
# UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

# Aromatic Plants as Additives for Farmed Fish Diet: Effects on the Immune System, Stress and Metabolism

# Plantas Aromáticas como Aditivos de la Dieta de Peces de Acuicultura: Efectos sobre el Sistema Inmunitario, el Estrés y el Metabolismo

**D. José María García Beltrán**

**2019**

“Dadme un punto de apoyo y moveré el mundo”

Arquimedes de Siracusa (287 a.C. – 212 a.C)

# Agradecimientos

Bueno, esta es esa parte de la Tesis que todo estudiante de doctorado quiere escribir debido a la oportunidad de expresar todos los sentimientos que afloran después de años de duro trabajo. Y es que esta Tesis Doctoral representa el fin de una laboriosa etapa de mi vida en la que he obtenido muchísima experiencia y conocimientos, pero del mismo modo, también representa el comienzo de otra etapa llena de ilusiones.

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"Dadme un punto de apoyo y moveré el mundo"

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# PROJECTS

This dissertation is submitted in fulfilment of the requirements for the degree of *Philosophiae Doctor* (PhD) at the Faculty of Biology, University of Murcia (UMU), Spain. The different studies compiled in this dissertation represent original research carried out over a period of around four years, as part of some projects and grants:

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# PUBLICATIONS

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García Beltrán J.M., Espinosa C., Guardiola F.A., Esteban M.A. (2017). Dietary dehydrated lemon peel improves the immune but not the antioxidant status of gilthead seabream (*Sparus aurata* L.). Fish and Shellfish Immunology, 64, 426-436.

García Beltrán J.M., Espinosa C., Guardiola F.A., Esteban M.A. (2018). *In vitro* effects of *Origanum vulgare* leaf extracts on gilthead seabream (*Sparus aurata* L.) leucocytes, cytotoxic, bactericidal and antioxidant activities. Fish and Shellfish Immunology, 79, 1–10.

García Beltrán J.M., Espinosa C., Guardiola F.A., Manuguerra S., Santulli A., Messina C.M., Esteban M.A. (2019). Effects of dietary dehydrated lemon peel on some biochemical markers related to general metabolism, welfare and stress in gilthead seabream (*Sparus aurata* L.). Aquaculture research, 00, 1–11.

## Other publications/collaborations (3):

Mansour A.T., Miao L., Espinosa C., García Beltrán J.M., Ceballos D.C., Guardiola F.A., Esteban M.A. (2018). Effects of dietary inclusion of *Moringa oleifera* leaves on growth and some systemic and mucosal immune parameters of seabream. Fish Physiology and Biochemistry, 44, 1223-1240.

Espinosa C., García Beltrán J.M., Esteban M.A, Cuesta A. (2018). *In vitro* effects of virgin microplastics on fish head-kidney leucocyte activities. Environmental Pollution, 235, 30-38.

de Mattos B.O., López-Olmeda J.F., Guerra-Santos B., Espinosa C., García Beltrán J.M., Esteban M.A., Sánchez-Vázquez F.J., Fortes-Silva R. (2019). Coping with exposure to hypoxia: modifications in stress parameters in gilthead seabream (*Sparus aurata*) fed spirulina (*Arthrospira platensis*) and brewer's yeast (*Saccharomyces cerevisiae*). Fish Physiology and Biochemistry. Doi: 10.1007/s10695-019-00677-8.

## Works submitted to conferences or congresses (15)

García Beltrán J.M., Espinosa C., Cordero H., Esteban M.A. (2016). Dehydrated lemon bark as dietary additive in gilthead seabream (*Sparus aurata* L.) Poster. *VI Congreso Ibérico de Ictiología (SIBIC)*. Murcia (España).

Mansour A., Miao L., Espinosa C., García Beltrán J.M., Ceballos D., Esteban M.A. (2017). The intestinal immune response (inflammation, tight junction protein and humoral immune genes expression) of gilthead seabream *Sparus aurata* fed moringa leaves supplemented diets. Oral. *Asian-Pacific Aquaculture*. Kuala Lumpur (Malaysia).

García Beltrán J.M., Esteban M.A. (2017). Plantas como aditivos en la dieta de peces marinos: efectos en el sistema inmunitario y el estrés. Oral. II *Congreso de Biodiversidad y Conservación de la Naturaleza.* Almería (España).

García Beltrán J.M., Espinosa Ruiz C., Esteban Abad M.A. (2017). Efectos de extractos de orégano y verdolaga en la línea celular SAF-1 y leucocitos de dorada. Poster. *XVI Congreso Nacional de Acuicultura.* Zaragoza (España).

Chaves Pozo E., Miao L., Campo V., Faggio C., Valero Y., García Beltrán J.M., Esteban Abad M.A., Cuesta A. (2017). Implicación de granzimas en la respuesta inmune de peces frente a nodavirus (NNV). Poster. *XVI Congreso Nacional de Acuicultura*. Zaragoza (España).

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García Beltrán J.M., Mahdhi A., Esteban M.A. (2018). Possible applications of date palm (*Phoenix dactylifera* L.) seeds in fish aquaculture. Oral. *VII Congreso Ibérico de Ictiología (SIBIC)*. Faro (Portugal).

García Beltrán J.M., Esteban M.A. (2018). *In vitro* cytotoxic, bactericidal and antioxidant activity or oregano and purslane aqueous and ethanolic extracts and its possible application in aquaculture. Poster. *World Aquaculture Society (AQUA)*. Montpellier (France).

Esteban M.A., Rossi B, García Beltrán J.M., Cuesta A., Tugnoli B., Piva A., Grilli E. (2018). *In vitro* study of a dose -and time- effect response of a mixture of organic acids and nature identical compounds on gilthead seabream head-kidney leucocytes. Oral. *World Aquaculture Society (AQUA)*. Montpellier (France).

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García Beltrán J.M., González-Silvera D., Mahdhi A., Esteban M.A. (2019). Efecto de la administración de hueso de dátil (*Phoenix dactylifera* L.) en dietas de dorada. Poster. *XVII Congreso Nacional de Acuicultura*. Cartagena (España).

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABTS | 2,2'-azino-bis-3-(ethylbenzothiazoline-6-sulphonic acid) |
| ADF | Acid detergent fiber |
| ADL | Acid detergent lignin |
| ALT/GPT | Alanine transaminase-glutamic pyruvic transaminase |
| AST/GOT | Aspartate aminotransferase-glutamic oxalacetic transaminase |
| ATP | Adenosine triphosphate |
| BHT | Butylated hydroxytoluene |
| BSA | Bovine serum albumin |
| CAT | Catalase |
| cDNA | Complementary DNA |
| COX | Cyclooxygenase |
| db | Dry matter basis |
| DLP | Dehydrated lemon peel |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| DPS | Date palm seeds |
| ECPs | Extracellular products |
| EU | European Union |
| FAO | Food and Agriculture Organization of the United Nations |
| FCS | Foetal calf serum |
| FDA | Food and Drug Administration |
| FITC | Fluorescein isothiocyanate |
| FRAP | Ferric reducing ability of plasma |
| GALT | Gut-associated lymphoid tissue |
| GR | Glutathione reductase |
| GSFPx | Glutathione peroxidase |
| $H_2O_2$ | Hydrogen peroxide |
| HBSS | Hank's balanced salt solution |
| HK | Head-kidney |
| HSP | Heat shock proteins |
| HSPs | Small heat shock proteins |
| Ig | Immunoglobulin |
| iNOS | Inducible nitric oxide synthase |
| K | Condition factor |

| | |
|---|---|
| LPS | Lipopolysaccharide |
| MENA | Middle East and North Africa |
| mRNA | Messenger RNA |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NDF | Neutral detergent solution |
| NICs | Nature identical compounds |
| NOx | NADPH oxidase |
| $O_2^-$ | Superoxide anion |
| OD | Optical density |
| OH | Hydroxyl group |
| -OH | Hydroxil radical |
| PAMPs | Pathogen-Associated Molecular Patterns |
| PBS | Phosphate buffer saline |
| PBS-T | PBS-Tween |
| PG | Peptidoglycan |
| PMA | Phorbol myristate acetate |
| qPCR | Real-time PCR |
| $-ROO^-$ | Peroxyl-radical |
| ROS | Reactive oxygen species |
| RNA | Ribonucleic acid |
| RNS | Reactive nitrogen species |
| RT | Room temperature |
| SGR | Specific growth rate |
| SOD | Superoxide dismutase |
| TAA | Total antioxidant activity |
| TBARS | Thiobarbituric acid reactive substances |
| TCA | Trichloroacetic acid |
| Th | Lymphocytes T helper |
| TLRs | Toll-like receptors |
| TMB | 3,3',5,5'-tetramethylbenzidine hydrochloride |
| Treg | Lymphocytes T regulators |
| US | United States |

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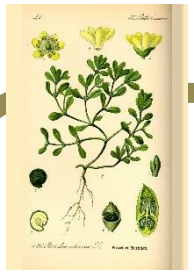

*In vitro*

## Chapter I

immunostimulant,
cytotoxic,
bactericidal
& antioxidant activities
of oregano
date palm,
purslane &
moringa extracts


II.A.

II.B.

II.C.

*Dietary*

immunostimulant & antioxidant activities
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# ABSTRACT

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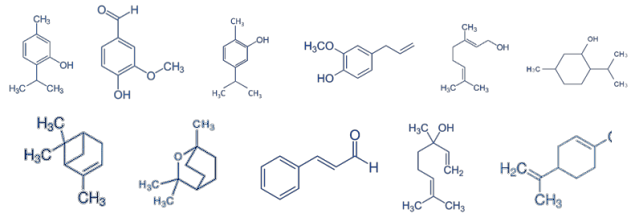
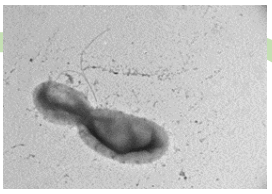
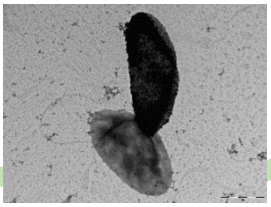




*V. harveyi*

*V. anguillarum*



*In vitro*
immunostimulant & antioxidant activities

*V. harveyi*
challenge

### III.B. *In vivo*
Immunostimulant
& antioxidant status

# SUMMARY

Aquaculture is the fastest growing animal food production sector worldwide, which has increased during the last decades and is expected to continue doing it contributing in the year 2030 almost two thirds of fish for food worldwide. However, this great activity that takes place in the fish farms also entails some problems among which are a large number of fish per cage, the worsening of the quality of the water, the handling of fish and the appearance of wounds, which implies the appearance of stress and negative effect on the immune system, provoking immunosuppression and the appearance of infections that can lead to the death of fish and therefore cause large economic losses. That is why fish are vaccinated and antibiotics have been used until a few years ago with the aim of improving the fish immune system to treat or prevent diseases or infections and their mortality, although both treatments may have negative effects in animals, humans and environment.

In this sense, phytotherapy, that is the use of medicinal plants for the prevention or treatment of a wide variety of diseases and infections, or to maintain good health, has become a very good and interesting alternative to the use of antibiotics due to biological compounds they contain (phytochemicals), which can replace them as immuno-prophylactic agents to stimulate the immune system and also do not present negative impact on animals, humans or the environment.

The aim of this Doctoral Thesis is to determine if some medicinal plants, or compounds present in them are possible for direct application in fish aquaculture, and in particular of gilthead seabream. To get our objective we divided this Doctoral Thesis in 3 parts or chapters.

First part (Chapter 1): we studied the *in vitro* effect of different concentrations (0.001 – 1 mg/mL) of aqueous and ethanolic extracts from four different plants (oregano, date palm, purslane and moringa) on several fish cell types and against bacteria as well as their antioxidant activity.

Toxicity and immunostimulatory capacity of plant extracts on gilthead seabream head-kidney (HK) leucocytes and a fibroblast cell line (SAF-1) was evaluated obtaining that adequate concentrations showed immunostimulatory capacity and promoted cell division. Afterwards, we demonstrate that plant extracts are able to inhibit the growth of tumor cells, as indicated by the toxicity against a fish hepato-carcinoma cell line (PLHC-1), as well as

their strong antibacterial activity against three pathogenic fish bacteria (*Vibrio harveyi*, *V. anguillarum* and *Photobacterium damselae* subsp. *piscicida*). Finally, antioxidant activity of plant extracts was also studied and was very high in some of the studied samples.

Second part (Chapter 2): we studied the dietary *in vivo* effect of some plants (oregano, date palm seeds and lemon peel) as feed additives on gilthead seabream. All the plant-supplemented diets failed to promote the seabream growth performance, and even one of them decreased it.

Afther that, we evaluated the effect of the addition of plants on the innate and adaptive immune system of gilthead seabream, including humoral (serum and skin mucus: immunoglobulin M (IgM) level, natural haemolytiyc complement, lysozyme, bactericidal, protease, antiprotease and peroxidase activities) and cellular (HK leucocyte phagocytic, respiratory burst and peroxidase activities) parameters. In turn, the effect of plants was also studied on the activity of liver antioxidant enzymes (glutathione reductase, superoxide dismutase and catalase) and the total antioxidant activity present in serum and skin mucus. Finally, the expression of numerous HK immune-related genes and liver antioxidant genes was also studied. Positive results were obtained at both humoral and cellular immune responses and gene expression, as well as a tendency to improve the oxidative status of the fish.

Finally, the effect of lemon peel on fish metabolism was also considered and it was observed that its addition had any negative or harmful effect in fish metabolism.

Third part (Chapter 3): we studied the *in vitro* and *in vivo* effects of compounds identical to natural (NICs) ones ("artificial phytochemicals").

First, phytochemicals showed, *in vitro*, a very strong antibacterial activity against *V. harveyi* and *V. anguillarum* but no adverse effects on gilthead seabream HK leucocyte viability or immune responses. Secondly, we tested the dietary effects of various concentrations of a mixture of NICs on gilthead seabream growth performance and immune and oxidative status after challenging with a bacterial infection or not. The mixture of NICs improved the fish growth and the immune parameters of gilthead seabream.

# INTRODUCTION

# I.1 World aquaculture

Aquaculture is the set of activities, techniques and knowledge of breeding aquatic species of plants and animals. It is an important economic activity of food production, raw materials for industrial and pharmaceutical use, and living organisms for resettlement or ornamentation, through techniques aimed at doing more efficient its performance. Aquaculture is a sector in constant growth that takes place in seawater and freshwater. Although many species of molluscs, crustaceans and other aquatic animals including amphibians and reptiles, invertebrates and marine algae are cultivated, fish aquaculture represents the half of world aquaculture production (FAO, 2018), which in 2017 exceeded to that of fishing by 18.3 million of tonnes (APROMAR, 2019) (Fig. 1).

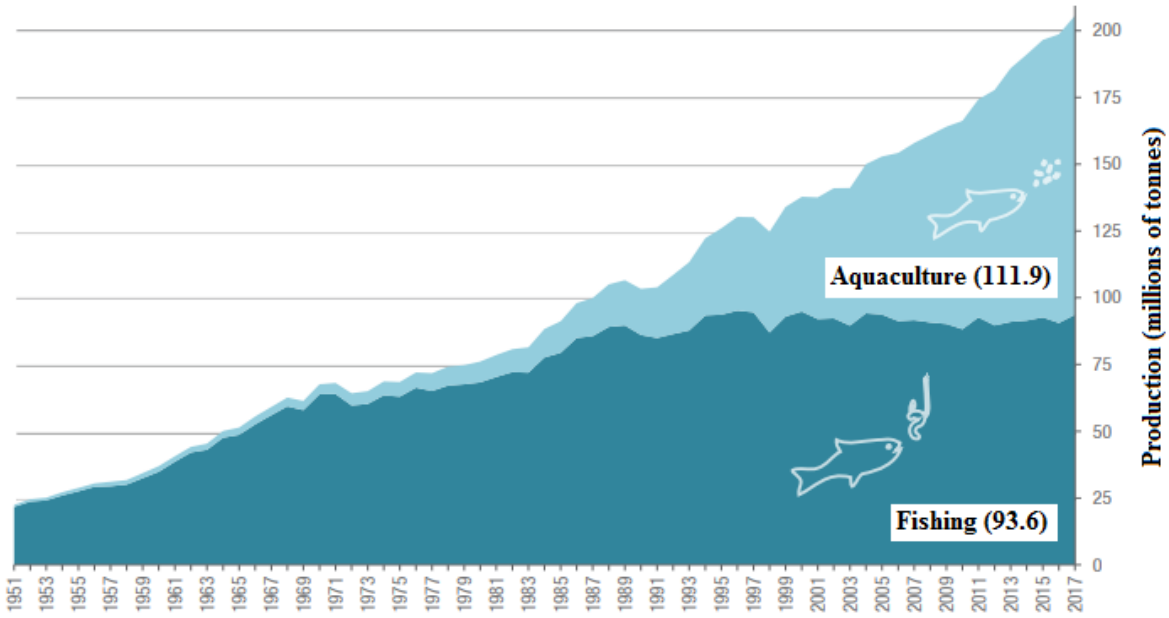



Figure 1: Evolution of world aquatic production (aquaculture plus fishing) in the period 1950-2017 (APROMAR, 2019).

Thanks to the continuous growth of aquaculture over the last decades and a stagnation of world fishing activity since the late 1980s, have led to aquaculture to be the most important source of aquatic food and nutrition (Fig. 2). Asian countries are the main producers, including China, India, Indonesia, Vietnam, Bangladesh and Philippines among others, although other non-Asian countries such as Egypt and Norway are also important producers. Another important fact to highlight is that aquaculture is even more important in

the Mediterranean and the Black Sea, where fishing catches have decreased by one third since 2007. In these regions, all hake, most red mullet, turbot, sole, seabream and small pelagic stocks are considered overfished. Due to this fact, General Fisheries Commission for the Mediterranean (GFCM) estimates that about 62.2% of fish stocks in this area are fished at unsustainable levels (FAO, 2018) (Fig. 3).

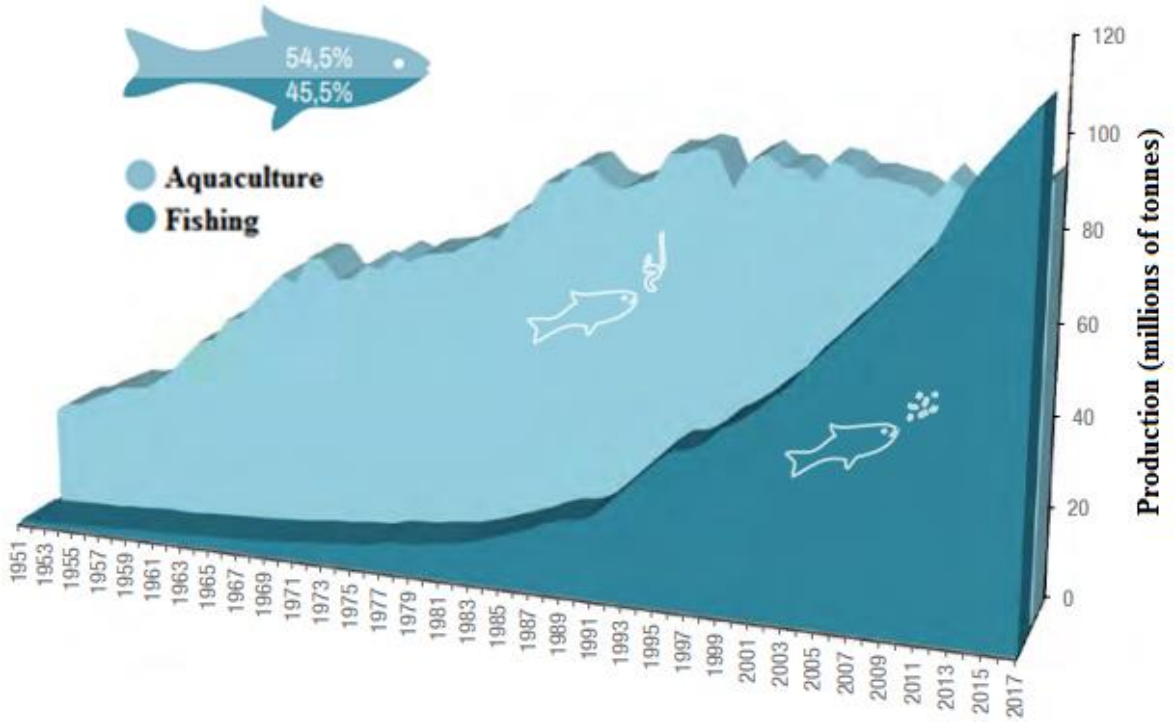



Figure 2: Evolution of world aquaculture and fishing in the period 1950-2017 (APROMAR, 2019).

In 2014, Spain ranked 22nd of world aquaculture producer (FAO, 2016) while was the first producer in the European Union (EU), with an aquaculture production of 311,032 tonnes in 2017 (APROMAR, 2019) (Fig. 4), being molluscs and especially mussels the main cultivated species and Spain the first producer. For its part, regarding fish production in 2017, Spain ranked in 3rd place of EU with a total production of 66,591 tonnes (Fig. 5). In 2018, aquaculture production in Spain was 348,395 tonnes, and the more cultivated species were mussels (273,600 tonnes), European sea bass (22,460 tonnes), rainbow trout (18,856 tonnes) and gilthead seabream (14,930) (APROMAR, 2019) (Fig. 6). Within Spain, the Region of Murcia, which exclusively produces fish, is the principal producer in terms of coastal territory and focuses its production on European sea bass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and red tuna (*Thunnus thunnus*) (MAPAMA, 2017).

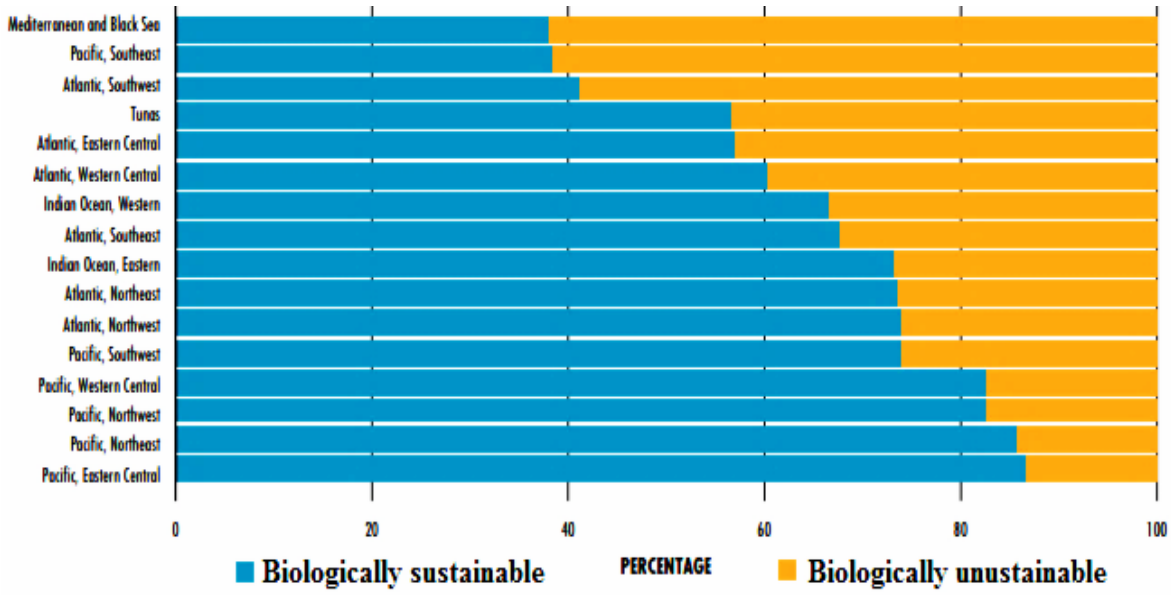



Figure 3: Percentages of stocks fished at biologically sustainable and unsustainable levels in 2015. (FAO, 2018).

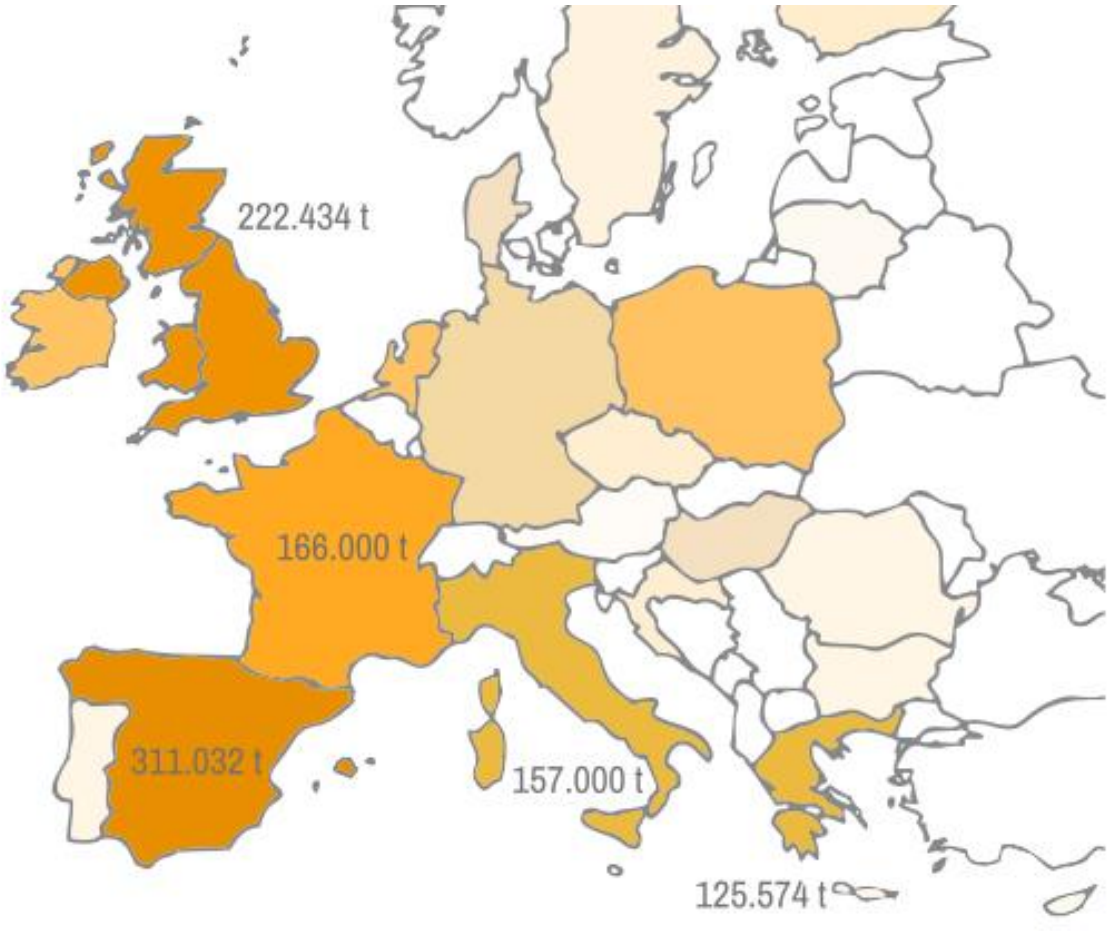



Figure 4: Distribution of aquaculture production in the Member States of the European Union by their quantity (tonnes) in 2017 (APROMAR, 2019).

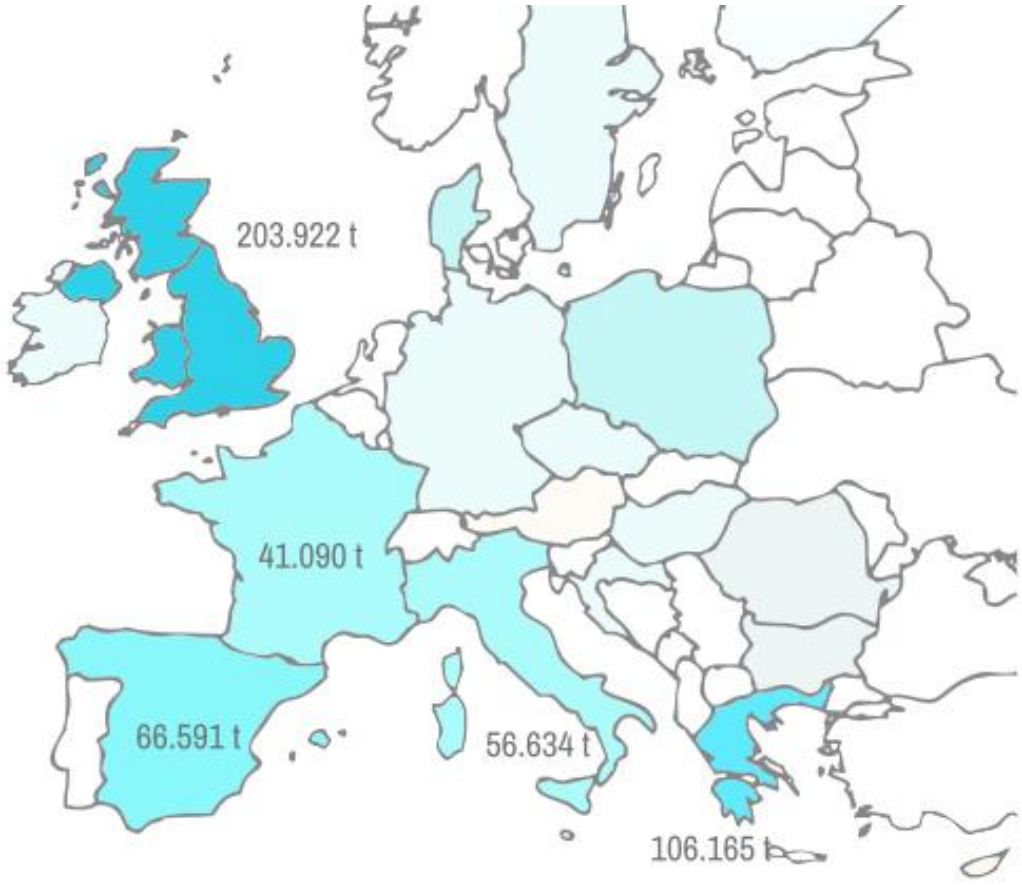



Figure 5: Distribution of aquaculture fish production in the Member States of the European Union by volume (tonnes) in 2017 (APROMAR, 2019).

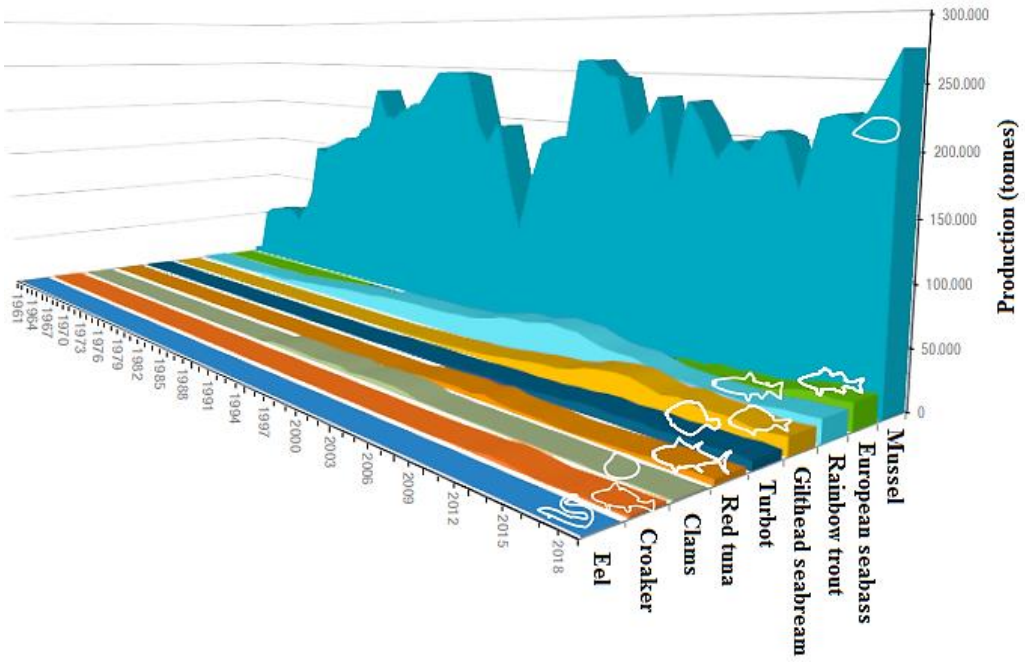



Figure 6: Evolution of the production of aquaculture in Spain, in tonnes and by species, in the period of 1960-2018 (APROMAR, 2019).

Consumption of aquatic products worldwide has exceeded 20 Kg/person-year thanks to the development of aquaculture. In the EU and Spain, the media of aquatic products consumption is almost 25 Kg/person-year, representing the consumption of fresh fish the 45% in Spain (APROMAR, 2019). The importance of aquaculture is also increased by noting that in 2013-2015, fish accounted almost a quarter (20%) of animal protein in the total intake of the world population and 7% of all proteins consumed. Fish in the diet is a rich source in proteins of easy digestion and high quality that contain all the essential amino acids, provides essential polyunsaturated long chain omega-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid), vitamins (D, A and B) and minerals (including calcium, iodine, zinc, iron and selenium). The content of unsaturated fatty acids of fish provides health benefits against cardiovascular diseases and help to the development of the nervous system, including the brain (FAO, 2018).

# I.2 Gilthead seabream

Gilthead seabream (*S. aurata*) is a teleost fish (bonny fish) that belongs to the order *Perciformes*, family *Sparidae*. It is naturally distributed in all the Mediterranean Sea, the Black Sea, and in the Eastern Atlantic Ocean, from the British Isles to the coast of Senegal. Gilthead seabream is a protandrous hermaphrodite species, first they are male individuals approximately 2 years (APROMAR, 2019). It is an euryaline fish species and prefers mild temperature water of about 25ºC, while cold or hot water temperatures (below 11ºC or above 30ºC, respectively) affect it. It is mainly a carnivorous species, although occasionally it can be herbivorous (Sola et al., 2007; Studer, 2015).

Its cultivation goes back to pre-roman periods (Sola et al., 2007; Studer, 2015). In terms of total production in the world, it is of vital importance because the aquaculture of this species accounts for 95.2% of its total supply. Gilthead seabream is cultivated in all the Mediterranean area and represents the 59$^{th}$ most cultivated species of world aquaculture and the 3$^{rd}$ of the EU. Within Spain, as it happens worldwide, the production of gilthead seabream takes place almost exclusively by the aquaculture industry (Fig. 7). The total production of juvenile gilthead seabream in 2018 in Europe was estimated to be 734,299 million units, where the principal producer was Turkey (260 million units), followed by Greece (250 million units), Italy (80 million units), France (65.6 million units) and Spain (37.5 million units). Regarding data of Spain, where production of juvenile gilthead seabream production is estimated of 40 million units in 2018, it is concentrated in Valencian Community, Balearic Islands, Cantabria and Andalusia. On the other hand, 20 countries have developed aquaculture production of adult gilthead seabream in 2018, with a total production of 246,839 tonnes in Europe and the rest of Mediterranean, being the main producers Turkey (83,000 tonnes, 33.6% of total production), Greece (61,000 tonnes, 24.7 %), Egypt (36,000 tonnes, 14.6 %) and Spain (14,930 tonnes, 6 %) (Fig. 8). Within Spain, where gilthead seabream aquaculture production in 2018 has been 14,930 tonnes, Valencian Community has led the production (7,806 tonnes, 52.3% of total production) followed by the Region of Murcia (3,184 tonnes, 21.3%), Canary Islands (2,380 tonnes, 16%) and Andalusia (1,560 tonnes, 10.4%) (APROMAR, 2019) (Fig. 9).

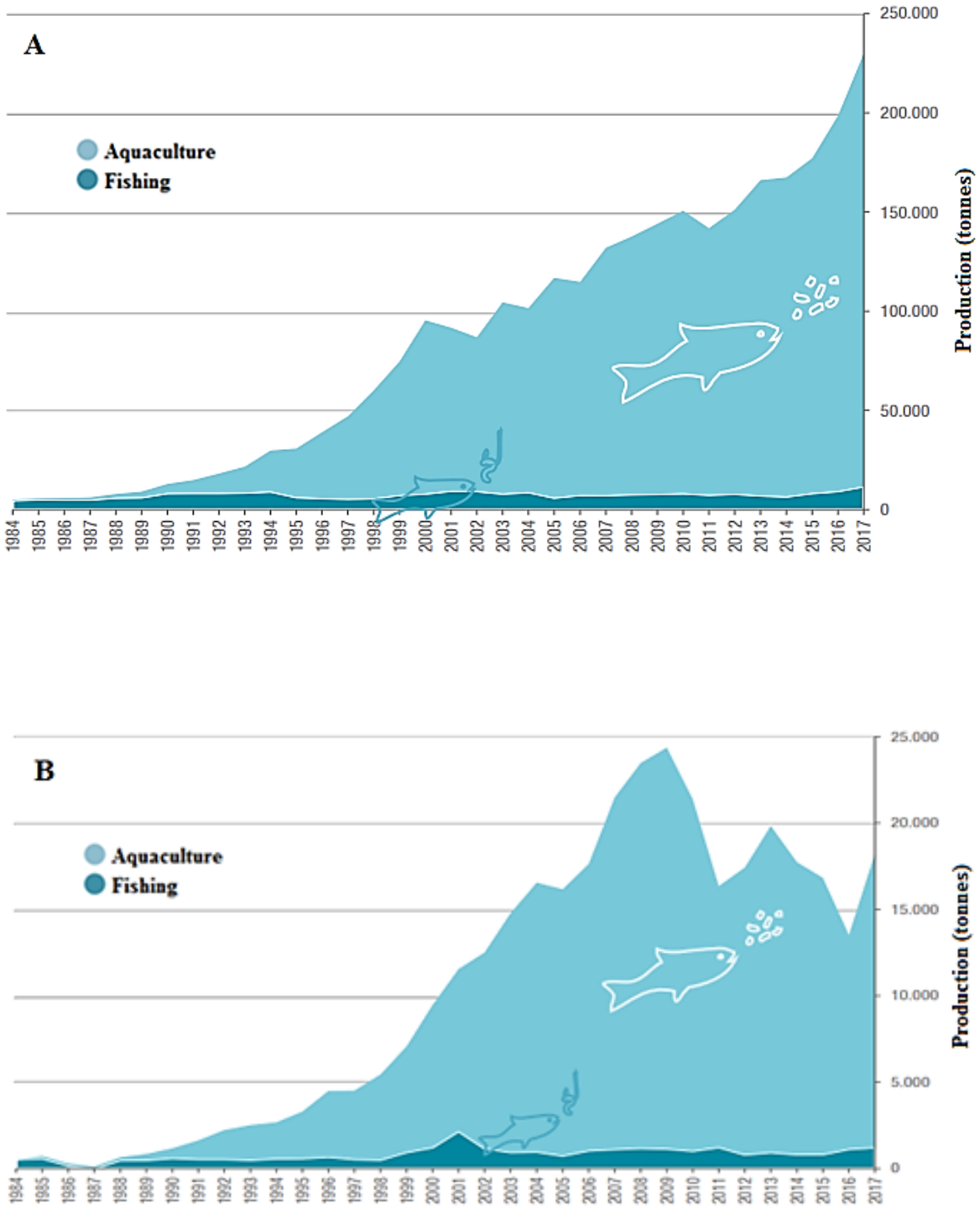



Figure 7: Evolution of gilthead seabream production through aquaculture and fishing in the period of 1984-2017. A) Worldwide. B) Spain (APROMAR, 2019).

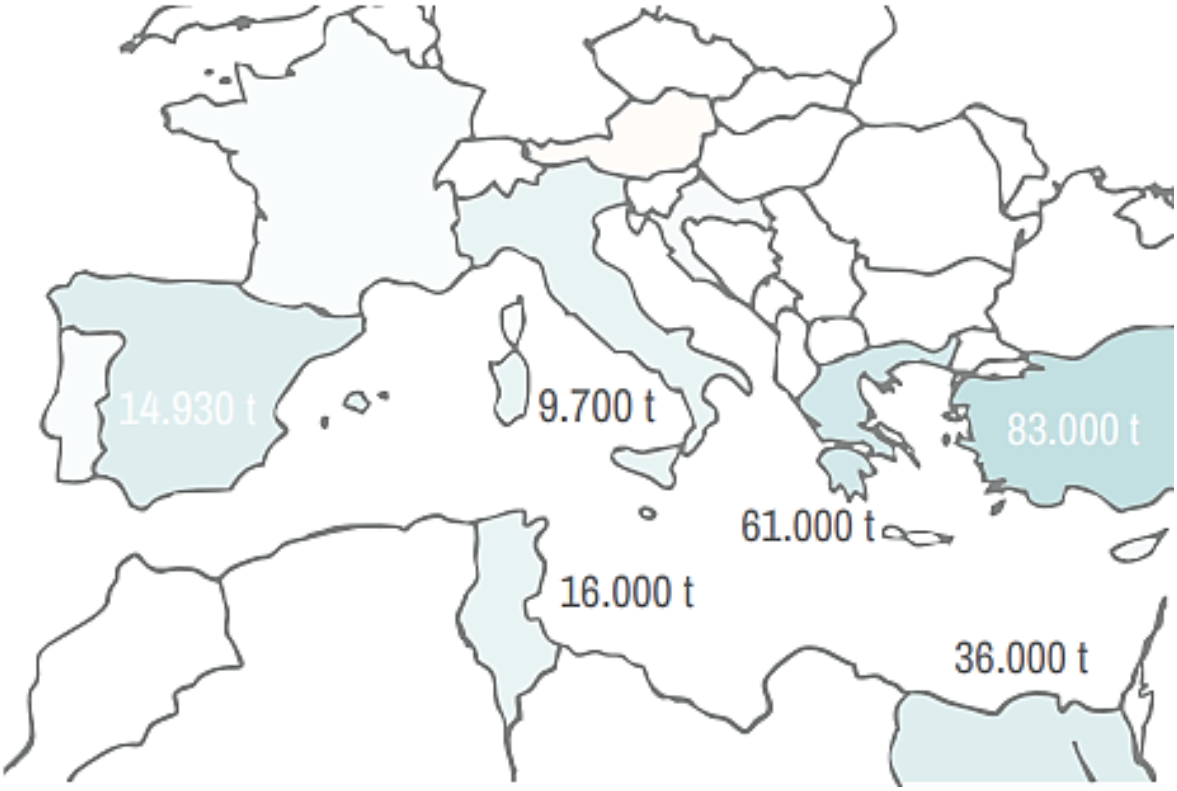



Figure 8: Distribution of gilthead seabream aquaculture production in the Mediterranean area in 2018 in volume (tonnes) (APROMAR, 2019).

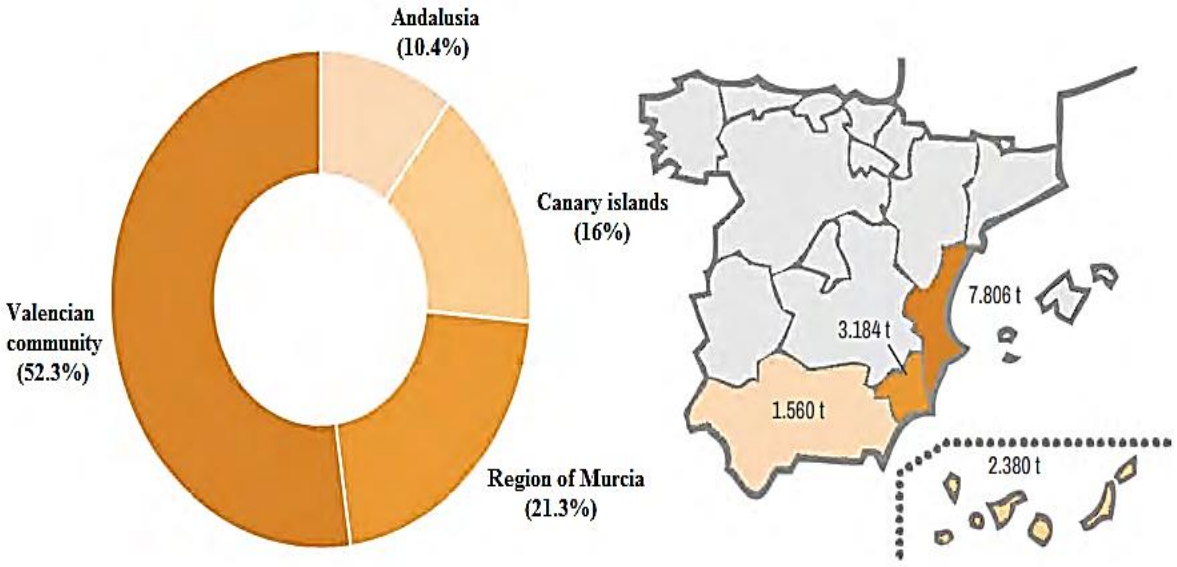



Figure 9: Distribution of gilthead seabream aquaculture production in Spain by Autonomous Community in 2018 in volume (tonnes) (APROMAR, 2019).

# I.3 Teleost immune system

The immune system is the set of structures and biological processes of an organism that allow it to protect itself from external pathogens whether they are biological (pathogenic agents) or physical-chemical (such as contaminants or radiation) agents. Fish, like all vertebrates, have specialized organs that intervene in the immune response (Fig. 10) (for review see Tort et al., 2003; Uribe et al., 2011; Esteban, 2012; Kiron, 2012; Rauta et al., 2012). The anterior, cephalic or head-kidney (HK) and the thymus are the primary lymphoid organs. The HK is the main hematopoietic organ and is the equivalent to the bone marrow of mammals (Meseguer et al., 1995; Zapata et al., 2006). In the HK there are granulocytes, macrophages and T and B cells, and it is the main site of antibody production (Press et al., 1994; Zwollo et al., 2005; Whyte, 2007). The thymus is the main place of production of T cells (Davis et al., 2002), although it also contains macrophages, myeloid cells and granular eosinophilic cells (Zapata et al., 2006). In addition to the primary lymphoid organs, the fish also have secondary lymphoid organs. The spleen is a secondary organ and plays an important role in hematopoiesis, antigen degradation and antibody production (Manning and Nakanishi, 1996).

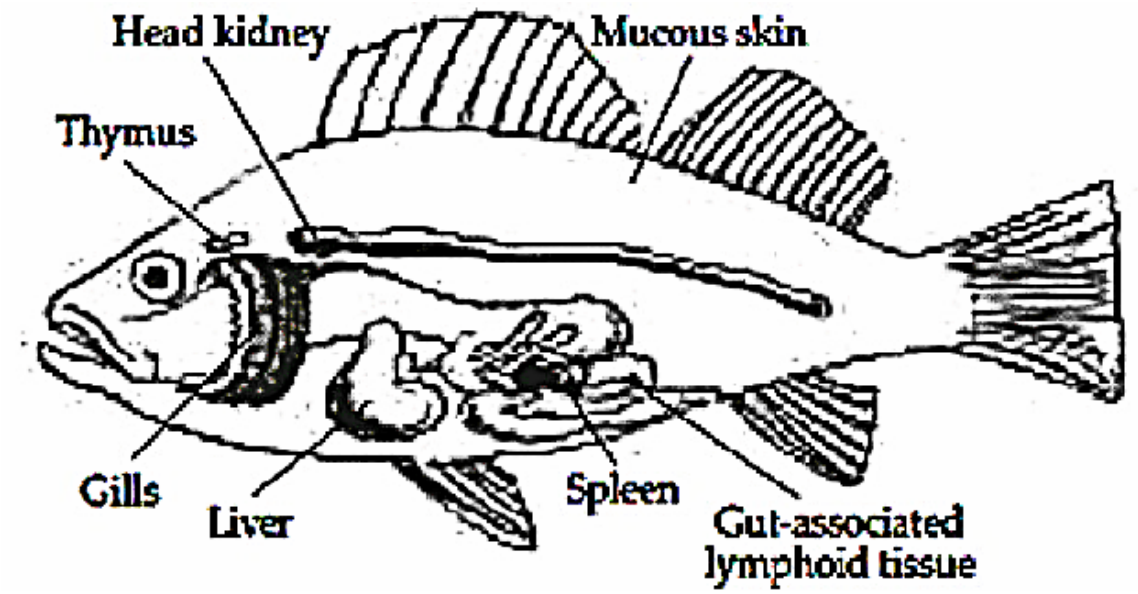



Figure 10: Immune organs present in fish (Tort et al., 2003).

Together with cellular and humoral factors, fish immunity is composed by physical barriers (Magnadottir, 2006, 2010), which represent other secondary organs known as mucosal-associated lymphoid tissues (MALT). Fish are the oldest group of vertebrates that have this kind of defence, which is composed of skin- (SALT), gut- (GALT), gill- (GIALT) and nasal-associated lymphoid tissue (NALT) (Salinas et al., 2011, 2015) (Fig. 11). These tissues form the barriers of the fish, which are part of the immune system and constitute the first line of defence between the organism and the environment that surrounds it, preventing the entry of pathogens. These tissues are composed of cellular and humoral components of both the innate and adaptive immune systems (Boyton and Openshaw, 2002; Bruce at al., 2002; Magnadottir, 2010).

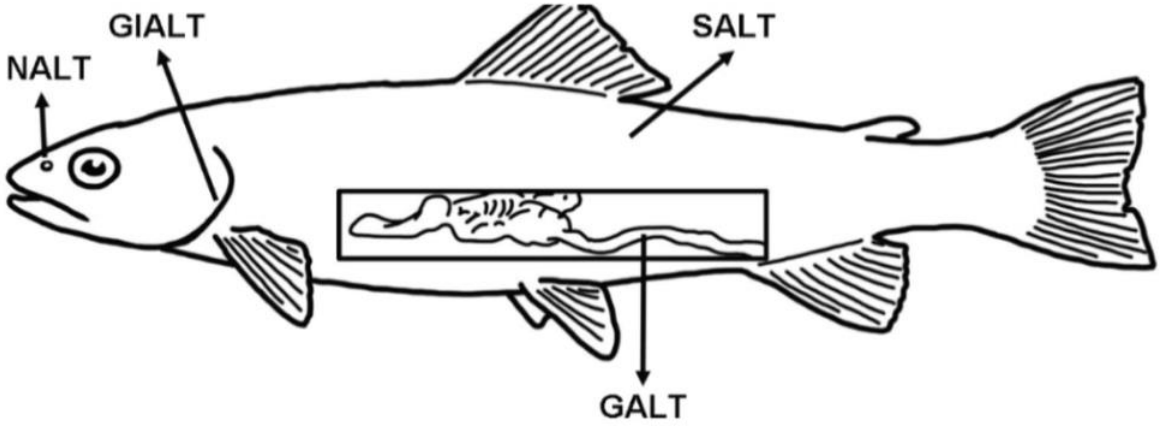



Figure 11: Physical immunological barriers present in fish (Salinas, 2015).

The teleost fish, like all the gnatostomates, have an innate immune system (natural or non-specific) highly conserved and an adaptive immune system (acquired or specific) less evolved, existing a cooperation between both and being the innate immune system the main defensive system of the fish (Tort et al., 2003). The innate immune system defends the organism in a non-specific manner that is, recognizing pathogens in a generic way (Kurtz, 2005) through the recognition of molecular patterns associated with pathogens (Medzhitov and Janeway, 1998; Magnadottir, 2006). The cellular factors that make up this immune system are leucocytes including, among others, monocytes/macrophages, granulocytes (neutrophils, eosinophils and basophils) and non-specific cytotoxic cells (NCCs) (Frøystad et al., 1998; Seternes et al., 2002; Li et al., 2006; Reite and Evensen, 2006).

As for humoral factors, which are secreted to serum and mucus, are among many others complement system, lysozyme (Yano, 1996; Saurab and Sahoo, 2008), antibacterial peptides (Ellis, 2001; Maier et al., 2008), proteases and antiproteases (Bowden et al., 1997; Bayne and Gerwick, 2001). All these components have an important role in innate immune defence protecting organisms against bacteria and viruses (Uribe et al., 2011; Rauta et al., 2012).

In addition to the innate immune system, fish are the first vertebrate group to develop an adaptive immune system (Warr, 1995), which confers a specific response for each pathogen and a greater speed and efficiency in the elimination of pathogens in recurrent infections through of memory generation (Mayer, 2006) (Fig. 12).

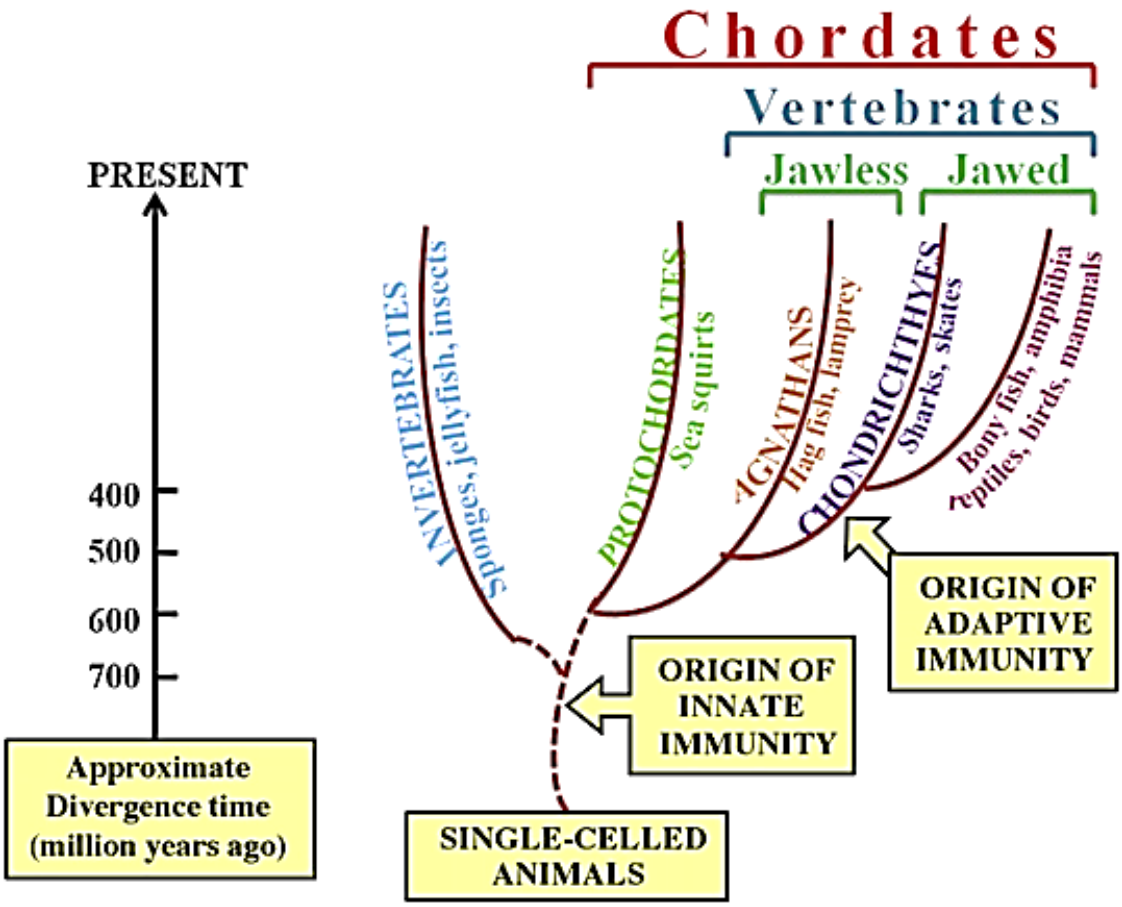



Figure 12: Evolution of immune system (Rauta et al., 2012).

Like the innate immune system, the adaptive system is formed by cellular and humoral factors. Among the cellular factors, the most important are B lymphocytes, which produce immunoglobulins (Igs or antibodies), and T lymphocytes, which mediate the immune response by eliminating infected cells by intracellular pathogens (Laing and Hansen, 2011). On the other hand, the humoral factors are Igs, which are IgM (the most abundant), IgD (Acton et al., 1971; Wilson et al., 1997; du-Pasquier and Litman, 2000; Janeway et al., 2005; Ohta and Flajnik, 2006), and IgT/IgZ (predominant in mucosal secretions) (Hansen et al., 2005).

Finally, cytokines are secreted by cells involved in the immune response and collaborate in both, innate and adaptive immunity. Some of these cytokines are pro-inflammatory, and are involved in the development of the inflammatory response against pathogens through the activation of macrophages and T lymphocytes, while other cytokines are anti-inflammatory and help to control the activation of the immune response (Uribe et al., 2011; Rauta et al., 2012) (Fig. 13).

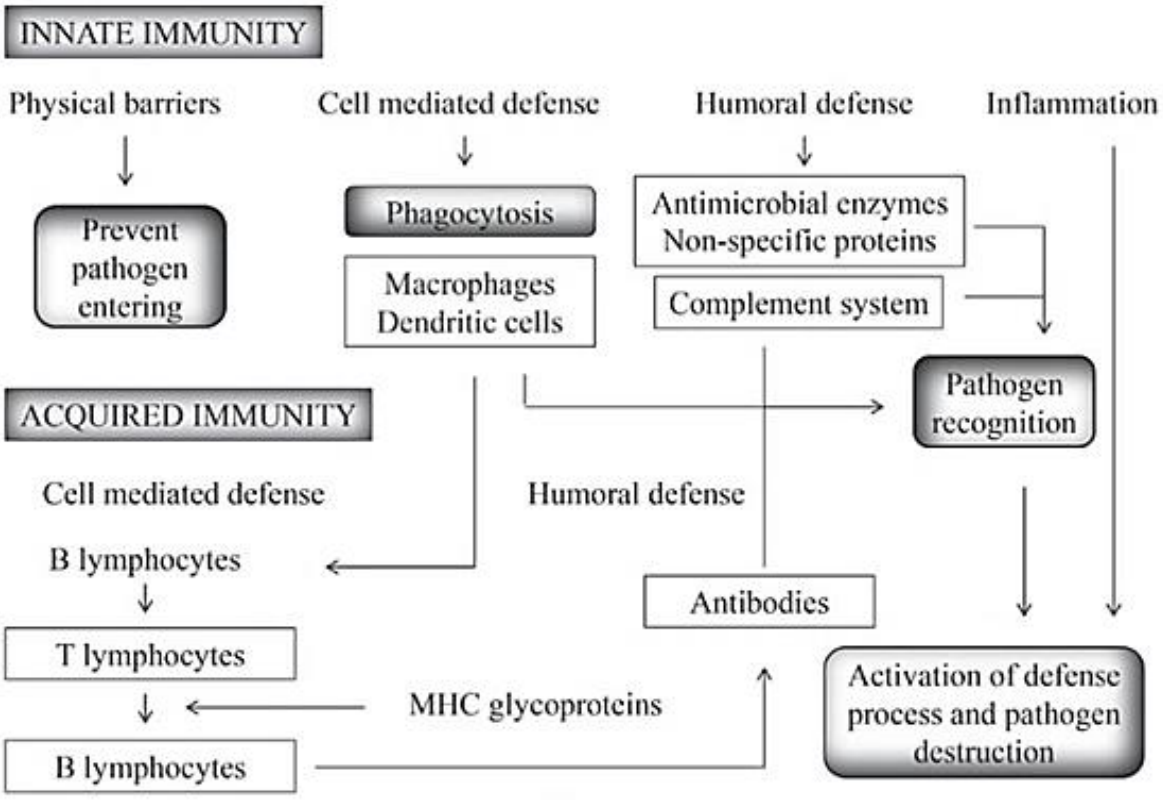



Figure 13: Relationship between innate and adaptive immunity (Biller-Takahashi and Urbinati, 2014).

# I.4 Use of vaccines and antibiotics

Thanks to the great development of aquaculture fish production, the world population can stock up on fish. However, this great activity entails problems due to that fish are subjected to some stressful situations that can cause stress, the apparition of infections and even, fish death, leading to great economic losses (Harikrishnan et al., 2011). The reason is that stress exerts a negative effect on fish immune system, being unable to properly protect fish from pathogens, which are normal inhabitants of the marine ecosystems. Among the factors that cause stress, and the consequent immunodepression in farmed fish, are included the high density of fish per cage/net, poor water quality, changes in temperature, salinity and pH, manipulation, absence of oxygen, and the presence of ammonium, nitrites, carbon dioxide, heavy metals, pesticides, toxins and pathogens (Newman, 2000). For these reasons, vaccination and antibiotics have been used until few years ago to treat or avoid fish diseases or infections trying to improve or strength the immune system of fish or to decreasing levels of the pathogens and fish mortality. In both situations, they try to allow fish to fight or even avoid such diseases or infections (Cabello, 2006), although the possibility to have negative effects in fish is high.

Vaccination (reviewed by Gudding and Van Muiswinkel, 2013), although is the most effective method, has not had succeeded in controlling intracellular pathogens. Furthermore, vaccination is not efficient at present against many important bacterial and viral diseases and commercial vaccines are very limited and only effective against one type of pathogen (pathogen-specific). Besides this, vaccination is too expensive and also stressful for fish (Raa et al., 1992; Robertsen, 1999; Sakai, 1999; Kennedy et al., 2006).

For its part, chemotherapy, that is, the use of antibiotics, as well as food additives in the feed, through baths or dives, or by injection have been used not only as prophylactic but as growth promoters and therapeutics (Cabello, 2006; Hoa et al., 2011; Rico et al., 2013). However, antibiotics present a large number of negative effects for fish, humans and the environment, due to the fact that they cause environmental contamination of fauna and flora, immunodepression, accumulation in fish tissues (and subsequent transfer to humans once food is ingested) and appearance of bacterial strains resistant to antibiotics (reviewed by Hansa et al., 2015; O´Neill, 2015; Elbashir et al., 2017; Joy et al., 2017) (Fig. 14).

In first place, antibiotics are not biodegradable and they can remain in the fish tissues that are destined to human consumption, being also harmful to consumers (Cabello, 2006; Santos and Ramos, 2016). In second place, antibiotics alone, or antibiotics in food or fish faeces, remain in the environment for a long time, so marine bacteria are long time in contact with low or sub-therapeutic doses of them (Cabello, 2006; Gullberg et al., 2011; Chen et al., 2015). Finally, these bacteria can led to a development of bacterial multi-resistance and spread this resistance to the microorganisms present in the ecosystem (Smith et al., 1994; Petersen et al., 2002; Cabello, 2006; Yang et al., 2013), and what is more important, can propagate these resistance genes to fish and human pathogens through the food chain (Gauthier, 2015 Kumar et al., 2017).

