC form hour 4 to 24.

Regardless of the time factor, Hb concentration was found superior for WO_F 0.8 and WO_U 0.4 treatments when compared with the control diet. Significant increase in Hb concentration between hour 4 and 24 after IP injection was registered, irrespectively the dietary treatment.

Hematocrit values for the WO_U 0.4 treatment 24 hours after IP injection were not registered. A statistically significant reduction in Ht values was found between hour 4 and 24 after IP injection for WO_F 0.4 and 0.8 diets. Hour 24 after IP injection HT values showed a significant reduction for WO_U 0.4 compared with the control diet.

Since MCV and MCHC calculation is dependent on the Ht values, WO_U 0.4 hour 24 value does not exist. A decrease in MCV values were found for control, WO_F 0.4 and WO_F 0.8 between hour 4 and 24 after IP injection. Since no interactions were found, all diets exhibit a reduction in MCV value from hour 4 to 24.A significant increase of MCHC in fish fed WO_F 0.4 treatment was found between hour 4 and 24.

MCH levels were significantly higher in those fed WO_F 0.4 than those fed the control treatment, regardless of the time factor. A decrease in MCH was also registered for the WO_U 0.4 dietary treatment from hour 4 to 24 after IP injection.

Table 8. Hematocrit, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cells (RBC), and white blood cells (WBC) in European seabass fed dietary treatments during 10 weeks after chemically inactivated *Photobacterium damselae subsp. Damselae* bacterial challenge sampled 4 and 24 hours after IP injection.

Parameters	**Dietary treatment**							
	CTRL		**WO_F 0.4**		**WO_F 0.8**		**WO_U 0.4**	
	4h	**24h**	**4h**	**24h**	**4h**	**24h**	**4h**	**24h**
WBC (×10^4 µl^{-1})	1.02 ± 0,13	1.00 ± 0,16	1.23 ± 0,15	1,13± 0,16	1.20 ± 0,19	0,90 ± 0,23	1.24 ± 0,14	1,27 ± 0,11
RBC (×10^6 µl^{-1})	0,81 ± 0,04	0,99 ± 0,14	0,77 ± 0,12	0,89 ± 0,27	1,02 ± 0,12	1,08 ± 0,22	1,05 ± 0,12	1,38 ± 0,13
Hemoglobin (g dl)	0,72 ± 0,14	0,95 ± 0,14	0,98 ± 0,17	0,99 ± 0,17	1,17 ± 0,09	1,15 ± 0,37	1,20 ± 0,19	1,21 ± 0,17
Hematocrit (%)	1,03 ± 0,09	0,98^b ± 0,08	1,15* ± 0,08	0,8^a ± 0,09	1,20* ± 0,28	0,86^{ab} ± 0,09	0,98 ± 0,15	-
MCV (µm^3)	1,22 ± 0,15	0,98 ± 0,12	1,42 ± 0,11	0,98 ± 0,41	1,15 ± 0,21	0,82 ± 0,18	0,98 ± 0,12	-
MCHC (g 100 ml^{-1})	1,07 ± 0,43	1,00^a ± 0,10	0,82* ± 0,10	1,40^b ± 0,19	1,06 ± 0,27	1,28 ± 0,38^{ab}	1,13 ± 0,23	-
MCH (pg cell^{-1})	0,92 ± 0,22	0,94 ± 0,11	1,28 ± 0,18	1,2 ± 0,48	1,02 ± 0,27	1,03 ± 0,23	1,08± 0,13	0,88 ± 0,17

Two-way ANOVA

Parameters				**Treatment**			
	Treatment	**Time**	**Treatment x time**	**CTRL**	**WO_F 0.4**	**WO_F 0.8**	**WO_U 0.4**
WBC	0,002	0,047	ns	A	AB	A	B
RBC	≤0.001	0.001	ns	AB	A	BC	C
Hemoglobin	0.001	ns	ns	A	AB	B	B
Hematocrit	ns	≤0.001	0.025	-	-	-	-
MCV	0.012	≤0.001	ns	nd.	nd.	nd.	nd.
MCHC	ns	0,014	0,025	-	-	-	-
MCH	0,028	ns	ns	A	B	AB	AB

Values are presented as means of fold changes (fish injected with chemically inactivated PhDP: fish injected with PBS) ± SD (n = 9). Different lowercase letters in the same row mean significant differences among dietary treatments ($p < 0.05$) for a specific time, while an asterisk stands for significant differences between times for the same diet. Different capital letters indicate differences among diets regardless of time and nd. stands for no significant differences were detected between diets by the Tuckey post-hoc test..

