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Amino acids as key mediators of immune status and nutritional condition in fish.

Lourenço de Oliveira Coelho e Ramos Pinto

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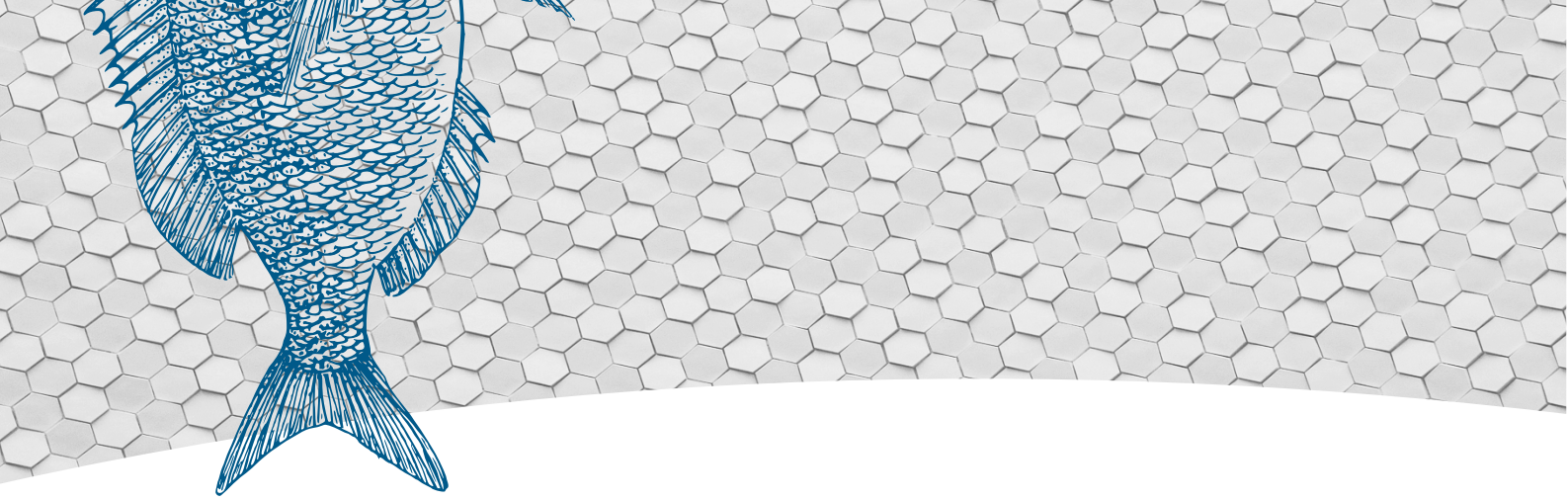

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Amino acids as key mediators of immune status and nutritional condition in fish

Thesis for applying to a Doctor degree in Animal Sciences, Specialty in Nutrition, submitted to the Institute of Biomedical Sciences Abel Salazar of the University of Porto.

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Summary

Given the fast growth and intensification of aquaculture industry, regardless of the specific fish husbandry practices, this activity has some inherent and inevitable features that may compromise fish health and well-being. In this regard, and to ensure a sustained shift away from chemotherapeutic and antibiotic treatments, the aquaculture sector has recognized the need for alternative solutions that combine better husbandry practices with the development of alternative feeding solutions containing health-promoting additives, such as amino acids (AA).

This thesis describes the results of a series of studies designed to evaluate the dietary effect of specific AA — taurine, threonine, histidine, tryptophan, arginine and citrulline — in the improvement of the immune response of gilthead seabream juveniles, when supplied at levels beyond the nominal nutritional requirements of this species, given their direct or indirect roles in different physiological and immune mechanisms.

Through a combination of classical indicators and high-throughput molecular biology techniques, the thesis demonstrates that tryptophan supplementation can improve the immune condition of gilthead seabream juveniles on a short-term basis without compromising long-term fish growth, displaying an immunomodulatory and potentially antioxidant role when seabream are subjected to an explicit inflammatory challenge. Another result is the observation that arginine or citrulline supplementation induce similar effects, seemingly stimulating the innate immune system of gilthead seabream after a short feeding period. Finally, only mild effects were observed in fish fed increased levels of histidine, threonine and taurine in a non-stressful context.

In conclusion, this thesis demonstrates that AA inclusion in aquafeeds can boost the gilthead seabream immune system, particularly through the use of tryptophan, arginine and citrulline. Further, it illustrates that both “dosage” and “feeding period” are central factors determining their health-promoting properties, and that these become particularly apparent under explicitly challenging situations. Thus, tailor-made feed formulations leveraging the functional properties of tryptophan, arginine and citrulline are a promising tool as immune modulators for the farmed fish contributing to a more sustainable sector both at economic and environmental level.

Resumo

Dada a intensificação e rápido crescimento da indústria da aquacultura, esta atividade apresenta aspetos que podem comprometer a saúde e o bem-estar dos peixes. Deste modo, e reconhecida a necessidade de garantir uma redução sustentada dos tratamentos quimioterapêuticos e do recurso a antibióticos, o sector da aquacultura tem vindo a estudar soluções que procuram conciliar as melhores práticas de cultivo com o desenvolvimento de alimentos contendo aditivos que promovem a saúde dos peixes, tais como os aminoácidos.

A presente Tese descreve os resultados de estudos desenvolvidos com o objetivo de avaliar o efeito da suplementação de alguns aminoácidos (AA) em específico — taurina, treonina, histidina, triptofano, arginina e citrulina — na melhoria da imunidade inata de juvenis de dourada, quando fornecidos em níveis que ultrapassam os requisitos nutricionais desta espécie, tendo em conta os seus papéis diretos ou indiretos em diferentes mecanismos fisiológicos e imunológicos.

Os resultados demonstram que a suplementação de triptofano pode melhorar, a curto prazo, a condição imunológica de juvenis de dourada, sem comprometer o seu crescimento a longo prazo, exibindo um papel imunomodulador e potencialmente antioxidante quando os peixes são submetidos a um desafio inflamatório explícito. A observação da indução de efeitos semelhantes através da suplementação com arginina ou citrulina e a aparente estimulação do sistema imunológico da dourada após um curto período de alimentação é outro resultado importante a ter consideração. Por fim, é importante referir o facto de apenas efeitos leves terem sido observados nos peixes alimentados com níveis de histidina, taurina e treonina acima do requisito num contexto desprovido de stress.

Em suma, o presente estudo pretende demonstrar que a inclusão de AA nas rações pode beneficiar o sistema imunológico da dourada, sendo ainda possível concluir que tanto a “dose” como o “período de alimentação” se destacam enquanto fatores centrais e determinantes nas suas propriedades promotoras de saúde, o que se torna particularmente aparente em contextos mais exigentes (e.g. sob condições de stress ou quando expostos a agentes patogénicos). Desta forma, rações formuladas para tirar partido das propriedades funcionais do triptofano, arginina e citrulina são ferramentas promissoras para melhorar o estado imune dos peixes de aquacultura, contribuindo para um setor mais sustentável, tanto a nível económico como ambiental.

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Chapter 1

General Introduction

1.1 Sustainable aquaculture

Aquaculture is the fastest growing food production sector in the world (FAO, 2018). Fish and seafood provide around 17 % of the world's animal protein supply and the demand for these type of food products is growing more rapidly than the human population, currently outpacing the demand for meat (FAO, 2018). Hence, a major responsibility of this sector is to provide food and nutritional security to the world's growing population in a sustainable way, which constitutes a big challenge. Carnivorous fish in particular, such as European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), often require protein sources from wild-caught fish, increasing the economic and environmental costs of production. While aquafeed production used to largely rely on the use of fishmeal (FM) and fish oil (FO) as main ingredients, these raw materials cannot be seen as sustainable, since their production is highly dependent upon marine capture fisheries (Hardy, 2010; Oliva-Teles, et al., 2015; Tacon, Metian, 2008), which constitute a limited resource. Furthermore, feed costs represent the largest fraction of fish farming production costs (up to 60% of total costs), which constitutes a great challenge for the future. Hence, new feed formulations must be crafted to be well-balanced and less expensive (Olmos Soto, et al., 2015).

In brief, FM diets for carnivorous fish are costly and potentially both environmentally unsustainable and unsafe, due to the possibility for bioaccumulation of toxic compounds (Brill, et al., 2019).

FM and FO are mainly used as ingredients in feed production for aquaculture and agriculture, with part of it being used by the pet food industry. Currently, about one-third of FM and FO goes to the agricultural sector, being used as fertiliser for crops and inputs for animal feed (5 % of it being used for broilers, and 23 % for pigs), but aquaculture is rapidly becoming the dominant user since the early 2000s and now accounts for 70 % of the demand for FM and FO. In 2016, 69 % of FM and 75 % of FO production went directly to the fish and seafood farming sector, particularly driven by China's huge aquaculture sector and the salmon industry of Norway, Scotland and Chile (Bachis, 2017; Naylor, et al., 2009). Overfishing down food webs is unstainable both at an economic and ecological level, and can have large impacts on the ecosystem (Smith, et al., 2011). So, the inclusion of wild fish catch in modern aquafeed

formulations must be partially or totally eliminated, increasing the inclusion of alternative economical vegetable products.

The replacement of FM and FO by alternative, more sustainable, sources of protein and energy is clearly recognized as a priority by the industry. However, in order for alternative ingredients to be viable, they must be widely available, price-competitive, suitable for feed production and have certain nutritional characteristics, like low levels of fibre, starch, and non-soluble carbohydrates and antinutrients, plus have an adequate amino acid profile (Kaushik, Hemre, 2008; Nates, 2016), which constrains available options. Plant feedstuffs are widely used as an alternative to FM since they are available in large quantities (Gatlin, et al., 2007). Naturally, omnivorous species have been the easiest to convert to low or no FM diets. Additionally, replacement of fishmeal by plant by-products also requires attention regarding the availability of mineral and trace elements (Kaushik, Hemre, 2008). In carnivorous fish species, the complete substitution of FM has been reported to be a challenge, in terms of reaching an adequate growth performance and fish health status (Desai, et al., 2012; Estruch, et al., 2018; Krogdahl, et al., 2005). Nowadays, a variety of novel ingredients and compounds are being studied and used as important feed additives to overcome the inherent limitations of these alternative feedstuffs. Moreover, the use of processed animal proteins (PAP), such as insect meals, blood meal, meat and bone meal, feather meal and other poultry by-products, in aquafeeds is already a common practice (Gasco, et al., 2018). Finally, the development of other alternative sources of protein, leveraging biomasses obtained from single-celled (e.g. bacteria, yeasts, microalgae) or other simple organisms (e.g. fungi, macroalgae), is also an interesting path, since organisms from lower trophic levels tend to display higher biomass conversion efficiencies (Ritala, et al., 2017).

The gilthead seabream (*Sparus aurata,* Linnaeus, 1758) is one of the main marine carnivorous teleost fish farmed in Mediterranean aquaculture, having a high commercial value due to its savoury meat. In fact, sparids were one of the first families of marine fish for which farmers were able to develop good larval techniques and completely close the life cycle. Since hatching, each fish takes about 2 years to reach 400 g, with its commercial size ranging from 250 g to more than 1500 g (Pavlidis, Mylonas, 2011). The largest Mediterranean productions of seabream come from intensive farming both inland and in floating cages at sea (Pavlidis, Mylonas, 2011; Trujillo, et al., 2012). In the case of Portugal, gilthead seabream farms operate mostly

in the south (Algarve) and in Madeira Island. With a production of around 450 tonnes per year, about half of Portugal's gilthead seabream production volume (around 1 038 tonnes), aquaculture in Madeira is presently undergoing a period of expansion (European_Parliament, 2017; INE/DGRM, 2019). Currently in Madeira Island, there are two companies in business, both dedicated to the production of gilthead seabream (*Sparus aurata*).

Current fish production facilities are designed and built to optimize growth performance and welfare (Føre, et al., 2018). However, pathogens (e.g. bacteria or viruses) can become a serious problem under stressful conditions which can occur when fish are reared in captivity, particularly under intensive systems (e.g. aquaculture facilities and public aquariums). These can lead to health problems and even mortality at any stage of the production process (Conte, 2004). For this reason, the use of sub-therapeutic doses of antibiotics in animal feeds has been common practice to protect them from diseases and to improve production performance in modern animal husbandry (Cheng, et al., 2014). However, the excessive use of antibiotics has lead to the development of antibiotic-resistant bacteria (Done, et al., 2015), with serious implications for human health (Stanton, 2013). Non-therapeutic antimicrobial uses (e.g. to promote animal growth and feed efficiency) are also linked to the propagation of multidrug resistance (Marshall, Levy, 2011). Research that demonstrates the risks associated with these practices have led to regulatory changes, at least at the European Union (EU) level. The Feed Additives Regulation -1831/2003/EC banned the use of antibiotics as growth promoters in animal feed, effectively since January 2006 (EU, 2003). Since then, academic researchers and the aquaculture sector as a whole have recognized the need for alternative solutions that combine better husbandry practices (to minimize health problems due to poor welfare) with the development of alternative feeding solutions containing health-promoting additives, to ensure a sustained shift away from chemotherapeutic and antibiotic treatments.

1.2 Functional diets in aquaculture

Functional diets are those that extend beyond satisfying the basic nutritional requirements of the farmed fish, contributing towards optimal growth, health and survival, through the inclusion of specific additives with health and growth-promoting

properties (Encarnação, 2016). A large number of supplements (e.g. amino acids, prebiotics, probiotics, glucans, nucleotides, methyl donors, essential fatty acids) or feed ingredients, with a wide range of potentially positive effects on fish physiology and health, are available for inclusion in functional feeds (Andersen, et al., 2016; Ringø, et al., 2011; Trichet, 2010).

A well-balanced nutrition provides nutrients and energy required for an optimal growth, reproduction and general homeostasis. As such, developing feed formulations that not only meet fish requirements but also provide additional benefits (e.g. in terms of improved fish health and disease resistance) has been recognized as a promising strategy to improve the sector's profitability. The prospect is that, for example, these functional diets can boost the fish innate immune system, enabling them to better cope with and resist pathogens such as virus and bacteria (Martin, Krol, 2017), thus reducing potential economic losses due to e.g. pathogen outbreaks.

During an immune response (an energy-demanding state), the supply of nutrients and energy must be sufficient to cover the costs associated to this response as well as the usual metabolic processes. Furthermore, other types of physiological challenges also often contribute towards the depletion of essential nutrients, e.g. the nutritional requirements of several amino acids can increase (Aragão, et al., 2008; Conceição, et al., 2012; Costas, et al., 2008). Thus, the inclusion of certain nutrients (such as amino acids) at dosages above their nominal nutrient requirement levels (e.g. the levels required to ensure maximum growth performance, under normal rearing conditions) can have a functional aim (i.e. a role that goes beyond the direct nutritional functions of these additives).

Recent studies by our research group have clarified the potential use of some essential amino acids as nutraceutical additives for aquaculture fish: i) Costas, et al. (2011) observed that dietary arginine supplementation enhanced most assessed aspects of the innate immune mechanisms (NO levels, ACH50 and peroxidase) and increased disease resistance at the maximum level of supplementation (6.9 g 16 g^{-1} N); ii) Machado, et al. (2018) observed an enhanced immune status in European seabass fed a methionine-rich diet, which translated into an improved immune response to infection, since higher cellular differentiation/proliferation and recruitment to the inflammatory focus, improved plasma humoral immune parameters and modulation of key immune-related genes were observed, along with higher survival after a bacterial challenge; iii) Azeredo, et al. (2019) observed that a tryptophan-rich

diet can counteract stress-induced cortisol production in Senegalese sole, thereby rendering fish better prepared to cope with disease, which could translate into a promising strategy to overcome chronic stress-induced disease susceptibility in farmed Senegalese sole.

1.3 The immune response in teleost fish

Immunity covers all mechanisms and responses used by the organism to protect itself from the threats of pathogens. Fish immune system is physiologically similar to that of higher vertebrates: it is divided into physical barriers and cellular and humoral immune responses. The immune system of fish, posses non-specific and specific immune defence mechanisms. In fact, immunity acts like a combination of these two: innate response usually precedes, activates and regulates the nature of the adaptive response, being crucial in disease resistance, while the adaptive response is usually delayed, but crucial for long-lasting immunity (Secombes, Wang, 2012).

In comparison with higher vertebrates, fish have a less-specific immune system, characterized by a shorter response, a limited immunoglobulin repertoire, a weak memory and a limited mucosal response. As free-living organisms, from early stages they rely mainly on their innate immunity for survival (Rombout, et al., 2011).

The thymus, kidney (anterior/head and middle) and spleen are the main and largest lymphoid organs in teleosts (Zapata, et al., 2006). The kidney, and particularly the head-kidney, assume haematopoietic functions in teleost fish (Secombes, Wang, 2012), displaying a role equivalent to the bone marrow in vertebrates. The head-kidney is responsible for leucocyte production, being also reponsible for some endocrine functions, including the release of corticosteroids and other hormones (Tort, et al., 2003). Structurally, the head-kidney is a network of several reticular fibres that support lymphoid tissue. Macrophages are the main cells present in head-kidney, which aggregate into structures called melanomacrophage centres (MMCs), existing mostly as Ig+ cells (B cells) (Geven, Klaren, 2017; Mokhtar, 2017).

1.3.1 Immune system physical barriers

Fish scales, and mucosal surfaces, such as skins, gut, gills and nasal cavities, act as first barriers against pathogens (Smith, et al., 2019). The mucosal barriers in particular have an assembly of immune cells (lymphocytes) which constitute the lymphoid tissue. An important component of the lymphoid tissue is the mucosal-associated lymphoid tissue (MALT). MALT is present in fish mucosal surfaces, a very large area for the possible pathogens invasion, and possesses several defence mechanisms (both innate and adaptive) (Gomez, et al., 2013). In teleosts, the MALT is subdivided into skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT, only present in fish), gut-associated lymphoid tissue (GALT) and the nasopharynx-associated lymphoid tissue (NALT) (Salinas, 2015).

The SALT is an assembly of multiple cell types that includes secretory cells (e.g goblet cells and dendritic epidermal T cells), lymphocytes (T and B cells), granulocytes, macrophages and Langerhans-like cells (Esteban, 2012; Xu, et al., 2013). Besides its obvious role as a physical barrier, skin has the ability to secrete mucus. The skin mucus of teleost fish plays key roles in the defence against microorganisms, since it contains important immune components, such as: i) lectins, proteins which bind sugars with high specificity and are involved in recognition processes both at molecular and cellular levels, like opsonization and activation of complement; ii) antimicrobial peptides, which present antimicrobial activity against several pathogens; iii) lysozyme, an ubiquitous bactericidal enzyme; iv) proteases, crucial to kill pathogens by cleaving their proteins or by activating immunological mechanims; and v) antimicrobial IgM (Dash, et al., 2018; Esteban, 2012).

The gastrointestinal tract (GI) also has an important function of preventing pathogen invasion through its epithelial layer, besides its role in nutrient absorption. For instance, if microorganisms are ingested, they will face the GI tract, which, similarly to what happens with the gills and skin, possesses a GALT. Fish GALT, consists of several leucocyte populations located both intraepithelially and in the gut lamina propria, with no structural organization. GALT comprises both innate and adaptive immune cellular components essential to fight pathognes, like macrophages, mast/eosinophilic granular cells, dendritic cells, B and T cells (Smith, et al., 2019).

1.3.2 Innate immunity

Despite being the first phylogenetic group of animals with a completely developed adaptive immune system, fish mostly rely on their innate response. The innate immune system is at the forefront of the immune defence that responds to initial infection and/or disease and does not retain memory of previous responses. Teleosts highly rely on innate immune mechanisms to recognise pathogens as well as to develop the corresponding immune tolerance, leading or not to inflammatory responses.

The innate immune system is responsible for the recognition of an infection, initiation and resolution of an inflammation, and wound repair, in a complex network of several pro- and anti-inflammatory elements. These are usually characterised as cellular – natural killer cells, phagocytes thrombocytes – and humoral components – hormones, cytokines, protease inhibitors, lysins, precipitins and agglutinins (Ellis, 2001; Scapigliati, et al., 2001; Secombes, 1999; Secombes, et al., 2001).

Various other common immunological elements are also involved, such as growth inhibitors, lytic enzymes, the classic, the alternative and the lectin complement pathways, cytokines, chemokines and antibacterial peptides (Secombes, Wang, 2012). Recognition of pathogens and the responses of innate immune cells are accomplished via transmembrane receptors, Pattern Recognition Receptors (PRRs), of the circulating leucocytes (lymphocytes, neutrophils and monocytes/macrophages). Leucocytes communicate between them, resulting in innate and adaptive responses. The most diverse and well-characterized PRRs are the Toll-like receptors (TLRs) family of cell receptors, that are able to recognize several prevalent and conserved biomolecules of pathogens known as Pathogen-associated molecular patterns (PAMPs), including lipopolysaccharides (LPS) or peptidoglycans (PGN) on bacterial cell walls, double-stranded RNA (dsRNA) or unmethylated CpG DNA on viruses, β-glucan on fungi, and other dangerous endogenous molecules (Sasai, Yamamoto, 2013). PRRs are able to recognize and bind to PAMPs, triggering an inflammatory response. Upon inflammation, a production of several immune mediators by the several circulating pathognes occurs, such as cytokines (e.g IL-1β, IL-8, IL-10, IL-34) that mediate the immune response either with pro- or anti-inflammatory actions (Reyes-Cerpa, et al., 2012). Morever, in response to an inflammatory state, leucocytes produce several immune components like anti-proteases and proteases, peroxidase,

nitric oxide (NO) and reactive oxygen species (Secombes, et al., 2001). Additionally, neutrophils and monocytes/macrophages play a crucial role on the resolution of inflammation. For instance, neutrophils have a potent anti-microbial mechanism contributing to an effective pathogen killing and a control of the acute inflammation (Havixbeck, Barreda, 2015).

1.3.3 Adaptive immunity

The adaptive or acquired arm of the immune machinery is usually activated for the resolution of infections and re-establishment of fish homeostasis if a pathogen evades or overpowers the innate immune response(Secombes, Wang, 2012).

The adaptive immune system produces effector cells and molecules that are specific to the antigens (structures that bind to fish antibodies) of invading pathogens. This way, the adaptive defence can “learn” (i.e. can adapt to) and “remember” (i.e. can maintain a memory of) the antigens of pathogens, providing a long-lasting defence and protection against recurrent infections, leading to a faster inflammatory resolution (Morris, 2010).

The adaptive immune response is mediated by the actions of two major lymphocyte groups: T cells, that are the key elements of cellular mediated immunity whereas B cells, are responsible for the antibody (humoral) response (Secombes, Wang, 2012). The T cell receptor (TCR) is membrane-bound and, once stimulated, can activate the T cell to function either as a helper CD4 T cell or a cytotoxic CD8 T cell (Pennock, et al., 2013; Scapigliati, et al., 2000). B cells produce immunoglobulins (Ig), high-affinity antigen-binding proteins, also acting as professional antigen-presenting cells to trigger T cells (Smith, et al., 2019). Teleost fish can present up to three immunoglobulin (Ig) isotypes (i.e IgM, IgT and IgD). The IgM class of antibody has long been considered the most ancient and is the most prevalent Ig in fish plasma, contributing to both innate and adaptive immunity in fish. IgT is the most important immunoglobulin of mucosal surfaces in bony fishes, if not a dedicated mucosal immunoglobulin isotype, while IgD induces the production of antimicrobial, opsonizing, pro-inflammatory, B cell activating factors (Mashoof, Criscitiello, 2016).

1.4 Amino acids in immune mechanisms

Amino acids (AA) are precursors for the synthesis of important biologically active peptides and molecules, besides being the building blocks of proteins (Li, et al., 2009). Among them, essential AA are important regulators of key metabolic pathways that are essential for growth, reproduction, immunity and maintenance of the organisms (Conceição, et al., 2012). AA requirements may also increase as consequence of stressful conditions (e.g. handling, density) and metabolic changes associated with inflammation and infection (Conceição, et al., 2012; Melchior, et al., 2004; Sakkas, et al., 2013). In fact, the dependence of the immune system upon the availability of AA is, at least in part, related to their role as signalling molecules essential for cellular function (Li, et al., 2009). As such, AA present important immune functions in higher vertebrates as they regulate, for instance: i) activation of T- and B-lymphocytes, natural-killer cells and macrophages; ii) cellular redox stage, gene expression and lymphocyte proliferation; and iii) production of antibodies, cytokines and cytokine substances such as NO and superoxide (Wu, 2013).

This thesis is focused on six key amino acids: taurine, histidine, threonine, tryptophan, arginine and citrulline, most of which (i.e. except taurine and citrulline) are essential for protein synthesis. Therefore, a brief review on the main roles of each on the innate immune response and other physiological processes is presented separately.

1.4.1 Taurine

Taurine is a conditionally-essential nutrient that, although technically being considered an aminosulfonic acid, due to its chemical structure (NRC, 2011; Salze, Davis, 2015), is often referred to as an “amino acid” (in the sense of, “a small aminated acid”) in practice. The description and characterization of taurine biosynthesis was reviewed by Salze, Davis (2015). Figure 1 illustrates the two predominant pathways of taurine biosynthesis in teleosts. Pathway 1 – the cyteinesulfinate-dependent pathway – is the central pathway for taurine metabolism in mammals. Regarding this pathway, it is important to highlight that the flux of cysteinesulfinate to taurine is mainly determined by the highly-regulated cysteine dioxygenase (CDO), while the oxidation of the hypotaurine into taurine can also be enzymatically regulated (Vitvitsky, et al.,

2011). CDO plays an important role in maintaining balanced intracellular cysteine levels, with its activity being highly reactive to changes in dietary cysteine intake levels. In mammals, taurine biosynthesis occurs mainly in the liver, because cysteinesulfinate decarboxylase (CSD) activity is high in hepatocytes, when compared to muscle or kidney (Salze, Davis, 2015). Pathway 2 – the cysteic acid-dependent pathway – is a secondary pathway for the synthesis of taurine from cysteine, being dependent on cysteic acid decarboxylase (CAD) activity to convert cysteic acid into taurine.

In fish, the dominant pathway for taurine biosynthesis seems to be species-dependent. While some suggest that rainbow trout relies on pathway 1 (high CDS activity), other research in common carp and Japanese flounder indicates that taurine is produced not through pathway 1, but through pathway 2 (low CSD activity) (Yokoyama, et al., 2001). Gibson, et al. (2007) suggested also that rainbow trout benefit from taurine dietary supplementation, due to their scarce levels of CSD activity which are unable to provide the necessary amount of taurine for an optimal growth.

Even though taurine can be synthesized from methionine and cysteine in fish, the rate of synthesis is often too low to satisfy their nutritional requirements, particularly in the case of carnivorous fish fed dietary formulations with a low fishmeal inclusion (Goto, et al., 2003). This is particularly relevant in modern aquafeed formulations, since plant protein sources lack taurine (Brill, et al., 2019).

Taurine's role across numerous biological processes in fish has already been described, with a range of physiological problems and histological changes having been reported when Tau levels are reduced in the diet. One of the first clear signs observed as a result of taurine deficiency is growth depression, which can be accompanied by green liver syndrome, reduced haematocrit, anaemia and reduced disease resistance (Salze, Davis, 2015). In fact, research has shown that taurine supplementation in rainbow trout fed a fishmeal-free diet can improve growth and feed efficiency (Gibson, et al., 2007). Moreover, taurine has been described to play an important osmoregulatory role in fish (Takagi, et al., 2006). At the moment, information suggesting that taurine plays a role in fish health and immunity is still very fragmented. Nevertheless, Maita, et al. (2006), reported that yellowtail (*Seriola quinqueradiata*) fed a fishmeal-free diet supplemented with taurine displayed improved fish survival in response to an artificial bacterial challenge, reaching similar levels as those fed a fishmeal-containing control diet, suggesting immunoregulatory properties in fish.

Taurine plays a key role in bile acid metabolism, since it is the only amino acid conjugated with bile acids (which are of great importance for the emulsification, digestion and absorption of lipids in the intestine) in the liver of several fish species (Goto, et al., 1996). Briefly, bile salts are derived from cholesterol, synthesized by the liver and released into the intestine to emulsify lipids, enhancing the absorption of dietary lipids and fat-soluble vitamins (El-Sayed, 2014). Hence, in fish, as in mammals, it was demonstrated that taurine plays an important role in fat digestion as conjugator with bile acids (Kim, et al., 2008).

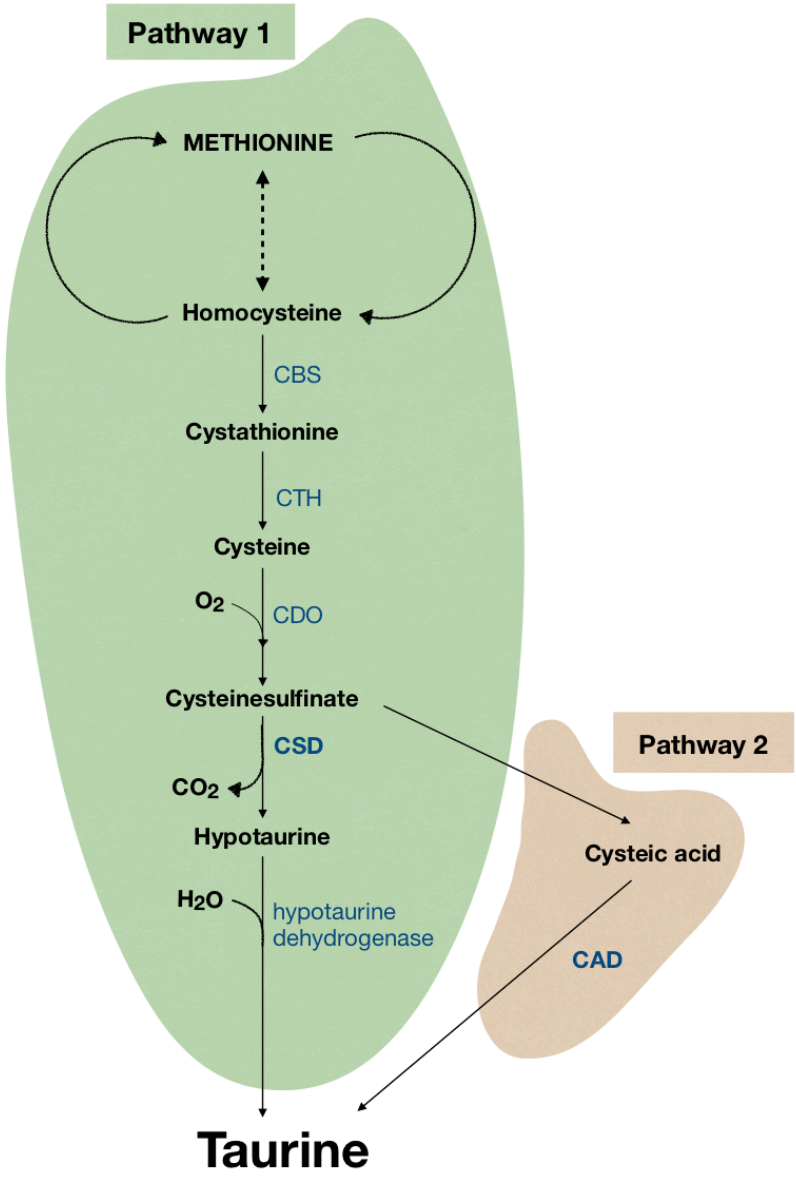

Fig. 1 Schematic illustration of the two predominant pathways of taurine biosynthesis in teleosts. **CBS**: cystathionine β-synthase; **CTH**: cystathionine gamma-lyase; **CDO**: cysteine dioxygenase; **CSD**: cysteinesulfinate decarboxylase; **ADO**: cysteamine dioxygenase; **CAD**: cysteic acid decarboxylase. Adapted from (Bender, 2012; Salze, Davis, 2015).

1.4.2 Histidine

Histidine is an essential AA abundant in plasma albumin and skeletal muscle in fish (NRC, 2011), which participates in one-carbon unit metabolism, consequently affecting DNA and protein synthesis (Li, et al., 2009). Histidine can be metabolized either to glutamate, anserine or carnosine (Fig. 2). Excess histidine is degraded to urocanate by the enzyme histidase (HAL) in the liver and further to glutamate. Briefly, histidine is metabolized to glutamate by a pathway that involves the elimination of a one-carbon group. In this reaction, 4-imidazolone-5-propionate is transferred to N-formiminoglutamate (FIGLU) by imidazolone propionase (IP), yielding glutamate.

Directly or through its derivative compounds, histidine plays important roles in homeostatic maintenance, osmoregulation, muscle pH buffering and detoxification of reactive carbonyl species (Farhat, Khan, 2013; Waagbø, et al., 2010). In humans, histidine contributes also significantly to the buffering capacity of plasma and tissue proteins (Bender, 2012). In fish, histidine has been described to have the ability to bind to and modulate the absorption of metals (zinc, copper and iron), affecting the distribution and excretion of essential elements (Wade, Tucker, 1998). Moreover, various studies indicated that histidine and its derivatives may act as antioxidants or can mitigate the impact of oxidative stress (Bellia, et al., 2008; Hobart, et al., 2004; Wade, Tucker, 1998). Histidine and its derivatives, such as carnosine and anserine, have been proven to scavenge reactive oxygen species, and contribute also to improve taste, texture and overall fillet quality (Bender, 2012; Farhat, Khan, 2013; Gao, et al., 2016).

The most evident and acute pathology observed when salmon are fed at suboptimal histidine levels is the development of cataracts (Remø, et al., 2014), which seems to be related to the antioxidant capacity of histidine and its derivatives. For example, it has been reported that, in the human lens, N-acetylcarnosine inhibits oxidative damage and consequently reduces the risk of cataract development (Babizhayev, et al., 2004). While the presence of this specific metabolite in fish lens has not been demonstrated, there are evidences that similar imidazole antioxidants derived from histidine (such as N-acetyl-histidine) are synthesized in the lens of Atlantic salmon (Remø, et al., 2011).

Anserine can be synthesised by incorporating β-alanine to 1-methyl histidine or by methylation of carnosine in the cells. Substantial levels of anserine are found in marine and animal products, while it is practically absent in plant protein sources used in aquafeeds (Andersen, et al., 2016). In mammals, carnosine and anserine are found mainly in muscle (Fig. 2). However, the effects of dietary supplementation of aquafeeds with these particular dipeptides are not well characterized yet.

Histidine and its derivates, beside displaying a clear antioxidant function, serve also as an energy fuel during starvation and can be a major component of non-carbonate buffers which protect fish against changes in pH resulting from hypoxia, burst-swimming, and increased lactic acid build-up (Bender, 2012; Li, et al., 2009). As a direct precursor of histamine, histidine plays a prominent role in allergic and immune responses as well (Ahmed, Khan, 2005). Studies in mice demonstrated the influence of histamine on wound healing, circulatory disease, immunology and infectious disease (Ohtsu, 2012). Additionally, a study with juvenile grass carp fed a His-deficient diet demonstrated significantly increased osmotic fragility of erythrocytes (Gao, et al., 2016).

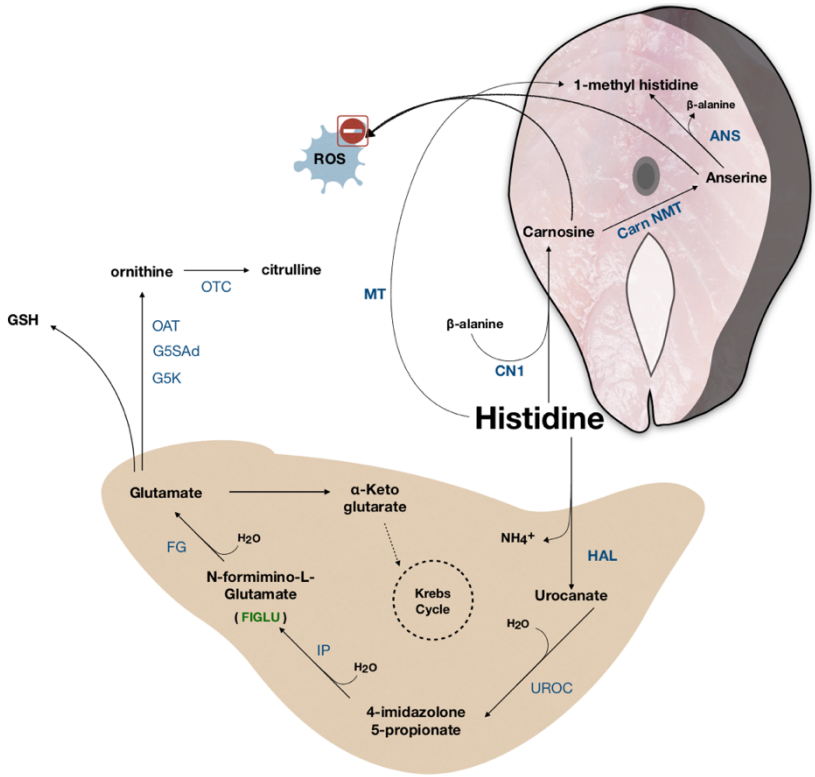

Fig. 2 Schematic illustration of histidine metabolism. Anserine can be synthesised by methylation of carnosine in the cells. **MT** – methyl transferase, **CN1** – carnosinase, **carn NMT** – carnosine N-methyl transferase, **ANS** – anserinase. Excess histidine is degraded to urocanate by the enzyme histidase in the liver. **HAL**: histidine ammonia lyase; **UROC**: urocanase; **IP**: imidazolone propionase; **FG**: formimidoylglutamase; **G5K**: glutamate-5-kinase; **G5SAd**: glutamate-5-semialdehyde dehydrogenase; **OAT**: ornithine aminotransferase; **OTC**: ornithine transcarbamylase. Adapted from (Andersen, et al., 2016; Azeredo, 2017; Marchese, et al., 2018)

1.4.3 Threonine

Threonine is considered the third limiting AA, after lysine and methionine, for growing fish, when these are fed low fishmeal feed formulations (Small, Soares, 1999; Tibaldi, Tulli, 1999). In mammals, three main pathways have been described for threonine catabolism. These pathways depend on which enzyme mediates the reaction: i) threonine dehydratase, ii) threonine aldolase or iii) threonine dehydrogenase (Fig. 3).

Threonine is a major component of mucin in the small intestine in mammals, which suggest its involvement in the regulation of intestinal barrier integrity and function (Faure, et al., 2005; Feng, et al., 2013). It is also involved in many physiological and biochemical processes, including in growth and immune functions (Fatma Abidi, A Khan, 2008; Gao, et al., 2014; Tibaldi, Tulli, 1999). In fact, studies have shown that threonine deficiency in rats decreased gut weight, which likely reflects the fact that the digestive and absorption functions of fish are largely dependent on the proper growth and development of digestive organs (Faure, et al., 2005). Furthermore, it has been shown to be involved in the stimulation of lymphocyte proliferation in mammals (Li, et al., 2007). In fish, Feng, et al. (2013) studied the effects of threonine both *in vivo* and *in vitro* in juvenile Jian carp and found that it was able to improve growth, digestive and absorptive capacity, enterocyte proliferation and differentiation, and protein synthesis. Likewise, dietary threonine improved growth, digestive and absorptive capacity, and the antioxidant status in intestine and hepatopancreas of sub-adult grass carp up to 13.9 mg threonine/g diet inclusion (Hong, et al., 2015). Therefore, an adequate provision of threonine may contribute to an ideal intestinal function and maintenance of the oxidative status.

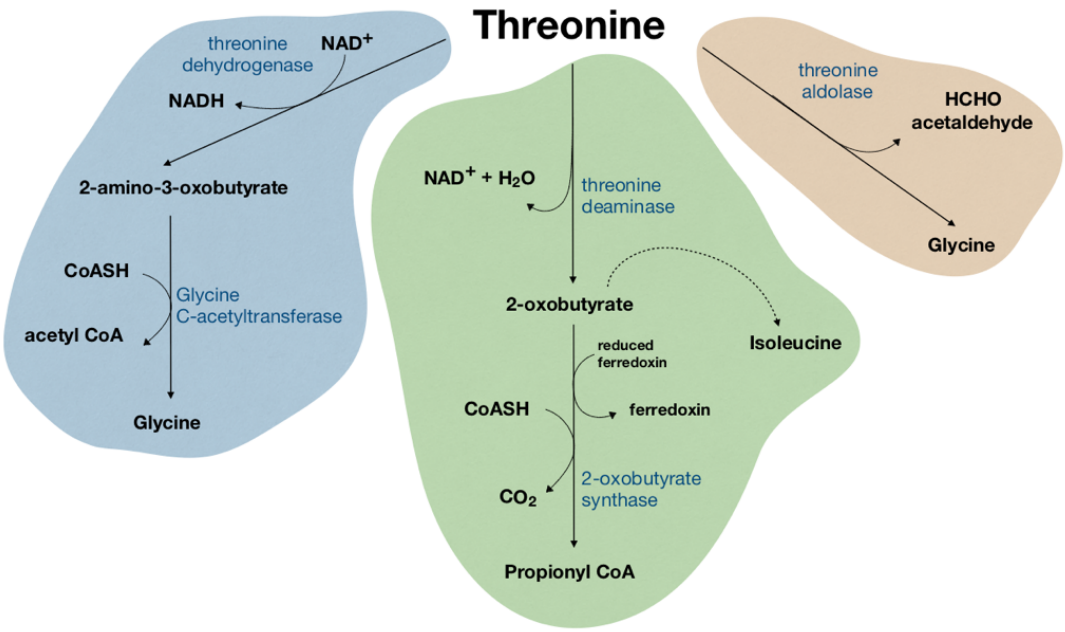

Fig. 3 Schematic illustration of threonine catabolism. Adapted from (Bender, 2012)

1.4.4 Tryptophan

Tryptophan is an essential AA in all animals. In addition to being required for protein synthesis, it is a precursor to bioactive molecules and neurotransmitters such as serotonin (5-HT), kynurenine and melatonin (Hoseini, et al., 2017). In vertebrates, upon dietary uptake, tryptophan can enter one of the two described pathways (Fig. 4): (i) catabolism through the kynurenine pathway (KP) and (ii) serotonin (5-HT) synthesis.

In fish, the routes leading to the production of serotonin are well-established and known to be similar to those of other vertebrates. Though less data is available about fish kynurenine pathway, it is known that this pathway is mainly directed to degrade excess tryptophan, regulate immune response and produce sexual pheromones. Since tryptophan is the precursor of serotonin, its intake modulates behaviour, mood and stress response in fish (Herrero, et al., 2007; Lepage, et al., 2002; Lepage, et al., 2005). Practically all tryptophan is degraded along the KP pathway, and complete tryptophan degradation mainly occurs in the liver, triggered by an oxidation step mediated by the tryptophan-2,3-dioxygenase (TDO).

Kynurenine pathway also occurs in macrophages. Yet, in these immune cells, the role of TDO is played by the indoleamine-2,3-dioxygenase (IDO). This enzyme grants tryptophan its role in immune response. It has higher affinity to tryptophan than TDO

does, concomitantly suppressing TDO's activity. The activation of IDO occurs upon an inflammatory stimulus, such as the presence of bacterial lipopolysaccharides (LPS) or IFN-γ, observed both in mammals and teleost fish (Cortés, et al., 2016).

In fact, in humans, plasma tryptophan concentration, its brain bioavailability and the associated serotonergic activity in the raphe nuclei are influenced by diet composition, stress, physical activity and immune system activation (Russo, et al., 2009). Dietary tryptophan deficiency causes growth retardation, scoliosis and interference in mineral metabolism in fish (Murthy, Varghese, 1997; Walton, et al., 1984). Akhtar, et al. (2013) and Ciji, et al. (2015), for instance, showed that growth performance of *Labeo rohita* under stressful conditions and water pollution is improved when fish are fed diets containing 0.75 and 1.5 % tryptophan of the dietary protein, compared to feeds attaining the estimated tryptophan requirement for this species (0.36-0.38 % of the dietary protein). Furthermore, available evidence shows that dietary tryptophan supplementation successfully mitigates stress response to different stressors (e.g. social/crowding, temperature, salinity) in several vertebrates (Akhtar, et al., 2013; Hoseini, Hosseini, 2010; Liu, et al., 2015; Tejpal, et al., 2009).

Primary stress responses, which involve the initial neuroendocrine responses, include the release of catecholamines from chromaffin tissue and the stimulation of the hypothalamus-pituitary-interrenal (HPI) axis, ending in the release of corticosteroid hormones into circulation. This is followed by secondary (e.g. changes in plasma and tissue ion and metabolite levels) and tertiary responses, effects on whole-animal performance like changes in growth, nutritional condition, overall resistance to disease and ultimately survival (Barton, 2002; Tort, 2011). Cortisol, a mediator of the primary stress response, is known to influence several aspects of immune defence mechanisms in fish (Hoseini, et al., 2017). For instance, cortisol inhibits LPS-induced expression of pro-inflammatory cytokines and inducible nitric oxide (NO) synthase (Stolte, et al., 2008; Verburg-van Kemenade, et al., 2011; Verburg-Van Kemenade, et al., 2009). Cortisol was also reported to induce apoptosis and inhibit proliferation of immune cells. For example, cortisol inhibited the proliferation of rainbow trout monocyte/macrophage cell line and *in vivo* studies in common carp revealed that stress reduced the number of circulating B-lymphocytes (Engelsma, et al., 2003; Pagniello, et al., 2002).

Serotonin is a monoamine and neurotransmitter involved in activating and inhibitory mechanisms related to the HPI axis, ultimately modulating cortisol production. 5-HT

can either induce or inhibit adrenocorticotropic hormone (ACTH) production in the pituitary, and consequently increase or decrease interrenal cortisol synthesis (Lepage, et al., 2003). Therefore, tryptophan supplementation has the potential to indirectly (through 5-HT) affect cortisol levels and, thus, the physiological response to stress.

Overall, given the metabolic pathways downstream of tryptophan, its use as a dietary supplement can both directly affect stress responses while also indirectly affecting fish immunity.

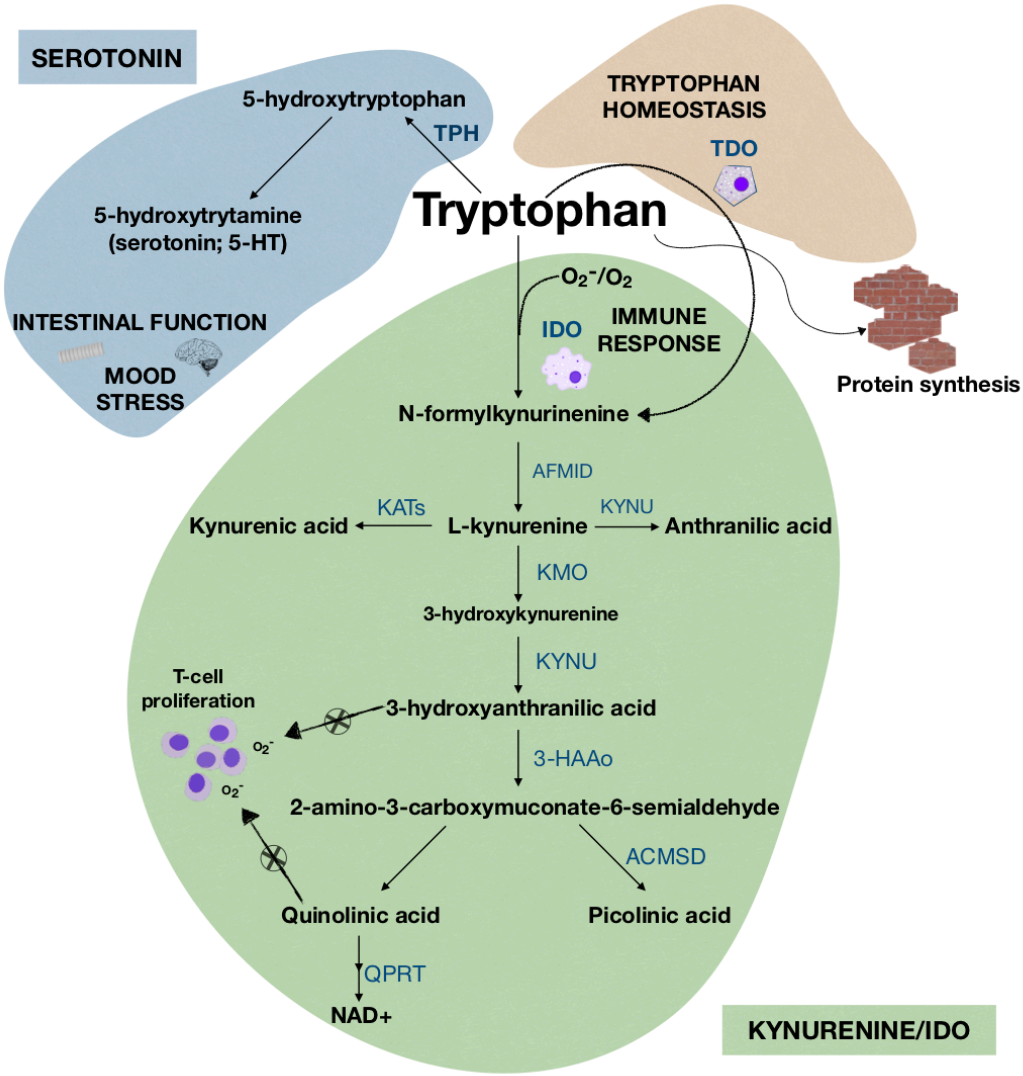

Fig. 4 Tryptophan metabolism in mammals and its main functions in key tissues. **TPH**: tryptophan hydroxylase; **TDO**: tryptophan 2, 3-dioxygenase**; IDO**: 2, 3-indoleamine dioxygenase; **NAD+**: nicotinamide adenosine dinucleotide; **AFMID**: arylformamidase; **KATs**: kynurenine amino-transferase; **KYNU**: kynureninase; **KMO**: kynurenine 3-hydroxylase; **KYNU**: kynureninase; **3-HAAo**: 3- hydroxyanthranilic acid oxygenase; **ACMSD**: picolinate carboxylase; **QPRT**: quinolinic acid phosphoribosyltransferase. Adapted from (Azeredo, 2017; Hoglund, et al., 2019; Le Floc'h, et al., 2011; Simon P., et al., 2015).

1.4.5 Arginine

Arginine is the precursor for several compounds, such as: nitric oxide (NO), urea, polyamines, proline, glutamate, creatine and agmatine in terrestrial animals, being considered one of the most versatile AA (Wu, Morris, 1998). The importance of arginine in the immune system is related with two main pathways, i) prompting NO and citrulline production, via the inducible nitric oxide synthase (iNOS) or ii) prompting ornithine production, via arginase (Fig. 5). Upon an inflammatory state, triggered by LPS and IFNγ, fish macrophages (M1) express iNOS, followed by a high NO production against infectious agents (e.g. bacteria) (Buentello, Gatlin, 1999; Mills, 2001; Mills, 2015; Nitz, et al., 2019). M1 macrophage activity depends on extracellular arginine levels: in the case of sufficient supply, M1 exports citrulline but, under arginine depletion, macrophages import citrulline and show an increase in argininosuccinate synthase activity to sustain NO output (Qualls, et al., 2012). On the other hand, arginase activity is observed in M2 macrophages. These cells are activated in response to anti-inflammatory T-cell-produced cytokines (that occur in a later stage of the inflammatory process, supressing the activity of other immune cells)(Mills, 2015). These arginase-expressing macrophages mainly mediate regulatory functions and play a key-role on the resolution of the immune response and tissue repair (Fig. 5).