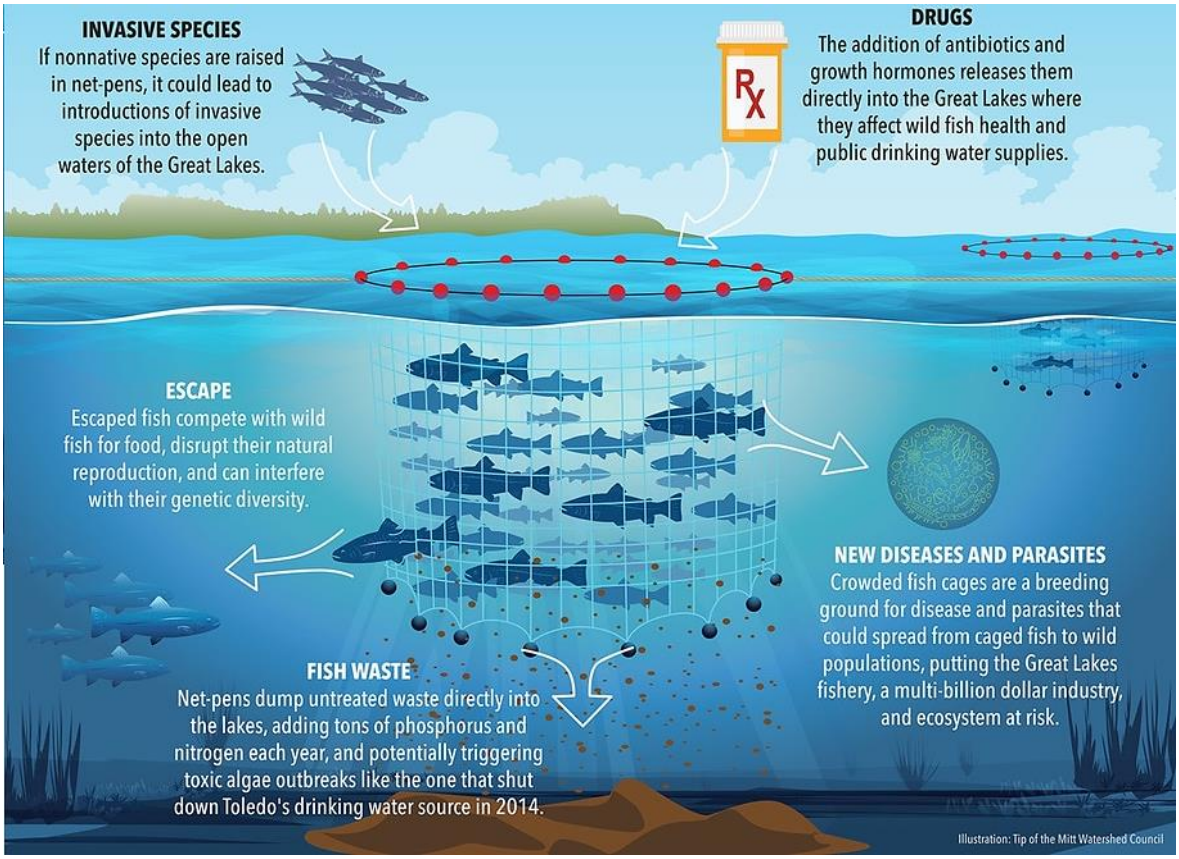



Figure 14: Problems in aquaculture (https://impakter.com/farming-in-water-the-future-and-challenges-of-aquaculture/).

Although the large number of harmful effects associated with the use of antibiotics are clear, the fate of global aquaculture continues to be annoying, and the regulation of the use of antibiotics varies widely from one country to another, and this regulation does not exist in many of them (Smith, 2008; Pruden et al., 2013). For this reason, World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO) regulated the use of antibiotics in aquaculture and their substitution by natural treatments in order to avoid the bacteria resistance and to prevent future problems on environment, animal and human health, although each country has its own legislation (da-Cunha et al., 2018). Therefore, the use of antibiotics is increasingly restricted, especially in the United States (US) [by the Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) guidance] and Europe (CE Regulations), where the number of antibiotics that can currently be used in aquaculture is very limited and strictly controlled (Hansa et al., 2015; Joy et al., 2017), especially in the EU (Official Regulatory Document (EC) No 1831/2003 of the European Parliament and of the Council on additives for use in animal nutrition). It was established that antimicrobials can be used in human and veterinary medicine (except coccidiostats and histomonostats) and their use as growth promoters and as additives, even for experiments with scientific purposes should be progressively eliminated until their total elimination on January 1, 2006, and being replaced by alternative products (Ng and Koh, 2016). However, in the Asian developing countries (e.g. China, India and in many other developing countries, except Japan), where the vast majority of world aquaculture production is generated, there is no law prohibiting or regulating the use of antibiotics (Ganguly et al., 2011; Maron et al., 2013; Chuah et al., 2016). Therefore, regulatory agencies for controlling the use of all drugs legally used in aquaculture have been established. Furthermore, the use of drugs must be done always under veterinary prescription, including dose rates, times, route of delivery and limitations (Hansa et al., 2015; Joy et al., 2017).

# I.5 Eco-friendly alternatives to the use of chemotherapeutic compounds

The very high intensity that is carried out in the sector of aquaculture and this serious phenomenon that the use of antibiotics entails, have attracted growing global public health concern and justified the need for investigation in alternative forms of infectious disease treatment, being prevention and management measures the central concern to overcome such outbreaks of diseases (Marti et al., 2014). Therefore, it is necessary to take measures that guarantee the total economic, social and environmental sustainability activity. For this reason, FAO established the "Code of Conduct for Responsible Fisheries" (FAO, 1995) and promoted the Blue Growth initiative (FAO, 2013), within the framework of the sustainable development goals, with the purpose of carrying out sustainable management of living aquatic resources, balancing its use and conservation, avoiding overexploitation and pollution to restore the services of aquatic ecosystems and the productive capacity of the oceans (FAO, 2016, 2018). Therefore, one of the fundamental pillars of FAO, which is crucial for the development of modern aquaculture in order to prevent disease and to preserve it sustainably, is the use of natural probiotics and immunostimulants through prophylactic administration in order to strength and stimulate fish immune system (Raa et al., 1992; Robertsen, 1999; Magnadottir, 2010) as alternative to chemotherapy and vaccines (Anderson, 1992; Secombes, 1994; Magnadottir, 2010) being in addition cheaper than synthetic drugs (Gudding and Van Muiswinkel, 2013; Chakraborty et al., 2014; Hoseinifar et al., 2016).

One way of administration is to incorporate the immunostimulants into the fish diets. For this reason, "immunonutrition" (improvement of the health of animals through food) is based in the fact that an appropriate feed regime gives optimum health to fish, enhancing its immune response and reducing the chance of being infected (Kiron, 2012) (Fig. 15). The immunonutrients can directly stimulate the immune system or stimulate the growth of the commensal microbiota (Kuhlwein et al., 2014). The former hypothesis has gained importance and interest in the last decade.

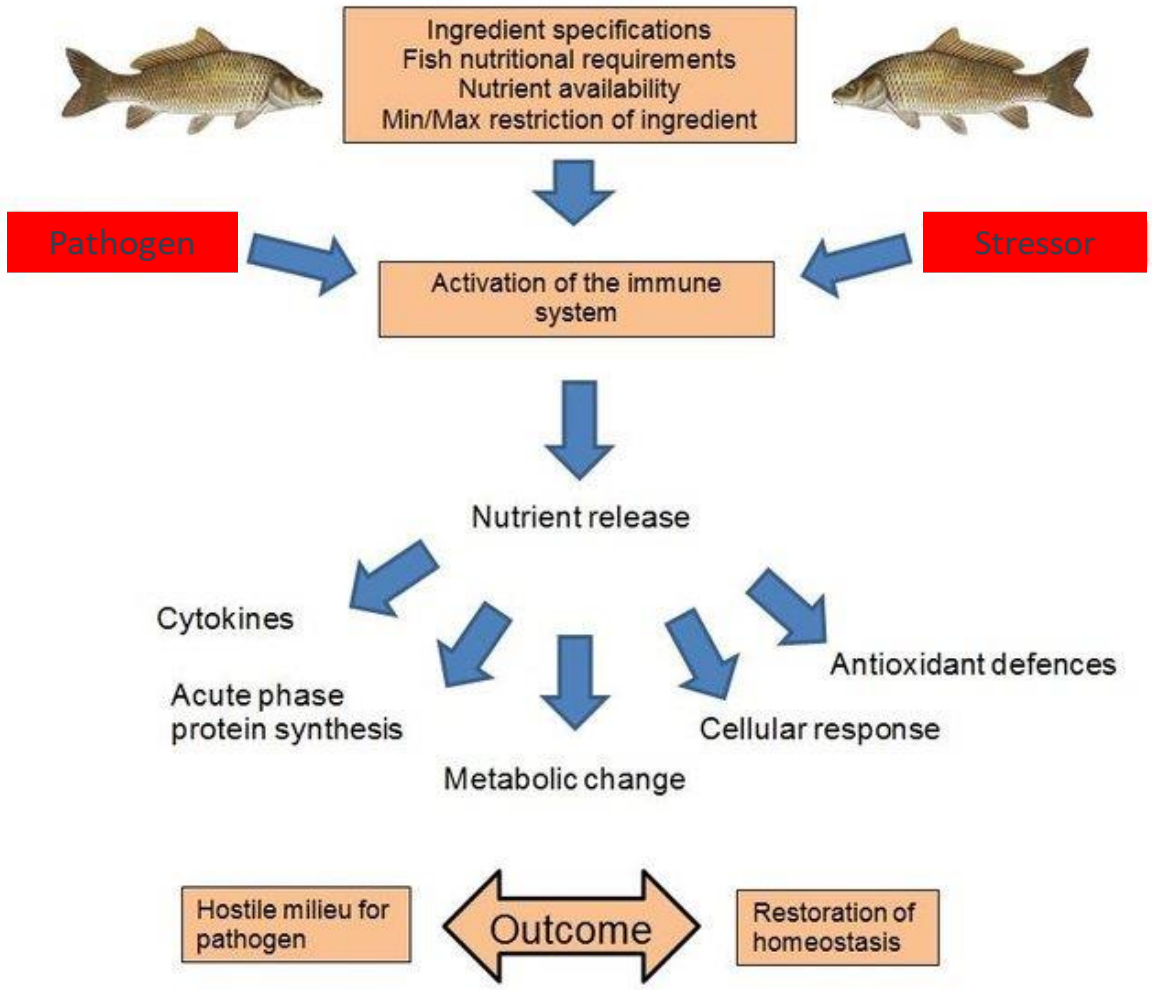



Figure 15: Fish immunonutrition (Rawling, 2013).

Regarding its definition, an immunostimulant is a substance that increases the ability of the immune system (both innate and specific) to fight infections and diseases (Anderson, 1992). Due to one of the objectives of their use is the elimination of the employment of antibiotics in aquaculture, different kinds of natural immunostimulants and growth promoters (biodegradable and friendly for environment, fish and humans) have been added to feed as prophylactic methods to increase the immune response and disease resistance of fish (reviewed by NRC, 2011; Newaj-Fyzul et al., 2015; Vallejos-Vidal et al., 2016; Wang et al., 2016a; Dawood et al., 2017). According to these reviews, among the immunostimulants tested are probiotics (*Bacillus sp.*, *Lactobacillus sp.* …), prebiotics (inulin, oligofructose, oligosaccharides, lactulose), polysaccharides (chitosan and alginate), vitamins (C and E), organic acids (short-chain fatty acids, volatile fatty acids and weak

carboxylic acids), amino acids, antioxidant micronutrients (vitamins C and E, and carotenoids), trace minerals, hormones, nucleotides, other substances (such as lactoferrin, chitin, fucoidan, lectin and thyroxine), antibacterial peptides, and finally, algae, herbs and plants.

On the other hand, Pathogen-Associated Molecular Patterns (PAMPs) have also been used as feed additives in order to activate the immune response, trough recognition by Pathogen-Recognition Receptors (PRRs) such asToll-Like Receptors (TLRs), leading to promote a pro-inflammatory response, thus modulating innate and adaptive immune responses. Among these PAMPs are included bacterial compounds (lipopolysaccharide [LPS], peptidoglycan [PG], muramyldipeptide [MDP], flagelin and unmethylated CpG deoxyribonucleic acid [DNA]), virus compounds (double-stranded ribonucleic acid [RNA]) and fungal compounds (β-glucans and zymosan) (Vallejos-Vidal et al., 2016). In addition to their biological effects, the use of these natural substances increases the consumer confidence of farmed fish (Dawood et al., 2017).

In this sense, phytotherapy, which is a natural therapy that consists on the use of products of plant origin (usually medicinal plants) for the prevention, cure or relief of a wide variety of diseases and infections, or to maintain health, has become a very interesting friendly and immunostimulant alternative to antibiotics due phytochemicals could replace chemotherapeutic agents as immunoprophylactics and allow the possibility to administer plants orally in the diet (Fig. 16).

Figure 16: Phytotherapy as immunoprophylactic (https://www.mivell.com/en/phytotherapy/).

# I.6 Medicinal plants

By definition, medicinal plants are those plants that can be used, whole or by specific parts (leaves, flowers, fruits, barks, stems, seeds or roots), to treat people or animal diseases. The effects of medicinal plants on humans have been known for thousands of years due they have been used worldwide through several generations in human traditional medicine to treat several diseases and infections playing a very important role in the healthcare (Tan and Vanitha, 2004; Hao and Xiao, 2015; Yuan et al., 2016). Even now, around 80% world population use these plants, especially in developing countries such as Latin America, Africa and Asia (Mahady, 2001; Jaradat, 2015; Ajlan, 2016) where conventional therapies are relatively expensive and accessibility is a major concern in many rural populations. Furthermore, due the content of carbohydrates, proteins, fatty acids (higher amount of polyunsaturated omega-3 and omega-6 fatty acids than saturated fatty acids), amino acids, minerals, vitamins (A, B1, B2, B3, B6, B9, C and E) and fibers present in these plants (Chang, 2000; Vayalil, 2012; Zhou et al., 2015; Alegbeleye, 2018; Falowo et al., 2018), they have also an important nutritional value and are consumed by millions of people for thousands of years worldwide, representing a very good alternative to combat malnutrition and several diseases that take place in poor nations where the hunger is a big problem especially in Asian and African countries (Vayalil, 2012; Saini et al., 2016; Iranshahy et al., 2017; Alegbeleye, 2018). Furthermore, purslane and moringa are considered mother`s friend due they can increase milk production in lactating mothers and in postpartum bleeding (Iranshahy et al., 2017; Alegbeleye, 2018).

In addition, production of pharmaceutically active drugs based on plant resources has turn into an essential aspect in developing countries, bordering on high rate of health and environmental problems, and related infectious diseases due these plants provide many secondary metabolites (phytochemicals), also called active principles or bioactive components (Madi et al., 2016; Saini et al., 2016; Yuan et al., 2016) among which are phenols, polyphenols, flavonoids, alkaloids, terpenoids, tannins, saponins, glycosides, steroids, quinones, lectins, polypeptides and polysaccharides (Sivaram et al., 2004; Citarasu, 2010; Chakraborty and Hancz, 2011), which are the responsible of biological activities of plants and are synthesized by them as a response to environmental stresses such as ultra-violet (UV) light, heat or cold, osmotic stress and high salinity, water deficit/dehydration,

nutrient deprivation, infection by fungi, bacteria and viruses, defense against herbivores and as signaling compounds to attract seed-dispersing animals (Aniya et al., 2018; Pohl and Lin, 2018).

Besides this, due to the alarming increase in the number of pathogenic microorganisms which are resistant to drugs and, therefore, compromise the existing antibiotic and antifungal agents, updating of antibiotic and antifungal formula or addition of a new active agent has become a challenging research field (Boulenouar et al., 2009). Therefore, the therapeutic properties of plants have become a crucial element of healthcare all over the world (Sashi et al., 2003). Consequently, new bioactive molecules merit consideration for their best therapeutic uses to preserve the environment and people's health. Similarly, in the field of fish aquaculture, there is a growing awareness in screening natural substances mainly obtained from plants with the intention of exploit new biocompounds of natural origin which could be used to prevent and/or control diseases (Reverter et al., 2014). In this sense, many medicinal plants are a promising alternative to antibiotics and can be considered good candidates for use in farmed fish for several reasons including their known uses in traditional medicine, their beneficial properties that their biological compounds present, their absence of negative impacts on fish, environment and human health, low cost and eco-friendly origin (Direkbusarakom, 2004; Citarasu, 2010; Harikrishnan et al., 2011; Bulfon et al., 2013; Reverter et al., 2014; van-Hai, 2015; Awad and Awaad, 2017).

For all these reasons, at present there is an intense and active research into natural immunostimulant products for fish (Barman et al., 2011), and their use in fish diets has been a common practice for many years (Vallejos-Vidal et al., 2016). In addition to the immunostimulant properties, medicinal plants can be also a source of nutrients for fish which allow them to have other positive effects on fish, such as the stimulation of fish growth and early maturation of cultured species (Newaj-Fyzul and Austin, 2015).

On the other hand, it is well known that chemical composition of plants depends on many factors (Koldas et al., 2015; Rodríguez-García et al., 2016) such as chemotypes, geographical origin, harvest time, growth conditions, genetic, cultivation technique and photoperiod (Lim and Quah, 2007; Aranha and Jorge, 2012; de-Falco et al., 2013; Uddin et al., 2014; Leone et al., 2015; Stohs and Hartmand, 2015; Petropoulos et al., 2016; Yan et al., 2016; Davidenco et al., 2017; Pezzani et al., 2017; Chuang et al., 2018).

Furthermore, chemical composition can change even with growth stages and different parts of the plant (Masoodi et al., 2011; Leone et al., 2015; Zhou et al., 2015; Syed et al., 2016; Iranshahy et al., 2017).

Therefore, due to the necessity of searching for new alternatives to the use of antibiotics in aquaculture to enhance immune status and control fish diseases, the use of medicinal plants as additives in fish diet has gained importance and numerous studies have been carried out since the last decade and this is the main focus of the present Doctoral Thesis.

Gilthead seabream

*Sparus aurata* L.


Drawing from Flora von Deutschland Österreich und der Schweiz. Otto Wilhelm Thomé. 1885. Not included in figures.

# OBJECTIVES

The present Doctoral Thesis tries to determine if it is possible the use of plants, or some compounds present on them, for direct application in fish aquaculture in general, and on gilthead seabream in particular.

## The specific objectives of the present work are:

1. Evaluate the *in vitro* effects of different plant extracts on gilthead seabream head-kidney leucocyte activities, as well as their cytotoxic, bactericidal and antioxidant properties.

2. Study the *in vivo* effects of several plants as dietary additives for gilthead seabream.

3. Estimate the *in vitro* bactericidal activity of natural identical compounds (NICs) and their effects on gilthead seabream head-kidney leucocyte viability and functions.

4. Assess the *in vivo* effects of NICs as dietary additives for gilthead seabream.

# EXPERIMENTAL CHAPTERS

# CHAPTER I.

*In vitro* immunostimulant, cytotoxic, bactericidal and antioxidant activities of oregano, date palm, purslane and moringa extracts

# CHAPTER II.

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Nature-identical compounds. *In vitro* and *in vivo* properties

III.A. *In vitro* study of NICs

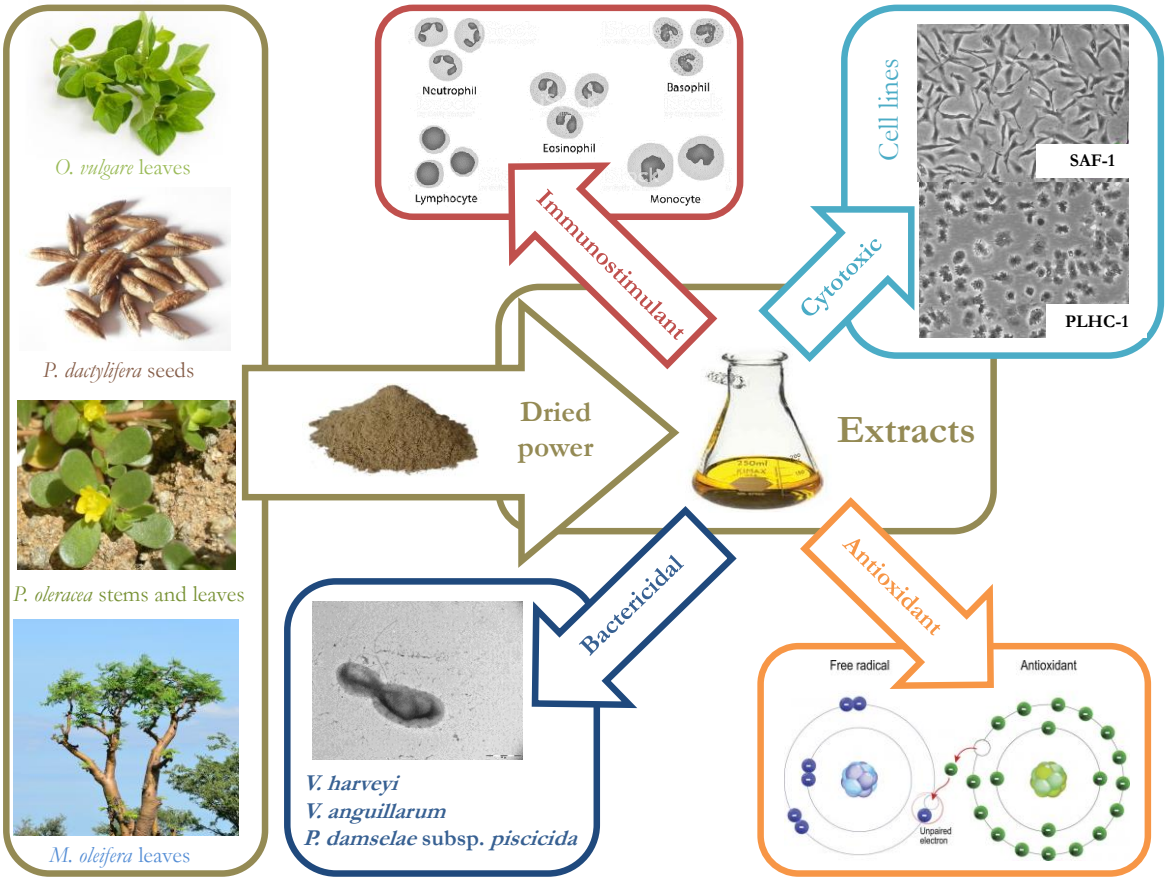
III.B. *In vivo* study of NICs


Drawing not included in figures.

# CHAPTER I.

# *In vitro* immunostimulant, cytotoxic, bactericidal and antioxidant activities of oregano, date palm, purslane and moringa extracts

## I.1 Graphical abstract



## I.2. Introduction

Oregano (*Origanum vulgare*), which belongs to the *Lamiaceae* family, is the most important and variable species of aromatic genus *Origanum* and is widespread throughout the world (Davidenco et al., 2017; Sakkas and Papadopoulou, 2017; Oniga et al., 2018) and is particularly abundant in the Mediterranean areas, in temperate and arid zones of Eurasia and the North of Africa (Kokkini, 1997; Alma et al., 2003; Loizzo et al., 2009). Oregano is a very common plant in Spain and especially in southern regions, like Murcia due the climate and land.

Date palm (*Phoenix dactylifera*) is considered among the most important species of the *Arecaceae* family, which encompasses about 200 *genera* and more than 2,500 species (El-Hadrami and El-Hadrami, 2009). Date palm is an important and one of the oldest trees (5,500–3,000 before Christ [BC]) cultivated by man and still continues being the main crop cultivated in arid regions such as Middle East and North Africa (MENA) regions (Vayalil, 2012). During more than 5,000 years date palm has represented a traditional and socio-economic importance for local populations (Jain et al. 2011). In addition, date palm is a very common plant in Spain and especially in region of Murcia or province of Alicante, which presents the highest concentration of date palm of Europe.

As to purslane (*Portulaca oleracea*), this plant is a herbaceous weed belonging to family *Portulacaceae* and also represents a warm-climate plant with a cosmopolitan distribution including tropical and subtropical areas worldwide and can be found in Europe and North Africa, North America, Asia, Australia and New Zealand (Masoodi et al., 2011; Uddin et al., 2014; Zhou et al., 2015; Syed et al., 2016). Curiously, this plant is classified by WHO as one of the most used medicinal plants and it has been given the term "Global Panacea" (Dweck, 2001; Xu et al., 2006), and is known as "vegetable for long life" (Jin et al., 2013).

For its part, moringa (*Moringa oleifera*) is the most important and most cultivated species of *Moringaceae* family and *Moringa* genus (Kou et al., 2018). This plant is native of sub-Himalayan Mountains of Northern India, Pakistan, Afghanistan, Bangladesh and Nepal but actually is grown and produced on tropical and subtropical countries worldwide from Africa, Asia, Central and South America and in the Pacific and Caribbean Islands (Brilhante et al., 2017). Due its fast growth, resistance to drought and longevity, this plant

has received several popular names such as superfood tree, drumstick tree, horseradish tree, miracle tree, tree of life or benzoil tree (Falowo et al., 2018).

According to the medicinal use of these plants, all of them are well known and very used since ancient times in folk and traditional medicine to treat pains, inflammations, infections and diseases including respiratory, gastrointestinal, genito-urinary, cardiovascular, cerebrovascular, skin, kidney, bladder, liver, uterine, prostate, ear, dental, eye, hematological, musculoskeletal and nervous disorders (Syed et al., 2016; Ansari and Alzohairy, 2018; Falowo et al., 2018; Oniga et al., 2018). For this purpose, plants are used in infusion or tea (Alegbeleye, 2018; Ansari and Alzohairy, 2018; Oniga et al., 2018), juice (Syed et al., 2016) or some forms of powder or pulp (Ansari and Alzohairy, 2018). Other times, their essential oil (Coccimiglio et al., 2016) are used or eaten fresh in salads or directly as fruits (Syed et al., 2016; Alegbeleye, 2018).

Moreover, these plants are used in food industry as aromatic spice herb for flavoring food and drink products (Kaurinovic et al., 2011), to preserve food (Falowo et al., 2018; Oniga et al., 2018) or in the production of food products for human consumption (Zhou et al., 2015; Bentrad et al. 2017a; Iranshahy et al., 2017; Falowo et al., 2018), although their use in pharmaceutical, cosmetic (Hossain et al., 2014; Karim et al., 2016; Mehdizadeh et al., 2018; Oniga et al., 2018) and agricultural industry (Kaurinovic et al., 2011; Alegbeleye, 2018) is also very known.

As to biological activities, these plants present many of them including antimicrobial (antibacterial, antifungal, antiviral and antiparasitic), anticancer, antioxidant, anti-inflammatory, immunomodulatory, tissue protective, antinociceptive and wound healing among much others (Iranshahy et al., 2017; Abdel-Magied et al., 2018; Alegbeleye, 2018; Falowo et al., 2018; Kou et al., 2018; Oniga et al., 2018), which are due to their wide variety of secondary metabolites, most of them phenolic compounds such as phenolic acids, flavonoids, terpenes, alkaloids, tannins and coumarins, among much others, in the case of oregano (Teixeira et al., 2013; Han et al., 2017; Oniga et al., 2018), date palm (Taleb et al., 2016; Agostini-Costa, 2018), purslane (Uddin et al., 2014; Zhou et al., 2015; Iranshahy et al., 2017) and moringa (Brilhante et al., 2017; Paikra et al., 2017; Falowo et al., 2018).

All these reasons aimed us to consider *a priori* that they could be good sources of natural compounds employed to treat, prevent and/or control fish diseases in aquaculture.

Furthermore, regarding literature and to the best of our knowledge, the number of studies about the *in vitro* effects of these plants, their extracts or essential oils on fish cells is very scarce. Therefore, the abundance of these plant species in Spain, their content of bioactive compounds, the absence of *in vitro* studies on fish cells and the possibility to increase or potentiate the valorization of these abundant natural products on the Mediterranean and MENA areas we decided to develop the present work.

The aim of our study was to evaluate the *in vitro* effects of different aqueous and ethanolic (absolute ethanol in the case of oregano, date palm and purslane or 80% ethanol in the case of moringa) extracts on gilthead seabream (*S. aurata*) HK leucocytes. Firstly, we determined their effects on the leucocyte viability, and then on immune activities including phagocytosis and respiratory burst and peroxidase content. Gilthead seabream was selected as a representative species of marine Mediterranean aquaculture and due its economic importance in Spain and in our Region. Furthermore, the possible cytotoxic activity of such extracts on SAF-1 cell line (*S. aurata* fin) and PLHC-1 cell line (*Poeciliopsis lucida* hepato-carcinoma, pahotgens bacteria) were determined. Beside this, the bactericidal activity against three important bacterial pathogens for fish (*V. harveyi, V. anguillarum* and *P. damselae* subsp. *piscicida*) was also evaluated. Finally, the antioxidant activity of the extracts was also determined.

## I.3. Material and methods

### *I.3.1. Fibre determination and chemical analysis of date palm seeds (DPS)*

Date palm (Deglet Nour variety) was obtained on Monastir (Tunisia) at the ripeness stage and used to dissect the seed. The DPS moisture content was measured gravimetrically in unground seeds (AOAC, 2010). Crude proteins were determined using a Behr analysis equipment (Behr Labor TechnikGmbh). The Kjeldahl nitrogen content (AOAC, 2005a) was converted to a protein concentration with a specific factor of 6.25. Fats were solubilized with petroleum ether using a Soxhlet apparatus (AOAC, 1997) and total ash was determined by calcination at 550°C (AOAC, 2005b).

Dietary fibers were estimated according to the Van Soest method (Nabili et al., 2016) using a Raw Fiber Extractor 6 Channel (VELP Scientifica). The first step of the analysis involved chemical extraction with a neutral detergent solution (NDF) leading to unsolvable fraction containing cellulose, hemicellulose and lignin. Then, the acid detergent fiber (ADF) was estimated throw the digestion of the degradable hemicelluloses and some proteins with acid solution. The acid detergent lignin (ADL) was based on solubilization of the cellulose in 72% sulfuric acid ($H_2SO_4$). Hemicellulose was calculated as NDF – ADF and cellulose as ADF – ADL.

### *I.3.2. Plant extracts*

Oregano (natural and ecological) was bought in a local market (Murcia, Spain) while the other three plants were obtained in nature. Date palm (Deglet Nour variety) was obtained on Monastir (Tunisia) at the ripeness stage, purslane was collected in Orihuela (Alicante, Spain) and moringa was collected from the experimental farm of the Botany Department, Faculty of Agriculture (Saba Basha), Alexandria University (Egypt).

Plants were dried and the selected parts were crashed until to be powder with an electric grinder. Only leaves were used in the assays carried out with oregano and moringa, leaves were used together with stems in those assays carried out with purslane, while in those carried out with date palm, the seeds were employed to obtain the extracts. DPS were ground into powder (32 meshes) with a microphyte disintegrator FZ102 (Huanghua Faithful Instrument). One g of powder and forty mL of water or absolute ethanol (1:40 w/v) were

used for extracts preparation. To prepare the aqueous extracts, the powder was macerated and shaken with boiling water (initially) and maintained for 4 h at 25ºC. The mixture was filtered twice using a nylon net filter with a 100 mm pore size and then freeze-dried (lyophilized). Prior to use in the assays, the extracts were filtered using sterile filters of 0.22 mm diameter. For the preparation of ethanolic extracts, dry leaves were macerated and shaken with pure ethanol (1:40 w/v, 48 h, 25ºC). The resulting mixture was then filtered twice as described above, and evaporated at 40ºC until dryness. The ethanolic extract of moringa was obtained according to Mekonnen et al. (2005). Briefly, powdered air-dried leaves were extracted using 80% ethanol (plant material: solvent, 1:5 w/v) under reflux for 20 h. The extract was then concentrated using rotary evaporator and freeze-dried (lyophilized) and stored at -20°C.

### *I.3.3. Animals*

Five specimens of the seawater teleost gilthead seabream (*S. aurata* L.), obtained from a local farm (Murcia, Spain), were used for each one of the studies carried out with each plant already mentioned. Fish were kept in re-circulating seawater aquaria (250 L) in the Marine Fish Facilities at the University of Murcia. The water temperature was maintained at 20 ± 2ºC with a flow rate of 900 L/h and 28‰ salinity. The photoperiod was 12 h light:12 h dark. Fish were allowed to acclimatize for 15 days before the start of the trial, where they were fed with a commercial pellet diet (Skretting, Spain) at a rate of 2% body weight/day. The fish were killed after starving for 24 h by using an overdose of MS-222 (Sandoz, 100 mg/mL water). All experimental protocols were approved by the Ethical Committee of the University of Murcia.

### *I.3.4. HK leucocyte isolation and incubation with extracts*

Before the dissection of the HK, the specimens were bled. Blood was collected from the caudal vein and afterwards fish were dissected to obtain HK fragments, isolating the leucocytes according to Esteban et al. (1998). Briefly, HK were cut into small fragments and transferred to 12 mL of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride (to adjust the medium's osmolarity to gilthead seabream plasma osmolarity of 353.33 mOs), 3% foetal calf serum (FCS, Gibco), 2 mM L-glutamine (Gibco), 100 i.u./mL penicillin (Flow) and 100 mg/mL streptomycin (Flow)]. HK leucocytes were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 mm),

washed twice (400 g, 10 min), counted in an automatic counting chamber (BioRad) and adjusted to $2\times10^7$ cells/mL in sRPMI. Cell viability was determined by the trypan blue exclusion test.

To study the possible effects of aqueous and ethanolic extracts on HK leucocyte viability and immune activities, aliquots of 50 µL of the HK leucocytes suspension were dispensed into glass tubes (Falcon) to ascertain viability and phagocytic activity, 50 µL into a flat-bottomed 96-well plates to assess respiratory burst activity and 5 µL into a flat-bottomed 96-well plates for peroxidase activity. Afterwards, same aliquots of aqueous (directly dissolved in sRPMI) or ethanolic extracts [dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and then further diluted in sRPMI] were added at a final concentration of 0.001, 0.1, 0.5 and 1 mg/mL. For controls, sRPMI replaced the aqueous extract and 1% DMSO in sRPMI replaced the ethanolic extracts. Leucocytes were incubated in the presence of the extracts for 24 h at 21ºC in an incubator with 5% $CO_2$ and 85% humidity.

#### *I.3.5. HK leucocyte viability*

After incubation, HK leucocyte viability was studied adding 50 µL of propidium iodide (PI) (400 mg/mL, Sigma-Aldrich) to each 100 µL aliquot of HK leucocytes (previously incubated with the extracts, as described above). The tubes were gently mixed before analysis in a FACScan flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 5,000 cells, which were acquired at a rate of 300 cells/s. Data were collected in the form of two-parameter side scatter (granularity, SSC) and forward scatter (size, FSC), and green fluorescence (FL1) and red fluorescence (FL2) dot plots or histograms were made on a computerized system. Dead cells were estimated as the percentage of cells with propidium iodide (red-PI fluorescent cells). A quantitative study of the flow cytometric results was made using the statistical option of the Lysis Software Package (Becton Dickinson).

#### *I.3.6. HK leucocyte immune activities*

##### *I.3.6.1. Phagocytosis*

The phagocytic activity of gilthead seabream HK leucocytes was studied by flow cytometry according to Rodríguez et al. (2003a). Heat killed (30 min, 60ºC) and lyophilized

*Saccharomyces cerevisiae*, strain S288C, were washed twice, counted and adjusted to $10^8$ yeast cells/mL in sRPMI. To label yeast cells with fluorescein isothiocyanate (FITC, Sigma-Aldrich) they were incubated with 5 mg/mL FITC at 22ºC with constant stirring (40 cycles/min) and in darkness for 15 min (Rodríguez et al., 2003b). After labelling, free FITC was removed by washing twice in phosphate buffer saline (PBS) and the yeast cells were resuspended in sRPMI. FITC-labelled yeast cells were acquired for flow cytometric study. The staining uniformity was examined and then the yeast cell suspensions were aliquoted and stored at -80ºC.

Phagocytosis samples consisted of 60 µl of labelled-yeast cells and 100 ul of HK leucocytes (previously incubated as described above). Samples were mixed, centrifuged (400 g, 5 min, and 22°C), resuspended and incubated for 30 min at 22°C. At the end of the incubation time, samples were placed on ice to stop phagocytosis and 400 ul ice-cold PBS was added to each sample. The fluorescence of the extracellular yeasts was quenched by adding 50 ul ice-cold trypan blue (0.5% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or HK leucocytes were included in each phagocytosis assay. All samples were analysed in a FACScan flow cytometer set to analyse the phagocytic cells, which show the highest SSC and FSC values. Data of 5,000 phagocytic cells were collected and the phagocytic ability, defined as the percentage of phagocytic cells with one or more ingested bacteria (green-FITC fluorescent cells), and phagocytic capacity, defined by their mean fluorescence intensity, equivalent to the relative number of ingested yeast cells per cell, were assessed.

#### *I.3.6.2. Respiratory burst activity*

The respiratory burst activity of HK leucocytes was studied by a chemiluminescence method (Bayne and Levy, 1991). Briefly, 100 ul of Hank's balanced salt solution (HBSS, Gibco) containing 1 mg/mL phorbolmyristate acetate (PMA, Sigma-Aldrich) and $10^{-4}$ M luminol were added to the 100 ul of HK leucocytes (previously incubated as described above).

The plates were shaken and immediately read in a chemiluminometer (BMG, FluoStar Galaxy). Measurements were performed in 30 cycles of 2 min each. The kinetic of the reactions was analyzed and the maximum slope of each curve was calculated. Control samples containing HK leucocytes without PMA and luminol were also analyzed.

#### I.3.6.3. Peroxidase activity

The total peroxidase activity of HK leucocytes was measured according to Quade and Roth (1997). To do this, HK leucocytes (previously incubated as described above) were lysed for 10 min with 0.002% cetyltrimethylammonium bromide (CTAB, Sigma-Aldrich) at 60 rpm. Afterwards, 100 µL of 10 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, Sigma-Aldrich) and 5 mM hydrogen peroxide ($H_2O_2$) (both substrates prepared daily) were added and after 2 min, 50 µL of 2 M $H_2SO_4$ were also added to stop the reaction. The absorbance of the samples was measured at 450 nm in a microplate reader. Control samples containing leucocytes without substrates were also analyzed.

### I.3.7. Cytotoxicity of plant extracts on fish cell lines

The established cell line SAF-1 (ECACC nº 00122301) was seeded in 25 $cm^2$ plastic tissue culture flasks (Nunc) in L-15 Leibowitz medium (Life Technologies), supplemented with 10% FCS, 2 mM L-glutamine, 100 i.u./mL penicillin and 100 mg/mL streptomycin. Cells were grown at 25ºC in a humidified atmosphere (85% humidity). Exponentially growing cells were detached from culture flasks by brief exposure to trypsin (0.25% in PBS, pH 7.2-7.4), according to the standard trypsinization methods. The detached cells were collected by centrifugation (200 g, 5 min, 25ºC) and cell viability was determined by the trypan blue exclusion test. The established cell line PLHC-1 (ATCC® CRL2406™) was seeded in 25 $cm^2$ plastic tissue culture flasks in Minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's salts adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 5% FCS, 100 i.u./mL penicillin and 100 mg/mL streptomycin. Cells were grown at 30ºC in a humidified atmosphere (85% humidity) with 5% $CO_2$. Exponentially growing cells were detached from the culture flasks by brief exposure to of trypsin (0.05% in PBS, pH 7.2-7.4), according to the standard trypsinization methods. The detached cells were collected by centrifugation (200 g, 5 min, 30ºC) and cell viability was determined by the trypan blue exclusion test.

A cytotoxicity assay of each cell type was performed in five replicates at each concentration of each extract. When cell lines were approximately 80% confluent, cells were detached from the flasks with trypsin (as described before), and aliquots of 100 µL containing 50,000 cells/well were dispensed into 96-well tissue culture plates and incubated (24 h, at the temperature for each cell line). This cell concentration was previously

determined in order to obtain satisfactory absorbance values in the cytotoxic assay and to avoid cell overgrowth. After that, the culture medium was replaced by 100 µL/well of the aqueous or ethanolic extracts at 0.001, 0.05, 0.1, 0.25, 0.5, 0.75 and 1 mg/mL. Control samples received the same volume of culture medium (for the aqueous extracts) or 1% DMSO (for the ethanolic extracts). Cells were incubated for 24 h and then their viability was determined using the MTT assay, which is based on the reduction of the yellow soluble tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT, Sigma-Aldrich) to a blue, insoluble formazan product by mitochondrial succinate dehydrogenase (Denizot and Lang, 1986; Berridge and Tan, 1993). For this, cells were washed with PBS and 200 µL/well of MTT (1 mg/mL) were added. After 4 h of incubation, cells were washed again and the formazan crystals were solubilized with 100 µL/well of DMSO. Plates were shaken (5 min, 100 rpm) in dark conditions and the absorbance at 570 nm and 690 nm determined in a microplate reader.

### *I.3.8. Bactericidal activity of plant extracts*

Three pathogenic bacteria for fish (*V. harveyi*, *V. anguillarum* and *P. damselae* subsp. *piscicida*) were used in the bactericidal assays. They were cultured for 48 h at 25ºC in Triptic Soy Agar (TSA, Difco Laboratories), and then single colonies inoculated in Triptic Soy Broth (TSB, Difco Laboratories), both supplemented with NaCl to a final concentration of 1% (w/v), and growth with continuous shaking (100 rpm) for 24 h. Exponentially growing bacteria were washed and resuspended in sterile PBS at $10^8$ colony forming units (c.f.u.)/mL.

Bactericidal activity was determined following the method of Stevens et al. (1991) with some modifications. For this, aliquots of 20 µL of bacteria were added to wells of a flat-bottomed 96-well plate (in six replicates) and incubated with the same volume of aqueous or ethanolic extracts, ranging from 0.001 to 1 mg/mL, for 5 h at 25ºC. PBS solution was added to some wells instead of the extracts and served as positive control. Then, 25 µL of MTT (1 mg/mL) were added to each well and the plates were newly incubated for 10 min at 25ºC to allow the formation of formazan. Plates were then centrifuged (2,000 g, 10 min), and the precipitates dissolved in 200 µL of DMSO and transferred to a flat-bottomed 96-well plate. The absorbance of the dissolved formazan was measured at 570 nm. Bactericidal activity was expressed as percentage of non-viable bacteria, calculated as the difference

between absorbance of surviving bacteria in test samples compared to the absorbance of bacteria from positive controls (100%).

### I.3.9. Total antioxidant activity (TAA) of plant extracts

The TAA of aqueous or ethanolic extracts was analysed by the 2,2'-azino-bis-3-(ethylbenzothiazoline-6-sulphonic acid) (ABTS) method described by Arnao et al. (1999), which is based on the ability of the antioxidants in the sample to reduce the radical cation of ABTS, as determined by the decolouration of ABTS+, and measuring the quenching of the absorbance at 730 nm. This activity is calculated by comparing the values of the sample with a standard curve of ascorbic acid and expressed as ascorbic acid equivalents (mmol)/mg protein. Samples of 50 µL of aqueous or ethanolic extracts at 0.1, 0.5 and 1 mg/mL were added to 950 µL of ABTS+ and the decrease of absorbance was measured in a spectrophotometer (BOECO S-22 UV/Vis) using as blank of reaction with PBS. The samples were analyzed in triplicate. A standard curve was done with ascorbic acid (Sigma-Aldrich) and the antioxidant capacity of aqueous and ethanolic extracts interpolated from the adjusted curve.

### I.3.10. Statistical analyses

The results are expressed as means ± SEM. The normality of the variables was confirmed by the Shapiro–Wilk test and homogeneity of variance by the Levene test. Statistical differences among the four groups of treatments were assessed by one-way ANOVA analyses, followed by the Tukey or Games-Howell test, depending on the homogeneity of the variables. The significance level was 95% in all cases ($P < 0.05$). All the data were analysed by the computer application SPSS for Windows® (version 15.0).

## I.4. Results

### *I.4.1. Fibre determination and chemical analysis of DPS*

Fibre and chemical composition of selected DPS was determined and the results indicated that hemicelluloses were the most abundant fibres (26.5 ± 0.001% dry-matter basis, db) followed by celluloses (24.1 ± 0.1% db) and lignin (21.2 ± 0.001% db). Results from the chemical analysis revealed that fat was more abundant (11.2 ± 0.1% db) than proteins (6.2 ± 0.01% db) while ash represents the 1.5 ± 0.1% db.

### *I.4.2. Effects of plant extracts on leucocyte viability*

Leucocyte viability was evaluated in order to discard cytotoxic effects that could affect the following immune activities analysed. Regarding aqueous extracts, no significant effect was observed on HK leucocyte viability after being incubated with oregano (Fig. 17A) or purslane (Fig. 17C) extracts, respect to control leucocytes. When the leucocytes were incubated with 0.5 mg/mL of aqueous extract of date palm their viability increased significantly, compared to control leucocytes (Fig. 17B). Curiously, the highest tested concentration of aqueous moringa extracts showed the opposite effects, producing a significant decrease and then resulting toxic for the fish leucocytes (Fig. 17D). Regarding ethanolic extracts, a significant decrease of the HK leucocyte viability was recorded when cells were incubated with the highest tested concentration of oregano (Fig. 17A) or purslane (Fig. 17C) and 0.5 mg/mL or higher date palm extracts (Fig. 17B) in comparison with control group. However, after incubation with moringa ethanolic extracts the viability of HK leucocytes was always increased, respect to the viability recorded for control HK leucocytes (Fig. 17D).

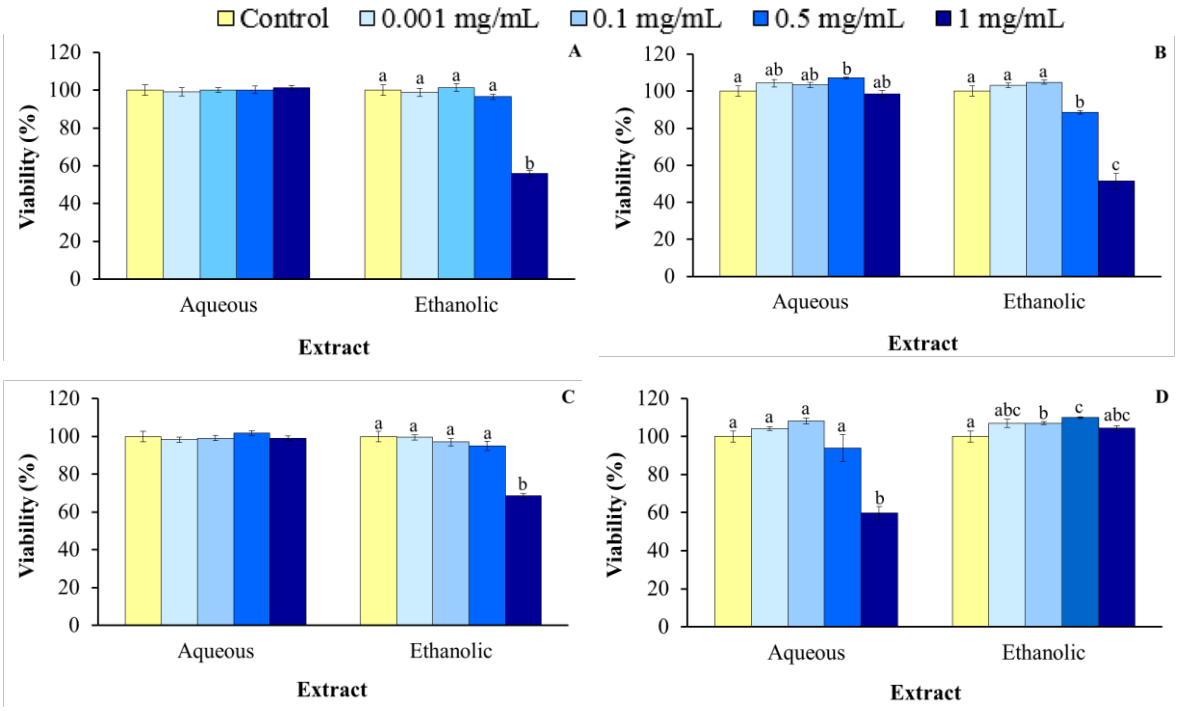



Figure 17: Viability of gilthead seabream head-kidney leucocytes after 24 h of incubation with aqueous or ethanolic extracts from oregano (A), date palm (B), purslane (C) or moringa (D). The results are expressed as mean ± SEM (n = 5). Different letters denote significant differences between treatment groups ($P < 0.05$).

### I.4.3. Effects of plant extracts on leucocyte phagocytosis

#### I.4.3.1. Phagocytic ability

Phagocytic ability of HK leucocytes after being incubated with aqueous extracts of oregano (Fig. 18A) or purslane (Fig. 18C) was significantly increased in a dose-dependent manner respect to control leucocytes. Contrarily, when leucocytes were incubated with aqueous moringa extracts, a significant and drastic reduction of the activity, in comparison with control leucocytes, was obtained (Fig. 18D). However, no significant effects were observed after incubation with date palm extracts respect to control leucocytes (Fig. 18B). Regarding ethanolic extracts, incubation of HK leucocytes with low concentrations of oregano (Fig. 18A) or purslane (Fig. 18C) increased the leucocytes phagocytic ability, although only in the case of oregano this was significant in comparison with control leucocytes. However, a dose-dependent decrease of the activity was obtained in HK leucocytes incubated with intermediate to high concentrations of oregano (Fig. 18A), date palm (Fig. 18B) or purslane (Fig. 18C) extracts, respect to control leucocytes, although the

results were only statistically significant in those HK leucocytes incubated with oregano or date palm. Finally, no significant effects were detected for this activity after incubation of leucocytes with ethanolic extracts of moringa compared with control leucocytes (Fig. 18D).

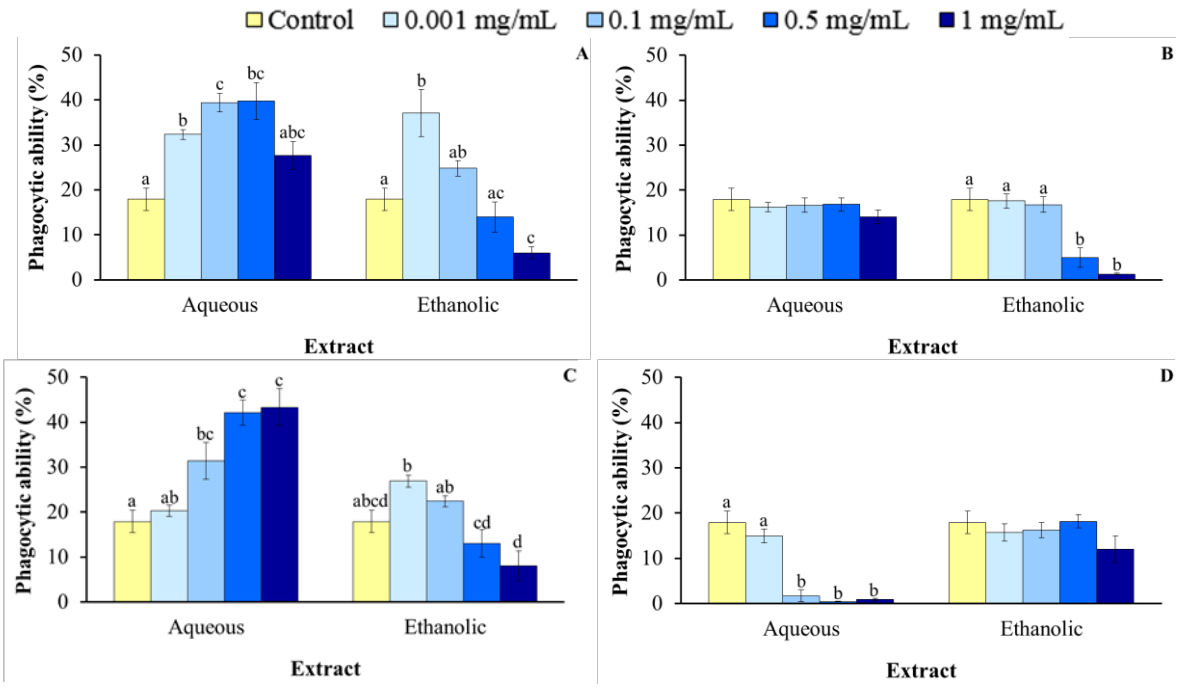



Figure 18: Phagocytic ability of gilthead seabream head-kidney leucocytes after 24 h of incubation with aqueous or ethanolic extracts from oregano (A), date palm (B), purslane (C) or moringa (D). The results are expressed as mean ± SEM (n = 5). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *I.4.3.2. Phagocytic capacity*

High concentrations of aqueous extracts of oregano (Fig. 19A) and date palm (Fig. 19B) showed a significant decrease of HK leucocyte phagocytic capacity respect to control leucocytes, while some concentrations of moringa extracts (Fig. 19D) also decreased it. For its part, incubation with purslane extracts (Fig. 19C) showed no effects in comparison with control leucocytes. Regarding ethanolic extracts, although low concentrations of oregano and purslane increased leucocyte phagocytic capacity in a not significant manner, high concentrations of oregano (Fig. 19A), date palm (Fig. 19B) and moringa (Fig. 19D) decreased this activity at significant level respect to control leucocytes.

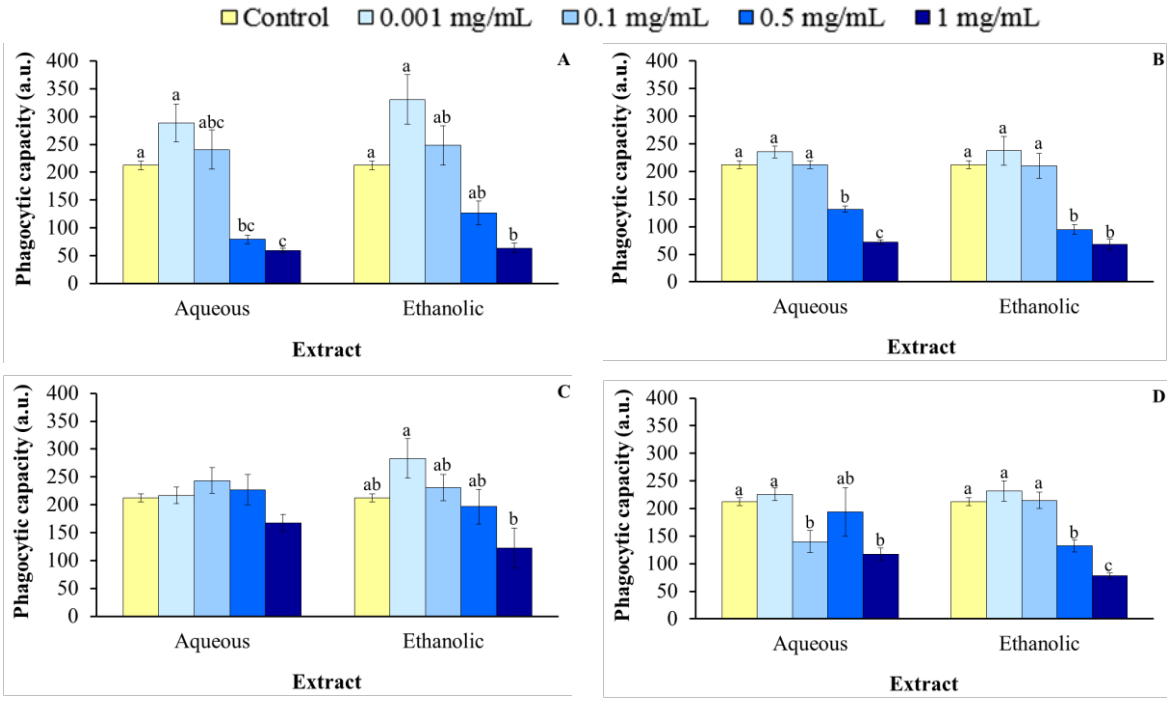



Figure 19: Phagocytic capacity of gilthead seabream head-kidney leucocytes after 24 h of incubation with aqueous or ethanolic extracts from oregano (A), date palm (B), purslane (C) or moringa (D). The results are expressed as mean ± SEM (n = 5). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *1.4.4. Effects of plant extracts on leucocyte respiratory burst activity*

Aqueous extracts of oregano (Fig. 20A), date palm (Fig. 20B) and moringa (Fig. 20D) showed a significant decrease in the respiratory burst activity respect to control leucocytes, especially in the case of moringa. However, no significant differences were observed after incubation with purslane extracts (Fig. 20C). Focusing on ethanolic extracts, all of them showed a significant decrease in this activity respect to control leucocytes, especially in the case of date palm (Fig. 20).

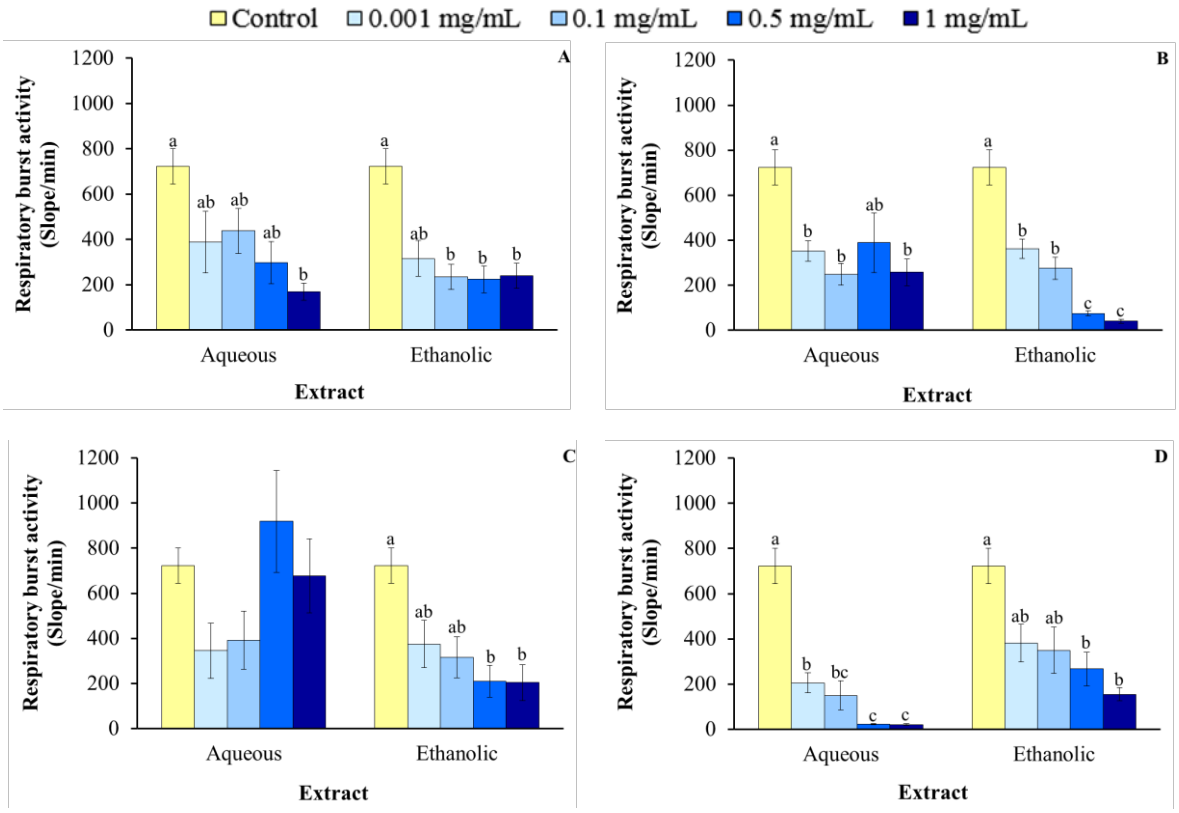



Figure 20: Respiratory burst activity of gilthead seabream head-kidney leucocytes after 24 h of incubation with aqueous or ethanolic extracts from oregano (A), date palm (B), purslane (C) or moringa (D). The results are expressed as mean ± SEM (n = 5). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *I.4.5. Effects of plant extracts on leucocytes peroxidase activity*

Regarding results for aqueous and ethanolic extracts, no significant differences were obtained in the peroxidase activity of leucocytes upon incubation with any plant, although both aqueous and ethanolic extracts of date palm (Fig. 21B) and moringa (Fig. 21D) increased it.

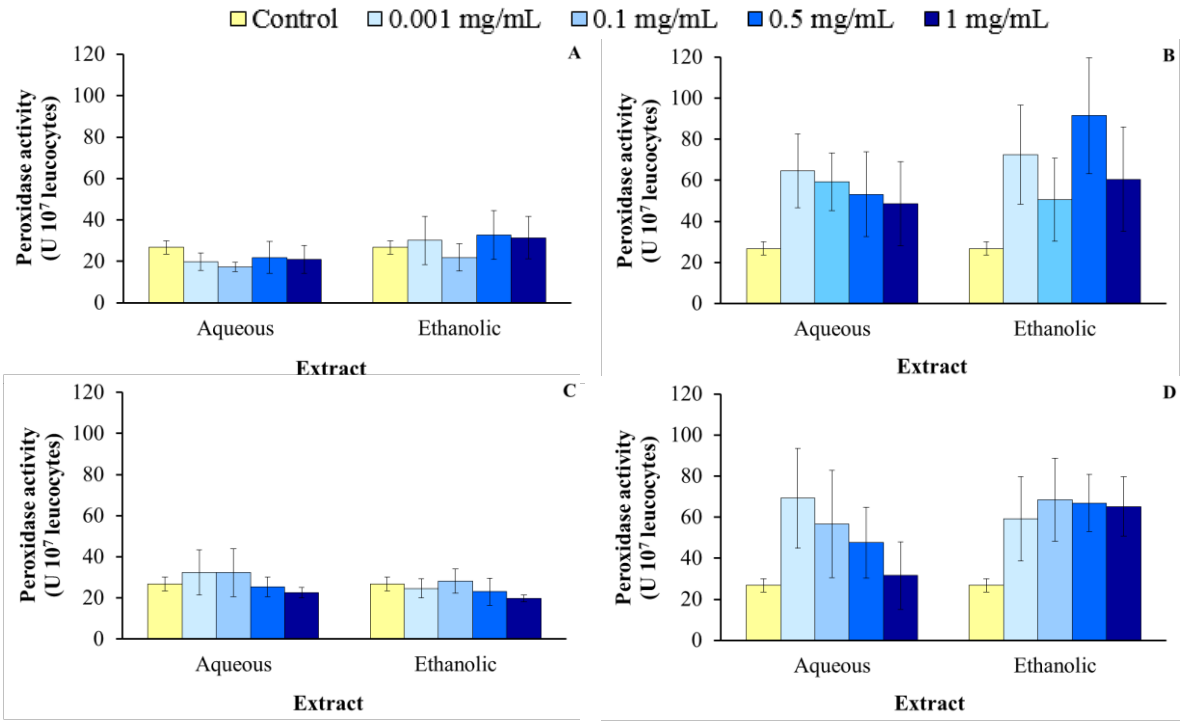



Figure 21: Peroxidase activity of gilthead seabream head-kidney leucocytes after 24 h of incubation with aqueous or ethanolic extracts from oregano (A), date palm (B), purslane (C) or moringa (D). The results are expressed as mean ± SEM (n = 5).

