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Effects of Ygeia+ on the European seabass immune response and disease resistance: New tools and opportunities - Y+Health

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André Pereira da Cunha

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Abstract

Aquaculture industry has been growing rapidly past few years. However, along the way the fast growth of the industry starts to be associated with the emergence of some new challenges. To face this cycle, companies and researchers worldwide, are working in diverse aspects of the production to make the final product a better, more sustainable and economically viable product. The present study was conceived to investigate the impact of one fortified diet (Ygeia+) in the health condition of European seabass (*Dicentrarchus labrax*). It aimed particularly to: i) study the effects of Ygeia+ short-term feeding on immune-condition and oxidative stress of European seabass; and ii) assess the interactive effects of short-term feeding with Ygeia+ and vaccination on the immune response and disease resistance of European seabass.

Two trials were performed with European seabass weighing 12-36 g in recirculating seawater systems. In both trials, fish were fed for 5 and 10 days a control diet or the Ygeia+ diet, both commercially available, produced and provided by Sorgal. In the first trial, fish were inoculated with an inactivated pathogen after each feeding time and the inflammatory response was assessed at 4, 24 and 48 h after it. In the second trial, fish were vaccinated with AVAC VR/PD/TM (HIPRA) or sham injected after each feeding period. Following vaccination procedures, fish were fed the control diet for 3 weeks and sampled for the assessment of immune parameters and oxidative stress biomarkers. Fish were also bath challenged with *Tenacibaculum maritimum* to evaluate if Ygeia+ can induce protection and its synergistic effects with AVAC VR/PD/TM.

The first trial pointed to a positive effect of Ygeia+ with a tendency to increase circulating monocyte and neutrophil numbers following inflammation in fish fed Ygeia+ for 5 and 10 days, consequently to an increase of plasma lysozyme and peroxidase activities. During the second trial, specific IgM augmented significantly in all vaccinated groups, which showed improved survival. Moreover, seabass fed Ygeia+ tended to have increased oxidative stress related enzymatic activity in the liver, compared to their counterparts fed the control diet. In summary, functional diets such as Ygeia+ are important sustainable prophylactic strategies that deserve further attention. Future studies should consider, other feeding times to fine-tune Ygeia+ as a feeding strategy to improve European seabass robustness.

Keywords: European seabass, Ygeia+, functional feeds, immunomodulation, vaccination

Resumo

A indústria da aquacultura tem crescido acentuadamente nos últimos anos. Em resultado desse rápido crescimento, têm surgido alguns desafios. Assim, empresas e investigadores têm trabalhado em conjunto com intuito de melhorar a produção, através do aprimoramento do produto final. Para tal, pesquisa tem sido conduzida no sentido de obter um produto de elevada qualidade, ambientalmente e economicamente sustentável. O presente trabalho insere-se precisamente nesta temática, visando perceber o impacto de uma dieta específica (Ygeia+) no estado de saúde e resposta imunológica do robalo.

Em particular, pretendemos: i) estudar os efeitos da dieta Ygeia+ durante curtos espaços de tempo na condição imunológica e stress oxidativo do robalo; e ii) perceber a interação entre a dieta Ygeia+ e vacinação no sistema imune, bem como na resistência a doenças, em robalos.

Dois ensaios foram realizados em sistemas de reciculação fechados utilizando robalos com o peso médio de 12 a 36 gramas. Em ambos os ensaios, os peixes foram alimentados com uma dieta controlo ou com a dieta Ygeia+ (ambas as dietas são comercializadas) durante 5 e 10 dias. Ambas as dietas foram produzidas e fornecidas pela Sorgal. No primeiro ensaio, após o período de alimentação os animais foram inoculados com um patogénio inativado para se proceder à avaliação da resposta inflamatória às 4, 24 e 48 horas. No segundo ensaio, após o período de alimentação os animais foram ora vacinados com uma vacina AVAC (efetiva contra *Vibrio anguillarum*, *Photobacteria damselae* e *Tenacibaculum maritimum*) ora tratados com placebo (PBS). Após este procedimento, os animais foram igualmente mantidos com dieta controlo por 3 semanas. No final desse período, parte dos animais foi amostrado para avaliação dos parâmetros imunes e do estado oxidativo, enquanto os restantes peixes foram desafiados através de banho com *Tenacibaculum maritimum* com o intuito de se verificar os efeitos sinergéticos da vacina e da dieta.

No primeiro ensaio foi possível verificar um efeito positivo da dieta Ygeia+ com o aumento dos monócitos e neutrófilos circulantes após infeção quer aos 5 como aos 10 dias. Esse aumento refletiu-se numa maior atividade da lisozima e peroxidase no plasma. Com o segundo ensaio, foi possível verificar a eficiência da vacina com o aumento das IgM específicas nos animais vacinados. Em resumo, dietas como a Ygeia+ são estratégias profiláticas sustentáveis que merecem maior atenção. Estudos futuros devem testar outros tempos de alimentação de forma a otimizar o uso desta dieta como estratégia para melhorar a saúde geral dos robalos de produção.

Palavras-chave: Robalo, Ygeia+, Dietas funcionais, imunomodulação, vacinação

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Aim

The aim of the present study was to understand the role of Ygeia+ as a fortified feed for European seabass (*Dicentrarchus labrax*), by evaluating its effects on the fish immune status, inflammatory response and disease resistance.

Therefore, this project can increment the conceptual knowledge of multi-action (immunization and feed supplementation) approaches to fish immunological enhancement in aquaculture, using functional commercial diets which, in the last instance, can reduce chemotherapeutic and antibiotics administrated (further ahead explored).