This AA is also an intermediate in the urea cycle. In this pathway, arginine is cleaved by arginase to liberate urea, and ornithine is formed. Ornithine is then converted to proline or polyamines. Polyamines are important for lymphocyte proliferation and differentiation (Nitz, et al., 2019).

Considerable evidence from studies in diverse animal models indicates that adequate amounts of arginine are required for lymphocyte development and that a dietary arginine surplus enhances immune function during immunological challenges (Hoseini, et al., 2020; Li, et al., 2007). Nevertheless, arginine was also reported to mediate immunosuppressive mechanisms. In mammals, T-cell activation and function is dictated by arginine metabolism in myeloid suppressor cells (Bronte, Zanovello, 2005). Sharma, et al. (2004) observed that L-arginine, by its conversion to NO, was able to modulate the immune response in rats and mice under restraint stress (RS), antagonizing the immuno-suppressive effect of RS on humoral as well as cell-mediated immune responses. In a similar way to higher vertebrates, fish produce NO and

ornithine from arginine via iNOS and arginase, respectively (Buentello, Gatlin, 1999; Zhou, et al., 2015). Indeed, upon inflammatory circumstances, fish phagocytes produce NO, acting as an oxidant against pathogens compromising its structures and function (Andersen, et al., 2015; Bronte, Zanovello, 2005).

In studies with fish, different outcomes have been observed. A positive effect of feeds supplemented with arginine on disease resistance has been reported in several teleosts (Costas, et al., 2011; Li, et al., 2009), whereas a negative effect was observed in Jian carp (*Cyprinus carpio var. Jian*), where both *in vivo* and *in vitro* arginine supplementation counteracted LPS-induced inflammatory responses (Jiang, et al., 2015). A similar detrimental effect was also observed in European seabass (*Dicentrarchus labrax*) fed arginine-enriched diets (Azeredo, et al., 2015). New insights of a recent study revealed that arginine supplementation could compromise to some extent the seabass cell-mediated immune response, decreasing the circulating numbers of neutrophils and monocytes (Azeredo, et al., 2020). Therefore, despite current knowledge about arginine metabolism, opposing effects from different studies point to a species-specific role of arginine in the fish immune status, a topic that deserves further attention.

Arginine and its metabolites (L-ornithine and L-proline) have been also suggested to play a relevant role in the stress response, acting towards stress amelioration in different animals. In fact, dietary arginine supplementation decreased the serum level of cortisol in growing-finishing pigs and weaned piglets (Ma, et al., 2010; Yao, et al., 2011). Experiments with Senegalese sole (*Solea senegalensis*) under chronic stressful conditions (e.g. high densities and handling), reported that stress can affect amino acids requirements (Aragão, et al., 2008). Arginine and histidine concentration were significantly lower in fish under crowding stress (Costas, et al., 2008). This confirms that during stressful conditions, essential amino acids involved in metabolic pathways have an important role. In this context, arginine-rich diets led to decreased plasma cortisol levels and an enhancement of several aspects of the innate immune response (i.e. circulating monocytes, NO production and humoral parameters) in Senegalese sole under chronic stress, resuting in increased disease resistance upon a possible bacterial infection (Costas, et al., 2013; Costas, et al., 2011).

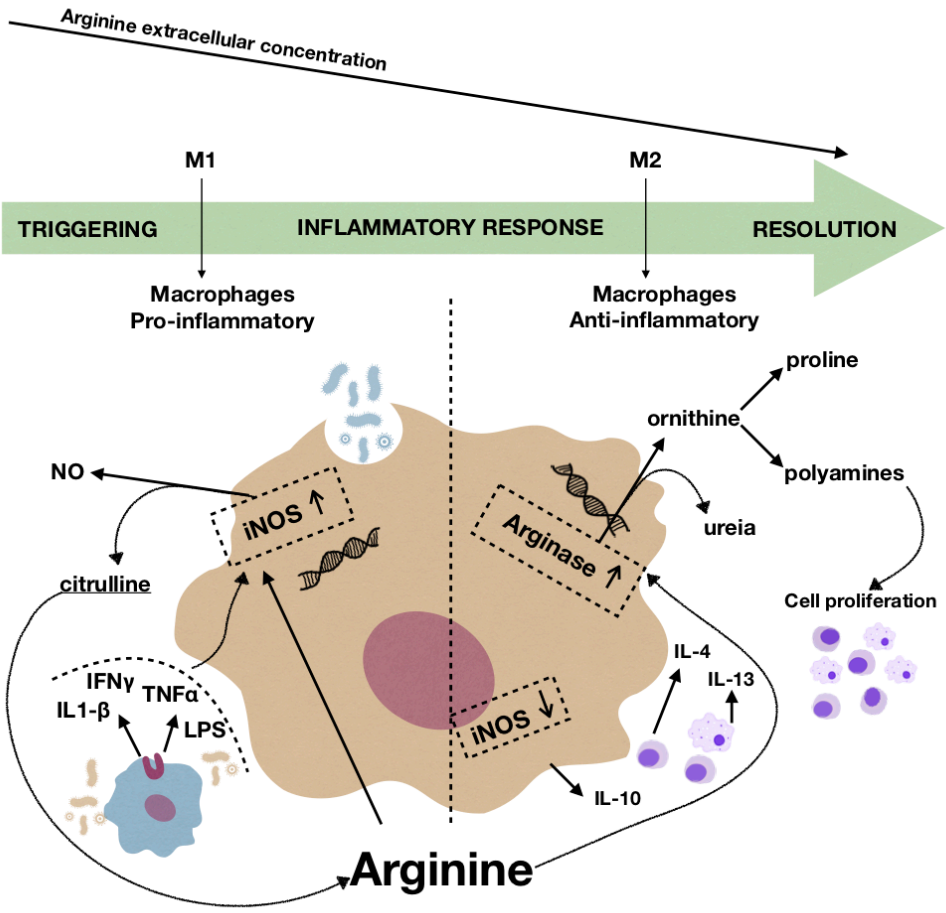

Fig. 5 Macrophage activation, phenotype determination and arginine metabolism during the inflammatory response. **M1**: classically activated macrophage; **M2**: alternatively activated macrophage; **IFNγ**: interferon γ; **LPS**: lipopolysaccharide; **IL-1β**: interleukin-1β; **TNFα**: tumor necrosis factor-α; **iNOS**: inducible nitric oxide synthase; **NO**: nitric oxide; **IL-10**: interleukin-10; **IL-4**: interleukin-4; **IL-13**: interleukin-13. Adapted from (Azeredo, 2017; Nitz, et al., 2019)

1.4.6 Citrulline

Citrulline is the precursor of arginine, through a two-step reaction mediated by argininosuccinate synthase and argininosuccinate lyase. It is also a by-product of arginine upon its conversion to NO (Breuillard, et al., 2015) (Fig. 5). In mammals, it has been reported that citrulline might offer a safe alternative to arginine for improving macrophage function under certain metabolic conditions. Moreover, Batista, et al. (2012) found that a citrulline-enriched diet improved gut function by decreasing intestinal permeability and positively affecting bacterial translocation and immune function in mice. It also stimulated intestinal production of secretory immunoglobulin A, which is the first line of host defences against environmental pathogens.

Indeed, few studies have approached the effect of a citrulline surplus on immune responses in higher vertebrates, and to the best of our knowledge there is scarce

information about this in fish. Nonetheless, Buentello, Gatlin (1999) revealed that *in vitro* NO production was improved in peritoneal macrophages of channel catfish, *Ictalurus punctatus*, upon addition of citrulline to the culture media. A pathogen challenge performed by the same authors revealed that dietary arginine surplus (2 % inclusion of the diet) enhanced the ability of channel catfish to survive exposure to *Edwardsiella ictaluri* (Buentello, Gatlin, 2001).

1.5 Thesis main objectives

This thesis' main goal is to evaluate the dietary effects of different amino acids — taurine, threonine, histidine, tryptophan, arginine and citrulline — when supplied at levels beyond the nominal nutritional requirements, in improving the innate immunity of gilthead seabream juveniles, given their direct or indirect roles in different physiological and immune mechanisms, using a combination of classical indicators (e.g. haematological and humoral parameters) and high-throughput molecular biology techniques (e.g. transcriptomics and proteomics), and with particular focus on immunologically-relevant targets (head-kidney, gut, blood/plasma).

Furthermore, this thesis also seeks to clarify how contextual factors such as "presence of an inflammatory stimulus", "dosage" and "exposure time" can interact with (and thus affect) the functional effects of these amino acids in a practical setting.

Finally, complementary information regarding the impact of these amino acids on other aspects of fish physiology and health (e.g. growth performance, stress response and nutritional condition indicators) was also collected in order to contribute towards a more complete understanding and more widespread adoption of amino acids as functional additives in aquafeeds.

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Chapter 2

Dietary histidine, threonine or taurine supplementation affects gilthead seabream (*Sparus aurata*) immune status

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Abstract

The concept of supporting animal health through the best possible nutrition is well-accepted in modern aquaculture, and functional amino acids (AA) appear to be good candidates to improve health and growth performance. For instance, Histidine (His), Taurine (Tau) and Threonine (Thr) appear to play important roles in homeostatic maintenance, detoxification of reactive species and immune function. The present study aimed to evaluate the effects of His, Tau and Thr supplementation (at 0.40 %, 0.50 % and 0.75 % of feed, respectively) on the gilthead seabream (*Sparus aurata*) immune status. Triplicate groups of fish (8.77 ± 0.13 g) were either fed a control diet (CTRL) with a balanced AA profile, or the CTRL diet supplemented with His, Tau orThr. After 2 and 4 weeks of feeding, fish were euthanized and blood was collected for blood smears, plasma and mucus for humoral immune parameters, and head-kidney for health-related gene expression (29 biomarkers). A decrease in total white blood cells (WBC) was observed in fish fed Thr supplemented diet after 2 weeks compared to the other dietary treatments. In contrast, peripheral lymphocytes augmented in fish fed Thr comparted to those fed His dietary treatment after 4 weeks. Plasma antiprotease activity increased in fish fed Thr compared to those fed CTRL and Tau dietary treatments, regardless of sampling time. The bactericidal activity in skin mucus decreased in fish fed Tau and His dietary treatments compared to those fed the CTRL diet after 2 weeks. Mucus antiprotease activity significantly increased in fish fed His regardless of sampling time. The membrane IgT (*mIgT*) was up-regulated in fish fed Tau after 4 weeks, while C-type lectin (*clec10a*) was down-regulated in fish fed Thr after 2 weeks of feeding. By comparing the molecular signatures of head-kidney by means of a PLS-DA, it is possible to visualise that the main difference is between the two sampling points, regardless of diets. Altogether, these results suggest that dietary supplementation with His, Tau or Thr at the tested levels causes mild immune-modulation effects in gilthead seabream, which should be further studied under disease challenge conditions. Still, plasma antiprotease activity increased in fish fed Thr supplemented diet which, together with the decrease in clec10 transcript and total WBC at 2 weeks contrasting the enhancement of acquired immune condition at 4

weeks, reinforces the importance of supplementation duration when aiming to improve immune alertness. In addition, His supplementation led to an increase of total antiproteases activity measured in skin mucus, while some stimulation of dietary Tau levels on neutrophil numbers could be seen.
Keywords: Amino acids; immune-modulation; mucosal immunity; blood leucocytes.

2.1 Introduction

In a fish farming context, fish are susceptible to a wide range of pathogens, since seawater is a good growing media for many bacteria and virus that can ruin an entire fish stock. Once present in water, pathogens are in direct contact with the first lines of defence of fish and can easily spread, particularly if the animals are not well-nourished and prepared (Oliva-Teles, 2012; Quesada, et al., 2013). As such, health maintenance is of utmost importance in modern fish farming, and establishing strategies to improve fish immune status and welfare is essential.

Recently, there has been an effort to adopt the use of supplements that can boost fish immune status (e.g. yeast extracts, probiotics, prebiotics and amino acids) in the development of functional aquafeeds which can contribute to significantly reduce the abusive use of antimicrobials (e.g. antibiotics) and disinfectants (Leal, Calado, 2019). Moreover, it is known that amino acids (AA) requirement are expected to increase under challenging rearing conditions, so dietary AA supplementation beyond the nominal requirements for each species could benefit animals, as already reviewed elsewhere (Conceição, et al., 2012; Herrera, et al. (2019). Thus, research on dietary AA supplementation as potential functional additives (i.e. supplied at levels beyond the species' requirements) in fish, with particular emphasis on their effects on the immune system, should be better explored.

Histidine (His) is an essential AA (EAA) abundant in plasma albumin and skeletal muscle in fish (NRC, 2011), which participates in one-carbon unit metabolism, consequently affecting DNA and protein synthesis (Li, et al., 2009). Directly or through its derivative compounds, histidine plays important roles in homeostatic maintenance, osmoregulation, muscle pH buffering and detoxification of reactive carbonyl species (Farhat, Khan, 2013; Waagbø, et al., 2010). In humans, His contributes also significantly to the buffering capacity of plasma and tissue proteins (Bender, 2012). Proteins buffer effect is the result of their dissociable side groups. For most proteins, including hemoglobin, the most important of these dissociable groups is the imidazole ring of His residues. The His metabolite carnosine (beta-alanyl-L-histidine) also combats intramuscular acidosis by maintaining intracellular and extracellular buffering in muscle tissue pH (Lancha Junior, et al., 2015). Moreover, various studies indicate that His and its derivatives act as antioxidants or can mitigate the impact of oxidative

stress (Bellia, et al., 2008; Hobart, et al., 2004; Wade, Tucker, 1998). Histidine and its imidazole derivatives such as carnosine and anserine have been proven to scavenge reactive oxygen species, and contribute also to improve taste, texture and overall fillet quality (Bender, 2012; Farhat, Khan, 2013; Gao, et al., 2016). Additionally, a study with juvenile grass carp fed a His-deficient diet demonstrated significantly increased osmotic fragility of erythrocytes (Gao, et al., 2016).

Threonine (Thr) is often considered the third limiting AA after lysine and methionine for growing fish fed low fishmeal feed formulations (Small, Soares, 1999; Tibaldi, Tulli, 1999). It is also involved in many physiological and biochemical processes, including growth and immune functions (Fatma Abidi, A Khan, 2008; Gao, et al., 2014; Tibaldi, Tulli, 1999). Duval, et al. (1991) discovered that Thr-enriched cell culture medium prevented apoptosis, stimulated mouse hybridoma cell growth and promoted antibody production in lymphocytes, through protein synthesis and cellular signalling mechanisms. Also, Thr deficiency up-regulated nitric oxide levels in blood monocytes of broilers (Corzo, et al., 2007). Dietary Thr inclusion at 13.9 mg/g diet improved growth, digestive and absorptive capacity, as well as the antioxidant status in intestine and hepatopancreas of sub-adult grass carp (Hong, et al., 2015). Thr is also a major component of mucin in the small intestine in animals, which suggests its importance in the regulation of intestinal barrier integrity and function (Faure, et al., 2005; Feng, et al., 2013).

Taurine (Tau) is a conditionally essential nutrient that, although technically being considered an amino sulfonic acid, due to its chemical structure (NRC, 2011; Salze, Davis, 2015), is often referenced to as an amino acid (in the sense of a small aminated acid). Tau can be synthesized from methionine and cysteine in fish, but the rate of synthesis is usually too low to satisfy the nutritional requirements of (at least) carnivorous fish (Goto, et al., 2003). Hence, growth depression is one of the first clear observed signs reported during Tau deficiency (Salze, Davis, 2015). In fact, Tau supplementation in rainbow trout (*Oncorhynchus mykiss*) fed a fishmeal-free diet improved growth and feed efficiency (Gibson, et al., 2007). Tau's role across numerous biological processes in fish has already been described, with a range of physiological problems and histological changes having been reported when Tau levels are reduced in the diet, namely green liver syndrome, reduced haematocrit, anaemia and reduced disease resistance (Salze, Davis, 2015). Tau has also been described to play an important osmoregulatory role in fish (Takagi, et al., 2006). At the moment, information

suggesting that Tau plays a role in fish health and immunity is still very fragmented. Nevertheless, Maita, et al. (2006), reported that yellowtail (*Seriola quinqueradiata*) fed a fishmeal-free diet supplemented with Tau displayed improved fish survival in response to an artificial bacterial challenge, reaching similar levels as those fed a fishmeal-containing control diet, suggesting it may have immunoregulatory properties in fish.

The main goal of this study was to evaluate the effects of His, Thr and Tau on gilthead seabream immune function when added as a supplement to a practical aquafeed formulation.

2.2 Material and Methods

2.2.1 Diets Formulation

Extruded feeds were based on plant proteins sources, and limiting fish meal inclusion to 12 %. This mimics most of the currently used commercial diets for gilthead seabream. Using this basal formulation, three other experimental diets were produced at SPAROS Lda. (Olhão, Portugal) through the inclusion of crystalline His, Thr and Tau.

Briefly, a control (CTRL) diet was formulated to meet current commercial formulations for this species, as well as its known nutritional requirements. The three other diets were identical to the CTRL diet but supplemented with 0.4 % His, 0.75 % Thr or 0.5 % of Tau of feed (Table 1). These inclusion levels were chosen to be at least 50 % above the requirement levels established for gilthead seabream (Kaushik, 1998; Peres, Oliva-Teles, 2009). Main ingredients were ground (below 250 µm) in a Hosakawa, model #1 micropulverizer hammer mill (Hosokawa Micron Ltd., United Kingdom). These ground ingredients were then mixed according to the target formulation in a Double-helix Mixture TGC, model 500L (TGC Extrusion, France), to attain a basal mixture (no oils were added at this stage). All diets were manufactured by extrusion (pellet size 2.0 mm) by means of a pilot-scale twin-screw extruder CLEXTRAL BC45 (Clextral, France) with a screw diameter of 55.5 mm and temperature ranging 105 – 110 ºC. Upon extrusion, all batches of extruded feeds were dried in a convection oven (OP 750-EF, LTE Scientifics, United Kingdom) for 2 h at 60 ºC. After this process, pellets were left to cool at room temperature, and subsequently

the AA were mixed with the fish oil fraction according to each target formulation and added under vacuum coating conditions in a Pegasus vacuum mixer (PG-10VCLAB, DINNISEN, The Netherland).

Table 1. Ingredients of the experimental diets.

	Experimental diets			
Ingredients (% feed basis)	**CTRL**	**HIS**	**TAU**	**THR**
Fishmeal LT70 (NORVIK) [a]	12.00	11.95	11.94	11.94
Poultry meal 65 [b]	5.00	4.98	4.98	4.98
Soy protein concentrate (Soycomil) [c]	8.50	8.47	8.46	8.46
Wheat gluten [d]	7.37	7.34	7.33	7.33
Corn gluten [e]	8.00	7.97	7.96	7.96
Soybean meal 48 [f]	7.68	7.65	7.64	7.64
Soybean meal 44 [g]	15.00	14.94	14.93	14.93
Rapeseed meal [h]	5.00	4.98	4.98	4.98
Wheat meal [i]	14.30	14.24	14.23	14.23
Sardine oil [j]	4.65	4.63	4.63	4.63
Rapeseed oil [k]	10.85	10.81	10.80	10.80
Vit & Min Premix INVIVO 1% [l]	1.00	1.00	1.00	1.00
Antioxidant [m]	0.20	0.20	0.20	0.20
Sodium propionate [n]	0.10	0.10	0.10	0.10
MCP [o]	0.20	0.20	0.20	0.20
DL-Methionine [p]	0.15	0.15	0.15	0.15
L-Threonine [q]				0.75
L-Histidine [r]		0.40		
Taurine [s]			0.50	
Proximate analyses				
Dry matter (% feed)	94.6	94.3	94.2	94.3
Crude protein (% dry weight)	42.62	41.92	42.24	42.23
Crude lipid (% dry weight)	18.3	19.1	18	18.3
Ash (% dry weight)	6.4	6.1	6.3	6.1
Gross Energy (kJ g-1 DM)	21.83	21.75	21.37	22.00

[a] *Fish meal LT70: 71.9%CP, 6.8% CF, Norvik Sopropêche, France*
[b] *Poultry meal: 65%CP, 14.4% CF, SAVINOR UTS, Portugal*
[c] *Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands*
[d] *Wheat gluten: 80.4% CP; 5.6% CF, VITAL Roquette, France*
[e] *Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal.*
[f] *Soybean meal 48: Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, CARGILL, Spain*
[g] *Soybean meal 44: Solvent extracted dehulled soybean meal: 44% CP, 1.8% CF, CARGILL, Spain*
[h] *Rapeseed meal: Defatted rapeseed meal: 37.7% CP, 2.3% CF, Premix Lda, Portugal*
[i] *Wheat meal: 11.7% CP; 1.6% CF, Casa Lanchinha, Portugal*
[j] *Sardine oil, 98.1% CF, Norvik Sopropêche, France*
[k] *Rapeseed oil, 98.2% CF Henry Lamotte Oils GmbH, Germany*
[l] *Vitamin and mineral premix: INVIVONSA Portugal SA, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 500mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; excipient wheat gluten.*
[m] *Antioxidant: VERDILOX, Kemin Europe NV, Belgium*
[n] *Sodium propionate: Disproquímica, Portugal*
[o] *Monocalcium phosphate: Premix Lda, Portugal*
[p] *DL-Methionine: DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany*
[q] *L-Threonine: ThreAMINO 98.5%, Evonik Nutrition & Care GmbH, Germany*
[r] *L-Hisdidine: L-Histidine 98%, Ajinomoto Eurolysine SAS, France*
[s] *L-Taurine: L-Taurine 98%, ORFFA, The Netherlands*

Table 2. Amino acid composition (g AA.100 g^{-1} diet) of the experimental diets.

	Experimental diets			
	CTRL	**HIS**	**TAU**	**THR**
Arginine	7.6	6.1	6.3	5.7
Histidine	2.1	3.4	2.6	2.4
Lysine	5.3	6.3	6.7	6.2
Threonine	3.8	3.5	3.6	4.3
Isoleucine	4.5	4.2	4.3	4.1
Leucine	8.4	7.2	7.1	6.9
Valine	4.8	4.9	5.0	4.7
Methionine	2.3	2.5	2.9	2.2
Phenylalanine	5.3	4.0	4.0	3.8
Cystine	0.6	1.3	1.3	1.3
Tyrosine	4.0	3.4	3.5	3.1
Aspartic acid + Asparagine	7.4	9.6	10.1	9.5
Glutamic acid + Glutamine	19.0	21.8	19.0	21.9
Alanine	4.8	4.8	5.0	4.6
Glycine	5.1	4.4	4.9	4.2
Proline	6.7	6.6	6.5	6.4
Serine	4.5	4.4	4.5	4.4
Taurine	0.3	0.3	1.0	0.2

*Tryptophan was not analysed

2.2.2 Rearing conditions

The current trial was conducted by trained scientists (following FELASA category C recommendations) and according to the animal experimentation guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE at the experimental facilities of I3S (Instituto de Investigação e Inovação em Saúde, Porto, Portugal).

Gilthead seabream (*Sparus aurata*) juveniles with average weight of 8.77 ± 0.44 g were acquired from a certified hatchery (SONRÍONANSA; Cantabria, Spain) and were randomly distributed into 12 tanks (200 L; n = 70) for an acclimatization period of 3 weeks being fed the CTRL diet. Fish were held in a recirculation seawater system in which oxygen saturation (7.3 ± 0.01 mg L^{-1}) and photoperiod were automatically controlled (10 h dark and 14 h light). Temperature was maintained by a water heater/cooler system at 20 ± 0.5 ºC and kept unchanged throughout the experiment. Both nitrite and ammonium levels were daily recorded, and its levels controlled by a water ozoniser system. Water renovations and system cleanings were performed twice a week. After the quarantine period, the experiment was started by feeding each group with the respective feed three times a day *ad libitum*. The control (CTRL) group was fed a control diet, which met the EAA requirement levels estimated for gilthead seabream (Kaushik, 1998; Peres, Oliva-Teles, 2009). The other 3 groups were fed diets with the inclusion of Thr, Tau or His (THR, TAU and HIS dietary treatments, respectively), as stated in Table 2.

2.2.3 Experimental procedures

An initial sampling point was set, and 10 fish fed the CTRL diet were euthanized and designated as time zero (TØ) to assess their immune status prior to the feeding trial.

The feeding trial lasted 4 weeks in order to assess the effect of short and mid-term dietary AA supplementation. Feed intake was recorded daily and body weight of fish fed dietary treatments was measured at the beginning and at the end of the feeding trial. Growth was monitored by obtaining the initial body weight (IBW) and final body weight (FBW) and used to calculate growth performance parameters.

At 2 and 4 weeks after feeding the experimental diets, 36 fish from each group (i.e. 12 per replicate) were euthanized by anaesthetic overdose with 2-phenoxyethanol and individually weighed. Samples were obtained for immunological and transcriptomic studies. Skin mucus was collected by gently scraping the fish dorsal-lateral surface using a cell scraper with enough care to avoid contamination with blood, urogenital and intestinal excretions, snap frozen in liquid nitrogen and stored at -80 ºC. Blood was collected from the caudal vein using heparinized syringes, centrifuged at 10,000 × *g* for 10 min and plasma pools were stored. Blood from 4 fish was also used to perform blood smears. Head-kidney was collected and snap frozen for gene expression analysis. All samples were immediately kept at -80 ºC until further processing.

2.2.4 Haematological procedures

Blood was collected from the caudal vein using heparinized syringes. The haematological profile consisted of total white (WBC) and red (RBC) blood cells counts and blood smears for differential WBC counting as described by Machado, et al. (2015).

Blood smears were initially fixed with formol-ethanol (10 % of 37 % formaldehyde in absolute ethanol) and afterwards stained with Wright's stain (Haemacolor; Merck). Neutrophils were identified according to their peroxidase activity, which was detected using the method described by Afonso, et al. (1998). The slides were examined under oil immersion (1000 ×) and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes, and neutrophils. The relative proportion of each cell type was subsequently calculated.

2.2.5 Immune parameters

Plasma bactericidal activity was measured using *Vibrio anguillarum* according to Graham, et al. (1988) adapted by Machado, et al. (2015), with some modifications. Succinctly, 20 µl of plasma were added to duplicate wells of a U-shaped 96-well plate. Hanks' Balanced Salt solution (HBSS), was added to some wells instead of plasma and served as positive control. To each well, 20 µl of *V. anguillarum* (1×10^6 cfu ml^{-1}) were added and the plate was incubated for 3 h at 25 ºC. To each well, 25 µl of iodonitrotetrazolium chloride, INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride; 1 $mg.ml^{-1}$; Sigma) to allow the formation of formazan. Plates were

then centrifuged at 2000 x *g* for 10 min. and the precipitate was dissolved in 200 µl of dimethyl sulfoxide (Sigma). The absorbance of the dissolved formazan was measured at 490 nm in a Synergy HT microplate reader (Biotek). Total bactericidal activity is expressed as the percentage of killed bacteria, calculated from the difference between the samples (surviving bacteria) and the positive control (100 % living bacteria).

IgM in plasma was measured by an ELISA assay. Succinctly, 4 µl of plasma were previously diluted (1:100) in 396 µl of Na_2CO_3 (50 mM, pH = 9.6) buffer and then 100 µl of these diluted plasma samples were added to the 96 well in duplicates, using 100 µl of buffer (Na_2CO_3) as a negative control. The samples (antigen) were allowed to stand at 22 °C for 1 hour, being subsequently removed by means of an aspirator with a pipet tip. Then, 300 µl of blocking buffer (5 % low fat milk powder in 0.1 % Tween 20) was added to each well and incubated for 1 h at 22 ºC. This mixture was then removed by aspiration and followed by three consecutive washes with 300 µl of T-TBS (0.1 % Tween 20). After properly cleaning and drying the wells, 100 µl of the anti-gilthead seabream (*Sparus aurata*) primary IgM monoclonal antibody previously diluted in blocking buffer (1:100) was added to each well and incubated for 1 hour at 22 ºC. The primary antibody was then removed by aspiration, with three consecutive washes being performed. Afterwards, the anti-mouse IgG-HRP, secondary antibody diluted 1:1000 in blocking buffer, was added and incubated for 1 hour at 22 ºC, being then recovered by aspiration. The wells were then washed three times and 100 µl of TMB substrate solution for ELISA (BioLegend #421101), previously prepared, was added to each well and incubated for 5 min. The colour change reaction was stopped after 5 min by adding 100 µl of 2 M sulphuric acid and the optical density was read at 450 nm.

The antiprotease activity was determined as described by Ellis (1990) adapted by Machado, et al. (2015). Briefly, 10 µl of plasma were incubated with the same volume of trypsin solution (5 $mg.ml^{-1}$ in $NaHCO_3$, 5 $mg.ml^{-1}$, pH 8.3) for 10 min at 22 ºC in polystyrene microtubes. To the incubation mixture, 100 µl of phosphate buffer (NaH_2PO_4, 13.9 $mg.ml^{-1}$, pH 7.0) and 125 µl of azocasein (20 $mg.ml^{-1}$ in $NaHCO_3$, 5 $mg.ml^{-1}$, pH 8.3) were added and incubated for 1 h at 22 ºC. Finally, 250 µl of trichloroacetic acid were added to each microtube and incubated for 30 min at 22 ºC. The mixture was centrifuged at 10,000 × *g* for 5 min at room temperature. Afterwards, 100 µl of the supernatant was transferred in duplicates to a 96-well plate that previously contained 100 µl of NaOH (40 $mg.ml^{-1}$) per well. The OD was read at 450 nm.

Phosphate buffer was added to some wells instead of plasma and trypsin and served as blank, whereas the reference sample was phosphate buffer instead of plasma. The percentage of inhibition of trypsin activity compared to the reference sample was calculated.

Total peroxidase activity in plasma was measured according to the procedures described by Quade, Roth (1997). Briefly, 15 µl of plasma in duplicates were diluted with 135 µl of HBSS without Ca^{2+} and Mg^{2+} in flat-bottomed 96-well plates. Then, 50 µl of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB; Sigma) and 50 ml of 5 mM H_2O_2 were added. The colour change reaction was stopped after 2 min by adding 50 ml of 2 M sulphuric acid and the optical density was read at 450 nm. Wells without plasma were used as blanks. One unit of peroxidase activity (units.ml^{-1} plasma) was defined by the quantity of peroxidase that produces an absorbance change of 1 OD.

2.2.6 Gene expression analysis

Head-kidney were taken from fish fed the experimental diets for 2 and 4 weeks. Genes were analysed using the seabream PCR-array platform of the IATS Nutrigenomics group (http://nutrigroup-iats.org). Total RNA was extracted using a MagMAX™-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, USA). RNA yield was 50–100 µg with 260 to 280 nm UV absorbance ratios (A260/280) of 1.9–2.1. Reverse transcription (RT) of 500 ng total RNA was performed with random decamers using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions.

Negative control reactions were run without reverse transcriptase and real-time quantitative PCR was carried out on a CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using a 96-well PCR array layout designed for simultaneously profiling a panel of 29 genes for head-kidney (summarized by biological processes in Table 3). Overall, the simultaneous profiling of a panel of 29 transcripts were analysed in the head kidney, associated with health biological processes, like interleukins and cytokines (9), macrophages and monocytes chemokines (3), immunoglobulins (4), antiprotease (1), antimicrobial peptide/iron recycling (1), T-cell markers (6) and pattern recognition receptors (5). Controls of general PCR performance were included on each array, being performed all the pipetting operations

by means of the EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany). Briefly, RT reactions were diluted to convenient concentrations and the equivalent of 660 pg of total input RNA was used in a 25 µl volume for each PCR reaction.

PCR-wells contained a 2× SYBR Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.9 µM were used to obtain amplicons of 50–150 bp in length (Table S1). The program used for PCR amplification included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. The efficiency of PCR reactions was always higher than 90 %, and negative controls without sample templates were routinely performed for each primer set. The specificity of reactions was verified by analysis of melting curves (ramping rates of 0.05 ºC/s over a temperature range of 55–95 °C), and linearity of serial dilutions of RT reactions. Fluorescence data acquired during the PCR extension phase were normalized using the delta–delta Ct method (Livak and Schmittgen, 2001). β-Actin was tested for gene expression stability using GeNorm software (M score = 0.21) and it was used as housekeeping gene in the normalization procedure. Fold-change calculations were done in reference to the mean response of CTRL fish. To compare the mRNA gene expression level of a panel of genes in a given dietary treatment, all data values were expressed in reference to the expression level of *casp3* in CTRL fish, which was arbitrarily assigned a value of 1.

Table 3. Genes included in head kidney pathway-focused PCR array.

Gene name/category	Symbol
Interleukins and cytokines	
Interleukin-1 beta	*il-1β*
Interleukin-6	*il-6*
Interleukin-7	*il-7*
Interleukin-8	*il-8*
Interleukin-10	*il-10*
Interleukin 12 subunit beta	*il12*
Interleukin-15	*il-15*
Interleukin-34	*il-34*
Tumor necrosis factor-alpha	*tnf α*
Macrophages and monocytes chemokines	
Macrophage colony-stimulating factor 1 receptor 1	*csf1r1*
C-C chemokine receptor type 3	*ccr3*
C-C chemokine CK8 / C-C motif chemokine 20	*ck8/ccl20*
Immunoglobulins	
Immunoglobulin M	*sIgM*
Immunoglobulin M membrane-bound form	*IgM-m*
Immunoglobulin T	*sIgT*
Immunoglobulin T membrane-bound form	*IgT-m*
Apoptosis	
Caspase 3	*casp3*

Gene name/category	Symbol
T-cell markers	
Cluster of differentiation 3 epsilon chain	*cd3e*
Cluster of differentiation 3 zeta chain	*cd3x*
CD4-full	*cd4-full*
Cluster of differentiation 8 alpha	*cd8a*
Cluster of differentiation 8 beta	*cd8b*
Zeta-chain-associated protein kinase 70	*zap70*
Pattern recognition receptors	
Toll-like receptor 2	*tlr2*
Toll-like receptor 5	*tlr5*
Toll-like receptor 9	*tlr9*
Macrophage mannose receptor 1	*mrc1*
Antimicrobial peptide/Iron recycling	
Hepcidin	*hepc*
Complement pathways	
Complement factor 3	*c3*
C-type lectin receptor signalling	
C-type lectin domain family 10 member A	*clec10a*

2.2.7 Statistical analysis

All results are expressed as means ± standard error (SE). Univariate statistic evaluation of the data was accomplished by two-way ANOVA with sampling time and dietary treatment as main factors. A significance of $p<0.05$ was applied to all statistical tests. Gross deviations from the ANOVA assumptions of error normality and homoscedasticity were evaluated through residual analysis (using QQ-plots and "residuals vs. fitted" scatter plots). All tests were run with SPSS statistical analysis software (SPSS ver.26.0; Chicago, USA).

For gene expression data, unsupervised principle component analysis (PCA) was first performed on data as an unbiased statistical method to observe intrinsic trends in the dataset, using EZ-INFO® v3.0 (Umetrics, Sweden). To achieve the maximum separation between groups, supervised partial least-squares discriminant analysis (PLS-DA) was subsequentially applied. Potential differential genes were selected according to the Variable Importance in the Projection (VIP) values. Variables with VIP > 1 were considered to be influential for the separation of samples in PLS-DA analysis (Kieffer, et al., 2016; Li, et al., 2012; Wold, et al., 2001).

2.3 Results

2.3.1 Growth performance

No differences among dietary groups were observed neither for final body weight (FBW) nor relative growth rate (RGR) at the end of the feeding trial (Table 4).

2.3.2 Immune parameters

Total red blood cells (RBC) and white blood cells (WBC) counts increased between the first and second sampling point, regardless of dietary treatments. Lower WBC counts were observed for fish fed THR after 2 weeks compared to their counterparts fed the other dietary treatments (Table 5). Similarly, peripheral lymphocytes were less abundant in fish fed HIS than those fed THR after 4 weeks, while circulating neutrophils increased in seabream fed TAU compared to fish fed His, regardless of sampling time.

2.3.3 Plasma humoral parameters

Most plasma humoral immune parameters changed between sampling points, regardless of dietary treatments. Bactericidal and antiprotease activities increased after 4 weeks, while IgM levels had the opposite pattern. Regarding dietary effects, plasma antiprotease activity augmented in fish fed THR compared to seabream fed CTRL and TAU dietary treatments, regardless of sampling time (Table 6).

2.3.4 Skin mucus humoral parameters

The bactericidal activity in skin mucus decreased in seabream fed TAU and HIS dietary treatments compared to their counterparts fed the CTRL diet after 2 weeks of feeding, displaying levels of mucosal bactericidal activity similar to ones measured in TØ fish. Mucus antiprotease activity increased in seabream fed HIS compared to fish fed the CTRL diet regardless of sampling time (Table 6).

Table 4. Body weight (BW, g fish^{-1}) and relative growth rate (RGR, % day^{-1}) of gilthead seabream fed the experimental diets for 2 and 4 weeks.

Parameters	CTRL	CTRL		THR		TAU		HIS	
	TØ	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
IBW (g fish^{-1})	8.77 ± 0.13								
FBW (g fish^{-1})		11.07 ± 0.23	14.49 ± 2.10	10.89 ± 0.30	14.04 ± 1.49	11.08 ± 0.33	13.89 ± 1.08	11.13 ± 0.49	13.70 ± 0.10
RGR (RGR, % day^{-1}),		1.63 ± 0.28	1.76 ± 0.50	1.65 ± 0.51	1.73 ± 0.35	1.54 ± 0.18	1.58 ± 0.32	1.84 ± 0.48	1.67 ± 0.09

Two-way ANOVA

Parameters				Time		Diet			
	Diet	Time	Time x diet	2 weeks	4 weeks	CTRL	THR	TAU	HIS
IBW	-	-	-	-	-	-	-	-	-
BW	0.925	<0.001	0.893	A	B	-	-	-	-
RGR	0.814	0.911	0.898	-	-	-	-	-	-

Values are presented as means ± SD (n = 12). P-values from two-way ANOVA ($p \leq 0.05$). Tukey *post-hoc* test was used to identify differences in the experimental treatments. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets.

Table 5. Haemoglobin, red blood cells (RBC) and white blood cells (WBC) of gilthead seabream fed dietary treatments during 2 and 4 weeks. Values are presented as means ± SE (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences between dietary treatments for the same time. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets. Different symbols indicate difference between times for the same dietary treatment.

Parameters		CTRL	CTRL		THR		TAU	
		TØ	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Haemoglobin	(g dl^{-1})	0.60 ± 0.10	0.60 ± 0.06	0.60 ± 0.05	0.65 ± 0.09	0.65 ± 0.04	0.57 ± 0.03	0.56 ± 0.05
WBC	(x10^4 µl)	4.66 ± 0.33^{b}	4.53 ± 0.60^{b}	6.21 ± 0.36	3.34 ± 0.20^{a}	7.11 ± 0.56	4.85 ± 0.28^{b}	7.00 ± 0.40
RBC	(x10^6 µl)	1.55 ± 0.15	1.14 ± 0.09	1.44 ± 0.10	1.28 ± 0.11	1.58 ± 0.04	1.00 ± 0.06	1.42 ± 0.09
Absolute peripheral blood leucocytes								
Thrombocytes	(x10^4µl)	3.31 ± 0.23	3.46 ± 0.57	4.54 ± 0.26	2.46 ± 0.18	4.77 ± 0.36	3.62 ± 0.27	5.19 ± 0.40
Lymphocytes	(x10^4µl)	1.21 ± 0.23^{ab}	0.93 ± 0.14	1.37 ± 0.18 ^{ab}	0.70 ± 0.34*	1.95 ± 0.49 $^{b\#}$	0.98 ± 0.46	1.49 ± 0.48 ^{ab}
Monocytes	(x10^4µl)	0.02 ± 0.01	0.02 ± 0.01	0.06 ± 0.02	0.02 ± 0.01	0.06 ± 0.02	0.04 ± 0.01	0.08 ± 0.02
Neutrophils	(x10^4µl)	0.13 ± 0.03	0.07 ± 0.02	0.22 ± 0.04	0.08 ± 0.03	0.18 ± 0.02	0.18 ± 0.03	0.25 ± 0.02

Parameters		HIS	
		2 weeks	4 weeks
Haemoglobin	(g dl^{-1})	0.54 ± 0.06	0.67 ± 0.10
WBC	(x10^4 µl)	4.94 ± 0.47^{b}	5.94 ± 0.56
RBC	(x10^6 µl)	1.24 ± 0.11	1.66 ± 0.12
Absolute peripheral blood leucocytes			
Thrombocytes	(x10^4µl)	3.56 ± 0.35	4.60 ± 0.47
Lymphocytes	(x10^4µl)	1.22 ± 0.49	1.11 ± 0.43 ^{a}
Monocytes	(x10^4µl)	0.07 ± 0.02	0.06 ± 0.02
Neutrophils	(x10^4µl)	0.09 ± 0.01	0.15 ± 0.04

Two-way ANOVA

Parameters					Time			Diet			
		Diet	Time	Time x diet	TØ	2 weeks	4 weeks	CTRL	THR	TAU	HIS
Haemoglobin	(g dl^{-1})	0.633	0.538	0.699	-	-	-	-	-	-	-
WBC	(x10^4 µl)	0.406	<0.001	0.017	A	A	B	-	-	-	-
RBC	(x10^6 µl)	0.286	<0.001	0.898	B	A	B	-	-	-	-
Absolute peripheral blood leucocytes											
Thrombocytes	(x10^4µl)	0.214	<0.001	0.298	A	A	B	-	-	-	-
Lymphocytes	(x10^4µl)	0.74	<0.001	0.003	AB	A	B	-	-	-	-
Monocytes	(x10^4µl)	0.071	0.003	0.116	AB	A	B	-	-	-	-
Neutrophils	(x10^4µl)	0.006	<0.001	0.287	A	A	B	AB	AB	B	A

Table 6. Plasma and mucus humoral parameters of gilthead seabream fed dietary treatments during 2 and 4 weeks. Values are presented as means ± SE (n = 12). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences between dietary treatments for the same time. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets. Different symbols indicate difference between times for the same dietary treatment.

Parameters		CTRL	CTRL		THR		TAU		HIS	
		TØ	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Plasma										
Bactericidal activity	(%)	28.57 ± 7.31	43.75 ± 2.14	47.56 ± 3.01	37.65 ± 2.56	48.25 ± 2.76	38.47 ± 2.20	39.92 ± 3.30	42.39 ± 3.40	43.54 ± 2.90
Antiprotease	(%)	⊗	92.02 ± 0.99	92.69 ± 1.43	94.60 ± 0.59	95.45 ± 1.01	90.06 ± 0.85	94.56 ± 0.59	91.71 ± 1.04	95.97 ± 0.50
Peroxidase	(U/mL)	⊗	6.29 ± 0.71	7.07 ± 0.71	6.60 ± 0.79	7.18 ± 0.55	6.34 ± 0.64	6.44 ± 0.43	6.95 ± 0.43	7.94 ± 1.00
IgM	(abs)	0.80 ± 0.11	0.85 ± 0.02	0.72 ± 0.05	0.77 ± 0.04	0.68 ± 0.06	0.66 ± 0.04	0.74 ± 0.04	0.81 ± 0.05	0.66 ± 0.05
Mucus										
Bactericidal activity	(%)	60.53 ± 1.02^{a}	66.41 ± 0.63^{*b}	55.83 ± 1.76$^{\#}$	64.57 ± 0.89^{ab}	59.11 ± 1.59	60.80 ± 1.34^{a}	59.08 ± 1.19	59.40 ± 1.26^{a}	57.69 ± 1.49
Antiprotease	(%)	29.45 ± 2.37	25.52 ± 2.70	26.99 ± 1.33	28.10 ± 1.97	26.88 ± 1.31	28.36 ± 2.10	28.69 ± 2.49	27.52 ± 0.90	30.62 ± 2.6
Peroxidase activity	(U/mg)	0.66 ± 0.06	0.73 ± 0.09	0.69 ± 0.09	0.89 ± 0.10	0.78 ± 0.11	1.00 ± 0.12	0.71 ± 0.08	0.81 ± 0.11	0.78 ± 0.41

⊗ There was not enough sample to perform this analysis due to fish size constraints

Two-way ANOVA

Parameters					Time			Diet		
	Diet	Time	Time x diet	TØ	2 weeks	4 weeks	CTRL	THR	TAU	HIS
Plasma										
Bactericidal activity (%)	0.151	0.037	0.343	A	AB	B	-	-	-	-
Antiprotease (%)	0.013	<0.001	0.059	-	A	B	A	B	A	AB
Peroxidase (U/mL)	0.471	0.208	0.926	-	-	-	-	-	-	-
IgM (abs)	0.302	0.023	0.045	B	B	A	-	-	-	-
Mucus										
Bactericidal activity (%)	0.053	<0.001	0.001	AB	B	A	-	-	-	-
Antiprotease (%)	0.012	0.149	0.102	-	-	-	A	AB	AB	B
Peroxidase activity (U/mg)	0.452	0.1	0.541	-	-	-	-	-	-	-

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2.3.5 Gene expression

Gene expression seems to be consistent with the humoral parameters measured in plasma and skin mucus. IgT-m decreased significantly in seabream fed TAU compared to those fed THR dietary treatments for a 4-week feeding period (Figure 1A and S. Table 2). The C-type lectin domain family domain 10 member (clec10a) decreased in seabream fed THR dietary treatment compared to fish the CTRL diet after 2 weeks of feeding (Figure 1B and S. Table 2).

Through the PLS-DA analysis considering all nine groups resulting from the combinations of all experimental factors (S. Figure 1A) and the one with only four groups (diet effect) (S. Figure 1B), a low degree of explained Y-variance is observed, coupled with a low prediction capacity: with 9 groups we obtain R2Y of 11 % and Q2 of 0 %, while reducing to 4, increases the R2Y to 14 % and the Q2 remains 0 %. In fact, by reducing the number of groups to two (2W & 4W), the discriminant analysis (S. Figure 1C) is able to explain 54 % of variance (R2 Y) and to predict more than 32 % of the total variance (Q2). In order to understand and interpret the contribution of the different genes to these components, a table of the variable importance projection (VIP) score of the genes is presented in S. Figure 1D, ordered by their importance. The VIP values suggest that only 11 out of the 29 genes are important for the obtained projection (VIP>1). It is clear that most genes (8/11) are modulated by component 1, which we attributed to “time” effect (since it clearly separates the two sampling points).

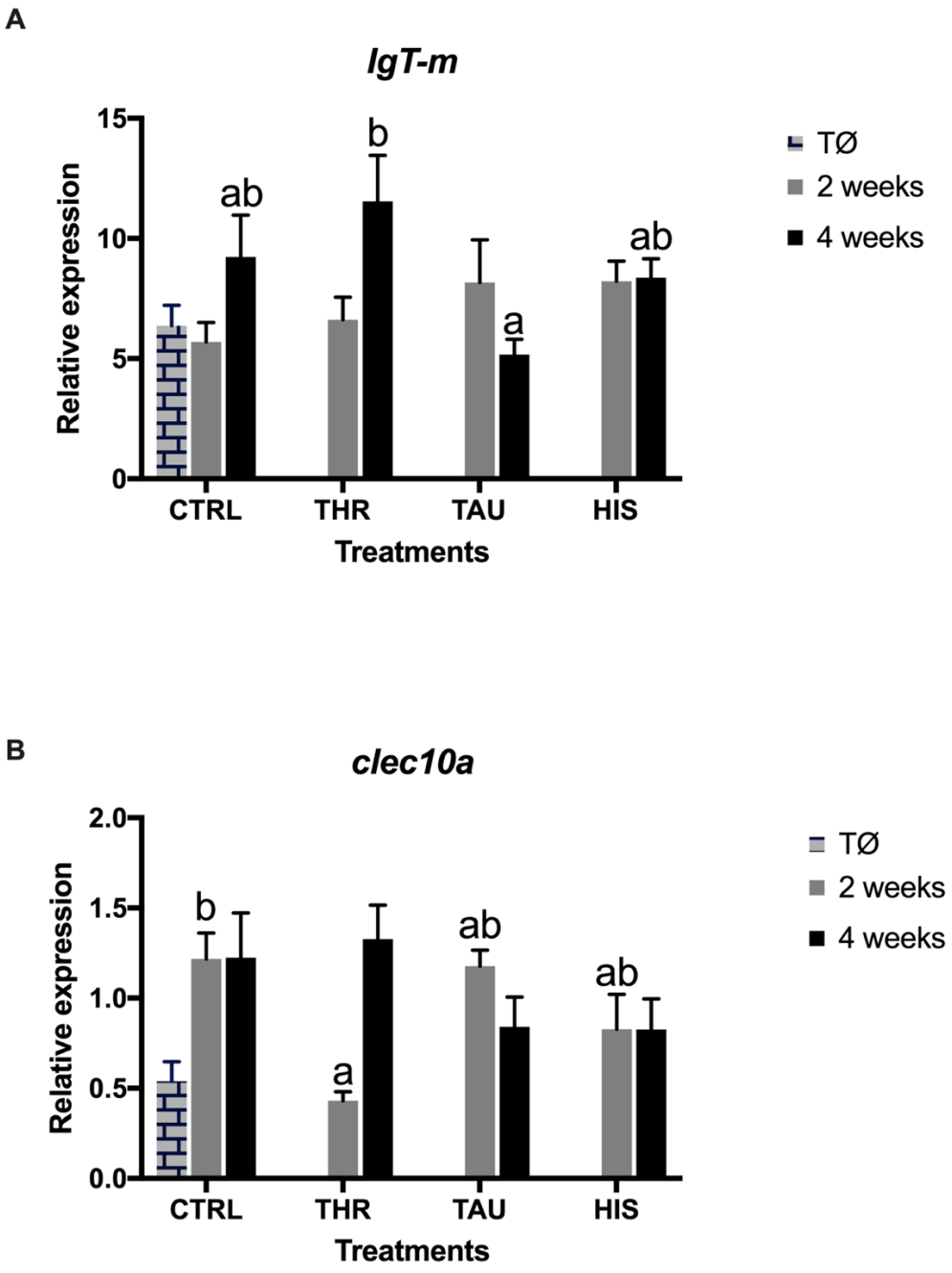

Figure 1. Relative expression of Immunoglobulin T membrane-bound form (*IgT-m*, **A**), C-type lectin domain family 10 member A (*clec10a*, **B**), genes in the head-kidney of gilthead seabream at time Ø and fed the dietary treatments during 2 and 4 weeks. Values are presented as means ± SE (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same sampling time.

