UNIVERSIDAD DE ALMERÍA

Departamento de Biología y Geología

Valorización de biomasa de macroalgas y subproductos agroindustriales como fuentes de ingredientes nutricionales y funcionales en piensos para acuicultura

Valorization of macroalgal biomass and agro-industrial by-products as sources of nutritional and functional ingredients for aquafeeds

TESIS DOCTORAL

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MEMORIA PARA OBTENER EL GRADO DE DOCTOR

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HACEN CONSTAR

Que la presente memoria titulada “Valorization of macroalgal biomass and agro-industrial by-products as sources of nutritional and functional ingredients for aquafeeds” que presenta Dña. Francisca Purificación Martínez Antequera para optar al grado de Doctor por la Universidad de Almería, ha sido realizada bajo su dirección, y autorizan su presentación y defensa.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ADC	Apparent digestibility coefficient
AGC	Automatic gain control
ANEU	Apparent net protein utilization
AWCD	Average well color development
BCA	Bicinchoninic acid
CAT	Catalase
CF	Condition factor
DAD	Diode array detection
DIA	Data independent analysis
DM	Dry matter
DNS	3,5-dinitrosalicylic acid
DOE	Design of experiments
E	Shannon evenness
EC	Epicatechin
ECG	Epicatechin gallate
EGCG	Epigallocatechin
ESI	Electrospray ionization
EU	European Union
FCR	Feed conversion ratio
FE	Feed efficiency
FI	Feed intake
FW	Final weight
FWHM	Full width at half maximum
GPx	Glutathione peroxidase
H’	Shannon index or Functional biodiversity
Hb	Haemoglobin
Hc	Hematocrit
HCD	Higher collisional dissociation
HRMS	High resolution mass spectrometry
HSI	Hepatosomatic index
K	Condition factor
LC	Liquid chromatography
LSD	Least significant difference
MDA	Malondialdehyde
MWCO	Molecular weight cut-off
NFE	Nitrogen free extract
NPU	Net protein utilization

NSPs	Non-starch polysaccharides
OD	Optical density
OPA	Orthophthaldehyde
P	Phosphorus
PER	Protein efficiency ratio
R	Functional richness
RAS	Recirculation aquaculture system
RF	Radio frecuency
RFU	Relative fluorescence units
ROS	Reactive oxygen species
SAWCD	Substrate average well color development
SD	Standard deviation
SGR	Specific growth rate
SOD	Superoxide dismutase
SSH	Solid-state hydrolysis
SW	Seawater
TAG	Triacylglyceride
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox equivalent antioxidant capacity
TG	Triglycerides
U	Unit of enzyme activity
UHPLC	Ultra-high performance liquid chromatography
VFI	Voluntary feed intake
WG	Weight gain

ABSTRACT

In current aquaculture, increased attention is paid in searching for new unconventional ingredients that can be used in the development of sustainable feeds able to provide essential nutrients or bioactive compounds with positive effects on the health and welfare of fish. In this sense, two categories of these ingredients, such as macroalgae biomass and agro-industrial by-products, present a great potential, not only for their nutritional value, but also for their contents in compounds with biological activity, mainly polyphenols. However, many of these ingredients have significant nutritional limitations, linked to the presence content of different antinutritive factors such as non-starch polysaccharides (NSPs), phytic acid, and in some cases inhibitors of digestive proteases. However, to counteract the potential negative effects of such compounds, different treatments (physical, chemical and enzymatic) can be used to improve the bioaccessibility of nutrients. Considering this, this Doctoral Thesis includes different experiments aimed to evaluate the potential of several unconventional ingredients that may be included in diets for different aquaculture species from a double perspective: i) as sources of nutrients, improving their nutritional value through enzymatic treatments, and ii) as a supply of compounds with possible beneficial effects on metabolism.

Chapter 1 deals with the use of a macroalgae meal (the chlorophyte *Ulva ohnoi*) when included in feeds for two relevant species in European aquaculture (*Dicentrarchus labrax* and *Sparus aurata*), focusing on both improving the bioavailability of nutrients through a pretreatment with carbohydrases and on its possible supply of bioactives. The results show the possibility of using up to 5 % of *U. ohnoi* meal in the feed without compromising the zootechnical parameters, being the enzymatic pretreatment ineffective to improve its nutritional value. However, the inclusion of *U. ohnoi* determined an immunostimulatory effect, evidenced by an increase in the lysozyme activity of the mucus in the two mentioned species.

Chapter 2 was oriented towards assessing the effect of an enzyme pretreatment with a multienzymatic complex (glucanases + phytase) of the vegetable ingredients used in two feeds for mullet (*Mugil cephalus*) on the digestive use of proteins and phosphorus. Assays were performed both *in vitro* and *in vivo*. The results of the *in vitro* test confirmed that in both feeds the enzymatic treatment significantly modified the potential

bioavailability of some nutrients. The *in vivo* trial showed an improvement in protein digestibility in one of the feeds, but also a reduction in the net phosphorus digestibility in both, probably because the hydrolysis produced by phytase increased the amount of this nutrient in the feed digestive system above the intestinal absorption capacity of such nutrient.

Chapter 3 evaluated the effect of enzymatic treatment of the same plant by-products used in the previous trial (brewer's spent grain and rice bran), carried out by solid state hydrolysis (SSH) on growth and feed efficiency when they were included in a feed for *M. cephalus*. In a first test on a laboratory scale, fish that received the enzyme-treated feed showed a significant improvement in zootechnical parameters compared to those fed on the untreated feed, although not comparable to those of the commercial feed used as control. However, when carrying a test under real production conditions, values of growth and feed efficiency showed an equivalence between the enzyme treated and the commercial feeds and confirmed that the enzymatic pretreatment of plant by-products by SSH improves their nutritional value.

Chapter 4 was proposed as a preliminary study aimed at evaluating the most determining factors affecting the digestive bioavailability of phenolic compounds present in bagasse and wine lees by means of *in vitro* digestive simulation models of two species with different feeding habits (*S. aurata* and *M. cephalus*). The results indicated that the presence of the feed matrix and the type of wine by-product have a significant effect on the digestive release of total and specific polyphenols, while the species had a considerable influence only for some specific polyphenols.

Chapter 5 focused on the determination of the potential effects of the inclusion of two wine by-products (grape pomace and lees) in the feed on the growth, general metabolism, oxidative and immunological status of juvenile mullets (*Liza aurata*). The results showed a significant positive effect of grape pomace on feed efficiency, as well as on different indicators of the metabolic and immunological status of the fish, but also a dramatic change in the composition of the intestinal microbiota associated with lees consumption. In addition, a general improvement was found in the productive efficiency, physiological and immunological status of the fish when the feed was supplemented with any of the two by-products, both

under normal production conditions and also after challenging subjecting the fish with a moderate acute hypoxia, largely due to its content in phenolic compounds.

RESUMEN

En la acuicultura actual se presta cada vez más atención a la búsqueda de nuevos ingredientes no convencionales para el desarrollo de piensos sostenibles que puedan aportar nutrientes esenciales o compuestos bioactivos con efectos positivos sobre la salud y el bienestar de los peces. En este sentido, dos categorías de estos ingredientes, tales como la biomasa de macroalgas y los subproductos agroindustriales, presentan un gran potencial no solo por su valor nutritivo sino también por su riqueza en compuestos con actividad biológica, principalmente polifenoles. Sin embargo, gran parte de estos ingredientes presentan limitaciones nutricionales importantes, vinculadas principalmente con su alto contenido en diferentes factores antinutritivos tales como polisacáridos no amiláceos (NSPs), ácido fítico, y en algunos casos inhibidores de proteasas digestivas. No obstante, para contrarrestar los potenciales efectos negativos de tales compuestos se pueden emplear diferentes tratamientos (físicos, químicos y enzimáticos) destinados a mejorar la bioaccesibilidad de los nutrientes. Partiendo de esta base, la presente Tesis Doctoral evalúa el potencial de diversos ingredientes no convencionales potencialmente utilizables en piensos para diferentes especies acuícolas desde una doble perspectiva: i) como fuentes de nutrientes, mejorando para ello su valor nutritivo mediante tratamientos enzimáticos, y ii) como aportes de compuestos con posibles efectos beneficiosos sobre el metabolismo.

El **Capítulo 1** aborda el uso de la harina de una macroalga (la clorofita *Ulva ohnoi*), centrándose en la mejora de la biodisponibilidad de nutrientes mediante un pretratamiento con carbohidrasas y en su posible aporte de bioactivos al ser incluida en piensos para dos especies relevantes en la acuicultura europea (*Dicentrarchus labrax* y *Sparus aurata*). Los resultados muestran la posibilidad de utilizar hasta un 5 % de harina de *U. ohnoi* en los piensos sin comprometer los parámetros zootécnicos, siendo en todos los casos el pretratamiento enzimático ineficaz para mejorar su valor nutricional. No obstante, la inclusión de *U. ohnoi* determinó un efecto inmunoestimulador, evidenciado por un aumento de la actividad lisozima del mucus en las dos especies mencionadas.

El **Capítulo 2** se orientó hacia la evaluación del efecto del pretratamiento de los ingredientes vegetales utilizados en dos piensos para mújol (*Mugil cephalus*) con un complejo multienzimático (glucanasas + fitasa) sobre el

uso digestivo de proteínas y fósforo. Los ensayos se realizaron tanto *in vitro* como *in vivo*. Los resultados del ensayo *in vitro* confirmaron que en ambos piensos el tratamiento enzimático modificaba significativamente la biodisponibilidad potencial de algunos nutrientes. El ensayo *in vivo* evidenció una mejora en la digestibilidad de la proteína en uno de los piensos, pero también la reducción en la digestibilidad neta del fósforo en ambos, debido probablemente a que la hidrólisis producida por la fitasa incrementó la cantidad de este nutriente en el digestivo por encima de la capacidad de absorción intestinal de dicho nutriente.

El **Capítulo 3** evaluó el efecto del tratamiento enzimático de los mismos subproductos vegetales utilizados en el ensayo anterior (bagazo de cerveza y salvado de arroz), realizado mediante hidrólisis en estado sólido (SSH) sobre el crecimiento y eficiencia alimenticia cuando eran incluidos en un pienso para *M. cephalus*. En un primer ensayo a escala de laboratorio, los peces que recibieron el pienso tratado con enzimas mostraron una mejora significativa en los parámetros zootécnicos en comparación con los obtenidos con pienso sin tratar, aunque no comparables a los del pienso comercial usado como control. Sin embargo, al realizar una prueba de engorde en condiciones reales de producción los resultados de crecimiento y eficiencia alimenticia evidenciaron la equivalencia entre los piensos experimental y comercial, y confirmaron que el pretratamiento enzimático de los subproductos vegetales por SSH mejora su valor nutritivo.

El **Capítulo 4** se planteó como un estudio preliminar destinado a evaluar los factores más determinantes en la biodisponibilidad digestiva de los compuestos fenólicos presentes en el bagazo y las lías del vino para dos especies de diferente hábito alimenticio (*S. aurata* y *M. cephalus*) mediante modelos de simulación digestiva *in vitro*. Los resultados indicaron que la presencia de la matriz del pienso y el tipo de subproducto del vino tienen un efecto significativo en la liberación digestiva de los polifenoles totales y específicos, mientras que la especie influyó significativamente solo para algunos polifenoles específicos.

El **Capítulo 5** se enfocó hacia la determinación de los potenciales efectos de la inclusión en el pienso de dos subproductos del vino (bagazo de uva y lías) sobre el crecimiento, estado inmunológico, metabolismo general y estatus oxidativo de juveniles de lisas (*Liza aurata*). Los resultados

evidenciaron un efecto positivo significativo del bagazo de uva en la eficiencia alimenticia, así como en diferentes indicadores del estado metabólico e inmunológico de los peces, pero también una drástica modificación de la composición de la microbiota intestinal asociada al consumo de las lías. Además, se constató una mejora general en la eficiencia productiva, estado fisiológico e inmunológico de los peces cuando los piensos se suplementaron con cualquiera de los dos subproductos, tanto bajo condiciones normales de producción como tras someter a los ejemplares a un proceso de hipoxia aguda moderada, en buena medida debido a su contenido en compuestos fenólicos.

I. GENERAL INTRODUCTION

1. CURRENT STATUS AND CHALLENGES FOR A SUSTAINABLE AQUACULTURE

In recent decades, aquaculture has become the most developed primary sector activity worldwide (Naylor et al., 2021). It currently represents a production volume of 88 million tons per year, equivalent to that of extractive fisheries (FAO, 2022). This great development has been based mainly on three points: **a) the use of artificial feeds, b) the development of intensive production systems** and **c) the cultivation of high market value species**, many of them with a carnivorous feeding habit. Although these aspects have led to the success of aquaculture as a food producing activity, it has also generated several problems related to both the environmental impact and the provision of the resources needed to feed an increasing number of farmed organisms.

As indicated above, most of the world's aquaculture production is based on the use of large quantities of artificial feeds, mostly made from ingredients that are either difficult to renew (fish meal and oils, or more recently, krill meal) or whose production consumes significant quantities of energy, such as different meals and vegetable concentrates. In addition, in many cases, the use of these ingredients in aquafeed competes with their use in human food or feeds for terrestrial animals, thus reducing their availability, leading to price fluctuations and determining a growing concern about the sustainability of current aquaculture production (van Riel et al., 2023).

In relation to the second point mentioned above, it should be noted that a large part of the current aquaculture is carried out under highly productive intensive systems that, in many cases, amplify environmental stress agents, reduce water quality and facilitate the appearance of pathologies (Segner et al., 2012; Sneddon et al., 2016). Thus, the use of biologically active compounds that may increase the ability of cultivated organisms to deal with such negative aspects is becoming a priority subject of research (Ringø et al., 2012; Dawood et al., 2018).

Regarding the latter aspect, it should be noted that in economically developed areas, (European Union (EU), North America, Australia, etc.) marine fish farming focuses on the production of carnivorous species such as salmon (*Salmo salar*), cobia (*Rachycentrum canadum*), European sea bass (*Dicentrarchus labrax*), greater amberjack (*Seriola dumerili*),

turbot (*Scophtalmus maximus*) or gilthead sea bream (*Sparus aurata*), whose diet requires high amounts of good quality proteins. In contrast, the production of marine species of low trophic levels in these countries is still uncommon, despite their importance in the sustainable development of aquaculture (Tacon et al., 2009; FAO, 2019). In this regard, it should be noted the importance of promoting the sustainable development of aquaculture, an issue that in the EU has been recognized by the European Commission. This Commission indicates that the protection of environment must be the basis for the development of aquaculture activities, so that the sector can simultaneously increase its productivity and environmental commitment (European Commission, 2002, 2009, 2013). In fact, current policies of the EU favor the shift from a linear to a circular and sustainable economy, which requires striking a balance between the growth of economic activities, the protection of natural resources and the increasing needs of a growing world population (European Commission, 2018). According to such approaches, aquaculture production should follow circular economic strategies based on the better use of resources and lower waste production. In line with this, **the future of aquafeed production should be based mainly on the use of non-conventional ingredients** to avoid competition with human food, considering mainly the valorization of by-products or co-products generated by the agri-food industry. In this sense, aquaculture should follow the steps of terrestrial animal production to promote the inclusion of by-products in the feed formulas of different species in order to reduce the consumption of finite resources (Gatlin et al., 2007; Jobling, 2016).

It should also be noted that agro-industrial by-products contain a wide range of active molecules (flavonoids, terpenoids, tocopherols and phenolic compounds) with a widely demonstrated ability to protect organisms against several types of environmental stress owing to their antioxidant properties (Dawood et al., 2022). In this sense, the potential interest in many of these by-products is double, since they can serve both as nutritional ingredients, providing macronutrients in significant quantities, and also as sources of functional compounds that can improve specific biological functions. These functions are mainly related to the reinforcement of the immune system and the optimization of general metabolism, which can be an important way to reduce the use of antibiotics and to generate more biologically-efficient aquafeeds.

Finally, the diversification of farmed species is another major challenge facing by aquaculture. The EU is therefore starting to orientate efforts aimed to promote the production of species with a low trophic level, such as mullets, form the mugilidae family (e.g., the grey mullet *Mugil cephalus* or the golden gray mullet *Liza aurata*) due to their good adaptation to a wide range of temperatures and geographical locations, rapid growth and feeding habits. These species require less protein in their feeds during the growth stages (Huntington & Hasan, 2009), this being in line with the philosophy of sustainable aquaculture, because of the use of non-conventional ingredients and the reduction of production costs.

2. NON-CONVENTIONAL INGREDIENTS IN AQUAFEEDS

Due to the great expansion of aquaculture production, different ingredients initially considered as non-conventional, such as cereal by-products (brans and glutens), animal meals (from meat and bones, blood, or krill), legume meals (guar), etc., are now commonly used in feed formulations (Aladetohun & Sogbesan, 2013; Ullah et al., 2016; Liu et al., 2020; Yu et al., 2020). For this reason, an updated definition of non-conventional ingredients is required and these should be all resources or by-products that are not regularly used for animal feed, including aquaculture species. These ingredients, besides not being used for human consumption, usually present a low economic value because most of them are generated as by- or co- products from agriculture and fisheries as well as from food processing industries (Gatlin et al., 2007; Dawood et al., 2022). In some other cases, they are also obtained as by-products of specific industrial processes (such as *Corynebacterium glutamicum* biomass obtained from the production of glutamic acid) or are available in significant amounts as a result of primary production in specific ecosystems (e.g., macroalgal biomass). A final category would group the non-conventional ingredients generated by specific production processes, such as microalgae, insect meals and oils (Quang Tran et al., 2022), fungal biomass produced from wood waste (Solberg et al., 2021) or methane growing bacteria (Øverland et al., 2010).

2.1. Agro-industrial by-products

As mentioned above, the agri-food industry generates large volumes of by-products and wastes resulting from processing different types of vegetables and manufacturing of processed foods. These ingredients present highly variable contents in macronutrients, so their potential

levels of inclusion in aquafeeds are also remarkably diverse and strongly conditioned by the type of organism and its digestive physiology (Dawood et al., 2022). Also, as indicated, a number of agro-industrial by-products contain a wide range of biologically active molecules (mainly phenolic compounds) that can benefit the health and resistance of species to some of the adverse effects present in aquaculture practice (Leyva-López et al., 2020; Dawood et al., 2022). Several studies have demonstrated such benefits by including by-products from different origins such as apple (Ahmadifar et al., 2019), banana (Giri et al., 2016), orange (Van Doan et al., 2019), grape (Mousavi et al., 2021), date palm (Kari et al., 2022) or brewer's spent grain (Fernandes et al., 2022).

2.2. Natural biomass

This category included varied species of macroalgae that are naturally available in appreciable quantities in different coastal areas of the planet, where they are collected regularly for different uses (Charlier et al., 2008; Sudhakar et al., 2018). In general, the use of macroalgae as a nutritional ingredient is limited by their low protein and lipid contents; however, they are highly appreciated as sources of bioactive compounds because of their contents of pigments, polysaccharides, polyphenols and vitamins (Moutinho et al., 2018). It should be noted that the content of such compounds is determined mainly by the species and harvest season. At the same time, their digestibility is strongly conditioned by the presence of antinutritional factors and mainly by the nature of the polysaccharides they contain (Garcia-Vaquero & Hayes, 2016). This last aspect limits the quantities to be used in aquafeed, since it has been shown that the incorporation of macroalgae at feed concentrations below 10 % improves zootechnical parameters such as SGR, FCR and WG (Wassef et al., 2013), while values above 10 % can compromise the optimal performance in various species (Azaza et al., 2008; Dantagnan et al., 2009).

2.3. Specifically generated ingredients

Microalgae are unicellular organisms that present a high nutritional interest owing to their adequate content of proteins, lipids and carbohydrates (Shah et al., 2018). In addition, they usually present an ideal amino acid profile that avoids the need to supplement amino acids, which can involve a high diet cost. Several studies have confirmed that the inclusion of low or moderate quantities of microalgae in aquafeeds (maximum of 5 % by weight) has positive effects on zootechnical

parameters of different species, such as sea bream, sole and salmon (Vizcaíno et al., 2014; Vizcaíno et al., 2018; Wei et al., 2022).

In addition, it is worth noting the importance of other unicellular proteins, like yeasts and bacteria, whose usefulness has been widely demonstrated. Yeasts, specifically *Saccharomyces cerevisiae*, present an amino acid profile resembling that of fishmeal (Vidakovic et al., 2016) and many studies support their usefulness as a source of protein in aquafeeds (Nasseri et al., 2011; Huyben et al., 2017). Similarly, bacteria are of great interest as sources of protein and essential micronutrients (Wan-Mohtar et al., 2022). However, single-cell proteins are not only rich in protein, but also contain other compounds (e.g., enzymes) that can help to improve the digestibility of nutrients present in diets. Their inclusion in experimental diets in many cases has a positive effect on the performance of aquaculture species (Blomqvist et al., 2018; Wan-Mohtar et al., 2022).

Another ingredient to consider within this group are insect meals generated mainly from larvae of the mealworm beetle (*Tenebrio molitor*) or the soldier fly (*Hermetia illucens*). Insects are protein-rich organisms presenting an ideal amino acid profile that are also a source of vitamins and bioactive compounds, such as chitin and antimicrobial peptides (Gasco et al., 2018). In general, insect farming conditions and biomass processing methods can affect the nutritional composition of the product, digestibility, shelf life and level of insect inclusion required by aquaculture species (Maulu et al., 2022). Several studies have shown promising results when including insect meal in the diets of various aquaculture species, such as Nile tilapia (*Oreochromis niloticus*) (Were et al., 2022), Japanese sea bass (*Lateolabrax japonicus*) (Wang et al., 2019), Atlantic salmon (*S. salar*) (Lock et al., 2016; Stenberg et al., 2019), gilthead sea bream (*S. aurata*) (Randazzo et al., 2021) and rainbow trout (*Oncorhynchus mykiss*) (Cardinaletti et al., 2019). The results obtained showed improvements not only in growth parameters but also in the immune response to diseases.

3. LIMITATIONS AND TREATMENTS FOR IMPROVING THE NUTRITIONAL USE OF INGREDIENTS

Practical use of many non-conventional ingredients is conditioned by some characteristics of their composition that limit the bioavailability of their nutrients and/or bioactive compounds (Dawood & Koshio, 2020).

To counteract these limitations, physical, chemical or enzymatic treatments are often used to improve the bioaccessibility of nutrients and to reduce or eliminate antinutritional compounds that may affect the health of fish (Manach et al., 2005; Kokou & Fountoulaki, 2018). These treatments are routinely applied when processing legumes and cereals to obtain several types of meals and concentrates (Klinger & Naylor, 2012). To a lesser extent, there is information available on similar treatments applied to other types of ingredients, such as other vegetable by-products, insects, micro-and macroalgae, yeasts and bacteria (Table 1).

Most of the treatments conducted on the ingredients are intended to partially hydrolyze the fraction of complex polysaccharides present in plant fruits and seeds, macroalgae tissues or cell walls in microalgae and bacteria. These complex carbohydrates are usually known as non-starch polysaccharides (NSPs), and they present a wide range of differences in physical chemical properties (chemical composition, water solubility, water retention capacity, etc.) as well as in the ways they interact with other feed ingredients and the intestinal microbiota. As example, many by-products of vegetable origin are rich in cellulose (an insoluble polymer of glucose) as well as in other heteropolymers formed by other sugars such as mannose, xylose and arabinose. In an equivalent manner, macroalgae also present a great diversity of specific polysaccharides like alginates, agar, carrageenan, xylans, sulfated galactans, porphyrans and ulvan (Makkar et al., 2016). Similarly, cell walls of some fungi and insect skeletons present chitin, other type of indigestible polysaccharide present (Rahman et al., 2023). Depending on the amount and type of NSPs present in the feed, significant effects on intestinal transit, nutrient absorption and microbial diversity have been observed (Sinha et al., 2011). In many cases, NSPs configures a matrix that hinders the access of digestive enzymes to the protein and starch present in cereal and legume seeds. For this reason, the use of enzyme complexes capable of completely or partially degrading them has shown positive effects on the nutritional use of feeds including these ingredients (Castillo & Gatlin, 2015).

Table 1. *Examples of antinutritional factors present in ingredients used in the formulation of aquafeeds.*

Ingredients	Antinutritional factors	Treatments	References
Soy meal, corn gluten, wheat meal, rapeseed meal, wheat gluten, soy protein concentrate, guar meal, pea meal	Protease inhibitors, lectins, phytic acid, saponins, polyphenols, alkaloids, NSPs	Physical (extrusion, thermal), enzymatic (phytase, microbial fermentation), chemical (acidic, alkaline, ethanolic extraction, aqueous extraction)	Gestetner et al.(1968); Storebakken et al. (1998); Vielma et al. (1998); Cheng & Hardy (2002); Maitra & Ray (2003); Drew et al. (2007); Gatlin et al. (2007); Krogdahl et al. (2010); Mandal & Ghosh (2010); Dalsgaard et al. (2012); Goda et al. (2012); Zamini et al. (2014); Kokou & Fountoulaki (2018)
Moringa oleifera by-products, brewer's spent grain, date by-products	Phytic acid, NSPs, lignin	Enzymatic (enzyme complex, phytase, fungal fermentation)	Cooray & Chen (2018); San Martin et al. (2020); Dawood & Koshio (2020); Shahzad et al. (2021)
Ulva rigida, Gracilaria gracilis	Algal NSPs	Enzymatic (fungal fermentation, hydrolysis), physical (MW, ultrasound, autoclave, vibrating mill), chemical (acidic, alkaline)	Batista et al. (2020); Fernandes et al. (2022)
Nannochlorosis oceanica, Chlorella vulgaris, Tetraselmis sp.	Algal NSPs	Physical (vibrating mill), enzymatic (hydrolysis)	Batista et al. (2020)
Brewer's yeast	NSPs	Enzymatic (enzyme complex)	San Martin et al. (2020)
Larvae of soldier-black fly (*Hermetia illucens*)	Chitin	Physical (thermal temperature, high pressure)	Campbell et al. (2020)

In addition to the hydrolysis of NSPs, one of the greatest benefits of using enzyme mixtures is the reduction in the viscosity of the digesta, the release of oligosaccharides and therefore, the improvement in the utilization of nutrients such as starch and fat (Adeola & Bedford, 2004).

The improvement in fat digestibility is particularly noticeable because NSPs increase the hydrolysis of bile salts and thus reduce lipid digestion (Vahjen et al., 2007). They also increase protease accessibility and total energy availability in the proximal gut, which reduces nutrient competition between the fish and its gut microbiota, ensuring greater nutrient availability where absorption is more efficient (Adeola & Cowieson, 2011). In addition, exogenous carbohydrases can promote the development of beneficial microbiota, resulting in a better health status of fish (Adeola & Cowieson, 2011). However, the inclusion of phytase in such enzyme mixtures has shown positive effects in different aquaculture species, since by fully or partially hydrolyzing phytate, the nutritional use of different ingredients is improved and the discharge of phosphorus to the medium is reduced (Cao et al., 2007).

Nevertheless, the efficiency of hydrolysis by such enzymes is largely dependent on several factors. For example, interactions between gastric and intestinal proteases produced by fish and exogenous enzymes can adversely affect their potential beneficial effects (Fernandes et al., 2021). In addition, the efficacy of exogenous enzymes can be greatly reduced by the high temperatures reached during feed preparation and mechanical shear forces that denature the enzymes (Jacobsen et al., 2018). Therefore, they should be highly resistant to this process or applied later in the final coating of the grain (Ai et al., 2007; Dalsgaard et al., 2012; Jiang et al., 2014). These enzymes may be affected by the time available for enzymatic action within the digestive system of the species, which is closely related to the intestinal transit rates. An interesting alternative to overcome the aforementioned limitations is to carry out the pretreatment of ingredients with multi-enzymatic complexes before the granulation of the feed, for example, by the solid-state hydrolysis (SSH) procedure. SSH works with a percentage of solid substrate greater than 15 %; therefore, there is little free water (Chen & Liu, 2016) (Figure 1). This process is routinely used to obtain specific products, such as glucose or other sugars, or directly to increase the nutritional value of vegetable ingredients by reducing their NSPs content (Opazo et al., 2012). Using SSH, hydrolysis can be carried out under optimal conditions for enzymes and their activity is not affected by the elevated temperatures reached during the preparation of feed or by the biochemical conditions present in the digestive system of the fish.

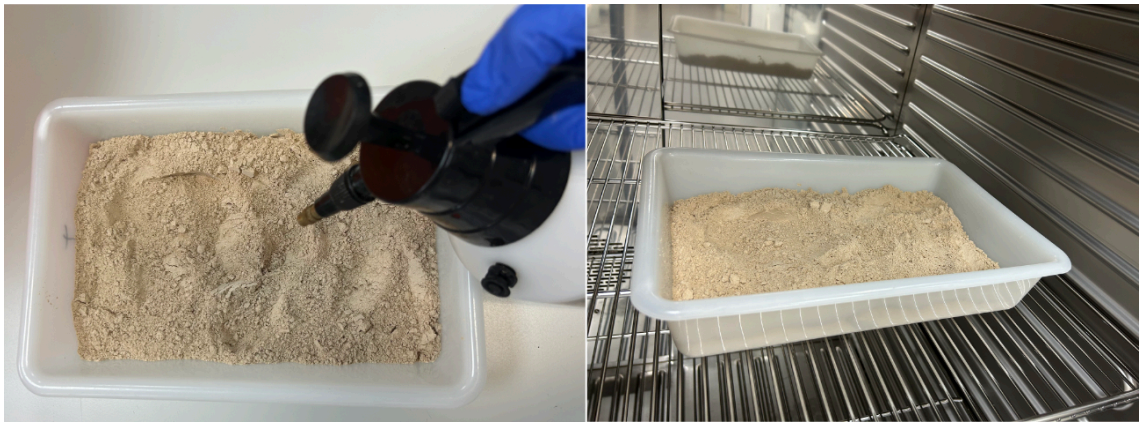

Figure 1. *Treatment of fibrous ingredients with multi-enzymatic complexes by the solid-state hydrolysis (SSH) procedure. Application of the enzymatic complex (left) and hydrolysis incubation under optimal conditions (right).*

4. NON-CONVENTIONAL INGREDIENTS AS SOURCES OF BIOACTIVE COMPOUNDS

As mentioned above, numerous non-conventional ingredients, mainly those derived from vegetables, contain different compounds with biological activity, being polyphenols the most abundant and important category. These are secondary vegetable metabolites that are usually esterified or glycosylated (Vermerris & Nicholson, 2006) being composed primarily by one aromatic ring (hydrophobic domain) and one or more attached hydroxyl groups (hydrophilic domain). This group of compounds includes phenolic acids (hydroxycinnamics and hydroxybenzoids), flavonoids, coumarins, xanthones, chalcones, stilbenes, lignins and lignans (Ferreira et al., 2017). The biological properties of these phenolic compounds, specifically their antioxidant activities, are related to their chemical and structural characteristics. The number and position of hydroxyl groups, the presence of double bonds and the ability to delocalize electrons play key roles in the ability of phenolic compounds to scavenge free radicals and donate hydrogen atoms (Leicach & Chludil, 2014). Another relevant factor is the possible interaction of phenolic compounds with cell membrane components, enzymes, transcription factors and receptors (Fraga et al., 2010). In addition, the interaction between ingested polyphenols and gut microbiota seems to play a crucial role in their potential health benefits (Crozier et al., 2010; Marín et al., 2015; Man et al., 2020). The microbiota present in the gastrointestinal tract can metabolize polyphenols with different physiological meanings (Gowd et al., 2019; Sun et al., 2020), while polyphenols can modify the

composition, diversity and/or activity of intestinal bacterial communities (Bustos et al., 2012).

Considering the aforementioned, it is worth mentioning that the digestive bioavailability of polyphenols is another key factor for understanding their biological activity. Since it differs between various phenolic compounds and depends on the characteristics of the digestive process and the feed matrix, greater knowledge in this subject is required to explain their expected biological activity. Within this context, the study of the possible interactions polyphenol-matrix is a prerequisite to develop formulation of feeds that may improve the bioavailability of polyphenols present in the ingredients (D'Archivio et al., 2010).

Table 2. *Effects of including of vegetable-derived polyphenols in aquafeeds.*

Type by-products	Effects	References
Sorghum distillery	Improves antioxidant status Negatively affects growth	Lee et al. (2009)
Orange	Improves antioxidant capacity Maintains hematological profile	Vicente et al. (2019)
Banana	Improves SGR, FCR and HSI	Yossa et al. (2022)
Grape	Improves immunomodulatory and anti-inflammatory activities	Magrone et al. (2016)
	Maintains SGR Increases FCR	Peña et al. (2020)
Date palm	Improves the SGR	Sotolu et al. (2014); Kamali-Sanzighi et al. (2019)

In addition to their high antioxidant capacity, these secondary vegetable metabolites are characterized by their immunostimulatory capacity (Long et al., 2017; Ji et al., 2018) (Table 2). The inclusion of vegetable-based polyphenols as immunostimulants has led to promising results, such as strengthening the health status of fish and improving their resistance to pathogens (Abdel-Latif et al., 2020; Mohammadi et al., 2020). They can also be considered natural antibiotics with possible applications as additives in aquafeed (Dawood et al., 2018; Farha et al., 2020).

5. INGREDIENTS CONSIDERED IN THE PRESENT THESIS

Considering the above, this Thesis has addressed the evaluation of the possible use of different non-conventional ingredients in aquafeed from the perspectives of their nutritional value and their contents in bioactive

compounds. The ingredients used were one macroalgae (*Ulva ohnoi*) and three types of by-products generated in the production of alcoholic beverages: brewer's spent grain, wine bagasse and wine lees.

5.1. Macroalgae

Macroalgae are a diverse group of marine organisms comprising more than 10,000 different species (Collins et al., 2016). Depending on their pigments, macroalgae are classified as brown (Phaeophyta), red (Rhodophyta), or green (Chlorophyta). Marine macroalgae can adapt to changing and extreme environmental conditions, producing secondary metabolites as an adaptive response. These metabolites include functional proteins (Cruces et al., 2012), peptides (Harnedy & FitzGerald, 2011), mycosporin like amino acids (Carreto & Carignan, 2011), carotenoids, phenolics (Dethier et al., 2005), fatty acids (Alamsjah et al., 2007; Wang et al., 2008), vitamins (Pinto et al., 2003), functional carbohydrates (Karsten et al., 1996) and other secondary metabolites (Oliveira et al., 2013; Svensson et al., 2013).

As described, the chemical composition of macroalgae is mainly determined by the species and the harvesting season. The protein content is generally low in brown algae compared to green (10-26 %) and red algae (35-47 %) (Garcia-Vaquero & Hayes, 2016). On the other hand, the lipid fraction of algae is also of great interest from a nutritional point of view due to its high content of polyunsaturated fatty acids (PUFAs). In addition, the total concentration of polysaccharides in macroalgae varies from 4 % to 76 % of dry weight and is composed mainly of non-starch polysaccharides that can act as prebiotics, exerting a relevant role in the improvement and increase of the beneficial microbiota of the individual (Roberfroid, 2005). On the other hand, the polysaccharides present in algae show great diversity: brown algae contain alginates and polymers containing sulfated fucose and laminarin; red algae are a rich source of agar, carrageenans, xylans, sulfated galactans and porphyrans; and green algae contain sulfated ulvan, xylans and galactans (Makkar et al., 2016).

Table 3. *Effect of the inclusion of different species of macroalgae in aquafeeds.*

Macroalgae	Species	Effects	References
Chlorophyta	*Ulva lactuca*	Increases FW, WG, and the value of the productive protein, but decreases with the % of inclusion FCR increases when the % of inclusion increases FI and PER remain unchanged	Wassef et al. (2013)
	Ulva rigida	Decreases FW, WG and PER at 30 % inclusion FCR increases at 30 % inclusion	Azaza et al. (2008)
	Ulva spp.	The FW, SGR, FI, FCR and PER are maintained.	Silva et al. (2014)
Rhodophyta	*Eucheuma denticulatum*	Parameters are maintained: FW, WG, SGR, Total FI, FCR, PER, NPU and CF	Shapawi & Zamry (2016)
	Gracilaria sp.	Parameters are maintained: FW, Daily WG, FCR, PER, VFI	Valente et al. (2006)
	Palmaria palmata	Parameters are maintained: FW, WG, CF, FCR and SGR	Wan et al. (2016)
	Pterocladia capillacea	Increases FW, WG to 5 % Decreases FW, WG at 10 % and 15 % Increases FCR by 15 %	Wassef et al. (2013)
Phaeophyceae	*Cystoseira barbata*	Higher utilization of dietary protein and energy at 15 % FW, WG, SGR, ANEU and FCR are maintained	Kut Güroy et al. (2007)
	Sargassum polycystum	FW, WG, SGR, FCR, PER, NPU and CF are maintained	Shapawi & Zamry (2016)
	Ecklonia cava	WG, FCR, PER, FI and CF are maintained	Kim et al. (2014)
	Macrocystis pyrifera	Decreases FW and SGR when % inclusion increases	Dantagnan et al. (2009)

Results obtained when testing different species of macroalgae in fish feeds are summarized in Table 3. In the case of the different species of the genus *Ulva,* great interest has been paid to the effects of ulvan and their oligosaccharides. The results confirm the immunostimulant power

of the polysaccharide ulvan. In contrast, the oligosaccharides obtained do not exert a stimulating effect under the conditions evaluated (Fernández-Díaz et al., 2017). In addition to immunostimulation, ulvan has been shown to have hepatoprotective (Sathivel et al., 2014), antihyperlipidemic, antihypercholesterolemic (Pengzhan et al., 2003; Qi et al., 2012; Godard et al., 2009), antioxidant (Qi et al., 2005; Qi et al., 2006; Shao et al., 2014), antibacterial (Gadenne et al., 2013), antiviral (Aguilar-Briseño et al., 2015) and antitumor (Shao et al., 2014) activities. For these reasons, the use of several extracts of these species are of great interest to improve the welfare of cultured fish (Figure 2).

Figure 2. *Processing of* Ulva ohnoi *to obtain meal (cultivated biomass, sun drying, kild dried and trituration, respectively).*

5.2. Brewer's spent grain

Among the great variety of by-products generated by the agri-food industry, it is worth mentioning the residues derived from production of alcoholic beverages, such as beer, which is of great interest due to the large volumes generated, interesting profiles of chemical composition and easy availability worldwide. The brewing industry generates more than 7 million tons of brewer spent grain (BSG) and yeast, with BSG being the most abundant by-product, accounting by approximately 85 % of the total by-products obtained. Approximately 20 kg of wet BSG is produced per 100 L of brewed beer. Brewer spent grain (BSG) is a heterogeneous lignocellulosic material comprising considerable amounts of lignin (12–28 % dry matter) and non-starch polysaccharides (NSPs) (30–50 % dry matter) derived from barley husk cell walls. The NSPs component consisted of approximately equal amounts of cellulose and hemicellulose, with arabinoxylans being the most abundant hemicellulose. In addition to lignocellulose, protein is the second most predominant component of brewer spent grain, accounting for up to 30 % of dry matter content (Lynch et al., 2016). However, the use of BSG as ingredient in aquafeeds

presents some limitations, such as amino acid imbalances, high contents of NSPs and low phosphorus availability (Belyea et al., 2004). As described in Section 3, different pretreatments (physical, chemical, or enzymatic) can be used to counteract the negative effects of these limitations to increase the bioaccessibility of nutrients (Kokou & Fountoulaki, 2018).

Several studies have investigated the potential use of brewer's spent grain as a non-conventional ingredient in aquaculture (Table 4). However, the more suitable levels of inclusion of such by-products in aquafeeds are species-specific and depend on several factors, such as individual amino acid requirements and feeding habits. Therefore, inclusions not exceeding 20 % have been recommended to avoid compromising the zootechnical parameters of the species (Estévez et al., 2021; Tidwell et al., 2021).

