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Immune defence mechanisms presented in liver homogenates and bile of gilthead seabream (*Sparus aurata*)

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ABSTRACT

Because the role of the liver of fishes in providing possible immunity remains largely unknown, the aim of this work was to identify and characterize different humoral defence mechanisms in the liver homogenates and bile of gilthead seabream (*Sparus aurata*) for the first time. Total protein levels and several immune parameters (complement activity, lysozyme and immunoglobulin M level) were studied. Furthermore, the activity of some lytic (proteases, antiproteases, esterase, alkaline phosphatase) and antioxidant (superoxide dismutase, catalase and peroxidase) enzymes was determined. Finally, bacteriostatic activity on three opportunist fish pathogens (*Vibrio harveyi*, *Vibrio angillarum* and *Photobacterium damselae*) was measured. Lysozyme and antiprotease activity were undetected in liver and bile, while natural haemolytic complement activity was only detected in bile, and immunoglobulin M was detected in both samples. The levels of proteases, esterase and antioxidant enzymes were greater in bile than in liver homogenates, while the level of alkaline phosphatase was very low in both samples. In addition, while no bacteriostatic activity was detected on liver homogenates, the bile revealed a very potent bacteriostatic activity against all the tested pathogenic bacteria. These results corroborate that fish liver – especially fish bile – contains many factors involved in innate immunity that could be useful for better understanding the role of the liver as an organ involved in fish immune functions as well as the possible contribution of bile to gut mucosal immunity.

KEYWORDS
antioxidant enzymes, aquaculture, bacteriostatic activity, bile, gilthead seabream (*Sparus aurata*), innate immunity, liver

1 | INTRODUCTION

The human liver is the largest glandular organ engaged primarily in vascular, metabolic and nutrient storage and detoxification activities, playing an important role in the metabolization and/or excretion of drugs and other exogenous substances. While the liver is frequently considered a nonimmunological organ, in fact, the healthy liver is a site of complex immune activity facilitated by nonhaematopoietic cell populations and a diverse immune cell repertoire (reviewed by Knolle & Gerken, 2000). At present, knowledge about the liver of fish in relation to immunity is particularly scarce.

The liver of fish is a discrete organ, ventrally positioned in the cranial area of the general cavity. Its size, shape and volume are adapted to the available space between other organs. Hepatocytes (the main

cell types present in the liver) of many vertebrates continuously produce and secrete bile, a yellowish, brownish or olive-green liquid secretion (vulgarly known as hiel). Bile is released into the bile ducts and canaliculi, and is stored in the gallbladder during interdigestive periods. After a meal, the stored bile acids are released into the intestine. Bile is actively involved in the digestion processes (functioning as an emulsifier of fatty acids), in the excretion of metabolites (such as bile acids) and in the neutralization of duodenal acid pH (Dosch *et al*., 2019). After promoting nutrient digestion and absorption in the proximal intestine, bile acids travel down the length of the small intestine where they are then carried back to the liver *via* the portal circulation for uptake and re-secretion into bile, although those bile acids that escape absorption pass into the colon before being eliminated in the faeces (reviewed by Trauner & Boyer, 2003).

Bile comprises approximately 97% water, and also contains electrolytes, colic and chenodeoxycholic bile acids (produced from cholesterol when conjugated with glycine and taurine), biliary pigments (especially bilirubin, which comes from the metabolism of the heme group of haemoglobin), cholesterol and phospholipids. Research over the past 80 years has demonstrated the role of bile acids in intestinal fat absorption, hepatic bile formation and cholesterol homeostasis (Hofmann & Hagey, 2014; Marin *et al*., 2015). However, more recent studies revealed that bile acids act as pleotropic signalling metabolites that regulate not only diverse metabolic processes but also inflammatory pathways in multiple cell types and tissues in mammals. In fact, they modulate the intestinal and liver innate immune system and interact with the microbiota (Chen *et al*., 2019; Fiorucci *et al*., 2018; Liu & Wang, 2019). This has generated considerable renewed interest in the study of bile acids and their metabolism (Ferrebee & Dawson, 2015).

The immunological characteristics of bile among teleost have been little studied compared to those of serum or mucus. Available studies (from different species of teleost) demonstrate the presence of different immunoglobulins (Ig) in bile – mainly of the IgA and IgM types – indicating that the levels of bile antibodies increase in the presence of certain bacteria (Brown & Kloppel, 1989). Similarly, the antimicrobial capacity of bile in the intestine has also been described as able to modulate the immune response to a bacterial infection in the digestive tract (Ruangsri *et al*., 2010). Taking these considerations into account, the aim of this work was to characterize either the presence (or absence) of different molecules and enzymes involved in the immune and antioxidant status of healthy specimens of gilthead seabream (*Sparus aurata*) and the bacteriostatic activity in liver homogenates. Furthermore, the results were compared with those obtained for the same activities studied in bile. The possible implications for gut immunity as well as the success of oral vaccination are discussed.