2.4 Discussion

The present study showed that some modulation of leucocyte response was observed in fish fed TAU dietary treatment, with the number of circulating neutrophils being increased regardless of sampling time. There is no published information about Tau involvement in differentiation of this cell-type, but it is known that neutrophils contain very high concentrations of taurine due to its uptake from the blood (Bouckenooghe, et al., 2006). Hence, some stimulation of Tau levels on neutrophil numbers could be postulated and it could also be speculated that Tau requirement may increase upon infection. Neutrophils from the reserve pools are quickly attracted to infectious foci by microbial products and chemotactic substances released by host cells (do Vale, et al., 2002).

Moreover, plasma antiprotease increased in fish fed THR dietary treatment compared to those fed the CTRL diet, which could indicate a potential capacity of this particular AA to boost host's innate immune defences. The observed effect of a dietary Thr surplus may also translate into an improved resistance against bacteria in animals fed higher levels of this AA. Antiproteases play a crucial role in the inhibition of certain proteases either by "trapping" proteases to avoid protein hydrolysis or by binding to their active sites (Agbowuro, et al., 2018; Laskowski, Kato, 1980). Also, several pathogens (mainly bacteria) express a variety of non-specific proteases which degrade important proteins involved in fish innate immunity (Gonzalez-Silvera, et al., 2018; Potempa, Pike, 2009). Hence, the presence of stronger antiprotease activity in seabream fed the THR dietary treatment may indicate an improvement in fish immune condition.

Skin mucus is an important physical and chemical barrier against invading pathogens. It serves as an important component of the innate immune machinery by: i) its continuously renewal, preventing stable colonization of the skin by pathogens and; ii) containing a number of innate immune proteins and enzymes (e.g. lysozyme, complement proteins and antibacterial proteins and peptides) including antiproteases (Dash, et al., 2018). In the present study, seabream fed HIS dietary treatment showed an increase of total antiprotease activity measured in skin mucus. In contrast, fish fed the HIS diet had a lower bactericidal activity in skin mucus compared to those fed the CTRL diet after 2 weeks, which seems to point to a differential immune status in fish

fed this diet. One should take into account that the bactericidal activity is a multifactorial indicator since it evaluates a wide range of innate immune mechanisms and molecular defences against bacterial invasion, such as proteins of the complement system, antimicrobial peptides, acute phase proteins, immunoglobulins, lysozyme and cytokines (Ellis, 1999; Graham, et al., 1988). Therefore, and considering the decrease in lymphocyte numbers observed at 4 weeks, it could be suggested that dietary His supplementation seems to improve certain key aspects of innate immunity in detrimental of factors contributing to acquired immunity at the skin mucus level.

The results obtained in terms of bactericidal activity are comparable with those of previous works where the impact of a Tau-rich diet on lysozyme and alternative complement system was evaluated. Han, et al. (2014), observed that Tau supplementation did not influence blood parameters (e.g. hematocrit, hemoglobin, glucose, total protein, total cholesterol, total bilirubin and triglycerides) of Japanese flounder (*Paralichthys olivaceus*). This was corroborated by Magalhães, et al. (2019) in a similar study with white seabream (*Diplodus sargus*) juveniles, where no differences were observed in plasma alternative complement pathway and lysozyme levels. Nonetheless, a diet rich in taurine boosted the lysozyme activity and other immune parameters, such as phagocytic index, respiratory burst, and total immunoglobulin content, in yellow catfish (*Pelteobagrus fulvidraco*) (Li, et al., 2016). This highlights the importance of further studies to clarify the effects of Tau on fish immune system, since it could be species and context-dependent.

In the present study, dietary treatments did not induce strong transcriptional changes, as the observed differences were mostly time-dependent. Nonetheless, some mild effects were observed with the downregulation of *clec10,* a member of the C-type lectin (CTLs) superfamily, in seabream fed the THR dietary treatment for 2 weeks.

Lectins are a family of glycoprotein pattern-recognition receptors highly represented in fish, being typically multivalent proteins that recognize and bind specific carbohydrate recognition domains (CRD) (Lieschke, Trede, 2009). The presence of several CRDs, in combination with other proteins domains, enable not only the recognition of specific carbohydrates on pathogens surface, but also on the surface of immunocompetent cells. Some lectins induce the synthesis of pro-inflammatory cytokines, including *il1-β1*, *il1-β2*, *tnf-α1*, *tnf-α2* and *il8* in rainbow trout macrophages

and fibroblast-like cells (Perez-Sanchez, et al., 2015). Lectins play an active role in innate immunity in PAMP recognition, opsonisation, phagocytosis and complement activation (Vasta, 2009; Vasta, et al., 2004). C-type lectins, in particular, have several functions in innate immunity and contribute to the homing of leucocytes and immune cell trafficking, as well as pathogen recognition and subsequent T cell activation (Mayer, et al., 2017). As such, CLEC10 is likely to play a role in regulating adaptive and innate immune responses and, thus, in this study it could be suggested the downregulation of this CTL in seabream fed THR diet for 2 weeks, and the concomitant decrease in blood total WBC, as a possible inhibition of immune alertness (e.g. pathogen recognition and innate immunity).

In the present study, data from the analysed responses of fish fed supplemented diets suggest that the effects of Thr, His and Tau supplementation on fish immune system seem to be of an indirect nature, when compared with other EAAs previously studied, such as tryptophan, methionine or arginine (Azeredo, et al., 2020; Machado, et al., 2018; Ramos-Pinto, et al., 2019a), which present stronger and more direct effects. In a previous work where the effects of dietary tryptophan supplementation were also explored in gilthead seabream, only a transient immune enhancement was observed in fish fed an extreme formulation (no FM inclusion) over a short-term feeding period (2 weeks), suggesting that these putative advantageous effects seem to disappear over a longer feeding period (13 weeks) (Ramos-Pinto, et al., 2019b). Therefore, the importance of “exposure time” as a central factor in determining the apparent functional effects of additives seems to be of major importance. In the present study, this fact seems to be particularly relevant for the THR dietary treatment. While a decrease in total WBC numbers and *clec10* transcripts was observed after 2 weeks of feeding, the acquired arm of the immune system was stimulated by dietary Thr supplementation with an enhancement of peripheral lymphocytes and *IgT-m* mRNA expression level in the head-kidney after 4 weeks of feeding.

In conclusion, this study suggests that dietary supplementation with His, Tau or Thr at the tested levels causes mild immune-modulation effects in gilthead seabream, which should be further studied under disease challenge conditions. Still, plasma antiprotease activity increased in fish fed THR dietary treatment which, together with the decrease in *clec10* transcripts and total WBC at 2 weeks contrasting the enhancement of acquired immune condition at 4 weeks, what reinforces the

importance of feeding time when aiming to improve immune alertness. In addition, HIS dietary treatment led to an increase of total antiproteases activity measured in skin mucus, while some stimulation of dietary Tau on peripheral neutrophil numbers could also be seen. Hence, further studies with other supplementation levels and eventually duration of supplementation could help to clarify the potential immunomodulatory role of these AA for the gilthead seabream.

2.5 Ethics statement

The experiments were approved by the i3S Animal Welfare Committee and carried out in a registered installation (license number 0421/000/000/2018). Experiments were performed by trained scientists in full compliance with national rules and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

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Chapter 3

Dietary tryptophan supplementation induces a transient immune enhancement of gilthead seabream (*Sparus aurata*) juveniles fed fishmeal-free diets

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Abstract

European aquaculture is an industry with a high sustainability profile contributing to the supply of safe seafood. However, several diseases can affect farmed fish and it is imperative to find alternatives for chemotherapeutic treatments when disease outbreaks occur. Maintenance of health through nutrition is well-establish in modern animal farming, and amino acids (AA) are promising candidates as functional additives to improve fish health. Therefore, the goal of this research is to provide a better understanding of the influence of tryptophan supplementation on nutritional condition and immune mechanisms in fish. Triplicate groups of fish (13.3±0.3g) previously fed with a fishmeal-based diet were either fed a control diet with an extreme formulation (0% fishmeal) but meeting the AA requirements (CTRL), or the SUP diet, formulated as the CTRL with an increase in tryptophan (TRP) content. After 2 and 13 weeks of feeding, head-kidney (HK), liver (L) and white skeletal muscle (WSM) were collected for gene expression, whereas plasma was suited for humoral immune parameters. A holistic approach using transcriptomic, humoral and zootechnical parameters was undertaken. The expression of 29-31 genes for WSM, L or HK confirms an effect due to the treatment across time. A two-way ANOVA analysis revealed that 15-24 genes varied significantly depending on the tissue, and the multivariate analysis by means of PLS-DA explained (R2) and predicted (Q2) with four components up to 93% and 78% of total variance, respectively. Component 1 (R2=50.06%) represented the time effects, whereas components 2 (24.36%) and 3 (13.89%) grouped fish on the basis of dietary treatment, at early sampling. The HK results in particular suggest that fish fed SUP diet displayed an immunostimulated state at 2 weeks. No major differences were observed in plasma humoral parameters, despite an increase in antiprotease and peroxidase activities after 13 weeks regardless of dietary treatment. These results suggest that tryptophan supplementation may improve the seabream immune status after 2 weeks. Hence, the use of functional feeds is especially relevant during a short-term feeding period before a predictable stressful event or disease outbreak, considering that these putative advantageous effects seem to disappear after a 13-week feeding period.

3.1 Introduction

A sustainable and profitable aquaculture relies on the production of healthy fish, which requires balanced feeds manufactured with high-quality ingredients. Research in last years has focused on the reduction of fish meal and fish oil use in aquafeeds, in order to mitigate the strong reliance of the aquaculture sector on these limited resources. In practice, plant-based ingredients are currently the most studied and immediately available alternatives to reduce the dependence of carnivorous species on marine fisheries resources (Benedito-Palos, et al., 2007; Benedito-Palos, et al., 2016; Izquierdo, et al., 2005; Mourente, Bell, 2006; Simó-Mirabet, et al., 2018), though many other types of ingredients are also increasingly considered (e.g. processed animal proteins, insect meals, algae and yeast biomasses). Thus, good practices in diet formulation are imperative, given the significant impact that nutrition can have on fish performance and health. Moreover, it is important to find feed ingredients and/or additives to compensate for the replacement of traditional marine feed ingredients with more sustainable unconventional feedstuffs (Hardy, 2010), as this replacement sometimes brings fish health and welfare issues (Conceição, et al., 2012). Such a fish health and welfare dimension in fish feeds formulation can also help to reduce even further the use of medicated feeds (Novais, et al., 2018; Watts, et al., 2017). Although there is a wide range of studies involving the successful replacement of marine ingredients with alternative (usually plant-based) ingredients in gilthead seabream, most studies are focused on growth performance, with some drawback effects on welfare and immunity (Matos, et al., 2017). Still, important advances are expected in next years in the use of functional or fortified feeds. This term describes feeds that go beyond satisfying the minimum nutritional requirements, improving growth performance and overall health status and stress resistance of farmed animals (Encarnação, 2016). This new concept in modern animal nutrition can be achieved through the inclusion of a wide range of possible ingredients, additives and purified nutrients (e.g., amino acids, prebiotics, probiotics, glucans, nucleotides, methyl donors, essential fatty acids).

Amino acids (AA) are fundamental nutrients in aquafeeds formulation, their importance going well beyond their role as the building blocks of proteins. AA are also functional compounds, since AA are precursors for the synthesis of other important

biologically active molecules such as nitric oxide and polyamines being essential for reproduction, immunity and organism maintenance (Li, et al., 2009b). AA requirements may increase as consequence of stressful conditions (e.g. handling) and metabolic changes associated with inflammation and infection (Conceição, et al., 2012; Melchior, et al., 2004; Sakkas, et al., 2013). In fact, the dependence of the immune system on the availability of AA is related to their role as signalling molecules which are essential for cellular function (Li, et al., 2009a). For instance, AA have essential roles in higher vertebrates as they regulate i) activation of T- and B-lymphocytes, natural-killer cells and macrophages; ii) cellular redox stage, gene expression and lymphocyte proliferation; and iii) production of antibodies, cytokines and cytokine substances as nitric oxide (NO) and superoxide (Wu, 2013).

The importance of AA supplementation in aquafeeds is generally acknowledged, and AA such as lysine and methionine are routinely added to commercial diets to compensate their relatively low presence in e.g. most vegetable ingredients. Contrarily, supplementing other essential AA (such as tryptophan, threonine and histidine) when alternative ingredients are used is not as established as with lysine and methionine. As such, it is likely that the supply of these AA is sometimes compromised when low fish meal diets are used, leading to potential negative effects in terms of fish health.

Tryptophan (Trp), in particular, is an essential amino acid with recognized roles in both neuroendocrine and immune systems (Hoseini, et al., 2017). Trp is a precursor of bioactive molecules and neurotransmitters such as serotonin, kynurenine and melatonin. Products of tryptophan catabolism can enhance host immunity by inhibiting the production of superoxide, scavenging free radicals and minimizing the production of pro-inflammatory cytokines (Hoseini, et al., 2017; Perianayagam, et al., 2005). Nonetheless, the modulatory effects of Trp dietary supplementation on fish immune responses seems to depend on species, level of supplementation, time of administration and prior stress condition (Hoseini, et al., 2017).

The present study aimed to explore the effects of tryptophan supplementation on the health status and growth performance of gilthead seabream juveniles in the context of fish-meal free diets. Since there is a current need to gather deeper knowledge on the time-dependent modulatory effects of functional diets, the effects of experimental diets were assessed after short and long-term feeding periods.

3.2 Material and Methods

3.2.1 Experimental diets

Two different diets were formulated and manufactured by SPAROS Lda. (Olhão, Portugal) to fulfil the known nutritional requirements of gilthead seabream juveniles: a control diet (CTRL) which contained 0 % fishmeal, while meeting the estimated AA requirements for this species (Kaushik, 1998; Peres, Oliva-Teles, 2009), along with a supplemented diet (SUP), with the same basal formulation, but with an additional tryptophan supplement. Since the formulated diets were extreme (0% fishmeal), five essential AA were added to all diets in order to fulfil the gilthead seabream AA requirements and obtain balanced diets (Table 1). The level of supplementation was chosen based on available data (Conceição, et al., 2012; Hoseini, et al., 2017; Li, et al., 2009a). Main ingredients were ground (below 250 μm) in a Hosakawa micropulverizer hammer mill, model #1 (Hosokawa Micron Ltd., United Kingdom). Powdered ingredients were then mixed according to the target formulation in a TGC double-helix mixer, model 500L (TGC Extrusion, France), to attain a basal mixture (no oils were added at this stage). All diets were manufactured by extrusion (pellet size 2.0 mm) by means of a CLEXTRAL BC45 pilot-scale twin-screw extruder (Clextral, France) with a screw diameter of 55.5 mm and temperature ranging 105 – 110 ºC. Upon extrusion, all batches of extruded feeds were dried in a convection oven (OP 750-EF, LTE Scientifics, United Kingdom) for 2 h at 60 ºC. After this process, pellets were left to cool at room temperature, and subsequently the essential AA were mixed with the fish oil fraction according to the formulation and added under vacuum coating conditions in a Pegasus vacuum mixer (PG-10VCLAB, DINNISEN, The Netherlands) respective mixture. Throughout the duration of the trial, experimental feeds were stored at room temperature. Formulation and proximal composition of experimental diets are presented in Table 1. Proximate composition analysis was performed by the following methods: dry matter, by drying at 105 ºC for 24 h; ash, by combustion at 550 ºC for 12 h; crude protein (N × 6.25), by a flash combustion technique followed by gas chromatographic separation and thermal conductivity detection (LECO FP428); fat, after petroleum ether extraction, by the Soxhlet method; total phosphorus, according to the ISO/DIS 6491 method, using the vanado-molybdate reagent; gross energy, in an adiabatic bomb calorimeter (IKA).

Total AA content of diets was determined by hydrolysation in 6 M HCl at 116 ºC for 2 h in nitrogen-flushed glass vials. Samples were then pre-column derivatised with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). Analyses were done by ultra-high-performance liquid chromatography in a Waters reverse-phase AA analysis system, using norvaline as an internal standard. During acid hydrolysis asparagine is converted to aspartate and glutamine to glutamate, so the reported values for these AA represent the sum of the respective amine and acid. The resultant peaks were analysed with EMPOWER software (Waters, USA). Tryptophan was independently determined by HPLC, after alkaline hydrolysis (Silliker Portugal, S.A.) The AA profiles of the experimental diets are presented in Table S1.

Table 1. Ingredients and proximate composition of the experimental diets

Ingredients (%)	CTRL	SUP
Poultry meal 65 [a]	10.00	10.00
Soy protein concentrate [b]	14.00	14.00
Wheat gluten [c]	10.44	8.88
Corn gluten [d]	11.00	11.00
Guar korma 60 [e]	9.00	9.00
Soybean meal 48 [f]	15.20	15.20
Rapeseed meal [g]	3.00	3.00
Wheat meal [h]	5.50	5.40
Fish oil [i]	9.20	9.30
Soybean oil [j]	3.00	3.00
Rapeseed oil [k]	3.00	3.00
Vit & Min Premix [l]	1.00	1.00
Binder (Kieselghur) [m]	0.20	0.20
Antioxidant powder (Paramega) [n]	0.20	0.20
Sodium propionate [o]	0.10	0.10
Monocalcium phosphate [p]	3.00	3.00
L-Histidine [q]	0.30	1.00
L-Lysine [r]	1.20	1.20
L-Threonine [s]	0.25	0.95
L-Tryptophan [t]	0.11	0.27
DL-Methionine [u]	0.30	0.30
Proximate analyses (% Dry Weight)		
Dry matter (% as fed)	93.94	93.49
Crude protein (% DW)	49.88	50.56
Crude lipid (% DW)	20.70	18.40
Ash (% DW)	6.60	6.48
Gross Energy (kJ g^{-1} DM)	21.88	21.97
AA composition (g AA 100 g^{-1} diet)		
Tryptophan	0.60	0.93

[a] *Poultry meal: 65%CP, 14.4% CF, SAVINOR UTS, Portugal*
[b] *Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands*
[c] *VITAL: 83.7% CP, 1.6% CF, ROQUETTE Frères, France.*
[d] *Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal.*
[e] *Guar meal: KORFEED 60: 60.2% CP, 6.9% CF, India*
[f] *Soybean meal 48: Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, CARGILL, Spain*
[g] *Rapeseed meal: Defatted rapeseed meal: 37.7% CP, 2.3% CF, Premix Lda, Portugal*
[h] *Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal*
[i] *Fish oil: SAVINOR UTS, Portugal*
[j] *Soybean oil: Henry Lamotte Oils GmbH, Germany*
[k] *Rapeseed oil: Henry Lamotte Oils GmbH, Germany*
[l] *Vitamin and mineral premix: PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 500mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; excipient wheat middlings;*
m Binder: Kieselguhr (natural zeolite), LIGRANA GmbH, Germany
[n] *Antioxidant: Paramega PX, Kemin Europe NV, Belgium*
[o] *Sodium propionate: Disproquímica, Portugal*
[p] *Monocalcium phosphate: 22% P, 18% Ca, Fosfitalia, Italy*
[q] *L-Hisdidine: L-Histidine 98%, Ajinomoto Eurolysine SAS, France*
[r] *L-Lysine: L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France*
[s] *L-Threonine: ThreAMINO 98.5%, Evonik Nutrition & Care GmbH, Germany*
[t] *L-Tryptophan: TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany*
[u] *DL-Methionine: DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany*

3.2.2 Fish and rearing facilities

The trial was conducted by trained scientists (following FELASA category C recommendations) and according to the European Economic Community animal experimentation guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE at the Ramalhete research station of CCMAR (University of Algarve, Portugal), from August to November 2016. Fish with an initial body weight of 13 g were randomly distributed in 1000 L tanks and fed a (fishmeal based) commercial diet for 3 weeks to ensure acclimation to the experimental conditions. Triplicate groups of 150 gilthead seabream per treatment were hand-fed *ad libitum* twice a day (except Sundays, when fish were fed once a day) each experimental diet (i.e. CTRL and SUP) for 92 days. Through the trial, fish were subjected to a natural temperature regime, which was logged every day (Suppl. Fig. 1). Seawater was supplied at 2 L/min (mean temperature 23 °C ± 2.6; mean salinity 34 ± 0.7 ppt) in a flow-through system (mean dissolved oxygen above 5 $mg.L^{-1}$). All physical and chemical water parameters were evaluated daily during the experiment.

3.2.3 Experimental procedures

The growth trial was designed to have two sampling points (short and long-term feeding periods). The first stage of the feeding trial lasted 15 days, which was used to assess the effects of a short-term dietary supplementation with tryptophan on gilthead seabream growth- and health-related biomarkers. After a 24 h fasting period, 19 fish per tank were randomly selected and sacrificed with a tricaine methanesulfonate lethal dose (200 µg/L). All fish were sampled for mucus and blood collection. Briefly, skin mucus was collected by gentle scraping the fish dorso-lateral surface using a cell scraper with enough care to avoid contamination with blood, urogenital and intestinal excretions according to (Guardiola, et al., 2014), snap frozen in liquid nitrogen and stored at -80 ºC. Tissue samples (i.e. head kidney, liver and dorsal skeletal muscle) were also obtained from 16 of the sampled fish, snap frozen in liquid nitrogen, and stored at -80 °C until further analyses. The remaining three fish were stored at -20 °C for subsequent analysis to determine body proximal composition. At 36 and 70 days, intermediate biometric samplings of all fish were undertaken to evaluate growth

performance, along with cleaning of all rearing tanks. At the end of the trial (92 days), and after a 24 h fasting period, 19 fish per tank were sampled as described for the 15 days sampling point.

3.2.4 Haematological procedures

Blood was collected from the caudal vein using heparinized syringes. The haematological profile consisted of total white (WBC) and red (RBC) blood cells counts, haematocrit (Ht) and haemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain) as described by Machado, et al. (2015). The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated as follows:

- MCV (μm^3) = (Ht/RBC) x 10
- MCH ($pg.cell^{-1}$) = (Hb/RBC) x 10
- MCHC ($g.100\ mL^{-1}$) = (Hb/Ht) x 100

Blood smears were performed right after blood collection, air dried and fixed with formol-ethanol (10% of 37% formaldehyde in absolute ethanol). Afterwards they were stained with Wright's stain (Hemacolor; Merck). Detection of peroxidase activity was carried out as described by Afonso, et al. (1998) to facilitate the identification of neutrophils. The slides were examined (1000×), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. The absolute amount ($\times 10^4\ \mu L^{-1}$) of each cell type was calculated. The remaining blood was centrifuged at 8000 × g during 8 min at room temperature and plasma was stored at -80 °C until assayed.

3.2.5 Plasma cortisol and humoral immune parameters

Plasma cortisol levels were measured by means of a commercial enzyme-linked immunosorbent assay (ELISA) kit (RE52611 for human serum and saliva; IBL, Hamburg, Germany), as previously described by López-Olmeda, et al. (2009). Plasma was first diluted (1:20) in diethyl ether. After centrifugation, the recovered supernatant

was isolated and, once evaporated, the same amount of phosphate buffer containing 1 $g.L^{-1}$ gelatine (pH 7.6) was added. Afterwards, 20 µL of the samples, standards and controls, in duplicate, were added to the respective wells of the microtiter plate. After adding 200 µL of the enzyme conjugate, the plate was incubated during 1 h at room temperature. At the end of the incubation period, the plate was washed 3 times with a wash buffer and 100 µL of TMB (3,3',5,5'-tetramethylbenzidine hydrochloride) substrate solution was pipetted in each well. Subsequently, a 15 min incubation (18-25 °C) was performed and the reaction stopped by the addition of 100 µL of TMB stop solution. Absorbance was read at 450 nm after 10 min. The concentration of the samples was read directly from the standard curve after Logit-Log adjustment.

Plasma bactericidal activity was measured according to Graham, et al. (1988) adapted by Machado, et al. (2015). *Photobacterium damselae* subsp. *piscida* (*Phdp*), strain PP3 was used. Succinctly, 20 µL of plasma were added to duplicate wells of a U-shaped 96-well plate. The medium used for growing the bacteria strain, Tryptic soya broth (TSB), was added to some wells instead of plasma and served as positive control. To each well, 20 µL of *Phdp* (1×10^6 $cfu.mL^{-1}$) were added and the plate was incubated for 2.5 h at 25 ºC. To each well, 25 µL of 3-(4,5dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (1 $mg.mL^{-1}$; Sigma) to allow the formation of formazan. Plates were then centrifuged at 2000 × g for 10 min and the precipitate was dissolved in 200 µL of dimethyl sulfoxide (Sigma). The absorbance of the dissolved formazan was measured at 560 nm in a Synergy HT microplate reader (Biotek). Total bactericidal activity is expressed as the percentage of killed bacteria, calculated from the difference between the samples (surviving bacteria) and the positive control (100% living bacteria).

The protease activity was determined using the azocasein hydrolysis assay according to the method of Guardiola, et al. (2014) with modifications. Briefly, 10 µL of plasma was incubated with 100 µl of sodium bicarbonate buffer (5 $mg.mL^{-1}$ $NaHCO_3$, pH 8.3) and 125 µL of azocasein (20 $mg.mL^{-1}$ in $NaHCO_3$, 5 $mg.mL^{-1}$, pH 8.3) for 24 h at 22º C in polystyrene microtubes with continuous shaking. The reaction was stopped by adding 10% trichloroacetic acid (TCA) (100 $mg.mL^{-1}$) and the mixture centrifuged (6000 × g, 5 min). Afterwards, 100 µL of the supernatant was transferred in duplicates to a 96-well plate that previously contained 100 µL of NaOH (40 $mg.mL^{-1}$) per well. The OD was read at 450 nm in a Synergy HT microplate reader. Phosphate buffer was added to some wells instead of plasma and served as blank (0% of protease activity),

whereas the reference sample was replaced by trypsin solution (5 $mg.mL^{-1}$ in $NaHCO_3$, 5 $mg.mL^{-1}$, pH 8.3) instead of plasma (100% activity). The percentage of inhibition of trypsin activity compared to the reference sample was calculated.

The anti-protease activity was determined as described by Ellis (1990) adapted by Machado, et al. (2015). Briefly, 10 µL of plasma were incubated with the same volume of trypsin solution (5 $mg.mL^{-1}$ in $NaHCO_3$, 5 $mg.mL^{-1}$, pH 8.3) for 10 min at 22 ºC in polystyrene microtubes. To the incubation mixture, 100 µL of phosphate buffer (NaH_2PO_4, 13.9 $mg.mL^{-1}$, pH 7.0) and 125 µL of azocasein (20 $mg.mL^{-1}$ in $NaHCO_3$, 5 $mg.mL^{-1}$, pH 8.3) were added and incubated for 1 h at 22 ºC. Finally, 250 µL of trichloroacetic acid were added to each microtube and incubated for 30 min at 22 ºC. The mixture was centrifuged at 10000 × g for 5 min at room temperature. Afterwards, 100 µL of the supernatant was transferred in duplicates to a 96-well plate that previously contained 100 µL of NaOH (40 $mg.mL^{-1}$) per well. The OD was read at 450 nm. Phosphate buffer was added to some wells instead of plasma and trypsin and served as blank, whereas the reference sample was phosphate buffer instead of plasma. The percentage of inhibition of trypsin activity compared to the reference sample was calculated.

Total peroxidase activity in plasma was measured according to the procedures described by Quade, Roth (1997). Briefly, 15 µL of plasma in duplicates were diluted with 135 µL of HBSS without Ca^{2+} and Mg^{2+} in flat-bottomed 96-well plates. Then, 50 µL of 20 mM TMB (Sigma) and 50 mL of 5 mM H_2O_2 were added. The colour change reaction was stopped after 2 min by adding 50 mL of 2 M sulphuric acid and the optical density was read at 450 nm. Wells without plasma were used as blanks. One unit of peroxidase activity (units.mL^{-1} plasma) was defined by the quantity of peroxidase that produces an absorbance change of 1 OD.

Alternative complement pathway (ACP) activity was estimated as described by Oriol Sunyer, Tort (1995). 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. Horse red blood cells (HorRBC; Probiologica Lda, Portugal) were used for ACP determination. HorRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5 × 10^8 cells.mL^{-1}. Ten mL of HorRBC suspension were then added to 40 mL of serially diluted plasma in Mg-EGTA-GVB buffer. Samples were incubated at room temperature for 100 min with regular shaking. The reaction was stopped by

adding 150 mL 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 Synergy HT microplate reader (Biotek). The ACH50 units were defined as the concentration of plasma giving 50% haemolysis of HorRBC. All analyses were conducted in duplicates.

Total plasma nitrite and nitrate content was measured using a Nitrate/Nitrite colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany) by adapting it to a 96-well plate and by following manufacturer's instructions. Since both these compounds are derivatives of endogenously produced NO, they are indicative of NO amount in plasma. Briefly, 10 µL of plasma were diluted in 90 µL of distilled water in duplicate and then 50 µL of reduced nicotinamide adenine dinucleotide phosphate (NADPH) were added, followed by the addition of 4 µL of nitrate reductase. A blank was produced by adding distilled water instead of plasma. Absorbance at 540 nm was read after 30 min incubation at 25 °C. Afterwards, 50 µL of sulfanilamide and an equal volume of N-(1-naphthyl)-ethylenediamine dihydrochloride were added to each well. The mixture was allowed to stand at 25° C for 15 min and absorbance was read at 540 nm. Total nitrite levels were calculated from a previously prepared sodium nitrite standard curve.

3.2.6 Gene expression analysis

Total RNA from target tissues (liver, head kidney and white skeletal muscle) was extracted with a MagMax^{TM}-96 for microarrays total RNA isolation kit (Life Technologies, Carlsbad, CA, USA) after tissue homogenization in TRI reagent following manufacturers' instructions. RNA quantity and purity was determined by Nanodrop (Thermo Scientific) with absorbance ratios at 260 nm/280 nm of 1.9-2.1. Reverse transcription (RT) of 500 ng of total RNA was performed with random decamers using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) following manufacturers' instructions. RT reactions were incubated for 10 min at 25 °C and 2 h at 37 °C. Negative control reactions were run without reverse transcriptase.

Real-time quantitative PCR was carried out with the CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), using 96-well PCR array

layouts designed for simultaneously profiling a panel of 31 genes for liver samples, and 29 genes for head kidney and muscle samples (summarized in Table 2). Overall, the genes comprised in the liver array covered different biological processes such as GH/IGF system (10), energy sensing and oxidative metabolism (4), respiration uncoupling (1), antioxidant defence and molecular chaperons (11) and cytoplasmatic and lysosomal proteases (5). Transcripts analysed in muscle were associated with the GH/IGF system (10), energy sensing and oxidative metabolism (10), respiration uncoupling (1) and muscle growth and cell differentiation (8). Transcripts analysed in the head kidney were interleukins and cytokines (9), macrophages and monocytes chemokines (3), immunoglobulins (4), antiprotease (1), antimicrobial peptide/iron recycling (1), T-cell markers (6) and pattern recognition receptors (5). Specific primer pair sequences are listed in Sup. Table S2. Controls of general PCR performance were included on each array, being performed all the pipetting operations by means of the EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany). Briefly, RT reactions were diluted to convenient concentrations and the equivalent of 660 pg of total input RNA was used in a 25 µL volume for each PCR reaction. PCR-wells contained a 2× SYBR Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.9 µM were used to obtain amplicons of 50–150 bp in length.

The program used for PCR amplification included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 sec at 95 °C and annealing/extension for 60 s at 60 °C. The efficiency of PCR reactions was always higher than 90%, and negative controls without sample templates were routinely performed for each primer set. The specificity of reactions was verified by analysis of melting curves (ramping rates of 0.05 ºC/sec over a temperature range of 55–95 °C), and linearity of serial dilutions of RT reactions. Fluorescence data acquired during the PCR extension phase were normalized using the delta–delta Ct method (Livak, Schmittgen, 2001). β-Actin was tested for gene expression stability using GeNorm software (M score = 0.21) and it was used as housekeeping gene in the normalization procedure. Fold-change calculations were done in reference to the mean response of CTRL fish. For comparing the mRNA gene expression level of a panel of genes in a given dietary treatment, all data values were in reference to the expression level of a specific gene in CTRL fish. In liver, gene expression was in reference to the expression level of *cpt1*, whereas in head kidney and white skeletal muscle was in reference of *il7* and *igfr2*, respectively, which were arbitrarily assigned a value of 1.

Table 2. Genes included in the liver (†), head kidney (‡) and white muscle (#) pathway-focused PCR arrays.

Gene name/category	Symbol
GH/IGF system	
Growth hormone receptor I	*ghr-i* †#
Growth hormone receptor II	*ghr-ii* †#
Insulin-like growth factor-I	*igf-i* †#
Insulin-like growth factor-II	*igf-ii* †#
Insulin-like growth factor binding protein 1a	*igfbp1* †
Insulin-like growth factor binding protein 2b	*igfbp2* †
Insulin-like growth factor binding protein 3	*Igfbp3* #
Insulin-like growth factor binding protein 4	*igfbp4* †
Insulin-like growth factor binding protein 5b	*Igfbp5b* #
Insulin-like growth factor binding protein 6b	*Igfbp6b* #
Insulin-like growth factor receptor I	*igfr1* †#
Insulin-like growth factor receptor II	*igfr2* †#
Insulin receptor	*insr* †#
Energy sensing and oxidative metabolism	
Sirtuin 1	*sirt1* #
Sirtuin 2	*sirt2* #
Sirtuin 5	*sirt5* #
NADH-ubiquinone oxidoreductase chain 2	*nd2* #
NADH-ubiquinone oxidoreductase chain 5	*nd5* #
Cytochrome c oxidase subunit I	*coxi* #
Cytochrome c oxidase subunit II	*coxii* #
Carnitine palmitoyltransferase 1A	*cpt1a* †#
Citrate synthase	*cs* †#
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1α* †#
Hypoxia inducible factor-1 alpha	*hif-1α* †
Respiration uncoupling	
Uncoupling protein 1	*ucp1* †
Uncoupling protein 3	*ucp3* #
Interleukins and cytokines	
Interleukin-1 beta	*il-1β* ‡
Interleukin-6	*I-6* ‡
Interleukin-7	*il-7* ‡
Interleukin-8	*il-8* ‡
Interleukin-10	*il-10* ‡
Interleukin 12 subunit beta	*il12* ‡
Interleukin-15	*il-15* ‡
Interleukin-34	*il-34* ‡
Tumor necrosis factor-alpha	*tnf α*

Gene name/category	Symbol
Muscle growth and cell differentiation	
Myoblast determination protein 1	*myod1* #
Myogenic factor MYOD2	*myod2* #
Myogenic factor 5	*myf5* #
Myogenic factor 6	*myf6/mrf4/ herculin* #
Myostatin/Growth differentiation factor 8	*mstn/gdf-8* #
Myocyte-specific enhancer factor 2A	*mef2a* #
Myocyte-specific enhancer factor 2C	*mef2c* #
Follistatin	*fst* #
Antioxidant defence and molecular chaperons	
Catalase	*cat* †
Glutathione peroxidase 1	*gpx1* †
Glutathione peroxidase 4	*gpx4* †
Glutathione reductase	*gr* †
Peroxiredoxin 3	*prdx3* †
Peroxiredoxin 5	*prdx5* †
Superoxide dismutase [Mn]	*Mn-sod/sod2* †
Fatty acid binding protein, heart	*h-fabp* †
Glucose-regulated protein, 170 kDa	*grp-170* †
Glucose-regulated protein, 94 kDa	*grp-94* †
70 kDa heat shock protein, mitochondrial	*mthsp70/grp-75/mortalin* †
Cytoplasmatic and lysosomal proteases	
Calpain 1	*capn1* †
Calpastatin	*cast* †
Cathepsin B	*ctsb* †
Cathepsin D	*ctsd* †
Cathepsin L	*ctsl* †
Macrophages and monocytes chemokines	
Macrophage colony-stimulating factor 1 receptor 1	*csf1r1* ‡
C-C chemokine receptor type 3	*ccr3* ‡
C-C chemokine CK8 / C-C motif chemokine 20	*ck8/ccl20* ‡
Immunoglobulins	
Immunoglobulin M	*IgM* ‡
Immunoglobulin M membrane-bound form	*mIgM* ‡
Immunoglobulin T	*IgT* ‡
Immunoglobulin T membrane-bound form	*IgT-m* ‡

Table 2. (continued)

Antiprotease		***Antimicrobial peptide/Iron recycling***	
Alpha-2-macroglobulin	*a2m* ‡	Hepcidin	*hepc* ‡
T-cell markers		***Pattern recognition receptors***	
Cluster of differentiation 3 epsilon chain	*cd3e* ‡	Toll-like receptor 1	*tlr1* ‡
Cluster of differentiation 3 zeta chain	*cd3x* ‡	Toll-like receptor 2	*tlr2* ‡
CD4-full	*cd4-full* ‡	Toll-like receptor 5	*tlr5* ‡
Cluster of differentiation 8 alpha	*cd8a* ‡	Toll-like receptor 9	*tlr9* ‡
Cluster of differentiation 8 beta	*cd8b* ‡	Macrophage mannose receptor 1	*mrc1* ‡
Zeta-chain-associated protein kinase 70	*zap70* ‡		

3.2.7 Calculations

Zootechnical performance measures were calculated as:

Feed conversion ratio (FCR) = apparent feed intake/weight gain, where wet weight gain is: FBW – IBW. FBW, final body weight; IBW, initial body weight.

- Daily growth index (DGI) = 100 × ((Final body weight)$^{1/3}$ – (Initial body weight)$^{1/3}$)/days
- Relative growth rate (RGR, %.day^{-1} = (e^{g}-1) x 100, where g = (ln(Final body weight) – ln(Initial body weight))/days
- Voluntary feed intake (VFI) = 100 × crude feed intake/(average body weight × days), where ABW was calculated as: (IBW + FBW)/2
- Protein efficiency ratio (PER) = weight gain / crude protein ingested
- Nutrient or energy gain (g or kJ.kg ABW^{-1}.day^{-1}) = (final carcass nutrient or energy content − initial carcass nutrient or energy content)/(average body weight × days), where ABW was calculated as: (IBW + FBW)/2
- Nutrient retention (%) was calculated as: 100 × nutrient gain/nutrient intake.

3.2.8 Statistical analysis

Statistic evaluation of the data was accomplished by mixed effect ANOVA, using the SPSS statistical analysis software (SPSS ver. 23.0; Chicago, USA). The dependent variable was the corresponding response, the fixed factors were

"treatment" and "sampling time" and the random factor was "tank" nested within treatment. A significance threshold of $p<0.05$ was applied to all statistical tests. For data expressed in Table 3, a one way ANOVA Tukey HSD's post-test for pairwise comparisons between means of different groups were performed. All variables were checked for normality and homogeneity of variance, by using the Shapiro-Wilk and the Levene's test, respectively. For plasma ACH50, peroxidase, MCV, MCHC index, plasma bactericidal activity, nitric oxide, thrombocytes and monocyte counts, data were transformed by means of $\log_{10}$. For gene expression data, a $\mathrm{Log}2$ transformation was applied to all expression values. Unsupervised multivariate analysis by principle component analysis (PCA) was first performed on data as an unbiased statistical method to observe intrinsic trends in the dataset, using EZ-INFO® v3.0 (Umetrics, Sweden). To achieve the maximum separation among the groups, supervised multivariate analysis by partial least-squares discriminant analysis (PLS-DA) was sequentially applied, using EZ-INFO® v3.0 (Umetrics, Sweden). Potential differential genes were selected according to the Variable Importance in the Projection (VIP) values. Variables with VIP > 1 were considered to be influential for the separation of samples in PLS-DA analysis (Kieffer, et al., 2016; Li, et al., 2012; Wold, et al., 2001).

3.3 Results

3.3.1 Growth performance

No differences were observed between diet groups in final body weight (FBW), daily growth index (DGI), relative growth rate (RGR) and voluntary feed intake (VFI) either after 15 or 92 days of feeding the experimental diets. Fish fed the SUP diet showed a tendency for higher feed conversion ratio (FCR) compared to those fed the CTRL diet, after 15 days of feeding, whereas this trend was not observed after the long-term feeding period (Table 3).

3.3.2 Haematological profile

In general, total WBC and RBC counts, as well as Hb, MCH and MCHC values, were similar between dietary treatments and sampling times, whereas Ht and MCV levels increased between first and final sampling points regardless of experimental diets (Table 4). While the concentration of peripheral thrombocytes remained unchanged throughout the trial, circulating lymphocytes, neutrophils and monocytes showed a decrease over time regardless of diet (Table 4). Moreover, peripheral monocyte concentration decreased in fish fed the SUP diet compared to those fed CTRL, regardless of feeding time.

3.3.3 Cortisol and immune parameters in plasma

Both plasma bactericidal activity and nitric oxide levels showed a decrease over time regardless of dietary treatments, whereas antiprotease activity showed the opposite pattern (Table 5). Cortisol, ACH50, peroxidase and protease values were not affected by either sampling point or dietary treatment.

3.3.4 Humoral innate immune parameters in mucus

Peroxidase activity decreased over time in skin mucus regardless of dietary treatments, whereas no differences were observed in ACH50, bactericidal and protease activities (Table 5).

Table 3. Final body weight (FBW, g fish^{-1}), relative growth rate (RGR), daily growth index (DGI), feed conversion ratio (FCR), feed efficiency (FE), voluntary feed intake (VFI) and protein efficiency ratio (PER) of gilthead seabream fed the experimental diets for 15 and 92 days. Values are expressed as mean ± SE (n=3).

	15 Days		92 Days	
	CTRL	**SUP**	**CTRL**	**SUP**
BW	20.07 ± 0.97	20.65 ± 0.73	68.45 ± 4.46	64.30 ± 3.31
RGR (%)	2.78 ± 0.34	2.94 ± 0.35	1.80 ± 0.07	1.72 ± 0.07
DGI	2.32 ± 0.29	2.46 ± 0.30	1.87 ± 0.10	1.77 ± 0.09
FCR	1.52 ± 0.02	1.66 ± 0.17	1.41 ± 0.05	1.55 ± 0.12
FE	0.64 ± 0.04	0.67 ± 0.12	0.71 ± 0.03	0.65 ± 0.05
VFI	4.17 ± 0.21	4.21 ± 0.25	1.96 ± 0.02	2.10 ± 0.09
PER	1.38 ± 0.10	1.44 ± 0.24	1.55 ± 0.51	1.39 ± 0.10
Intake (g.Kg ABW $^{-1}$.days^{-1})				
Dry matter	39.23 ± 2.14	39.44 ± 2.41	18.94 ± 0.15	20.20 ± 0.89
Protein	19.57 ± 1.06	19.94 ± 1.22	9.45 ± 0.75	10.21 ± 0.45
Lipids	8.64 ± 0.47	7.76 ± 0.47	4.17 ± 0.29	3.97 ± 0.18

Table 4. Haematocrit, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cells (RBC), white blood cells (WBC) and absolute values of peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes and neutrophils in gilthead seabream fed dietary treatments during 15 and 92 days. Values are expressed as means ± SE (n=9).

Parameters		Dietary treatments						
		15 Days		92 Days		Two-way ANOVA		
		CTRL	SUP	CTRL	SUP	Time	Diet	Time x diet
Haematocrit	(%)	31.00 ± 1.40	34.77 ± 0.68	36.36 ± 1.27	37.08 ± 1.36	<0.005	0.722	0.418
Haemoglobin	(g dl^{-1})	2.08 ± 0.18	2.34 ± 0.07	2.19 ± 0.16	2.49 ± 0.11	0.690	0.947	0.749
MCH	(pg $cell^{-1}$)	7.61 ± 0.60	8.46 ± 0.50	7.87 ± 0.63	9.17 ± 0.57	0.439	0.581	0.721
MCV	(μm^3)	114.22 ± 2.90	116.54 ± 1.35	131.25 ± 1.06	136.60 ± 9.12	0.038	0.910	0.859
MCHC	(g 100 ml^{-1})	6.63 ± 0.33	6.79 ± 0.34	5.99 ± 0.32	6.97 ± 0.27	0.364	0.713	0.331
RBC	($x10^6$ µl)	2.75 ± 0.14	3.05 ± 0.11	2.84 ± 0.17	2.75 ± 0.15	0.462	0.284	0.205
WBC	($x10^4$ µl)	6.66 ± 0.90	5.80 ± 0.19	5.15 ± 0.47	4.99 ± 0.37	0.437	0.212	0.286
Absolute peripheral blood leucocytes								
Thrombocytes	($x10^4$µl)	4.11 ± 0.50	3.90 ± 0.15	3.61 ± 0.20	3.65 ± 0.30	0.730	0.474	0.217
Lymphocytes	($x10^4$µl)	1.85 ± 0.32	1.38 ± 0.12	0.32 ± 0.05	0.61 ± 0.04	< 0.001	0.261	0.622
Monocytes	($x10^4$µl)	0.25 ± 0.05	0.12 ± 0.01	0.10 ± 0.02	0.06 ± 0.01	0.010	0.048	0.269
Neutrophils	($x10^4$µl)	0.32 ± 0.06	0.37 ± 0.07	0.83 ± 0.09	0.66 ± 0.08	< 0.001	0.637	0.168

P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments.

Table 5. Plasma and mucus humoral parameters of gilthead seabream fed dietary treatments during 15 and 92 days.

Parameters		Dietary treatments						
		15 Days		92 Days		Two-way ANOVA		
		CTRL	SUP	CTRL	SUP	Time	Diet	Time x diet
Plasma								
Cortisol	(ng/ml)	29.03 ± 4.23	27.72 ± 2.48	32.94 ± 4.35	28.46 ± 4.36	0.552	0.356	0.751
Bactericidal activity	(%)	48.55 ± 0.71	49.86 ± 1.45	7.76 ± 2.38	20.73 ± 5.73	<0.001	0.016	0.073
Protease activity	(%)	5.37 ± 0.16	5.18 ± 0.13	5.31 ± 0.16	5.43 ± 0.20	0.167	0.957	0.352
Antiprotease activity	(%)	93.26 ± 0.29	93.48 ± 0.37	97.76 ± 0.10	97.48 ± 0.07	<0.001	0.962	0.403
Peroxidase activity	(units/ml)	41.61 ± 8.42	26.72 ± 4.09	47.46 ± 9.61	42.02 ± 8.39	0.074	0.456	0.760
ACH50	(units/ml)	23.41 ± 3.11	31.80 ± 4.36	30.59 ± 7.02	33.60 ± 3.60	0.323	0.204	0.546
Nitric Oxide	(µM)	123.33 ± 16.60	89.04 ± 14.96	49.55 ± 4.63	51.38 ± 9.89	<0.001	0.454	0.173
Mucus								
Bactericidal activity	(%)	33.38 ± 3.02	33.51 ± 7.51	35.65 ± 1.33	35.09 ± 1.18	0.439	0.931	0.889
Protease activity	(%)	7.40 ± 0.79	6.74 ± 1.04	7.50 ± 0.54	7.91 ± 0.47	0.360	0.857	0.445
Peroxidase activity	(units/ml)	26.05 ± 8.20	23.40 ± 7.03	11.60 ± 2.50	16.71 ± 2.67	0.022	0.782	0.386
ACH50	(units/ml)	2.43 ± 0.56	2.85 ± 0.43	3.55 ± 0.14	2.77 ± 0.21	0.130	0.599	0.081

Values are expressed as means ± SE (n=9). P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments

3.3.5 Gene expression

Most of the analysed genes in liver (25 out of 31) showed differential expression (up- or down-regulated) between the two sampling points (Table 6). Tryptophan supplementation induced a down-regulation of *igfr1*, *igfr2* and *insr* after 15 days of feeding, but these differences were not maintained after long-term feeding. Significant differences were also observed in muscle gene expression at the two different sampling points (Table 7), with a clear trend of up-regulation of transcript levels (18 out of 21 differentially expressed genes) at 92 days after feeding. Regarding dietary effects*, igfbp3* expression was up-regulated in fish fed the SUP diet after the short-term feeding period, and *capn1* was down-regulated after 92 days of feeding the SUP diet. In head kidney tissue, 14 out of 29 genes were differentially expressed after 92 days of feeding regardless of dietary treatment (Table 8). Up-regulated genes were *il-34*, *ccr3*, *ck8/ccl20*, *tlr9*, *sIgM*, *mIgM,* and *tlr5*), whereas *il-1β, il-8*, *sIgT*, *hepc* and *cd8b* were down-regulated after 92 days of feeding. Regarding a diet effect on health-related biomarkers in the head kidney, three transcripts (*cd3x*, *tlr2* and *Il-10*) were up-regulated after a short-term feeding period in fish fed the SUP diet compared to those the CTRL diet, although a general but non-significant trend to gene expression increase was observed for almost all analysed genes at 15 days when fed the SUP diet. This effect was not retained after 92 days of feeding.

In order to get a clearer picture of the time and diet effect on tissue gene expression, an overall multivariate analysis combining raw data from the three tissues (using PLS-DA) was performed to discriminate the tissues' molecular signatures of fish fed the experimental diets both at short and long-term feeding periods (Fig. 1). This approach showed that overall expression patterns can be summarized through 3 main components that explain 88.31% of total variance (Fig. 1A). Component 1 (50.06% of total variance, X-axis) was able to clearly separate fish sampled at early and late on the feeding trial. Dietary groups after 15 days of feeding evidenced a good separation along component 2 (24.36% of total variance, Y-axis) (Fig. 1B). Component 3 (13.89% of total variance, Y-axis) appeared to be also related to diet effect, contributing to further separate CTRL and SUP groups at early sampling (Fig. 1C). The variable importance projection (VIP) score of the genes after three components is presented in Fig. 1D. VIP contribution after 1 and 2 components are presented as Sup. Table S4.

Biomarkers with a VIP > 1 that appeared after the first component (highlighted in yellow), were mostly represented by liver and muscle genes (22 out of 26). By contrast, genes explaining the most variance associated to component 2 (highlighted in blue) were mostly from head kidney (7 genes) and liver (4 genes). Only 6 new genes, with a representation of the 3 analysed tissues (3 from muscle, 2 from liver, 1 from head kidney), were reported as main contributors to variance when the component 3 (highlighted in orange) was also considered. When the VIP contribution was ranked after the 3 components on the analysis, the highest contribution corresponded to hepatic *insr*, *igfr1* and *igfr2* genes, which were down-regulated in fish fed the SUP diet at short-period sampling (Table 6).

Table 6. Hepatic gene expression in gilthead seabream fed dietary treatments during 15 and 92 days. All values are reported as mean ± SE (n=9). Different lowercase letters stand for significant differences among dietary treatments for the same time, while symbols stand for significant differences between times for the same diet.