Table 4. *Effect of the inclusion of brewer's spent grain (BSG) meal on different aquaculture species.*

% BSG	Species	Effects	References
7.5-15 %	*Sparus aurata*	Maintains zootechnical parameters Maintains fillet quality	Estévez et al. (2021)
20 %	*Sparus aurata*	Maintains protein digestibility	San Martin et al. (2020)
0-26.22-39.32-52.43 %	*Oreochromis niloticus*	No negative effects on zootechnical parameters up to an inclusion of 39.32 %	Hessein et al. (2013)
0-27-55 %	*Oreochromis niloticus and Icatalurus punctatus*	Reduction of growth when the % of inclusion is increased Reduction of weight gained from the inclusion level 27 %	Tidwell et al. (2021)
7.5-15 %	*Oreochromis niloticus*	Final weight gain including 15 % Maintains growth rate Low protein digestibility at 15 %	Estévez et al. (2022)
20 %	*Oreochromis mykiss and Sparus aurata*	Maintains protein and lipid digestibility Maintains growth rate	Nazzaro et al. (2021)

5.3. Wine by-products

Considering the large volumes generated in the production of alcoholic beverages, the by-products generated in wine production have potential as ingredients in aquafeeds. Wine production generates grape pomace or skin as main by-product, which constitutes nearly 15-20 % of the total weight of the grape (Zhu et al., 2015) (Figure 3). It is composed of the solid residues remaining after pressing of the grapes (seeds, skin and a low pulp content). Grape skin is a complex lignocellulosic material that also contains a large amount of hemicellulosic sugars, capable of generating xylose and glucose monomers that can be used after hydrolysis. Oligosaccharides have recently been characterized as carbohydrates formed by 3-10 monosaccharide residues, which fit the definition of prebiotics, since they are not digested or absorbed in the intestinal tract and can act as nutrients for the colon microbiota. In addition, grape pomace contains significant amounts of polyphenols such as anthocyanins, hydroxybenzoic acids, hydroxycinnamic acids, flavonols and stilbenes. Among the flavonols, kaempferol, quercetin, myricetin and isorhamnetin stand out and resveratrol is the main stilbene (Bordiga et al., 2013). On the other hand, wine lees are the residues settled after fermentation or during storage at the bottom of the barrels where the wine is contained, being formed largely by dead yeasts, lactic bacteria, fatty acids and phenolic compounds. Wine lees have attracted the attention of several sectors and industries, such as pharmaceuticals, cosmetics and chemistry, to produce ethanol, tartrates and polyphenols as well as supplements for microorganisms (Hwang et al., 2009; Cechini et al., 2016).

***Figure 3**. Processing of the main by-product generated in the wine industry (red wine grape pomace). Fresh product (left), and grape pomace meal (right).*

Despite all their possible applications, there are few publications testing the potential use of either grape pomace or lees as functional ingredients for aquaculture feeds. Nevertheless, studies testing their application in species like the Peruvian anchovy (Solari-Godiño et al., 2017), carp (Souza et al., 2019) and rainbow trout (Peña et al., 2020; Pulgar et al., 2021), and have reported beneficial effects on the oxidative status of the individuals.

6. *IN VITRO* MODELING AS A TOOL TO ASSESS NUTRIENT BIOAVAILABILITY IN AQUAFEED

In recent years, *in vitro* digestive simulation systems have attracted great interest for the evaluation of nutritional, functional and pharmaceutical ingredients mainly because of the ethical, technical and economic problems associated with *in vivo* assays. Thus, *in vitro* digestion models have been extensively used to simulate the digestive processing of ingredients within the gastrointestinal tract of humans and terrestrial animals because they are simple, standard, reproducible, quick and unethically restricted methods (Li et al., 2020; Mulet-Cabero et al., 2020).

It is important to bear in mind that *in vitro* and *in vivo* digestibility assays do not measure the same parameters and, for that reason, a direct correlation between them is not always clear or possible. To understand these differences, two specific terms that describe what happens to nutrients and other compounds in the digestive tract must be considered: bioaccessibility and bioavailability. Bioavailability is a complex but key indicator for assessing ingredient efficacy, which, in turn, is closely dependent on bioaccessibility. Bioaccessibility is the fraction of a compound that is released from the feed matrix into the digestive lumen by mechanical and chemical action and is therefore available for intestinal absorption. After being released from the feed matrix and becoming bioaccessible, nutrients can be absorbed from the gastrointestinal tract, determining their bioavailability as the amount of a nutrient or compound available for metabolic use after digestion and absorption. Bioaccessibility depends on several factors linked mainly to the structure and composition of the feed matrix, while bioavailability largely depends on intestinal absorption processes, interaction with the microbiota and subsequent cellular metabolism (Rein et al., 2013). Taking this into account, it follows that *in vitro* assays are based on the use of bioreactors with different complexities that simulate the

physicochemical conditions present in the digestive system of the target species to observe their effect on the bioavailability of nutrients, drugs, or bioactive compounds. They also make it possible to study not only the biochemical conditions of the digestive system that influence bioavailability, but also how different components of the feed matrix interact with each other to facilitate access or possible availability of both nutrients and bioactive compounds.

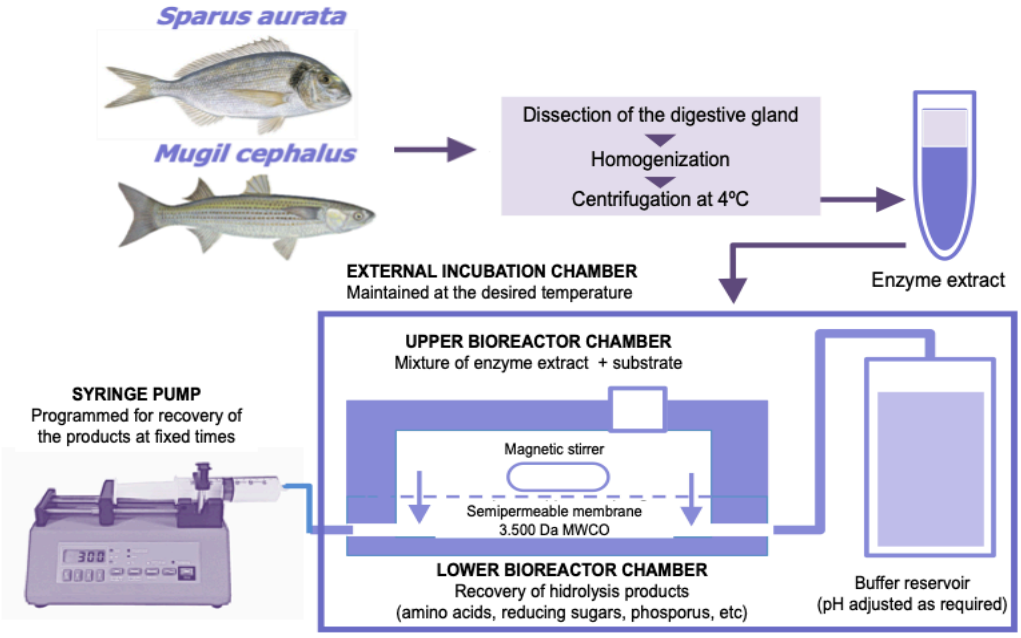

Figure 4. *Scheme of the open system* in vitro *simulation of fish digestion using a membrane bioreactor.*

In an equivalent manner to what applied with humans and terrestrial mammals, different types of *in vitro* digestion models have been used in nutritional studies with aquatic species using different configurations, such as pH-stat (Dimes et al., 1994), modified pH-stat including an acid pre-digestion step that simulates stomach action (Alarcón et al., 2002), closed-chamber reaction systems (Bassompierre et al., 1997; Rungruangsak-Torrissen, et al., 2002) and open systems in which the products of the reaction are continuously withdrawn when they occur, simulating more closely what happens in the digestive tract of animals (Moyano & Savoie, 2001) (Figure 4). The main orientation of such studies has been the evaluation of the protein quality of feed ingredients (Alarcón et al., 2002; Hamdan et al., 2009), the study of factors affecting the efficiency of enzymatic hydrolysis during digestion (Gilannejad et al., 2018) or the effect of digestive biochemistry on toxic compounds (Nogueira et al., 2022). In spite of this wide range of applications, to date,

they have not been used to evaluate the potential interactions and beneficial effects of including bioactive compounds (like phenolics) in aquafeeds.

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II. HYPOTHESIS AND OBJECTIVES

The **general hypothesis** of the present Thesis is:

There are unconventional ingredients whose use in aquafeeds can be justified either by a nutritional improvement linked to an enzymatic treatment or because they provide bioactive compounds.

To demonstrate such hypothesis, the nutritional and functional evaluation of two types of ingredients was proposed: macroalgae biomass and agro-industrial fibrous by-products. This was developed through different specific objectives.

Objective 1. *Aimed to determine the putative effects of enzymatic pretreatment with carbohydrases on the availability of nutrients and bioactive compounds in the biomass of the macroalgae* Ulva ohnoi *when used as a feed ingredient for two marine species, as European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*).*

To date, no studies have evaluated the potential benefits of including *Ulva* in aquafeeds, from both nutritional and functional perspectives. An experiment was designed to evaluate the biological responses obtained when using *U. ohnoi* as an ingredient in diets for two marine fish, the Euopean sea bass (*D. labrax*) and the gilthead sea bream (*S. aurata*). The study developed different experiments focused on the evaluation of nutritional aspects using both *in vitro* and *in vivo* assays, as well as the potential benefits derived from the presence of bioactive compounds on the immunological parameters and oxidative status of the individuals.

Objective 2. *Aimed to evaluate the possible improvement in the growth performance and feed efficiency resulting from enzymatic pretreatment of brewer's spent grain with an enzyme mixture of carbohydrases and phytase when included in feeds for grey mullet (*Mugil cephalus*).*

This experiment was conceived to evaluate whether a pretreatment of vegetable ingredients containing high proportions of phytate and non-starch polysaccharides (NSPs), using a mixture of enzymes applied under a solid-state hydrolysis system (SSH), could improve their nutritional value when used as ingredients in feed for grey mullet (*Mugil cephalus*). Two trials were conducted to achieve this goal.

Experiment 1. A first short-term trial for the preliminary evaluation of growth performance, feed efficiency and energy metabolism of fish fed these aquafeeds.

Experiment 2. A second long-term feeding trial focused on the evaluation of differences in growth and feed efficiency produced by aquafeeds under real production conditions.

Objective 3. *Aimed to estimate the value of wine by-products (grape pomace and lees) as potential sources of functional compounds in aquafeeds.*

This objective was developed through two different experiments:

Experiment 1. This study was conducted to assess the effects of different factors on the potential bioavailability of phenolic compounds present in two types of wine by-products (grape pomace and lees) when included in feeds for two fish species: the gilthead sea bream (*S. aurata*) and the grey mullet (*M. cephalus*). Both species were selected to illustrate the expected differences linked to particular characteristics of their digestive physiology (mainly determined by both the amount and types of digestive enzymes and by the presence of an acid stage in the digestion of gilthead sea bream, which is absent in the case of mullet). The study was based on *a)* the use of an *in vitro* model adapted to simulate the digestion of both fish species; *b)* a factorial experimental design, aimed at evaluating the effect of different factors involved in digestion; and *c)* a precise analytical methodology of phenolic compounds released after simulated digestion using ultra-high performance liquid chromatography (UHPLC) coupled to high-performance mass spectrometry (HRMS).

Experiment 2. The aim was to evaluate the potential benefits of including the two above mentioned wine by-products as functional ingredients in feed for juveniles of golden mullet *Liza aurata*. This study was designed to evaluate both the general effects on metabolism derived from the intake of bioactive compounds present in by-products and the potential protective effect against induced stress (hypoxia) by evaluating variations in oxidative status.

It is noteworthy that the objectives of this Doctoral Thesis, oriented to valorize some agri-food products within a circular economy approach, are closely related to some of the Sustainable Development Goals (SDGs) of the United Nations. In particular, they would be aligned with Goals 2 (innovative study to reduce extreme hunger and nutritional depletion among societies) and 12 (Ensure sustainable consumption and production patterns) and more indirectly with Goals 13 (Take urgent action to combat climate change and its effects) and 14 (Conserve and sustainably use the oceans, seas and marine resources).

III. EXPERIMENTAL WORKS

CHAPTER 1

Evaluation of the inclusion of the green seaweed *Ulva ohnoi* as an ingredient in feeds for gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*)

Martínez-Antequera, F. P., Martos-Sitcha, J. A., Reyna, J. M., & Moyano, F. J. (2021). Animals, 11(6), 1684.

ABSTRACT

This study evaluated the use of *Ulva ohnoi* as an ingredient in feeds for aquaculture in three different experiments. Experiment 1 was oriented to confirm the negative effect of *U. ohnoi* on fish digestion. Experiment 2 assessed the effect on growth, feed efficiency, and immune status of juvenile sea bass (*Dicentrarchus labrax*) fed on diets including *U. ohnoi*, previously treated or not with carbohydrases used to partially hydrolyze indigestible polysaccharides. Experiment 3 was aimed to evaluate the potential protective effect of *U. ohnoi* on the oxidative status of sea bream (*Sparus aurata*) challenged by the consumption of a feed formulated with the oil fraction completely oxidized. Results show a negligible effect of *U. ohnoi* meal on protein digestion when included in feeds at levels of 10 % or less. Moreover, results of growth and feed use evidenced the possibility of using up to 5 % inclusion of algal meal in feeds without adverse effects on the zootechnical parameters, while the enzyme pretreatment was ineffective to improve its nutritional use. Finally, the inclusion of *U. ohnoi* in feeds determined both an immunostimulatory effect, evidenced by an increase in skin mucus lysozyme in the two mentioned fish species, and a positive influence on the oxidative metabolism of sea bream when fed on a diet including rancid oil.

Keywords: aquaculture feeds; bioactive compounds; *Ulva ohnoi*

1. INTRODUCTION

Ulva are green macroalgae belonging to the phylum Chlorophyta that presents a great environmental polymorphism, and genetic analysis suggests that the different described species for the genus (*U. armoricana, rigida, prolifera, pertusa, fasciata,* or *ohnoi*) are only environmental variants or clades (Dominguez & Loret, 2019). *Ulva* blooms are frequent and described in several parts of the world associated with an excess of dissolved nutrients in coastal waters resulting from fields fertilization or human wastes (Teichberg et al., 2010). The potential use of *Ulva* biomass provided by these blooms as a source of fertilizer or bioenergy has been widely assessed (Saqib et al., 2013). Several studies have tested the potential inclusion of *Ulva* in aquaculture feeds, although previous results pointed that the observed effects are closely related to the level of dietary inclusion. Hence, positive effects on growth and feed efficiency have been reported at incorporation rates accounting for less than 10 % of the dry weight of the feed (Valente et al., 2006; Wassef et al., 2013). In contrast, levels exceeding this amount either do not result in significant effects (Silva et al., 2015; Abdel-Warith et al., 2016; Vizcaíno et al., 2016) or produce negative results (Siddik et al., 2015; Shpigel et al., 2017). In this sense, it has been suggested that the presence of protease inhibitors may limit digestive use of this seaweed in several fish species such as the Senegalese sole (*Solea senegalensis),* the gilthead sea bream *(Sparus aurata),* or the European sea bass *(Dicentrarchus labrax*,) and hence limiting growth performance and feed use (Vizcaíno et al., 2020).

On the other hand, the role of seaweeds as sources of bioactive compounds is widely recognized (Cho et al., 2007; Øverland & Skrede, 2017). In the case of *Ulva* species, the polysaccharide ulvan may present beneficial effects on the immunological status in some fish species such as *Solea senegalensis* (Fernández-Díaz et al., 2017), and they also contain compounds with reported antioxidant effect (Yildiz et al., 2012; Trigui et al., 2013; Magnoni et al., 2017). Nevertheless, results are somewhat contradictory, and some authors did not find such positive effects when including this seaweed in diets for marine species such as *D. labrax* (Lobo et al., 2018) or *S. senegalensis* (Fumanal et al., 2020). For this reason, the possibility of testing the potential positive effect of using *Ulva* as a protective agent against conditions determining oxidative stress was considered within the framework of this general evaluation. The selected challenge was the consumption of rancid oil since this has

been reported as one factor with a high impact on the growth and oxidative metabolism of fish (Koshio et al., 1994; Baker & Davies, 1997; Peng et al., 2009).

Considering all the aforementioned information, it is clear that the potential inclusion of seaweeds in fish feeds must consider different aspects related to their potential role, either as nutritional or functional ingredients. To date, no comprehensive study has assessed the potential benefits of including *Ulva* in fish feeds from both perspectives. The present work was intended to evaluate biological responses obtained when using *Ulva ohnoi* as an ingredient in feeds for two important marine fish for European aquaculture, the European sea bass (*D. labrax*) and the gilthead sea bream (*S. aurata*). The study developed different experiments focused on the evaluation of nutritional aspects by using both *in vitro* and *in vivo* approaches, as well as the potential benefits derived from the presence of bioactive compounds on immunological parameters and oxidative status of fish.

2. MATERIALS AND METHODS

2.1. Algal biomass

The biomass of *U. ohnoi* used in the different experiments was collected from external tanks of the facilities of the Aquaculture Technology Center CTAQUA (El Puerto de Santa María, Spain). After washing with fresh water, the biomass was partially desiccated using a solar dryer until it reached a moisture content of nearly 20 %. Once received in the laboratory, the biomass was subjected to an additional drying in an oven for 24 h at 60 °C and subsequently finely chopped until obtaining a fine powder that was used in all the assays.

2.2. Description of the experiments

As indicated in the previous section, 3 different experiments were designed based on the flow diagram and decision criteria detailed in Figure 1 and described below.

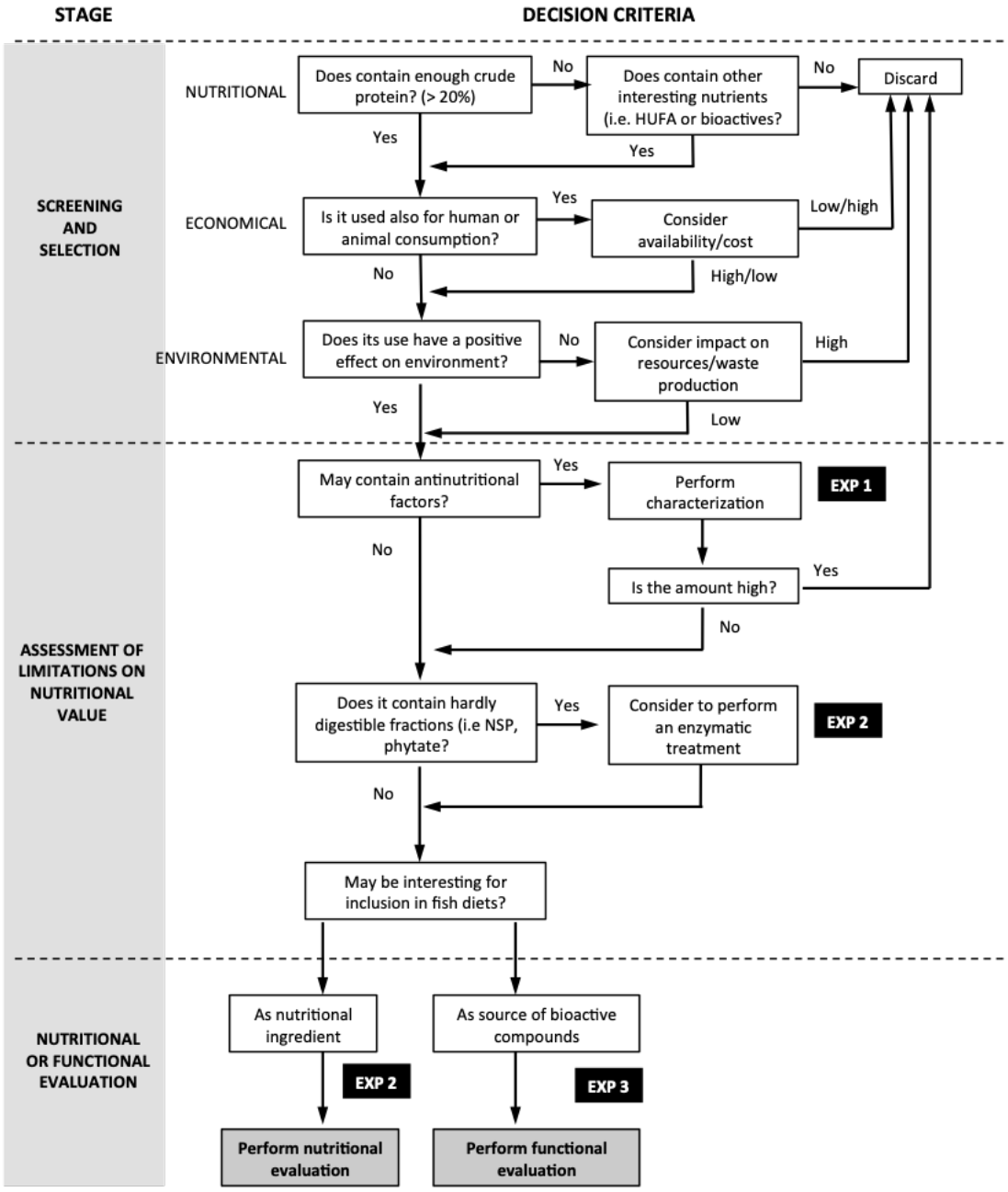

Figure 1. *Flow diagram used to design the experiments developed in the present study.*

2.3. Experiment 1

This preliminary assay was oriented to confirm the potential negative effect of *U. ohnoi* on fish digestion.

According to the study of Vizcaíno et al. (2020), *U. ohnoi* contains a protease inhibitor of fish digestive proteases that would inactivate up to 60 % of the alkaline protease activity present in the gut of different fish

species such as European sea bass or Senegalese sole. However, such a study did not consider the amounts of algal meal and enzyme that could be really present in the digestive tract of fish consuming a feed enriched with the seaweed. The present experiment was designed to quantify on a physiological basis such expected inhibition by; (a) developing an inhibition assay after establishing accurately the expected relationship between the amount of algae and enzyme activity present in the digestive system of juvenile sea bass fed on a standard ration, and (b) using an *in vitro* assay to confirm the effect of the protease inhibitor on the digestive hydrolysis of protein present in the feed.

To develop point (a), the total amount of protease activity in the gut of juvenile sea bass during digestion was measured on fish sampled 3 h after being fed on a commercial feed (n = 10; 40.6 ± 2.8 g). Digestive enzyme extracts were prepared after manual homogenization of dissected tissues, and the determination of acid and alkaline protease activities was carried out using hemoglobin and casein as substrates, respectively (Anson, 1938; Walter, 1984). On the other hand, the expected amount of *Ulva* present in the digestive tract was estimated considering the average amount of feed consumed per meal in a fish of the above-indicated size and an inclusion level of *Ulva* in the feed of 80 g/kg. Both data (total activity produced by a fish and total amount of algae ingested per meal) were used to determine the % inhibition of protease activity potentially derived by using such amount of algae. The inhibition assay was carried out similarly to that described by Vizcaíno et al. (2020) by mixing 1 mL of intestinal enzyme extract of known activity to the required amount of seaweed meal to achieve the E:S ratio calculated above. The mixture was incubated for 60 min, and the observed reduction in activity was expressed as % of that determined using an extract preincubated only in the presence of water as a reference.

In a second approach, the potential effect of protease inhibitor on digestive protein hydrolysis was estimated more precisely by an *in vitro* digestive simulation test. The assay was developed using the membrane bioreactor and general procedure described in Gilannejad et al. (2017). In short, the device consists of two chambers separated by a semi-permeable membrane of 3,500 kDa MWCO (ZelluTrans/Roth1). Enzyme extracts and feed samples were placed in the upper chamber and maintained under continuous agitation using a magnetic stirrer. Amino

acids passing across the membrane into the lower chamber were recovered at different time intervals during the reaction time. During the acid phase of digestion, the upper chamber contained the substrate dissolved in water and adjusted to pH 4.0 as well as the crude enzyme extract from the sea bass stomach, while the lower chamber contains distilled water. During the alkaline phase, the pH of the upper chamber was raised to pH 8.5 prior to the addition of the intestinal enzyme extracts, being the lower chamber filled with 100 mM Tris-maleate buffer at the same pH (supplemented with 100 mM $CaCl_2$ and 50 mM NaCl). The complete arrangement was maintained at 25 °C. Total amino acids released during the hydrolysis were measured using the o-phtaldialdehyde method (Church et al., 1983). The assay developed using this configuration tested differences in protein hydrolysis obtained using the E:S ratio described above with samples of feeds C (without *Ulva* meal) and U8 (containing 80 g/kg *Ulva*) formulated for Experiment 2 (Table 1).

Table 1. *Ingredients and proximal composition of the five feeds used in Experiment 2. The number (5, 8) indicates the % inclusion of the* Ulva ohnoi *meal, while the enzyme treatment is indicated by "E". (*) Gross energy was estimated from nutrient analysis.*

Ingredients (g/100 g)	C	U5/UE5	U8/UE8
Fish meal	32.0	32.0	32.0
Soybean meal	8.0	8.2	8.7
Guar meal	10.0	6.0	5.0
Soy concentrate	20.0	20.2	21.1
Corn gluten	15.0	15.0	16.0
Dried *Ulva* meal	0.0	5.0	8.0
Wheat meal	3.7	0.5	0.0
Fish oil	4.9	4.9	5.0
Sunflower oil	3.9	4.0	4.0
Soy lecithin	0.4	0.4	0.4
Vit/min premix	0.5	0.5	0.5
Taurine	0.5	0.5	0.5
Palatability enhancer	0.1	0.1	0.1
Cr_2O_3	1.0	1.0	1.0
Crude protein (%)	52.0	51.6	50.8
Fat (%)	14.0	14.2	14.0
Ash (%)	7.0	7.2	7.4
Moisture (%)	10.6	11.2	11.0
Gross Energy (MJ/kg) *	20.8	20.9	20.6

2.4. Experiment 2

This experiment was oriented to assess the effect on growth, feed efficiency, and immune status of juvenile sea bass fed on diets including *Ulva*, previously treated or not with a mixture of carbohydrases used to partially hydrolyze the fraction of indigestible polysaccharides.

2.4.1. Enzyme pretreatment of Ulva

A certain amount of the *Ulva* meal described in Section 2.1. was enzymatically pre-treated with a commercial mixture of carbohydrases (Rovabio Advance Max L), which presented a high activity of glucanases and pectinase. To carry out the treatment, the meal was mixed carefully with citrate buffer (pH 5.0, 0.1 M; 1:3 w/v) to obtain a moist mass with the optimal conditions for the action of the enzymes, which were previously solubilized in a small amount of the same buffer and added by spraying. The enzyme mixture was added using the dose indicated by the producer (0.2 mL/kg) and allowed to act by keeping the mixture at 45 °C for 6 h, with manual stirring every hour to ensure the homogeneity of the reaction. After this time, the reaction was stopped by placing the mixture in a cold chamber at 4 °C until being used as an ingredient in the preparation of the experimental diets. Besides the chemical analysis described in the next section, scanning electron microscopy was used to assess the potential effect of enzyme treatment on the tissue structure of *Ulva*. Several images were obtained from different areas of the samples to guarantee the representativeness of the results (Figure 2).

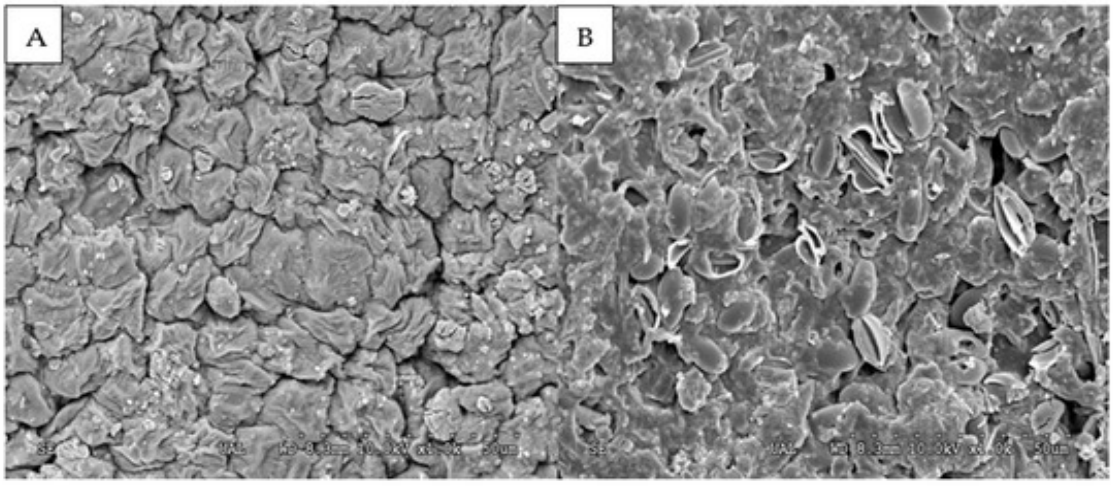

Figure 2. *Scanning electron microscopy of a sample of the meal of* Ulva ohnoi *(**A**) before enzymatic hydrolysis and (**B**) after 24 h of enzymatic hydrolysis.*

2.4.2. Diet and analysis

Five experimental feeds were formulated, including either 5 % or 8 % of *Ulva* meal, enzymatically treated (UE5, UE8) or not (U5, U8). A diet without alga meal was used as a control (C) (Table 1). Cr_2O_3 was included in all diets as an inert marker to evaluate digestibility. Feeds were prepared using a lab-scale extrusion machine provided with a mesh size of 2 mm, dried, and stored at 4 °C until used. Besides proximate analysis, samples of each feed were used for the analysis of some compounds that could be affected by the enzyme pretreatment, as soluble protein, reducing sugars, free pentoses, total phenolics, and total antioxidant capacity. Soluble protein was analyzed by the Bradford method (Bradford, 1976) using the SIGMA Total Protein Kit (TP0100). Reducing sugars were measured using 3,5-dinitrosalicylic acid (DNS) following the method described by Miller (1959). Free pentoses were measured by the phloroglucinol method described by Douglas (1981). Total phenolics were determined by the method described by Graça et al. (2005). Trolox equivalent antioxidant capacity (TEAC) was determined following the DPPH method described by Brand-Williams et al. (1995). All the analyses were performed in triplicate samples from each diet.

2.4.3. Animals and facilities

A total of 450 juvenile European sea bass (*Dicentrarchus labrax*) (16.4 ± 1.2 g) were distributed into 15 tanks (120 L; n = 30 fish per tank) in the facilities of CTAQUA (El Puerto de Santa María, Spain). The tanks were provided with a settling column for stool removal (Guelph method). Each of the five experimental feeds was evaluated in triplicate. Each experimental diet was offered to visual satiety two times per day and 6 days per week in a 67-day feeding trial. The amount of food ingested by each experimental unit was recorded on a weekly basis using gravimetric methods (g of feed consumed / tank). Water quality of the system was continuously monitored; temperature, dissolved oxygen, and survival data were controlled daily, whereas ammonium, nitrite, and salinity levels were checked weekly. For the digestibility assay, feces were removed daily for 3 weeks, dried, and processed to determine their nitrogen contents. Fecal samples obtained on three different days were pooled to form one sample, and three different samples were obtained from each tank. The determination of the total chromium of feeds and

feces was carried out using the diphenylcarbazide method (Divakaran et al., 2002).

2.4.4. Parameters evaluated

The following growth parameters were evaluated:

Specific Growth Rate (SGR) = (100 × (ln final body weight—ln initial body weight))/days
Weight Gain (WG) = (100 × (body weigh increase))/initial body weight
Feed Conversion Ratio (FCR) = total feed intake/weight gain

Moreover, apparent digestibility coefficients (ADC) were calculated as follows:

$$ADC\ nutrient = 100 - [\left[\frac{(\%\ of\ indicator\ in\ food)}{(\%\ of\ indicator\ in\ feces)}\right]\left[\frac{(\%\ of\ nutrient\ in\ feces)}{(\%\ of\ nutrient\ in\ food)}\right] * 100]$$

The potential variations in the immunological status of the fish after being fed on diets including *Ulva* were assessed by measuring lysozyme and alkaline phosphatase activities in skin mucus (12 fish per diet) sampled at the end of the growth experiment. Lysozyme was measured using a commercial kit (Ref. E22013; Thermo Fisher Scientific, Waltham, Massachusetts, USA), adapted to 96-well microplates. Alkaline phosphatase activity was determined using pNp-phosphate disodium salt (Sigma-Aldrich M8168) as substrate following the method described by Gee et al. (1999).

2.5. Experiment 3

This experiment was aimed to evaluate the potential protective effect of *Ulva* on the oxidative status of fish challenged by the consumption of a feed including oxidized oil.

2.5.1. Ingredients and feeds

The *U. ohnoi* meal was the same as in previous experiments. Four experimental feeds were elaborated (Table 2); two of them included 10 % of *U. ohnoi* meal and either fresh or oxidized oil (U/UO). The other two control feeds followed the same scheme and also included the two types of oil (C/CO). Oxidation of the mixture of fish and sunflower oils used in the elaboration of CO and UO feeds was produced by heating at 60 °C with intermittent air injection (10 min of injection and 30 min of rest) for

24 h until the peroxide value (POV) reached 101.25 meq/kg (the value for untreated oil was 0.60 meq/kg). Feeds were prepared using a lab-scale extrusion machine provided with a mesh size of 2 mm, dried, and stored at 4 °C until used. Samples of each feed were used for the analysis of soluble protein, reducing sugars, free pentoses, total phenolics, and antioxidant capacity, according to the methodologies described in Section 2.4.

Table 2. *Ingredients and proximal composition of the four feeds used in Experiment 3. (*) Gross energy was estimated from nutrient analysis.*

Ingredients (g/100 g)	C/CO	U/UO
Fish meal	25.0	25.0
Soybean meal	13.3	13.1
Guar meal	10.0	10.0
Soy concentrate	10.0	10.0
Corn gluten	15.0	15.0
Dried *Ulva* meal	0.0	10.0
Defatted rice bran	8.0	0.0
Wheat starch	5.3	3.3
Fish oil (oxidized or not)	6.1	6.2
Sunflower oil (oxidized or not)	4.9	5.0
Soy lecithin	1.2	1.2
Vit/min premix	0.8	0.8
Taurine	0.2	0.2
Attractant	0.2	0.2
Crude protein (%)	45.0	44.8
Fat (%)	12.1	12.4
Ash (%)	7.42	8.03
Moisture (%)	8.12	8.77
Gross Energy (MJ/kg)*	19.7	19.8

2.5.2. Animals and feeding schedule

A total of 400 juvenile gilthead sea bream (*Sparus aurata*) (46.31 ± 0.29 g) were distributed into 12 tanks (330 L; *n*= 32 fish per tank) in the facilities of CTAQUA (El Puerto de Santa María, Spain). The water quality of the system was continuously monitored as described in the previous section. Each of the four experimental feeds was evaluated in triplicate. Each experimental diet was offered to visual satiety two times per day and 6 days per week. The amount of feed ingested by each of the experimental units was recorded on a weekly basis using gravimetric methods (g of feed

consumed/tank). The trial ran for 28 days, being this period divided into three stages: preliminary feeding (7 days), challenge (14 days), and recovery (7 days). During the preliminary feeding, all the fish were fed on a commercial feed to normalize their nutritional status. During the challenge, triplicate groups of 30 fish received each of the 4 types of experimental feeds. After this period, during recovery, the fish groups that were fed on feeds containing rancid oil (CO and UO) received the feed containing *Ulva* (U), while the other two groups maintained the same feeds consumed during the previous stage.

2.5.3. Evaluated parameters

At the end of the trial, overnight fasted fish (5 fish per tank, 15 per experimental condition) were randomly sampled and anesthetized with 2-fenoxiethanol for liver and skin mucus collection. Previously, fish were bled out with heparinized syringes and killed by the cervical section, and their livers were extracted and weighed to calculate the hepatosomatic index (HSI). Samples of liver and skin mucus were rapidly taken, snap-frozen in liquid nitrogen, and stored at −80 °C until used in biochemical analyses.

Livers were homogenized (1:10, w/v) in 100 mM potassium phosphate buffer (pH 7.4) at 4 °C using a mini handheld homogenizer (Ref. MT-13K; Hangzhou Miu Instruments Co., Ltd., Hangzhou, China) for 1 min. Homogenates were centrifuged at 12,000 *g* for 15 min at 4 °C, and supernatants were used to determine different enzyme activities: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Superoxide dismutase was measured using the commercial kit (Ref. CS0009; Sigma-Aldrich, St. Louis, MO, USA). Catalase was measured using a commercial kit (Ref. EIACATC; Thermo Fisher Scientific, Waltham, MA, USA). Glutathione peroxidase was measured using a commercial kit (Ref. 703102; Cayman Chemical, Ann Arbor, MI, USA). Lipid peroxidation was assessed by measuring total thiobarbituric acid reactive substances (TBARS) using the method of Buege & Aust 1978). In addition, mucus lysozyme was measured (5 fish/tank) as described in Section 2.4.

2.6. Statistical analysis

The normality of the data was performed using the Shapiro–Wilk test, and homoscedasticity analysis was conducted using the Brown–Forsythe test. Statistical analysis of the data was carried out by one or two-way ANOVA, followed by the Bonferroni test where appropriate. The significance level was established at $p < 0.05$. When required, data expressed in percentage were previously arc-sin transformed. All the analyses were performed using the software Statgraphics Centurion (Statgraphics Corp. CA. EE.UU.).

3. RESULTS

3.1. Inhibitory effect of *U. ohnoi* meal on protein digestion in European sea bass

The activity of digestive alkaline proteases measured in juvenile sea bass was 62 U/g fish. Accordingly, a 40 g fish (representative size for a juvenile fish) should produce about 2,500 U of enzyme in each feeding episode. On the other hand, a fish of such size receives two meals daily (1.5 % of the weight/meal) this accounting for 0.6 g feed/meal. If such feed contains 80 g/kg of *U. ohnoi* meal (an average amount estimated from the studies cited in the Introduction section), the estimated intake of seaweed per meal should be around 50 mg/meal. This should result in a relative proportion of 0.02 mg *U. ohnoi* per unit enzyme activity in each feeding episode. When such value is represented in the plot published by Vizcaíno et al. (2020), it results in less than 10 % protease inhibition (Figure 3). This was coincident with the result obtained in the inhibition assay, which produced a 10.7 % decrease in the activity of sea bass alkaline proteases. This negligible effect was confirmed by results obtained with the *in vitro* assay, on which no visible reduction in the hydrolysis of the protein fraction associated with the presence of *U. ohnoi* meal in the feed was appreciated (Figure 4).

