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Effects of purslane (*Portulaca oleracea* L.) and *Shewanella putrefaciens* probiotic enriched diet on gilthead seabream (*Sparus aurata* L.)

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(Maria Camara Ruiz)

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RESUMO/ABSTRACT

Resumo

A intensificação da aquicultura tem vindo a produzir uma série de efeitos prejudiciais, tanto para o meio ambiente como para a saúde humana. Um grande problema da intensificação dos sistemas de aquicultura têm sido as perdas económicas causadas pelas altas taxas de mortalidade devido às doenças. A fim de prevenir e / ou tratar estas doenças, grandes quantidades de medicamentos veterinários foram administrados nas últimas décadas em explorações piscícolas. No entanto, a administração excessiva de antibióticos e outros produtos químicos mostrou causar efeitos adversos. Um dos métodos mais promissores de controlo de doenças em peixes é através da estimulação dos seus mecanismos naturais de defesa com a administração de imunoestimulantes. O objetivo do presente estudo foi avaliar os potenciais efeitos da suplementação dietética de uma planta medicinal (*Portulaca oleracea* L.), vulgarmente conhecida como beldroega, isoladamente ou em combinação com um probiótico (*Shewanella putrefaciens*, SpPdp11) sobre o desempenho no crescimento e no estado imunológico (tanto sistémico como a nível da mucosa) da dourada (*Sparus aurata* L.). Os peixes foram alimentados com uma dieta controlo (CD) ou com uma das dietas suplementadas (PD e MIXD) durante 30 dias. Após 15 e 30 dias de tratamento, o desempenho no crescimento e nos parâmetros celulares e humorais foram determinados em leucócitos do rim cefálico, soro, muco da pele e homogeneizados intestinais. Concomitantemente, a expressão de vários genes (*ef1a, il-1b, igm, hep, bd, tcrb, csfr1, alp)* relacionados com o sistema imune foi também avaliada no rim cefálico, pele e intestino. Os resultados demonstraram que a suplementação dietética com beldroega (*P. oleracea*) durante 30 dias aumentou a capacidade fagocítica dos leucócitos, os níveis de imunoglobulina M totais no muco da pele e a atividade de certas enzimas nos homogeneizados de soro (protease) e intestino (protease e antiprotease). Além disso, curiosamente a expressão de *igm* foi regulada no rim, principal após 15 dias de administração da dieta. Estes resultados sugerem que a suplementação dietética com beldroega tem a capacidade de modular vários parâmetros do sistema imunitário e da mucosa da dourada.

Palavras-chave: *Portulaca oleracea*, *Shewanella putrefaciens*, plantas medicinais, probióticos, imunoestimulantes, imunidade, dourada (*Sparus aurata*), aquicultura.

Abstract

The intensification of aquaculture has resulted in a series of detrimental effects to both the environment and human health. A major setback of the intensification of aquaculture systems has been the economic losses caused by the high mortality rates due fish diseases. In order to prevent and/or treat fish diseases, large amounts of veterinary drugs have been administered over the last few decades in fish farms. However, the excessive administration of antibiotics and other chemicals has been shown to cause adverse effects. One of the most promising methods of controlling fish diseases is by enhancing their natural defence mechanisms with the administration of immunostimulants. The aim of the present study was to evaluate to potential effects of dietary supplementation of a medicinal plant (*Portulaca oleracea* L.) alone or in combination with a probiotic (*Shewanella putrefacien*s, SpPdp11) on growth performance and the immune status (at both systemic and mucosal level) of gilthead seabream (*Sparus aurata* L.). Fish were fed a control (CD) or one of the supplemented diets (PD and MIXD) for 30 days. After 15 and 30 days of the feeding trial, growth performance, and cellular and humoral parameters were determined in head-kidney leucocytes, serum, skin mucus and intestine homogenates. Concomitantly, gene expression of several immune-related genes (*ef1a, il-1b, igm, hep, bd, tcrb, csfr1, alp*) were also evaluated in head kidney, skin and intestine. Our results demonstrated that the dietary administration of purslane (*P. oleracea*) for 30 days increased phagocytic capacity in head kidney leucocytes, total immunoglobulin Mlevels in skin mucus and the activity of certain enzymes in serum (protease) and intestine homogenates (protease and antiprotease). Moreover, interestingly *igm* expression was up-regulated in head kidney after 15 days of diet administration. These results suggest that the dietary administration of purslane has the ability to modulate several immune parameters of the systemic and mucosal immunity of gilthead seabream.

Keywords: *Portulaca oleracea*, *Shewanella putrefacien*s, medicinal plants, probiotics, immunostimulants, immunity, gilthead seabream (*Sparus aurata*), aquaculture.

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List of Abbreviations

% WG- weight gain percentage
BSA- bovine serum albumin
CD- control diet
º C- celsius
cfu – colony forming unit
DNA-deoxyribonucleic acid
DNAse I-deoxyribonuclease I
FAO- Food and Agriculture Organization of the United Nations
FCS-fetal calf serum
FSC- forward scatter
GALT- gut-associated lymphoid tissue
GIALT-gill-associated lymphoid tissue
GVB- isotonic veronal buffered saline
H_2O_2- hydrogen peroxide
HBSS- hanks balances salt solution
HEWL- hen egg white lysozyme
HK- head kidney
IgD – Immunoglobulin D
IgM – Immunoglobulin M
IgT- Immunoglobulin T
LAB-lactic acid bacteria
MALT- mucosa-associated lymphoid tissue
MIXD- mixture diet
MMCs- melanomacrophage centers
MS-222- tricaine mesylate
NaCl- sodium chloride
NALT- nasopharynx-lymphoid tissue
PBS-phosphate buffered saline
PCR- polymerase chain reaction
PD- purslane-supplemented diet
PMA-phorbol myristate acetate
RaRBC- rabbit red blood cells
RNA- ribonucleic acid
SALT- skin associated lymphoid tissue
SE- standard error
SGR-specific growth rate
sRPMI-RPMI-1640 culture medium
SSC- side scatter
TCA-trichloro acetic acid
TMB-3,3’,5,5’- tetramethylbenzidine hydrochloride

TSB- trypticase soya broth
Wf- final weight
Wi -initial weight

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LITERATURE REVIEW

1. Literature review

1.1. Introduction

Aquaculture, also known as aquafarming, is defined as the breeding, rearing, and harvesting of aquatic animals and/or plants (fish, shellfish, seaweed), in natural or controlled marine or freshwater environments. Aquaculture is currently the fastest growing animal food-producing sector in the world. With capture fisheries remaining relatively static since the late 80s, aquaculture has experienced an impressive growth because of the high demand of fish products for human consumption (Fig. 1). Thus, this industry represents an important source of food, healthy protein, income and livehoods for millions of people worldwide. Aquaculture currently provides approximately half of all the fish for human consumption (even higher than wild catch) for the first time ever while relieving some pressure of certain fish stocks at the same time (FAO, 2016).

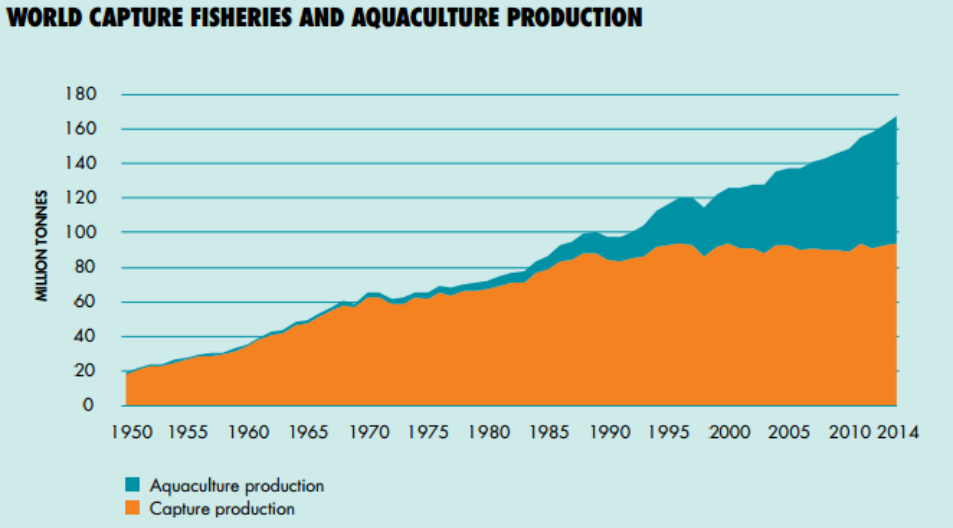

Figure 1. World capture fisheries and aquaculture production (FAO, 2016).

In numbers, aquatic animals harvested from aquaculture reached 73.8 million tonnes with an estimated first-sale value of US $ 160.2 billion in 2014, consisting of 49.8 million tonnes of finfish (US $ 99.2 billion), 16.1 million tonnes of molluscs (US $ 19 billion), 6.9 million tonnes of crustaceans (US $ 36.2 billion), and 7.3 million tonnes of other aquatic animals including frogs (US $ 3.7 billion) (FAO, 2016). China, the world top aquaculture producer, accounted for 45.5 million tonnes in 2014, which represents more than 60 percent of global fish production from aquaculture. Other major producers include India, Vietnam, Bangladesh and Egypt (FAO, 2016).

More than 500 species are currently being farmed all over the world. The most harvested species in aquaculture in recent years have been the Pacific cupped oyster

(*Crassostrea gigas* L.) and the silver carp (*Hypophthalmichthys molitrix*). In the Mediterranean, gilthead seabream (*S. aurata* L.), belonging to the *Sparidae* family, is one of the most important fish species being cultured. World production was reported around 160,000 tonnes per year. This species is very common in the Mediterranean Sea, being present along the Eastern Atlantic coasts from Great Britain to Senegal. Most production occurs in the Mediterranean, with Greece (49%) being the largest producer, followed by Turkey, Spain and Italy. There is also considerable production in Croatia, Cyprus, Egypt, France, Malta, Morocco, Portugal and Tunisia (FAO, 2016).

Nowadays, the world is facing one of the greatest challenges ever: how to feed more than 9 billion people by 2050 in a very complicated context of climate change and economic uncertainty between other factors. According to FAO, approximately 52% of the 600 wild fish species with economic value are heavily depleted, 17% overfished, and 7% fully exploited. Taking this into account, capture fisheries production will remain relatively static for the next decades while fish production from aquaculture is expected to experience a vigorous growth (as it has already been) for the next decades in order to continue to supply protein to the overgrowing population.

The aquaculture industry still needs to overcome several challenges in order to become more productive, feasible, and sustainable at this impressive rate. One of the main issues associated with intensive aquaculture is the sudden outbreak of diseases, which comes together with the necessity of its fast development and super-intensification. The maintenance of large numbers of fish crowed together causes alterations in behaviour, growth, development and provides a propitious environment for the development and spread of diseases, resulting in partial or total loss of production (Bondad-Reantaso et al., 2005). Global estimation of the economic losses related to diseases by the World Bank in their report from 2014 was estimated to be approximately US $6 billion per year.

Natural and synthetic chemicals such as antibiotics, anti-parasitic agents or disinfectants have become necessary in order to prevent and treat bacterial and parasitic diseases in aquaculture. The use of these substances has contributed to the productivity of the aquaculture sector but also has raised criticism and negative reactions towards the industry. In fact, chemical residues in cultured organisms represent a potential hazard to the consumer (Heuer et al., 2009). Furthermore, continuous application of such

compounds has been associated to the development of drug-resistant bacteria (inside and outside the farms) (Le et al., 2005) and to the potential degradation of the ecosystems near by the aquaculture facilities (Rico et al., 2012).

In conclusion, diseases have become one of the major constraints to sustainable aquaculture production and trade. In general terms, disease outbreaks are associated with fish fitness and health, being most pathogens opportunistic and taking advantage of immunocompromised or stressed fish, breaking the equilibrium between host, environment and pathogen (Defoirdt et al., 2011).

1.2.Fish immune system

The immune system of fish is very similar to other higher vertebrates. However, there are some important differences that must be taken into account. In contrast to other vertebrates, fish are free-living organisms from early stages and thus, they heavily depend on their immune system. Fish are always in contact with a variety of non-pathogenic and pathogenic microorganisms and have developed a variety of mechanisms in order to defend themselves and guarantee their survival (Rombout et al., 2005). The fish immune system is divided into innate (natural or non-specific) and adaptive (acquired or specific) immune system and they are both composed of many different cells and molecules. In particular, non-specific immunity is a fundamental defence mechanism in fish. Nevertheless, every component of the immune system has its own protective value and the final combination of these components is more likely to be related to a satisfactory immune response (Whyte, 2007).

1.2.1. Innate immune system

The innate immune system of fish and other vertebrates, which constitutes the basis of immune defence, is the first line of defence against invading pathogens (Narnaware et al., 1994). The innate immune system's response is determined by the evolutionary lineage and genetic make-up, which has been shaped through time by environmental factors and pathogenic associations (Janeway & Medzhitov, 1998; Carroll & Janeway, 1999; Alvarez-Pellitero, 2008). The innate immunity is characterized by being non-specific, meaning that it does not depend upon previous recognition of the surface structures of the invader. It also has the advantages of being inducible by external molecules, reacts in a very short time scale and induces an inflammatory response. In addition, it also plays a key role in the acquired immune response and homeostasis

through a system of receptor proteins (Magnadottir, 2006).