		15 Days		92 Days		Two-way ANOVA (p<0.05)		
Biological Process	**Gene symbol**	**CTRL**	**SUP**	**CTRL**	**SUP**	**Time**	**Diet**	**Time x diet**
GH/IGF System	*ghr-i*	1.17 ± 0.22	1.37 ± 0.24	1.96 ± 0.18	2.31 ± 0.32	<0.001	0.732	0.773
	ghr-ii	1.39 ± 0.11	1.46 ± 0.11	0.88 ± 0.09	0.78 ± 0.09	<0.001	0.636	0.416
	igf-i	5.76 ± 0.69	5.55 ± 0.32	8.77 ± 1.17	8.17 ± 0.78	<0.001	0.864	0.806
	igf-ii	2.01 ± 0.26	2.62 ± 0.27	4.71 ± 0.53	5.25 ± 1.02	<0.001	0.579	0.352
	igfbp1a	0.06 ± 0.01	0.09 ± 0.02	0.04 ± 0.00	0.04 ± 0.00	<0.001	0.373	0.217
	igfbp2b	2.12 ± 0.15	2.52 ± 0.19	1.42 ± 0.13	1.63 ± 0.15	<0.001	0.330	0.863
	igfbp4	0.68 ± 0.06	0.63 ± 0.06	0.54 ± 0.04	0.49 ± 0.05	0.006	0.537	0.794
	igfr1	0.10 ± 0.01 ^{b}	0.05 ± 0.01 ^{a}	0.08 ± 0.01	0.07 ± 0.01	0.354	0.145	0.003
	igfr2	0.28 ± 0.03 ^{b}	0.12 ± 0.01 ^{a*}	0.22 ± 0.02	0.26 ± 0.03 $^{\#}$	0.012	0.275	<0.001
	insr	0.96 ± 0.08 ^{b*}	0.47 ± 0.01 ^{a*}	0.69 ± 0.08 $^{\#}$	0.84 ± 0.06 $^{\#}$	0.139	0.246	<0.001
Cytoplasmic and lysosomal proteases	*capn1*	0.15 ± 0.02	0.19 ± 0.02 $^{\#}$	0.12 ± 0.01	0.09 ± 0.01 *	<0.001	0.957	0.013
	cast	0.28 ± 0.02	0.29 ± 0.02	0.53 ± 0.05	0.50 ± 0.05	<0.001	0.867	0.626
	ctsb	1.76 ± 0.14	1.98 ± 0.20	1.86 ± 0.16	2.01 ± 0.15	0.523	0.668	0.884
	ctsd	0.17 ± 0.02	0.18 ± 0.02	1.03 ± 0.19	1.06 ± 0.14	<0.001	0.780	0.939
	ctsl	6.74 ± 0.53	8.21 ± 0.65	11.50 ± 0.86	12.20 ± 1.30	<0.001	0.499	0.293
Energy sensing and oxidative metabolism	*pgc1α*	0.32 ± 0.04	0.39 ± 0.04	0.17 ± 0.01	0.16 ± 0.02	<0.001	0.584	0.162
	cpt1α	0.93 ± 0.06	0.90 ± 0.04	1.09 ± 0.09	1.16 ± 0.09	0.001	0.813	0.228
	cs	0.43 ± 0.03	0.49 ± 0.02	0.81 ± 0.06	0.89 ± 0.07	<0.001	0.429	0.668
	hif-1α	0.55 ± 0.03	0.50 ± 0.03	0.36 ± 0.03	0.35 ± 0.02	<0.001	0.464	0.525

Table 6 (continued)

Antioxidant effects	*mthsp70/grp-75*	0.53 ± 0.08	0.54 ± 0.04	0.70 ± 0.07	0.76 ± 0.09	0.005	0.704	0.831
	grp-170	1.24 ± 0.15	1.33 ± 0.18	1.13 ± 0.15	1.23 ± 0.14	0.602	0.429	0.907
	grp-94	3.82 ± 0.68	3.65 ± 0.58	1.47 ± 0.24	2.47 ± 0.52	0.013	0.574	0.117
	cat	10.86 ± 0.95	11.63 ± 1.33	13.15 ± 1.24	13.24 ± 0.67	0.051	0.744	0.924
	gpx1	1.08 ± 0.06	1.17 ± 0.06	0.96 ± 0.05	0.92 ± 0.08	0.006	0.914	0.263
	gpx4	4.08 ± 0.65	4.52 ± 0.75	13.82 ± 2.19	14.60 ± 0.81	<0.001	0.521	0.978
	gr	0.24 ± 0.01	0.25 ± 0.02	0.35 ± 0.02	0.35 ± 0.03	<0.001	0.996	0.824
	prdx3	0.45 ± 0.03	0.47 ± 0.04	0.68 ± 0.06	0.71 ± 0.05	<0.001	0.817	0.994
	prdx5	0.29 ± 0.04	0.25 ± 0.03	1.13 ± 0.12	1.11 ± 0.09	<0.001	0.624	0.674
	Mn-sod/sod2	0.80 ± 0.07	0.76 ± 0.06	0.77 ± 0.06	0.75 ± 0.07	0.822	0.620	0.952
	h-fabp	26.47 ± 2.04	27.89 ± 1.66	45.78 ± 4.31	58.29 ± 6.00	<0.001	0.620	0.952
Respiration uncoupling	*ucp1*	15.18 ± 1.17	17.26 ± 1.08	8.77 ± 0.88	10.39 ± 0.74	<0.001	0.248	0.746

Values are expressed as means ± SE (n=9). P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same time while symbols stand for significant differences between times for the same diet

Table 7. Muscle gene expression in response in gilthead seabream fed dietary treatments during 15 and 92 days. All values are reported as mean ± SE (n=9). Different lowercase letters stand for significant differences among dietary treatments for the same time while symbols stand for significant differences between times for the same diet.

		15 Days		92 Days		Two-way ANOVA (p<0.05)		
Biological Process	**Gene symbol**	**CTRL**	**SUP**	**CTRL**	**SUP**	**Time**	**Diet**	**Time x diet**
GH/IGF System	*ghr-i*	3.16 ± 0.33	3.22 ± 0.35	9.99 ± 1.27	10.86 ± 0.83	<0.001	0.669	0.620
	ghr-ii	4.72 ± 0.71	8.35 ± 3.11	3.97 ± 0.66	2.66 ± 0.36	0.012	0.928	0.104
	igf-i	0.16 ± 0.01	0.25 ± 0.08	0.29 ± 0.04	0.22 ± 0.04	0.089	0.729	0.167
	igf-ii	1.34 ± 0.12	2.00 ± 0.35	2.60 ± 0.24	2.27 ± 0.20	0.001	0.336	0.058
	igfbp3	3.69 ± 0.30 a#	5.42 ± 0.52 b#	1.80 ± 0.18 *	1.58 ± 0.10 *	<0.001	0.275	0.004
	igfbp5b	1.64 ± 0.11	2.09 ± 0.14	3.57 ± 0.38	3.23 ± 0.24	<0.001	0.051	0.538
	igfbp6b	0.28 ± 0.03	0.34 ± 0.03	0.32 ± 0.04	0.28 ± 0.03	0.647	0.827	0.109
	insr	1.65 ± 0.16	2.22 ± 0.45	2.25 ± 0.19	2.62 ± 0.72	0.131	0.374	0.505
	igfr1	1.40 ± 0.09	1.72 ± 0.26	2.85 ± 0.20	3.13 ± 0.32	<0.001	0.421	0.699
	igfr2	0.98 ± 0.10	1.48 ± 0.38	1.34 ± 0.09	2.01 ± 0.54	0.025	0.296	0.883
Muscle growth & cell differentiation	*myod1*	10.86 ± 0.53	12.92 ± 1.88	13.90 ± 1.60	13.59 ± 1.17	0.136	0.635	0.582
	myod1	2.04 ± 0.41	1.95 ± 0.23	2.28 ± 0.22	1.92 ± 0.25	0.347	0.908	0.277
	myf5	0.47 ± 0.03	0.50 ± 0.04	0.48 ± 0.03	0.53 ± 0.12	0.854	0.625	0.890
	myf6	0.45 ± 0.03	0.55 ± 0.04	0.75 ± 0.06	1.23 ± 0.41	<0.001	0.074	0.712
	mstn	2.16 ± 0.23	2.54 ± 0.34	6.38 ± 1.65	6.94 ± 1.88	<0.001	0.749	0.972
	mef2a	15.43 ± 1.14	19.37 ± 2.38	42.76 ± 3.10	48.54 ± 4.43	<0.001	0.158	0.669
	mef2c	5.94 ± 0.22	5.88 ± 0.51	12.08 ± 1.21	12.85 ± 1.51	<0.001	0.819	0.595
	fst	0.67 ± 0.08	0.88 ± 0.15	0.57 ± 0.07	0.55 ± 0.06	0.013	0.540	0.301

Table 7(continued)

Energy sensing & oxidative metabolism	*sirt1*	0.37 ± 0.02	0.42 ± 0.05	0.56 ± 0.06	0.59 ± 0.10	0.002	0.638	0.682
	sirt2	0.48 ± 0.02	0.56 ± 0.07	0.75 ± 0.06	0.76 ± 0.05	<0.001	0.350	0.549
	sirt5	1.03 ± 0.08	1.16 ± 0.17	1.16 ± 0.10	0.97 ± 0.12	0.809	0.373	0.220
	cpt1α	10.72 ± 0.37	12.32 ± 1.18	22.94 ± 1.91	22.87 ± 2.26	<0.001	0.507	0.536
	cs	25.09 ± 1.58	29.77 ± 3.84	36.41 ± 2.49	35.23 ± 2.49	0.004	0.359	0.419
	nd2	44.81 ± 3.28	45.38 ± 5.75	88.01 ± 15.02	82.28 ± 6.00	<0.001	0.849	0.887
	nd5	26.63 ± 1.85	30.03 ± 3.09	45.55 ± 7.20	36.86 ± 3.23	0.003	0.798	0.246
	cox i	239.75 ± 17.83	333.71 ± 48.73	320.82 ± 27.86	253.56 ± 20.87	0.758	0.827	0.048
	cox ii	123.85 ± 6.90	163.49 ± 27.34	146.09 ± 23.72	112.52 ± 8.34	0.349	0.997	0.094
Respiration uncoupling	*ucp3*	14.43 ± 1.93	17.25 ± 3.13	29.40 ± 4.92	30.68 ± 3.38	<0.001	0.520	0.933
	pgc1α	0.58 ± 0.15	0.37 ± 0.06	2.47 ± 0.43	3.00 ± 0.61	<0.001	0.727	0.249

Values are expressed as means ± SE (n=9). P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same time while symbols stand for significant differences between times for the same diet

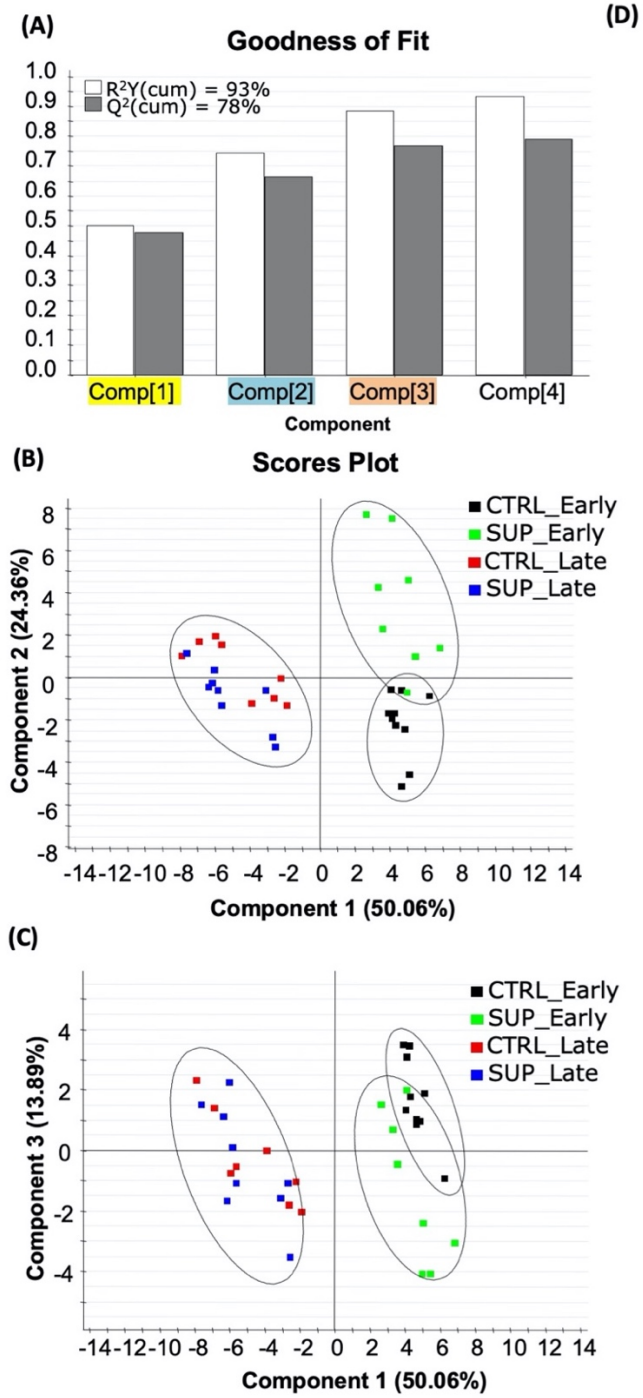
Table 8. Head kidney expression in response in gilthead seabream fed dietary treatments during 15 and 92 days.

		15 Days		92 Days		Two-way ANOVA (p<0.05)		
Biological Process	**Gene symbol**	**CTRL**	**SUP**	**CTRL**	**SUP**	**Time**	**Diet**	**Time x diet**
Interleukins & Cytokines	*il-1β*	0.09 ± 0.02	0.11 ± 0.02	0.03 ± 0.00	0.03 ± 0.01	<0.001	0.403	0.576
	il-6	0.02 ± 0.00*	0.04 ± 0.01	0.05 ± 0.01#	0.04 ± 0.01	0.104	0.580	0.015
	il-7	1.03 ± 0.15	1.17 ± 0.09	1.00 ± 0.08	0.80 ± 0.12	0.105	0.625	0.078
	il-8	0.05 ± 0.01	0.06 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.013	0.842	0.487
	il-10	0.47 ± 0.03[a]	0.66 ± 0.04[b]	0.67 ± 0.05	0.52 ± 0.04	0.601	0.731	<0.001
	il-12	0.06 ± 0.01	0.08 ± 0.01	0.05 ± 0.00	0.04 ± 0.00	0.137	0.993	0.077
	il-15	0.23 ± 0.02*	0.24 ± 0.02	0.33 ± 0.02[#]	0.27 ± 0.02	<0.001	0.693	0.021
	il-34	1.11 ± 0.12	1.26 ± 0.07	2.18 ± 0.15	2.02 ± 0.14	<0.001	0.736	0.132
	tnf-α	0.14 ± 0.02	0.16 ± 0.01	0.17 ± 0.02	0.16 ± 0.01	0.361	0.659	0.220
Macrophages and monocytes chemokines	*csf1r1*	1.73 ± 0.19*	1.99 ± 0.11	2.90 ± 0.22[#]	2.41 ± 0.15	<0.001	0.958	0.015
	ccr3	4.85 ± 0.59	4.55 ± 0.38	5.71 ± 0.35	5.65 ± 0.49	0.012	0.873	0.996
	ck8 / ccl20	0.36 ± 0.06	0.46 ± 0.04	0.62 ± 0.10	0.52 ± 0.06	0.019	0.620	0.047
Immunoglobulins	*sIgM*	76.46 ± 7.40	65.68 ± 10.87	129.14 ± 16.21	103.47 ± 14.30	0.008	0.043	0.817
	mIgM	12.86 ± 1.24	13.73 ± 1.14	17.73 ± 1.17	15.12 ± 1.14	0.017	0.748	0.594
	sIgT	0.67 ± 0.42	1.11 ± 0.72	4.81 ± 1.48	2.73 ± 0.85	0.140	0.701	0.463
	mIgT	9.16 ± 0.96	10.91 ± 1.67	8.35 ± 1.06	7.76 ± 0.70	0.011	0.728	0.124

Table 8 (continued)

Anti-protease	*a2m*	0.10 ± 0.04	0.12 ± 0.02	0.09 ± 0.02	0.09 ± 0.02	0.655	0.725	0.132
Antimicrobial peptide/ iron recycling	*hepc*	67.75 ± 10.00	103.42 ± 17.42	10.91 ± 1.88	10.20 ± 1.69	<0.001	0.349	0.202
T-cell markers	*cd3e*	2.33 ± 0.35	3.89 ± 0.70	3.04 ± 0.13	2.85 ± 0.25	0.567	0.420	0.150
	cd3x	2.00 ± 0.23^{a}	3.27 ± 0.49^{b}	2.62 ± 0.19	2.55 ± 0.18	0.601	0.137	0.028
	cd4-full	1.51 ± 0.23	2.59 ± 0.54	2.05 ± 0.13	1.84 ± 0.12	0.491	0.389	0.021
	cd8a	1.28 ± 0.24	2.06 ± 0.53	1.19 ± 0.10	0.98 ± 0.13	0.133	0.876	0.118
	cd8b	0.61 ± 0.16	1.14 ± 0.34	0.35 ± 0.04	0.28 ± 0.04	0.002	0.810	0.119
	zap70	1.55 ± 0.19	1.95 ± 0.20	2.01 ± 0.18	1.93 ± 0.13	0.108	0.590	0.130
Pattern recognition receptors	*tlr1*	1.15 ± 0.05	1.20 ± 0.07	1.26 ± 0.06	1.04 ± 0.08	0.617	0.041	0.059
	tlr2	1.44 ± 0.12^{a*}	1.95 ± 0.06^{b*}	3.04 ± 0.15$^{\#}$	3.02 ± 0.18$^{\#}$	<0.001	0.104	0.007
	tlr5	0.32 ± 0.04	0.33 ± 0.02	0.48 ± 0.07	0.48 ± 0.04	0.001	0.463	0.796
	tlr9	0.25 ± 0.03*	0.34 ± 0.04*	0.79 ± 0.10$^{\#}$	0.62 ± 0.05$^{\#}$	<0.001	0.666	0.050
	mrc1	5.18 ± 0.63	5.39 ± 0.47	5.83 ± 0.32	5.59 ± 0.57	0.373	0.889	0.552

Values are expressed as means ± SE (n=9). P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same time while symbols stand for significant differences between times for the same diet.



(D)

Order	Gene (tissue)	VIP > 1 (after component 3)
1	*insr*_Liv	1.738
2	*igfr1*_Liv	1.534
3	*igfr2*_Liv	1.530
4	*tlr2*_HK	1.437
5	*hif-1α*_Liv	1.386
6	*igfbp3*_Mus	1.348
7	*cd3x*_HK	1.344
8	*prdx5*_Liv	1.319
9	*hepc*_HK	1.304
10	*mef2a*_Mus	1.285
11	*ghr-i*_Mus	1.269
12	*igfbp2b*_Liv	1.254
13	*cs*_Liv	1.244
14	*gpx4*_Liv	1.243
15	*il-10*_HK	1.26
16	*capn1*_Liv	1.222
17	*cd8b*_HK	1.205
18	*ctsd*_Liv	1.203
19	*il-34*_HK	1.191
20	*igfbp5b*_Mus	1.189
21	*nd2*_Mus	1.185
22	*cpt1a*_Mus	1.180
23	*cd3e*_HK	1.177
24	*ucp1*_Liv	1.137
25	*mef2c*_Mus	1.129
26	*cd4-full*_HK	1.126
27	*igfr1*_Mus	1.121
28	*h-fabp*_Liv	1.119
29	*tlr9*_HK	1.109
30	*cd8a*_HK	1.109
31	*sirt2*_Mus	1.101
32	*igfbp1a*_Liv	1.096
33	*cast*_Liv	1.090
34	*IgM*_HK	1.083
35	*pgc1α*_Mus	1.070
36	*ctsl*_Liv	1.063
37	*cs*_Mus	1.062
38	*gpx1*_Liv	1.041
39	*il-6*_HK	1.036
40	*ucp3*_Mus	1.020
41	*gr*_Liv	1.009
42	*igf-ii*_Mus	1.006
43	*coxi*_Mus	1.006
44	*coxii*_Mus	1.003

Fig. 1. Discriminant analysis (PLS-DA) of liver, muscle and head kidney molecular signatures of fish fed the experimental diets. Relative expression data of the 89 genes included in the array can be found on tables 6, 7 & 8. (**A)** Cumulative coefficients of goodness of fit (R^2, white bars) and prediction (Q^2, grey bars) by each component; the three first components explained 88.31% of total variance. (**B and C)** PLS-DA score plots of all biomarkers analysed in the three target tissues along the three main components. **(D)** Ordered list of markers by variable importance (VIP) in projection of PLS-DA model for group differentiation. Markers with VIP values > 1 after the first, second and third components are highlighted in yellow, blue and orange, respectively.

3.4 Discussion

The present study represents the first attempt to explore and compare the putative short and long-term effects of a functional diet, in the context of an extreme formulation (0% fishmeal) supplemented with tryptophan, on the growth and health condition of the gilthead seabream juveniles. It should be noted that the first weeks of the study were conducted at high temperature for gilthead seabream, what may elicit an increase in amino acid requirements (Costas, et al., 2012), and both diets were just above the lysine and methionine requirements estimated by Peres, Oliva-Teles (2009).

In the present study, no differences were observed between dietary treatments after a long-term feeding period, both at zootechnical, physiological and transcriptional level. Nevertheless, this holistic approach revealed interesting results about the impact of a supplemented diet at different sampling points. The fact that “time” seemed to be the main modulatory factor on the humoral immune parameters analysed underlines the sensitivity of some of these measures to contextual factors. For instance, plasma ACH50 levels tended to increase in fish fed the SUP diet after a short-feeding period. Machado, et al. (2015) and Azeredo, et al. (2019) also observed a tendency for increased plasma ACH50 values in European seabass and Senegalese sole juveniles fed a tryptophan supplemented diet for a period of 15 or 38 days, respectively. In the present study, the absence of an immune-priming effect, such as antigen recognition, may explain the lower response / variation in most humoral parameters and the health biomarkers panel analysed. In this context, Machado, et al. (2015) showed that slight short-term feeding effects on the European seabass immune response can be enhanced upon stimulation with an inflammatory agent as observed by a general increase in innate immune parameters in fish fed diets supplemented with essential AA.

Simultaneous gene expression analysis using a customized PCR-array platform offers the possibility to identify over time and at a high level of confidence the most responsive tissues and biomarkers in fish fed different experimental diets. This was inferred from discriminant analysis (PLS-DA) integrating all expression data from liver, skeletal muscle and head kidney in fish fed CTRL and SUP diets, which showed the existence of three major groups in terms of gene expression: two groups corresponding to each diet group for the early sampled fish plus a merged group

combining all fish from the late sampling. In this discriminant model, half of the total variance was explained by the first component (~50% of observed variance), corresponding to the effects of time on gene expression, whereas the second and third components (~40% of observed variance) mostly described the short-term diet effects. The sources of variance due to sampling time would reflect the increase of fish size and the change of experimental conditions throughout the 92-day feeding trial, with a decrease of daily water temperature from 25 to 18.5 ºC (Fig. S1). Regarding the specific effects of diet, it is noteworthy that, though VIP analysis with the first three components highlighted that the top contributing genes were liver markers of GH/IGF axis (*insr, igfr1, igfr2*), after the same analysis with the first two components points towards a high importance of head kidney biomarkers at the early sampling point. Therefore, the effect of dietary tryptophan supplementation is particularly important after a short-term feeding period, which seems particularly evident for some immune-related genes (*il-10, cd3x, trl2*).

The up-regulation of immune-related genes can be considered a beneficial effect as reported by other authors in response to administration of AA or derivates. For instance, Cuesta, et al. (2008) have reported the up-regulation of lymphocyte markers and other immune-relevant genes in the head kidney of gilthead seabream upon intraperitoneal injection of melatonin (synthesized from serotonin and Trp). Research in Japanese flounder, also revealed that TLR2 expression was up-regulated in blood leukocytes after treatments with Poly:I(C) and peptidoglycan (Hirono, et al., 2004). Moreover, the *il-10* transcript, which is produced by activated monocytes (T cells) seems to be a crucial factor for some forms of peripheral tolerance and a major suppressor of immune system and inflammation (Reyes-Cerpa, et al., 2012). This type of immune enhancement can be especially important in cases of stressful conditions (e.g. temperatures changes, handling, crowding, transport), known to have immunosuppressive effects.

Stimulation of the immune status of gilthead seabream after 2 weeks of feeding with the SUP diet was concomitant with an early and transient decrease in feed conversion (higher FCR). This may be related to the down-regulated expression of hepatic markers (i.e. *igfr1*, *igfr2* and *insr*) of the GH/IGF system, which is highly responsive to nutritional and environmental stimuli (Pérez-Sánchez, et al., 2018). IGFs directly stimulate cell proliferation, differentiation, and hypertrophy and inhibit muscle atrophy, with the effects of IGF1 on muscle being mediated by the specific binding with IGF1

receptor (IGFR1) (Fuentes, et al., 2013). Montserrat, et al. (2012) reported that, in gilthead seabream myocytes, IGF2 (mediated by IGFR2) activates the MAPK/ERK and PI3K/AKT pathways in a stronger way than IGF1, suggesting that IGF2 is powerful in stimulating their muscle growth (Rius-Francino, et al., 2011). Likewise, insulin signalling via its receptor induce complex effects on metabolism, cell growth and differentiation, including macrophages and endothelial cells. The major tissues targeted by insulin's effects on metabolism include: i) muscle, where it promotes glucose uptake and protein synthesis, and ii) liver, where insulin promotes glucose utilization, suppresses glucose production, and promotes triglyceride synthesis (Haeusler, et al., 2017). Thus, the transient observed down-regulation of *insr* could negatively influence protein synthesis, and it would be consistent with the trend for a higher FCR in fish fed the SUP diet at an early stage. Overall, these observations support the idea that a wide panel of biomarkers helps to better explore in a consistent manner the modulation and effects of functional aquafeeds in terms of fish growth performance and its health status along time.

3.5 Conclusions

This study provides additional information about the effect of dietary supplementation with essential AA in aquafeeds, particularly regarding the supplementation with tryptophan both over short- and long-term feeding periods. It was demonstrated that, in the context of a challenging fishmeal-free diet, supplementation with tryptophan seems to improve gilthead seabream juveniles' immune status on a short-term basis without compromising long-term fish growth. Hence, we consider that the use of functional feeds can be a promising approach for boosting fish immune status, particularly through a short-term feeding period before a predictable stressful event or disease outbreak, considering that these putative advantageous effects are not retained on a long-term basis. Nonetheless, further studies focusing on disease resistance and other stressors must be planned to improve the knowledge on the modulation of growth and immune status in fish through the dietary use of AA.

3.6 Ethics statement

CCMAR facilities and their staff are certified to house and conduct experiments with live animals (Group-C licenses by the Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal). The protocol was approved by the CCMAR Animal Welfare Committee.

3.7 Authors Contributions

LC, SE, TSS and BC conceived the experiment and contributed with both reagents and goods. LR-P conducted the main experimental work. RA, SF-B and BR assisted with analytical procedures. LR-P directed most laboratory techniques and wrote the manuscript under the supervision of JAM-S, JA C-G, JP-S, BC and TSS. L-RP, JAM-S. All authors contributed to and approved the manuscript. The authors also acknowledge Rita Colen and Denise Schrama (UAlg – CCMAR), for their help and support throughout the experimental trial. Authors also thank M. A. González (IATS-CSIC) and M. Machado (CIIMAR) for excellent assistant and help in gene expression and laboratory procedures.

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Chapter 4

Immunomodulatory effects of dietary tryptophan supplementation in gilthead seabream (*Sparus aurata*)

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Immunomodulatory effects of dietary tryptophan supplementation in gilthead seabream (*Sparus aurata*)

Abstract

Amino acids, besides being building blocks for protein synthesis, are known to be essential for the synthesis of many key metabolites. As such, their inclusion in modern feed formulations with functional purposes seems to have potential. Tryptophan (Trp) in particular is precursor of several important metabolites that can act by regulating immune response, modulate behaviour, mood and stress response in fish (e.g. serotonin, melatonin). The present study aimed to evaluate the effects of dietary Trp supplementation on the gilthead seabream (*Sparus aurata*) immune status and inflammatory response. Triplicate groups of fish (6.28 ± 0.28 g) were either fed a control diet (CTRL) with a balanced AA profile, or the CTRL diet supplemented with graded levels of Trp (i.e. 0.5 % and 1 % of feed, TRP1 and TRP2, respectively) for a 4-week feeding period. After 2 and 4 weeks, fish were euthanized and blood was collected for blood smears, plasma for humoral immune parameters, whole gut for oxidative stress biomarkers and anterior gut for the measurement of health-related transcripts. After the 4-week feeding period, fish were intraperitoneal injected with inactivated bacteria and the inflammatory insult was monitored with samplings at 4-, 24- and 48-hours post-injection. During the feeding trial, the analysed responses were not significantly altered by dietary Trp supplementation. In contrast to the lack of effects observed during the feeding trial, the immune response seems to be slightly boosted by dietary tryptophan upon the inflammatory insult, supported by a general increase in several of the analysed transcripts (i.e. *il-34*, *IgM* and *cd8a*), in particular in fish fed the highest Trp inclusion level (TRP2). Data from this study point to an immunomodulatory effect of tryptophan, particularly upon an immune stimulation, evidenced through alterations of the gilthead seabream oxidative stress and health-related biomarkers in the anterior gut in a dose-dependent manner.

Keywords: tryptophan; immune-modulation; oxidative stress; inflammatory insult.

4.1 Introduction

The effect of partial replacement of fish meal and fish oil in modern aquafeed formulations has been the focus of recent research, not only in terms of its impact on growth performance but also of its effects on immune and defence mechanisms (Martin, Krol, 2017). To overcome some aquafeed limitations, amino acids (AA) have been added to these formulations, being an important factor on fish health management in aquaculture. Particularly when subjected to several physiological challenges, fish AA requirements are known to increase as a result of the metabolic adaptations associated with the stress response or inflammatory processes (Sakkas, et al., 2013).

The inclusion of feed grade tryptophan (Trp) in aquafeeds has already been studied and proposed as a growth modulator or nutraceutical ingredient in several fish species. In fact, the Commission Implementing Regulation (EU) 2020/229 of 19 February 2020 has recently published in the Official Journal of the EU a regulation concerning the suitability of L-tryptophan (produced by *Escherichia coli*) as a feed additive for all animal species. Therefore, its use as a functional additive in animal feeds is currently authorized (EU, 2020).

In vertebrates, upon dietary uptake, Trp is either used for protein synthesis or can enter one of the two described pathways: (i) catabolism through the kynurenine pathway (KP) or (ii) serotonin (5-HT) synthesis. Trp is then a precursor to bioactive molecules and neurotransmitters such as 5-HT, kynurenine and melatonin (Hoseini, et al., 2017). Dietary Trp deficiency is known to cause growth retardation, scoliosis and interference in mineral metabolism in fish (Murthy, Varghese, 1997; Walton, et al., 1984). Available evidence shows that dietary Trp supplementation successfully mitigates the stress response to different stressors (e.g. social/crowding, temperature, salinity) in several vertebrates (Akhtar, et al., 2013; Hoseini, Hosseini, 2010; Liu, et al., 2015; Tejpal, et al., 2009). For instance, Akhtar, et al. (2013) and Ciji, et al. (2015) showed that growth performance of *Labeo rohita* under stressful conditions and water pollution was improved when fish were fed diets containing 0.75 and 1.5 % Trp of the dietary protein, compared to those fish fed the tryptophan requirement for this species (0.36-0.38 % of feed).

The mechanisms by which Trp and its metabolites exert the observed stress-mitigating effects are still not totally clear, since there are many related processes that are affected by Trp and its metabolites. One line of research seeks to clarify the relationship between dietary Trp intake and primary indicators of a physiological stress response (e.g. cortisol, glucose, lactate). Results suggest that, though Trp seems to directly affect (e.g.) cortisol levels, the type of effect observed depends strongly on contextual factors, such as the prior stress state of fish (Lepage, et al. (2002), Hoseini, et al., 2017). Also, given that 5-HT can either induce or inhibit adrenocorticotropic hormone production in the pituitary, and consequently increase or decrease interrenal cortisol synthesis (Lepage, et al., 2003), it seems plausible that the putative effects of Trp on cortisol levels are, at least to some degree, mediated through this metabolite.

Another important aspect of Trp and its metabolites is their effects on mood and behaviour, which can potentially improve fish welfare by mitigating the negative psychological effects of exposure to stress factors. 5-HT, in specific, is a neurotransmitter commonly associated to animal behaviour, appetite and aggression (Hoglund, et al., 2005). Trp administration itself was also reported in several teleost species to affect and modulate their behaviour (e.g. aggressive-suppressive behaviours) via the serotonergic system (Basic, et al., 2013; Herrero, et al., 2007; Winberg, et al., 2001), and reduce stress-induced anorexia by improving aspects of appetite (Hoglund, et al., 2007).

Besides its effects on physiological stress, there is evidence that dietary Trp (either directly or indirectly) can have also a mitigating effect in regard to cellular stress. For instance, Wen, et al. (2014) reported an intestinal upregulation of superoxide dismutase 1 (SOD1) and glutathione peroxidase (GPx), the major antioxidant enzymes in fish, in rainbow trout fed Trp-supplemented diets, suggesting that Trp could improve the enzymatic antioxidant capacity in fish. Consistent observations were obtained in rats fed a Trp-deficient diet, where GPx activity in the hepatic tissues decreased (Raju, et al., 2007).

Metabolism and immunity are affected when fish are under stress, so the mitigation of stress responses can lead to reduction of the immunosuppressive effects under chronic stress (Tort, 2011). As such, dietary supplementation with Trp, by mitigating the negative effects of stress factors, can indirectly ameliorate fish immune status. In fact, inhibition of post-stress immunosuppression in fish through an increase in dietary Trp has been previously reported (Hoseini, et al., 2017).

Trp metabolism in intestine has a central physiological role through its different pathways, particularly the 5-HT route. This pathway is highly active in the gut (which contains about 95% of total body serotonin content), particularly in the wall, where the myenteric plexus is a major monoamine producer and acts as a major neuromodulator involved in intestinal contraction and secretory mechanisms (Caamano-Tubio, et al., 2007; Le Floc'h, et al., 2011). As the presence of enterochromaffin cells in teleost species is not transversal to all, there is a serious lack of studies on the function of 5-HT-producing enterochromaffin cells. Still, given the homology between the serotonergic systems of fish and mammals, it is likely that 5-HT released by these cells might display the same pro-inflammatory and motility roles they do in higher vertebrates (Bertrand, Bertrand, 2010; Gershon, 2013). Mardones, et al. (2018) observed that diets supplemented in Trp caused increases in the intestinal 5-HT content in *S. salar* tissues, but there were no such differences in the 5-HT intestinal content in *O. kisutch* fed diets with the same Trp inclusion level.

Besides its use in protein synthesis, practically all tryptophan is degraded along the kynurenine pathway, with complete tryptophan degradation mainly occurring in the liver through an oxidation step mediated by tryptophan-2,3-dioxygenase (TDO). Even though the kynurenine pathway is also active in macrophages, the role of TDO is played by the indoleamine-2,3-dioxygenase (IDO) instead (Cortés, et al., 2016). The IDO is also expressed in the gut of many species, but its role in the intestine is not well described. Available knowledge suggests that IDO could be involved in the control of inflammatory response in the gut due to the regulatory effect of IDO on T cells and immune response (Frumento, et al., 2001; Grohmann, et al., 2003).

It is therefore plausible that dietary Trp supplementation can influence intestinal kynurenine and serotonergic pathways in fish. Since gut is considered an important organ orchestrating animal welfare, it is instrumental to study possible mechanisms through which Trp can exert health benefits. The present study was thus designed to evaluate a possible immunomodulatory effect of tryptophan in the gilthead seabream following a short-term feeding trial and an inflammatory insult.

4.2 Material and Methods

4.2.1 Diets Formulation

Extruded feeds were based on plant proteins with a 15 % inclusion of fish meal. Using this formulation as control, two other experimental diets with graded levels of Trp were produced at SPAROS Lda. (Olhão, Portugal).

Briefly, a control (CTRL) diet was formulated to mimic a current commercial diet formulation for this species, while meeting its estimated AA requirements (i.e. five essential AA were added to obtain a balanced diet) (Kaushik, 1998; Peres, Oliva-Teles, 2009). Two other diets were identical to the CTRL diet, but supplemented with tryptophan at 0.5 % and 1 % (w/w feed) levels (TRP1 and TRP2, respectively) (Table 1). Main ingredients were ground (below 250 μm) in a Hosakawa, model #1 micropulverizer hammer mill (Hosokawa Micron Ltd., United Kingdom). These ground ingredients were then mixed according to the target formulation in a Double-helix Mixture TGC, model 500L (TGC Extrusion, France), to attain a basal mixture (no oils were added at this stage). All diets were manufactured by extrusion (pellet size 2.0 mm) by means of a pilot-scale twin-screw extruder CLEXTRAL BC45 (Clextral, France) with a screw diameter of 55.5 mm and temperature ranging 105 – 110 ºC. Upon extrusion, all batches of extruded feeds were dried in a convection oven (OP 750-EF, LTE Scientifics, United Kingdom) for 2 hours at 60 ºC. After this process, pellets were left to cool at room temperature, and subsequently the essential amino acids were mixed with fish oil fraction according to each target formulation and added under vacuum coating conditions in a Pegasus vacuum mixer (PG-10VCLAB, DINNISEN, The Netherland).

Table 1. Ingredients of the experimental diets.

	Experimental diets		
Ingredients (% feed basis)	**CTRL**	**TRP1**	**TRP2**
Fishmeal Super Prime [a]	15.40	15.40	15.40
CPSP 90 [b]	4.90	4.90	4.90
Squid meal 80 [c]	8.70	8.70	8.20
Fish gelatin [d]	5.00	5.00	5.00
Soy protein concentrate [e]	11.00	11.00	11.00
Wheat gluten [f]	10.00	9.50	9.50
Wheat meal [g]	19.60	19.60	19.60
Fish oil [h]	11.40	11.40	11.40
Soy lecithin – Powder [i]	3.80	3.80	3.80
Vit & Min Premix PV01 [j]	2.00	2.00	2.00
Lutavit E50 [k]	0.10	0.10	0.10
Betaine HCl [l]	0.20	0.20	0.20
Antioxidant powder [m]	0.20	0.20	0.20
MAP (Monoammonium phosphate) [n]	3.80	3.80	3.80
L-Lysine [o]	1.90	1.90	1.90
L-Threonine [p]	0.50	0.50	0.50
L-Tryptophan [q]	0.00	0.50	1.00
DL-Methionine [r]	0.50	0.50	0.50
L-Taurine [s]	1.00	1.00	1.00
Proximate analyses			
Dry matter (% feed)	94.60	93.69	94.26
Crude protein (% dry weight)	53.71	53.65	53.59
Crude lipid (% dry weight)	19.32	19.52	19.83
Ash (% dry weight)	9.54	9.18	9.46
Gross Energy (kJ g-1 DM)	21.57	22.79	22.80

[a] *Fish meal Super Prime: 66.3%CP, 11.5% Pesquera Diamante, Peru*
[b] *CPSP: 82%CP, 9% CF, Sopropêche, France*
[c] *Squid meal 80: 79% CP, 5% CF, Sopropêche, France*
[d] *Fish gelatin: 93.3% CP, WEISHARDT International, Slovakia*
[e] *Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands*
[f] *Wheat gluten: 80.4% CP; 5.6% CF, VITAL Roquette, France*
[g] *Wheat meal: 11.7% CP; 1.6% CF, Casa Lanchinha, Portugal*
[h] *Fish oil: SAVINOR UTS, Portugal*
[i] *Soybean lecithin: P700IPM, Lecico GmbH, Germany*
[j] *Vitamin and mineral premix: PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 500mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; excipient wheat middlings;*
[k] *Lutavit E50, 50% Vit. E, ORFFA Vitamin E 50%, The Netherlands*
[l] *Betaine HCL, Beta-Key 95%, ORFFA, The Netherlands*
[m] *Antioxidant: VERDILOX, Kemin Europe NV, Belgium*
[n] *Monoammanum phosphate: Windmill Aquaphos: 26% P, ALIPHOS ROTTERDAM B.V, The Netherlands*
[o] *L-Lysine: L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France*
[p] *L-Threonine: ThreAMINO 98.5%, Evonik Nutrition & Care GmbH, Germany*
[q] *L-Tryptophan: TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany*
[r] *DL-Methionine: DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany*
[s] *L-Taurine: L-Taurine 98%, ORFFA, The Netherlands*

4.2.2 Rearing conditions

The current trial was conducted by trained scientists (following FELASA category C recommendations) and according to the animal experimentation guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE at the experimental facilities of I3S (Instituto de Investigação e Inovação em Saúde, Porto, Portugal). Juvenile gilthead seabream (*Sparus aurata*) were acquired from a certified hatchery (SONRÍONANSA; Cantabria, Spain). Fish with an initial body weight of 6.28 ± 0.28 g were randomly distributed into 12 tanks (200 L; n = 70) for an acclimatization period of 3 weeks being fed the CTRL diet. Fish were held in a recirculation seawater system in which oxygen saturation (7.3 ± 0.01 mg L^{-1}) and photoperiod (10 h dark and 14 h light) were automatically controlled. Temperature was maintained by a water heater/cooler system at 20 ± 0.5 ºC and kept unchanged throughout the experiment. Both nitrite and ammonium levels were daily recorded, and its levels controlled by a water ozoniser system. Water renovations and system cleanings were performed twice a week. After the quarantine period, the experiment was started by feeding each group with the respective feed three times a day *ad libitum*. The control (CTRL) group was fed the CTRL diet whereas the other two groups were fed diets TRP1 and TRP2, as stated in Table 2.

Table 2. Amino acid composition (g AA 100 g^{-1} diet) of the experimental diets.

	Experimental diets		
	CTRL	**TRP1**	**TRP2**
Arginine	8.14	7.37	7.17
Histidine	2.09	1.96	1.81
Lysine	6.83	8.38	8.28
Threonine	5.02	4.48	4.51
Isoleucine	3.59	3.58	3.70
Leucine	5.92	5.84	5.91
Valine	3.89	3.81	3.93
Methionine	4.07	3.69	3.39
Phenylalanine	4.57	3.87	3.72
Cystine	0.51	0.43	0.58
Tyrosine	3.92	3.37	3.23
Aspartic acid + Asparagine	4.18	6.51	6.33
Glutamic acid + Glutamine	12.41	15.99	16.40
Alanine	4.37	4.93	5.03
Glycine	8.45	7.82	8.38
Proline	7.11	6.91	7.47
Serine	4.71	4.34	4.38
Taurine	2.92	2.62	2.61
Tryptophan	0.69	1.52	2.12

4.2.3 Experimental procedures

An initial sampling point was set, where 10 fish fed the CTRL diet at the end of the acclimation period, were scarified and designated as time zero (TØ) to assess their immune status prior to the feeding trial.

The feeding trial lasted 4 weeks in order to assess the effect of short and mid-term tryptophan supplementation. Feed intake was recorded daily and the body weight of fish fed the dietary treatments was measured before the trial and at each sampling point. Growth was monitored by obtaining the initial body weight (IBW) and final body weight (FBW) and used to calculate growth performance parameters.

At 2 and 4 weeks after feeding the experimental diets, and at 4h, 24h and 48 hours post i.p. injection (see section 2.4 below), 36 fish from each group (12 per replicate) were euthanized by anaesthetic overdose with 2-phenoxyethanol (1 ml L-1) and individually weighed. Samples were obtained for immunological and gene expression studies. Blood was collected from the caudal vein using heparinized syringes, centrifuged at 10,000 × *g* for 10 min and plasma pools from two fish were stored. Blood from 4 fish was also used to perform haematological procedures. Anterior gut was collected and stored in RNA later for gene expression. The remaining gut sampled was also collected and snap frozen for posterior homogenization to evaluate gut oxidative stress parameters. All samples were immediately kept at -80 ºC until assessment.

4.2.4 Bacterial growth and inoculum preparation

Vibrio anguillarum strain PC696.1 was kindly provided by Alicia Toranzo (University of Santiago de Compostela). Bacteria were routinely cultured at 22 ºC in thiosulfate-citrate-bile salts-sucrose agar (TCBS; Difco Laboratories) or tryptic soy broth (TSB; Difco Labs) supplemented with NaCl to a final concentration of 2 % (w/v) (TSB-2) and stored at -80 ºC in TSB-2 supplemented with 15 % (v/v) glycerol. To prepare the inoculum for injection into the fish peritoneal cavities, 100 µl of stocked bacteria were cultured overnight at 22 ºC on TCBS. Exponentially growing bacteria were collected from the TCBS and re-suspended in sterile TSB-2. Thereafter, absorbance was read, and concentration adjusted against its growth curve to 6.5 x 10^7 colony forming units (CFU) ml^{-1}, to achieve an intended bacterial concentration to kill 30 % of the fish (LD30)

according to preliminary trials. Bacterial concentration was confirmed by plating the resulting cultures on TCBS plates and counting of the colony forming units (cfu) ml^{-1}. Bacteria was then killed by sonication for 2 × 30 s on ice. Loss of bacterial viability was confirmed by plating resulting cultures on TCBS plates and failing to see any bacterial growth.

4.2.5 Haematological procedures

Blood was collected from the caudal vein using heparinized syringes. The haematological profile consisted of total white (WBC) and red (RBC) blood cells counts and haemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain) as described by Machado, et al. (2015).

Blood smears were firstly fixed with formol-ethanol (10 % of 37 % formaldehyde in absolute ethanol) and afterwards stained with Wright's stain (Haemacolor; Merck). Neutrophils were identified according to their peroxidase activity, which was detected using the method described by Afonso, et al. (1998). The slides were examined under oil immersion (1000 ×) and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes, and neutrophils. Each cell type relative proportion was subsequently calculated.

4.2.6 Plasma cortisol and immune parameters

Plasma cortisol levels were measured by means of a commercial enzyme-linked immunosorbent assay (ELISA) kit (RE52611 for human serum and saliva; IBL, Hamburg, Germany), as previously described by López-Olmeda, et al. (2009). Plasma was first diluted (1:20) in diethyl ether. After centrifugation, the recovered supernatant was isolated and, once evaporated, the same amount of phosphate buffer containing 1 g L^{-1} gelatine (pH 7.6) was added. Afterwards, 20 µl of the samples, standards and controls, in duplicate, were added to the respective wells of the microtiter plate. After adding 200 µl of the enzyme conjugate, the plate was incubated during 1 h at room temperature. At the end of the incubation period, the plate was washed 3 times with a wash buffer and 100 µl of TMB (3,3',5,5'-tetramethylbenzidine hydrochloride) substrate solution was pipetted in each well. Subsequently, a 15 min incubation (18 -25 ºC) was

performed and the reaction stopped by the addition of 100 µl of TMB stop solution. Absorbance was read at 450 nm after 10 min. The concentration of the samples was read directly from the standard curve after Logit-Log adjustment.

Plasma bactericidal activity was measured according to Graham, et al. (1988) adapted by Machado, et al. (2015), with some modifications. *Vibrio anguillarum* was used. Succinctly, 20 µl of plasma were added to duplicate wells of a U-shaped 96-well plate. Hanks' Balanced Salt solution (HBSS), was added to some wells instead of plasma and served as positive control. To each well, 20 µl of *V. anguillarum* (1×10^6 cfu ml^{-1}) were added and the plate was incubated for 3h at 25 ºC. To each well, 25 µl of Iodonitrotetrazolium chloride, INT (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride; 1mg ml^{-1}; Sigma) to allow the formation of formazan. Plates were then centrifuged at 2000 x g for 10 min and the precipitate was dissolved in 200 µl of dimethyl sulfoxide (Sigma). The absorbance of the dissolved formazan was measured at 490 nm in a Synergy HT microplate reader (Biotek). Total bactericidal activity is expressed as the percentage of killed bacteria, calculated from the difference between the samples (surviving bacteria) and the positive control (100 % living bacteria).

Plasma antiprotease activity was determined as described by Ellis (1990) adapted by Machado, et al. (2015). Briefly, 10 µl of plasma were incubated with the same volume of trypsin solution (5 mg ml^{-1} in $NaHCO_3$, 5 mg ml^{-1}, pH 8.3) for 10 min at 22 ºC in polystyrene microtubes. To the incubation mixture, 100 µl of phosphate buffer (NaH_2PO_4, 13.9 mg ml^{-1}, pH 7.0) and 125 µl of azocasein (20 mg ml^{-1} in $NaHCO_3$, 5 mg ml^{-1}, pH 8.3) were added and incubated for 1 h at 22 ºC. Finally, 250 µl of trichloroacetic acid were added to each microtube and incubated for 30 min at 22 ºC. The mixture was centrifuged at 10,000 × *g* for 5 min at room temperature. Afterwards, 100 µl of the supernatant was transferred in duplicates to a 96-well plate that previously contained 100 µl of NaOH (40 mg ml^{-1}) per well. The OD was read at 450 nm. Phosphate buffer was added to some wells instead of plasma and trypsin and served as blank, whereas the reference sample was phosphate buffer instead of plasma. The percentage of inhibition of trypsin activity compared to the reference sample was calculated.

Total plasma nitrite and nitrate content was measured using a Nitrate/Nitrite colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany) by adapting it to a 96-well plate and by following manufacturer's instructions. Since both these compounds are derivatives of endogenously produced NO, they are indicative of NO

amount in plasma. Briefly, 10 µl of plasma were diluted in 90 µl of distilled water in duplicate and then 50 µl of reduced nicotinamide adenine dinucleotide phosphate (NADPH) were added, followed by the addition of 4 µl of nitrate reductase. A blank was produced by adding distilled water instead of plasma. Absorbance at 540 nm was read after 30 min incubation at 25 ºC. Afterwards, 50 µl of sulfanilamide and an equal volume of N-(1-naphthyl)-ethylenediamine dihydrochloride were added to each well. The mixture was allowed to stand at 25 ºC for 15 min and absorbance was read at 540 nm. Total nitrite levels were calculated from a previously prepared sodium nitrite standard curve.

4.2.7 Gut oxidative stress

Tissue samples were homogenized 1:10 (m/v) in 0.1 M phosphate buffer (pH 7.4). Part of the tissue homogenate was used to determine the extent of endogenous LPO by measuring thiobarbituric acid-reactive substances (TBARS) as suggested by Bird, Draper (1984). Total protein concentrations were determined in 1:10 (v/v) diluted gut homogenate samples using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce #23225, Rockford, USA) for microplates. Bovine serum albumin served as a standard.

The remaining tissue homogenate was centrifuged for 20 min at 12,000 rpm (4 ºC) to obtain the post-mitochondrial supernatant fraction (PMS). CAT activity was determined in PMS by measuring consumption of the substrate H2O2 at 240 nm (Clairborne, 1985).

Total glutathione (TG) content was determined with PMS fraction at 412 nm using a recycling reaction of reduced glutathione (GSH) with 5,50-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase (GR) excess (25 ml from stock with 1 U/ml) (Baker, et al., 1990; Tietze, 1969). TG content was calculated using a calibration curve of GSH standards.