### 1.4.6. Cytotoxicity of plant extracts on SAF-1 and PLHC-1 cell lines

#### 1.4.6.1. SAF-1 cell line

Regarding the effects of aqueous extracts on SAF-1 cells, high concentrations of oregano (Fig. 22A) and date palm (Fig. 22B) extracts significantly increased cell viability in comparison with control cells, contrarily to results obtained for moringa, where all concentrations decreased in a significant manner cell viability (Fig. 22D), then resulting toxic for gilthead seabream cells. For its part, no significant differences were observed after SAF-1 incubation with purslane extracts (Fig. 22C). Focusing on ethanolic extracts, high concentrations of oregano (Fig. 22A) and purslane (Fig. 22C) extracts resulted cytotoxic for SAF-1 cells, showing a significant dose-dependent decrease of cell viability respect to control cells. On the other hand, low-moderated concentrations of moringa extracts also decreased in a significant manner cell viability in comparison with control cells, while high concentrations increased it significantly (Fig. 22D). Finally, no effects on cell viability were observed after incubation of cells with date palm extracts (Fig. 22B).

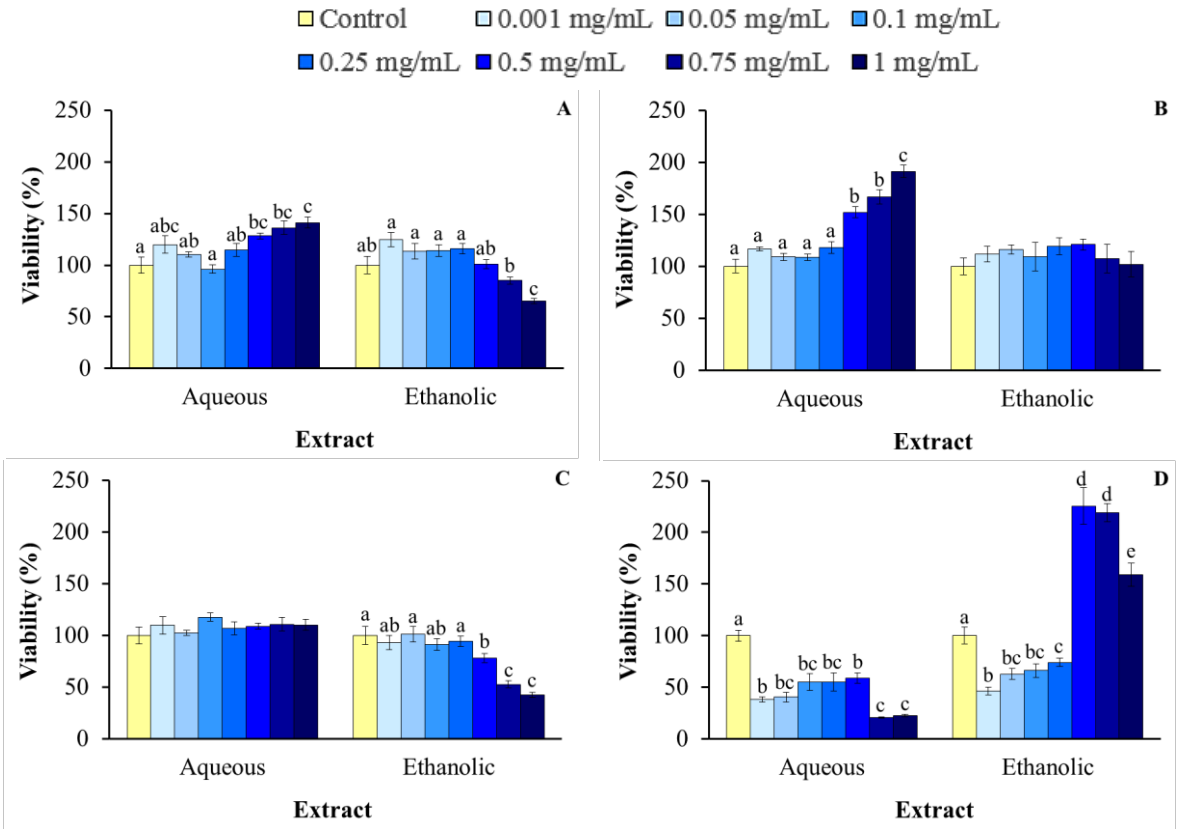



Figure 22: Viability of SAF-1 cells after 24 h of incubation with aqueous or ethanolic extracts from oregano (A), date palm (B), purslane (C) or moringa (D). The results are expressed as mean ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

### I.4.6.2. PLHC-1 cell line

For aqueous extracts, moderate-high concentrations of oregano (Fig. 23A) and moringa (Fig. 23D) produced a significant dose-dependent decrease of cell viability respect to control group, then resulting both cytotoxic, especially in the case of moringa. For its part, only the highest concentration of date palm extracts (Fig. 23B) significantly decreased cell viability in comparison with control cells, while no differences were obtained after incubation with purslane extracts (Fig. 23C).

Regarding results of ethanolic extracts, moderate-high concentrations of oregano (Fig. 23A), date palm (Fig. 23B) and purslane (Fig. 23C) decreased in a significant manner cell viability in comparison with control cells. Curiously, no effects were obtained after incubation of cells with moringa extracts (Fig. 23D).

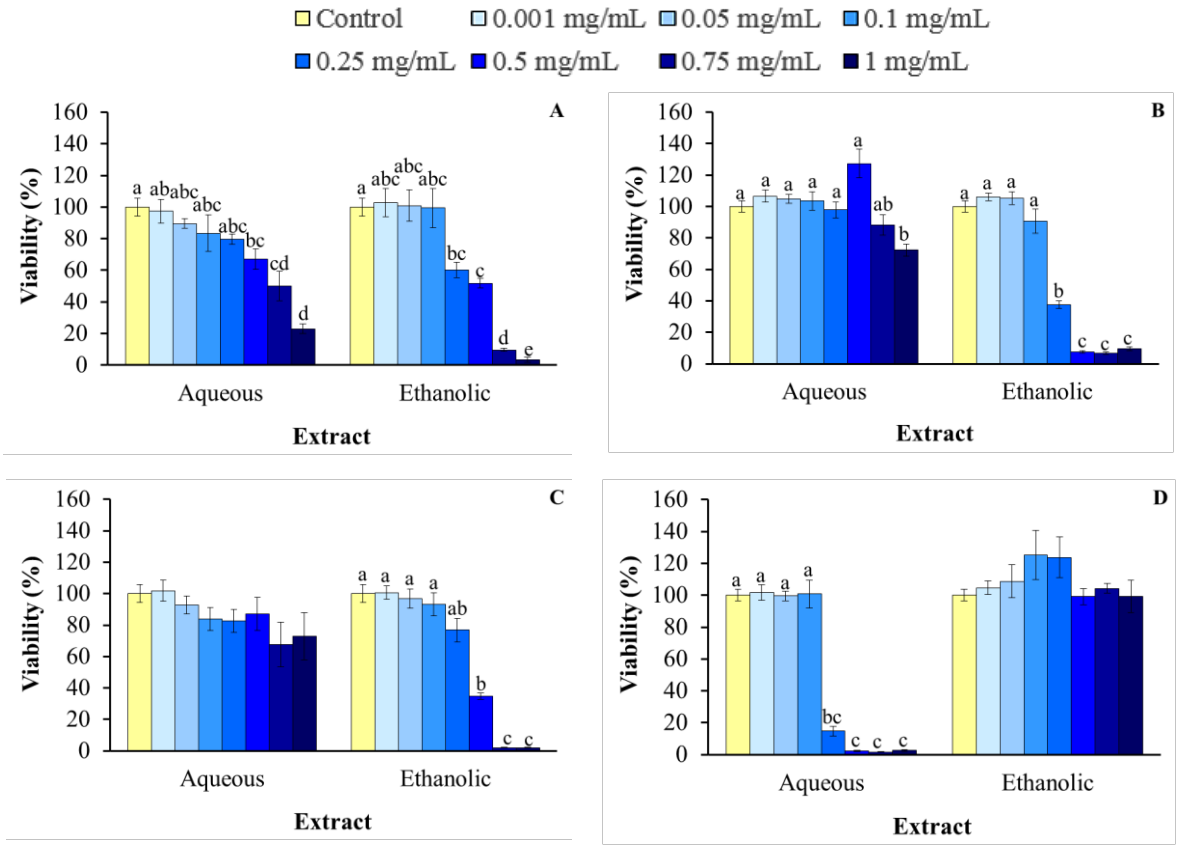



Figure 23: Viability of PLHC-1 cells after 24 h of incubation with aqueous or ethanolic extracts from oregano (A), date palm (B), purslane (C) or moringa (D). The results are expressed as mean ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

### I.4.7. Bactericidal activity of plant extracts

#### I.4.7.1. Vibrio harveyi

Regarding results of aqueous extracts, only purslane extracts (Fig. 24C) showed bactericidal activity, while no effects were obtained after bacterial incubation with date palm (Fig. 24B) and moringa (Fig. 24D) extracts. Contrarily, incubation with oregano extracts (Fig. 24A) increased bacteria viability respect to control.

Focusing on the activity of ethanolic extracts, oregano (Fig. 24A) and purslane (Fig. 24C) extracts showed a significant dose-dependent increase of bactericidal activity respect to control, especially in the case of purslane where low concentrations decreased already bacteria viability. Curiously, the lowest concentration of oregano extracts increased significantly bacteria viability (Fig. 24A). For its part, no activity was obtained after incubation with date palm (Fig. 24B) or moringa (Fig. 24D) extracts.

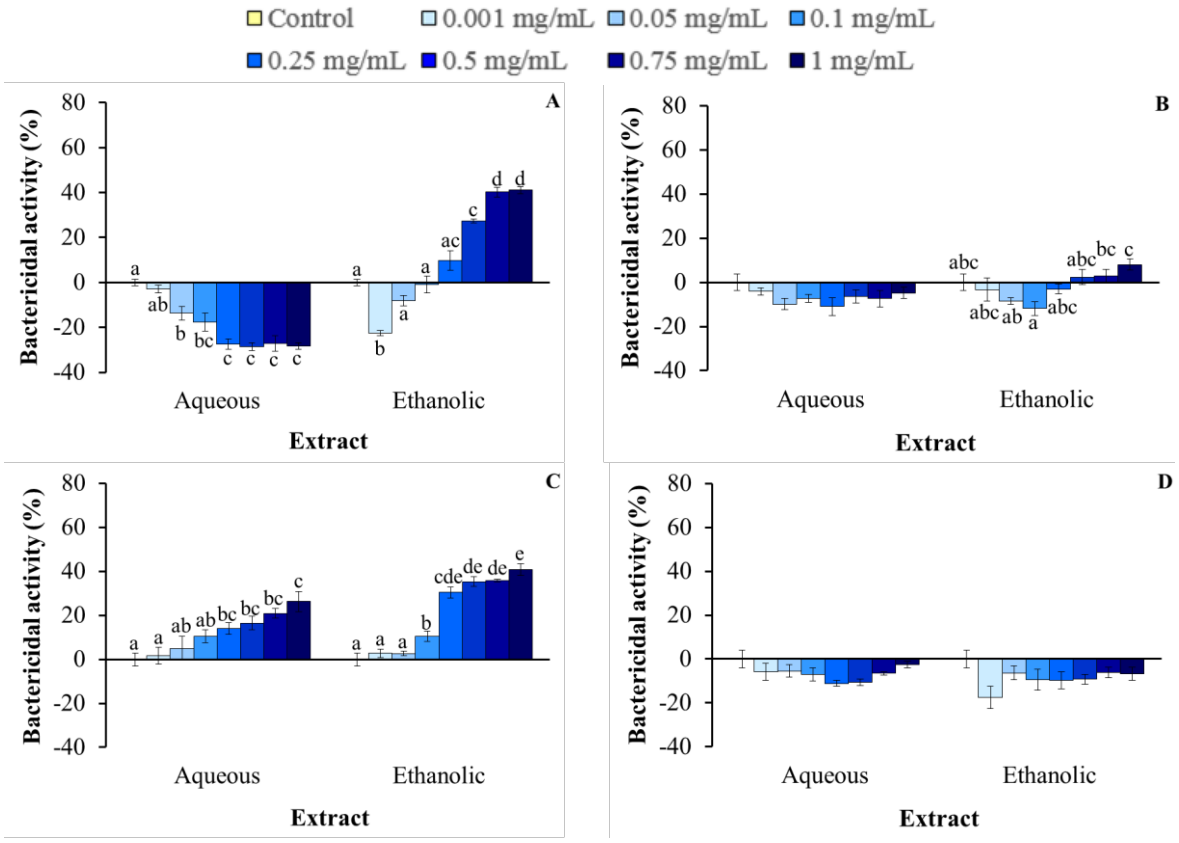



Figure 24: Bactericidal activity of aqueous or ethanolic extracts from oregano (A), date palm (B), purslane (C) or moringa (D) against *Vibrio harveyi* after 5 h of incubation. Control bacteria show 100% viability and 0 % bactericidal activity. The results are expressed as mean ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *I.4.7.2. Vibrio anguillarum*

Aqueous extracts of oregano (Fig. 25A) and date palm (Fig. 25B) showed no bactericidal activity respect to control, while purslane (Fig. 25C) and moringa (Fig. 25D) extracts showed a dose-dependent increase of this activity, being significant for moderate-high concentrations. Curiously, the lowest concentration of purslane extracts increased bacteria viability in a significant manner (Fig. 25C).

Focusing on ethanolic extracts results, a significant dose-dependent increase of bactericidal activity was observed after incubation of bacteria with oregano (Fig. 25A), purslane (Fig. 25C) and moringa (Fig. 25D) extracts, where low-moderate concentrations decreased bacteria viability. For its part, the highest concentration of date palm extract (Fig. 25B) also showed bactericidal activity.

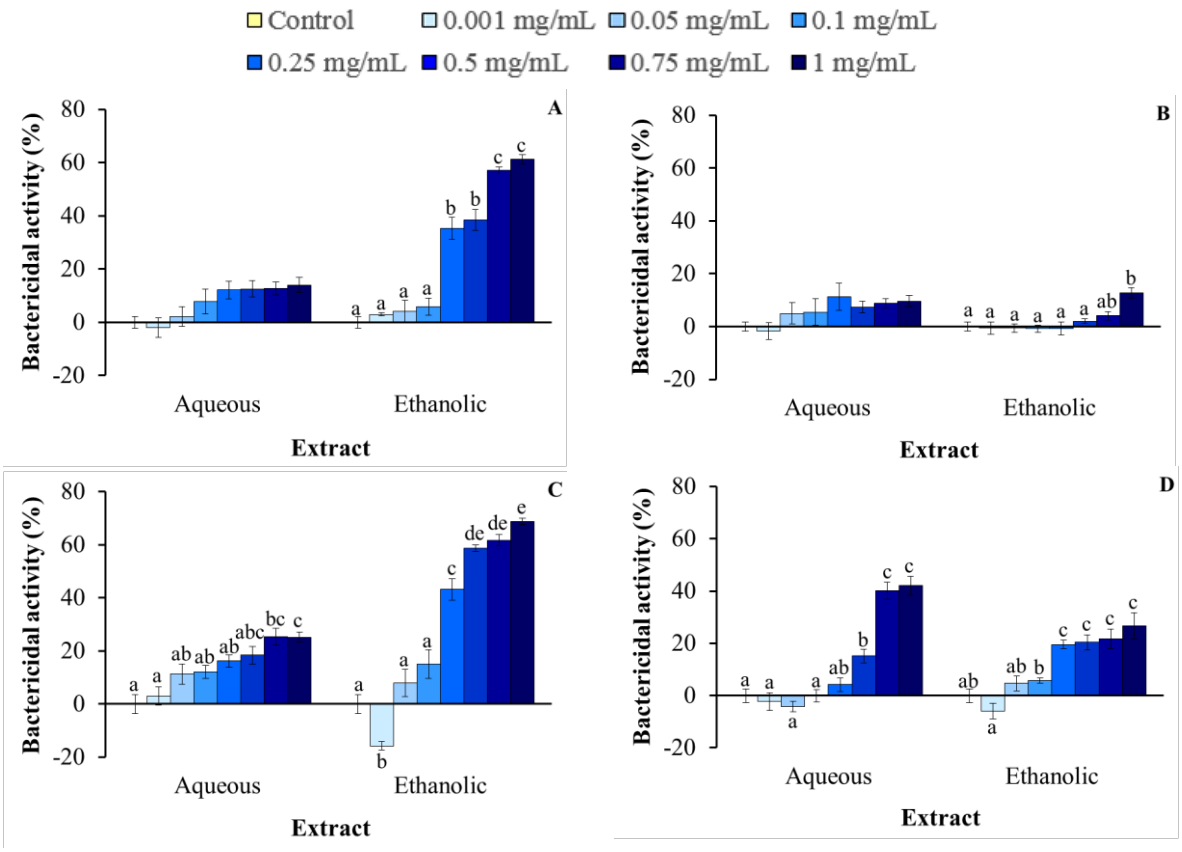



Figure 25: Bactericidal activity of aqueous or ethanolic extracts from oregano (A), date palm (B), purslane (C) or moringa (D) against *Vibrio anguillarum* after 5 h of incubation. Control bacteria show 100% viability and 0 % bactericidal activity. The results are expressed as mean ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *I.4.7.3. Photobacterium damselae* subsp. *piscicida*

Aqueous extracts of purslane (Fig. 26C) and moringa (Fig. 26D) showed a significant dose-dependent increase of bactericidal activity respect to control, especially in the case of purslane, where low concentrations already showed significant activity. For its part, the highest concentration of oregano extracts also showed bactericidal activity (Fig. 26A), while no effects were observed after incubation with date palm extracts (Fig. 26B).

Focusing on the activity of ethanolic extracts, oregano (Fig. 26A), purslane (Fig. 26C) and moringa (Fig. 26D) showed a significant dose-dependent bactericidal activity respect to control, while no effects were obtained after incubation with date palm extracts (Fig. 26B).

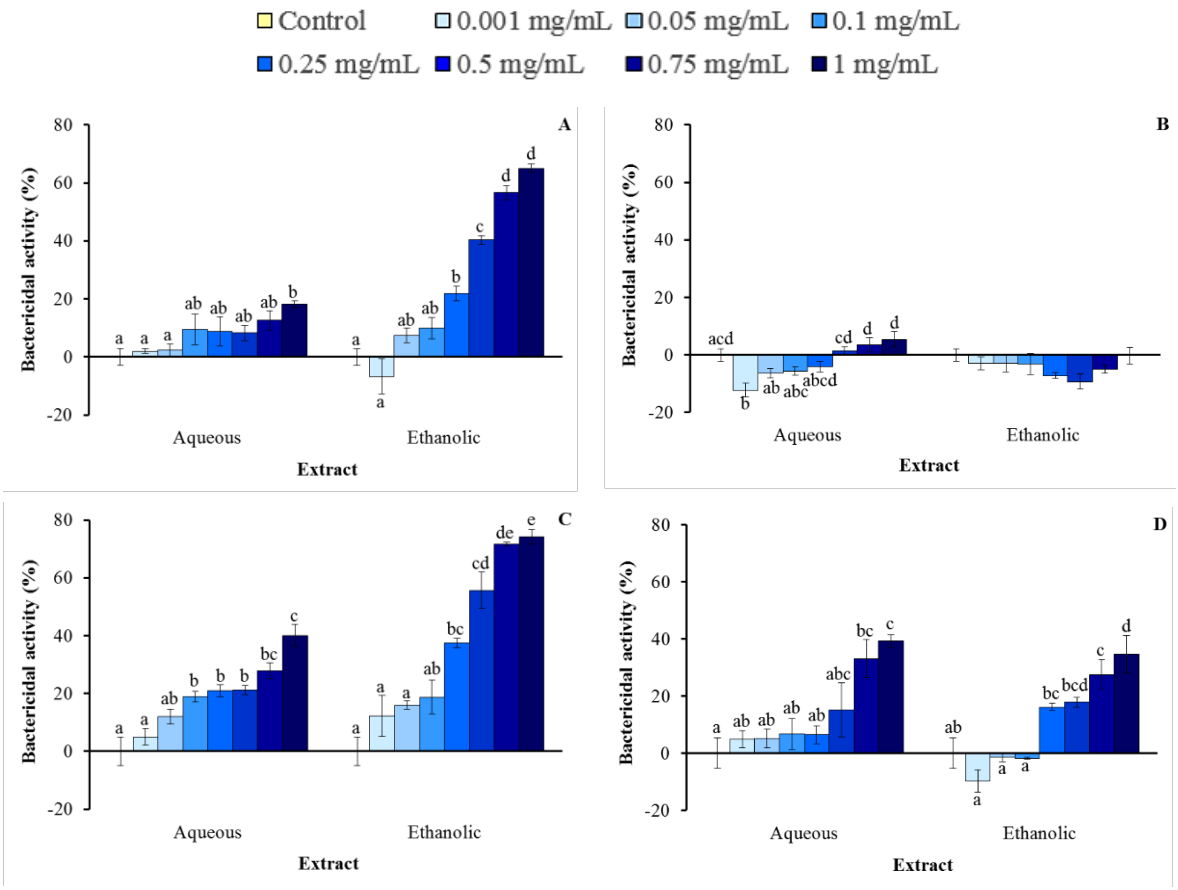



Figure 26: Bactericidal activity of aqueous or ethanolic extracts from oregano (A), date palm (B), purslane (C) or moringa (D) against *Photobacterium damselae* subsp. *piscicida* after 5 h of incubation. Control bacteria show 100% viability and 0 % bactericidal activity. The results are expressed as mean ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *I.4.8. TAA of plant extracts*

The TAA of plant extracts was also tested due to the importance of oxidative stress in the maintenance of fish health. Results show that both, aqueous and ethanolic plant extracts present antioxidant activity in a dose-dependent manner, although aqueous extracts presented more activity than ethanolic in all cases (Fig. 27).

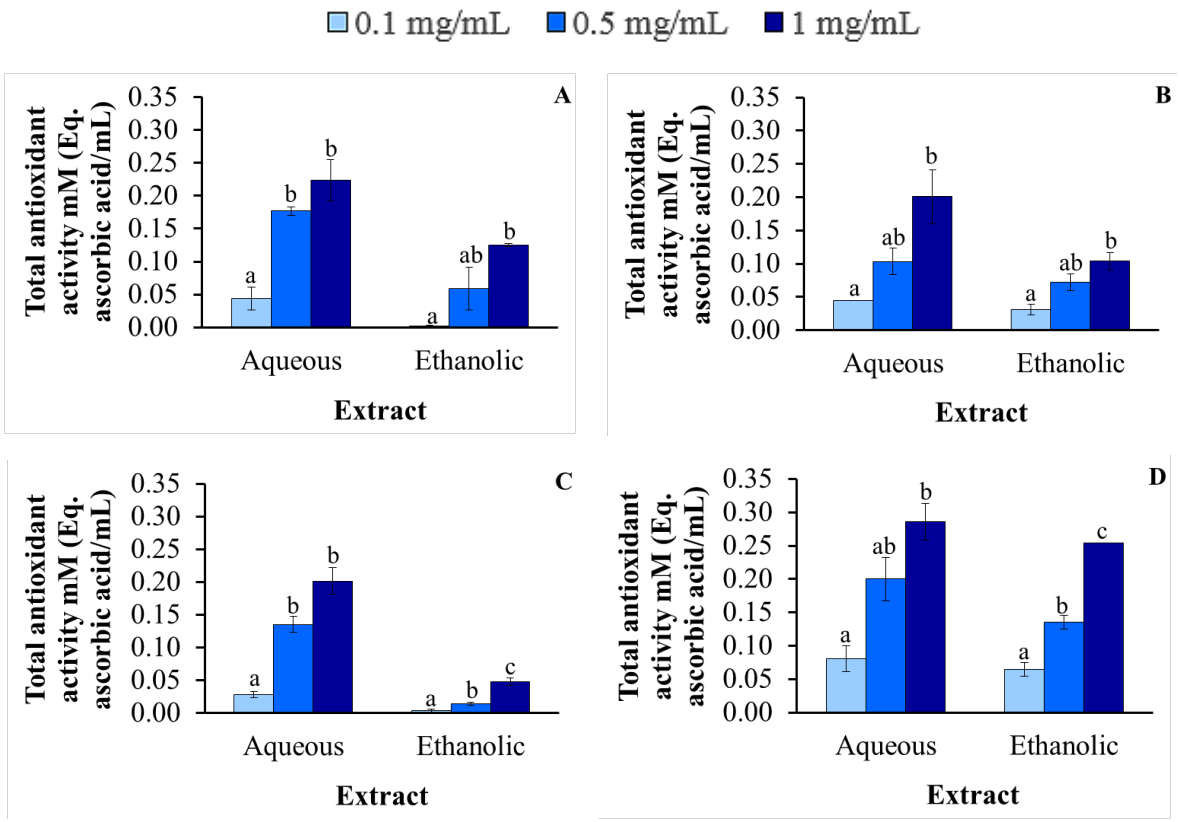


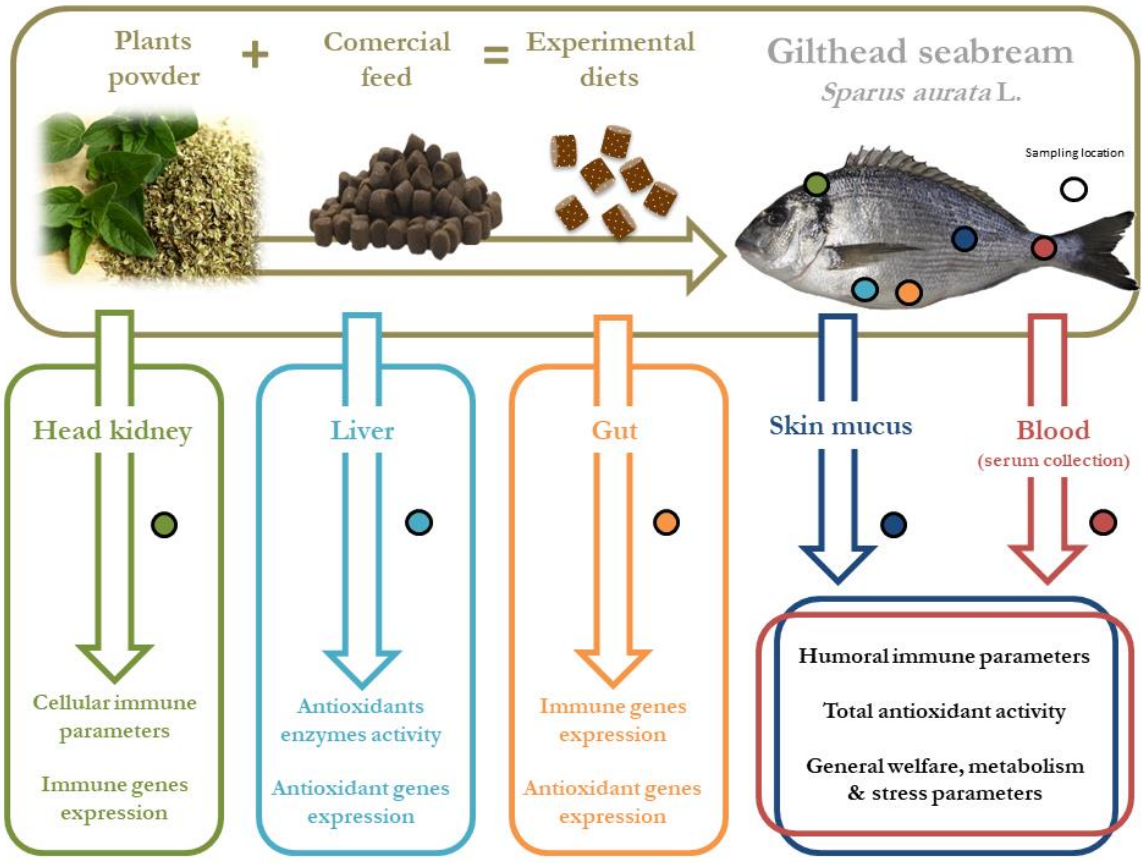

Figure 27: Antioxidant activity of aqueous or ethanolic extracts from oregano (A), date palm (B), purslane (C) or moringa (D). The results are expressed as mean ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).


Drawing from Flora von Deutschland Österreich und der Schweiz.
Otto Wilhelm Thomé. 1885. Not included in figures.

# CHAPTER II.

# Dietary immunostimulant and antioxidant activities of different plants on gilthead seabream (*Sparus aurata* L.)

## II.1 Graphical abstract



## II.2. Introduction

Fish aquaculture has been predicted that it will continue increasing, supplying almost two-thirds of food fish worldwide (World Bank, 2013; FAO, 2018). However, aquaculture management carries a series of risks such as high concentration of fish, bad water conditions, transport or manipulation that can help the apparition of some problems such as skin lesions and stress, which can compromise the immune status of fish and makes them susceptible to infections and diseases and even die, causing finally heavy economic losses (Bulfon et al., 2013; Holmes et al., 2016). Therefore, an environmentally sustainable development of the aquaculture maintaining an adequate level of welfare is crucial to ensure its success in the future by good growth performance, health and quality by rendering fish less susceptible to disease and infections (World Bank, 2013). In this sense, phytotherapy has begun to be a very interesting friendly and immunostimulant alternative to antibiotics and vaccines due to that phytochemicals could replace chemotherapeutic agents as immunoprophylactics contributing to fish welfare, without a negative impact on animal, human or environment (Direkbusarakom, 2004; Citarasu, 2010; Harikrishnan et al., 2011; Bulfon et al., 2013; Reverter et al., 2014; van-Hai, 2015; Awad and Awaad, 2017). Furthermore, thanks to the importance of these plants as food sources due to their content of vitamins, minerals, fibers, carbohydrates, proteins and fatty acids (Anwar et al., 2007; Saini et al., 2016), they have the ability to stimulate the appetite of the fish and their growth when they are administered in the food (Immanuel et al., 2004; Sivaram et al., 2004; Citarasu, 2010; Charakborty and Hancz, 2011; Pavaraj et al., 2011), besides promoting the maturation of the cultivated species (Chakraborty and Hancz, 2011; Harikrishnan et al., 2011; Bulfon et al., 2013).

For these reasons, studies where medicinal plants, their extracts or their essential oils have been used *in vivo* as additives in fish diet has gained importance since the last decade (reviewed by Sakai, 1999; Dugenci et al., 2003; Direkbusarakom, 2004; Galina et al., 2009; Citarasu, et al., 2010; Chakraborty and Hancz, 2011; Harikrishnan et al., 2011; Bulfon et al., 2013; Chakraborty et al., 2014; Reverter et al., 2014; Newaj-Fyzul and Austin, 2015; van-Hai, 2015; Awad and Awaad, 2017). According to these available reviews, the reduction of costs, easy availability and preparation, biodegradability, biocompatibility and the absence of side effects on animals or environment are crucial to their use in aquaculture, as well as they are capable to stimulate both, innate and adaptive immune system including humoral and cellular responses.

Taking into account the known traditional uses of plants, their stable abundance during all the year, the great quantity of demonstrated pharmacological properties, the *in vivo* studies in which plants, their extracts or essential oils have been used as additives in fish feed, the increasing importance of antioxidant additives in feed, and our previous experience in testing different supplemented diets in fish (Cerezuela et al., 2012, 2013, 2016; Esteban et al., 2014; Adel et al., 2015a, 2015b; Awad et al., 2015; Espinosa et al., 2016; Guardiola et al., 2016; 2017a, 2017b, 2018; Mansour et al., 2018) we decided to carry out the present research. Oregano leaves, DPS and dehydrated lemon peel (DLP) powder were used as dietary supplement to gilthead seabream.

# Chapter II.A.

## Dietary administration of oregano leaves powder

## II.A.3. Results

## II.A.1. Introduction

Oregano (*O. vulgare*) is well known for its aromatic and medicinal properties (Oniga et al., 2018), as well as from a culinary and agricultural point of view (Goncearіuc et al., 2015). Due to the beneficial effects in human health, oregano has been used in traditional medicine since ancient times (Barros et al., 2010; Chishti et al., 2013; Oniga et al., 2018) and is very common in Mediterranean areas including Spain (Ietswaart, 1980; Kokkini, 1997), where is especially abundant in the southern regions such as the region of Murcia.

Regarding its use as additive to animal diets there are some studies available focused on the effects of dietary administration of organo essential oils on animal diets including lambs (Dudko et al., 2017), pigs (Ariza-Nieto et al., 2011; Ranucci et al., 2015; Zou et al., 2016; Cheng et al., 2017; Zou et al., 2017), broiler chickens (Giannenas et al., 2003; Fotea et al., 2015; Ghazi et al., 2015; Mohiti-Asli and Ghanaatparast-Rashti, 2015), rats (Akilli and Eraslan, 2016), mice (Bukovska et al., 2007) and boar (Liu et al., 2017), while different extracts have been used in rats (Srihari et al., 2008; Vujicic et al., 2015; Abd et al., 2016; Hassanzadeh-kiabi and Negahdari, 2017), broiler chickens (Scocco et al., 2017) and rabbits (Nosal et al., 2014). Furthermore, the dried entire plant or some parts have been used as fed additive as powder in ducks (Park et al., 2015), while dried powder enriched with essential oil was used in pigs (Walter and Bilkei, 2004). Finally, regarding studies carried out in fish, oregano essential oil was administered to Tilapia zillii (*Coptodon zillii*) (Mabrok and Wahdan, 2018), oregano leaves 80% ethanolic leaves extract was administered to rainbow trout (*Oncorhynchus mykiss*) (Haghighi and Rohani, 2015) and dried powder was administered to Nile tilapia (*Oreochromis niloticus*) (Seden et al., 2009). In most of these studies, oregano used as dietary additive has showed antioxidant, anti-stress, antimicrobial, protective and inmunostimulatory activities (Walter and Bilkei, 2004; Park et al., 2015).

Therefore, due to the abundance of the genus *Origanum* in Spain, its widespread use in many industrial sectors, and considering the previous studies carried out with oregano, we decided to carry out the present research, which is the first that studied the effect of dried oregano leaves as dietary additive in marine fish such as gilthead seabream. The aim of the present work was to study the effects of dietary supplementation with dried powder from oregano leaves on gilthead seabream growth performance, humoral and cellular immune parameters and liver antioxidant status.

## II.A.2. Material and methods

### *II.A.2.1. Animals*

Sixty specimens of gilthead seabream were obtained from a local farm and maintained as in Chapter 1. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

### *II.A.2.2. Preparation of diets and experimental design*

Dried oregano leaves were bought in a local market (Murcia, Spain) and crused to be power. A commercial pellet diet (Skretting) was crushed and mixed with tap water before adding the correct amount of oregano powder and pelleting to obtain diets supplemented with 0% (control), 0.5% and 1% oregano. All the experimental diets were allowed to dry and stored at 4°C.

Fish were randomly assigned and divided into six tanks (n = 10 in each) thus establishing three groups and two replicates by group: control (0% or non-supplemented diet), 0.5% and 1% oregano supplemented diet. Animals were fed at a rate of 2% body weight/day for a month. Fifteen and thirty days after the beginning of the treatment, ten animals from each experimental group (five of each replicate tank) were sampled after sacrificing with an overdose of MS-222.

### *II.A.2.3. Sample collection*

Samples of blood, skin mucus, HK and liver were obtained. Blood samples were collected from the caudal vein with an insulin syringe. The blood samples were left to clot at 4°C for 4 h and later the serum was collected by centrifugation (10,000 g, 10 min, and 4°C) and stored at -80°C. Skin mucus samples were collected using the method described by Guardiola et al. (2014a). Briefly, skin mucus was collected by gentle scraping the dorso-lateral surface of specimens using a cell scraper with enough care to avoid contamination with blood and urine-genital and intestinal excretions. The skin mucus was vigorously shaken and centrifuged (400 g, 10 min, 4ºC). The supernatant was collected and stored at -80ºC until use. HK leucocytes were isolated as previously described in Chapter 1 and adjusted to $10^7$ cells/mL in sRPMI. Cell viability was determined by the trypan blue exclusion test. Fragments of liver for antioxidant enzymes analysis were collected and stored at -80°C until use.

#### II.A.2.4. Growth performance

The body weight of each fish was measured before the trial. Growth was monitored by obtaining the weight gain (WG), specific growth rate (SGR) and condition factor (CF), which were calculated for each of the treatments according to Silva-Carrillo et al. (2012).

$$WG\% = [(\text{final weight} - \text{initial weight})/\text{initial weight}] \times 100$$

$$SGR\% = [(\ln \text{final weight} - \ln \text{initial weight})/\text{number days}] \times 100$$

$$CF\% = [(\text{weight}/\text{length}^3)] \times 100$$

#### II.A.2.5. Total protein in skin mucus and liver homogenates

Skin mucus and liver protein concentration was determined by the dye binding method of Bradford (1976), using bovine serum albumin (BSA, Sigma-Aldrich) as the standard. Briefly, 2 mg/mL solution of BSA was prepared and serial dilutions made with PBS as standards. Dilutions of 5 µL of skin mucus or liver homogenates (see below) with 15 µL of PBS were prepared. Then 250 µL of Bradford reagent (Sigma-Aldrich) were added to BSA and skin mucus and liver dilutions and incubated at room temperature (RT) for 10 min. The absorbance of each sample was then read at 595 nm and the total protein content of skin mucus and liver interpolated from the standard curve.

#### II.A.2.6. Immune status

##### II.A.2.6.1. IgM level

Serum and skin mucus IgM levels were analysed using the enzyme-linked immunosorbent assay (ELISA) (Cuesta et al., 2004). For this, 100 µL per well of 1/500 diluted serum and 100/500 diluted skin mucus with 50 mM carbonate-bicarbonate buffer (35 mM $NaHCO_3$ and 15 mM $Na_2CO_3$, pH 9.6) were placed in flat-bottomed 96-well plates in triplicate, and proteins coated by overnight incubation at 4ºC. After three rinses with 200 µL per well of PBS-T (0.1 M PBS with 0.05% Tween 20, pH 7.3) the plates were blocked for 2 h at RT with 200 µL of blocking buffer (3% BSA in PBS-T), followed by three new rinses with PBS-T. The plates were then incubated for 1 h with 100 µL/well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd., 1/100 in blocking buffer). Following rising, plates were incubated for 1 h with 100 µL/well of secondary antibody anti-mouse IgG-HRP (1/1,000 in blocking buffer, Sigma-Aldrich). After exhaustive rising with PBS-T the plates were developed using 100 µL/well of a 0.42 mM solution of TMB,

prepared daily in a 100 mM citric acid/sodium acetate buffer (pH 5.4) containing 0.01% $H_2O_2$. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 µL 2 M $H_2SO_4$ and the plates were read at 450 nm in a plate reader. Serum and skin mucus samples of gilthead seabream previously tested were used as a positive control. Negative controls consisted of samples without serum and skin mucus or without primary antibody, whose optical density (OD) values were subtracted for each sample value.

#### *II.A.2.6.2. Natural haemolytic complement activity*

The activity of the alternative complement pathway of serum was assayed using sheep red blood cells (SRBCs, Biomedics) as targets (Ortuño et al., 1998). Equal volumes of SRBC suspension (6%) in phenol red-free HBSS containing $Mg^{+2}$ and ethylene glycoltetraacetic acid (EGTA, Sigma-Aldrich) were mixed with serially diluted serum to give final serum concentrations ranging from 10% to 0.078%. After incubation for 90 min at 22ºC, the samples were centrifuged at 400 g for 5 min at 4ºC to avoid unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their OD at 550 nm in a plate reader. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 100 µL of distilled water or HBSS to100 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) against the volume of serum added (mL) on alog-log scale graph. The volume of serum producing 50% haemolysis ($ACH_{50}$) was determined and the number of $ACH_{50}$ units/mL obtained for each experimental fish.

#### *II.A.2.6.3. Lysozyme activity*

Serum and skin mucus lysozyme activity was measured according to the turbidimetric method described by Parry (1965) with some modifications. To do this, 20 µL of serum and skin mucus diluted 1:10 with 0.04 M PBS buffer, pH 6.2, were placed in a flat-bottomed 96-well plate. To each well, 200 µL of freeze-dried *Micrococcus lysodeikticus* in the above buffer (0.3 mg/mL, Sigma-Aldrich) was added as lysozyme substrate. The reduction in absorbance at 450 nm was measured over 15 min at 3 min intervals at RT in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001/min. The units of lysozyme present in skin mucus and serum were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma-Aldrich), and the results were expressed as U/mL for the serum samples or as U/mg protein for the skin mucus samples.

#### II.A.2.6.4. Bactericidal activity

Serum and skin mucus bactericidal activity was studied against *V. harveyi*, *V. anguillarum* and *P. dampselae* subsp. *piscicida* as previously described in Chapter 1.

#### II.A.2.6.5. Protease activity

Serum and skin mucus protease activity was quantified using the azocasein hydrolysis assay according to the method described by Ross et al. (2000). For this, 10 µL of serum or 100 µL of skin mucus were incubated overnight at RT and in agitation with 100 µL of ammonium bicarbonate buffer and 125 µL of 2% (serum samples) or 0.7% (skin mucus samples) azocasein (Sigma-Aldrich) in sterile eppendorfs. The reaction was stopped by adding 250 µL of 10% (serum samples) or 4.6% (skin mucus samples) trichloroacetic acid (TCA). The mixtures were centrifuged (6,000 g, 5 min), 100 µL of the supernatants transferred to a flat-bottomed 96-well plate, and 100 µL of 1 N (serum samples) or 0.5 N (skin mucus samples) NaOH added. OD was read at 450 nm using a plate reader. Serum or skin mucus were replaced by trypsin (5 mg/mL, Sigma-Aldrich) for the positive controls (100% of protease activity) or by ammonium bicarbonate buffer for the negative controls (0% of protease activity). Activity for each sample was expressed as % protease activity in relation to the controls.

#### II.A.2.6.6. Antiprotease activity

Serum antiprotease activity was determined by the ability of serum to inhibit trypsin activity (Hanif et al., 2004). Briefly, 10 µL of serum samples were incubated (10 min, 22ºC) with the same volume of standard trypsin solution (5 mg/mL). After adding 100 µL of 100 mM ammonium bicarbonate buffer and 125 µL of buffer containing 2% azocasein (Sigma-Aldrich), samples were incubated (2 h, 30ºC) and, following the addition of 250 µL of 10% TCA, a new incubation (30 min, 30ºC) was done. The mixture was then centrifuged (10,000 rpm, 10 min) being the supernatants transferred to a 96-well plate in triplicate containing 100 µl/well of 1 N NaOH, and the OD read at 450 nm using a plate reader. For a positive control, buffer replaced serum and trypsin, and for a negative control, buffer replaced the serum. The antiprotease activity was expressed interms of percentage of trypsin inhibition according to the formula:

%Trypsin inhibition =100 × (Trypsin OD – Sample OD)/Trypsin OD.

#### II.A.2.6.7. Peroxidase activity

Serum and skin mucus peroxidase was determined diluting 5 µL of serum and 10 µL of skin mucus were diluted with 45 µL and 40 µL, respectively, of HBSS without $Ca^{2+}$ or $Mg^{2+}$ in flat-bottomed 96-well plates. As substrates, 100 µL of 20 mM TMB and 5 mM H2O2 were added. The colour-change reaction was stopped after 2 min by adding 50 µL of 2 M $H_2SO_4$ and the optical density was read at 450 nm in a plate reader. Standard samples without serum or skin mucus were used as blanks.

#### II.A.2.6.8. HK leucocyte immune activities

The phagocytosis, respiratory burst and peroxidase activities of HK leucocytes were determined as explained in Chapter 1.

### II.A.2.7. Antioxidant status

#### II.A.2.7.1. Liver antioxidant enzyme activities

Samples of liver were homogenized in 50 mM potassium phosphate buffer, pH 7, for 5 min and centrifugued (400 g, 10 min, 4ºC). Supernatants were collected and stored at 80ºC for later determination of glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT) activities. GR activity was measured by the method modified by Carlberg and Mannervik (1975). The reaction was initiated by adding 0.1 mM nicotinamide adenine dinucleotide phosphate (NADPH) to the samples in 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 mM glutathione disulphide or oxidized (GSSG). The change in absorbance was monitored at 340 nm for 3 min by a UV–Vis spectrophotometer (Thermo Scientific, model Evolution 300 dual beam). One unit of GR activity is defined as the amount of enzyme that catalyzes the reduction of 1 µmol of NADPH per minute (ε340 nm for NADPH/6.22 mMcm).

SOD activity was determined by the method of McCord and Fridovich (1969) based on the inhibition of the reduction of cytochrome C in the presence of SOD at 550 nm. The SOD-like activity of the complexes was studied at 25ºC by an indirect method using cytochrome C as superoxide oxidant (indicator). The superoxide radical anion was generated *in situ* by the xanthine oxidase reaction, and detected spectrophotometrically by monitoring the formation of the reduced form of cytochrome C at 550 nm in a spectrophotometer.

CAT activity was measured by the method of Aebi (1984) monitoring the consumption of $H_2O_2$ at 240 nm. This method is based on the principle that the absorbance will decrease due to the decomposition of $H_2O_2$ by catalase at 240 nm. $H_2O_2$ solution (10 mM), liver extract and 50 mM phosphate buffer, pH 7 were pipetted into a cuvette. The reduction of $H_2O_2$ was followed at a wavelength of 240 nm for 4 min against a blank containing 50 mM phosphate buffer.

All the enzyme activities were expressed in units of enzyme/mg protein.

#### *II.A.2.7.2. TAA of serum and skin mucus*

TAA of serum and skin mucus was analysed by the ABTS method as described in Chapter 1.

### *II.A.2.8. Statistical analyses*

The results are expressed as means ± SEM. The normality of the variables was confirmed by the Shapiro–Wilk test and homogeneity of variance by the Levene test. Statistical differences among the four groups of treatments were assessed by one-way ANOVA analyses, followed by the Tukey or Games-Howell test, depending on the homogeneity of the variables. The significance level was 95% in all cases ($P < 0.05$). All the data were analysed by the computer application SPSS for Windows®.

# II.A.3 Results

## II.A.3.1. Growth performance

No significant differences were obtained for WG, SGR or K in fish fed 0.5% or 1% oregano diets for 15 or 30 days compared with the control group (Fig. 28).

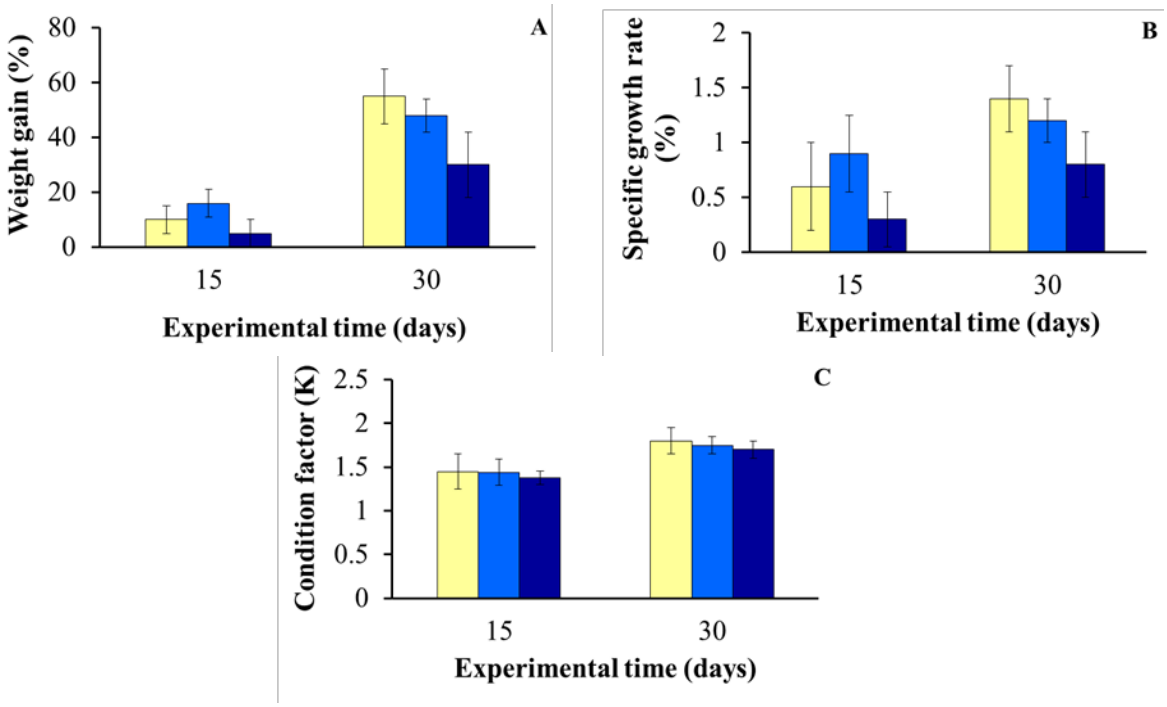



Figure 28: Weight gain (A), specific growth rate (B) and condition factor (C) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 0.5% (light blue) or 1% (dark blue) oregano for 15 and 30 days. Data are presented as means ± SEM (n = 10).

## II.A.3.2. Humoral immune parameters

### II.A.3.2.1. IgM level

No significant variations compared with the control group were observed in serum or skin mucus at 15 days of feeding with oregano diets; however, at 30 days serum IgM was significantly lower in fish fed 0.5% oregano diet, while skin mucus IgM level was significantly higher in fish fed 1% oregano diet compared with the control group (Fig. 29).

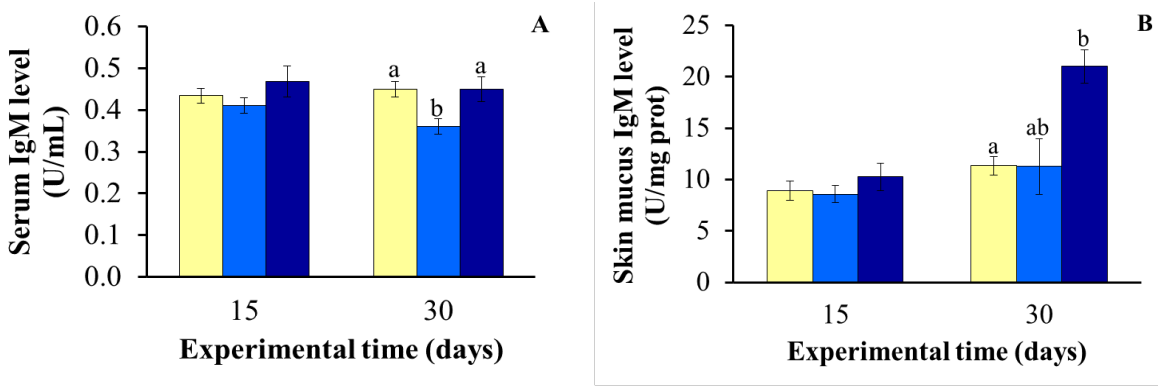



Figure 29: IgM levels in the serum (A) and skin mucus (B) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 0.5% (light blue) or 1% (dark blue) oregano for 15 and 30 days. Data are presented as means ± SEM (n = 10). Different letters denote significant differences between treatment groups ($P < 0.05$)..

### *II.A.3.2.2. Natural haemolytic complement activity*

No significant differences were observed in the natural haemolytic complement activity determined in serum of fish fed any experimental diet for 15 or 30 days, but there was a trend to increase in fish fed with oregano diets in a dose-dependent manner at 30 days compared with control fish (Fig. 30).

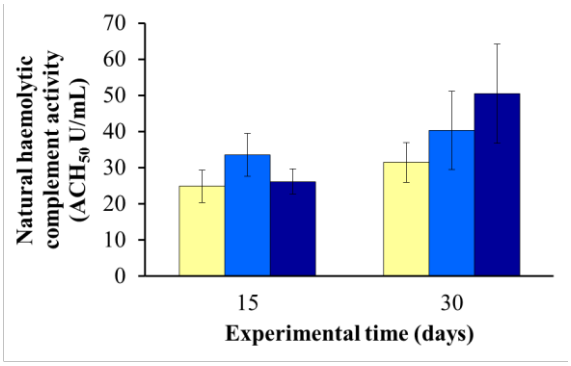



Figure 30: Serum natural haemolytic complement activity of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 0.5% (light blue) or 1% (dark blue) oregano for 15 and 30 days. Data are presented as means ± SEM (n = 10).

### *II.A.3.2.3. Lysozyme activity*

No differences were observed in serum or skin mucus of fish fed 0.5% or 1% oregano diets for 15 or 30 days compared with control fish (Fig. 31).

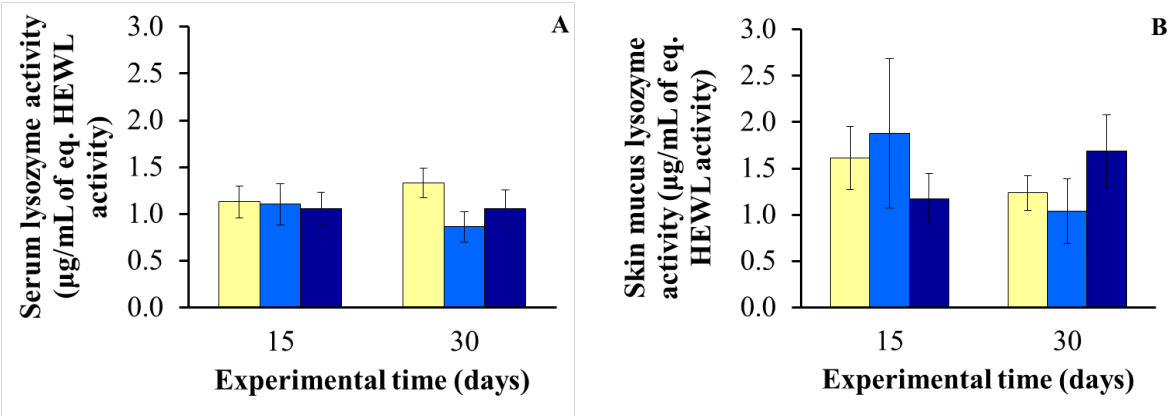



Figure 31: Lysozyme activity in the serum (A) and skin mucus (B) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 0.5% (light blue) or 1% (dark blue) oregano for 15 and 30 days. Data are presented as means ± SEM (n = 10).

### II.A.3.2.4. Bactericidal activity

Bactericidal activity was measured against three fish pathogens (*V. harveyi, V. anguillarum* and *P. damselae* subsp. *piscicida*). No significant variations were observed in the bactericidal activity of serum against any of the tested bacteria from fish fed for 15 or 30 days with oregano supplemented diets compared with control fish (Fig. 32A, 32B, 32C). However, skin mucus of fish fed 0.5% oregano diet for 30 days or 1% oregano diet for 15 or 30 days showed a significant increased bactericidal activity against *P. damselae* compared with control fish (Fig, 32D, 32E, 32F).

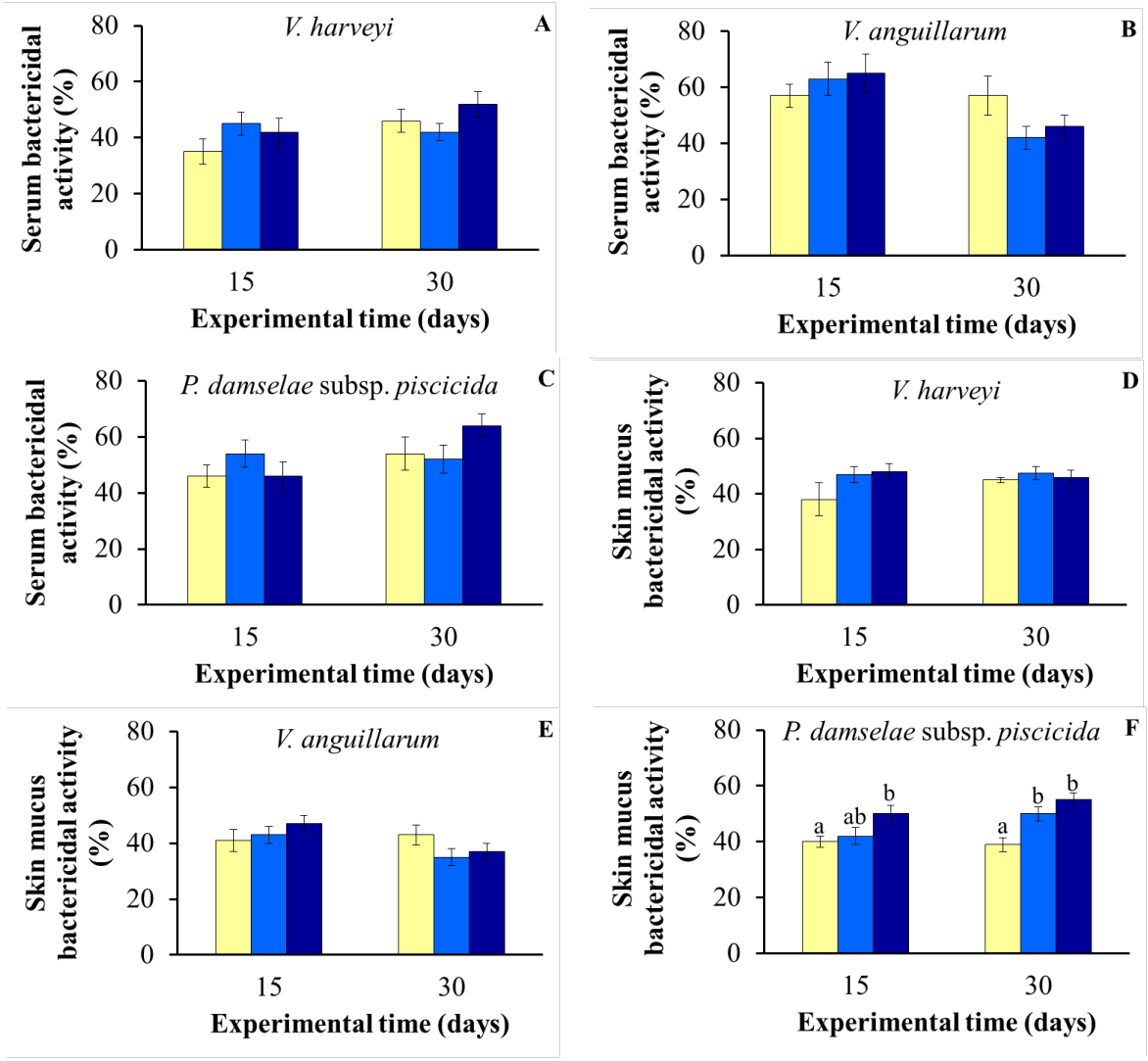



Figure 32: Bactericidal activity against *Vibrio harveyi* (A, D), *Vibrio anguillarum* (B, E) or *Photobacterium damselae* subsp. *piscicida* (C,F) in the serum (A, B, C) and skin mucus (D, E, F) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 0.5% (light blue) or 1% (dark blue) oregano for 15 and 30 days. Data are presented as means ± SEM (n = 10). Different letters denote significant differences between treatment groups ($P < 0.05$).

### II.A.3.2.5. Protease and antiprotease activities

A significantly higher protease activity in serum of fish fed 0.5 or 1% oregano diets compared with control fish was observed only at 30 days (Fig. 33A). However, no significant variations were observed in skin mucus of fish fed 0.5% oregano diet for 15 or 30 days compared to control group. Nevertheless, fish fed 0.5% oregano diet for 30 days showed a significant decrease in the protease activity of skin mucus compared with fish fed 1% oregano diet (Fig. 33B).

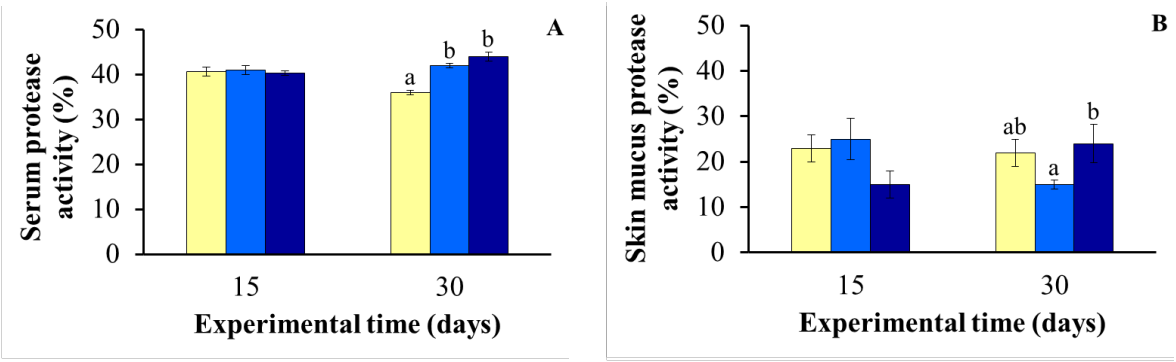



Figure 33: Protease activity in the serum (A) and skin mucus (B) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 0.5% (light blue) or 1% (dark blue) oregano for 15 and 30 days. Data are presented as means ± SEM (n = 10). Different letters denote significant differences between treatment groups ($P < 0.05$).

In the case of serum antiprotease activity, no significant differences were observed in serum of fish fed with oregano supplemented diets for 15 days compared with the activity found in control group. However, in serum from fish fed with 1% oregano diet for 30 days significant decreases were observed in such activity, compared with control fish (Fig. 34).

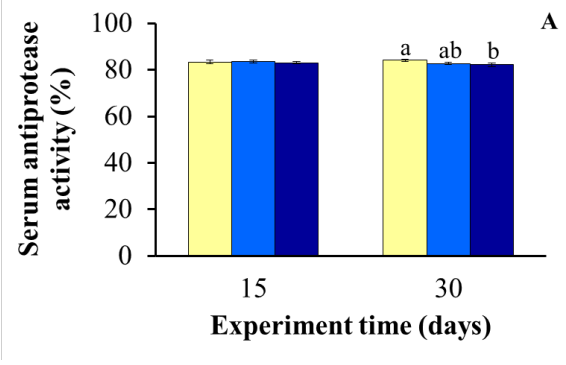



Figure 34: Antiprotease activity in the serum of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 0.5% (light blue) or 1% (dark blue) oregano for 15 and 30 days. Data are presented as means ± SEM (n = 10). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *II.A.3.2.6. Peroxidase activity*

No significant differences were observed in the peroxidase activity in serum or skin mucus of fish fed 0.5% or 1% oregano diets for 15 or 30 days, compared with control fish (Fig. 35).