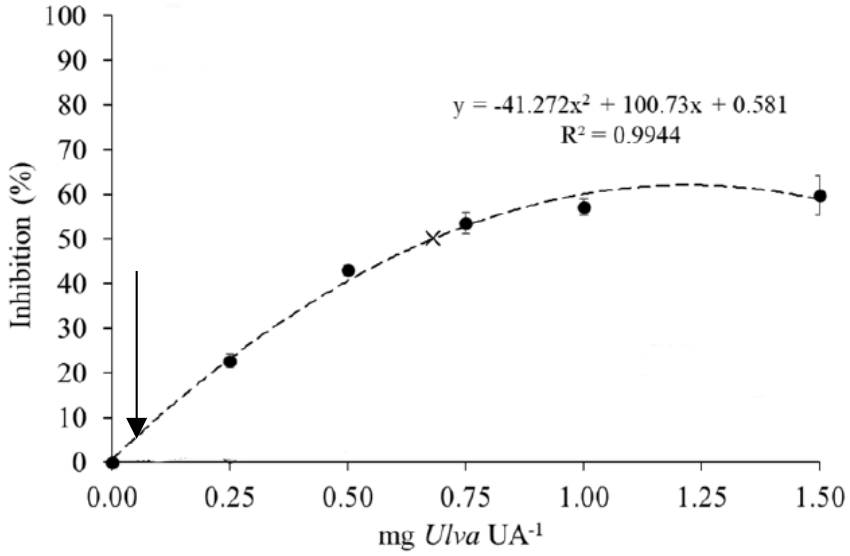

Figure 3. *Inhibitory response of European sea bass intestinal proteases after incubation with increasing doses of raw or heat-treated* Ulva *extracts (graph adapted from (Vizcaíno et al., 2020). The inhibition value (% over a control) corresponding to 0.02 mg Ulva/U activity is represented by an arrow.*

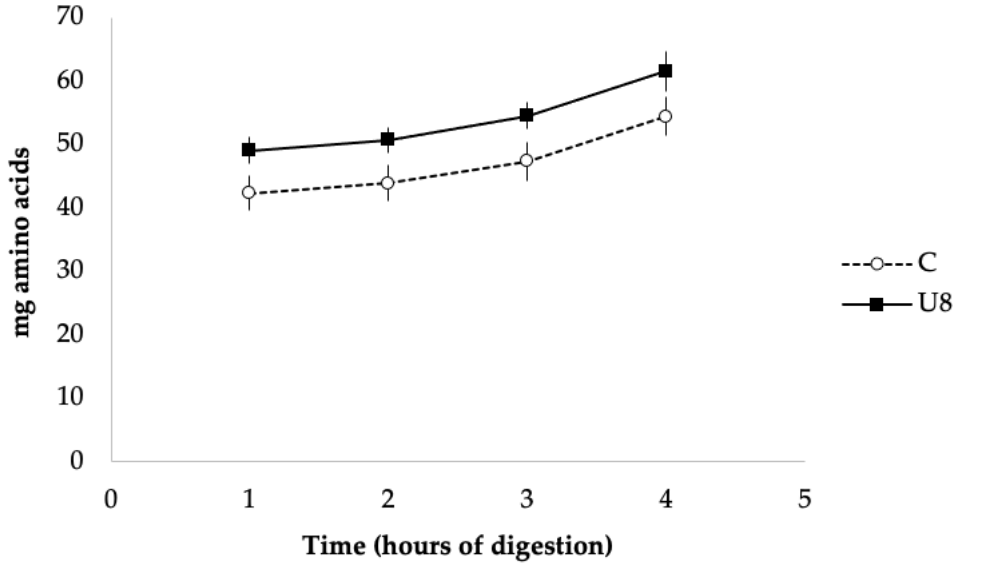

Figure 4. *Time release of total amino acids in the* in vitro *simulation of the digestion of feed, including 8 %* U. ohnoi *meal (U8) and without macroalgae (C). Each point represents the average value of three determinations ± SD.*

3.2. Effect on nutritional efficiency and immune status of juvenile European sea bass fed on diets including *U. ohnoi* previously treated or not with a mixture of carbohydrases

The potential differences in the amounts of some readily bioavailable nutritional compounds (soluble protein, reducing sugars, pentoses) or bioactives (total phenols, TEAC) in the different experimental diets are

detailed in Table 3. Results evidenced a significant reduction in the amount of soluble protein in diets including *Ulva* when compared to the control, as well as a negative effect of the enzyme treatment on this parameter. The amount of reducing sugars was also significantly lower in feeds containing *Ulva* meal when compared to the control diet, although no effect of the enzyme treatment was observed in this case. In addition, neither the presence of *Ulva* nor the enzyme treatment influenced the amount of pentoses or total phenols, while TEAC was significantly increased in feeds containing *U. ohnoi* in relation to the control.

Table 3. *Experiment 2. Nutrient content of the feeds expressed in g/100 d.m. Values are presented as mean ± SD. Values in columns not sharing the same letter differ significantly with $p < 0.05$. Comparisons between inclusion levels (0 %, 5 %, 8 %) are indicated with capital letters, while paired comparisons between enzyme treatment (U5/UE5; U8/UE8) are indicated with lowercase letters.*

Feed	Soluble protein	Reducing sugars	Pentoses	Totals phenols	TEAC (mM)
C	2.16 ± 0.25^{A}	0.43 ± 0.09^{A}	0.30 ± 0.04	0.13 ± 0.01	0.47 ± 0.03^{A}
U5	0.87 ± 0.10^{Ba}	0.25 ± 0.04^{Ba}	0.32 ± 0.06	0.13 ± 0.01	1.02 ± 0.03^{B}
U8	0.60 ± 0.03^{Ca}	0.20 ± 0.01^{B}	0.32 ± 0.05	0.12 ± 0.01	0.97 ± 0.01^{B}
UE5	0.62 ± 0.01^{b}	0.16 ± 0.02^{b}	0.40 ± 0.04	0.14 ± 0.03	0.94 ± 0.01
UE8	0.13 ± 0.05^{b}	0.28 ± 0.01	0.37 ± 0.04	0.12 ± 0.01	1.01 ± 0.00

Results regarding growth performance and feed use obtained when providing the experimental feeds for 65 days to juvenile European sea bass are summarized in Table 4. During the experimental period, fish grew adequately and multiplied their weight by a factor of ~2.45, with an overall SGR ~1.3–1.4 % day^{-1}. There were no significant mortalities, and they maintained healthy and active. Overall, the results were quite homogeneous, and no clear effect of the inclusion of *Ulva* meal, treated or not, was evidenced. No significant differences were found in FCR between groups, but they were present in growth rates, with significantly lower rates obtained for fish fed on the feeds, including the higher amount of *U. ohnoi* meal, irrespective of enzyme treatment. Values of apparent digestibility for protein were significantly higher for these same diets and also for the diet, including the lower amount of algal meal enzymatically treated.

Table 4. *Experiment 2. Growth and efficiency in the use of food in experimental feeds. Values are presented as mean ± SD. Values in a row not sharing the same letter are significantly different, with p < 0.05. FCR: feed conversion ratio; SGR: specific growth rate; ADC: apparent digestibility coefficient.*

Parameter	C	U5	UE5	U8	UE8
Initial weight (g)	508.37 ± 0.89	507.69 ± 6.07	510.22 ± 3.43	512.72 ± 1.43	508.05 ± 4.57
Final weight (g)	1265.35 ± 52.72	1261.73 ± 27.67	1249.66 ± 23.29	1217.24 ± 39.55	1195.20 ± 12.79
Weight increase	756.98 ± 52.45	754.05 ± 25.07	739.43 ± 26.71	704.52 ± 39.34	687.16 ± 17.00
Feed intake (g)	998.45 ± 46.46	1005.59 ± 14.41	1007.84 ± 7.77	964.41 ± 12.97	945.72 ± 8.04
FCR	1.31 ± 0.06	1.32 ± 0.03	1.35 ± 0.05	1.37 ± 0.06	1.37 ± 0.03
SGR (% day^{-1})	1.38 ± 0.04^{a}	1.38 ± 0.04^{a}	1.35 ± 0.02^{a}	1.29 ± 0.05^{b}	1.29 ± 0.02^{b}
ADC Protein	82.79 ± 0.03^{a}	81.76 ± 0.52^{a}	84.92 ± 0.52^{b}	84.30 ± 0.87^{b}	84.86 ± 0.33^{b}

Values of the mucosal enzymes measured at the end of the growth period as indicators of the immune status of fish fed on the control diet and those including untreated *Ulva* meal are resumed in Table 5. Significantly higher values of both lysozyme and alkaline phosphatase were measured in fish receiving the higher amount of *U. ohnoi*.

Table 5. *Experiment 2. Activities of mucosal enzymes (in U/mg protein) measured in juveniles of* D. labrax *fed on diets including different amounts of* U. ohnoi *meal. Values presented as mean ± SD. Values in a row not sharing the same letter differ significantly with p < 0.05.*

	C	U5	U8
Lysozyme	52.99 ± 18.33^{a}	37.81 ± 5.23^{a}	82.75 ± 30.01^{b}
Alkaline phosphatase	2,376.13 ± 428.48^{a}	2,798.81 ± 718.83^{ab}	3,221.11 ± 857.98^{b}

3.3. Experiment 3. Evaluation of the effect of *Ulva* on the oxidative status of fish challenged by the consumption of a feed including rancid oil

As in the previous experiment, the potential differences in some bioactive compounds between the diets were evaluated by measuring total phenols and TEAC (Table 6). While the content of total phenols was not affected by the addition of macroalgae, these feeds presented a slight but significant increase in total antioxidant capacity. Although this was not a growth experiment, weight changes were also monitored in order to assess possible effects on the nutritional status of the fish. No significant differences in weight were observed between groups either after the challenge or the recovery periods. In addition, no mortality was recorded during the whole experiment. Values of the hepatosomatic index (HSI) measured at the end of the challenge showed significantly lower values

in fish receiving feeds containing *Ulva*, regardless they included rancid oil or not, and the general trends were maintained after the recovery period (Table 7).

Table 6. *Experiment 3. Bioactive compounds content of the experimental feeds expressed in g/100 m.s. Values presented as mean ± SD. Values in a column not sharing the same letter differ significantly with p < 0.05.*

Feed	Total phenols	TEAC (mM)
C	0.79 ± 0.08	0.87 ± 0.00 [a]
CO	0.85 ± 0.05	0.88 ± 0.02
U	0.81 ± 0.08	0.92 ± 0.00 [b]
UO	0.85 ± 0.09	0.92 ± 0.03

Table 7. *Experiment 3. Biometric parameters obtained after the two phases of the experiment. Values presented as mean ± SD. Values in a column not sharing the same letter differ significantly with p < 0.05.*

Parameter	C	CO	U	UO
Initial weight (g)		46.31 ± 0.29		
		Challenge		
Final weight (g)	57.55 ± 2.05	56.95 ± 3.45	57.39 ± 4.43	59.32 ± 6.59
HSI (%)	1.21 ± 0.15[a]	1.28 ± 0.10[a]	1.11 ± 0.11[b]	1.11 ± 0.12[b]
		Recovery		
Final weight (g)	62.84 ± 2.70	63.25 ± 2.23	61.27 ± 3.73	64.64 ± 6.14
HSI (%)	1.31 ± 0.22[a]	1.18 ± 0.12[a]	1.10 ± 0.04[b]	1.08 ± 0.10[b]

The immunological and oxidative status of the fish is detailed in Table 8. After the challenge, only fish fed the control diet, including rancid oil, showed a significantly lower value of mucus lysozyme. After the recovery period, these same fish evidenced a significant increase in the activity of lysozyme while the rest of the groups showed homogeneous values. In addition, the oxidative status of fish measured through the activities of different enzymes present in the liver evidenced some clear differences between the experimental groups. While no significant differences in the activity of SOD were observed between groups either during the challenge or after recovery, the activity of GPx was significantly lower in fish receiving feeds, including *Ulva*, irrespective if they included rancid oil or not. On the other hand, no effect was observed after the feed change in any of the two groups (C or U) that initially were fed on feeds, including rancid oil. In the case of CAT, high values were measured in all groups after the challenge, with the exception of fish receiving the control diet with fresh oil that showed a significantly lower value. Nevertheless, values

of this enzyme measured after recovery showed a different pattern, with higher values observed in fish fed any of the diets, including *Ulva,* when compared with their equivalents in control fish. Moreover, fish initially fed on any of the diets, including rancid oil (UO, CO), showed a significant reduction in the values during the recovery period. Lipid peroxidation measured as MDA was evidenced by significantly higher values in fish consuming rancid oil in any of the two feeds (C or U) during the initial challenge period. After the recovery, only those fish initially fed on *Ulva* + rancid oil (UO) showed a significant decrease in lipid peroxidation, while the effect was not evident in those that received the control + rancid oil (CO).

Table 8. *Experiment 3. Immunological and oxidative status measured in mucus and liver as a response to oxidation associated with the consumption of a feed enriched with macroalgae. Comparisons of enzyme activities between feeds within each stage (challenge/recovery) are indicated with capital letters, while those made between each feeding phase for the same diet are detailed with a small letter. Values presented as mean ± SD. Values not sharing the same superscript differ significantly with p < 0.05.*

	C	CO	U	UO
		Challenge		
In mucus				
Lysozyme (U/mg protein)	50.81 ± 14.35^{A}	38.25 ± 3.67^{Ba}	48.28 ± 7.66^{AB}	44.12 ± 4.17^{AB}
In liver				
GPx (U/mg protein)	75.08 ± 5.55^{A}	65.98 ± 648^{AB}	54.41 ± 11.93^{C}	57.10 ± 11.79^{BC}
SOD (U/mg protein)	44.96 ± 7.51	43.91 ± 6.40	38.28 ± 3.64	44.96 ± 2.46
CAT (U/mg protein)	246.72 ± 1.23^{A}	580.92 ± 31.25^{BCa}	541.45 ± 32.58^{B}	626.55 ± 59.57^{C}
MDA (nmol/mg protein)	77.72 ± 15.40^{A}	144.60 ± 67.62^{B}	97.55 ± 30.26^{A}	111.71 ± 15.54^{ABa}
		Recovery		
In mucus				
Lysozyme (U/mg protein)	50.81 ± 10.21	59.89 ± 15.96^{b}	48.28 ± 23.40	55.77 ± 18.47
In liver				
GPx (U/mg protein)	75.08 ± 15.34^{A}	76.70 ± 15.58^{A}	54.41 ± 14.16^{C}	61.25 ± 12.47^{AB}
SOD (U/mg protein)	44.96 ± 2.82	44.88 ± 8.14	38.28 ± 7.49	58.56 ± 25.02
CAT (U/mg protein)	246.72 ± 51.44^{A}	333.67 ± 66.25^{Ab}	541.45 ± 80.04^{B}	529.70 ± 93.31^{B}
MDA (nmol/mg protein)	77.72 ± 11.86^{B}	188.18 ± 35.66^{C}	97.55 ± 32.56^{B}	42.58 ± 10.35^{Ab}

4. DISCUSSION

4.1. Inhibitory effect of *U. Ohnoi* meal on protein digestion in European sea sass

A number of papers evaluate the potential inhibitory effect of some ingredients on the digestive proteases of different species of aquatic animals. These studies, developed using a wide range of relative

concentrations of both the enzymes and the potential inhibitors, provide useful information on the sensitivity of such enzymes on a species-specific basis (Alarcón et al., 2001; López-López et al., 2005). Nevertheless, results must be carefully interpreted considering the real physiological conditions existing in the gut of the organisms. When doing this, as in the present study, an almost negligible effect was evidenced, and values of 60 % inhibition, as those reported for proteases of marine fish by Vizcaíno et al. (2020), appear to be out of range. Moreover, instead of the presence of a Bowman-Birk protein inhibitor suggested in such study, the effects should be better interpreted as a result of the interaction between digestive enzymes and polyphenols present in *Ulva* species, that may reach 75 mg/100 g (Yildiz et al., 2012) and which negative interactions have been previously reported (Mcdougall & Stewart, 2005; Tan & Chang, 2017). On the other hand, the closer simulation of the digestion process performed in the present study, including the acid stage, resulted in no effect on the hydrolysis of feed protein in the presence of *U. ohnoi* meal, suggesting that a similar response could be obtained *in vivo*. Results attained in Experiment 2 regarding the digestibility of protein in feeds, including *U. ohnoi* meal, confirmed this hypothesis.

4.2. Effect on nutritional efficiency and immune status of juvenile sea bass fed on diets including *Ulva* previously treated or not with a mixture of carbohydrases

A first point to consider in the evaluation of different types of seaweeds as ingredients in fish feeds is the level of inclusion. In this sense, positive effects on weight gain, specific growth rate, and feed use efficiency have been notified for most species when rates are less than 10 % of the dry weight of the feed (Wassef, 2001; Valente et al., 2006; Ergün et al., 2009; Wassef et al., 2013). In contrast, levels of around 10 % do not determine changes in the mentioned parameters (Diler et al., 2007; Silva et al., 2015; Abdel-Warith et al., 2016; Vizcaíno et al., 2016), but higher amounts of 20 % or more negatively affect growth parameters (Diler et al., 2007; Siddik et al., 2015; Abdel-Warith et al., 2016; Shpigel et al., 2017). Considering all these preliminary results, in the present work two levels of dietary inclusion, i.e., 5 % and 8 %, were evaluated.

The first observed result after the inclusion of *U. ohnoi* meal in the feeds was a modification in the potential bioaccessibility of some readily soluble

nutrients. The significant reduction in the amount of soluble protein in diets, including the seaweed, when compared to the control could be related to the presence of complex polysaccharides, which may represent more than 50 % of its composition (Alves et al., 2013; Rasyid, 2017). These molecules could interfere with the release of soluble protein carried out in an aqueous medium and also modify the rates of hydrolysis of the whole digesta. On the other hand, the significant reduction in soluble protein associated with the enzyme treatment of the seaweed meal could be explained considering that commercial products are obtained from the fermentation of fungi or yeasts with different degrees of purification. Therefore, although they are enriched in a series of main enzymatic activities (carbohydrases in this case), they can also have residual proteases and lipases (Fasuyi & Kehinde, 2009), the former acting as hydrolyzing agents with an effect on the soluble protein fraction. On the other hand, no significant increase in the amount of reducing sugars or pentoses was evidenced, suggesting that the potential effect of the enzyme mixture could result in hydrolysis, mostly rendering oligosaccharides and not the above-indicated smaller compounds. The content of potential bioactives, measured as total phenols or TEAC, was significantly higher in the diet, including *U. ohnoi* when compared to those of the control diet, and no negative effect of the enzyme treatment was evidenced. This suggests that if the intended use of seaweeds as *U. ohnoi* is focused as sources of both nutrients and bioactive ingredients, an enzyme pretreatment could be carried out without affecting the potential effectiveness of these latter.

In a similar way to what was described by several authors (Zhu et al., 2016; Fernandes et al., 2019), the enzymatic pretreatment of the seaweed meal was oriented to increase the bioavailability of nutrients by partially hydrolyzing the polysaccharide matrix. In the present study, such a positive effect was neither evidenced by changes in the bioavailability of key compounds (soluble protein, sugars, phenols) nor in the nutritive use of the feeds. The limited efficiency of the enzyme treatment was confirmed by the scanning images that evidenced the maintenance of the integrity of tissues to a great extent (Figure 2). It can be suggested that more effective hydrolysis could have been carried out using a different enzymatic compound to that used in the present study (Rovabio Max L) since its combination of enzymes was specifically designed to hydrolyze the fraction of non-starch polysaccharides present in terrestrial plants,

but not in seaweeds such as *Ulva*. In spite of containing a significant fraction of xylan (Bobin-Dubigeon et al., 1997; Maneein et al., 2018) potentially susceptible to hydrolysis, its high contents in the sulfopolysaccharide ulvan seem to make it especially resistant to chemical rupture (Kidgell et al., 2019). In fact, enzymatic hydrolysis of this compound without including an aggressive phase of acid treatment requires specific enzymes (ulvan-lyases or ulvanases) identified to date only in some bacteria of marine origin (Collén et al., 2011; Konasani et al., 2018).

The reported effects of the inclusion of *Ulva* in feeds for aquaculture are highly conditioned by the amount of algae used, the specific species of fish, and the species of *Ulva*. In the case of sea bass, reported results are contradictory. The inclusion of up to 10 % of *Ulva lactuca* in feeds containing 65 % fishmeal used for early juveniles (0.22 g average weight) produced results equivalent to those obtained with the control feed (Wassef et al., 2013). In contrast, the inclusion of 5–10 % of *Ulva rigida* in a feed with 60 % fishmeal for juveniles (4.7 g of average weight) determined a decrease in rates of growth and feed use (Valente et al., 2006). Peixoto et al. (2016) reported better growth rates, weight, and food use in fish of 24 g of average weight when including up to 7.5 % of an unidentified *Ulva* meal in a feed with 30 % fishmeal and a greater variety of ingredients. Results obtained in the present work when including 5 % or 8 % of *U. ohnoi* meal evidenced no significant effect on growth or feed efficiency, so they would be in line with the first two studies and not so much with the last one. However, it must be considered that the huge variability in the composition in macro- and micronutrients of the different *Ulva* species may greatly influence these results. Even results obtained when using the same species of algae in different species may also be variable. In the specific case of *U. ohnoi* used in the present work, its inclusion at 5 %, despite having some positive effects on the integrity of the intestinal epithelium, impaired growth rates, and feed efficiency when included in feeds for *Solea senegalensis* (Vizcaíno et al., 2019), and hence limited use of this alga in feeds is recommended for this species. In contrast, an equivalent level of inclusion in a feed for *Salmo salar* did not produce significant differences in growth or feed use (Norambuena et al., 2015), although in this case, the evaluation was not carried out with a raw algal meal but using a derived product (Verdemin©), which could

potentially have different physical-chemical and nutritional characteristics.

Few studies provide data on protein digestibility in feeds, including *Ulva*, but these point out important species-related differences. Norambuena et al. (2015) reported a negative effect of *Ulva* on protein digestibility and suggested this could be determined by the limited capacity in the hydrolysis of complex polysaccharides. Nevertheless, after considering that the inclusion of *Ulva* represented only 1–2 % of the total protein in their diets, the authors suggested that the reduction in protein digestibility could be due to a negative interaction between some components of the products of algae and proteolytic enzymes. In contrast, values of protein digestibility for juvenile sea bass obtained in the present study were not negatively affected by the presence of *U. ohnoi*, regardless of the level or enzyme treatment, this supporting the previously indicated negligible effect of compounds that could negatively affect the activity of enzymes. This should be in line with results reported by Valente et al. (2006) when testing 5–10 % *Ulva rigida* in sea bass feeds, suggesting that in this species, the digestive capacity is sufficient to adequately hydrolyze these amounts of algae and could also explain why no significant effect was obtained from the enzyme pretreatment.

Immunomodulation has been observed in many studies using seaweed extracts. Although the actual phytoimmunostimulant compounds are unknown, some studies suggest that polysaccharides present in seaweeds may activate the non-specific immune responses in both teleost and shrimps (Kang et al., 2008), and they may be more effective in enhancing mucosal immunity than systemic immunity (Hoseinifar et al., 2016). Nevertheless, results obtained with different species may be somewhat contradictory. As an example, the use of a seaweed mix including *Fucus sp., Gracilaria sp.* and *Ulva sp.* as a supplement in diets for European sea bass, subjected to either combined salinity and temperature oscillations, did not mitigate the negative effects of such environmental changes on growth performance and innate immune responses (Lobo et al., 2018). In addition, lack of skin and gill mucosal immune stimulation has been reported when testing a 5 % dietary inclusion of *U. ohnoi* in diets for *S. senegalensis* (Fumanal et al., 2020), but the authors suggest it could be due to the low inclusion level used. In contrast, supplementation with 5 % *Ulva spp.* increased resistance to

infection by *Pasteurela piscicida* in red sea bream (Satoh et al., 1987) and extracts obtained from *Ulva spp.* and *Chondrus crispus* have shown to increase respiratory burst and immune system stimulation in turbot and Atlantic salmon phagocytes (Castro et al., 2004). In the present study, a significant increase in the activity of mucus lysozyme and alkaline phosphatase was evidenced in sea bass fed on the higher level of *U. ohnoi*. This result is in line with the increase in plasma lysozyme described in other species fed on feeds supplemented with different seaweeds such as kelp (*Ecklonia cava*) in olive flounder (Vizcaíno et al., 2019), *Gracilaria sp.* in sea bass (Norambuena et al., 2015), or *Ulva* in sea bream (Collén et al., 2011).

4.3. Experiment 3. Evaluation of the effect of *U. ohnoi* on the oxidative status of fish challenged by the consumption of a feed including rancid oil

The initial characterization performed in Experiment 2 of the present study (Table 2) indicated that although the inclusion of *U. ohnoi* in diets did not increase significantly the contents of total phenolic compounds when compared to a control diet, they determined a significant increase in Trolox equivalent antioxidant capacity (TEAC). For this reason, a specific experiment was designed to assess the potential protective effect of *U. ohnoi* against oxidative stress derived from intake of feeds, including rancid oil, using, in this case, a different species (gilthead sea bream) than that used in previous experiments.

Surprisingly, no negative effects on food acceptance or growth performance were observed in fish fed on feeds, including this altered oil. The present results are similar to those reported in this same species by Mourente et al. (2002) and by other authors in different species such as European sea bass *D. labrax* (Messager et al., 1992), Atlantic halibut (*Hippoglossus hippoglossus*) (Martins et al., 2007), or the Chinese longsnout catfish (*Tachysurus dumerilii)* (Dong et al., 2011). The lack of response in growth and feed intake in the present study suggests that the species apparently is not very sensitive to oxidized fish oil, or maybe the experimental duration was not long enough to obtain an effect in such parameters. Nevertheless, metabolic indicators pointed to internal effects not readily evidenced as growth responses. After the initial period of challenge, values of HSI indicated a significantly smaller size of livers in fish fed on diets including *Ulva* and also a decrease in the case of fish

initially fed on the control diet with rancid oil after the recovery period. This suggests changes in hepatic lipid metabolism that determined a lower deposit or higher mobilization of certain lipid classes associated with the consumption of *Ulva*. The same lowering effect on HSI has been previously reported in European sea bass fed with *Gracilaria gracilis* supplemented diets at 8 % (Batista et al., 2020). This fact can be associated with reduced total lipids content, including triglycerides or cholesterol both in plasma or in the liver, as previously demonstrated in red sea bream (*Pagrus major*) fed *Spirulina sp.*, which may reflect a high activity of key enzymes related to fatty acid β-oxidation to activate lipid mobilization (Nakagawa et al., 2000). Moreover, controverted or not so clear results have been reported in the evaluation of hepatosomatic index as a mirror of energetic balance mainly related to lipid metabolism in different fish cultured species after different challenges (Magnoni et al., 2018; Guerreiro et al., 2019; Peixoto et al., 2019), so the protective role of this seaweed regardless of the state of the oil present in aquafeeds could not be ruled out.

On the other hand, changes in the immunological and oxidative status were evidenced in the present study. A decreased activity of mucus lysozyme was associated with the consumption of rancid oil, but normal levels seemed to be restored during recovery. This result should be in line with the increased activity of this enzyme measured in sea bass associated with the consumption of feeds, including *U. ohnoi* in Experiment 2. Regarding the oxidative status, it is clear that variations in the different parameters evaluated (lipid peroxidation and antioxidant enzymes) were influenced to a different extent either by the consumption of rancid oil, by the presence of *Ulva* in the diet, or by the interaction between them. Various studies have indicated that the extracts derived from seaweeds and microalgae are natural sources of antioxidants that neutralize free radicals (Goiris et al., 2012). SOD and CAT are important enzymes in the antioxidant defense system, as they play a key role in removing free radicals and toxicity of drugs and chemicals (Farombi et al., 2007). In the present study, the values of MDA evidenced the effect of consuming rancid oil in any of the two feeds (C or U) during the initial challenge period, being in agreement with results obtained in studies with other species (Hamre et al., 2001; Tocher et al., 2003). The important point is that such values were reverted during the recovery period, but only in those fish that initially have received a feed, including *Ulva*. This

suggests that some compounds present in the seaweed may exert a hepatoprotective role that enhances the active metabolism of oxidized lipids that could be accumulated in the liver (Sen et al., 2006).
The observed reduction in the activity of liver GPx associated with the consumption of *U. ohnoi* is in agreement with results reported when including *Gracilaria sp.* in diets for the same species (Silva-Brito et al., 2020) even at lower levels (2.5 %). In addition, high values of CAT associated with the inclusion of seaweeds in diets have been described in a number of studies (Peixoto et al., 2019; Sharma et al., 2019; Shi et al., 2019). Again, the observed reduction in the activity of CAT in fish initially fed on any of the diets, including rancid oil (UO, CO), that after receiving the feed with *U. ohnoi* during the recovery period suggests the onset of a metabolic response oriented to reduce the amount of oxidation products. A similar effect was reported by Tocher et al. (2003) in different marine fish such as turbot *S. maximus*, halibut (*H. hippoglossus*), and gilthead sea bream (*S. aurata*) fed on rancid oil when received supplementation of vitamin E, suggesting a protective role against oxidative metabolic misbalances. In addition, the reduced (although not significant) activity of SOD measured in the present study in fish receiving the U diet should be in line with results reported by Safavi et al. (2019), who found significantly lower levels of this enzyme in livers of rainbow trout (*Oncorhynchus mykiss*) fed on extracted polysaccharides from *Ulva* and *Gracillaria.*

5. RESUME AND CONCLUSIONS

From all the previously described experiments, it can be concluded that:

Contrarily to previous reports, no significant negative effect on protein digestion could be expected when using *U. ohnoi* as an ingredient in feeds when included at levels usually used for this kind of products (10 % of the diet or less).

U. ohnoi meal presents a reduced value as a nutritional ingredient when used in aquafeeds, even after being enzymatically treated to partially hydrolyze its carbohydrate fraction. Nevertheless, different results could be obtained if enzymes specifically capable of hydrolyzing ulvan could be used.

In contrast to the above-mentioned, the role of *U. ohnoi* meal as a source of bioactive compounds is confirmed. An immunostimulatory effect was evidenced by an increase in mucus lysozyme in two different species (sea bass and sea bream). In addition, some compounds present in *U. ohnoi* perhaps positively influence the oxidative metabolism of the fish, being able to counteract the negative effects resulting from acute stress produced by the consumption of rancid oil.

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CHAPTER 2

Evaluation of enzyme additives on the nutritional use of feeds with a high content of plant ingredients for *Mugil cephalus*

Martínez, F. P., Bermúdez, L., Aznar, M. J., & Moyano, F. J. (2019). Fishes, 4(4), 56.

ABSTRACT

The Mugilidae are a group of fish with a great interest for aquaculture due to their omnivorous profile, rapid growth, and resistance to environmental variations. The selection of feed ingredients for these species is currently focused on an extensive use of plant by-products, with this being limited by their content in anti-nutritive factors (mainly phytate and non-starch polysaccharides; NSPs). Nevertheless, specific enzymes can be used to counteract some of those negative effects. In the present study, the effect of pretreating two high-plant feeds with a mixture of enzymes (glucanases + phytase) on the digestive use of protein and phosphorus by juvenile mullets (*Mugil cephalus*) was assessed using both *in vitro* and *in vivo* assays. The enzymatic treatment significantly modified the potential bioavailability of some nutrients, such as a reduction of sugars, pentoses, and phytic phosphorus. Also, it increased the digestibility of protein in one of the feeds but reduced that of phosphorus in both of them. The potential usefulness of enzyme treatment and the information provided by the two types of assays are discussed.

Keywords: aquaculture; plant ingredients; enzymes

1. INTRODUCTION

Increasing concerns about the environmental and economic sustainability of aquaculture indicate that its future development cannot rely on intensive production systems of carnivorous species, due to the high impact linked to the need of using large quantities of fishmeal and oils in their feeds (Tacon & Metian, 2008). Therefore, multiple instances promote the development of semi-intensive and integrated aquaculture systems based on omnivorous or herbivorous species, as well as an extended use of plant ingredients and by-products (Naylor et al., 2009). This orientation is increasing in Asia and South America, but to date, it is not so extensive in Europe, where fish aquaculture is still primarily focused on the intensive production of carnivorous species with a high market value. Nevertheless, the need for diversification in Mediterranean marine aquaculture has promoted an increased interest in developing alternative models of aquaculture (aquaponics, recirculation systems, multitrophic aquaculture, etc.) as well as the cultivation of some omnivorous species. The common feature of these systems is the use of species placed at low trophic levels showing less demanding nutritional requirements, in terms of total amount or quality of feed ingredients, and whose feeding can be carried out largely using low-cost ingredients.

Within this context, it is worth noting the high potential presented by the species of the family Mugilidae (mullets), a group of fish living in temperate and subtropical coastal waters in both hemispheres. Mullets, which include species like *Mugil cephalus, Chelon labrosus, Liza aurata, Liza saliens*, and *Liza ramada*, present a great adaptability to different culture conditions, with their potential for aquaculture being determined by their omnivorous profile, rapid growth, and resistance to environmental variations (Abellán & Arnal, 2013). The culture of Mugilidae, particularly of the grey mullet (*Mugil cephalus*), is considered a priority within current strategies of European aquaculture. As an example, the EU Program DIVERSIFY considers the establishment of a basis for reproduction in captivity and the development of low-cost feeds adapted to its nutritional requirements as a main objective for this species. In contrast to the great amount of information available on the natural feeding habits of mullets, data on their nutritional requirements and use of artificial feeds are scarce (Brusle, 1981). This is due to the fact that the culture of mullets takes place under extensive or semi-intensive systems, implying that a great part of the food is naturally produced, and

only supplemented with low-quality feeds (Oren, 1981; Biswas et al., 2012). However, the renovated interest to develop the culture of this species requires the development of suitable and more species-specific formulations. In this sense, the possibility of using high amounts of plant ingredients, even in feeds used during early stages of development, has been well demonstrated (Oren, 1981; Wassef et al., 2001; Kalla et al., 2003; Jana et al., 2012; Gisbert et al., 2016).

For many years, studies aimed at evaluating the incorporation of plant ingredients into feeds for aquaculture species has been one of the most active and productive research lines. Different reviews have identified the potentials and limitations of using these ingredients (Naylor et al., 2009; El-Dahhar et al., 2014). One is the deficiencies in essential amino acids in plant proteins, which has to be compensated for to obtain a protein profile for the feed that is suitable for the requirements of the species. Another key aspect is the presence of a wide variety of antinutritional factors in plant ingredients, such as alkaloids, protease inhibitors, saponins, lectins, as well as non-starch polysaccharides (NSPs) and phytate (Gatlin et al., 2007). NSPs is the fraction of carbohydrates present in plant ingredients commonly named “undigestible fiber”. It is formed by cellulose (glucose insoluble polymer) and a group of other complex sugar heteropolymers, such as mannose, xylose, arabinose, etc., which present important differences in their solubility, water retention capacity, and interaction with other feed ingredients or the intestinal microbiota. Significant effects on intestinal transit, nutrient absorption, or microbial diversity can be observed depending on the amount and type of NSP present in a plant ingredient (Olsen et al., 2009). On the other hand, phytate is an organic acid present in plant sources, particularly in some seeds and also in the fiber fraction. Phytate phosphorus is not bioavailable to monogastrics because they lack phytase, the enzyme able to hydrolyze such a compound; hence, it passes through the gastrointestinal tract and is finally eliminated in the feces. In addition, phytic acid combines with several nutritionally important minerals, such as calcium, magnesium, iron, and zinc, that become insoluble and are not absorbed in the intestine. Also, it is well known that phytate inhibits proteolytic enzymes (Morales et al., 2011; Navarro et al., 2019).

The use of enzyme additives may be a powerful tool to counteract the potential negative effects derived from the presence of phytate and NSP,

and thus increase the nutritional value of plant ingredients. Since, in many cases, NSPs form a matrix that hinders the access of digestive enzymes to the protein and starch present in cereal and leguminous seeds, the use of glucanases capable of totally or partially hydrolyzing them has shown positive effects on the nutritive use of feeds, including those feedstuffs (Humer et al., 2015). In a similar way, the use of phytase has shown positive effects on different fish species in terms of their ability to improve the whole nutritional use of different ingredients and to reduce phosphorus discharge into the environment (Cain & Garling, 1995; Cao et al., 2007; Castillo & Gatlin, 2015).

To date, all studies aimed at testing the potential effect of enzyme supplementation on fish feeds have been based on mixing with the rest of the ingredients, with their potential activity in the fish gut being affected by the processing conditions, mostly the high temperatures reached during pelleting. This could explain why in several cases, the results obtained were non-significant (Sugiura et al., 2001). A possible alternative could be the external addition of the enzyme in the oil used to cover the pellets after extrusion; this has offered interesting results in the case of phytase added to trout feeds (Yigit & Olmez, 2011). Another method, on which there are not published references, is the pre-treatment of the plant ingredients with specific enzymatic additives under controlled conditions prior to the preparation of the feed pellets. This solid-state hydrolysis (SSH) operates with a percentage of solid substrate greater than 15 %, so little or no free water is present (Forster et al., 1999). The procedure is routinely used in different industrial processes aimed at obtaining specific products, such as glucose or other sugars, or directly to increase the nutritional value of plant ingredients for human or terrestrial animal consumption by reducing the content of NSP (Chen & Liu, 2016).

On the other hand, the preliminary selection of the suitability of a given ingredient and/or enzyme treatment for a given species can be performed using *in vitro* assays simulating the digestion process of such species. These assays, extensively used in the evaluation of the nutritional quality of foods and feeds for humans and terrestrial animals and more recently adapted to aquatic animals, may help to predict differences in the potential bioavailability of main nutrients (protein, carbohydrates, and fats) as well some minerals (Opazo et al., 2012).

Considering the above-mentioned factors, the main objective of the present work was to evaluate the effect of an SSH enzymatic treatment of plant ingredients used in feeds for grey mullet on the potential bioavailability of nutrients. Changes in the bioavailability of sugars, amino acids, and phosphorus were evaluated using both *in vitro* and *in vivo* digestibility assays.

2. MATERIALS AND METHODS

2.1. Ingredients and experimental feeds

The different ingredients used in the fabrication of the experimental feeds were supplied by DIBAQ S.A. (Segovia, Spain). Two different diets, detailed in Table 1, were designed considering the following points: (a) the inclusion of a low amount of fish meal (10–15 g/100 g); (b) use of some different ingredients in both diets (diet 1 contained rapeseed meal while diet 2 included rice bran); (c) adaption of the diets to the nutritional requirements of juvenile mullets (thus they contained 45 % crude protein and 13 % total fat); and (d) contain a similar amount of NSP (24–5 g/100 g) and phytate (0.3 %).

Each of the two diets was prepared with or without enzyme treatment, resulting in a total of four experimental diets. The enzymes used were Viscozyme™ (Sigma-Aldrich RN V2010), a mixture of xylanase, cellulase, and hemicellulose containing 100 fungal beta glucanase units/g at a dose of 10 g/kg feed and Quantum™ phytase (AB Enzymes, Germany), containing 2,500 phytase units/g at a dose of 0.4 g/kg feed. To carry out the treatment, the vegetable meals in each diet were milled to a mesh size of 0.5 mm and mixed carefully with citrate buffer (pH 5.0, 0.1 M; 1:3 w/v) to obtain a moist mass with the optimal conditions for the action of the enzymes, which were solubilized in water and added to the feed mixtures by spraying. The enzymes were allowed to act by keeping the mixture at 45 °C for 4 h, with manual stirring every hour to ensure the homogeneity of the reaction. After this time, the reaction was stopped by placing the mixture in a cold chamber at 4 °C until the addition of the rest of diet ingredients and preparation of feed pellets. Cr_2O_3 was included in all diets as an inert marker to evaluate digestibility. The feeds were prepared using a lab-scale extrusion machine provided with a mesh size of 2 mm, and dried and stored at 4 °C until used.

2.2. Analytical techniques used

Samples of each feed were used for the analysis of total protein, soluble protein, reducing sugars, free pentoses, total phosphorus, and phytic phosphorus according to the following methodologies:

Total nitrogen was analyzed by CNHS elementary analysis, with the contents in N (g/100 g sample) transformed into protein using a conversion factor of 6.25. Soluble protein was analyzed by the Bradford (1976) using the SIGMA Total Protein Kit (Sigma-Aldrich TP0100). Reducing sugars were measured using 3,5-dinitrosalicylic acid (DNS) following the method described by Miller (1959). Free pentoses were measured by the phloroglucinol method described by Douglas (1981). Total phosphorus in feeds and feces was determined by the molybdovanadate method after total digestion of the organic matter with concentrated nitric acid (AOAC, 1995). Phytic acid was determined following the bipyridine method described by Haug & Lantzsch (1983). All the analyses were performed in triplicate samples from each diet.