SOD catalyses the reduction of cytochrome c that occur in the presence of superoxide radicals and express the amount of enzyme required to inhibit in 50 % the rate of reduction of cytochrome c (Flohé, ötting, 1984) adapted to microplate (Lima, et al., 2007). In the microplate wells, 0.2 ml of the reaction solution [10 ml of 0.7 mM xanthine solution in NaOH 1 mM and 100 ml of 0.03 mM cytochrome c solution in phosphate buffer 50 mM pH 7.8 with 1 mM Na-EDTA] was added to 0.05 ml of gut

PMS with protein concentrations of 0.25 mg ml-1. The optical density was measured at 550 nm in a microplate reader (BioTek Power Wave 340) each 20 s during 3 min at 25 °C.

4.2.8 Gene expression analysis

Total RNA isolation of anterior gut was conducted with NZY Total RNA Isolation kit (NZYTech, Lisbon, Portugal) following manufacturer's specifications. Retro-transcription was performed with 1600 ng of total RNA, using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal). Quantitative PCR assays were performed with an Eppendorf Mastercycle ep realplex, using 1 µl of diluted cDNA (1:5 v/v) mixed with 10 µl of NZYSpeedy qPCR Master Mix and 0.4 µl (10 µM) of each specific primer in a final volume of 20 µl. cDNA amplification was carried out with specific primers for genes that have been selected for their involvement in immune responses and arginine metabolism. Primers were designed with NCBI Primer Blast Tool according to known qPCR restrictions (amplicon size, Tm difference between primers, GC content and self-dimer or cross-dimer formation). Efficiency of primer pairs was analysed in serial, 5-fold dilutions of cDNA by calculating the slope of the regression line of the cycle thresholds (Ct) vs. the relative concentration of cDNA. Accession number, efficiency values, annealing temperature, product length, and primers sequences are presented in Table 3 Melting curve analysis was also performed to verify that no primer dimers were amplified. The standard cycling conditions were 94 ºC initial denaturation for 2 min, followed by 40 cycles of 94 ºC denaturation for 30 s, primer annealing temperature for 30 s and 72 ºC extension for 30 s. All reactions were carried out as technical duplicates. The expression of the target genes was normalized using the expression of gilthead seabream elongation factor 1 α (*ef1α*).

4.2.9 Statistical analysis

All results are expressed as means ± standard deviation (SD). Univariate statistic evaluation of the data was accomplished by two-way ANOVA with sampling time and dietary treatment as main factors. A significance of $p < 0.05$ was applied to all statistical tests. Gross deviations from the ANOVA assumptions of error normality and homoscedasticity were evaluated through residual analysis (using QQ-plots and "residuals vs. fitted" scatter plots). All tests were run with SPSS statistical analysis software (SPSS ver.26.0; Chicago, USA). For gene expression data, a transformation by Log2 was applied to all expression values.

Table 3. Biomarkers analysed in gut by real-time PCR.

Acronym	Gene Bank ID	Eff[1]	AT[2]	Product lenght[3]	Forward primer sequence
il-10	EF625901	134.62	57	65	**F:** AACATCCTGGGCTTCTATCTG
					R: GTGTCCTCCGTCTCATCTG
il-34	JX976629.1	96.70	60	214	**F:** CATCAGGGTTCATCACAACG
					R: GACTCCCTCTGCATCCTTGA
il-1β	AJ277166.2	81.17	60	245	**F:** TCTTCAAATTCCTGCCACCA
					R: CAATGCCACCTTGTGGTGAT
cd8α	AJ878605	115.44	60	287	**F:** CTCGACTGGTCGGAGTTAA
					R: TCCATCAGCGGCTGCTCGT
tnf-α	AJ413189.2	104.87	60	245	**F:** TGAACAGAGGCGACAAACTG
					R: GCCACAAGCGTTATCTCCAT
IgM	AM493677	115.44	59	136	**F:** CAGCCTCGAGAAGTGGAAAC
					R: GAGGTTGACCAGGTTGGTGT
csfr	AM050293	127.58	60	129	**F:** ACGTCTGGTCCTATGGCATC
					R: AGTCTGGTTGGGACATCTGG
ido2	XM_030440944.1	112.51	60	177	**F:** CATCATTGTCCCCGGTAATC
					R: TCGCTTTCTCTCGACACAGA
hsp70	DQ524995.1	99.20	55	124	**F:** ACGGCATCTTTGAGGTGAAG
					R: TGGCTGATGTCCTTCTTGTG
sod(Mn)	JQ308833	99.02	60	134	**F:** CCTGACCTGACCTACGACTATGG
					R: AGTGCCTCCTGATATTTCTCCTCTG
gpx1	DQ524992	89.61	60	129	**F:** GAAGGTGGATGTGAATGGAAAAGATG
					R: CTGACGGGACTCCAAATGATGG
hep	EF625901	89.57	60	382	**F:** GCCATCGTGCTCACCTTTAT
					R: CCTGCTGCCATACCCCATCTT
*ef1α**	AF184170	105.35	58	87	**F:** CTGTCAAGGAAATCCGTCGT
					R: TGACCTGAGCGTTGAAGTTG

1 Efficiency of PCR reactions were calculated from serial dilutions of tissue RT reactions in the validation procedure.
2 Annealing temperature (°C)
3 Amplicon size (nt)

4.3 Results

4.3.1 Growth performance

No differences were observed in final body weight (FBW) and relative growth rate (RGR) among dietary treatments (Table 4).

4.3.2 Blood immune parameters

4.3.2.1 Feeding trial

White blood cells (WBC) count decreased between 2 weeks and 4 weeks sampling points, regardless of dietary treatment, returning to initial counts in fish sampled at 4 weeks, while no differences were observed for red blood cells (RBC). Haemoglobin increased in fish sampled at 2 weeks compared to those sampled at TØ and 4 weeks (Table 5).

4.3.2.2 Inflammatory response

Main differences were observed between sampling points after the inflammatory insult: a general increase in cell numbers (WBC and RBC) and in the haemoglobin concentration measured in blood (Table 6).

4.3.3 Plasma cortisol and humoral immune parameters

4.3.3.1 Feeding trial

No differences were observed either in plasma cortisol or nitric oxide levels. Plasma antiprotease activity decreased in fish sampled at 4 weeks compared to those sampled at 2 weeks, regardless of dietary treatment. Plasma bactericidal activity presented the same pattern, but fish fed TRP1 had decreased plasma bactericidal activity from the first (2W) to the final sampling point (4W) (Table 7).

4.3.3.2 Inflammatory response

No differences were observed among dietary treatments. An increase over sampling time was observed in plasma bactericidal activity and cortisol levels post-injection, regardless of dietary treatment (Table 8).

Table 4. Body weight (BW, g fish^{-1}), feed conversion ratio (FCR) and relative growth rate (RGR) of gilthead seabream at TØ and fed the experimental diets for 2 and 4 weeks.

Parameters		CTRL	CTRL		TRP1		TRP2	
		TØ	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
BW	(g)	6.28 ± 0.28	10.30 ± 0.78	14.08 ± 1.14	10.32 ± 0.81	13.77 ± 0.12	10.59 ± 0.49	13.31 ± 1.00
FCR	(g)	x	0.94 ± 0.23	1.13 ± 0.14	0.92 ± 0.16	1.16 ± 0.10	0.91 ± 0.12	1.32 ± 0.19
RGR		x	3.82 ± 0.69	2.90 ± 0.27	4.18 ± 0.43	2.99 ± 0.12	4.16 ± 0.65	2.74 ± 0.16

Two-way ANOVA

Parameters				Time		Diet		
	Time	Diet	Time x diet	2 W	4 W	CTRL	TRP1	TRP2
BW	<0.001	0.871	0.516	A	B	-	-	-
FCR	0.003	0.645	0.47	A	B	-	-	-
RGR	<0.001	0.683	0.626	B	A	-	-	-

Values are presented as means ± SD (n = 12). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets.

Table 5. Haemoglobin, red blood cells (RBC) and white blood cells (WBC) of gilthead seabream at TØ and fed the experimental diets for 2 and 4 weeks

Parameters		CTRL	CTRL		TRP1		TRP2	
		TØ	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
WBC	($x10^4$ µl)	2.07 ± 0.68	4.96 ± 0.57	2.53 ± 0.42	5.11 ± 0.99	2.28 ± 0.67	4.65 ± 0.80	2.33 ± 0.88
RBC	($x10^6$ µl)	1.59 ± 0.33	1.93 ± 0.20	1.66 ± 0.36	1.90 ± 0.16	1.60 ± 0.47	1.81 ± 0.24	1.84 ± 0.37
Hb	(g dl^{-1})	1.71 ± 0.11	2.25 ± 0.11	1.90 ± 0.12	2.17 ± 0.25	1.92 ± 0.20	2.14 ± 0.19	2.05 ± 0.20
Absolute peripheral blood leucocytes								
Thrombocytes	($x10^4$ µl)	1.41 ± 0.40	3.46 ± 0.38	1.93 ± 0.42	3.64 ± 0.73	1.71 ± 0.35	4.14 ± 1.94	1.98 ± 0.78
Lymphocytes	($x10^4$ µl)	0.42 ± 0.26	0.89 ± 0.33	0.46 ± 0.23	0.71 ± 0.22	0.52 ± 0.23	0.85 ± 0.25	0.28 ± 0.22
Monocytes	($x10^4$ µl)	0.03 ± 0.03	0.04 ± 0.05	0.00 ± 0.01	0.04 ± 0.04	0.01 ± 0.01	0.08 ± 0.07	0.04 ± 0.06
Neutrophils	($x10^4$ µl)	0.21 ± 0.16	0.59 ± 0.48	0.36 ± 0.43	0.90 ± 0.50	0.34 ± 0.32	0.46 ± 0.25	0.27 ± 0.13

Two-way ANOVA

Parameters					Time			Diet		
		Time	Diet	Time x diet	TØ	2 W	4 W	CTRL	TRP1	TRP2
WBC	($x10^4$ µl)	<0.001	0.566	0.547	A	B	A	-	-	-
RBC	($x10^6$ µl)	0.042	0.768	0.229	A	B	AB	-	-	-
Hb	(g dl^{-1})	<0.001	0.712	0.076	A	C	B	-	-	-
Absolute peripheral blood leucocytes										
Thrombocytes	($x10^4$ µl)	<0.001	0.755	0.805	A	C	B	-	-	-
Lymphocytes	($x10^4$ µl)	<0.001	0.576	0.177	A	B	A	-	-	-
Monocytes	($x10^4$ µl)	0.003	0.276	0.545	AB	B	A	-	-	-
Neutrophils	($x10^4$ µl)	0.003	0.437	0.669	A	B	A	-	-	-

Values are presented as means ± SD (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets.

Table 6. Plasma humoral parameters of gilthead seabream fed the experimental diets for 2 and 4 weeks

Parameters		CTRL		TRP1		TRP2	
		2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Bactericidal activity	(%)	19.20 ± 6.21	11.28 ± 6.14	19.73 ± 4.93 [b]	8.73 ± 6.00 [a]	15.30 ± 7.94	14.96 ± 5.26
Antiprotease activity	(%)	86.35 ± 2.75	84.55 ± 3.53	86.97 ± 1.27	83.42 ± 1.79	84.40 ± 3.04	84.10 ± 2.02
Nitric Oxide	(µM)	577.89 ± 73.63	549.84 ± 35.34	508.04 ± 118.36	524.46 ± 68.07	520.67 ± 106.97	524.46 ± 68.07
Cortisol	(ng ml^{-1})	13.81 ± 7.92	17.60 ± 8.63	18.36 ± 8.33	15.75 ± 6.19	13.69 ± 6.44	10.98 ± 7.07

Two-way ANOVA

Parameters					Time		Diet		
		Time	Diet	Time x diet	2 W	4 W	CTRL	TRP1	TRP2
Bactericidal activity	(%)	0.001	0.878	0.050	B	A	-	-	-
Antiprotease activity	(%)	0.02	0.062	0.526	B	A	-	-	-
Nitric Oxide	(µM)	0.927	0.352	0.81	-	-	-	-	-
Cortisol	(ng ml^{-1})	0.837	0.28	0.481	-	-	-	-	-

Values are presented as means ± SD (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences between dietary treatments for the same time. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets. Different symbols indicate difference between times for the same dietary treatment.

Table 7. Haemoglobin, red blood cells (RBC) and white blood cells (WBC) of gilthead seabream during the challenge period

Parameters		Dietary treatments							
		CTRL				**TRP1**			
		4W	4 h	24 h	48 h	4W	4 h	24 h	48 h
WBC	($x10^4$ µl)	2.53 ± 0.42	2.75 ± 1.13	2.80 ± 0.70	3.35 ± 1.14	2.28 ± 0.67	2.62 ± 0.55	3.08 ± 0.69	4.47 ± 0.76
RBC	($x10^6$ µl)	1.66 ± 0.36	1.75 ± 0.36	1.67 ± 0.15	1.82 ± 0.23	1.60 ± 0.47	1.58 ± 0.25	1.75 ± 0.23	2.07 ± 0.36
Hb	(g dl^{-1})	1.90 ± 0.12	1.92 ± 0.09	1.95 ± 0.05	1.98 ± 0.17	1.87 ± 0.15	1.89 ± 0.07	2.14 ± 0.15	2.03 ± 0.11
Absolute peripheral blood leucocytes									
Thrombocytes	($x10^4$ µl)	1.93 ± 0.42	1.46 ± 0.54	1.78 ± 0.53	2.02 ± 0.35	1.71 ± 0.35	1.31 ± 0.29	1.71 ± 0.47	2.81 ± 0.66
Lymphocytes	($x10^4$ µl)	0.46 ± 0.23	0.25 ± 0.10	0.51 ± 0.19	0.34 ± 0.10	0.52 ± 0.23	0.26 ± 0.10	0.63 ± 0.22	0.65 ± 0.32
Monocytes	($x10^4$ µl)	0.00 ± 0.01	0.02 ± 0.02	0.01 ± 0.01	0.01 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.02	0.04 ± 0.03
Neutrophils	($x10^4$ µl)	0.36 ± 0.43	1.02 ± 0.59	0.54 ± 0.21	0.56 ± 0.18	0.34 ± 0.32	1.15 ± 0.49	0.69 ± 0.26	0.88 ± 0.18

Parameters		Dietary treatments			
		TRP2			
		4W	4 h	24 h	48 h
WBC	($x10^4$ µl)	2.33 ± 0.88	2.48 ± 0.87	3.37 ± 1.12	3.46 ± 0.94
RBC	($x10^6$ µl)	1.84 ± 0.37	1.36 ± 0.18	1.72 ± 0.32	1.78 ± 0.33
Hb	(g dl^{-1})	1.99 ± 0.09	1.86 ± 0.11	1.99 ± 0.14	2.00 ± 0.13
Absolute peripheral blood leucocytes					
Thrombocytes	($x10^4$ µl)	1.98 ± 0.78	1.30 ± 0.55	2.15 ± 1.33	2.46 ± 1.21
Lymphocytes	($x10^4$ µl)	0.28 ± 0.22	0.22 ± 0.08	0.46 ± 0.36	0.27 ± 0.18
Monocytes	($x10^4$ µl)	0.04 ± 0.06	0.01 ± 0.01	0.03 ± 0.04	0.01 ± 0.02
Neutrophils	($x10^4$ µl)	0.27 ± 0.13	0.95 ± 0.32	0.46 ± 0.35	1.07 ± 0.47

Two-way ANOVA

Parameters					Time				Diet		
		Time	Diet	Time x diet	4W	4h	24h	48h	CTRL	TRP1	TRP2
WBC	($x10^4$ µl)	<0.001	0.514	0.272	A	A	AB	B	-	-	-
RBC	($x10^6$ µl)	0.41	0.72	0.171	AB	A	AB	B	-	-	-
Hb	(g dl^{-1})	0.002	0.82	0.428	AB	A	C	BC	-	-	-
Absolute peripheral blood leucocytes											
Thrombocytes	($x10^4$ µl)	0.001	0.712	0.548	AB	A	AB	B	-	-	-
Lymphocytes	($x10^4$ µl)	0.004	0.001	0.584	AB	A	B	AB	AB	B	A
Monocytes	($x10^4$ µl)	0.49	0.165	0.287	-	-	-	-	-	-	-
Neutrophils	($x10^4$ µl)	<0.001	0.301	0.978	A	B	B	B	-	-	-

Values are presented as means ± SD (n = 12). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences between dietary treatments for the same time. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets. Different symbols indicate difference between times for the same dietary treatment.

Table 8. Plasma humoral parameters of gilthead seabream during the challenge period

Parameters		Dietary treatments							
		CTRL				TRP1			
		4W	4 h	24 h	48 h	4W	4 h	24 h	48 h
Bactericidal activity	(%)	11.28 ± 6.14	20.49 ± 3.67	4.94 ± 4.54	6.42 ± 7.28	8.73 ± 6.00	16.48 ± 6.17	3.67 ± 0.64	7.69 ± 5.78
Antiprotease activity	(%)	85.62 ± 1.56	83.76 ± 2.73	85.63 ± 1.27	84.85 ± 2.89	83.42 ± 1.79	85.32 ± 1.88	87.71 ± 1.49	84.40 ± 4.93
Cortisol	(ng ml^{-1})	17.60 ± 8.63	28.50 ± 5.48	27.06 ± 2.64	15.74 ± 5.89	15.75 ± 6.19	27.90 ± 10.20	15.71 ± 5.21	12.05 ± 5.10

Parameters		Dietary treatments			
		TRP2			
		4W	4 h	24 h	48 h
Bactericidal activity	(%)	14.96 ± 5.26	17.93 ± 9.47	5.12 ± 1.19	12.76 ± 10.19
Antiprotease activity	(%)	84.10 ± 2.02	84.69 ± 3.45	83.54 ± 4.02	85.79 ± 1.83
Cortisol	(ng ml^{-1})	10.98 ± 7.07	22.05 ± 3.72	19.70 ± 10.00	25.44 ± 9.19

Two-way ANOVA

Parameters					Time				Diet		
		Time	Diet	Time x diet	4W	4h	24h	48h	CTRL	TRP1	TRP2
Bactericidal activity	(%)	<0.001	0.262	0.804	BC	C	A	AB	-	-	-
Antiprotease activity	(%)	0.646	0.685	0.166	-	-	-	-	-	-	-
Cortisol	(ng/ml)	<0.001	0.221	0.038	A	AB	A	B	-	-	-

Values are presented as means ± SD (n = 12). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences between dietary treatments for the same time. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets. Different symbols indicate difference between times for the same dietary treatment.

4.3.4 Gut oxidative stress

4.3.4.1 Feeding trial

No differences where observed in the parameters measured in homogenised gut, either in catalase, SOD or tGSH (Table 9).

4.3.4.2 Inflammatory response

Main changes were observed between sampling points after the inflammatory insult, with no differences among dietary treatments. A general increase in SOD upon the immune stimulation with killed bacteria was observed, throughout the three sampling times, while the opposite pattern was observed for catalase and tGSH. Catalase levels were increased in fish fed either CTRL or TRP2, 4 hours after the inflammatory insult, and gradually decreased after 24 hours, reaching a minimum level 48 hours after i.p. injection with inactivated bacteria, while this effect was not clear in fish fed the TRP1 diet (Table 10).

Table 9. Biochemical biomarkers responses of gilthead seabream fed the experimental diets for 2 and 4 weeks.

Parameters		CTRL		TRP1		TRP2	
		2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Protein	(mg/ml)	6.18 ± 0.55	6.78 ± 0.91	5.58 ± 1.03	6.59 ± 0.99	6.18 ± 0.48	5.97 ± 1.49
Catalase	(U Cat/mg prot.)	53.69 ± 7.75	55.91 ± 12.45	55.87 ± 13.17	56.38 ± 14.01	57.81 ± 15.44	53.86 ± 8.88
SOD	(U SOD/mg prot.)	5.83 ± 2.88	5.50 ± 1.81	4.90 ± 1.92	6.09 ± 3.33	4.71 ± 1.27	6.46 ± 2.56
tGSH	(nmol/mg prot.)	43.86 ± 2.32	40.60 ± 8.59	46.50 ± 14.26	37.96 ± 9.75	47.64 ± 5.76	38.30 ± 9.02

Two-way ANOVA

Parameters					Time		Diet		
		Time	Diet	Time x diet	2 W	4 W	CTRL	TRP1	TRP2
Protein	(mg/ml)	0.17	0.53	0.329	-	-	-	-	-
Catalase	(U Cat/mg prot.)	0.924	0.96	0.835	-	-	-	-	-
SOD	(U SOD/mg prot.)	0.302	0.99	0.573	-	-	-	-	-
tGSH	(nmol/mg prot.)	0.034	0.98	0.691	-	-	-	-	-

Values are presented as means ± SD (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments.

Table 10. Biochemical biomarkers responses of gilthead seabream during the challenge period

Parameters		Dietary treatments							
		CTRL				**TRP1**			
		4W	4 h	24 h	48 h	4W	4 h	24 h	48 h
Protein	(mg/ml)	6.78 ± 0.91	5.19 ± 1.23	6.68 ± 0.86	5.91 ± 0.54	6.59 ± 0.99	5.01 ± 1.62	6.49 ± 0.78	5.94 ± 0.68
Catalase	(U Cat/mg prot.)	55.91 ± 12.45^{ab}	77.57 ± 17.94^{c}	49.41 ± 9.26^{ab}	40.84 ± 12.82^{a}	56.38 ± 14.01	57.47 ± 12.49	49.66 ± 13.80	55.44 ± 10.37
SOD	(U SOD/mg prot.)	5.50 ± 1.81	12.92 ± 2.28	14.07 ± 5.86	12.81 ± 3.84	6.09 ± 3.33	13.39 ± 4.68	14.24 ± 4.06	11.26 ± 2.06
tGSH	(nmol/mg prot.)	40.60 ± 8.59	47.54 ± 9.89	34.79 ± 6.06	29.95 ± 7.75	37.96 ± 9.75	42.56 ± 11.53	29.47 ± 9.88	31.32 ± 10.02

Parameters		Dietary treatments			
		TRP2			
		4W	4 h	24 h	48 h
Protein	(mg/ml)	5.97 ± 1.49	5.30 ± 0.81	4.89 ± 0.76	5.61 ± 1.06
Catalase	(U Cat/mg prot.)	61.61 ± 20.57^{b}	69.52 ± 15.37^{c}	53.40 ± 10.26^{ab}	32.22 ± 10.82^{a}
SOD	(U SOD/mg prot.)	6.46 ± 2.56	11.36 ± 1.87	14.57 ± 0.51	14.07 ± 4.49
tGSH	(nmol/mg prot.)	38.30 ± 9.02	48.52 ± 13.04	43.21 ± 15.56	27.96 ± 14.86

Two-way ANOVA

Parameters					Time				Diet		
		Time	Diet	Time x diet	4W	4h	24h	48h	CTRL	TRP1	TRP2
Protein	(mg/ml)	0.005	0.06	0.309	B	A	AB	AB	-	-	-
Catalase	(U Cat/mg prot.)	<0.001	0.91	0.024	BC	C	AB	AB	-	-	-
SOD	(U SOD/mg prot.)	<0.001	0.93	0.767	A	B	B	B	-	-	-
tGSH	(nmol/mg prot.)	<0.001	0.41	0.606	AB	B	A	A	-	-	-

Values are presented as means ± SD (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences between dietary treatments for the same time. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets. Different symbols indicate difference between times for the same dietary treatment.

4.3.5 Gut gene expression

4.3.5.1 Feeding trial

Out of the twelve health and antioxidant biomarkers analysed in fish anterior gut, only the colony stimulating factor-1 receptor (*csf1r*) was modulated by the dietary treatments: fish fed the TRP1 diet displayed significantly higher expression of this marker compared to those fed the CTRL diet, regardless of sampling time (Fig. 2A).

4.3.5.2 Inflammatory response

When analysing the expression of the same biomarker panel after an inflammatory insult, stronger variations in gene expression were observed, particularly in fish fed the highest tryptophan inclusion level (TRP2) compared to those fed the CTRL diet (Fig. 3 and 4). During the immune stimulation trial, interleukin 10 (*il-10*, Fig.3A), interleukin 34 (*il-34*, Fig.3B), immunoglobulin M (*IgM*, Fig. 3D), cluster of differentiation 8α (*cd8*α, Fig.3F) and glutathione peroxidase 1 (*gpx1*, Fig.4D) were up-regulated in fish fed TRP2 diet, regardless of sampling point.

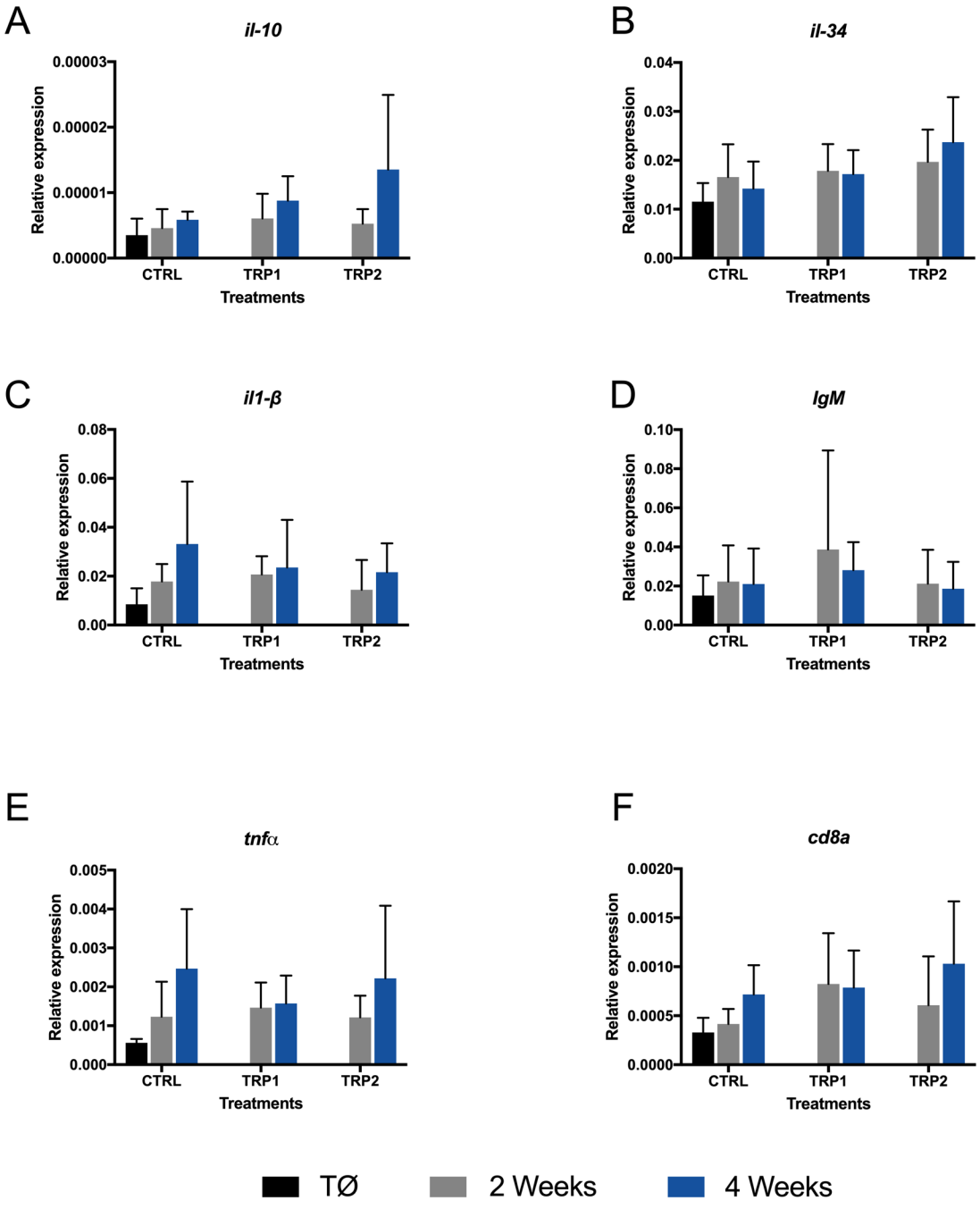

FIGURE 1. Relative expression of interleukin 10 (*il-10*, A), interleukin 34 (*il-34*, B), interleukin 1 β (*il1-β*, C), immunoglobulin M (*IgM*, D), tumor necrosis factor α (*tnfα*, E) and cluster of differentiation 8α (*cd8α*, F) g genes in the head-kidney of gilthead seabream at time Ø and fed the dietary treatments during 2 and 4 weeks .Values are presented as means ± SD (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments.

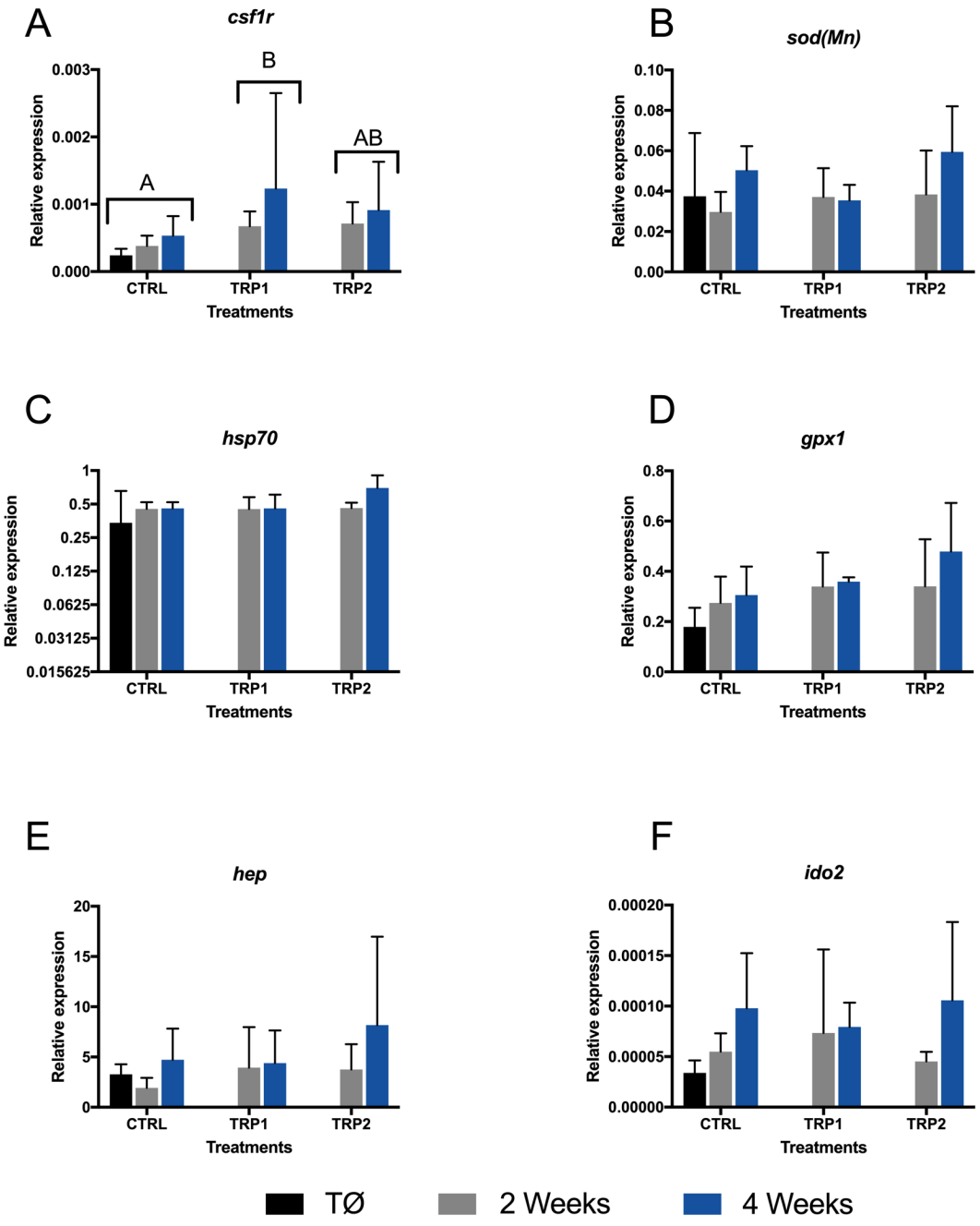

FIGURE 2. Relative expression of colony stimulating factor-1 receptor (*csfr*, A), superoxide dismutase (*sod(Mn),* B), 70 kilodalton heat shock proteins (*hsp70*, C), glutathione peroxidase 1 (*gpx1*, D), hepcidin (*hep*, E) and indoleamine 2,3-dioxygenase (*ido2*, F) genes in the head-kidney of gilthead seabream at time Ø and fed the dietary treatments during 2 and 4 weeks. Values are presented as means ± SD (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different capital letters indicate differences among diets regardless of time. Different capital letters indicate differences among diets regardless of time.

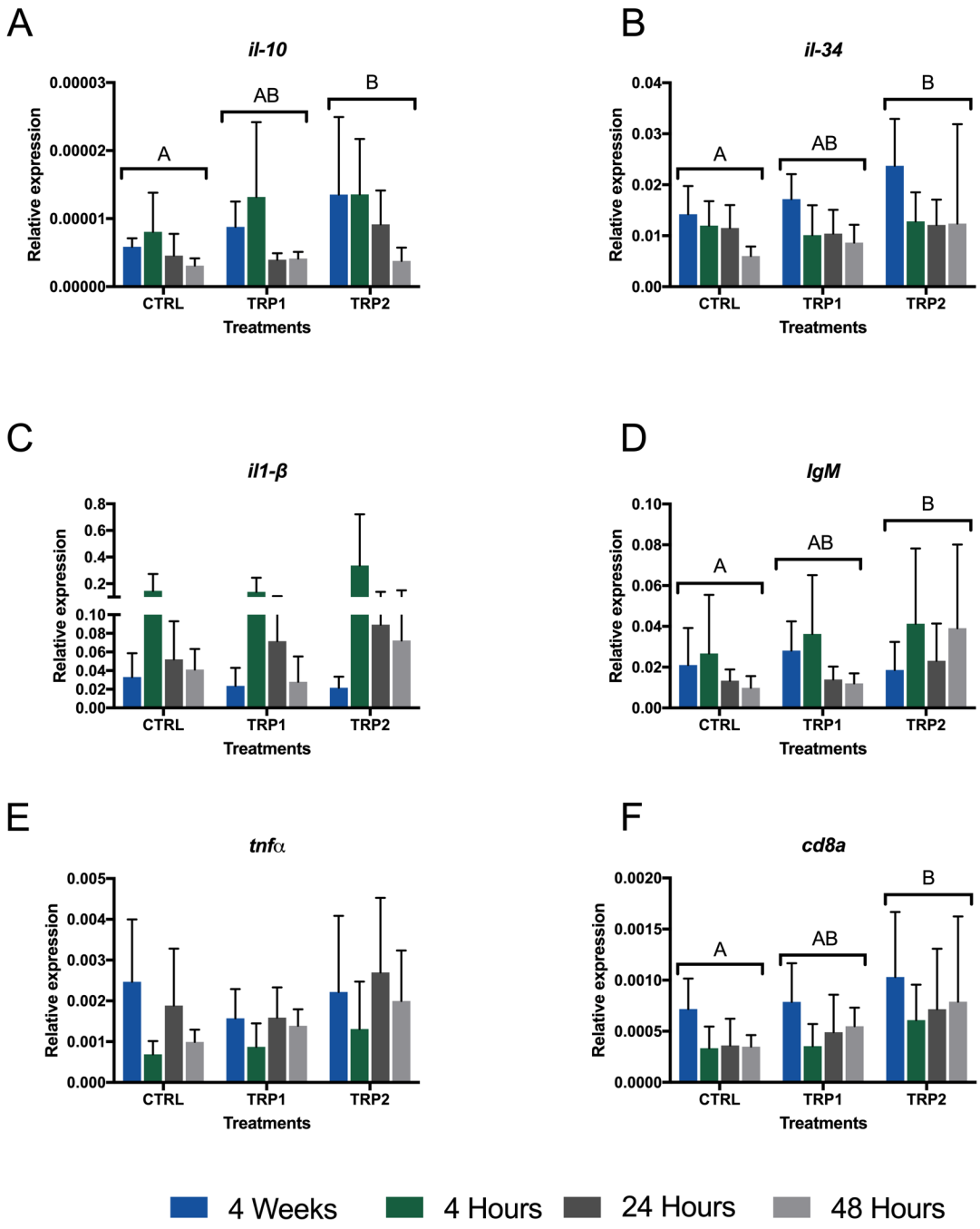

FIGURE 3. Relative expression of interleukin 10 (*il-10*, A), interleukin 34 (*il-34*, B), interleukin 1 β (*il1-β*, C), immunoglobulin M (*IgM*, D), tumor necrosis factor α (*tnfα*, E) and cluster of differentiation 8α (*cd8α*, F) genes in the head-kidney of gilthead seabream at 4 weeks (prior to the inflammatory insult), 4h, 24h and 48 hours post-injection. Values are presented as means ± SD (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different capital letters indicate differences among diets regardless of time.

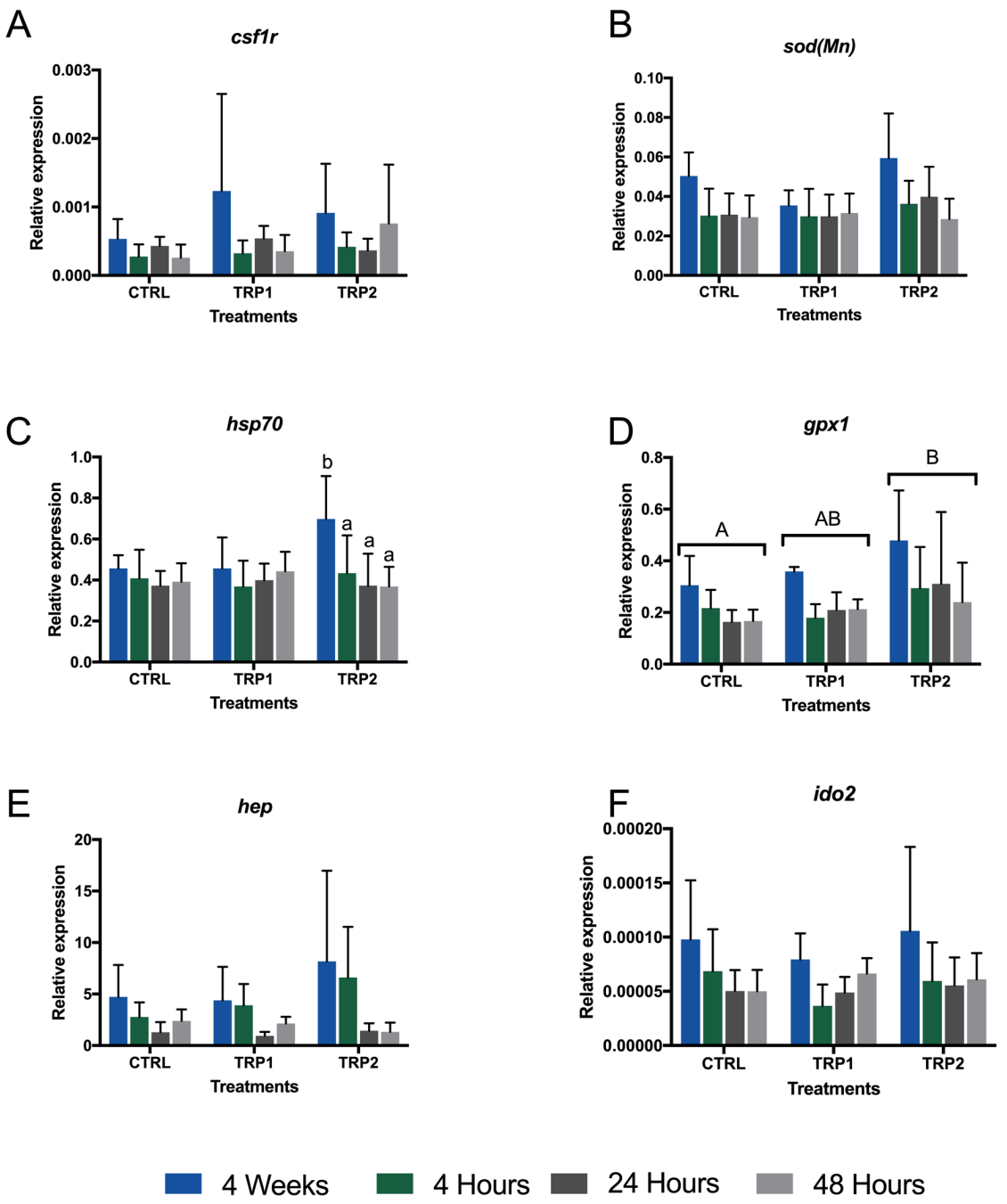

FIGURE 4. Relative expression of colony stimulating factor-1 receptor (*csfr*, A), superoxide dismutase (*sod(Mn),* B), 70 kilodalton heat shock proteins (*hsp70*, C), glutathione peroxidase 1 (*gpx1*, D), hepcidin (*hep*, E) and indoleamine 2,3-dioxygenase (*ido2*, F) genes in the head-kidney of gilthead seabream 4 weeks (prior to the inflammatory insult), 4h, 24h and 48 hours post-injection. Values are presented as means ± SD (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences between dietary treatments for the same time. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets.

4.4 Discussion

In the present study, dietary Trp supplementation did not affect cortisol and humoral innate immune parameters as well as the panel of health and oxidative stress-related biomarkers analysed in the anterior gut throughout the feeding trial. The exception was colony stimulating factor-1 receptor (*csf1r*), which was up-regulated in fish fed TRP1. Even though the function of CSF1-R proteins is related with cellular proliferation and the pathways of differentiation of macrophages in bony fish (Roca, et al., 2006), no differences were observed in peripheral monocyte numbers during the feeding trial. Data from the present study seem to suggest that effects of dietary Trp supplementation on immune status are not evident in the absence of an insult, at least at the tested levels.

Likewise, in a similar experimental design, Machado, et al. (2019) found that the immune status of European seabass (*Dicentrarchus labrax*) fed diets supplemented with Trp for a feeding period of 2 and 4 weeks was not altered. Furthermore, previous works have demonstrated that tryptophan supplementation (i.e. 0.5 % DM) for 2 weeks feeding period was not able to improve the immune status of either European seabass or Persian sturgeon (*Acipenser persicus*) (Hoseini, et al., 2016; Machado, et al., 2015). Some reports have suggested a possible antioxidant effect of tryptophan by binding highly reactive free radicals and ameliorating hepatic oxidative stress in terrestrial animals (Liu, et al., 2015; Mao, et al., 2014; Perez-Gonzalez, et al., 2014). Similar effects were also observed in fish. Akhtar, et al. (2013) and Kumar, et al. (2014), observed that SOD and CAT activities in liver and gills of *L. rohita* under thermal stress were reduced when using tryptophan-enriched diets. Nonetheless, the results of the present study indicated that the gut oxidative status (SOD, CAT and tGSH) remained unchanged throughout the feeding trial.

Tryptophan requirements under stressful conditions were reported to increase with a concomitant decrease in plasma tryptophan levels (Costas, et al., 2008). In this study, plasma cortisol levels were evaluated during the feeding trial (i.e. resting conditions) and following the inflammatory insult, which is known to induce a stress response including an increase in plasma cortisol levels (Tort, 2011). Indeed, plasma cortisol levels increased in gilthead seabream as a response to the i.p. injection, but changes in plasma cortisol levels due to dietary Trp supplementation were not

observed. These observations are in line with previous studies that reported an unclear (or at least strongly context-dependent) effect of dietary Trp on cortisol levels (Hoseini, et al., 2017; Lepage, et al., 2002). Those studies further suggested that Trp effects could be related to other stress-related processes and not directly linked to regulatory effects on cortisol release.

In this study, changes in blood leucocyte numbers were observed once inflammatory mechanisms were triggered by inoculating inactivated *V. anguilarum*. Thus, a clear response of the immune system upon the inflammatory insult was observed regardless of dietary treatment. For instance, a significant increase in the concentration of lymphocytes was observed 24h post-injection and an increase in the numbers of neutrophils was recorded from the 4h sampling point onwards. On the other hand, blood leucocyte numbers were unaffected by dietary Trp levels, which could prevent clear immunomodulatory effects due to gross alterations in immune cell dynamics at the systemic level. However, taking into consideration the gut gene expression results, the immune response seems to be triggered in fish fed the Trp-enriched diets after exposure to an inflammatory insult. This immunomodulatroy effect of dietary Trp was evidenced through a general increase in several of the analysed transcripts (i.e. *il-34*, *IgM* and *cd8a*), particularly in fish fed the highest Trp inclusion level (TRP2). Interleukin-34 is a newly characterized cytokine that participates in several signalling pathways that regulate major cellular functions, including proliferation, differentiation, survival, metabolism, and cytokine/chemokine expression in addition to cellular adhesion and migration (Baghdadi, et al., 2018). Immunoglobulin M plays a vital role in adaptive immune responses, reacting with a particular antigen and binding with receptors on the surface of leucocytes, enhancing phagocytosis (Mashoof, Criscitiello, 2016). The CD8 protein is an important biomarker of the CD8+ T cells (often called cytotoxic T lymphocytes), key for immune defence against pathogens, including both viruses and bacteria (Nakanishi, et al., 2015). As such, it seems that fish fed a diet supplemented with 2.8 times the Trp requirement for gilthead seabream presented an enhanced capacity to deal with pathogens.

In this study, the anti-inflammatory cytokine IL-10 was also upregulated in the gut of fish fed TRP2. IL-10 is a cytokine with potent anti-inflammatory properties that plays a central role in limiting the host's immune response to pathogens, thereby preventing damage to the host and maintaining normal tissue homeostasis (Iyer, Cheng, 2012). Moreover, IL-10 is often increased upon the proliferation and differentiation of M2-type

macrophages, often to counteract a pro-inflammatory state and mostly found in inflammatory-resolution scenarios (Mills, 2015). Therefore, the enhancement of gut *il10* transcripts from this study may be interpreted as a sign that, though dietary Trp supplementation may promote a faster resolution of the inflammatory process.

Fish have evolved effective defence mechanisms (such as glutathione peroxidase) to counteract the negative effects of ROS overproduction, often formed under stressful or inflammatory condition. Serotonin and melatonin can, at least to some degree, possibly account for tryptophan's effects on antioxidant capacity, acting by scavenging directly free radicals, mainly superoxide anion, hydrogen peroxide and hydroxyl radicals which in high amounts significantly inflicts cell damage (Cabeza, et al., 2001; Gülçin, 2008). On the other hand, it is also possible that Trp directly affects the antioxidant capacity through mechanisms independent of serotonin or melatonin. In the current study, the transcript of an antioxidant enzyme, glutathione peroxidase 1 – *gpx1*, was significantly upregulated in fish fed TRP2 compared to those fed CTRL. A similar effect has been previously reported in rainbow trout (Wen, et al. (2014)), supporting the hypothesis that Trp can (directly or indirectly) affect the antioxidant status of fish by increasing the expression of proteins with antioxidant functions. Nonetheless, no differences were observed in the oxidative parameters analysed in the homogenised gut (CAT, SOD and tGSH activity). This could be a reflection of the fact that the fish were not challenged with an active pathogen, thus leading to a relatively mild inflammatory process with low levels of associated oxidative stress.

Tryptophan is first oxidized by one of two enzymes in the kynurenine pathway (TDO or IDO), following then different routes. IDO's affinity to tryptophan is higher than TDO's, so this pathway is naturally favoured. Also, IDO is induced by inflammatory stimulus like LPS or IFN-γ, with its expression being particularly strong in fish mucosal tissues (skin, gills and gut), representing the relevance of the IDO-mediated tryptophan metabolism in immune tolerance, particular in tissues in close contact with microorganisms (Cortés, et al., 2016). However, in the present study, the inflammatory insult was not able to modulate IDO2 expression in the gut. Furthermore, no clear effects on IDO2 expression were observed as a consequence of increased dietary Trp. This suggests that, if IDO activity is increased as a consequence of an inflammatory insult and/or higher tryptophan availability, this putative increase in activity is not likely to result from an increased expression of IDO2. Overall, the main results of this study indicate that dietary Trp supplementation seems to particularly affect fish immune

status during an inflammatory insult, which is in accordance with previous works by Azeredo, et al. (2019).

In summary, the present study suggests that tryptophan inclusion has an immunomodulatory role of gilthead seabream immune response upon an immune stimulation, evidenced through a modulation of the gilthead seabream oxidative stress and health-biomarkers in the anterior gut. The present study points to a dose-dependent effect, since the indicators were overall more affected in fish fed the diet with a higher inclusion level.

4.5 Ethics statement

The experiments were approved by the i3S Animal Welfare Committee and carried out in a registered installation (license number 0421/000/000/2018). Experiments were performed by trained scientists in full compliance with national rules and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

4.6 Acknowledgments

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Chapter 5

Short-term supplementation of dietary arginine and citrulline modulates gilthead seabream (*Sparus aurata*) immune status

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Abstract

Several amino acids (AA) are known to regulate key metabolic pathways that are crucial for immune responses. In particular, arginine (ARG) has important roles regarding immune modulation since it is required for macrophage responses and lymphocyte development. Moreover, citrulline (CIT), ARG precursor, was reported as an alternative for improving macrophage function in mammals. This study aimed to explore the effects of dietary ARG and CIT supplementation on the gilthead seabream immune status. Triplicate groups of fish (23.1 ± 0.4 g) were either fed a control diet (CTRL) with a balanced AA profile, or the CTRL diet supplemented with graded levels of ARG or CIT (i.e. 0.5 % and 1 % of feed; ARG1, CIT1, ARG2 and CIT2, respectively). After 2 and 4 weeks of feeding, fish were euthanized and blood was collected for blood smears, plasma for humoral immune parameters and shotgun proteomics, and head-kidney tissue for the measurement of health-related transcripts. A total of 94 proteins were identified in the plasma. Among them, components of the complement system, apolipoproteins, as well as some glycoproteins were found to be highly abundant. After performing a PLS of the expressed proteins, differences between the two sampling points were observed. In this regard, component 1 (61%) was correlated with the effect of sampling time, whereas component 2 (18%) seemed associated to individual variability within diet. Fish fed ARG2 and CIT2 at 4 weeks were more distant than fish fed all dietary treatments at 2-weeks and fish fed the CTRL diet at 4-weeks. Therefore, data suggest that the modulatory effects of AA supplementation at the proteome level were more effective after 4-weeks of feeding and at the higher inclusion level (i.e. 1% of feed). The bactericidal activity increased in fish fed the highest supplementation level of both AAs after 4-weeks. Peripheral monocyte numbers correlated positively with NO, which showed an increasing trend in a dose-dependent manner. The *csfr1* receptor tended to be up-regulated at the final sampling point regardless of diets. Data from this study point to an immunostimulatory effect of dietary ARG or CIT supplementation after 4-weeks of feeding, particularly when supplemented at a 1 % inclusion level.

