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Dietary tryptophan supplementation and its modulatory role in juvenile European seabass (*Dicentrarchus labrax*) during chronic inflammation

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Tese de Candidatura ao grau de Mestrado em Ciências do Mar - Recursos Marinhos submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Porto 2021

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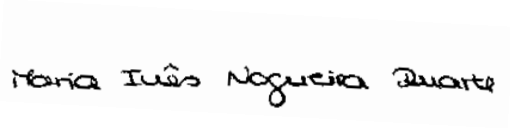

Maria Inês Nogueira Duarte

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Abstract

Amino acids (AA) in addition to their roles in protein synthesis, habe been shown to influence central metabolic pathways, which are critical in the establishment of an effective immune response. Tryptophan complex metabolism results in several metabolites that, directly or indirectly, regulate a wide array of physiological functions, including immune-related processes. Since the immune system is responsible for fish homeostasis through the stimulation and mobilization of various cellular and humoral parameters, it is of utmost importance to investigate the capacity of immunomodulators such as tryptophan to better equip the animal against potential hazardous scenarious. The present study aimed to gather new insights on the modulatory effects of tryptophan during chronic inflammation in European seabass (*Dicentrarchus labrax*).

A total of 192 fish (34.55 ± 7.84 g) were randomly distributed into 12 tanks of a recirculating seawater system. Two dietary treatments were evaluated: a control diet, meeting the nutritional requirements of seabass (CTRL) and a CTRL-based diet supplemented with tryptophan (TRP). After the acclimatization period being fed CTRL, fish started to be fed also on TRP, and an inflammatory response was induced by intraperitoneally injecting fish with Freund's Incomplete Adjuvant (FIA). A control group was instead injected with a sham saline solution (Hank's Balanced Salt Solution, HBSS). Fish were fed twice a day for 28 days and were sampled at 7, 14, 21 and 28 days post-injection. The haematological profile, plasma humoral parameters as well as gut immune and oxidative stress parameters were assessed.

Regarding response to inflammation, inhibition of gut immunity was observed while leucocyte response was locally enhanced in FIA-injected fish. Data also pointed out a redistribution of peripheral energy towards the inflamed site. Results also suggest that fish immune response was altered under higher dietary tryptophan availability. When TRP was provided, fish humoral bactericidal activity and cell response shown to be compromised, highlighting a possible tryptophan-mediated immune suppression. Further studies need to be conducted regarding the neuroendocrine response (i.e. plasma cortisol levels), since no significant modulatory effects of tryptophan nor inflammation were observed.

Tryptophan modulatory effects during the inflammatory response are important indicators of this AA's role in fish immunity, thereby pointing out tryptophan as a potential functional ingredient to be used as regulator of the immune response.

Key-words: European Seabass, Amino acids, Chronic inflammation, Immunomodulation, Tryptophan

Resumo

Os aminoácidos (AA), além do seu papel na síntese proteica, influenciam vias metabólicas centrais, que são críticas no estabelecimento de uma resposta imune eficaz. O metabolismo complexo do triptofano resulta em vários metabolit*os* que, direta ou indiretamente modulam uma ampla gama de funções fisiológicas, incluindo processos relacionados com o sistema imunológico. Uma vez que o sistema imunológico é responsável pela homeostase dos peixes por meio da estimulação e mobilização de vários parâmetros celulares e humorais, é de extrema importância investigar a capacidade de imunomodeladores como o triptofano em melhor equipar o animal contra potenciais cenários de risco. O presente estudo teve como objetivo reunir novos conhecimentos sobre os efeitos modeladores do triptofano durante a inflamação crónica de robalo (*Dicentrarchus labrax*).

Um total de 192 peixes (34,55 ± 7,84 g) foram distribuídos aleatoriamente em 12 tanques de recirculação. Foram avaliados dois tratamentos dietéticos: uma dieta controlo, atendendo às exigências nutricionais do robalo (CTRL) e uma dieta à base da dieta CTRL suplementada com triptofano (TRP). Após uma semana de aclimatação sendo alimentados com CTRL, os peixes começaram a ser também alimentados com TRP, e foram submetidos a uma inflamação peritoneal ao serem injetados intraperitonealmente com Adjuvante Incompleto de Freund (FIA). Em paralelo, um outro grupo de peixes foi injetado com uma solução salina (Solução Salina Equilibrada de Hank, HBSS) para servir como grupo controlo. Os peixes foram alimentados duas vezes ao dia durante 28 dias e amostrados aos 7, 14, 21 e 28 dias após a injeção. Foram avaliados o perfil hematológico, parâmetros humorais do plasma, bem como parâmetros imunológicos e do stress oxidativo no intestino anterior.

Em relação à resposta à inflamação, a inibição da imunidade intestinal foi observada enquanto a resposta dos leucócitos foi localmente aumentada em peixes injetados com FIA. Os resultados deste trabalho também podem refletir uma redistribuição da energia periférica em direção ao local inflamado. Relativamente ao efeito da dieta experimental, os resultados sugerem que o estado imunológico dos peixes alimentados com TRP foi alterado. Quando a dieta TRP foi fornecida, a atividade bactericida humoral dos peixes e a resposta celular mostraram-se comprometidas, indicando uma possível supressão imunológica. Mais estudos precisam ser feitos em relação à resposta neuroendócrina (ou seja, níveis de cortisol plasmático), uma vez que não foram observados efeitos modulatórios significativos do triptofano nem da inflamação.

Este efeito modelador do triptofano durante a resposta inflamatória é mais um importante indicador do seu papel na imunidade dos peixes. Assim, este estudo evidencia o potencial deste aminoácido como suplemento para dietas funcionais para peixes, em estratégias de regulação da resposta imunitária.

Palavras-chave: Robalo, Aminoácidos, Inflamação crónica, Imunomodelação, Triptofano

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List of abbreviations

5-HT	Serotonin
AA	Amino acids
ACTH	Adrenocorticotropic hormone
CFU	Colony forming units
CTRL	Control
DNA	Deoxyribonucleic acid
DTNB	5,5'- dithiobis-2-nitrobenzoic acid
FAO	Food and Agriculture Organization
FIA	Freund's Incomplete Adjuvant
GSH	Total glutathione
GSSG	Oxidized glutathione
HBSS	Hank's balanced salt solution
HPI	Hypothalamus-pituitary-interrenal
Ht	Haematocrit
i.p.	Intraperitonal injection
IDO	Indoleamine 2,3-dioxygenase
IgD	Immunoglobulin D
IgM	Immunoglobulin M
IgT	Immunoglobulin T
IgZ	Immunoglobulin Z
KP	Kynurenine pathway
LPO	Lipid peroxidation
LPS	Lipopolyssacharide
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MHC	Major histocompatibility complex
MPA	Metaphosphoric acid solution
NADPH	β- nicotinamide adenine dinucleotide phosphate
NKC	Natural killer cells
PAMP	Pathogens-associated molecular patters
Phdp	*Photobacterium damselae* subsp. *piscicida*

PRP	Pattern recognition protein
PRR	Pattern recognition receptor
RAS	Recirculating aquaculture system
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SDG	Sustainable development goals
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TDO	Tryptophan 2,3-dioxygenase
TLR	Toll-like receptor
TMB	3,3’,5,5’-tetramethylbenzidine hydrochloride
TNFα	Tumor necrosis factor α
TRP	Tryptophan-supplemented diet
TSA	Tryptic soy agar
UV	Ultra violet
WBC	White blood cell

Introduction

1. World Aquaculture and Fisheries

1.1. Current state of affairs

The current global context allied with the ever-increasing world population, climate crises, competition for natural resources, along with the aggravation of the economic instability due to the covid-19 pandemic, has resulted in one of the world's most challenging task, providing food to over 7 billion people. Taking this into account, in 2015 the 2030 Agenda was adopted by the United Nations General Assembly for a more Sustainable Development. Although all sustainable development goals are applicable to a wide range of sectors within the aquaculture industry, the ones specifically involved are as follows: SDG 8 Decent work and economic growth; SDG 12 Responsible Consumption and Production; SDG 13 Climate Action; SDG 14 Life Bellow Water (Assembly, 2015). Together these goals aim to stimulate a sustainable development within the aquaculture industry and to counteract bottlenecks associated with intensive fish production.

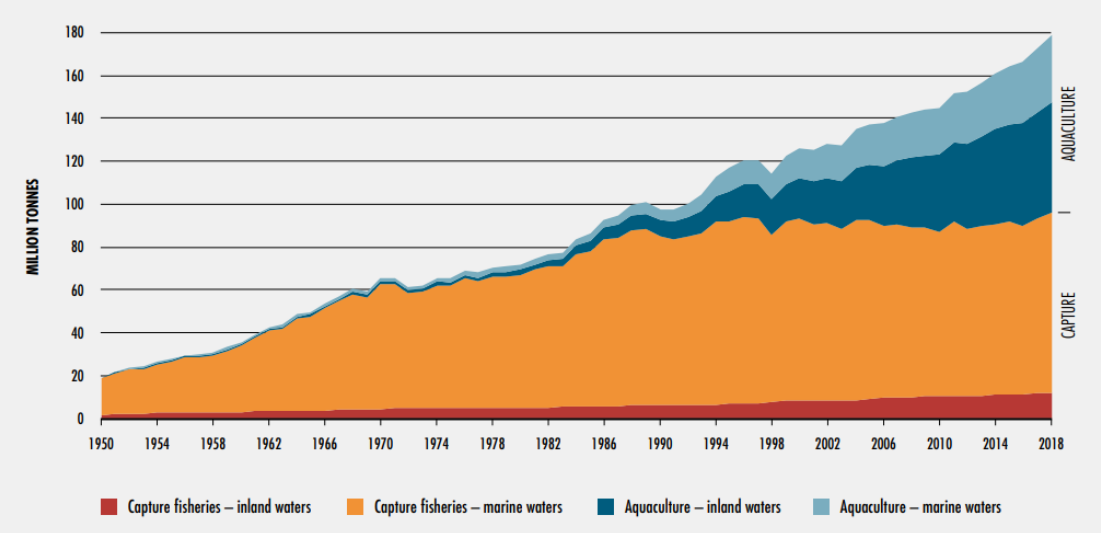

Figure 1 Evolution of capture fisheries and aquaculture production from 1950 to 2018. Source: (FAO, 2020).

As time passes, aquaculture has proven to be a reliable and indispensable source of fish for human consumption, as the demand for seafood and fish-related goods keeps increasing. Nonetheless, in order to bridge the supply-demand gap, aquaculture has undergone a rapid growth (Figure 1) (Ahmed & Thompson, 2019). This growth has been fuelled by a number of factors that together contribute to a rising per capita fish supply as well as feed consumption (FAO, 2020). Some examples of these factors include the expansion of global fish trade, stagnant fisheries, competitive fish pricing, salary increases,

and increased knowledge of fish health benefits (Naylor *et al.*, 2021). By 2018, global per capita fish supply amounted to 20.5 kg in contrast with 9.0 kg amounted in 1961. In addition to this, global fish consumption increased at an average rate of 3.1 percent from 1961 to 2017, surpassing the overall population growth (FAO, 2020). Nonetheless, there is still a long way to go and the need to adopt new measures capable to fulfil the ongoing demand for fish supply, when acting in accordance with the 2030 Agenda, persists. By the period of 2019-2028 is to be expected a 10 percent increase in fish production, as well as 9 percent rise in the world population over the same period. Moreover, fish per capita consumption is estimated to be 21.3 kg, which translates to 25 million tonnes of fish destined for human intake, which aquaculture is likely to provide (OECD-FAO, 2019). These estimates are indicative of how relevant aquaculture will continue to be when it comes to the supply of fish and seafood to the world.

According to FAO, despite the attempts to counter the pattern seen in previous years, the overall state of the world's fish marine stocks keeps declining. Recently, despite being a popular issue, the sustainability and recovery of the fishing industry is still unknown (FAO, 2020). As the recovery of overexploited or exploited stocks at the threshold of sustainability are still uncertain, it is still prudent to expect and rely in aquaculture to meet the majority of the world's fish needs. Nonetheless, the aquaculture's growth arises new problems and other challenges, as in any other industry that experienced a rapid growth and progress (Rosenthal, 1985).

2. European seabass (*Dicentrarchus labrax*)

The European seabass, *Dicentrarchus labrax* (Linnaeus, 1758) (Figure 2) is a marine perciform fish species with a strong representation in Mediterranean aquaculture, being naturally distributed in the Mediterranean, Black Sea and along the Eastern Atlantic coast (FAO, 2009).This species is both eurythermal (5-28 °C) and euryhaline (3 ppm to full-length seawater), which enables it to be found in the wild in coastal areas, in estuaries, brackish-water lagoons and occasionally in freshwater rivers (Moretti *et al.*, 1999).

In natural settings, sexual maturity occurs at 3 years in males and 4 years in females, however this values differ when under farming conditions to 2 years for males and 3 years for females (Gorshkov *et al.*, 2004).Considering the Mediterranean population, the female gonadal maturation initiates in September with spawning taking place in winter, December to March. Seabass are voracious predators and in the wild their feeding range includes crustaceans, molluscs and fish (FAO, 2009).

Figure 2 European seabass, *Dicentrarchus labrax* (Linnaeus, 1758). Photograph: Inês Duarte, 2021

Currently, *Dicentrarchus labrax* is one of the most relevant marine fish species reared in the Mediterranean and in Europe aquaculture industry. The growth of the industrial production remained low until the 1980s, having experienced a rapid growth during the 1990s with the introduction of intensive and semi-intensive production systems in the industry (Barazi-Yeroulanos, 2010). Presently, Turkey is the main producer of European seabass, followed by Greece, Spain and Egypt (FAO, 2009).