2 | MATERIALS AND METHODS

2.1 | Ethic statements

The experimental protocols carried out were approved by the Ethics Committee of the University of Murcia (permit number A13150104), and follow the guidelines of the European Union for the handling of animals (2010/63/EU).

2.2 | Fish and sampling

Gilthead seabream (*S. aurata*) specimens (110 g mean body weight) from the same cohorts were obtained from a local farm (Murcia, Spain). After 40 days to ensure the absence of disease, 30 fish were distributed in three 400 l recirculating tanks in the Marine Fish Facility (University of Murcia, Spain). The fish were fed a commercial diet (Skretting) at a rate of 3% body weight per day. Daily, the water temperature was controlled at 22 ± 1°C with a flow rate of 800 l h^{-1} and 28‰ salinity, and an artificial photoperiod of 12 h light:12 h dark was maintained.

Prior to sampling, fish were starved for 24 h and anesthetized with clove oil (50 mg l^{-1} water). Fish were weighed, and after blood withdrawal they were dissected to remove the liver and gall bladders. Liver samples were homogenized in 1 M sodium phosphate buffer in a sample:buffer ratio of 1:4 (w/v), using a T10 basic Ultra-Turrax homogenizer (IKA, Staufen, Germany). Bile samples were obtained directly by puncturing each one of the gallbladders with an insulin syringe for individual analysis. Bile samples were centrifuged (900 × *g* for 5 min) to eliminate any possible debris. Each bile sample was separated into two aliquots, one of which was diluted 1:10 in phosphate buffer saline (PBS). Due to the small volume of bile obtained from each fish and the quantities required to perform certain analyses, catalase activity and bacteriostatic activity were only tested in the liver and bile samples of 1:10 and 1:100 diluted in PBS. After being obtained, the samples were analysed.

2.3 | Total protein levels

The total protein concentration in liver homogenates and bile samples was estimated using the Coomassie Brilliant Blue G-250 method with Bradford reagent (Sigma-Aldrich, Darmstadt, Germany). Briefly, 5 μl of sample was incubated with 250 μl of Bradford reagent in flat-bottomed 96-well plates (in triplicate). Serial dilutions of bovine serum albumin (BSA; Sigma-Aldrich) were used as a standard. The samples were incubated during 10 min at room temperature and in darkness and then the absorbance was read at 550 nm in a plate reader (BMG, Fluostar Omega, De Meern, Holland). The total protein concentration existing in each sample was expressed as mg ml^{-1} and used to standardize the immune parameters and activities present on liver homogenates and bile samples.

2.4 | Immune parameters

2.4.1 | Natural haemolytic complement activity

Alternative complement pathway activity was assayed in liver homogenates and bile using sheep red blood cells (SRBC; Biomedics, Madrid, Spain) as targets (Ortuño *et al*., 1998). Equal volumes of SRBC suspension (6%) in phenol red-free Hank's buffer (HBSS) containing Mg^{+2}

and ethylene glycol tetraacetic acid (EGTA) were mixed with serially diluted serum to give final serum concentrations ranging from 10% to 0.078% in round (U)-bottomed 96-well plates. After being incubated (90 min, 22°C), the samples were centrifuged (400 × *g*, 5 min, 4°C) to avoid unlysed erythrocytes. The relative haemoglobin content present in the supernatants was calculated according their optical density at 550 nm in a plate reader (BMG Labtech, Champigny, France). The maximum (100%) and minimum (spontaneous) haemolysis values were achieved by adding 100 μl of distilled water or HBSS to 100 μl samples of SRBC, respectively.

The degree of haemolysis (*Y*) was estimated and the lysis curve for each specimen was obtained by plotting $Y\ (1 - Y)^{-1}$ against the volume of serum added (ml) on a log–log scaled graph. The volume of serum producing 50% hemolysis (ACH_{50}) was determined and the number of ACH_{50} units ml^{-1} was obtained for each experimental fish.

2.4.2 | Lysozyme activity

Lysozyme activity was measured according to the Swain *et al.* (2007) method. Briefly, 20 μl of liver or bile samples were placed in flat-bottomed 96-well plates in triplicate. To each well was added 180 μl of freeze-dried *Micrococcus lysodeikticus* (0.2 mg ml^{-1}, Sigma-Aldrich, Darmstadt, Germany) in 40 mM sodium phosphate (pH 6.2). As blanks of each sample, 20 μl of liver homogenate or bile was added to 180 μl of sodium phosphate buffer. The absorbance was measured at 450 nm after 20 min at 35°C in a microplate reader (Synergy HT, Biotek, Cedez, France). A standard curve was made with hen egg white lysozyme (HEWL; Sigma) through serial dilutions, allowing the quantities of lysozyme existing in each sample to be determined. Lysozyme values are expressed as μg ml^{-1} equivalent of HEWL activity.