5.1 Introduction

Feeds can have significant health implication in farmed fish and thus good practices in diet formulation are imperative, since it also represents a significant expenditure to the aquaculture industry. Therefore, in recent years, the industry is willing to explore the so called “functional feeds”, fortified diet formulations that have added benefits besides meeting fish essential nutritional requirements, being both health and growth boosters (Encarnação, 2016; Li, et al., 2009). The application of such diets can provide a healthier and more sustainable alternative to chemotherapeutic and antibiotic treatments. Amongst a wide range of candidate functional ingredients, little attention has been paid to the role of individual amino acids as potential immunomodulators in fish. In this regard, arginine (ARG) is one of the most versatile amino acids by serving as the precursor for the synthesis of protein, nitric oxide (NO), urea, polyamines, proline, glutamate, creatine and agmatine in terrestrial animals (Wu, Morris, 1998). Polyamines are important for lymphocytes proliferation and differentiation, and NO is a strong bactericidal agent synthetized by activated phagocytes.

Considerable evidence from studies in diverse animal models indicates that adequate amounts of arginine are required for lymphocyte development and that a dietary arginine surplus enhances immune function during immunological challenges (Li, et al., 2007). Nevertheless, arginine was also reported to mediate immunosuppressive mechanisms. In mammals, T-cell activation and function is dictated by arginine metabolism in myeloid suppressor cells (Bronte, Zanovello, 2005). Sharma, et al. (2004) observed that L-arginine, by its conversion to NO, was able to modulate the immune response in rats and mice under restraint stress (RS), antagonizing the immunosuppressive effect of RS on humoral as well as cell-mediated immune responses. In a similar way to higher vertebrates, fish produce NO and ornithine from arginine *via* the inducible NO synthase (iNOS) and arginase, respectively (Buentello, Gatlin, 1999; Zhou, et al., 2015). Indeed, upon inflammatory circumstances, fish phagocytes produce NO, acting as an oxidant against pathogens compromising its structures and function (Andersen, et al., 2015; Bronte, Zanovello, 2005). In fish, different outcomes have been observed. A positive effect of feeds supplemented with arginine on disease resistance has been reported in several teleosts (Costas, et al., 2011; Li, et al., 2009), whereas an inhibitory effect was

observed in Jian carp (*Cyprinus carpio var. Jian*) where both *in vivo* and *in vitro* arginine supplementation counteracted LPS-induced inflammatory responses (Jiang, et al., 2015). A similar detrimental effect was also observed in European seabass (*Dicentrarchus labrax*) fed arginine-enriched diets (Azeredo, et al., 2015). New insights of a recent study revealed that arginine supplementation could compromise to some extent the seabass cell-mediated immune response, decreasing the circulating numbers of neutrophils and monocytes (Azeredo R., et al., 2020). Therefore, despite current knowledge about arginine metabolism, opposing effects from different studies point to a species-specific role of arginine in the fish immune status, a topic that deserves further attention.

Arginine and its metabolites (L-ornithine and L-proline) have also been suggested to play a relevant role in the stress response, promoting stress mitigation in different animals. In fact, dietary arginine supplementation decreased the serum level of cortisol (around -33% than the CTRL fed group) in growing-finishing pigs and weaned piglets (Ma, et al., 2010; Yao, et al., 2011). Experiments with Senegalese sole (*Solea senegalensis*) under chronic stressful conditions (e.g. high densities and handling), reported that stress can affect amino acids requirements (Aragão, et al., 2008) Arginine and histidine concentration were significantly lower in fish under crowding stress (Costas, et al., 2008). This confirms that during stressful conditions, essential amino acids involved in metabolic pathways have an important role. In this regard, ARG-rich diets proved to decrease plasma cortisol levels and enhance several aspects of the innate immune response (i.e. circulating monocytes, NO production and humoral parameters) in Senegalese sole under chronic stress, increased disease resistance upon a possible bacterial infection (Costas, et al., 2011; Costas, et al., 2013).

Citrulline (CIT) is the precursor of arginine, a two-steps reaction mediated by argininosuccinate synthase and argininosuccinate lyase. It is also a by-product of arginine upon conversion to NO (Breuillard, et al., 2015), a mechanism also described in fish (Pederzoli, et al., 2007). In mammals, it has been reported that citrulline supplementation might offer a safe alternative to arginine for improving macrophage function under certain metabolic conditions. Moreover, Batista, et al. (2012) found that a citrulline-enriched diet improved gut function by decreasing intestinal permeability and decreasing bacterial translocation improving protection against bacterial, and immune function in mice; and it stimulated intestinal production of secretory

immunoglobulin A, which is the first line of host defenses against environmental pathogens.

Indeed, few studies have approached the effect of citrulline surplus on immune responses in higher vertebrates, and to the best of our knowledge there is scarce information about this in fish. Nonetheless, Buentello, Gatlin (1999) revealed that *in vitro* NO production was improved in peritoneal macrophages of channel catfish, *Ictalurus punctatus*, upon addition of citrulline to the culture media. A pathogen challenge performed by the same authors revealed that dietary arginine surplus (2 % inclusion of the diet) enhanced the ability of channel catfish to survive after exposure to *Edwardsiella ictaluri* (Buentello, Gatlin, 2001).

Presently, the potential use of these amino acids as dietary supplements in fish health management is not fully developed. Citrulline could present itself as an additive to improve macrophage-mediated immune responses, but few studies have been performed in fish so far. Therefore, the potential immunomodulatory role of arginine and citrulline in fish deserves further attention, and efforts should be driven to ascertain local and systemic immune responses and disease resistance for each particular species of interest. The present study aimed to explore the response of gilthead seabream (*Sparus aurata*) juveniles to arginine- and citrulline-enriched diets in terms of their health status, in the context of a practical feed formulation.

5.2 Material and Methods

5.2.1 Rearing conditions

The feeding trial was carried out at the experimental facilities of the IU-ECOAQUA of the Universidad de Las Palmas de Gran Canaria (Las Palmas, Canary Islands, Spain). Gilthead seabream juveniles (*Sparus aurata*), originated from the natural spawning of wild broodstock were reared according to standard larval and juvenile rearing protocols at the experimental facilities of GIA (Grupo de Investigación en Acuicultura) until achieving the desired size (IU-ECOAQUA).

Fish with an initial body weight of 23.08 ± 0.33 g (mean ± SD) were randomly distributed in 500 L tanks and fed a commercial diet for 3 weeks to ensure acclimation to the experimental conditions. Triplicate groups of 80 gilthead seabream per treatment were hand-fed ad libitum three times a day (except Sundays, when fish were fed once

a day) each experimental diet for 1 month. The trial was carried out in a RAS system with aerated seawater (temperature: 22 ± 0.5 °C, salinity of 37 ± 1 ‰; pH of 8.2 ± 0.2; photoperiod: 12L/12D). Water flow was 15-20 L/min, oxygen content in water effluents was always higher than 90 % saturation and unionized ammonia was regularly recorded and remained below toxic levels (<0.05 mg/L). All physical and chemical water parameters were evaluated daily during the experiment.

The animal experiments described complied with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals.

5.2.2 Diets formulation

Extruded feeds were based on plant proteins with 5 % of fish meal inclusion and a partial replacement (30-35 %) of fish oil. This diet formulation mimics most of the currently used commercial diets for gilthead seabream. Based on this formulation, 5 experimental diets with varying concentrations of arginine and citrulline were produced at SPAROS Lda. (Olhão, Portugal).

A control (CTRL) diet was formulated similar to commercial diet for this species. The four other diets were identical to the CTRL diet but supplemented with graded levels of arginine and citrulline at 0.5 % (ARG1 and CIT1) and 1 % (ARG2 and CIT2) of feed (Table 1). Main ingredients were ground (below 250 µm) in a Hosakawa, model #1 micropulverizer hammer mill (Hosokawa Micron Ltd., United Kingdom). These ground ingredients were then mixed according to the target formulation in a Double-helix Mixture TGC, model 500 L (TGC Extrusion, France), to attain a basal mixture (no oils were added at this stage). All diets were manufactured by extrusion (pellet size 2.0 mm) by means of a pilot-scale twin-screw extruder CLEXTRAL BC45 (Clextral, France) with a screw diameter of 55.5 mm and temperature ranging 105 – 110 ºC. Upon extrusion, all batches of extruded feeds were dried in a convection oven (OP 750-EF, LTE Scientifics, United Kingdom) for 2 hours at 60 ºC. After this process, pellets were left to cool at room temperature, and subsequently the essential amino acids were mixed with fish oil fraction according to each target formulation and added under vacuum coating conditions in a Pegasus vacuum mixer (PG-10VCLAB, DINNISEN, The Netherland).

Table 1. Ingredients of the experimental diets.

	Experimental diets				
Ingredients (%)	**CTRL**	**ARG1**	**CIT1**	**ARG2**	**CIT2**
Fishmeal Super Prime (Diamante) [a]	5.00	5.00	5.00	5.00	5.00
Haemoglobin powder [b]	2.00	2.00	2.00	2.00	2.00
Poultry meal 65 [c]	5.00	5.00	5.00	5.00	5.00
Wheat gluten [d]	17.00	17.00	17.00	17.00	17.00
Corn gluten [e]	30.00	30.00	30.00	30.00	30.00
Rapeseed meal [f]	5.00	5.00	5.00	5.00	5.00
Corn meal [g]	12.95	12.95	12.95	12.95	12.95
Fish oil [h]	14.50	14.50	14.50	14.50	14.50
Vit & Min Premix PV01 [i]	1.00	1.00	1.00	1.00	1.00
Soy lecithin [j]	1.00	1.00	1.00	1.00	1.00
Binder [k]	1.00	1.00	1.00	1.00	1.00
Antioxidant powder [l]	0.20	0.20	0.20	0.20	0.20
Sodium propionate [m]	0.10	0.10	0.10	0.10	0.10
MCP [n]	3.00	3.00	3.00	3.00	3.00
L-Arginine [o]		0.50		1.00	
L-Citrulline [p]			0.50		1.00
L-Histidine [q]	0.20	0.20	0.20	0.20	0.20
L-Lysine [r]	1.50	1.50	1.50	1.50	1.50
L-Threonine [s]	0.30	0.30	0.30	0.30	0.30
L-Tryptophan [t]	0.15	0.15	0.15	0.15	0.15
DL-Methionine [u]	0.10	0.10	0.10	0.10	0.10
Proximate analyses (% dry weight)					
Dry matter (%)	91.61	91.79	92.54	91.94	93.15
Crude protein	46.91	47.19	47.61	47.33	48.08
Crude lipid	16.72	17.01	16.94	17.01	16.96
Ash	5.81	5.85	5.75	5.77	5.78
Gross Energy (kJ g^{-1} DM)	21.57	21.52	21.79	21.66	21.89

[a] *Fish meal Super Prime: 66.3%CP, 11.5% Pesquera Diamante, Peru*
[b] *Porcine haemoglobin powder: 91%CP, 1% CF, SONAC BV, The Netherlands*
[c] *Poultry meal: 65%CP, 14.4% CF, SAVINOR UTS, Portugal*
[d] *Wheat gluten: 80.4% CP; 5.6% CF, VITAL Roquette, France*
[e] *Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal.*
[f] *Rapeseed meal: Defatted rapeseed meal: 37.7% CP, 2.3% CF, Premix Lda, Portugal*
[g] *Corn meal: 10% CP, 4% CF, Ribeiro e Sousa Lda., Portugal.*
[h] *Fish oil: SAVINOR UTS, Portugal*
[i] *Vitamin and mineral premix: PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 500mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; excipient wheat middlings;*
[j] *Soybean lecithin: P700IPM, Lecico GmbH, Germany*
[k] *Binder: Kieselguhr (natural zeolite), LIGRANA GmbH, Germany*
[l] *Antioxidant: VERDILOX, Kemin Europe NV, Belgium*
[m] *Sodium propionate: Disproquímica, Portugal*
[n] *Monocalcium phosphate: ALIPHOS MONOCAL, Belgium*
[o] *L-Arginine: L-Arginine 95%, Premix Lda., Portugal*
[p] *L-Citrulline: L-Citrulline fermentative, Denk, Germany*
[q] *L-Hisdidine: L-Histidine 98%, Ajinomoto Eurolysine SAS, France*
[r] *L-Lysine: L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France*
[s] *L-Threonine: ThreAMINO 98.5%, Evonik Nutrition & Care GmbH, Germany*
[t] *L-Tryptophan: TrypAMINO 98%, Evonik Nutrition & Care GmbH, Germany*
[u] *DL-Methionine: DL-METHIONINE FOR AQUACULTURE 99%, EVONIK Nutrition & Care GmbH, Germany*

Table 2. Amino acid composition (g amino acid 100 g-1 diet) of the experimental diets.

	Experimental Diets				
	CTRL	**ARG1**	**ARG2**	**CIT1**	**CIT2**
Arginine	3.52	3.96	5.12	3.42	3.52
Citrulline	0.0174	*	*	0.39	0.81
Histidine	1.08	1.06	1.13	1.02	1.04
Isoleucine	1.68	1.69	1.77	1.70	1.76
Leucine	4.99	5.21	5.06	5.07	5.17
Lysine	2.32	2.31	2.35	2.16	2.20
Threonine	1.65	1.61	1.65	1.67	1.67
Valine	1.99	2.06	2.08	2.05	2.09
Methionine	1.05	1.05	1.04	1.06	1.09
Cystine	0.34	0.34	0.36	0.31	0.33
Methionine + Cystine	1.39	1.39	1.41	1.37	1.42
Phenylalanine	2.22	2.16	2.35	2.27	2.31
Tyrosine	1.77	1.86	1.97	1.79	1.82
Phenylalanine + Tyrosine	3.99	4.02	4.32	4.05	4.14
Taurine	0.04	0.05	0.04	0.03	0.03
Aspartic acid + Asparagine	2.36	2.37	2.39	2.32	2.42
Glutamic acid + Glutamine	10.15	10.01	10.06	9.95	10.12
Alanine	2.36	2.48	2.54	2.46	2.55
Glycine	1.71	1.67	1.76	1.62	1.66
Proline	3.79	3.78	3.84	3.62	3.70
Serine	2.15	2.10	2.11	2.00	2.04

* not measured, assumed to be the same as the CTRL
# Tryptophan was not measured

5.2.3 Experimental procedures

Samplings were performed at the end of two and four weeks of feeding in order to assess the effect of short and mid-term dietary supplementation of these amino acids. Feed intake was recorded daily and body weight was measured before the trial and at each sampling point. Growth was monitored by taking the initial body weight (IBW) and final body weight (FBW) in each sampling point.

At the end of each feeding period, four fish from each dietary replicate (12 fish per dietary group) were sacrificed by anaesthetic overdose with clove oil and individually weighed. Blood was collected from the caudal vein using heparinized syringes, centrifuged at 10000 × *g* for 10 min at 4 ºC and plasma pools were stored at −80 ºC. Blood was also used to assess blood smears. Head-kidney were also obtained, snap frozen in liquid nitrogen, and stored at −80 °C for gene expression.

5.2.4 Haematological procedures

Blood smears were firstly fixed with formol-ethanol (10 % of 37 % formaldehyde in absolute ethanol) and afterwards stained with Wright's stain (Haemacolor; Merck). Neutrophils were identified according to their peroxidase activity, which was detected using the method described by Afonso, et al. (1998). The slides were examined under oil immersion (1000 ×) and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes, and neutrophils (Machado, et al., 2015). Each cell type relative proportion was subsequently calculated.

5.2.5 Innate immune parameters

The anti-protease activity was determined as described by Ellis (1990) adapted by Machado, et al. (2015). Briefly, 10 µl of plasma was incubated with the same volume of trypsin solution (5 mg ml^{-1} in $NaHCO_3$, 5 mg ml^{-1}, pH 8.3) for 10 min at 22 ºC in polystyrene microtubes. To the incubation mixture, 100 µl of phosphate buffer (NaH_2PO_4, 13.9 mg ml^{-1}, pH 7.0) and 125 µl of azocasein (20 mg ml^{-1} in $NaHCO_3$, 5 mg ml^{-1}, pH 8.3) were added and incubated for 1 h at 22 ºC. Finally, 250 µl of trichloroacetic acid was added to each microtube and incubated for 30 min at 22 ºC. The mixture was centrifuged at 10000x *g* for 5 min at room temperature. Afterwards, 100 µl of the supernatant was transferred in duplicates to a 96 well-plate that previously contained 100 µl of NaOH (40 mg ml^{-1}) per well. The OD was read at 450 nm. Phosphate buffer was added to some wells instead of plasma and trypsin and served as blank, whereas the reference sample was phosphate buffer instead of plasma. The percentage of inhibition of trypsin activity compared to the reference sample was calculated.

Total plasma nitrite and nitrate content was measured using a Nitrate/Nitrite colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany) by adapting it to a 96-well plate and by following manufacturer's instructions. Since both these compounds are derivatives of endogenously produced NO, they are indicative of NO amount in plasma. Briefly, 10 µl of plasma was diluted in 90 µl of distilled water in duplicate to which was then added 50 µl of reduced nicotinamide adenine dinucleotide

phosphate (NADPH) and 4 µl of nitrate reductase. A blank was determined by adding distilled water instead of plasma. Absorbance at 540 nm was read after 30 min incubation at 25 °C. Afterwards, 50 µl of sulfanilamide and an equal volume of N-(1-naphthyl)-ethylenediamine dihydrochloride were added to each well. The mixture was allowed to stand at 25 °C for 15 min and absorbance was read at 540 nm. Total nitrite levels were calculated from a previously prepared sodium nitrite standard curve.

Plasma bactericidal activity was measured according to Graham, et al. (1988) adapted by Machado, et al. (2015), with some modifications. Succinctly, 20 µl of plasma was added to duplicate wells of a U-shaped 96-well plate. Hanks' Balanced Salt solution (HBSS) was added to some wells instead of plasma and served as positive control. To each well, 20 µl of *Vibrio anguillarum* (1 x 10^6 cfu ml^{-1}) was added and the plate was incubated for 3 h at 25 ºC. To each well, 25 µl of iodonitrotetrazolium chloride, INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride; 1 mg ml^{-1}; Sigma) were added to allow the formation of formazan. Plates were then centrifuged at 2000x *g* for 10 min and the precipitate was dissolved in 200 µl of dimethyl sulfoxide (Sigma). The absorbance of the dissolved formazan was measured at 490 nm in a Synergy HT microplate reader (Biotek). Total bactericidal activity is expressed as the percentage of killed bacteria, calculated from the difference between the samples (surviving bacteria) and the positive control (100 % living bacteria).

Plasma Immunoglobulin M (IgM) was measured by an ELISA assay. Briefly, 4 µl of plasma was previously diluted (1:100) in 396 µl of Na_2CO_3 buffer (50 mM, pH=9.6) and 100 µl of the diluted plasma was added to the 96 wells plate in duplicates. 100 µl of Na_2CO_3 buffer was used as a negative control. Samples (antigen) were allowed to adhere to the plate at 22 °C for 1 hour, and afterwards the samples were removed by means of an aspirator. 300 µl of blocking buffer (5 % low fat milk powder in T-TBS (0.1 % Tween 20)) was added to each well and left to stand for 1 hour incubation period at 22 ºC. Blocking buffer was then removed by aspiration and wells were washed thrice with 300 µl of T-TBS (0.1 % Tween 20). After properly cleaned and dried, 100 µl of the anti-gilthead seabream primary IgM monoclonal antibody previously diluted in blocking buffer (1:100) was added to each well followed by 1 hour incubation at 22 ºC. After removing the primary antibody by aspiration and having washed the wells thrice, 100 µl of the anti-mouse IgG-HRP secondary antibody diluted in blocking buffer (1:1000) was added to the wells and plate was incubated for 1 hour at 22 ºC. 100 µl of previously prepared TMB substrate solution for ELISA was added to each well after the plate had

been aspirated and washed, and plates were incubated for 5 min. The colour change reaction was stopped after 5 min by adding 100 µl of 2 M sulphuric acid and the optical density was read at 450 nm.

5.2.6 Plasma proteomics

Protein identification and quantitation was performed by nanoLC-MS/MS. This equipment is composed by an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Samples were loaded onto a trapping cartridge (Acclaim PepMap C18 100Å, 5 mm x 300 µm i.d., 160454, Thermo Scientific) in a mobile phase of 2 % acetonitrile (ACN), 0.1 % formic acid (FA) at 10 µl min^{-1}. After 3 min loading, the trap column was switched in-line to a 50 cm by 75 µm inner diameter EASY-Spray column (ES803, PepMap RSLC, C18, 2 µm, Thermo Scientific, Bremen, Germany) at 300 nl min^{-1}. Separation was generated by mixing A: 0.1 % FA, and B: 80 % ACN, with the following gradient: 5 min (2.5 % B to 10 % B), 120 min (10 % B to 30 % B), 20 min (30 % B to 50 % B), 5 min (50 % B to 99 % B) and 10 min (hold 99 % B). Subsequently, the column was equilibrated with 2.5 % B for 17 min. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.9 software (Thermo Scientific, Bremen, Germany).

The mass spectrometer was operated in data-dependent (dd) positive acquisition mode alternating between a full scan (m/z 380-1580) and subsequent HCD MS/MS of the 10 most intense peaks from full scan (normalized collision energy of 27 %). ESI spray voltage was 1.9 kV. Global settings: use lock masses best (m/z 445.12003), lock mass injection Full MS, chrom. peak width (FWHM) 15 s. Full scan settings: 70k resolution (m/z 200), AGC target 3e6, maximum injection time 120 ms. dd settings: minimum AGC target 8e3, intensity threshold 7.3e4, charge exclusion: unassigned, 1, 8, >8, peptide match preferred, exclude isotopes on, dynamic exclusion 45s. MS2 settings: microscans 1, resolution 35k (m/z 200), AGC target 2e5, maximum injection time 110 ms, isolation window 2.0 m/z, isolation offset 0.0 m/z, spectrum data type profile.

The raw data was processed using Proteome Discoverer software (Thermo Scientific) and searched against a database for *Sparus aurata* provided by Pauletto, et al. (2018).

The Sequest HT search engine was used to identify tryptic peptides. The ion mass tolerance was 10 ppm for precursor ions and 0.02 Da for fragmentations. Maximum allowed missing cleavage sites was 2. Cysteine carbamidomethylation was defined as constant modification. Methionine oxidation and protein N-terminus acetylation were defined as variable modifications. Peptide confidence was set to high. The processing node Percolator was enabled with the following settings: maximum delta Cn 0.05; decoy database search target FDR 1 %, validation based on q-value. Protein label free quantitation was performed with the Minora feature detector node at the processing step. Precursor ions quantification was performing at the processing step with the following parameters: Peptides to use unique plus razor, precursor abundance was based on intensity, normalization mode was based on total peptide amount, pairwise protein ratio calculation, hypothesis test was based on t-test (background based).

The software PANNZER2 (Protein ANNotation with Z-scorRE) was used to fully automatically annotate the unknown protein functions (Toronen, et al., 2018).

5.2.7 Gene expression

Total RNA isolation of head-kidney was conducted with NZY Total RNA Isolation kit (NZYTech, Lisbon, Portugal) following manufacturer's specifications. First-strand cDNA was synthesized from a total RNA per sample (280 ng), which was performed using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal). Quantitative PCR assays were performed with an Eppendorf Mastercycle ep realplex, using 1 µl of diluted cDNA (1:5 dilution) mixed with 10 µl of NZYSpeedy qPCR Master Mix and 0.4 µl (10 µM) of each specific primer in a final volume of 20 µl. cDNA amplification was carried out with specific primers for genes that have been selected for their involvement in immune responses and arginine metabolism. Primers were designed with NCBI Primer Blast Tool according to known qPCR restrictions (amplicon size, Tm difference between primers, GC content and self-dimer or cross-dimer formation). Efficiency of primer pairs was analysed in serial, 5-fold dilutions of cDNA by calculating the slope of the regression line of the cycle thresholds (Ct) vs. the relative concentration of cDNA. Accession number, efficiency values, annealing temperature, product length, and primers sequences are presented in Table S1. Melting curve analysis was also performed to verify that no primer dimers were

amplified. The standard cycling conditions were 94 °C initial denaturation for 2 min, followed by 40 cycles of 94 °C denaturation for 30 s, primer annealing temperature for 30 s and 72 °C extension for 30 s. All reactions were carried out as technical duplicates. The expression of the target genes was normalized using the expression of gilthead seabream elongation factor 1 α (*ef1α*).

5.2.8 Statistical analysis

All results are expressed as means ± standard deviation (SD). Univariate statistic evaluation of the data was accomplished by Two-way ANOVA with sampling time and dietary treatment as main factors. A significance of $p<0.05$ was applied to all statistical tests. Gross deviations from the ANOVA assumptions of error normality and homoscedasticity were evaluated through residual analysis (using QQ-plots and "residuals vs. fitted" scatter plots). All tests were run with SPSS statistical analysis software (SPSS ver.26.0; Chicago, USA).

For gene expression data, a transformation by Log2 was applied to all expression values. Unsupervised principle component analysis (PCA) was first performed on data as an unbiased statistical method to observe intrinsic trends in the dataset, using EZ-INFO® v3.0 (Umetrics, Sweden). To achieve the maximum separation between groups, supervised partial least-squares discriminant analysis (PLS-DA) was subsequentially applied. Potential differential genes were selected according to the Variable Importance in the Projection (VIP) values. Variables with VIP > 1 were considered to be influential for the separation of samples in PLS-DA analysis (Wold, et al., 2001; Li, et al., 2012; Kieffer, et al., 2016). For proteomics data, a transformation by Log2 was applied to all abundance values. Effects were evaluated through linear modelling, using "time" and "supplementation" coded as binary factors (and their interaction) as factors. Effect significance was evaluated using an F-test. Multiple comparison correction, to control the FDR, was performed using the q-value method, as implemented in Bioconductor's "qvalue" package. Additional multivariate analyses were performed by means of principal component analysis (PCA) and partial least squares regression (PLSR) using the R statistical software (v3.3.2) with the “factoextra” and “pls” packages.

5.3 Results

5.3.1 Growth performance

No differences were observed in final body weight (FBW), daily growth index (DGI), relative growth rate (RGR), voluntary feed intake (VF), feed efficiency (FE), or feed conversion ratio (FCR) among the dietary treatments (Table 4).

5.3.2 Peripheral blood leucocytes

With the exception of monocytes, the relative proportion of peripheral blood leucocytes (thrombocytes, lymphocytes and neutrophils) of gilthead seabream fed dietary treatments during 2 and 4 weeks were similar among dietary treatments and sampling times (Table 5). The proportion of monocytes was observed to be higher in fish fed diets with the highest supplementation level (i.e. ARG2 and CIT2) compared to their respective counterparts fed the lower supplementation level (Fig. 1). Overall, fish fed CIT2 had the highest relative proportion of circulating monocytes regardless of sampling point.

5.3.3 Plasma immune parameters

Bactericidal activity increased in fish fed ARG2 compared to fish fed the CTRL diet after 4 weeks of feeding (Table 6). Plasma NO levels were enhanced in fish fed the highest supplementation levels regardless of sampling time (Table 6). A decrease in antiprotease activity was observed in dish fed ARG2 from the first to final sampling point. No differences were observed in IgM levels (Table 6). Moreover, monocytes from fish fed ARG2 and CIT2 were also found to be positively correlated with NO levels measured in plasma and showed and increasing trend in a dose-dependent manner (R^2=0.339, $p < 0.001$) (Fig.1).

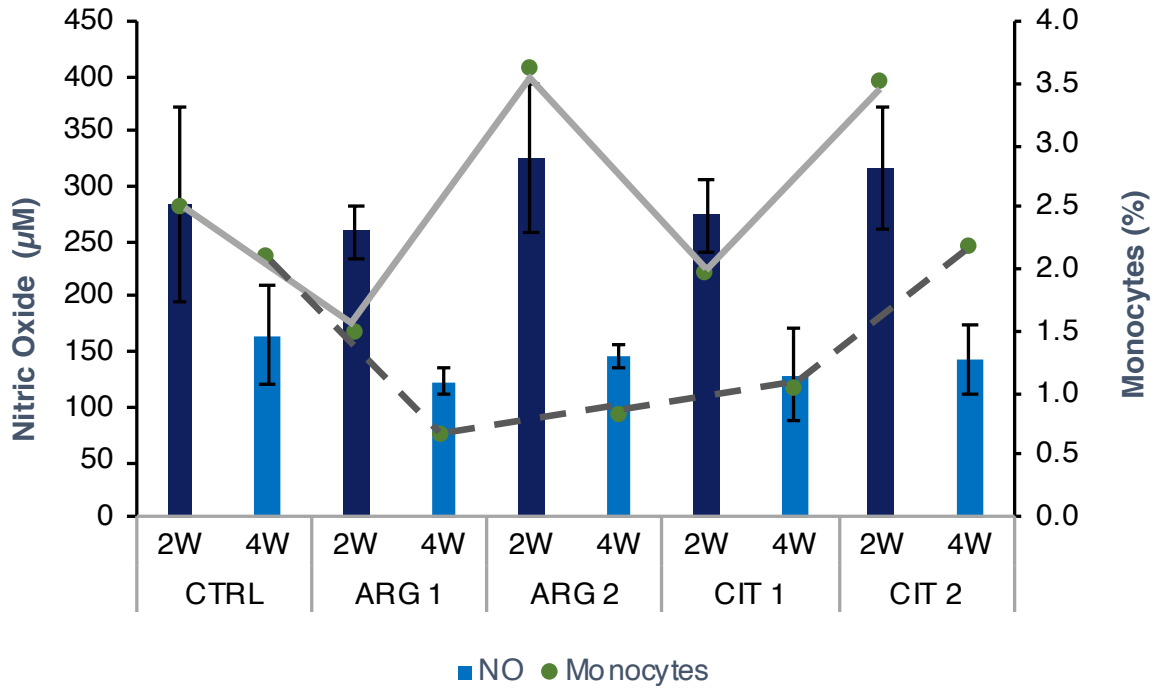

FIGURE 1. Nitric oxide levels and monocytes relative values (trend lines) of gilthead seabream fed dietary treatments during the feeding trial are positively correlated ($R^2=0.339$, $p < 0.001$). Values are presented as means ± SD (n = 9)

Table 4. Body weight (BW, g fish^{-1}), Relative growth rate (RGR), daily growth index (DGI), feed conversion ratio (FCR), feed efficiency (FE) and voluntary feed intake (VFI) of gilthead seabream fed the experimental diets for 2 and 4 weeks.

		CTRL		ARG1		ARG2	
		2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
BW	(g)	26.58 ± 1.65	35.34 ± 1.41	26.92 ± 0.29	36.32 ± 0.48	27.70 ± 0.69	36.31 ± 1.93
RGR	(% day^{-1})	0.97 ± 0.33	1.46 ± 0.17	1.16 ± 0.06	1.60 ± 0.04	1.32 ± 0.28	1.58 ± 0.17
DGI	(% day^{-1})	0.94 ± 0.34	1.48 ± 0.16	1.11 ± 0.06	1.62 ± 0.04	1.29 ± 0.27	1.60 ± 0.19
FCR		2.63 ± 0.91	1.79 ± 0.20	2.01 ± 0.11	1.73 ± 0.05	1.78 ± 0.37	1.71 ± 0.12
FE		0.41 ± 0.15	0.56 ± 0.07	0.50 ± 0.03	0.58 ± 0.02	0.58 ± 0.13	0.58 ± 0.04
VFI	(% BW)	2.33 ± 0.06	2.93 ± 0.06	2.30 ± 0.01	2.97 ± 0.02	2.27 ± 0.05	2.96 ± 0.06

		CIT1		CIT2	
		2 weeks	4 weeks	2 weeks	4 weeks
BW	(g)	26.55 ± 0.73	36.37 ± 2.75	28.23 ± 1.01	34.46 ± 2.15
RGR	(% day^{-1})	0.95 ± 0.22	1.55 ± 0.29	1.49 ± 0.24	1.41 ± 0.16
DGI	(% day^{-1})	0.92 ± 0.21	1.58 ± 0.31	1.45 ± 0.24	1.42 ± 0.18
FCR		2.56 ± 0.56	1.84 ± 0.25	1.56 ± 0.31	1.85 ± 0.11
FE		0.41 ± 0.10	0.55 ± 0.08	0.66 ± 0.12	0.54 ± 0.03
VFI	(% BW)	2.33 ± 0.04	2.95 ± 0.10	2.24 ± 0.04	2.91 ± 0.06

Table 5. Relative proportion of peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes and neutrophils) of gilthead seabream fed dietary treatments during 2 and 4 weeks.

Parameters		CTRL		ARG1		ARG2	
		2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Thrombocytes	(%)	57.94 ± 6.97	64.73 ± 9.09	57.83 ± 7.87	61.63 ± 12.95	61.44 ± 8.73	69.63 ± 10.71
Lymphocytes	(%)	30.51 ± 6.96	26.36 ± 7.60	32.89 ± 9.24	31.48 ± 10.42	29.65 ± 6.47	24.38 ± 9.24
Monocytes	(%)	2.50 ± 2.37	2.09 ± 1.69	1.49 ± 0.78	0.65 ± 0.67	3.61 ± 2.50	0.81 ± 1.53
Neutrophils	(%)	9.04 ± 5.07	6.81 ± 3.95	7.78 ± 5.62	6.25 ± 4.11	5.31 ± 3.10	5.19 ± 4.96

Parameters		CIT1		CIT2	
		2 weeks	4 weeks	2 weeks	4 weeks
Thrombocytes	(%)	60.89 ± 8.80	66.24 ± 10.31	55.77 ± 5.67	62.33 ± 13.63
Lymphocytes	(%)	29.80 ± 8.71	26.05 ± 7.92	31.38 ± 8.33	25.47 ± 13.49
Monocytes	(%)	1.95 ± 1.48	1.04 ± 0.96	3.50 ± 1.69	2.17 ± 2.28
Neutrophils	(%)	7.36 ± 3.07	6.67 ± 5.75	9.36 ± 4.76	10.03 ± 7.14

Two-way ANOVA

Parameters					Time		Diet				
		Time	Diet	Time x diet	2 Weeks	4 Weeks	CTRL	ARG1	ARG2	CIT1	CIT2
Thrombocytes	(%)	0.002	0.188	0.963	A	B	-	-	-	-	-
Lymphocytes	(%)	0.021	0.414	0.943	B	A	-	-	-	-	-
Monocytes	(%)	<0.001	0.014	0.236	B	A	AB	A	AB	AB	B
Neutrophils	(%)	0.411	0.072	0.883	-	-	-	-	-	-	-

Values are presented as means ± SD (n = 9). P-values from Two-way ANOVA ($p \leq 0.05$). Tukey *post-hoc* test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences between dietary treatments for the same time. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets. Different symbols indicate difference between times for the same dietary treatment.

Table 6. Plasma humoral parameters of gilthead seabream fed dietary treatments during 2 and 4 weeks

Parameters	CTRL		ARG1		ARG2	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Antiprotease activity (%)	93.41 ± 1.51	93.65 ± 1.46	94.45 ± 1.04	93.58 ± 2.33	95.22 ± 0.68*	92.16 ± 2.02$^{\#}$
Bactericidal activity (%)	62.41 ± 8.03	63.31 ± 6.57^{ab}	59.18 ± 9.22	61.58 ± 6.19^{a}	53.70 ± 9.17	71.29 ± 4.84^{c}
IgM (abs)	0.80 ± 0.27	0.91 ± 0.41	0.74 ± 0.30	0.76 ± 0.38	0.69 ± 0.29	0.61 ± 0.24
Nitric Oxide (µM)	283.40 ± 87.24	165.17 ± 45.40	259.19 ± 23.73	122.26 ± 11.72	325.70 ± 68.01	145.64 ± 10.59

Parameters	CIT1		CIT2	
	2 weeks	4 weeks	2 weeks	4 weeks
Antiprotease activity (%)	95.00 ± 1.36	93.58 ± 1.59	94.80 ± 0.94	92.18 ± 2.81
Bactericidal activity (%)	59.46 ± 7.63	69.52 ± 6.40^{bc}	56.64 ± 11.51$^{\#}$	69.92 ± 4.31^{bc*}
IgM (abs)	0.57 ± 0.26	0.73 ± 0.21	0.68 ± 0.25	0.78 ± 0.30
Nitric Oxide (µM)	273.44 ± 31.76	128.59 ± 41.82	317.08 ± 54.86	142.76 ± 32.78

Two-way ANOVA

Parameters		Time	Diet	Time x diet	Time		Diet				
					2 Weeks	4 Weeks	CTRL	ARG1	ARG2	CIT1	CIT2
Antiprotease activity	(%)	<0.001	0.486	0.016	B	A	-	-	-	-	-
Bactericidal activity	(%)	<0.001	0.473	0.002	A	B	-	-	-	-	-
IgM	(abs)	0.261	0.113	0.635	-	-	-	-	-	-	-
Nitric Oxide	(μM)	<0.001	0.016	0.257	B	A	AB	A	B	AB	B

Values are presented as means ± SD (n = 9). *P*-values from two-way ANOVA ($p \leq 0.05$). Tukey *post-hoc* test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences between dietary treatments for the same time. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets. Different symbols indicate difference between times for the same dietary treatment.

5.3.4 Plasma proteomics

A total of 92 proteins were identified in the plasma of all treatments and a detailed list is provided in Table S1. Among them, components of the complement system, apolipoproteins, as well as some glycoproteins were found to be highly abundant (Figure S1). The PLSR analysis of the expressed proteins showed differences between the two sampling points independently of dietary treatment. In this regard, component 1 (61%) explained the effect of sampling time, whereas the interpretation of component 2 (18%) is not as clear. Nonetheless, it can be observed that the plasma proteome profile of fish fed the supplemental diets is seemingly affected after 4 weeks of feeding, compared to fish fed CTRL (Figure 2). Overall, only 19 out of 92 proteins were significantly modulated in fish fed diets with the highest inclusion of arginine and citrulline after 4 weeks, compared to fish fed other treatments for 2 weeks (Table 7 & Figure S2). Proteins of the complement system (highlighted in green) were highly modulated in fish fed the supplemented diets after 4 weeks compared to fish fed other dietary treatments over a shorter period of 2 weeks, which is in accordance with previous immune-related data analysed in this trial (Table 5 & 6).

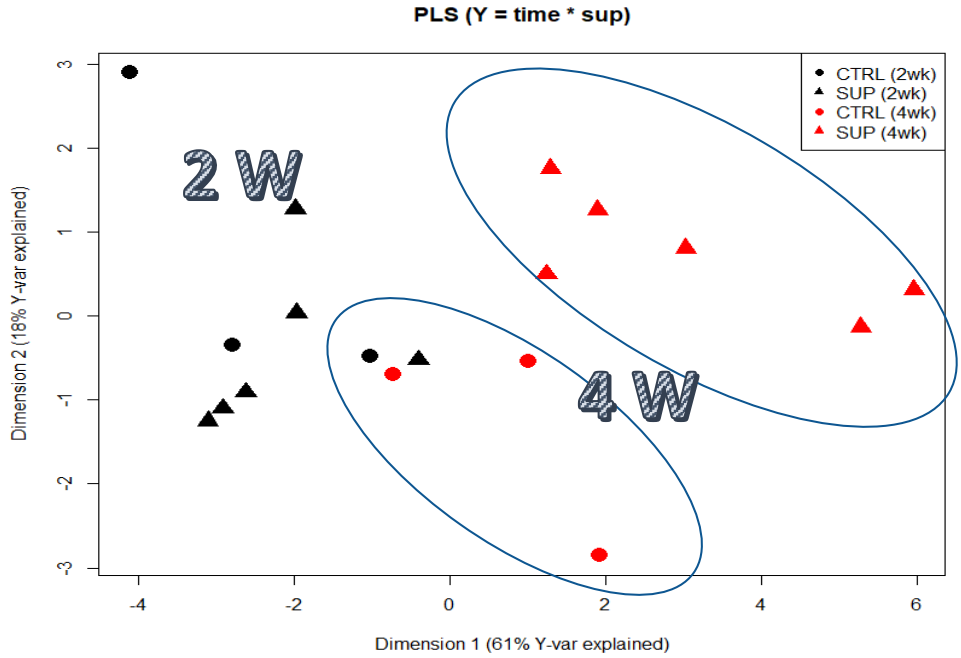

FIGURE 2. Partial least squares regression analysis (PLSR) of plasma proteomic signatures of fishes fed the experimental diets. PLS score plots of all proteins analysed along the two main components, considering all supplemented diets as a pool designated as SUP. The Y-matrix contained factors “time”, “supplemented” and the interaction between these two factors

Table 7. Differently expressed proteins in fish fed the highest supplementation levels of both ARG and CIT in study after 4 weeks of feeding.

Accession	Description	Biological process	Molecular function	p-values
Sa_23268.2.1	Complement component 7a	complement activation	chitin binding	0.0037
Sa_4795.1.1	Phosphoglucomutase 1	carbohydrate metabolic process	intramolecular transferase activity, phosphotransferases	0.0045
Sa_46414.1.1	Complement component 7b	complement activation	chitin binding	0.0078
Sa_9379.3.3	Alpha-1,4 glucan phosphorylase	carbohydrate metabolic process	glycogen phosphorylase activity	0.0094
Sa_48024.9.1	Tributyltin binding protein type			0.0157
Sa_18106.7.1	Leucine zipper-EF-hand containing transmembrane protein 1	Ca export from the mitochondrion ribosome binding	mitochondrion	0.0171
Sa_24913.2.4	Coagulation factor IXa	blood coagulation	calcium ion binding	0.0205
Sa_27134.1.1	Uncharacterized protein	semaphorin-plexin signaling pathway	semaphorin receptor activity	0.0226
Sa_13814.1.1	intelectin	induction of bacterial agglutination	oligosaccharide binding	0.0246
Sa_33122.3.1	apolipoprotein C-II	lipid transport	enzyme activator activity	0.0274
Sa_25136.3.1	Phosphoglycerate mutase	ATP generation from ADP/pyruvate metabolic process	bisphosphoglycerate mutase activity	0.0282
Sa_26882.2.1	Uncharacterized protein	complement activation	endopeptidase inhibitor activity	0.0317
Sa_19875.1.1	Complement factor D	complement activation, alternative pathway	serine-type endopeptidase activity	0.0319
Sa_12582.3.1	Sex hormone-binding globulin type-II	response to estradiol	androgen binding	0.0410
Sa_32547.2.1	Uncharacterized protein			0.0416
Sa_1942.4.1	Fucose mutarotase	monosaccharide metabolic process	monosaccharide binding	0.0417
Sa_44333.2.1	Kininogen	negative regulation of endopeptidase activity	cysteine-type endopeptidase inhibitor activity	0.0420
Sa_12688.1.1	Thyroid hormone receptor interactor 10	vesicle-mediated transport	lipid binding	0.0423
Sa_41650.2.1	Plasminogen activator inhibitor 2	negative regulation of fibrinolysis	serine-type endopeptidase inhibitor activity	0.0441

The colors stand for the graded level of p-values, greener is lower that light green of yellow.

5.3.5 Gene expression

No differences were observed in gene expression between supplemented diets and the control group, but differences were observed between sampling points (Table S2). Nonetheless, the *il-10* transcript was up-regulated in fish fed ARG1 for 4 weeks compared to those fed CIT2 (Fig. 3C). Moreover, *il-34* was up-regulated in fish fed CIT1 when compared to CIT2 regardless of sampling time (Fig. 3D). Besides the classical ANOVA analysis approach, an overall multivariate analysis (using PLS-DA) was performed (Fig. 5). PLS-DA analysis focused on the "diet" factor suggests that the expression data could not extensively explain all differences between diets (R2Y of 28 %, Fig. 5A), with the obtained prediction capacity being also low (Q2 of 14 %). Despite this fact, it was observed that, when samples are plotted along the first two components (Fig. 5A), supplemented samples tend to lie closer to the upper-left quadrant, while non-supplemented samples lie closer to the bottom-right quadrant. When performing the PLS-DA analysis focusing on the "time" factor (Fig. 5B), it was able to explain 45 % of Y-variance R2Y and to predict more than 24 % of the total variance (Q2). In this particular analysis, component 1 represented sampling time effects (C1, 40.9 %), whereas the interpretation of component 2 (4.6 %) was not clear (Figure 5B), thus confirming a clear time effect on the biomarkers panel analysed. In order to understand and interpret the contribution of the different genes to these components, a table of the variable importance projection (VIP) score of the genes ordered by its importance is presented in Fig. 5B. It is clear that all biomarkers were highly affected by sampling time (Component 1, highlighted in blue).

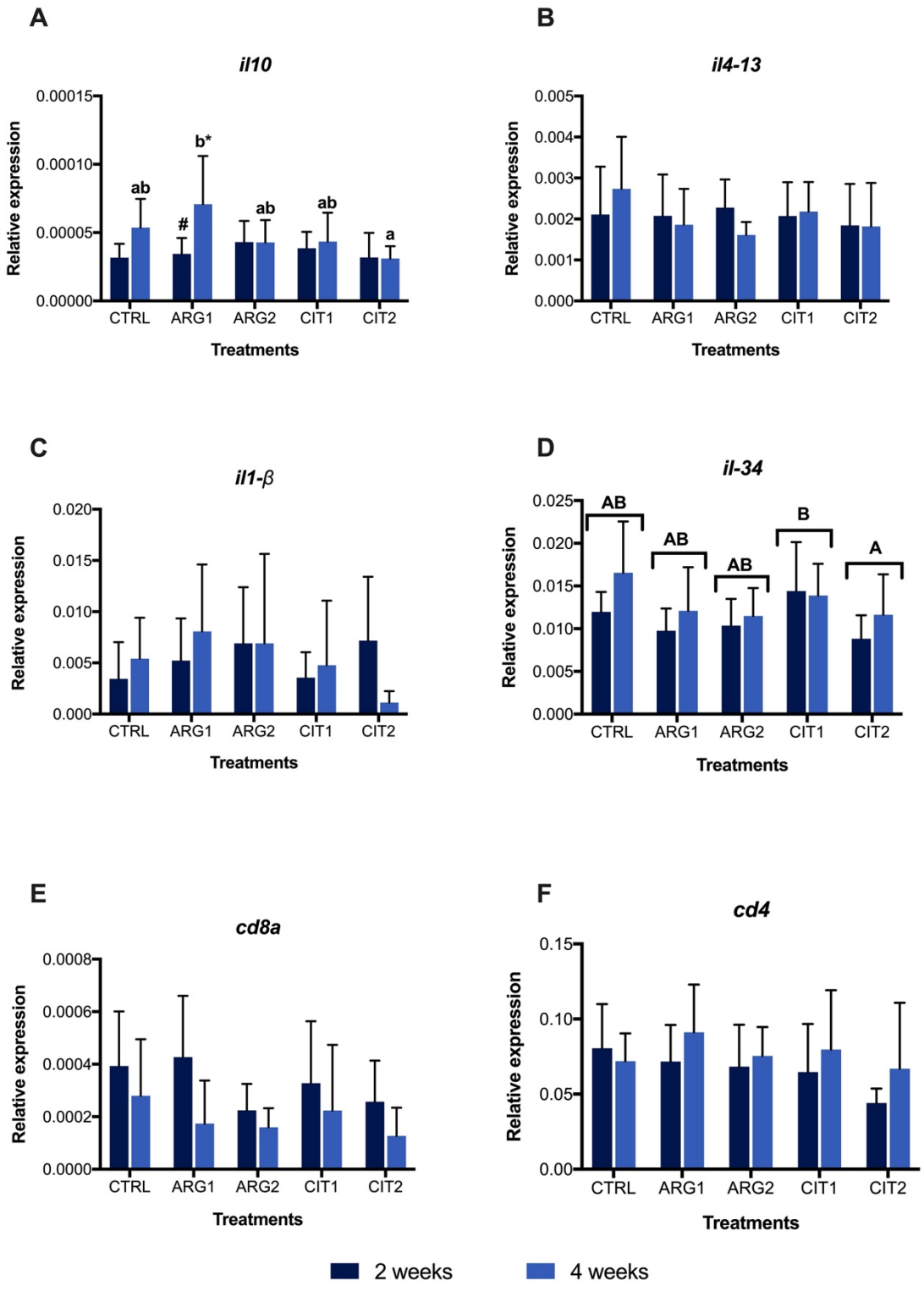

FIGURE 3. Relative expression of interleukin 1 β (*il1-β*, **A**), interleukin 4-13b (*il4-13* **B**), interleukin 10 (*il-10*, **C**), interleukin 34 **(***il-34,* **D**), cluster of differentiation 8α (*cd8α*, **E**) cluster of differentiation 4 (*cd4*, **F**) genes in the head-kidney of gilthead seabream fed dietary treatments during 2 (■) and 4 weeks (■). Values are presented as means ± SD (*n* = 9). *P*-values from two-way ANOVA ($p \leq 0.05$). Tukey *post-hoc* test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments≤ for the same time. Different capital letters indicate differences among diets regardless time. Different symbols indicate difference among time for the same dietary treatment.