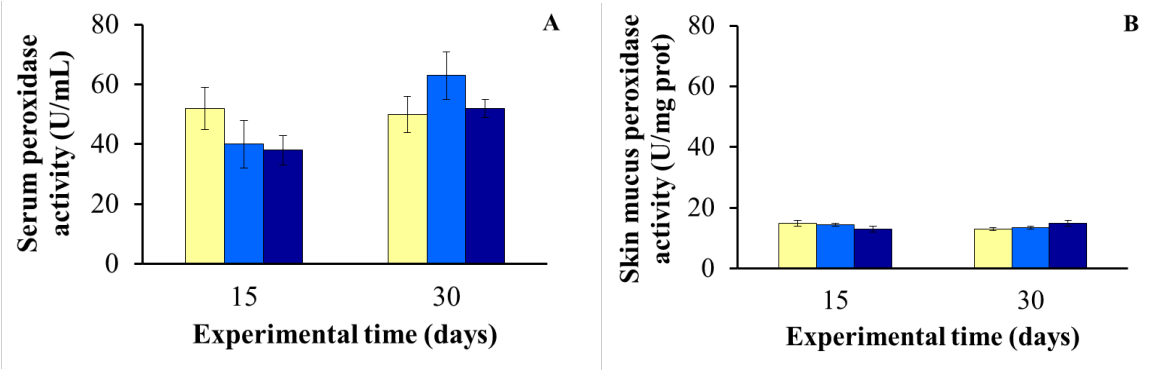



Figure 35: Peroxidase activity in the serum (A) and skin mucus (B) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 0.5% (light blue) or 1% (dark blue) oregano for 15 and 30 days. Data are presented as means ± SEM (n = 10).

### II.A.3.3. Cellular immune parameters

HK leucocyte phagocytic ability was significantly higher only in fish fed 0.5% oregano diet compared with control fish at 15 days (Fig. 36A), while no significant variations were observed in phagocytic capacity of fish fed 0.5% or 1% oregano diets at 15 or 30 days compared with control fish (Fig. 36B). Respiratory burst and peroxidase activities did not show any significant difference between fish fed oregano diets and control fish (Fig. 36C, 36D).

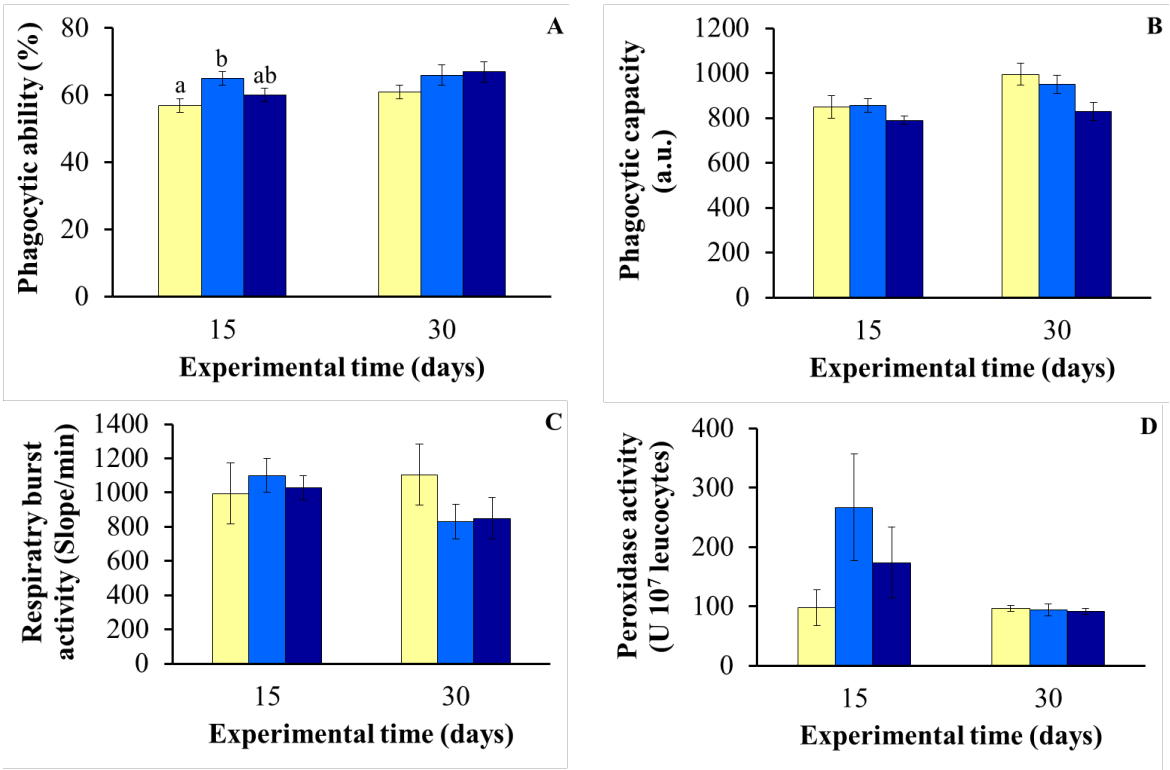



Figure 36: Phagocytic ability (A) and capacity (B), and respiratory burst (C) and peroxidase (D) activities in the head-kidney leucocytes of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 0.5% (light blue) or 1% (dark blue) oregano for 15 and 30 days. Data are presented as means ± SEM (n = 10). Different letters denote significant differences between treatment groups ($P < 0.05$).

### II.A.3.4. Liver antioxidant enzyme activities

No significant effects were observed in any of the three studied enzymes in fish fed oregano supplemented diets respect to the values obtained in liver from control group at any assayed time (Fig. 37), although fish fed 1% oregano diet showed a significant decrease of SOD activity respect fish fed 0.5% oregano diet at 15 days (Fig. 37B).

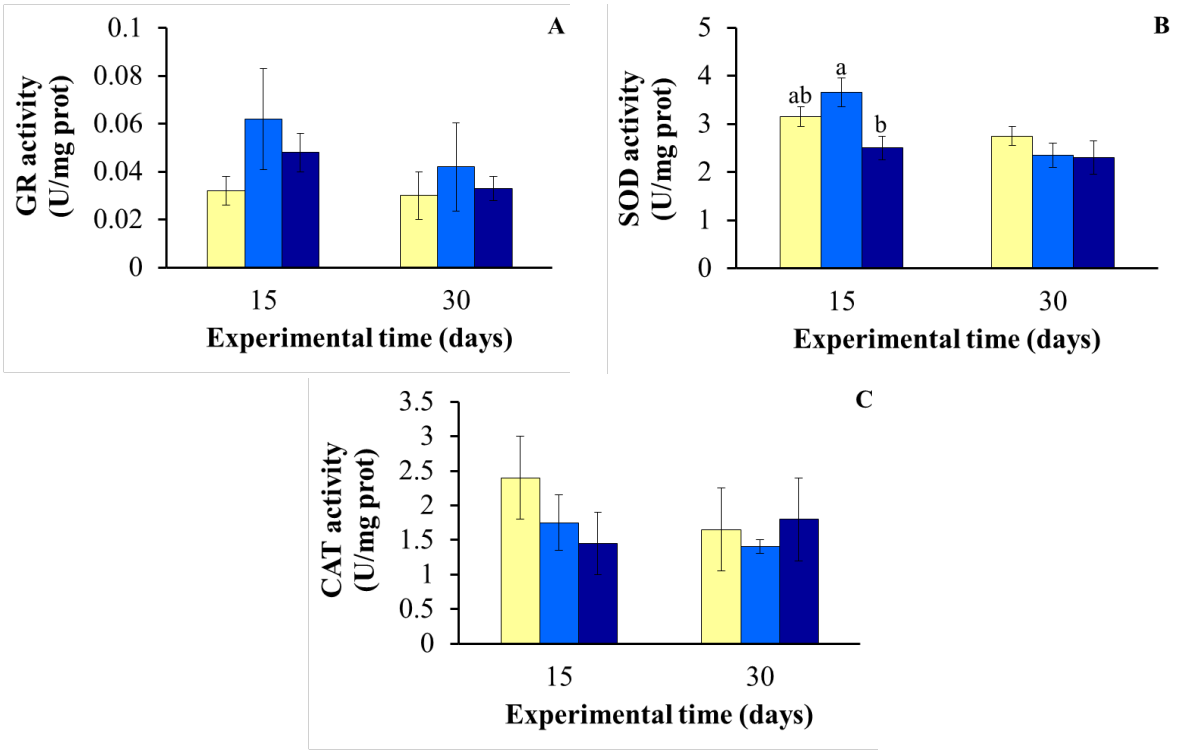



Figure 37: Glutathione reductase (A), superoxide dismutase (B) and catalase (C) activities in the liver of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 0.5% (light blue) or 1% (dark blue) oregano for 15 and 30 days. Data are presented as means ± SEM (n = 10). Different letters denote significant differences between treatment groups ($P < 0.05$).

### II.A.3.5. TAA of serum and skin mucus

No significant differences were observed in the TAA of serum or skin mucus obtained from fish fed any diet at 15 or 30 days, but there was a trend to increase in both serum and skin mucus in fish fed with oregano supplemented diets in a dose-dependent manner at 30 days compared with control fish (Fig. 38).

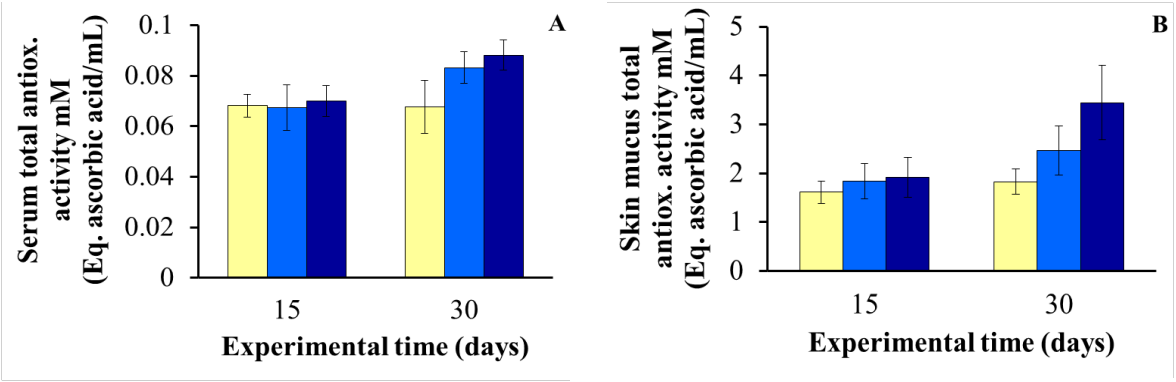
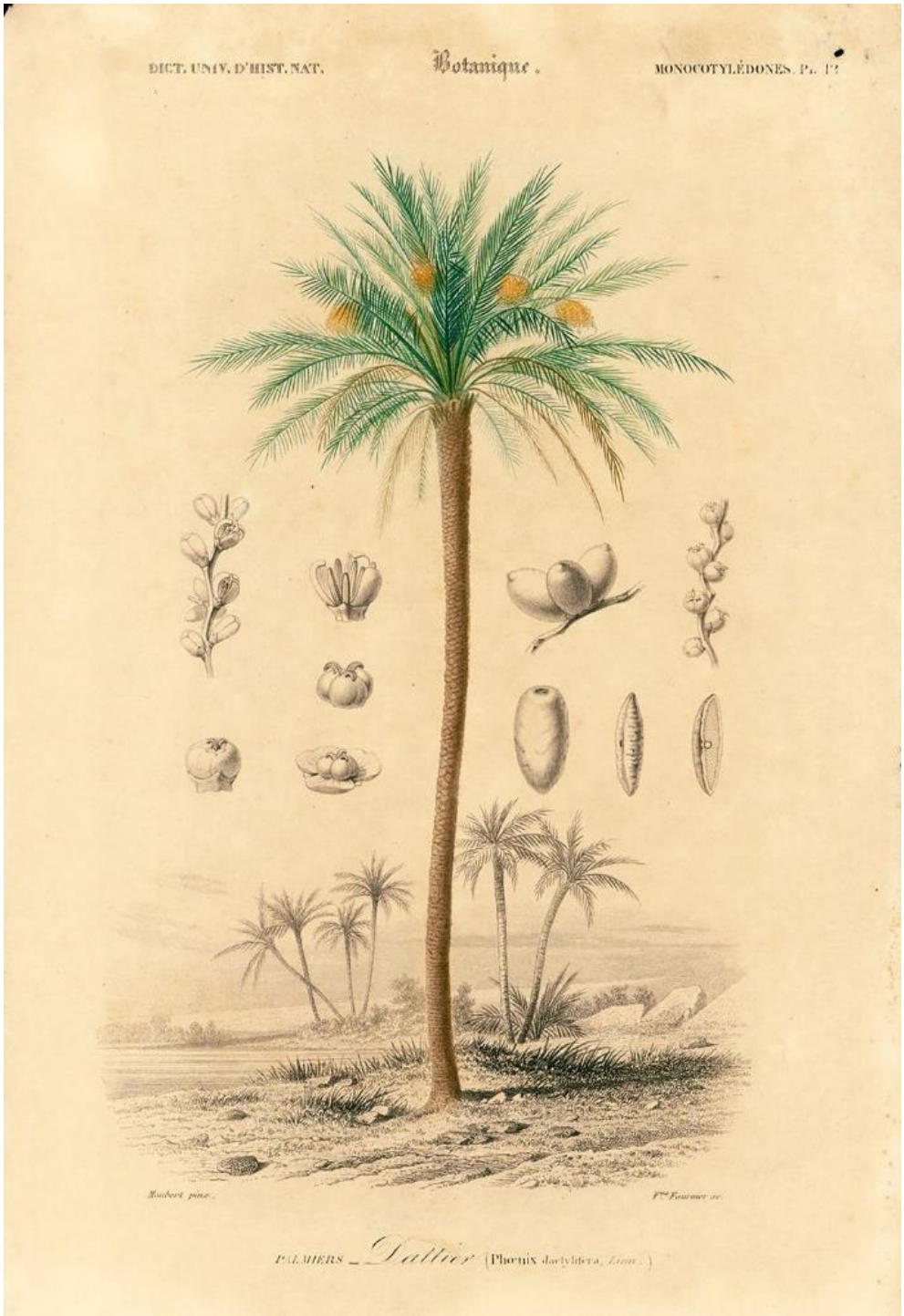



Figure 38: Total antioxidant activity in the serum (A) and skin mucus (B) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 0.5% (light blue) or 1% (dark blue) oregano for 15 and 30 days. Data are presented as means ± SEM (n = 10).




Drawing from Dictionnaire Universel d'Histoire Naturelle.
Charles Henry Dessalines d'Orbigny. 1861. Not included in figures.

# Chapter II.B.

## Dietary administration of date palm seeds powder

## II.B.1. Introduction

Date palm (*P. dactylifera*) is a very important plant species from an economical (Jain et al., 2011) and medicinal (El-Hadrami and Al-Khayri, 2012) point of view in MENA countries. Furthermore, this plant presents a very high food value (Zaid and de-Wet, 1999) due to the fact that its fruit is consumed and very appreciated because of its content of vitamins, minerals, fibers, carbohydrates, proteins, fatty acids and energy (Anwar et al., 2007; Saini et al., 2016).

DPS, which are considered a by-product or waste and discarded most times have been used in form of extracts in many studies in rats and antioxidant and tissue-protective effects have been observed in all of them as well as inhibition of DNA damage and antidiabetic, anti-hyperglycemic and anti-inflammatory activity after induction of oxidative stress (Al-Qarawi et al., 2004; Al-Qarawi et al., 2005; Habib and Ibrahim, 2011; Abdelaziz and Ali, 2014; Ahmed et al., 2015; Waly et al., 2015; Attia et al., 2016; Hasan and Mohieldein, 2016; Khan et al., 2017; Abdel-Magied et al., 2018; Abdel-Salam et al., 2018; Khan et al., 2018). More interesting is the use of DPS in livestock. DPS are habitually softened by soaking in water to feed livestock or animals (e.g. camels, sheep, goats and horses), or crushed dry and added to chicken feed, although cows, pigs, broilers, lambs and fish (Belal, 2008; El-Hadrami et al., 2011; Hossain et al., 2014) have been also used to study the effect of DPS as dietary additive and very positive results have been obtained.

Therefore, due to the fact that date palm is a very common plant in Spain and especially in region of Murcia and province of Alicante, which presents the highest concentration of date palm of European Union, its economical and medicinal properties and the common use of DPS in livestock and their positive previous results as dietary additive, we developed the present research using powder of DPS as dietary supplement to gilthead seabream in a try also to develop a sustainable aquaculture in the MENA countries using a natural product which are discarded after the fruit is consumed, helping also to these countries to create job, help meet global demand, and achieve their own food security aspirations (AquaMe, 2016). The main aim of our study was to analyse the effect of dietary DPS on gilthead seabream growth performance, immune status and liver antioxidant defense.

## II.B.2. Material and methods

### *II.B.2.1. Animals*

Seventy-two specimens of gilthead seabream were obtained from a local and maintained as in Chapter 1.

### *II.B.2.2. Preparation of diets and experimental design*

Date palm dates, Deglet Nour variety, at the ripeness stage were collected on Monastir (Tunisia). Seeds were isolated and grounded into powder (32 meshes). A commercial pellet diet (Skretting, Spain) was crushed and mixed with tap water before adding the correct amount of crushed DPS powder and pelleting to obtain diets supplemented with 0% (control), 1.5% and 3% DPS. All the experimental diets were allowed to dry and stored at 4°C.

Fish were randomly assigned and divided into nine tanks (n = 8 in each) thus establishing three groups and three replicates by group: control (non-supplemented diet), 1.5% and 3% DPS diet. Animals were fed at a rate of 2% body weight/day for a month. Fifteen and thirty days after the beginning of the treatment, nine animals from each experimental group (three of each replicate tank) were sampled after sacrificing as previously. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

### *II.B.2.3. Sampling and analysis*

Samples were obtained and growth parameters, immune stauts and antioxidant activity determined as in the material and methods of Chapter II.A.

# II.B.3. Results

## II.B.3.1. Growth performance

Although a decrease in WG and SGR of fish fed 3% DPS diet was observed, no significant differences were obtained for any parameter at any assayed time compared with the control group (Fig. 39).

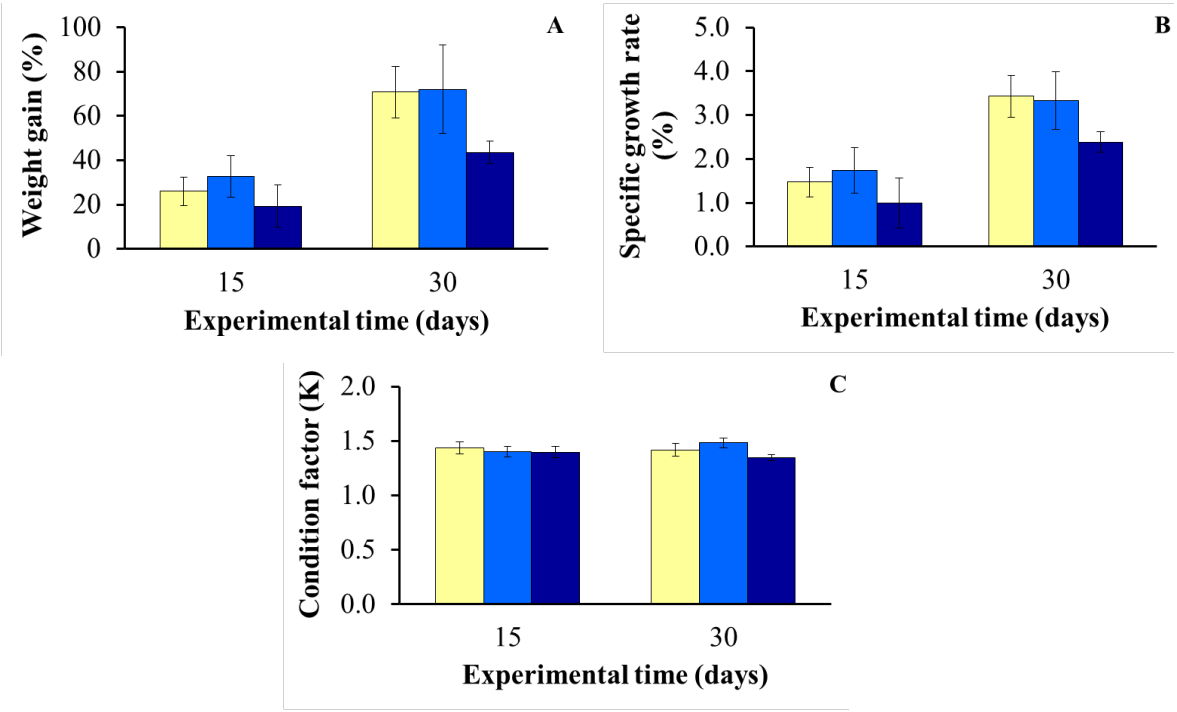



Figure 39: Weight gain (A), specific growth rate (B) and condition factor (C) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) date palm seeds (DPS) for 15 and 30 days. Data are presented as means ± SEM (n = 9).

## II.B.3.2. Humoral immune parameters

### II.B.3.2.1. IgM level

No significant variations, compared with the control group, were observed in serum or skin mucus IgM levels at 15 or 30 days after feeding. However, no significant increases were observed in skin mucus IgM level at 15 days in fish fed 3% DPS diet, respect to control (Fig. 40B).

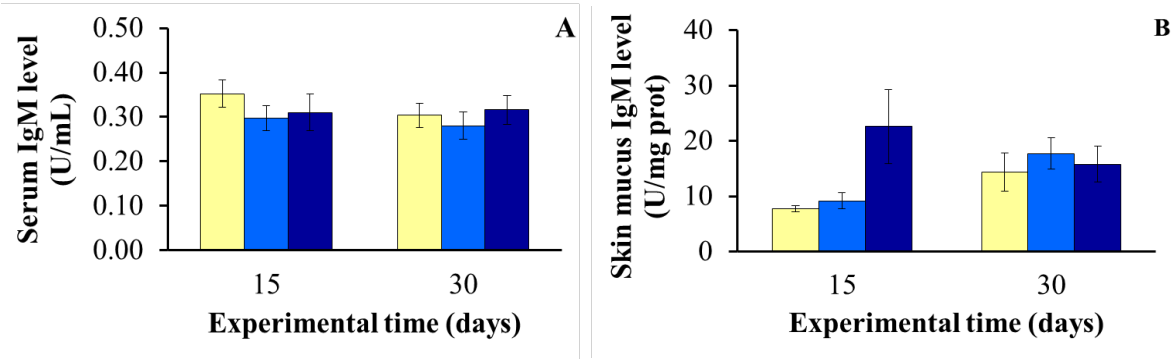



Figure 40: IgM levels in the serum (A) and skin mucus (B) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) date palm seeds (DPS) for 15 and 30 days. Data are presented as means ± SEM (n = 9).

### II.B.3.2.2. Lysozyme activity

Fish fed 3% DPS diet showed an increase in serum lysozyme activity at 15 days while fish fed with 1.5% and 3% DPS diets showed a dose-dependent decrease in skin mucus lysozyme activity. However, no significant differences were observed in serum or skin mucus at 15 or 30 days compared with control group (Fig. 41).

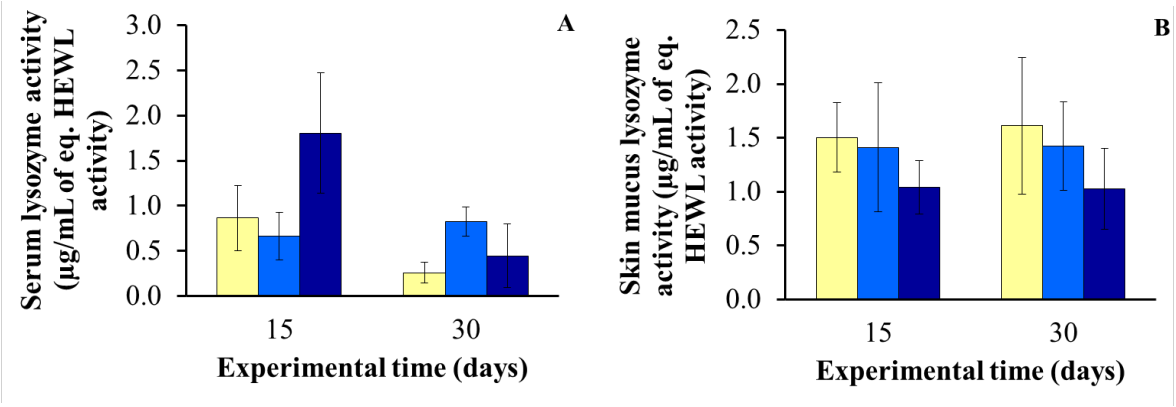



Figure 41: Lysozyme activity in the serum (A) and skin mucus (B) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) date palm seeds (DPS) for 15 and 30 days. Data are presented as means ± SEM (n = 9).

### II.B.3.2.3. Bactericidal activity

Bactericidal activity against *V. harveyi* and *V. anguillarum* was studied in skin mucus. No significant differences were detected against any bacteria assayed at any time comparing the results with control group (Fig. 42), although a trend to increase bactericidal activity against *V. harveyi* at 15 days with the percentage of DPS in diets was observed.

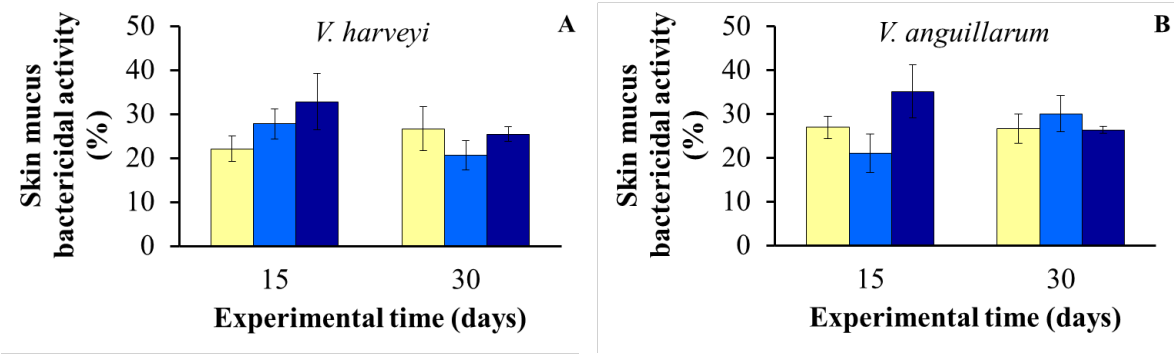



Figure 42: Bactericidal activity against *Vibrio harveyi* (A) and *Vibrio anguillarum* (B) in the skin mucus of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) date palm seeds (DPS) for 15 and 30 days. Data are presented as means ± SEM (n = 9).

### II.B.3.2.4. Protease and antiprotease activities

Fish fed 1.5% DPS diet showed a significant increase of serum protease activity in comparison with control group, while antiprotease activity did not show any difference between any different treatments at any assayed time (Fig. 43)..

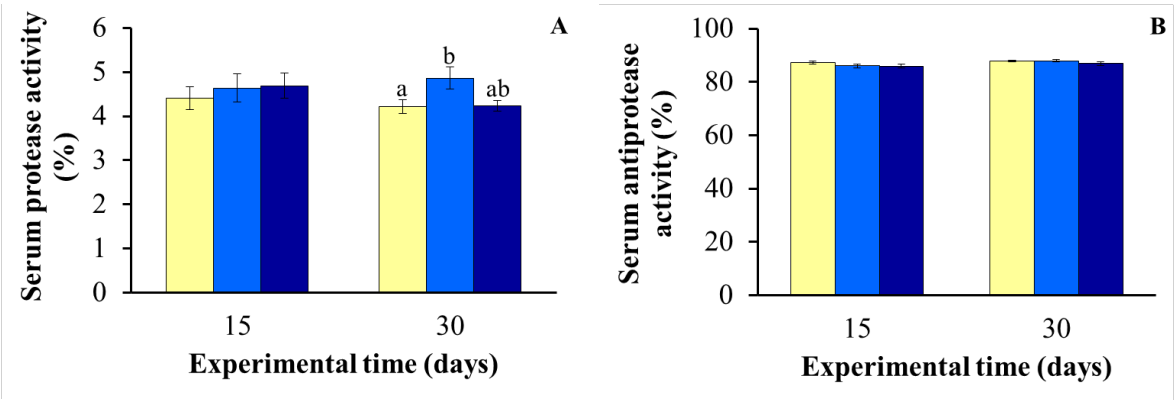



Figure 43: Protease (A) and antiprotease (B) activities in the serum of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) date palm seeds (DPS) for 15 and 30 days. Data are presented as means ± SEM (n = 9). Different letters denote significant differences between treatment groups ($P < 0.05$).

### II.B.3.2.5. Peroxidase activity

Peroxidase activity was also studied in both serum and skin mucus. In any case we observed significant variations at 15 or 30 days when results were compared with control groups (Fig. 44). However, fish fed 1.5% DPS diet showed higher serum peroxidase activity at 15 and 30 days, while fish fed 3% DPS diet showed only higher activity at 30 days. For its part, fish fed 3% DPS diet also showed higher skin mucus peroxidase activity at 15 days.

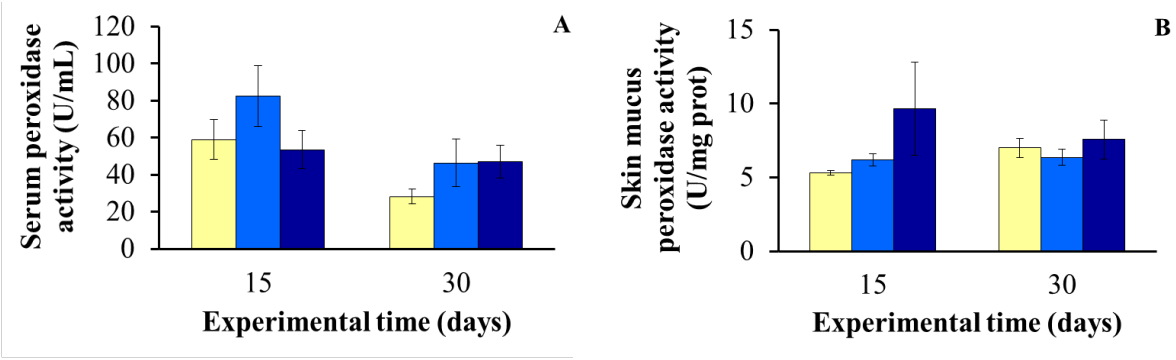



Figure 44: Peroxidase activity in the serum (A) and skin mucus (B) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) date palm seeds (DPS) for 15 and 30 days. Data are presented as means ± SEM (n = 9).

### *II.B.3.3. Cellular immune parameters*

Fish fed 1.5% DPS diet showed a significant increase of HK leucocyte phagocytic ability when compared with control group at 15 days, while fish fed 3% DPS diet showed also a significant increase of phagocytic ability at 15 and 30 days in comparison with control and 1.5% groups (Fig. 45). No differences in phagocytic capacity and respiratory burst activity were observed between groups at any assayed time. Regarding peroxidase activity, although fish fed 1.5% and 3% DPS diets showed higher activity at 30 days, no significant variations were obtained in comparison with control group (Fig. 45).

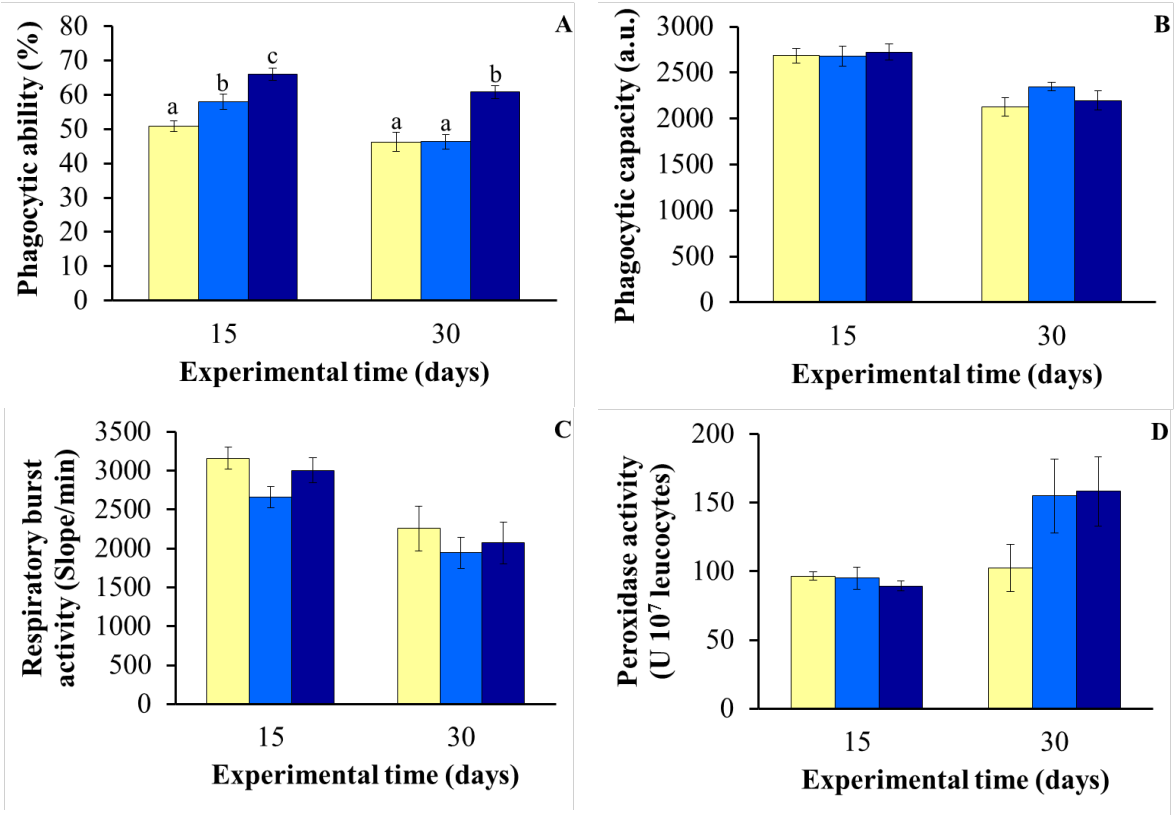



Figure 45: Phagocytic ability (A) and capacity (B), and respiratory burst (C) and peroxidase (D) activities in the head-kidney leucocytes of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) date palm seeds (DPS) for 15 and 30 days. Data are presented as means ± SEM (n = 9). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *II.B.3.4. Liver antioxidant enzyme activities*

No differences in any enzyme activity were showed between groups at any assayed time (Fig. 46). However, in the case of GR activity, a trend dose-dependent increase is observed at 15 days, while 1.5% DPS diet showed strong but not significant increase at 30 days. Curiously, a trend of dose-dependent increase is also observed in CAT activity at 15 and 30 days.

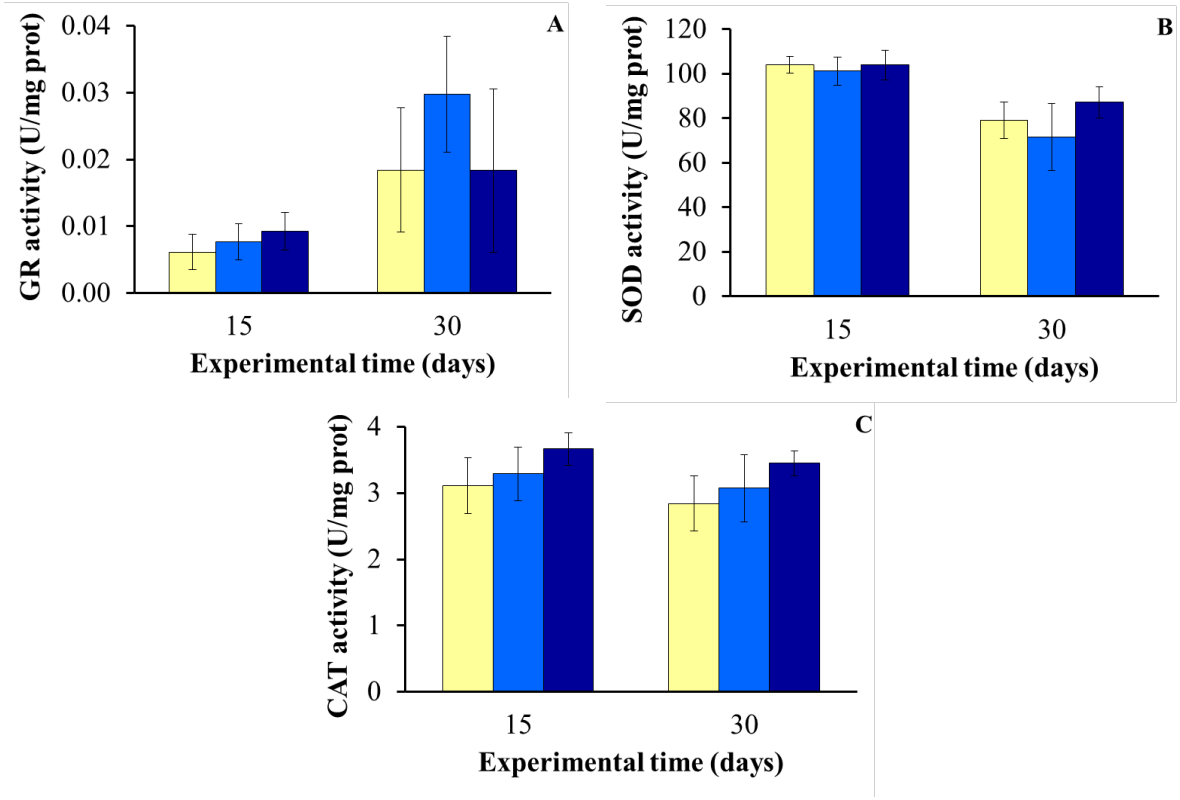



Figure 46: Glutathione reductase (A), superoxide dismutase (B) and catalase (C) activities in the liver of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) date palm seeds (DPS) for 15 and 30 days. Data are presented as means ± SEM (n = 9).

### *II.B.3.5. TAA of skin mucus*

Although no significant differences were observed in the skin mucus TAA, fish fed 3% DPS diet for 30 days showed an increased activity respect to control fish (Fig. 47).

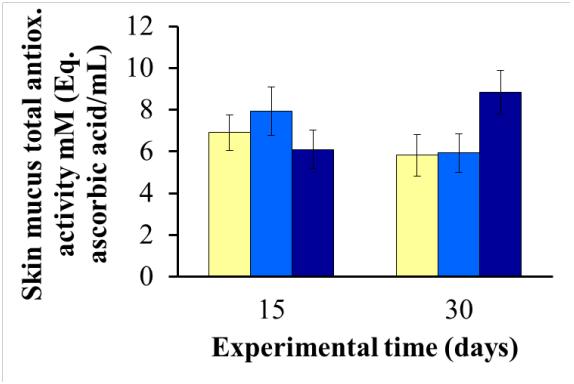



Figure 47: Total antioxidant activity in the skin mucus of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) date palm seeds (DPS) for 15 and 30 days. Data are presented as means ± SEM (n = 9).


Drawing from Dictionnaire Universel d'Histoire Naturelle.
Charles Henry Dessalines d'Orbigny.1861. Not included in figures.

# Chapter II.C.

## Dietary administration of dehydrated lemon peel powder

## II.C.1. Introduction

Lemon (*Citrus limon*) is the third most important species of citrus in the world, behind orange and mandarin (González-Molina et al., 2010). Thanks to climatic conditions, Spain is the first producer of lemon inside the European countries and one of the first producers worldwide (FAO, 2007). Spain is also the main lemon producer in the Mediterranean Basin, with a production of 961,000 tonnes, surpassing Italy and Turkey and is also the leading exporting country in the world. In Murcia (south-eastern Spain), lemon has been cultivated since the fifteenth century because the conditions generated by the Mediterranean climate make this region ideal for production, especially along the entire Vega River Segura (González-Molina et al., 2010; Bouzenna et al., 2016).

The fruits, consumed in the form of juice, provides a wide variety of beneficial effects to health, which have allowed it being used in traditional medicine (González-Molina et al., 2010; Bouzenna et al., 2016). Thanks to their biological activities such as immune-enhancing, antioxidant, antibacterial, anti-inflammatory, antitoxic, hepato-protective, neuro-protective and kidney-protective (Padilla-Camberos et al., 2014; Ou et al., 2015; Cho et al., 2015; Chowdhury et al., 2015; Nouri and Shafaghatlonbar, 2015; Ramadan et al., 2015; Sridharan et al., 2015; Wu et al., 2015; Gomez et al., 2016; Shetty et al., 2016), lemon leaves have been used in traditional medicine worldwide to treat obesity, diabetes, hypercholesterolemia, cardiovascular diseases, brain disorders, inflammation, cancer, microbial infections, digestive disorders, vitamin deficiencies and bronchial, asthmatic and degenerative diseases among others (González-Molina et al., 2010; Padilla-Camberos et al., 2014; Lim et al., 2015; Ou et al., 2015; Bouzenna et al., 2016; Park et al., 2016; Shetty et al., 2016). Moreover, lemon fruit presents high amounts of nutrients such as dietary fibre, vitamin C, potassium, citric acid, ascorbic acid and minerals. In addition, lemon peel essential oil is used in the food industry as well as in perfumes and cosmetics (Bouzenna et al., 2016).

As to the bioactive compounds, lemon as well as all citrus plants present a high concentration and variety of phytochemicals including phenolic acids, flavonoids, carotenoids, aldehydes, terpenes, tannins, coumarins and glucosides such as saponins and alkaloids (Park et al., 2014; Chowdhury et al., 2015; Lee et al., 2015; Lim et al., 2015;

Okuyama and Yakugaku, 2015; Ou et al., 2015; Wu et al., 2015; Gómez et al., 2016; Gualdani et al., 2016; Nakajima et al., 2016; Park et al., 2016; Shetty et al., 2016).

Regarding literature, there are two *in vivo* studies where the effect of dietary lemon juice (Khan and Riaz, 2016) and aqueous-methanol peel extract (Sridharan et al., 2015) have been analysed in rats and anxiolytic, antidepressant, preventive and curative activities have been obtained.

To the best of our knowledge, in aquaculture, the effect of lemon peel essential oil has been studied in juvenile Ningu (*Labeo victorianus*) (Ngugi et al., 2016) and Mozambique tilapia (*Oreochromis mossambicus*) (Baba et al., 2016), as well as DLP powder in Nile tilapia (*O. niloticus*) and African catfish (*C. gariepinus*) (Rahman et al., 2019), and improvements in the immune status were observed.

Taking into account all these considerations, we decided to carry out the present research using as feed additive DLP, with the possibility to increase the economic potential of this by-product from the lemon industry, which is discarded. No previous treatment was done, thus avoiding the use of other chemicals, while adding to the value of this waste. The aim of the present work was to study the effects of DLP dietary supplementation on gilthead seabream growth performance, immune and antioxidant status, and its effect on some serum metabolic parameters related to health, welfare and stress. Due to the effects of medicinal plants on some biochemical responses related to health and metabolism, they need to be carefully evaluated through feeding trials before their definitive inclusion as ingredients/supplements in fish diets. To the best of our knowledge, this is the first study in fish where the influence of lemon on fish metabolic status was also studied (including glucose, lactate, urea, transaminases, etc.).

# II.C.2. Material and methods

## *II.C.2.1. Animals*

Forty-two specimens of gilthead seabream were obtained from a local and maintained as in Chapter 1.

## *II.C.2.2. Chemical characterization of DLP*

DLP was generously provided by the local industry *Lemon King Miguel Parra e hijos* (Santomera, Murcia, Spain). A chemical analysis of DLP composition was provided by the local industry and is represented in Table 1.

Table 1: Chemical composition of two dehydrated lemon peel (DLP) samples.

| Analysed parameter | Dehydrated lemon peel sample a | Dehydrated lemon peel sample b | Units |
|---|---|---|---|
| Humidity | 9.43 | 9.29 | g/100g |
| Dry residue | 90.57 | 90.71 | g/100g |
| Ashes | 3.15 | 3.12 | g/100g |
| Proteins | 5.29 | 6.08 | g/100g |
| Fat | 2.54 | 4.31 | g/100g |
| Total dietary fiber | 71.29 | 67.33 | g/100g |
| Carbohydrates | 8.29 | 9.87 | g/100g |
| Energy (Kcal) | 220 | 237 | Kcal/100g |
| Energy (Kjul) | 895 | 969 | Kjul/100g |
| Fructose | 1.25 | 1.2 | g/100g |
| Glucose | 1.62 | 1.73 | g/100g |
| Galactose | <0.05 | <0.05 | g/100g |
| Sacarose | 0.75 | 0.72 | g/100g |
| Maltose | <0.05 | <0.05 | g/100g |
| Lactose | <0.05 | <0.05 | g/100g |
| Total sugars | 3.62 | 3.65 | g/100g |

## *II.C.2.3. Preparation of diets and experimental design*

A commercial pellet diet (Skretting, Spain) was crushed and mixed with tap water before adding the correct amount of crushed DLP powder and pelleted to obtain diets supplemented with 0% (control), 1.5% and 3% DLP.

All diets were made in order to be isoenergetics, isolipidics and isoproteics (Table 2).

Table 2: Composition of dehydrated lemon peel (DLP) and diets administered to control group (control diet), 1.5% (control diet supplemented with 1.5 % DLP) and 3% (control diet supplemented with 3% DLP) groups. Data are expressed as g/100g total.

| Nutrients | DLP | Control diet | 1.5% DLP diet | 3% DLP diet |
|---|---|---|---|---|
| Carbohydrates | 8.29 | 28.4 | 28.5 | 28.6 |
| Proteins | 5.74 | 55.3 | 55.4 | 55.5 |
| Lipids | 3.42 | 15 | 14 | 13 |
| Fibers | 69.30 | 1.3 | 2.1 | 2.9 |
| Energy value (kcal/100g) | 228 | 444.125 | 447.54 | 451.96 |

Fish were randomly assigned and divided into three tanks (n = 12 in each) thus establishing three groups: control (non-supplemented diet), 1.5% and 3% DLP supplemented diet. Animals were fed at a rate of 2% body weight/day for a month. Fifteen and thirty days after the beginning of the treatment, six animals from each experimental group were sampled after sacrificed as previously. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

### *II.C.2.4. Sampling and analysis*

Samples were obtained and growth parameters, immune status and antioxidant activity determined as in the material and methods of Chapter 2.1.

### *II.C.2.5.* Real-time PCR (qPCR)

HK and liver samples were stored in TRIzol® reagent (Life Technologies) and processed as indicated by the manufacturer's instructions in order to extract the total RNA. The RNA present in samples was then quantified and the purity was assessed by spectrophotometry; the 260:280 ratios were 1.8-2.0. The RNA was then treated with DNase I (Promega) to remove genomic DNA contamination. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the SuperScript IV reverse transcriptase (Life Technologies) with an oligo-dT18 primer. The expression of the selected genes was analysed by real-time PCR (qPCR), which was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) and using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). Reaction mixtures (containing 10 µl of SYBR Green supermix, 5 µl of primers [0.4 µM each] and 5 µl of cDNA template) were incubated for 10 min at 95ºC, followed by 40 cycles of 15 sec at 95ºC, 1 min at 60ºC and, finally, 15 sec at 95ºC, 1 min at 60ºC and 15 sec at 95ºC. In all cases, each PCR was

performed with triplicate samples. For each messenger RNA (mRNA), gene expression was corrected by both the elongation factor 1α (*ef1a*) and ribosomal protein S18 (*18s*) RNA content in each sample. The selected pro-inflammatory genes were natural killer enhancing factor A (*nkefa*), interleukins 1beta (*il1β*) and 6 (*il6*), cyclooxygenase 2 (*cox2*) and colony-stimulating factor receptor 1 (*csfr1*). Adaptive immune genes selected were IgM heavy chain and Ig-T heavy chain (*igmh*, *igth*) and T-cell receptor B chain (*tcrb*). The antioxidant genes selected were glutathione reductase (*gr*), superoxide dismutase (*sod*) and catalase (*cat*). Furthermore, stress related genes such as heat shock proteins 70 and 90 (*hsp70*, *hsp90*) and antimicrobial related genes such as hepcidin (*hamp*) and β-defensin (*bdef*) were also studied (Table 3).

Table 3: Primers used for real-time PCR analysis.

| Gene | Abbreviation | GenBank ID | **Primer sequence (5'→3')** |
|---|---|---|---|
| **Elongation factor 1 α** | *ef1a* | AF184170 | Fw - CTGTCAAGGAAATCCGTCGT<br>Rev - TGACCTGAGCGTTGAAGTTG |
| 18S ribosomal | *18s* | AM490061 | Fw - CGAAAGCATTTGCCAAGAAT<br>Rev - AGTTGGCACCGTTTATGGTC |
| Natural Killer enhancing factor A | *nkefa* | GQ252679 | Fw - CTCCAAGCAATAATAAGCCCAAAG<br>Rev - TCACTCTACAGACAACAGAACAC |
| T-cell receptor B chain | *tcrb* | AM261210 | Fw - AAGTGCATTGCCAGCTTCTT<br>Rev - TTGGCGGTCTGACTTCTCTT |
| **Interleukin 1β** | *il1b* | AJ277166 | Fw - GGGCTGAACAACAGCACTCTC<br>Rev - TTAACACTCTCCACCCTCCA |
| Interleukin 6 | *il6* | AM749958 | Fw - AGGCAGGAGTTTGAAGCTGA<br>Rev - ATGCTGAAGTTGGTGGAAGG |
| Cyclooxygenase 2 | *cox2* | AM296029 | Fw - GAGTACTGGAAGCCGAGCAC<br>Rev - GATATCACTGCCGCCTGAGT |
| Colony-stimulating factor receptor 1 | *csfr1* | AM050293 | Fw - ACGTCTGGTCCTATGGCATC<br>Rev - AGTCTGGTTGGGACATCTGG |
| IgM heavy chain | *igmh* | AM493677 | Fw - CAGCCTCGAGAAGTGGAAAC<br>Rev - GAGGTTGACCAGGTTGGTGT |
| IgT heavy chain | *igth* | FM145138 | Fw - TGGCAAATTGATGGACAAAA<br>Rev - CCATCTCCCTTGTGGACAGT |
| Glutathione reductase | *gr* | AJ937873 | Fw - CAAAGCGCAGTGTGATTGTGG<br>Rev - CCACTCCGGAGTTTTGCATTTC |
| Superoxide dismutase | *sod* | AJ937872 | Fw - CCATGGTAAGAATCATGGCGG<br>Rev - CGTGGATCACCATGGTTCTG |
| Catalase | *cat* | FG264808 | Fw - TTCCCGTCCTTCATTCACTC<br>Rev - CTCCAGAAGTCCCACACCAT |
| Heat shock protein 70 | *hsp70* | EU805481 | Fw - AATGTTCTGCGCATCATCAA<br>Rev - GCCTCCACCAAGATCAAAGA |
| Heat shock protein 90 | *hsp90* | DQ524994 | Fw - GGAGCTGAACAAGACCAAGC<br>Rev - AGGTGATCCTCCCAGTCGTT |
| Hepcidin | *hamp* | CB184616 | Fw - GCCATCGTGCTCACCTTTAT<br>Rev - CTGTTGCCATACCCCATCTT |
| **β**-defensin | *bdef* | FM158209 | Fw - CCCCAGTCTGAGTGGAGTGT<br>Rev - AATGAGACACGCAGCACAAG |

### II.C.2.6. Biochemical parameters related to general welfare, metabolism and stress in serum

Glucose and lactate levels were determined spectrophotometrically by a commercial kit (Sentinel) as previously reported (Santulli et al., 1999; Messina et al., 2013). The urea content and transaminase activity were assessed by a kit from Globe Diagnostics. Aspartate aminotransferase-glutamic oxalacetic transaminase (AST/GOT) and alanine transaminase-glutamic pyruvic transaminase (ALT/GPT) were used as reference biomarkers.

### II.C.2.7. Analysis of biomolecular markers in skin mucus

Biomolecular markers were analysed by immunoblotting. Equivalent amounts of protein (20 μg) were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane using a Trans Blot Turbo Transfer System (Bio-Rad). Filters were then used to detect levels of Hsp70, Hsp25 and Phospo-c-Jun (Sigma-Aldrich), and the appropriate anti-mouse or anti-rabbit horseradish peroxidase–conjugated secondary antibodies (GAR/M-HRP, Bio-Rad). Immune-reactive signals were detected using enhanced chemo-luminescent (ECL) reagents (Bio-Rad). Correct protein loading was confirmed by red Ponceau staining. Images were obtained, visualized, photographed and digitalized with Chemi Doc XRS (Bio-Rad), and further analyzed with Image Lab software for relative quantification of the bands (Bio-Rad). The results were expressed as fold increase of each treatment in relation to the respective control; the images shown are representative of almost three different immunoblots, for which the mean quantification is reported in each figure, together with the significance of the differences ($P < 0.05$).

### II.C.2.8. Statistical analyses

The results were expressed as means ± SEM. The normality of the variables was confirmed by the Shapiro–Wilk test and homogeneity of variance by the Levene test. Statistical differences among the four groups of treatments were assessed by one-way ANOVA analyses, followed by the Bonferroni or Games-Howell test, depending on the homogeneity of the variables. The significance level was 95% in all cases ($P < 0.05$). All the data were analysed by the computer application SPSS for Windows®.

## II.C.3. Results

### II.C.3.1. Growth performance

No significant deviations compared with the control were obtained for WG or SGR in fish fed 1.5% DLP for 15 or 30 days (Fig. 48). However, in the case of the fish fed 3% DLP, while no significant differences were observed in WG and SGR compared to the values recorded for the control group after fifteen days, significant decreases were obtained for both parameters after 30 days (Fig. 48).

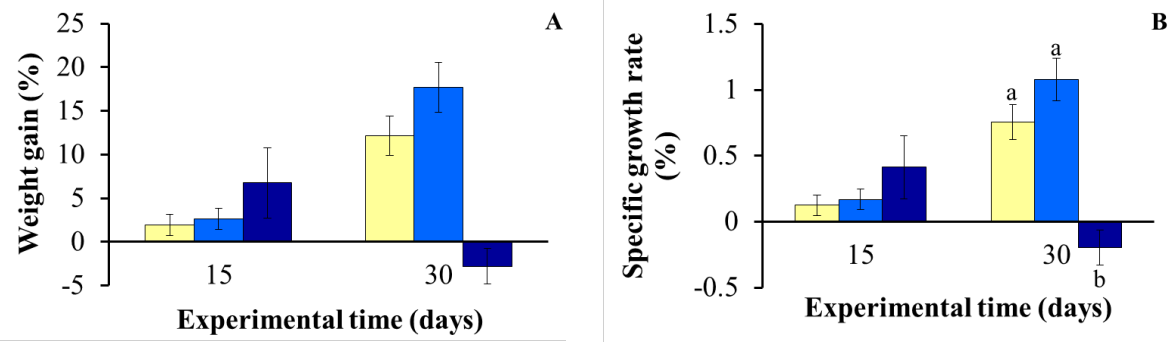



Figure 48: Weight gain (A) and specific growth rate (B) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) dehydrated lemon peel (DLP) for 15 and 30 days. Data are presented as means ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

### II.C.3.2. Humoral immune parameters

#### II.C.3.2.1. IgM level

No significant variations compared with the values recorded for control fish (fed non-supplemented diet) were observed in the IgM levels measured in serum and skin mucus of gilthead seabream specimens, except at 15 days when significantly higher serum IgM levels were measured (Fig. 49).

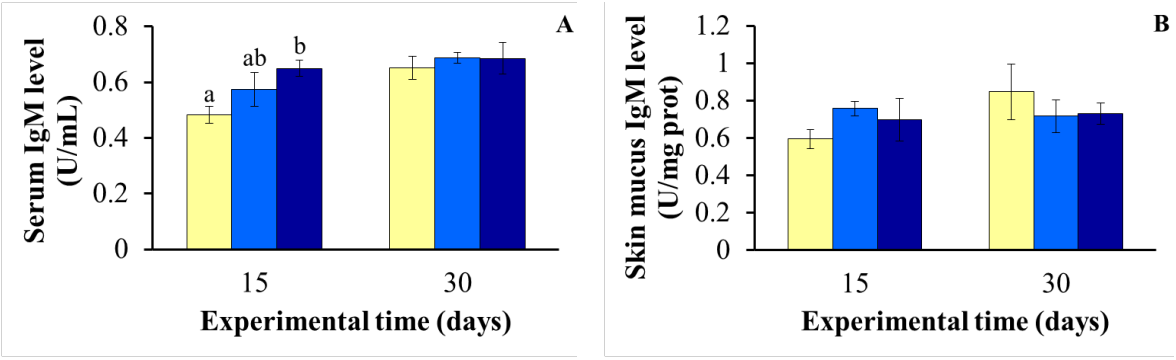



Figure 49: IgM levels in the serum (A) and skin mucus (B) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) dehydrated lemon peel (DLP) for 15 and 30 days. Data are presented as means ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

### II.C.3.2.2. Peroxidase activity

No significant variations were observed in the peroxidase activity in the serum or skin mucus of fish fed the different diets for 15 or 30 days compared with the values determined in control fish (Fig. 50).

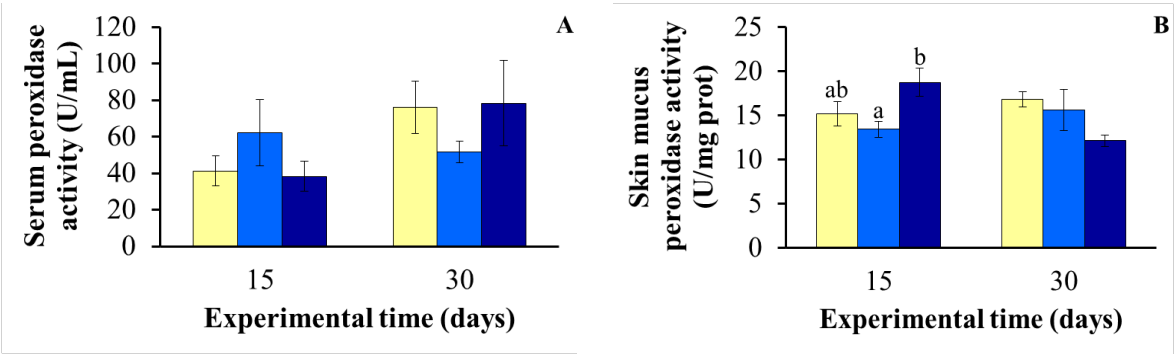



Figure 50: Peroxidase activity in the serum (A) and skin mucus (B) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) dehydrated lemon peel (DLP) for 15 and 30 days. Data are presented as means ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

### II.C.3.3. Cellular immune parameters

The phagocytic ability of HK leucocytes increased in a dose-dependent manner in fish fed the DLP supplemented diets although the increase was only statistically significant in leucocytes from fish fed the 3% DLP diet (Fig. 51A). However, no significant effects were observed in the phagocytic capacity of leucocytes from fish fed the DLP supplemented diets compared with control fish (Fig. 51B).

As regards respiratory burst activity, no significant effects were observed between leucocytes from fish fed the DLP supplemented diets and those from the control group at any assayed time (Fig. 51C). Peroxidase activity showed significant increases in leucocytes collected from fish fed 15 days with the 1.5% and 3% DLP diets compared to the values recorded for leucocytes from control fish. However, no significant differences were recorded in peroxidase activity from fish for the 1.5% DLP diet for 30 days or the 3% DLP diet for 15 or 30 days (Fig. 51D).

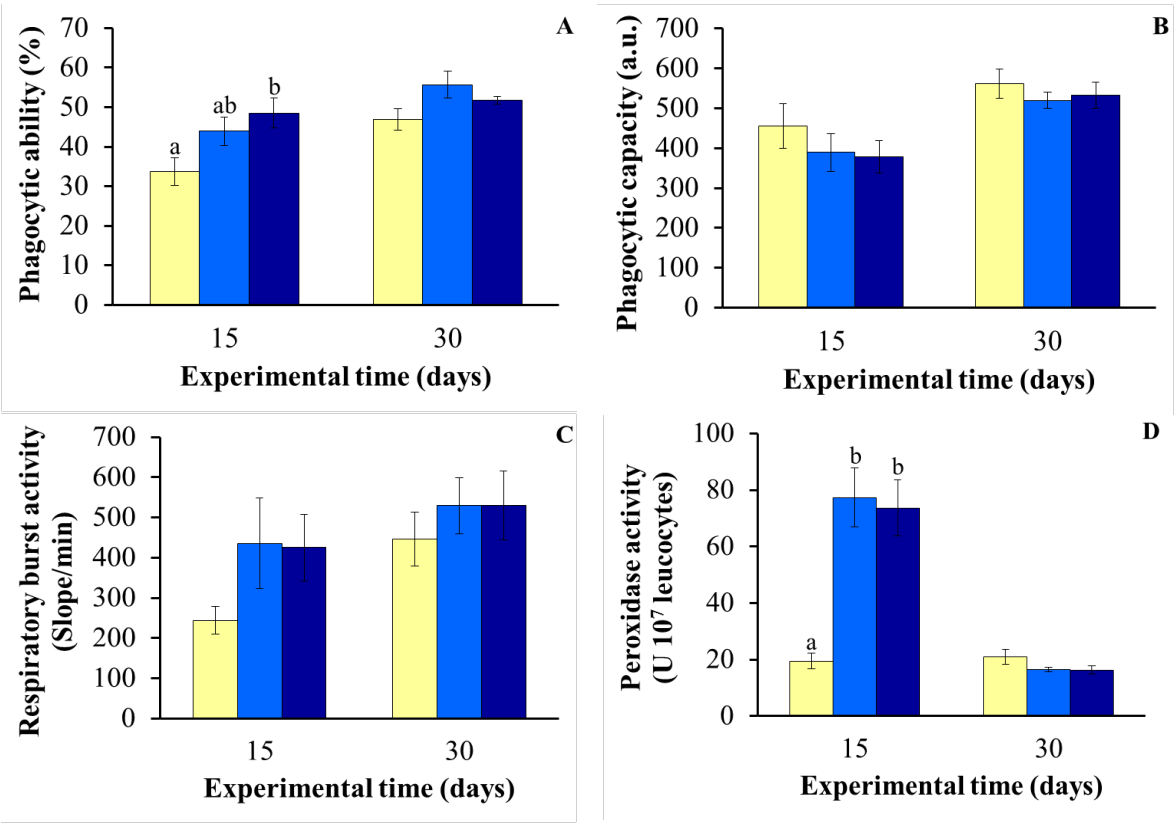



Figure 51: Phagocytic ability (A) and capacity (B) and respiratory burst (C) and peroxidase (D) activities in the head-kidney leucocytes of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) dehydrated lemon peel (DLP) for 15 and 30 days. Data are presented as means ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *II.C.3.4. Liver antioxidant enzyme activities*

No significant effects were observed in any of the enzymes in fish fed the DLP supplemented diets respect to the values obtained in liver from control group at any assayed time (Fig. 52).