2.3. *In vitro* digestive hydrolysis assay

Changes in the potential bioavailability of the nitrogen fraction, pentoses, and P present in feeds, enzymatically treated or not, were evaluated *in vitro* under conditions simulating the digestive tract of a mullet. The assays were performed using membrane bioreactors modified from that described in Morales & Moyano (2010). The device consists of two chambers separated by a semi-permeable membrane of 3,500 kDa MWCO (Molecular Weight Cut- Off). Fish enzyme extracts and feed samples were placed in the upper chamber and maintained under continuous agitation using a magnetic stirrer. Hydrolysis products passing across the membrane into the lower chamber were recovered at different time intervals during the reaction time. The whole system was maintained at 25 °C within a temperature-controlled chamber.

The enzyme extracts used in the assays were obtained from adult individuals of mullet (*M. cephalus*) (n = 4, 2.2 kg average weight) on which intestinal pH and total protease activity were measured according to the method of Kunitz as modified by Walter (1984). The operation of the *in vitro* simulation required the use of an enzyme:substrate ratio close to that existing in the gut of live fish. This was estimated considering, on

the one hand, the average total protease production measured in the intestine of the fish and on the other hand, the mean protein intake of such fish in a single meal. As a result of the aforementioned estimation, an enzyme:substrate ratio of 20 U/mg protein and a pH of 8.5 were used in the assays. All the assays were carried out in triplicate. The release of products from feeds in the absence of enzyme hydrolysis was assessed by running assays on which the enzyme extracts were heat inactivated (placed in a water bath at 100 °C for 5 min). Hydrolysis products were analyzed using the same methodologies described for the feeds while the release of amino acids was quantified by the orthophthaldehyde (OPA) method described by Church et al. (1983).

2.4. *In vivo* digestibility test

A total of 1,200 fish (9.3 ± 1.1 g) were distributed into 12 tanks (120 L; 100 fish per tank) provided with a settling column for stool removal (Guelph method). Each of the four experimental feeds were evaluated in triplicate. The fish were fed manually every day in two meals. Feces were removed daily for 3 weeks, dried, and processed to determine their nitrogen, total phosphorus, and phytate contents as previously detailed. Fecal samples obtained on three different days were pooled to form one sample and three different samples were obtained from each tank (a total of 3 × 3 = 9 samples per diet). The determination of the total chromium of feeds and feces was carried out using the diphenylcarbazide method (Divakaran et al., 2002). Apparent digestibility coefficients (ADC) were calculated as follows:

$$ADC\ nutrient = 100 - [\left[\frac{(\%\ of\ indicator\ in\ food)}{(\%\ of\ indicator\ in\ feces)}\right]\left[\frac{(\%\ of\ nutrient\ in\ feces)}{(\%\ of\ nutrient\ in\ food)}\right] * 100]$$

2.5. Statistical analysis

Data were analyzed by ANOVA followed by the Bonferroni test using the software Statgraphics Centurion (Statgraphics Technologies, The Plains, VI. EE.UU.). The significance level was established at $p < 0.05$. When required, data expressed in percentage were previously arc-sin transformed.

3. RESULTS

The effect of the enzymatic treatment on the potential availability of nutrients of the experimental feeds (composition detailed in Table 1) is shown in Table 2. The results show that enzyme pretreatment significantly increased the contents of the available reducing sugars and pentoses while it reduced that of phytate. The release of nutrients due to the action of the digestive enzymes of mullet under conditions simulating the digestion of the species is summarized in Table 3. A significantly higher amount of amino acids was released from feed 1 than 2. On the other hand, the enzymatic treatment determined a significant increase in the release of amino acids and pentoses from both feeds while, in contrast, the release of total P remained unaffected. The values of the apparent digestibility coefficients for protein, total phosphorus, and phytic phosphorus are detailed in Table 4. The digestibility values for both protein and P were within the normal ranges determined for these nutrients in other species; in fact, the values were high, considering the high content of vegetable ingredients used in the feeds. There were no significant differences in the digestive utilization of protein between both untreated feeds, but the enzyme addition resulted in a significant improvement in the protein digestibility of feed 1. In the absence of enzymatic treatment, the digestibility of total P was significantly higher for feed 2. Enzymatic treatment reduced the digestibility values in both feeds, with this reduction being significant in the case of feed 2. A similar result was obtained for phytate.

Table 1. *Ingredients and proximate composition of the diets used in the experiment.*

Ingredients (in g/100 g d.w.)	FEED 1	FEED 2
Fishmeal 67/10	15.0	10.0
Soybean meal 47	15.0	21.8
Rapeseed meal	15.0	-
Defatted rice bran	-	12.0
Soybean protein concentrate	8,0	10.0
Corn gluten meal 60	16.0	15.0
Guar meal (Korma)	15.0	20.0
Fish oil	4.9	4.9
Sunflower oil	3.9	3.9
Soy lecithin	1.0	1.0
Vitamin/mineral premix	0.1	0.1
Taurin	0.5	0.5
Yeast	0.7	0.8
Cr_2O_3	1.0	1.0
Starch	4.4	-
Proximate composition (in g/100 g)		
Crude protein	45.00	45.01
Crude fat	13.00	13.00
Digestible carbohydrates (starch + oligosaccharides)	5.85	9.00
NSP	23.90	25.17
Ash	5.95	6.42
Phosphorus	0.89	0.87
Phytate P	0.28	0.33

Table 2. *Differences in nutrient content of experimental feeds (g/100 d.m). Statistical comparisons between feeds (3 samples per feed) prior the enzyme treatment is detailed in capital letters while those made within each feed, with or without enzymatic treatment are detailed in lowercase. Values not sharing the same letter differ significantly with $p < 0.05$.*

	Total Protein	Soluble protein	Reducing sugars	Pentoses	Phosphorus	Phytate
FEED 1	46.85 ± 1.18	6.19 ± 0.66^{Aa}	0.67 ± 0.07^{Aa}	0.24 ± 0.02^{Aa}	1.33 ± 0.03	0.44 ± 0.04^{Aa}
FEED 1+enz	46.85 ± 1.18	4.12 ± 0.24^{b}	5.94 ± 0.34^{b}	0.83 ± 0.04^{b}	1.30 ± 0.01	0.21 ± 0.00^{b}
FEED 2	46.96 ± 0.84	2.94 ± 0.44^{Ba}	1.12 ± 0.04^{Ba}	0.28 ± 0.01^{Aa}	1.55 ± 0.20	0.96 ± 0.02^{Ba}
FEED 2+enz	46.96 ± 0.84	4.82 ± 0.37^{b}	5.61 ± 0.29^{b}	0.82 ± 0.04^{b}	1.41 ± 0.10	0.56 ± 0.02^{b}

Table 3. *Nutrients released after* in vitro *hydrolysis of the experimental diets. Data expressed in total amount or as a percentage of the nutrient initially present in sample (protein, NSP (non-starch polysaccharide), or total P). Statistical comparisons between feeds (3 samples per feed) prior the enzyme treatment is detailed in capital letters, while those made within each feed, with or without enzymatic treatment, are detailed in lowercase. Values not sharing the same letter differ significantly with p < 0.05.*

	Amino acids (mg)	Amino acids (%)	Pentoses (mg)	Pentoses (%)	P (mg)	P (%)
FEED 1	110.65 ± 0.87^{Aa}	39.36 ± 0.31^{Aa}	1.32 ± 0.13^{Aa}	0.99 ± 0.09^{Aa}	4.63 ± 0.50^{A}	58.0 ± 6.21^{A}
FEED 1+enz	115.76 ± 1.02^{b}	41.18 ± 0.36^{b}	3.32 ± 0.34^{b}	2.31 ± 0.27^{b}	4.62 ± 0.07	59.2 ± 0.85
FEED 2	72.73 ± 0.19^{Ba}	25.81 ± 0.07^{Ba}	1.14± 0.11^{Aa}	0.76 ± 0.08^{Aa}	3.07 ± 0.02^{B}	33.0 ± 0.26^{B}
FEED 2+enz	85.91 ± 13.07^{b}	30.49 ± 4.63^{b}	3.48± 0.08^{b}	2.31 ± 0.05^{b}	2.51 ± 0.27	39.7 ± 3.15

Table 4. *Apparent digestibility coefficients (ADC) in g/ 100 g of protein total P and phytate for the experimental diets. Statistical comparisons between feeds (9 samples per feed) prior the enzyme treatment are detailed in capital letters, while those made within each feed, with or without enzymatic treatment, are detailed in lowercase. Values not sharing the same letter differ significantly with p < 0.05.*

	Protein	Phosphorus	Phytate
FEED 1	89.6 ± 1.0^{Aa}	47.9 ± 7.1^{Aa}	55.2 ± 2.5^{Aa}
FEED 1+enz	92.3 ± 0.4^{b}	38.5 ± 3.9^{a}	54.6 ± 5.0^{a}
FEED 2	91.9 ± 0.1^{Aa}	70.1 ± 3.0^{Ba}	62.4 ± 1.5^{Ba}
FEED 2+enz	91.9 ± 0.5^{a}	61.0 ± 6.0^{b}	52.9 ± 5.7 ^{b}

4. DISCUSSION

Most studies testing the effect of enzyme addition to improve the nutritional use of plant ingredients have been carried out in freshwater fish species (tilapia, carp, catfish, sturgeon, rainbow trout) but few have investigated marine fish like the Japanese sea bass or mullet (Humer et al., 2015). This could be explained considering the greater presence of herbivorous species in fresh water, which implies a preferential use of low-value plant ingredients in their feeds that would justify the use of enzymes. In contrast, most marine species are carnivorous; although plant ingredients are also routinely used in their feeds, they are mostly high-quality protein concentrates with a reduced content of NSP. The present study was developed using grey mullet, an omnivorous marine fish, with the feeds designed to include a significant amount of plant ingredients (around 70 g/100 g diet) with a presumed high content in several antinutritional factors like NSP and phytic acid. This is justified considering that the profile of ingredients to be used in feeds for mullets

should mostly be based on the use of low-cost meals (guar, rapeseed, etc.) and by-products (cereal brans, distiller's dried grains with soluble, etc.). It is noteworthy that most studies aimed at evaluating enzymes as additives do not reproduce the real conditions used during manufacturing, since experimental feeds are usually prepared by cold granulation (Ai et al., 2007; Dalsgaard et al., 2012; Moyano et al., 2015). Nevertheless, most feeds currently used in aquaculture are extruded at high temperatures, so the enzymes to be used should be highly resistant to this process. As previously indicated, one possible alternative is to add the enzymatic mixtures in the final coating of the grain (Yigit & Olmez, 2011). Another one, used in the present work, is to carry out enzymatic pretreatment of the ingredients before preparation of the pellets. As shown in Table 2, the enzyme pretreatment demonstrated a clear modification of the nutritional profile of the diets, which showed an increased amount of reducing sugars and pentoses, as well as a decrease in the amount of phytate phosphorus. To date, only one published study developed a similar approach (Jiang et al., 2014) and presents results on the biochemical transformation of the ingredients used in trout feeds after enzymatic action. In that study, plant ingredients made up 45 % of the feed (34 % crude protein); this resulted in NSPs accounting for 8 % of the proximate composition. These figures are considerably different to those of the feeds used in the present study, where the content of plant ingredients was much higher (60 %–70 %) and the estimated NSP contents (including cellulose) exceeded 20 %. The enzymatic action in the work of Denstadli et al. (2011) determined a reduction in the NSP content between 10 % and 13 % when using soy flour as the main ingredient and only 4 % to 6 % when using rapeseed flour. Additionally, the authors did not obtain significant changes in the contents of pentoses and reducing sugars. In the present study, changes in the amount of NSP were not measured directly, but, as previously indicated, the products of hydrolysis multiplied their concentrations by three to four times, suggesting that the enzymatic hydrolysis was remarkably higher.

The *in vitro* model of gut hydrolysis by the digestive enzymes of mullet, which was used to evaluate differences in the potential bioavailability of some nutrients, showed some interesting results when compared to those obtained in the analysis of the feeds. As an example, a significantly lower amount of available amino acids was measured for feed 2 when compared to feed 1 despite both feeds presenting the same crude protein contents.

This could be explained considering the higher proportion of plant ingredients included in the former (78 vs. 68 g/100 g diet). Interestingly, the enzyme treatment significantly increased the amount of available amino acids in both diets, but the increase was higher for feed 2 than for feed 1 (4 % and 18 %, respectively). As identified by Castillo & Gatlin (2015), this increased accessibility of protein to the action of digestive enzymes could be explained considering that NSP are present as part of the cell wall, thus shielding substrates from contact with the digestive enzymes, or as part of cell content, where their presence may interfere with digestion and absorption due to their chemical nature. This is in line with the observed significant increase in the amount of pentoses released from the enzyme-treated feeds. On the other hand, a low potential bioavailability of total P was determined for the experimental feeds, irrespective of the enzyme treatment. This could be explained considering that phytate accounted for more than 60 % of total P present in feed 2 but represented only one third of that present in feed 1. This was also reflected by the lower bioavailability of this element observed in the digestive simulation despite the observed reduction in phytate produced by the enzyme treatment.

The results obtained in the *in vivo* evaluation of digestibility were somewhat surprising. No significant differences were measured between ADC values of protein in untreated diets, and the enzyme treatment only significantly improved the value obtained for feed 1. The higher bioaccessibility of the protein fraction found in the *in vitro* assay for such feed could explain this result to a certain extent. An opposite result was obtained for ADC of total P or phytate, since a much higher digestive utilization of this element was measured for feed 2 in untreated feeds and the enzyme treatment resulted in a decreased digestibility in both feeds. The observed reduction in the efficiency of the digestive utilization of P after enzyme treatment of phytate could be explained considering that the increase in available P resulting from phytate hydrolysis was not in parallel with an equivalent ability for its absorption at the intestinal level. It must be considered that the experiment was performed using very young fish, on which the functionality of the intestine was still under development, and this could limit the absorption of the extra amount of P produced by the action of phytase. Intestinal transport of P is complex and proceeds via two distinct mechanisms: One component is developed by a sodium-phosphorus co-transporter (Na-Pi-II) and appears to

saturate at low P concentrations, whereas the second mechanism relies on the luminal P concentration and does not show saturation, resembling passive diffusion (Knöpfel et al., 2019). The presence of divalent cations in sea water, which is continuously ingested by young marine fish, could lead to the formation of insoluble phosphate compounds that may limit gastrointestinal phosphorus bioavailability (Bakke et al., 2010). Also, it has been reported that an increase in the availability of dietary P typically decreases the efficiency of P utilization, thereby increasing the amount of P excreted (Coloso et al., 2003).

In any case, the correlation between the results obtained with *in vitro* and *in vivo* assays was not directly clear. This can be explained considering that both types of assays measure different things. *In vitro* tests allow an estimation of the potential bioavailability of nutrients; that is, that fraction that would be available for biological functions once absorbed by the intestine (something that may not occur in practice, as indicated in the case of P). In contrast, *in vivo* digestibility assays estimate the net result of such intestinal absorption (Faulks & Southon, 2005). The results obtained in the present work support such differences in the approaches; the significantly lower values of potential P bioavailability measured *in vitro* for feed 2 should reflect a much slower release from the feed matrix. As a result, a better digestive efficiency and decreased fecal loss was observed *in vivo*, probably due to the decreased saturation of the intestinal transporters. Hence, the assessment of *in vivo* digestibility could be considered as a post-absorption method to estimate bioavailability while *in vitro* assays can provide a pre-absorption estimation of this parameter (Dimes et al., 1994; Opazo et al., 2012; Moyano et al., 2015). Moreover, *in vitro* models can simulate the physical or chemical transformations of food components but not other aspects, such as the effect of antinutritional factors or the influence of carbohydrates, transformed or not, on the intestinal microbiota. In the case of mullet, this last aspect is of great importance because it is an omnivorous fish with a well-developed intestine in which microbiota are presumed to play a fundamental role in the transformation of nutrients. This could explain why the clear differences in amino acid release measured between feeds *in vitro* were not equally reflected in the ADC of their protein fraction. It follows that *in vitro* bioavailability assays can correlate better with other indicators of biological efficiency like the

conversion index or the specific growth rate, as suggested by the results obtained by Dimes et al. (1994) or Rungruangsak-Torrissen et al. (2002).

5. CONCLUSIONS

In conclusion, the results obtained in the present study suggest that:

Pretreatment of plant ingredients prior to feed elaboration with enzyme mixtures is a suitable way to modify their nutritional profile, increasing the potential bioavailability of different nutrients.

The characteristics of enzyme treatment should be carefully adapted to the physiological features of the target species, mostly in the case of non-adult fish that present a still underdeveloped digestive tract. Excessive hydrolysis of some substrates (i.e., phosphorus) causes an increase in their concentration at the gut level that may impair absorption, resulting in decreased digestibility due to fecal loss.

In vitro assays oriented to assess potential differences in the bioavailability of nutrients derived from enzyme treatments may help to explain and predict results obtained *in vivo* to a certain extent. Refinement of the conditions for developing such assays could represent a powerful tool to gain a better understanding of variations in gut nutrient availability.

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CHAPTER 3

Solid-State Hydrolysis (SSH) improves the nutritional value of plant ingredients in the diet of *Mugil cephalus*

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ABSTRACT

The possibility of improving the nutritional quality of plant byproducts (brewer's spent grain and rice bran) through an enzyme treatment was tested in a formulated feed for grey mullet (*Mugil cephalus*). The enzyme treatment was carried out by Solid-State Hydrolysis (SSH) using a commercial preparation including carbohydrases and phytase. A feed prepared without the treatment and a commercial feed for carp were used as controls. In a preliminary short-term trial carried out at laboratory facilities, fish receiving the enzyme-treated feed showed significant improvement in both FCR and SGR when compared to those obtained with the untreated diet, although both experimental diets presented worse values than those obtained with the commercial feed. Different metabolic indicators including higher values of muscle glycogen and plasmatic triglycerides supported the positive effect of the enzyme treatment on the nutritional condition of the fish over those fed on the diet containing non-treated ingredients. Results of growth and feed efficiency that were obtained in a second long-term trial developed for 148 days under real production conditions evidenced the equivalence among the experimental and commercial diets and confirmed that enzyme pretreatment of plant ingredients by SSH may be a useful procedure to improve the nutritive value of high fiber plant by-products when included in practical diets for this species and others with similar nutritional features.

Keywords: aquaculture feeds; plant byproducts; enzymatic pretreatment

1. INTRODUCTION

Although in the last years important efforts have been carried out to reduce the levels of the conventional marine resources, i.e., fish meal and fish oil, in the diets of cultured fish, the sustainable development of marine aquaculture requires species that can be produced without the need to use high amounts of such ingredients, the availability of which is growing progressively more limited. Mugilidae (mullets) are a group of fish gaining increasing interest for aquaculture due to their rapid growth, resistance to a wide range of environmental conditions, and omnivorous profile. Over the past few years, the culture of these species, particularly of the grey mullet (*Mugil cephalus*), is considered a priority within the current strategies of aquaculture diversification in different parts of the world, with particular interest in some Mediterranean countries (Christou et al., 2013). In addition to specific research aimed at completing its reproduction in captivity, the culture of grey mullet requires the development of suitable species-specific diets, the availability of which represents a bottleneck for their production under intensive systems. Recent studies show good results when testing highly nutritive diets based in the use of zooplankton species (Abo-Taleb, Ashour, et al., 2021; Abo-Taleb, El-Feky, et al., 2021) and several others support the possibility of using high amounts of plant byproducts in feeds for these species, even during early stages of their development (Wassef et al., 2001; Kalla et al., 2003; Jana et al., 2012; El-Dahhar et al., 2014; Gisbert et al., 2016). Nevertheless, in this latter case the selected products presented a high nutritional quality (high protein, low fibre contents) and hence the potential of using other vegetable ingredients with a more limited nutritional value has not been properly tested.

As indicated previously, due to their low trophic level and opportunistic nature, mullets are ideal candidates to take advantage of feeds including high percentages of alternative products and byproducts, many of which have high interest for local use within the framework of the circular economy. Nevertheless, from a nutritional point of view, such plant byproducts may present important limitations linked to both their amino acid imbalances and reduced digestibility due to the presence of a wide variety of antinutritional compounds including alkaloids, lectins, digestive enzyme inhibitors, indigestible carbohydrates (mainly non-starch polysaccharides, NSPs) and phytate (Gatlin et al., 2007). In this sense, the use of enzyme additives may be a powerful tool to counteract

the potential negative effects derived from the presence of some of these compounds, such as phytate and NSP, thus increasing the whole nutritional value of the ingredients. A number of commercially available multienzyme complexes have been developed to improve the use of carbohydrates and phytate present in plant ingredients used in feeds for terrestrial animals. Nevertheless, they have been designed for optimal functioning under the body temperature and pH conditions existing in the digestive systems of pigs and poultry, which are notably different from those present in aquatic species. This may explain the limited effectiveness and somewhat contradictory results obtained when such products are tested in some fish species, such as Japanese sea bass, carp or rainbow trout (Ai et al., 2007; Dalsgaard et al., 2012; Jiang et al., 2014; Castillo & Gatlin, 2015). The efficiency of hydrolysis produced by such enzymes is greatly conditioned by several aspects. As an example, there may be interactions between gastric and intestinal proteases produced by fish and the exogenous enzymes that can negatively affect their potential beneficial effects (Fernandes et al., 2021). Furthermore, the effectiveness of the exogenous enzymes may be greatly reduced by the high temperatures reached during feed preparation or, in the case of being applied post-extrusion via oil top coating or spraying, by the time available for the enzymatic action inside the digestive system of the species, which is closely related to gut transit rates linked to water temperature.

An interesting alternative to overcome the aforementioned limitations is the pre-treatment of plant ingredients with the enzyme compound before the preparation of the feed pellets, using Solid-State Hydrolysis (SSH). SSH operates with a percentage of solid substrate greater than 15 %, so there is little or no free water (Chen & Liu, 2016). This process is routinely used to obtain specific products such as glucose or other sugars, or directly to increase the nutritional value of plant ingredients by reducing the content of NSP (Opazo et al., 2012). By using SSH, hydrolysis can be carried out under optimal conditions for the enzymes, and their activity is not affected either by the high temperatures reached during feed preparation or by the biochemical conditions present in the guts of fish.

Considering all the above, the aim of the present work was to assess whether pre-treatment of plant ingredients containing high proportions of NSP and phytate using a mixture of enzymes applied under an SSH

protocol could improve nutritional value when used in the diet of *M. cephalus*. To achieve this objective two different trials were carried out: an initial short-term trial aimed at preliminary evaluation of the growth performance, feed efficiency and energy metabolism of fish fed on such diet, and a second long-term trial focused only on the evaluation of differences in growth and feed efficiency produced by the diet when evaluated under field production conditions.

2. MATERIALS AND METHODS

2.1. Experiment 1. Short-Term trial

2.1.1. Ingredients and experimental feeds

The experimental (EXP) diet was formulated taking the proximate composition (Table 1) of a commercial diet as a reference (AQUASOJA, SORGAL, Ovar, Portugal). This commercial diet (COM) was routinely used as maintenance feed in the culture of *M. cephalus* by the company providing the fish. It contains several animal ingredients (fishmeal, fish hydrolysate, feather meal, meat and bone meal, poultry fat) and plant ingredients (soybean, wheat, bean and sunflower meals) to give a total amount of 35 g/100 g crude protein and 9 g/100 g crude fat. The EXP diet was formulated including more than 75 % plant ingredients, of which 30 % were high-fibre by-products such as rice bran and brewer's spent grain. To prepare the enzyme treated diet (EXP/enz), all plant ingredients were milled to a mesh size of 0.5 mm and mixed with citrate buffer (pH 5.0, 0.1 M) to obtain a moist mass (1:2 w/v), providing the optimal conditions for the action of the multienzyme complex under SSH. The product used was Rovabio®, a mixture of xylanases, glucanases, arabinofuranosidases and phytase produced by Adisseo (Auvergne, France). It was added to the mixture of plant meals by dissolving the dose recommended for terrestrial species by the manufacturer (0.2 mL/kg) in a certain amount of citrate buffer (0.1 M, pH 5.5) that was then carefully sprayed and mixed. The enzymes were allowed to act, keeping the mixture at 45 °C for 6 h with manual stirring every hour to ensure the homogeneity of the reaction. After this time, the reaction was stopped by placing the mixture in a cold chamber at 4 °C until addition of the rest of the diet ingredients and preparation of feed pellets. The feeds were prepared using an extrusion machine with a mesh size of 2 mm, dried, and stored at 4 °C until use.

Table 1. *Ingredients and proximate composition of the experimental feed used in the experiments.*

Ingredients (in g/100 g d.w.)	EXP	COM
Fishmeal 67/10	10.00	
Soybean meal 47	18.83	
Defatted rice bran	10.00	
Soybean protein concentrate	8.00	
Corn gluten meal 60	8.00	
Guar meal (Korma)	11.16	
Brewer's spent grain	20.00	
Fish oil	3.25	
Sunflower oil	2.60	
Soy lecithin	0.65	
Vitamin/mineral premix	0.05	
Taurin	0.30	
Yeast	3.00	
Squid hydrolysate	1.50	
Proximate composition (in g/100 g)		
Crude protein	35.60	35.00
Crude fat	9.03	9.00
Digestible carbohydrates (starch + oligosaccharides)	10.56	4.00
NSP	27.73	
Ash	6.21	8.00
Phosphorus	0.85	
Phytate P	0.35	
Gross Energy (MJ kg^{-1})	17.80	17.10

Declared ingredients in the commercial diet: Meat and bone meal, feather meal, fishmeal, fish hydrolysate, wheat meal, horse bean meal, sunflower meal, dehulled soybean meal, rice bran, fish oil, poultry fat, brewer's yeast. EXP: experimental; COM: commercial; NSP: Non-starch polysaccharides; P: phosphorus. Vitamins and mineral premix (IU or mg kg−1 diet); DL-alpha tocopherol acetate, 200 mg; sodium menadione bisulphate, 10 mg; retinyl acetate, 16,650 IU; DL-cholecalciferol, 2000 IU; thiamine, 25 mg; riboflavin, 25 mg; pyridoxine, 25 mg; cyanocobalamin, 0.1 mg; niacin, 150 mg; folic acid, 15 mg; L- ascorbic acid monophosphate, 750 mg; inositol, 500 mg; biotin, 0.75 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg; copper sulphate heptahydrate, 25 mg; ferric sulphate monohydrate,100 mg; potassium iodide, 2 mg; manganese sulphate monohydrate, 100 mg; sodium selenite, 0.05 mg; zinc sulphate monohydrate, 200 mg.

The effect of SSH on the feeds was assessed by chemical analysis of some specific compounds of which the relative concentrations were expected to be modified as a result of enzyme treatment: soluble protein, reducing sugars, pentoses, total phosphorus and phytate phosphorus. Soluble protein was analysed by the Bradford method (Bradford, 1976) using a SIGMA Total Protein Kit (TP0100). Reducing sugars were measured using

3,5-dinitrosalicylic acid (DNS) following the method described by Miller (1959). Pentoses were measured by the phloroglucinol method described by Douglas (1981). Total phosphorus was determined by the molybdovanadate method after total digestion of the organic matter with concentrated nitric acid. Phytic acid was determined following the bipyridine method described by Haug & Lantzsch (1983). All the analyses were performed in triplicate on samples from each diet. The rest of compounds (total crude protein and lipids, moisture, ash) were analysed using AOAC protocols (AOAC, 1995). In brief, crude protein (N × 6.25) was evaluated using the Kjeldahl method, lipid content was determined by petroleum ether extraction (40–60 °C) using a Soxhlet System, moisture content was calculated by drying at 105 °C for 24 h, and ash content was determined using a muffle furnace at 550 °C for 5 h. In addition to these chemical analyses, change in the water retention capacity of feed pellets as a result of partial hydrolysis of the carbohydrate fraction was evaluated as described in Heywood et al. (2002).

2.1.2. Feeding trial, samples collection and data recording

Juvenile grey mullets (*Mugil cephalus*) were provided by PIMSL (Sevilla, Spain), transferred to the experimental facility (CTAQUA, Centro Tecnológico de la Acuicultura de Andalucía, El Puerto de Santa María, Cádiz, Spain) and acclimated to laboratory conditions for two weeks. Then, the fish (12.24 ± 1.05 g body weight) were randomly distributed in triplicate groups in nine 400 L tanks (n = 100 fish per tank, 300 fish per experimental diet) coupled to a recirculation aquaculture system (RAS) and equipped with physical and biological filters and programmable temperature and O_2 suppliers. Water flow of 5–6 L/tank/min ensured a daily renewal of ten times total volume, and was maintained at 20.3 ± 1.0 °C during the experiment. Experimental diets were offered to apparent satiation three times per day, with the orientative daily ration adapted according to weight controls carried out every 14 days. The experiment lasted six weeks. Total feed intake was recorded for each experimental unit to calculate growth performance parameters. At the end of the trial, overnight fasted fish (four fish per tank, twelve per experimental conditions) were randomly sampled and deeply anaesthetized with 2-fenoxiethanol in a lethal dose (1 mL/L SW) to obtain blood and tissue samples. Blood was drawn from caudal vessels with heparinized syringes and centrifuged at 3,000 *g* for 15 min at 4 °C to

separate plasma, which was then snap-frozen in liquid nitrogen and stored at −80 °C until used for biochemical analysis. Fish were cervically sectioned in order to obtain biopsies of different tissues; samples of liver were rapidly taken and weighed to calculate the hepatosomatic index (HSI) and, together with samples of white skeletal muscle, were snap-frozen in liquid nitrogen and stored at −80 °C for subsequent biochemical analysis. Maintenance and sampling of the fish was carried out in compliance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

2.1.3. Growth performance and biometric parameters

The following growth parameters were evaluated:

Specific Growth Rate (SGR) = (100 × (ln final body weight − ln initial body weight)/days
Weight Gain percent (WG) = (100 × (body weigh increase)/initial body weight
Feed Conversion Ratio (FCR) = total feed intake/weight gain
Condition Factor (K) = (100 × body weight)/fork length^3
Hepatosomatic Index (HSI) = (100 × liver weight)/fish weight

2.1.4. Biochemical parameters

Glucose (Ref. 1001200), lactate (Ref. 1001330) and triglycerides (Ref. 1001311) in plasma and tissues were measured using commercial kits (Spinreact, St. Esteve d'en Bas, Girona, Spain) adapted to 96-well microplates. Plasma total protein concentration was determined with a BCA Protein Assay Kit (Ref. 23225, Thermo Fisher Scientific Pierce, Waltham, MA, USA,) using BSA as the standard. Glycogen concentration was quantified using the method described by Decker & Keppler (1974), while glucose obtained after glycogen breakdown with amyloglucosidase (Ref. A7420; Sigma-Aldrich, St. Louis, MO, USA) was determined using the same commercial kit described above. To analyse biochemical parameters in liver and muscle, frozen tissues were homogenized by ultrasonic disruption in 7.5 volumes ice-cold 0.6 N perchloric acid, neutralized using 1 M KCO_3, and centrifuged (30 min, 3,220 *g* and 4 °C); the supernatants were then isolated to determine tissue metabolites. All assays were performed using a PowerWaveTM 340 microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) using the KCjunior™ data analysis software (Bio-Tek Instruments, Winooski, VT, USA) for Microsoft®.

2.2. Experiment 2. Field trial

Experimental feeds, feeding trial, samples collection and data recording

The same three experimental feeds described and evaluated in Experiment 1 were used in this trial. In this case, the experiment was carried out in the facilities of PIMSL (Sevilla, Spain); 1,200 juvenile fish with a 39.63 ± 1.14 g initial mean body weight were randomly distributed in six 8 m^3 concrete outdoor tanks in duplicate groups (n = 200 fish per tank) coupled to a recirculation aquaculture system (RAS) equipped with physical and biological filters. Water temperature varied within a wide range during the 148 days of the experimental period according to season, from June to December 2020 (19.5 ± 3.14 °C). Fish were fed twice a day at an initial ration of 2 % b.w. that was adjusted after one weight control carried out at an intermediate point during the experiment. Total feed intake and weight increase were recorded for each experimental group in order to calculate FCR and SGR at the end of the experiment, as indicated in Section 2.1.4.

2.3. Statistical analysis

After a preliminary evaluation to determine the normality of the data using the Shapiro–Wilk test, homoscedasticity analysis was conducted using the Brown–Forsythe test. Due to the different composition of the two diets used as a reference (commercial and experimental without enzyme), separate comparisons were carried out among the diets, on one hand comparing the commercial diet with each of the two experimental diets, and on the other comparing only these latter between themselves. The objective was to assess whether any of the two experimental feeds were equivalent to the commercial diet, while in the second case the objective was to check whether the enzyme treatment could improve on the performance obtained with the experimental diet. The first was carried out by one-way ANOVA followed by the Bonferroni test, and the second was performed using Student *T*-test. The significance level was established at $p < 0.05$. Data expressed in percentage were previously arc-sin transformed. All analyses were carried out using Statgraphics Centurion software (Statgraphics Technologies, Inc., The Plains, VA, USA).

3. RESULTS

3.1. Experiment 1

The analysis of the diets showed significantly higher values of some compounds (soluble protein, reducing sugars and phytate) and lower values of total phosphorus in EXP feed when compared to COM feed, reflecting differences in the ingredients used in their elaboration. The Solid-State enzymatic Hydrolysis significantly increased the amount of potentially available reducing sugars and pentoses, and reduced the amount of phytate (Table 2). Furthermore, physical transformation resulting from the enzyme treatment determined a significant reduction in water retention capacity in the EXP/enz diet (Table 2).

Table 2. *Differences in the nutrient content and water retention of experimental feeds (g/100 d.m.). Statistical comparisons between COM and any of the EXP diets is noted in capital letters, while comparisons between EXP and EXP/enz are detailed in lowercase. Each assay was performed in triplicate. Values not sharing the same letter differ significantly with $p < 0.05$. COM: commercial; EXP: experimental; EXP/enz: experimental enzyme treated.*

	Soluble protein	Reducing sugars	Pentoses	Phosphorus	Phytate	Water retention
COM	3.13 ± 0.05^{A}	0.45 ± 0.00^{A}	0.29 ± 0.01^{A}	1.31 ± 0.02^{A}	0.22 ± 0.00^{A}	305.77 ± 6.89^{A}
EXP	6.83 ± 1.13^{Ba}	2.08 ± 0.02^{Ba}	0.28 ± 0.00^{Aa}	0.83 ± 0.10^{Ba}	0.34 ± 0.01^{Ba}	305.45 ± 3.87^{Aa}
EXP/enz	5.58 ± 0.24^{Ba}	2.83 ± 0.03^{Bb}	0.36 ± 0.05^{Bb}	0.76 ± 0.03^{Ba}	0.21 ± 0.02^{Bb}	280.94 ± 9.29^{Bb}

3.1.1. Growth performance

Results of growth performance and feed utilisation are presented in Table 3. No mortality occurred during the experiment, and all groups presented an increase in body mass, accounting for 11 % to 33 % of their initial mean body weight. Fish presented a normal condition factor for the species (K = 1.07–1.12). The results presented in Table 4 show that the experimental diet, irrespective of being enzymatically treated or not, clearly offered worse results in growth and feed efficiency than the control diet. Nevertheless, it was also clear that the Solid-State enzymatic Hydrolysis significantly improved the same parameters when comparing fish fed on EXP/enz to those obtained with EXP.

Table 3. *Growth and feed efficiency measured in fish fed on the experimental diets. Statistical comparisons between COM and any of the EXP diets is noted in capital letters, while comparisons between EXP and EXP/enz are detailed in lowercase. COM: commercial; EXP: experimental; EXP/enz: experimental enzyme treated; FCR: feed conversion ratio; SGR: specific growth rate; HSI: hepatosomatic index.*

Parameter	COM	EXP	EXP/enz
Initial body mass (g/fish)	12.02 ± 0.33^{A}	11.89 ± 0.55^{Aa}	12.38 ± 0.16^{Aa}
Final body mass (g/fish)	16.60 ± 0.60^{A}	13.21 ± 0.49^{Ba}	15.36 ± 0.04^{Ab}
Feed consumption (g/fish)	10.02 ± 0.17^{A}	8.65 ± 0.45^{Ba}	9.23 ± 0.17^{Ab}
FCR (g feed/g fish)	2.19 ± 0.11^{A}	5.89 ± 0.60^{Ba}	3.11 ± 0.19^{Bb}
SGR (%/day)	0.75 ± 0.02^{A}	0.25 ± 0.05^{Ba}	0.50 ± 0.03^{Bb}
HSI (%)	1.27 ± 0.07^{A}	0.93 ± 0.18^{Ba}	0.83 ± 0.20^{Bb}
Condition factor (K)	1.11 ± 0.12^{A}	1.07 ± 0.12^{Ba}	1.07 ± 0.17^{Ba}

3.1.2. Biochemical parameters

Data on blood and tissue biochemistry are detailed in Table 4. The parameters measured in plasma show significantly lower values of lactate in fish fed any of the experimental diets when compared to those obtained in fish fed the COM diet.

Table 4. *Metabolites measured in plasma and tissues of fish fed on the different experimental diets. Statistical comparisons between COM and any of the EXP diets is noted in capital letters, while comparisons between EXP and EXP/enz are detailed in lowercase.*

Parameter	COM	EXP	EXP/enz
In plasma (mg/dL)			
Glucose	80.54 ± 26.21^{A}	92.12 ± 29.61^{Aa}	76.76 ± 14.81^{Aa}
Lactate	67.28 ± 32.89^{A}	56.71 ± 11.07^{Ba}	47.13 ± 22.73^{Bb}
Protein	32.60 ± 5.46^{A}	34.62 ± 10.08^{Aa}	38.23 ± 4.35^{Aa}
TAG	38.09 ± 7.60^{A}	34.13 ± 4.76^{Aa}	42.53 ± 6.48^{Bb}
In liver (mg/g ww)			
Glucose	1.41 ± 0.48^{A}	1.37 ± 0.56^{Aa}	1.34 ± 0.29^{Aa}
Glycogen	7.26 ± 1.26^{A}	2.61 ± 1.52^{Ba}	3.31 ± 1.32^{Ba}
In muscle (mg/g ww)			
Glucose	0.77 ± 0.30^{A}	0.84 ± 0.28^{Aa}	0.72 ± 0.30^{Aa}
Glycogen	0.78 ± 0.45^{A}	0.38 ± 0.14^{Ba}	0.79 ± 0.31^{Ab}
Lactate	24.57 ± 6.63^{A}	26.35 ± 7.62^{Ba}	24.96 ± 3.90^{Aa}
TAG	10.48 ± 5.14^{A}	10.79 ± 3.83^{Aa}	11.17 ± 7.15^{Aa}

Additionally, the amount of TAG measured in fish fed the EXP/enz diet was significantly higher than in fish fed the EXP diet. Liver glycogen

measured in fish fed any of the experimental diets was also significantly lower than in fish fed the COM diet. Indeed, muscle glycogen measured in fish fed on the EXP diet was significantly reduced compared to the other two groups.

3.2. Experiment 2. Field trial

The results on growth performance and feed efficiency in the field trial are presented in Table 5. Average fish growth during the whole experimental period ranged from 0.32 to 0.43 %, although it was low or even absent during a great part of the period due to low winter temperatures. Nevertheless, all of the experimental groups doubled their initial weight by the end of the experiment. Moreover, all groups presented reasonably good FCR values, ranging from 2.33 to 2.67, and in contrast to those obtained in experiment 1, the values of FCR were significantly enhanced when fish were fed on the EXP/enz diet.