2.4.3 | Total IgM levels

Total IgM levels in liver and bile were analysed using the enzyme-linked immunosorbent assay (ELISA) (Cuesta *et al.*, 2004). For this, 20 μl per well of 1/100 diluted samples were placed in flat-bottomed 96-well plates in triplicate and incubated for 1 h with 100 μl per well of mouse anti-gilthead *S. aurata* IgM monoclonal antibody (Aquatic Diagnostics Ltd, Stirling, UK) (1/100 in blocking buffer). The secondary antibody was anti-mouse IgG-HRP (1/1000 in blocking buffer; Sigma). Samples without liver, bile or primary antibody were used as negative controls.

2.5 | Enzymatic activities

2.5.1 | Proteases

Protease activity was quantified using the azocasein hydrolysis assay according to the method described by Ross *et al.* (2000). Briefly, aliquots of 10 μl of liver or bile samples (diluted 1/10 in 100 mM ammonium bicarbonate buffer) were incubated for 24 h at 30°C in a 96-well flat-bottomed plate with 125 μl of 100 mM ammonium bicarbonate buffer containing 2% azocasein (Sigma-Aldrich, Darmstadt, Germany). The OD of the samples were read at 450 nm using a plate reader. Liver homogenate or bile were replaced by trypsin (5 mg ml^{-1}; Sigma) as a positive control (100% of protease activity) or by the buffer as a negative control (0% activity).

2.5.2 | Antiproteases

Total antiprotease activity was determined in liver and bile samples by the ability of serum to inhibit trypsin activity (Hanif *et al.*, 2004). Briefly, 10 μl of samples was incubated (10 min, 22°C) with the same volume of standard trypsin solution (5 mg ml^{-1}) in a 96-well flat-bottomed plate. For a positive control, buffer replaced liver or bile and trypsin, and for a negative control, buffer replaced the liver or bile. The antiprotease activity was expressed in terms of the percentage of trypsin inhibition according to the formula: % trypsin inhibition = (trypsin OD – sample OD)/trypsin OD × 100.

2.5.3 | Esterase

Esterase activity was determined according to the method described in Guardiola *et al.* (2016). Equal volumes of liver homogenates or bile and 0.4 mM *p*-nitrophenylmyristate substrate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, 30°C) were incubated. The OD was continuously measured at 1 min intervals during 1 h at 405 nm in a plate reader (FLUOstar Omega, BMG Labtech, De Meern, Holland). The initial rate of the reaction was used to calculate the activity. One unit of activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenylmyristate product in 1 min and the results were expressed as units ml^{-1}.

2.5.4 | Alkaline phosphatase

Alkaline phosphatase activity was measured by incubating an equal volume of liver homogenate or bile with 4 mM i-nitrophenyl liquid phosphate (Sigma) in 100 mM ammonium bicarbonate buffer containing 1 mM MgCl2 (pH 7.8, 30°C) as described by Ross *et al.* (2000). One unit of activity was defined as the amount of enzyme required to release 1 mmol of *p*-nitrophenol product in 1 min.

2.6 | Antioxidant enzymes

2.6.1 | Superoxide dismutase

The superoxide dismutase (SOD) activity of the liver and bile samples was studied at 25°C by an indirect method using cytochrome

C as superoxide oxidant (which acts as indicator) (Espinosa *et al*., 2014). A volume of 50 μl of samples was added to 950 μl of 50 mM potassium phosphate buffer pH 7.0 containing EDTA (2 mM), xanthine (100 μM) and cytochrome C (12 μM) (Sigma-Aldrich, Madrid, Spain). The SOD activity was expressed in U × mg protein^{-1}.

2.6.2 | Catalase

The activity of catalase (CAT) was measured by monitoring the consumption of hydrogen peroxide (H_2O_2) at 240 nm (McCord and Fridovich 1969). This method is based on the principle that the absorbance will decrease due to the decomposition of H_2O_2. The catalase activity was expressed in U × mg protein^{-1}.

2.6.3 | Peroxidase activity

Peroxidase activity in liver homogenates and bile was measured according to Quade and Roth (1997). Briefly, 10 μl of sample was diluted with 45 μl of HBSS without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates before adding 100 μl of 20 mM 3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma-Aldrich) and 5 mM H_2O_2. Standard samples without liver or bile were used as blanks.