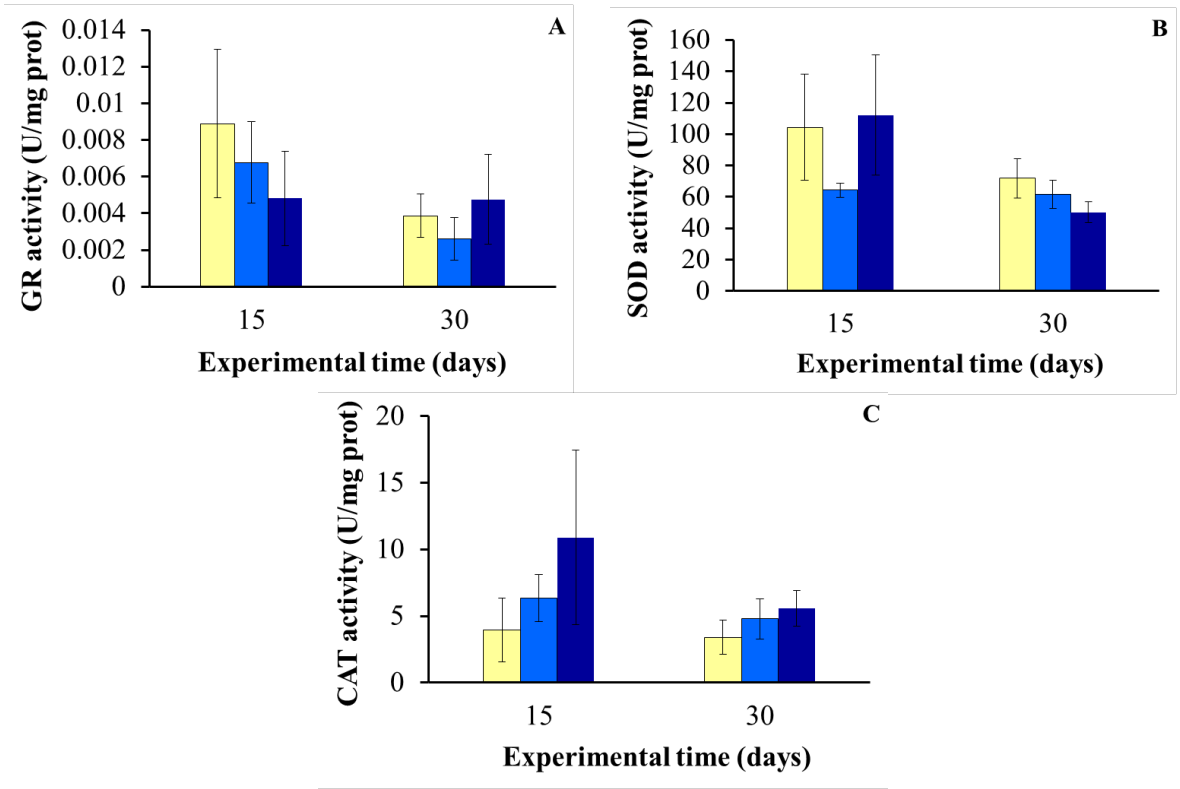



Figure 52: Glutathione reductase (A), superoxide dismutase (B) and catalase (C) enzyme activities in the liver of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) dehydrated lemon peel (DLP) for 15 and 30 days. Data are presented as means ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

### II.C.3.5. TAA of serum

The serum TAA showed a general increase in fish fed the diets enriched with DLP compared to the values obtained for fish fed the control diet (Fig. 53).

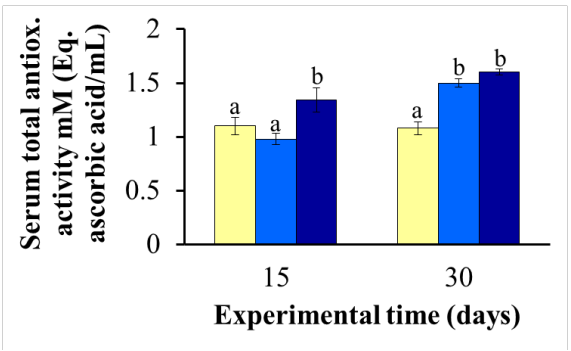



Figure 53: Total antioxidant activity in the serum of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) dehydrated lemon peel (DLP) for 15 and 30 days. Data are presented as means ± Standard Deviation (n = 6). Different superscripts letters indicate a significant difference respect to the control ($P < 0.05$).

### II.C.3.6 Gene expression

The gene expression of several genes related to immune and antioxidant status was studied in HK and liver of specimens, respectively.

#### II.C.3.6.1. Expression of immune-related genes

No significant effects were observed in the expression of the studied genes in the HK of fish fed the 1.5% DLP diet for 15 or 30 days compared with the expression recorded for control fish (Fig. 54). However, a significant up-regulation was detected for *nkefa*, *csfr1*, *il1b* and *ight* genes in HK from fish for 15 days with the 3% DLP supplemented diet (Fig. 54A).

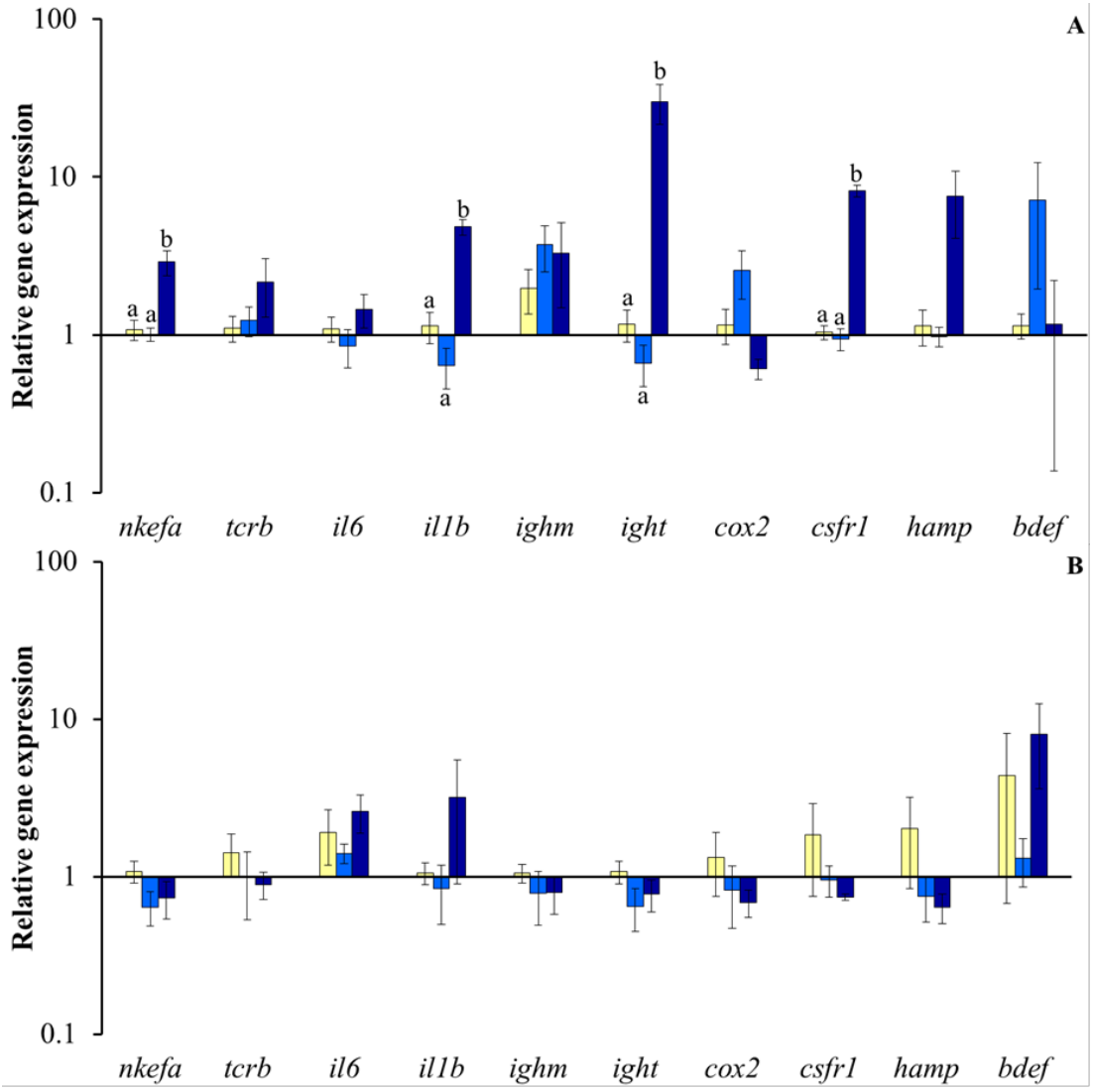



Figure 54: Expression of immune-related genes in the head-kidney of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) dehydrated lemon peel (DLP) for 15 (A) and 30 (B) days. Data are presented as means ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

### II.C.3.6.2. Expression of antioxidant-related genes

No significant effects were observed in the expression of any studied gene in the liver from fish fed the DLP diets compared with control values at any assayed time (Fig. 55).

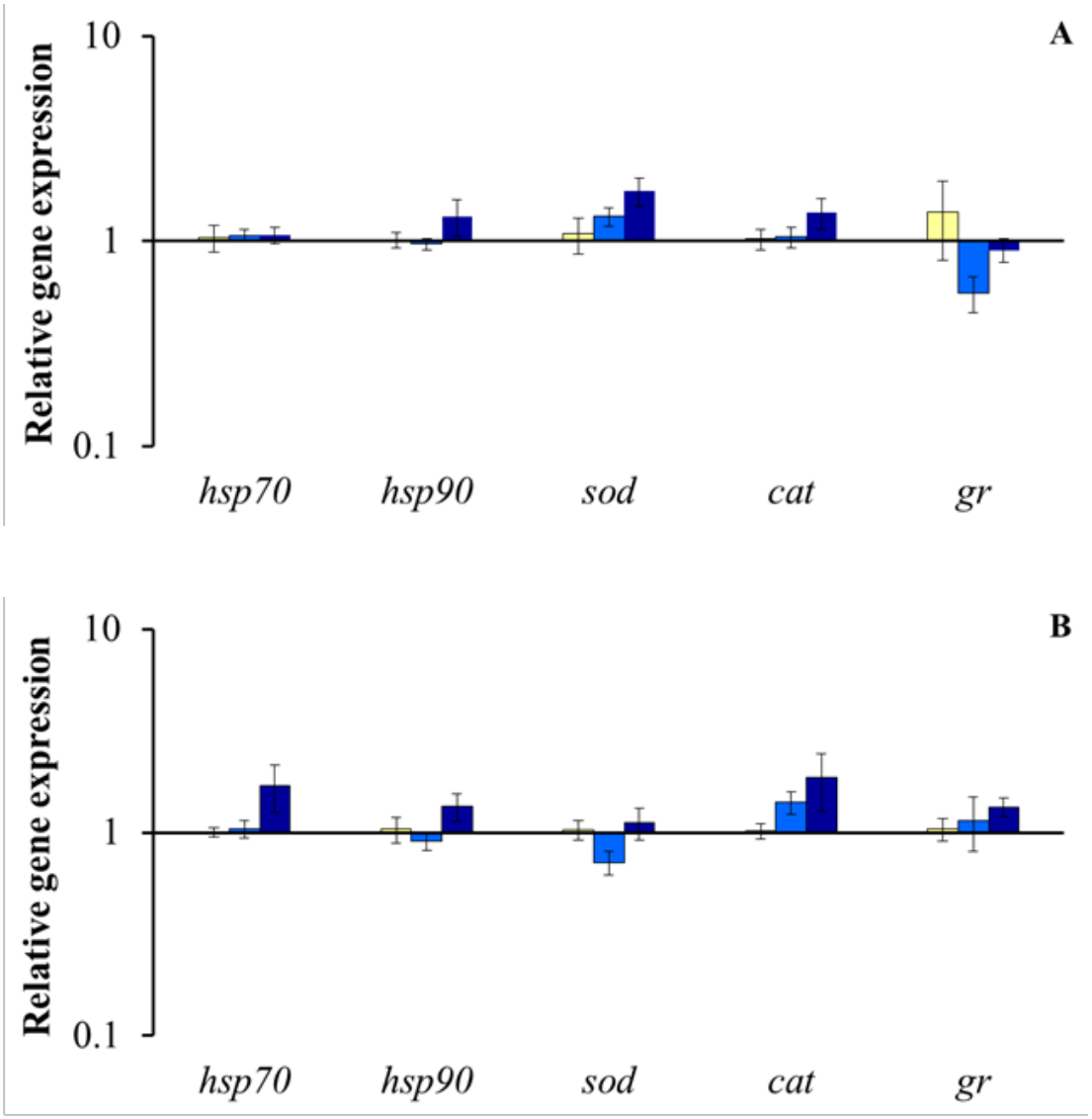



Figure 55: Expression of antioxidant-related genes in the liver of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) dehydrated lemon peel (DLP) for 15 (A) and 30 (B) days. Data are presented as means ± SEM (n = 6).

### *II.C.3.7. Biochemical parameters related to general welfare, metabolism and stress in serum*

The glucose and lactate levels in serum showed a similar trend of variation during the experiment: both parameters decreased at 15 days in specimens fed with the 1.5% DLP (Fig. 55A, 55B) but after 30 days values resulted similar to the control fish. The levels of urea increased after 30 days in fish fed with both experimental diets respect to the control (Fig. 56C). Finally, the transaminases AST/GOT and ALT/GPT, showed an increase in fish fed with 1.5 % DLP after 30 days, while the inclusion of 3% DLP determined an increase at 15 and 30 days of administration, respect to the control (Fig. 56D, 56E).

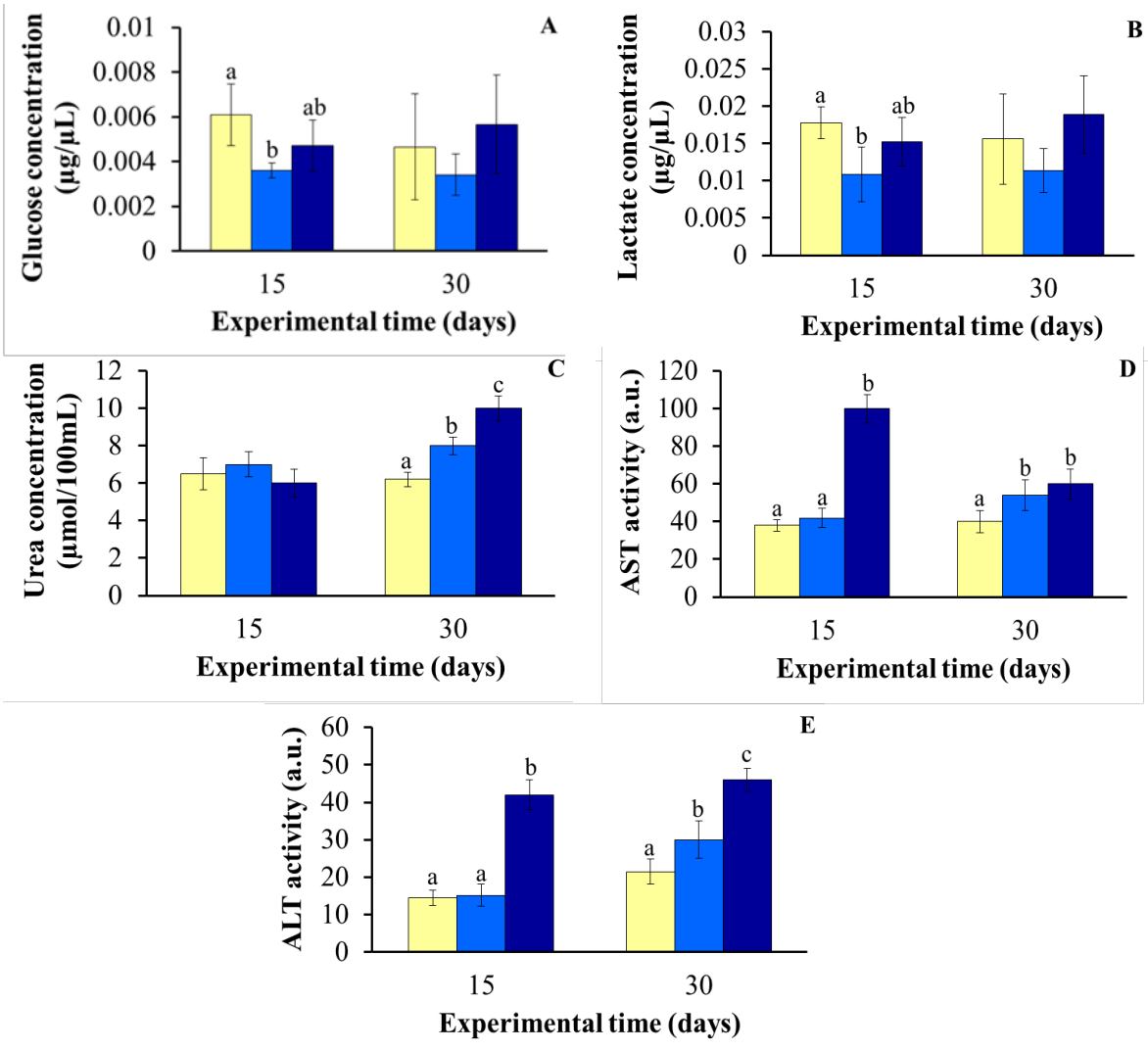



Figure 56: Glucose (A), lactate (B) and urea (C) concentrations and aspartate aminotransferase (D) and alanine aminotransferase (E) activities in the serum of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) dehydrated lemon peel (DLP) for 15and 30 days. Data are presented as means ± SD (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *II.C.3.8. Analysis of biomolecular markers in skin mucus by immunoblotting*

In the skin mucus, we assessed, for the first time, the levels of some molecular markers related to the overall response of fish to stress, oxidative stress and apoptosis, such as Hsp70, Hsp25 and Phospho-c-Jun (Fig. 57). Compared with the controls, Hsp70 increased after 15 days of receiving both DLP diets but, after 30 days, the levels fell sharply in fish fed the 3% DLP diet (Fig. 57A). Hsp25 generally showed the same trend as Hsp70, showing a transient increase after 15 days in fish fed both DLP diets compared with the control group, and a reduction after 30 days (Fig. 57B). Finally, Phospho-c-Jun also showed an increase in the protein phosphorylation level after 15 days with respect to the control, followed by a decrease after 30 days of the trial, regardless of the inclusion level of DLP in the diet (Fig. 57C).

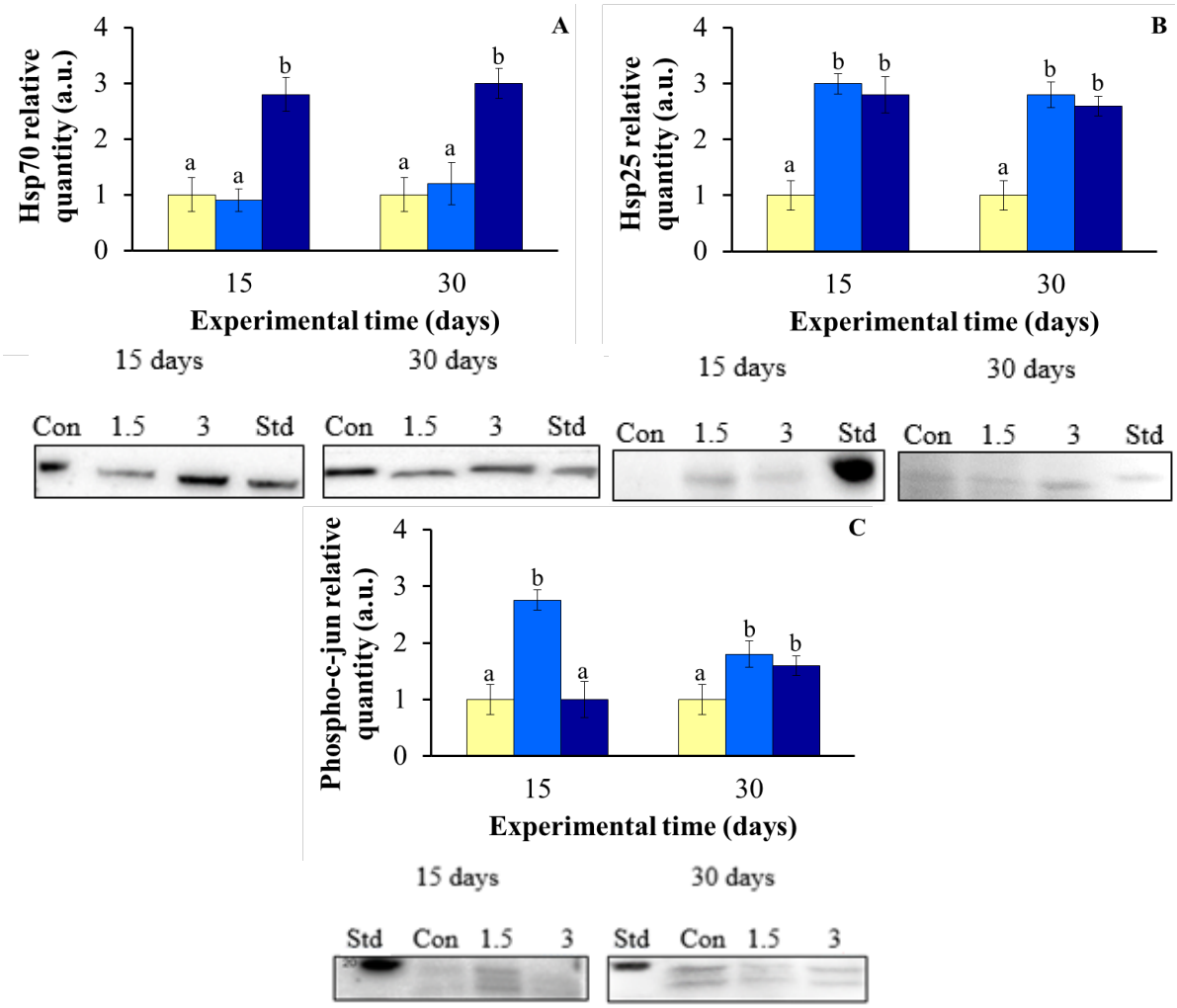
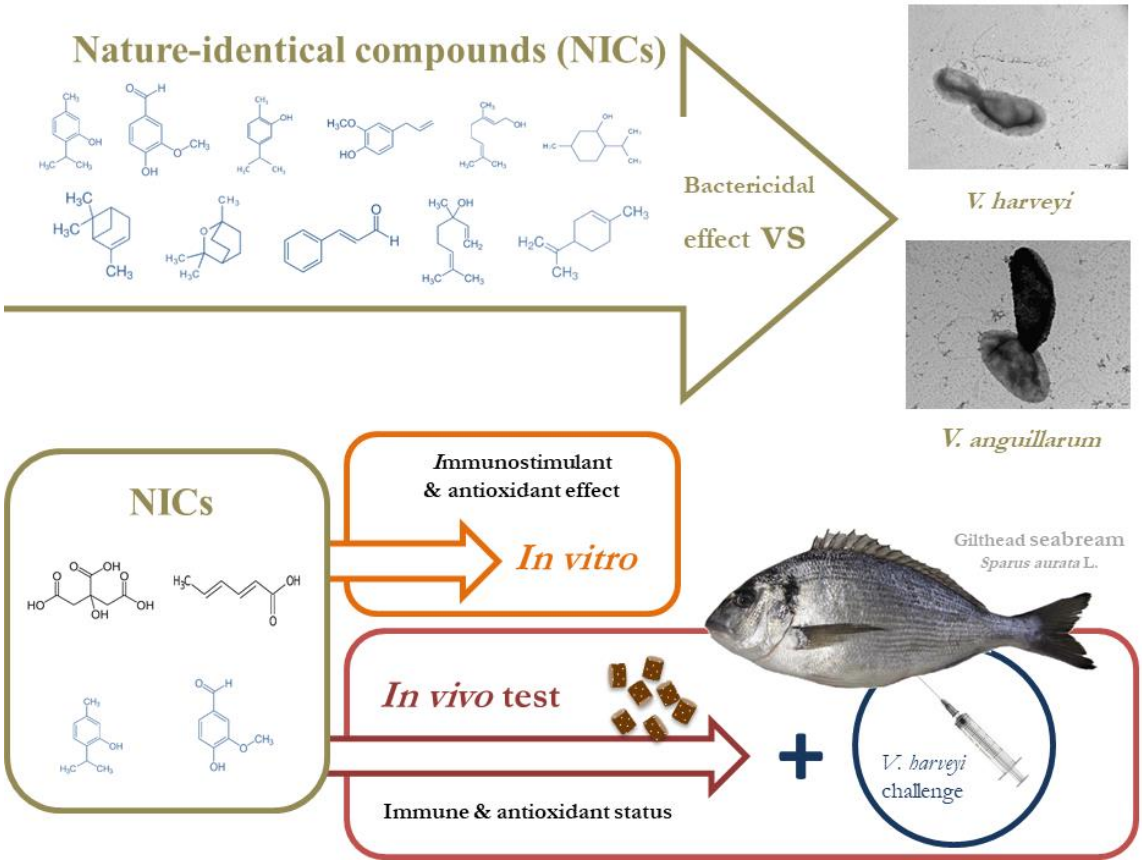



Figure 57: Representative immunoblots, and their quantification, of Hsp70 (A), Hsp25 (B) and Phospho-c-Jun (C) in the skin mucus of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 1.5% (light blue) or 3% (dark blue) dehydrated lemon peel (DLP) for 15 and 30 days. Relative quantities of each protein were normalized to the control. Data are presented as means ± SD (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$). Each immunoblot image is representative of almost three separate immunoblotting analyses.

# CHAPTER III.

## Nature-identical compounds. *In vitro* and *in vivo* properties

### III.1 Graphical abstract



## III.2. Introduction

Plants have become a great source of bioactive chemicals for natural drug development, which is an essential aspect in developing countries, in part because the increasing number of pathogenic microorganism resistant to conventional drugs (Boulenouar et al., 2009). As we have previously highlighted, chemical compounds of the plant secondary metabolism, including phenolic compounds such as flavonoids, terpenes, phenolic acids, alkaloids, tannins and coumarins, among others, are the ones responsible for most of the plant properties and activities, and are naturally present in plant essential oils (Koldas et al., 2015; Han et al., 2017). Most of these substances, which present antimicrobial, antioxidant, anti-inflammatory and growth promoting properties as well as improving gut function (Ibrahim et al., 2018), are extracted from plants by distillation in variable amounts. However, at present, nature-identical compounds (NICs), which can be defined as flavouring substances obtained by chemical synthesis or isolated by chemical processes and chemically identical to substances naturally present in material of vegetable or animal origin, have appeared. Their use has many advantages to the use of plant extracts, for example, they can be used pure and in the required concentration.

Thanks to the development of NICs it is possible to obtain chemical compounds of plants that are not present in some country or region or even outside of the harvesting period. Furthermore, it is easier to make the desired compounds synthetically, which results much cheaper and sostenible than obtain the same substances directly from plants. For these important reasons we found interesting to study the biological activities of several NICs both *in vitro* and *in vivo*.

Regarding *in vitro* studies, first of all, we evaluated the bactericidal activity against *V. harveyi* and *V. anguillarum* of selected NICs. Then we studied the immunostimulant and antioxidant activity of a mix of NICs on gilthead seabream HK leucocyte activities.

On the other hand, two *in vivo* experiments were developed. In the first one, we carried out a feeding trial by using different concentrations of a mix of NICs as dietary supplement to gilthead seabream and growth performance, immune and antioxidant status were analysed. In a second trial, two selected concentrations from the first trial were used again to feed fish, which were challenged with *V. harveyi*, and growth performance, immune and antioxidant status were again analysed before and after the bacterial challenge.

# Chapter III.A.

## *In vitro* study of NICs

# III.A.1.1 *In vitro* effects of NICs

## III.A.1.1 Material and methods

### *III.A.1.1.1. NICs used in the study*

Up to 21 different pure NICs were used in a blind test for the bactericidal activity. A blend of different NICs (containing two organic acids, a monoterpene and a phenolic aldehyde) was used for the rest of the *in vitro* studies. NICs nature, composition or purity are confidential and were supplied by the company Vetagro S.p.A.

### *II.A.1.1.2. Bactericidal activity*

Two pathogenic bacteria for fish (*V. harveyi* and *V. anguillarum*) were used in the bactericidal assays as in Chapter 1.

Bactericidal activity was determined by evaluating the effects of 21 NICs on the bacterial growth curves using the method of Sunyer and Tort (1995) with some modifications. Aliquots of 100 µL of each one of the bacterial dilutions (1/10) were placed in flat-bottomed 96-well plates and cultured with equal volumes of NICs in a range of concentrations from 0.12 to 7.50 mM in bacteria culture medium. The OD of the samples was measured at 620 nm at 2 h intervals during 24 h at 22ºC in a plate reader. Results were expressed as OD values. Wells with medium without bacteria (100% bactericidal activity and 0% growth) and wells with bacteria without NICs (0% bactericidal activity, 100% bacterial growth) were included. Samples and controls were conducted in triplicate.

### *III.A.1.1.3. Animals*

Six specimens of the seawater teleost gilthead seabream were obtained from a local farm and mantained as in Chapter 1.

### *III.A.1.4. HK leucocyte isolation and incubation with NICs*

Gilthead seabream HK leucocytes, isolated as described in Chapter 1, were incubated for 0 min, 30 min, 2 and 4 h with a blend of NICs in a range of concentrations of 50, 100, 250, 500 or 1,000 mg/L. After incubation, leucocyte viability, phagocytic, respiratory burst and peroxidase activities were determined and the obtained results were compared with control group (leucocytes incubated in culture medium without NICs) (see Chapter 1 for materials and methods).

### *III.A.1.5. Real-time PCR (qPCR)*

The relative gene expression of HK leucocytes after incubation with the blend of NICs was also studied by qPCR. After incubation, leucocytes were stored at - 80ºC in TRIzol reagent until total RNA isolation (see material and methods Chapter 2.3.).

The selected pro-inflammatory genes were interleukin 1beta, 6, 7, 8, 15 and 18 (il1β, *6, 7, 8, 15, 18)*, while anti-inflammatory genes were interleukin 10 (*il10*) and transforming growth factor β (*tgfβ*). The expression of antioxidant genes as nuclear factor erythroid 2 (*nrf2*), glutathione reductase (*gr*) superoxide dismutase (*sod*), and catalase (*cat*) was also determined (Table 4).

Table 4: Primers used for real-time PCR analysis.

| Gene | Abbreviation | GenBank ID | **Primer sequence (5'→3')** |
|---|---|---|---|
| Elongation factor 1 alpha | *ef1a* | AF184170 | Fw - CTGTCAAGGAAATCCGTCGT<br>Rev - TGACCTGAGCGTTGAAGTTG |
| 18S ribosomal | *18s* | AM490061 | Fw - CGAAAGCATTTGCCAAGAAT<br>Rev - AGTTGGCACCGTTTATGGTC |
| Interleukin 1 beta | *il1b* | AJ277166 | Fw - GGGCTGAACAACAGCACTCTC<br>Rev - TTAACACTCTCCACCCTCCA |
| Interleukin 6 | *il6* | AM749958 | Fw - AGGCAGGAGTTTGAAGCTGA<br>Rev - ATGCTGAAGTTGGTGGAAGG |
| Interleukin 7 | *il7* | JX976618 | Fw - GATCTGGAAAACACCGGAGA<br>Rev - TGGACGTGCAGTTCTGTAGC |
| Interleukin 8 | *il8* | AM765841 | Fw - GCCACTCTGAAGAGGACAGG<br>Rev - TTTGGTTGTCTTTGGTCGAA |
| Interleukin 15 | *il15* | JX976625 | Fw - CTACTGGACCGGGATCAATG<br>Rev - TCGTCTATGATCTGCGCAAC |
| Interleukin 18 | *il18* | JX976626 | Fw - TTGAGGGGTTGTCCTGTTTC<br>Rev - AGTTTTTACCCCAGCCCTGT |
| Interleukin 10 | *il10* | FG261948 | Fw - CTCACATGCAGTCCATCCAG<br>Rev - TGTGATGTCAAACGGTTGCT |
| Transforming growth factor beta | *tgfb* | AF424703 | Fw - GCATGTGGCAGAGATGAAGA<br>Rev - TTCAGCATGATACGGCAGAG |
| Nuclear factor erythroid 2 | *nrf2* | FP335773 | Fw - GTTCAGTCGGTGCTTTGACA<br>Rev - CTCTGATGTGCGTCTCTCCA |
| Glutathione reductase | *gr* | AJ937873 | Fw - CAAAGCGCAGTGTGATTGTGG<br>Rev - CCACTCCGGAGTTTTGCATTTC |
| Superoxide dismutase | *sod* | AJ937872 | Fw - CCATGGTAAGAATCATGGCGG<br>Rev - CGTGGATCACCATGGTTCTG |
| Catalase | *cat* | FG264808 | Fw - TTCCCGTCCTTCATTCACTC<br>Rev - CTCCAGAAGTCCCACACCAT |

# III.A.1.2 Results

## *III.A.1.2.1. Bactericidal activity*

Some NICs tested showed a dose-dependent bactericidal activity against *V. harveyi* (Fig. 58) and *V. anguillarum* (Fig. 59). Among NICs, there are some (upper panels) such as phenylpropanoid, a phenolic aldehyde, a flavonoid and, especially, monoterpene phenols showing potent bactericidal activity since some are able to inhibit bacterial growth at very low concentrations (0.94 mM). By contrast, other NICs (lower panels) show much lower to negligible bactericidal activity.

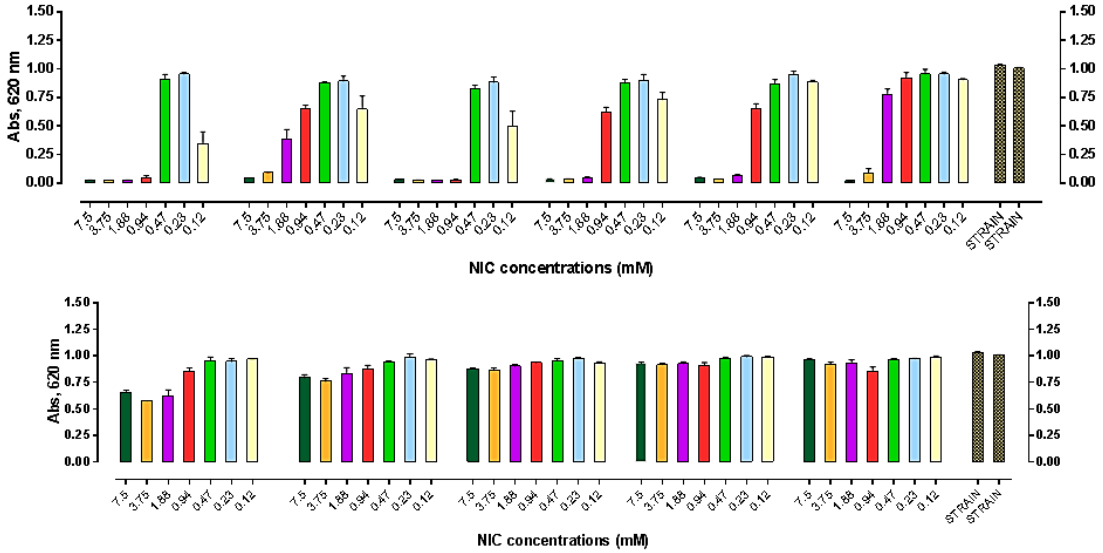



Figure 58: Bactericidal activity of NICs against *V. harveyi*. Bacteria were grown in culture medium alone (Control; STRAIN) or containing NICs and 24 h later the optical density of the bacterial cultures determined and presented as the mean ± SD (n = 3).

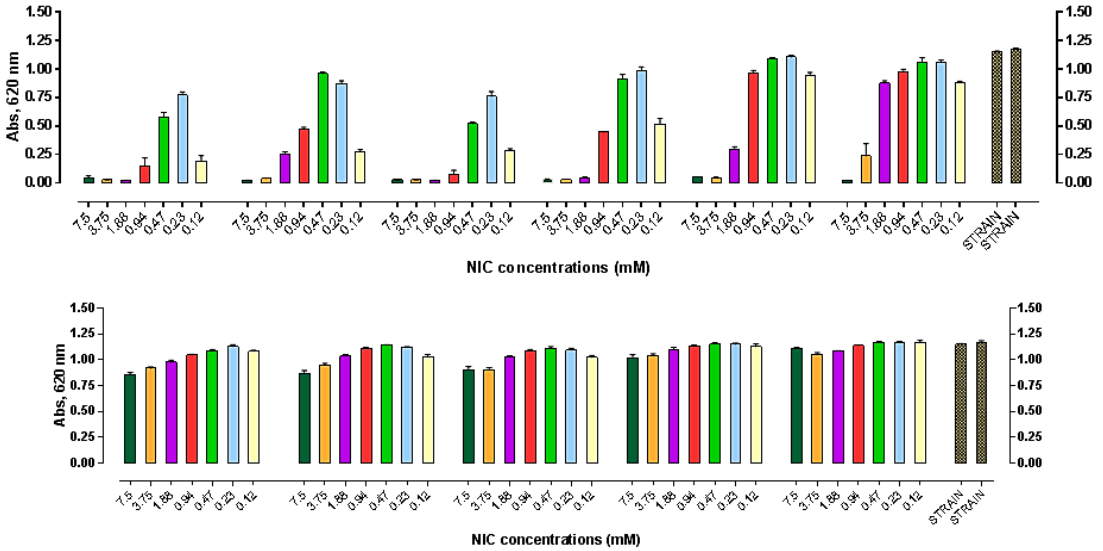



Figure 59: Bactericidal activity of NICs tested against *V. anguillarum*. Bacteria were grown in culture medium alone (Control; STRAIN) or containing NICs and 24 h later the optical density of the bacterial cultures determined and presented as the mean ± SD (n = 3).

### *III.A.1.2.2. HK leucocyte viability and activities*

The incubation of HK leucocytes with the NICs mixture did not affect the leucocyte viability at any assayed time (Fig. 60A). Similarly, the phagocytic properties of the HK leucocytes were not significantly affected by the incubation of differents concentrations of the mixture at any assayed time (Fig. 60B, 60C). Regarding respiratory burst, incubation of leucocytes with different mixture concentrations produced an inverse dose-dependent increment of this activity but it never reached a significant extent (Fig. 60D).

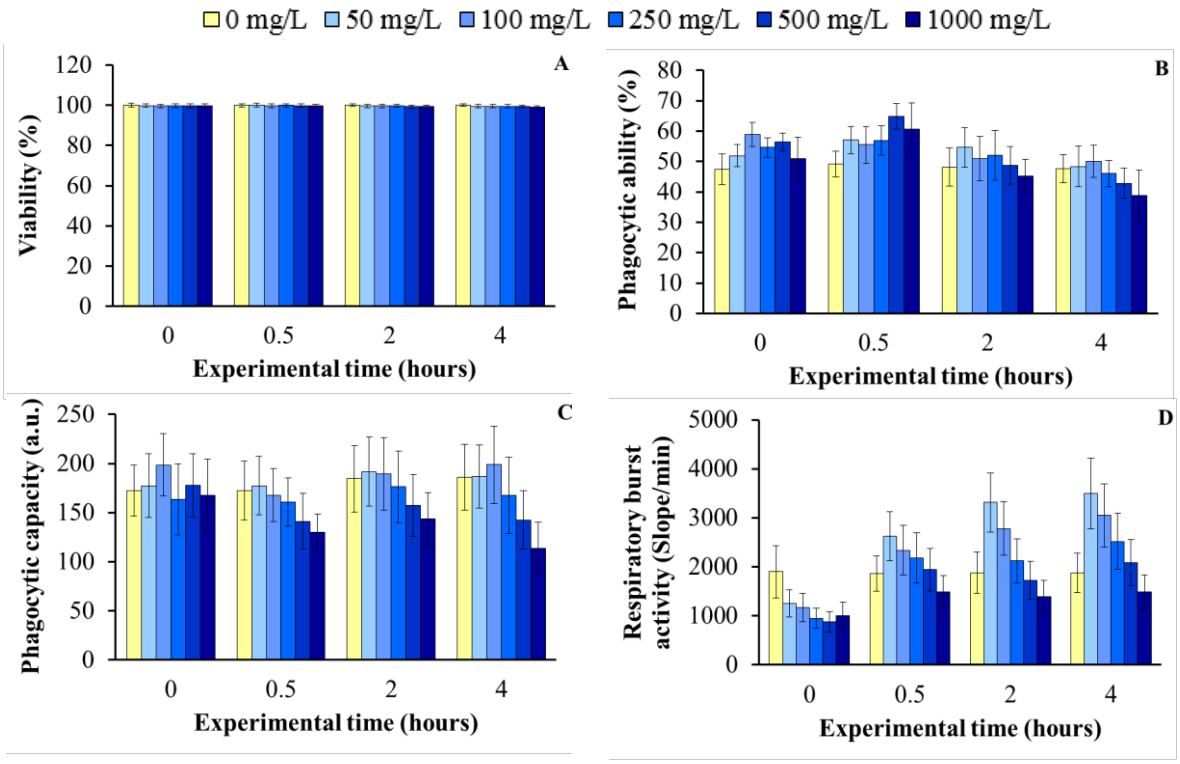



Figure 60: Viability (A), phagocytic ability (B), phagocytic capacity (C) and respiratory burst activity (D) of gilthead seabream head-kidney leucocytes after incubation with NICs. The results are expressed as mean ± SEM (n = 6).

### *II.A.1.2.3. HK leucocyte gene expression*

Different significant variations were detected in the expression of all the studied genes in HK leucocytes after being incubated with the different concentrations of the NICs mixture. The increases or decreases recorded respect to control samples depended on the mixture concentration and on the incubation time.

Regarding pro-inflammatory gene expression, no differences in comparison with control group were observed at 0 h of incubation between any tested concentrations in any

of the studied genes (Fig. 61A). Results at 0.5 hours of incubation were very different. *Il1b* expression increased with NICs concentrations from 50 to 250 mg/L in comparison with control group, but it was statistically significant only at 50 and 250 mg/L. The NICs dose of 50 mg/L was the one which increased in a significant manner *Il6* expression compared with results of control group. For its part, 1,000 and 100 mg/L of NICs significantly decreased *Il15* and *Il18* gene expression, respectively respect to control samples (Fig. 61B). Similarly, at 2 h, 100 and 1,000 mg NICs/L increased in a significant manner the gene expression of *Il15*, while 100 mg/L also increased *Il18* expression when results are compared with control samples (Fig. 61C). After 4 h of incubation, concentrations from 50 to 250 mg/L increased *Il1b* gene expression, but only the increase produced by 100 and 250 mg/L was statistically significant in comparison with control group. For its part, all concentrations decreased the gene expression of *Il7* in a significant manner, with the exception of 100 mg/L, which showed a not significant decrease respect control samples (Fig. 61D).

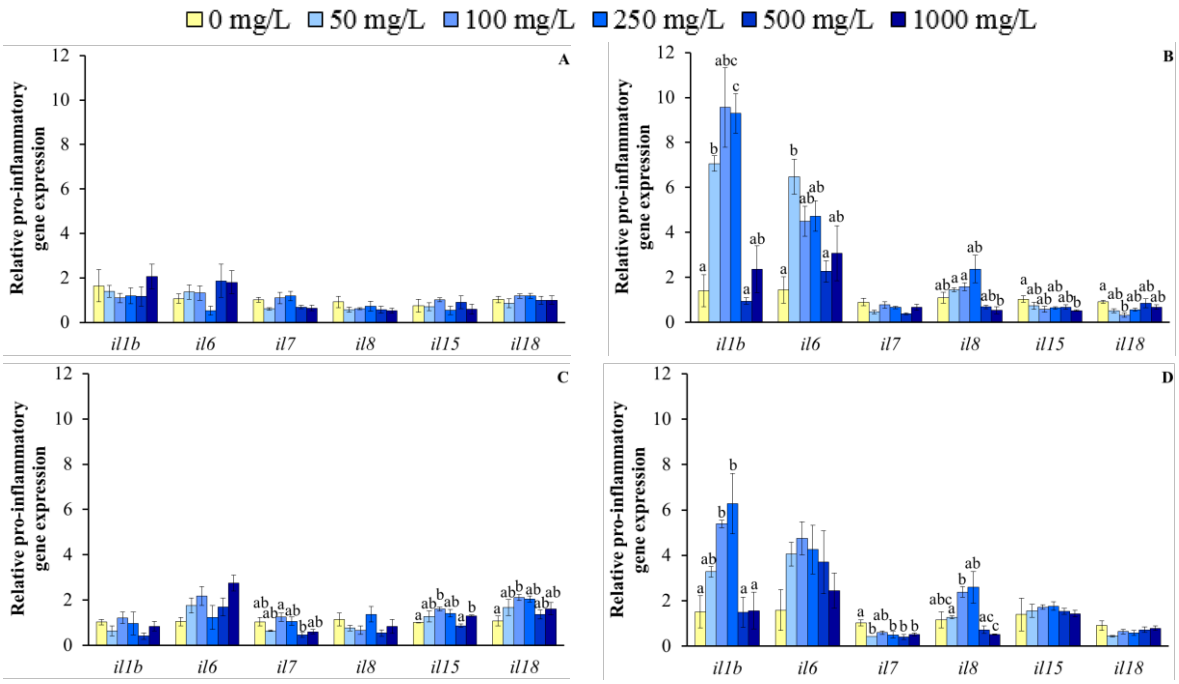



Figure 61: Relative pro-inflammatory gene expression of gilthead seabream head-kidney leucocytes after incubation with NICs for 0 (A), 0.5 (B), 2 (C) or 4 h (D). The results are expressed as mean ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

As to anti-inflammatory gene expression, no significant differences were observed at 0 h, although there is an increase of *il10* gene expression using 1,000 mg NICs/L respect to the control group (Fig. 62A). Expression of *tgfb* gene decreased using all concentrations of NICs in comparison with control group at 0.5 h, but only for 100 and 1,000 mg/L was statistically significant (Fig. 62B). As happened at 0 h, no significant variations were observed with control group at 2 h of incubation (Fig. 62C). Finally, after 4 h of incubation, all tested concentrations decreased *il10* expression in comparison with control group, being only statistically significant for 500 mg/L (Fig. 62D).

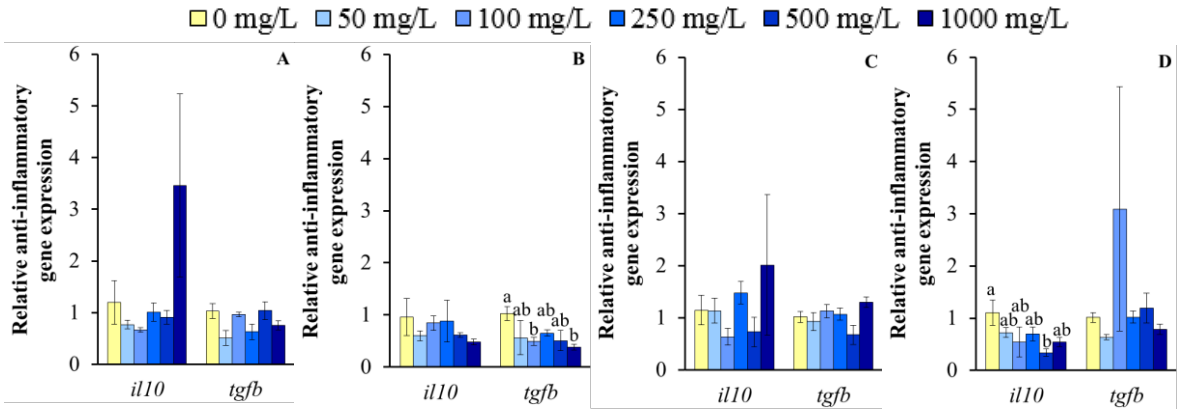



Figure 62: Relative anti-inflammatory gene expression of gilthead seabream head-kidney leucocytes after incubation with NICs for 0 (A), 0.5 (B), 2 (C) or 4 h (D). The results are expressed as mean ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

To finalize, the antioxidant gene expression results were very variable. At 0 h of leucocytes incubation any concentration of the NICs mixture showed significant variations of any gene studied respect control group (Fig. 63A). At 0.5 h, NICs concentrations from 250 to 1,000 mg/L decreased in a significant manner the gene expression of *nrf2* respect control samples. For its part, *gr* gene expression increased using 100 and 250 mg/L while decreased using 500 and 1,000 mg/L (Fig. 63B). Incubation for 2 h with the mixture, as happened at 0 h, showed no differences between any assayed concentration respect control (Fig. 63C). Finally, at 4 h of incubation, NICs concentrations of 50 and 100 mg/L significantly increased *gr* gene expression (Fig. 63D).

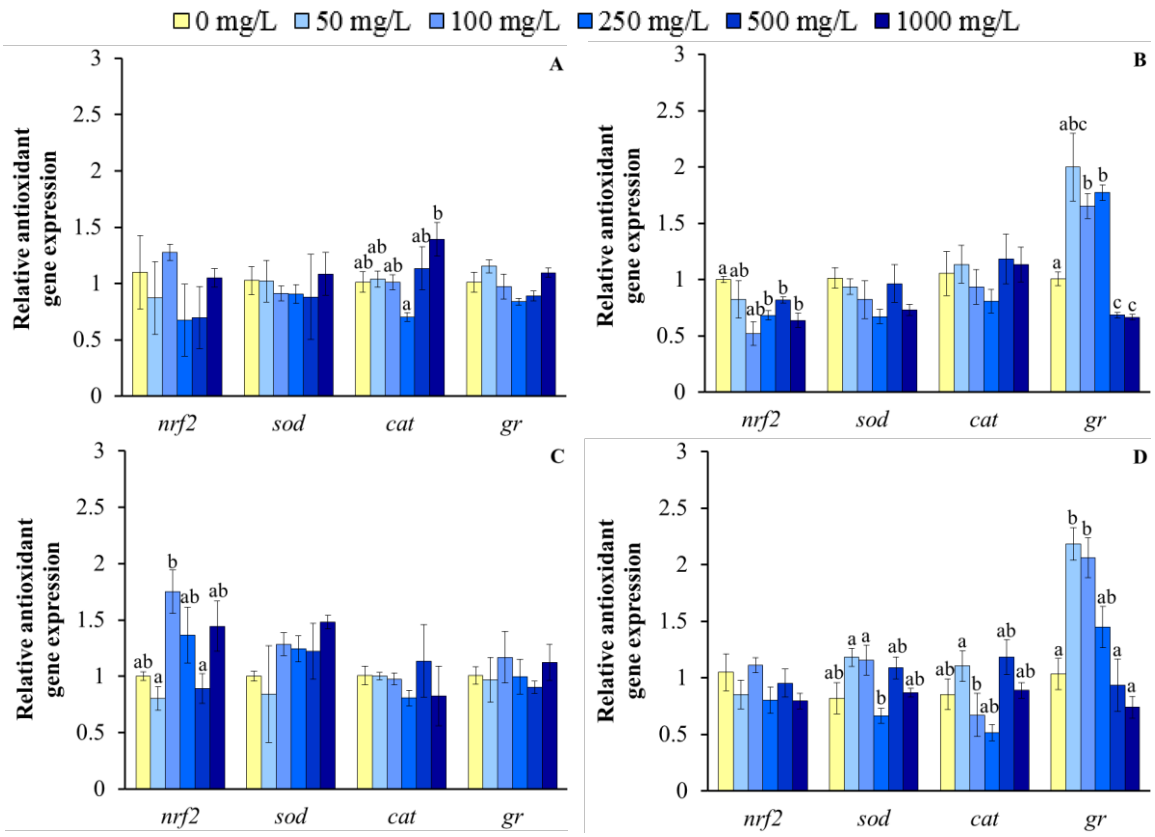



Figure 63: Relative antioxidant gene expression of gilthead seabream head-kidney leucocytes after incubation with NICs for 0 (A), 0.5 (B), 2 (C) or 4 h (D). The results are expressed as mean ± SEM (n = 6). Different letters denote significant differences between treatment groups ($P < 0.05$).

# Chapter III.B.

## *In vivo* effects of NICs

### III.B.1 First experiment

#### III.B.1.1 Material and metods

##### *III.B.1.1.1. Animals*

##### *III.B 1.1.2. Preparation of diets and experimental design*

##### *III.B.1.1.3. Sample collection and determinations*

#### III.B.1.2. Results

##### *III.B.1.2.1. Growth performance*

##### *III.B.1.2.2. Humoral immune parameters*

##### *III.B.1.2.3. Cellular immune parameters*

##### *III.B.1.2.4. Gut gene expression*

### III.B.2 Second experiment

#### III.B.2.1 Material and metods

##### *III.B.2.1.1. Animals*

##### *III.B.2.1.2. Preparation of diets and experimental design*

##### *III.B.2.1.3. Sample collection and determinations*

#### III.B.2.2. Results

##### *III.B.2.2.1. Growth performance*

##### *III.B.2.2.2. Humoral immune parameters*

##### *III.B.2.2.3. Cellular immune parameters*

##### *III.B.2.2.4. Proximal gut gene expression*

## III.B.1 First experiment

Fish were fed during sixty days with a feed enriched with different concentrations of a blend of NICs.

### III.B.1.1 Material and metods

#### *III.B.1.1.1. Animals*

One hundred and sixty specimens of gilthead seabream were obtained from a local farm and maintained as in Chapter 1.

#### *III.B 1.1.2. Preparation of diets and experimental design*

Four experimental diets were supplied by the international company Vetagro S.p.A. and consisted on non-supplemented diet (control) and three diets supplemented with different concentrations of a blend of NICs.

Fish were randomly assigned and divided into eight groups and tanks (n = 20 in each one) thus establishing four groups and two replicates by group: control, 25 NICs supplemented diet, 50 NICs supplemented diet and 100 NICs supplemented diet. Animals were fed *ad libitum* for two months. After fifteen, thirty and sixty days of trial, ten animals from each experimental group (five of each replicate tank) were sampled after sacrificing with an overdose of MS-222. All the experimental protocols were approved by the Ethical Committee of the University of Murcia.

#### *III.B.1.1.3. Sample collection and determinations*

Samples of blood and HK were obtained from each animal (see material and methods of Chapter 2.1). Fragments of proximal and distal gut were also sampled for gene expression and stored at -80°C in TRIzol Reagent as in Chapter 2.3.

Growth performance (WG and SGR) and immune status (serum IgM levels, natural haemolytic complement activity, HK leucocyte phagocytic and respiratory burst activities) were studied as described in Chapter 2.1. qPCR to study antioxidant, pro-inflammatory and anti-inflammatory gene expression on proximal and distal gut was also analysed as in Chapter 2.3. For the statistical analyses see material and methods of Chapter 2.1.

## III.B.1.2. Results

### *III.B.1.2.1. Growth performance*

WG and SGR were the two selected parameters to study growth performance of fish fed different diets. Similar results were obtained for both parameters. No differences were observed at 15 or 60 days between fish fed any tested diet. However, fish fed 50 NICs diet for 30 days increased significantly both WG and SGR respect control group (Fig. 64).

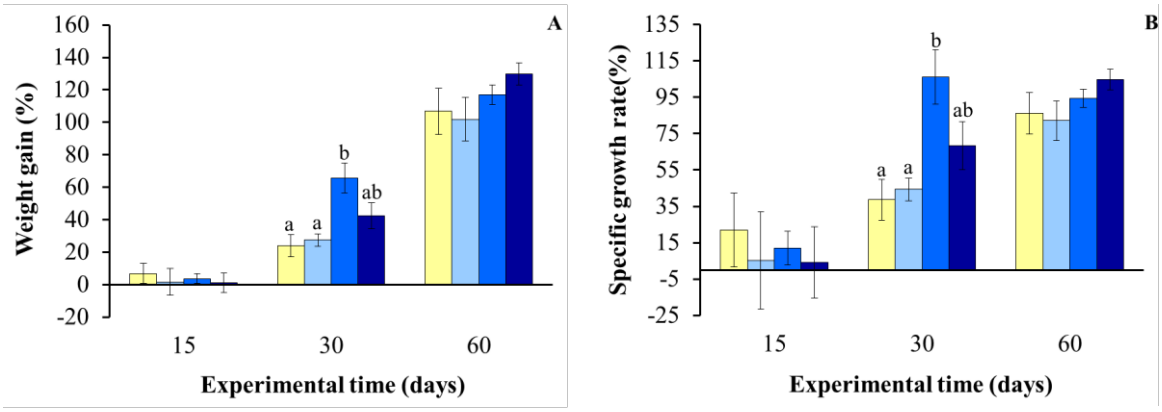



Figure 64: Weight gain (A) and specific growth rate (B) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 25 (light blue), 50 (blue) or 100 (dark blue) NICs for 15, 30 or 60 days. Data are presented as means ± SEM (n = 10). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *III. B.1.2.2. Humoral immune parameters*

Humoral immune parameters were studied in serum. IgM level was higher in the serum of fish fed with NICs diets than in fish fed control diet for 15, 30 and 60 days (Fig. 65A). However, only fish fed 100 NICs diet for 15 days showed a significant increase of IgM level in serum in comparison with control fish (Fig. 65A). For its part, no significant differences between any tested diets at any assayed time were found for natural haemolytic complement activity, although fish fed 25 and 50 NICs diets for 60 days showed an increase in this parameter in comparison with values obtained for control group (Fig. 65B).

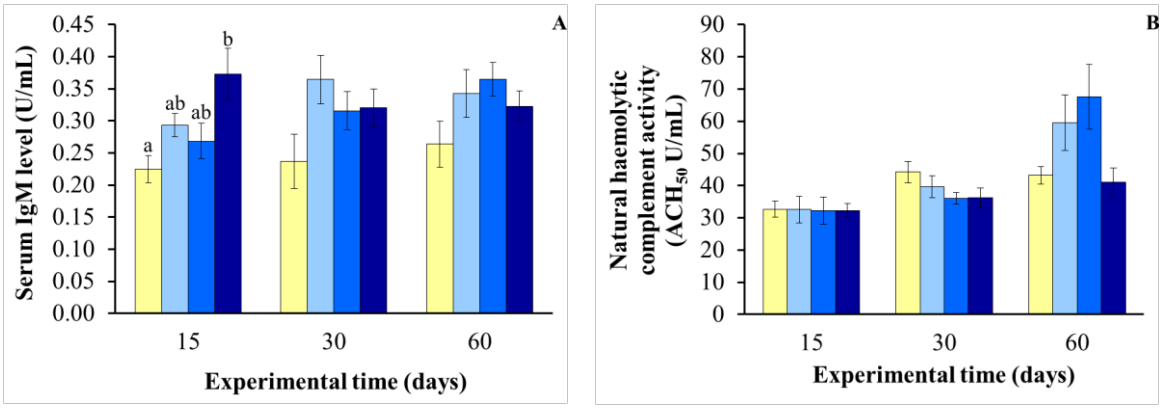



Figure 65: IgM level (A) and natural haemolytic complement activity (B) in the serum of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 25 (light blue), 50 (blue) or 100 (dark blue) NICs for 15, 30 or 60 days. Data are presented as means ± SEM (n = 10). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *III.B.1.2.3. Cellular immune parameters*

Results of HK leucocyte phagocytic ability revealed that fish fed with 50 NICs diet for 15 or 60 days showed a significant increase respect control fish (Fig. 66A), while all fish fed with NICs diets increased significantly leucocytes phagocytic capacity at 60 days (Fig. 66B). Finally, regarding leucocytes respiratory burst activity, no significant differences were obtained in comparison with control group at 15 or 30 days, although at 15 days, fish fed 25 NICs diet showed a decrease and fish fed 50 and 100 NICs diets showed an increase. However, 25 NICs diet significantly decreased the respiratory burst activity of HK leucocytes respect control fish at 60 days of experiment (Fig. 66C).

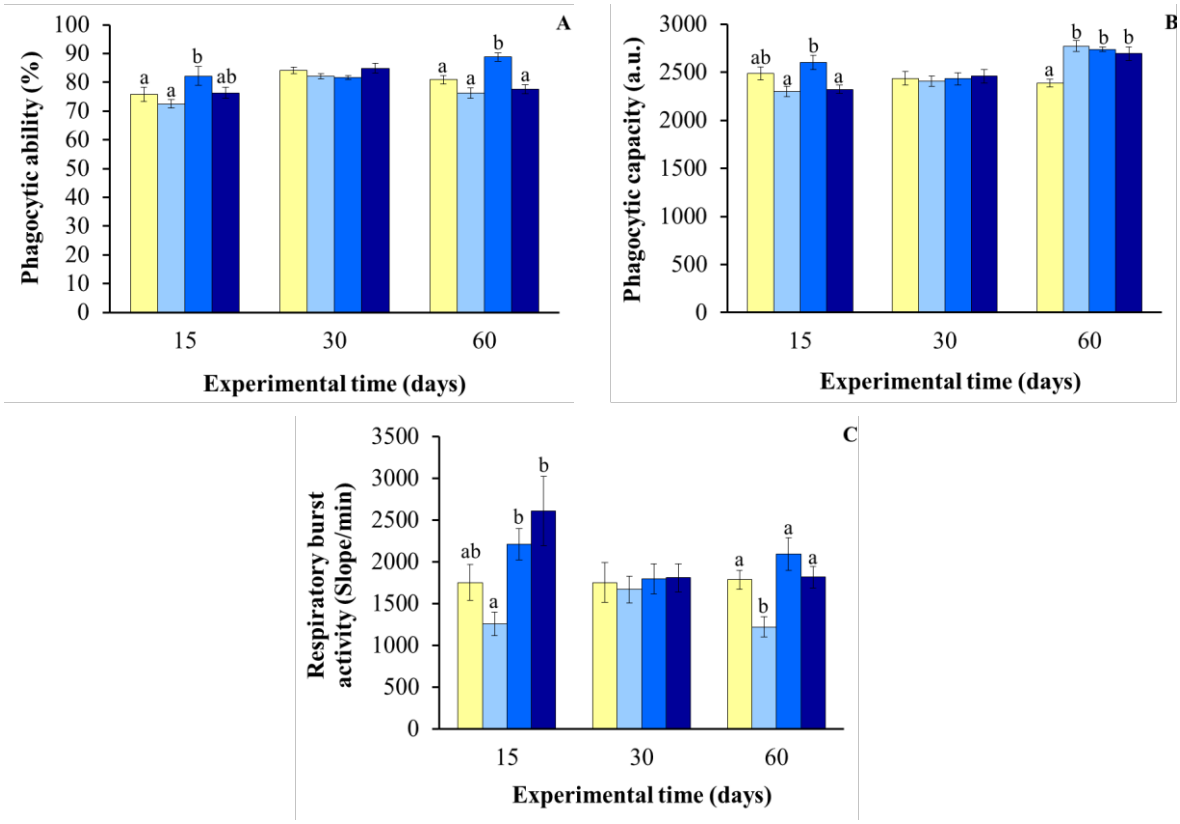



Figure 66: Phagocytic ability (A) and capacity (B), and respiratory burst activity (C) in the head-kidney leucocytes of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 25 (light blue), 50 (blue) or 100 (dark blue) NICs for 15, 30 or 60 days. Data are presented as means ± SEM (n = 10). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *III.B.1.2.4. Gut gene expression*

The expression of pro-inflammatory, anti-inflammatory and antioxidant genes was studied in the proximal and distal gut. Pro-inflammatory gene expression in the proximal gut of fish fed NICs diets did not show any significant difference with fish fed control diet at any assayed time (Fig. 67).