Table 5. *Growth and feed efficiency in fish fed on the experimental diets in the field trial. Statistical comparisons between COM and any of the EXP diets is noted in capital letters, while comparisons between EXP and EXP/enz are detailed in lowercase.*

Parameter	COM	EXP	EXP/enz
Initial body mass (g/fish)	40.13 ± 0.18^{A}	38.25 ± 0.35^{Ba}	40.50 ± 0.71^{Ab}
Final body mass (g/fish)	88.05 ± 11.53^{A}	94.65 ± 13.08^{Aa}	104.70 ± 13.86^{Aa}
Feed consumption (g/fish)	18.58 ± 0.96^{A}	17.83 ± 1.68^{Aa}	17.76 ± 0.01^{Aa}
FCR (g feed/g fish)	2.65 ± 0.12^{A}	2.67 ± 0.29^{Aa}	2.33 ± 0.58^{Bb}
SGR (% /day)	0.32 ± 0.08^{A}	0.38 ± 0.09^{Aa}	0.43 ± 0.09^{Aa}

4. DISCUSSION

The “extreme” experimental feed (EXP) used in the present work was designed to assess the limits of including a high amount of plant ingredients of limited nutritional value in practical diets for *M. cephalus*, as well as the potential benefits derived from the enzymatic treatment of such plant ingredients. For this reason, it included a low proportion of fishmeal (10 %), a high amount of fibrous byproducts containing significant levels of phytate and NSP (10 % rice bran, 20 % brewer’s spent grain) and no supplementation with lysine or methionine. Although Experiment 1, developed with small juveniles, was not maintained for enough time to allow the fish to at least double their weight, it offered

preliminary insight on the potential performance of the diets and two clear results: on the one hand, it demonstrated that the EXP diet did not fulfill the nutritional requirements of the species at this early stage of development; on the other, it showed that enzyme pretreatment of the plant ingredients had a significant positive effect on the nutritional value of such a diet.

Related to the first point, despite the values of FCR and SGR obtained with diets EXP being not directly comparable to those obtained with diet COM (as both types of diets were differently formulated and contained quite different ingredients in terms of acceptance, digestibility and nutritional value), it was clear that the use of the EXP diet impaired the growth of the fish. Similarly, El-Gendy et al. (2016) reported a clear reduction in FCR and SGR when feeding juveniles of *M. cephalus* with diets prepared with increasing amounts of plant ingredients (from 20 to 100 %), in that case including a significant proportion of cereal bran. Certainly, a different selection of plant ingredients with a higher nutritional value should have produced much better results; in this way, Gisbert et al. (2016) reported good results in terms of SGR, digestive physiology and fish condition when feeding small (0.2 g) juveniles of *M. cephalus* on feeds including a blend of corn and wheat gluten and soy protein concentrate, supplemented with crystalline L-lysine and DL-methionine. Nevertheless, as mentioned previously, the objective of the present study was to assess to what extent feeds for *M. cephalus* could include high amounts of plant byproducts and no specific supplementation to minimize the final cost of the diet without compromising growth.

Regarding the second point, it is clear that Solid-State enzymatic Hydrolysis of the EXP diet with a commercial mixture of xylanases, β- glucanases and pectinases resulted in a significant improvement in both FCR and SGR compared to the values obtained with the untreated diet, suggesting a clear positive effect of the enzyme complex. Similar positive effects have been reported in terrestrial species such as pigs (Sun et al., 2020) and poultry (Poernama et al., 2021), being associated to a great extent with the modification of the structure of carbohydrates present in plant ingredients, which increases the bioaccessibility of nutrients to the action of digestive enzymes. In the present study, such modification was indirectly evidenced by the significant reduction in the

water retention capacity, as well as by the significant increase in bioavailable monosaccharides and the reduction in phytate measured in the EXP/enz feed (Table 2). The improvement observed in the FCR and SGR over those obtained in fish fed the EXP diet was higher than 50 %, suggesting that partial hydrolysis of the antinutritive factors (NSP and phytate) exerted a positive impact on the nutritional value of the feed, and hence on the performance indicators.

In the present study, the measurement of different metabolites was intended to evaluate the potential impact of the EXP diet on fish energy orchestration, as well as whether enzyme treatment yielded significant effects on metabolic homeostasis. As presented herein, no significant differences were observed in glucose levels measured in plasma, muscle or liver irrespective of the diet or enzyme treatment, even when this latter treatment significantly increased the amount of available dietary sugars measured in the fish receiving the EXP/enz diet. In contrast to what has been described for carnivorous fish (Polakof et al., 2012), this suggests a good ability of the grey mullet to use carbohydrates as a source of energy, demonstrating a homeostatic load of this metabolite that might be considered sufficient for the potential growth of this species. The unfavourable nutritional status of fish fed on the EXP diet was reflected by several indicators. First, a significantly lower accumulation of liver glycogen was correlated with the lower weight of this organ, which determined decreased values of HSI as well as lower plasma levels of lactate and could reflect an impairment between the total energy incorporated through the feed and the demand for physiological processes such as growth. Furthermore, a significantly lower concentration of muscle glycogen was measured in fish receiving the EXP diet. Under normal conditions, if the feed is able to cover nutritional needs excess glucose may be stored as glycogen (glycogenesis) or converted into lipids (lipogenesis) instead of being oxidized for energy; however, under conditions of food deprivation or nutritionally unbalanced feeding, glucose requirements are satisfied either by glycogen depletion to glucose (glycogenolysis) or by de novo glucose synthesis through gluconeogenesis from lactate, glycerol or certain amino acids (Enes et al., 2009). In contrast, the comparatively improved nutritional status associated with consumption of the EXP/enz feed was supported by significantly higher values of muscle glycogen and plasma TAG. Moreover, the results also suggest that de novo gluconeogenesis in the

muscle may contribute to higher glycogen content, although to elucidate whether this fact is a cause or a consequence of better feed utilisation would require further investigation related to the key role of several metabolic enzymes in this and other tissues.

The results obtained in Experiment 2 were noticeably different, for two main reasons: the initial size of the juvenile fish was much higher (nearly 40 g average weight) and the experimental period was long enough that the fish were able to double their initial weight (Table 5). In addition, the environmental conditions were different, as the experiment was carried on outdoors in standard facilities used for rearing the intermediate stages of growing fish. Under such conditions, both the EXP and EXP/enz diets appeared to be equivalent to the COM diet, and Solid-State enzymatic Hydrolysis confirmed its positive effect on the nutritional value of the ingredients. Although the growth rates of *M. cephalus* were relatively low at the end of the 28 week feeding period, they were comparable to those reported in previous studies carried out with this and other similar species that evidence the slow growth rate of mugilids (Varlet et al., 1970; Chervinski, 1976; Richard et al., 2010). Legarda et al. (2019) reported an SGR of 0.56 %/day for *Mugil cephalus* juveniles fed 1 % bw daily in a biofloc system maintained at 28 ± 1 °C. Karapanagiotidis et al. (2014) also recorded low SGR values (around 0.45 %/day) in juveniles of *Liza aurata* fed once per day at 2 % of their biomass at temperatures ranging from 12 to 26 °C.

Values of FCR obtained with any of the EXP diets were clearly improved in relation to those obtained in Experiment 1, being in this case equivalent or even better than obtained with the COM diet. This could be thanks to for two main reasons: on the one hand, as indicated previously, the experiment was carried out using older fish, and presumably their ability to digest fibrous materials improved greatly with age, this being associated with the maturation and development of the digestive function. These changes in digestive capability with development have been previously reported in other mullet species including the thick-lipped grey mullet, *Chelon labrosus* (Pujante et al., 2017; Gilannejad et al., 2020). This suggests that the possibility of using a high amount of fibrous plant byproducts in diets for this species is strongly conditioned by the age of the fish, and probably linked to the ability, acquired with development, to properly digest such ingredients. In addition, it must be

considered that the conditions in which the fish were maintained in this latter experiment, mainly a lower stocking density, could result in lower stress and a positive impact on feeding behaviour, resulting in better food utilization. Although there are few studies on the nutrition of mullets under field conditions, the study carried out by Karapanagiotidis et al. (2014) on *Liza aurata* showed that growth and feed utilization of the fish was not significantly affected by variations in the dietary protein level. Such absence of a clear effect could be due to the possible complementary effect of natural food present in the water mass. In the present study, the only source of nutrients was the artificial feeds, and no other food source was available; hence, the observed response was representative of the nutritional value of the feeds.

The results further confirmed the positive effect of the enzyme treatment already pointed out in Experiment 1, which is in line with other previous studies. A 10 % improvement in FCR was reported by Maas et al. (2019) when including enzymes in diets for tilapia formulated solely using vegetable ingredients, and a 14 % improvement in FCR was obtained after addition of multi-enzyme complexes (Natuzyme® or Hemicell®) in diets for Caspian salmon (Zamini et al., 2014). As previously indicated, a number of beneficial effects have been reported resulting from the hydrolysis of NSP (Adeola & Bedford, 2004; Sinha et al., 2011). In addition, the significant reduction in phytate derived from Solid-State enzymatic Hydrolysis (Table 2) could result in not only an increased availability of phosphorus, but also in a decrease in some other negative effects associated with the presence of phytate on the digestive bioavailability of proteins and some minerals, as described by several authors (Sugiura et al., 2001; Cao et al., 2007). Furthermore, the citrate buffer used to develop the process of SSH could enhance the solubilisation of certain minerals, such as Fe or Mn (Sugiura et al., 1998).

The results support the suitability of SSH as a method for proper application of exogenous enzymes, as the hydrolysis is performed under optimal conditions and the enzyme activity is not affected by either the high temperatures reached during feed preparation or by the biochemical conditions present in the gut of the fish. This overcomes most of the physiological and technical limitations described when enzymes are included in the feed. To date, only one study, carried out by Denstadli et al. (2011) on trout feeds, has tested this way of performing enzyme

treatment. In that study, the feeds included 450 g/kg feed of plant ingredients and an estimated amount of NSPs accounted for 80 g/kg feed, values much lower than those used in the present study where the feed contained nearly 700 g plant ingredients per kg of feed and the estimated amount of NSP exceeded 200 g/kg. While in that work the enzyme treatment determined a 10–13 % reduction of NSP content when using soybean meal as the main ingredient and of 4–6 % when using rapeseed meal, in the present study the hydrolysis of NSP was not measured directly; however, the observed changes in the bioavailability of pentoses and reducing sugars suggested a modification of the nutritional value of the feed that impacted the results obtained on growth and nutritive utilization. From a practical point of view, performing such treatment by SSH allows adaptation of the more suitable operative conditions (dose, reaction time, etc.) to the specific features of different plant ingredients. In addition, because the enzyme mixture is used prior to pelleting, inactivation due to thermal processing should eliminate any further undesired effects. Although the potential application of such procedures requires further research, it offers interesting possibilities for a wider utilization of different inexpensive byproducts in feeds for herbivorous/omnivorous fish species such as *M. cephalus*, which has highly positive features from an environmental perspective.

5. CONCLUSIONS

Considering the aforementioned, the present study strongly suggests that enzyme pretreatment of highly fibrous plant ingredients by SSH using a commercial mixture of different carbohydrases and phytase may be a useful procedure to improve the nutritive value of high fiber plant byproducts for inclusion in practical diets for *M. cephalus* and other fish with similar nutritional features.

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CHAPTER 4

Assessing differences in the bioaccessibility of phenolics present in two wine by-products using an *in-vitro* model of fish digestion

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ABSTRACT

Increasing attention is currently being paid to the protective role of polyphenols in health and oxidative status in fish. For this reason, the potential use of different natural sources of such compounds, like wine by-products, is under study. One key step required to gain a better understanding on the biological roles of polyphenols for a given species is to assess the different factors affecting their digestive bioaccessibility, and a great number of such studies is based in the use of *in vitro* digestion models. In the present study the potential digestive bioavailability of the phenolic compounds present in wine bagasse and lees was evaluated for two fish species showing great differences in their digestive phisyiology: the omnivorous gilthead sea bream (*Sparus aurata*) and the herbivorous flathead grey mullet (*Mugil cephalus*). The study was developed using *in vitro* models adapted to simulate their digestion and a factorial experimental design that simultaneously evaluated the effects of the ingredient used as source of polyphenols, presence or absence of feed matrix, fish species and digestion time. The release of the phenolic compounds was evaluated using ultra-high performance liquid chromatography (UHPLC) coupled to high resolution mass spectrometry (HRMS) detection. Both the presence of feed matrix and the type of wine by-product showed a significant effect on the digestive release of both total and specific types of polyphenols while fish species showed to be significant only for some specific compounds, like eriodyctiol or syringic acid. The time of digestion was not identified as a statistically significant factor in the release of phenolic compounds due to the great variability in the patterns observed that were classified as early, sustained and late. The observed great variations in the patterns of release of different types of phenolic compounds with time suggest an important effect of gut transit rates on the net bioavailability of a given phenolic compound in the live fish. The present study is, to our knowledge, the first one on which an *in vitro* approach was applied to assess to what extent the possible complexation of wine polyphenols present in wine by-products with either digestive enzymes or components of the feed matrix could limit their bioaccessibility if included in diets of two different fish species.

Keywords: polyphenols, UHPLC-Q-Orbitrap-MS, wine by-products, fish species, *in vitro* digestion

1. INTRODUCTION

Increasing attention is being paid to the inclusion of biologically active ingredients in aquaculture feeds that can benefit fish health and resistance to different stressors (changes in environmental parameters - temperature, salinity, etc., alterations in water quality, manipulation, etc.) as well as disease outbreaks. Within this context, it is of great interest to investigate the potential use of bioactive molecules with antioxidant and immunostimulatory functions present in a great number of agro-industrial by-products. Indeed, the valorization of some of these materials as a source of active compounds would be in line with the principles of the circular economy. In this sense, by-products from the winemaking process represent a cheap and exceptionally rich source of valuable compounds that could be used as natural additives and functional ingredients (Hang, 1988; Arvanitoyannis et al., 2006; Galanakis, 2012; Galanakis & Schieber, 2014; Prokopov et al., 2015). Wine by-products have traditionally been used in feeding terrestrial animals mainly as a source of fiber, carbohydrates and minerals, but more recently, different studies have highlighted their role as a source of different chemical compounds, mostly phenolics, with positive effects in the production of pigs, poultry or ruminants (Chedea et al., 2019; Turcu et al., 2020; Costa et al., 2022). In the case of fish, the few studies published to date suggest that grape polyphenols may also produce beneficial effects. The more remarkable are the prevention of liver diseases related to oxidative stress (Souza et al., 2019), reduction in the deterioration of cellular energy homeostasis (Baldissera et al., 2019), improvement in the growth and feed digestibility (Peña et al., 2020) and changes in the composition of the intestinal microbiota (Pulgar et al., 2021).

The study of the intestinal absorption and bioavailability of dietary phenolic compounds in humans and animals is a complex issue that involves several factors. In the one hand, they are influenced by physicochemical properties of the different molecules (chemical structure, molecular size, configuration, lipophilicity, solubility, pKa, etc.) and in the other by their interactions with other components of the digesta (food/feed matrix and digestive enzymes), as well as with the colonic microbiota. All those factors determine great differences in the rates of absorption and hence in bioavailability of phenolics present in different vegetable sources (plants and fruits, either raw or processed and also in by-products) and hence in their observed biological effects. In the

case of humans, and to a lesser extent in terrestrial animals, many of the above mentioned aspects have been extensively addressed using a wide array of *in vitro* assays (Sengul et al., 2014; Mandalari et al., 2016; Seczyk et al., 2021). Although there is a great difference between biological properties of polyphenols observed *in vitro* and their bioactivity *in vivo*, simulations of the physiological conditions present in the digestive tract become a valuable tool that helps to reach a greater understanding on how such factors may influence potential bioavailability of phenolics (Tagliazucchi et al., 2010). *In vitro* digestive simulations of aquatic animals have been used with different purposes; the evaluation of protein quality of feed ingredients (Alarcón et al., 2002; Hamdan et al., 2009), the study of factors affecting the efficiency of enzyme hydrolysis in the digestion (Gilannejad et al., 2017) or the effect of the digestive biochemistry on toxic compounds (Nogueira et al., 2022). Nevertheless, to date they have not been used to evaluate the potential beneficial effects of including phenolic compounds in diets.

A key aspect required for a proper evaluation of the release of phenolic compounds in experiments simulating digestion is to apply methodologies allowing and accurate quantitative and qualitative detection of the highly diverse profiles that can be obtained. The analysis of phenolic compounds present in wine and its by-products has been routinely developed using liquid chromatography (LC) coupled to diode array detection (DAD), due to its high sensitivity and easy operation (Revilla & Ryan, 2000; Sun et al., 2007). Nevertheless, some problems occur when ultraviolet (UV) spectrum is studied, since the UV spectrum of phenolic compounds is quite similar, being the identification ambiguous. Due to this, the best option for the analysis of phenolic compounds is the use of LC coupled to mass spectrometry (MS) using tandem MS/MS stages and an Electrospray Ionization (ESI). Of course, due to the physical–chemical properties of phenolics the ionization mode was in positive and negative (Sun et al., 2007; Vrhosek et al., 2012; López-Gutiérrez et al., 2016; Liu et al., 2020). In addition, in the last years the use of high-resolution mass spectrometry (HRMS) has emerged as a revolutionary way for screening samples in a short time to obtain a complete characterization of them. In this case, a complete profile of phenolic compounds can be achieved using the capacity of full scan acquisition, exact mass resolution and the used of HRMS spectral libraries that provide the possibility to detect 1000s of compounds without using analytical standards (Liu et al., 2020).

Considering all the aforementioned, the objective of the present study was to assess how different factors can affect the potential bioavailability of the phenolic compounds present in two types of wine by-products (bagasse -WB- and lees -WL-) when provided in the feed of two fish species. The species chosen were the gilthead sea bream (*Sparus aurata*) and the flathead grey mullet (*Mugil cephalus*) and they were selected considering that both of them are commonly aquacultured species that could potentially benefit from the protective effect of a dietary reinforcement in phenolics. Also, this may help to illustrate the expected differences linked to particular features of their digestive physiology (mainly determined both by the amount and types of digestive enzymes and by the presence of an acid stage in the digestion of sea bream that is absent in the case of mullet).

The study was based in (a) the use of an *in vitro* model adapted to simulate the digestion of both fish species; (b) a factorial experimental design, oriented to evaluate the effect of different factors involved in the digestion and (c) an accurate analytical methodology of the phenolic compounds based in using ultra-high performance liquid chromatography (UHPLC) coupled to high resolution mass spectrometry (HRMS) detection. The study was run as a preliminary step to the development of an *in vivo* test currently in progress, since we expect that results obtained would provide valuable information related to the selection of the most suitable ingredient, as well as on the biological responses derived from its dietary inclusion.

2. MATERIALS AND METHODS

2.1. Origin and characterization of the wine by-products (bagasse and lees)

Two types of by-products were obtained from red grape varieties: (i) wine bagasse (WB) came from an artisanal winery located in Fondón (Almería, Spain) and (ii) wine lees (WL) came from a winery located at Chiclana de la Frontera (Cádiz, Spain). Upon collection the two products were stored at −20 °C until processed or used for the different analysis. Processing consisted in the case of WB in a drying at low temperature (28 °C) followed by milling until obtaining a fine powder. The WL were simply thawed prior to being used in liquid form. The composition of both WB and WL is detailed in Table 1.

Table 1. *Proximal composition and total phenolics in red wine bagasse (WB) and lees (WL).*

Proximal composition (g/kg)	WB	WL
Total fat	54 ± 8	27 ± 4
Saturated fatty acids	8 ± 2	8 ± 1
Total carbohydrates	320 ± 60	130 ± 20
Reducing sugars	162 ± 7	3.4 ± 2.8
Dietary fiber	470 ± 90	480 ± 100
Crude protein	83 ± 12	180 ± 20
Total minerals	35 ± 7	160 ± 30
Na	< 0.05	0.11 ± 0.02
Bioactive compounds		
Total phenolics	53.8 ± 0.7	21.4 ± 0.9
Antioxidant capacity (μmol TEAC/g d.m.)	337.4 ± 14.1	134.4 ± 16.0

2.2. Wine by-products experiments

2.2.1. Experiment 1

The assay was aimed to assess the potential inhibitory effect of phenolics present in WB on the digestive proteases of one of the species used in the study; the gilthead sea bream (*Sparus aurata*). It was developed following a protocol previously applied by our group (Moyano et al., 1998). In brief, the assays were carried out incubating for 1 h a fixed amount of enzyme extracts obtained from the stomach or intestine of fish specimens in the presence of variable amounts of WB. After this time, residual protease activity was measured and expressed in relation to a control on which the enzymes were incubated in the presence of distilled water. The enzyme extracts were prepared by manual homogenization of tissues obtained after dissection of fish weighing about 100 g, followed by cold centrifugation (20,000 *g*; 4 °C) and separation of clear supernatants. After this process, the activities of stomach and intestinal proteases were determined in the extracts; acid protease was measured at pH 2.5 using hemoglobin as substrate (Anson, 1938) while alkaline protease was measured at pH 8.5 using casein (Walter, 1984).

2.2.2. Experiment 2

The objective was to evaluate changes in the potential digestive bioavailability of phenolics present in both WB and WL either when used pure or included in a feed matrix. Such experimental feed matrix was prepared using some pure ingredients in proportions (in g/100 g)

reflecting the composition of a standard fish feed: bovine albumin (45 %), sunflower oil (18 %), potato starch (10 %) and carboxy methyl cellulose (15 %). The matrix was prepared by mixing the ingredients with some distilled water in order to obtain a moist paste that was used as substrate to which WB or WL were added to reach a 10 % in dry weight. *In vitro* simulation of fish digestion was carried using a protocol developed by our group, based on the use of semi-permeable membrane bioreactors (Morales & Moyano, 2010). Each device consists of two chambers separated by a membrane of 3,500 kDa MWCO (ZelluTrans/Roth). Fish enzyme extracts and substrates are placed in the upper chamber and maintained under continuous agitation using a magnetic stirrer. To develop the acid phase of digestion (only in the case of *S. aurata*) the upper chamber contained the desired substrate dissolved in water and adjusted to pH 4.0 by addition of a few drops of HCl 0.1 M, as well as the crude enzyme extract from the stomach of the selected species while the lower chamber contained distilled water. During the alkaline phase, pH of the upper chamber was raised to pH 8.2 using borate buffer (0.1 M supplemented with 20 mM $CaCl_2$, sodium taurocholate 45 µM and NaCl 50 mM) prior to the addition of the intestinal enzyme extracts. Products released during the reaction time and passing across the membrane into the lower chamber can be recovered at different time intervals by a constant flow of the same alkaline buffer and used to determine the release of products contained in the substrate (phenolics in our case). The complete arrangement (formed by several experimental units) is maintained within a thermal chamber set at the desired temperature.

Digestive enzyme extracts of sea bream were obtained after dissection from two different sections (stomach and proximal intestine) of 15 individuals of approximately 50 g, while in the case of mullets they were obtained only from the intestinal portion of 5 adult specimens of approximately 3 kg. Specimens of both species were supplied by Central Research Services in Marine Cultures (SCI-CM, Operational Code REGA ES11028000312) of the University of Cádiz. The extracts were prepared by mechanical homogenization of the tissues in distilled water (1:10 w/v) followed by centrifugation (3,220 *g*, 20 min, 4 °C). The supernatant was then filtered through a dialysis system with a MWCO of 10 kDa (Pellicon XL, Millipore) and the concentrated extracts were freeze-dried until being required for the assays. Activities of acid and alkaline proteases were determined as indicated in Experiment 1. The values of protease activities were used as indicators to estimate the number of extracts required to provide physiological enzyme:substrate ratios in the assays developed for

each species. These were calculated considering, on one hand, the average total production of enzyme measured in a few fish in relation to their live weight and on the other, the average intake per meal of fish of such size, obtained from commercial ration tables. This information resulted in values of 33.3 and 16.7 U/mg protein for stomach and intestinal digestion in sea bream and 18.3 U/mg protein for intestinal digestion in mullet.

Different experiments were developed following a factorial design that simultaneously evaluated the effect of type of product, presence or not of feed matrix, fish species and digestion time. Details of this design are presented in Table 2 and the combination of the different factors resulted in a set of 16 different runs.

Table 2. *Conditions used and parameters measured on the* in vitro *experiment aimed to test the effect of feed matrix in the bioavailability of wine polyphenols.*

Fish species	Gilthead sea bream (*Sparus aurata*) Flathead mullet (*Mugil cephalus*)
Factors evaluated	Feed matrix (presence/absence) Type of product (bagasse/lees) Fish species (sea bream/mullet) Time of intestinal digestion (3/6 h)
Parameters measured in the digestate	Type and amount of specific phenolic compounds (UHPLC-Q-Orbitrap-MS)

2.3. UHPLC-Q-Orbitrap-MS analysis

Once the hydrolysis experiments were conducted, the obtained samples were analyzed to determine the profile of the phenolic compounds released. Sample treatment and UHPLC-Q-Orbitrap-MS analysis was based on a previous study developed in the research group by López-Gutiérrez et al. (2016). A previous extraction was carried out by mixing 2 mL of the dialysate samples with a mixture composed of methanol:water (80:20, *v/v*). After they were shaken for 1 min in a vortex and 2 h on a rotary shaker. They were then centrifuged at 5,000 rpm for 10 min to collect the supernatant that was diluted in a proportion 1:10 (*v/v*) with extraction solvent. Once the samples were extracted, they were injected and analyzed by UHPLC-Q-Orbitrap-MS. The chromatographic

separation was performed on a Vanquish Flex Quaternary LC (Thermo Fisher Scientific, San Jose, CA, United States) was used equipped with a reverse-phase C18 column, Hypersil Gold (100 mm × 2.1 mm, 1.9 µm, Thermo Fisher Scientific) at flow rate of 0.2 mL/min. The compounds were separated with gradient elution using water (A) and methanol (MeOH) (B) containing both 0.1 % formic acid as eluents. The step gradient was as follows: 0–1 min 95 % of A; then, it was linearly decreased to 70 % in 2.5 min, to 0 % in 2.5 min and remained constant during 8 min. Finally, it increased to 95 % in 2 min and remained constant during 5 min. The total running time was 20 min. The injection volume was 10 µL and column temperature was 30 °C (López-Gutiérrez et al., 2016).

The LC system was coupled to a hybrid mass spectrometer Q-Orbitrap Thermo Fisher Scientific (Q-Exactive™, Thermo Fisher Scientific, Bremen, Germany) using ESI (HESI-II, Thermo Fisher Scientific, San Jose, CA, United States) in positive and negative ion mode. ESI parameters were as follows: spray voltage, 4 kV; sheath gas (N_2, 95 %), 35 (arbitrary units); auxiliary gas (N_2, 95 %), 10 (arbitrary units); S-lens RF level, 50 (arbitrary units); heater temperature, 305 °C, and capillary temperature, 300°C. The mass spectra were acquired employing four alternating acquisition functions: (1) full MS, ESI +, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 70,000 Full Width at Half Maximum (FWHM); AGC target = 1e6, scan time = 250 ms; (2) full MS, ESI -, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 70,000 FWHM; AGC target = 1e6, scan time = 250 ms; (3) data independent analysis (DIA), ESI +, setting higher energy collisional dissociation (HCD) on, and collision energy = 30 eV, mass resolving power = 35,000 FWHM, scan time = 125 ms; (4) DIA, ESI - (setting HCD on, and collision energy = 30 eV), mass resolving power = 35,000 FWHM, scan time = 125 ms. The mass range in the full scan MS experiments was set to m/z 50–750. LC chromatograms were acquired using the external calibration mode.

The raw files obtained from each analysis were processed using the software Trace Finder 4.0 (Thermo Fisher Scientific, Les Ulis, France) with an in-house database composed of around 100 polyphenols. This database involved the name of the compounds and their molecular formula, theoretical exact mass of the precursor ion and theoretical exact mass of two fragments. Moreover, full-scan data of each sample was

carefully studied using Xcalibur TM version 3.0, with Qualbrowser to monitor the spectra of the detected compounds.

2.4. Statistics

The values of enzyme inhibition were subjected to arcsin transformation prior to be evaluated by one-way ANOVA followed by a Fisher's LSD test at a confidence level of 95 %. The design and further evaluation of the data of the factorial experiment (relative surface of the different peaks corresponding to each polyphenol, obtained in the UHPLC-MS assays) were carried out using the DOE module of the Minitab software 17 (Minitab Inc., State College, PA. United States).

3. RESULTS

A total of 13 main phenolic compounds were identified by UHPLC-Q-Orbitrap-MS for wine by-products in the digested samples, used in the present study, being classified in relation to their chemical structure as detailed in Table 3. In the case of WB it showed a major presence of flavan-3-ols (catechin, gallocatechins and procyanidins), flavonols (kaempferol, quercetin) and hydroxybenzoic acids like syringic and chlorogenic acids while the phenolic profile of WL showed the presence of flavanols such as quercetin, quercitrin and kaempferol, as well as flavanols like catechin, epicatechin, and procyanidin B2. Figure 1 shows an example of extracted ion chromatogram of the flavonols and flavanols catechin, epicatechin, quercetin 3-O-glucoside and epicatechin gallate.

The results of the inhibition of activity of the proteases present in stomach and intestine of sea bream (*S. aurata*) when incubated in the presence of WB are detailed in Figure 2. The values reached in both cases pointed to a maximum reduction of either 8 and 4 % of the activities of stomach or intestinal proteases, respectively. Results obtained in assays simulating digestive hydrolysis of matrix including any of the two wine byproducts by fish enzymes are summarized in Table 4 and Figures 3–6. As shown in Table 4, the presence of feed matrix was the only factor with a significant effect on the digestive release of both total and specific types of polyphenols (from $p < 0.001$ to $p < 0.037$ for different compounds). The type of wine by-product was also a significant factor affecting the digestive release of most polyphenols and in consequence, the interaction of both factors showed also to be significant for most of them. In contrast, fish species was not a significant factor affecting the total release of

polyphenols, although it showed to be significant for some specific compounds, like eriodyctiol or syringic acid.

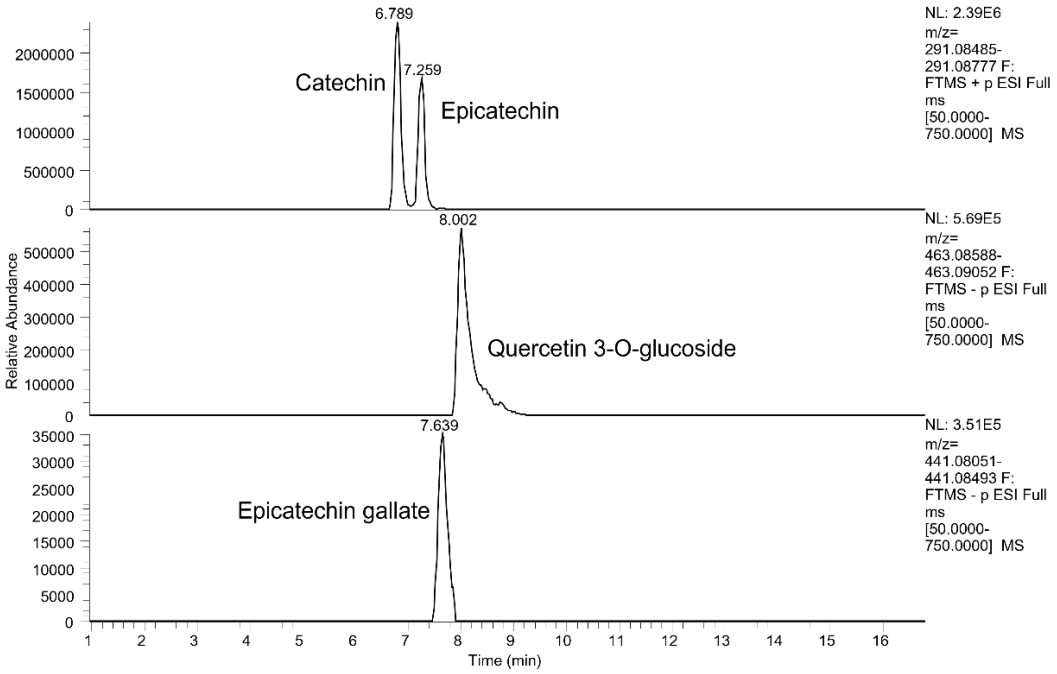


Figure 1. *Example of an extracted ion chromatogram of the flavonols and flavanol catechin, epicatechin, quercetin 3-O-glucoside and epicatechin gallate in samples of WB.*

***Table 3**. List of the main phenolic compounds identified in the wine by-products used in the study by UHPLC-Q-Orbitrap-MS.*

Name	Type	Chemical formula	Precursor ion (*m/z*)	Mass error (ppm)	Product ion (*m/z*)	Retention time (min)	Ionization mode
Catechin	Flavanol	$C_{15}H_{14}O_6$	291.08631	-2.64	139.03895	6.99	Positive
Epicatechin (EC)	Flavanol	$C_{15}H_{14}O_6$	291.08631	-2.85	123.04491	7.45	Positive
Epicatechin gallate (ECG)	Flavanol	$C_{22}H_{18}O_{10}$	441.08272	-4.67	169.01304	2.27	Negative
Epigallocatechin (EGCG)	Flavanol	$C_{15}H_{14}O_7$	305.06668	-0.19	255.92270	2.93	Negative
Kaempferol-3-O-glucoside	Flavonol	$C_{21}H_{20}O_{11}$	447.09328	-0.89	255.02924	8.42	Negative
Quercetin 3-O-glucoside	Flavonol	$C_{21}H_{20}O_{12}$	463.08820	0.19	302.03696	8.25	Negative
Quercetin-3-O-rhamnoside (quercitrin)	Flavonol	$C_{21}H_{20}O_{11}$	447.09328	-0.18	230.98517	8.40	Negative
Quercetin-3-O-rutinoside (rutin)	Flavonol	$C_{27}H_{30}O_{16}$	609.14611	-0.48	301.03474	8.20	Negative
Eriodyctiol	Flavanone	$C_{15}H_{12}O_6$	287.05611	-3.68	151.00235	3.48	Negative
Naringenin	Flavanone	$C_{15}H_{12}O_5$	271.06120	-0.61	119.04879	8.82	Negative
Chlorogenic acid	Phenolic acid	$C_{16}H_{18}O_9$	353.08781	-0.38	191.05610	7.55	Negative
Syringic acid	Phenolic acid	$C_9H_{10}O_5$	197.04555	-2.57	123.00734	7.96	Negative
Procyanidin B1	Non hidrolizable tannin	$C_{30}H_{26}O_{12}$	577.13515	-0.46	289.07154	6.64	Negative

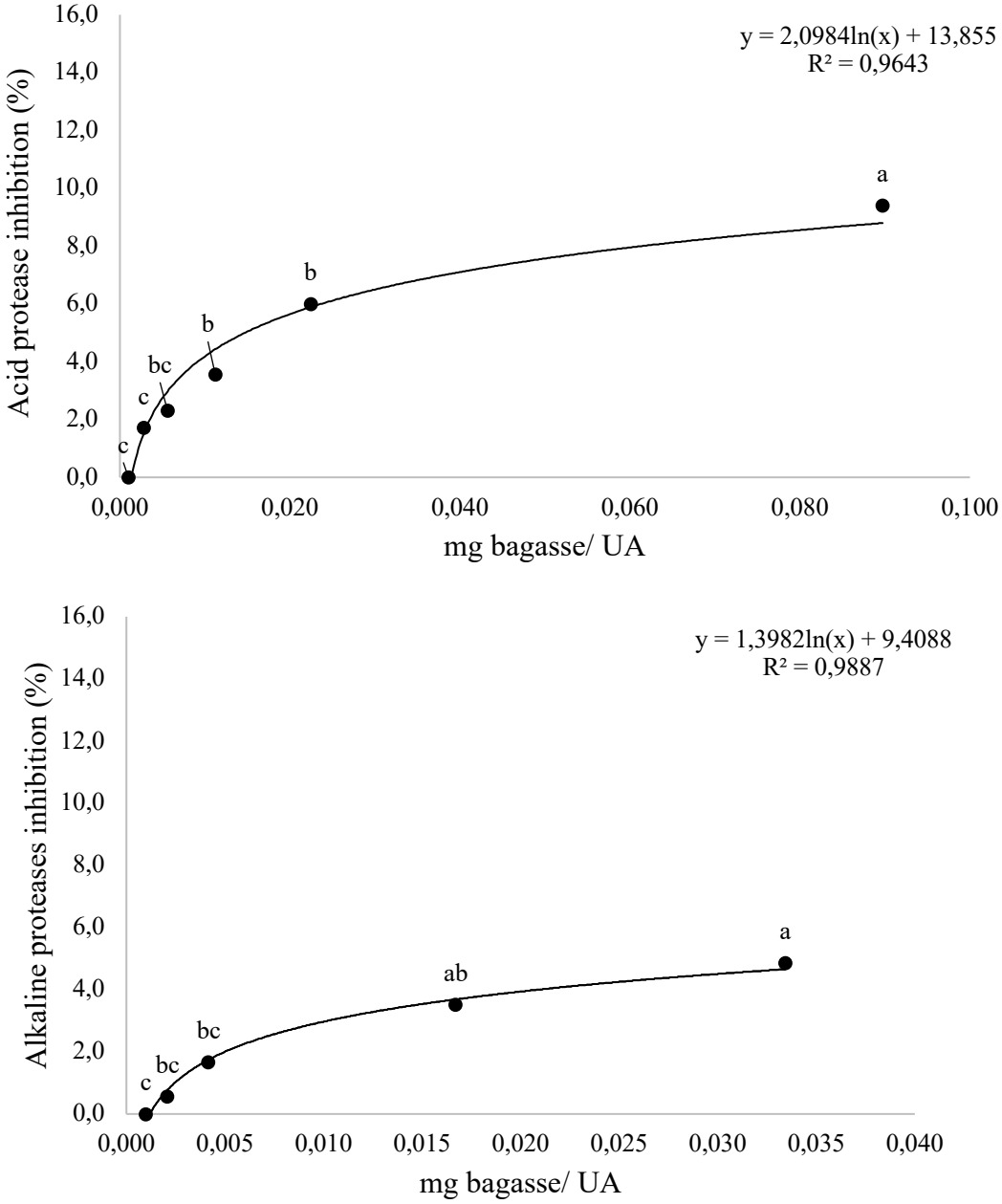


Figure 2. *Inhibition curve of acid and alkaline protease activity obtained after 1 h incubation of digestive extracts of gilthead sea bream in the presence of increasing concentrations of wine bagasse. Each point is the mean of three replicate measures. Points not sharing a common letter are statistically different with $p < 0.05$.*

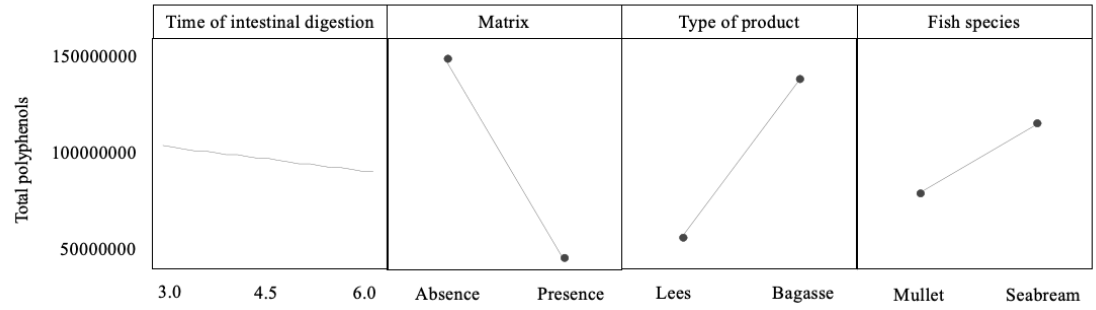
Table 4. *Significance of the evaluated factors on the amounts of total and some selected phenolic compounds released from wine by-products under conditions simulating fish digestion. Significant (p < 0.05) factors and interactions are indicated in bold letters.*

	TOTAL	Catechin	Eriodyctiol	Epicatechin	Kaempferol-3-glucoside	Quercentin-3-o-glucoside	Syringic acid
				p value			
Model	0.008	0.031	0.007	0.040	0.053	0.003	0.082
Lineal	0.003	0.017	0.003	0.023	0.032	0.001	0.053
Total digestion time (TDT)	0.438	0.751	0.002	0.664	0.144	0.112	0.790
Presence of matrix (M)	**0.001**	**0.012**	**0.011**	**0.016**	**0.021**	**0.001**	**0.037**
Type of wine by-product (WP)	**0.004**	**0.012**	0.547	**0.019**	**0.021**	**0.001**	0.099
Fish species (FS)	0.076	0.065	**0.004**	0.058	0.344	0.389	**0.037**
Interactions	**0.031**	0.073	**0.024**	0.088	0.108	**0.015**	0.142
TDT * M	0.937	0.670	0.508	0.593	0.144	0.078	0.790
TDT * WP	0.498	0.849	0.192	0.733	0.144	0.125	0.619
TDT * FS	0.389	0.827	**0.002**	0.823	0.607	0.803	0.790
M * WP	**0.005**	**0.014**	0.300	**0.021**	**0.021**	**0.001**	0.099
M * FS	0.053	0.075	0.515	0.066	0.344	0.720	**0.037**
WP * FS	0.056	0.071	0.368	0.075	0.344	0.481	0.099
R-square	95.71 %	92.28 %	96.00 %	91.32 %	90.16 %	97.06 %	88.03 %
R-square fitted	87.12 %	76.84 %	88.00 %	73.95 %	70.48 %	91.18 %	64.09 %

The magnitude and trend of the above mentioned significant effects can be clearly evaluated in Figures 3–5. The plot of the main effects presented in Figure 3 indicates that the presence of feed matrix significantly decreased the release of phenolic compounds, irrespective of the species and type of product, and that total amount of available phenolics was significantly higher in WB. Also, the effect of fish species (determined both by the source of enzymes and by the presence of an acid stage in the digestion of sea bream, that was absent in the case of mullet) was the opposite for catechin and eriodyctiol, being the release of this latter significantly influenced by the time of digestion but not in the case of catechin. These points, as well as some additional aspects like the different (and sometimes opposite) effect of matrix on the release of specific phenolics present in both WB and WL when digested by the enzymes of the two fish species can be appreciated in more detail in Figures 4-5. As shown in the mentioned figures, fish species significantly influenced the profiles of phenolics release, being the differences both quantitative and qualitative. Quantitative differences were evident since, in absence of matrix, the simulated digestion of mullet released around 50 % less compounds from WB than that of sea bream, while in contrast, the total amount released from WL was 40 % higher. Nevertheless, such

differences disappeared when any of the two by-products were included in the feed matrix. Regarding the effect on specific compounds, it was noticed that sea bream enzymes released mainly catechin, epicatechin and quercetin from WB, while in the case of mullet, besides quercetin and catechin, the main product released was eriodyctiol. In a similar way, kaempferol was practically the only product released from WL by the digestion of mullet, while in the case of sea bream a more complex profile including naringenin, epicatechin or syringic acid was observed. Also, in both species, the diversity of phenolic compounds released after inclusion of WL in the feed matrix was significantly reduced when compared to that produced by WB.