Gene expression

As represented in **Figure 8** no statistically significant differences were registered for the normalized mRNA expression levels of pro-inflammatory cytokines IL-B, IL-8, and TNF-α and pro-apoptotic Casp-9.

Downregulation of the expression levels of Cox-2 gene was found for WO_F 0.8 treatment when compared with the control diet after IP injection with chemically inactivated PhdP.

Pro-inflammatory cytokine IL-6 and proapoptotic Casp-3 levels were found upregulated for the WO_F 0.4 treatment, regardless of the time factor. On hour four after IP injection a decrease in IL-6 expression levels was also found in WO_F 0.8 treatment and WO_U 0.4 treatment.

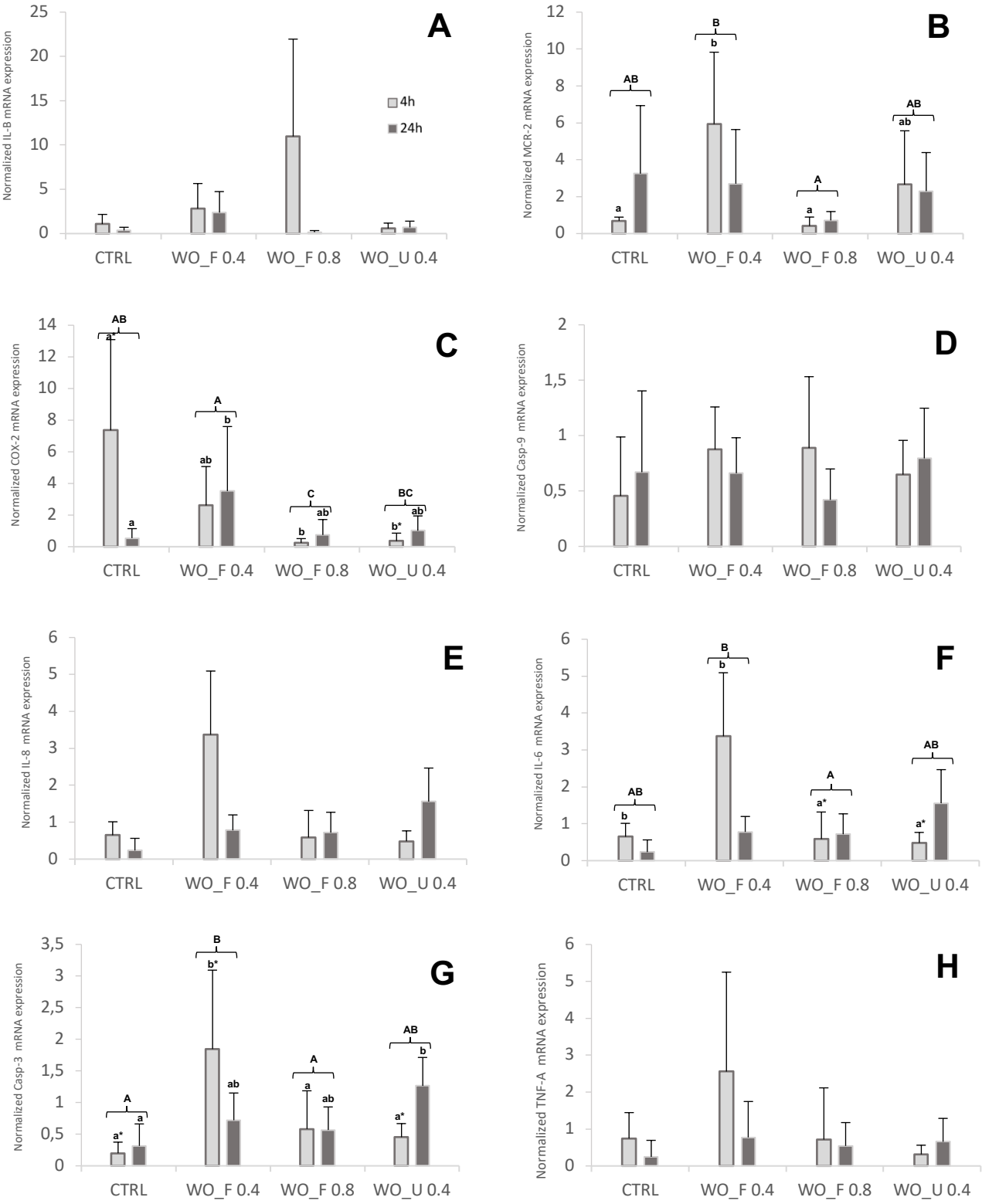

Figure 8. Quantitative expression of (A)Interleukin 1 β, (B) Melanocortin 2 receptor, (C) Ciclo-oxigenase-2, (D) Caspase-9, (E) Caspase-3, (F) Tumor necrosis factor α, (G) Interleukin 8 and (H) Interleukin 6 genes in the distal intestine of European sea bass fed dieatary treatments at 4 and 24h after IP injection with chemically inactivated PhDP. Values are presented as means ± SD (n=6). Different lowercase letters stand for significant differences among dietary treatments for the same time while different capital letters indicate differences among times regardless diets and among diets regardless time. Asterisk stands for significant differences between times for the same diet.

Discussion and conclusions

Vegetable extracts are known to have different aquaculture applications such as stress reducer agents, growth promotion, appetite stimulation, immunostimulant and prevent diseases in fish aquaculture. These activities are produced in part by alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics, steroids, and essential oils present in the plants (Reverter, Tapissier-Bontemps et al. 2014). Vegetable agroindustry by-products, such as those from winery, olive oil and brewery industries, constitute a financial and environmental responsibility for corporations, recently they have received some attention, since they may constitute a low-cost and circular source of functional ingredients for aquafeeds formulations.