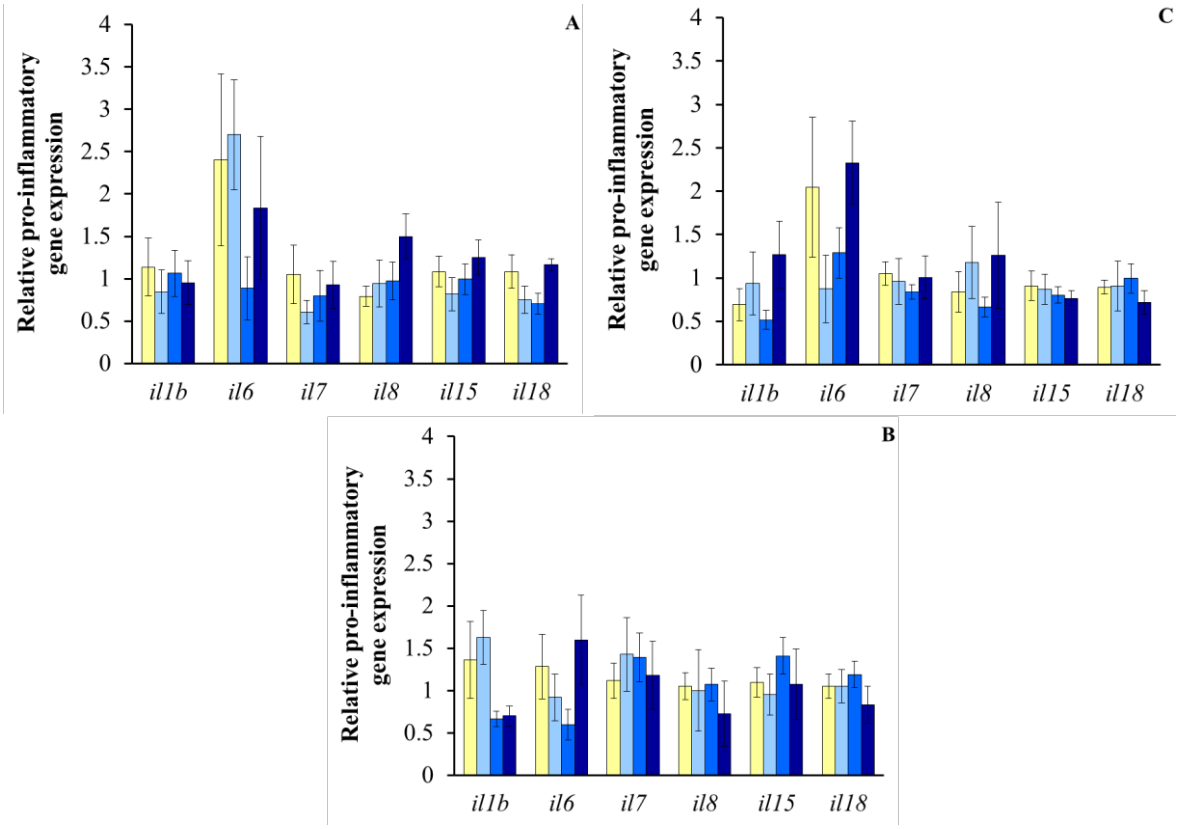



Figure 67: Relative expression of pro-inflammatory genes in the proximal gut of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 25 (light blue), 50 (blue) or 100 (dark blue) NICs for 15 (A), 30 (B) or 60 (C) days. Data are presented as means ± SEM (n = 10).

As happened with pro-inflammatory genes, the expression of anti-inflammatory genes in the proximal gut of fish fed NICs diets did not show any significant difference with fish fed control diet at any assayed time (Fig. 68). Similar results were obtained for antioxidant gene expression, which did not show any variations at any assayed time in comparison with control fish (Fig. 69).

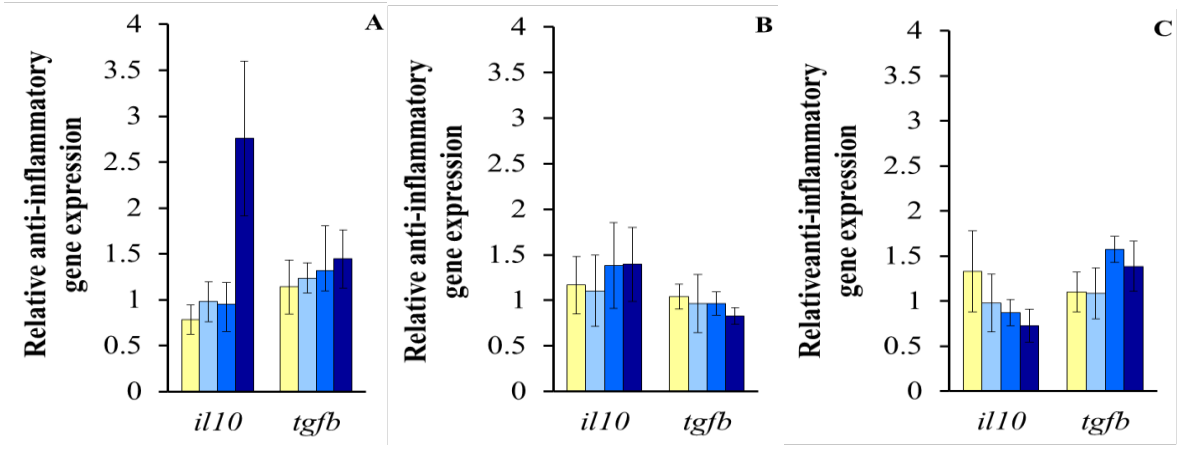



Figure 68: Relative expression of anti-inflammatory genes in the proximal gut of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 25 (light blue), 50 (blue) or 100 (dark blue) NICs for 15 (A), 30 (B) or 60 (C) days. Data are presented as means ± SEM (n = 10).

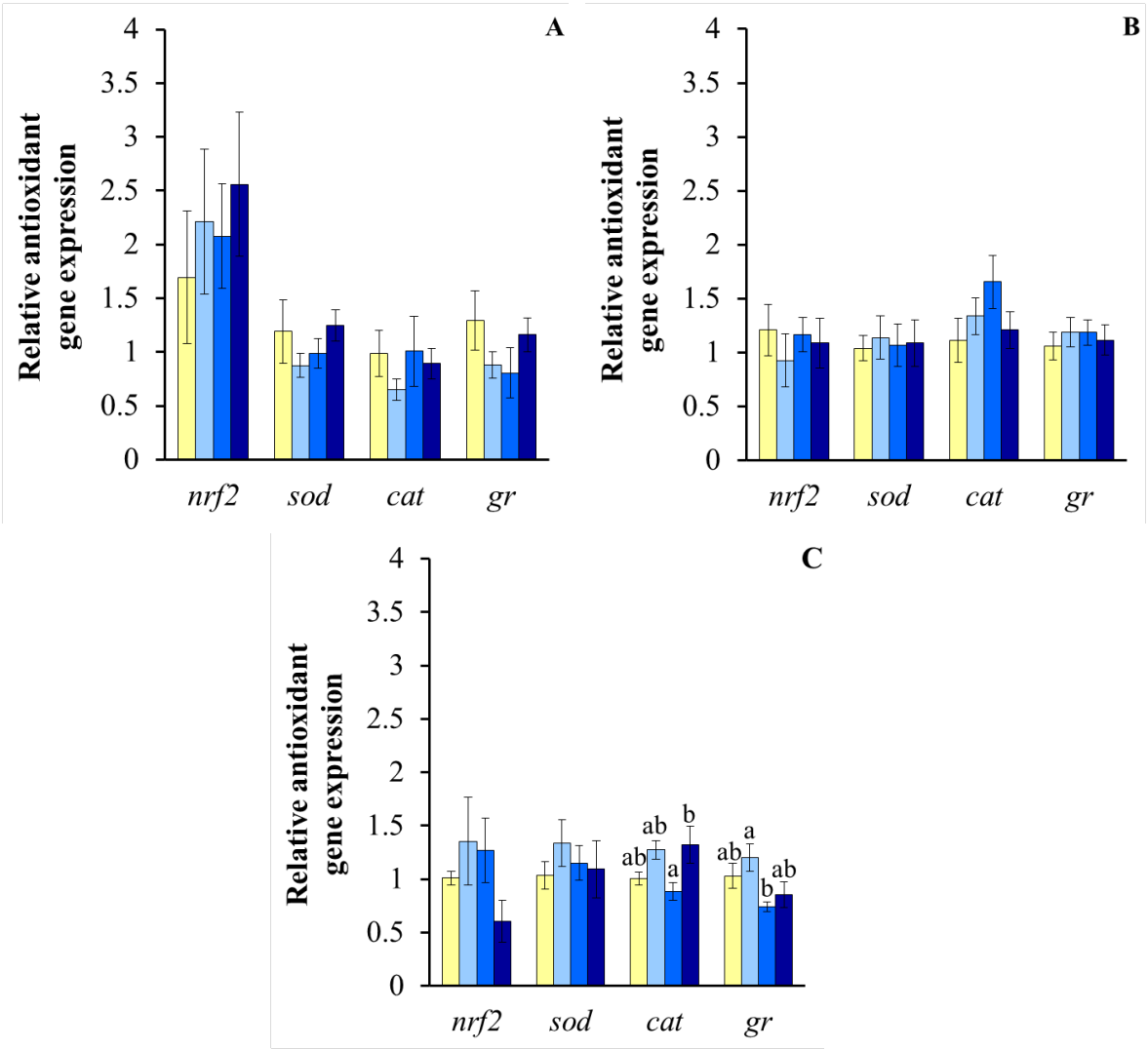



Figure 69: Relative expression of antioxidant genes in the proximal gut of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 25 (light blue), 50 (blue) or 100 (dark blue) NICs for 15 (A), 30 (B) or 60 (C) days. Data are presented as means ± SEM (n = 10). Different letters denote significant differences between treatment groups ($P < 0.05$).

In the case of the distal gut, at 15 days all pro-inflammatory studied genes showed lower expression in fish fed NICs diets in comparison with control fish, although only in the case of *il6* gene the decrease was significant (Fig. 70A). For its part, at 30 or 60 days of experiment there were not statistically significant differences between fish fed NICs diets and fish fed control diet (Fig. 70B, 70C), although fish fed 50 NICs diet showed a trend to increase *il6* gene expression at 60 days (Fig. 70C). It is important to underline that fish fed 25 NICs diet did not show practically expression of *il1b*, *6*, *15* and *18*, while *il8* had not expression in any group (Fig. 70C).

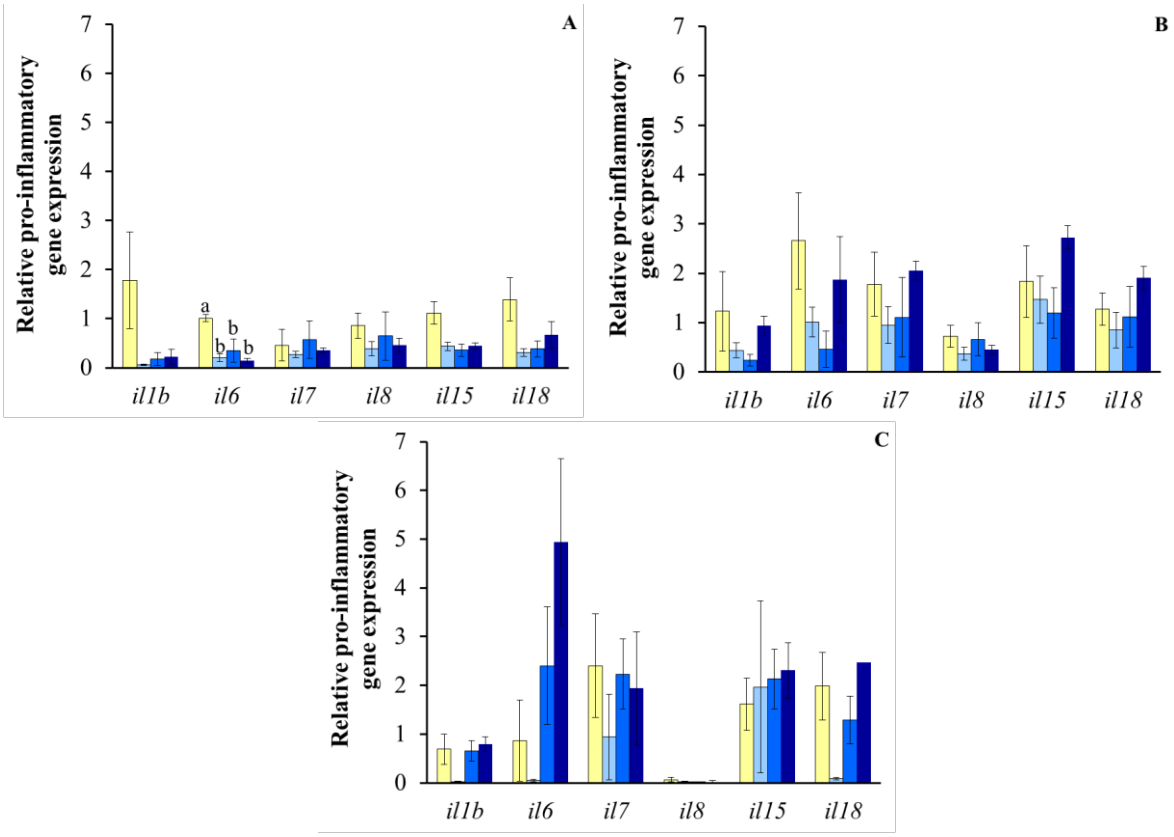



Figure 70: Relative expression of pro-inflammatory genes in the distal gut of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 25 (light blue), 50 (blue) or 100 (dark blue) NICs for 15 (A), 30 (B) or 60 (C) days. Data are presented as means ± SEM (n = 10). Different letters denote significant differences between treatment groups ($P < 0.05$).

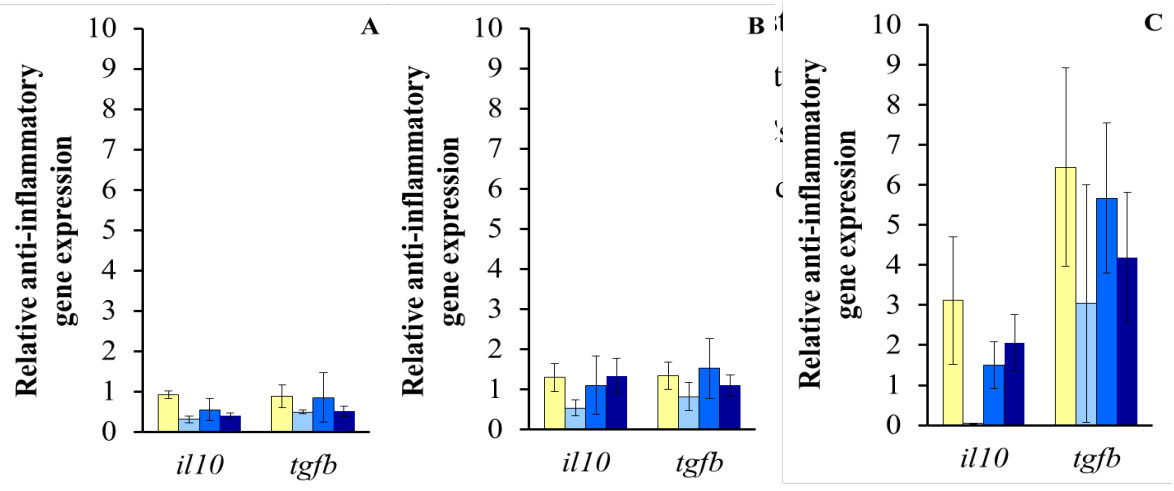



Figure 71: Relative expression of anti-inflammatory genes in the distal gut of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 25 (light blue), 50 (blue) or 100 (dark blue) NICs for 15 (A), 30 (B) or 60 (C) days. Data are presented as means ± SEM (n = 10).

As to antioxidant gene expression, as in the case of proximal gut, no significant differences were obtained in fish fed NICs diets in comparison with control fish (Fig. 72). However, it is important to highlight fish fed NICs diets showed lower expression of antioxidant genes than fish fed control diet at 15 days, while at 30 or 60 days gene expression increase together with NICs concentration, although no differences with control fish were obtained (Fig. 72B, 72C).

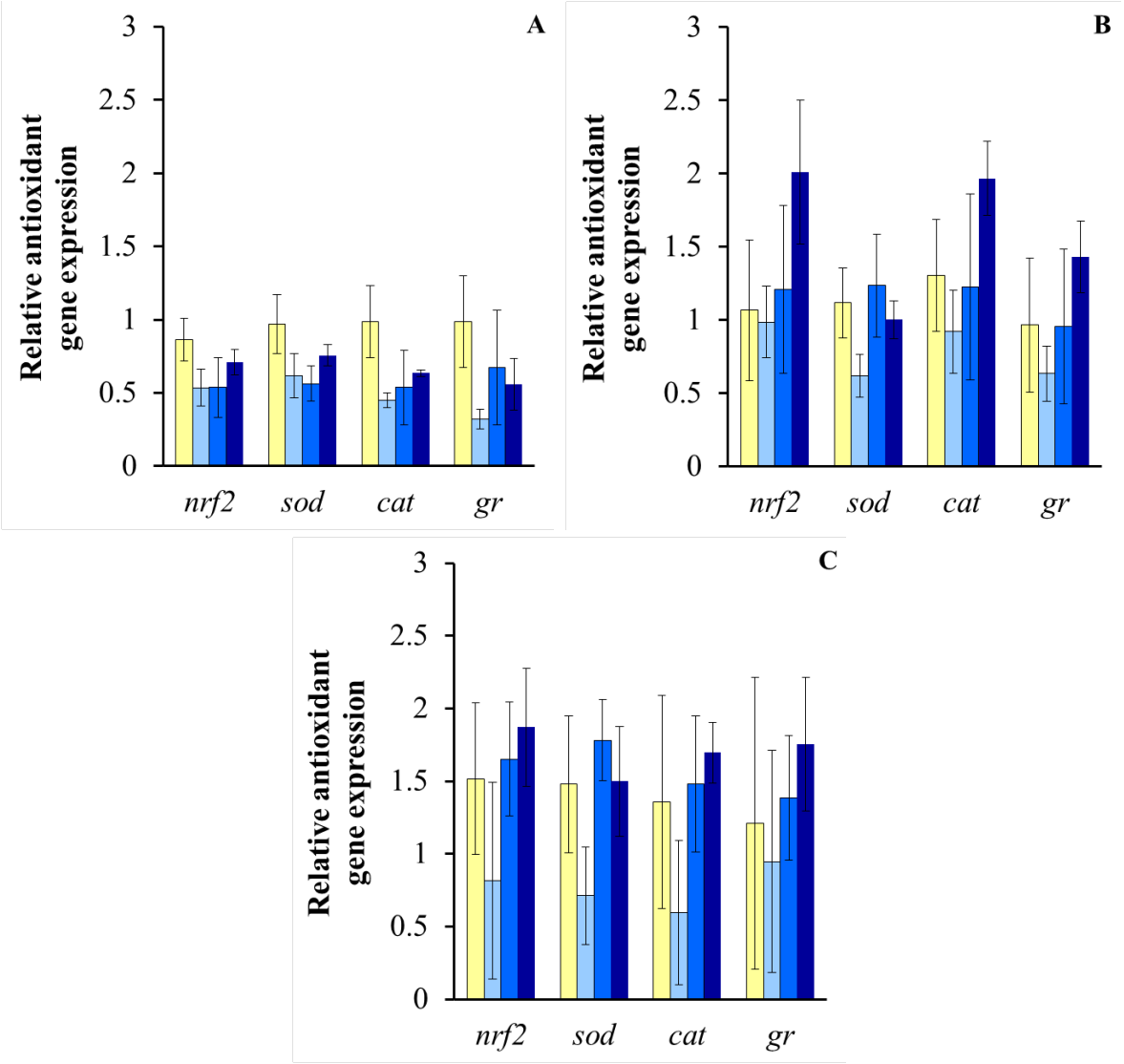



Figure 72: Relative expression of antioxidant genes in the distal gut of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 25 (light blue), 50 (blue) or 100 (dark blue) NICs for 15 (A), 30 (B) or 60 (C) days. Data are presented as means ± SEM (n = 10).

## III.B.2 Second experiment

Fish were fed during sixty days with two selected concentrations of the blend NICs from the first trial before being challenged with *V. harveyi*. Growth performance, immune and antioxidant status were again analysed before and after the bacterial challenge.

### III.B.2.1 Material and metods

#### *III.B.2.1.1. Animals*

One hundred and twenty specimens of gilthead seabream were obtained from a local farm as in Chapter 1.

#### *III.B.2.1.2. Preparation of diets and experimental design*

Fish were randomly assigned and divided into eight tanks (n = 20 in each) thus establishing three groups and two replicates by group: control (non-supplemented diet), 50 NICs supplemented diet and 100 NICs supplemented diet. Thirty days after the beginning of the treatment, six animals from each experimental group (three of each replicate tank) were sampled after sacrificing with an overdose of MS-222. After that, fish were challenged with *V. harveyi* using an intraperitoneal injection containing 100 µL with a concentration of $10^7$ u.f.c./mL and fish were sampled at one and fifteen days post-infection.

#### *III.B.2.1.3. Sample collection and determinations*

Samples of blood, HK and gut were obtained from each animal (see material and methods of Chapters 2.1. and 2.3.). Growth performance (WG, SGR and K), immune status (serum IgM levels, natural haemolytic complement activity, HK leucocyte phagocytic and respiratory burst activities) and gene expression in the gut were studied as described in Chapters 2.1.and 2.3.

## III.B.2.2. Results

### *III.B.2.2.1. Growth performance*

WG, SGR and K were studied to analyse the growth performance of fish. Both 50 and 100 NIC diets increased in a significant manner the WG and SGR of fish in comparison with control diet at 30, 31 and 45 days (Fig. 73A, 73B). No significant differences between fish fed NICs and control diets for 30 days were detected on K, while after 45 days (15 days post-infection), K of fish fed 100 NICs diet increased significantly respect to the values obtained for fish fed control diet (Fig. 73C).

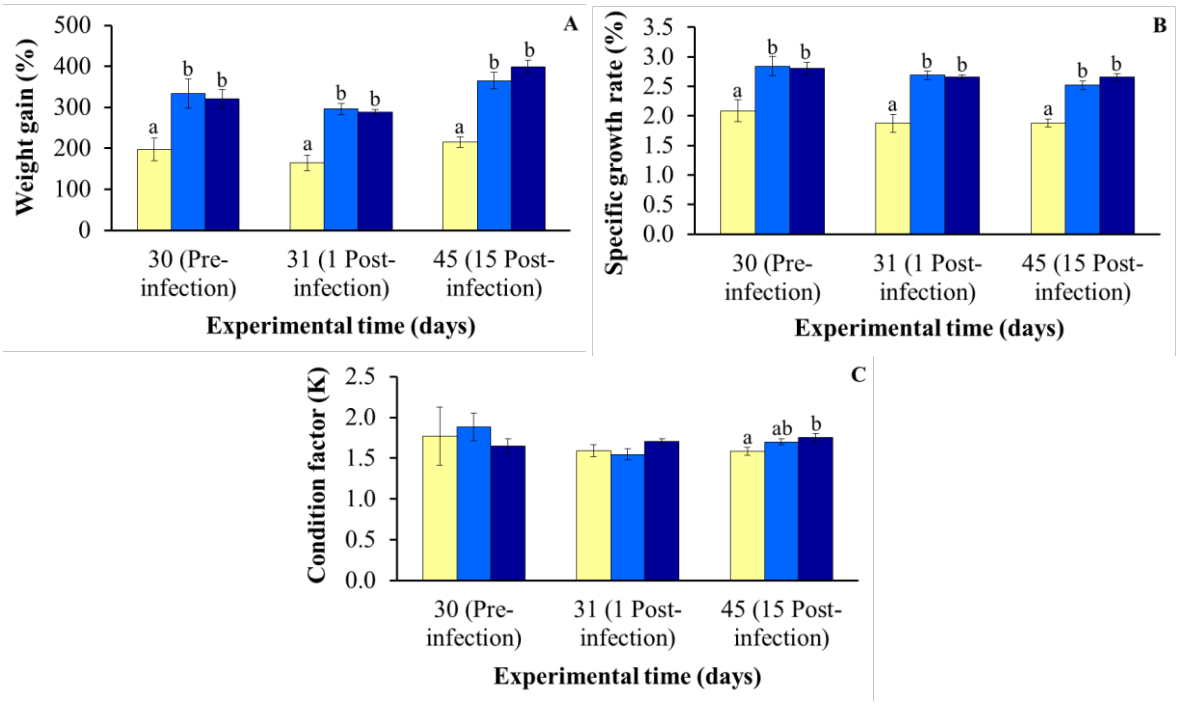



Figure 73: Weight gain (A), specific growth rate (B) and condition factor (C) of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 50 (blue) or 100 (dark blue) NICs for 30 days and then challenged with *Vibrio harveyi* and sampled 1 and 15 days later (31 and 45 days of feeding, respectively). Data are presented as means ± SEM (n = 6 at 30 and 31 days, n = 9 at 45 days). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *III.B.2.2.2. Humoral immune parameters*

As in the first trial, IgM level and natural haemolytic complement activity were studied in serum. No significant differences between fish fed NICs and control diets at any assayed time were detected for the IgM levels (Fig. 74A). Regarding natural haemolytic complement activity, fish fed 50 NICs diet showed a significant decrease respect to fish fed control diet for 31 days (1 day post-infection) (Fig. 74B).

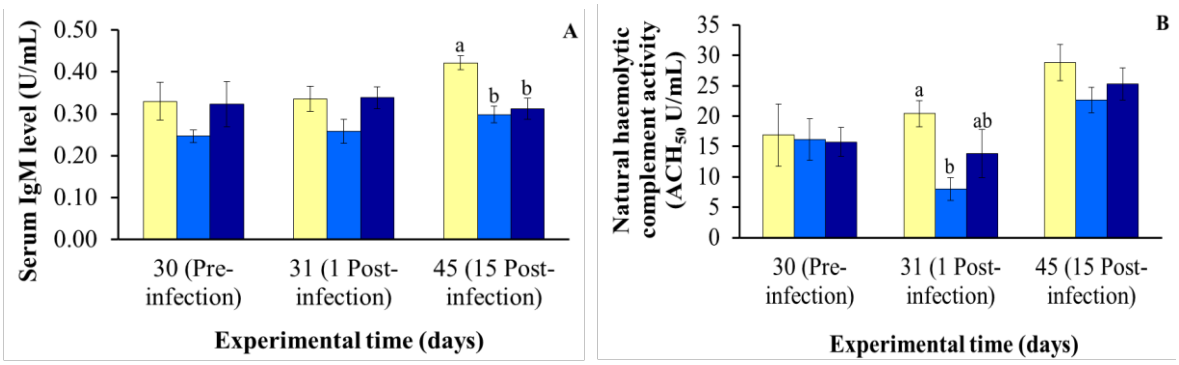



Figure 74: IgM level (A) and natural haemolytic complement activity (B) in the serum of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 50 (blue) or 100 (dark blue) NICs for 30 days and then challenged with *Vibrio harveyi* and sampled 1 and 15 days later (31 and 45 days of feeding, respectively). Data are presented as means ± SEM (n = 6 at 30 and 31 days, n = 9 at 45 days). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *III.B.2.2.3. Cellular immune parameters*

Phagocytic ability and capacity and respiratory burst activities of HK leucocytes were analysed in our study. Phagocytic ability of leucocytes from fish fed NICs diets was higher than fish fed control diet at every assayed time, although only at 45 days (15 days post-infection) differences were statistically significant (Fig. 75A). For its part, phagocytic capacity of leucocytes from fish fed 100 NICs diet showed a significant decrease in comparison with fish fed control diet at 30 days (pre-infection) (Fig. 75B). No statistically significant differences were observed in the respiratory burst activity between fish fed NICs and control diets at any assayed time although at 31 days (1 day post-infection), fish fed 100 NICs diet showed an important increment in comparison with values obtained for fish fed control diet (Fig. 75C).

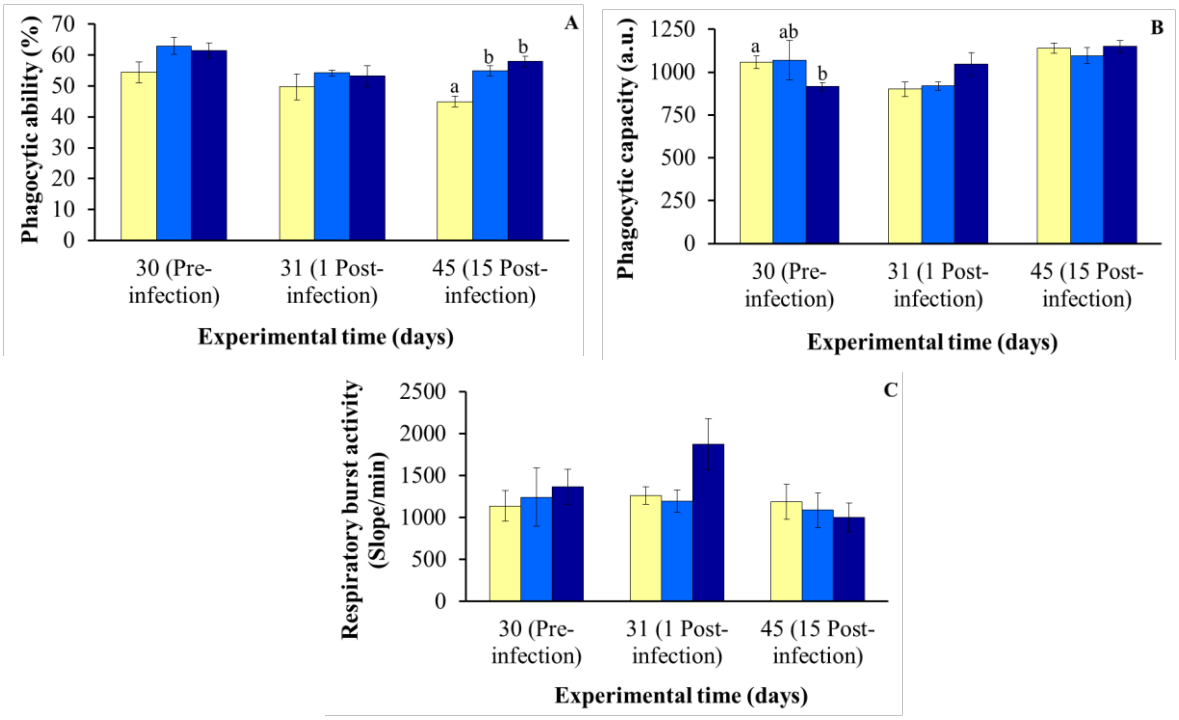



Figure 75: Phagocytic ability (A) and capacity (B) and respiratory burst activity (C) in the head-kidney leucocytes of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 50 (blue) or 100 (dark blue) NICs for 30 days and then challenged with *Vibrio harveyi* and sampled 1 and 15 days later (31 and 45 days of feeding, respectively). Data are presented as means ± SEM (n = 6 at 30 and 31 days, n = 9 at 45 days). Different letters denote significant differences between treatment groups ($P < 0.05$).

### *III.B.2.2.4. Proximal gut gene expression*

Pro-inflammatory genes expression did not show significant differences between fish fed with NICs diets and fish fed control diets at any assayed time (Fig. 76). However, there is an important increase in the expression of some genes at 31 and, especially, 45 days. At 31 days (1 day post-infection), overexpression of *il7* and *8* genes in fish fed 50 and 100 NICs diets respectively was observed in comparison with fish fed control diet (Fig. 76B). For its part, at 45 days (15 days post-infection), fish fed 50 NICs diet showed an overexpression of *il7* gene in comparison with control fish, while fish fed 100 NICs diet showed overexpressed the *il6*, *7* and *8* genes (Fig. 76C).

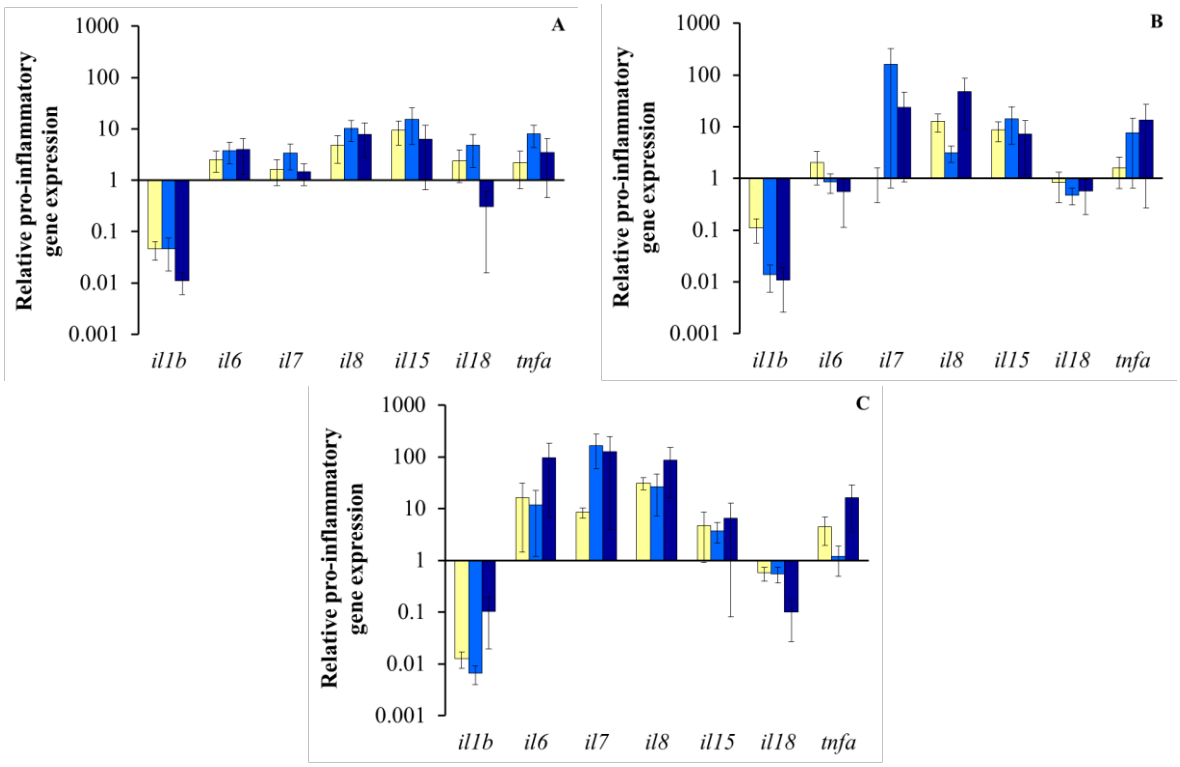



Figure 76: Relative expression of pro-inflammatory genes in the proximal gut of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 50 (blue) or 100 (dark blue) NICs for 30 days (A) and then challenged with *Vibrio harveyi* and sampled 1 (B) and 15 (C) days later (31 and 45 days of feeding, respectively). Data are presented as means ± SEM (n = 6 at 30 and 31 days, n = 9 at 45 days). Data are presented as means ± SEM [(n = 6 at 30 and 31 days), (n = 9 at 45 days)].

Regarding the anti-inflammatory gene expression, as in the case of pro-inflammatory genes, no significant differences were obtained between any tested diet at any assayed time (Fig. 77). The only remarkable fact was a very strong induction of *tgfb* gene expression in fish fed NICs diets in comparison with the values recorded for fish fed with the control diet (Fig. 77).

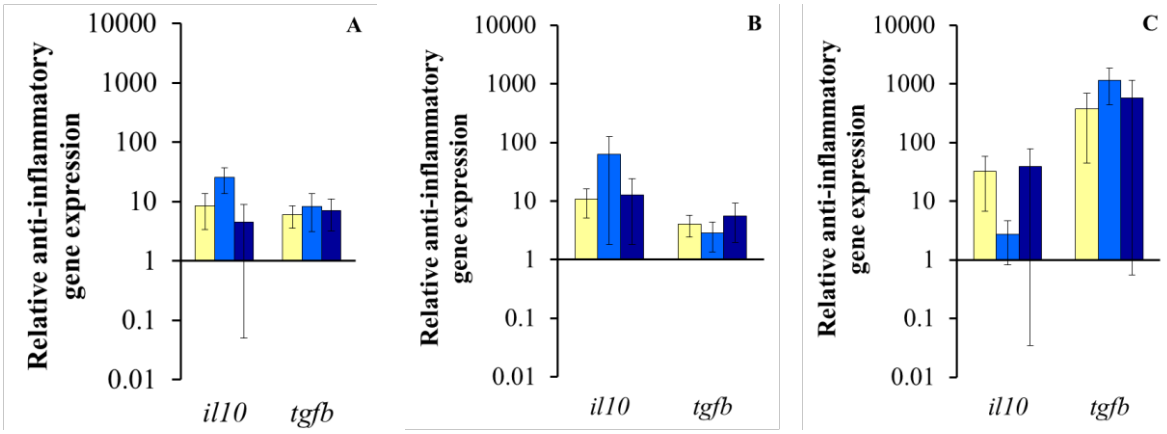



Figure 77: Relative expression of anti-inflammatory genes in the proximal gut of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 50 (blue) or 100 (dark blue) NICs for 30 days (A) and then challenged with *Vibrio harveyi* and sampled 1 (B) and 15 (C) days later (31 and 45 days of feeding, respectively). Data are presented as means ± SEM (n = 6 at 30 and 31 days, n = 9 at 45 days). Data are presented as means ± SEM [(n = 6 at 30 and 31 days), (n = 9 at 45 days)].

Finally, fish fed NICs diets did not show any significant variations at any assayed time in comparison with the values obtained for control fish (Fig. 78). Furthermore, an increase of *cat* gene expression was observed at 45 days (15 days post-infection) in fish fed all the experimental diets (Fig. 78).

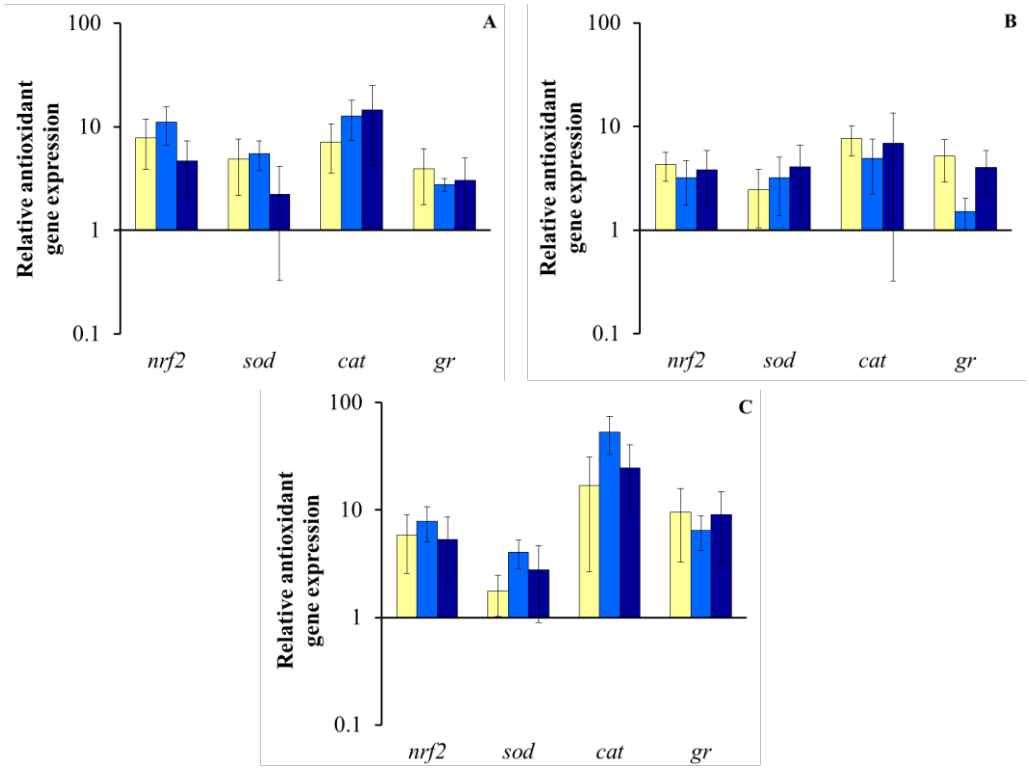



Figure 78: Relative expression of antioxidant genes in the proximal gut of gilthead seabream specimens fed diets supplemented with 0 (control, light yellow), 50 (blue) or 100 (dark blue) NICs for 30 days (A) and then challenged with *Vibrio harveyi* and sampled 1 (B) and 15 (C) days later (31 and 45 days of feeding, respectively). Data are presented as means ± SEM (n = 6 at 30 and 31 days, n = 9 at 45 days). Data are presented as means ± SEM [(n = 6 at 30 and 31 days), (n = 9 at 45 days)].

# Discussion

Globally, fish consumption and fish aquaculture sector activity, possibly the fastest-growing animal food producing sector worldwide (Newman, 2000), have increased in the last decades, with Spain being the first producer in the EU (OESA, 2015; APROMAR, 2019). This rapid increase is due in part to the reduction of natural stocks and the increase in consumer demand for healthy proteins (Hoseinifar et al., 2017). Furthermore, the World Bank model predicts that 62% of fish for food will be produced by aquaculture by 2030, after which, the aquaculture sector will likely dominate future global fish supply (World Bank, 2013). However, the super-intensive practices developed in fish farms imply various problems, including environmental harm (e.g. bad water quality), increased number of opportunistic pathogen microorganisms and stress conditions, which may compromise fish growth and health and make animals more susceptible to infections and diseases, resulting in deaths and substantial economic losses (Bulfon et al., 2013; Holmes et al., 2016). In addition, until few years ago antibiotics were used with little or no control to treat or prevent fish diseases, but, due their negative impact such as their tendency to accumulate in fish, the appearance of multi-resistant pathogens and the possibility that they may reach humans through the food chain, they have been banned in the EU since 2006 (Cabello, 2006; Ng and Koh, 2016).

Therefore, due in part to the accelerated growth of aquaculture, it is necessary to establish an agenda for the global economy ensuring its successful and sustainable development both environmentally and economically (World Bank, 2013; FAO 2018). In this sense, the use of medicinal plants is a very interesting alternative to chemotherapeutics in aquaculture (Direkbusarakom, 2004; Citarasu, 2010; Harikrishnan et al., 2011; Bulfon et al., 2013; Reverter et al., 2014; van-Hai, 2015; Awad and Awaad, 2017).

Finally, due to the need to find new compounds of natural origin with potential applications in fish aquaculture, the absence of i*n vitro* studies about the effect of plants on fish cells and the good results obtained to date in *in vivo* studies using different medicinal plants, we have undertaken developed the present *in vitro* and *in vivo* work using several plants, most of them naturally present in Spain.

## *In vitro* studies with fish cells: effects on cell viability and activities

Due the very few studies carried out on plant extracts in fish cells, this *in vitro* study can be regarded as an attempt to reduce the number of animals used in research activities and to select the most appropriate extracts to be included as feed additives for fish in subsequent *in vivo* studies.

For the plant extraction strategies, factors such as part of the plant, solvent used, method of extraction, time and temperature of extraction are crucial variables to obtain extracts with powerful biological activities. Most scientists prefer to use dried plant material because the water content may affect the solubility of subsequent separation by liquid-liquid extraction and the stability of secondary metabolic compounds (Ncube et al., 2008). Furthermore, the drying method is also important because it can affect the phytochemicals that can be extracted (Leone et al., 2015). In addition, drying helps in the storage, handling and use in manufacturing for industrial use.

The compounds in question can be obtained from the whole plant or from a specific part of a plant, including fruits, seeds, flowers, roots, stems or leaves (Burt, 2004; Viuda-Martos et al., 2010; Chinelo et al., 2014; Zhou et al., 2015; Iranshahy et al., 2017). Although the presence and concentration of the different components may vary according to the part of the plant, it has been demonstrated that they are usually more varied and present in higher concentrations in the aerial parts of the plants (Burt, 2004; Barros et al., 2010; Kunicka-Styczyńska, 2011; Syed et al., 2016, Iranshahy et al., 2017). During extraction, solvents diffuse into the solid plant material and solubilize compounds of similar polarity (Green, 2004), thus chemical compounds extracted from a plant also depend on the solvent used (concentration and polarity) and time and method of extraction (Lim and Quah, 2007; Roldán-Gutiérrez et al., 2008; Sultana et al., 2009; Aranha and Jorge, 2012; Chinelo et al., 2014; Koldas et al., 2015; Leone et al., 2015; Rodríguez-Solana et al., 2015; Valdez-Solana et al., 2015; Zhou et al., 2015; Baranauskaité et al., 2016; Petropoulos et al., 2016; Syed et al., 2016; Iranshahy et al., 2017). In general, alcoholic or organic solvents are better than water solvents to obtain high phytochemical concentrations (Bulfon et al., 2013).

Because the aerial parts present more variety and higher concentrations of phytochemicals, in the present work we used plant leaves in the case of oregano and moringa, and leaves and stems in the case of purslane. Moreover, in an attempt to add value to date palm we used seeds because they are discarded once the fruit has been eaten. In the same way, since solvents influence the type and concentrations of bioactive compounds obtained, for the *in vitro* studies we used solvents with different polarities: water and ethanol.

The first activity was to verify the effects of plant extracts on gilthead seabream HK leucocyte viability so that those plant extracts with a negative impact on cells could be discarded. Secondly, the effects of such extracts were studied on the main innate cellular immune activities of HK leucocytes, including phagocytosis, respiratory burst and peroxidase (myeloperoxidase). Phagocytosis is a self-protective reaction against infection and invasion of the animal body by foreign substances, and is considered a crucial mechanism for limiting the growth of fish pathogens (Divyagnaneswari et al., 2007) and is an important reaction against infection (Esteban et al., 2015). As a consequence of the activation of the phagocytic cells they increase their oxygen consumption and respiratory burst activity occurs, which generates superoxide anion and $H_2O_2$ through the activity of an NADPH-oxidase (NOx) (Crocnan et al., 2000). Afterwards, these reactive oxygen species (ROS) have a strong anti-microbial activity, but may also cause some damage *in situ* such as DNA damage, destruction of surrounding tissue and apoptosis in other immune cells. In addition, although less studied, immune cells present peroxidases - lysosomal proteins stored in cytoplasmic granules that are also involved in antimicrobial mechanisms via hypohalous acid production (Klebanoff, 2005). These enzymes are released into the extracellular space during cell activation and degranulation (Spitznagel et al., 1983; Rodríguez et al., 2003).

## Biological activities of plants on *in vitro* studies: cytotoxic, bactericidal and antioxidant activities

Cytotoxicity is a very important and powerful activity of plants. In fact, various medicinal plants are considered toxic and can cause serious damage to health. For this reason, assessment of the toxicity of medicinal plants or plant extracts is essential to determine their applicability as pharmacological drugs (Monteiro et al., 2014). Therefore, the first step in our *in vitro* study was to analyse the effect of the selected plant extracts on SAF-1, obtained from gilthead seabream fin (Béjar et al., 1997), and PLHC-1, from topminnow, cell lines. In connection with their cytotoxic activity, one of the most common uses of medicinal plants in traditional medicine is in cancer treatment and prevention due the demonstrated antitumor activity of many natural compounds and because they are safety (Chavan et al., 2013; Monteiro et al., 2014; Solowey et al., 2014; Greenwell and Rahman, 2015; Reyad-ul-Ferdous et al., 2016; Ijaz et al., 2018; Rahman, 2018). These properties allow them to be used as adjuvant with cancer treatments for patients (Monteiro et al., 2014; Rahman, 2018) due they help to inhibit angiogenesis, metastasis, metabolism and severe side effects (Jeong et al., 2011). Among these mechanisms are the arrest of cell division and cell growth, loss of mitochondrial membrane potential, down regulation of anti-apoptotic proteins, and, especially, the up regulation of pro-apoptotic proteins (Monteiro et al., 2014; Solowey et al., 2014; Greenwell and Rahman, 2015; Ijaz et al., 2018).

Infections by pathogenic bacteria is one of the principal problems in fish farms, and can lead to significant economic losses (Direkbusarakom, 2004; Harikrishnan et al., 2011; Bulfon et al., 2013; Reverter et al., 2014; van-Hai, 2015; Awad and Awaad, 2017). For this reason, plant extracts were also tested for their antibacterial activity. Normally, the pathogenic bacteria responsible for causing infections are naturally present in the aquatic environment and even in wild fish populations but they rarely cause disease. However, the conditions of stress that occur in aquaculture encourage the appearance of bacterial infections due the decreased activity of the host immune system (da-Cunha et al., 2018), which is used by bacteria to increase their virulence (Lyte, 2004; Toranzo et al., 2005). This is the case of *Vibrio* species, and, although some are considered primary pathogens (Lavilla-Pitogo et al., 1990), most are opportunistic pathogens that take advantage of host immunosuppression to infect them (Alderman and Hastings, 1998). These bacteria are halophilic Gram-negative gammaproteobacteria widely distributed in a great range of

habitats in the ocean (Thompson et al., 2004; Austin and Zhang; 2006: Ishimaru and Nakai, 2017), and can exist in different forms, as planktonic, in biofilms or associated to hosts (Nguyen and Jacq, 2014). According to the review made by Kalatzis et al. (2018), the number of incidents caused by these bacteria (also known as "vibriosis") in marine aquaculture has increased globally in recent years due to the intense activity that takes place in fish farms. High water temperatures also favor their increase, meaning that climat change will also favor the increase of this infection even in temperate or cold regions (Rowley et al., 2014; Vezzulli et al., 2016). In addition, vibriosis also usually appears with stressors such as poor water quality, poor diet composition, salinity or low dissolved oxygen levels, physical stressors such as transport stress or handling, and overcrowding (Austin and Austin, 2012).

Vibriosis, which occurs especially in marine fish and invertebrates, is characterised by haemorrhages, superficial skin sores, ulcers and necrosis, and in most cases there is general septicaemia (Austin and Austin, 2007), although eyes and swim bladder problems are also frequently observed (Thompson et al. 2004; Toranzo et al., 2005; Chatterjee and Halda, 2012). This disease, which affects most warm and cold water fish species and many economically important marine farmed fish species worldwide, causing multibillion-dollar losses to the aquaculture industry, represents the major cause of mortality and the most important limiting factor for the development of intensive marine aquaculture (Thompson et al., 2004; Toranzo et al., 2005; Austin and Zhang, 2006; Rivas et al., 2013; Nguyen and Jacq, 2014). Although many species of *Vibrio* such as *V. harveyi*, *V. anguillarum*, *V. damsela*, *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. cholera* and *V. salmonicida* among many others, are known to be responsible for fish diseases (Austin and Austin, 2012; Scarano et al., 2014; Ishimaru and Nakai, 2017; Kalatzis et al., 2018) other strains are avirulent (Austin and Zhang, 2006; Ruwandeepika et al., 2010). Treating vibriosis with chemotherapeutics is the preferred option since only one commercial vaccine for *V. anguillarum* is available (Kalatzis et al., 2018).

Regarding the importance of vibriosis in gilthead seabream, *Vibrio* genus represent one of the main pathogens affecting this fish species (Balebona et al., 1998; Toranzo et al., 2005), which, moreover, displays multi-resistance to antibiotics (Zorrilla et al., 2003; Scarano et al., 2014; Zouiten et al., 2017). Furthermore, the prevalence of *V. harveyi* in gilthead seabream in the Spanish Mediterranean coast occurs almost exclusively in warm months (June to November) (Pujalte et al., 2003). *V. harveyi* and *V. anguillarum* are normal

habitants of the aquatic environment, including seawater and sediments, and part of the normal flora of animals (Frans et al., 2011). They are probably the most important pathogens for aquaculture since they affect economically relevant marine vertebrates and invertebrates (reviewed by Austin and Zhang, 2006; Frans et al., 2011; Ruwandeepika et al., 2012; Hickey and Lee, 2017), gilthead seabream being one of the most affected organisms (Toranzo et al., 2005).

High salinity and temperature are two factors that increase the growth, motility (Groberg et al. 1983; Larsen, 1984; Kao et al. 2009) and virulence of these bacteria (Diggles et al., 2000; Alavandi et al., 2006), which leads to a great increase in the number of infections (Austin et al., 2005). In most cases of infection by species of *Vibrio*, they adhere to the host's skin, which they penetrate, taking advantage of wounds on the surface and mucous layer (Spanggaard et al., 2000; Croxatto et al., 2007; Weber et al., 2010), although they can also initiate infection through the gut through oral or anal entry (O´Toole et al., 2004).

Among the virulence mechanisms of these bacteria is the secretion of extracellular products (ECPs), most of which are proteases with hydrolytic, hemolytic and cytotoxic activity that degrade fish tissues (Balebona et al., 1998), although LPSs, siderophores binding iron proteins, bacteriocin-like substances (BLIS), plasmids, flagella, quorum sensing and biofilms are also mechanisms (reviewed by Austin and Zhang, 2006; Cano-Gómez et al., 2009; Frans et al., 2011; Ruwandeepika et al., 2012; Hickey and Lee 2017).

The third bacteria that we focus on in this work is *P. damselae* subsp. *piscicida*. This bacterium belongs to the genus or clade *Photobacterium*, which is within the family *Vibrionaceae* (Osorio et al., 2000; Sawabe et al., 2007; Pérez-Cataluña et al., 2016), and *P. damselae* was formerly called *V. damselae* (Fouz et al., 1992). The bacterial members of this genus are distributed worldwide in marine environments and can be present in seawater, sea sediments, saline lake waters and animals, where they may establish different relationships, varying from symbiotic ones, such as commensalism or mutualism, to pathogenic interactions (Dunlap, 2009).

*P. damselae* subsp *piscicida* is the etiological agent of "pasteurellosis" or "photobacteriosis", which is a bacterial septicemia characterized by chronic cases in several internal organs, giving rise to whitish tubercles consisting of bacterial accumulations and multifocal necrosis in liver, spleen and kidney (Margariños et al., 1996a, b; Osorio et al.,

2000; Romalde et al., 2002). Pasteurellosis affects a wide variety of aquatic organisms, including fish, mollusks and crustaceans, corals and sponges (Romalde et al., 2002; Moi et al., 2017) and represents a limiting factor for aquaculture worldwide causing high mortality rates and severe economic impact (Barnes et al., 2005), especially in Japan and the Mediterranean area, where it affects gilthead seabream farms (Romalde et al., 2002; Zorrilla et al., 2003; Toranzo et al., 2005). Moreover, pasteurellosis appears more in summer months, when temperatures are high, as is salinity, and the water quality is low (Margariños et al., 1996a). This bacterium, then, can be considered as an opportunist pathogen for gilthead seabream. Furthermore, *P. damselae* subsp. *piscicida* has shown an increased resistance to antibacterial drugs (Zorrilla et al., 2003).

As regards the virulence mechanisms of *P. damselae* subsp. *piscicida*, unlike *V. harveyi* and *V. anguillarum*, the site of attachment and host adhesion is the gut (Margariños et al., 1996b). According to the reviews made by Romalde et al. (2002), Andreoni and Magnani (2014) and Labella et al. (2017), among these mechanisms are the secretion of ECPs with phospholipase, cytotoxic and hemolytic activities that help in the invasion of the host. According to the reviews made by Citarasu (2010), Nazzaro et al. (2013), Pandey (2013), Borges et al. (2015) and da-Cunha et al. (2018), most of the phytochemical compounds exert their action on the cell surface, affecting membranes and/or the cell wall. Furthermore, these compounds can affect intracellular adenosine triphosphate (ATP) production and concentration and induce protein and enzyme denaturation as well as the inhibition of nucleic acids synthesis and metabolism among other mechanisms. In addition, another possible method of action is to render substrates unavailable to the microorganism, inhibiting biofilm formation (affecting motility, ECPs production, adhesion and quorum sensing) and bacterial growth by binding of sulfhydryl (-SH) groups, which leads to reactive radical formation.

In this respect, previous results have demonstrated that Gram-positive marine bacteria are generally more susceptible to herbal extracts than Gram-negative ones, like *Vibrionaceae* (Trombetta et al. 2005; Dubber and Harder, 2008), which is due to the differences in the cell wall structure. In their cell wall, Gram-positive bacteria present only PG, which allows penetration of hydrophobic molecules and acts both on the cell wall and within the cytoplasm, whereas Gram-negative bacteria present a double layer of phospholipids linked to PG by LPSs, which only allows the penetration of some hydrophobic

molecules through porin proteins (Nazzaro et al. 2013). Nevertheless, some herbal compounds act as inhibitors of the quorum-sensing pathways in *Vibrio* sp. (Citarasu, 2010).

Stress is another important factor that can appear in fish farms due the high intensity of cultivation and activity of aquaculture. Fish can develop oxidative stress and increase ROS production, which negatively affects animal health and performance (Jooyandeh and Aberoumand, 2007). ROS, which are produced normally as by-products of the normal oxygen metabolism, can be overproduced by exogenous stressors, increasing their levels dramatically and producing an imbalance between ROS formation and cellular antioxidant capacity (Lee et al., 2017a; Aniya, 2018; Pohl and Lin, 2018). During inflammation, leucocytes recruited to the site of damage perform the phagocytosis of infected cells and respiratory burst activity which increases oxygen consumption and ROS production, helping to neutralise the invading organisms (de-Lavor et al., 2018). Therefore, in normal conditions, ROS production by mitochondrial metabolism and redox processes are important in order to signal specific physiological pathways or functions, while an overproduction and an uncontrolled balance is toxic for the organism (Amorati and Valgimigli, 2018; de-Lavor et al., 2018; Pohl and Lin, 2018). In this case, ROS are highly reactive and allow the appearance of diseases and infections, and furthermore, can produce DNA, protein, membrane lipid and tissue damage, apoptosis and even fish death, finally decreasing production and having an economic impact (Jooyandeh and Aberoumand, 2007; Lee et al., 2017a; Aniya, 2018; de-Lavor et al., 2018; Pohl and Lin, 2018). Furthermore, combating or treating oxidative stress is of vital importance since it is related with the origin of other diseases and disorders such as inflammation (de-Lavor et al., 2018; Yahfoufi et al., 2018), cancer (Greenwell and Rahman, 2015; Oyenihi and Smith, 2019) or infections (Martelli and Giacomini, 2018). Fortunately, phytochemicals are natural antioxidants, and present a strong ability to decrease the free radicals that cause oxidative stress. In this sense, ROS production leads to the expression and secretion of transcriptional factors and signal transduction cascades, which, in turn, leads to the expression of pro-inflammatory cytokines (de-Lavor et al., 2018). However, polyphenols including phenolic acids, flavonoids and terpenes, among many others, are able to regulate the expression and secretion by immune cells of these cytokines and, furthermore, decrease the level of enzymes involved in ROS production such as xanthine-oxidase (XOx) and NOx, as well as increase levels of enzymes related with ROS elimination such SOD, CAT, and glutathione peroxidase (GSHPx) (Yahfoufi et al., 2018).

Therefore, phytochemicals are interesting alternatives for the treatment of inflammatory diseases (chronic or not) (de-Lavor et al., 2018; Yahfoufi et al., 2018). On the other hand, as we previously commented, ROS are able to produce DNA damage leading to epigenetic alterations and mutations and cancer, although they can also induce apoptosis. Furthermore, inflammation can lead to cancer development through the activation and secretion of prostaglandins, cytokines, chemokines and nitric oxide (NO) (Karim et al., 2016). In this case, polyphenols, and also alkaloids, decrease cancer by decreasing ROS production. In addition, they are able to delay cancer progression by stimulating apoptosis, autophagy and stopping the cell cycle and cell proliferation, angiogenesis and metastasis (Coa et al., 2013; Greenwell and Rahman, 2015; Oyenihi and Smith, 2019). Finally, according to the review made by Martelli and Giacomini (2018), ROS production can contribute to the appearance of resistant bacteria, and phytochemicals are also a strong tool to combat these problems due their antioxidant and antibacterial activities. In the literature, phytochemicals have demonstrated high *in vitro* antioxidant activity through their radical scavenging and metal chelator activities (Lee et al., 2017a; Amorati and Valgimigli, 2018; Aniya et al., 2018; de-Lavor et al., 2018; Pateiro et al., 2018; Pohl and Lin, 2018; Oyenihi and Smith, 2019).

## Effects of oregano leaf extracts

As regards the effect of oregano extracts on gilthead seabream HK leucocyte viability, the present results show that only the most concentrated ethanolic extract used (1 mg/mL) significantly decreased leucocyte viability since it was toxic for cells, confirming the affirmations made by Martins et al. (2014). Our results agree with three studies where the cytotoxic effect of ethanolic extracts on human peripheral blood cells were studied (Zhamanbayeva et al., 2016), lymphocytes (Arami et al., 2013) and monocytes (Chuan et al., 2018). In all cases, when cells were incubated with ethanolic extracts from aerial parts, no toxic effects were observed at low concentrations (up to 0.2 mg/mL) as occurred in our study, where only the highest concentration tested (1 mg/mL) showed toxicity. However, in the case of oregano essential oil, very low concentrations decreased the viability of human macrophages (Ocaña-Fuentes et al., 2010). These results underline the fact that both the concentration and type of extract are crucial for obtaining positive results.

Furthermore, concentrations up to 0.4 mg/mL of ethanolic extracts showed a protective effect against DNA damage induced by ROS in human lymphocytes (Arami et al., 2013) and against ionizing radiation in mouse bone marrow cells (BMCs) (Habibi et al., 2015), which could be related to their content of phenolic compounds such as flavonoids, terpenes, thymol and carvacrol (Arami et al., 2013), which are rich in hydroxyl (OH) groups and can scavenge the free radicals (Habibi et al., 2015).

As regards the effects on HK leucocyte activities the incubation of leucocytes with both tested extracts increased their phagocytic ability, with the exception of 1 mg/mL ethanolic extracts, which induced a decrease in this activity and also in the phagocytic capacity, probably due to a decrease in the number of viable leucocytes, as previously indicated. However, the stimulant properties of the extracts disappeared at high concentrations. More specifically, a decrease in phagocytic capacity was detected when leucocytes were incubated with 0.5 or 1 mg/mL aqueous extracts. In general, a positive effect on the phagocytic activity of leucocytes was recorded after incubation of the cells with plant extracts. More studies are needed to understand which substances present in the extracts may be responsible for these results.