2.7 | Bacteriostatic activity

Three pathogenic bacteria for fish (*Vibrio harveyi*, *Vibrio angillarum* and *Photobacterium damselae*) were used in the bacteriostatic assay. All bacterial strains were grown from 1 ml of stock culture that had been previously frozen at −80°C. The bacteria were cultured (48 h, 25°C) in Triptic Soy Agar (TSA; Difco Laboratories) and then inoculated in Triptic Soy Broth (TSB; Difco Laboratories, Franklin Lakes, NJ), both supplemented with NaCl to a final concentration of 1% (w/v). Exponentially growing bacteria were resuspended in sterile PBS and adjusted to 10^8 colony forming units (c.f.u.) ml^{-1}.

Bacteriostatic activity was determined following method of Stevens *et al*. (1991). Samples of 20 μl of liver homogenate or bile (previously diluted 1:10 or 1:100 in PBS) were added in quadruplicate wells of a U-shaped 96-well plate (Thermo Fisher Scientific, Massachusetts, Unites States). Hank's balanced solution was added to some wells instead of the extracts and served as positive control. Aliquots of 20 μl of the bacteria previously cultured were added and the plates were incubated for 2.5 h at 25°C (in the case of *V. harveyi* and *V. anguillarum*). Bacteriostatic activity was expressed as percentage of no viable bacteria, calculated as the difference between absorbance of bacteria surviving and the absorbance of bacteria from positive controls (100%).

2.8 | Statistical study

The results are expressed as means ± S.E.M. The normality of the variables was established by the Shapiro–Wilk test and the homogeneity of variance by the Levene test. Statistical differences among the groups were evaluated by one-way ANOVA analyses, followed by the Tukey or Games Howell test, depending on the homogeneity of the variables (SPSS for Windows® version 15.0, SPSS Inc., Chicago, USA). Statistical differences were set at $P < 0.05$.

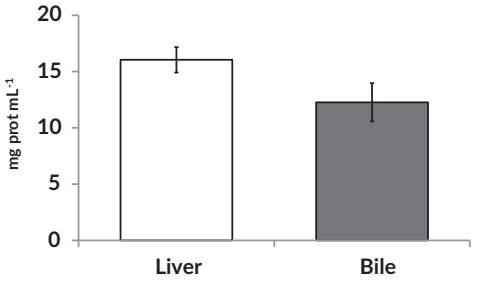

FIGURE 1 Total proteins (mg protein ml^{-1}) determined in liver homogenates (white) and bile (grey) of gilthead seabream. Values are means ± S.E. ($n = 9$, three replicates)

3 | RESULTS

Total protein levels, main humoral immune parameters, some enzymatic and antioxidant activities related to immunity and antioxidant status, and bacteriostatic activity were assayed in samples of liver homogenate and bile of gilthead seabream specimens. More proteins were present in liver homogenates than in bile (Figure 1).

Natural haemolytic complement activity was not detected in gilthead seabream liver homogenates, although it was present in bile (Figure 2a). Lysozyme activity was not detected in either liver homogenates or bile, but IgM was detected in both liver and bile samples. The titre of IgM was higher in bile than in liver, although the presence of IgM in bile demonstrated greater variability among the different specimens than the IgM levels determined in liver (Figure 2b).

Protease activity in liver was approximately 20%, significantly less than diluted bile samples (approximately 80%) or undiluted bile (around 100%) (Figure 3a). In contrast, no antiprotease activity was detected in either liver or bile samples. Very low activity was noticed in gilthead seabream liver homogenates for both esterase and alkaline phosphatase enzymes (Figure 3b). However, while esterase activity was present in bile (medium activity of 16 units per mg of protein), alkaline phosphatase was detected only at very low concentrations (Figure 3c).

No SOD activity was detected in liver homogenates, with very low levels of catalase being obtained and levels of peroxidase at approximately 60 units per ml recorded (Figure 4). These three antioxidant activities were detected in bile at medium concentrations of 35, 108 and 168 units per mg of protein for SOD, catalase and peroxidase, respectively (Figure 4).

Finally, bacteriostatic activity in relation to three fish pathogens relevant to aquaculture (*P. damselae*, *V. angillarum* and *V. harveyi)* was tested in liver and bile samples. It was found that the presence of liver