Previously, OP has been used with the aim of partially replace fish oil used for animal feeds, with increase biological activity against platelet aggregation and a cardioprotective effect (Nasopoulou, Stamatakis et al. 2011, Nasopoulou, Smith et al. 2014, Sioriki, Smith et al. 2016). A goldfish in vivo trial, has reported that polyphenols derived from residual water from olive mill reduced steatosis damage (Alesci, Cicero et al. 2014). Polyphenolic extract from red grapes (*Vitis vinifera*) has been administrated to farmed European sea bass juveniles decreasing in intestinal IL-1β and IL-6, pro-inflammatory cytokines and an increase protective natural immunity and adaptive immune response leading to a reduced fish mortality in comparison to controls (Magrone, Fontana et al. 2016). BSG is rich in phenolic acids, especially ferulic and p-coumaric acids, that have antioxidant, antimicrobial and anti-inflammatory properties (Rachwał, Waśko et al. 2020). Plus, BSG has a high β-glucan content and its oral administration in fish at low concentration has reported to have an immunostimulatory effect (Vetvicka, Vannucci et al. 2013).

The antibacterial potential of plants extracts has been previously described against high incidence aquaculture fish pathogens. Indeed, Indian lettuce *(Lactuca indica)* and rosemary (*Rosmarinus officinalis)* have been reported to manifest antibacterial activity against *Streptococcus iniae* in kelp grouper (*Epinephelus bruneus*) and tilapia (*Oreochromis spp.*) (Abutbul, Golan et al. 2004, Harikrishnan, Kim et al. 2011). Green chiretta *(Andrographis paniculata)* was effective against *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus)* (Rattanachaikunsopon and Phumkhachorn 2009). Vibriosis effects could be counteracted with treatment application of ginger (*Zingiber officinale)*(Talpur, Ikhwanuddin et al. 2013), Mongolian milkvetch (*Astragalus membranaceus),* Chinese skullcap *(Scutellaria baicalensis), Forsythia spp.* (Pan, Yan et al. 2013) in fish. Chinese skullcap treatment has revealed to protect rock

bream (*Oplegnathus fasciatus*) against *Edwardsiella tarda* (Harikrishnan, Kim et al. 2011).