Although European seabass has become one of the most important fish species in the Mediterranean aquaculture industry, its production faces a number of challenges that ultimately result in an impact on the production efficiency, often related to environmental conditions, feed and nutrition quality and disease outbreaks (Kousoulaki *et al.*, 2015). FAO reports describe the European seabass as vulnerable to a wide range of diseases often linked to the stressful rearing conditions they are subject to, despite its sturdiness (FAO, 2009). As there is a shortage of appropriate and approved chemotherapeutics in the aquaculture industry, the urgency of alternate methods and procedures to enhance the response of fish to stress and immune challenge conditions is called for (FAO, 2009).

3. Aquaculture constraints: threats to fish health and welfare

An intensive production has inherent dangers regarding the environment, farmed fish welfare and ultimately, final consumers. Although hardly unavoidable, they are seen as the biggest fish production bottlenecks. A good example of this is the alarming rise in antibiotic resistance and tolerance (Defoirdt *et al.*, 2011), whether through the overuse of therapeutics or by the under dosing of pharmaceuticals, has resulted in the presence of antibiotic residues in fish, as well as in the surrounding environment (Haya *et al.*, 2001; Miller & Harbottle, 2018), which ultimately affects the final consumer. Both of these cases are commonly seen in aquaculture farms as a result of the lack of available and licensed therapies (FAO, 2009) and thus the recurrent use of the same chemotherapy drugs are

hardly unavoidable. This combined with the confinement of fish, by the high stocking density or through the handling and transportation performed by fish farms operatives (Barton, 2002; Miranda *et al.*, 2018), create without a doubt a stressful enviornment. These conditions, which are commonly associated with efforts to improve productivity and profitability, favour the emergence and spread of bacterial infections within aquaculture units (Cabello *et al.*, 2013).

Even though producers are legally and ethically required to assure the health and welfare of the fish under their care, it is hard to get rid of all stress-inducing factors associated with the production process. As stress threatens and disrupts the homeostatic balance, as an adaptive response in order to overcome these challenges, fish diverts energy required from normal growth and metabolism to counteract stressors (Bonga, 1997). As a result, the immune response shows a decline in effectiveness that eventually will result in immune suppression and predisposition to disease and infection (Conceição *et al.*, 2012). Stressful rearing conditions are also known to affect fish amino acid requirements and metabolism, thus a rise in the demand of certain indispensable amino acids may occur, likely due to the synthesis of proteins and specific molecules related with the immune response (Aragão *et al.*, 2008; Conceição *et al.*, 2012; Costas *et al.*, 2008; Costas *et al.*, 2011b).

4. Teleost immune system

4.1. Fish immune system

The teleost immune system is composed of innate and adaptive immune responses, which together provide a complex network of molecules and signalling pathways in order to shield the host against pathogenic organisms. Considering an evolutionary point of view, fish are the earliest phylogenetic group exhibiting both innate and adaptive immunity (Verburg-van Kemenade *et al.*, 2009), with the latter defence mechanism not being as developed as the ones found in higher vertebrates (Warr, 1995). Both branches of the fish immune system, even though they operate together, differ in specificity and speed response. Initially, kicks in the innate immunity that functions as a broad response, involving generic and non-specific methods of recognition of pathogens in order to prevent their dispersal (Ellis, 2001). The rapid manner of such response incapacitates the pathogenic organism to attach and multiply in the host; however, due to lack of specificity, there is a risk of damaging healthy tissue (Parkin & Cohen, 2001). If the infection/inflammation continues unresolved, the specific defence mechanism is activated, which is known to be limited when compared to mammals, due to the poikilothermic nature of fish (Tort *et al.*, 2003). Since the adaptive immunity is temperature-dependent its activation state and

readiness of response is sacrificed and, as a result, there is a limited antibody repertoire and slow immune cell proliferation (Ellis, 2001; Magnadóttir, 2006).

4.2. The innate immune system

Fish, as aquatic animals, are found in diverse and extreme aquatic environments, being in constant and close interaction with a high microbial load milieu, with fish skin, skin mucus, gills and gut, continuously being challenged by external stressors. Fish highly rely in robust defence mechanisms that enable them to achieve tolerance and an effective inflammatory response (Figure 3). The innate immune system is commonly made up of three compartments: the epithelial and mucosal barrier, the humoral compounds and the cellular components (Table 1). The first line of defence of fish against pathogenic organisms and external stressors is represented by the epithelia that involves the gills, skin and gut (Cabillon & Lazado, 2019). In addition to being an extremely important physical and mechanical layer of protection, fish mucus contains several non-specific and specific immune defence parameters, such as antimicrobial peptides, lectins, lysozymes, complement factors and immunoglobulins (Ig) (Bonga, 1997; Castro & Tafalla, 2015; Magnadottir, 2010; Magnadóttir, 2006; Palaksha *et al.*, 2008; Rakers *et al.*, 2013; Whyte, 2007).

The teleost immune system relies on different strategies of immune recognition in order to maintain homeostasis: recognition of nonself (pathogens, microbes, etc.), recognition of self and ultimately the recognition of abnormal self (Charles A. Janeway & Medzhitov, 2002). These strategies are of utmost importance for animals because they enable them to distinguish between potentially infectious nonself and self, as well as differentiate pathogens from commensals organisms. This recognition strategy is focused on the recognition of conserved molecular patterns characteristic of microbial and pathogen physiology (Mogensen, 2009). Innate immune recognition is based on the detection of molecular structures that are exclusive to microorganisms, enabling the host to recognize pathogen associated molecular patterns (PAMPs) (Medzhitov, 2007). Hence, infection is recognized by germline-encoded pattern recognition receptors (PRR) or pattern recognition proteins (PRP), which function as molecular sentinels capable of recognizing conserved microbial components and inducing the production of pro-inflammatory mediators (Barton, 2008; Palti, 2011). Examples of PAMPs are peptidoglycans and lipopolysaccharides (LPS) present in cell walls of gram-positive and gram-negative bacteria (respectively), fungal β 1, 3- glucan, viral double stranded RNA and bacterial DNA (Magnadóttir, 2006). PRRs can either be found as soluble components just like the complement protein C3 and lectins (Magnadóttir, 2006; Verburg-van Kemenade *et al.*, 2009), or expressed as membrane receptors on cells of the immune system, such as Toll-like receptors (TLR), that has

received significant attention, being the best characterized of the PRRs families described as present in fish (Magnadóttir, 2006; Palti, 2011).

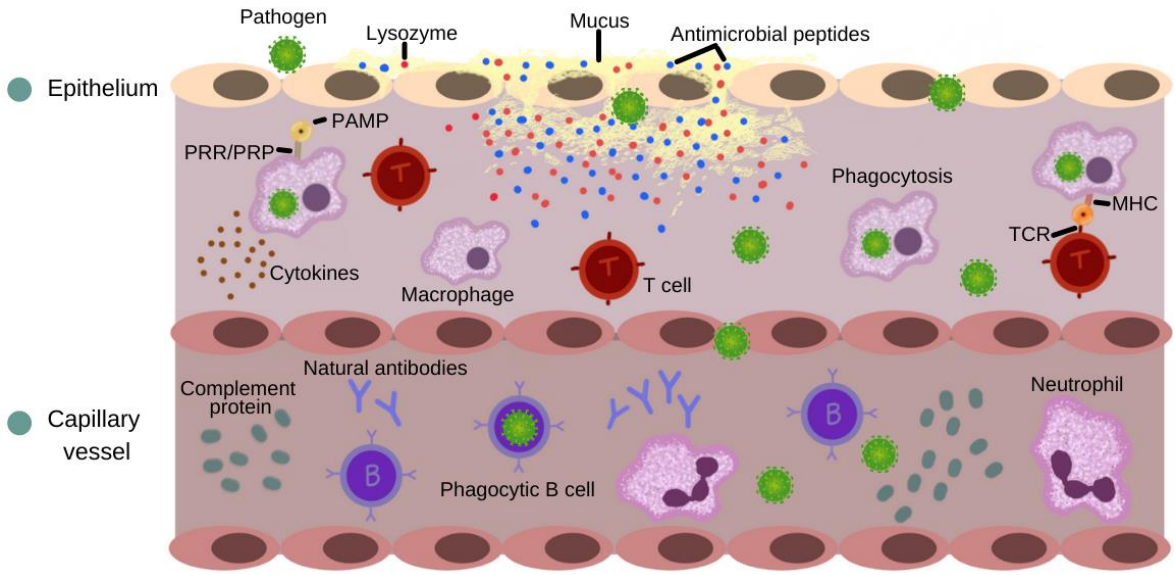

Figure 3 Illustration of the general mechanisms involved in the immune response. Adapted from (Castro & Tafalla, 2015).

4.2.1. Inflammation

The recognition of PAMPs by TLRs induces an increase in the expression of pro-inflammatory cytokines (IL-6, IL-1β, IL-8 and TNF-α), eicosanoids, and prostaglandin (Malaviya *et al.*, 1996; Verburg-van Kemenade *et al.*, 2009; Zhang & An, 2007), responsible to mediate, coordinate and orchestrate a local and systemic inflammatory response to pathogenic agents. Thus, the inflammatory response is an integral part of the innate immune response, with inflammatory mediators providing a connection between non-specific and specific immune systems (Calder, 2006). The first host response to injury besides the local activity of different factors such as enzymes and antimicrobial peptides is a systemic reaction, induced by chemokines that is characterized by increased vascular permeability and blood flow (Suzuki & Iida, 1992), as well as the migration and gathering of phagocytes and activation of lytic mediators (Alexander & Ingram, 1992; Uribe *et al.*, 2011).

The role of cellular components in the inflammatory response in teleosts has been described as biphasic, with neutrophils arriving first at the inflamed site, recruited by chemokines, followed by monocytes/macrophages and lymphocytes (Reite & Evensen, 2006). At the site of injury phagocytosis is triggered, with neutrophils and macrophages being the primary cells involved. Phagocytosis in addition to its contribution to pathogen

clearance, at the same time constitutes a relevant and crucial step for antigen presentation by these cells (Castro & Tafalla, 2015). Upon pathogen internalization, phagocytes induce the production of oxygen and nitrogen radicals, otherwise called as reactive oxygen species (ROS) (Ellis, 2001), a process associated to an increase in oxygen consumption and generally known as respiratory burst. This mechanism results in oxygen depletion. The activity of superoxide dismutase serves an important antioxidant role, acting over the superoxide anion generating hydrogen peroxide, which is then degraded by other enzymes such as catalase. In short, through the development of ROS during respiratory burst, phagocytic cells are responsible for the killing and destruction of pathogenic agents. In addition to this, phagocytic cells are capable of inhibiting the adherence and settlement of bacteria through antimicrobial agents such as lysozyme, complement factors, antiproteases and nitric oxide (Alexander & Ingram, 1992).

Eventually, the inflammation diminishes, cellular and tissue debris are recovered, and healing processes lead the host to a new homeostasis. However, if this is not the case and the infection persists, adaptive immune machinery is activated.

Table 1 Summary of the immune elements and their role in the immune response

		Elements	Main Function	References
Innate Immunity	Physical	Mucosal barriers (Mucus, scales, epithelium, gills and gut)	-Acts as a first layer of protection, blocking and limiting potential pathogenic intruders; -The protective mucus layer is equipped with mucins, lysozymes, lectins, immunoglobulins, complement proteins and antimicrobial peptides.	(Cabillon & Lazado, 2019; Ellis, 2001)
	Cellular	Natural killer cell (NK)	- Effector cells that kill altered, infected and cancerous cells in order to maintain homeostasis.	(Castro & Tafalla, 2015)
		Phagocytic cells	-Neutrophils: Phagocytic cells that destroy invading pathogens by producing ROS; have a significant antibacterial capacity; and produces proinflammatory cytokines;	(Do Vale *et al.*, 2002; Havixbeck & Barreda, 2015;

			-Monocytes: differentiate into macrophages and dendritic cells during inflammatory conditions; -Macrophages: Responsible for cytokine production and phagocytic activity; two existing phenotypes according to the inflammation stage (M1 and M2).	Hodgkinson *et al.*, 2015; Lu & Chen, 2019; Mills & Ley, 2014; Tafalla & Novoa, 2000)
	Humoral	Lysozymes	-Lytic enzymes that act on the peptidoglycan of bacterial walls.	(Magnadóttir, 2006)
		Protease inhibitors	-Primary role in body fluids homeostasis; -They are involved in acute phase reactions and secrete proteolytic enzymes.	(Magnadóttir, 2006; Uribe *et al.*, 2011)
		Natural antibodies	-Polyreactive and with low-binding affinity; -Important role in acquired immune defence; -Mostly IgM.	(Castro & Tafalla, 2015; Whyte, 2007)
		Antimicrobial peptides (AMPs)	-Host defence peptides through disruptive lytic actions; -The most conserved peptides include: Defensins, Cathelicidins and Hepcidins.	(Castro & Tafalla, 2015; Whyte, 2007)
		Complement	-Kills invading pathogens, induces an inflammatory response, clears apoptotic cells, and modulates the adaptive immune response; -Activated by means of three pathways: Classical, Alternative and Lectin.	(Magnadóttir, 2006; Uribe *et al.*, 2011; Watts *et al.*, 2001; Whyte, 2007)

Adaptive Immunity	Cellular	B lymphocytes	-Production of high affinity Ig/antibodies against aggressors (mainly IgM, IgD, IgT/Z).	(Magnadottir, 2010; Smith *et al.*, 2019)
	Humoral	T lymphocytes	-T cytotoxic cells kills/destroys infected cells and cancerous cells; -T helper cells assist and mediate B lymphocytes through the production of cytokines and/or by antigen presentation.	(Magnadottir, 2010; Nakanishi *et al.*, 2015)

5. Immunonutrition

As described above, the teleost immune system is a well-developed and an effective defensive mechanism against potentially hazardous scenarios for the host, which can be induced by the presence of pathogenic organisms or stress factors. With the intensification of production methods, aquaculture has become, undoubtedly, a highly stressful environment which, in addition to being highly prone to disease, represents a challenging environment for fish to thrive. Upon pathogenic invasion, several biological pathways are activated, generating a hostile milieu for pathogens and culminating in the restoration of host homeostasis (Kiron, 2012) (Figure 4). However, this does not come without a cost. In fact, the metabolic cost that results with the activation of the immune system has to be provided by endogenous sources of nutrients (Kiron, 2012). Diets that not only supply the nutrients and energy required for fish growth and development, but that also potentiate a certain physiological trait (e.g. the efficacy of the immune response) are generally known as functional diets. As fish feeds represent the highest production cost (Kiron, 2012; Trichet, 2010) optimizing them is key to improve the health and welfare of fish, which represents a major importance in production profitability. These special feeds aim to promote additional health benefits beyond traditional feeds, by being supplemented with specific ingredients known to modulate, directly or indirectly the effectiveness of the immune response (Li *et al.*, 2009).