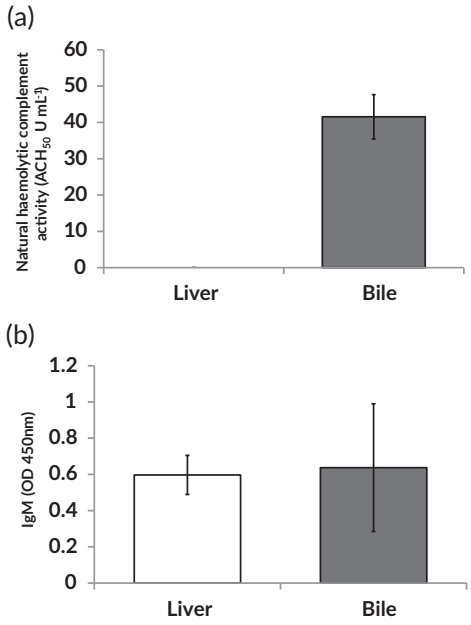

FIGURE 2 Natural haemolytic complement activity (ACH_{50} units ml^{-1}) (a) and immunoglobulin M (OD 450 nm) (b) determined in liver homogenates (white) and bile (grey) of gilthead seabream. Values are means ± S.E. ($n = 9$, three replicates)

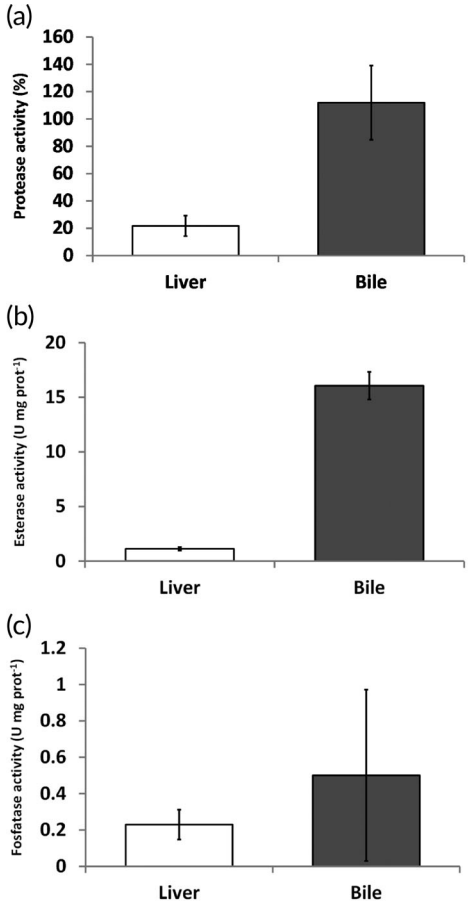

FIGURE 3 Protease (%) (a), esterase (U mg of protein) (b) and phosphatase alkaline (U mg^{-1} of protein) (c) determined in liver homogenates (white) and bile (grey) of gilthead seabream. Values are means ± S.E. ($n = 9$, three replicates)

homogenates did not affect bacteria viability in a significant manner (Figure 5). On the contrary, gilthead seabream bile (1:10) samples demonstrated very strong bacteriostatic activity (approximately 98%) against the three tested pathogens. When similar assays were developed with bile diluted at 1:100, it was possible to detect that the bacteriostatic activity was low for *P. damselae* (58%), medium for *V. anguillarum* (63%) and high (approximately 82%) for *V. harveyi* (Figure 5).

4 | DISCUSSION

The liver of vertebrates is a central immunological organ (reviewed by Weiskirchen & Tacke, 2014) and it is also supposed to be an important immune organ in teleosts. However, more basic data are needed related to deciphering the role played by fish liver in the immune status of both healthy and diseased fish. This absence of evidence was the primary reason for developing the present study on liver homogenates and bile from gilthead seabream, a species selected as a marine fish model due to interest in their role as a farmed fish, especially in the Mediterranean area (FAO, 2020).

To address the lack of baseline information on fish liver immunology, several recent works have been developed to better understand fish gut-liver immunity. These works focus on genomic, transcriptomic and proteomic studies and demonstrate that important immune-related pathways (such as coagulation and complement cascades, toll-like receptors, T- and B-cell receptors, and signalling pathways) are present in the liver (Baumgarner *et al*., 2013; Tafalla *et al*., 2016; Wang *et al*., 2016). However, these studies were carried out with the aim of studying the immune response under several conditions, such as short-term starvation (Baumgarner *et al*., 2013) of fish exposed to environmental toxicants (Li *et al*., 2013), during soybean-induced enteritis (De Santis *et al*., 2015), or on parasitic or bacterial infections (Wang *et al*., 2016). To the best of our knowledge, no study has focused on understanding the immune activities of the liver and bile of healthy fish, which is the aim of the present study. As the innate immune response involves a concerted network of induced gene products, preformed immune effectors, biochemical signalling cascades and specialized cells, our study focused on some humoral immune parameters, as well as enzymes involved in immunity and in the antioxidant status, and on bacteriostatic activity.