To the authors knowledge, this is the first study assessing the antimicrobial potential of SSFed and unfermented winery, olive oil and brewery byproducts extracts. The antimicrofial potential of WO_F, WO_U, BSG_F and BSG_U was assayed against well documented aquaculture fish pathogens namely, *Aeromonas bestiarium, Aeromonas bivalvium, Aeromonas hydrophila, Aeromonas salmonicida, Aeromonas veronii and Aeromonas bivalvium; Edwardsiella tarda; Shigella sonnei; Photobacterium damselae subs. damselae* and PhDP*; Tenacibaculum maritimum; Vibrio anguillarum, Vibrio harveyi, Vibrio parahaemolyticus,* and *Vibrio vulnificus and Streptococcus agalactiae, Streptococcus iniae and Streptococcus parauberis.*

Both WO fermented and unfermented extracts have revealed promising results suggesting its antimicrobial potential. The WO_U extract at 200mg.ml^{-1} has antimicrobial potential against a *Aeromonas bestiarium, Aeromonas Hydrophila and Aeromonas Bivalvium; Edwardsiella tarda; Photobacterium damselae* subs*. Damselae* and PhDP: *Tenacibaculum maritimum; Vibrio anguillarum, Vibrio harveyi, Vibrio parahaemolyticus* and *Vibrio vulnificus and Streptococcus agalactiae,* in its optimum growth conditions. Interestingly, the WO_F extract was effective against PhDP similarly to the WO_U results and against *Streptococcus iniae* for which WO_U extract has not shown any antimicrobial activity. Despite BSG extracts have a higher phenolic content, revealed no antimicrobial potential in well diffusion assays which might be related with BSG high carbohydrates content that support the growth of microorganisms (Rachwal et al., 2020). Thus, the low antimicrobial potential of BSG extract may possibly be linked with the low amount of ferulic and p-coumaric acids, its chemical properties that may be suitable for the growth of microorganisms, or lack of other bioactive compounds with antimicrobial properties (Rachwał, Waśko et al. 2020). A better characterization of phenolic compounds and/or other bioactive compounds in WO and BSG extracts may help to ascertain the different antimicrobial potentials obtained for both extracts. In particular for *Streptococcus iniae* for which results have suggested that the antimicrobial activity could be prevenient from the SFF process in the specific WO matrix.

The preliminary in vitro studies are important to prevent time and resource-intensive labor, and ethical concerns over the use of animals in laboratory that are inherent to an in vivo trial, and to ground the posterior results that could provide from them. The previous well-diffusion assays results opened a new door to posterior investigation in the WO antimicrobial in vivo potential. Despite that, we are able now to increase our

expectations about these extracts incorporation as low cost and sustainable alternative to the conventional chemical synthesized antimicrobial compounds, which are responsible to affect not only fish but also consumer health (Lulijwa, Rupia et al. 2020). Based on the results obtained in the in vitro studies, demonstrating a strong evidence of the WO_U extract antimicrobial potential against a wide spectrum of microorganisms from *Aeromonas, Edwardsiella*, *Photobacterium, Vibrio and Streptococcus* and WO_F extract antimicrobial potential against PhdP and *Streptococcus iniae* it was decide to further explore their application potential, through their incorporation in a fish diet and test in a feeding trial with European seabass. For that, the immune modulation action of both extract were evaluated in European seabass, fed diets supplemented with both extracts for 10 weeks and, at the end, challenged with chemically inactivated PhdP. It was decided to use this pathogenic due to its economic importance for aquaculture and the WO fermented and unfermented extracts had the most promising antimicrobial potential against PhDP in the previous well diffusion assays.

Previously plant bioactive compounds have also been reported to improve specific and nonspecific immune responses in fish (Reverter, Tapissier-Bontemps et al. 2014). The innate immune system consists of macrophages, monocytes, granulocytes, and lysozyme, and phagocytic cells are the most important components (Van Hai 2015). Reactive oxygen and nitrogen species produced by WBC have the ability to kill bacterial pathogens causing illnesses in fish, constituting a primary element of non-specific defense in fish (Alexander, John Wesly Kirubakaran et al. 2010). RBC, WBC, Hb act as a meter of the blood profile in fish linked to the innate immune defence and regulation of immunological function (Ballarin, Dall'Oro et al. 2004). WBC are responsible for providing protection or resistance to disorders caused by infectious pathogens and non-infectious factors such as nutrition, temperature and handling (Harikrishnan and Balasundaram 2005). Total value of WBC also describes the health status and immune system of the fish. Additionally, Hb decrease may be prevenient from RBC swelling and poor Hb mobilization of the spleen and other haematopoesis organs (Lie, Evensen et al. 1989).