The fish innate response is commonly divided into three components: physical bariers, cellular and humoral factors which include humoral and cellular receptor molecules that are soluble in plasma and other fluids (Magnadottir, 2006)

1.2.1.1. Physical barriers

Flakes, skin and gills act as the first barrier against infectious agents (Ellis, 2001). It is known that fish skin mucus contains lectins, pentraxins, lysozymes, complement proteins, antibacterial peptides and immunoglobulin M (IgM), which have an important role in inhibiting the entry of pathogens (Alexander & Ingram, 1992; Rombout et al., 1993). Furthermore, the epidermis is able to react to different attacks and its integrity is essential for osmotic balance and to prevent the entry of foreign agents (Hibiya, 1994). Several cells are also present in such physical barriers, such as lymphocytes, macrophages and eosinophilic granular cells (Ellis, 2001; Fischer et al., 2006).

1.2.1.2. Cellular and humoral components

The major components of the innate immune system are classified in cellular (cells) and humoral (molecules) components. The cellular components include phagocytes (macrophages, monocytes, neutrophils), lymphocyte populations that are analogous to T cells and B cells, non-specific cytotoxic cells (similar to mammalian natural killer cells, NK cells), mast cells and dendritic cells (Magnadottir, 2006). The humoral components include lysozyme, complement system and cytokines, among others. In fish, the innate immune system consists of neutrophil activation, production of peroxidase and oxidative radicals, and the initiation of other inflammatory radicals (Ellis, 1977; Ainsworth et al., 1991). Out of all of them, phagocytosis is one of the main mediators of innate immunity to pathogens (Secombes & Fletcher, 1992)

Several internal and external factors can influence innate immune response parameters. For instance, temperature changes, stress management and density may have suppressive effects on this type of response, while several food additives and immunostimulants can enhace their efficiency (Magnadottir, 2006; 2010).

1.2.2. Adaptive immune system

If a pathogen evades the innate defence mechanism, an adaptive immune response will

be initiated. The specific immune system, often referred as adaptive immunity or acquired immunity, is characterized by being able to recognize specific pathogens more efficiently after exposure. In general terms, it is a primary response to a specific pathogen providing an enhanced response on secondary encounters with the same pathogen. However, activation of the acquired immune system is relatively slow in fish (Ellis, 1998).

The adaptive immune response, as the innate system, has humoral and cellular components. The humoral components of the adaptive response are antibodies and cytokines while the cellular components are lymphocytes (T and B cells). Immunoglobulins (to date only IgM, IgD, and IgT described in fish) are a major component of the vertebrate humoral immune system. IgM, mostly present in serum, is the main immunoglobulin present in teleosts (Ellis, 1998).

1.2.3. Lymphoid organs

The fish immune system includes lymphoid organs that are considered either primary or secondary lymphoid organs. Most of the primary and secondary lymphoid organs present in mammals are also found in fish, except from the lymphatic nodules and the bone marrow (Fig. 2) (Evensen, 1999). Instead, the head kidney assumes hematopoietic functions and it is the principal immune organ responsible for phagocytosis (Danneving et al.,1994), antigen processing and formation of IgM (Brattgjerd & Evensen, 1996), and immune memory (Kaattari & Irwin,1985).

As it was previously mentioned, there are two types of lymphoid organs in fish. Primary lymphoid organs include thymus and head kidney that produce and mature stem cells. Secondary lymphoid organs include kidney, spleen, and mucosa-associated lymphoid tissue (MALT). Besides, liver, skin and intestine are also important organs that take part in the immune response (Zapata et al., 2006).

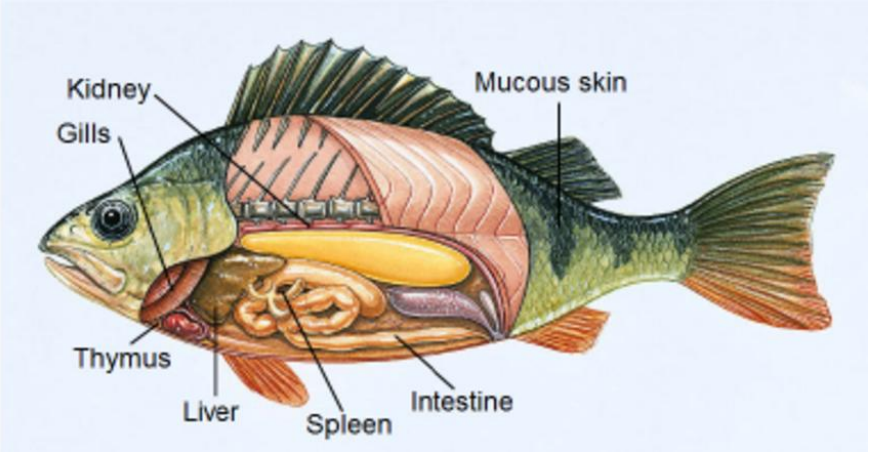

Figure 2. Immune organs in teleost fish (Kum & Sekkin, 2011)

1.2.3.1. Primary lymphoid organs

1.2.3.1.1. Thymus

The thymus is a paired bilateral organ (two lobes), homogeneous, and it is represented by a thin sheet of oval lymphoid tissue that is arranged subcutaneously in the dorsal comissure of the operculum. This organ is situated beneath the pharyngeal epithelium. The structure that characterizes the thymus of fish is a capsule that surrounds the lymphoid bark tissue (Ellis, 2001).

It is defined as a primary lymphoid organ. The thymus can be considered as an aggregation of macrophages that promote the encapsulated proliferation of T cells. It mainly contains T cells and few populations of B cells. Furthermore, myeloid cells and eosinophilic granular cells can be found in this organ (Davis et al., 2002).

1.2.3.1.2. Head kidney

The anterior part of the kidney, often referred to as the head kidney, is significantly important in haematopoiesis and immunity in fish. The head kidney in teleost fish is the equivalent to the bone marrow in vertebrates. Moreover, it is the largest site of haematopoiesis from early development until adulthood (Zapata et al., 2006).

The head kidney is formed by two Y arms, which penetrate underneath the gills. Regarding structure, the anterior kidney is composed of a network of reticular fibres that provide support for lymph tissue. These fibres are found scattered among hematopoietic cells immerse conforming a reticular stroma. The main cells found in the anterior kidney are macrophages, which aggregate into melanomacrophage centers (MMCs),

and lymphoid cells, which are found at all developmental stages and exist mostly as Ig+ cells (B cells) (Press et al., 1994).

In conclusion, the head kidney is a valuable organ with key regulatory functions, the central organ for immune-endocrine and even neuro-immune-endocrine interactions (Evensen, 1999; Tort et al., 2003)

1.2.3.2. Secondary lymphoid organs

Secondary lymphoid organs include kidney, spleen, and mucosa-associated lymphoid tissue.

1.2.3.2.1. Kidney

The kidney also works as a secondary lymphoid organ. The head kidney is a major organ where antibody producing cells are formed (Zapata et al., 2006).

1.2.3.2.2. Spleen

The spleen is classified as a secondary lymphoid organ in fish. It is composed of a system of splenic ellipsoids, MMCs and lymphoid tissue. In most species, ellipsoids are clustered together and are organized around the other two components (Ferguson, 1989). The ellipsoids are thick-walled capillaries that open in the pulp and result from the division of the splenic arterioles. The cells along the walls are actively involved in the macrophage phagocytosis of antigens, usually in the form of antibodies or metabolic products. Antigens may be detained for long periods of time, which has an important role in immunological memory (Zapata et al., 2006).

1.2.3.2.3. Mucosa-associated lymphoid tissue

One of the secondary lymphoid organs is the MALT (mucosa-associated lymphoid tissue). According to anatomical location, the MALT in teleost fish is subdivided into gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT) and nasopharynx-associated lymphoid tissue (NALT) (Salinas, 2015).

In general, fish MALT has defence mechanisms (both innate and adaptive) that constitute the first line of defence against infectious agents and work together to maintain homeostasis at the mucosa (Esteban, 2012). In particular, B cells, plasma cells and Igs have specialized to defend the complex environment that defines mucosal

barriers (Salinas, 2015).

1.3. Prophylactic measures against diseases outbreaks

1.3.1. Chemotherapy

In order to cope with the problem of disease outbreaks in aquaculture, fish farms have routinely administered excessive numbers of antimicrobial agents, such as antibiotics, as additives in fish food and sometimes in baths and injections (Rico et al., 2013). Antibiotics are drugs of natural or synthetic origin, that are able to kill or inhibit the growth of different microorganisms. Several antibiotics have been successfully applied to treat fish diseases including amoxicillin, erythromycin or oxytetracycline between others (Smith et al., 1994; Agnew & Barnes, 2007).

However, this practice might result in antibiotics entering into the environment by leaching from uneaten foods, or unabsorbed parts in aquatic animals and potentially could have detrimental side effects. The application of antibiotics and chemotherapeutics in aquaculture has several negative impacts like immunosuppression and residue accumulation in tissues (Rijkers et al., 1980; Harikrishnan et al., 2009a,b). Another big issue associated to this practice is the emergence of antibiotic-resistant bacteria, making treatments less effective. Furthermore, the transference of resistant genes between bacteria could also potentially affect human health negatively (Kesarcodi-Watson et al., 2008). In fact, the impact of antibiotic-resistant bacteria on human health has become a major international concern. In order to avoid these problems and to prevent the dependence of aquaculture on antibiotics, vaccines have been considered as an alternative to chemotherapeutics.

1.3.2. Vaccines

Currently, vaccination might be the most effective prophylactic measure for controlling fish diseases. Several vaccines are already commercially available against some bacterial and viral diseases affecting cultured fish. However, they are significantly expensive and they are only effective towards a single pathogen because of the complex antigenic structure of the pathogens (Raa et al., 1996; Robertsen, 1999; Sakai, 1999). For these reasons, vaccination is somehow limited in aquaculture farming. Also, this approach only induces the adaptive/specific immunity against a particular pathogen, and has not been so far successful for intracellular pathogens (Sakai, 1999). Furthermore, to

our days, the causal agent(s) of some diseases or syndromes has/have not been determined yet. In this context, immediate control of all fish diseases is impossible due to the existence of a wide range of pathogens in the aquatic environment. Thus, monovalent vaccines are an insufficient method for controlling diseases.

To sum up, antibiotics and vaccines are used for the treatment (therapeutic use) and prevention (prophylaxis) of fish diseases. In general, the current methods applied in aquaculture to treat microbial diseases are highly problematic, and neither effective nor cost efficient. With chemotherapeutics, large amounts of chemotherapeutic agents are administered and then discharged into the environment, having negative impacts. Regarding vaccines, there are several problems associated with the cost and their relative efficiency, which makes their use limited. In this context, the industry demands alternative preventive practices that can potentially help maintaining animal welfare and that also do not damage the environment while obtaining better production and higher profits.

1.4.Alternatives to chemotherapeutics and vaccines

As an alternative to the use of chemical agents and vaccines, dietary administration of probiotics, prebiotics and natural immunostimulants has been considered in aquaculture. By definition, immunostimulants or immunostimulators, are substances (chemical drugs, nutrients) that are able to enhance both non-specific and specific immune response by inducing activation or increasing activity of any of its components and increase the host's resistance against diseases that in most circumstances are caused by pathogens (Cao et al., 1999). For instance, vaccines are considered immunostimulants although they only enhance the specific immune system. Nowadays, the enhancement of the immune system is the most promising method of preventing fish diseases.

Immunostimulants can be divided into several groups depending on their source: bacterial, algae-derived, nutritional factors and hormones/cytokines. However, this grouping is independent of their mode of action (Sakai, 1999).

1.4.1. Probiotics

Probiotic administration has been evaluated as a potential alternative to antibiotics and chemotherapeutics in aquaculture. Probiotics are harmless bacteria that help the well being of the host animal and contribute, directly or indirectly, to protect the host animal

against harmful pathogens. Probiotics act by producing inhibitory compounds, boosting immune competence, contributing to the intestinal microbial balance and providing nutritional benefits (Balcazar et al., 2006).

Several probiotics have been evaluated in the last decade in aquaculture. The most popular ones are lactic acid bacteria (LAB) such as *Lactobacillus casei*, or others from the genus *Bacillus* sp. They have been included in supplementary diets for a huge variety of organisms and eventually included in commercial probiotic formulations (Cordero et al., 2014).

The benefits of probiotic supplementation include improvements in feed values, contribution to enzymatic digestion, inhibition of pathogenic microorganisms, anti-mutagenic and anti-carcinogenic activity, growth-promoting factors and increase of the immune response (Wang & Xu, 2006)

The effects of probiotics differ depending on the species. For instance, *Shewanella putrefaciens* strain (SpPdp11) is a probiotic naturally isolated from skin of healthy gilthead seabream, which has been shown to have positive effects (Verschuere et al., 2000). In particular, previous studies demonstrate that SpPdp11 inhibits the attachments to skin mucus and acompetitive exclusion properties against *Photobacterium damselae* subsp. *piscicida* (Chabrillon et al., 2005a) and *Vibrio harvey* (Chabrillon et al., 2005b). The application of probiotics in aquaculture shows potential, but still needs considerable efforts of research.

1.4.2. Plant products

Aside from probiotics, the use of plant products has been also considered as an alternative to antibiotics and chemotherapeutics in aquaculture, being an eco-friendly approach for the control of pathogens. A lot of attention has been given to the use of plants in dietary supplementation in many countries, leading to the belief that they could be used as immunoprophylactic in the aquaculture industry. In fact, there have been an increasing number of published articles highlighting the potential application of natural products including plants as immunostimulants in the last years (Fig. 3).