Immunonutrition refers to the potential to modulate the activity of the immune system or the consequences related with its activation (Calder, 2003), by specific food items fed in amounts above those normally required by the animal. This type of approach can be applied preceding critical times inherent to the production process: before the occurrence of potential outbreaks, handling, vaccination and expected variation of external factors.

Example of successful feed additives that act as prophylactic measures include: probiotics, prebiotics, nucleotides, pigments, vitamins and amino acids (AA) (Oliva-Teles, 2012).

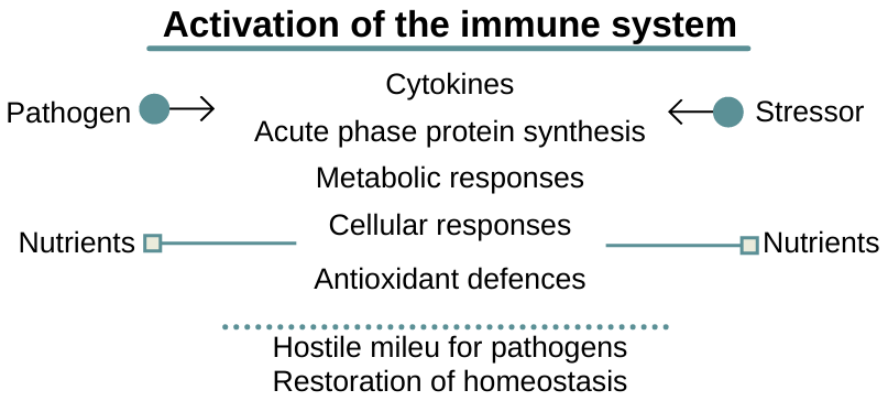

Figure 4 Notion of nutritional immunology. Adapted from (Kiron, 2012).

5.1. Amino acids and the immune response

It is now well known that nutrition has great potential to mitigate the emergence of diseases and stress that are typically associated with an intensive production system (Kiron, 2012). With this in mind, the scientific community has been investigating novel and innovative immune-nutritional strategies in order to sustain fish health through the optimal nutrition available (Azeredo *et al.*, 2017b). It is generally established that healthy fish are more likely to respond successfully to potential infections, simply because in not being developing any other especially energy-demanding physiological process, a perfectly adequate and efficient immune response will be mounted.

Any type of physiological reaction requires not only energy but also precursors in order to assemble an efficient response, implying that nutrient requirements may fluctuate as a result of external factors that may jeopardize the wellbeing of fish (Aragão *et al.*, 2008; Aragão *et al.*, 2010; Costas *et al.*, 2008). The immune response is a particularly demanding biological process that involves the modulation of different molecules, protein synthesis, as well as cell proliferation and migration. As a result, it is only natural that an efficient immune response is highly reliant on a variety of precursors as well as nutrients, with special emphasis on AA.

Being the building units of proteins, AA are naturally associated with fish growth. However, AA's destiny is not limited to fish development, as they function as subtract for a variety of enzymes and are precursors of key immune molecules (Andersen *et al.*, 2016). Despite the fact that AA play multiple roles in various biological processes, nutritional provision may not satisfy the fish needs, as nutrient requirements levels from diets may be dismissing the energy cost associated with the immune response. When assembling

various biological functions, such as an inflammatory response, the need for certain nutrients and AA may change, as a result of a greater demand for protein synthesis and the production of essential modulators, precursors, and immune factors (Costas *et al.*, 2012). Therefore, when confronted with a critical situation, such as temperature variation, handling, vaccination, or bacterial infection, AA dietary provision may not be sufficient when taking into account the fish needs, as stress may cause an increase in nutrient requirements (Aragão *et al.*, 2008; Aragão *et al.*, 2010; Costas *et al.*, 2008). These higher requirements are easily recognised by the drastic reduction in certain AA plasma levels upon stressful situations (Costas *et al.*, 2008).

With the rise of a more proactive sustainable consciousness and as the pressure on producer's increases, the aquaculture industry has tried to distance itself from fish meal diets, adopting vegetable ingredients instead (Li *et al.*, 2009). Nonetheless, these vegetable based diets often lack all the nutrients required, and are characterized by AA imbalances (Jobling, 2016). Furthermore, the use of such ingredients may also be impacting the overall fish growth and health (Bonaldo *et al.*, 2011) with the presence and incorporation of anti-nutritional factors (Francis *et al.*, 2001).

This thesis in particular has its focus on tryptophan, a dietary AA that has been proven to modulate the immune response.

5.2. Tryptophan

In addition to being an indispensable component of protein synthesis, tryptophan, after dietary uptake, undergoes a number of complex metabolic routes, resulting in the production of many important bioactive substances that fall into two pathways: (1) The pathway where the indole ring is left intact, such as serotonin (5-HT), melatonin and N-acetyl-HT; (2) the metabolic route where the indole ring is destroyed, which initiates the kynurenine-niacin pathway. The equilibrium among the two pathways is dependent on physiological conditions and pathological status (Le Floc'h *et al.*, 2011), as well as susceptible to external factors (Hoseini *et al.*, 2019), as different metabolites are originated from distinct tryptophan biotransformations in order to react to the different stimuli. These compounds are known to have a wide range of physiological effects, as they are involved in the modulation of the stress response, antioxidant system, immune system and behavioural response (Hoseini *et al.*, 2019; Le Floc'h & Seve, 2007).

The kynurenine-niacin pathway (KP) is responsible for most of the available tryptophan degradation. Moreover, it has been mentioned that this metabolic route (i.e. the produced metabolites) regulates the immune response (Cortés *et al.*, 2016).

The KP is present in all main tissues, being initiated by the oxidation of tryptophan by one of two enzymes: indoleamine 2,3 dioxygenase (IDO) or tryptophan 2,3- dioxygenase (TDO). These two enzymes differ in tissue distribution, with TDO being present on the liver and brain, while IDO can be found in the peripheral tissues and immune system cells, such as leucocytes (Grohmann *et al.*, 2003; Moffett & Namboodiri, 2003) and plays a crucial role in the activation of dendritic cells (Azeredo *et al.*, 2017a; Hwang *et al.*, 2005).

In mammals, the KP starts with the oxidization of tryptophan by IDO/TDO (Figure 5), producing N-formylkynurenine, which is then converted to kynurenine by arylformamidase. At that point kynurenine is metabolized by kynurenine aminotransferase, resulting in its conversion to kynurenic acid. Nevertheless, this route of the KP needs tryptophan supplementation in order to occur. As an alternative, kynurenine can be transformed in 3-hydroxykynurenine, followed by 3-hydroxyanthranilic acid. Subsequently, 3-hydroxyanthranilic acid will be oxidized into 2- amino-3-carboxymuconate semialdehyde. At this moment in the KP, 2- amino-3-carboxymuconate semialdehyde may undergo two distinct metabolic routes: it can be fully oxidized to picolinic acid and further in acetyl coenzyme A or it can be transformed into quinolinic acid and afterwards, in nicotinic acid. Although most of the KP steps are well documented in mammals, only some of the elements have been described as present in teleosts (Cortés *et al.*, 2016; Francis *et al.*, 2001; Hoseini *et al.*, 2019), therefore there is a need for further investigation in order to ascertain and determine if the same metabolic steps are present in lower vertebrates.

Inflammatory stimuli, such as LPS, IFN- γ and cytokines (Cortés *et al.*, 2016), induce IDO expression and activity in immune cells, such as monocytes and macrophages, leading to tryptophan depletion. This phenomenon is also a protective measure, as it prevents its use by pathogens and microorganisms (Le Floc'h *et al.*, 2011). In addition to this, tryptophan metabolites (3-hydroxyanthranilic and quinolinic acid) are known to be able to regulate T cell function and modulate the oxidative status, by removing superoxide radicals, as well as it lessens the production of pro-inflammatory cytokines (Francis *et al.*, 2001; Hoseini *et al.*, 2019; Perianayagam *et al.*, 2005; Ramos-Pinto *et al.*, 2019).

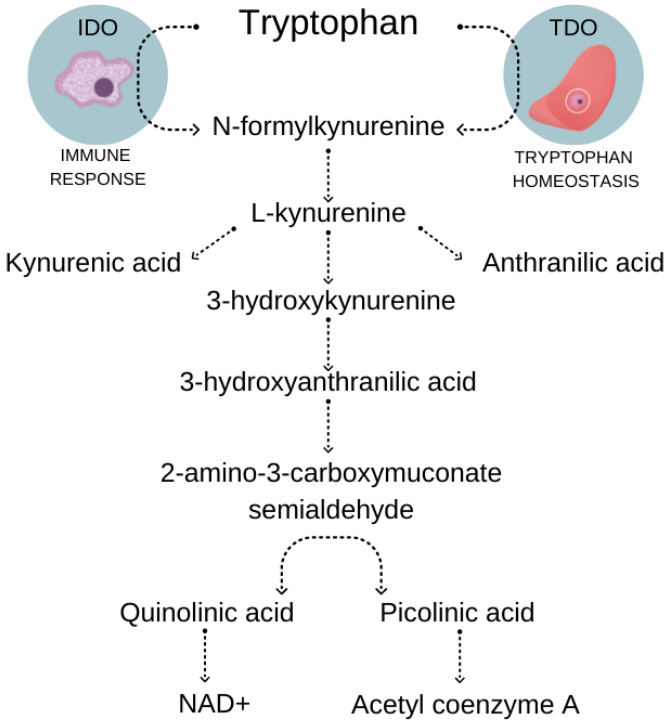

Figure 5 Tryptophan metabolism and its role in the immune response. **IDO**: 2,3-indoleamine dioxygenase; **TDO**: tryptophan 2,3-dioxygenase; **NAD+**: nicotinamide adenosine dinucleotide. Adapted From (Azeredo, 2017; Hoseini *et al.*, 2019; Le Floc'h *et al.*, 2011)

Tryptophan is also known to indirectly modulate the immune response through a neuroendocrine pathway. In teleost fish, monoamine 5-HT is limited by the availability of its precursor, tryptophan. Tryptophan is firstly converted to 5-HT by the activity of tryptophan hydroxylase, followed by the decarboxylation of 5-hydroxytryptophan. 5-HT is a monoamine neurotransmitter that regulates the release of adrenocorticotropic hormone (ACTH) (Fernstrom, 2016). This modulation could be achieved by stimulating or suppressing ACTH production in the pituitary, which in turn controls interrenal cortisol synthesis (Lepage *et al.*, 2002; Spinedi & Negro-Vilar, 1983). In addition, 5-HT is often linked with the modulation of aggressive behaviour, mood and stress responses in fish (Höglund *et al.*, 2005) (Figure 6). Stress situations often induce an increase of cortisol levels in plasma, which leads to a modification in the AA metabolism in teleosts (Kiron, 2012). Moreover, as already stated, stressful rearing conditions result in additional AA requirements, as fish experience a shift in energy to the synthesis of stress-related proteins. Taking this into account, tryptophan may be a key element to increase fish stress resistance by modulating cortisol levels, as it is the precursor of the neurotransmitter serotonin (Conceição *et al.*, 2012).

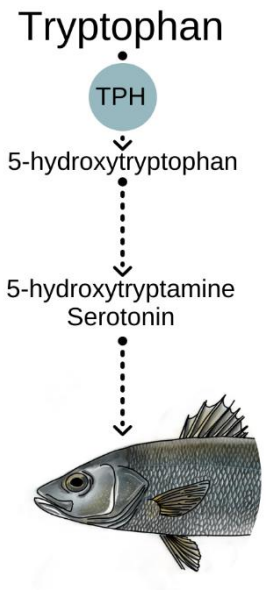

Figure 6 Tryptophan neuroendocrine pathway. **TPH**: tryptophan hydroxylase. Adapted From: (Azeredo, 2017; Hoseini *et al.*, 2019)

The relationship between the neuroendocrine and immune systems is well established. In fact, this interaction allows for a more complex response to potentially harmful situations being sustained by shared tissues, receptors, and mediators (Maier, 2003), all of which lead to a more powerful and efficient resolution of inflammation. Since tryptophan has been shown to partake directly or indirectly in both systems, its supplementation may be an important factor in the rapid resolution of inflammation. However, conflicting data and findings involving tryptophan supplementation have been published, highlighting the need for additional research on the topic (Azeredo *et al.*, 2019; Machado *et al.*, 2015; Machado *et al.*, 2019).