Under infection or stress condition, the total WBC levels usually are reduced which may affect the fish defense capacity against pathogens (Peres, Costas et al. 2014). In present study, after an inflammatory insult with chemically inactivated PhDP, fish fed a diet fortified with 0.4% of WO_U extract reveled higher total WBC, RBC and Hb count than those fed the control diet, suggesting a higher capacity to cope with the bacterial challenge, being less susceptive to the disease (Shahi and Singh 2011). The increment

in this hematological parameters also revealed an rapid nonspecific and innate inflammatory response against chemically inactivated PhDP for this dietary treatment.

The cytokines, il-1β and tnf-α, are determinant mediators of pro-inflammatory responses and in the activation of B and T cells (Sher and Coffman 1992) and very often co-expressed with other macrophage-derived inflammatory mediators such as il-6, il-8 and cox-2 in pathogen mediated infections (de Bruijn, Belmonte et al. 2012) .

Caspase-3 is responsible for the catalysis of protein degradation during the apoptosis process and the expression of this gene has been described as a good indicator of apoptosis in fish leucocytes (Reyes-Becerril, Sanchez et al. 2018). Caspase gene expression may be highly modulated by different inflammatory stimuli. For example, in Japanese flounder, caspase gene expression was modulated by a bacterial challenge, suggesting an important role against bacterial infection (Li, Li et al. 2019). Moreover, the response of caspase-3 and 6 to infection was also reported in yellow-striped grunt (Haemulon flavolineatum) grunt fin cells (Miwa, Mano et al. 2002, Imajoh, Sugiura et al. 2004) and of caspase-8 to bacterial infection in sea bass (Reis, Costa-Ramos et al. 2010).

The presence of pro-inflammatory cytokines as IL-1β, TNF-α and IL-6 and it involvement in the inflammatory response has been described for most fish species (Zou and Secombes 2016). IL-6 is mainly produced by T cells and macrophages, stimulating the immune response to inflammation and has been described different fish species (Iliev, Castellana et al. 2007); (Castillo, Teles et al. 2009) including seabream (Boltaña, Castellana et al. 2017)). Cyclooxygenase (Cox) catalyzes the first step in the synthesis of prostanoids, from the precursor arachidonic acid (AA). Under infection, the pro-inflammatory enzyme Cox-2 gene expression is up-regulated in several fish species (. In in sea bream (Sparus aurata) challenged with Photobacterium damselae Cox-2 gene expression was also increased (Grasso, Padilla et al. 2015). In this work we found that WO_F at 0.8 % dietary treatment was responsible for the inhibition of the expression of the pro-inflammatory cytokine Cox-2, which may indicate a much decreased inflammatory response from the fish submitted to this dietary treatment. On the other end, WO_F 0.4 treatment seemed to increase the expression of proinflammatory IL-6 and Casp-3 when compared with the control diet, suggesting a higher inflammatory response and higher macrophage activity in distal intestine of the fish (de Bruijn, Belmonte et al. 2012) (Reyes-Becerril, Sanchez et al. 2018).

The results indicate that WO_U 0.4 extract may be responsible for a much faster innate response to this PhDP chemically inactivated bacterial challenge due to its increased WBC count when compared with the control diet, while WO_F 0.4 suggest having a much more adaptative response to the inflammatory insult prevenient from the PhDP bacterial challenge, indicated by its increment in Casp-3 and IL-6 expression without an increment in WBC. Interestingly, WO_F 0.8 has not shown any indicators of the innate or adaptative immune response to the inflammatory challenge suggesting an overall improved fish health status (Magrone, Fontana et al. 2016).

In summary, this study suggests that WO_F extract has antimicrobial potential against *Streptococcus iniae, and Photobacterium damselae subs. Piscicida,* WO _U extract has proved to have in vitro antimicrobial potential against *Aeromonas bestiarium, Aeromonas hydrophila and Aeromonas bivalvium; Edwardsiella tarda; Photobacterium damselae subs. Damselae; Tenacibaculum maritimum; Vibrio anguillarum, Vibrio harveyi, Vibrio parahaemolyticus* and *Vibrio vulnificus*. More studies are required in order to assay the WO__F and WO_U dietary treatment anti-inflammatory potential in European sea bass with higher incorporation rates and a bacterial challenge with live PhDP in order to relate the immunological information with mortality rates. Despite that here we report strong evidence suggesting that WO extracts could have antimicrobial and immunostimulant properties in European sea bass and that they may act as an alternative for the use of antibiotics in aquaculture.

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