In the case of the respiratory burst, incubation with oregano extracts had a depressing effect. The decrease of this activity could be related to the presence of antioxidant compounds in both extracts, which can eliminate ROS produced in respiratory burst activity, although a better understanding of the molecular phenomena involved in the regulation of NOx in fish could help us to understand how the respiratory burst, which is also a crucial effector mechanism of fish immunity, is regulated. Besides the above, no significant variations were detected in the peroxidase activity of leucocytes after incubation with oregano extracts. All these results suggest that the effect of the extracts depended on the leucocyte activity tested and the type and concentration of the extract used. The results also indicate the optimum range of concentrations of aqueous and ethanolic extracts of oregano that can be used as immunostimulants without risk of toxicity for cells when used in *in vitro* studies.

Furthermore, oregano presents a particular composition of fatty acids (Viuda-Martos et al., 2010; Chishti et al., 2013; Martins et al., 2014; Han et al., 2017) (principally $\alpha$-linolenic, linoleic and palmitic acids) (Han et al., 2017), which could be responsible for immunostimulant effects on gilthead seabream leucocytes since they are very important for normal growth and health maintenance, improving the immune status and preventing diseases (Fritsche, 2006). Furthermore, this plant contains high levels of vitamins, including A, C, E and B-complex, which also present immunostimulant properties and high antioxidant activity (Aranha and Jorge, 2012). In this sense, oregano ethanolic extracts improved the post-thawed quality of bull semen possibly due to an increase in antioxidant enzymes activity and reduction of lipid peroxidation (Kia et al., 2016).

Regarding the effects of oregano extracts on SAF-1 and PLHC-1 cell lines, this is the first study of the *in vitro* effect of oregano extracts on a fish cell line. The incubation of SAF-1 cells with aqueous extracts increased cell viability, while incubation with ethanolic extracts had the opposite effect, decreasing viability, as in the case of HK leucocytes. The present work allowed us to compare the effects of oregano extracts on the viability of a primary culture of HK leucocytes and on a cell line obtained from the same fish species. The results demonstrate that moderate or high concentrations of ethanolic extracts have cytotoxic effects, while aqueous extracts increase SAF-1 cell viability. Therefore, ethanolic extracts at moderate or high concentrations were toxic for both cell types (leucocytes and SAF-1 cells). Regarding the increase of mitosis and viability of the SAF-1 cells observed after incubation

with the aqueous extracts of oregano, it might be interesting to study whether the dietary administration of such extracts or of the whole leaves could be used to treat wounds or the scarring of fish tissues after injury, taking into account that the SAF-1 cells were obtained from fin (Béjar et al., 1997).

As regards the cytotoxic activity of oregano extracts against the PLHC-1 tumour cell line, while only the highest concentration of aqueous extracts showed cytotoxic activity, low-moderate to high concentrations of ethanolic extracts also decreased cells viability Our results agree with those of similar studies, where both aqueous and ethanolic extracts from leaves showed dose-dependent cytotoxic activity against different human tumour cell lines, including cervical-uterine carcinoma, rhabdo-myo sarcoma, myeloblastic leukemia,breast, colon and lung adenocarcinoma cell lines (Al-Kalaldeh et al., 2010; Dhahir et al., 2012; Alshami et al., 2013; Koldas et al., 2015; Coccimiglio et al., 2016; Zhamanbayeva et al., 2016; Hassanzadeh-kiabi and Negahdari, 2017; Nile et al., 2017). Therefore, phenolic compounds including flavonoids (such as apigenin, luteolin and quercetin), tannins (Dahir et al., 2012; Zhang et al., 2014; Marrelli et al., 2015), and especially, terpenoids carvacrol and thymol are the main compounds responsible for this activity (Kozlowska et al., 2015; Coccimiglio et al., 2016; García-Beltrán and Esteban, 2016; Baranauskaite et al., 2017), and, it is known that, in general, they are more abundant in ethanolic extracts (Kozlowska et al., 2015; Coccimiglio et al., 2016), which could be due to the dielectric constant of ethanol as organic solvent. In fact, oregano ethanolic extracts showed a very high content of phenolic compounds, which was correlated with powerful antitumor activity (Nile et al., 2017).

However, although oregano has previously shown bactericidal activity, our knowledge about the bactericidal activity of oregano as a natural treatment for fish bacterial pathogens is limited. It is important to emphasise that in the present study, both extracts showed bactericidal activity in a dose-dependent manner against the three fish pathogens tested. Again, our results demonstrated that ethanolic extracts showed higher bactericidal activity than aqueous extracts. Low-moderate and high concentrations of ethanolic extracts showed bactericidal activity against the three pathogens tested, while only the highest concentration of the aqueous extract showed significant activity against *P. damselae*. The present results agree with other studies that demonstrated that ethanolic extracts have stronger bactericidal activity than aqueous ones (Teixeira et al., 2013; Karaboduk et al., 2014; Coccimiglio et al., 2016; Klūga et al., 2017).

The different bactericidal activity observed in this study between aqueous and ethanolic extracts may also be due to the different components extracted in each case. As in the case of antitumor activity, flavonoids, phenolic acids and specially the terpenoids thymol and carvacrol present bactericidal activity (Goncearіuc et al., 2015; Coccimiglio et al., 2016; Sakkas and Papadopoulou, 2017) and are much more abundant in ethanolic than in aqueous extracts. When ethanol, methanol and hexane extracts from *Ocimum basilicum* were investigated for their *in vitro* antimicrobial properties against 146 microbial organisms including aquaculture pathogens, the hexane extract showed a stronger and broader spectrum of antibacterial activity (Adigüzel et al., 2005). This suggests that hexane extracts of oregano might exhibit even higher bactericidal activity than the activities obtained in the present work.

Curiously, in the case of *V. harveyi*, aqueous extracts did not show any bactericidal activity against this bacterium, and the number of viable bacteria increased in a dose-dependent manner. Previous studies indicated that aqueous extracts of oregano had no (Teixeira et al., 2013; Karaboduk et al., 2014) or low (Masood et al., 2007; Ashraf et al., 2011; Teixeira et al., 2013; Martins et al., 2014; Akrayi et al., 2015) bactericidal activity against different human bacteria. In addition, González and Marioli (2010) also showed the low activity of oregano aqueous extracts against *Paenibacillus larvae*. Furthermore, the available results seem to indicate that the temperature is also important for the composition of the obtained extracts and may be a very significant factor in terms of bactericidal activity. In this sense, an aqueous decoction prepared by boiling oregano leaves in sterile distilled water for 15 min resulted in an extract without bactericidal activity (Masood et al., 2007; Saeed and Tariq, 2009). However, when an aqueous infusion was prepared by soaking oregano leaves in sterile distilled water for two days with occasional shaking, the obtained infusion had bactericidal activity against many bacteria including Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa, Proteus mirabilis, Aeromonas hydrophila, Serratia marcescens, Shigella dysenteriae, Klebsiella sp., Salmonella sp., Citrobacter sp.* and *Flavobacterium sp.* and Gram-positive *Staphylococcus sp., Enterobacter aerogenes, Micrococcus sp.* and *Bacillus sp.* (Masood et al., 2007; Saeed and Tariq, 2009; Martins et al., 2014).

Finally, their antioxidant activity is probably the most important activity of plants if they are to be used in aquaculture due its capacity to prevent lipid peroxidation (Chishti et

al., 2013; Kia et al., 2016; Sakkas and Papadopoulou, 2017). Furthermore, oregano is one of the *Lamiaceae* plant species with the highest content of phenolic compounds with antioxidant activity (Yan et al., 2016; Nile et al., 2017). Oregano has an antioxidant capacity (Zheng and Wang, 2001; Kaurinovic et al., 2011; Habibi et al., 2015) that allows it to scavenge free radicals, and thus inhibit the oxidative mechanisms that lead to degenerative diseases. In our study, both tested oregano extracts showed a dose-dependent antioxidant activity, which was particularly evident in the aqueous extracts. There are many studies where aqueous (Ivanova et al., 2005; Kim et al., 2011), ethanolic (Bendini et al., 2002; Arami et al., 2013; Coccimiglio et al., 2016; Baranauskaite et al., 2017; Nile et al., 2017) or both extracts (Teixeira et al., 2013; Karaboduk et al., 2014; Martins et al., 2014; Koldas et al., 2015) showed antioxidant activity, depending on the technique used.

As to the phenolic compounds responsible for this activity, although the terpenoids thymol and carvacrol are important (Arami et al., 2013; Chishti et al., 2013; Coccimiglio et al., 2016; Baranauskaite et al., 2017; Davidenco et al., 2017; Sakkas and Papadopoulou, 2017), the antioxidant activity of oregano extracts is attributable principally to the flavonoids, phenolic acids and vitamins present in them (Kaurinovic et al., 2011; Kim et al., 2011; Aramis et al., 2013; Teixeira et al., 2013; Karaboduk et al., 2014; Zhang et al., 2014; Koldas et al., 2015), where rosmarinic acid, ursolic acid, caffeic acid, quercetin, kaempferol and origanol A and B could be of special importance (Kaurinovic et al., 2011; Habibi et al., 2015; Kia et al., 2016; Baranauskaite et al., 2017). In this sense, aqueous extracts present a higher content of flavonoids and phenolic acids than ethanol extracts (Kaurinovic et al., 2011; Karaboduk et al., 2014; Habibi et al., 2015).

## *Effects of date palm seed extracts*

Despite the many reports on the nutritional value of dates, many other potentialities of the fruits remain to be explored. Between 10% and 15% of date fruit corresponds to seed, which is considered a by-product and waste (Habib and Ibrahim, 2008; Hossain et al., 2014; Sirisena et al., 2017). As to their composition, a study performed using 18 different varieties of DPS established that protein, fat, ash, carbohydrate and fiber levels were 4.81 - 5.83%, 5.71 - 7.92%, 0.82 - 1.14%, 2.43 - 4.65% and 67.56 - 74.20%, respectively (Habib and Ibrahim, 2008; Ardekani et al., 2010; Hossain et al., 2014). For this work, we used the variety Deglet nour, which is one of the most appreciated varieties of date palms. Results from the fibre and chemical composition of the DPS used in the present study agree with results obtained in other date palm varieties (Lee et al., 2017b; Nehdi et al 2018) showing that hemicelluloses are the most abundant fibres (26.5 + 0.001% db) followed by celluloses (24.1 + 0.1% db) and lignin (21.2 + 0.001% db). Furthermore, fats were more abundant (11.2 + 0.1% db) than proteins (6.2 + 0.01% db). The mean oil content of the seeds was about 7% and oleic acid was the main fatty acid (48.67%), followed by lauric acid (17.26%), stearic acid (10.74%), palmitic acid (9.88%), and linolenic acid (8.13%). These results confirm that DPS have great nutritional value, which makes them valid ingredients for different food applications (Nehdi et al., 2018). The overall results for their composition are similar to those obtained by Hossain et al. (2014), which showed that Deglet Nour variety had 83.1% of carbohydrates, 10.1% of fat and 5.6% of protein. Furthermore, other interesting properties of DPS oils are that they have high thermal and oxidative stabilities, which are very important properties when looking for ingredients to be incorporated into feed, since they guarantee their stability throughout the fabrication processes (Nehdi et al., 2018).

Regarding proteins, date fruits are well known to be very nutritious. Nevertheless, the protein contents of the fruit, particularly the seed and flesh, are still very poorly studied, largely due to their difficult physical characteristics (Lee et al., 2017b). A variety of protein extraction methods have been used and compared to identify the proteins and to perform proteomic analysis. The phenol extraction method obtained the highest protein yields for both seed (8.26 mg/g) and flesh (1.57 mg/g). Liquid chromatography-tandem mass spectrometry analysis showed that about 50-64% of the extracted proteins could be identified as having known functions, including those involved in glycolysis, the Krebs cycle, storage

and also defence (Lee et al., 2017b). Although more studies are needed to properly identify the proteins present in seed extracts, the scarce available results seem to indicate that palm fruit seeds could have a high nutritional value and represent a considerable potential for the development of functional foods for fish.

The present results indicate that DPS aqueous extracts (mainly when used at 0.5 mg/mL) significantly increased the viability of gilthead seabream HK leucocytes. By contrast, a decrease in HK leucocyte viability was recorded after incubation with 0.5 or 1 mg/mL ethanolic extracts, pointing to a toxic effect on such cells. A possible explanation for these results could be that the lipids present in ethanolic extracts would affect and alter the gilthead the cell membrane of seabream leucocytes and also, cell viability, as has been demonstrated in human monocytes treated *in vitro* with lipid emulsions. Treated monocytes differentially incorporated fatty acids after lipid emulsion challenge and subsequently disruption of the membrane lipid order was detected (Boisramé-Helms et al., 2014). The assumption that ethanolic extracts of DPS are supposed to contain higher levels of lipids than aqueous one seems to corroborate this hypothesis although future studies along these lines are needed.

However, the present *in vitro* results indicate that, in terms of gilthead seabream HK leucocyte phagocytosis, respiratory burst and peroxidase activities, no immunostimulant effects could be attributed to DPS extracts, whether aqueous or ethanolic, at any of the assayed doses or incubation times tested in the present work. Our results for DPS aqueous extracts are very similar to those obtained by Mahaldashtian et al. (2015, 2016), which showed that incubation with date palm pollen aqueous extracts had no effect on the proliferation rate or even most concentrated extract increased colony formation of isolated spermatogonial stem cells and Sertoli cells. The antioxidant compounds such as flavonoids, alkaloids and carotenoids present in date aqueous extract reduced cell apoptosis, while oestrogenic compounds promoted cell division and growth (Mahaldashtian et al., 2015, 2016), possibly due to vitamin A (in the form of retinoic acid), which can induce cell differentiation (Mahaldashtian et al., 2016). Future studies would determine whether similar or opposite results to the present ones could be obtained when DPS extracts are administered *in vivo* to fish. In addition, two studies have shown the protective effect against oxidative stress induced by $H_2O_2$ in human melanocytes and keratinocytes pre-incubated with DPS oil, where an increase in the viability of pre-treated cells after incubation with $H_2O_2$ was

observed due an increase in antioxidant enzymes GSHPx, SOD and CAT and a decrease in lipid peroxidation (Yasin et al., 2015).

Interestingly, our study is also the first to demonstrate the cytotoxic effect of DPS extracts on fish cell lines. In the case of the SAF-1 cells, viability was increased or not affected by incubation with aqueous or ethanolic extracts, respectively, contrary to the results obtained by Dhaouadi et al. (2010), who showed that an aqueous-acetone polyphenolic extract of date palm syrup decreased the viability of mouse fibroblasts. Dates are used in traditional medicine as a remedy to treat breast cancer of women in Palestine thanks to their properties to delay cancer progression related with angiogenesis (Taleb et al., 2016). We confirmed this antitumor activity, where highly cytotoxic effects of DPS extracts were observed against PLHC-1 tumour cells, especially in the case of ethanolic extracts. Our results confirm previous data obtained in human tumour cell lines including aqueous-acetone polyphenolic extract against neuroblastoma (Dhaouadi et al., 2010), ethanolic and methanolic extracts against breast adenocarcinoma (Khan et al., 2016; El-Abed et al., 2018), polyphenol-rich and acetone and chloroform extracts against human colorectal adenocarcinoma (Eid et al., 2014; Sundar et al., 2017), and acetone extract against cervical-uterine carcinoma (Kchaou et al., 2016). However, it is necessary underline that we used a much lower concentrations than other studies (Kchaou et al., 2016; Khan et al., 2016; El-Abed et al., 2018), and that the Deglet Nour variety is the most active (Kchaou et al., 2016), its activity being due to the inhibition of cell proliferation through cell cycle arrest and apoptosis rather than the result of cytotoxic activity (Khan et al., 2016; El-Abed et al., 2018). Phenolic acids such as coumaric, gallic, vanillic acid, cinnamic acid, caffeic acid and its derivates (Dhaouadi et al., 2010; Kchaou et al., 2016), sulfated flavonoids linked to the sugars of flavonol glycosides (Yasin et al., 2015; Kchaou et al., 2016; El-Abed et al., 2018), tannins and carotenoids (Kchaou et al., 2016) could be the phytochemicals mainly responsible for this activity. Furthermore, β-glucans are possibly very important in the antitumor activity of dates, as various *in vivo* studies have shown (Rahmanil et al., 2014; Yasin et al., 2015).

It is well known date palm plays an important role in the prevention and treatment of infections by bacteria (Rahmanil et al., 2014) and DPS represent an important source of antimicrobial compounds (Bentrad et al., 2017b). However, regarding the bactericidal activity studied herein, no activity of DPS aqueous extracts was observed against any

bacteria, while only the highest concentration of ethanolic extracts showed activity against *V. anguillarum*. There have been several studies where the antibacterial activity of DPS, fruit, bark or syrup has been analysed and different growth or maturation states exhibited different antibacterial properties, being therefore extremely important to obtain a good antibacterial activity (Saleh and Otaibi, 2013; Bentrad et al., 2017b).

Concerning studies using DPS, higher concentrations of aqueous, ethanolic, methanolic, aqueous-methanolic, chloroform and acetone extracts have shown dose-dependent activity against Gram-negative *E. coli*, *P. aeruginosa*, *P. fluorescens*, *Klebsiella pneumoniae*, *Salmonella typhi*, *S. paratyphi* and *Proteus vulgaris* and Gram-positive *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus subtilis* and *B. cereus* (Mossa et al., 1986; Ammar et al., 2009; Saddiq and Bawazir, 2010; Aamir et al., 2013; Saleh, 2016). Again, the solvent used for extraction is extremely important to obtain good activity (Saleh, 2016; Sundar et al., 2017) since aqueous extract showed low or no activity (Saleh, 2016), as did a chloroform extract in the study of Sundar et al. (2017). Furthermore, organic and aqueous fractions obtained using diethyl ether after extraction with hydrochloric acid of DPS and pollen, as well as lipophilic extracts and pure substances such as catechol, hydroxyquinone, vanillin, benzoic, gallic, resorcylic, syringic cinnamic and ferulic acids also showed antibacterial activity against multi-resistant *E. coli*, *P. aeruginosa*, *E. faecalis*, *S. aureus* and *S. aureus* multi-resistant (Bentrad et al. 2017a, b), the Deglet Nour variety having the highest antibacterial activity of all the tested varieties (Zehra et al., 2015; Bentrad et al., 2017b).

The antibacterial activity of date fruits (Zehra et al., 2015; Kchaou et al., 2016; Samad et al., 2016), barks (Zehra et al., 2015; Ravishanker and Raut, 2016) or syrup extracts (Ravishanker and Raut, 2016) has also been studied and, and, while the results are very diverse, they all seem to have less activity than DPS.

Due to the differences between the different solvents used, it is established that very polar molecules are obtained with water, while ethanol allows both polar and non-polar substances to be dissolved, and chloroform can dissolve compounds that range from moderately polar to moderately non-polar (Saleh, 2016). Furthermore, chloroform extract has aromatic molecules while acetone extract presents aliphatic molecules (Sundar et al., 2017). In addition, as in the case of the solvent used, it is important to stress that the

maturation stage is crucial for this activity, as in Saleh and Otaibi (2013), who showed that aqueous, ethanol and ether extracts of three different varieties of date fruit in three different stages exert different levels of antibacterial activity. Furthermore, although all extracts showed similar activity, the ethanol extract contained the highest amount of phenolic compounds (carotenoids and anthocyanins) than the other extracts (Saleh and Otaibi, 2013). Therefore, the antibacterial activity depend on the variety, the solvent used and the state of maturity in which the fruit is found. Furthermore regarding the concentrations used in other studies, which were much higher than in our study, it is possible stronger antibacterial activity would be obtained by increasing the concentration of our extracts. These results could be useful to the nutraceutical and pharmaceutical industries for the development of natural compound-based products.

As phytochemicals responsible for the antibacterial activity, the phenolic acids (coumaric, ferulic, gallic, vanillic, syringic, cinnamic, sinapic and caffeic acids and derivates) (Dhaouadi et al., 2010; Kchaou et al., 2016) and flavonoids (flavones, flavonols, flavanones, anthocyanidins and flavans, among them isoquercetin, luteolin, acacetin, apigenin, genistein, catechin and rutin) (Ammar et al., 2009; Kchaou et al., 2016) could be mainly responsible. Furthermore, it has been suggested that, together with phenolic acids and flavonoids, coumarins and tannins present strong activity (Kchaou et al., 2016), as do terpenes like triterpene β-amiyrin acetate (Ravishanker and Raut, 2016). As to the method of action, it has been suggested that there are interactions between phytochemicals and the bacteria wall, resulting in inhibition of microbial growth (Kchaou et al., 2016). This activity could be due to the action of compounds on the membrane, which would allow the entry or exit of certain constituents and proteins to change (Saddiq and Bawazir, 2010).

Finally, it is important to highlight another capacity which may make DPS a good candidate for use in aquaculture, since digested date fruit extract and its polyphenol-rich extract are able to significantly increase the growth of gut microbiota, especially the whole extract, due to the presence of polyphenols and fibers (Eid et al., 2014).

Dates are very rich in phenolics, both in quality and quantity (Yasin et al., 2015; Samad et al., 2016), which has been suggested as being due to the extreme temperatures and climate where this plant grows in comparison to others (Yasin et al., 2015), opening up many fields of investigation in terms of new potential uses (Samad et al., 2016). In this sense, dates

constitute a very good source of antioxidants and some studies have established them to have the highest concentration of polyphenols among dried fruits (Rahmanil et al., 2014). For this reason, dates have strong antioxidant activity (Samad et al., 2016), which was evaluated in our study and although both tested extracts showed powerful dose-dependent activity, aqueous extracts had stronger antioxidant activity than ethanolic extracts. Indeed, several studies have demonstrated that extracts of DPS present antioxidant activity. For example, aqueous, methanol, acetone, aqueous-methanol, aqueous-acetone, DMSO, and aqueous-methanol-acetone-formic acid extracts of 14 date varieties showed powerful antioxidant ABTS, 2,2-diphenyl-1-picrylhydrazyl (DPPH [hydrogen donating ability]) and ferric-reducing ability in plasma (FRAP), reduced thiobarbituric acid reactive substances (TBARS) activity and had considerable phenolic content (Ardekani et al., 2010; Thouri et al., 2017), which was especially higher in aqueous extracts. Furthermore, DPS petroleum ether (Mohamed and Al-Okbi, 2005) and hydroacetone extract (Ahmed et al., 2015) also had activity. Finally, DPS oil exhibited very weak DPPH activity (Boukouada et al., 2014).

For their part, it is quite probably date fruit extracts have more antioxidant power than DPS. In this sense, aqueous, aqueous-ethanolic and aqueous-acetone extracts showed free radical scavenging activity, lipid peroxidation and protein oxidation (Vayalil, 2002; Mohamed and Al-Okbi, 2005; Zhang et al., 2013; El-Arem et al., 2014; Esteban et al., 2014; Zehra et al., 2015; El-Abed et al., 2018), as did methanolic, ethyl acetate and petroleum ether extracts (Mohamed and Al-Okbi, 2005; Biglari et al., 2008; Zhang et al., 2013). Furthermore, aqueous-acetone extract from date bark (Zehra et al., 2015) and date syrup (Dhaouadi et al., 2010) also had excellent antioxidant activity.

It is important to highlight that extracts from fresh fruit present higher activity than extracts from dried fruit, making them unstable (Vayalil, 2012). Also, in the case of antioxidant activity, it the maturation stage of date fruits seems very important. For example, in the case on Deglet Nour variety, the besser stage contains important amounts of polyphenolic compounds and therefore higher antioxidant capacity (El-Arem et al., 2014). Finally, as regards the solvent used (Ardekani et al., 2010; Hossain et al., 2014; Thouri et al., 2017), water is the most polar and the best for obtaining polyphenol compounds (Thouri et al., 2017). Although Al-Farsi and Lee (2008) optimized the extraction of total phenols using a water-acetone mixture as a solvent, it has also been demonstrated that most antioxidant components in dates are water-soluble (Esteban et al., 2014). This was

demonstrated in an experiment by Zhang et al. (2013), where different solvents were used to extract different compounds, and methanol and water extracts provided glycosides, glucopyranosides, β-sitosterol and proteins, which presented much higher activity than hexane and ethyl acetate fractions (Zhang et al., 2013). Phenolic acids such as coumaric, gallic, ferulic vanillic, cinnamic, chlorogenic, protocatechuic, syringic, 3-hydroxybenzoic, phenylacetic, and caffeic acids and derivates (Dhaouadi et al., 2010; El-Arem et al., 2014), flavonoids including proanthocyanidins (Ahmed et al., 2015) and catechin (El Arem et al., 2014), carotenoids (Yassin et al., 2015), tocopherols (especially α- and γ-tocopherol) (Mohamed and Al-Okbi, 2005; Boukouada et al., 2014) and unsaturated fatty acids (Boukouada et al., 2014), especially oleic acid (Ardekani et al., 2010) could be involved in this activity.

## Effects of purslane leaf and stem extracts

Effects of purslane extracts on gilthead seabream HK leucocytes showed that only the most concentrated ethanolic extract used (1 mg/mL) significantly decreased leucocyte viability, showing its toxic effect for cells. The toxicity of purslane aqueous and ethanolic extracts has been tested in human lymphocytes (Behravan et al., 2011; Askari et al., 2016). Purslane polysaccharides increased rat spleen T and B lymphocyte proliferation in a dose-dependent manner (Chen et al., 2009). According to Askari et al. (2016), polysaccharides present in purslane extracts could be responsible for the cytotoxicity that induces apoptosis and sub-G1 phase cell cycle arrest, although their concentrations are presumably of great importance since the opposite results were obtained using only polysaccharides (Chen et al., 2009).

Regarding phagocytic activity, the results demonstrated that the incubation of leucocytes with aqueous extracts increased their phagocytic ability in a dose-dependent manner, and did not affect the phagocytic capacity, while incubation with ethanolic extracts did not affect phagocytic ability or capacity. These results suggest that components present in the aqueous extracts have the ability to activate phagocytosis, and this could be due to several processes, such as the stimulation of the expression of cellular receptors that recognize PAMPs, the production of cytokines, antibodies or increasing the emission of pseudopods. Therefore, aqueous extracts of purslane used at a suitable concentration enhance the immune response by activating phagocytosis activity and can help fish to combat infections by removing pathogens, although further studies are needed to know what substances are responsible for this effect.

However, the results of respiratory burst activity showed that while purslane aqueous extracts did not have any effect on this activity, depressing effects were observed after being incubated with high concentrations of ethanolic extracts (0.5 or 1 mg/mL). Besides, no variations were detected in the peroxidase activity of leucocytes after being incubated with purslane extracts. These results concerning respiratory burst and peroxidase activities could be due to the presence of antioxidant compounds in both the aqueous and ethanol extracts, which may be able to eliminate the ROS produced by activation of immune cells. This might mean that purslane extracts could activate the phagocytosis activity of immune system cells and, at the same time, help to eliminate the ROS that can cause damage and oxidative stress

to fish. Therefore, aqueous extracts showed better immunostimulant results than ethanolic extracts, the phagocytic ability being increased by moderated-high concentrations of aqueous extracts, which could be related to the vitamin C and B-complex present in them.

The literature mentions that incubation with purslane ethanolic extracts has an immunostimulatory effect by increasing the human T helper 1 - T regulators/T helper 2 lymphocytes ratio (Th1-Treg/Th2), which could be due to the presence of alpha-linolenic acid (Askari et al., 2016), tannins and alkaloids (Catap et al., 2018).

Furthermore, when the protective effect of purslane extract was studied by Behravan et al. (2011), following the incubation of human lymphocytes with $H_2O_2$, aqueous extracts decreased DNA damage and allowed cell proliferation, while 80% ethanolic extracts had no effect. Finally, purslane polysaccharides (glucose and galactose) also decreased RBC hemolysis induced by $H_2O_2$ in a dose-dependent manner (Chen et al., 2009). According to Beharavan et al. (2011), the protective effect of purslane extracts is related to omega-3 fatty acids, gallotannins, flavonoids (kaempferol, quercetin, apigenin), vitamins C and E and glutathione (Behravan et al., 2011), although polysaccharides have also demonstrated their importance in protection (Chen et al., 2009).

To the best of our knowledge, this is the first study of the *in vitro* effects of purslane extracts on a fish cell line. The incubation of SAF-1 cells with aqueous extracts did not have any effect on cell viability, while the incubation with moderate-high concentrations of ethanolic extracts decreased the viability of cells, as occurred with leucocytes. Therefore, the effects on HK leucocyte and SAF-1 cells demonstrated that aqueous extracts did not affect viability of any cells, while moderated-high concentrations of ethanolic extracts are toxic for both kind of cell, decreasing their viability in a significant manner. The absence of any effect in our study of purslane aqueous extract on cell viability confirms the results obtained by Mohammad et al. (2011) with rat embryo fibroblasts cells. Our results also corroborate the results obtained by Soliman et al. (2017), where low concentrations of ethanolic extracts from aerial parts of purslane did not affect human erythrocytes. Furthermore, aqueous and ethanolic extracts from aerial parts showed the cytoprotective effects of RBCs against oxidative stress (Karimi et al., 2011). Omega-3 fatty acids, flavonoids and vitamins C and E could be mainly responsible for this cytoprotective effect on cells, especially flavonoids and vitamin C.

As regards PLHC-1 cells, incubation with purslane aqueous extract did not affect cell viability significantly, while incubation with ethanolic extracts led to a significant decrease in cell viability at moderate-high concentrations. The cytotoxic activity of purslane aqueous and ethanolic extracts from whole plant and seeds has been previously been recorded ain the case of murine mammary adenocarcinoma (Mohammad et al., 2011) as has the toxicity of ethanolic extracts against human colorectal, breast and hepatocellular adenocarcinoma cells (Farshori et al., 2014; Marrelli et al., 2015; Jin et al., 2017; Nile et al., 2017). Furthermore, purslane oil has also revealed cytotoxic activity against human lung epithelial adenocarcinoma cells (Al-Sheddi et al., 2015), as have purslane water-soluble polysaccharides and their sulfated derivates, which inhibited mouse cervical carcinoma and human cervical-uterine adenocarcinoma cells proliferation (Chen et al., 2010; Zhao et al., 2013).

Therefore, ethanolic extracts show better cytotoxic activity against tumour cells than aqueous extracts, which could be due to the inhibition of the production of the endogenous factors required for cell proliferation and differentiation or the induction of apoptosis. In this sense, purslane extracts inhibit tumor cell proliferation through cell cycle arrest, DNA damage and apoptosis (Chen et al., 2010; Zhao et al., 2013; Jin et al., 2017). This effect may be due to the modulation of mRNA and protein expression (Ji et al., 2015). The high content of phenolic compounds present in purslane ethanolic extracts may well be responsible for its cytotoxic activity (Nile et al., 2017), among them, flavonoids such as kaempferol and apigenin, terpenoids (Mohammad et al., 2011; Marrelli et al., 2015; Zhou et al., 2015), alkaloids, tannins and saponins (Mohammad et al., 2011; Zhou et al., 2015).

Although acting as bactericidal is one of the principal activities of purslane (Uddin et al., 2014; Zhou et al., 2015; Syed et al., 2016; Iranshahy et al., 2017), there are no studies about this activity of purslane against fish pathogens. In the present study, both extracts showed dose-dependent bactericidal activity against the three fish pathogens tested. Our results demonstrated that ethanolic extracts show higher bactericidal activity than aqueous extracts. Low, medium and high concentrations of ethanolic extracts show bactericidal activity, while only medium and high concentrations of aqueous extracts do so. These results agree with other studies where aqueous and ethanolic extracts showed strong bactericidal activity against Gram-negative and Gram-positive bacteria (Cho et al., 2008; Ma et al., 2013; Soliman et al., 2017). The stronger effect of ethanolic extracts observed in this study may

have been due to the different components extracted in each case, which would explain their different properties. Again, ethanolic extracts seem to have a greater quantity and concentration of phenolic compounds than aqueous extracts although polysaccharides could play a fundamental role in the antibacterial activity (Zhou et al., 2015).

To finalize, both aqueous and ethanolic purslane extracts showed a dose-dependent antioxidant activity, which was particularly evident in the aqueous extracts. Previous studies have demonstrated this activity in aqueous and ethanolic extracts of purslane (Cho et al., 2008; Taha and Osman, 2015; Nile et al., 2017). To repeat, regarding our results for antioxidant activity and the rest of the activities assessed, they may be attributable to the high content of phenolic compounds in the extracts (Nile et al., 2017), including flavonoids present in the aqueous extract, tannins, alkaloids (Zhou et al., 2015; Catap et al., 2018), phenolic acids and, especially, vitamin C (Uddin et al., 2014; Zhou et al., 2015), which is a water-soluble vitamin with high antioxidant activity. Interestingly, in the study of Silva and Carvalho (2014), stems of purslane showed higher values for antioxidant activity than leaves and flowers, probably due to the total phenolic content.

## *Effects of moringa leaf extracts*

The highest concentration (1 mg/mL) of moringa aqueous extracts significantly decreased HK leucocyte viability, while intermediate concentrations of ethanol extracts (0.1 - 0.5 mg/mL) increased it. As regards leucocyte phagocytic and respiratory burst activities, both, ethanol and, especially, aqueous extracts of low concentration (0.1 mg/mL), decreased both activities. However, the results obtained for peroxidase activity showed that aqueous and ethanol extracts increased it although not in a significant manner.

Studies of the effects of moringa extracts on primary cultures are scarce; however, some of these studies were carried out on leucocytes, for which the results are variable. In this sense, low concentrations of hot-aqueous leaf extracts produced a dose-dependent increase of human lymphocyte viability (Nair and Varalakshmi, 2011), while low concentrations of 70% ethanol leaf extracts and pure compounds (phenolic acids and flavonoids) decreased the viability, respiratory burst activity and chemotaxis of human lymphocytes, macrophages and neutrophils (Vongsak et al., 2013). For their part, 95% ethanol leaf extract combined from moringa and *Centella asiatica* had no cytotoxic effect on human dermal fibroblasts (Hisam et al., 2018). Furthermore, the effect of moringa extracts has been also studied in rat cells. The 95% ethanol leaf extract at low concentrations protected rat neurons against natural death, significantly promoting neuron viability, differentiation, dendritic complexity, axonal development and synaptogenesis, with the B-carotenes (lutein and B-sitosterol), vitamin C, flavonoids, phenolic acids and carotenoids the compounds being responsible for (Hannan et al., 2014). Similar results were obtained in the study carried out by Fernandes et al. (2016) using cold-aqueous leaf extracts, where no effect on rat fibroblasts was observed but the viability of rat bone marrow derived mesenchymal stem cells (BMMSCs) and rat hepatocytes increased after treatment with $CCl_4$.

According to the reviews made by Karim et al. (2016), Khor et al. (2018) and Kou et al. (2018), one of the strongest biological activities and most common uses of moringa is in anticancer treatment. According to these reviews, seeds, barks, roots, stems and, especially, aqueous, ethanol, methanol, dichloromethane, hexane, chloroform, petroleum ether extracts and essential oil from leaves have demonstrated anticancer activity by many different pathways (Anwar et al., 2007; Leone et al., 2015; Karim et al., 2016; Paikra et al., 2017; Alegbeleye, 2018; Falowo et al., 2018; Khor et al., 2018; Kou et al., 2018). In this sense,

there are many *in vitro* studies demonstrating this activity in moringa. Similarly, results obtained in the present study show that both aqueous and ethanol extracts have cytotoxic activity against both SAF-1 and PLHC-1 cell lines. All concentrations tested of aqueous extracts and low-moderate concentrations (0.001 - 0.25 mg/mL) of ethanol extracts significantly reduced SAF-1 cell viability, while moderate-high concentrations (0.5 - 1 mg/mL) of ethanol extracts increased it significantly. For its part, moderate-high concentrations (0.25 - 1 mg/mL) aqueous extracts significantly decreased PLHC-1 cell viability, while ethanol extracts had no effect against this tumor cell line.

The effects of moringa extracts on non-tumor cell lines has also been analysed and their cytotoxic activity observed, corroborating the results obtained in our study. Aqueous leaf extracts decreased the viability of African green monkey kidney cells at low concentrations (Jung, 2014) and embryonic cells of human kidney (Madi et al., 2016). Furthermore, in the study of Jaafaru et al. (2018), low-moderate concentrations of a glucomoringin-isothiocyanate rich extract decreased murine fibroblast cell viability. For its part, in the study of Zhou et al. (2018), 70% ethanol stem extract did not affect human skin keratinocytes viability. Therefore, although moringa extracts can also affect normal cells, they are normally less affected than tumor cells, so the compounds present in moringa can protect normal cells against extreme cytotoxicity (Jung, 2014; Karim et al., 2016).

The literature cites how both, aqueous and ethanolic extracts present strong cytotoxic activity against human tumor cell lines, although there are no studies using fish cells, this being the first such study to analyze the effect of moringa extracts on fish cells. In this sense, both moringa extracts showed cytotoxic activity by decreasing the viability of many tumour cells (Khalafalla et al., 2010; Pamok et al., 2010; Jung, 2014; Diab et al., 2015; Madi et al., 2016). There are several mechanisms whereby moringa exerts antitumor activity and inhibits cell proliferation, including cell cycle arrest (Berkovich et al., 2013; Jung, 2014; Al-Asmari et al., 2015; Diab et al., 2015; Karim et al., 2016; Kou et al., 2018), the up-regulation of cell apoptosis (Nair and Varalakshmi, 2011; Sreelatha et al., 2011; Diab et al., 2015; Karim et al., 2016; Madi et al., 2016; Jaafaru et al., 2018; Kou et al., 2018), suppressing angiogenesis and metastasis (Berkovich et al., 2013; Karim et al., 2016; Kou et al., 2018), increasing ROS and DNA fragmentation and decreasing antioxidant enzymes production (Nair and Varalakshmi, 2011; Sreelatha et al., 2011; Jung, 2014; Diab et al., 2015; Madi et al., 2016).

Furthermore, phenolic acids, flavonoids, vitamins, and essential fatty acids may be responsible for the enhanced proliferative effect on normal cells (Fernandes et al., 2016). However, although the final activity is due to the mix of all the above, some with a synergistic effect (Berkovich et al., 2013; Diab et al., 2015), glucosinolates (glucomoringin) and their derivate isothiocyanates (4[α-L-rhamnosyloxy]-benzyl isothiocyanate) and thiocarbamates (O-Ethyl4-(α-L-rhamnosyloxy)-benzyl carbamate, niazimicin and niaziminin), and b-sitosterol-glucopyranoside seem to be mainly responsible for anticancer activity (reviewed by Anwar et al., 2007; Tiloke et al., 2013; Leone et al., 2015; Karim et al., 2016; Paikra et al., 2017; Alegbeleye, 2018; Falowo et al., 2018; Khor et al., 2018; Kou et al., 2018; Tiloke et al., 2018). In addition, Tiloke et al. (2013) and Jung (2014) reported that the absence of glycosidic compounds results in a less pronounced antitumor effect. Furthermore, it seems that compounds with sugar moiety, aromatic rings and long chain hydrocarbons have strong anticancer activity (Tiloke et al., 2018). Due the high polarity of glucosinolate hydrolysis products, they are easily dissolved in ethanol (Khalafalla et al., 2010), and, especially, in water solutions, which explains why they are present in higher concentrations in aqueous extracts (Jaafaru et al., 2018; Tiloke et al., 2018).

According to the reviews made by Wang et al. (2016), Paikra et al. (2017) and Kou et al. (2018), leaves, flowers, roots, stem, pod, bark and seed aqueous, ethanol, methanol hexane, ethyl acetate and chloroform extracts have strong antibacterial activity against Gram-negative and Gram-positive bacteria. Our results showed that moringa extracts, both, aqueous and ethanolic, showed activity against *V. anguillarum* and *P. damselae* subsp. *piscicida* but not against *V. harveyi*. In the literature, the both aqueous and ethanolic extracts were seen to have antibacterial activity, with the exception of the studies carried out by Doughari et al. (2007) and Dholvitayakhun et al. (2012), who pointed out that aqueous or ethanolic leaf extracts generally exhibit little or no antimicrobial activity. For its part, aqueous extracts from leaves (Peixoto et al., 2011; Muhammad et al., 2016) or seeds (Caceres et al., 1991), as well as 50% ethanolic extract from leaves (Peixoto et al., 2011) showed activity against some Gram-negative *E.coli*, *P. aeruginosa* and *Aeromonas caviae* and Gram-positive *S. aureus* and *E. faecalis*, but not against Gram-negative *Shigella flexneri* and *Salmonella enteritidis* and Gram-positive *S.* pyogenes, the extraction temperature being very important (highest activity at 56ºC or even less) (Caceres et al., 1991). Therefore, the compounds that are responsible for the antibacterial activity must be sensitive to high temperatures.

As regards the antibacterial activity of ethanolic extracts, Pal et al. (1995), Nikkon et al. (2003) and Elgamily et al. (2016) showed that ethanol extract from leaves had bactericidal activity. In addition, compounds isolated from moringa also showed antibacterial activity, as in the study of Nikkon et al. (2003), who showed that deoxy-niazimicine had more activity than crude extracts, as well as water soluble constituents of seeds (Miller et al., 2017).

Although the antibacterial activity of moringa against *Vibrio sp.* has been studied, no studies have been made with *V. harveyi*, *V. anguillarum* or *P. damselae* subsp. *Piscicida*, although extracts from leaves and pods possessed higher activity than extracts from stem and seeds against *V. cholera*, *V. vulnificus* and *V. mimicus* isolated from aquatic animals and hatchery water, which showed low activity (Brilhante et al., 2015). Furthermore, very low concentration of ethanol seed extract showed activity against *V. cholera*, *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. navarrensis*, *V. brasiliensis*, *V. xuii*, *V. corallilyticus*, *V. neptunis* and *V. diazotrophicus* (Costa et al., 2017), while very high concentrations of aqueous and, especially, ethanolic extracts from seeds showed activity against *V. cholera* (Vieira et al., 2010). Finally, methanol and hexane seed extracts also presented activity against *V. cholera* (Atieno et al., 2011).

As to the phytochemicals that present antibacterial activity, bioactive compounds are highly polar and so soluble in polar solvents, such as ethanol and, especially, water, and they are easily dissolved at high temperature (Dholvitayakhun et al., 2012). In this sense, although most studies revealed that, as in the case of anticancer activity, flavonoids (quercetin, kaempferol and rutin), alkaloids, saponins, phenolic acids, terpenoids (especially triterpenes), sterols, antrhaquinones and coumarins have antibacterial activity (Doughari et al., 2007; Dholvitayakhun et al., 2012; Elgamily et al., 2016; Muhammad et al., 2016; Wang et al., 2016b), water-soluble tannins (Surendra et al., 2016) and glucosinolates and their derived isothiocyanates (pterygospermin and 4-α-L-rhamnosyloxy benzyl isothiocyanate), thiocarbamates (aglycone of deoxy-niazimicine, benzyl thiocarbamate, niazirine or benzylnitrile, benzyl carbamate and benzyl thiocarboxamide) are the main compounds responsible for antibacterial activity (Jahn et al., 1986; Caceres et al., 1991; Anwar et al., 2007; Padla et al., 2012; Wang et al., 2016b; Costa et al., 2017; Arévalo-Híjar et al., 2018). These compounds can pass through cell membranes easily due to the presence of ethyl group (Wang et al., 2016b), inhibit essential cellular membrane enzymes (Arévalo-Híjar et al., 2018) and the synthesis of PG (Dzotam et al., 2016), and generate negatively charged free

radical compounds, which disrupt the bacteria membrane (Surendra et al., 2016). Finally, water-soluble lectins (antimicrobial peptides) and flocculating cationic proteins from seeds can also damage the cell membrane, affecting cell permeability, growth and survival (Ferreira et al., 2011; Moura et al., 2015).

Finally, antioxidant activity is possibly the most powerful activity of moringa due to the presence of a great quantity of antioxidant phytochemicals, which give it a wide range of medicinal and therapeutic properties, mitigating oxidative stress by scavenging free radicals (Paikra et al., 2017; Kou et al., 2018) and metal chelating activities (Valdez-Solana et al., 2015). In this sense, it has been established that the total phenolic and flavonoid content of moringa leaves and flowers is twice that found in other vegetables (Pakade et al., 2013). Furthermore, antioxidant activity of moringa has been reported to be higher than the synthetic antioxidant counterparts such as butylated hydroxytoluene (BHT), rutin and ascorbic acid (Falowo et al., 2018). In our study, both aqueous and ethanolic extracts had a very high and dose-dependent antioxidant activity, which was a slightly higher in aqueous extracts.

In the literature, aqueous and 50% - 100% ethanolic leaf extracts have been shown to have ABTS, DPPH, FRAP, inhibition of TBARS, reducing power activity and inhibition of lipid peroxidation and radical and anion scavenging activity (Chumark et al., 2008; Khalafalla et al., 2010; Sreelatha and Padma, 2011; Moyo et al., 2012; Aa et al., 2017). For their part, 50-100% methanol leaf extracts also showed a high reducing power (Aa et al., 2017), as did aqueous and ethanol leaf extracts after petroleum ether extraction (Shariar et al., 2012) and acetone leaf extract (Moyo et al., 2012). As to the method by which phytochemicals exert their antioxidant activity, they can act as direct scavengers, neutralizing free radicals, or decompose peroxides because they are very good electron donors (hydrogen), which allows them to stabilizing free radicals or peroxyl-radicals ($-ROO^{-}$) and inhibiting the formation of hydroxyl (-OH) radical (Chumark et al., 2008; Sreelatha and Padma, 2011; Sharma et al., 2011; Moyo et al., 2012; Nwidu et al., 2018).

Tannins, saponins, alkaloids, triterpenoids, sterols (β-sitosterol), proanthocyanidins, anthocyanins, anthraquinones, carotenoids, coumarins, and especially phenolic acids (such as chlorogenic, ellagic, ferulic, protocatcheuic, coumaric, caffeic and salicylic acids) and flavonoids (such as quercetin, kaempferol, rutin, apigenin, catechin, epicatechin,

isorhamnetin and myricetin) are mainly responsible for the antioxidant activity (Siddhuraju and Becker, 2003; Khalafalla et al., 2010; Sharma et al., 2011; Sreelatha and Padma, 2011; Moyo et al., 2012; Alhakmani et al., 2013; Dzotam et al., 2016; Maiyo et al., 2016; Surendra et al., 2016; Kou et al., 2018; Hisam et al., 2018; Nwidu et al., 2018; Zhou et al., 2018; Yan et al., 2019), although glycosides (glucosinolates and their derivates isothiocyanates and thiocarbamates) and carbohydrates are also very strong antioxidants (Khalafalla et al., 2010; Luqman et al., 2012; Shahriar et al., 2012; Alhakmani et al., 2013; Surendra et al., 2016; Yan et al., 2019). Furthermore, vitamins such as ascorbic acid also present strong antioxidant activity (Kou et al., 2018). At this point we should mention the very high amount of vitamin C in moringa leaves (Leone et al., 2015), as well as folates (such as tetrahydrofolic and formylfolic acids), which are water-soluble vitamins (Saini et al. 2016). Finally, organic acids such as critric, malic, benzoic, linolenic, linoleic and decanoic acids are also important for antioxidant activity (Yan et al., 2019).

## Phytotherapy: effects of incorporation of plants in fish feed

There are three ways to administer plant extracts in aquaculture: by injection (intraperitoneal or intramuscular), bathing/immersion or orally (in the diet) (Reverter et al., 2014; van-Hai, 2015). Although intraperitoneal injection is considered the most rapid and efficient way of administering immunostimulants compared with immersion, bathing or diet, it is also the most expensive and stressful way of administration (Bulfon et al., 2015). However, the incorporation of these plants orally as feed additives allows treating a greater number of fish at the same time and it is not stressful for them (Sakai, 1999; Harikrishnan et al., 2011). Furthermore, plants can be administered singly or in combination (Wang et al., 2016a) and can be used in several forms, including crude, extracts or extracted active compounds (van-Hai, 2015; Awad and Awaad, 2017). Moreover, the effect of plants as feed additives is usually dose-dependent, but it is important to highlight that an overdose could lead to immunosuppression (Kajita et al., 1990; Jian and Wu, 2003; 2004). Generally, there is no positive correlation between concentration and effect, and low concentrations are the most practical (Awad and Awaad, 2017).

It has been established that medicinal plants can enhance digestive enzymes, improving the digestibility and availability of nutrients and, therefore, increasing food utilization, protein synthesis, growth rates and the survival of aquatic animals (Nya and Austin, 2009; Citarasu, 2010; Takaoka et al., 2011; Awad et al., 2012). For this reason, we first evaluated the effect of the addition of plants in feed on the growth performance of gilthead seabream, including WG, SGR and K.

Regarding the effects of plants on the immune system and resistance to diseases, reviews made by Harikrishnan et al. (2011), Bulfon et al. (2013), Reverter et al. (2014), van-Hai, (2015) and Awad and Awaad (2017) established that plants are capable of potentiating the immune system at both systemic and mucosal levels; they can stimulate and promote antimicrobial responses and decrease stress levels, thereby improving survival. According to these reviews, plants strengthen both the innate and adaptive immune responses although best results were obtained for the innate immune response, including cellular and humoral parameters. Therefore, we evaluated the effect of plants on the immune system.

Finally, due the high intensity and activity of fish farms, fish can develop oxidative stress and increase ROS and reactive nitrogen species (RNS) production, which can damage many biological molecules such as proteins and DNA, playing a crucial role in the process of several diseases, infections and even the death of fish (Jooyandeh and Aberoumand, 2007). For this reason, the incorporation of antioxidant compounds to the feed is increasingly important (Salami et al., 2016) to avoid lipid peroxidation of the feed and oxidative stress in animals. In this sense, reviews have already established that medicinal plants also enhance antioxidant activity, and therefore, show anti-oxidative stress properties in fish by inhibiting the generation of oxygen anions and scavenging the free radicals which may oxidize nucleic acids, proteins and lipids and can initiate degenerative diseases (Citarasu, 2010). Such plants may also act by increasing antioxidant enzymes such as GSHPx, GR, SOD, CAT and phenoloxidase. Among these ROS are $H_2O_2$, -OH, superoxide anion ($O_2^-$), and -$ROO^-$ such as lipid peroxyl radical (-$LOO^-$). However, living organisms present an antioxidant system to keep a balance between ROS and the antioxidant status of the body, and these antioxidant enzymes are able to neutralize ROS production and combat oxidative stress (de-Lavor et al., 2018; Pohl and Lin, 2018). In this sense, $O_2^-$ is converted to $H_2O_2$ by SOD, $H_2O_2$ is converted to $H_2O$ and $O_2$ by CAT and GSHPx, and lipid peroxide (LOOH) is converted to alkoxyl radical (LOH) and $H_2O$ also by GSHPx (Aniya, 2018; de-Lavor et al., 2018). However, it is well known that antioxidant systems cannot be considered separately, but as integrated systems able to act in a cooperative manner (Wang et al., 2006). For this reason, it is better to refer to the TAA of body fluids and tissues rather than measure the antioxidant levels or ROS production separately. Therefore, TAA can be determined in specialized organs such as liver, although body fluids such as serum or skin mucus are good indicators of the individual antioxidant network, and of the oxidative stress situation in particular (Wang et al., 2006). Therefore, in the present study, liver enzyme activities were determined including GR, SOD and CAT, as well as serum and skin mucus TAA by the ABTS method and skin mucus Hsp70, Hsp25 and Phospo-c-Jun levels. Furthermore, both the immune and antioxidant status were also evaluated in different tissues, including HK, liver and gut by reference to gene expression.

Finally, the effect of medicinal plants used as dietary additives on metabolic and oxidative stress (glucose, lactate, urea, AST/GOT and ALT/GPT) was also analysed.

## Effects of oregano leaves incorporated in gilthead seabream diet

In previous studies carried out in fish, oregano essential oil was administered to Tilapia zillii (*Coptodon zillii*) for 2 weeks before challenge with *V. anguillarum* (Mabrok and Wahdan, 2018), 80% ethanolic oregano leaf extract was administered to rainbow trout (*Oncorhynchus mykiss*) for 8 weeks (Haghighi and Rohani, 2015) and dried powder was administered to Nile tilapia (*Oreochromis niloticus*) for 13 weeks before challenge with *A. hydrophila* (Seden et al., 2009). However, our study is the first to analyse the effect of the incorporation of oregano in the diet of a marine fish, gilthead seabream.

In terms of growth performance, our results did not identify significant differences for WG, SGR or K in fish fed with oregano-supplemented diets for 15 or 30 days compared with the control group. Similarly, Ariza-Nieto et al. (2011) and Ranucci et al. (2015) found no differences in WG or SGR of pigs fed oregano essential oil or extracts, although most of studies of this kind found an increase in these parameters in mice (Bukovska et al., 2007), rabbits (Nosal et al., 2014), broiler chickens (Fotea et al., 2015; Ghazi et al., 2015; Mohiti-Asli and Ghanaatparast-Rashti, 2015; Scocco et al., 2017) and pigs (Walter and Bilkei, 2004; Zou et al., 2017). However, our results are contrary to the results obtained by Seden et al. (2009), where the addition of 1% oregano powder to a Nile tilapia diet significantly increased WG, SGR and feed use compared with a control diet, which may be due to the difference between the species in question. Nile tilapia is a herbivorous freshwater species and gilthead seabream is a carnivorous marine species. However, using only dried powder in ducks (Park et al., 2015), as in our case, WG and feed use were not altered compared with a control group. Therefore, it seems that the compounds of essential oils or extracts are responsible for the stimulation of growth rate and the optimal use of feed, while using only dried powder could be related with the anti-lipogenesis activity of genus *Origanum*, which prevents the formation or accumulation of fat and therefore can affect the growth rate (Liolios et al., 2010). Another possible explanation for this lack of effect could be related to the restricted feeding of fish, since fish were not fed *ab libitum* (Ranucci et al., 2015), while Giannenas et al. (2003) established that compounds of oregano could stimulate the enhancement of performance under challenging environmental and stress situations.

Regarding the results obtained for the immune system, while serum IgM level fell in fish fed the 0.5% oregano supplemented diet compared to the control group at 15 days, the skin mucus IgM level was significantly higher in fish fed the 1% oregano supplemented diet compared with the control group at 30 days, thereby improving the immune response of fish to fight pathogens. These results seem to suggest that the principal antibody immune response is linked to skin mucus activity and to the percentage of oregano in the diet. As with IgM, fish fed the oregano supplemented diet showed a trend to increase dose-dependent serum natural haemolytic complement activity compared with control fish at 30 days, also enhancing the innate immune system against pathogens. For its part, lysozyme activity did not show any difference between any groups at any assessed time. However, Haghighi and Rohani (2015) observed a significant increase in serum lysozyme activity in rainbow trout fed an 80% oregano ethanolic extract for 6 weeks. Similarly, Mabrok and Wahdan (2018) found increased plasma lysozyme activity in Tilapia zilii fed oregano essential oil after 12 and 24 h of challenge by *V. anguillarum*.

However, although not in serum, we observed a significant increase in the antibacterial activity of skin mucus against *P. damselae* in fish fed the oregano supplemented diets compared to control fish at 15 and 30 days, also improving the activity against bacteria and agreeing with the results obtained by Mabrok and Wahdan (2018), who showed a significant increase in plasma bactericidal activity in fish fed oregano essential oil after 6, 12 and 24 h of challenge with *V. anguillarum*. Another previous study demonstrated the use of oregano extracts as pig dietary additive induced antibacterial activity against coliform bacteria (Scocco et al., 2017). In this sense, Nile tilapia fed oregano diets (0.5 - 2%) showed no mortality after challenge with *A. hydrophila*, while control fish showed a high percentage of mortality (Seden et al., 2009). Other studies have also showed that the inclusion of oregano essential oils or extracts as dietary additive reduced *Eimeria tenella* parasitic infection (Giannenas et al., 2003; Nosal et al., 2014; Mohiti-Asli and Ghanaatparast-Rashti, 2015; Dudko et al., 2017), also confirming its antiparasitic activity.

In the case of protease activity, the serum of fish fed the oregano supplemented diets showed a significant increase compared to control fish at 30 days, while the antiprotease activity of fish fed the most concentrated oregano supplemented diet showed a decrease of this activity. For its part, the peroxidase activity of serum or skin mucus did not show any difference between any diets at any assayed time. The results obtained in our study partially

coincide with previous studies where a significant increase in plasma proteases and antiproteases was recorded in fish fed oregano essential oil after challenge with *V. anguillarum* (Mabrok and Wahdan, 2018).

All these results of humoral immune response in serum and skin mucus of fish suggest that the defence against pathogens mainly takes place in the skin mucus, improving both innate (natural haemolytic complement and antibacterial activity) and adaptive (IgM) immune responses. The addition of oregano to the diet could be responsible for the type and concentration of compounds present in skin mucus. For its part, the immune response that takes place in the serum mainly involves protease activity, directly inhibiting the activity of the pathogen. In this sense, if fish already have this ability to directly inhibit the pathogen, their capacity to activate antiprotease activity and inhibit proteins of pathogens could be diminished, since the concentration of the pathogen is lower because it is already being attacked by the components of the serum. Therefore, the use of oregano may induce the activation of immune system cells and the secretion of compounds with immune activity to the serum.

In the present work, we also study the effects of oregano on cellular immune parameters. Our results showed a significant increase in the phagocytic ability of leucocytes from fish fed the 0.5% oregano supplemented diet for 15 days. Therefore, oregano at a suitable concentration in fish feed can help fish to combat infections, removing the pathogen through the activation of HK leucocyte phagocytic ability. These results agree with the study of Haghighi and Rohani (2015), who showed that 80% ethanolic extract of oregano increased rainbow trout HK leucocyte phagocytic activity at 2 weeks. Furthermore, in the study by Mabrok and Wahdan (2018), a significant increase in white blood cells (WBCs) was observed in fish fed oregano essential oil after 12 and 24 h challenge with *V. anguillarum*.

Respiratory burst activity for its part did not show any difference between any diets at any assayed time, contrary to the results obtained by Haghighi and Rohani (2015), which showed diet containing 80% ethanolic extracts of oregano increased respiratory burst activity of rainbow trout leucocytes after 4 and 8 weeks. As occurred in serum and skin mucus, the peroxidase activity of HK leucocytes showed no differences between any diet at any assayed time. It is important to underline that in both activities, respiratory burst and peroxidase, the oxygen metabolites produced by the immune system cells give rise to other ROS that have

strong anti-microbial activity, but which may also cause some damage *in situ* by destroying surrounding tissue and inducing apoptosis in other immune cells. Therefore, these results suggest a balance between ROS and its elimination caused by the antioxidant activity of the compounds present in oregano and transferred to serum, skin mucus and the immune system cells of fish.

As in the case of diseases and infections that occur in fish farms due to the high intensity of activity, fish can develop stress, and their organism may produce ROS, which can even lead to the death of fish and heavy economic losses. Oregano belongs to the plant species with the highest content of antioxidants (García-Beltrán and Esteban, 2016; Yan et al., 2016), which highlights the potential importance of adding oregano to the feed as an antioxidant compounds. For this reason, we studied the antioxidant activity of three liver enzymes (GR, SOD and CAT) and the TAA of serum and skin mucus of fish. Unexpectedly, none of the liver antioxidant enzymes studied showed differences in fish fed the oregano supplemented diets compared with control fish at 15 or 30 days, while serum and skin mucus TAA showed a trend to increase, although not significantly, at 30 days. Our results did not agree with those of previous studies where the use of dried powder, essential oils or extracts induced an increase in antioxidant enzyme activity, including SOD, CAT, GR, GSHPx and glutathione-S-transferase (GST) (Srihari et al., 2008; Park et al., 2015; Ranucci et al., 2015; Vujicic et al., 2015; Liu et al., 2017) and TAA (Cheng et al., 2017; Liu et al., 2017) and a decrease in ROS, RNS, oxidative stress, DNA damage (Vujicic et al., 2015; Akilli and Eraslan, 2016; Liu et al., 2017) and lipid peroxidation (Liu et al., 2017). Therefore, these results could mean that the antioxidant defence of fish is not related to the enzymatic activity of the liver, but to the antioxidant capacity present in the compounds of serum and skin mucus, which may can help to prevent oxidative stress. It is possible that the compounds present in oregano are transferred to the serum and skin mucus and then act by removing ROS, contributing to decreasing oxidative stress.

## Effects of date palm seeds incorporated in gilthead seabream diet

As we have previously said, DPS is rich in dietary fibres. The significance of dietary fibres in animal feeding is their influence on the speed of passage, mucosal functionality and the crucial role they play as substrate for gut microbiota, which is related to performance and digestive health (Gidenne, 2015). Taking into account that, at present, most marine farmed fish are carnivores (FAO, 2016) and that indigenous microbiota play a critical role in the lives of their vertebrate hosts (reviewed by Llewellyn et al., 2014), it would be very interesting to carry out *in vivo* studies to determine the possible effects of dietary fibre supplementation on fish gut microbiota composition and also to support the hypothesis that fish gut microbiota could make the fermentation of these fibres found in DPS extracts with potential benefits to the host.

Previous studies in which DPS has been incorporated in animal diets provided variable results as regards growth performance (Belal, 2008; El Hadrami et al., 2011; Hossain et al., 2014). In studies using sheep as animal model, the incorporation of DPS and date fruits increased growth performance by improving feed utilization (Al-Kinani and Alwash, 1975; Elgasim et al., 1995; Al-Owaimer et al., 2011). Its use in cows did not present adverse effects (Alwash and de-Peters, 1982; Rezaeenia et al., 2018) or even had antioxidant effects, increasing TAA in milk and blood (Sharifi et al., 2017). Some studies in broilers found a negative effect of DPS on growth performance and feed utilization (Najib et al., 1994; Al-Bowait and Al-Sultan,2007), while other studies showed no effect (Vandepopuliere et al., 1995) or positive results (El-Far et al., 2016). In this respect, it was established that the way DPS are added and the age of broilers are crucial for obtaining positive or negative results (Hussein et al., 1998). Similarly, as regards the effect of DPS on fish growth performance, the concentration used seems to be important. In this sense, Yousif et al. (1996) demonstrated that the incorporation of dates and DPS at 15% in tilapia (*Oreochromis aureus*) decreased WG, SGR and feed utilization compared with fish fed a control diet containing no date pulp or DPS. Similar results were obtained in Nile tilapia, where low concentrations of DPS increased WG and high concentrations (15% - 30% or more) decreased it (Belal and Al-Owaifer, 2005; Belal, 2008; Gabber et al., 2012; Assem et al., 2014; Gabber et al., 2014). Low concentrations (0.5% - 2%) of DPS also increased WG, SGR and feed utilization in African catfish (*Clarias*

*gariepinus*) (Sotolu et al., 2014). However, the results for common carp (*Cyprinus carpio*) are contradictory since 0.25% DPS diet decreased WG, SGR and feed utilization, while a 0.5% DPS diet increased them (Ahmed et al., 2017).