A)



B)

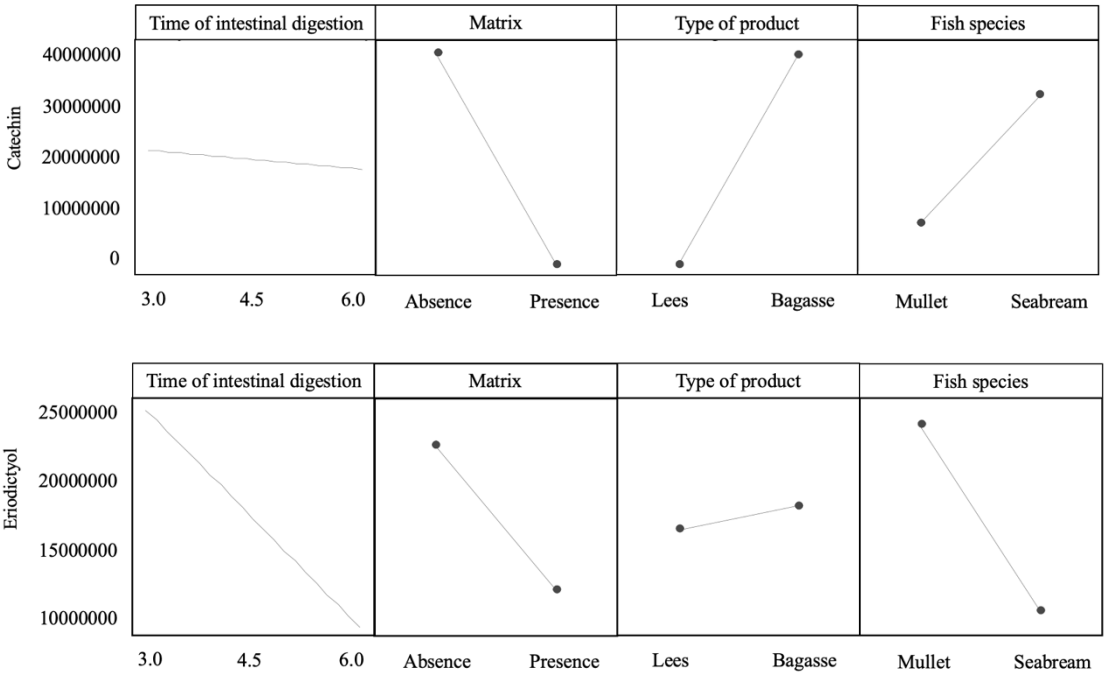


Figure 3. *Plot of the main effects evaluated on the release of A) total polyphenols and B) two specific types of phenolic compounds (catechin and eriodictyol).*

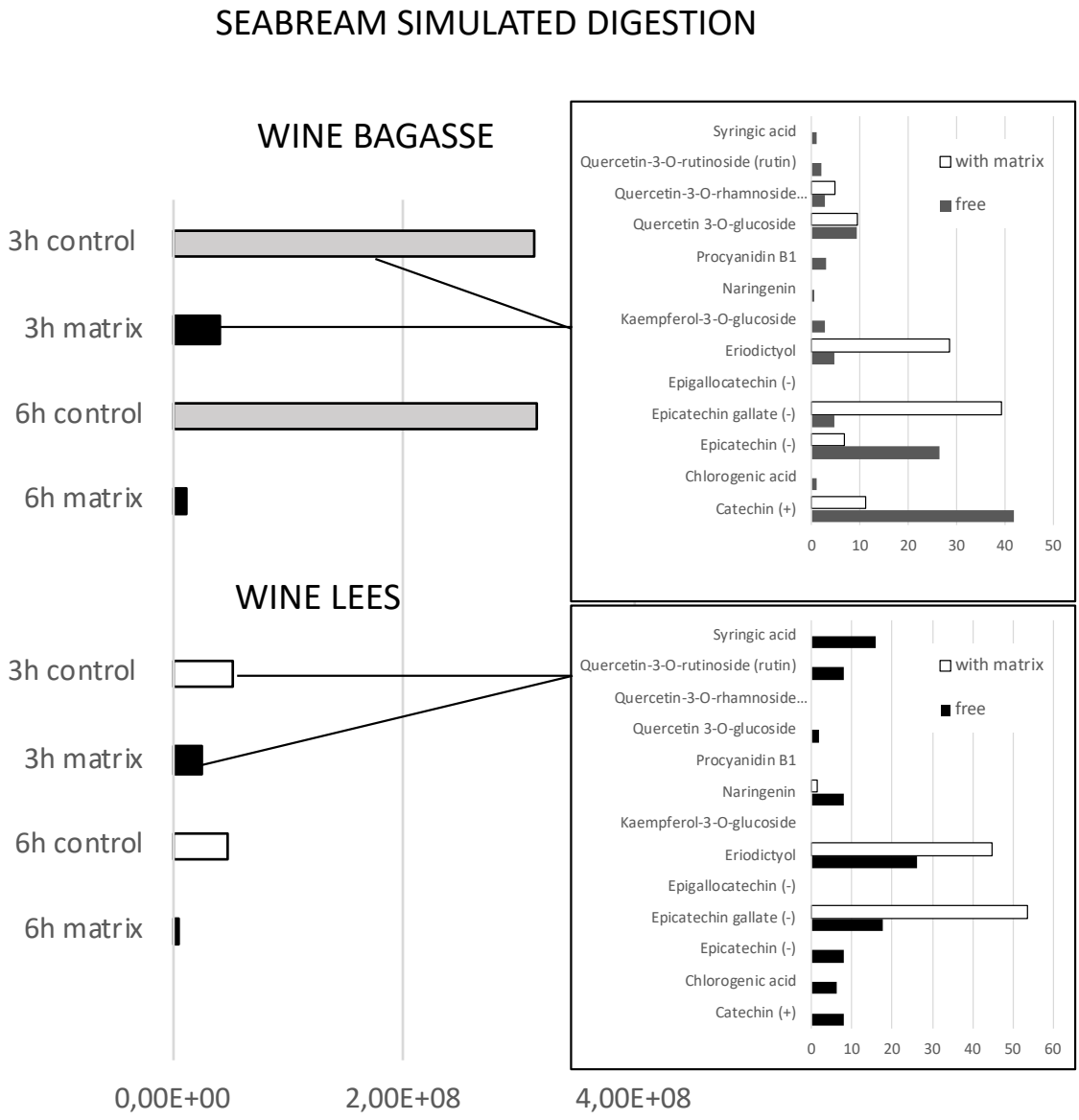


Figure 4. *Effect of feed matrix on the release of total phenolic compounds from either wine bagasse or lees, measured at two different digestion times (3 and 6 h) during simulated digestion of sea bream. Values are expressed as the sum of all peak areas detected in samples measured by UHPLC. The release of specific types of phenolics after 3 h digestion of either bagasse or lees are shown in the small graphs, being values expressed as % of the total amount of phenolics measured in the presence or absence of feed matrix.*

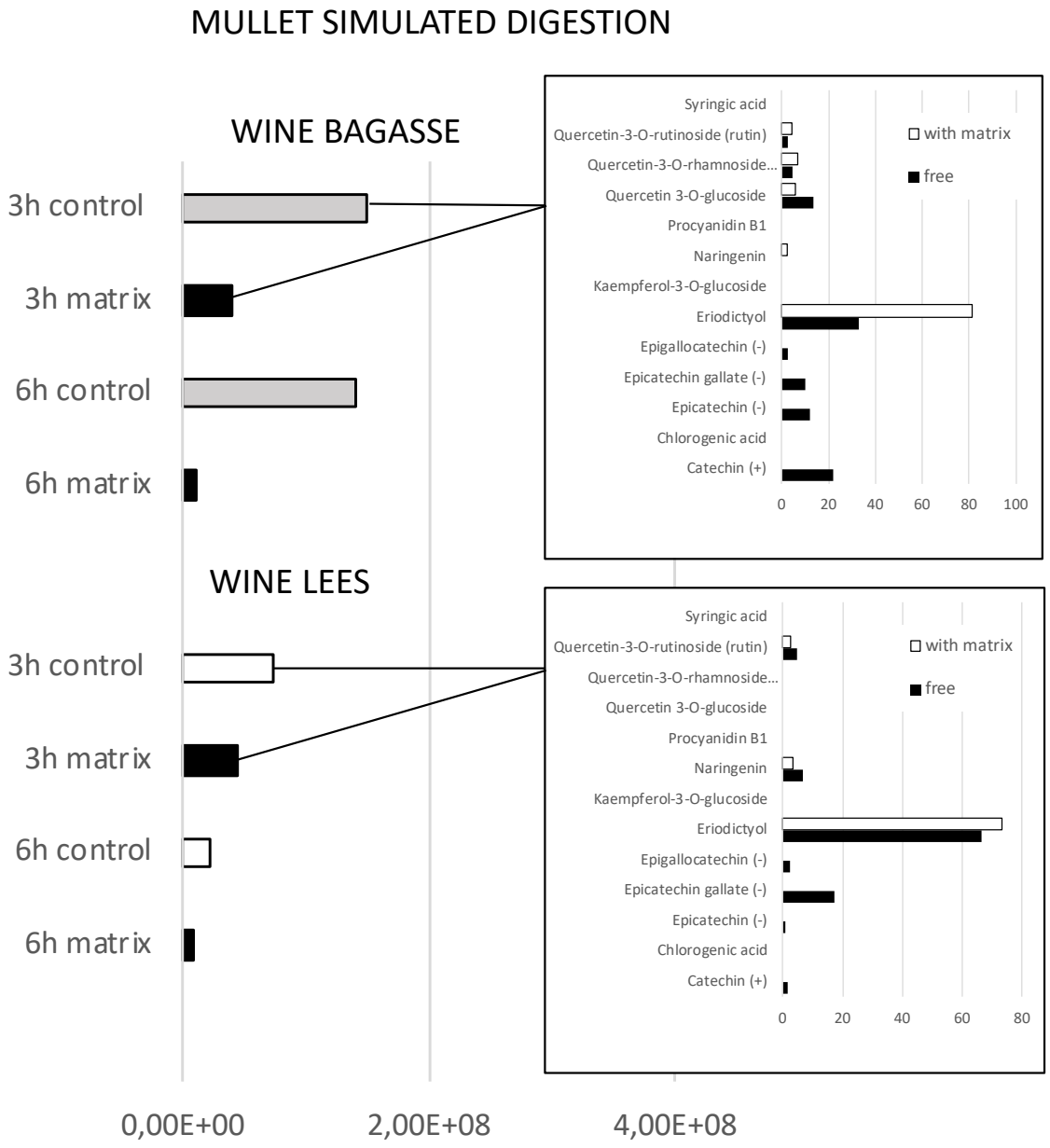


Figure 5. *Effect of feed matrix on the release of total phenolic compounds from either wine bagasse or lees, measured at two different digestion times (3 and 6 h) during simulated digestion of flathead mullet. Values are expressed as the sum of all peak areas detected in samples measured by UHPLC. The release of specific types of phenolics after 3 h digestion of either bagasse or lees are shown in the small graphs, being values expressed as % of the total amount of phenolics measured in the presence or absence of feed matrix.*

Figure 6. *Profiles of release of phenolic compounds from either wine bagasse (WB) or lees (WL) measured at two different times (3 and 6 h) during simulated digestion of A) sea bream and B) flathead mullet. Values are expressed as the sum of all peak areas detected.*

The time of digestion was not identified as a statistically significant factor in the release of phenolic compounds because some of them showed opposite patterns of release as a function of time. Nevertheless, a detailed

analysis of the peaks identified in the profiles (Figure 6) offers important information, allowing to identify three different patterns of release:

i) *Early* (when most part of the compound was measured in the digestate after 3 h of intestinal digestion). This would be the case of eriodictyol released for WB in the digestion of mullet, or from WL in the digestion of sea bream and for kaempferol and epigallocatechin after the digestion of WL by the mullet.

ii) *Sustained* (when significant amounts of the product are measured both 3 h and 6 h after the digestion). This was the case of catechins and epicatechins present in WB or of syringic acid in WL when digested by both species.

iii) *Late* (when the compound was detected only or mainly after 6 h of alkaline digestion). This was the case for quercetin present in WB for both fish species or for chlorogenic acid or naringenin present in WL when digested by sea bream.

4. DISCUSSION

The profile of phenolic compounds identified in both WB and WL was similar to that described previously by other authors (González-Manzano et al., 2004; Makris et al., 2006; Beres et al., 2017). Nevertheless, important differences between the WB and WL were evidenced during the *in vitro* study of release. The phenolic profile of WL depends on the type of crushed grapes and other factors that are present during wine production (i.e., maturation time, material of the barrel, etc.), since they are transferred to the yeast due to the adsorption capacity of their cell wall (Mena et al., 2014). WL have been pointed out as a good source of flavanols such as quercetin, quercitrin, kaempferol, and myricetin although also flavanols, namely catechin, epicatechin, and procyanidin B2, were also identified (Jara-Palacios, 2019).

An accurate estimation of the extent and relevance of potential positive effects associated to the inclusion of wine polyphenols in fish feeds needs a detailed assessment of the bioavailability of the different compounds. This is required to correctly establish dose–response relationships. Bioavailability, defined as the fraction of a nutrient or compound released from the food/feed matrix after the intestinal digestion and absorption process is much more important to assess the potential functionality of

a given compound than the simple measurement of its gross concentration. As indicated in the Introduction section, a number of different studies developed using *in vitro* models of human digestion have demonstrated that phenolic compounds may strongly interact within the digestive tract both with enzymes and the components of the food matrix, this resulting in important modifications of their potential bioavailability. Regarding the first point, results obtained in the present study evidenced a slight inactivation of sea bream proteases in the presence of the phenolic compounds present in WB. Partial inactivation of digestive enzymes in the presence of phenolic compounds has been reported for different enzymes present either in human stomach or intestine (Martínez-González et al., 2017). Several studies evidence that polyphenols may form complexes by multiple weak interactions (primarily hydrophobic) between amino acid side chains and their aromatic rings. These covalent binding of flavonoids and proteins is usually the result of the reaction between functional groups, such as amino groups of proteins and the quinones formed by oxidation of flavonoids, may prevent the enzymes from interacting with their substrates (Barrett et al., 2018). Some of these interactions have been identified for specific types of catechins and the active catalytic site of trypsin (Cui et al., 2015). In spite of this, the reduction in the activity of both stomach and intestinal proteases of sea bream evidenced in the present study was not high when compared to that produced by specific inhibitors in plant ingredients (Moyano et al., 1998). Hence, it is presumed that this negative effect on digestion associated to the intake of WB (at the levels assayed in the present study) could be easily overcome in the live fish, especially considering that the physiological impact of such partial inactivation of proteases should be modulated by other factors like the total intake of the ingredient or the extension of the feeding period. In addition, other species-specific responses like a compensation of protease inhibition by overproduction of enzymes must be also considered, although it has been reported in salmonids (Haard et al., 1996) but not in tilapia (Anderson et al., 1991).

The *in vitro* approach used in the present study also evidenced that the potential bioavailability of grape polyphenols contained either in WB or WL was significantly affected by interactions with the feed matrix, as well as by species-specific features of the digestion process (presence/absence of stomach digestion, enzyme profile). Important differences in the profile of the released polyphenols were evidenced as a result of the interaction with the components of the feed matrix. In example, some phenolic

compounds as kaempferol, quercetin, catechin and epicatechin showed a significantly reduced release in the presence of food matrix, while others like quercetin 3-glucoside were not affected in the same manner. The feed matrix can be defined as the continuous medium of either cellular origin (i.e., in plant or animal meals) or formed by complex microstructures resulting from processing (i.e., in compound feeds) in which nutrients and bioactive compounds are contained and interact (Aguilera, 2019). In this sense, it is important to evaluate the potential effects of the food matrix on the bioaccessibility of phenolic compounds and other antioxidants, since only the compounds released and/or absorbed in the intestine are potentially bioavailable and capable of exerting their beneficial effects. The relevance of such interactions has been highlighted by several authors (Zhang et al., 2014; Oliveira et al., 2018; Aguilera, 2019).

The estimated reduction in the number of phenolic compounds released under simulated digestion was very high in the case of WB (73 and 86 % for phenolics for or either mullet and sea bream) and also evident in the case of WL (40 and 53 %, respectively). As previously indicated, polyphenols have a significant affinity for proteins, this leading to the formation of insoluble complexes of a higher molecular size that can precipitate (Ozdal et al., 2013). Nevertheless, they can also interact with other macromolecules present in the feed matrix, like carbohydrates and lipids, reducing absorption of such nutrients and also of the phenolic compounds themselves (Zhang et al., 2014). In fact, it has been demonstrated that the presence of carbohydrates in the digesta determines interactions with some types of phenolic compounds through the formation of protein-tannin-carbohydrate ternary structures (De Freitas et al., 2003; Mateus et al., 2004).

There are few studies dealing with the effects of food matrix components and their interactions with specific phenolic compounds according to their typology in human nutrition. It has been reported that isoflavones are the best absorbed dietary flavonoids, flavanols, flavanones and flavonol glycosides are intermediate, whereas proanthocyanins, flavanol gallates and anthocyanins are the worst absorbed (Beres et al., 2017). However, it is clear that the absorption of specific types of dietary flavonoids may be influenced by the particular types of interactions with the components of the matrix in which they are consumed. As an example, Latruffe et al. (2014) reported that quercetin and rutin were more strongly bound to bovine serum albumin than catechin and

epicatechin. On the other hand, reductions in the amounts of lysine, cysteine and tryptophan present in soy proteins after interacting with different phenolic compounds like flavonoids, apigenin, kaempferol, quercetin and myricetin have been reported (Rawel et al., 2002).

The differences observed in the present study related to species could be explained considering some key factors characterizing the digestion process in both fish, mainly the absence of an acid stage of hydrolysis in the digestion of mullet and the differences in their digestive biochemistry. In relation to the first point, is worth mentioning that stomach of mullets is formed by a thin-walled cardiac and thick-walled pyloric portion, adapted to function as a mill for feed particles similar to the gizzards of birds, but it does not produce hydrochloric acid or pepsin (Payne, 1978). In contrast, the fully functional stomach present in sea bream is able to perform an acid stage of the digestion (Márquez et al., 2012). It has been demonstrated that the chemical structure of the phenolic compounds is closely related to their susceptibility to pH. An acid environment can positively affect the solubilization of some types of phenolic compounds like caffeic, chlorogenic, and gallic acids that are not stable to high pH, while catechin, epigallocatechin, ferulic acid, rutin, and trans-cinnamic acid resist high pH-induced degradation (Friedman & Jurgens, 2000). In addition, it is clear that the existence of quantitative and qualitative differences in the type of digestive enzymes between both species may determine a different hydrolysis of the different ingredients of the feed matrix, as well as of the WB and WL, this resulting in a different profile of the released compounds. Finally, is worth to mention that the observed great variations in the patterns of release of different types of phenolic compounds with time suggest an important effect of gut transit rates on the net bioavailability of a given phenolic compound in the live fish. While a fast release could result in a high availability for intestinal absorption, a longer time may determine a lower availability, higher fecal excretion and hence limited biological effects.

5. CONCLUSION

This is, to our knowledge, the first study in which an *in vitro* approach was applied in a similar manner as for humans and terrestrial animals, to assess to what extent the possible complexation of wine polyphenols present in wine by-products with either digestive enzymes or components of the feed matrix could limit their bioaccessibility if included in diets of two different fish species. It is clear that this kind of *in vitro* assays

represent a highly valuable tool that can be considered a preliminary step to ascertain results obtained when testing *in vivo* practical use of different types of agri-food by-products that may be used as sources of antioxidant compounds in fish nutrition. Their main advantages arise when considering the wide diversity of phenolic compounds present in any of such products (as occurs in WB and W), as well as the important variations existing between the conditions existing in the digestive tract of fish species with different feeding habits (in terms of digestive enzymes, pH variations or gut transit rates). However, *in vivo* experiments are required in order to validate the results obtained with the present *in vitro* tests since it cannot be ruled out that other factors not identified/tested in the study could affect the rate of release respect to digestion time.

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CHAPTER 5

Feed supplementation with winery by-products improves the physiological status of juvenile *Liza aurata* during a short-term feeding trial and hypoxic challenge

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ABSTRACT

The search of bioactive compounds obtained from natural sources with beneficial effects in growth and health is an increasing trend in aquaculture. Wine by-products are an excellent source of such compounds, mostly phenolics, with demonstrated antioxidant and immunostimulant activities in vertebrates. The present study evaluated the effects of dietary inclusion (100 g/kg) of two wine by-products (grape pomace and lees) on growth, immune status and metabolism of juvenile golden gray mullet (*Liza aurata*), as well as the potential protective effect of compounds present in the two by-products against induced stress produced by moderate hypoxia. Results evidenced a significant positive effect of grape pomace on feed efficiency, as well as in different indicators of metabolic and immunological status of the fish. Also, a significant negative effect of wine lees on the functional diversity of intestinal microbiota was evidenced. Fish fed on diets containing any of the two by-products evidenced significantly lower levels of cortisol when challenged by hypoxia, this pointing to a protective effect mediated by their contents in phenolic compounds and suggesting an interesting and practical application for these agricultural by-products.

Keywords: aquafeeds; winery by-products; bioactive compounds; capacity antioxidant; polyphenols

1. INTRODUCTION

A great part of aquaculture activity is currently based on the use of intensive systems which implies the existence of stressful situations for farmed animals as well as a reduction in water quality that facilitates the appearance of pathologies. On the other hand, prolonged temperature stress associated to global warming may affect the neuroendocrine and osmoregulatory systems, altering cardiorespiratory performance and aerobic scope as well as immune responses of several economically important species (Brodie et al., 2014; Gazeau et al., 2014; Paukert et al., 2016; Stévant et al., 2017; Stewart et al., 2019; Zhang et al., 2019). Taking this into account, increasing attention is being paid to the inclusion in aquafeeds of ingredients with biological activity that can benefit the health and resistance of fish against the aforementioned adverse effects. Within this context, it is of great interest to consider the great diversity of by-products generated by the agri-food industry that can be an important source of bioactive molecules with antioxidant capacity (terpenes, carotenoids, phenolic compounds, etc.). A good example of this situation is the wine production, which takes place worldwide (EU, USA, South America, China, Australia, etc.) and generates a great volume of residues accounting by 31–40 % of the total grapes harvested (Lavelli et al., 2016). These include grape pomace (skins and seeds), grape stalks and lees (Bonamente et al., 2015; Lavelli et al., 2016). Grape pomace is the waste originated when pressing whole grapes, and nine million tons of this waste are produced per year in the world, which constitutes about 20 % w/w of the total grape biomass used for wine production (Teixeira et al., 2014). In contrast, wine lees are a sludge material made of intact or partially degraded yeast cells and other insoluble particles that accumulates at the bottom of wine tanks after the alcoholic fermentation (Fia, 2016). These by-products are a cheap and exceptionally rich source of functional ingredients (Hang, 1988; Arvanitoyannis et al., 2006; Galanakis, 2012; Galanakis & Schieber, 2014; Prokopov et al., 2015). In this context, grape pomace constitutes a source of phenolic compounds like anthocyanins (e.g., malvidin, peonidin), flavan-3-ols (e.g., catechin, proanthocyanidins), flavonols (e.g., quercetin, myricetin), stilbenes, and phenolic acids (Negro et al., 2003; Makris et al., 2007), whereas wine lees contain significant amounts of polysaccharides, proteins, lipids and other organic species, as well as between 1.9 and 16.3 g of polyphenols/kg depending on the wine type and processing (Bustamante et al., 2008).

Nevertheless, in spite of their easy availability worldwide and the above-mentioned interesting chemical composition, wine by-products are not currently considered economically useful and most of them are disposed, although small amounts have traditionally been used in feeding terrestrial animals mainly as a source of fiber, carbohydrates and minerals. Recently, different studies have highlighted the positive effect of phenolics present in grape pomace, in the production of pigs, poultry or ruminants (Chedea et al., 2018; Turcu et al., 2018; Costa et al., 2022). In the case of fish, the few studies published to date suggest that grape polyphenols may also produce beneficial effects, as the prevention of liver diseases related to oxidative stress (Souza et al., 2019), reduction in the deterioration of cellular energy homeostasis (Baldissera et al., 2019), improvement in the growth and feed digestibility (Peña et al., 2020), or even changes in the composition of the intestinal microbiota (Pulgar et al., 2021).

The Mugilidae is a family of omnivorous and euryhaline marine species of great commercial interest that contribute considerably to coastal fisheries in many countries in Asia-Pacific, Africa and Europe (Saleh, 2006; Biswas et al., 2012; Whitfield et al., 2012). This family, which includes species such as *Mugil cephalus, Chelon labrosus, Liza aurata, Liza saliens,* and *Liza ramada*, has a relevant role in marine and coastal aquaculture production, being the third most produced group of species worldwide (FAO, 2022). For this reason, the Mugilidae have been recognized as a family of potential species for the diversification of aquaculture in the Mediterranean region, as well as in other regions of the world (South Korea, China, South Africa, etc.), due to its good adaptation in a wide range of temperatures and geographic locations, rapid growth, and feeding habits. In addition, due to their nutritional needs, they require a lower amount of protein in feed during the growth phase (Huntington & Hasan, 2009), giving rise to a possible sustainable aquaculture thanks to the use of alternative ingredients and the reduction of production costs. These features make the different species of mullets excellent candidates for testing the potential beneficial effects of including any kind of agri-food by-product in diets.

Considering this, the present work was intended to evaluate the potential benefits of including red grape pomace or lees as functional ingredients in aquafeeds for juveniles of *Liza aurata*. The study was conceived in two

different experiments focused on evaluating specific effects in metabolism, and also the putative protective effect against stress produced by moderate hypoxia induced as a short and acute challenge. The information obtained could be highly valuable to reinforce the orientation of aquaculture towards greater sustainability, both for the use of species with a low trophic level and for the recovery of by-products within a circular economy approach.

2. MATERIAL AND METHODS

2.1. Processing and composition of wine by-products

The two wine by-products, red grape pomace and lees, used in this study were obtained from two artisanal wineries placed in Fondón (Almería, Spain) and Chiclana de la Frontera (Cádiz, Spain), respectively. The grape pomace (GP) was dried in an oven for 72 h at 60 ºC and subsequently finely grounded until obtaining a fine powder that was used in the assays while the wine lees (WL) were employed in liquid form. A characterization of phenolic compounds and antioxidant capacity was carried out in both products using specific methods (Brand-Williams et al., 1995; Graça et al., 2005). In addition, proximal and mineral composition were determined by accredited laboratories: KUDAM laboratory (Alicante, Spain) and Central Research Services (University of Almería, Spain), respectively, using standard methodologies.

2.2. Experimental diets

The three experimental feeds consisted in one control (C) and two diets that included either GP or WL at 100 g/kg. This dose was higher than that used in a previous study performed in rainbow trout (Peña et al., 2020), but it was adopted considering the possibility of feeding mullets with a high amount of vegetable by-products as well as their comparatively lower intake rates (Quirós-Pozo et al., 2023). Feeds were prepared at the Service of Experimental Diets (CEIMAR, University of Almería, Spain) using a lab-scale extrusion machine provided with a mesh size of 3 and 4 mm, being dried and stored at 4 ºC until used. The composition of the diets is detailed in Table 1.

Table 1. *Ingredients and proximate composition (in g/ 100 g) of the experimental diets used in the study.*

Ingredients (g/100g)	C	GP	WL
Fish meal LT94	8.0	8.0	8.0
Pea protein concentrate	8.0	8.0	8.0
Soybean meal 48	15.0	15.0	15.0
Soybean protein concentrate 60	15.0	15.0	15.0
Wheat gluten	5.0	5.0	5.0
Sunflower seed meal	10.0	10.0	10.0
Wheat meal	27.5	27.5	27.5
Potato starch	5.0	5.0	5.0
Wheat bran	10.0	-	-
Red grape pomace	-	10.0	-
Red wine lees	-	-	10.0
Fish oil	4.0	4.0	4.0
Sunflower oil	2.0	2.0	2.0
Vitamin/mineral premix	1.0	1.0	1.0
Guar gum	1.5	1.5	1.5
Proximate composition (g/100 g)			
Crude Protein	36.0	35.4	36.8
Total Fat	8.4	9.0	8.8
NFE	48.0	47.1	44.3
Ash	6.2	7.4	8.9

2.3. Animals and facilities

Juveniles of golden gray mullet (*Liza aurata)* were provided by the Servicios Centrales de Investigación en Cultivos Marinos (SCI-CM, CASEM, University of Cádiz, Puerto Real, Cádiz, Spain; Facilities for Breeding, Supplying and Users of Experimental Animals; Spanish Operational Code REGA ES11028000312). Fish were kept in the SCI-CM facilities in a flow-through 5 m^3-tank provided with seawater (SW) in controlled conditions of salinity (38 ppt) and temperature (19 ºC), and under natural photoperiod (10:14 h, light:dark, LD; 36°31′45″ N, 6°11′31″ W). Maintenance and handling of the fish followed the guidelines for experimental procedures in animal research from the Ethics and Animal Welfare Committee of the University of Cádiz, according to the Spanish (RD53/2013) and European Union (2010/63/UE) legislation. The Ethical Committee from the Autonomous Andalusian Government approved the experiments under the reference number 15/09/2021/132.

2.4. Experiment 1. Short-term trial

A total of 180 juvenile fish with average body mass of 65.45 ± 0.08 g were distributed into 9 tanks (400 L; n = 20 fish per tank; initial stocking density: ~3.0 kg m^{-3}) in the experimental facilities of the SCI-CM under similar environmental conditions of salinity, temperature and photoperiod described above. Each of the three experimental feeds was evaluated in triplicate. Daily ration was fixed at 2 % b.w. being distributed in 5 daily meals using automatic feeders. The feeding-trial lasted 6 weeks. Water quality of the system was continuously monitored; temperature, dissolved oxygen, and survival data were controlled daily, whereas ammonium, nitrite, and salinity levels were checked weekly.

This experiment was oriented to assess the effect of feeding juvenile mullets during a limited time on diets including the two wine by-products on their basal metabolism, immune status and gut microbiota. In addition, although the experimental period was too short to be considered for evaluation of effects on growth performance, some zootechnical and somatic indices were also estimated.

2.4.1. Sampling

At the end of the short-feeding trial, overnight fasted fish (four fish per tank, twelve per experimental conditions) were randomly sampled and deeply anaesthetized with 2-fenoxiethanol in a lethal dose (1 mL/L SW) prior to obtain samples of skin mucus, blood and tissues. Mucus samples were taken by scraping with a cell scraper on both dorso-lateral sides of each individual. Blood was drawn from caudal vessels with heparinized syringes and centrifuged at 3,000 *g* for 5 min at 4 ºC to separate plasma, which was then snap-frozen in liquid nitrogen and stored at -80 ºC until used for biochemical analysis. After this, fish were cervically sectioned to obtain biopsies of different tissues and were snap-frozen in liquid nitrogen and stored at -80 ºC for subsequent biochemical analysis.

2.4.2. Growth performance and biometric parameters

The following growth parameters were evaluated:

Specific Growth Rate (SGR) = (100 × (ln final body weight - ln initial body weight)/days
Weight Gain (WG) = (100 × (body weigh increase)/initial body weight
Feed Efficiency (FE) = weight gain/total feed intake

Condition Factor (K) = (100 × body weight)/fork length^3
Hepatosomatic index (HSI) = (100 × liver weight)/fish weight

2.4.3. Biochemical and immunological parameters

Prior to analyze biochemical parameters in liver, samples of frozen tissue were homogenized by ultrasonic disruption in 7.5 volumes ice-cold 0.6 N perchloric acid, neutralized using 1 M KCO_3, and centrifuged (30 min, 3,220 *g* and 4 ºC); the supernatants were then isolated to determine tissue metabolites. Glucose, lactate, triglycerides and total cholesterol in plasma and/or liver were analyzed using commercial kits (Refs. 1001200, 1001330, 1001311 and 41021, respectively; Spinreact, St. Esteve d'en Bas, Girona, Spain), adapted to 96-well microplates. Plasma total protein concentration was determined with a BCA Protein Assay Kit (Ref. 23225; Thermo Fisher Scientific Pierce, Waltham, MA, USA) using BSA as the standard. Glycogen concentration was quantified in liver homogenates using the method described by Decker & Keppler (1974), while glucose obtained after glycogen breakdown with amyloglucosidase (Ref. A7420; Sigma-Aldrich, St. Louis, MO, USA) was determined using the same commercial kit described above. Plasma cortisol levels were measured with the commercial Cortisol Enzyme Immunoassay Kit (Ref. K003-H1W; Arbor Assays) according to the manufacturer's indications. All assays were performed using a PowerWaveTM 340 microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) using the KCjuniorTM data analysis software (Bio-Tek Instruments, Winooski, VT, USA) for Microsoft®.

The potential variations in the immunological status were assessed by measuring lysozyme and alkaline phosphatase activities in skin mucus. Lysozyme was measured using a commercial kit (Ref. E22013; Thermo Fisher Scientific, Waltham, Massachusetts, USA), adapted to 96-well microplates. One unit of activity was defined as one Relative fluorescence units (RFU). Alkaline phosphatase activity was determined using 4- Methylumbelliferyl phosphate disodium salt (Ref. M8168; Sigma-Aldrich, St. Louis, MO, USA) as substrate following the method described by Fernley & Walker (1965). One unit of activity was defined as 10^3 RFU.

2.4.4. Functional diversity of intestinal microbiota

Functional biodiversity was determined by analyzing the physiological profile at the community level using Biolog EcoPlate ™ microplates (Biolog, USA) according to Feigl et al. (2017) with some modifications. Samples from the distal intestine (the section between the distal end of the midgut and the anus) were taken from two individuals per tank under sterile conditions (6 fish per experimental feed). Intestinal samples were suspended and homogenized in sterile saline solution and diluted up to 10^{-3} maintaining sterile conditions. From this dilution, fresh samples from each tank were homogenized to obtain a pool per tank, and then 150 µL were pipetted into each well of the Biolog EcoPlate™ microplate and incubated at 30 °C for 96 h. After incubation, the optical density (OD) at 590 nm was determined using Gen5 software 5 by Cytation (Biotek, USA). The ODi values and number of substrates was used to calculate: i) functional activity intensity as Average Well Color Development (AWCD=ΣODi/N); ii) functional richness (R= sum of the number of cells where ODi>0.15); iii) functional biodiversity as Shannon index (H'=- Σpi In(pi), where pi=ODi/ΣODi), and Shannon evenness (E=H'/lnR). The results were also expressed as substrate average well color development (SAWCD) for each of substrate categories (Sala et al., 2010).

2.5. Experiment 2. Challenge trial

This experiment was aimed to test whether supplementation in the diet with the phenolic compounds provided by either GP or WL may determine protection against oxidative stress generated by adverse situations that may take place in aquaculture practice, as it may be oxygen shortage (hypoxia). The experiment was developed as a continuation of Experiment 1, with that fish that were previously fed with the experimental diets for 6 weeks. These fish were maintained in their tanks and allowed to recover from the stress of manipulation for one week under the same feeding regimen and environmental conditions. During this time, each group received the same diet established during the initial phase of the study prior to be challenged by a temporary mid-hypoxia.

2.5.1. Experimental setup of hypoxia conditioning

All the individuals from 2 of the 3 tanks of each experimental diet (C, GP, or WL) were challenged to a non-lethal decrease in oxygen (mid-hypoxia) maintained for a time of 3.5 h at levels close to the limiting oxygen saturation (2.8 mg O_2/L, Figure 1) taking into account water temperature and salinity according to Remen et al. (2016) and Martos-Sitcha et al. (2017); this point was achieved to assure that aerobic metabolism was not replaced by anaerobic processes. The remaining tank of the triplicates served as a control (normoxia) without any manipulation in oxygen level. Survival, metabolic, oxidative and stress status were evaluated after their recovery to oxygen levels above to 85 % O_2 saturation (normoxia) through sequential samplings after 3.5 h of mid-hypoxia, as well as during the following 18 h of recovery (2 and 18 h post-hypoxia). At the end of each experimental time, four or six fish per tank (6-8 animals per experimental conditions) were randomly sampled as described in Section 2.4.1.