Scope of the thesis

The notions of maintaining and modulating fish health through the best possible nutrition are well-accepted in modern aquaculture, being both in line with the SDG established by the United Nations to be achieved by 2030. It is also recognized that healthy fish are more likely to successfully respond to potential infections, which results in less energy being redirected from metabolism to the inflammation process. This correlates to higher-quality fish, with more nutritional value, which leads to an increase in aquaculture's profitability. To accomplish this, producers rely on functional feed additives, such as AA. Considering their involvement in many immune-related pathways, AA stand out as ideal candidates to be used as immunomodulators, not only to enhance fish immunity, but also to improve the efficacy of other prevention tools. Tryptophan, in particular, has been shown to modulate the immune response to infection, and recent research suggests that its availability influences many immune mechanisms. Nonetheless, the use of tryptophan supplementation for animal health management is still being explored. Therefore, the primary aim of this research is to gather new insights on the modulatory effects of TRP during chronic inflammation in European seabass (*Dicentrarchus labrax*). To do so, it was intended i) to evaluate the effects of a chronic inflammatory process on the activation of hypothalamus-pituitary-interrenal axis, and ii) to assess the effects of tryptophan dietary supplementation on both neuroendocrine and immune responses.

Material and Methods

1. Fish Rearing Conditions

The present study was conducted under the supervision of accredited researchers in laboratory animal science by the Portuguese veterinary authority and accordingly to all guidelines on the protection of animals used for research purposes. This trial was conducted at BOGA (Bioterium of Aquatic Organisms), a CIIMAR (Interdisciplinary Centre of Marine and Environmental Research - Porto University) facility in Matosinhos, Portugal.

A total of 192 juvenile seabass (34.55 ± 7.84 g) were randomly distributed into 12 recirculating tanks (n= 16; photoperiod 12h light/ 12h dark) and were acclimatized for one week being fed a control diet, as described below (Figure 7). The physicochemical parameters such as oxygen saturation (7.5 ± 0.5 mg l^{-1} and salinity (27.6 ± 3.0) were monitored and registered on a daily basis. In addition to those, both temperature (19.4 ± 0.9 ºC) and nitrite levels (0.4 ± 0.1 mg/l) were also recorded daily and kept constant throughout the entire trial period. When necessary, water renovation and systems cleaning were also performed.

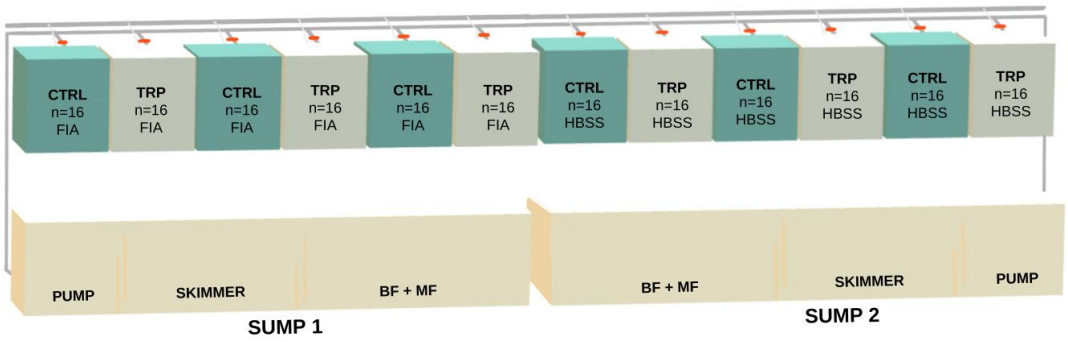

Figure 7 Experimental design. **BF**: biofilter; **MF**: mechanical filter

2. Experimental diets

Two diets were formulated and manufactured by Sparos Lda. (Olhão, Portugal). A non-supplemented diet was used as a control diet (CTRL) meeting the amino acid dietary requirement of European seabass. The supplemented diet was identical to the CTRL but supplemented with 0.3% L-Tryptophan (feed weight), at the expenses of wheat meal. This diet is considered as a dietary treatment and will be mentioned as TRP (tryptophan-supplemented diet). More detailed information on diets composition and proximate analysis can be seen in Table 1. Amino acids analysis is given in Table 2.

Table 2 Ingredient and chemical composition of the experimental diets.

Ingredients (%)	CTRL	TRP
CPSP 90[1]	5.00	5.00
Fish gelatin[2]	2.00	2.00
Soy protein concentrate[3]	25.00	25.00
Pea protein concentrate[4]	6.00	6.00
Wheat gluten[5]	10.00	10.00
Corn gluten meal[6]	15.00	15.00
Wheat meal[7]	15.80	15.50
Vit & Min Premix[8]	1.00	1.00
Antioxidant[9]	0.20	0.20
Sodium propionate[10]	0.10	0.10
MCP[11]	3.00	3.00
L-Lysine HCl 99%[12]	0.60	0.60
L-Tryptophan[13]	**0.00**	**0.30**
DL-Methionine[14]	0.20	0.20
Soy lecithin[15]	1.00	1.00
Fish oil[16]	15.10	15.10
Total	100.00	100.00
Proximate analyses (% dry weight)		
Crude protein (%)	45.70	46.00
Crude fat (%)	18.00	18.00
Fiber (%)	1.70	1.70
Starch (%)	13.40	13.20
Ash (%)	6.80	6.80
Energy (MJ/kg)	21.90	21.90

Table 3 Amino acid composition of experimental diets.

Amino acids (g AA 100 g^{-1} DW)	CTRL	TRP
Arginine	2.42	2.81
Histidine	1.69	1.16
Lysine	15.74	14.08
Threonine	0.85	0.59
Isoleucine	0.31	0.37

Leucine	0.32	0.41
Valine	3.91	3.62
Tryptophan	**0.76**	**1.05**
Methionine	0.75	0.79
Phenylalanine	0.76	0.98
Cysteine	0.09	0.09
Tyrosine	0.68	0.72
Aspartic acid	1.45	1.28
Asparagine	0.09	0.10
Glutamic acid	3.21	3.82
Glutamine	1.54	1.94
Alanine	1.01	1.00
Glycine	0.39	0.56
Proline	0.67	0.69
Serine	0.37	0.49
Taurine	1.34	1.31
Ornithine	0.58	0.51
gamma-Amino-n-butyric acid	0.50	0.56
Hydroxyproline	0.03	0.04
beta-Alanine	0.43	0.48

[1]CPSP 90: 82.6% crude protein (CP), 9.6% crude fat (CF), Sopropêche, France;

[2]Fish gelatin: 88% CP, 0.1% CF, LAPI Gelatine SPA, Italy;

[3]Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands;

[4]NUTRALYS F85F: 78% CP, 1% CF, ROQUETTE Frères, France;

[5]VITAL: 83.7% CP, 1.6% CF, ROQUETTE Frères, France;

[6]Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal;

[7]Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal;

[8]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;

[9]Paramega PX, Kemin Europe NV, Belgium;

[10]PREMIX Lda., Portugal;

[11]MCP: 22% phosphorus, 16% calcium, Fosfitalia, Italy;

[12]Lysine HCl 99%, Ajinomoto Eurolysine SAS, France.

[13]L-Tryptophan 98%, Ajinomoto Eurolysine SAS, France;

[14]DL-Methionine for Aquaculture: 99% Methionine, Evonik Nutrition & Care GmbH, German

[15]Lecico P700IPM, LECICO GmbH, Germany;

[16]SAVINOR UTS, Portugal;

3. Experimental design and sampling procedures

After one week of acclimatization to the system, fish started to be fed also on TRP and were intraperitoneally injected with either an inflammatory insult (Freund's Incomplete Adjuvant, FIA) or a sham saline solution (Hank's Balanced Salt Solution, HBSS). Fish were fed the experimental diets in triplicate tanks by hand twice a day for 28 days, 2 % of their biomass. All groups (FIA-CTRL, FIA-TRP, HBSS-CTRL, and HBSS-TRP) were sampled at 7, 14, 21 and 28 days post-injection. Three fish per tank were randomly sampled at each time point (n=6) and euthanized using 2-phenoxyethanol (0.5 ml/l) (Figure 8). Blood samples were collected from the caudal vein using 1 ml heparinized syringes, and later placed in 1.5 ml heparinized tubes and gently homogenised for haematological analysis (described in the following topic). The remaining blood was centrifuged for 10 min at 10,000 x g at 4 ºC and afterwards plasma was collected and stored at -80 ºC. The anterior gut was also collected for immune and oxidative stress analysis. In addition, peritoneal exudates collection was also performed as described by Machado *et al.* (2018).

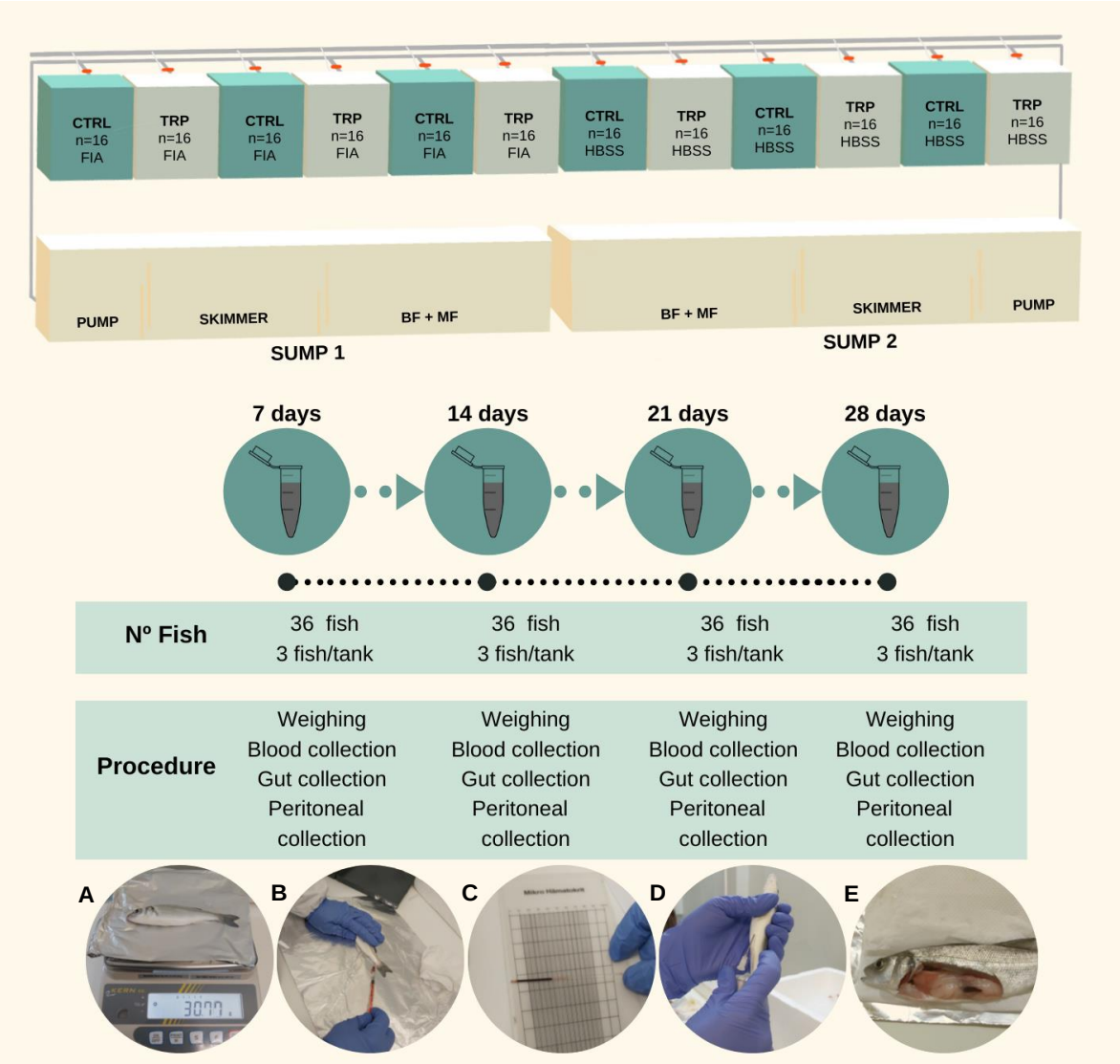

Figure 8 Experimental design and sampling procedures. **A**: Weighing; **B**: Blood collection; **C**: Haematocrit test; **D**: Peritoneal exudates collection; **E**: Gut collection

3.1. Haematological and analytical procedures

The haematological profile was carried out according to Machado *et al.* (2015), including total white (WBC) and red (RBC) blood cells counts, haematocrit (Ht) and haemoglobin concentration. Thereupon, the mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) as well as mean corpuscular haemoglobin concentration (MCHC) were calculated (Machado *et al.*, 2015). WBC and RBC counts were obtained from a dilution 1/20 and 1/200 (respectively) of homogenized blood in HBSS with heparin. Shortly after fish euthanasia and blood collection, cold HBSS supplemented with 30 units heparin ml^{-1} was injected into the fish's peritoneal cavity in order to collect the peritoneal exudate. Then, WBC, RBC and total peritoneal cells counts were carried out using a microscope and a Neubauer chamber. Values of WBC and total peritoneal cells are given in a concentration of $10^4\ \mu l^{-1}$, while RBC has a concentration of $10^6\ \mu l^{-1}$.