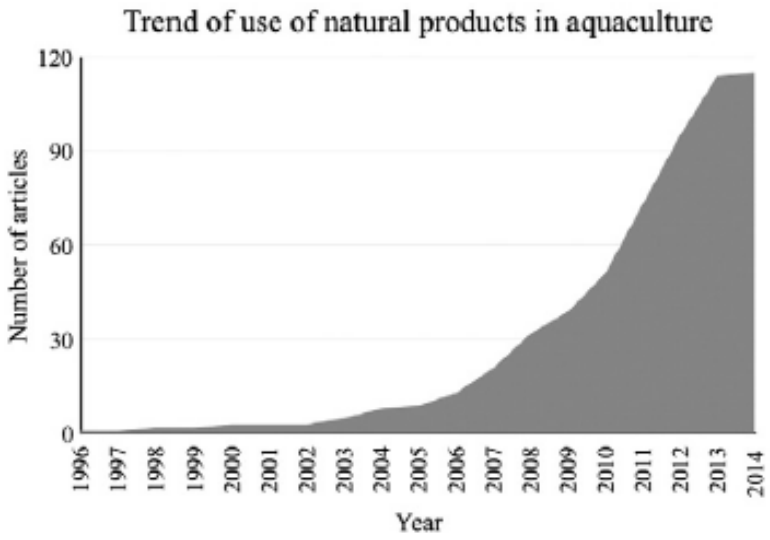

Figure 3. Number of published articles about the use of plant, algae, or natural products in aquaculture (Reverter et al., 2014).

Medicinal plants have been used as immunostimulants for thousands of years, especially in traditional Chinese human medicine (Tan & Vanitha, 2004). Medicinal plants contain many types of active components like polysaccharides, alkaloids or flavonoids that enhance the immune response of fish via lysozyme, complement, antiprotease, phagocytosis, respiratory burst, etc. **(**Harikrishnan et al., 2011). Various types of plant active compounds substances have been reported to enhance the innate immunity of fishes, such as aloe (*Aloe vera*) (Kim et al., 1999). Unfortunately, the mode of action is not always addressed, especially at the molecular mechanism levels.

Plant products have also been reported as anti-stress, appetite stimulators, and to possess aphrodisiac and antimicrobial properties (Citarasu et al., 1998, 1999, 2001, 2002). Another advantages of the use of these plants are that they are cheap, relatively easy to prepare, have fewer side effects during treatment and cause no environmental problems (Citarasu, 2010), which makes them perfectly suitable for the industry. However, they have been studied mostly in chicken, mice, or human cell lines (Zhou et al., 2015).

A wide range of medicinal plants have shown the potential to improve both growth parameters and the survival of aquatic organisms by enhancing their immune system (Immanuel et al., 2004). In particular, more than 60 different medicinal plant species have been studied so far for the improvement of fish health and disease management in aquaculture (Bulfon et al, 2015), including aloe (*A. vera*) (Kim et al., 1999), almond

(*Terminalia catappa*) (Chitmanat et al., 2005) or cinnamon (*Cinnamomum zeylanicum*) (Ahmad et al., 2011) among others.

Plant products can be administered by injection, bathing or orally (diet), being the last one the most practicable (Jeney & Anderson, 1993; Sakai, 1999; Yin, et al., 2006). They have the potential to increase a vaccine's effect, thereby reducing the necessary dose in the first place (Jeney & Anderson, 1993). They can be administered as a whole plant or parts (leaf, root or seed) or extract compounds, via water routine or feed additives, either singly or as a combination of extract compounds, or even as a mixture with probiotics or other immunostimulants (Van Hai, 2015).

1.4.2.1. Plant products as growth promoters

Several plants have been reported as appetite stimulators and to promote weight gain when they were administered to cultured fish (Pavaraj et al., 2011; Takaoka et al., 2011). For instance, a study on Nile tilapia showed that food intake, specific growth rate and final weight increased when garlic was incorporated in the diet (Diab et al., 2002; Shalaby et al., 2006). In another study, grouper (*Ephinephelus tauvina*) fed with a diet supplemented with a mixture of methanolic herb extracts (Bermuda grass (*Cynodon dactylon*), Long pepper (*Piper longum*), stonebreaker (*Phyllanthus niruri*), coat buttons (*Tridax procumbens*) and ginger (*Zingiber officinalis*) displayed 41% higher weight than fish fed with the control diet (Punitha et al., 2008). Furthermore, plant products have shown to improve digestibility and availability of nutrients leading to an increase in feed conversion and higher protein synthesis (Citarasu, 2010; Talpur et al., 2013).

1.4.2.2.Plant products as immunostimulants

There has been an increasing interest in the use of plant products as fish immunostimulants in the last decade. Several studies have monitored the immunological parameters after either intraperitoneal injection or orally administered plant products on distinct fish species. They have found that treated fish showed increased lysozyme activity, phagocytic activity, complement activity, increased respiratory burst activity and increased plasma protein (Dügenci et al., 2003; Wu et al., 2010).

1.4.3. Purslane as a medicinal plant

Portulaca oleracea L. (Fig. 4), commonly known as purslane (US and Australia), but also called rigla (Egypt), pigweed (England), little hogweed, red root or pursle, is a

valuable plant that, besides being a medicinal plant, possesses many other exploitable characteristics (Elkhayat et al., 2008). It is a common weed that grows all over the world. Purslane is a warm-climate plant and it is widely distributed in the tropical and subtropical areas of the world. Unfortunately, it has been considered as a bad weed, as it has happened with many other medicinal plants. However, it is still possible to find it in markets in some countries where it is sold as vegetables. Recently, it has received renewed interest due to its many exploitable characteristics as it has been described as 'power food of the future' (Levey, 1993).

Figure 4. *Portulaca oleracea* in the wild.

P. oleracea has been used as a folk medicine in many countries, acting as a febrifuge, antiseptic, and so forth (Lee et al., 2012). It exhibits a wide range of pharmacological effects, including antibacterial (Zhang et al., 2002), antiulcerogenic (Karimi et al., 2004), anti-inflammatory (Chan et al., 2000), antioxidant (Chen et al., 2012), and wound-healing (Rashed et al., 2003) properties. The World Health Organization lists this plant as one of the most used medicinal plants, and it has been given the term "Global Panacea" (Xu et al., 2006). Also, the Chinese folklore described it as "vegetable for long life" (Chen et al., 2009). *P. oleracea* has a high potential to be used as human and animal food and to be utilized as a pharmacological agent in medicine as well.

Aside from possessing medicinal properties, this plant also provides a source of nutritional benefits owing it to being rich in omega-3 fatty acids, α-linolenic acid and antioxidants (α-tocopherol, β-carotene , ascorbic acid, and glutathione) (Palaniswamy et al., 2001).

Many constituents of *P. oleracea* have been isolated, including flavonoids, alkaloids,

fatty acids, terpenoids, polysaccharides, vitamins, sterols, proteins, and minerals. Flavonoids possess a wide range of pharmacological properties such as antibacterial, antivirus, anti-inflammation, and antioxidation properties. The levels of flavonoids vary according to the part of the plant. The highest levels of flavonoids are present in the roots, followed by stem and leafs. Also, different types of flavonoids are present in this plant, including kaempferol, myricetin, luteolin, apigenin, quercetin, genistein, and genistin (Zhu et al., 2010).

In summary, dietary manipulation plays an important role in the contribution to improve fish overall health. The application of additives in the diets as plants or extracts of plants as natural and innocuous compounds seems like a potential alternative to the use antibiotics and chemotherapeutic agents in aquaculture. It has been also suggested that a combination of probiotics and natural immunostimulants could have more beneficial effects to fish than a single administration of one of them. However, this has rarely been studied (Esteban, 2014). Furthermore, the effects of purslane as a natural immunostimulant and/or growth promoter has not been studied in fish yet.

OBJECTIVES

2. Objectives

The general objective of the present study is to determine the effects of *P. oleracea* either alone or in combination with a naturally isolated probiotic (*S. putrefaciens*, SpPdp11) on gilthead seabream (*S. aurata* L.) growth performance and immune status. In order to perform this, growth parameters, cellular innate immune parameters in head kidney leucocytes, and different immune parameters in serum, skin mucus and intestine homogenates will be analyzed. Moreover, the expression of several immune-related genes will also be analyzed in head kidney, skin and intestine.

METHODOLOGY

3. Material and methods

3.1.Diet additives

3.1.1. Purslane

Purslane was obtained from the neighborhoods of the Faculty of Biology (Espinardo Campus, University of Murcia, Spain) and its identification was done according to standard methods by our Botany Department. Plants were carefully washed using distilled water and dried on an incubator at 60ºC for 2 days. Afterwards, one kilogram of the aerial part was taken and then the plants were ground into fine powder using a grinder (Restsch, MM2000)

3.1.2. Probiotic

S. putrefaciens (SpPdp11) was grown in tubes containing trypticase soya broth (TSB, Sigma), supplemented with 1.5% sodium chloride (NaCl) (TSBs) at 25ºC, and were continuously shaken for 18 h. Dilutions were performed in order to quantify the number of bacteria present in the culture tubes. The absorbance of 1 ml aliquots of bacteria cell culture dilutions was measured at 625 nm in a spectrophotometer (Boeco, Germany). The original number of bacteria was adjusted using dilutions in order to administer to correct amount of bacteria to each experimental group of fish. Bacterial cell cultures were centrifuged (4,000 g, 15 min, 4ºC). Culture medium was removed after centrifugation and bacteria were re-suspended in the least possible amount of cod oil, which was then sprayed on the pellets before feeding the animals.

S. putrefaciens (SpPdp11) was generously provided by Prof. M.A. Moriñigo from the University of Málaga (Spain).

3.2. Experimental diets

Commercial diet (Skretting, Spain) was crushed and mixed with the obtained purslane powder and/or the SpPdp11 into the appropriate concentrations to get four different experimental diets: non-supplemented (control diet, CD), 2% *P. oleracea* (PD) and 2% *P. oleracea* + 10^9 cfu g^{-1} *S. putrefaciens* (MIXD). The diets were remade into pellets, allowed to dry and stored in a light protected environment at 4ºC until use.

3.3. Fish maintenance and experimental design

Thirty-six specimens (50.56 ± 1.6 g weight and 14.26 ± 0.17 cm length) of the hermaphroditic protrandous teleost gilthead seabream (*S. aurata* L.), obtained from a local fish farm (Cádiz, Spain), were kept in re-circulating seawater aquaria (200 L) in the Marine Fish Facility at the University of Murcia. The water temperature was maintained at 20 ± 2ºC with a flow rate of 900 L h^{-1} and 28‰ salinity. The photoperiod was 12 h light: 12 h dark and fish were fed with commercial pellet diet at a rate of 2% body weight day^{-1}. Fish were allowed to acclimatize for 15 days before the start of the experimental trial. All experimental protocols were approved by the Ethical Committee of the University of Murcia (Permit Number: A13150104).

Fish were weighed, measured and randomly divided into 6 aquaria (n=6) where 3 groups were established (two replicates per group). Each group received one of the experimental diets (CD, PD or MIXD) at 2% of body weight day^{-1} for 30 days. Three fish were sampled from each aquarium (six fish from each experimental diet) after 15 or 30 days. At the end of the feeding trial, fish were killed by an overdose of anaesthetic (MS-222, 100 mg L^{-1}) before sampling.

3.4. Sample collection

Blood samples were collected from the caudal vein with an insulin syringe. Blood samples were left to clot at 4ºC for 4 h, and later the serum was collected after centrifugation (10,000 g, 5 min, 4ºC) and stored at -80ºC until use.

Head kidney (HK) samples were cut into small fragments and transferred to 8 ml of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% NaCl, 2% fetal calf serum (FCS, Gibco), 10 u. ml ^{1} penicillin (Flow) and 100 mg ml ^{1} streptomycin (Flow) (Esteban et al., 1998)]. Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 μm), washed twice (400 g, 10 min), counted (Z2 Coulter Particle Counter) and adjusted to 10^{7} cell ml^{-1} in sRPMI. Cell viability was higher than 98%, as determined by the trypan blue exclusion test (Esteban et al., 1998).

Skin mucus samples were collected from specimens using the method described by Guardiola et al. (2014). Briefly, skin mucus was collected by gentle scraping the dorso-

lateral surface of seabream specimens using a cell scraper with sufficient care to avoid contamination with blood and urogenital and intestinal excretions. Collected mucus samples were vigorously shaken and then centrifuged (2,000 g, 10 min, 4ºC). The protein concentration in the supernatant of each sample was determined by Bradford's dye binding method (Bradford, 1976) using bovine serum albumin (BSA, Sigma) as the standard.

Whole intestine samples were collected, homogenized (Homogenizer, T10 basic, IKA, Germany) in cold sodium phosphate buffer (0.05 M PBS; pH 6.2) and centrifuged (3,000 x g, 10 min, 4ºC). The supernatants were collected, filtered and then kept at -80ºC until further analysis. Samples of HK, skin, and intestine were stored in TRIzol Reagent (Invitrogen) at -80ºC for gene expression analysis.

3.5. Growth parameters

Body weight and length of each fish were measured before the trial and all fish were weighed and measured at the beginning of each sampling. Growth was monitored by obtaining the initial weight (Wi), final weight (Wf), weight gain (%WG), and specific growth rate (SGR), which were calculated for each group according to Silva-Carrillo et al. (2012); SGR = [(Ln final weight ─ Ln initial weight) number of days^{-1}] ×100; and %WG = ((Wf ─ Wi) Wi^{-1}) × 100.