Among the immune parameters, complement and lysozyme activities and IgM levels were selected for this study due to their crucial roles. The complement system consists of a family of approximately 30 soluble proteins involved in the elimination of pathogens. The liver produces most of the complement proteins for systemic release into the bloodstream (Cravedi *et al*., 2013). In mammals, the complement system has been extensively studied and is very important for liver homeostasis in immune responses as well and interacts with other effector systems of both innate and adaptive immunity (Thorgersen *et al*., 2019). In humans, it is crucial to control the complement in very

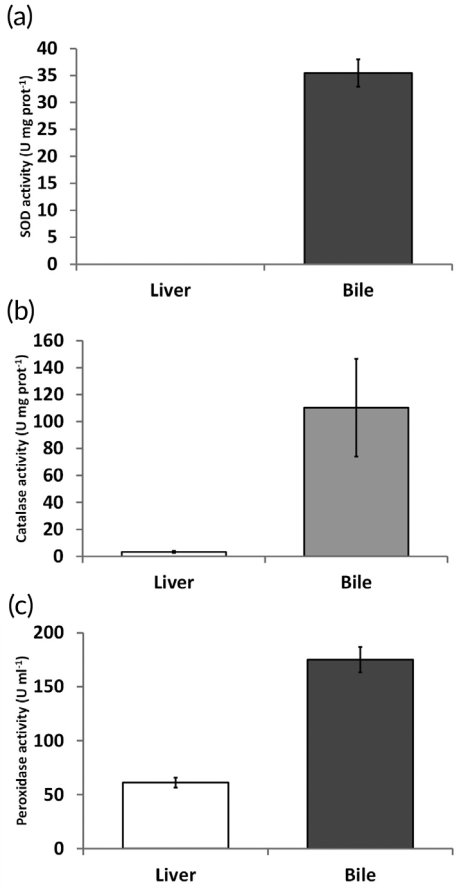

FIGURE 4 Superoxide dismutase (SOD) (U mg^{-1} of proteins) (a) catalase (U mg^{-1} of proteins) (b) and peroxidase (U mg^{-1} protein) (c) determined in liver homogenates (white) and bile (grey) of gilthead seabream. Values are means ± S.E. ($n = 9$, three replicates)

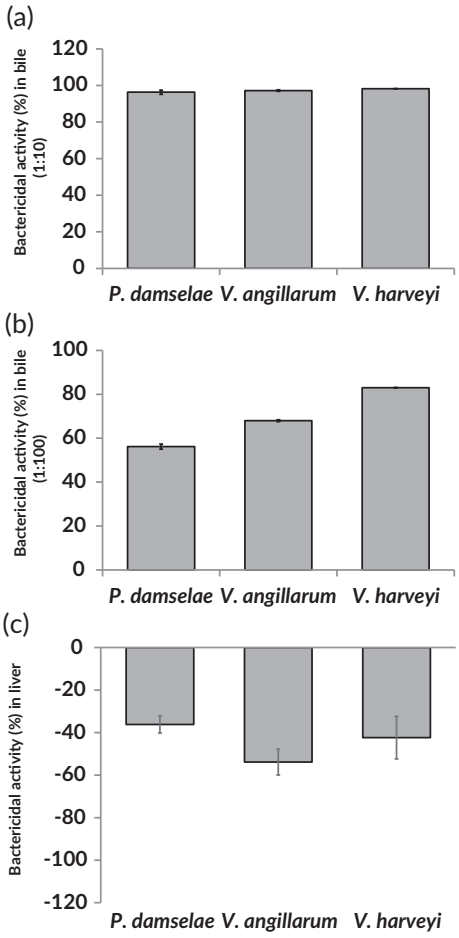

FIGURE 5 Bacteriostatic activity (%) against *Photobacterium damselae*, *Vibrio angillarum* and *Vibrio harveyi* determined in bile diluted 1:10 (a) or 1:100 (b) in saline phosphate buffer and liver homogenates (c) of gilthead seabream. Values are means ± S.E. ($n = 9$, three replicates)