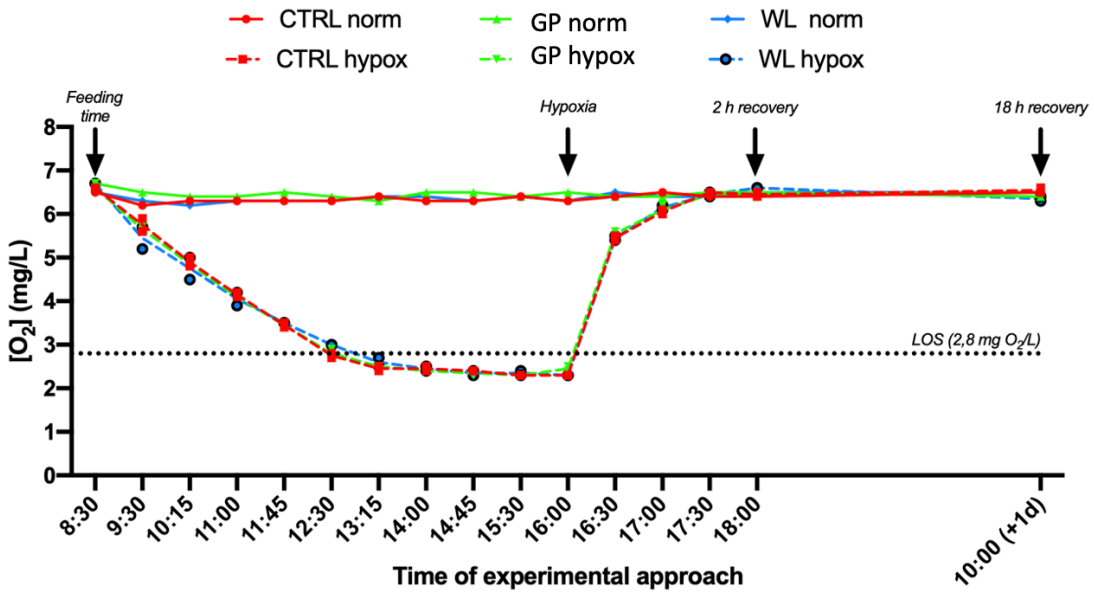

Figure 1. *Graphical representation of the experimental setup of hypoxia conditioning.*

2.5.2. Biochemical parameters

Parameters evaluated were the same described in Section 2.4.2. In addition, the hematocrit (Hc) was measured after centrifugation of blood in heparinized capillary tubes at 13,000 *g* for 5 min. Hemoglobin (Hb) in plasma was measured using commercial kits (Ref. 1001230; Spinreact, St. Esteve d'en Bas, Girona, Spain) adapted to 96-well microplates.

2.5.3. Oxidative status

Livers were homogenized (1:10, w/v) in 100 mM phosphate-buffered saline (pH 7.4) at 4 ºC using a mini handheld homogenizer (Ref. MT-13K; Hangzhou Miu Instruments Co., Ltd., Hangzhou, China) for 1 min. Homogenates were centrifuged (12,000 *g* for 15 min at 4 ºC) and supernatants were used to determine different enzyme activities: superoxide dismutase (SOD), catalase (CAT) and lipid peroxidation (TBARS). Superoxide dismutase was measured using the commercial kit (Ref. CS0009; Sigma-Aldrich, St. Louis, MO, USA). Catalase was measured using a commercial kit (Ref. EIACATC; Thermo Fisher Scientific, Waltham, MA, USA). Glutathione peroxidase was measured using a commercial kit (Ref. 703102; Cayman Chemical, Ann Arbor, MI, USA). Lipid peroxidation was assessed by measuring total thiobarbituric acid reactive substances (TBARS) using the method of Buege & Aust (1978).

2.6. Statistical analysis

The normality of the data was performed using the Shapiro–Wilk test, and homoscedasticity analysis was conducted using the Brown–Forsythe test. Statistical analysis of the data was carried out by one-way (Experiment 1) or two-way (Experiment 2) ANOVA, followed by Fisher's LSD test where appropriate. The significance level was established at $p < 0.05$. When required, data expressed in percentage were previously arc-sin transformed. All the analyses were performed using the software Statgraphics Centurion (Statgraphics Corp. CA. EE.UU.) for Windows.

3. RESULTS

3.1. Experiment 1. Short-term trial

3.1.1. Growth performance and biometric parameters

Growth performance, feed efficiency and biometric parameters of the experimental groups are resumed in Table 2. Significantly higher values of final body mass, SGR, FE and PER were found in GP group when compared to the C, while fish receiving the WL diet showed intermediate values. No significant differences were observed for values of K and HSI between experimental groups.

***Table 2**. Growth, feed efficiency, as well as biometric and somatic parameters measured in fish fed on the experimental diets (C: control; GP: 10 % grape pomace; WL: 10 % wine lees). Values presented as mean ± SD. Values not sharing a common letter are significantly different with p < 0.05. SGR: specific growth rate; FE: feed efficiency; PER: protein efficiency ratio; K: condition factor; HSI: hepatosomatic index.*

Parameter	C	GP	WL	p-value
Initial body mass (g/fish)	65.41 ± 0.05	65.45 ± 0.06	65.51 ± 0.12	0.402
Final body mass (g/fish)	83.11 ± 0.50^{a}	87.73 ± 1.16^{b}	85.66 ± 1.41^{ab}	0.006
SGR (% / day)	0.83 ± 0.02^{a}	1.01 ± 0.05^{b}	0.92 ± 0.06^{ab}	0.009
FE	0.55 ± 0.02^{a}	0.73 ± 0.04^{c}	0.63 ± 0.03^{b}	0.001
PER	1.62 ± 0.05^{a}	2.13 ± 0.12^{c}	1.86 ± 0.11^{b}	0.002
K	1.30 ± 0.12	1.29 ± 0.10	1.25 ± 0.12	0.586
HSI (%)	0.84 ± 0.21	0.89 ± 0.24	0.79 ± 0.14	0.490

3.1.2. Biochemical and immunological parameters

Plasma and liver metabolites measured in fish after being fed for 6 weeks with experimental diets are showed in Table 3. Significantly lower plasma lactate levels were observed in the GP and WL groups. However, no significant differences were observed in the rest of circulant parameters assessed. Also, significantly lower values of liver glycogen were observed in fish receiving the GP diet when compared to those observed in the WL group. Regarding the immune status, lysozyme activity in mucus enhanced significantly in GP group respect to the other experimental groups.

***Table 3**. Biochemical and immunological parameters measured in plasma, liver and mucus of fish fed on the different experimental diets (C: control; GP: 10 % grape pomace; WL: 10 % wine lees). Values presented as mean ± SD. Values not sharing a common letter differ significantly with p < 0.05.*

Plasma	**C**	**GP**	**WL**	**p-value**
Glucose (mM)	3.49 ± 0.54	3.19 ± 0.51	3.27 ± 0.69	0.437
Lactate (mM)	3.33 ± 0.76^{a}	2.38 ± 0.53^{b}	2.60 ± 0.42^{b}	0.001
Triglycerides (mM)	3.36 ± 1.05	2.90 ± 1.04	2.85 ± 1.04	0.453
Total cholesterol (mM)	8.28 ± 1.20	8.95 ± 1.61	8.48 ± 1.48	0.503
Total proteins (mg/mL)	38.73 ± 4.12	37.40 ± 4.88	38.95 ± 6.31	0.733
Cortisol (ng/mL)	3.24 ± 1.91	2.73 ± 0.54	4.41 ± 1.67	0.087
Liver				
Glucose (mM/g w.w.)	11.87 ± 2.79	11.05 ± 3.24	11.35 ± 2.53	0.777
Glycogen (mM/g w.w.)	22.20 ± 7.19^{ab}	19.51 ± 7.88^{b}	27.66 ± 7.58^{a}	0.038
Lactate (mM/g w.w.)	43.36 ± 20.34	50.34 ± 15.92	44.49 ± 14.30	0.566
Triglycerides (mM/g w.w.)	46.01 ± 16.45	37.54 ± 14.92	43.34 ± 8.53	0.316
Mucus				
Alkaline phosphatase (U/mg protein)	11.17 ± 2.99	12.27 ± 2.73	11.03 ± 2.82	0.526
Lysozyme (U/mg protein)	11.79 ± 2.51^{b}	30.08 ± 22.05^{a}	13.45 ± 7.29^{b}	0.037

3.1.3. Functional profile of intestinal microbiota

The different indicators of functional diversity in the aerobic bacterial communities present in the gut of *L. aurata* are resumed in Figure 2. As a general trend, no significant differences were found between fish receiving C and GP diets, while fish fed WL diet decreased significantly in average well color development (AWCD), functional richness (R) and shannon index (H') values. In addition, this group showed significantly higher shannon evenness (E). This suggesting a negative effect of the consumption of lees on gut microbial populations. The relative abundance of metabolic activities grouped according to the type of substrates is shown in Figure 3. The profiles obtained for fish fed C or GP diets presented a predominance of bacterial groups using carbohydrates (33-45 %) and polymers (14-47 %) as major metabolic substrates, being followed by those using amino acids and carboxylic acids (both with values of 12-17 %), while the use of amines and phenolic compounds was scarcely represented in both groups (about 1 %). In contrast, samples from fish fed WL diet evidenced a completely different profile,

characterized by a major use of polymers (47 % of all the evaluated substrates) and a significantly reduced use of amino acids as a substrate with respect to C and GP groups.

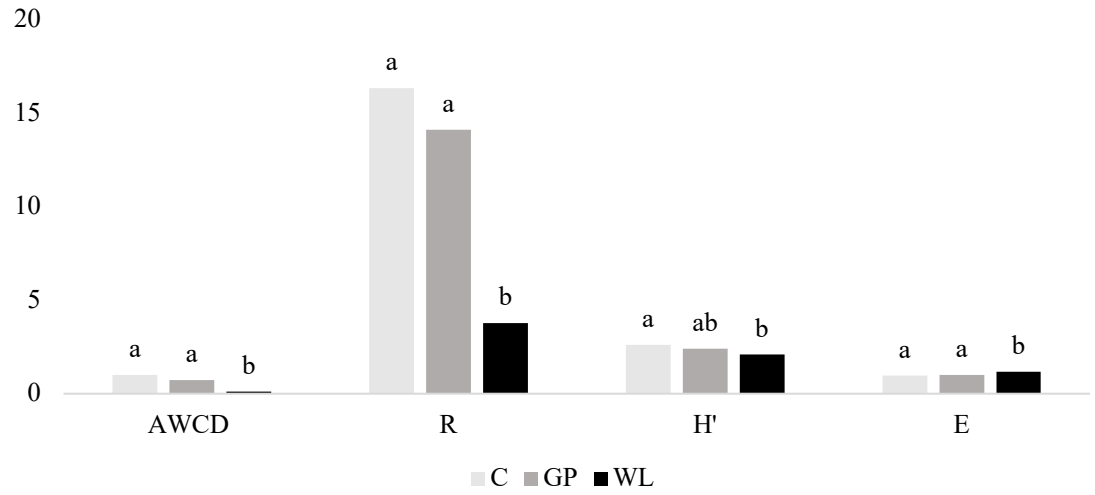

***Figure 2**. Variations in functional biodiversity of gut microbiota in fish fed on the different experimental diets (C: control; GP: 10 % grape pomace; WL: 10 % wine lees). Values not sharing a common letter differ significantly with p < 0.05. R: number of substrates oxidized (substrate richness). AWCD: Average color development is an index of the total bioactivity; H´: Shannon index (H) is the functional biodiversity index; E: Shannon evenness.*

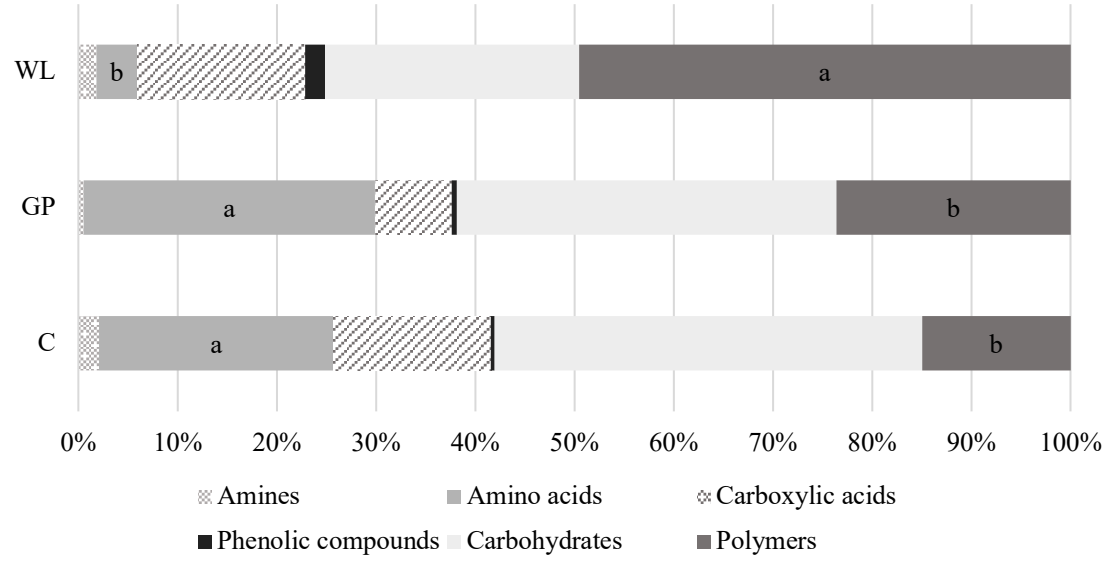

***Figure 3**. Preferred use of the different types of metabolic substrates by the aerobic intestinal microbiota of fish fed on the experimental diets, expressed as relative abundance (SAWCD). Values presented as mean ± SD. Values not sharing a common letter differ significantly with p < 0.05.*

3.2. Experiment 2. Challenge trial

Values of biochemical parameters in blood, plasma and liver of fish maintained in normoxia were used as a reference to compare with those from fish subjected to hypoxia, and P values obtained after their evaluation on the effects produced by time post-challenge and diet are shown in Table 4. In the case of plasma, cortisol and cholesterol were the only parameters showing significant variations related to the sampling moment, while lactate was the only parameter significantly affected by the type of feed. At hepatic level, significant variations with time were observed in glucose and glycogen, while the amount of triglycerides was influenced by the feed.

Table 4*. P values obtained after evaluation of the effects of time and diet on the parameters measured in blood, plasma and liver of fish maintained in normoxia using two-way ANOVA. Not significant; n.s.*

	Plasma			***Liver***		
Parameter	**Time**	**Diet**	**Interaction**	**Time**	**Diet**	**Interaction**
Hematocrit (%)	0.040	n.s	n.s			
Hemoglobin (g/dL)	n.s	n.s	n.s			
Glucose (mM)	n.s	n.s	n.s	<0.001	n.s	n.s
Lactate (mM)	n.s	<0.001	n.s	n.s	n.s	n.s
Cortisol (ng/mL)	<0.001	n.s	n.s			
Triglycerides (mM)	n.s	n.s	n.s	n.s	<0.001	n.s
Total cholesterol (mM)	0.001	n.s	n.s			
Total proteins (g/dL)	n.s	n.s	n.s			
Glycogen (mM/g w.w.)				<0.001	n.s	n.s
U CAT/mg S.P.				n.s	n.s	n.s
U SOD/ mg S.P.				<0.001	n.s	n.s
U GPx/mg S.P.				n.s	n.s	n.s
U (nmol) MDA /mg S.P.				n.s	n.s	n.s

The effect of hypoxia and further recovery on the same parameters measured in blood and plasma is detailed in Table 5. This was evaluated in two forms; a) comparing values measured along time course between fish challenged with hypoxia or maintained in normoxia for a given diet and b) comparing values measured only in hypoxia challenged fish at different moments (prior, during and after the challenge) within a given diet and between diet groups. The following main results observed when comparing fish challenged with hypoxia to those in normoxia were observed: i) significant lower values of cortisol in C and GP groups (but

not in the WL group), ii) significant higher values of cholesterol in C and WL groups and iii) lower levels of total proteins in all groups.

The evaluation of time modifications during the challenge showed a significant increase in plasma glucose in all groups during hypoxia, maintaining higher values during a longer time in C and WL groups than in GP group, prior all of them reached normal values after 18 h recovery (Figure 4). Also, cortisol enhanced significantly in all groups while TG decreased throughout the different stages of the experiment in all the experimental groups. In addition, challenged fish presented a similar pattern for cholesterol irrespective of the diet characterized by a significant decrease in values associated to the hypoxia, followed by an increase of values during recovery. On the other hand, while no significant variations were observed in lactate in the C group during all the experiment, although both GP and WL groups presented significantly lower initial levels of this parameter that increased during hypoxia and decreased during recovery.

Values of hepatic metabolic parameters (Table 6) evidenced a generalized increase in glucose and glycogen in the three experimental groups after exposure to hypoxia and recovery, although these variations were less pronounced in the C group. In contrast, TG significantly decreased in C and GP groups, observed in lactate in those fish fed the GP and WL diets during the recovery stage.

Changes in the oxidative status of the experimental fish are detailed in Table 7. No variations in CAT were measured in relation to normoxia, time course or diet type. On the other hand, values of SOD presented significant time variations in fish maintained in normoxia, as well as in challenged fish in GP and WL groups. The only significant effect associated to diet was the lower value measured 18 h after recovery in fish of GP group when compared to the C. Finally, MDA values did not present variations associated to the evaluated factors (hypoxia, time or diet) with the exception of the significant time variations measured in fish fed on WL diet.

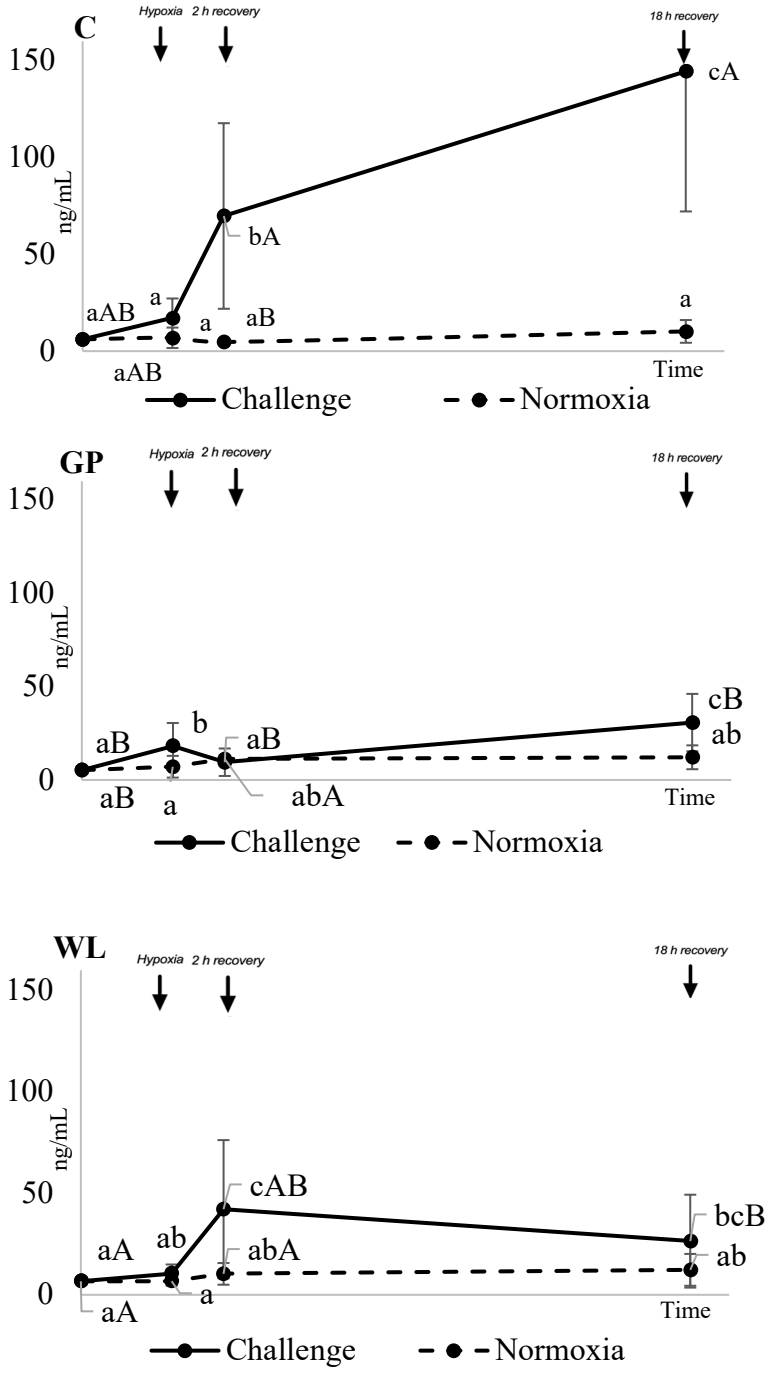

Figure 4. *Effect of mid-hypoxia and recovery time on plasma cortisol levels measured in juveniles of* Liza aurata *fed on the experimental diets (C: control; GP: 10 % grape pomace; WL: 10 % wine lees). Comparisons of values between normoxia and challenge within each diet are noted with an asterisk; comparisons among different sampling points within the same diet are noted with small letter; comparisons of the same sampling points among different diets are noted with capital letter. Values presented as mean ± SD. Values not sharing the same superscript differ significantly with* $p < 0.05$.

***Table 5**. Time course of biochemical parameters measured in blood (hematocrit and hemoglobin) and plasma (glucose, lactate, triglycerides, total cholesterol and protein) of fish receiving the different experimental diets (C: control; GP: 10 % grape pomace; WL: 10 % wine lees) after short time hypoxia challenge. Comparisons of values between normoxia and challenge within each diet are noted with an asterisk; comparisons among different sampling points within the same diet are noted with small letter; comparisons of the same sampling points among different diets are noted with capital letter. Values presented as mean ± SD. Values not sharing the same superscript differ significantly with p < 0.05.*

	C		GP		WL	
Hematocrit (%)	**Normoxia**	**Challenge**	**Normoxia**	**Challenge**	**Normoxia**	**Challenge**
0 h						
3.5 h Hypoxia	29 ± 5	34 ± 3^{B*}	33 ± 1^{a}	40 ± 3^{aA*}	35 ± 4^{a}	34 ± 4^{B}
2 h Recovery	33 ± 4	31 ± 4^{B}	28 ± 4^{b}	31 ± 6^{bB}	31 ± 2^{b}	36 ± 4^{A*}
18 h Recovery	34 ± 5	34 ± 1	33 ± 2^{a}	32 ± 3^{b}	35 ± 4^{ab}	33 ± 4
Hemoglobin (g/dL)						
0 h						
3.5 h Hypoxia	1.84 ± 0.39	1.97 ± 0.20	1.73 ± 0.26	2.13 ± 0.61	1.91 ± 0.20	1.94 ± 0.58
2 h Recovery	1.80 ± 0.22	1.85 ± 0.18^{AB}	1.81 ± 0.33	1.70 ± 0.35^{B}	1.81 ± 0.35	2.00 ± 0.24^{A}
18 h Recovery	1.71 ± 0.54	2.02 ± 0.29	1.88 ± 0.14	1.91 ± 0.24	2.06 ± 0.40	1.86 ± 0.24
Glucose (mM)						
0 h	3.49 ± 0.54^{a}		3.19 ± 0.52^{a}		3.27 ± 0.69^{a}	
3.5 h Hypoxia	3.53 ± 0.32	4.39 ± 0.86^{bc}	3.24 ± 0.17	5.49 ± 1.69^{c*}	3.38 ± 0.27	5.61 ± 0.98^{b*}
2 h Recovery	3.58 ± 0.09	4.82 ± 1.30^{cAB}	3.31 ± 0.13	4.24 ± 0.86^{bB*}	3.46 ± 0.28	5.33 ± 0.52^{bA*}
18 h Recovery	3.53 ± 0.13	3.73 ± 0.47^{ab}	3.34 ± 0.17	3.81 ± 0.34^{ab*}	3.29 ± 0.08	3.74 ± 0.53^{a}
Lactate (mM)						
0 h	3.33 ± 0.76^{A}		2.38 ± 0.53^{aB}		2.60 ± 0.42^{aB}	
3,5 h Hypoxia	3.18 ± 0.59^{A}	3.26 ± 0.61	2.20 ± 0.67^{B}	3.84 ± 1.25^{b*}	2.19 ± 0.76^{B}	3.91 ± 1.12^{b*}
2 h Recovery	2.97 ± 0.26^{A}	3.25 ± 0.47	2.23 ± 0.52^{B}	2.92 ± 0.52^{a*}	2.13 ± 0.44^{B}	3.14 ± 0.69^{a*}
18 h Recovery	3.15 ± 0.42^{A}	3.04 ± 0.52^{A}	2.24 ± 0.28^{B}	2.47 ± 0.29^{aB}	2.15 ± 0.40^{B}	2.69 ± 0.45^{aAB*}
Triglycerides (mM)						
0 h	3.36 ± 1.05^{a}		2.91 ± 1.05^{a}		2.85 ± 1.04^{a}	
3.5 h Hypoxia	3.31 ± 0.67	2.81 ± 0.80^{aA}	2.55 ± 0.87	2.03 ± 0.49^{bB}	2.88 ± 0.74	2.53 ± 0.77^{abAB}
2 h Recovery	3.22 ± 0.16	2.75 ± 0.60^{a}	3.00 ± 0.14	2.19 ± 0.98^{ab}	2.91 ± 0.50	3.12 ± 1.28^{a}
18 h Recovery	3.12 ± 0.30	1.81 ± 0.26^{b*}	3.11 ± 0.40	1.73 ± 0.38^{b*}	2.95 ± 0.32	1.82 ± 0.67^{b*}
Total cholesterol (mM)						
0 h	8.28 ± 1.20^{ab}		8.95 ± 1.60^{a}		8.48 ± 1.48^{ab}	
3.5 h Hypoxia	7.31 ± 1.43^{b}	7.40 ± 1.30^{b}	7.82 ± 1.46^{b}	7.38 ± 1.14^{b}	7.31 ± 1.36^{b}	7.54 ± 1.08^{b}
2 h Recovery	7.16 ± 0.74^{b}	9.15 ± 1.53^{a*}	7.63 ± 0.88^{b}	8.05 ± 1.36^{ab}	7.36 ± 1.00^{b}	8.96 ± 0.63^{a*}
18 h Recovery	7.14 ± 0.41^{b}	8.63 ± 1.44^{ab*}	7.68 ± 0.51^{b}	8.30 ± 1.46^{ab}	7.42 ± 0.38^{b}	9.21 ± 0.43^{a*}
Total proteins (g/dL)						
0 h	38.73 ± 4.12		37.40 ± 4.88		38.95 ± 6.31	
3.5 h Hypoxia	38.47 ± 1.38	37.01 ± 2.97	37.66 ± 7.85	38.15 ± 4.88	37.95 ± 3.57	39.18 ± 4.84
2 h Recovery	39.08 ± 0.91	36.74 ± 0.63*	38.62 ± 1.74	34.34 ± 3.17*	37.90 ± 0.65	36.18 ± 3.75
18 h Recovery	39.17 ± 1.51	35.89 ± 2.97^{B*}	38.18 ± 0.63	37.96 ± 1.13^{A}	38.55 ± 1.32	35.84 ± 1.31^{B*}

Table 6. *Time course of biochemical parameters measured in liver of fish receiving the different experimental diets (C: control; GP: 10 % grape pomace; WL: 10 % wine lees) after short time hypoxia challenge. Comparisons of values between normoxia and challenge within each diet are noted with an asterisk; comparisons among different sampling points within the same diet are noted with small letter; comparisons of the same sampling points among different diets are noted with capital letter. Values presented as mean ± SD. Values not sharing the same superscript differ significantly with p < 0.05.*

	C		GP		WL	
Glucose (mM/g w.w.)	**Normoxia**	**Challenge**	**Normoxia**	**Challenge**	**Normoxia**	**Challenge**
0 h	11.87 ± 2.79^{a}		11.05 ± 3.24^{a}		11.35 ± 2.53^{a}	
3.5 h Hypoxia	11.50 ± 1.47	13.13 ± 0.98^{ab*}	12.53 ± 2.16^{ab}	13.57 ± 1.99^{ab}	13.50 ± 1.96^{a}	13.11 ± 2.15^{ab}
2 h Recovery	12.35 ± 2.18	15.23 ± 3.02^{bc}	15.19 ± 1.26^{b}	14.27 ± 2.50^{b}	13.90 ± 2.76^{a}	14.93 ± 1.24^{bc}
18 h Recovery	13.44 ± 2.34	16.29 ± 3.20^{c*}	13.84 ± 1.68^{ab}	17.48 ± 2.85^{c*}	18.62 ± 2.62^{b}	16.63 ± 3.21^{c}
Glycogen (mM/g w.w.)						
0 h	22.20 ± 7.19^{aAB}		19.51 ± 7.88^{aB}		27.66 ± 7.58^{aA}	
3.5 h Hypoxia	41.12 ± 14.15^{b}	40.90 ± 4.14^{b}	42.84 ± 9.15^{b}	42.08 ± 9.99^{b}	38.54 ± 5.37^{b}	42.86 ± 10.31^{b}
2 h Recovery	35.64 ± 11.90^{b}	41.68 ± 8.62^{b}	45.28 ± 7.65^{b}	40.16 ± 15.59^{b}	42.13 ± 4.22^{b}	46.81 ± 8.39^{b}
18 h Recovery	32.61 ± 6.89^{ab}	40.29 ± 6.32^{b}	45.08 ± 7.79^{b}	37.47 ± 5.89^{b}	37.55 ± 5.45^{b}	39.09 ± 13.15^{b}
Lactate (mM/g w.w.)						
0 h	43.36 ± 20.34		50.34 ± 15.92^{a}		44.49 ± 14.30^{a}	
3.5 h Hypoxia	57.72 ± 33.85	41.30 ± 13.20	43.75 ± 18.94	48.01 ± 19.92^{ab}	40.33 ± 17.02	43.38 ± 12.56^{ab}
2 h Recovery	39.75 ± 17.03	43.59 ± 17.50^{A}	33.61 ± 9.39	20.57 ± 6.58^{cB*}	47.24 ± 16.37	32.34 ± 13.52^{bAB}
18 h Recovery	26.64 ± 9.26	31.94 ± 7.53	38.94 ± 11.19	35.75 ± 12.42^{b}	47.64 ± 8.29	35.69 ± 10.37^{ab}
Triglycerides (mM/g w.w.)						
0 h	46.01 ± 16.45^{a}		37.54 ± 14.92^{a}		43.34 ± 8.53^{a}	
3.5 h Hypoxia	52.45 ± 18.69^{A}	34.57 ± 10.62^{b*}	30.39 ± 9.97^{B}	32.71 ± 12.73^{ab}	35.57 ± 15.46^{AB}	28.94 ± 16.48^{b}
2 h Recovery	37.64 ± 10.96	34.25 ± 3.90^{bB}	25.06 ± 10.10	21.54 ± 6.17^{bC}	31.16 ± 8.64	40.18 ± 4.39^{aA*}
18 h Recovery	40.87 ± 9.38	34.29 ± 9.66^{b}	38.49 ± 14.11	28.00 ± 11.99^{ab}	33.65 ± 2.55	35.30 ± 6.47^{ab}

Table 7. *Time course of oxidative status measured in liver of fish receiving the different experimental diets (C: control; GP: 10 % grape pomace; WL: 10 % wine lees) after short time hypoxia challenge. Comparisons of values between different sampling points within the same diet are noted with small letter; comparisons between different dietary groups at the same sampling points are noted with capital letter. Values presented as mean ± SD. Values not sharing the same superscript differ significantly with p < 0.05.*

	C		GP		WL	
U CAT/mg S.P.	**Normoxia**	**Challenge**	**Normoxia**	**Challenge**	**Normoxia**	**Challenge**
0 h	8.00 ± 2.58		7.42 ± 0.15		7.56 ± 2.27	
3.5 h Hypoxia	8.81 ± 2.20	6.89 ± 0.97	7.22 ± 1.23	6.78 ± 2.02	7.06 ± 0.39	7.24 ± 0.86
2 h Recovery	9.17 ± 0.34	8.63 ± 1.92	7.34 ± 0.65	8.77 ± 2.49	7.18 ± 0.41	8.65 ± 1.49
18 h Recovery	9.28 ± 1.23	7.67 ± 5.32	7.92 ± 1.05	8.20 ± 1.41	9.55 ± 0.10	7.83 ± 1.85
U SOD/ mg S.P.						
0 h	49.23 ± 7.59^{a}		45.82 ± 20.57^{ab}		36.38 ± 4.41^{a}	
3.5 h Hypoxia	28.48 ± 2.63^{a}	57.36 ± 27.32	32.33 ± 3.37^{a}	35.98 ± 4.46^{a}	38.74 ± 3.88^{ab}	60.29 ± 17.82^{ab}
2 h Recovery	51.27 ± 8.41^{ab}	35.59 ± 9.39	47.04 ± 0.79^{a}	74.84 ± 39.81^{b}	57.44 ± 14.51^{b}	73.78 ± 44.01^{b}
18 h Recovery	74.59 ± 29.22^{b}	44.98 ± 11.93^{A}	106.69 ± 18.41^{b}	31.83 ± 2.18^{aB*}	87.46 ± 24.51^{c}	39.28 ± 4.60^{aAB*}
U (nmol) MDA /mg S.P.						
0 h	437.12 ± 156.65		391.33 ± 175.92		540.61 ± 313.71^{ab}	
3.5 h Hypoxia	634.17 ± 532.61	653.45 ± 498.24	703.01 ± 102.58	550.64 ± 296.15	641.15 ± 41.01	1050.66 ± 657.07^{b}
2 h Recovery	463.43 ± 96.98	550.37 ± 295.81	465.27 ± 29.39	433.98 ± 85.80	373.38 ± 126.69	469.28 ± 90.90^{a}
18 h Recovery	742.09 ± 583.25	406.44 ± 197.64	548.75 ± 74.68	447.54 ± 123.22	434.79 ± 195.57	633.91 ± 243.28^{ab}

4. DISCUSSION

Results evidenced that the inclusion of a relatively high amount of wine making by-products (100 g/kg) in the diets of juvenile *L. aurata* did not negatively affect growth or feed efficiency, at least in a short-term feeding trial (6 weeks). The values of SGR and FE not only were in agreement to those reported when vegetable ingredients and by-products are included in diets for mullets (El-Gendy et al., 2016; Gisbert et al., 2016; Quirós-Pozo et al., 2021; Martínez-Antequera et al., 2022), but also improved significantly in the case of using GP, as demonstrate the present study. This could be explained considering either that a) irrespectively of the nutritional value of the wine by-products, the amount of nutrients provided by the rest of ingredients used in the feed was enough to sustain growth properly, b) the two by-products were nutritionally equivalent to the wheat bran they substituted in the C feed, and/or c) besides any of the two former, some components present in GP exerted a beneficial effect on the intestinal functioning and general metabolism of the fish.

The absence of negative effect after inclusion of GP or WL in the diets was also supported by the similarities observed in the values of most metabolic indicators measured either in plasma (glucose, total proteins, triglycerides, cholesterol) or liver (glucose, lactate, triglycerides) in fish fed those diets including any of the two by-products and those fed the control diet. Moreover, is worthwhile to state that the above-mentioned positive effect on growth and feed efficiency associated to the inclusion of GP in the diet was supported by significantly lower values of plasma lactate and liver glycogen measured in these fish, or the low (although not significant) values of cortisol observed in fish fed both of the GP or WL diet. Since lactate is a secondary indicator of stress (Moran et al., 2008), while cortisol is a hormone that affects various physiological processes such as growth or the immune response (Guardiola et al., 2016; Sadoul & Vijayan, 2016) as well as intermediary metabolism (Jerez-Cepa et al., 2019), low levels of both metabolites should be indicative of a good welfare status. On the other hand, the low liver glycogen levels could be indicative of a more active metabolism oriented to growth, as it has been reported in a previous study carried out in *Chelon labrosus*, a closely related species (de las Heras et al., 2015). In addition, the significantly higher levels of lysozyme measured in the mucus of fish fed on the GP diet points to an improved immunological status, this being in line to results reported in some freshwater fish

species when extracts rich in bioactives obtained from some by-products of wine, olive and chestnut wood, were included in the diets (Hoseinifar et al., 2020, Jahazi et al., 2020, Mehrinakhi et al., 2021).

Gut microbiota plays an essential role in the health and physiology of fish (Egerton et al., 2020), and the inclusion of new ingredients in aquaculture diets has an impact on its composition and diversity (Batista et al., 2016; Ringø et al., 2016; Panteli et al., 2021) although the evaluation of alternative raw-materials as those assessed in the present study is mandatory to evaluate the putative modifications carried out at physiological level (Sullam et al., 2012; Dehler et al., 2017). In this sense, it is worth mentioning that the phenolic compounds present in grape by-products inhibit the growth of different pathogenic microorganisms (Papadopoulou et al., 2005; Radovanovic et al., 2009). Several techniques have been used to investigate the diversity of animal gut microbial community, mainly high throughput sequencing coupled with bioinformatics analysis. Nevertheless, a proper analysis of the gut microbiota should include information regarding metabolic activity. This can be carried out using Ecoplates (Biolog Inc., Hayward, CA, USA), which enables characterization of microbial communities through their patterns of carbon substrate utilization (Stefanowicz, 2006). This technique has been widely applied to assess functional metabolic diversity of manure composting and wastewater (Gryta et al., 2014; Wang et al., 2018) and more recently of pig fecal microbiota (Checcucci et al., 2021). In the present study, Ecoplates were used as a tool to evaluate potential changes in gut microbiota associated to the consumption of either GP or WL, and our results evidenced that dietary inclusion of WL produced both quantitative and qualitative effects on the bacterial community. The quantitative effect was observed by a significant reduction in the diversity of microbial population, while the qualitative effect was denoted by a modification on the profile of the main substrates used as carbon sources by the microbial population. This was evidenced by a decrease in the relative use of simple carbohydrates and amines and by an increase in the employ of complex carbohydrates and phenolic compounds. These changes could be due to either i) the presence of a higher concentration of active polyphenols in the digesta of fish fed on WL that negatively affected the microbial populations, ii) interactions with the yeast cells provided with the WL or iii) a combination of both effects. Regarding the first aspect, it is widely demonstrated that

polyphenols can modulate the gut microbiota in several ways, either by direct and indirect interactions, stimulating or inhibiting bacterial growth. This depends on the polyphenol structure, the dosage and the strain of microorganism (Catalkaya et al., 2020), being Gram-positives more sensitive to polyphenols than Gram-negatives (Corrêa et al., 2019). Considering this, it follows that not only the growth of pathogenic bacteria can be inhibited by the different polyphenols (catechin, epicatechin and quercetin mainly), but also that some beneficial microorganisms, such as lactic acid bacteria and probiotic strains (Duda-Choak, 2012; Makarewicz et al., 2021) can induce an improvement in the welfare and general physiological status of fish. On the other hand, it has been demonstrated that different types of yeasts may exert strong effects on the gut microbiome (Zhou et al., 2009; Ringø et al., 2016). In relation to the modification in the profile of main substrates, the less consumed carbon sources vary depending on the microbial community. Human and animal gut microbes may extensively utilize amino acids for the synthesis of proteins in a species-dependent manner, but only a few strains of lactic acid bacteria (LAB) have been proven to degrade amines (Callejón et al., 2014). As a conclusion, the measured reduction in the ability of using amino acids, as well as the change in the profile of carbohydrate substrates, points to an impoverishment of the bacterial community in fish fed on diet including WL. This could result in a lower ability to process diets including a high amount of protein or vegetable ingredients with a diversity of carbohydrate components.