3.6. Immune parameters

3.6.1. Cellular parameters

3.6.1.1. Leucocyte peroxidase activity

The peroxidase activity in HK leucocytes was measured according to Quade & Roth (1997). Briefly, 15 µl of HK leucocytes were diluted with 135 µl of Hank's buffer salt solution (HBSS) without Ca^{2+} or Mg^{2+} in flat-bottomed 96-well plates. 50 µl of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, Sigma) and 5 mM hydrogen peroxide (H_2O_2) were added. To determine the leucocyte peroxidase content, 10^6 HK leucocytes in sRPMI were lysed with 0.002% cetyltrimethylammonium bromide (Sigma) and, after centrifugation (400 g, 10 min), 150 µl of the supernatants were transferred to a fresh 96-well plate containing 25 µl of 10 mM TMB and 5 mM hydrogen peroxide (H_2O_2). In both cases, the colour-change reaction was stopped after 2 min by adding 50 µl of 2 M sulphuric acid (H_2SO_4) and the optical density was read at

450 nm in a plate reader. Standard samples without leucocytes, respectively, were used as blanks.

3.6.1.2. Respiratory burst activity

The respiratory burst activity of gilthead seabream HK leucocytes was studied by a chemiluminescence method described by Bayne & Levy (1991). Briefly, samples of 10^6 leucocytes in sRPMI were placed in the wells of a flat-bottomed 96-well microtiter plate, to which 100 µl of HBSS containing 1 µg ml^{-1} phorbol myristate acetate (PMA, Sigma) and 10^{-4} M luminol (Sigma) were added. The plate was shaken and luminescence immediately read in a plate reader (BMG labtech) for 1 h at 2 min intervals. The kinetics of the reactions were analysed and the maximum slope of each curve was calculated. Luminescence backgrounds were calculated using reagent solutions containing luminol but not PMA.

3.6.1.3. Phagocytic activity

The phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by gilthead seabream HK leucocytes was studied by flow cytometry according to Rodríguez et al. (2003). Heat-killed and lyophilized yeast cells were labelled with fluorescein isothiocyanate (FITC, Sigma), washed and adjusted to $5x10^7$ cells ml^{-1} of sRPMI. Phagocytosis samples consisted of 125 µl of labelled-yeast cells and 100 µl of HK leucocytes in sRPMI (6.25 yeast cells:1 leucocyte). Samples were mixed, centrifuged (400 x g, 5 min, 22ºC), resuspended and incubated at 22ºC for 30 min. At the end of the incubation time, samples were placed on ice to stop phagocytosis and 400 µl ice-cold PBS was added to each sample. The fluorescence of the extracellular yeasts was quenched by adding 40 µl ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or HK leukocytes were included in each phagocytosis assay.

All samples were analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 3,000 cells, which were acquired at a rate of 300 cells s^{-1}. Data were collected in the form of two-parameter side scatter (granularity) (SSC) and forward scatter (size) (FSC), and green fluorescence (FL1) dot plots or histograms were made on a computerized system. The fluorescence histograms represented the relative fluorescence on a logarithmic scale. The cytometer was set to analyse the phagocytic cells, showing highest SSC and FSC values. Phagocytic ability was defined as the percentage of cells with one or more ingested bacteria (green-FITC

fluorescent cells) within the phagocytic cell population while the phagocytic capacity was the mean fluorescence intensity. The quantitative study of the flow cytometric results was made using the statistical option of the Lysis Software Package (Becton Dickinson).

3.6.2. Humoral parameters in serum, mucus and intestine homogenates

3.6.2.1. Natural haemolytic complement activity

Natural haemolytic complement activity was measured in serum according to Sunyer & Tort (1995) with some modifications. The following buffers were used: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatin; EDTA-GVB, as previous one but containing 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mg^{2+} and 10 mM EGTA. Rabbit red blood cells (RaRBC; Probiologica Lda, Portugal) were used for natural haemolytic complement determination. RaRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5×10^8 cells ml^{-1}. Twenty µl of RaRBC suspension were then added to 40 µl of serially diluted serum in Mg-EGTA-GVB buffer. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 40 µl of distilled water or Mg-EGTA-GVB buffer to 20 µl samples of RaRBC, respectively. Samples were incubated at room temperature for 100 min with regular shaking every 20 min. The reaction was stopped by adding 150 µl of cold EDTA-GVB. Samples were then centrifuged and the extent of haemolysis was estimated by measuring the optical density of the supernatant at 414 nm in a microplate reader (Synergy HT). The degree of haemolysis (Y) was estimated and the lysis curve for each specimen was obtained by plotting $Y\ (1\text{-}Y)^{-1}$ against the volume of serum added (µl) on a log-log scaled graph. The volume of serum producing 50% haemolysis (ACH_{50}) was determined and the number of ACH_{50} units ml^{-1} obtained for each experimental fish.

3.6.2.2. Lysozyme activity

Lysozyme activity was measured according to the turbidimetric method described by Swain et al. (2007) with some modifications. Briefly, 20 µl of serum or skin mucus were placed in flat-bottomed 96-well plates. To each well, 180 µl of freeze-dried *Micrococcus lysodeikticus* (0.2 mg ml^{-1}, Sigma) in 40 mM sodium phosphate (pH 6.2) was added as lysozyme substrate. As blanks of each sample, 20 µl of serum or skin mucus were added to 180 µl of sodium phosphate buffer. The absorbance at 450 nm was

measured after 20 min at 35ºC in a microplate reader (Synergy HT). The amounts of lysozyme present in serum and skin mucus were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma) through serial dilutions in the above buffer. Serum and skin mucus lysozyme values are expressed as µg ml^{-1} equivalent of HEWL activity.

3.6.2.3. Peroxidase activity

The peroxidase activity in serum and skin mucus was measured according to Quade and Roth (1997) with some modifications. Briefly, 30 µl of skin mucus, 15 µl of serum and 10 µl of intestine homogenate were diluted with 120 µl, 135 µl or 140 µl of Hank's buffer (Hank's Balanced Salt Solution, HBSS) without Ca^{2+} or Mg^{2+} in flat-bottomed 96-well plates, respectively. Fifty µl of 20 mM TMB and 5 mM H_2O_2 were then added to each well and serves as substrates. After 2 min the reaction was secured by adding 50 µl of 2 M sulphuric acid and the OD was measured at 450 nm in a plate reader. Samples without skin mucus, serum, intestine homogenate respectively, were used as blanks. Absorbance alteration more than one can be defined as one unit of peroxidase. The final results were expressed as units ml^{-1}.

3.6.2.4. Protease activity

Protease activity was measured in serum, skin mucus and intestine homogenates using the azocasein hydrolysis assay according to Guardiola et al. (2014) with some modifications. Briefly, 100 µl of skin mucus, serum and intestine homogenates were incubated with equal volume of 115 mM phosphate buffered saline (PBS, pH 7.0) containing 2% azocasein (Sigma) for 24 h at 30ºC. The reaction was stopped by adding 10% trichloro acetic acid (TCA) and the mixture centrifuged (10,000 g, 10 min). The supernatants were transferred to a 96-well plate in triplicate containing 100 µl $well^{-1}$ of 1 N NaOH, and the OD read at 450 nm using a microplate reader (Synergy HT). Serum, skin mucus and intestine homogenate were replaced by trypsin (5 mg ml^{-1}, Sigma), as positive control (100% of protease activity), or by buffer, as negative controls (0% activity). The percentage of trypsin activity compared to the positive control was calculated.

3.6.2.5. Antiprotease activity

Total antiprotease activity was determined by the ability of serum, skin mucus and intestine homogenates inhibit trypsin activity with some modifications (Guardiola et al.,

2014). Briefly, 10 µl of skin mucus, 10 µl of intestine homogenate or 10 µl of serum were incubated for 10 min at 22ºC with 10 µl of standard trypsin solution (5 mg ml^{-1}, in 100 mM sodium bicarbonate). Afterwards, 100 µl of 0.7%, 0.7%, or 2% azocasein (in 100 mM sodium bicarbonate) for skin mucus, intestine homogenates and serum samples, respectively were added and the samples incubated for 60 min at 22ºC. Finally, 250 µl of 4.6%, 4.6% and 10% of TCA (trichloroacetic acid) were added for skin mucus, intestine homogenates and serum respectively and a new incubation for 30 min at 22ºC was done. The mixture was then centrifuged (10,000 g, 5 min) being the supernatants transferred to a 96-well plate in triplicate containing 100 µl $well^{-1}$ of 0.5N, 0.5N and 1N NaOH for skin mucus, intestine homogenates and serum respectively, and the OD read at 450 nm using a microplate reader (Synergy HT). Sodium bicarbonate in place of skin mucus, intestine homogenates, serum and trypsin served as blank whereas the reference sample was sodium bicarbonate in place of skin mucus, intestine homogenates and serum. The percentage inhibition of trypsin activity compared to the reference sample was calculated.

3.6.2.6. Total IgM levels

Total IgM levels were analyzed using the enzyme-linked immunosorbent assay (ELISA) (Cuesta et al., 2004). Briefly, 100 µl of skin mucus (diluted 1:5 with 50 mM carbonate-bicarbonate buffer, pH 9.6) or serum (diluted 1:500 with the above buffer) were placed in flat-bottomed 96-well plates in triplicate and coated overnight at 4°C. Samples were rinsed 3 times with PBS-T [20 mM phosphate buffer (PBS) and 0.05% Tween 20, pH 7.3], blocked for 2 h at room temperature with blocking buffer (PBS-T containing 3% bovine serum albumin BSA) and rinsed again. The plates were then incubated for 1 h with 100 µl per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with the secondary antibody anti-mouse IgG-HRP (1/1,000 in blocking buffer, Sigma). After exhaustive rinsing with PBS-T, the samples were developed using 100 µl of a 0.42 mM solution of 3,3,5,5 - tetramethyl benzidine hydrochloride (TMB, Sigma), 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, stopped by the addition of 50 µl of 2 M H_2SO_4 and the plates read at 450 nm in a plate reader (FLUO star Omega, BMG Labtech). Negative controls consisted of samples without skin mucus, serum or primary

antibody, whose optical density (OD) values were subtracted for each sample value. Data are presented as the OD at 450 nm for each sample value.

3.6.2.7. Alkaline phosphatase activity

Alkaline phosphatase activity in skin mucus samples was measured by mixing an equal volume of samples with 4 mM p-nitrophenyl liquid phosphate (Sigma) in 100 mM ammonium bicarbonate buffer containing 1 mM $MgCl_2$ (pH 7.8, 30 ºC) as described by Guardiola et al. (2014) with slight modifications. The OD was continuously measured at 1 min intervals over 1 h at 405 nm in a plate reader. Standard samples without intestine homogenates were used as blanks. The initial rate of the reaction was used to calculate the activity because the reaction follows of a linear correlation. The activity was expressed as U ml^{-1} which was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol product in 1 min. In the case of the plasma samples, several dilutions were tested but the alkaline phosphatase activity was not possible to detect.

3.6.2.8. Esterase activity

Esterase activity in serum, skin mucus and intestine homogenates samples was determined according to the method of Guardiola et al. (2014), with slight modifications. Then, 40 and 80 µL of plasma and intestine homogenates samples were mixed with 160 and 120 µL of 0.4 mM p-nitrophenyl myristate, respectively, as substrate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, 30 ºC). The OD was continuously measured at 1 min intervals over 1 h at 405 nm in a plate reader. Standard samples without plasma and intestine homogenates were used as blanks. The initial rate of the reaction was used to calculate the activity because the reaction follows of a linear correlation. The activity was expressed as U ml^{-1}, which was defined as the amount of enzyme required to release 1 µmol of p-nitrophenyl myristate product in 1 min.

3.6.3. Gene expression analysis

After 15 and 30 days of feeding, total RNA was extracted from gilthead seabream HK, skin, and intestine using TRIzol Reagent. It 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 III reverse transcriptase (Invitrogen) with an oligo-dT_{18} primer.

The expression of genes involved in immunity were analyzed by real-time qPCR on an ABI PRISM 7500 (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) using the 2 ΔCt method (Livak and Schmittgen 2001) and dividing the normalized expression values by the mean of the normalized expression values of the control ones. Reaction mixtures (containing 10 µl of 2 x SYBR Green supermix, 5 µl of primers (0.6 µM each) and 5 µl of cDNA template) were incubated for 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1 min at 60ºC, and finally 15 s at 95ºC, 1 min at 60ºC and 15 s at 95ºC. For each mRNA, gene expression was corrected by the elongation factor 1α (*ef1a*) RNA content in each sample. Negative qPCR controls using double-distilled water instead of cDNA were included in the assays for each primer pair.Gene names follow the accepted nomenclature for zebrafish (http://zfin.org/). In all cases, each PCR was performed with triplicate samples.

Table 1. Primers used for real-time qPCR.

Gene name	**Gene abbreviation**	**GenBank number**	**Primer sequences (5´→3´)**
Elongation factor 1α	*ef1α*	AF184170	F: CTGTCAAGGAAATCCGTCGT R: TGACCTGAGCGTTGAAGTTG
Interleukin-1β	*il-1β*	AJ277166	F: GGGCTGAACAACAGCACTCTC R: TTAACACTCTCCACCCTCCA
Immunoglobulin M	*Igm*	AM493677	F: CAGCCTCGAGAAGTGGAAAC R: GAGGTTGACCAGGTTGGTGT
Hepcidine	*Hep*	CB184616	F: GCCATCGTGCTCACCTTTAT R: CTGTTGCCATACCCCATCTT
β-defensin	*Bd*	FM158209	F: CCCCAGTCTGAGTGGAGTGT R: AATGAGACACGCAGCACAAG
T cell receptor	*Tcrβ*	AM261210	F: AAGTGCATTGCCAGCTTCTT R: TTGGCGGTCTGACTTCTCTT
Colony stimulating factor 1 receptor	*csfr1*	AM050293	F: ACGTCTGGTCCTATGGCATC R: AGTCTGGTTGGGACATCTGG
Alkaline phosphatase	*Alp*	AY266359	F: TTACTGGGCCTGTTTGAACC R: ATCCTTGATGGCCACTTCCAC

3.6.4. Statistical analyses

All analyses were conducted in triplicates and the results are expressed as means ± standard error (SE). Data were statistically analyzed by one-way ANOVA followed by Tukey tests to identify significantly different between groups. Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed prior to analysis and a non-parametric Kruskal-Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. All statistical analyses were conducted using SPSS software for WINDOWS. The level of significance used was $P < 0.05$ for all statistical tests.