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, prepared immediately after blood collection and homogenization, were air dried and fixed with formol-ethanol (10% formaldehyde in absolute ethanol). The detection of peroxidase was carried out as described in Afonso *et al.* (1997), to allow an easier neutrophil detection. Blood smears were then stained with Wright's stain (Haemacolor; Merck) and slides were examined in the microscope (1,000×). At least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. Absolute concentration ($\times 10^4$ μl^{-1}) of each cell type was calculated based on total blood WBC counts.

Peritoneal cells were collected according to Afonso *et al.* (1997), adapted from Silva *et al.* (1989). Cytospin preparations were then made with a THARMAC Cellspin apparatus and stained as specified above for blood smears. The lymphocytes, macrophages and neutrophils in the peritoneal exudates were differentially counted, and the number of each cell type was established after counting a minimum of 200 cells per slide. Once again, the concentration ($\times 10^4$ μl^{-1}) of each leucocyte type was posteriorly calculated.

3.2. Humoral parameters

3.2.1. Plasma lysozyme activity

Lysozyme activity was measured using a turbidimetric assay as described by Costas *et al.* (2011a) A solution of *Micrococcus lysodeikticus* (0.5 mg ml^{-1}, 0.05 M sodium phosphate buffer, pH 6.2) was promptly prepared. In a microplate, 250 µl of the solution mentioned above was added to 15 µl of plasma. The reaction was carried out at 25 ºC and the absorbance (450 nm) was measured after 0.5 and 4.5 min in a Synergy HT microplate reader, Biotek. Lyophilized hen egg white lysozyme (Sigma) was serially diluted in sodium phosphate buffer (0.05 M, pH 6.2) and used to create a standard curve. The lysozyme concentration in the sample was calculated using the formula of the standard curve. All analyses were conducted in triplicates.

3.2.2. Plasma peroxidase activity

Peroxidase activity was measured following the procedure described by Quade and Roth (1997), for plasma samples (Figure 9). A dilution was performed according to preliminary tests, 15 µl for plasma, which were diluted in 135 µl of HBSS without Ca^{+2} and Mg^{+2} in flat-bottomed 96-well plates, respectively. Afterwards, 50 µl of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB; Sigma) and 50 µl of 5 mM H_2O_2 were added to the plates. The colour-change reaction was stopped after 2 min by adding 50 µl of 2 M sulphuric acid. The optical density was read at 450 nm in a Synergy HT microplate reader, Biotek. The wells without plasma/gut homogenates were used as blanks, containing 150 µl of HBSS without Ca^{+2} and Mg^{+2}. Peroxidase activity was determined using the premise that one unit of peroxidase generates a one-unit change in optical density absorbance.

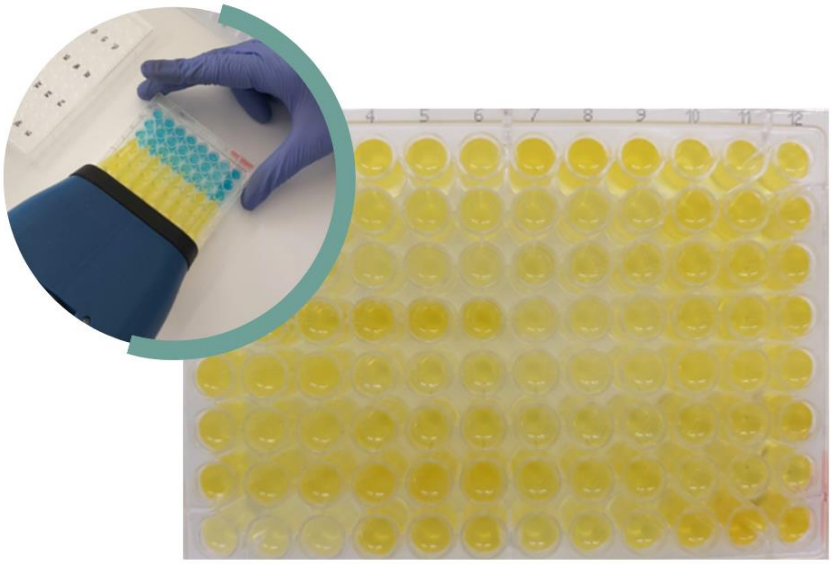

Figure 9 Microplate after adding sulphuric acid

3.2.3. Bactericidal Activity

Photobacterium damselae subsp. *piscicida*, strain PP3 (*Phdp*) was used in the bactericidal activity assay. Bacteria were cultured for 48 h at 25 °C on tryptic soy agar (TSA; Difco Laboratories) and then inoculated into tryptic soy broth (TSB; Difco Laboratories), both supplemented with NaCl to a final concentration of 1% (w/v) (TSA-1 and TSB-1, respectively). Bacteria in TSB-1 medium were then cultured during 24h at the same temperature, with continuous shaking (100 rpm). Exponentially growing bacteria were collected by centrifugation at 3500 × g for 30 min, resuspended in sterile HBSS and adjusted to 1×10^6 CFU ml^{-1}.

Plasma bactericidal activity was determined following the method of Graham *et al.* (1988) with some adjustments (Machado *et al.*, 2015) (Figure 10). Briefly, 20 µl of plasma was added to duplicate wells of a U-shaped 96- well plate. HBSS 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}) was added and the plate was incubated for 2.5 h at 25 ºC. Right after, 25 µl of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (1 mg ml^{-1}; Sigma) was added and the plate was once again incubated for 10 min at 25 ºC 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. Bactericidal activity is expressed as percentage, calculated from the difference between bacteria surviving compared to the number of bacteria from positive controls (100%).

% Viable bacteria	Sample Abs. x 100／Abs. of the reference sample
No viable bacteria	100 - % Viable bacteria

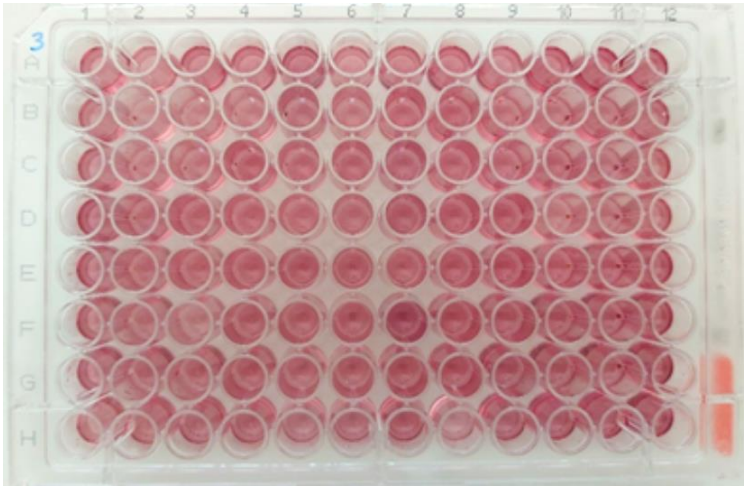

Figure 10 U-shaped 96-well microplates used for bactericidal activity assay after reading

3.2.4. Cortisol

Cortisol was firstly extracted by adding 20 µl of plasma to 180 µl of diethyl ether (Sigma). Cortisol levels in plasma were determined using a commercial ELISA kit (RE52061, IBL International GMBH, Germany), already validated for European seabass (Azeredo *et al.*, 2017a). Manufacturer's instructions were followed. Briefly, 20 µl of standard control and

sample were added to duplicate wells. Enzyme conjugate (200 μl) was added and the plate was incubated for 1h at 25 ºC. Afterwards, the plate was washed 3 times with 300 μl of wash buffer. To each well, 100 μl of TMB was added and incubated for 15 minutes (25 ºC) to allow the formation of formazan. The reaction was stopped with TMB stop solution. The optical density of the dissolved formazan was measured at 450 nm (Figure 11). Subsequently, the necessary calculations were performed.

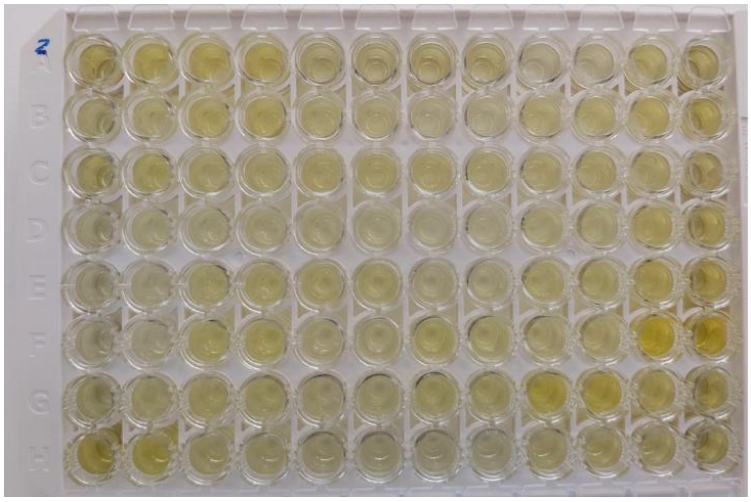

Figure 11 U-shaped 96-well microplates used for cortisol assay after reading

3.3. Gut Immune and Stress Oxidative parameters

3.3.1. Gut sample homogenization

Tissue samples were weighed and homogenized 1:10 (m/v) in 0.1M K-phosphate buffer (pH=7.4). Then, 200 μl of the tissue homogenate was placed in 2ml tube with 4 μl BHT 4% in methanol to be used to determine lipid peroxidation. The remaining tissue homogenate was centrifuged at 10,000 x g (4ºC) for a total of 20 minutes. All samples were promptly frozen at -80 ºC.

3.3.2. Lipid peroxidation (LPO)

This assay method is characterized by the oxidative degradation of unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters and cholesterol and is based on the reaction with thiobarbituric acid (TBA) in an acidic pH.

To the gut homogenate samples (200 μl of homogenate), 100 μl of cold TCA (trichloroacetic acid) 100% and 1 ml TBA 0.73% (2-thiobarbituric acid - Tris-HCl and DTPA solution) were added, at room temperature (RT). Following that, the samples were incubated for 1h at 100 ºC, before being centrifuged for 5 min at 11,500 rpm (RT). Afterwards, 200 μl of the

supernatant was transferred to a 96-well microplate. All analysis were conducted in triplicates and the wells that did not contain any sample served as blanks. Absorbance was read at 535 nm in a Synergy HT microplate reader, Biotek. Later, the appropriate calculations were carried out.

3.3.3. Gut peroxidase activity

Gut peroxidase activity was tested using the same approach as described earlier for plasma, with the exception in the amount of gut sample used (10 µl).

3.3.4. Catalase

This assay method is based on the measurement of the decrease of hydrogen peroxide concentration after the action of catalase.

Previously a dilution of the homogenate was performed in order to achieve a concentration of 0.7 mg ml^{-1}. In a 96-well UV microplate, 10 µl of sample was pipetted. Following that, 140 µl of K-phosphate buffer (0.005 M, pH=7) was added to each well. With the help of a multichannel pippete, 150 µl of reaction buffer (Hydrogen peroxide solution) was immediately added, achieving a final volume of 300 µl. The absorbance (240 nm) was measured in a Synergy HT microplate reader, Biotek, for 2 min (1 read every 15 seconds). All analysis were conducted in triplicates and the wells that did not contain any sample served as blanks. Subsequently, the necessary calculations were performed.

3.3.5. Superoxide dismutase

This method evaluates the superoxide dismutase (SOD), which is an antioxidant enzyme involved in the defence system against reactive oxygen species (ROS) (Flohé & ötting, 1984; Lima *et al.*, 2007).

Previously, gut homogenates were diluted in order to achieve a concentration of 0.3 mg ml^{-1} of protein. The standard was prepared and was added to a 96-well microplate, as well as 50 µl of sample. Then, 200 µl of working solution was added to all wells. Following that, 50 µl of xantina oxidase was added, resulting in the production of superoxide anions. The absorbance of the formazan (550 nm) was measured in a Synergy HT microplate reader, Biotek, for a total of 3 minutes (1 read every 20 seconds) (Figure 12). The stronger the SOD activity in the sample, the less formazan dye is produced. Later, the necessary calculations were performed.

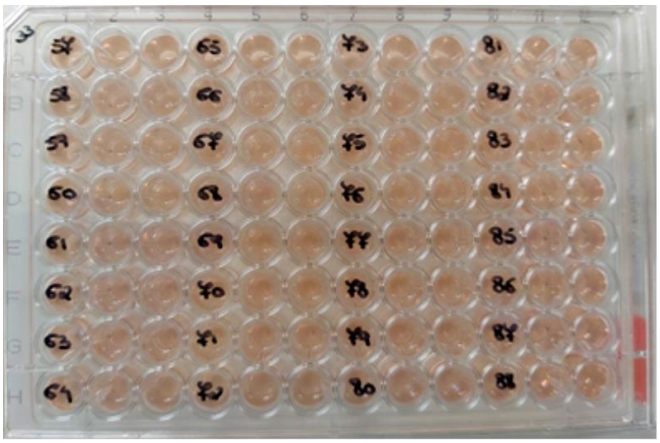

Figure 12 Superoxide dismutase microplate after reading

3.3.6. Total and oxidized glutathione

For total glutathione (tGSH) assessment, 50 μL of gut homogenate was diluted in 350 μl of 5% metaphosphoric acid solution (MPA) in a microtube which was centrifuged at 1000 g at 4 ºC for 10 minutes. Afterwards, 12.5 μl of the supernatant was diluted in 750 μl of assay buffer (Na-K phosphate buffer 0.2 M, pH=8.0) in a new microtube.