critical clinical situations such as liver damage or regeneration after major surgery, or the maintenance of the liver itself during transplantation (Thorgersen *et al*., 2019). Similarly, the complement system has a crucial role in fish, not just to alert the host to potential pathogens (bacteria, viruses, fungi and parasites) but also in their elimination. In addition, activation of the complement system can imply the triggering of the acquired immune response (Boshra *et al*., 2006). Of interest in our study, natural haemolytic complement activity was only detected in gilthead seabream bile but not in liver homogenates. It might be that these results denote a tight regulation of the complement system in the liver. Moreover, under 'normal' conditions, their proteins are in an inactive form, but a specific signal can cause the first to be activated, followed by the other proteins in the cascade. Conversely, lysozyme (or 1,4-β-*N*-acetylmuramidase) is a cornerstone of innate immunity with several functions, particularly those that are anti-inflammatory and antibacterial (Sahoo *et al*., 2012). Although lysozyme is an enzyme present in various mammal body fluids and external body secretions, such as tears, saliva and gastric juices (Sahoo *et al*., 2012), in the present study lysozyme was not detected in either liver homogenates or bile of gilthead seabream. Interestingly, lysozyme is present in gilthead seabream skin mucus at levels similar to those observed in serum (Guardiola *et al*., 2014). These results support the fact that the main functions attributed to complement and lysozyme could be carried out in the liver and bile by other molecules, such as bile acids, which will be discussed below. In mammals, five isotypes of Ig (IgM, IgD, IgG, IgA and IgE) have been described, with the predominant Ig in bile being secretory IgA, while IgM and IgG exist at much lower levels (Kagnoff, 1987). However, in lower vertebrates, a smaller number of antibody classes have been reported. Thus far, three different Ig heavy chain isotypes have been identified among teleost fish: IgM, IgD and IgT/Z (Mashoof & Criscitiello, 2016). In the present study, only IgM was studied due to the availability of specific antibodies. The results confirm that IgM, which is the most abundant and widely distributed Ig in fish, was present in the liver and bile. Although the titre of IgM was higher in bile than in liver, its level in bile was more variable among specimens than in liver. Perhaps this can be explained by the fact that continuous exposure to blood antigens or intestinal microbial products suffered by liver cells causes each of the individuals studied to present a different local immunological environment, which was already suggested by Bode *et al*. (2012).

In agreement with the present study, the presence of IgM and specific antibodies for a nematode was detected in the bile of rockcod (*Trematomus bernacchii*) (Abelli *et al*., 2005; Coscia & Oreste, 2000). Likewise, immunization of European eel (*Anguilla anguilla* L.) increased the bile antibody titres (Nagel *et al*., 2012). By contrast, a study of Atlantic cod (*Gadus morhua* L.) could not demonstrate the existence of antibodies in the gall bladder (Lund *et al*., 2006). Because of the scarce results on this topic, future studies are needed to demonstrate whether other Ig types are also present in fish liver and bile.

Different enzymes with a putative role in immune functions were also included in this study. Among them, proteases, antiproteases, esterase and alkaline phosphatase were considered. In general, the obtained values were low when compared with the values of these same activities in gilthead seabream skin mucus and serum (Guardiola *et al*., 2014). Proteases form a large family of protein-cleaving enzymes with a crucial role in many processes related to the immune system, such as inflammation, cell renewal and apoptosis (Heutinck *et al*., 2010). Proteases exert their protective function against pathogens both directly, by being able to directly break their proteins (Subramanian *et al*., 2007), and/or indirectly, by preventing or hindering the mechanisms involved in colonization and invasion (Aranishi *et al*., 1998). Another important role related to immunity is in the production of other innate immune components, such as complement, Igs or antibacterial peptides (Hjelmeland *et al*., 1983). Proteolysis also contributes to defence systems, as the recognition of peptide fragments of foreign proteins triggers the immune response, being the proteases, the enzymes involved in these important reactions (Barrett, 2001). Perhaps these significant functions of proteases explain the low levels detected in antiproteases, which should be low enough to maintain the proteases present in liver homogenates or bile activity. Conversely, the esterase family of enzymes (hydrolyse ester bonds) is the reason why they are involved in many detoxification reactions. They could also be involved in the survival, colonization and virulence of some pathogens (Kahya *et al*., 2017). Nonetheless, further studies are needed to elucidate the precise function of phosphatases and esterases because the possible relationships of these enzymes with immunity are not yet well understood (Nigam *et al*., 2012). New data from fish challenged by some stressors or pathogens is desirable to be able to determine whether (under these conditions) the levels of these enzymes increase in liver homogenates, bile or both, as occurs in fish serum and skin mucus during tissue regeneration (Rai & Mittal, 1991), and stressor agents and parasitic infection (Ross *et al*., 2000). Furthermore, esterase and alkaline phosphatase also increased in fish skin mucus after immunostimulant administration (Sheikhzadeh, Heidarieh, *et al*., 2012b; Sheikhzadeh, Karimi Pashaki, et al., 2012a). These data could help to determine the importance of such enzymes in gilthead seabream liver and bile.