RESULTS

4. Results

Results from the experimental trial are presented in the following section. Firstly, results regarding growth parameters (weight gain, specific growth rate) are shown. Secondly, results regarding immune cellular parameters (peroxidase activity, respiratory burst, phagocytosis) are exposed. Thirdly, results in relation to immune humoral parameters are displayed in the following order: serum, skin mucus and intestine homogenates. Lastly, results regarding gene expression of specific genes in head kidney, skin and intestine related to immunity and antioxidant status are shown.

4.1. Growth parameters

Regarding growth parameters, weight gain and specific growth rate values did no differ significantly among the groups (CD, PD, MIXD) at either 15 or 30 days of diet administration (Table 1). Thus, neither purslane nor the mixture had any significant effects on gilthead seabream growth parameters.

Table 2. Growth performance of *gilthead seabream* specimens fed different diets [control diet, non-supplemented (CD); *Portulaca oleracea* (PD); *Portulaca oleracea* + *Shewanella putrefaciens* probiotic (MIXD)] after 15 and 30 days of experimental trial. Values are expressed as mean ± SE (n=5). Different letters denote significant differences between treatment groups ($P \leq 0.05$).

Experimental groups	SGR_{0-15}	SGR_{15-30}	$\%WG_{0-15}$	$\%WG_{15-30}$
CD	**1.08 ± 0.19**	**0.64 ± 0.10**	**18.01 ± 3.45**	**10.21 ± 1.71**
PL	**0.85 ± 0.19**	**0.56 ± 0.10**	**13.92± 3.43**	**8.91± 2.57**
MIX	**0.62 ± 0.13**	**0.62 ± 0.11**	**9.86 ± 2.16**	**9.97 ± 1.81**

SGR_{0-15}: specific growth rate between 0-15 days; SGR_{15-30}: specific growth rate between 15-30 days; WG_{0-15} %: percentage of weight gain between 0-15 days; WG_{15-30}%: percentage of weight gain between 15-30 days.

4.2. Cellular parameters

From the three cellular immune parameters studied on leucocytes from gilthead seabream fed supplemented diets, only one of them was affected by dietary administration of purslane (Fig. 5). No significant variations were recorded in leucocyte peroxidase activity, respiratory burst and phagocytic ability at either 15 or 30 days

among different groups. Phagocytic capacity of leucocytes was significantly affected after 30 days of diet administration. Concretely, this activity was increased in leucocytes from fish fed PD diet in comparison to the other experimental groups (CD and MIXD) (Fig. 5). However, a significant decrease on the phagocytic capacity of HK leucocytes from fish fed the MIXD diet was detected compared to the values found in leucocytes from fish fed control diet.

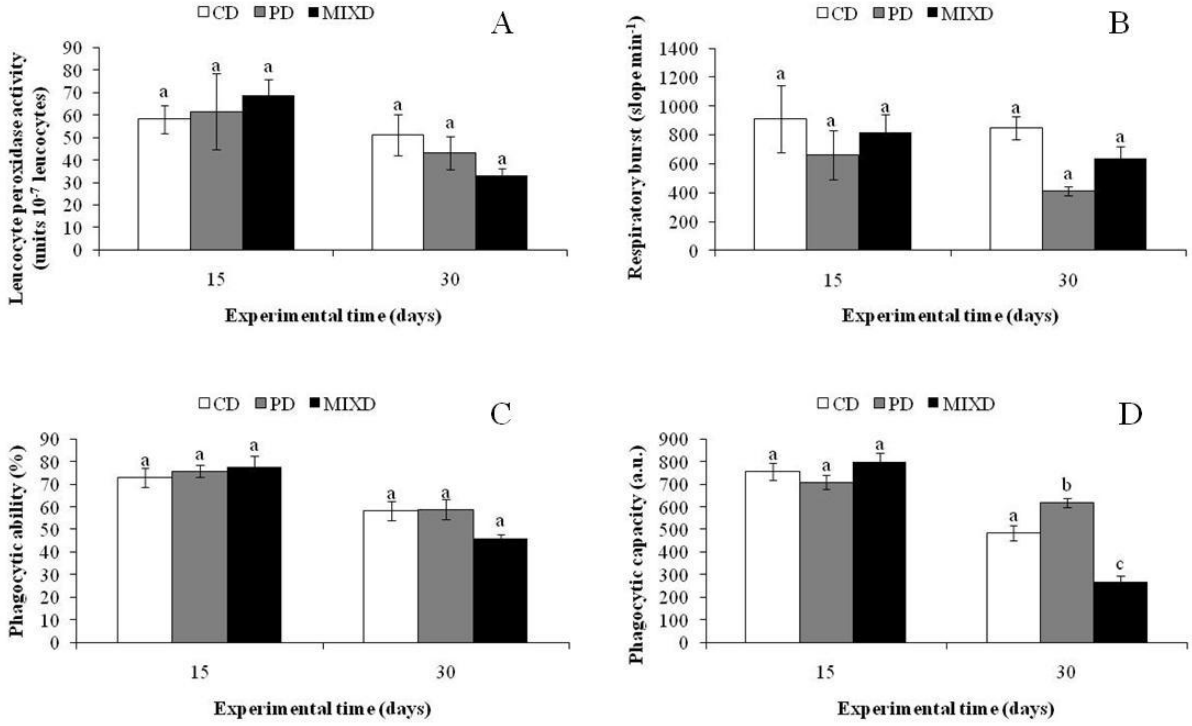

Figure 5. Peroxidase activity (A), respiratory burst (B), phagocytic ability (C) and phagocytic capacity (D) of head kidney leucocytes of gilthead seabream fed different experimental diets [control diet, non-supplemented (CD); *P. oleracea* (PD); *P. oleracea* + *S. putrefaciens* (MIXD)] for 15 and 30 days. Results are expressed as mean ±S.E (n=6). Different letters denote significant differences when $p<0.05$.

4.3. Humoral parameters

54.3.1. Serum

Regarding humoral immune parameters in serum, no significant differences were observed in haemolytic complement, peroxidase, lysozyme, antiprotease, and esterase activities between experimental groups at either 15 or 30 days of trial (Figs. 6 & 7). However, protease activity, fish fed PD diet showed significant increments after 15 days of administration respect to the control group (Fig. 6). Contrarily, protease activity decreased in serum from fish fed MIXD for 15 days compared to the values observed in serum from fish of the control group. However, after 30 days of diet administration, no

significant differences were observed in this activity among any of the experimental groups. Finally, no significant differences among any experimental groups after 15 or 30 days of administration of the diets were observed on the total IgM levels on serum (Fig. 7).

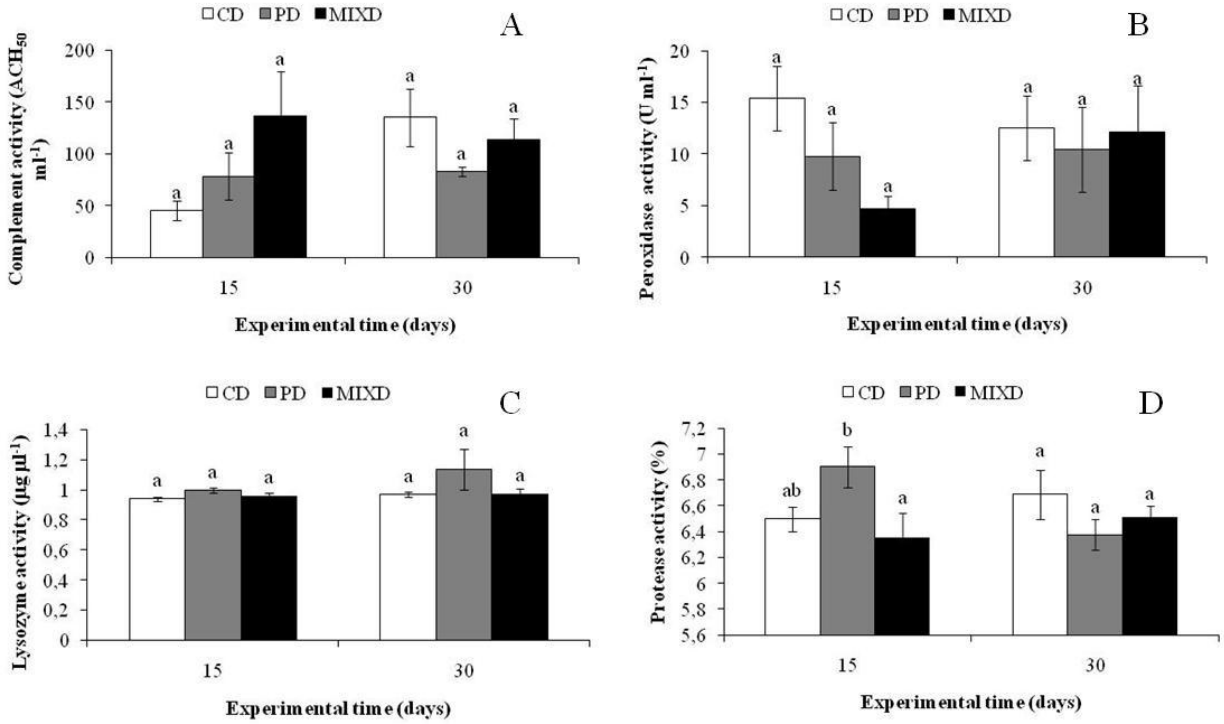

Figure 6. Complement (A), peroxidase (B), lysozyme (C) and protease (D) activity in serum of gilthead seabream fed different experimental diets [control diet, non-supplemented (CD); *P. oleracea* (PD); *P. oleracea* + *S. putrefaciens* (MIXD)] for 15 and 30 days. Results are expressed as mean ±S.E (n=6). Different letters denote significant differences when $p<0.05$.

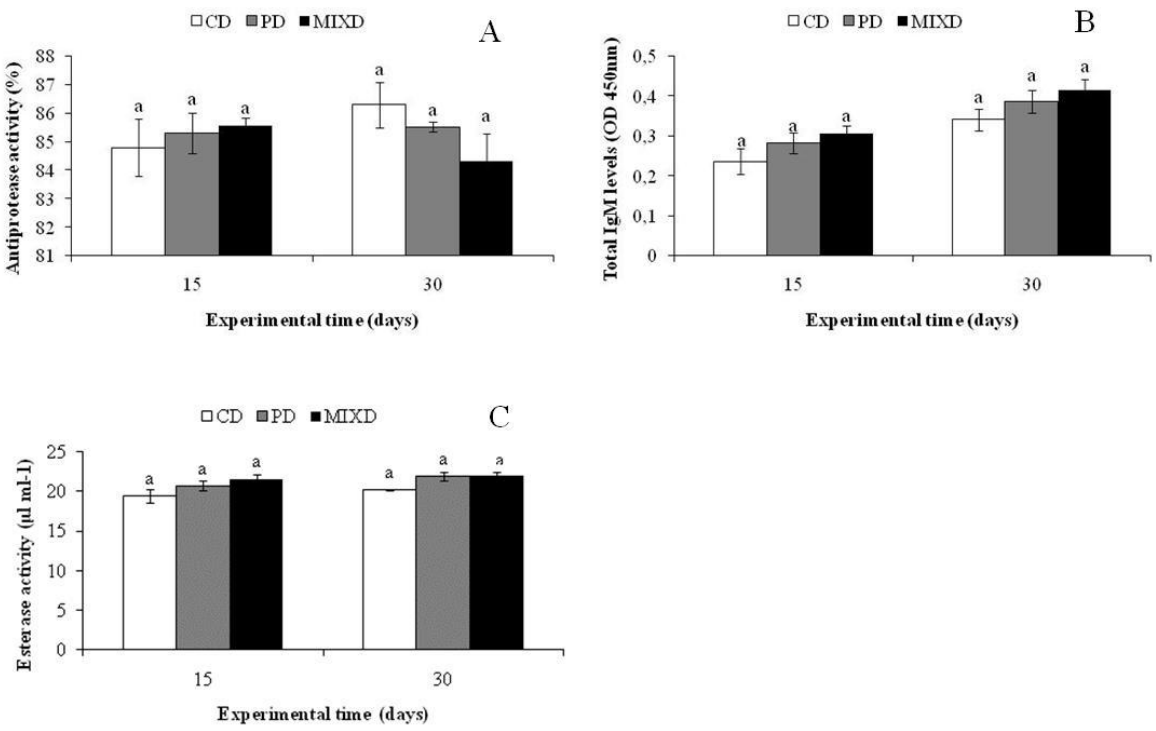

Figure 7. Antiprotease (A), total IgM levels (B) and esterase activity (C) in serum o gilthead seabream fed different experimental diets [control diet, non-supplemented (CD); *P. oleracea* (PD); *P. oleracea* + *S. putrefaciens* (MIXD)] for 15 and 30 days. Results are expressed as mean ± S.E (n=6). Different letters denote significant differences when $p<0.05$.

4.3.2. Skin mucus

No significant differences were recorded in the total protein concentration in skin mucus samples from gilthead seabream fed the different experimental diets for 15 or 30 days (Fig. 8A). Regarding IgM levels, the IgM present in skin mucus of gilthead seabream specimens fed 15 days with PD were significantly higher than the IgM levels detected in the other experimental groups (CD, MIXD) (Fig. 8B). However, no significant differences were observed in the IgM levels present in mucus of fish fed 30 days with any of the experimental diets, respect to the values recorded in mucus from fish fed CD. Regarding protease, antiprotease, lysozyme, peroxidase, alkaline phosphatase and esterase activities (Figs. 8C, 8D & 9), no significant differences were observed in skin mucus of gilthead seabream after either 15 or 30 days of trial between experimental groups.