Regarding the analysis of oxidized glutathione (GSSG), 15 μl of scavenger (thiol scavanger) was added to 50 μl of gut homogenate in another microtube, which was then incubated at room temperature for 10 minutes. 135 μl of cold MPA was added and microtubes were subsequently centrifuged at 1000 x g at 4 ºC for a total of 10 minutes. Following that, 25 μl of the supernatant was diluted in 350 μl of Assay buffer in a new microtube.

Fifty μl of standard, tGSH and GSSG samples and blanks were added to duplicated wells, followed by the addition of 50 μl of lyophilized 5,5'- dithiobis-2-nitrobenzoic acid (DTNB), and 50 μl of reductase solution (recombinant glutathione reductase). The plate was then mixed by an orbital shaker and incubated for 5 minutes at room temperature. Lastly, 50 μl of lyophilized β- nicotinamide adenine dinucleotide phosphate (NADPH) was added. The absorbance (412 nm) was measured in a Synergy HT microplate reader, Biotek, for a total of 10 minutes (1 read every minute) (Figure 13). Subsequently, the necessary calculations were performed.

GSH_t **GSSG**	(Net Rate-Intercept ⁄ Slope) x Dilution Factor

Figure 13 96-well plates after reading

4. Data Analysis

Data is presented as mean ± standard deviation. Statistical analysis was done by multifactorial ANOVA, with diet, stimulus and sampling time as factors. Data was tested for normality by the Shapiro-Wilk test and for homogeneity of variances by the Levene test. When normality was not verified, data was transformed prior to ANOVA. Significant differences among groups were determined by the Tukey multiple range test. One-way ANOVA was used when significant differences within conditions were verified. The probability level of 0.05 was used for rejection of the null hypothesis. All the statistical analysis were done using the SPSS 27 software package for windows.

Results

1. Haematological profile

Variations in time were noted for RBC, MCH, MCV, and MCHC (Table 4). MCH, MCV, and MCHC grew in value as they reached the two-week sampling time, then decreased over time. RBC counts, on the other hand, increased from 7 to 14 days remaining high throughout the following sampling times.

WBC showed an interactive effect between all three variables studied. FIA-injected fish that were fed TRP showed a decrease in total peripheral WBC concentration at 21 days of feeding, but at 28 days levels were similar to those observed at 14 days.

When looking at haemoglobin values, a peak was observed for both diets studied two weeks after the inflammatory insult. Regarding the haematocrit, fish injected with FIA presented higher levels than those injected with HBSS, irrespective of sampling time and dietary treatment. Moreover, the haematocrit of fish fed TRP was also lower than that of fish fed CTRL, regardless of sampling time and stimulus.

Total peritoneal cells decreased from 14 to 21 days regardless of stimuli or dietary treatment. In addition to time variation, it was also shown that FIA injected fish presented higher peritoneal cell counts than those injected with HBSS.

Peripheral neutrophil counts (Table 5) showed no significant variation with regard to time, stimuli, or diet. Regarding monocytes concentration, fish undergoing inflammation showed higher concentrations compared to those of the sham group. Fish fed TRP for 14 days showed higher monocytes counts than those fed the same diet for 7 days regardless stimulation, and this was followed by a decrease over time. In CTRL-fed fish, a time-dependent increase in monocytes was observed up to 21 days post-injection. Furthermore, while TRP-fed fish exhibited higher monocyte counts at 14 days compared to CTRL-fed fish, at 21 days, the opposite occurred, with TRP-fed fish showing lower counts relative to the CTRL group. Regarding lymphocytes, the interaction between time and stimulus was shown to be significant. In FIA-injected fish, lymphocytes were highest in the first week to decrease over time, while recovering at 28 days post-injection. Moreover, at 7 and 14 days, these numbers were higher than those observed in HBSS counterparts. Thrombocytes increased in TRP-fed fish undergoing inflammation from 14 to 28 days whereas in those injected with HBSS, thrombocyte counts decreased at first from 7 to 14 days and then peaked at 28 days with concentrations higher than those measured at 7 or 14 days post injection.

In regards to the differential counts of peritoneal leucocytes (Table 6), all cell types were observed to be higher in the peritoneal cavity of fish injected with FIA relative to fish injected with HBSS, irrespective of sampling time and dietary treatment. Furthermore, in what neutrophils are concerned, a time-dependent decrease was observed in all fish, regardless of dietary treatment or stimulus.

Table 4 Haemoglobin, mean corpuscular haemoglobin (MCH), red blood cells (RBC), white blood cells (WBC) and total peritoneal cells in European seabass fed dietary treatments and sampled at 7, 14, 21 and 28 days post-injection.

Multifactorial ANOVA	7 days				14 days				21 days				28 days			
	FIA		HBSS		FIA		HBSS		FIA		HBSS		FIA		HBSS	
	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL
WBC	7.55[ab] ± 1.21	8.63 ± 0.81	6.45 ± 1.59	5.62 ± 1.09	10.45 [a] ± 1.13	9.53 ± 1.55	5.90 ± 1.00	5.38 ± 1.00	6.40 [b] ± 1.30	8.75 ± 2.40	6.17 ± 0.82	5.77 ± 1.04	11.60 [a] ± 3.41	7.95 ± 1.55	7.10 ± 1.90	6.85 ± 1.98
RBC	2.68 ± 0.24	2.68 ± 0.37	2.40 ± 0.36	2.18 ± 1.23	2.20 ± 0.33	2.17 ± 0.47	2.27 ± 0.30	2.42 ± 0.35	2.85 ± 0.48	2.74 ± 0.46	2.38 ± 0.28	2.74 ± 0.36	2.81 ± 0.50	3.01 ± 0.38	2.72 ± 0.32	2.85 ± 0.35
Hematocrit	28.20 ± 3.27	31.40 ± 1.95	26.33 ± 5.96	32.17 ± 3.76	30.50 ± 2.26	31.17 ± 4.58	27.83 ± 5.31	31.20 ± 3.03	28.20 ± 3.19	31.50 ± 4.64	27.00 ± 1.41	27.67 ± 2.42	33.00 ± 5.33	33.00 ± 2.35	29.33 ± 3.98	28.83 ± 3.87
Hemoglobin	1.24 ± 0.19	1.19 ± 0.25	1.20 ± 0.24	1.18 ± 0.11	2.27 ± 0.26	1.94 ± 0.26	2.18 ± 0.26	1.98 ± 0.21	1.38 ± 0.14	1.30 ± 0.19	1.28 ± 0.27	1.33 ± 0.14	1.30 ± 0.21	1.42 ± 0.28	1.31 ± 0.15	1.34 ± 0.15
MCH	4.70 ± 1.04	4.48 ± 1.05	5.19 ± 1.83	4.84 ± 2.09	10.72 ± 2.67	9.25 ± 2.01	9.17 ± 2.18	8.38 ± 1.48	4.95 ± 0.93	4.82 ± 1.20	5.52 ± 1.61	4.95 ± 1.30	4.78 ± 1.24	4.78 ± 1.09	4.82 ± 0.33	4.74 ± 0.46
MCV	86.09 ± 43.24	98.64 ± 51.93	109.47 ± 15.87	131.76 ± 36.19	140.81 ± 20.76	150.11 ± 44.53	122.18 ± 8.40	109.29 ± 56.38	83.09 ± 42.61	117.43 ± 23.84	114.70 ± 14.77	102.92 ± 17.76	121.55 ± 33.57	88.49 ± 43.74	108.14 ± 9.95	101.55 ± 11.20
MCHC	4.40 ± 1.04	3.67 ± 1.07	4.89 ± 1.92	3.72 ± 0.65	7.60 ± 1.37	6.29 ± 0.87	7.59 ± 2.14	6.40 ± 0.76	4.80 ± 0.83	4.14 ± 0.69	4.73 ± 0.90	4.89 ± 0.62	4.08 ± 1.17	4.39 ± 0.84	4.50 ± 0.66	4.68 ± 0.25
Total peritoneal leucocytes	46.61 ± 22.45	42.40 ± 11.24	10.20 ± 5.55	8.18 ± 5.04	48.25 ± 31.52	55.55 ± 31.94	10.00 ± 3.51	8.15 ± 5.01	48.91 ± 24.82	41.27 ± 21.74	4.28 ± 1.95	5.03 ± 4.61	59.20 ± 37.59	68.08 ± 47.99	6.30 ± 3.90	5.20 ± 2.42

Multifactorial ANOVA	Time	Stimulus	Diet	Time x Stimulus	Time x Diet	Stimulus x Diet	Time x Stimulus x Diet	Time				Stimuli		Diet		Time x Stimulus								Time x Diet							
								7	14	21	28	FIA	HBSS	CTRL	TRP	FIA				HBSS				TRP				CTRL			
																7	14	21	28	7	14	21	28	7	14	21	28	7	14	21	28
WBC	0.11	<0.001	ns	0.022	ns	ns	0.025	ab	ab	b	a	#	*	-	-	ab#	a#	b	ab#	*	*	-	*	-	-	-	-	-	-	-	-
RBC	<0.001	ns	ns	ns	ns	ns	ns	b	a	a	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hematocrit	ns	0.013	0.012	ns	ns	ns	ns	-	-	-	-	#	*	A	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hemoglobin	<0.001	ns	ns	ns	0.04	ns	ns	b	a	b	b	-	-	-	-	-	-	-	-	-	-	-	-	b	a#	b	b	b	a*	b	b
MCH	<0.001	ns	ns	ns	ns	ns	ns	b	a	b	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

MCV	<0.001	ns	ns	ns	ns	ns	ns	b	a	b	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MCHC	<0.001	ns	ns	ns	ns	ns	ns	b	a	b	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total peritoneal leucocytes	0.049	<0.001	ns	ns	ns	ns	ns	ab	a	b	ab	#	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Values are presented as means ± SD (n=6). P-values from multifactorial ANOVA: ns: non-significant ($p \leq 0.05$). If interaction was significant, a Tukey post-hoc test was performed. Different capital letters represent significant differences between dietary treatments. Different lowercase letters represent changes between sampling time. Different symbols indicate significant differences between stimuli.

Table 5 Absolute values of peripheral blood leucocytes (neutrophils, monocytes, lymphocytes and thrombocytes) of European seabass fed dietary treatments and sampled at 7, 14, 21 and 28 days post-injection.

Multifactorial ANOVA	7 days				14 days				21 days				28 days			
	FIA		HBSS		FIA		HBSS		FIA		HBSS		FIA		HBSS	
	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL
Neutrophils	0.23 ± 0.12	0.26 ± 0.10	0.11 ± 0.05	0.17 ± 0.13	0.07 ± 0.12	0.10 ± 0.06	0.04 ± 0.04	0.02 ± 0.05	0.09 ± 0.06	0.19 ± 0.18	0.06 ± 0.09	0.09 ± 0.10	0.06 ± 0.11	0.06 ± 0.05	0.19 ± 0.20	0.00 ± 0.00
Monocytes	0.21 ± 0.11	0.61 ± 0.38	0.16 ± 0.12	0.22 ± 0.16	4.05 ± 1.45	1.48 ± 0.61	1.79 ± 0.52	1.22 ± 0.84	1.08 ± 0.79	2.61 ± 1.24	0.64 ± 0.34	1.21 ± 0.67	1.53 ± 0.55	1.54 ± 0.67	0.52 ± 0.22	0.69 ± 0.32
Lymphocytes	4.13 ± 0.93	4.04 ± 0.57	2.61 ± 0.82	2.20 ± 0.51	2.85 ± 0.85	4.15 ± 1.67	1.73 ± 0.30	1.55 ± 0.33	1.66 ± 0.66	2.03 ± 0.89	1.77 ± 0.24	1.39 ± 0.36	3.54 ± 1.72	2.28 ± 0.59	2.13 ± 0.70	1.64 ± 0.75
Thrombocytes	2.98 [b] ± 0.24	3.73 ± 0.55	3.57 [b] ± 0.84	3.02 ± 0.60	3.48 [b] ± 1.32	3.80 ± 0.86	2.33 [c] ± 0.53	2.60 ± 0.69	3.56 [ab] ± 0.70	3.92 ± 0.81	3.70 [ab] ± 0.65	3.07 ± 0.63	6.47 [a] ± 1.42	4.07 ± 0.90	4.26[a] ± 1.42	4.51 ± 1.14

Multifactorial ANOVA	Time	Stimulus	Diet	Time x Stimulus	Time x Diet	Stimulus x Diet	Time x Stimulus x Diet	Time				Stimuli		Time x Stimulus								Time x Diet							
								7	14	21	28	FIA	HBSS	FIA				HBSS				TRP				CTRL			
														7	14	21	28	7	14	21	28	7	14	21	28	7	14	21	28
Neutrophils	ns	ns	ns	ns	ns	ns	ns	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Monocytes	<0.001	<0.001	ns	ns	<0.001	ns	ns	c	a	b	b	#	*	-	-	-	-	-	-	-	-	c	Aa	Bb	b	b	Ba	Aa	ab
Lymphocytes	<0.001	<0.001	ns	0.12	ns	ns	ns	a	b	c	b	#	*	a#	ab#	d	abc	*	*	-	-	-	-	-	-	-	-	-	-
Thrombocytes	<0.001	0.003	ns	ns	ns	ns	0.021	-	-	-	-	#	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Values are presented as means ± SD (n=6). P-values from multifactorial ANOVA: ns: non-significant ($p \leq 0.05$). If interaction was significant, a Tukey post-hoc test was performed. Different capital letters stand for significant differences between dietary treatment. Different lowercase letters represent changes between sampling time. Different symbols indicate significant differences between stimuli.