Regarding on the Experiment 2, it was designed to assess the possible protective effect of wine polyphenols on the oxidative stress generated by a short-term mid-hypoxia event. The values of the different metabolic indicators measured in fish maintained in normoxia was used as a reference to assess the effect of feed type (by-product inclusion) and hypoxia in challenged fish. The significant variations with time observed in plasmatic cortisol measured in those normoxia fish could be explained either by the presence of internal rhythms and for this reason these sub-groups were evaluated to determine the presence any circadian rhythm that could mask those results obtained in challenged fish.

Significant variations with time were observed in plasmatic cortisol measured in those fish could be explained either by the presence of any kind of internal rhythms (Vera et al., 2014), or as a consequence of the

stress generated by the repeated sampling of the tanks. Such variations were considered a “basal level” of the indicator and were negligible when compared to the response obtained when fish were challenged with hypoxia and did not consume any of the wine byproducts (Figure 5). In fact, the different indicators evaluated in plasma and liver of challenged fish pointed to significant differences in the metabolic response to oxygen shortage when fed on WL and to a greater extent on GP when compared to the C group. Fish fed on the GP diet showed significantly higher values in hematocrit after hypoxia that could determine an increase in oxygen transport capacity, as previously described by other authors (Magnoni et al., 2017; Martos-Sitcha et al., 2017). Also, in these fish plasma glucose levels increased significantly after hypoxia and decreased during the recovery period, being this response linked to the observed modification in the levels of circulating cortisol (Arends et al., 1999). Although this effect was observed in the three treatments, the GP group showed the lowest glucose levels after 2 hours of recovery, which could be indicative of a greater reaction capacity compared to the other two groups. Indeed, although cortisol is a key stress indicator, circulant levels of this hormone showed a great variability within a population due to the great differences in the individual responses to the stressor, i.e., depending on the reactive or proactive nature of fish (Sneddon et al., 2016).

As an example, during hypoxia some fish may present spontaneous and explosive swimming to flee from the area with low oxygen (van Raaij et al., 1996), while others may decrease their activity to reduce energy expenditure (Pollock et al., 2007). In the present work, C and GP groups increased plasma cortisol just after hypoxia, this being in agreement to what described in other species such as trout (van Raaij et al., 1996) or cod (Herbert & Steffensen, 2005). Nevertheless, after 2 h of recovery, cortisol levels in the C group continued rising, while in the GP group decreased to pre-hypoxia levels, with the WL group showing an intermediate response. This points to a much more efficient regulation of cortisol in the GP group, which may be related to the presence of the bioactive compounds with antioxidant activity contained in the grape bagasse that counteracted ROS production during hypoxia (Pérez-Jiménez et al., 2012). After 18 h in normoxia, cortisol levels also dropped in the WL group, which could be related to the beneficial effects of some compounds present in yeast during stressful situations (de Mattos et al., 2019). In any case, both groups (GP and WL) showed significantly lower

levels of cortisol compared to the C group, which was not able of controlling the release of this hormone after hypoxia. This observation suggests that supplementation with either grape bagasse or wine lees provides antioxidant protection against hypoxia and that the different responses observed between the GP and WL groups after 2 hours of recovery could be related to the different profile of phenolic compounds with antioxidant capacity present in each product.

On the other hand, since lactate is produced during anaerobic glycolysis, it should be expected an increase in its levels during hypoxia and a further decrease during the recovery phase (Magnoni et al., 2017). This response was observed in fish receiving any of the two wine by-products, with a significantly higher reduction after 18 h recovery in group GP, this also pointing to a higher resilience to hypoxia induced by inclusion of GP by-products in aquafeeds.

In general, a decrease in plasma TAG levels was observed in the three treatments, probably due to a reduction in their metabolism, since they act as the main energy source in most teleost by producing ATP through β-oxidation but this requires a continuous supply of oxygen (Tocher, 2003), In addition, under hypoxia, lipid peroxidation enhancement has been observed due to the greater presence of ROS (Pérez-Jiménez et al., 2012), which could be another cause of the decrease in this metabolite observed in the three groups. Even so, the effect observed in other lipid source, as cholesterol, seems to be dependent on its physiological function. Indeed, this metabolite is considered not only as an energy source but also as the available source at plasma level acting as precursor of steroids, as cortisol (Sheridan, 1988). Our results suggest that patterns of changes observed could mirror the real need, and concentrations, of cortisol hormone after hypoxic conditions and its recovery in a short-time response, where fish from the control group were the most responsive in both parameters (see above).

In relation to the oxidative status, the only significant response observed was the increase in SOD observed 2 h after recovery measured in the GP and WL groups, as well as its reduction during recovery. As indicated previously for some other metabolites, this points to a higher ability to adapt to the stressor in the GP and to a lower extent in the WL groups through a more effective modulation of the production of the enzymes.

Although there are no studies evaluating the effect of the dietary inclusion of wine by-products in aquaculture species challenged with hypoxia conditions, some of them evaluated the effect of such by-products against other adverse conditions. Harikrishnan et al. (2021) evaluated the impact of including grape pomace at levels of 0.02 and 0.03 % in diets for *Labeo rohita* that were challenged with the pathogen *Flavobacterium columnaris*. Results indicated that both control and challenged fish treated with a 0.02 % inclusion presented significantly improved antioxidant status and immune defense mechanisms. Specifically, the SOD and GPx activities presented significantly higher values compared to those observed in fish fed with the control diet. In addition, it has been reported that antioxidants present in grape seed caused effective responses in abalone (*Haliotis laevigata*) during heat stress evidenced by an increased expression of oxidative defense genes (Shiel et al., 2017). On the other hand, Mousavi et al. (2020) evaluated the effects of including grape seed ethanolic extract (1 or 5 %) on the expression of antioxidant genes in the intestinal tissue of rainbow trout (*Oncorhynchus mykiss*). The results indicated that fish receiving the lower dose of the extract presented a higher expression of antioxidant genes in their intestine. These facts suggests that increased activity of antioxidant enzymes, i.e., SOD activity in GP and WL groups in our experiment, could maintain an efficient antioxidant balance, resulting in better general health status for individuals.

5. CONCLUSIONS

As a resume, the present study evidenced that inclusion of any of the two different wine by-products in diets for *L. aurata* at a relatively high level (10 %) improved growth performance or feed efficiency, even after a short-feeding trial. Nevertheless, other responses were different depending on the by-product; while GP produced a significantly improvement in the immune status, evidenced by an increase in mucus lysozyme, WL negatively affected the diversity and functional profile of intestinal microbiota. On the other hand, supplementation with any of the two products provided antioxidant protection against the challenge of mid-hypoxia, leading to lower levels of cortisol and modifications in the activities of some enzymes involved in the control of ROS, mainly in fish receiving GP. Considering this, it is concluded that the supplementation of diets for *L. aurata* with this latter by-product offers positive nutritional

and physiological results and can be also beneficial within the framework of circular economy.

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IV. GENERAL DISCUSSION

This section is oriented to offer a global evaluation of the main results obtained in the different experiments, although a detailed discussion of each experiment is included in the specific chapters. It is noteworthy that such experiments were carried out using a wide array of biological materials, analytical techniques and experimental designs. Such diversity can be considered a strong point that reinforces a multifactorial approach to the subject of study and is resumed in Table 1.

Also, it must be taken into account that the different experiments were developed within the framework of circular bioeconomy, since they were oriented to evaluate different non-conventional ingredients that could be potentially used in aquaculture feeds, both from the perspective of their nutritional value and also considering their contents of bioactive compounds as putative nutraceutics. As indicated in the General Introduction section, ingredients used were one macroalgae (*Ulva ohnoi*) and three types of by-products generated in the industry of alcoholic beverages: brewer's spent grain, wine bagasse and wine lees.

Several green seaweeds present nutritional limitations for inclusion in aquafeeds because of their contents in undigestible polysaccharides, although, in contrast, these may exert a positive effect on the immunological status of the fish. To date, it had not been considered to evaluate the potential benefits of including macroalgae in feeds from both perspectives simultaneously. For this reason, **Chapter 1** addressed this subject through three different experiments involving the use of the green seaweed *Ulva ohnoi* that were aimed to:

a) Re-evaluate the presence of protease inhibitors described for this macroalga by testing the inhibitory effect of *U. ohnoi* meal on protein digestion in European sea bass.

b) Assess its nutritional value as an ingredient, testing the effect of including *Ulva* previously treated or not with a mixture of carbohydrases on the nutritional efficiency and immune status of juvenile European sea bass.

c) Evaluate its potential protective effect on the oxidative metabolism of fish, evaluating the effect of *U. ohnoi* on the oxidative status of gilthead sea bream challenged by the consumption of a feed including rancid oil.

Table 1. *Summary of the different methodologies used in the experiments performed within the Thesis.*

	CHAPTERS	1	2	3	4	5
Diversity of biological materials						
Fish species	European sea bass	X				
	Gilthead sea bream	X			X	
	Grey mullet		X	X	X	
	Golden grey mullet					X
Ingredients	Green seaweed *Ulva ohnoi*	X				
	Brewer's spent grain		X	X		
	Wine by-products				X	X
Ingredients Modifications	SSH	X	X	X		
	Rancidity	X				
Diversity of techniques						
Analysis of compounds	Reducing sugars, pentoses, phytate, total phosphorus, total amino acids, etc.	X	X	X		
	Phenolic compounds (UHPLC-HRMS)				X	
Evaluation of fish status	Haematology and Metabolites (Hc, TAG, haemoglobin, glucose, lactate, cholesterol and glycogen)			X		X
	Oxidative status (CAT, GPx, SOD, TBARS)	X				X
	Inmunological status (lysozyme and alkaline phosphatase)	X				X
	Functional microbiota (Ecoplates)					X
In vitro digestive simulations			X		X	
In vivo assays	Digestibility (ADC)	X	X	X		
	Growth lab-scale	X	X			X
	Growth field conditions			X		
Physiological challenges	Rancid oil	X				
	Hypoxia					X
Diversity of experimental designs						
Experimental design	One factor	X	X	X		X
	Dose-response	X				
	Multifactorial				X	

Results showed a negligible effect of *U. ohnoi* meal on protein digestion when included in feeds for European sea bass at levels of 10 % or less. Moreover, results of growth and feed use evidenced the possibility of using up to 5 % inclusion of algal meal in feeds without adverse effects on the zootechnical parameters, although the low nutritional value of *U. ohnoi* was not improved significantly when subjected to an enzymatic

hydrolysis process. The peculiar composition of its polysaccharides (especially ulvan) makes difficult to achieve such hydrolysis with currently available commercial carbohydrase preparations. From a practical point of view, its use as a nutritional ingredient is also highly conditioned by the high amount of energy and economic costs associated with the different steps of collection, drying, grinding and subsequent treatments, so its potential use as an ingredient would only be justified as a source of antioxidant or immunostimulant compounds. These effects were evidenced in the present study by an increase in skin mucus lysozyme and the protective effect on the oxidative metabolism of gilthead sea bream when fed on a diet including a highly oxidized oil. However, since the content of compounds with these properties is notably higher in other groups of algae (e.g., brown or phaeophycean algae), it could be concluded that the potential interest of using *U. ohnoi* as an ingredient in aquaculture feeds is quite limited.

As indicated in the General Introduction section, plant by-products may present important limitations linked to both their amino acid imbalances and reduced digestibility due to the presence of a wide variety of antinutritional compounds including alkaloids, lectins, digestive enzyme inhibitors, indigestible carbohydrates (mainly non-starch polysaccharides, NSPs) and phytate. A number of commercially available multienzyme complexes have been developed to improve the use of carbohydrates and phytate present in plant ingredients used in feeds for terrestrial animals. Nevertheless, they have been designed for optimal functioning under the body temperature and pH conditions existing in the digestive systems of pigs and poultry, which are notably different from those present in aquatic species. This may explain the limited effectiveness and somewhat contradictory results obtained when such products are tested in fish species, mainly considering that the efficiency of hydrolysis produced by such enzymes is greatly conditioned by several aspects. As an example, there may be interactions between gastric and intestinal proteases produced by fish and the exogenous enzymes that can negatively affect their potential beneficial effects. Furthermore, the effectiveness of the exogenous enzymes may be greatly reduced by the high temperatures reached during feed preparation or, in the case of being applied post-extrusion via oil top coating or spraying, by the time available for the enzymatic action inside the digestive system of the

species, which is closely related to gut transit rates linked to water (environmental) temperature.

Chapters 2 and **3** describe the experiments performed using an enzymatic pretreatment of low-cost fibrous plant ingredients included in feeds for the grey mullet *(Mugil cephalus)* with a commercial mixture of enzymes (glucanases + phytase). In contrast to what described for *U. ohnoi*, results presented in **Chapter 2** evidenced that the enzymatic treatment of such ingredients (mainly brewer's spent grain and rice bran) with this commercial enzyme complex by SSH significantly modified the potential bioavailability of some nutrients, such as a reducing sugars, pentoses, and phytic phosphorus. To date, only one published study developed a similar approach (Denstadli et al., 2011) performing an enzyme pretreatment of plant ingredients to be used in trout aquafeeds. In that study, the content of vegetable ingredients represented 45 %, and NSPs constituted 8 % of the chemical composition of the evaluated feed. These figures were considerably different to those of the feeds used in the present study, where the content of plant ingredients was much higher (60 %–70 %) and the estimated NSPs contents (including cellulose) exceeded 20 %. The enzymatic action in the previously cited work determined a reduction in the NSPs content between 10 % and 13 % when using soy meal as the main ingredient and only 4 % to 6 % when using rapeseed meal. Additionally, the authors did not obtain significant changes in the contents of pentoses and reducing sugars. Even so, in the present study the enzymatic hydrolysis was remarkably higher since the concentrations of such products were increased by three to four times compared to those in the untreated ingredients.

Results of the growth trial using the enzyme treated plant ingredients are presented in **Chapter 3**. In the preliminary short-term trial carried out at laboratory scale, fish receiving the enzyme-treated feed showed significant improvement (higher than 50 %) in both FCR and SGR when compared to those obtained with the untreated diet, this suggesting that partial hydrolysis of the antinutritive factors (NSPs and phytate) exerted a positive impact on the nutritional value of the feed, and hence on fish performance. In addition, different metabolic indicators (i.e., higher values of muscle glycogen and plasmatic triglycerides) supported the positive effect of the enzyme treatment on the nutritional condition of the fish over those fed on the diet containing non-treated ingredients. Results

of growth and feed efficiency obtained in a second long-term trial developed for 148 days under real production conditions evidenced the equivalence among the experimental and commercial diets.

These trials confirmed that enzyme pretreatment of plant ingredients by SSH may be a useful procedure to improve the nutritive value of high fiber plant by-products when included in practical diets for grey mullet *M. cephalus*, as well as in other herbivorous/omnivorous species. In this sense, it should be noted that this approach only can be valuable when considering diets for such type of non-carnivorous fish, since the selection of feed ingredients for mullets and other herbivores (i.e. tilapia, carp, etc.) is currently focused on an extensive use of plant by-products with a nutritional value that may be frequently conditioned by their content in phytate and NSPs. In contrast, enzyme pretreatment of plant ingredients should present a more limited practical application in feeds for carnivorous species, since they are usually formulated with ingredients with a higher protein contents and lower amounts of carbohydrates. Regarding nutritional experiments carried out with mullets, it is important to consider some particularities of such fish group that must be taken into consideration. They are slow-growing species that present low intakes when water temperature is below 22-24 ºC, this resulting in long test periods required to achieve appreciable growth, or even differences among treatments. In the present study, for example, the fish reared in the laboratory facilities did not double their weight after several weeks and they barely did so in the field trial after 20 weeks. On the other hand, it is important to take into account some peculiarities related to feed preparation, i.e., the need of including specific attractants, since the organoleptic properties of the food seem to play an important role in consumption and, on the other hand, the physical presentation, since buoyancy of the pellets favors their consumption in the case of *M. cephalus*, a species that usually feeds on water surface.

Following an already well-established trend in the nutrition of humans and terrestrial animals, there is a growing interest in seeking ingredients rich in polyphenols for their use in aquafeeds. One key step required to gain a better understanding on the biological roles of polyphenols for a given species is to assess the different factors affecting their digestive bioaccessibility, and a great number of such studies is based in the use of *in vitro* digestion models. **Chapter 4** develops the first study of this

kind that has evaluated the potential digestive bioavailability of the phenolic compounds present in wine bagasse and lees in two fish species showing great differences in their digestive physiology (the omnivorous gilthead sea bream, *S. aurata* and the herbivorous flathead grey mullet, *M. cephalus*) using this type of simulation models. The study was carried out using *in vitro* models adapted to simulate their digestion combined to a factorial experimental design that, besides the fish species, simultaneously evaluated the effects of a) the ingredient used as source of polyphenols, b) the presence or absence of feed matrix, and c) the length of the digestion time. The release of the phenolic compounds was evaluated using ultra-high performance liquid chromatography (UHPLC) coupled to high resolution mass spectrometry (HRMS) detection. Both the presence of feed matrix and the type of wine by-product showed a significant effect on the digestive release of both total and specific types of polyphenols, while fish species showed to be significant only for some specific compounds, like eriodyctiol or syringic acid. Moreover, results obtained in this chapter demonstrate that the time of digestion was not identified as a statistically significant factor in the release of phenolic compounds due to the great variability in the patterns observed that were classified as early, sustained and late. These great variations in the patterns of release of different types of phenolic compounds with time suggest an important effect of gut transit rates on the net bioavailability of a given phenolic compound in the live fish.

The study was original not only for using an *in vitro* approach adapted to aquatic species, but also by assessing to what extent the possible complexation of polyphenols presents in wine by-products with either digestive enzymes or components of the feed matrix could greatly influence their bioaccessibility if included in the diets of any of the two fish species. In this sense, it is worthwhile to highlight the value of digestive simulation as a tool to understand in detail not only the great differences in the release of phenolics depending on their chemical characteristics and their interaction with feed components, but also depending on the capacity to extract and assimilate by different fish species. Related to this, it is also important to consider the need to adapt the simulation conditions to the peculiarities of the digestive physiology and biochemistry of the species studied, as it has been done in the present study, since only in this manner the results obtained may be representative of the expected responses in the fish. Also, the need to use

reliable and precise techniques for measuring phenolics is fundamental. Common and unspecific methodologies, such as the Folin-Ciocalteu colorimetric method, may overestimate the results providing positive reactions with other organic compounds that present hydroxylated aromatic rings, such as the case of some amino acids present in the digestate. In contrast, the precision provided by the UHPL-HMRS technique for the quantification of the different polyphenols has been confirmed in the present study.

The above mentioned results were used as a basis for the last experiment described in **Chapter 5** that was focused on: a) the evaluation of the effect of dietary inclusion of two types of wine by-products (grape bagasse and lees) on growth, immune status, metabolism and gut microbiota of juvenile golden gray mullet (*Liza aurata*) and b) the assessment of the potential protective effect of wine polyphenols against stress produced by a physiological challenge induced by a short and acute hypoxia. After a conditioning feeding period of 6 weeks employing a control diet and two diets supplemented with 100 g/kg of either grape pomace or lees, results evidenced that the inclusion of a relatively high amount of wine making by-products in the diets of juvenile *L. aurata* improves growth or feed efficiency. The fish also presented significantly lower values of plasma lactate and hepatic reserves of glycogen. Nevertheless, dietary inclusion of wine lees produced both quantitative and qualitative effects on the gut bacterial community. The quantitative effect was observed by a significant reduction in the diversity of microbial population, while the qualitative effect was denoted by a modification on the profile of the main substrates used as carbon sources by the microbial population (a decrease in the relative use of simple carbohydrates and amines together with an increase in the use of complex carbohydrates and phenolic compounds). These changes were explained as the result of a higher concentration of active polyphenols in the digesta of the fish that negatively affected the microbial populations, or even by the interactions with the yeast cells provided with the lees. In contrast, results demonstrated the existence of a general improvement in the productive efficiency, physiological and immune status when diets were supplemented with grape pomace.

The positive results obtained by including winemaking by-products, specifically grape bagasse, in the metabolism of the golden mullet (*Liza*

aurata), support its potential as a functional ingredient. However, it must be considered that the positive effects were obtained using relatively high levels of inclusion. In the experiment, the amounts of dry bagasse used were relatively high (100 g/kg feed), something that is only feasible in a feed formulation for an herbivorous species, but that would be difficult to achieve in a diet for a carnivorous species. This suggests that practical application of phenolics obtained from plant ingredients in diets for a variety of fish species should require some kind of product concentration. In this sense, during the realization of the present study, we learned about the existence of a commercially produced concentrate of grape bagasse (Winox®) produced by the company Phodé (Terssac, France), which is sold as an antioxidant additive for different terrestrial species. Given the interest in testing its efficacy in aquatic species, a collaboration was signed to evaluate its usefulness as an additive, an experiment that is currently undergoing in a carnivorous species (*D. labrax*).

In any case, it seems clear that grape bagasse presents a great potential as a functional additive in aquaculture feeds and that future research should be assess the better way to promote its practical application.

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V. CONCLUSIONS

Results from the present **Doctoral Thesis** led to draw the following conclusions:

1. The meal obtained from the seaweed *Ulva ohnoi* has a reduced value as a nutritional ingredient in aquafeeds, even after being enzymatically treated to partially hydrolyze its carbohydrate fraction. Nevertheless, it presents some bioactive compounds that positively influence the immune status and the oxidative metabolism of fish.

2. The pretreatment of highly fibrous vegetable ingredients in feeds for herbivorous species using a commercial mixture of different carbohydrases and phytase under a SSH protocol increases the potential bioavailability of different nutrients and significantly improves feed efficiency and growth under real production conditions.

3. *In vitro* digestive simulations have evidenced that the bioavailability of specific phenolic compounds present in wine by-products included in feeds is conditioned by the feed matrix, the type of wine by-product and the digestive physiology of the fish species used.

4. The inclusion of wine by-products (particularly grape bagasse) in the feed of an herbivorous species like *Liza aurata* determines different positive effects: besides improving zootechnical parameters and immune status, offers antioxidant protection against a stressful agent such as hypoxia.

5. Agro-industrial by-products have a great potential as ingredients in aquafeeds, either as sources of bioactive compounds or overcoming their nutritional limitations through enzymatic treatments aimed to increase the bioavailability of carbohydrates and minerals.

VI. CONCLUSIONES

Los resultados de la presente **Tesis Doctoral** permitieron extraer las siguientes conclusiones:

1. La harina obtenida del alga marina *Ulva ohnoi* tiene un valor reducido como ingrediente nutricional en piensos acuícolas, incluso después de haber sido tratada enzimáticamente para hidrolizar parcialmente su fracción de carbohidratos. No obstante, presenta algunos compuestos bioactivos que influyen positivamente en el estado inmunológico y el metabolismo oxidativo de los peces.

2. El pretratamiento de ingredientes vegetales altamente fibrosos en piensos para especies herbívoras utilizando una mezcla comercial de diferentes carbohidrasas y fitasa bajo un protocolo SSH aumenta la biodisponibilidad potencial de diferentes nutrientes y mejora significativamente la eficiencia alimenticia y el crecimiento durante condiciones reales de cultivo.

3. Las simulaciones digestivas *in vitro* han demostrado que la biodisponibilidad de compuestos fenólicos específicos presentes en los subproductos del vino incluidos en los piensos está condicionada por la matriz del pienso, el tipo de subproducto del vino y la fisiología digestiva de la especie de pez a alimentar.

4. La inclusión de subproductos del vino (particularmente bagazo de uva) en la alimentación de una especie herbívora como *Liza aurata* determina diferentes efectos positivos: además de mejorar los parámetros zootécnicos y el estado inmunológico, ofrece protección antioxidante frente a un agente estresante como la hipoxia.

5. Los subproductos agroindustriales tienen un gran potencial como ingredientes en piensos acuícolas, ya sea como fuentes de compuestos bioactivos o superando sus limitaciones nutricionales a través de tratamientos enzimáticos destinados a aumentar la biodisponibilidad de carbohidratos y minerales.

VII. ANNEXES

SCIENTIFIC CONTRIBUTIONS

National conferences. Oral communications

1. **Martínez-Antequera, F.P**., Fernández, L., & Moyano, F.J. (2021). "Aplicación biotecnológica de carbohidrasas de origen marino en la modificación nutricional de macroalgas". Paper presented in II International Congress of Young Marine Researchers, Motril, Spain.
2. **Martínez, F.P**., Bermúdez, L., Aznar, M.J., & Moyano, F.J. (2019). "Effect of an enzyme pretreatment on some anti nutritional factors present in plant ingredients potentially used in aquaculture feeds". Paper presented in VIII Simposio de Investigación en Ciencias Experimentales, Almería, Spain.
3. **Martínez, F.P**., Bermúdez, L., Aznar, M.J., & Moyano, F.J. (2019). "Efecto del pretratamiento enzimático de ingredientes vegetales sobre el uso nutricional de piensos para acuicultura". Paper presented in II Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias, Almería, Spain.

National conferences. Poster communications

1. **Martínez-Antequera, F.P**., Molina, L., De las Heras, V., Mancera, J.M, Moyano, F.J., & Martos-Sitcha, J.A. (2022). “Evaluación de la inclusión de subproductos de vinificación en piensos sobre el rendimiento productivo, metabolismo y estado inmunológico de la lisa (*Mugil cephalus*)”. Paper presented in XVIII Congreso Nacional de Acuicultura, Cádiz, Spain.
2. **Martínez-Antequera, F.P**., López, R., Martos-Sitcha, J.A., Mancera, J.M, Garrido, A., & Moyano, F.J. (2022). “Efecto de la matriz alimentaria sobre la biodisponibilidad potencial de polifenoles presentes en subproductos de vinificación suministrados en piensos para dorada o mújol”. Paper presented in XVIII Congreso Nacional de Acuicultura, Cádiz, Spain.
3. **Martínez-Antequera, F.P**., Martos-Sitcha, J.A., Mancera, J.M., & Moyano, F.J. (2021). "Los subproductos de vinificación como fuente de bioactivos de interés en acuicultura". Paper presented in X Simposio de Investigación en Ciencias Experimentales, Almería, Spain.
4. **Martínez Antequera, F.P**., Quirós, R., Aznar, M.J., Robaina, L., & Moyano, F.J. (2021). "Use of *in vitro* models to assess differences in

digestive efficiency between two closely related fish species". Paper presented in XIV Congreso Anual de Biotecnología, Zaragoza, Spain.

5. **Martínez, F.P**., Reyna, J.M., Aznar, M.J., & Moyano, F.J. (2020). "Evaluación de *Ulva ohnoi* como ingrediente potencial en piensos para acuicultura". Paper presented in IX Simposio de Investigación en Ciencias Experimentales, Almería, Spain.
6. **Martínez, F.P.**, Bermúdez, L., Aznar, M.J., & Moyano, F.J. (2019). "Effect of an enzyme pretreatment on some anti nutritional factors present in plant ingredients potentially used in aquaculture feeds". Paper presented in VIII Simposio de Investigación en Ciencias Experimentales, Almería, Spain.
7. **Martínez, F.P**., Bermúdez, L., Aznar, M.J., & Moyano, F.J. (2019). "Evaluation of enzyme additives on the nutritional use of feeds with a high contents of plant ingredients for juveniles of *Mugil cephalus*". Paper presented in II International Congress of Young Marine Researchers, Málaga, Spain.

International conferences. Poster communications

1. **Martínez-Antequera, F.P**., Martos-Sitcha, J.A., Mancera, J.M., & Moyano, F.J. (2022). “Vinification by-products as a source of bioactive products of interest in aquaculture”. Paper presented in Aquaculture Europe Congress, Rimini, Italy.
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3. **Martínez-Antequera, F.P**., Reyna, J.M., Aznar, M.J., & Moyano, F.J. (2021). "Efecto del pretratamiento enzimático de *Ulva ohnoi* como ingrediente potencial en piensos para acuicultura". Paper presented in X Foro Iberoamericano de los Recursos Marinos y la Acuicultura, online.
4. Barranco-Ávila, I., **Martínez-Antequera, F. P**., Mancera, J.M, Moyano, F.J., & Martos- Sitcha, J.A. (2021). "Evaluación de piensos de valor nutritivo mejorado sobre el rendimiento productivo y metabolismo de la lisa (*Mugil cephalus)".* Paper presented in X Foro Iberoamericano de los Recursos Marinos y la acuicultura, online.

Scientific papers in this Doctoral Thesis

1. **Martínez-Antequera, F. P.**, López-Ruiz, R., Martos-Sitcha, J.A., Mancera, J.M., & Moyano, F.J. (2023). Assessing differences in the bioaccessibility of phenolics present in two wine by-products using an *in-vitro* model of fish digestion. Frontiers in Veterinary Science, 10, 547. DOI: 10.3389/fvets.2023.1151045. IF: 3.47, Q1 (Veterinary Science).
2. **Martínez-Antequera, F. P**., Barranco-Ávila, I., Martos-Sitcha, J. A., & Moyano, F. J. (2021). Solid-State Hydrolysis (SSH) Improves the Nutritional Value of Plant Ingredients in the Diet of *Mugil cephalus*. Fishes, 7(1), 4. DOI: 10.3390/fishes7010004. IF: 3.17, Q2 (Aquatic Science).
3. **Martínez-Antequera, F.P**., Martos-Sitcha, J.A., Reyna, J.M., & Moyano, F.J. (2021). Evaluation of the inclusion of the green seaweed *Ulva ohnoi* as an ingredient in feeds for gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). Animals, 2021, 11(6), 1684. DOI: 10.3390/ani11061684. IF: 3.23, Q1 (Animal Science and Zoology).
4. **Martínez, F.P**., Bermúdez, L., Aznar, M.J., & Moyano, F.J. (2019). "Evaluation of enzyme additives on the nutritional use of feeds with a high content of plant ingredients for *Mugil cephalus*". Fishes 4(4). DOI: 10.3390/fishes4040056. IF: 3.17, Q3 (Aquatic Science).

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1. Imentai, A., Gilannejad, N., Martínez-Rodríguez, G., López, F. J. M., **Martínez, F. P**., Pěnka, T., & Policar, T. (2022). Effects of first feeding regime on gene expression and enzyme activity in pikeperch (*Sander lucioperca*) larvae. Frontiers in Marine Science, 9, 864536.
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Book Chapters

1. Moyano, F.J., & **Martínez-Antequera, F.P.** (2021). Marine bioactive compounds. A general view. In J. Maroto and E. Abollo (Ed.), Potential use of probiotics and micro/macro algaes as additives and major ingredients in aquafeeds (21-39). Fundación CETMAR.

animals

MDPI

Article

Evaluation of the Inclusion of the Green Seaweed *Ulva ohnoi* as an Ingredient in Feeds for Gilthead Sea Bream (*Sparus aurata*) and European Sea Bass (*Dicentrarchus labrax*)

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Simple Summary: The use of seaweeds in aquafeeds is receiving increasing attention due to their potential nutritional and functional benefits. However, several green seaweeds such as *Ulva* presents nutritional limitations because of the undigestible polysaccharides, although these may exert a positive effect on the immunological status of the fish. The present study developed three different experiments aimed to re-evaluate the presence of protease inhibitors described for *Ulva ohnoi*, to assess its nutritional value as an ingredient and also to evaluate its potential protective effect on the oxidative metabolism of fish, being experiments developed in two different fish species (European sea bass *Dicentrarchus labrax* and gilthead sea bream, *Sparus aurata*). Results indicate the absence of negative effects of *U. ohnoi* on protein digestion of sea bream but a limited value as a feed ingredient. In contrast, its contents in bioactives seem to be correlated to the observed positive effects on the immune status and oxidative metabolism when fish are challenged by the consumption of highly oxidized dietary oil.

Abstract: This study evaluated the use of *Ulva ohnoi* as an ingredient in feeds for aquaculture in three different experiments. Experiment 1 was oriented to confirm the negative effect of *U. ohnoi* on fish digestion. Experiment 2 assessed the effect on growth, feed efficiency, and immune status of juvenile sea bass (*Dicentrarchus labrax*) fed on diets including *U. ohnoi*, previously treated or not with carbohydrases used to partially hydrolyze indigestible polysaccharides. Experiment 3 was aimed to evaluate the potential protective effect of *U. ohnoi* on the oxidative status of sea bream (*Sparus aurata*) challenged by the consumption of a feed formulated with the oil fraction completely oxidized. Results show a negligible effect of *U. ohnoi* meal on protein digestion when included in feeds at levels of 10% or less. Moreover, results of growth and feed use evidenced the possibility of using up to 5% inclusion of algal meal in feeds without adverse effects on the zootechnical parameters, while the enzyme pretreatment was ineffective to improve its nutritional use. Finally, the inclusion of *U. onhoi* in feeds determined both an immunostimulatory effect, evidenced by an increase in skin mucus lysozyme in the two mentioned fish species, and a positive influence on the oxidative metabolism of seabream when fed on a diet including rancid oil.

Keywords: aquaculture feeds; bioactive compounds; *Ulva ohnoi*

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1. Introduction

Ulva are green macroalgae belonging to the phylum Chlorophyta that presents a great environmental polymorphism, and genetic analysis suggests that the different described species for the genus (*U. armoricana*, *rigida*, *prolifera*, *pertusa*, *fasciata*, or *ohnoi*) are only

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Article

Evaluation of Enzyme Additives on the Nutritional Use of Feeds with a High Content of Plant Ingredients for *Mugil cephalus*

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Abstract: The Mugilidae are a group of fish with a great interest for aquaculture due to their omnivorous profile, rapid growth, and resistance to environmental variations. The selection of feed ingredients for these species is currently focused on an extensive use of plant by-products, with this being limited by their content in anti-nutritive factors (mainly phytate and non-starch polysaccharides; NSPs). Nevertheless, specific enzymes can be used to counteract some of those negative effects. In the present study, the effect of pretreating two high-plant feeds with a mixture of enzymes (glucanases + phytase) on the digestive use of protein and phosphorus by juvenile mullets (*Mugil cephalus*) was assessed using both in vitro and in vivo assays. The enzymatic treatment significantly modified the potential bioavailability of some nutrients, such as a reduction of sugars, pentoses, and phytic phosphorus. Also, it increased the digestibility of protein in one of the feeds but reduced that of phosphorus in both of them. The potential usefulness of enzyme treatment and the information provided by the two types of assays are discussed.

Keywords: aquaculture; plant ingredients; enzymes

1. Introduction

Increasing concerns about the environmental and economic sustainability of aquaculture indicate that its future development cannot rely on intensive production systems of carnivorous species, due to the high impact linked to the need of using large quantities of fishmeal and oils in their feeds [1]. Therefore, multiple instances promote the development of semi-intensive and integrated aquaculture systems based on omnivorous or herbivorous species, as well as an extended use of plant ingredients and by-products [2]. This orientation is increasing in Asia and South America, but to date, it is not so extensive in Europe, where fish aquaculture is still primarily focused on the intensive production of carnivorous species with a high market value. Nevertheless, the need for diversification in Mediterranean marine aquaculture has promoted an increased interest in developing alternative models of aquaculture (aquaponics, recirculation systems, multitrophic aquaculture, etc.) as well as the cultivation of some omnivorous species. The common feature of these systems is the use of species placed at low trophic levels showing less demanding nutritional requirements, in terms of total amount or quality of feed ingredients, and whose feeding can be carried out largely using low-cost ingredients.

Within this context, it is worth noting the high potential presented by the species of the family Mugilidae (mullets), a group of fish living in temperate and subtropical coastal waters in both hemispheres. Mullets, which include species like *Mugil cephalus*, *Chelon labrosus*, *Liza aurata*, *Liza saliens*, and *Liza ramada*, present a great adaptability to different culture conditions, with their potential

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Article

Solid-State Hydrolysis (SSH) Improves the Nutritional Value of Plant Ingredients in the Diet of *Mugil cephalus*

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Abstract: The possibility of improving the nutritional quality of plant byproducts (brewers' spent grain and rice bran) through an enzyme treatment was tested in a formulated feed for grey mullet (*Mugil cephalus*). The enzyme treatment was carried out by Solid-State Hydrolysis (SSH) using a commercial preparation including carbohydrases and phytase. A feed prepared without the treatment and a commercial feed for carp were used as controls. In a preliminary short-term trial carried out at laboratory facilities, fish receiving the enzyme-treated feed showed significant improvement in both FCR and SGR when compared to those obtained with the untreated diet, although both experimental diets presented worse values than those obtained with the commercial feed. Different metabolic indicators including higher values of muscle glycogen and plasmatic triglycerides supported the positive effect of the enzyme treatment on the nutritional condition of the fish over those fed on the diet containing non-treated ingredients. Results of growth and feed efficiency that were obtained in a second long-term trial developed for 148 days under real production conditions evidenced the equivalence among the experimental and commercial diets and confirmed that enzyme pretreatment of plant ingredients by SSH may be a useful procedure to improve the nutritive value of high fiber plant byproducts when included in practical diets for this species and others with similar nutritional features.

Keywords: aquaculture feeds; plant byproducts; enzymatic pretreatment

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1. Introduction

Although in the last years important efforts have been carried out to reduce the levels of the conventional marine resources, i.e., fish meal and fish oil, in the diets of cultured fish, the sustainable development of marine aquaculture requires species that can be produced without the need to use high amounts of such ingredients, the availability of which is growing progressively more limited. Mugilidae (mullets) are a group of fish gaining increasing interest for aquaculture due to their rapid growth, resistance to a wide range of environmental conditions, and omnivorous profile. Over the past few years, the culture of these species, particularly of the grey mullet (*Mugil cephalus*), is considered a priority within the current strategies of aquaculture diversification in different parts of the world, with particular interest in some Mediterranean countries [1]. In addition to specific research aimed at completing its reproduction in captivity, the culture of grey mullet requires the development of suitable species-specific diets, the availability of which represents a bottleneck for their production under intensive systems. Recent studies show good results when testing highly nutritive diets based in the use of zooplankton species [2,3], and several others support the possibility of using high amounts of plant byproducts in feeds for these species, even during early stages of their development [4–8]. Nevertheless, in this latter

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Assessing differences in the bioaccessibility of phenolics present in two wine by-products using an *in-vitro* model of fish digestion

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Increasing attention is currently being paid to the protective role of polyphenols in health and oxidative status in fish. For this reason, the potential use of different natural sources of such compounds, like wine by products, is under study. One key step required to gain a better understanding on the biological roles of polyphenols for a given species is to assess the different factors affecting their digestive bioaccessibility, and a great number of such studies is based in the use of *in vitro* digestion models. In the present study the potential digestive bioavailability of the phenolic compounds present in wine bagasse and lees was evaluated for two fish species showing great differences in their digestive phisyiology: the omnivorous gilthead sea bream (*Sparus aurata*) and the herbivorous flathead grey mullet (*Mugil cephalus*). The study was developed using *in vitro* models adapted to simulate their digestion and a factorial experimental design that simultaneously evaluated the effects of the ingredient used as source of polyphenols, presence or absence of feed matrix, fish species and digestion time. The release of the phenolic compounds was evaluated using ultra-high performance liquid chromatography (UHPLC) coupled to high resolution mass spectrometry (HRMS) detection. Both the presence of feed matrix and the type of wine by-product showed a significant effect on the digestive release of both total and specific types of polyphenols while fish species showed to be significant only for some specific compounds, like eriodyctiol or syringic acid. The time of digestion was not identified as a statistically significant factor in the release of phenolic compounds due to the great variability in the patterns observed that were classified as early, sustained and late. The observed great variations in the patterns of release of different types of phenolic compounds with time suggest an important effect of gut transit rates on the net bioavailability of a given phenolic compound in the live fish. The present study is, to our knowledge, the first one on which an *in vitro* approach was applied to assess to what extent the possible complexation of wine polyphenols present in wine by-products with either digestive enzymes or components of the feed matrix could limit their bioaccessibility if included in diets of two different fish species.

KEYWORDS

polyphenols, UHPLC-Q-Orbitrap-MS, wine by-products, fish species, *in vitro* digestion