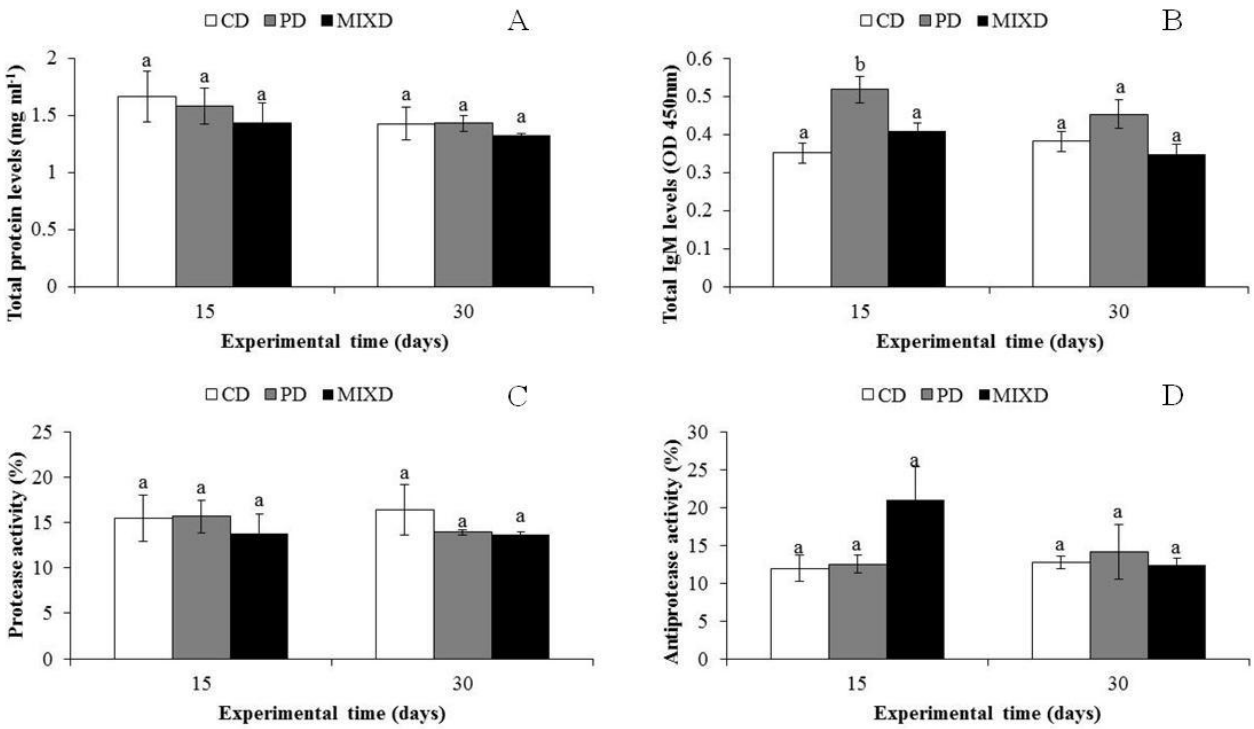

Figure 8. Total protein levels (A), total IgM levels (B), protease (C) and antiprotease (D) activity present in skin mucus of gilthead seabream fed different experimental diets [control diet, non-supplemented (CD); *Portulaca oleracea* (PD); *Portulaca oleracea* + *Shewanella putrefaciens* probiotic (MIXD)] for 15 and 30 days. Results are expressed as mean ±S.E (n=6). Different letters denote significant differences when $p<0.05$.

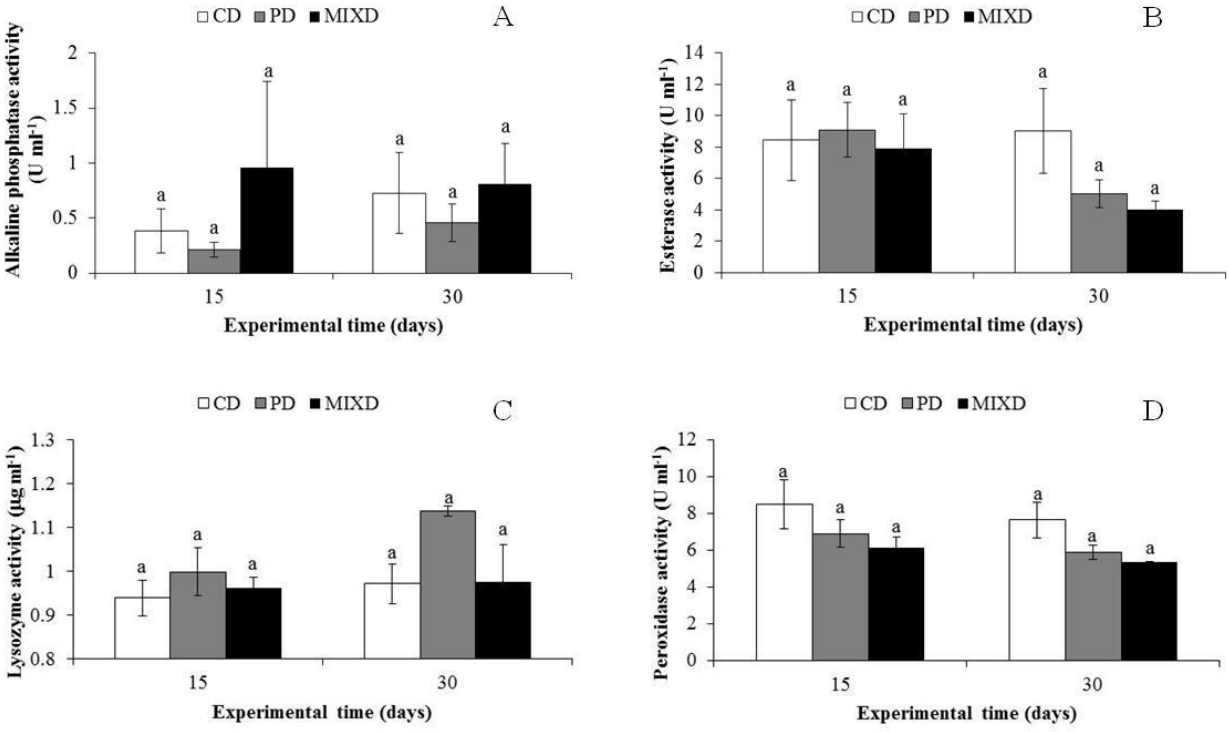

Figure 9. Alkaline phosphatase (A), esterase (B), lysozyme (C) and peroxidase (D) activity in skin mucus of gilthead seabream fed different experimental diets [control diet, non-supplemented (CD); *P. oleracea* (PD); *P. oleracea* + *S. putrefaciens* (MIXD)] for 15 and 30 days. Results are expressed as mean ±S.E (n=6). Different letters denote significant differences when $p<0.05$.

4.3.3. Intestine homogenates

Different enzymes related to the immune activity were determined in gilthead seabream intestine homogenates after being fed with the experimental diets. With respect to protease and antiprotease activities in intestine homogenates of gilthead seabream, no significant differences were observed between experimental groups after 15 days of administration. However, a significant increase in the fish fed purslane-supplemented diet (PD) after 30 days of experimental trial was observed. (Figs. 10A & 10B),

Esterase activity showed a significant decrease in intestine homogenates of gilthead seabream in fish fed the mixture diet (MIXD) after 15 days of administration (Fig. 10C). For this activity, no significant differences were found between experimental groups at the end of the trial.

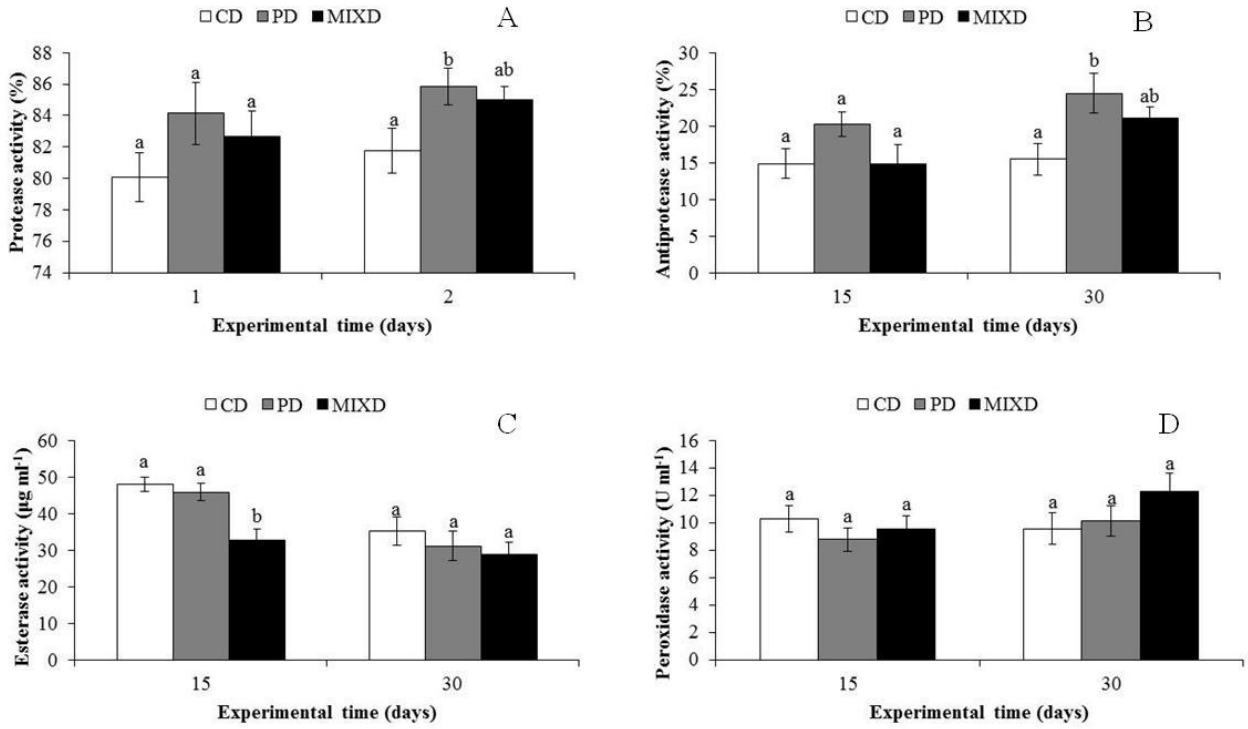

Figure 10. Protease (A), antiprotease (B), esterase (C) and peroxidase activity (D) in intestine homogenates of gilthead seabream fed different experimental diets [control diet, non-supplemented (CD); *P. oleracea* (PD); *P. oleracea* + *S. putrefaciens* (MIXD)]

for 15 and 30 days. Results are expressed as mean ±S.E (n=6). Different letters denote significant differences when $p<0.05$.

4.4. Gene expression

4.4.1. Head kidney

Several immune-related genes were analysed using qPCR in the HK of all fish fed experimental diets for 15 (Fig. 11A) and 30 (Fig. 11B) days. Among them, no significant differences on the expression of *il-lb, hep, bd, tcrb* and *csfr1* genes between any of experimental groups at either 15 or 30 days of trial were detected. However, the expression of *igm* gene was up-regulated in the HK of fish fed MIX after 15 days compared to the values observed in the HK of fish from the control group (CD) (Fig. 11A). No significant variations were observed in the transcription level of *igm* gene on HK of fish fed MIXD for 30 days respect to the values recorded for HK of fish fed CD (Fig. 11B).