However, studies analyzing the effect of a DPS diet on immune status are very scarce. For this reason, in recent years, our research team has evaluated many and varied effects caused by the dietary administration of date palm fruit extracts (alone or in combination with probiotic bacteria) on growth performance, mucosal and systemic immunity and antioxidant status in several fish species, finding marked immunostimulant effects on common carp (Hoseinifar et al., 2015), European sea bass (*D. labrax*) (Guardiola et al., 2016) and gilthead seabream (Esteban et al., 2014; Cerezuela et al., 2016). In the studies carried out in gilthead seabream, a commercial diet used as control diet was enriched with probiotic *Shewanella putrefaciens* (Pdp11), probiotic *Bacillus sp.*, aqueous date palm fruit extracts (4%) or a combination of Pdp11 + *Bacillus sp* + aqueous date palm fruit extracts and given to fish for 4 weeks. In the study carried out in European sea bass, the diet with probiotic *Bacillus sp.* was not used, while in the study carried out in common carp only date fruit aqueous extract at 200 mL/kg was tested for 8 weeks. However, the effect of DPS powder alone on immune and antioxidant status has not been studied. Therefore, the positive results obtained previously *in vivo* led us to develop the present work, in an attempt to ascertain whether DPS could potentially be considered as a functional food ingredient for farmed fish. Furthermore, the concentrations used in our study were low since, as mentioned above, previous studies, including those made in fish, demonstrated that low concentrations had different effects on animals. Although a slight decrease in growth performance of fish fed the 3% DPS diet was observed, no significant differences were observed between fish fed DPS diets and those fed the control diet.

As for humoral immune status, no differences were observed in serum or skin mucus IgM level, although skin mucus IgM in fish fed the 3% diet showed a non-significant increase at 15 days, which is in line with previous results pointing to non-significant variations in serum IgM level in European sea bass (Guardiola et al., 2016) or a significant increase in skin mucus IgM of gilthead seabream (Cerezuela et al., 2016) after 2 or 4 weeks of treatment with date fruit aqueous extracts alone or with probiotic, as well as in skin mucus total Ig of common carp (Hoseinifar et al., 2015) after 8 weeks of treatment with date fruit extract. Furthermore, skin of gilthead seabream specimens fed with date extract alone or with probiotic significantly

increased the expression of *ighm* and *ight* genes after 2 and 4 weeks, respectively (Cerezuela et al., 2016).

Regarding lysozyme activity, although results showed an increase of serum lysozyme activity in fish fed DPS diets at 15 and 30 days, neither serum nor skin mucus showed significant increases with respect to the control group at any assayed time. However, a marked increase in skin mucus lysozyme activity was observed after 8 weeks of treatment with date extract in common carp (Hoseinifar et al., 2015), as well as lysozyme gene expression in HK of European sea bass fed with date extract for 4 weeks (Guardiola et al., 2016).

As in the study of Guardiola et al. (2016), where European sea bass fed with date extract alone or with probiotic did not show any difference at any assayed time in bactericidal activity against *V. harveyi*, *V. anguillarum*, *P. damselae* and *E. coli* with respect to the control group, no differences in bactericidal activity of skin mucus samples against *V. harveyi* or *V. anguillarum* were obtained after feeding with DPS powder, although a non- significant dose-dependent increase was observed against *V. harveyi* at 15 days. In addition, fish fed 1.5% DPS diet showed a significantly increase in serum protease activity at 30 days. Th eresults obtained in our study are totally in line with and corroborated previous results, where skin mucus protease activity was significantly higher in gilthead seabream fed a diet enriched with date extract alone or with probiotic at 2 and 4 weeks (Cerezuela et al., 2016), as well as in common carp at 8 weeks (Hoseinifar et al., 2015).

For its part, serum antiprotease activity did not vary between the different treatments and control group at any assayed time, which is contrary to the results obtained in previous studies, where skin mucus of gilthead seabream specimens fed with date extract showed decreased antiprotease activity at 2 weeks and increased levels at 4 weeks compared with the control group (Cerezuela et al., 2016). Similar results were obtained by Guardiola et al. (2016), in whose study European sea bass specimens fed with date extract with probiotic showed a decrease in serum antiprotease activity at 2 weeks. Furthermore, although non-significant differences were obtained, fish fed with DPS diets showed higher peroxidase activity, contrary to previous results, in which diets enriched with date extract alone or with probiotic decreased peroxidase activity in skin mucus of gilthead seabream specimens at 2 and 4 weeks (Cerezuela et al., 2016) or had no effect in serum of European sea bass at any assayed time (Guardiola et al., 2016).

In our experiment, cellular immune status was also analysed and positive results were obtained. Starting with phagocytic activity of HK leucocytes, specimens fed with DPS enriched diets showed a significant increase in phagocytic ability at 15 and 30 days, especially with the 3% diet, while phagocytic capacity was not affected. Our results agreed with the results obtained by Guardiola et al. (2016), which showed that European sea bass specimens fed date extract with probiotic had higher phagocytic ability and capacity at 2 and 4 weeks. However, in the case of respiratory burst, no differences were obtained between different treatments and control group at any assayed time, while in the study of Guardiola et al. (2016) specimens fed date extract with probiotic showed a decrease in this activity at 4 weeks. Finally, fish fed the DPS diets showed a non-significant dose-dependent increase in HK leucocyte peroxidase activity at 30 days, as in Guardiola et al. (2016), where no differences were obtained between the different treatments and control group at any assayed time.

As to the effect of DPS on the antioxidant status of fish, although none of the antioxidant enzymes of liver showed significant differences between DPS diets and the control group, an increase in GR activity in fish fed the 1.5% diet at 30 days and a dose-dependent increase in CAT activity at 15 and 30 days was observed. In previous studies in fish, the expression of antioxidant enzymes GR, CAT and SOD was studied in gut, skin and gills (Esteban et al., 2014). In that study, no differences for any enzyme were obtained in gut samples of fish fed date extract with respect to the control fish. For their part, at 4 weeks, GR and CAT gene expression increased in skin of fish fed date extract alone while fish fed date extract with probiotic had increased GR but decreased SOD expression levels. In the case of gill samples, fish fed date extract alone or with probiotic showed a significant increase in GR and CAT expression at 4 weeks. SOD expression increased significantly in fish fed date extract alone or with probiotic at 2 weeks, while this increase was not significant at 4 weeks (Esteban et al., 2014), as in the study of Guardiola et al. (2016), where a significant up-regulation of SOD expression was recorded in HK from European sea bass specimens fed date extract.

Finally, 3% DPS diet increased TAA in skin mucus, although not significantly, which is in line with results obtained by Guardiola et al. (2016) in European sea bass, where fish fed date extract alone significantly increased serum biological antioxidant potential at 2 and 4 weeks.

## Effects of dehydrated lemon peel incorporated in gilthead seabream diet

The main reason for ising DLP in our study was that lemon peel, which is usually discarded, contains abundant biologically active compounds (Park et al., 2014; Wu et al., 2015; Nakajima et al., 2016). Regarding studies carried out in fish, there are only three previous works using lemon peel. For example, lemon peel essential oil was used as dietary additive in juvenile Ningu (*L. victorianus*) challenged with *A. hydrophila* (Ngugi et al., 2016) and Mozambique tilapia (*O. mossambicus*) challenged with *Edwardsiella tarda* (Baba et al., 2016), and hematological, immune and metabolic parameters were analysed. Moreover, DLP at 1%, 2% and 4% was added to feed of Nile tilapia and African catfish for 7 weeks (Rahman et al., 2019) and growth performance, humoral and cellular immune parameters, antioxidant enzymes (SOD, CAT and GSHPx) and metabolic parameters (such as glucose and cortisol) were analysed. Therefore, although the effect of lemon has been studied in freshwater fish species, this is the first study where the effect of lemon has been studied in a marine fish, the gilthead seabream.

Many properties related to improve digestive functions in mammals have been demonstrated using citrus plants, including lemon, such as increased appetite or prebiotic effects (Gómez et al., 2016). In the present work, no significant improvements were obtained for WG or SGR in fish fed the 1.5% DLP diet for 15 or 30 days or the 3% DLP diet for 15 days compared with the values recorded for the control group, although there was a dose-dependent increasing tendency in all three cases. However, a significant decrease in both growth parameters was obtained when gilthead seabream were fed 3% DLP for 30 days. Our results are in line with these obtained by Rahman et al. (2019), who observed a non-significant decrease in the growth performance of Nile tilapia and African catfish. One explanation for the observed decrease in fish growth could be related with the diuretic properties attributed to lemon in mammals, which means that it increases urine production in the kidneys, thereby helping to eliminate excess fluid (Padilla-Camberos et al., 2014; Menichini et al., 2015; Wu et al., 2015). Another possible explanation could be related with the anti-lipogenesis effect of lemon, and genus *Citrus* in general, observed in *in vivo* studies carried out in rats (Miyake et al., 2006; Menichini et al., 2015) and in other *in vitro* studies (Lee et al., 2015; Lim et al., 2015). New research might help to ascertain the exact reasons for these results as well as to demonstrate whether DLP also has diuretic or anti-lipogenesis effects in fish.

The present study demonstrated that the immunostimulant effects of dietary DLP were evident after 15 days of treatment but not after 30 days. Furthermore, the effect of DLP on the immune system was dose-dependent. Significant increases over the control values were seen in serum IgM levels, skin mucus peroxidase activity and HK leucocyte phagocytic ability. These results are in line with previously obtained results (Ngugi et al., 2016; Baba et al., 2016; Rahman et al., 2019), which showed increases in serum Ig levels, lysozyme activity and phagocytic, respiratory burst and myeloperoxidase activity of ningu and Mozambique tilapia fed with lemon peel essential oil and Nile tilapia and African catfish after fed 1% and 2% DLP powder. Furthermore, the present results agree with previous results obtained in mammals, in which the anti-inflammatory (Campolo et al., 2016; Gómez et al., 2016), antibacterial (Ou et al., 2015; Ramadan et al., 2015; Shetty et al., 2016) and antitumor (Zaletok et al., 2015; Park et al., 2016) effects of citrus plants were demonstrated. Further studies are needed to demonstrate whether the observed immunostimulant effects correlated or not with an increased defense against diseases.

As regards the antioxidant effect of DLP on GR, SOD and CAT liver antioxidant enzymes of gilthead seabream, unexpectedly, no significant effects were observed in any antioxidant enzyme at any assayed time, contrary to the results obtained in the study carried out by Rahman et al. (2019), where a significant increase in CAT was observed in Nile tilapia and African catfish fed 1% and 2% DLP diets, while SOD activity only increased in African catfish. By contrast, rats with kidney disease showed a decrease in CAT and SOD activity after treatment with aqueous methanol lemon peel extract (Sridharan et al., 2015). For its part, the TAA of gilthead seabream in the present study was significantly higher after 30 days than in fish fed the control diet, suggesting a positive effect of supplementation in the diet on general antioxidant protection. An increase in antioxidant power in various organisms and also in fish fed natural sources of antioxidants, has been widely reported (Wang et al., 2006; Kumar et al., 2007; Pan et al., 2011; Santos et al., 2014). Furthermore, as discussed above, significant increases were observed in peroxidase activity in skin mucus and HK leucocyte. These results suggest that the antioxidant effects of DLP are effective only on peroxidase, in other words, on enzyme functions that decompose $H_2O_2$ into $O_2$ and $H_2O$.

In order to confirm the results obtained concerning activities related to the immune and antioxidant status of fish, the expression of several genes was studied. Among the genes related to the immune status we selected some pro-inflammatory (*il1b, il6* and *cox2*),

antimicrobial (*hamp* and *bdef*), antioxidant (*nkefa*) and macrophage stimulator (*csf1r*) genes. Furthermore, it is known that *il6* and *nkefa* genes also contribute to adaptive immunity (B cells maturation and antiviral function of $CD8^+$ T cells, respectively). Other genes studied were related to specific immunity: the antibodies (*ighm* and *ight*) and antigen receptors of T cells (*tcrb*). As antioxidants, we studied the expression of several genes in gilthead seabream liver: two anti-stress genes corresponding to heat shock proteins 70 and 90 *(hsp70* and *hsp90)* and the genes of the three antioxidant enzymes studied (*gr*, *sod* and *cat)*. None of the studied genes was significantly down-regulated by dietary DLP administration. By contrast, *nkefa*, *il1b*, *ight* and *csfr1* were significantly up-regulated in fish fed the 3% DLP diet for 15 days. The up-regulation of these genes helps to explain some of the observed effects of dietary DLP (e.g. antioxidant implication of *nkefa*, possible inflammatory response of *il1β*, stimulation of macrophages by *csfr1* and immunostimulation of adaptive immunity by *ight*). However, after 30 days of receiving the same diet such up-regulation was not observable and the expression was similar to that of the genes in control fish. This suggests that the immunostimulant properties of dietary DLP in gilthead seabream are obtained at short times (15 days) since longer exposure (in this case 30 days) leads to the possible adaptation of the immune status. Finally, we studied the expression of several genes in gilthead seabream liver: two anti-stress genes corresponding to heat shock proteins 70 and 90 *(hsp70* and *hsp90)* and the genes of the three antioxidant enzymes studied (*gr*, *sod* and *cat)*. No significant differences from control levels were detected in the genes studied in liver after DLP administration at the doses and times used in the present study, which reflects the result for antioxidant enzymes in liver.

As to the second part of the study, and as we have already commented, the management and reduction of stress is one of the most pressing issues in aquaculture since it may compromise fish health, welfare, productivity and quality. It is well known that stress affects the fish metabolism and reallocates energy within the organism. In response to stress, fish undergo a series of biochemical and physiological changes in an attempt to cope with the stress (Soengas and Aldegunde, 2002; Tort, 2011; Conceiçao et al., 2012). In this sense, the metabolic status is one of the main factors of welfare in fish, and some serum indicators such as cortisol, glucose, lactate and other enzymes involved in energy management are widely used to assess and monitor fish health and condition in relation to stress (Santulli et al., 1999; Messina et al., 2013). The nutritional status of fish influences their metabolism

and stress response: dietary components ensure an adequate energy supply and that nutritional requirements are covered, which is useful for biological processes, physiological functions and the immune response. Therefore, the diet plays a crucial role in potential stress situations (Conceiçao et al., 2002) as changes in feeding activity or energy requirements affect the metabolic response to abiotic factors (Guderley, 2004). Hence, adequate and balanced diets containing immunostimulants can improve the health and disease resistance of fish (Mena et al., 2013). Unlike omnivorous and herbivorous fish, carnivorous fish such as gilthead seabream, European seabass and rainbow trout, use more lipids and proteins than carbohydrates. Therefore, carnivorous fish fed carbohydrates show poor use of dietary carbohydrate in muscle (Polakof et al., 2012; Enes et al., 2009) and, so may present a long period of postprandial hyperglycemia (Enes et al., 2009).

Fish can use glucose as energy source and have a system to detect circulating glucose levels (glucosensing) to modulate and maintain the homeostasis of these levels (Soengas and Aldegunde, 2002; Polakof et al., 2012). The glucose levels of teleost fish are very variable and depend on the metabolic rate (Polakof et al., 2012) and a wide variety of factors and physiological conditions. In normal conditions, the fish metabolism is aerobic, and exogenous (dietary) glucose is catabolized by glycolysis. Due to oxidation and oxidative phosphorylation in the Krebs cycle and the respiratory chain, most of the ATP required is provided. The excess of glucose is stored as glycogen (glycogenesis) or converted into lipids (lipogenesis). Glycogen is the largest energy reserve available to fish. On the other hand, during stress or in situations that require sudden bursts of energy, endogenous glycogen is depleted (glycogenolysis) or glucose is synthesized *de novo* from lactate, glycerol and some amino acids (gluconeogenesis) (Soengas and Aldegunde, 2002; Enes et al., 2009; Polakof et al., 2012). Teleost fish can also use other energy sources such as lactate. During stress situations, such as food deprivation, hypoxia, intense swimming or the presence of pollutants, white muscle produces lactate (Cao et al., 1998a,b). Glycogen is the principal fuel providing glucose (Soengas and Aldegunde, 2002; Guderley, 2004; Enes et al., 2009; Polakof et al., 2012), but when reserves are low, the oxidation of lactate in the Krebs cycle by anaerobic glycolysis occurs (Soengas and Aldegunde, 2002), while when stress finishes this lactate reset glucose levels by gluconeogenesis. This lactate cannot be quickly processed, like glucose, from red muscle, heart, gills and brain. Some fish, like trout, use lactate as principal oxidative fuel (Weber et al., 2016). Glucose and lactate are physiological

indicators of non-specific stress (Chase et al., 2016). In stress situations there are changes in the metabolism, and plasma glucose and lactate levels increase (Laiz-Carrion et al., 2002). In these situations, there is a release of glucose through glycogenolysis (Laiz-Carrion et al., 2002; Soengas and Aldegunde, 2002), lipolysis (Shirdel et al., 2016) and the activation of gluconeogenesis (Laiz-Carrion et al., 2002; Shirdel et al., 2016), and fish use less exogenous glucose. Lactate levels also increase due to glucose reset lactate (Tkachenko and Grudnieswska, 2016). In our study, we confirm the hypoglycemic activity of citrus peel suggested in previous studies (Padilla-Camberos et al., 2014; Menichini et al., 2016), since gilthead seabream fed for 15 days with DLP showed decreased glucose and lactate levels. However, the levels of both metabolites returned to normal levels when fish were fed the same diets for 30 days. So, DLP included in the diet of gilthead seabream for 15 days shows antidiabetic activity and could help to inhibit hyperglycemia and improve the stress response, while after 30 days fish have become totally adapted to the diets. Therefore, it can also be concluded that diets enriched with 3% DLP do not affect fish metabolism after feeding for a month. Rahman et al. (2019) showed serum glucose also decreased with increasing DLP percentages in the diet of Nile tilapia, while no effects were observed in African catfish. Moreover, Ngugi et al. (2016) and Baba et al. (2016) also showed a decrease in serum and plasma and glucose, as well as of cortisol, triglycerides and cholesterol in ningu and Mozambique tilapia, respectively.

Although most teleost fish are ammoniotelic, many marine fish in situations of stress or nitrogen imbalance or receiving supplementation through plant protein-based diets (Tulli et al., 2007) can direct the nitrogen catabolism towards the production of urea, for which reason it can be considered a marker of adaptation/stress situations (Weihrauch et al., 2009; McDonald et al., 2012; Rahman et al., 2015). Urea, in fact, is an alternative nitrogenous waste for fish, but its production is expensive from an energetic point of view and can reduce growth performance in fish (Tulli et al., 2007). An increase in protein/amino acid catabolism is related with the Krebs cycle (principally through the production of alpha-ketoglutarate), and ammonia and urea production (McDonald et al., 2012). Fish can detoxify ammonia to urea when ammonia excretion is slow. In the case of urea, our study pointed to an increase only in specimens fed diets supplemented with 3% DLP, compared to fish fed the control diet. This can be explained as an effect of the transient reduced levels of circulating glucose on the utilization of alternative carbon chains as fuel (mainly proteins) through the Krebs

cycle and the involvement of transaminases that showed a temporary increase in its activities. Furthermore, the levels of urea were not dangerous and the inclusion of DLP in the fish diet, at the level tested in the present study, did not affect the general metabolism of gilthead seabream. However, rats with kidney stone disease decreased urea excretion after treatment with aqueous methanol lemon peel extract (Sridharan et al., 2015).

This crucial aspect was also confirmed from the trend of AST and ALT, which provide information on liver welfare status, protein metabolism and energy management (Bulfon et al., 2013; Ismail and Mahboub, 2016; Shirdel et al., 2016) and are also indicators of stress (Ismail and Mahboub, 2016; Shirdel et al., 2016; Tkachenko and Grudnieswska, 2016). High values of AST and ALT for prolonged time are related with hepatic damage or a disturbance in the Krebs cycle, and may indicate cellular necrosis (Ismail and Mahboub, 2016). Furthermore, AST and ALT are also recognized as biomarkers related to the total antioxidant status, while a general trend in its modulation and adaptation can be positively related to an improvement in the general antioxidant status, as observed in fish fed a diet supplemented with different doses of the antioxidant astaxantin (Wang et al., 2006). These are enzymes involved in carbohydrate and protein metabolism and their modulation/turnover in stress situations: if alanine and aspartate mobilization is required for glucose production by gluconeogenesis, their activities increase (Shirdel et al., 2016). In the present study, only fish fed the diet supplemented with 3% DLP showed an increase in the activity of these enzymes at 15 and 30 days, while with the diet containing 1.5% DLP an increase was observed, only after 15 days. These data suggest that activity of these liver enzymes is only modulated by the highest concentration of DLP in the diet (3%). The present results agree with those obtained in a study where blood glucose concentrations and AST and ALT activity improved after the dietary administration of *Astragalus membranaceus* and *Glycyrrhiza glabra* (liquorice) in the diet of *Perca flavescens* (Elabd et al., 2016). A similar protective effect was observed in Buffalo calves fed diets supplemented with lemon (Ahmed et al., 2009), and point to the management of available energy through central metabolism pathways.

In addition, the use of lemon peel as a dietary supplement in fish could improve growth, have hepatoprotective and immunostimulating effects. Our results are in line with those reported by Deng et al. (2011), which showed a significant decrease of AST and ALT

activities in rainbow trout fed a diet supplemented with propolis, highlighting the role of natural products in the prevention of liver injury.

Our research group recently demonstrated that skin mucus is a suitable matrix for evaluating stress conditions in fish (Guardiola et al., 2014b), and that some of its responses are related to certain serum responses. Therefore, this work also evaluated some biomolecular markers in this tissue, focusing on some proteins that are generally related to general stress and oxidative status. To our knowledge, few reports exist on the biomolecular markers related to stress in fish skin mucus. Among those that exist, a proteomic analysis of epidermal mucus from sea lice-infected Atlantic salmon (*Salmo salar*) (Provan et al., 2013) showed that some proteins are up-regulated in skin mucus of stressed fish – for example, Hsp70, Hsp25 and Phospho–c-Jun. Since these three proteins are also related to oxidative stress, we directed our attention towards these biomolecular markers.

Hsp70 is well known as a molecular chaperone, and is expressed in normal and stressful conditions. In both conditions, intracellular Hsp70 ensures protein homeostasis (Radons, 2016; Zuo et al., 2016). Hsp70 is involved in the cellular stress response pathways (Han et al., 2016; Metzger et al., 2016), as induced by thermal, hypoxia and oxidative stress, nutrient deprivation, osmotic pressure, chemical agents, microbial infections (Han et al., 2016; Metzger et al., 2016; Radons, 2016; Zuo et al., 2016) and inflammation (Radons, 2016; Zuo et al., 2016). Hsp70 activates an immune response that facilitates antigen-presenting cells (APCs) maturation, pro-inflammatory cytokine release, and the induction of an inflammatory T cell response. In the present study, the presence of Hsp70 in skin mucus and its levels varied with DLP supplementation and the length of experimental feeding, according to all the other markers. In particular, we observed a reduction in Hsp70 levels in the skin mucus of fish fed for the longest time (30 days) with DLP, which exhibited an improvement in TAA, according to the function of the protein, which is reduced in organisms with a good balance of antioxidants and over-expressed in a situation of oxidative stress (Chopra et al., 2013; Messina et al., 2014, 2016).

It is well known that many oxidizing agents cause the induction of Hsp and that the treatment of cells with antioxidants attenuates the response to stress (Padmini et al., 2012). Our results are consistent with the observations described by di-Giancamillo et al. (2015) that showed the levels of Hsp70 were decreased in pigs fed a diet supplemented with a

natural verbascoside extract, compared with pig fed a high-fat diet, suggesting the role of Hsp70 in modulating hepatic oxidative stress. The small heat shock proteins (Hsps), such as Hsps27 and the homolog Hsps25, are protective factors against oxidative stress and their over-expression represent a strategy of defense against ROS, tumor necrosis factor-induced by ROS, and protein oxidation (Mehlen et al., 1996; Escobedo et al., 2004). Furthermore, Hsps25 can modulate the glutathione (GSH) system in cells, which affects the TAA (Escobedo et al., 2004). This is due to the ability of Hsps to induce the activity of glucose-6-phosphate dehydrogenase (G6PDH), which is responsible for production of the reducing power and of the recharging of GSH (Mehlen et al., 1996). Our results, which point to an increase in proteins related with the increased TAA, confirm the role in fish skin mucus. Further investigations are needed to ascertain whether Hsps25 may represent a possible biomarker in fish skin mucus, which would serve for the fast and easy monitoring of health status of farmed fish.

Finally, the evaluation of Phospho-c-jun pointed to an increase in protein phosphorylation in fish fed the experimental diets for 15 days, compared with the values recorded for control fish, although the same parameter decreased after 30 days. It is well known that the expression of this marker is associated with an inflammatory response, involving the nuclear transcription complex Activator Protein-1 (AP1), composed of subunit c-jun and c-fos (Chopra et al., 2013). Both proteins are influenced by modifications in the redox balance (Mehlen et al., 1996) and our results confirm the response of c-jun to a situation in which the antioxidant status undergoes variations due to the inclusion of DLP in the diet.

## *In vitro* effects of Nature-identical compounds

Plants have been widely used as source of new bioactive compounds in order to develop new drugs to combat several diseases and infections due to their known use in traditional medicine (Pandey, 2013; Greenwell and Rahman, 2015; Madi et al., 2016; Reyad-ul-Ferdous et al., 2016; Saini et al., 2016; Yuan et al., 2016; Pohl and Lin, 2018; Rahman, 2018). Even today, natural products are a very good source of compounds that are used to treat many diseases better than commercial drugs. Furthermore, these compounds have been recognized as Generally Recognized as Safe (GRAS) by the US FDA (Quintans et al., 2019).

In this sense, plants are formed by essential oils, which are extremely complex in their composition since they contain a very high quantity and variety of phytochemicals, the mixture of compounds being responsible for all the biological activities of such plants (van-Zyl et al., 2006). These essential oils are not considered essential for plant survival but are tremendously important for plant adaptation to their environment, being involved in activities which increase plant competitiveness, defensive activities such as protection against microbial and herbivores acting as repellents or toxicants, and attraction activities such as the attraction of pollinators (Paduch et al., 2007; Schwab et al., 2008; Ilc et al., 2016). Furthermore, they are responsible for the fragrance and flavor of plants (Paduch et al., 2007; Ilc et al., 2016; de-Lavor et al., 2018), and are present in fruits, vegetables and flowers (Paduch et al., 2007). The biological activities of these essential oils include antimicrobial, antioxidant, antitumor, anti-inflammatory activities (van-Zyl et al., 2006; Paduch et al., 2007; Schwab et al., 2008; D´Souza et al., 2017; Ibrahim at al., 2018; Pateiro et al., 2018), although they also promote growth and improve gut function (Ibrahim et al., 2018).

Therefore, due to their biological activities, essential oils are used in food, cosmetic and pharmaceutical industry (de-Lavor et al., 2018; Pateiro et al., 2018). Furthermore, the use in food industry of natural compounds to protect food products from biochemical (oxidation) and microbial deterioration and extending their shelf-life is every year more important, ito replace synthetic antioxidants (BHT, butylated hydroxyanisole [BHA] and tert-butylhydroquinone [TBHQ]), due to their greater acceptability by consumers and their application, single or combined with other essential oils, as ingredients or in preservation technologies where they have beneficial effects on meat products (Pateiro et al., 2018; Yahfoufi et al., 2018).

In this sense, NICs (substances identical to those naturally present in material of vegetable or animal origin obtained by chemical synthesis or isolated by chemical processes) are starting to be used instead of plant compounds or extracts. In this way, NICs allow the use of chemical compounds in countries where the plants in question do not exist or are outside the best harvesting time, as well as being cheaper and sustainable. Among tested NICs are various monoterpenols, a penylphropanoid (terpene derivate), a phenolic aldehyde, a flavonoid and some organic acids. Terpenes are the main constituents of essential oils (Schwab et al., 2008; Quintans et al., 2019), and have been widely studied due to their great quantity of biological activities, including antimicrobial (bacteria, fungi and virus), anti-cancer, antioxidant, analgesic, antiparasitic, anti-allergenic, antispasmodic, antihyperglycemic, anti-inflammatory and immunomodulatory actions, which allow them to be used in the prevention and therapy of several diseases and infections (Paduch et al., 2007; Quintans et al., 2019). For these reasons, terpenes are of huge economic importance in the food industry as flavoring agents, cosmetic as fragrance agents in perfumes, agriculture as insecticides, and in the pharmaceutical industry as antimicrobial, antiretroviral and anti-malarial compounds (Schwab et al., 2008; Ilc et al., 2016). However, other phenolic compounds such as phenolic aldehydes or flavonoids are also present in essential oils and present many beneficial activities such as antioxidant, antimicrobial, anti-inflammatory and immunomodulatory among many other activities (Ncube et al., 2008; Pandey, 2013; Borges et al., 2015; de-Lavor et al., 2018; Pateiro et al., 2018; Yahfoufi et al., 2018) and are also used to treat a great variety of diseases and disorders (Yahfoufi et al., 2018).

Taking into account all these considerations, and with a view to introducing them as feed additive in fish aquaculture, we studied the *in vitro* antibacterial and immunomodulatory activities of NICs. For this purpose, different concentrations of single NICs were tested against *V. harveyi* and *V. anguillarum*, while the immunostimulant effect was studied on gilthead seabream HK leucocytes using a mix of NICs containing organic acids, monoterpenols and phenolic aldehydes.

Regarding antibacterial activity, the results for both *V. harveyi* and *V. anguillarum* are practically identical and NICs showed a dose-dependent activity. Phenolic aldehyde, flavonoid, terpene derivate, and especially monoterpenes, had bactericidal activity, inhibiting bacterial growth even at very low concentrations. These results are totally in line with previous studies where terpenes and their derivatives, flavonoids and phenolic

aldehydes, demonstrated a strong and dose-dependent antibacterial activity against Gram-negative and Gram-positive human bacteria (Si et al., 2006; van-Zyl et al., 2006; D´Souza et al., 2017), and even against fish pathogenic bacteria such as *E. coli* multidrug-resistant, *A. hydrophila, Citrobacter freundii* and *Raoultella ornithinolytica* isolated from fish (Junior et al., 2018). Furthermore, although these compounds showed activity against beneficial *Lactobacilli plantarum*, *L. acidophilus* and *Bifidobacterium longum* and *B. breve* gut bacteria, these bacteria were less sensitive than pathogenic ones (Si et al., 2006). Terpenes can present OH, carbonyl (CO) or aldehyde (CHO) groups (Paduch et al., 2007) and the presence of OH groups and the lipophilic property of terpenes have been postulated as being responsible for their antibacterial activity (van-Zyl et al., 2006; Paduch et al., 2007). In this sense, monoterpenes can increase membrane permeability, affecting their proteins and the respiration chain (Paduch et al., 2007). As in the case of terpenes, many different kinds of phenolic compounds, including flavonoids, have shown strong antibacterial activity, acting through membrane disruption but also due to the presence of OH groups and especially their lipophilic property (Ncube et al., 2008; Pandey, 2013; Borges et al., 2015).

Regarding the effect of a blend of NICs containing organic acids, a monoterpene and a phenolic aldehyde on HK leucocyte activity, different concentrations failed to affect cell viability, and had no toxic effect at the concentration used (1g/L). This is important since high concentrations of terpenes can be toxic (Paduch et al., 2007). Furthermore, the blend of NICs did not have any significant effect on phagocytic activity, although phagocytic capacity decreased in dose- and time-dependent manner. For its part, respiratory burst activity was not affected significantly, although all concentrations of NICs decreased it at time 0 min of incubation, probably due the antioxidant power of NICs, which inhibits ROS production or eliminates the ROS produced. On the other hand, at higher incubation times, low concentrations showed a non-significant increase in ROS production and therefore the greater stimulation of macrophages, while high concentrations showed a non-significant decrease in ROS, which may be due to the antioxidant power of the NICs or their anti-inflammatory activity when concentration is optimum. These results were confirmed by immune (pro- and anti-inflammatory) and antioxidant related genes expression in HK of fish, where low concentrations of NICs led to a significant increase of *il1b* and *il6* pro-inflammatory gene expression, whereas high concentrations, led to a significant increase of *il10* and *tgfb* anti-inflammatory genes (especially at 30 min of incubation). No significant

differences were observed in antioxidant genes until 4 hours of incubation, when low concentrations of NICs increased *gr* gene expression, possibly to combat the ROS production induced by pro-inflammatory cytokines.

According to reviews made by de-Lavor et al. (2018), Yahfoufi et al. (2018) and Quintans et al. (2019), the phenolic compounds present in essential oils, including, principally, flavonoids and especially terpenes, present immune-modulatory properties regulating immune cell populations, as well as cytokine production and pro- and anti-inflammatory gene expression. Phenolic compounds inhibit inflammatory responses, inhibiting the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), mitogen-activated protein kinase (MAPK), phosphatidylinositide 3-kinases/protein kinase B (PI3K/AkT), the inhibitor of kappa kinase/c-Jun amino-terminal kinases (IKK/JNK), mammalian target of rapamycin complex 1 (mTORC1) and janus kinases/signal transducer and activator of transcription proteins (JAK/STAT) signaling pathways, and promote the decrease of pro-inflammatory genes expression and cytokine production, as well as the increase of anti-inflammatory genes expression and cytokine production. Furthermore, they can suppress TLR and inhibit phospholipase A2 (PLA2), cyclooxygenase (COX), lipoxygenase (LOX) and inducible nitric oxide synthase (iNOS), leading to a reduction in the production of prostaglandins and leukotrienes. Finally, phenolic compounds can inhibit certain enzymes involved in ROS production like XOx and NOx, while they up-regulate other endogenous antioxidant enzymes like SOD, CAT and GSHPx (de-Lavor et al., 2018; Yahfoufi et al., 2018; Quintans et al., 2019).

Therefore, the results obtained in our study are totally in line with previous results because a dose-dependent decrease in ROS production by HK leucocytes was observed, as well as immunomodulation through decreasing the expression of pro-inflammatory genes and increasing anti-inflammatory genes at higher concentrations of NICs, corroborating the results reviewed by Yahfoufi et al. (2018), where polyphenols inhibited COX and iNOS enzymes in macrophages resulted in a decrease of prostaglandins, leukotrienes, NO, tumor necrosis factor alpha (TNFα) production and *il1b* and *il6* genes expression. Furthermore, polyphenols have been seen to inhibit NOx as well XOx, decreasing ROS production in human monocytes (Yahfoufi et al., 2018). This reduction of ROS production by macrophages has been also reviewed by de-Lavor et al. (2018), although against a microbial infection, essential oils stimulated ROS production in human neutrophils (reviewed by Quintans et al., 2019).

## *In vivo* effects of Nature-identical compounds incorporated in gilthead seabream diet

Organic acids, which are used as food preservatives due their antibacterial and antifungal activities, have been used as feed additives as an alternative to antibiotics and growth promoters, especially in pigs (Partanen and Mroz, 1999; Mroz, 2003). They have also been used although in broilers (Pirgozliev et al., 2008) and positive growth promoting results were obtained, although generally lower than those obtained using antibiotics (Partanen and Mroz, 1999; Mroz, 2003).

In this sense, some studies have been done using organic acids alone (Pirgozliev et al., 2008), plant pure phytochemicals alone (Wei et al., 2017) and both organic acids and pure phytochemicals together (Piva et al., 2007b; Grilli et al., 2015), as dietary alternative to antibiotics (Grilli et al., 2015; Wei et al., 2017). Therefore, although some studies have been done using organic acids together with NICs, including monoterpenols and phenolic aldehyde, as dietary additives in pigs (Piva et al., 2007a; Grilli et al., 2010) and chickens (Grilli et al., 2011), we wished to test different concentrations of the same blend of NICs used *in vitro* with HK leucocytes as feed additive for gilthead seabream in two different trials, so fish growth and immune (humoral, cellular and gene expression) and antioxidant status (gene expression) were studied. In the first *in vivo* experiment three NIC diets (25, 50 and 100) were administered for 8 weeks. After analysis of the results, in the second *in vivo* trial only two selected NICs diets (50 and 100) were used for 4 weeks before a challenge with *V. harveyi*.

As to the effect of a NICs diet on growth performance, after 4 weeks of the experiment, an increase in WG and SGR using the 50 and 100 NICs diets was observed, although only in the case of 50 NICs diet was the increase significant. These results were corroborated in the second trial before and after challenge, where 50 and 100 NICs diets increased WG, SGR and K with respect to the control group. Although the use of organic acids (Partanen and Mroz, 1999; Mroz, 2003) and phytochemicals (Ibrahim et al., 2018) is related with improvements in growth promotion, studies using organic acids alone (Pirgozliev et al., 2008) or NICs (Piva et al., 2007b; Grilli et al., 2010, 2015) had no significant effect on growth performance of broilers or pigs, respectively, although in the study of Grilli et al. (2010) better growth performance was observed from weeks 2 to 6. The

significant improvement of growth performance in our study may have been due to the capacity of organic acids to decrease pH of the diet, increasing gastrointestinal acidity, pancreatic enzyme activity and secretion, and protein and nutrient digestibility, which would allow the promotion of beneficial bacteria and microbial metabolism, leading to the better absorption of nutrients and reducing the proliferation of pathogenic bacteria (Partanen and Mroz, 1999; Mroz, 2003). Furthermore, organic acids may improve feed quality (Partanen and Mroz, 1999), can be metabolized to supply energy, improve the peripheral glucose utilization, help to develop the intestinal mucosa (Grilli et al., 2010) and protect the epithelial integrity of the gastrointestinal tract, improving the ability of the enterocyte to absorb nutrients and enhancing growth (Grilli et al., 2015). However, factors such as type and dose of organic acids used, their characteristics and utilization by gut microflora, the age of the animals treated and their health status among many other factors can influence the final effect of organic acids in the diet (Mroz, 2003), as can the treatment time (one month in most cases) (Wei et al., 2017).

Regarding the results of humoral immune parameters, the three NICs diets used in the first trial increased serum IgM level after 2, 4 and 8 weeks, although this increase was only significant at 2 weeks in fish fed the 100 NICs diet. For its part, serum hemolytic complement activity was not significantly affected by any diet, although an increase in fish fed the 25 and 50 NICs diets was observed. Therefore, positive results were obtained for serum humoral immune response. However, in the second trial, both 50 and 100 NICs diets significantly decreased serum IgM level 2 weeks after vibriosis challenge, while the 50 NICs diet also significantly decreased serum hemolytic complement activity 1 day after challenge of fish. Therefore, the positive results of the first trial could not be confirmed in the second.

Regarding cellular immune response, the 50 NICs diet significantly increased HK leucocyte phagocytic ability after 8 weeks in the first trial, while the three NICs diets significantly increased phagocytic capacity at the same time. As to respiratory burst, although the 25 NICs diet decreased and the 50 and 100 NICs diets increased respiratory burst activity at 2 weeks, only the 25 NICs diet decreased it significantly at 8 weeks. In the second trial, phagocytic ability was increased by the 50 and 100 NICs diets at 4 weeks and 1 day post-challenge, although only significant so 2 weeks post-infection. For its part, the 100 NICs diet significantly decreased phagocytic capacity at 4 weeks and increased it in a non-significantl way 1 day post-infection. Therefore, the NICs diet improved the number of

macrophages and acidophils that are able to phagocytose and therefore, combat infections. In the literature, the effect of the administration of polyphenols *in vivo* has been associated with changes in the differentiation of specific immune cells such as Th1, 9 and 17, natural killer (NK), macrophages and dendritic cells (DCs), as well as Treg (suppressor T cells) (reviewed by Yahfoufi et al., 2018). In our study, a clear positive effect was observed before and, especially, after challenge of fish with *V. harveyi*, increasing the number of phagocytic leucocytes, improving fish defenses against bacterial infection.

Finally, immune and antioxidant status were studied by gene expression in gut (proximal and distal in the first trial, and only proximal in the second trial). As to immune related genes, no differences were observed in pro- or anti-inflammatory gene expression in the proximal gut of fish fed NICs diets with respect control fish at any assayed time, although a non-significant increase was observed in anti-inflammatory *il10* gene in fish fed the 100 NICs diet after 2 weeks. However, in the distal gut, all NICs diets produced a decrease in pro-inflammatory gene expression at 2 weeks, although only in the case of *il6* was the decrease significant. Of note is that the three NICs diets increased, although not significantly, *il6* gene expression at 8 weeks. No differences were obtained in anti-inflammatory gene expression. In the case of the second trial, proximal gut did not show significant differences in pro-inflammatory gene expression, although it is important to highlight an increase of *il6*, *il8, tnfα* and, especially, *il7* by both 50 and 100 NICs after vibriosis challenge. As to anti-inflammatory gene expression, the 100, and especially the 50 NICs diet increased *tgfb* gene expression 15 days post-challenge. The use of essential oils *in vivo* has already pointed to the anti-inflammatory properties of compounds present in them, which are able to modulate the immune status by increasing anti-inflammatory gene expression and cytokine production and decreasing pro-inflammatory gene expression and cytokine production by inhibiting the NF-κB signaling pathway and MAPK cascade (extracellular signal-regulated kinases [ERK], stress-activated protein kinase/c-Jun amino-terminal kinases [SAP/JNK] and p38MAPK) induced by TLRs (de-Lavor et al., 2018; Yahfoufi et al., 2018; Quintans et al., 2019). Similar results were obtained in our study, where a trend for pro-inflammatory genes expression to decrease and anti-inflammatory gene expression to increase was observed. Furthermore, the immunomodulatory capacity of phytochemicals (Paduch et al., 2007; de-Lavor et al., 2018; Pateiro et al., 2018; Yahfoufi et al., 2018; Quintans et al., 2019) was also observed in our study, where an increase in pro-inflammatory gene expression, together with an increase in

anti-inflammatory genes, were observed after challenge with *V. harveyi,* suggesting controlled development of the immune response to bacterial infection. This controlled immune response was also observed by Grilli et al. (2015), who described a decrease in the expression of gut pro-inflammatory *il6*, *il12* and *ifnγ* genes expression as well as anti-inflammatory *tgfb* gene in weaned pigs treated with organic acids and pure monoterpene and phenolic aldehyde, or by Wei et al. (2017), who observed that a blend of monoterpenes decreased *tnfα* and *il6* gene expression induced by weaning in pigs.

As regards antioxidant gene expression, no differences were observed in proximal gut at any assayed time in the first trial. In the case of distal gut, all NICs diet produced a lower expression of antioxidant genes expression although this was not significant at 2 weeks, while the 100 NICs diet increased, but not significantly, at 4 and 8 weeks. No differences were obtained in the second trial, although *cat* increased in a non-significant manner in fish fed the NICs diet 15 days post-challenge. Previous studies have shown that the addition of essential oils or some of their compounds as dietary additives play a role in the animal antioxidant status, increasing antioxidant enzyme gene expression and activity such as CAT, SOD and GSHPx as well as their own capacity to eliminate ROS by direct scavenging activity after induction of a pathological processes, such as inflammation, contributing in this way to combating inflammation (de-Lavor et al., 2018; Yahfoufi et al., 2018). A blend of monoterpenes decreased ROS and TBARS levels induced by weaning in pigs by increasing SOD and GSHPx (Wei et al., 2017). Therefore, although no significant differences were observed, the results suggest fish fed NICs diets are able to stimulate immune and antioxidant status to combat infection better than fish fed a control diet.

Finally, our last objective was to study the effect of a blend of NICs administered to gilthead seabream against *V. harveyi* infection. No mortalities were observed for any of the assayed diets although, as we have previously commented, fish fed NICs increased phagocytic ability and immune response after challenge. Regarding the literature, there has been only one study carried out using organic acids and NICs (monoterpene) against *Salmonella hadar* and *S. enteritidis* infection in chickens (Grilli et al., 2011). In that study, chicken fed with organic acids and (0.03 – 0.5%) NICs showed a decrease in the cecal content of *Salmonella*. Furthermore, pigs fed a diet containing organic acids and NICs (phenolic aldehyde) showed a decrease intotal coliforms in caudal jejunum and more lactic acid bacteria in cecum than control group (Piva et al., 2007a) as did pigs fed diets containing

organic acids and pure monoterpene, which showed a reduction in total coliforms in caecum compared with control pigs, while both organic acid or monoterpene alone did not have any effect (Piva et al., 2007b), suggesting synergistic activity. For its part, a blend of pure monoterpenes reestablished the oxidative stress induced by weaning (increasing *Lactobacillus* genus population and reducing populations of *E. coli* and *Enterococcus* genus in the jejunum of pigs). According to Wei et al. (2017), controlling perturbations in gut microbiota, which are associated with inflammatory response, is another way that phytochemicals control inflammatory process. On the other hand, in the study of Pirgozliev et al. (2008), chickens fed a dietary additive of organic acids alone showed less lactic acid bacteria and coliforms than the control group, demonstrating the strong antibacterial activity of organic acids and their effect on gut microbiota (Piva et al., 2007a). As to the antibacterial mechanism of organic acids, this is based on their molecular weight, pKa (acid dissociation constant measured logarithmically) and number and types of side chains (Grilli et al., 2011). Organic acids are able to decrease the pH of the diet, which liberates $H^+$ ions, contributing to preventing bacterial colonization and activating pepsinogen that inhibits bacterial growth. Furthermore, they can change from undissociated (entering cells by diffusing across cell membrane) to dissociated forms (suppressing cell decarboxylases, catalases, peroxidases, enzymes involved in the metabolism of carbohydrates [enolase and lactic dehydrogenase] in the trichloroacetic acid cycle or enzymes containing sulfhydryl (SH) groups, and nutrient transport systems, so inhibiting their growth) (Sofos et al., 1986; Partanen and Mroz, 1999; Mroz, 2003). However, since the compounds present in essential oils the present antibacterial activity, results obtained in these experiments are probably the consequence of synergistic activity between organic acids and phytochemicals, especially monoterpenes, which can also affect membrane permeability and facilitate the passage or organic acids and allow ion leakage and membrane disruption. Furthermore, these phytochemicals also present anti-inflammatory and antioxidant activity, which allow them to inhibit bacterial growth (Piva et al., 2007a; Grilli et al., 2011; Grilli et al., 2015).

# Conclusions

1. Extracts of oregano, purslane, date palm seeds and moringa are good candidates for incorporation as additives in functional diets for farmed fish. Aqueous extracts of oregano and purslane as well as ethanolic extracts of oregano increase phagocytic activity of gilthead seabream HK leucocytes, while aqueous extracts of date palm seeds and ethanolic extracts of moringa increase their viability.
2. Aqueous extracts of oregano and date palm seeds as well as ethanolic extracts of moringa increase the proliferation of the gilthead seabream fin SAF-1 cell line, suggesting they could promote fish wound healing.
3. Aqueous, and especially ethanolic, extracts of oregano, date palm seeds and purslane, as well as moringa aqueous extracts, show important activity against a fish tumour cell line and could be considered good candidates to be employed in the treatment of fish cancer.
4. Aqueous, and especially ethanolic extracts of oregano, purslane and moringa are very good alternatives for the treatment and control of vibriosis infections due strong antibacterial activity they present against these bacteria.
5. All tested plant extracts, mainly aqueous ones, have potent antioxidant properties and could be included to be included in fish diets in order to avoid lipid peroxidation and increase antioxidant status of fish.
6. Feeding gilthead seabream with diets containing oregano, date palm seeds or dehydrated lemon peel resulted in increased fish antioxidant and immune status. These additives could both aquaculture production and fish quality, fulfilling the objective proposed by FAO to develop a sustainable aquaculture without affecting the environment.
7. The inclusion of dehydrated lemon peel in gilthead seabream diets seems to positively modulate the central metabolism and welfare of fish.
8. Nature identical compounds had strong bactericidal activity *in vitro* against fish pathogenic bacteria and no adverse effects on gilthead seabream HK leucocytes.
9. Nature identical compounds are also interesting candidates as dietary additive because they improved growth performance and immune response to *V. harveyi* infection, especially at cellular level increasing phagocytic activity of HK leucocytes.

# Resumen en castellano

# Introducción

La acuicultura es el sector de producción animal para alimentación con mayor crecimiento a nivel mundial, el cual ha incrementado durante las últimas décadas y está previsto que siga aumentando, aportando casi dos tercios de pescado para alimentación a nivel mundial en el año 2030 (World Bank, 2013; FAO 2018), compensando así el estancamiento sufrido por la actividad pesquera desde la década de 1980 (FAO, 2018). En cuanto a España, en el año 2014 ocupaba la 22ª posición a nivel mundial de producción total de acuicultura (FAO, 2016), mientras que en el año 2017 ya fue el principal productor dentro de la Unión Europea (UE) (al margen de Noruega) (APROMAR 2019).

La dorada es cultivada en toda el área mediterránea y se trata de la 59ª especie más cultivada a nivel mundial y la 3ª en la UE, donde España es el 4º principal productor y la región de Murcia la 2ª comunidad autónoma con mayor producción (APROMAR 2019).

Gracias al gran desarrollo de la acuicultura y a la gran producción de peces, la población mundial puede abastecerse de pescado, sin embargo, esta gran actividad que se desarrolla en las piscifactorías conlleva también ciertos problemas entre los que están un número elevado de peces en las jaulas, empeoramiento de la calidad del agua, manipulación de los peces y aparición de heridas entre otros, lo que conlleva a la aparición de estrés y a un efecto negativo en el sistema inmunitario, provocando inmunodepresión y dando lugar a la aparición de infecciones que pueden llegar a provocar la muerte de los peces y, por tanto, causar grandes pérdidas económicas (Newman, 2000; Harikrishnan et al., 2011; Bulfon et al., 2013; Holmes et al., 2016). Por ello, los peces son vacunados y los antibióticos han sido usados (hasta hace pocos años) con el objetivo de mejorar el sistema inmunitario de los peces para tratar o evitar la aparición de enfermedades o infecciones y la mortalidad de los mismos, aunque ambos tratamientos pueden tener efectos negativos (Cabello, 2006). En este sentido, la vacunación, aunque es el método más efectivo, no tiene éxito frente a patógenos intracelulares, es patógeno-específica, muy cara, estresante para los peces debido a que tienen que ser manipulados manualmente uno a uno y hay muy pocas vacunas comerciales disponibles (Gudding and Van Muiswinkel, 2013). Por su parte, los antibióticos, que han sido usados como profilácticos, promotores del crecimiento y terapéuticamente, han sido añadidos al pienso de los peces, administrados disueltos en el agua o por inyección (Hoa et al., 2011; Rico et al., 2013). Sin embargo, el uso de antibióticos también presenta un gran

número de efectos negativos para los peces, para los humanos que los consuman y para el medio ambiente (Hansa et al., 2015; O´Neill, 2015; Elbashir et al., 2017; Joy et al., 2017). En primer lugar, los antibióticos se acumulan en los tejidos de los peces y pueden ser transmitidos a los humanos a través de la cadena alimentaria (Santos and Ramos, 2016). En segundo lugar, las heces de los peces contienen trazas de antibióticos, por lo que las bacterias marinas están mucho tiempo en contacto con concentraciones no letales de antibioticos (Gullberg et al., 2011; Chen et al., 2015), lo que favorece el desarrollo de bacterias multi-resistentes que acaban causando daño en los animales y en los humanos (Yang et al., 2013; Gauthier, 2015; Kumar et al., 2017).

Debido a los problemas derivados del uso de los antibióticos, uno de los pilares dundamentales de la FAO es el desarrollo sostenible económico, social y ambiental de la acuicultura, para lo que se buscan posibles sustitutos de los antibióticos, como por ejmplo, compuestos inmunoestimulantes naturales que puedan ser usados como medidas profilácticas para fortalecer el sistema inmunitario de los peces (FAO, 2018). En este sentido, la fitoterapia (uso de plantas medicinales para la prevención o tratamiento de una gran variedad de enfermedades e infecciones, o para mantener el buen estado de salud) se ha convertido en una muy buena e interesante alternativa al uso de antibióticos, debido a los compuestos biológicos presentes en las plantas (fitoquímicos) ya que además de los potentes efectos que poseen, no tienen impacto negativo ni sobre los animales ni sobre los humanos o el medioambiente y son de bajo costo (Direkbusarakom, 2004; Citarasu, 2010; Harikrishnan et al., 2011; Bulfon et al., 2013; Reverter et al., 2014; van-Hai, 2015; Awad and Awaad, 2017).

Las plantas medicinales se han utilizado durante miles de años en la medicina tradicional de todo el mundo para tratar muchas enfermedades e infecciones (Tan and Vanitha, 2004; Hao and Xiao, 2015; Yuan et al., 2016) e incluso, a día de hoy, casi el 80% de la población mundial, especialmente la de países en vías de desarrollo o subdesarollados, sigue usando plantas medicinales para el tratamiento de enfermedades (Mahady, 2001; Jaradat, 2015; Ajlan, 2016). Además, debido al contenido de hidratos de carbono, proteínas, ácidos grasos, amino ácidos, minerales, vitaminas y fibras que poseen las plantas medicinales (Chang, 2000; Vayalil, 2012; Zhou et al., 2015; Alegbeleye, 2018; Falowo et al., 2018), éstas tienen también un importantísimo valor nutricional y son consumidas por millones de personas en todo el mundo, representando también una alternativa para combatir

la malnutrición en países donde el hambre es un verdadero problema (Vayalil, 2012; Saini et al., 2016; Iranshahy et al., 2017; Alegbeleye, 2018). Esto último también puede ser interesante para los peces desde el punto de vista nutricional (Cuesta et al., 2005), pudiendo tener efectos positivos tales como la estimlación del crecimiento o la maduración temprana (Immanuel et al., 2004; Sivaram et al., 2004; Citarasu, 2010; Charakborty and Hancz, 2011; Harikrishnan et al., 2011a; Pavaraj et al., 2011; Bulfon et al., 2013; Newaj-Fyzul and Austin, 2015). Además, estas plantas medicinales pueden ser administradas oralmente a través de su adicción al pienso de los peces, lo que permite tratar a un elevado número de peces en muy poco tiempo sin provocarles estrés (Sakai, 1999; Harikrishnan et al., 2011).

## Objetivos

El objetivo de la presente Tesis Doctoral es determinar si es posible el uso de plantas, o algunos compuestos presentes en ellas, para la aplicación directa en la acuicultura de peces, y en particular de dorada.

Concretamente, los objetivos específicos del presente trabajo son:

1. Evaluar los efectos *in vitro* de diferentes extractos de plantas en las actividades de los leucocitos de riñón cefálico de dorada y evaluar su actividad citotóxica, bactericida y antioxidante.

2. Evaluar los efectos *in vivo* de distintas plantas como aditivos alimentarios para dorada.

3. Evaluar la actividad bactericida y el efecto *in vitro* sobre la actividad inmunitaria de leucocitos de riñón cefálico de dorada de compuestos idénticos a los naturales.

4. Evaluar el efecto *in vivo* de compuestos idénticos a los naturales como aditivos alimentarios para dorada.

## Principales resultados y discusión

Debido a que el objetivo final de esta Tesis Doctoral es determinar el posible uso de plantas, o de alguno de sus componentes, en la acuicultura de dorada y debido también a la ausencia de estudios *in vitro* sobre el efecto de las plantas en células de peces, esta Tesis Doctoral se ha dividido en 3 partes.

Primera parte (Capítulo 1): se evaluó el efecto *in vitro* de extractos acuosos y etanólicos de orégano, hueso de sátil, portulaca y moringa en varios tipos celulares, así como frente a bacterias, analizando también su actividad antioxidante. Con el objetivo de testar la toxicidad de diferentes dosis y su capacidad inmunoestimulante, se incubaron los diferentes extractos a diferentes concentraciones (0.001 mg/mL – 1 mg/mL) con células inmunitarias de dorada (leucocitos de riñón cefálico) y con células de la línea SAF-1 y se observó que si se emplean a las concentraciones adecuadas, poseen capacidad inmunoestimulante y promueven la división celular.

Debido a la demostrada actividad antitumoral de ciertas plantas (Chavan et al., 2013; Monteiro et al., 2014; Solowey et al., 2014; Greenwell and Rahman, 2015; Reyad-ul-Ferdous et al., 2016; Ijaz et al., 2018; Rahman, 2018), en segundo lugar se estudió la citotoxicidad de los extractos frente la línea celualr PLHC-1 (una línea celular tumoral de pez) y se confirmó la capacidad de inhibición del crecimiento de células tumorales que estas plantas poseen.

Como otros estudios ya han reflejado, la actividad antibacteriana de las plantas es de sobra conocida (Nazzaro et al., 2013; Pandey, 2013; Borges et al., 2015; da-Cunha et al., 2018), por lo que también fue analizada estudiando la capacidad de inhibición del crecimiento de tres bacterias patógenas de peces (*V. harveyi*, *V. anguillarum* y *P. damselae* subsp. *piscicida*), demostrando también su posible uso como agente bactericida.

Por último, debido a que la actividad antioxidante es posiblemente la actividad más conocida que poseen las plantas (Lee et al., 2017a; Amorati and Valgimigli, 2018; Aniya et al., 2018; de-Lavor et al., 2018; Pateiro et al., 2018; Pohl and Lin, 2018; Oyenihi and Smith, 2019), se estudió la actividad antioxidante de los extractos, que fué también puesta de manifiesto.

Segunda parte (Capítulo 2): se evaluó el efecto *in vivo* de orégano, hueso de dátil y piel de limón como aditivos alimentarios de dorada (incluidas en el pienso de dorada).

Debido a que hay estudios que establecen que el uso de plantas medicinales podría mejorar las enzimas digestivas, optimizando con ello la digestibilidad y la disponibilidad de nutrientes, y por lo tanto incrementar la utilización de la comida, la síntesis de proteínas y el crecimiento de los peces (Nya and Austin, 2009; Citarasu, 2010; Takaoka et al., 2011; Awad et al., 2012), estudiamos el efecto de las dietas experimentales sobre el crecimiento de los peces. En este caso, ninguna de las plantas estudiadas lo aumentó, quizás debido al corto plazo de tiempo que duraron los ensayos.

De acuerdo a las revisiones echas por Harikrishnan et al. (2011), Bulfon et al. (2013), Reverter et al. (2014), van-Hai (2015) y Awad and Awaad (2017), las plantas son capaces de potenciar el sistema inmunitario de los peces, tanto a nivel humoral como celular, mejorando la respuesta a infecciones, al estrés y mejorando la supervivencia de los peces. Por lo tanto, el siguiente paso fue la evaluación del efecto de la adición de las plantas en la dieta de dorada sobre el sistema inmunitario innato y adaptativo. Se estudiaron factores humorales (nivel de IgM, actividad del complemento, lisozima, bactericida, proteasa, antiproteasa y peroxidasa en suero y moco de la piel) y celulares (actividad fagocítica, explosión respiratoria y peroxidasa de leucocitos de riñón cefálico). A su vez, el efecto de las plantas también se estudió sobre la actividad de enzimas antioxidantes del hígado (GR, SOD y CAT) y la actividad antioxidante total en suero y moco de la piel. Finalmente, la expresión de genes inmunitarios en riñón cefálico y de genes antioxidantes en hígado también fue analizada. Resultados positivos fueron obtenidos tanto a nivel de respuesta inmunitaria humoral como celular, al igual que una tendencia a mejorar el estado oxidativo de los peces. Por su parte, a nivel de expresión génica, los genes relacionados con el sistema inmunitario fueron estimulados en peces alimentados con las dietas enriquecidas con plantas. Para acabar este capítulo, se estudió el efecto de la piel de limón sobre el metabolismo de los peces y se observó que tras ser alimentados con la dieta durante dos semanas, se observó una disminución de los niveles de estrés, aunque los efectos no se mantuvieron tras cuatro semanas. Se concluye que esta dieta no tuvo ningún efecto perjudicial en el metabolismo de los peces.

Tercera parte (Capítulo 3): se evaluó el efecto *in vitro* e *in vivo* de compuestos idénticos a los naturales ("fitoquímicos artificiales").

En primer lugar, se evaluó la actividad bactericida *in vitro* de estos "fitoquímicos artificiales" frente a *V. harveyi* y *V. anguillarum* y la toxicidad y actividad inmunoestimulante de varias concentraciones (50 mg/L – 1000 mg/L) de una mezcla de ellos sobre leucocitos de riñón cefálico de dorada. Los fitoquímicos mostraron una potente actividad antibacteriana y no tuvieron ningún efecto adverso sobre los leucocitos.

En segundo lugar, se evaluó el efecto *in vivo* de la adición al pienso de dorada de diferentes concentraciones de la misma mezcla de fitoquímicos usada en el ensayo in *vitro*. Determinamos sus efectos sobre el crecimiento de los peces, sobre la inmunidad y el estado oxidativo de los mismos, después de someter a los peces a una infección bacteriana. La mezcla de fitoquímicos empleada mejoró el crecimiento de los peces y resultó inmunoestimular a las doradas.

## Conclusiones

1. Los extractos de orégano, semillas de palmera datilera, verdolaga y moringa son buenos candidatos para su incorporación como aditivos en dietas funcionales para peces. Los extractos acuosos de orégano y verdolaga, así como extractos etanólicos de orégano, aumentan la actividad fagocítica de los leucocitos de riñón cefálico de dorada, mientras que extractos acuosos de semillas de palmera datilera y extractos etanólicos de moringa aumentan su viabilidad.

2. Los extractos acuosos de orégano y semillas de palmera datilera, así como extractos etanólicos de moringa, aumentan la proliferación de la línea celular de dorada SAF-1, lo que sugiere que podrían ser empleados para promover la curación de heridas de peces.

3. Los extractos acuosos, y especialmente etanólicos, de orégano, semillas de palmera datilera y verdolaga, así como los extractos acuosos de moringa, muestran una actividad importante contra una línea celular tumoral de peces y podrían considerarse buenos candidatos para ser empleados en el tratamiento del cáncer.

4. Los extractos acuosos, y especialmente etanólicos, de orégano, verdolaga y moringa son muy buenas alternativas para el tratamiento y control de la vibriosis en peces, debido a la fuerte actividad antibacteriana que presentan contra estas bacterias.

5. Todos los extractos vegetales probados, principalmente los acuosos, tienen potentes propiedades antioxidantes y son candidatos interesantes para ser incluidos en las dietas de peces a fin de evitar la peroxidación lipídica y mejorar el estado antioxidante de los peces.

6. La alimentación de dorada con dietas que contienen orégano, semillas de palmera datilera o piel de limón deshidratada dio como resultado una mejora en el estado antioxidante e inmunitario de los peces. Estos aditivos podrían mejorar tanto la producción acuícola como la calidad de los peces, cumpliendo el objetivo propuesto por la FAO para un desarrollo sostenible de la acuicultura sin afectar el medio ambiente.

7. La inclusión de la piel de limón deshidratada en las dietas doradas parece modular positivamente el metabolismo central y el bienestar de los peces.

8. Los compuestos idénticos a los naturales mostraron una fuerte actividad bactericida *in vitro* contra bacterias patógenas de peces y no tuvieron efectos adversos sobre los leucocitos de riñón cefálico de dorada.

9. Los compuestos idénticos a los naturales también son candidatos interesantes como aditivos dietéticos ya que mejoraron el crecimiento de los peces y la respuesta inmunitaria a la infección por *V. Harvey*, especialmente a nivel celular aumentando la actividad fagocítica de los leucocitos de riñón cefálico.

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