Table 6 Absolute values of peritoneal leucocytes (neutrophils, monocytes and lymphocytes) of European seabass fed dietary treatments and sampled at 7, 14, 21 and 28 days post-injection.

Multifactorial ANOVA	7 days				14 days				21 days				28 days			
	FIA		HBSS		FIA		HBSS		FIA		HBSS		FIA		HBSS	
	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL
Neutrophils	15.95 ± 6.30	15.41 ± 2.59	2.74 ± 2.20	1.79 ± 1.18	7.78 ± 4.80	7.74 ± 4.30	1.90 ± 1.18	1.89 ± 1.39	5.58 ± 4.68	5.58 ± 3.15	0.80 ± 0.40	1.11 ± 0.72	5.39 ± 4.21	6.74 ± 4.90	1.95 ± 2.19	1.38 ± 0.78
Macrophages	6.68 ± 4.13	7.02 ± 2.21	0.70 ± 0.65	0.41 ± 0.42	5.78 ± 3.70	5.60 ± 2.56	0.80 ± 0.45	0.58 ± 0.40	3.61 ± 3.08	3.54 ± 2.21	0.33 ± 0.20	0.50 ± 0.45	3.22 ± 2.55	4.72 ± 3.51	0.63 ± 0.60	0.54 ± 0.31
Lymphocytes	1.32 ± 0.55	1.10 ± 0.29	0.31 ± 0.24	0.20 ± 0.16	0.48 ± 0.26	0.44 ± 0.29	0.29 ± 0.17	0.25 ± 0.18	0.75 ± 0.59	0.67 ± 0.46	0.12 ± 0.06	0.13 ± 0.10	0.88 ± 0.75	0.79 ± 0.50	0.18 ± 0.15	0.13 ± 0.05

Multifactorial ANOVA	Time	Stimulus	Diet	Time x Stimulus	Time x Diet	Stimulus x Diet	Time x Stimulus x Diet	Time				Stimuli	
								7	14	21	28	FIA	HBSS
Neutrophils	0.002	<0.001	ns	ns	ns	ns	ns	a	ab	b	b	#	*
Macrophages	ns	<0.001	ns	ns	ns	ns	ns	-	-	-	-	#	*
Lymphocytes	ns	<0.001	ns	ns	ns	ns	ns	-	-	-	-	#	*

Values are presented as means ± SD (n=6). P-values from multifactorial ANOVA: ns: non-significant ($p \leq 0.05$). Different lowercase letters represent changes between sampling time. Different symbols indicate significant differences between stimuli.

2. Humoral parameters

The complete set of data is presented in the appendix.

Regarding plasma bactericidal activity (Figure 14. A), fish fed TRP presented lower activity levels than those in fish fed CTRL, regardless of sampling time or stimuli. In addition, in FIA-injected fish, bactericidal activity was lower in animals sampled at 14 and 28 days compared to those sampled at 7 days.

Both plasma lysozyme and peroxidase were unaffected by any of the variables tested, though a non-significant decreasing trend in peroxidase appeared to occur over time (Figure 14. B and C).

Lastly, cortisol levels only varied over time, with values increasing from 7 to 21 days post injection and then declined at 28 days post-injection (Figure 14. D).

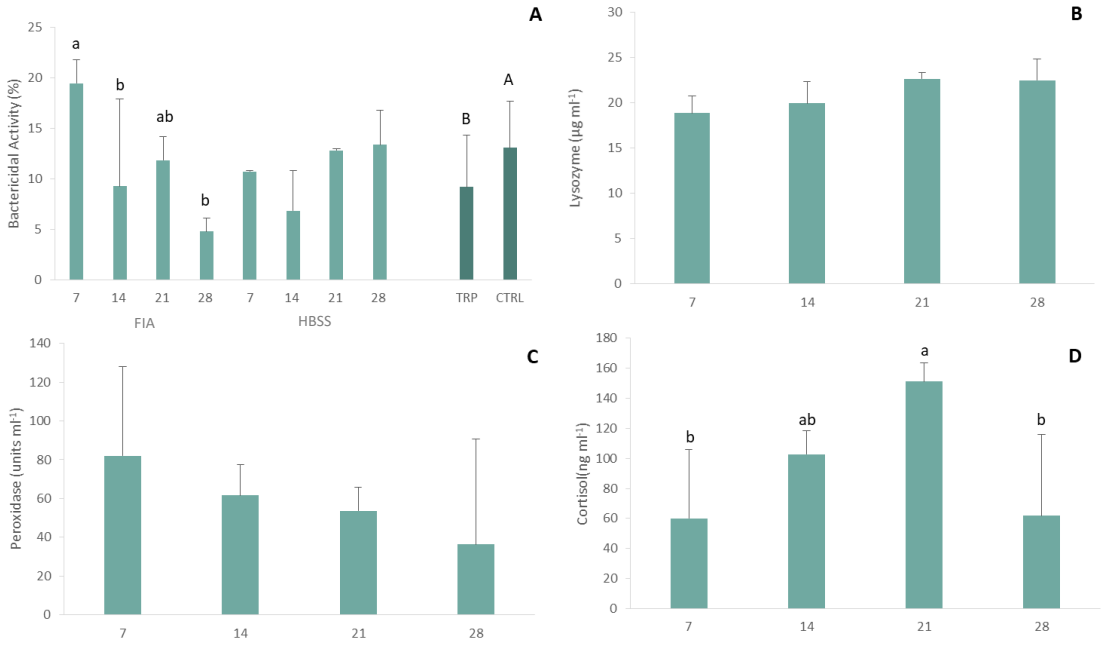

Figure 14 Humoral parameters in European seabass fed dietary treatments and sampled at 7, 14, 21 and 28 days post injection. Different low case letters indicate significant differences among sampling times. (Multifactorial ANOVA; Tukey post-hoc test; $p \leq 0.05$).

3. Gut immune and stress oxidative parameters

The complete set of data is presented in the appendix.

An interactive effect between sampling time and stimulus was observed on LPO, SOD, tGSH and GSSG values. LPO was higher in FIA-injected fish sampled at 7 days post-injection compared to those sampled 14, 21 and 28 days post-injection (Figure 15. A). SOD, tGSH and GSSG all increased over time in FIA-injected fish until 21 days post inflammatory

insult, returning to lower levels at 28 days post injection (Figure 15. B, C and D, respectively). No such a time-dependent effect was observed in HBSS-injected fish. Moreover, in FIA-injected fish, tGSH (at 14 and 21 days) and GSSG (at 14 days) were higher than in the corresponding HBSS-injected groups.

Catalase and peroxidase showed differences regarding not only sampling time but in inflammatory insult as well. Catalase levels were higher in fish sampled at 21 and 28 days post injection than in those sampled at 7 days, irrespective of stimulus or dietary treatment (Figure 15. E). In addition, fish injected with FIA presented higher values relative to the ones injected with HBSS.

Peroxidase values were lower at 14 days post injection compared to all other sampling times, regardless stimulation and dietary treatment (Figure 15. F). The same parameter was inhibited in FIA-injected fish relative to the HBSS-injected group.

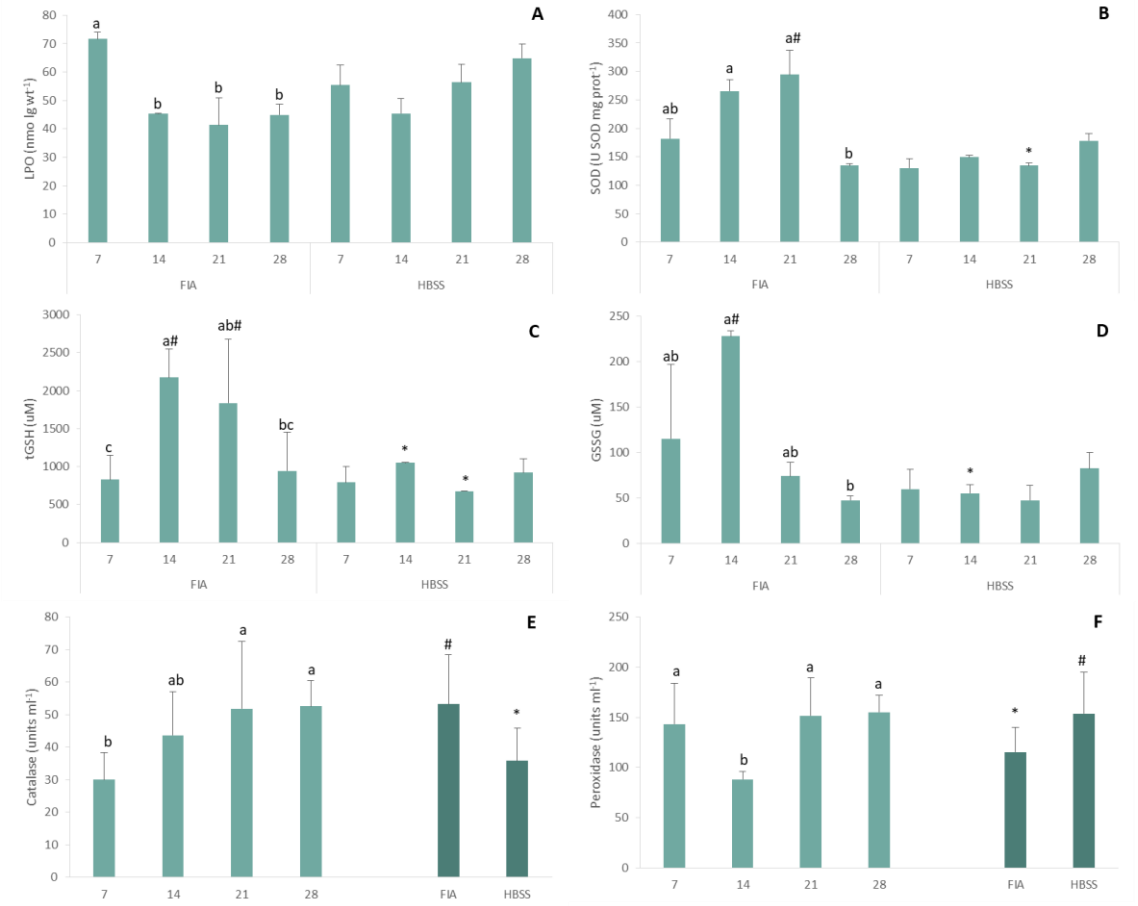

Figure 15 Gut immune and oxidative stress parameters in European seabass fed dietary treatments and sampled at 7, 14, 21 and 28 days post injection. Different low case letters indicate significant differences among sampling times. Different symbols indicate significant differences regarding stimulus (Multifactorial ANOVA; Tukey post-hoc test; $p \leq 0.05$)

Discussion

Nutritional strategies, such as the incorporation of AA in fish functional feeds, have grown in popularity in recent years as a means of alleviating disease and improving fish health (Kiron, 2012). Aside from being an essential amino acid that is required for fish protein synthesis, tryptophan participates in a number of metabolic processes linked to stress and immunological response (Le Floc'h *et al.*, 2011). Furthermore, tryptophan supplementation has been demonstrated to modulate a wide range of physiological functions (Hoseini *et al.*, 2019). Since most research have focused on the acute responses of fish's innate immune systems to infection and inflammation, there is still little information regarding their long-term responses. Thus, the present study aimed to contribute to understand the effects of chronic inflammatory process on the activation of HPI axis as well as to assess the effects of tryptophan dietary supplementation on both neuroendocrine and immune responses. A combination of an inflammatory model and a dietary intervention, as well as the assessment of humoral, immune, oxidative stress, and haematological parameters, were required to understand its effects.

Inflammation is a crucial part of the process by which the immune system defends the host homeostasis from potentially hazardous agents (Barton, 2008; Kiron, 2012). Thus, the recruitment of leucocytes to the inflamed site is a key step for the resolution of inflammation. Changes in blood leucocyte counts were seen in this study when inflammatory mechanisms were triggered after fish being i.p. injected with an inflammatory insult (FIA). This was expressed by higher monocytes and lymphocytes in FIA-injected fish, revealing a clear immune response. Regarding the inflammatory focus, neutrophils are the first cells arriving at the inflamed site (Castro & Tafalla, 2015; Verburg-van Kemenade *et al.*, 2009). Accordingly, in this study, despite no significant differences were detected at the peripheral level, peritoneal neutrophils peaked at 7 days, suggesting once more that an immune response was triggered after an inflammatory insult, with FIA-injected fish presenting greater values than the sham group. The same was also verified for the remaining peritoneal cells studied, that indicated significant differences with the inflammatory insult (peritoneal macrophages and lymphocytes), suggesting that a clear peripheral and local immune response was being assembled. The absence of a clear peripheral neutrophil response might be explained by the chosen sampling points. It is possible that, being the first and quickest cells to arrive and react in the inflammatory response, the first sampling point (one week after the insult) might have missed neutrophils response.