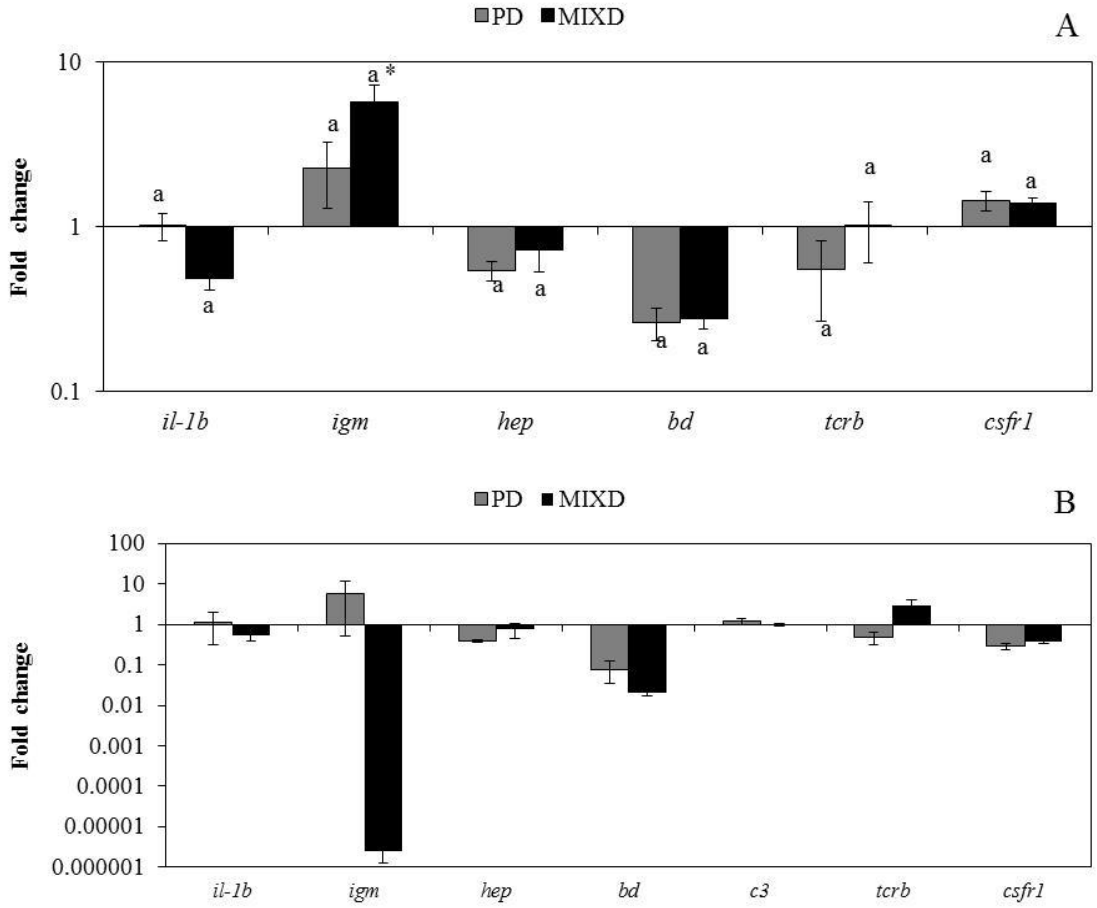

Figure 11. Gene expression, determined by qPCR, in head-kidney of gilthead seabream fed different experimental diets [*P. oleracea* (PD); *P. oleracea* + *S. putrefaciens* (MIXD)] for 15 (A) and 30 (B) days. Data are expressed as fold change relative to control diet specimens (means ± SE, n=6). Values higher than 1 express an increase

while values lower than 1 express a decrease in the indicated gene. Different letters and asterisks denote significant differences between experimental groups or each supplemented group respect to the control group, respectively, when $p<0.05$.

4.4.2. Skin and intestine

Several immune-related genes were analysed using real-time PCR in the skin (Fig. 12) and intestine (Fig. 13) of all fish fed experimental diets for 15 (A) and 30 (B) days. Among them, no variations on the expression of *il-lb, igm, alp, bd,* and *csfr1*genes between any of experimental groups at either 15 or 30 days of trial were detected.

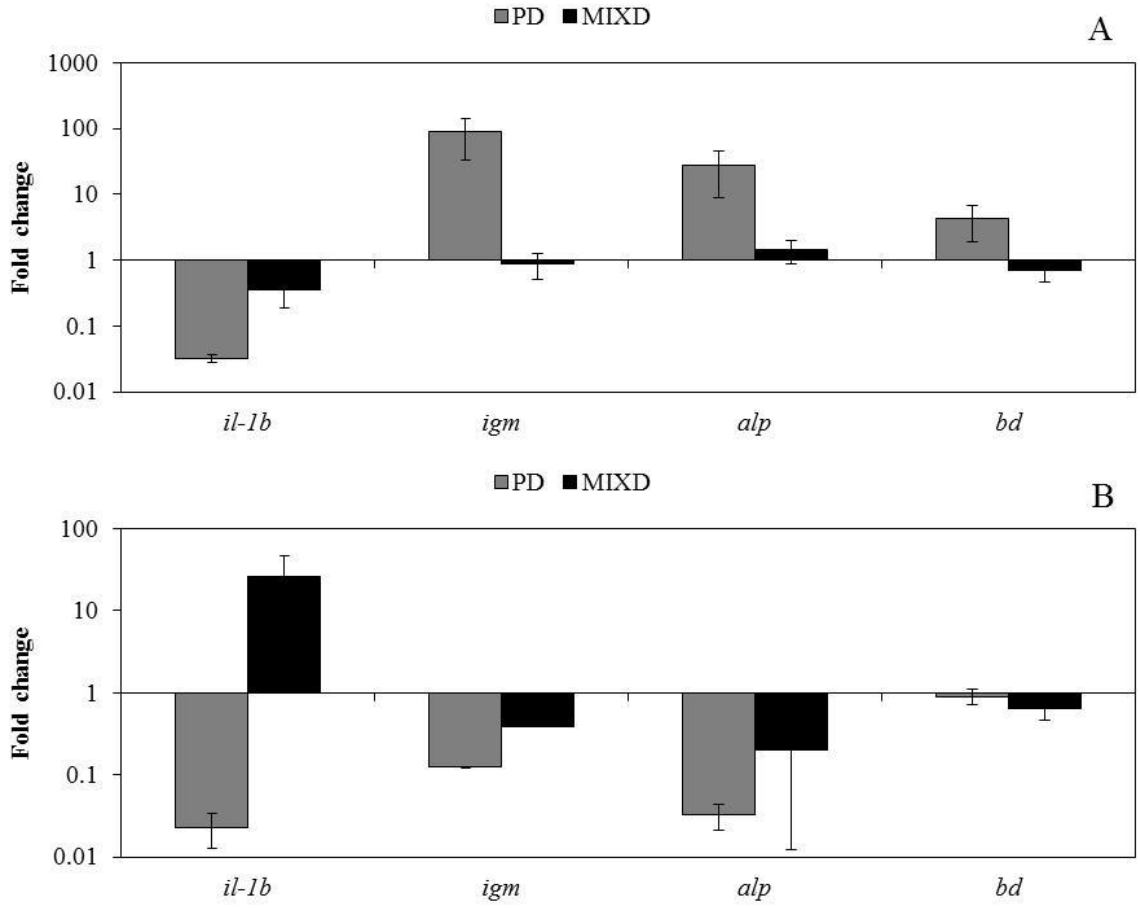

Figure 12. Gene expression, determined by qPCR, in skin of gilthead seabream fed different experimental diets [*Portulaca oleracea* (PD); *Portulaca oleracea* + *Shewanella putrefaciens* probiotic (MIXD)] for 15 (A) and 30 (B) days. Data are expressed as fold change relative to control diet specimens (means ± SE, n=6). Values higher than 1 express an increase while values lower than 1 express a decrease in the indicated gene.

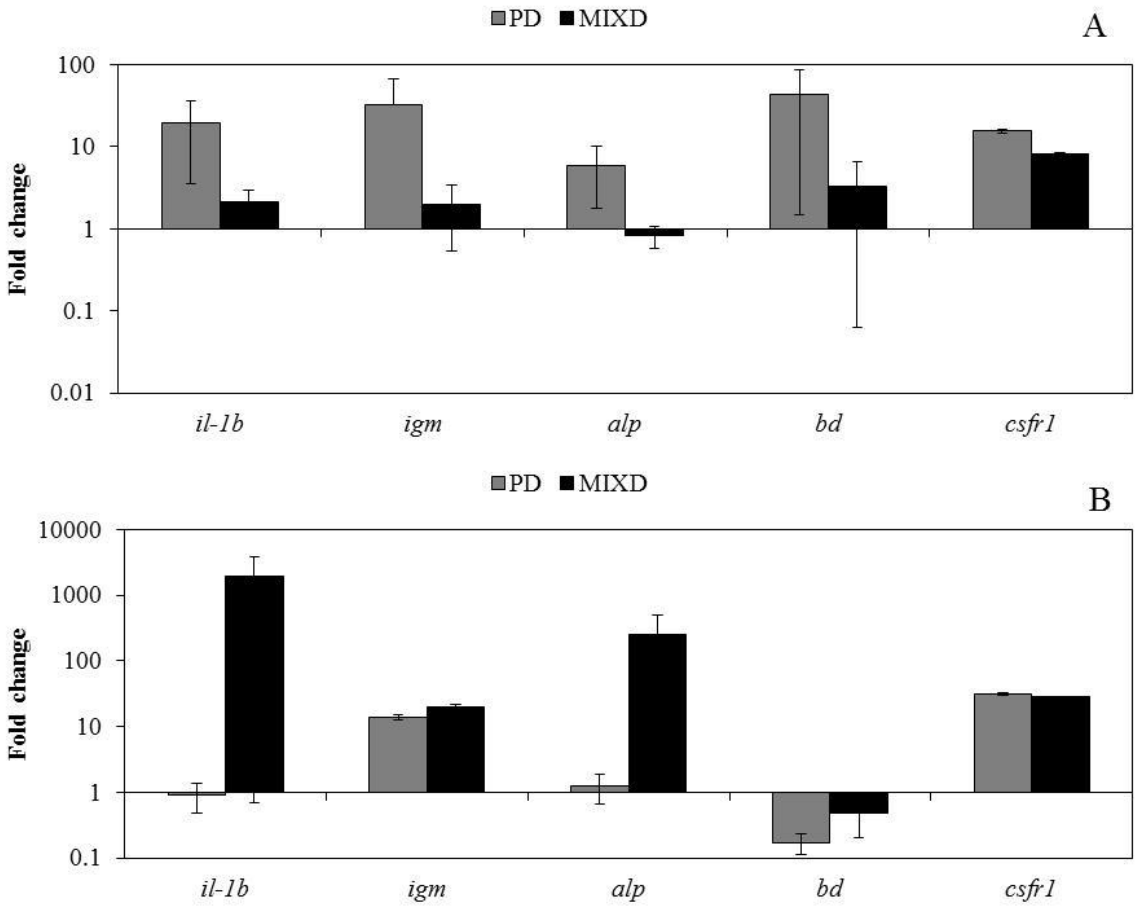

Figure 13. Gene expression, determined by RT-PCR, in intestine of gilthead seabream fed different experimental diets [*P. oleracea* (PD); *P. oleracea* + *S. putrefaciens* (MIXD)] for 15 (A) and 30 (B) days. Data are expressed as fold change relative to control diet specimens (means ± SE, n=6). Values higher than 1 express an increase while values lower than 1 express a decrease in the indicated gene.

DISCUSSION

5. Discussion

In recent years, there has been a growing attention in the effects of plant products in the immune system of farmed fish due to several reasons including that they are eco-friendly, cost-effective and also considered a potential alternative to chemotherapy (Jeney et al., 2009; Valenzuela et al., 2013). On the same note, the use of probiotics in fish aquaculture has also been suggested as a promising disease management strategy (Rico-Mora *et al.,* 1998; Martínez Cruz et al., 2012). Several studies have evaluated the effect, feasibility and efficacy of feed supplemented with a range of medicinal herbs and potential probiotics (Esteban et al., 2014; Adel et al., 2015). Previously, it has been hypothesized that a combination of specific immunostimulants and probiotics may be more effective on fish immune status than the administration of a single one. However, the impact of the established combinations can be counteractive if they are not properly selected.

In the present study, the potential immunostimulant effect of purslane alone or in combination with a probiotic (SpPdp11) on gitlhead seabream was evaluated. In the last years, the Fish Innate Immune Group (University of Murcia) has studied the use of several plants as natural additives to fish diets in order to determine if they could potentially be used in aquaculture (Esteban et al., 2014; Adel et al., 2015; Bahi et al., 2016). In this sense, purslane was selected for the present work due to its properties that have been already studied on mammals. This plant exhibits a wide range of pharmacological effects, including antibacterial (Zhang et al., 2002), anti-ulcerogenic (Karimi et al., 2004) anti-inflammatory (Chan et al., 2000) and wound-healing (Rashed et al., 2003) properties among others. Moreover, recently, it has also been evaluated that purslane has a very high nutritional quality. In fact, it possesses higher beta-carotene, ascorbic acid, and alpha-linolenic acid than any of the other cultivated vegetables and it is also one of the plants with highest omega-3 fatty acid levels (Liu et al., 2000). Furthermore, the fact that it has not been tested on fish and also that authors think that this plant is not as used and valued as it should be were enough reasons to choose purslane for the present study. On the other hand, the probiotic was chosen due to its already established positive effects not only in gilthead seabream but also on *Solea senegalensis* (Lobo et al., 2014; Cordero et al., 2015, 2016). This is the first study that looks at the use of purslane (*P. oleracea*) alone and in combination with a probiotic in fish diet. This first approach is an attempt to revalue this plant and, at the same time,

improve the health status of farmed gilthead seabream. In fact, the most significant results obtained were in the groups were the fish were fed purslane alone and not the combination of the plant and the probiotic.

Besides genetic and environmental factors, the nutritional status of fish is considered as a major aspect that influences the immune response and modulates the resistance to infection and disease occurrence. A large number of reviews have been published regarding the advantages of supplementing the aquafeeds with immunostimulants in fish (Sakai, 1999; Bricknell & Dalmo, 2005). Recently, in this sense, a lot of attention has been given to the possibility of using medicinal herbs as immunostimulants (Galindo-Villegas & Hosokawa, 2004). Plants seem to represent a promising source of bioactive molecules being at the same time readily available, inexpensive and biocompatible.

While the effects of immunostimulants on the systemic immune responses (humoral and adaptive) have been studied extensively (Ahmad et al., 2011; Abdel-Tawwab, 2015; Awad et al., 2015a,b), very few studies have been done to study the effects of these various immune-stimulating compounds on mucosal immunity of fish. However, it has recently been reported that administration of some herbal dietary supplements such as garlic (*Allium sativum*) (Salmanian-Ghehdarijani et al., 2016), peppermint (*Mentha piperita*) (Adel et al., 2015), date palm (*Phoenix dactylifera*) (Hoseinifar et al., 2015), fenugreek (*Trigonella foenum graecum*) (Guardiola et al., 2017) and myrtle (*Myrtus communis*) (Taee et al., 2017) were capable of improving the mucosal immune responses of different fish species including gilthead seabream, the fish species used in the present study.