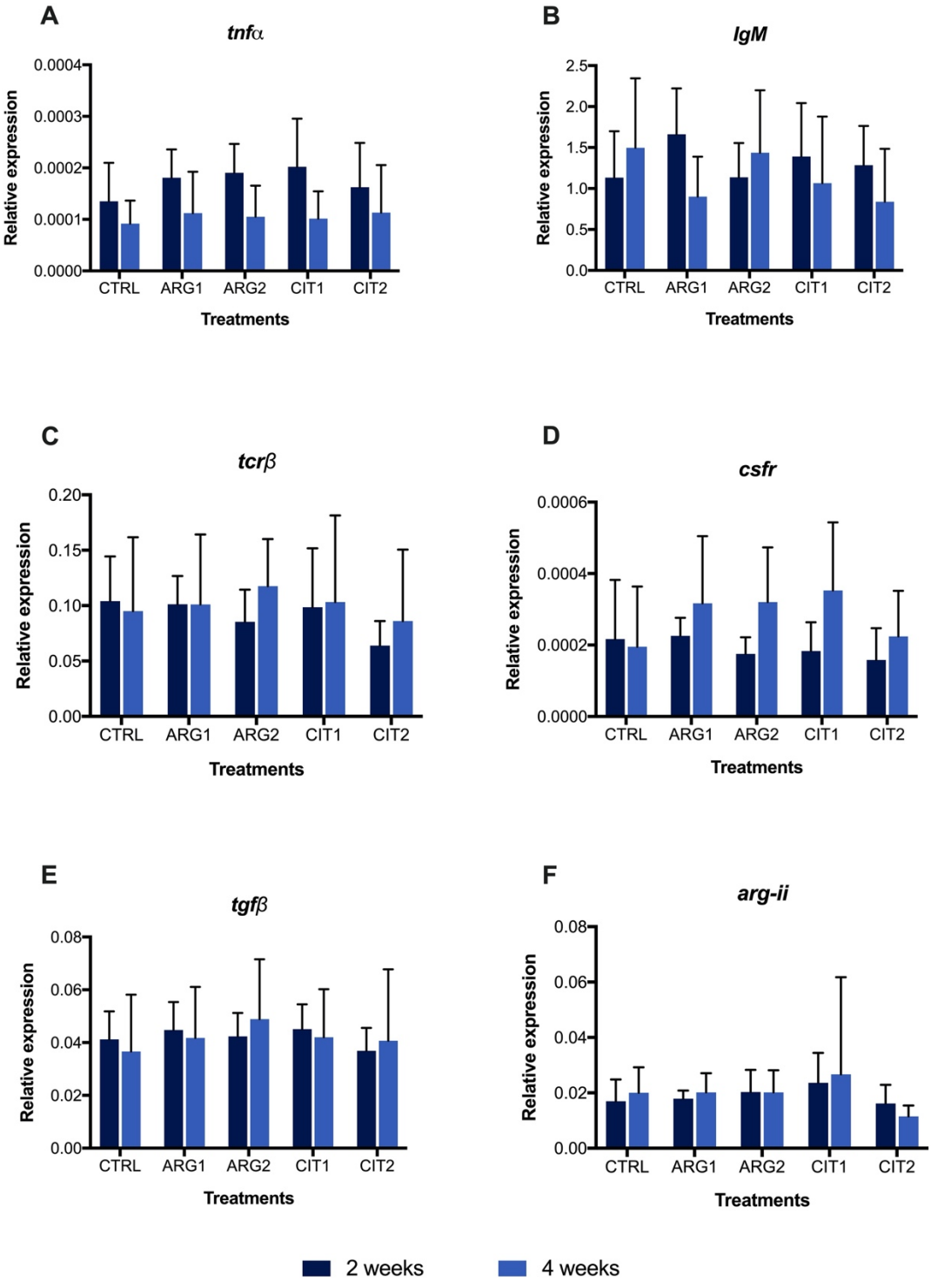

FIGURE 4. Quantitative expression of tumor necrosis factor α (*tnfα*, **A**), immunoglobulin M (heavy chain) (*IgM*, **B**), T cell receptor β (*tcrβ*, **C**), colony stimulating factor-1 receptor (*csfr*, **D**), transforming growth factor beta (*tgfβ*, **E**), arginase type II precursor (*arg-ii*, **F**) genes in the head-kidney of gilthead seabream fed dietary treatments during 2 (■) and 4 weeks (■). Values are presented as means ± SD (n = 9). *P*-values from Two-way ANOVA ($p \leq 0.05$). Tukey *post-hoc* test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same time. Different capital letters indicate differences among diets regardless time. Different symbols indicate difference among time for the same dietary treatment.

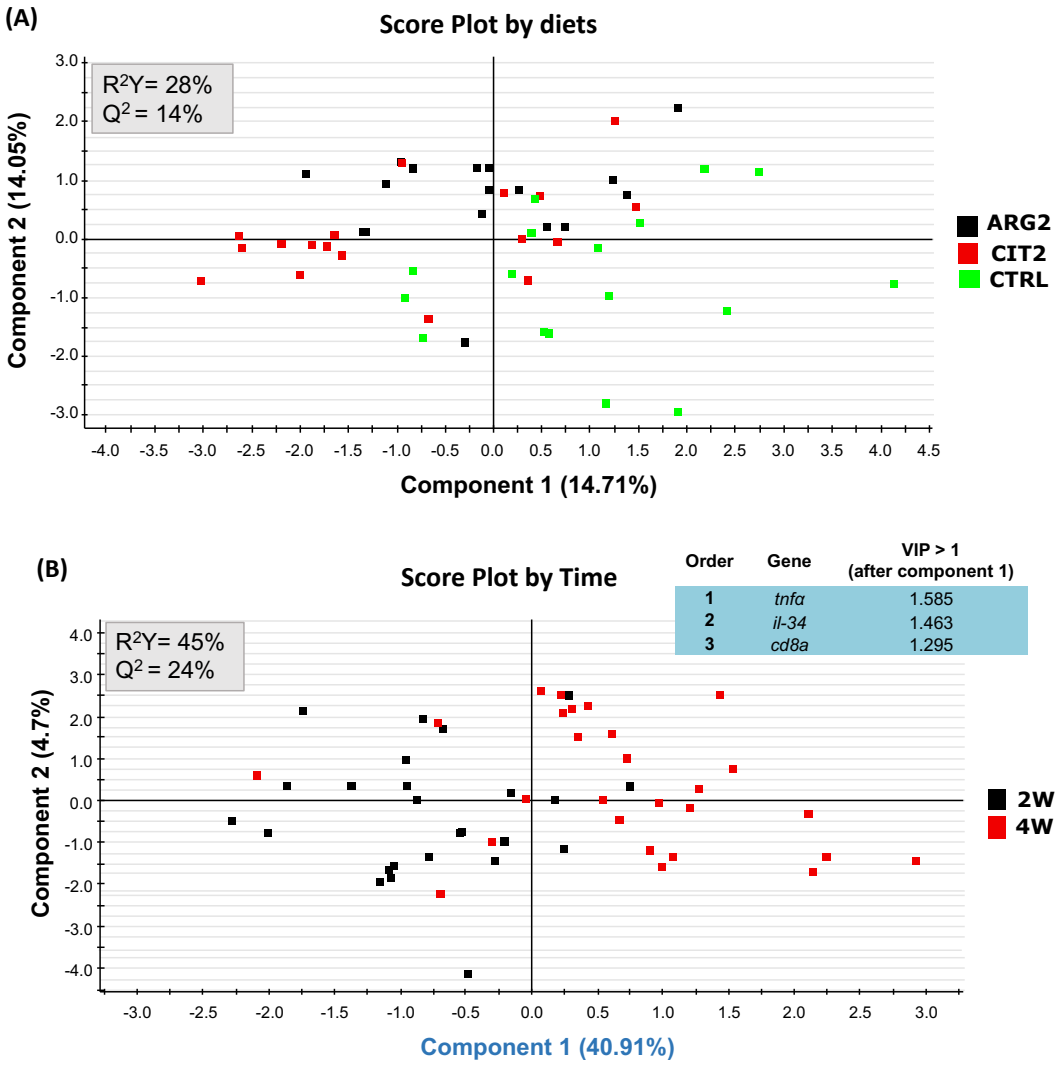

FIGURE 5. Discriminant analyses (PLS-DA) of head-kidney molecular signatures of gilthead seabream fed the experimental diets. Relative expression data of the 12 biomarkers can be found on table S2. (A) PLS-DA score plots of all biomarkers analysed in groups fed the highest supplementation level and the control, along the two main components, where the analysis was focused on the "diet" factor. (B) PLS-DA score plots of all biomarkers analysed along the two main components, where the analysis was focused on the "time" factor and ordered list of markers by variable importance (VIP) in projection of PLS-DA model for time differentiation. Markers with VIP values > 1 after the first component is highlighted in blue.

5.4 Discussion

Arginine surplus has proven to be a good strategy to modulate both the innate and adaptive immune fish response, where arginine may act through polyamines, directly by modulating gene expression, by regulating nutrient availability for immune cells through the endocrine control or through NO to fight pathogens (Andersen, et al., 2016). The present study represents the first attempt to explore the effects of dietary supplementation of arginine and citrulline on the gilthead seabream immune system. Results showed a light modulation of the immune status after 2 weeks of feeding, mainly in the relative proportion of monocytes, which decreased in time regardless of dietary treatments. Chen, et al. (2015) observed that in fish fed a diet rich in arginine (up to 21.9 g/kg diet) upon a challenge with *Aeromonas hydrophila* some health-related biomarkers like TNF-α and TGF-β where up-regulated in head-kidney, and decreased in higher inclusion levels, reiterating a dose-dependent effect. This may also point, that arginine supplementation efficacy on immune system, is dependent of an immune stimulus.

Noteworthy, fish fed the highest citrulline level had the highest relative proportion of monocytes regardless of sampling point. Monocytes from fish fed ARG2 and CIT2 were also found to be positively correlated with NO levels measured in plasma and showed an increasing trend in a dose-dependent manner. It is well known that NO is formed through the oxidation of $_{L}$-arginine in a reaction catalysed by the inducible NO synthases (iNOS), an enzyme mainly expressed in activated monocytes, macrophages and neutrophils, highlighting the importance of arginine availability upon immune stimulation (Bogdan, 2001; Yang, et al., 2013). Moreover, NO plays an important dual role in host defence against pathogens and cytotoxic actions in some pathological processes, acting as both pro-inflammatory or it can promote immunosuppression (Liew, 1995). Interestingly, Rapovy, et al. (2015) found that arginase-expressing macrophages preferred L-citrulline over L-arginine for the promotion of antimycobacterial activity. Briefly, NO production was not compromised in mice co-stimulated macrophages with with *Mycobacterium bovis* and interferon γ, expressing the arginase gene and cultured in citrulline-supplemented media, in contrast to those maintained in arginine-supplemented (Rapovy, et al., 2015).

Arginine being a major substrate for polyamine biosynthesis, essential for cell proliferation, when in surplus may increase total leukocytes, particularly macrophages-secreted cytokines (Buentello, et al., 2007). Hence, these results suggest that the inclusion of either amino acid at the highest level could boost to some extent the gilthead seabream immune response after a short feeding period of 2 weeks. Moreover, seabream fed ARG2 increased plasma bactericidal activity following 4 weeks of feeding. Bactericidal activity is a multifactorial indicator since it evaluates a wide range of innate immune mechanisms and molecular defences against bacterial invasion, such as proteins of the complement system, acute phase proteins and cytokines (Ellis, 1999; Graham, et al., 1988). Therefore, an increase of this parameter seems to indicate that fish fed ARG2 for 4 weeks have an enhanced immune status and might thereby develop a more efficient immune response.

Li, et al. (2013) showed that in an *in vitro* study with isolated carp erythrocytes, that the combination of several AA, among them citrulline (Gln, Ala, Cit and Pro) was able to confer protection to the cultured cells against oxidative damage (e.g. generation of ROS) induced by hydroxyl radical (•OH), leading probably to its protection from apoptosis. This action might be useful during an overproduction of radicals exceeding the antioxidant capacity of a cell.

In general, arginine and citrulline supplementation seemed to induce higher transcriptional changes, compared to the effect of the “time” factor. Taking into account the discriminant analysis (PLS-DA) that integrated the expression data from head-kidney, it can be seen that only data from fish fed the highest AA supplementation revealed a higher capacity to predict “time” instead of predict “diet”.

On the other hand, we consider that the low predicted variance (Q^2) values observed for the “diet” factor can, at least in part, be explained by the strong similarity between the ARG2 and CIT2 diets, in terms of their effects on the head kidney transcriptome.

High-throughput proteomics are gaining rapid traction in teleost physiology (Causey, et al., 2018), and it is well known that there should not be an expectation of a direct relationship between head-kidney transcriptomic response and plasma protein abundance. Hence, in order to better understand the modulation capacity of Arginine and Citrulline supplementation, plasma samples were evaluated in terms of their protein content. This approach was very useful to quantify a total of 92 plasma proteins, and particularly those that were modulated by the experimental diets. Among others, many proteins of the complement system, apolipoproteins, as well as some

glycoproteins were identified. A PLSR analysis of the expressed proteins showed that, despite “time” having a strong effect on plasma protein expression, a clear separation between fish fed the highest supplementation levels (ARG2 and CIT2) and CTRL was found at 4 weeks, while after a shorter feeding period of 2 weeks no differences were observed between experimental dietary treatments. A notable finding was the modulation of proteins of the complement system by the supplemented amino acids. These proteins are activated through the classical, alternative and lectin pathways, leading to bactericidal actions through pathogen opsonization, phagocytic activity and subsequent lysis (Merle, et al., 2015; Zhu, et al., 2013). Data from the present study highlighted two complement proteins subunits, C7a and C7b, proteins member of the lytic pathway. Along with C5b to C9, C7 is part of the formation of a porous transmembrane structure that is inserted into the lipid membrane of the microbial agent and causes cytolysis. C7 also possesses short-consensus repeats, tandem structural units that are found in plasma and membrane complement-regulatory proteins (Holland, Lambris, 2002). Nonetheless, recent evidences suggest that complement proteins functions go beyond the immune role, such as metabolic functions, particularly insulin-like roles and triglyceride metabolism facilitators (Merle, et al., 2015).

Arginine and its metabolites have been described to strongly affect tissue repair and cell replication – processes involved in animal growth and survival (Alexander, Supp, 2014; Popovic, et al., 2007; Wu, 2009). In the current study, no differences were observed in growth and feed conversion parameters. This is in line with results observed in a previous trial with juvenile red drum, where a diet supplemented with arginine did not alter growth performance (Cheng, et al., 2011). However, in that study the same basal diet supplemented with a combination of arginine and glutamine (at the same inclusion level) improved significantly feed efficiency, compared to those fed the basal diet, during a 7-week feeding trial (Cheng, et al., 2011). This suggests that arginine plays a co-operative role and is able to increment glutamine effect on feed efficiency, rather than a direct effect on fish growth performance. However, the mechanism for the beneficial effect of this combination is still unclear.

Moreover, Liang, et al. (2016) showed that juvenile blunt snout bream (*Megalobrama amblycephala*) fed an enriched arginine diet for 8 weeks had a better growth performance (optimal FCR, at 1.62% of dry diet), but in a dose-dependent manner, since either a higher or lower inclusion levels had negative impact on fish growth performance. Arginine (at 2.54% of diet) was also reported to improve feed

utilization (some improvements on FCR) in a 9-week feeding trial with juvenile red seabream (*Pagrus major*) (Rahimnejad, Lee, 2014). Taking into account that these trials had a longer feeding period, characteristic of a growth trial, the fact that no relevant growth performance-effect was observed in the present study could be time-related.

In summary, the present study unveiled a stimulation of the fish immune status for a short-term feeding period, verified mostly by a modulation of the gilthead seabream plasma proteome and health-biomarkers after 4 weeks of feeding diets rich in arginine and citrulline, particularly when supplemented at a 1% inclusion level. The concomitant increase of plasma bactericidal activity in fish fed ARG2 for the same period was also observed, and the peripheral monocyte numbers correlated positively with nitric oxide, which showed an increasing trend in a dose-dependent manner.

5.5 Ethics statement

The experiments were approved by the i3S Animal Welfare Committee and carried out in a registered installation (license number 0421/000/000/2018). Experiments were performed by trained scientists in full compliance with national rules and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

5.6 Authors Contributions

LC, SE, TSS and BC conceived the experiment and contributed with both reagents and goods. LR-P conducted the main experimental work. MA assisted with analytical procedures. LR-P directed most laboratory techniques and wrote the manuscript under the supervision of JAM-S, JA C-G, JP-S, BC and TSS. All authors contributed to and approved the manuscript.

5.7 Funding

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Chapter 6

General discussion, conclusions and future approaches

6.1 Discussion

The main purpose of this research was to evaluate the immune status of gilthead seabream fed diets supplemented with AA known to play relevant roles in several key health-related mechanisms and fish welfare. A holistic approach was then undertaken where transcriptomics, proteomics, haematology and humoral immune parameters were measured over the course of several growth/challenge trials, in order to assess not only the immunological impact of dietary AA supplementation, but also other potentially relevant effects (e.g. at the level of nutritional condition, growth performance and physiological/cellular stress). Overall, four trials were designed and executed to evaluate the effects of dietary AA supplementation under different farming scenarios and challenging conditions.

6.1.1 Amino acids without clear immunomodulatory effects in gilthead seabream juveniles

AA inclusion in modern aquafeeds has been a common practice to achieve balanced diets that meet all the nutritional requirements, but also with health-promoting goals (Encarnação, 2016). The dietary inclusion of essential AA (EAA) is becoming more commonplace because of their functional role as immune regulators and antioxidant function, besides their natural role in protein biosynthesis. The added value of several EAA have been demonstrated in experimental trials. As such, the inclusion of these additives in fish feeding may compensate, at least in some cases, the plausible increased costs for fish producers, since some of these functional diets can lead to a boost in the immune system of farmed fish and/or minimise the impact of stress, particularly important for reducing the risk of losses due to disease outbreaks.

Nonetheless, the use of threonine, taurine and histidine as functional additives is not a common practice, particularly in the context of immune-modulating additives. In this context, an exploratory trial with gilthead seabream juveniles in a recirculating aquaculture system (RAS) was designed to evaluate the effect of these AA inclusion in aquafeeds on the gilthead seabream immune status (**Chapter 2**). In general, the results from **Chapter 2** suggest that dietary supplementation with histidine, taurine or threonine above their nominal requirements for this species has relatively mild effects

on the seabream immune condition. Still, some effects of threonine and taurine supplementation on the fish immune system were observed, particularly after a longer feeding period (4 weeks), which reinforces the importance of feeding period when aiming to improve immune alertness.

The lack of clear functional effects observed during the trial could be attributed to several possible reasons: i) the chosen amino acids supplemented (particularly, His and Tau) simply do not have clear functional effects, at least at the immune system level; ii) the applied dosages and/or exposure times are not the most appropriate (for instance dietary threonine negatively affected innate immunity after 2 weeks whereas acquired immunity was enhanced at 4 weeks); iii) the specific experimental context (good rearing conditions devoid of any major sources of stress) may have concealed the potential effects of these additives on the immune system. These analyses suggest that, when evaluating functional effects of additives on the immune system, there may be a need for a specific (challenging) context, more than a change in the dosage or in the time of exposure, in order to make these effects evident.

6.1.2 Time-dependent nutraceutical effects of tryptophan

The effects of tryptophan supplementation were assessed in two different set-ups. First, in **Chapter 3**, the effects of a tryptophan surplus were evaluated in the context of an extreme diet formulation (0 % inclusion of FM) over a long feeding period. The overall results of this trial suggest that, in the context of a challenging fishmeal-free diet, supplementation with tryptophan seems to improve gilthead seabream juveniles' immune status on a short-term basis, with this effect disappearing after a long feeding period. It is important to have into account that this growth trial was designed to mimic the conditions that can be found on several aquaculture facilities, where multiple factors (biotic and abiotic) influence the farming conditions.

After **Chapter 3**, the observed effects of tryptophan-supplemented diets on a short-term basis were re-evaluated in another trial using two tryptophan inclusion levels over a shorter feeding period of 4 weeks, with an intermediate sampling after 2 weeks of feeding. This trial was conducted in a RAS system, where fewer variables are involved, in an attempt to isolate the effect of including tryptophan in the diet (**Chapter 4**). In contrast to **Chapter 3**, the results of **Chapter 4** revealed only mild effects during the 4

weeks feeding trial, with the effects of tryptophan on the immune system only becoming clear after an explicit immune challenge.

One possible justification for the different time dynamics of tryptophan supplementation in the two trials (short-term effect in **Chapter 3**, no clear effect after the same time period in **Chapter 4**) is precisely the fact that the first trial was performed under uncontrolled (and generally higher) temperatures (around 25 ºC), under a more challenging set-up for the fish. Either due to changes in AA requirements (as suggested by Conceição, et al. (2012)) or through other mechanisms, it seems that this particular context ellicited the observed short-term effect of tryptophan that is otherwise concealed when fish are reared under more controlled conditions (e.g. RAS).

Since gilthead seabream is, in practice, usually reared in set-ups resembling the one used in **Chapter 3** (more than set-ups like the one used in **Chapter 4**), it seems likely that its application as an immune-boosting additive in practical aquafeeds seems plausible. Nonetheless, given that the observed immune-boosting effect of tryptophan does not seem to be long-lasting, the application of such "tryptophan-enriched immune-boosting diets" seem to be more justified when fish are subjected to particularly challenging situations (e.g. temperature changes, exposure to pathogens, crowding, handling and transport).

6.1.3 Tryptophan and its antioxidant properties

In this thesis, no significant differences on markers of antioxidant capacity between different intake levels of tryptophan were observed when fish were not subjected to explicitly stressful conditions, neither in **Chapter 3** (after a short or long feeding period) nor before the challenge of **Chapter 4**. On the other hand, in **Chapter 4**, during the inflammatory insult, the expression levels of glutathione peroxidase 1 (*gpx1*) were significantly upregulated in fish fed a diet with high tryptophan inclusion. This reinforced the hypothesis that tryptophan may indirectly affect the fish immune response by increasing the expression of proteins with antioxidant functions through transcriptional mechanisms, beyond any other direct antioxidant property of tryptophan and its metabolites.

Overall, only minor effects on direct indicators of oxidative stress were observed in fish fed Trp-rich diets, even during a stressful event. This, again, reinforces the idea that the functional effects of Trp on the fish stress response may only be clearly

assessed in a situation where fish are not just exposed to mild and short-lived stress factors (e.g. an inflammatory challenge without resorting to the use of live pathogens, with low levels of associated oxidative stress), but already in a situation of distress (e.g. when actively suffering from a real infection process, in the field).

6.1.4 Tryptophan modulate the immune system of gilthead seabream upon an inflammatory insult.

After the short-term feeding trial with tryptophan, seabream were subjected to an immune stimulation with an i.p. injection of inactivated bacteria, to elicit an inflammatory response and study the role of tryptophan (**Chapter 4**) on the inflammatory response.

In **Chapter 4**, fish were i.p. injected with a lysed bacteria inoculum to evaluate the effect of a 4-week feeding period with a tryptophan-supplemented diet upon an immune stimulation, evaluating fish immune response parameters over the course of 48 h. In fact, as postulated after the first trial with tryptophan (**Chapter 3**), the results observed in fish sampled after i.p. injection in **Chapter 4** revealed that the overall parameters (gut gene expression and humoral parameters) were modulated by the higher inclusion level of tryptophan (TRP2) upon an inflammatory insult, particularly the health-related molecular signatures analysed in the anterior gut. Four gene transcripts associated with immune response (*il-10*, *il-34*, *IgM*, *CD8a*) were significantly upregulated in fish fed higher levels of Trp, upon an inflammatory insult. Also, changes in blood leucocyte numbers were observed, when inflammatory mechanisms were activated by killed bacteria i.p. injection. Thus, a clear immune system response was observed in diets supplemented with tryptophan. Overall, these results support the idea that a proper challenge scenario can help to identify possible nutraceutical effects of amino acids, since fish become more susceptible and their AA requirements may increase, which seems to be the case for tryptophan.

6.1.5 Arginine dietary inclusion: a good strategy to boost immune status.

In this Thesis, arginine (**Chapter 5**) was tested to determine its potential in improving the immune condition of gilthead seabream juveniles over a short feeding period (one month). The results of the study show an increase in the number of monocytes, accompanied by an increase in plasma bactericidal activity as a response to an increased dietary surplus of Arg, particularly at the highest levels, along with an increase in plasma NO levels.

Though it is not clear whether these should be only interpreted as signs of increased inducible NO synthase activity (given the increase in NO levels) or as signs of increased arginase activity (given the increased monocyte counts, which could indicate higher level of polyamine synthesis), it seems clear that dietary arginine induces increased arginine bioavailability, which in turn leads to a stimulated state of the immune system (reflected e.g. in the higher bactericidal activity and in the expression of proteins of the complement system in the plasma). Therefore, dietary supplementation with high levels of Arg seems to be a good strategy to improve gilthead seabream innate immune defences for a possible pathogen outbreak.

Furthermore, it is important to consider that this trial was conducted in a RAS system, where fish were subjected to a more controlled environment, similar to the experimental set-ups of **Chapter 2** and **4** (where Trp and His, Tau & Thr were tested, respectively). Comparing the results of arginine supplementation with the results obtained for the other AA, in a similar RAS context, we see that the immunostimulatory effect of arginine is particularly strong and directed to the innate arm of the immune system, being able to exert an effect even after a short feeding period, in the absence of any environmental or immunological disturbance.

6.1.6 Citrulline has similar immunomodulatory effects as those observed with arginine.

The observed effects of citrulline were in line with those observed upon arginine dietary supplementation, evaluated alongside in the same feeding trial (Chapter 5), particularly at high inclusion levels. Hence, results suggest that citrulline was well accepted and assimilated by seabream, and then possibly converted to arginine, similar to what was observed in seabass by Azeredo, et al. (2020).

Citrulline is a by-product of arginine upon conversion to NO, but also a precursor of arginine. Hence, supplementing citrulline as an alternative strategy to promote health and growth performance has been given considerable attention in studies with mammals. Contrary to what happens with arginine, supplemented citrulline bypasses the liver and is used in the endogenous synthesis of arginine in the kidney and not excreted as nitrogenous waste (Clark, et al., 2020). In this regard, these results suggest that supplementing citrulline might be a good alternative to arginine for boosting the gilthead seabream's immune system, by increasing endogenous availability of arginine through a more efficient mechanism than by providing arginine itself.

6.1.7 Time matters – Feeding time of functional feeds seems to be a key factor for their efficacy

A clear result of this Thesis, particularly from the work presented in **Chapters 2, 3**, **4** and **5** is that feeding time, besides dosage, seems to be a crucial factor in determining their efficacy. It seems clear that supplements, in this case AA, may lose their effectiveness after being available for a long feeding period. Two possible explanations can be assumed: either i) fish becomes adapted to this supplementation level through some physiological/metabolic readjustment; or ii) fish metabolism becomes exhausted after a longer feeding period with a certain supplement, reaching its maximum effect for a shorter period and leading to no effects afterwards.

However, more research is necessary regarding the optimal timing and dosage level for AA supplementation, to understand which combinations lead to optimal health and growth performance of farmed fish. Finally, examining the reproducibility of observed AA effects through multiple feeding trials seems advisable, given the dependence of observed effects on timing, dosage and the specific experimental context.

6.2 Conclusions

The main conclusions that can be drawn from the current Thesis are:

1) Dietary supplementation with threonine, histidine and taurine does not present a clear immunostimulatory effect after 4 weeks of feeding in seabream juveniles. Further studies with other supplementation levels and focusing on disease resistance and other stressors must be planned to confirm if some effect on immune system does indeed occur (**Chapter 2**).

2) In the context of a challenging fishmeal-free diet, supplementation with tryptophan seems to improve gilthead seabream juveniles' immune status on a short-term basis without compromising long-term fish growth. Functional feeds leveraging this effect would be especially relevant before/during a stressful event or disease outbreak, considering that these putative advantageous effects seem to disappear after a long-term feeding period (**Chapter 3**).

3) Tryptophan has an immunomodulatory role, particularly upon an immune stimulation, verified mostly by an improvement of the gilthead seabream oxidative status and health-biomarkers in anterior gut (**Chapter 4**).

4) Arginine and citrulline, particularly when supplemented at a 1% of feed inclusion level, induce a stimulation of the fish immune system after a short-term feeding period, verified mostly by a modulation of the gilthead seabream plasma proteome and health-biomarkers after 4 weeks of exposure (**Chapter 5**).

5) Citrulline supplementation seems to mimic arginine immunostimulatory effects, modulating plasma proteome and increasing peripheral monocytes (**Chapter 5**), suggesting that it can be used as a supplement in the same contexts that would benefit from arginine supplementation.

6) Collected data point to a dose-dependent effect of AA supplementation on health parameters (**Chapters 4 and 5**).

7) **Chapters 4** revealed that, upon an inflammatory insult, tryptophan modulates gilthead seabream immune response, highlighting the importance of a supplemented diet under challenging situations (e.g. bacterial challenge, exposure to stressors).

8) Feeding duration seems to be a key factor for the efficacy of fortified diets. In the context of this Thesis, it was observed that the use of functional feeds can be a promising approach for boosting fish immune status, particularly during a short-term feeding period before a predictable stressful event or disease outbreak, but most of these advantageous effects were not retained on a long-term basis (**Chapters 3, 4 and 5**)

9) Tailor-made feed formulations with functional proprieties, including the use of nutraceutical dosages of AAs such as tryptophan, arginine and citrulline, are a promising tool as immune modulators for fish reared in captivity, being a step forward for new feed formulation approaches. These new aquafeeds will play a key role in the path to more sustainable aquaculture practices, by contributing towards minimizing environmental footprint and successfully increasing economic farmers' profits.

6.3 Future perspectives and loose ends

The results and outcomes of this Thesis reveal new and relevant insights on the potential role of threonine, histidine, taurine, tryptophan, arginine and citrulline as supplements to fortify modern aquafeed formulations. Nonetheless, much more research is needed to better understand fish immunomodulation through functional diets, since several mechanisms and modes of action of AA in fish are still poorly understood. In Figure 1, a schematic summary of the main results is presented, along with the main questions that remained unanswered after this study, pointing for future research regarding the use of AAs as functional ingredients.

Although this Thesis suggests a high potential for using dietary AA administration to boost the gilhead seabream immune system, particularly through the use of tryptophan (**Chapters 3 and 4**), arginine and citrulline (**Chapter 5**), its revelance must be explored under different challenge scenarious, particularly in the case of tryptophan. Regarding this last AA, it seems plausible that its administration can improve fish capacity to maintain homeostais in response to chronic or acute stress conditions.

In **Chapter 4**, the potential of tryptophan as an immune modulator upon an inflammatory challenge was explored. Nonetheless, further research should be performed in the context of a live bacterial challenge, to study the disease resistance of fish fed these supplemented diets and then evaluate the ability of these putative additives in improving or not gilthead seabream survival. Hence, more in-depth studies are required to understand the role of these AA as immunomodulators during stress and infection, in order to contribute new knowledge towards better practices in the use of chemotherapeutic and antibiotic treatments in aquaculture.

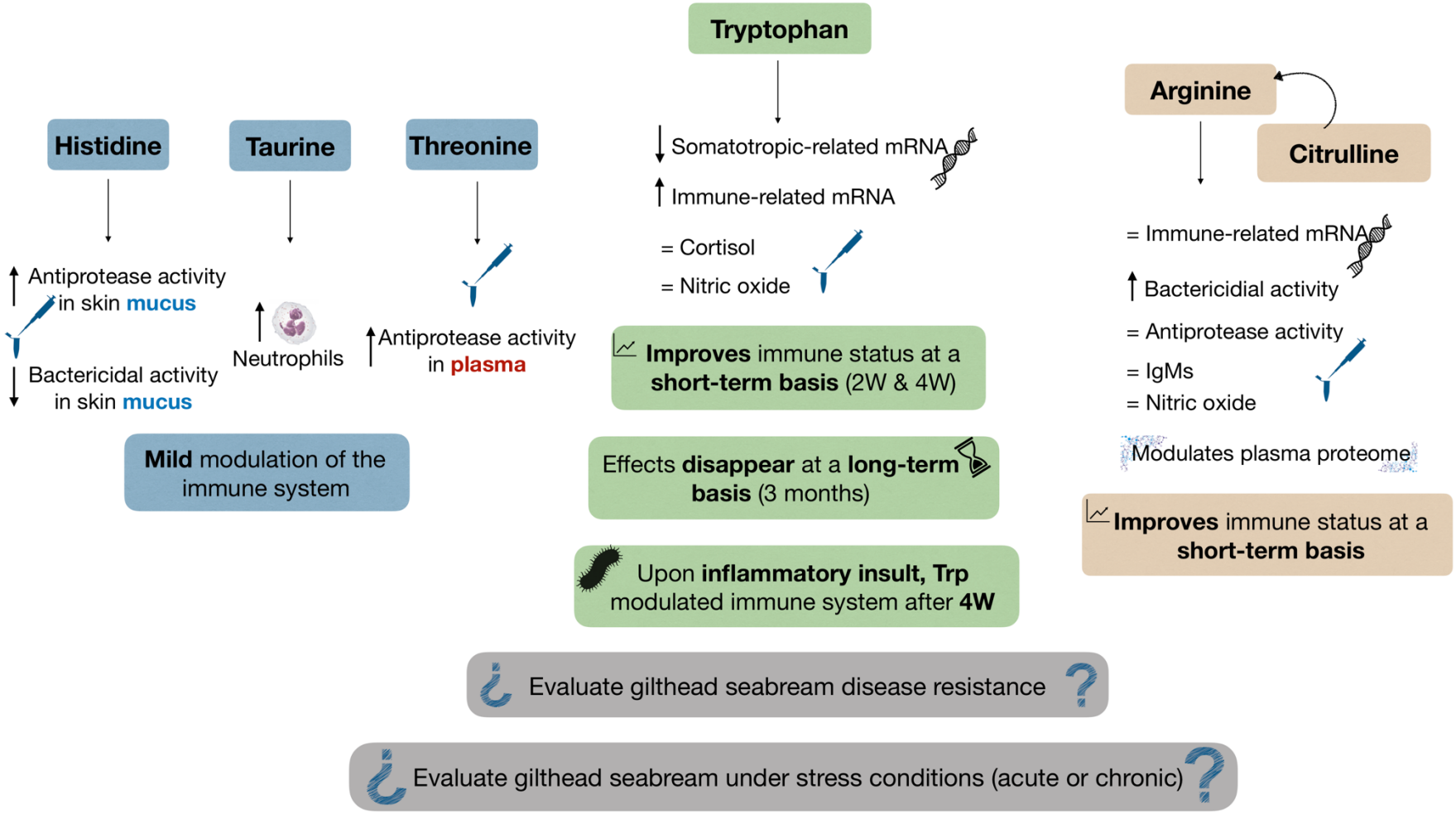

Figure 1. Amino acids impact in juvenile gilthead seabream's immune system and future approaches

6.4 References

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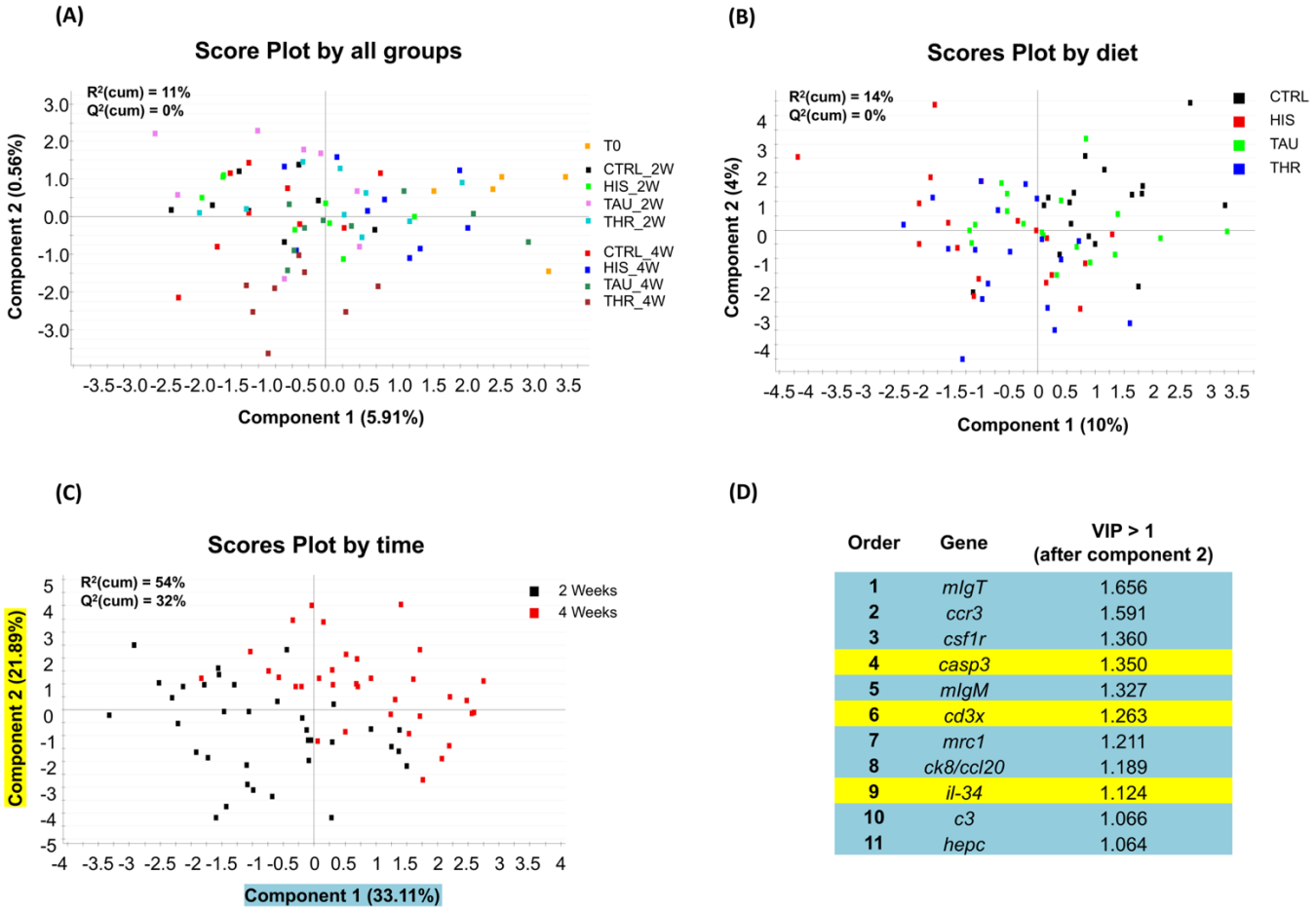
Appendix I

Dietary histidine, threonine or taurine supplementation affects gilthead seabream (*Sparus aurata*) immune status



(D)

Order	Gene	VIP > 1 (after component 2)
1	*mIgT*	1.656
2	*ccr3*	1.591
3	*csf1r*	1.360
4	*casp3*	1.350
5	*mIgM*	1.327
6	*cd3x*	1.263
7	*mrc1*	1.211
8	*ck8/ccl20*	1.189
9	*il-34*	1.124
10	*c3*	1.066
11	*hepc*	1.064

S. Figure 1. Discriminant analysis (PLS-DA) of head-kidney molecular signatures of fishes fed the experimental diets. Relative expression data of the 29 genes included in the array can be found on table 2. **(A)** PLS-DA scores plot of all biomarkers, using "experimental group" as target factor, for the two first components. **(B)** PLS-DA scores plot of all biomarkers, using "diet" as target factor, for the two first components. **(C)** PLS-DA scores plot of all biomarkers, using "time" as target factor, for the two first components **(D)** Ordered list of markers by variable importance (VIP) in projection of PLS-DA model for time differentiation. Markers with VIP values > 1 after the first and second components are highlighted in blue and yellow, respectively.

S. Table 1. Forward (F) and reverse (R) primers used for real-time PCR in head kidney.

Gene Name	Symbol	Acc. No.		Primer sequences (5' → 3')
ß-actin	*actb*	X89920	F	TCCTGCGGAATCCATGAGA
			R	GACGTCGCACTTCATGATGCT
C-C chemokineCK8 / C-C motifchemokine 20	*ck8/ccl 20*	GU181393	F	CCGTCCTCATCTGCTTCATACT
			R	GCTCTGCCGTTGATGGAAC
Caspase	*casp3*	EU722334	F	GCCAACGGACCTGGACCTG
			R	CCATCGCCTCTCCTCGCATCTA
C-C chemokine receptor type 3	*ccr3*	KF857317	F	CTACATCAGCATCACCATACGCATCCT
			R	TGGCACGGCACTTCTCCTTCA
CD4-full	*cd4-full*	AM489485	F	TCCTCCTCCTCGTCCTCGTT
			R	GGTGTCTCATCTTCCGCTGTCT
Cluster of differentiation 3 zeta chain	*cd3x*	MF175235	F	ATGGCGGTCCAGACGAGGGTTTC
			R	ACCAGCGAGGACAGGACCAGCAG
Cluster of differentiation 8 alpha	*cd8a*	EU921630	F	GCAGCAACGGTAACACGAACG
			R	CCAGTATGAGCGGAGTACAGAACA
Cluster of differentiation 8 beta	*cd8b*	KX231275	F	CCGAAATGTGGAAGACTGGAACTC
			R	CCAGTATGAGCGGAGTACAGAACA
Complement factor 3	*c3*	HM543456	F	GCTTACGCTCTTCTTGCTCTGGTGAA
			R	CATCTGACAACTGGTCTGGCATCGT
C-type lectin domain family 10 member A	*clec10a*	KF857329	F	CGACTCTGGACTCCCTCA
			R	CGTTGTTGATGGTGCGTTC
Hepcidin	*hepc*	AM749960	F	ACTCCTGGAAGATGCCGTATGC
			R	AACTTACACCTCCTGCGTCCAC
Immunoglobulin M	*igm*	JQ811851	F	ACCTCAGCGTCCTTCAGTGTTTATGATGCC
			R	CAGCGTCGTCGTCAACAAGCCAAGC
Immunoglobulin M membrane-bound form	*migm*	KX599199	F	GCTATGGAGGCGGAGGAAGATAACA
			R	CAGCGTCGTCGTCAACAAGCCAAGC
Immunoglobulin T	*igt*	KX599200	F	GCTGTCAAGGTGGCCCCAAAAG
			R	CAACATTCATGCGAGTTACCCTTGGC
Immunoglobulin T membrane-bound form	*igt-m*	KX599201	F	AGACGATGCCAGTGAAGAGGATGAGT

			R	CGAAGGAGGAGGCTGTGGACCA
Interleukin-1 beta	*il-1β*	AJ419178	F	GCGACCTACCTGCCACCTACACC
			R	TCGTCCACCGCCTCCAGATGC
Interleukin-6	*il-6*	EU244588	F	TCTTGAAGGTGGTGCTGGAAGTG
			R	AAGGACAATCTGCTGGAAGTGAGG
Interleukin-7	*il-7*	JX976618	F	CTATCTCTGTCCCTGTCCTGTGA
			R	TGCGGATGGTTGCCTTGTAAT
Interleukin-8	*il-8*	JX976619	F	CAGCAGAGTCTTCATCGTCACTATTG
			R	AGGCTCGCTTCACTGATGG
Interleukin-10	*il-10*	JX976621	F	AACATCCTGGGCTTCTATCTG
			R	GTGTCCTCCGTCTCATCTG
Interleukin 12 subunit beta	*il12*	JX976624	F	ATTCCCTGTGTGGTGGCTGCT
			R	GCTGGCATCCTGGCACTGAAT
Interleukin-15	*il-15*	JX976625	F	GAGACCAGCGAGCGAAAGGCATCC
			R	GCCAGAACAGGTTACAGGTTGACAGGAA
Interleukin-34	*il-34*	JX976629	F	TCTGTCTGCCTGCTGGTAG
			R	ATGCTGGCTGGTGTCTGG
Macrophage colony-stimulating factor 1 receptor 1	*csf1r1*	AM050293	F	TTGCGTGTGGTGAGGAAGGAAGGT
			R	AGCAGGCAGGGCAGCAGGTA
Macrophage mannose receptor 1	*mrc1*	KF857326	F	CTTCCGACCGTACCTGTACCTACTCA
			R	CGATTCCAGCCTTCCGCACACTTA
Toll-like receptor 2	*tlr2*	KF857323	F	CATCTGCGACTCTCCTCTCTTCCT
			R	GCGTGGATAGAGTTGGACTTGAG
Toll-like receptor 5	*tlr5*	KF857324	F	TCGCCAATCTGACGGACCTGAG
			R	CAGAACGCCGATGTGGTTGTAAGAC
Toll-like receptor 9	*tlr9*	AY751797	F	GCCTTCCTTGTCTGCTCTTTCT
			R	GCCGTAGAGGTGCTTCAGTAG
Tumor necrosis factor-alpha	*tnf-α*	AJ413189	F	CAGGCGTCGTTCAGAGTCTC
			R	CTGTGGCTGAGAGCTGTGAG
Zeta-chain-associated protein kinase 70	*zap70*	MF175239	F	TGGTGAAGGAGGAGATGATGAGG
			R	GCGAACGATGTAGCGGTTGT

S. Table 2. Head kidney expression in response in gilthead seabream at time ø and fed dietary treatments for 2 weeks and 4 weeks. All values are reported as mean ± SE (n=9) (Raw data). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences between dietary treatments for the same time. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets. Different symbols indicate difference between times for the same dietary treatment.

		TØ	**2 weeks**				**4 weeks**				**Two-way ANOVA (p<0.05)**		
Biological Process	Gene symbol	CTRL	CTRL	THR	TAU	HIST	CTRL	THR	TAU	HIST	Time	Diet	Time x diet
Interleukins & Cytokines	*il-1β*	0.08 ± 0.01	0.07 ± 0.01	0.10 ± 0.03	0.07 ± 0.01	0.05 ± 0.00	0.10 ± 0.03	0.07 ± 0.01	0.09 ± 0.02	0.10 ± 0.01	0.086	0.374	0.735
	il-6	0.04 ± 0.01	0.08 ± 0.02	0.06 ± 0.02	0.08 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.584	0.208	0.589
	il-7	1.06 ± 0.11	1.37 ± 0.16	1.14 ± 0.14	1.30 ± 0.21	1.07 ± 0.10	1.16 ± 0.09	1.14 ± 0.13	1.31 ± 0.14	1.08 ± 0.12	0.797	0.433	0.836
	il-8	0.07 ± 0.01	0.09 ± 0.01	0.08 ± 0.02	0.09 ± 0.01	0.08 ± 0.01	0.10 ± 0.02	0.08 ± 0.02	0.07 ± 0.01	0.08 ± 0.02	0.732	0.533	0.883
	il-10	0.69 ± 0.08	0.57 ± 0.04	0.68 ± 0.08	0.67 ± 0.07	0.61 ± 0.06	0.66 ± 0.03	0.62 ± 0.05	0.65 ± 0.06	0.69 ± 0.06	0.345	0.984	0.62
	il-12	0.09 ± 0.02	0.07 ± 0.01	0.05 ± 0.00	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.07 ± 0.02	0.06 ± 0.01	0.706	0.457	0.982
	il-15	0.25 ± 0.03	0.29 ± 0.04	0.26 ± 0.02	0.30 ± 0.03	0.28 ± 0.03	0.30 ± 0.02	0.28 ± 0.04	0.26 ± 0.03	0.27 ± 0.03	0.504	0.493	0.695
	il-34	1.57 ± 0.15	1.78 ± 0.12	1.76 ± 0.24	1.54 ± 0.10	1.37 ± 0.11	1.57 ± 0.13	1.39 ± 0.09	1.66 ± 0.07	1.66 ± 0.15	0.909	0.092	0.173
	tnf-α	0.25 ± 0.03	0.27 ± 0.03	0.24 ± 0.02	0.26 ± 0.02	0.24 ± 0.02	0.26 ± 0.01	0.22 ± 0.02	0.22 ± 0.01	0.23 ± 0.02	0.175	0.66	0.806
Macrophages and monocytes chemokines	*csf1r1*	4.30 ± 0.35	4.65 ± 0.39	4.40 ± 0.36	4.94 ± 0.48	4.48 ± 0.29	4.34 ± 0.32	4.09 ± 0.32	3.74 ± 0.14	4.59 ± 0.36	0.127	0.449	0.458
	ccr3	4.86 ± 0.55	5.94 ± 0.49	5.44 ± 0.40	5.17 ± 0.59	4.60 ± 0.40	5.71 ± 0.22	5.77 ± 0.25	5.83 ± 0.41	5.72 ± 0.49	0.094	0.038	0.548
	ck8/ccl20	0.76 ± 0.06	0.72 ± 0.08	0.81 ± 0.09	0.81 ± 0.06	0.74 ± 0.07	0.82 ± 0.07	0.78 ± 0.06	0.93 ± 0.11	0.84 ± 0.07	0.114	0.713	0.738
Immunoglobulins	*sIgM*	185.25 ± 27.90	188.57 ± 10.54	243.04 ± 28.52	225.26 ± 13.57	255.46 ± 23.91	223.21 ± 29.19	266.64 ± 25.54	249.75 ± 17.01	237.76 ± 13.26	0.224	0.23	0.817
	IgM-m	34.45 ± 3.82	25.76 ± 1.35	27.40 ± 1.86	32.16 ± 3.05	25.05 ± 1.39	31.24 ± 1.22	30.43 ± 2.00	29.85 ± 1.54	31.99 ± 2.74	0.03	0.579	0.283
	sIgT	0.28 ± 0.12	0.24 ± 0.15	0.07 ± 0.06	0.14 ± 0.09	0.24 ± 0.16	0.26 ± 0.13	0.82 ± 0.23	0.39 ± 0.16	0.17 ± 0.10	0.009	0.827	0.416
	IgT-m	6.67 ± 0.88	5.70 ± 0.81	6.62 ± 0.93	8.17 ± 1.76	8.21 ± 0.84	9.23 ± 1.74 ^{ab}	11.54 ± 1.92 ^{b}	5.16 ± 0.64 ^{a}	8.37 ± 0.79 ^{ab}	0.125	0.408	0.013
Complement factor	*c3*	0.02 ± 0.01	0.03 ± 0.02	0.00 ± 0.00	0.01 ± 0.00	0.02 ± 0.02	0.04 ± 0.03	0.04 ± 0.03	0.02 ± 0.01	0.02 ± 0.01	0.118	0.653	0.23

Iron recycling	hepc	15.00 ± 3.06	48.50 ± 9.73	49.69 ± 13.49	27.58 ± 4.61	43.92 ± 5.66	56.42 ± 9.67	53.29 ± 10.80	47.96 ± 11.24	45.01 ± 9.13	0.203	0.294	0.755
T-cell markers	*cd3x*	3.97 ± 1.22	2.43 ± 0.13	2.46 ± 0.12	2.55 ± 0.22	2.70 ± 0.23	2.41 ± 0.11	2.23 ± 0.14	2.31 ± 0.17	2.50 ± 0.28	0.302	0.714	0.955
	cd4-full	3.56 ± 1.41	1.97 ± 0.12	1.61 ± 0.05	1.77 ± 0.14	1.97 ± 0.15	1.94 ± 0.17	1.95 ± 0.14	1.86 ± 0.21	1.56 ± 0.20	0.762	0.476	0.29
	cd8a	4.28 ± 2.23	1.82 ± 0.25	1.69 ± 0.21	1.76 ± 0.17	1.73 ± 0.27	1.76 ± 0.17	1.78 ± 0.15	1.93 ± 0.17	1.96 ± 0.34	0.653	0.927	0.987
	cd8b	1.34 ± 0.81	0.47 ± 0.07	0.41 ± 0.05	0.42 ± 0.04	0.48 ± 0.10	0.44 ± 0.05	0.48 ± 0.07	0.57 ± 0.10	0.41 ± 0.08	0.776	0.58	0.697
	zap70	2.38 ± 0.44	1.83 ± 0.17	1.87 ± 0.10	1.95 ± 0.18	2.30 ± 0.30	2.05 ± 0.15	1.91 ± 0.17	1.67 ± 0.15	1.89 ± 0.21	0.472	0.427	0.422
Pattern recognition receptors	*tlr2*	3.93 ± 0.33	3.55 ± 0.31	3.10 ± 0.19	3.52 ± 0.37	3.13 ± 0.19	3.37 ± 0.25	3.20 ± 0.18	2.94 ± 0.27	3.64 ± 0.27	0.845	0.449	0.282
	tlr5	0.35 ± 0.05	0.33 ± 0.02	0.36 ± 0.02	0.31 ± 0.01	0.34 ± 0.02	0.32 ± 0.02	0.37 ± 0.02	0.35 ± 0.01	0.38 ± 0.03	0.409	0.041	0.622
	tlr9	1.70 ± 0.21	0.86 ± 0.12	0.97 ± 0.07	1.05 ± 0.14	0.92 ± 0.10	0.97 ± 0.08	0.81 ± 0.05	1.00 ± 0.13	1.11 ± 0.09	0.676	0.696	0.22
	mrc1	6.34 ± 0.75	7.62 ± 0.22	7.75 ± 0.57	7.27 ± 0.53	7.38 ± 0.82	8.08 ± 0.43	7.33 ± 0.43	8.22 ± 0.51	8.32 ± 0.44	0.212	0.791	0.57
Caspase	*casp3*	1.00 ± 0.06	1.02 ± 0.08	1.09 ± 0.07	1.11 ± 0.11	1.15 ± 0.08	1.04 ± 0.11	1.08 ± 0.09	0.99 ± 0.06	0.93 ± 0.09	0.252	0.709	0.762
Lectins	*clec10a*	0.54 ± 0.11	1.22 ± 0.14 ^{b}	0.43 ± 0.05 ^{a}	1.18 ± 0.09 ^{ab}	0.83 ± 0.19 ^{ab}	1.22 ± 0.25	1.33 ± 0.19	0.84 ± 0.17	0.83 ± 0.17	0.258	0.066	<0.001

Appendix II

Dietary tryptophan supplementation induces a transient immune enhancement of gilthead seabream (*Sparus aurata*) juveniles fed fishmeal-free diets

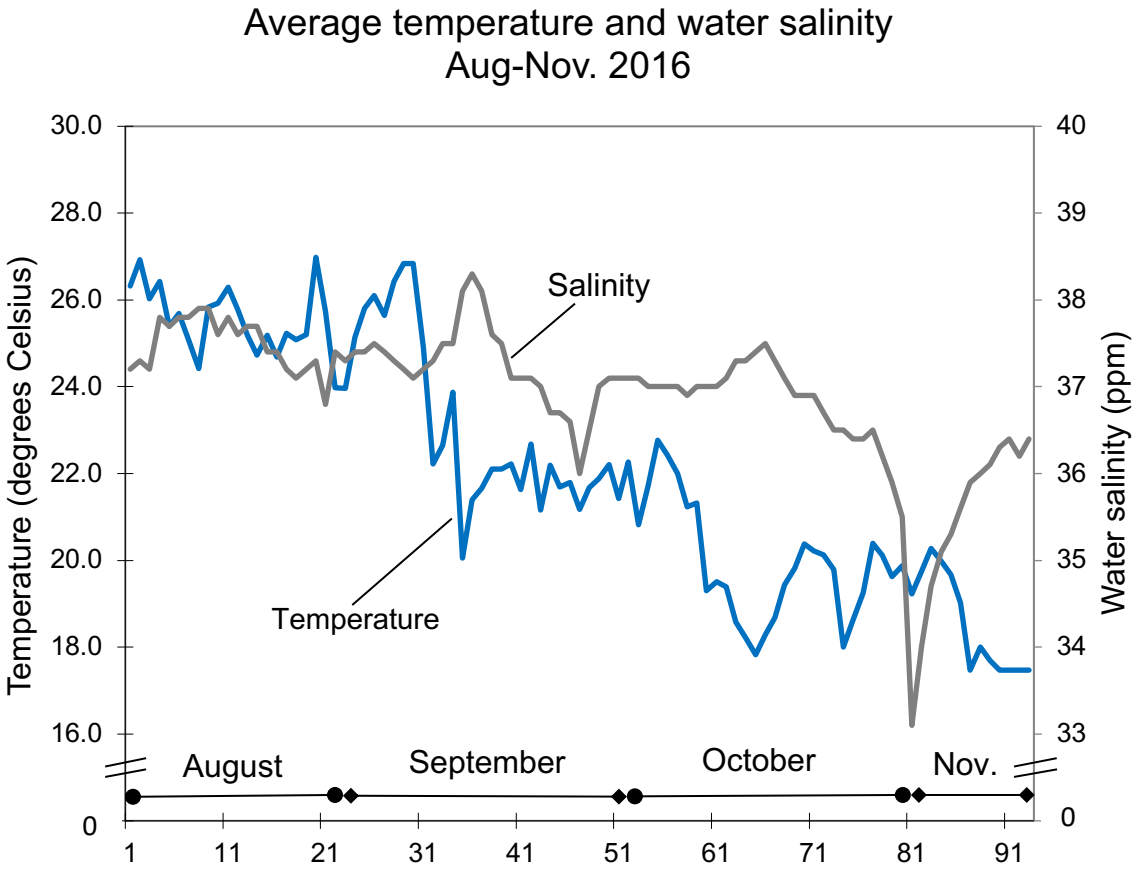


Fig. S1. Seawater temperature and salinity profiles during the experimental trial

Table S1. Amino acid composition (g AA 100 g^{-1} CP) of the experimental diet

	Experimental diets	
	CTRL	**SUP**
Arginine	7.45	7.75
Histidine	2.23	2.27
Lysine	5.15	5.26
Threonine	3.51	3.70
Isoleucine	3.11	3.31
Leucine	6.64	6.54
Valine	3.78	3.58
Methionine	2.02	2.00
Phenylalanine	4.46	4.49
Cystine	0.60	0.61
Tyrosine	2.93	3.03
Aspartic acid + Asparagine	6.31	6.34
Glutamic acid + Glutamine	14.48	14.46
Alanine	3.85	3.64
Glycine	3.81	3.89
Proline	5.57	5.44
Serine	3.58	3.79
Taurine	0.21	0.23
Tryptophan	0.60	0.93

Table S2. Effect of dietary treatments on whole body composition in gilthead seabream fed for 15 and 92 days (% wet weight, WW).