The effects of tryptophan dietary supplementation on leucocyte recruitment and migration to the inflammatory focus was not very incisive in the current study. Still, not only total WBC decreased from 14 to 21 days in FIA-injected fish fed TRP, but also monocytes response was inhibited in this group, compared to the CTRL-fed fish at 21 days. This regulatory aspect of tryptophan dietary supplementation, despite not very expressive, is in accordance with recent findings related to tryptophan-mediated immunosuppressive effects on seabass cellular response following infection with a bacterial pathogen (Machado *et al.*, 2019).

It is well established that an inflammatory process triggers a stress response, which manifests itself as an increase in plasma cortisol levels (Tort *et al.*, 2003). The current study's findings revealed that plasma cortisol levels did indeed rise over time, although irrespective of i.p. injection nature. At 7 days, though, despite no statistical significance was observed given the high intraspecific variability, there is a conspicuous difference between cortisol levels of fish undergoing inflammation and those that were not, suggesting the presence of an immune effect over the neuroendocrine system, particularly strong in the first stages of the inflammatory response. Dietary tryptophan supplementation, on the other hand, had no apparent influence on plasma cortisol levels. These findings are consistent with earlier research that found ambiguous effects of tryptophan on cortisol levels, stating that these effects are highly reliant on the context and duration of the experimental study (Hoseini *et al.*, 2019; Lepage *et al.*, 2002).

Neither tryptophan supplementation nor chronic inflammation significantly modulated plasma lysozyme and peroxidase. Neutrophils are the source of plasma peroxidase (Ellis, 1999), whereas lysozyme is a result of several leucocytes (neutrophils, monocytes and macrophages)(Saurabh & Sahoo, 2008). The lack of an effect of the dietary treatment and inflammatory insult on plasma peroxidase and lysozyme is congruent with the lack of effects on peripheral neutrophils. This lack of results was consistent with the findings of Machado *et al.* (2019), who observed that dietary treatments had no effect on peroxidase and lysozyme activities. Still, in parallel to the highest peripheral neutrophil concentration (observed at 7 days post-injection), peroxidase concentration was also the highest in fish sampled at the earliest sampling point. Differently, plasma total bactericidal activity decreased over time in FIA-injected fish. Bactericidal activity is an important immune indicator due to its multifactorial character, as it represents the sum of a wide range of immune mechanisms against a potential infectious agents (Ellis, 1999). Such a decrease in plasma overall defences might be related to cell migration to the inflammatory focus. Moreover, bactericidal activity decreased in fish fed TRP, which might imply that the immunological status of these fish was suppressed in some way. This result is in line with

Machado *et al.* (2015) study, who also observed a decrease in bactericidal activity in fish fed a tryptophan-supplemented diet compared to the ones fed a control diet.

Innate immunity serves as a first line of defence against infection, relying on both physical barriers as well as humoral and cellular responses (Castro & Tafalla, 2015). In fish, this nonspecific immunity gains great importance due to limitations of adaptive immune system. Similar to the skin, the gut is in direct contact with the external milieu and therefore, it is an important place of host-pathogen interaction that might result in the development of local immune responses. It is not surprising that it is extensively colonised by resident immune cells and by immune-related enzymes/proteins, in what is generally known as the gut-associated lymphoid tissue. Inflammation induced the activity of important gut stress oxidative-related enzymes and free-radical scavengers, such as SOD, tGSH and GSSG, which were found to increase over time in FIA-injected fish, during the first sampling points, only returning to basal activity levels at 28 days post-injection. Fish undergoing inflammation also showed higher CAT activity compared to the sham group. The immune cell response implies the activation of several cytotoxic mechanisms - e.g. respiratory burst and nitric oxide production – which are not only detrimental for pathogenic microorganisms but might also compromise self cellular integrity. Therefore, the activity of these antioxidant enzymes is critical in the inflammatory response efficiency. Hence, as expected, and even more so during an inflammatory process with a long-term profile, SOD activity in particular peaked at 21 days post-injection. In contrast, gut peroxidase activity was inhibited in FIA- relative to HBSS-injected group. Peroxidase, in addition to its antibacterial characteristics, transforms H_2O_2 into O_2 and H_2O. Given that H_2O_2 is produced from O_2^- by SOD, it is plausible that dropping peroxidase values are related to a decrease in H_2O_2 abundance. The observed rise in tGSH levels in FIA-injected fish, may justify the preceding, serving as a potential scavenger of O_2^-, making it less available for SOD to covert into H_2O_2.

Conclusion

Tryptophan is involved in a number of metabolic pathways that are associated with both stress and immune responses, and its supplementation has been shown to affect a variety of physiological functions. Thus, this study provides additional information about tryptophan modulatory effects in a chronic inflammation scenario in European seabass. The importance of this study relies on the fact that few studies were performed in a chronic inflammation scenario.

Overall, the findings of this study appear to indicate that tryptophan supplementation primes immunological suppression. This premise is supported by lower humoral bactericidal activity values and cellular response impairment in TRP-fed fish, which may be indicative of a supressive tryptophan modulatory effect. Therefore, tryptophan supplementation may compromise crucial inflammatory mechanisms. Regarding chronic inflammation, inhibition of gut immunity was seen while leucocyte response was locally enhanced in FIA-injected fish, which may be reflective of a redistribution of peripheral resources to the inflamed site. In what the neuro-endocrine response was concerned, no significant modulatory effects of tryptophan nor inflammation were observed, suggesting that tryptophan dietary surplus effects on the neuroendocrine-immune axis are highly dependent on each context (i.e. fish species, holding conditions, immune status).

Future studies should be performed on the sequence of this dissertation in order to improve the knowledge on the modulation of the immune status through dietary use of AA, in particular tryptophan, during chronic inflammatory conditions. Next step, should be the gene expression analysis, necessary to better understand and explain the findings and results this thesis presented.

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Appendix

Table 7 Absolute values of humoral parameters in European seabass fed dietary treatments and sampled at 7, 14, 21 and 28 days post injection.

Multifactorial ANOVA	7 days				14 days				21 days				28 days			
	FIA		HBSS		FIA		HBSS		FIA		HBSS		FIA		HBSS	
	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL
Bactericidal activity	17.77 ± 5.06	21.11 ± 7.77	10.59 ± 6.57	10.79 ± 10.78	3.16 ± 2.41	15.37 ± 6.78	3.97 ± 2.99	9.65 ± 7.06	10.18 ± 10.18	13.50 ± 10.19	12.94 ± 3.71	12.69 ± 4.77	3.82 ± 3.32	5.69 ± 2.64	10.93 ± 5.17	15.76 ± 6.24
Lysozyme	18.43 ± 14.49	16.01 ± 10.46	20.92 ± 11.05	20.07 ± 13.49	19.88 ± 6.58	20.49 ± 6.45	20.98 ± 1.75	18.39 ± 6.37	24.22 ± 4.57	27.47 ± 3.13	22.12 ± 2.96	16.73 ± 3.28	33.39 ± 6.24	26.01 ± 8.11	14.76 ± 5.86	15.69 ± 2.49
Peroxidase	83.65 ± 47.56	79.33 ± 30.41	65.25 ± 52.54	100.04 ± 60.76	47.40 ± 41.35	63.48 ± 74.81	50.24 ± 35.10	85.29 ± 57.50	60.18 ± 66.51	50.34 ± 44.52	63.20 ± 64.01	40.71 ± 33.21	43.03 ± 81.87	25.26 ± 30.57	41.05 ± 41.30	36.38 ± 42.05
Cortisol	105.33 ± 92.70	92.11 ± 87.69	32.93 ± 31.95	10.40 ± 12.43	86.06 ± 58.77	109.09 ± 74.24	93.90 ± 99.72	121.75 ± 130.44	135.12 ± 93.89	156.58 ± 90.11	149.90 ± 117.46	163.62 ± 132.66	17.52 ± 15.15	33.81 ± 23.93	56.19 ± 93.20	139.67 ± 242.25

Multifactorial ANOVA	Time	Stimulus	Diet	Time x Stimulus	Time x Diet	Stimulus x Diet	Time x Stimulus x Diet	Time				Diet		Time x Stimulus							
														FIA				HBSS			
								7	14	21	28	CTRL	TRP	7	14	21	28	7	14	21	28
Bactericidal activity	0.004	ns	0.008	0.002	ns	ns	ns	a	b	a	b	A	B	a	b	ab	b	-	-	-	-
Lysozyme	ns	ns	ns	ns	ns	ns	ns	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Peroxidase	ns	ns	ns	ns	ns	ns	ns	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cortisol	<0.001	ns	ns	ns	ns	ns	ns	b	ab	a	b	-	-	-	-	-	-	-	-	-	-

Values are presented as means ± SD (n=6). P-values from multifactorial ANOVA: ns: non-significant ($p \leq 0.05$). If interaction was significant, a Tukey post-hoc test was performed. Different lowercase letters represent changes between sampling time. Different capital letters indicate significant differences between diet.

Table 8 Absolute values of gut immune and oxidative stress parameters in European seabass fed dietary treatments and sampled at 7, 14, 21 and 28 days post injection.

Multifactorial ANOVA	7 days				14 days				21 days				28 days			
	FIA		HBSS		FIA		HBSS		FIA		HBSS		FIA		HBSS	
	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL	TRP	CTRL
LPO	70.08 ± 10.61	73.30 ± 25.85	60.36 ± 19.49	50.57 ± 9.67	45.44 ± 21.12	45.17 ± 38.34	41.82 ± 12.60	49.08 ± 14.16	34.54 ± 5.58	48.15 ± 28.27	60.89 ± 30.37	51.93 ± 9.82	42.16 ± 15.68	47.68 ± 19.32	61.26 ± 24.37	68.49 ± 31.40
SOD	206.47 ± 20.84	157.56 ± 70.44	119.04 ± 18.06	141.80 ± 51.40	279.96 ± 121.83	251.08 ± 53.63	151.95 ± 10.09	147.31 ± 42.86	263.80 ± 97.92	324.95 ± 114.42	131.77 ± 35.84	137.52 ± 23.92	136.97 ± 29.73	132.39 ± 43.02	168.81 ± 38.42	187.60 ± 83.19
GSH	1057.31 ± 378.05	596.41 ± 90.85	640.20 ± 311.21	941.67 ± 577.07	1909.62 ± 701.74	2436.44 ± 587.77	1055.67 ± 1332.76	1047.53 ± 500.10	1239.68 ± 661.86	2431.25 ± 996.90	674.95 ± 87.42	681.01 ± 517.91	582.20 ± 575.84	1305.27 ± 1630.93	795.34 ± 570.74	1050.45 ± 968.52
GSSG	172.92 ± 75.45	57.55 ± 27.55	44.37 ± 20.47	75.43 ± 74.47	232.06 ± 135.71	223.93 ± 130.35	48.73 ± 38.18	61.69 ± 67.59	63.41 ± 19.46	84.80 ± 55.11	36.05 ± 20.92	59.36 ± 67.87	50.65 ± 21.06	44.19 ± 25.51	69.96 ± 25.95	95.07 ± 115.26
Catalase	40.86 ± 12.28	30.13 ± 9.56	20.72 ± 5.41	28.31 ± 6.32	49.47 ± 18.05	59.40 ± 11.94	29.71 ± 3.91	36.13 ± 19.49	53.07 ± 20.44	80.57 ± 21.61	35.39 ± 10.88	38.15 ± 14.40	63.83 ± 25.01	48.17 ± 24.22	46.26 ± 15.57	52.05 ± 22.34
Peroxidase	121.25 ± 21.71	103.22 ± 41.08	153.27 ± 36.40	195.11 ± 77.63	82.18 ± 12.70	86.82 ± 20.71	83.43 ± 21.37	100.02 ± 31.02	135.96 ± 63.64	105.53 ± 42.16	185.17 ± 75.57	179.41 ± 67.37	141.90 ± 30.67	146.45 ± 46.72	179.63 ± 76.54	152.56 ± 62.87

Multifactorial ANOVA	Time	Stimulus	Diet	Time x Stimulus	Time x Diet	Stimulus x Diet	Time x Stimulus x Diet	Time				Stimuli		Time x Stimulus							
								7	14	21	28	FIA	HBSS	FIA				HBSS			
														7	14	21	28	7	14	21	28
LPO	0.010	ns	ns	0.039	ns	ns	ns	a	b	b	ab	-	-	a	b	b	b	-	-	-	-
SOD	ns	0.002	ns	<0.001	ns	ns	ns	-	-	-	-	#	*	ab	a	a#	b	-	-	*	-
GSH	0.002	<0.001	ns	0.010	ns	ns	ns	b	a	ab	b	#	*	b	a#	ab#	ab	-	*	*	-
GSSG	ns	<0.001	ns	0.001	ns	ns	ns	-	-	-	-	#	*	ab	a#	ab	b	-	*	-	-
Catalase	<0.001	<0.001	ns	ns	ns	ns	ns	b	ab	a	a	#	*	-	-	-	-	-	-	-	-
Peroxidase	<0.001	<0.001	ns	ns	ns	ns	ns	a	b	a	a	*	#	-	-	-	-	-	-	-	-

Values are presented as means ± SD (n=6). P-values from multifactorial ANOVA: ns: non-significant ($p \leq 0.05$). If interaction was significant, a Tukey post-hoc test was performed. Different lowercase letters represent changes between sampling time. Different symbols indicate significant differences between stimuli.