Fish are always in contact with a wide variety of microorganisms (pathogenic and non-pathogenic) that are present in the aquatic environment, thus they have developed robust defence mechanisms to survive. The fish immune system is divided into innate (natural or non-specific) and adaptive (acquired or specific) immune system and they are both composed of many different cells and molecules. The innate immune system of vertebrates, which constitutes the basis of immune defence, is the first line of defence against invading pathogens (Narnaware et al., 1994). Among the primary surfaces which are exposed to pathogenic agents, the skin is one of the main portals of entry of external microorganisms. Skin is the outermost organ of the body and the first line of

defence against external pathogens. It constitutes a crucial immune barrier, basically based on the fact that it possesses the skin-associated lymphoid tissue (SALT) (Ellis, 1999). Another fish mucosal surface that is highly important regarding the fight against pathogens is the gut (GALT). The intestinal tract is a complex system that plays a key role not only in digestion, absorption and osmoregulation, but also in the defence towards pathogens. In fact, all immune cells that are necessary for a local immune response are present in the gut mucosa of fish (Rombout et al., 2011). To sum up, the fish mucosal immune system is characterized by a diverse and unique repertory of innate and adaptive immune cells and molecules that contribute to the defence against infectious agents (Lazado et al., 2014).

As it was stated earlier, fish nutritional status is considered one of the most important factors to determine fish health status. In fact, generally speaking, there is a positive correlation between a proper diet and the ability to prevent the appearance of a disease. Some plants are known to enhance the production of some digestive enzymes, and thus improve growth rates. Previous studies using Chinese herbs (*Alteranthera sessilis*, *Eclipta alba* and *Cissus quadrangularis*) enhanced the activity of digestive enzymes (protease, amylase and lipase) of freshwater prawns (Radhakrishnan et al., 2014). Furthermore, American ginseng, green tea and cinnamon enhanced the growth performance and feed utilization of Nile tilapia (Abdel-Tawwab et al., 2010; Ahmad et al., 2011; Abdel-Tawwab et al., 2015). In the present study, none of the tested experimental diets affected significantly the growth parameters of gilthead seabream specimens, compared to the values recorded for fish from the control group (fed commercial diet). These results indicate that neither purslane nor the combination of both the plant and the probiotic tested at these concentrations and during these times had any particular effect on the growth parameters. However, the fact that the supplementation of the diet with purslane did not affect negatively to the fish growth, could also be considered as positive since it is known that some plants possess anti-nutritional factors. In fact, purslane contains phytate and oxalate which are considered as anti-nutritional factors, but the content of these factors are below the established toxic levels (Nikeshwori et al., 2015). Some of these factors might affect protein and/or mineral utilization and digestion, act as anti-vitamins, etc. For instance, a study using soybean in grass carp showed that the fish fed with soybean supplement had a lower growth performance than those fed fishmeal based diet (Dabrowski & Kozak, 1979).

Future studies are needed to understand the lack of positive effects of PD on gilthead seabream growth performance such as to test higher percentages of inclusion of purslane into the diet or longer administration times than those assayed in the present work.

The innate immune system of teleost fish is composed of both cellular and humoral components. Phagocytic cells are the most important cellular component of the fish innate immune system. In fact, phagocytosis is an essential mechanism of the innate immune system and the first line of defense against invading pathogens in all eukaryotic organisms (Haugland et al., 2012). For these reasons, phagocytosis in vertebrates has been recognized as a critical component of both innate and adaptive immune response to pathogens. In our study, HK leucocytes from fish fed purslane for 30 days showed a significantly increased phagocytic capacity respect to the values recorded for HK from fish fed control diet. This finding agrees with previous studies in gilthead seabream where *Cedrus deodara* administration also showed an increase in the leucocyte phagocytic activity (Awad et al., 2015). Similarly, another study using a combination of plants (*Astragalous membranaceous* and *Lonicera japonica*) in Nile tilapia also increased phagocytosis of blood leucocytes in response to a *Aeromonas. hydrophila* infection through injection (Ardó et al., 2008). Curiously in the present work, two parameters were studied to determine the phagocytic activity of HK leucocytes (phagocytic activity and capacity) and only one of them was increased in fish fed purslane diet. The results indicate that while the number of active phagocytes in HK were similar after been fed with the different experimental diets for 15 or 30 days, the phagocytes from HK of fish fed PD were more avid, which could represent a better defence of these fish in an eventual infection. Further assays will be developed in order to demonstrate this hypothesis as well as to determine the resistance of fish against different stressors.

Antibodies represent the major component of the humoral immune system and they are known to play an adaptive role in neutralizing and destroying invading pathogens in all class of vertebrates including fish (Verma et al., 2012). IgM is one of major component of teleost humoral immune system. It has been demonstrated that adaptive and innate immune factors are present in mucosal surfaces (Salinas, 2015). IgM is the most common immunoglobulin present in serum and mucus of fish and it plays a pivotal role

in systemic immune response (Parra et al., 2015). In particular, IgM participates in the opsonization of pathogens by facilitating their phagocytosis (Vallejos-Vidal et al., 2016). Previous studies using plant products demonstrated that IgM levels increased significantly in serum of fish fed supplementary artificial feed containing *Pontogammarus maeoticus* (Rufchaei et al., 2017). Similarly, in the present study, IgM increased in fish fed either purslane or mixture diets, although the recorded increments were not statistically significant respect to the values obtained in serum from fish fed control diet. These results seem to suggest that the observed effect on IgM level could be to the inclusion of puslane on diet more than to the probiotic one. Results in skin mucus revealed that, fish fed purslane supplemented diet for 15 days showed significantly increased IgM levels in skin mucus, compared to the values recorded for mucus from fish fed CD. In previous studies carried out by our group, experimental diets based on the combination of a plant and a probiotic increased the IgM levels in the skin mucus of gilthead seabream (Guardiola et al., 2017). Generally, the presence of high levels of IgM in skin mucus of gilthead seabream fed the purslane experimental diets could provide significant protection towards pathogens. Furthermore, as it is frequent with the effects caused by immunostimulant, similar significant increases were not observed in mucus from fish fed 30 days with PD, which seem to suggest an accommodation of the skin mucosal immunity to the dietary stimulus. Further studies are needed to know the substances present in this plant which could contribute to the detected stimulation of the immunologublin levels in skin mucus of gilthead seabream.

The role of proteases and antiproteases enzymes has been related with the defense towards bacterial or parasite infections (Subramanian et al., 2007). They are also known to enhance the production of other immunological components such as immunoglobulins and antimicrobial peptides (Cho et al., 2002a,b). Although there are still very few data available regarding this aspects in fish it could be really interesting as they play a protective role against pathogens. Proteases perform this task by directly degrading pathogens (Subramanian et al., 2007), hampering their colonization and invasion (Aranishi et al., 1998) or by enhancing the production of other innate components (Hjelmeland et al., 1983). In our study, protease activity in serum significantly increased in the group fed purslane-supplemented diet after 15 days of diet administration, comparing to the values obtained for fish fed control diet. On the other hand, serum antiprotease activity showed no significant variations among the serum of

the different experimental groups. Present results obtained in skin mucus of gilthead seabream showed that significant variations were observed neither in the antiprotease nor in the protease activity at any assayed time (15 or 30 days). However, just the opposite results were observed in samples of intestine homogenates, where significant increases in both protease and antiprotease activities were observed in fish fed for 30 days with purslane alone or in combination with the probiotic. Results obtained suggest that the purslane experimental diet increased both levels of protease and antiprotease on intestine homogenates, which means that it could potentially improve this barrier (GALT) against pathogens that would adhere to this mucosal surface. Antiproteases are blood proteins which act against pathogen proteolytic proteins. These results are very interesting because underline again the fact that fed enriched diet with purslane could help fish to fight against pathogens. Further studies should be performed in order to truly understand the effects of these natural products and, even more interesting, to understand their role in fighting natural infections.

Lysozyme, which is a bactericidal enzyme, was also studied. This enzyme is known to causes hydrolyzation of the b-1,4 glycosidic linkage between N-acetyl glucosamine and N-acetyl muramic acid of the bacterial cell wall peptidoglycan, thereby causing bacteriolysis and preventing bacterial growth (Saurabh & Sahoo, 2008). Lysozyme is also known to activate the complement system and phagocytes by acting as an opsonin, as well as to display anti-viral and anti-inflammatory properties (Magnadottir, 2006). Regarding lyzozyme in serum skin mucus no statistical differences were observed in the present study among the experimental groups at any of the studied times. Contrarily, a study using mushroom (*Agaricus bisporus)* as feeding supplement observed that lysozyme activity increased in skin mucus of carp (Khodadadiam-Zou et al., 2016).

Alkaline phosphatase is known to be present in skin mucus of fish, acting as an important enzyme that plays a potentially protective role during the initial stage of stress, skin regeneration (Sheikhzadeh et al., 2012b) or pathogenic infection (Fast et al., 2002). In the present study, no significant differences were observed in the levels of alkaline phosphatase in skin mucus of gilthead seabream during the experimental trial. However, in previous studies, the alkaline phosphatase activity in the skin mucus increased after dietary supplements of date palm fruit extracts in common carp (Roosta et al., 2014), ginger in roho labeo (*Labeo rohita*) (Sukumaran & Park, 2016) and garlic

in Caspian roach (Salmanian-Ghehdarijani et al., 2016). The increase in alkaline phosphatase activity may be attributed to an improved mucosal immunity. On the other hand, esterase activity, which is another hydrolytic enzyme, suffered significant decreases in the intestine homogenates of fish fed mixture diet for 15 days. In skin mucus, the levels of this enzyme were not significantly affected by dietary purslane, respect to the values recorded for fish fed control diet. Present results disagree with previous studies, which demonstrated that the levels of both enzymes (alkaline phosphatase and esterase) were increased in skin mucus of fish after the dietary administration of immunostimulants (Sheikhzadeh et al., 2012a,b).

It is known that peroxidase activity uses the antioxidant power of the hydrogen peroxide generated in other reactions in order to produce hypochlorite that leads to the production of chloramines. All of these compounds are oxidative substances that are able to attack microorganisms membranes (Ellis, 2001). Neither peroxidase activity of gilthead seabream HK leucocytes, serum, mucus or intestine homogenates showed any significant variations throughout the experiment in any experimental group, respect to the values found in fish from the control group. New studies are needed in order to precise the role of these enzymes in the fish immune system and the reasons induced the alteration of their levels depending on the diet.

In several studies, the expression of different immune-related genes has been studied to determine the immunostimulant properties of different compounds administering in fish with the diet. In the present study, qPCR was used to estimate the regulation of the expression of six immune-related genes (*il-1β, igm, hep, bd, tcrβ,* and *csfr1*) in HK of gilthead seabream, which is the main haematopoietic organ in fish. The selected genes were chosen based on their antibacterial function (*hep, bd)*, on being immune response activators (*il-1β* and *tcrβ*), or due to their role in the adaptive immune response (*igm*) and macrophage colony activator (*csfr1*). Out of all the genes analyzed, only *igm* was modulated by the mixture diet, showing an up-regulation after 15 days of dietary administration. Considering the results obtained regarding serum IgM levels and HK *igm* gene expression, it can be seen that while purslane does not increase the IgM levels in serum it is able of up-regulating its expression. If administration of purslane-supplemented diet for longer times than those used in the present work could imply or not significant increases on seric IgM levels deserve further attention.

Regarding skin and intestine, five immune-related genes (*il-1β, igm, alp, bd,* and *csfr1*) were studied in gilthead seabream. The selected genes were chosen based on their antibacterial function (*bd* and *alp)*, on being immune response activators (*il-1β* and *tcrβ*), or due to their role in the adaptive immune response (*igm*). The expression of such genes was no significantly affected by the experimental diets at any of the sampling times, neither in skin not in intestine. However, a trend was observed in the expression of *csfr1* gene, showing a non-significant increase in fish fed purslane alone or in combination with the probiotic for 15 and 30 days. Previous studies found that dietary supplementation of plants alone or in combination with probiotics up-regulated the expression of several genes. For instance, a study done with date palm fruit and probiotics showed an up-regulation of several genes including *il1b, csfr1,* and *igm* between others was observed in all diets, containing either the plant, the probiotics or a mixture of them, in gilthead seabream skin after two weeks of experimental trial (Cerezuela et al., 2015). Another study in which fish were fed microalgae, an up-regulation in *csfr1* was observed in the gut of gilthead seabream after two weeks of treatment (Cerezuela et al., 2012). Further investigation is strongly recommended in order to define the optimal doses and timings of administration as well as to isolate, characterize and quantify the bioactive compounds present in the used plants. Moreover, research on mode of action, stability of plant components and digestibility in fish as well as *in vitro* and *in vivo* toxicological tests are indispensable for their safe application in the aquaculture industry.

CONCLUSIONS

6. Conclusions

- Several immune parameters were enhanced mainly by purslane-supplemented diet.
- Phagocytosis of HK leucocytes significantly increased due to purslane supplementation, but decreased with the mixture after 30 days of trial. Protease activity in serum significantly increased in fish fed purslane-supplemented diet and the mixture diet showed an up-regulation in *igm* gene expression in the HK after 15 days of trial.
- IgM levels significantly increased due to purslane supplementation in skin mucus of gilthead seabream after 15 days of experimental trial.
- Protease and antiprotease activity significantly increased in intestine homogenates in fish fed both purslane-supplemented and mixture diet after 30 days of trial.
- Results obtained provide new evidence on the possible use of the entire aerial parts of purslane as an interesting natural immunostimulant for cultured gilthead seabream.
- Future research could isolate different constituents of purslane such as flavonoids, or use the parts with the most interesting and promising immunomodulatory compounds. Furthermore, more studies are needed in order to define optimal doses and timings of administration.

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