	Dietary treatments							
		CTRL		SUP		Two-way ANOVA		
Body composition (%WW)	**Initial**	**15 Days**	**92 Days**	**15 Days**	**92 Days**	**Time**	**Diet**	**Time x diet**
Moisture	72.69 ± 0.02	69.42 ± 0.11	66.15 ± 0.35	69.28 ± 0.07	65.36 ± 0.39	<0.001	0.296	0.415
Protein	14.11 ± 0.05	15.23 ± 0.13	17.04 ± 0.20	15.3 ± 0.17	17.15 ± 0.22	<0.001	0.759	0.951
Lipid	7.90 ± 0.06	9.93 ± 0.15	13.05 ± 0.46	10.09 ± 0.15	13.9 ± 0.27	<0.001	0.273	0.449
Ash	4.51 ± 0.05	3.87 ± 0.16	1.21 ± 0.04	4.15 ± 0.19	1.16 ± 0.03	<0.001	0.559	0.366
Retention (%)								
Dry matter	-	23.95 ± 1.32	25.76 ± 1.54	25.11 ± 3.65	24.16 ± 1.90	0.872	0.756	0.327
Protein	-	24.06 ± 0.42	27.54 ± 1.45	25.05 ± 2.23	25.09 ± 2.11	0.483	0.113	0.121
Lipids	-	43.55 ± 5.32	50.23 ± 6.51	52.21 ± 10.89	55.56 ± 5.09	0.270	0.137	0.704

Table S3. Forward (F) and reverse (R) primers used for real-time PCR in liver, head kidney and white muscle.

Gene Name	Symbol	Acc. No.		Primer sequences (5' → 3')	Amplification efficiency (%)
70 kDa heat shock protein, mitochondrial		DQ524993	F R	TCCGGTGTGGATCTGACCAAAGAC TGTTTAGGCCCAGAAGCATCCATG	99
Alpha-2-macroglobulin	*a2m*	AY358020	F R	TCCTGGGTGACATTCTGGGT CCGTATGGCATCCTCAGCAG	95
ß-actin	*actb*	X89920	F R	TCCTGCGGAATCCATGAGA GACGTCGCACTTCATGATGCT	95
C-C chemokineCK8 / C-C motifchemokine 20	*ck8/ccl20*	GU181393	F R	CCGTCCTCATCTGCTTCATACT GCTCTGCCGTTGATGGAAC	95
C-C chemokine receptor type 3	*ccr3*	KF857317	F R	CTACATCAGCATCACCATACGCATCCT TGGCACGGCACTTCTCCTTCA	95
Calpain 1	*capn1*	KF444899	F R	CAGAACCACAACGCCGTGAAGTTT AGGCACTGGGCTTTAAGACTCTCG	92
Calpastatin	*cast*	KM522786	F R	CCCAAACCCGAGCCCACCAT GACAAGAAGTCCAGAGCGTCTCCAGTA	96
Carnitine palmitoyltransferase 1A	*cpt1a*	JQ308822	F R	GTGCCTTCGTTCGTTCCATGATC TGATGCTTATCTGCTGCCTGTTTG	90
Catalase	*cat*	JQ308823	F R	TGGTCGAGAACTTGAAGGCTGTC AGGACGCAGAAATGGCAGAGG	100
Cathepsin B	*ctsb*	KJ524457	F R	TGGTCGAGAACTTGAAGGCTGTC GGGTCTACTGCCATTCACAT	91
Cathepsin D	*ctsd*	AF03619	F R	CACACTGGGAGACCTGCACTATGTCAATG ATTGCCAACTTGAAGTCCGTCCATACC	91
Cathepsin L	*ctsl*	KM522787	F R	GGGAACGGATGACCAGCCTTGT CGGTGTCATTGGCAGAGTTGTAGTTG	90
CD4-full	*cd4-full*	AM489485	F R	TCCTCCTCCTCGTCCTCGTT GGTGTCTCATCTTCCGCTGTCT	99
Citrate synthase	*cs*	JX975229	F R	TCCAGGAGGTGACGAGCC GTGACCAGCAGCCAGAAGAG	95
Cluster of differentiation 3 epsilon chain	*cd3e*	MF175240	F R	GGTGTGATGTTCGTCGTCTACAAGTG TGGCAGCGTGAGTGAGTCCT	96
Cluster of differentiation 3 zeta chain	*cd3x*	MF175235	F R	ATGGCGGTCCAGACGAGGGTTTC ACCAGCGAGGACAGGACCAGCAG	98
Cluster of differentiation 8 alpha	*cd8a*	EU921630	F R	GCAGCAACGGTAACACGAACG CCAGTATGAGCGGAGTACAGAACA	91

Cluster of differentiation 8 beta	*cd8b*	KX231275	F R	CCGAAATGTGGAAGACTGGAACTC CCAGTATGAGCGGAGTACAGAACA	98
Cytochrome c oxidase subunit I	*coxi*	KC217652	F R	GTCCTACTTCTTCTGTCCCTTCCTGTTCT AGGTTTCGGTCTGTAAGGAGCATTGTAATC	91
Cytochrome c oxidase subunit II	*coxii*	KC217653	F R	ACTGCCTACACAGGACCTTGCC GTCTGCTTCCAGGAGACGGAATTGT	95
Fatty acid binding protein, heart	*h-fabp*	JQ308834	F R	CTGGGTGTGGGCTTCGCTAC CTCTGTGTTCTTGATGGTGCTCTG	100
Follistatin	*fst*	AY544167	F R	GGACCAGACAAACAACGCATATTG CATAGATGATCCCGTCGTTTCCAC	90
Glucose-regulated protein, 170 kDa	*grp-170*	JQ308821	F R	CAGAGGAGGCAGACAGCAAGAC TTCTCAGACTCAGCATTTCCAGATTTC	91
Glucose-regulated protein, 94 kDa	*grp-94*	JQ308820	F R	AAGGCACAGGCTTACCAGACAG CTTCAGCATCATCGCCGACTTTC	96
Glutathione peroxidase 1	*gpx1*	DQ524992	F R	GAAGGTGGATGTGAATGGAAAAGATG CTGACGGGACTCCAAATGATGG	96
Glutathione peroxidase 4	*gpx4*	AM977818	F R	TGCGTCTGATAGGGTCCACTGTC GTCTGCCAGTCCTCTGTCGG	90
Glutathione reductase	*gr*	AJ937873	F R	TGCGTCTGATAGGGTCCACTGTC GTCTGCCAGTCCTCTGTCGG	100
Growth hormone receptor I	*ghr-i*	AF438176	F R	ACCTGTCAGCCACCACATGA TCGTGCAGATCTGGGTCGTA	97
Growth hormone receptor II	*ghr-ii*	AY573601	F R	GAGTGAACCCGGCCTGACAG GCGGTGGTATCTGATTCATGGT	98
Hepcidin	*hepc*	AM749960	F R	ACTCCTGGAAGATGCCGTATGC AACTTACACCTCCTGCGTCCAC	90
Hypoxia inducible factor-1 alpha	*hif-1α*	JQ308830	F R	CAGATGAGCCTCTAACTTGTGGAC TTAGCAAGAATGGTGGCAAGATGAG	95
Immunoglobulin M	*igm*	JQ811851	F R	ACCTCAGCGTCCTTCAGTGTTTATGATGCC CAGCGTCGTCGTCAACAAGCCAAGC	97
Immunoglobulin M membrane-bound form	*migm*	KX599199	F R	GCTATGGAGGCGGAGGAAGATAACA CAGCGTCGTCGTCAACAAGCCAAGC	95
Immunoglobulin T	*igt*	KX599200	F R	GCTGTCAAGGTGGCCCCAAAAG CAACATTCATGCGAGTTACCCTTGGC	100
Immunoglobulin T membrane-bound form	*igt-m*	KX599201	F R	AGACGATGCCAGTGAAGAGGATGAGT CGAAGGAGGAGGCTGTGGACCA	98
Insulin receptor	*insr*	KM522774	F	ACGGACAGCAAGAAGGCAGAGAATC	91

			R	CGAAGGAGGAGGCTGTGGACCA	
Insulin-like growth factor binding protein 1	*igfbp1*	KM522771	F	ACGGACAGCAAGAAGGCAGAGAATC	98
			R	CCGTTCCAAGAGTTCACACACCAG	
Insulin-like growth factor binding protein 2b	*igfbp2*	AF377998	F	AGCGATGTGTCCTGAGATAGTGAG	95
			R	GCACCGTGGCGTGTAGACC	
Insulin-like growth factor binding protein 3	*igfbp3**	MH577191 MH577192	F	ACA GTG CCG TCC ATC CAA	90
			R	GCT GCC CGT ATT TGT CCA	
Insulin-like growth factor binding protein 4	*igfbp4*	KM658998	F	GGCATCAAACACCCGCACAC	100
			R	ATCCACGCACCAGCACTTCC	
Insulin-like growth factor binding protein 5b	*igfbp5b*	MH577194	F	CGACAGGGCAGTCAAAGAAGCTAACC	96
			R	GTCTCGAAGGCATGTGAGCAGAAGG	
Insulin-like growth factor binding protein 6b	*igfbp6b*	MH577196	F	GAT TGC TCA CTG CGG ATC	99
			R	GGA GGG ACA GAC CTT GAA	
Insulin-like growth factor receptor I	*igfr1*	KM522775	F	TCAACGACAAGTACGACTACCGCTGCT	97
			R	CACACTTTCTGGCACTGGTTGGAGGTC	
Insulin-like growth factor receptor II	*igfr2*	KM522776	F	ACATTCGGGCAGCACTCCTAAGAT	92
			R	CCAGTTCACCTCGTAGCGACAGTT	
Insulin-like growth factor-I	*igf-i*	AY996779	F	TGTCTAGCGCTCTTTCCTTTCA	95
			R	AGAGGGTGTGGCTACAGGAGATAC	
Insulin-like growth factor-II	*igf-ii*	AY996778	F	TGGGATCGTAGAGGAGTGTTGT	93
			R	CTGTAGAGAGGTGGCCGACA	
Interleukin-1 beta	*il-1β*	AJ419178	F	GCGACCTACCTGCCACCTACACC	99
			R	TCGTCCACCGCCTCCAGATGC	
Interleukin-6	*il-6*	EU244588	F	TCTTGAAGGTGGTGCTGGAAGTG	90
			R	AAGGACAATCTGCTGGAAGTGAGG	
Interleukin-7	*il-7*	JX976618	F	CTATCTCTGTCCCTGTCCTGTGA	100
			R	TGCGGATGGTTGCCTTGTAAT	
Interleukin-8	*il-8*	JX976619	F	CAGCAGAGTCTTCATCGTCACTATTG	99
			R	AGGCTCGCTTCACTGATGG	
Interleukin-10	*il-10*	JX976621	F	AACATCCTGGGCTTCTATCTG	99
			R	GTGTCCTCCGTCTCATCTG	
Interleukin 12 subunit beta	*il12*	JX976624	F	ATTCCCTGTGTGGTGGCTGCT	100
			R	GCTGGCATCCTGGCACTGAAT	
Interleukin-15	*il-15*	JX976625	F	GAGACCAGCGAGCGAAAGGCATCC	98
			R	GCCAGAACAGGTTACAGGTTGACAGGAA	
Interleukin-34	*il-34*	JX976629	F	TCTGTCTGCCTGCTGGTAG	99
			R	ATGCTGGCTGGTGTCTGG	
Macrophage colony-stimulating factor 1 receptor 1	*csf1r1*	AM050293	F	TTGCGTGTGGTGAGGAAGGAAGGT	98
			R	AGCAGGCAGGGCAGCAGGTA	

Macrophage mannose receptor 1	*mrc1*	KF857326	F R	CTTCCGACCGTACCTGTACCTACTCA CGATTCCAGCCTTCCGCACACTTA	91
Myoblast determination protein 1	*myod1*	AF478568	F R	ATGGAGCTGTCGGATATCTCTTTC GAAGCAGGGGTCATCGTAGAAATC	96
Myocyte-specific enhancer factor 2A	*mef2a*	KM522777	F R	ATGGACGAGAGGAACAGGCAGGTTA GGCTATCTCACAGTCACATAGTACGCTCAG	97
Myocyte-specific enhancer factor 2C	*mef2c*	KM522778	F R	TAGCAACTCCCACTCTACCAGGACAAG GGAATACTCGGCACCATAAGAAGTCG	91
Myogenic factor 5	*myf5*	JN034420	F R	GCATGGTTGACAGCAACAGTCCAGTGT TGTCTTATCGCCCAAAGTGTCGTTCTTCAT	90
Myogenic factor 6	*myf6/mrf4/herculin*	JN034421	F R	GCAGCAATGACAAACCAGAGAGACGGAACA TGTCTTATCGCCCAAAGTGTCGTTCTTCAT	98
Myogenic factor MYOD2	*myod2*	AF478569	F R	CCAACTGCTCTGATGGCATGATGGATTTC GACCGTTTGCTTCTCCTGGACTCGTATG	100
Myostatin/Growth differentiation factor 8	*mstn/gdf-8*	AF258448	F R	AAGAGCAGATCATCTACGGCAAGATCC TCAAGAGCATCCACAACGGTCTACCA	96
NADH-ubiquinone oxidoreductase chain 2	*nd2*	KC217558	F R	TAGGTTGAATGACCATCGTA GGCTAAGGAGTTGAGGTT	97
NADH-ubiquinone oxidoreductase chain 5	*nd5*	KC217559	F R	CCTAAACGCCTGAGCCCTGG GCTGTAAACGAGGTGGCTAGAAGG	97
Peroxiredoxin 3	*prdx3*	GQ252681	F R	CCTAAACGCCTGAGCCCTGG ACCGTTTGGATCAATGAGGAACAGACC	90
Peroxiredoxin 5	*prdx5*	GQ252683	F R	GAGCACGGAACAGATGGCAAGG TCCACATTGATCTTCTTCACGACTCC	90
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1α*	JX975264	F R	CGTGGGACAGGTGTAACCAGGACTC TCCACATTGATCTTCTTCACGACTCC	92
Sirtuin 1	*sirt1*	KF018666	F R	GGTTCCTACAGTTTCATCCAGCAGCACATC CCTCAGAATGGTCCTCGGATCGGTCTC	90
Sirtuin 2	*sirt2*	KF018667	F R	GAACAATCCGACGACAGCAGTGAAG AGGTTACGCAGGAAGTCCATCTCT	98
Sirtuin 5	*sirt5*	KF018670	F R	CAGACATCCTAACCCGAGCAGAG CCACGAGGCAGAGGTCACA	99
Superoxide dismutase [Mn]	*mn-sod/sod2*	JQ308833	F R	CCTGACCTGACCTACGACTATGG AGTGCCTCCTGATATTTCTCCTCTG	93
Toll-likereceptor 1	*tlr1*	KF857322	F R	GGGACCTGCCAGTGTGTAAC AGTGCCTCCTGATATTTCTCCTCTG	92

Toll-likereceptor 2	*tlr2*	KF857323	F R	CATCTGCGACTCTCCTCTCTTCCT GCGTGGATAGAGTTGGACTTGAG	100
Toll-likereceptor 5	*tlr5*	KF857324	F R	TCGCCAATCTGACGGACCTGAG CAGAACGCCGATGTGGTTGTAAGAC	94
Toll-likereceptor 9	*tlr9*	AY751797	F R	GCCTTCCTTGTCTGCTCTTTCT GCCGTAGAGGTGCTTCAGTAG	100
Tumor necrosis factor-alpha	*tnf-α*	AJ413189	F R	CAGGCGTCGTTCAGAGTCTC CTGTGGCTGAGAGCTGTGAG	99
Uncoupling protein 1	*ucp1*	FJ710211	F R	GCACACTACCCAACATCACAAG CGCCGAACGCAGAAACAAAG	99
Uncoupling protein 3	*ucp3*	EU555336	F R	AGGTGCGACTGGCTGACG TTCGGCATACAACCTCTCCAAAG	99
Zeta-chain-associated protein kinase 70	*zap70*	MF175239	F R	TGGTGAAGGAGGAGATGATGAGG GCGAACGATGTAGCGGTTGT	100

(*) Acc. No. MH577191: *igfbp3a*; Acc. No. MH577192:*igfbp3b*. Primers used for *igfbp3* gene expression jointly amplify both *igfbp3a* and *igfbp3b* isoforms.

Appendix III

Immunomodulatory effects of dietary tryptophan supplementation in gilthead seabream (*Sparus aurata)*

Table S1. Anterior gut expression in gilthead seabream at time ø and fed dietary treatments for 2 weeks and 4 weeks. All values are reported as mean ± SD(n=9) (Raw data).

	TØ	**2 weeks**			**4 weeks**		
Gene symbol	CTRL	CTRL	TRP1	TRP2	CTRL	TRP1	TRP2
il-10	3.5E-06 ± 2.5E-06	4.6E-06 ± 2.9E-06	6.1E-06 ± 3.8E-06	5.3E-06 ± 2.2E-06	5.9E-06 ± 1.2E-06	1.4E+01 ± 3.8E+01	2.2E+01 ± 4.4E+01
il-34	0.0116 ± 0.0038	0.0166 ± 0.0067	0.0179 ± 0.0055	0.0197 ± 0.0066	0.0142 ± 0.0055	0.5346 ± 1.4636	0.0237 ± 0.0092
il-1β	0.009 ± 0.006	0.018 ± 0.007	0.021 ± 0.007	0.014 ± 0.012	0.033 ± 0.026	1.406 ± 3.911	0.082 ± 0.181
cd8α	0.0003 ± 0.0001	0.0004 ± 0.0002	0.0008 ± 0.0005	0.0006 ± 0.0005	0.0007 ± 0.0003	0.0008 ± 0.0004	0.0010 ± 0.0006
tnf-α	0.0006 ± 0.0001	0.0012 ± 0.0009	0.0015 ± 0.0006	0.0012 ± 0.0006	0.0012 ± 0.0009	0.0015 ± 0.0006	0.0012 ± 0.0006
IgM	0.0151 ± 0.0103	0.0223 ± 0.0185	0.0387 ± 0.0507	0.0212 ± 0.0174	0.0211 ± 0.0181	0.0281 ± 0.0143	0.0577 ± 0.1112
csfr1	0.0002 ± 0.0001	0.0004 ± 0.0002	0.0007 ± 0.0002	0.0007 ± 0.0003	0.0005 ± 0.0003	0.0012 ± 0.0014	0.0009 ± 0.0007
sod(Mn)	0.0375 ± 0.0313	0.0297 ± 0.0099	0.0371 ± 0.0143	0.0384 ± 0.0217	0.0504 ± 0.0119	0.0902 ± 0.1549	0.0594 ± 0.0226
hsp70	0.3393 ± 0.3178	0.4533 ± 0.0664	0.4512 ± 0.1260	0.4613 ± 0.0541	0.4571 ± 0.0640	3.9782 ± 9.9600	0.6979 ± 0.2094
gpx1	0.1788 ± 0.0762	0.2744 ± 0.1047	0.3394 ± 0.1355	0.3404 ± 0.1873	0.3055 ± 0.1134	1.0161 ± 1.7380	0.4788 ± 0.1932
hep	3.272 ± 1.010	1.918 ± 1.005	3.937 ± 4.035	3.755 ± 2.524	4.729 ± 3.100	4.396 ± 3.243	8.175 ± 8.804
ido2	3.4E-05 ± 1.2E-05	5.5E-05 ± 1.8E-05	7.3E-05 ± 8.3E-05	4.5E-05 ± 9.5E-06	9.8E-05 ± 5.5E-05	2.0E-01 ± 5.2E-01	1.1E-04 ± 7.8E-05

Different symbols indicate difference among time for the same dietary treatment.

Two-way ANOVA

Gene symbol	Time	Diet	Time x diet	Time			Diet		
				TØ	2 weeks	4 weeks	CTRL	TRP1	TRP2
il-10	0.003	0.111	0.617	A	AB	B	-	-	-
il-34	0.984	0.012	0.397	A	B	B	-	-	-
il-1β	0.152	0.474	0.468	A	B	B	-	-	-
cd8α	0.025	0.198	0.258	A	AB	B	-	-	-
tnf-α	0.007	0.961	0.239	A	B	B	-	-	-
IgM	0.509	0.565	0.733	-	-	-	-	-	-
csfr1	0.102	0.003	0.901	A	B	B	-	-	-
sod(Mn)	0.006	0.252	0.151	A	AB	B	-	-	-
hsp70	0.114	0.046	0.122	-	-	-	-	-	-
gpx1	0.05	0.057	0.586	A	B	B	-	-	-
hep	0.062	0.306	0.377	-	-	-	-	-	-
ido2	0.001	0.855	0.653	A	A	B	-	-	-

Values are presented as means ± SD (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences between dietary treatments for the same time. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets. Different symbols indicate difference between times for the same dietary treatment.

Table S2. Anterior gut expression in gilthead seabream at 4 weeks (prior to the inflammatory insult), 4h, 24h and 48h post-injection. All values are reported as mean ± SD(n=9) (Raw data).

	4 weeks			**4 Hours**		
Gene symbol	CTRL	CTRL	CTRL	CTRL	TRP1	TRP2
il-10	5.9E-06 ± 1.2E-06	5.9E-06 ± 1.2E-06	5.9E-06 ± 1.2E-06	8.1E-06 ± 5.7E-06	1.3E-05 ± 1.1E-05	1.4E-05 ± 8.2E-06
il-34	0.0142 ± 0.0055	0.0142 ± 0.0055	0.0142 ± 0.0055	0.0120 ± 0.0048	0.0101 ± 0.0059	0.0128 ± 0.0057
il-1β	0.033 ± 0.026	0.033 ± 0.026	0.033 ± 0.026	0.1466 ± 0.1261	0.1389 ± 0.1059	0.3367 ± 0.3850
cd8α	0.0007 ± 0.0003	0.0007 ± 0.0003	0.0007 ± 0.0003	0.0003 ± 0.0002	0.0004 ± 0.0002	0.0006 ± 0.0003
tnf-α	0.0012 ± 0.0009	0.0012 ± 0.0009	0.0012 ± 0.0009	0.0007 ± 0.0003	0.0009 ± 0.0006	0.0013 ± 0.0012
IgM	0.0211 ± 0.0181	0.0211 ± 0.0181	0.0211 ± 0.0181	0.0267 ± 0.0288	0.0363 ± 0.0288	0.0413 ± 0.0369
csfr1	0.0005 ± 0.0003	0.0005 ± 0.0003	0.0005 ± 0.0003	0.0003 ± 0.0002	0.0003 ± 0.0002	0.0004 ± 0.0002
sod(Mn)	0.0504 ± 0.0119	0.0504 ± 0.0119	0.0504 ± 0.0119	0.0303 ± 0.0136	0.0300 ± 0.0139	0.0363 ± 0.0117
hsp70	0.4571 ± 0.0640	0.4571 ± 0.0640	0.4571 ± 0.0640	0.4086 ± 0.1395	0.3687 ± 0.1255	0.4335 ± 0.1840
gpx1	0.3055 ± 0.1134	0.3055 ± 0.1134	0.3055 ± 0.1134	0.2168 ± 0.0706	0.1794 ± 0.0531	0.2942 ± 0.1597
hep	4.729 ± 3.100	4.729 ± 3.100	4.729 ± 3.100	2.7699 ± 1.4139	3.9143 ± 2.0672	6.6015 ± 4.9299
ido2	9.8E-05 ± 5.5E-05	9.8E-05 ± 5.5E-05	9.8E-05 ± 5.5E-05	6.9E-05 ± 3.9E-05	3.7E-05 ± 2.0E-05	6.0E-05 ± 3.6E-05

	24 Hours			48 Hours		
Gene symbol	CTRL	TRP1	TRP2	CTRL	TRP1	TRP2
il-10	4.6E-06 ± 3.2E-06	4.0E-06 ± 9.5E-07	9.2E-06 ± 5.0E-06	3.1E-06 ± 1.1E-06	4.1E-06 ± 9.7E-07	3.8E-06 ± 1.9E-06
il-34	0.0115 ± 0.0045	0.0104 ± 0.0047	0.0121 ± 0.0050	0.0060 ± 0.0019	0.0086 ± 0.0035	0.0124 ± 0.0195
il-1β	0.0521 ± 0.0409	0.0717 ± 0.0350	0.0893 ± 0.0503	0.0412 ± 0.0220	0.0280 ± 0.0272	0.0723 ± 0.0781
cd8α	0.0004 ± 0.0003	0.0005 ± 0.0004	0.0007 ± 0.0006	0.0003 ± 0.0001	0.0005 ± 0.0002	0.0008 ± 0.0008
tnf-α	0.0019 ± 0.0014	0.0016 ± 0.0007	0.0027 ± 0.0018	0.0010 ± 0.0003	0.0014 ± 0.0004	0.0020 ± 0.0012
IgM	0.0133 ± 0.0055	0.0140 ± 0.0064	0.0231 ± 0.0182	0.0098 ± 0.0058	0.0121 ± 0.0049	0.0391 ± 0.0410
csfr1	0.0004 ± 0.0001	0.0005 ± 0.0002	0.0004 ± 0.0002	0.0003 ± 0.0002	0.0004 ± 0.0002	0.0006 ± 0.0008
sod(Mn)	0.0308 ± 0.0108	0.0299 ± 0.0110	0.0399 ± 0.0151	0.0295 ± 0.0111	0.0316 ± 0.0099	0.0286 ± 0.0103
hsp70	0.3728 ± 0.0721	0.3994 ± 0.0807	0.3732 ± 0.1560	0.3924 ± 0.0893	0.4430 ± 0.0947	0.3690 ± 0.0954
gpx1	0.1627 ± 0.0465	0.2093 ± 0.0687	0.3103 ± 0.2777	0.1673 ± 0.0440	0.2132 ± 0.0375	0.2407 ± 0.1524
hep	1.2944 ± 0.9768	0.9393 ± 0.3896	1.4446 ± 0.7074	2.3910 ± 1.1098	2.1450 ± 0.6388	5.0289 ± 9.8390
ido2	5.0E-05 ± 1.9E-05	4.9E-05 ± 1.4E-05	5.5E-05 ± 2.6E-05	5.0E-05 ± 2.0E-05	6.6E-05 ± 1.4E-05	6.1E-05 ± 2.4E-05

Two-way ANOVA

Gene symbol	Time	Diet	Time x diet	Time			Diet		
				4 Weeks	4 Hours	24 Hours	48 Hours	CTRL	TRP1	TRP2
il-10	<0.001	0.005	0.489	B	B	A	A	A	AB	B
il-34	<0.001	0.095	0.436	C	B	B	A	A	AB	B
il-1β	<0.001	0.685	0.858	A	B	AB	A	-	-	-
cd8α	0.001	0.018	0.032	B	A	A	A	A	AB	B
tnf-α	<0.001	0.107	0.597	B	A	B	B	-	-	-
IgM	0.175	0.063	0.929	-	-	-	-	-	-	-
csfr1	<0.001	0.058	0.416	B	AB	A	A	-	-	-
sod(Mn)	<0.001	0.069	0.432	B	A	A	A	-	-	-
hsp70	<0.001	0.570	0.054	B	A	A	A	-	-	-
gpx1	<0.001	0.007	0.657	B	A	A	A	A	AB	B
hep	<0.001	0.392	0.736	C	BC	A	B	-	-	-
ido2	<0.001	0.576	0.254	B	A	A	A	-	-	-

Values are presented as means ± SD (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences between dietary treatments for the same time. Different capital letters indicate differences between diets regardless of time or difference between times regardless of diets. Different symbols indicate difference between times for the same dietary treatment.

Appendix IV

Short-term supplementation of dietary arginine and citrulline modulates gilthead seabream (*Sparus aurata*) immune status

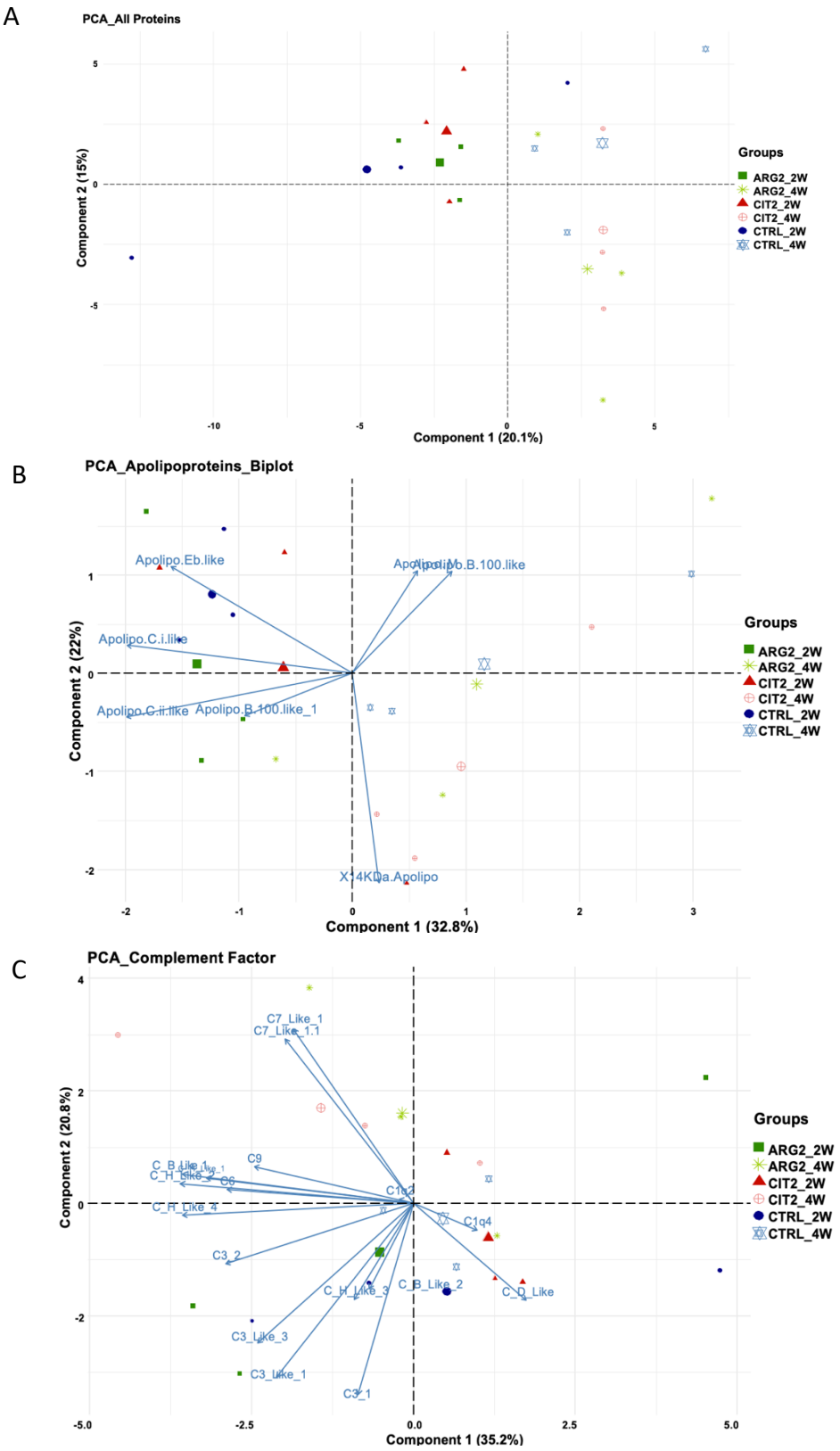


FIGURE S1. Principal component analysis (PCA) of plasma proteomics signatures of fishes fed the experimental diets. List of the 94 identified proteins can be found on table S3. **(A)** PCA score plots of all proteins analysed along the two main components, **(B)** PCA score plots of proteins belonging to the apolipoproteins family. **(C)** PCA score plots proteins belonging to the complement family.

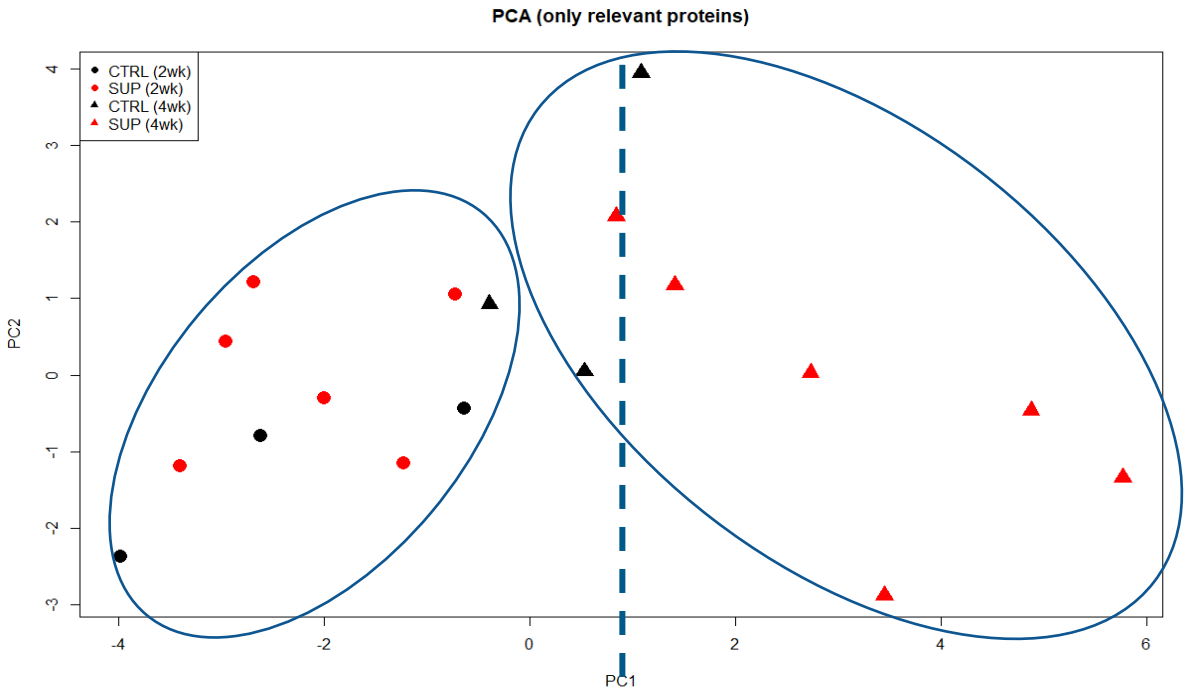


FIGURE S2. Principal component analysis (PCA) of plasma proteomic signatures of fish fed the experimental diets. PCA score plots of the 19 most relevant proteins analysed along the two main components, considering all supplemented diets as a pool designated as SUP.

Table S1. Proteins identified in plasma of fish fed all experimental diets.

Accession	Description
Sa_26882.2.1	complement C3-like
Sa_21997.2.1	fibrinogen beta chain
Sa_9899.8.1	ceruloplasmin-like
Sa_19340.12.5	complement factor B-like
Sa_33122.4.1	14 kDa apolipoprotein
Sa_44333.2.1	kininogen-1-like
Sa_19340.12.7	complement factor B-like
Sa_27286.2.1	beta-2-glycoprotein 1-like
Sa_26882.1.1	complement component C3
Sa_944.7.1	N-acetylmuramoyl-L-alanine amidase-like
Sa_23416.2.2	Angiotensinogen
Sa_32547.1.2	apolipoprotein Eb-like
Sa_22208.2.1	apolipoprotein B-100-like
Sa_1704.2.1	complement factor H-like
Sa_23268.1.1	complement component C6
Sa_12582.3.1	sex hormone-binding globulin
Sa_27317.1.1	complement component C9
Sa_48024.10.1	alpha-1-acid glycoprotein 1-like
Sa_2627.1.1	complement factor H-like
Sa_30666.1.1	proteoglycan 4-like isoform X2
Sa_27286.3.1	beta-2-glycoprotein 1-like
Sa_1704.3.2	complement factor H-like
Sa_16459.1.1	lumican
Sa_37816.2.2	cartilage acidic 1-like
Sa_32547.2.1	apolipoprotein C-I-like
Sa_1704.1.1	complement factor H-like
Sa_22432.2.1	complement C3-like
Sa_8250.3.1	serum amyloid P-component-like
Sa_48024.9.1	alpha-1-acid glycoprotein 1-like
Sa_41650.2.1	alpha-2-antiplasmin-like
Sa_27134.1.1	plexin-A4 isoform X1
Sa_13538.10.1	inter-alpha-trypsin inhibitor heavy chain H2
Sa_22432.3.1	Complement C3
Sa_736.1.1	immunoglobulin light chain isotype partial
Sa_19875.1.1	complement factor D-like
Sa_15521.3.1	catechol O-methyltransferase domain-containing 1-like
Sa_41650.1.1	pigment epithelium-derived factor
Sa_25136.3.1	phosphoglycerate mutase 2
Sa_16666.6.1	coagulation factor XIII A chain
Sa_46975.2.1	myelin zero 2
Sa_22208.1.1	apolipoprotein B-100-like
Sa_15740.1.1	Cytosolic phospholipase A2 gamma

Sa_17989.1.1	platelet glycoprotein V-like
Sa_33.3.1	Interleukin-4 receptor subunit alpha
Sa_8915.3.1	ependymin-1-like
Sa_33122.3.1	apolipoprotein C-II-like
Sa_18092.7.1	Z-dependent protease inhibitor-like
Sa_17271.1.1	triosephosphate isomerase
Sa_16733.6.1	apolipoprotein M
Sa_4480.1.2	beta-enolase
Sa_4774.6.1	peroxiredoxin 2
Sa_23268.2.1	complement component C7-like
Sa_34833.4.1	cytoplasmic 2
Sa_52479.3.1	fibrinogen 1
Sa_31172.5.1	glyceraldehyde-3-phosphate dehydrogenase
Sa_9379.3.3	glycogen muscle form
Sa_43047.2.1	complement C1q 2
Sa_7568.2.1	alpha-2-HS-glyco -like
Sa_45700.1.1	Ubiquitin-40S ribosomal S27a
Sa_46414.1.1	complement component C7-like
Sa_4795.1.1	phosphoglucomutase-1
Sa_4196.3.1	3-hydroxyanthranilate 3 4-dioxygenase
Sa_10407.1.1	plexin-A4
Sa_14444.2.1	histone H4- partial
Sa_18064.4.1	Peptide-N(4)-(N-acetyl-beta-D-glucosaminyl)asparagine amidase F
Sa_35438.1.1	glutathione peroxidase 3
Sa_17527.8.1	creatine kinase B-type isoform X1
Sa_20361.1.1	laminin subunit gamma-3-like
Sa_46557.1.1	lysyl oxidase homolog 4-like
Sa_19603.2.1	cadherin-5-like
Sa_4088.1.1	histone H3
Sa_20086.1.1	Carboxypeptidase Z
Sa_19293.4.1	coagulation factor VIII-like
Sa_13814.1.1	intelectin-like
Sa_10005.8.1	tubulin beta chain
Sa_24913.2.4	coagulation factor IX
Sa_14443.1.1	histone H2A-like
Sa_9500.7.1	thrombospondin-4-B-like
Sa_11647.2.1	vitronectin-like
Sa_8250.6.4	RIIa domain-containing 1 isoform X4
Sa_1942.4.1	fucose mutarotase isoform X1
Sa_18502.2.1	leucine-rich PPR motif-containing mitochondrial
Sa_28742.5.1	TBC1 domain family member 10A-like
Sa_40837.1.1	hyaluronan and proteoglycan link 1-like
Sa_13080.2.1	complement C1q 4
Sa_27877.1.1	threonine-protein kinase 494
Sa_17242.1.2	14-3-3 epsilon isoform X1
Sa_30036.2.1	nidogen-1

Sa_18106.7.1	LETM1 and EF-hand domain-containing mitochondrial isoform X1
Sa_5819.3.1	78 kDa glucose-regulated
Sa_12688.1.1	cdc42-interacting 4 homolog isoform X2
Sa_20976.2.1	elongation factor 2-like

Table S2. Quantitative expression of immune-related genes in the head-kidney of gilthead seabream fed the dietary treatments during 2 and 4 weeks.

	2 weeks					4 weeks				
Gene symbol	CTRL	ARG1	ARG2	CIT1	CIT2	CTRL	ARG1	ARG2	CIT1	CIT2
il-10	0.0003 ± 0.0001	0.0003 ± 0.0001$^{\#}$	0.0004 ± 0.0002	0.0004 ± 0.0001	0.0003 ± 0.0002	0.0001 ± 0.0000^{ab}	0.0001 ± 0.0000^{b*}	0.0000 ± 0.0000 ^{ab}	0.0000 ± 0.0000 ^{ab}	0.0000 ± 0.0000^{a}
il-34	0.0120 ± 0.0023	0.0098 ± 0.0026	0.0104 ± 0.0031	0.0144 ± 0.0057	0.0088 ± 0.0028	0.0166 ± 0.0060	0.0121 ± 0.0051	0.0115 ± 0.0033	0.0139 ± 0.0037	0.0116 ± 0.0047
Il4-13	0.0021 ± 0.0012	0.0021 ± 0.0010	0.0023 ± 0.0007	0.0021 ± 0.0008	0.0018 ± 0.0010	0.0027 ± 0.0013	0.0019 ± 0.0009	0.0016 ± 0.0003	0.0022 ± 0.0007	0.0018 ± 0.0011
il-1β	0.0034 ± 0.0036	0.0052 ± 0.0041	0.0069 ± 0.0055	0.0036 ± 0.0025	0.0072 ± 0.0062	0.0054 ± 0.0040	0.0081 ± 0.0065	0.0069 ± 0.0087	0.0048 ± 0.0063	0.0011 ± 0.0011
cd8α	0.0004 ± 0.0002	0.0004 ± 0.0002	0.0004 ± 0.0002	0.0004 ± 0.0002	0.0004 ± 0.0002	0.0003 ± 0.0002	0.0002 ± 0.0002	0.0002 ± 0.0001	0.0002 ± 0.0003	0.0001 ± 0.0001
cd4	0.0805 ± 0.0294	0.0717 ± 0.0243	0.0683 ± 0.0278	0.0647 ± 0.0320	0.0441 ± 0.0096	0.0719 ± 0.0186	0.0912 ± 0.0317	0.0754 ± 0.0192	0.0796 ± 0.0395	0.0669 ± 0.0438
tnf-α	0.0001 ± 0.0001	0.0002 ± 0.0001	0.0002 ± 0.0001	0.0002 ± 0.0001	0.0002 ± 0.0001	0.0001 ± 0.0000	0.0001 ± 0.0001	0.0001 ± 0.0001	0.0001 ± 0.0001	0.0001 ± 0.0001
IgM	1.1329 ± 0.5657	1.6613 ± 0.5610	1.1371 ± 0.4185	1.3900 ± 0.6531	1.2833 ± 0.4807	1.4978 ± 0.8463	1.4978 ± 0.8463	1.4978 ± 0.8463	1.4978 ± 0.8463	1.4978 ± 0.8463
tcr	0.1041 ± 0.0403	0.1012 ± 0.0255	0.0855 ± 0.0290	0.0987 ± 0.0531	0.0639 ± 0.0221	0.0951 ± 0.0665	0.1011 ± 0.0632	0.1176 ± 0.0425	0.1032 ± 0.0782	0.0861 ± 0.0645
csfr	0.0002 ± 0.0002	0.0002 ± 0.0001	0.0002 ± 0.0000	0.0002 ± 0.0001	0.0002 ± 0.0001	0.0002 ± 0.0002	0.0003 ± 0.0002	0.0003 ± 0.0002	0.0004 ± 0.0002	0.0002 ± 0.0001
tgfb	0.0412 ± 0.0106	0.0447 ± 0.0106	0.0424 ± 0.0089	0.0451 ± 0.0094	0.0369 ± 0.0087	0.0366 ± 0.0216	0.0418 ± 0.0193	0.0489 ± 0.0227	0.0421 ± 0.0182	0.0407 ± 0.0270
arg-II	0.0170 ± 0.0079	0.0179 ± 0.0029	0.0203 ± 0.0080	0.0236 ± 0.0108	0.0161 ± 0.0068	0.0201 ± 0.0092	0.0202 ± 0.0069	0.0202 ± 0.0080	0.0267 ± 0.0351	0.0115 ± 0.0039

Two-way ANOVA

Gene symbol				Time		Diet				
	Time	**Diet**	**Time x diet**	**2 weeks**	**4 weeks**	**CTRL**	**ARG1**	**ARG2**	**CIT1**	**CIT2**
il-10	0.005	0.037	0.021	-	-	-	-	-	-	-
il-34	0.039	0.021	0.577	-	-	AB	AB	AB	B	A
Il4-13	0.867	0.425	0.445	-	-	-	-	-	-	-
il-1β	0.997	0.392	0.101	-	-	-	-	-	-	-
cd8α	0.004	0.163	0.738	B	A	-	-	-	-	-
cd4	0.105	0.161	0.635	-	-	-	-	-	-	-
tnf-α	<0.001	0.544	0.755	B	A	-	-	-	-	-
IgM	0.245	0.791	0.088	-	-	-	-	-	-	-
tcr	0.384	0.506	0.786	-	-	-	-	-	-	-
csfr	0.003	0.306	0.284	A	B	-	-	-	-	-
tgfb	0.991	0.721	0.851	-	-	-	-	-	-	-
arg-II	0.815	0.234	0.908	-	-	-	-	-	-	-

Values are presented as means ± SD (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). Tukey *post-hoc* test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same time. Different capital letters indicate differences among diets regardless time or time regardless diets. Different symbols indicate difference among time for the same dietary treatment.