Reactive oxygen and nitrogen species (RONS) are produced by several endogenous and exogenous processes, and their negative effects are neutralized by antioxidant defences (Liguori *et al*., 2018). To control the formation of RONS, as well as to repair oxidative damage to macromolecules and tissues, aerobic organisms possess a complex armoury of antioxidants (Halliwell & Gutteridge, 1999). This includes SOD, peroxidase and catalase enzymes that scavenge RONS. The results presented here indicate that gilthead seabream live homogenates demonstrate low levels of the three antioxidant enzymes tested (SOD, catalase and peroxidase), while their presence was higher in bile than in liver. However, it is known that bile distributes antioxidants throughout the gut. Other substances may be present that perform this function. In mammals, this is the case for bile pigments, biliverdin and bilirubin, which are reducing species and hence potential antioxidants formed by the action of heme oxygenase and biliverdin reductase (Perez & Briz, 2009; Stocker, 2004). However, the precise role and contribution of gilthead seabream bile acids as cellular antioxidants deserve further investigation. Furthermore, other natural bile acids (or their derivatives) could arouse pharmacological interest owing to their expected protective properties.

The liver is in close contact with the gut, which (from an immunological perspective) is one of the main components of mucosa-associated lymphoid tissue (MALT). The gut health of farmed teleost fish is challenged by a number of factors, including microbial infection, nutritional and environmental stress, and other host-related factors (Zhang *et al*., 2020). These factors can alter or attack the intestinal epithelium and then induce intestinal mucosal barrier damage and inflammation among farmed fish (Torraca & Mostowy, 2018). An altered gut mucosal provides an entry portal for opportunistic or pathogenic microorganisms, most of which are bacteria (Gao *et al*., 2016). In the present study, the bacteriostatic activity on liver homogenates and bile of gilthead seabream was determined. As expected, potent bacteriostatic activity was detected on bile. Bile salts are known to exert a direct antimicrobial activity in the intestinal lumen based on their detergent property and have an influence on the gut microbiota. Besides bile acids, other antimicrobial compounds could be present in fish liver and bile, among them antimicrobial peptides (AMP), which are also very important as components of the innate immune system in metazoans. They prevent the colonization of opportunistic and pathogenic microorganisms, and have been studied in the liver from several fish species (Maier *et al*., 2008; Wang *et al*., 2007). Interestingly, the presence and spectra of the activity of AMPs were demonstrated in 13 tissues (mucus, gills, skin, intestine, rectum, head kidney, spleen, blood, gall bladder, liver, ovary, muscle and peritoneal wall) of Atlantic cod and the most potent activity was obtained in the gall bladder, which had a very strong antimicrobial activity against Gram-positive and Gram-negative bacteria, including the cod pathogen *V. anguillarum* (Ruangsri *et al*., 2010).

While very little is known about the antimicrobial components present in the gall bladder of teleost fish, the complex interactions between bile salts, gut microbiota and hepatic innate immunity are being considered in mammals (Schubert *et al*., 2017). Although pathogenic microorganisms should be efficiently eliminated, the large number of antigens derived from the gastrointestinal tract should be tolerized. From experimental observations, there is clear evidence that the liver favours the induction of tolerance rather than the induction of immunity (Knolle & Gerken, 2000). In terms of interaction between fish gut and liver, bile could provide different immune proteins to the intestinal mucus, which could be involved in facilitating immune tolerance that characterizes the gut of vertebrates, or in the activation of

an inflammatory response or a disease (Rombout *et al*., 2014). Acid-activated receptors regulate innate immunity by the modulation of the intestinal and liver innate immune system and contribute to the maintenance of a tolerogenic phenotype in enterohepatic tissues (Fiorucci *et al*., 2018). These highly complex mechanisms are perhaps the reason for the limited number of oral vaccines presently approved for use in humans and veterinary species, which clearly indicates that the development of effective and safe oral vaccines remains a challenge for immunologists. The insufficient efficacy of oral vaccines is partly due to antigen breakdown in the harsh gastric environment, but also to the high tolerogenic gut environment, as well as inadequate vaccine design (Embregts & Forlenza, 2016). Recently acquired knowledge of fish mucosal immunology could be used to develop effective mucosal vaccines in the near future.

To conclude, the current study reveals that gilthead seabream liver homogenates has low levels of immune parameters (only IgM), enzymes related to immunity (protease, esterase and alkaline phosphatase activities) and antioxidant enzymes, as well as almost absent bacteriostatic activity. By contrast, although lysozyme and antiprotease activity was undetected, the remaining activities studied were present in bile samples—some in very high concentrations—such as IgM, protease activity and, especially, bacteriostatic activity against important fish pathogenic bacteria. These results seem to suggest that bile should be considered a key component of the innate immune system of gilthead seabream. This preliminary study contributes to understanding the normal values of these important immune activities present in gilthead seabream bile and will enable comparison with those values present in fish under different environmental situations, and infection and disease conditions.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

M.Á.E. designed the experiment. D.G.S. performed the experiment and data generation. D.G.S analysed the experimental data. D.G.S. and M.Á.E. wrote the draft. D.G.S, A.C. and M.Á.E reviewed the manuscript, and M.Á.E manage the funding. All authors have approved the final version of the manuscript.

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