Introduction

Aquaculture

Aquaculture is defined as the production of aquatic organisms with human intervention in the process (FAO, 1988). Production includes the totality of life cycle or, in some cases, just part of it. Products retrieved from aquaculture can be grouped into vertebrates (fish), invertebrates and algae. Human intervention can range from just restocking to modulate the entire lifecycle. Aquaculture also implies a sense of ownership, whether for space or stock. With ownership also comes the need for protection (against predators and humans) and investment.

World Aquaculture

Aquaculture industry is one of the fastest growing food production industries, with an annual growth of 3.1%, only surpassed by poultry (4.7%) and is responsible for the maintenance of 20,533 direct employments worldwide. Following the growth rates, in 2018 aquaculture industry was evaluated at 263.4 billion dollars, representing an increment from previous years of 30 billion dollars. From the total worth value, 250.1 billion dollars were resultant from aquatic animals, which, by itself, account for 17% of the total animal protein ingested in the world.

Aquaculture steady's growth is contrasting with the stagnation of fisheries, observed since 1986. Wild captures have been stable at about 90 million tons each year, with a slight increase in 2017 and 2018, where captures reached 93 and 96 million tons, respectively.

World aquaculture is unevenly distributed worldwide, with only one country (China) representing 57.93% of all production. China's production is followed by far from the rest of Asia with 30.76%, America with 4.63%, Europe with 3.75%, Africa with 2.67% and, finally, Oceania with only 0.25%. In terms of products produced, these numbers translate into 54.3 million tons of finfish, with only 7.3 million represented by marine species. Invertebrates account for 27.6 million tons, which can be further divided into mollusks with 17.7 million tons, crustaceans with 9.4 million tons and 500 thousand tons for the remaining invertebrates. Finally, algae are responsible for 32.4 million tons of aquaculture products. (FAO, 2020)

Mediterranean Aquaculture

Environmental conditions of the Mediterranean Sea are characterized by its oligo or ultra-oligotrophic structure (Lacoue-Labarthe et al., 2015), with mild, wet winters and dry worm

to hot summers (Lionello et al 2006). Conditions are ideal to the production of species like European seabass (*Dicentrarchus labrax*). Therefore, aquaculture in the Mediterranean basin has been evolving towards the sea. Until 1990, production of salt-water species was mainly in land-based facilities and, since then and until 2010, more than 82% of the total salt-water species production has already moved to sea-cages. As expected with technology and industry evolution, an even larger percentage of the industry is expected to change from land-based to sea-based facilities. In Europe, the most produced salt-water species are the European seabass and gilthead seabream (*Sparus aurata*). They are predominantly produced by Greece, Turkey, Egypt, Italy and Spain. In this regard, since the above species are the most produced, it is rather drastic the methodology and changes in the production from 1990 to 2010, with the great majority being produced in the sea instead of land (Massa et al., 2017).

European seabass Aquaculture

European seabass was the first non-salmonid marine species commercially produced in Europe (Bagni, 2005). Production of European seabass was first recorded as an industry in 1973, with the production of 10 tons of fish. The flourishment started to occur at the end of the 80's and in 1987, it was recorded for the first time a production superior to one thousand tons. From 2007 to 2016, the production almost doubled with an increase of almost one hundred thousand tons, reaching a value of 191 thousand tons (FAO, 2018). Growth was so representative that it has become the 5th most produced species in European aquaculture (Ciccant, 2017). Production of European seabass can be divided into major and smaller producers. The major ones include Greece, Turkey, Spain, Italy, and France, and the minor ones are Portugal, Croatia, Cyprus, Israel, Egypt, Tunisia, and Morocco (Lees & Thomas, 2008). Once again, technological advancements like cooling systems and chain of transports, as well as new rearing techniques, have opened new horizons for the commercialization of this species. Traditionally consumed in the Mediterranean basin (due to its availability), it is now exported abroad into new emergent markets. With this ascendance of new economic markets, production must be adapted to the regional consumer perspective, which will induce the producer to adjust. For instance, in Portugal, client perception of good quality fish is entire fresh animals, whereas those perceptions change to fillets or even frozen fish in other countries. Commercialization of European seabass has traditionally been entire fresh fish and, mainly in Mediterranean basin countries, which correspond to the main producers for the species (Bagni, 2005).

Bringing the Portuguese case to the spotlight and considering the scope of this thesis, we will now frame European seabass into the Portuguese aquaculture scenario.

Portugal was the second country to produce European seabass, with a contribution rounded 10% of the total, only behind Italy. However, contrary to other countries that developed steadily over the years, Portuguese production raised until 2006 and decayed 75% in the next 10 years. Contradicting the descendant growth tendency, the past few years have seen a new invigorating boost in production, since in 2017 was registered a total production grew of 57% compared to the previous year, reaching a total of 700 tons (Apambiente, 2019). Considering the total production of the aquaculture industry in Portugal (12,487 tons), European seabass accounts for 5.9% of total production.

Dicentrarchus labrax (European seabass)

Biology

European seabass (*Dicentrarchus labrax* (Linnaeus, 1758)) (Figure 1) is a teleost species belonging to the moronidae family. The species inhabits coastal waters from north Africa up to the shores of Great Britain (Figure 2), including Mediterranean Sea and Black Sea. The large tolerance to different temperatures (2 to 32 ºC) and salinities (full strength sea water to 3‰) allows it to inhabit estuaries all along the coast, and incursion inshore. Nevertheless, is also observed a seasonal migration during flood and dry periods. During periods of flood, with the decrease of salinity in estuaries, the animal will go into deeper oceanic water. While in periods of dry, they will adventure further ahead into the higher parts of estuaries (Picket & Pawson, 1994).

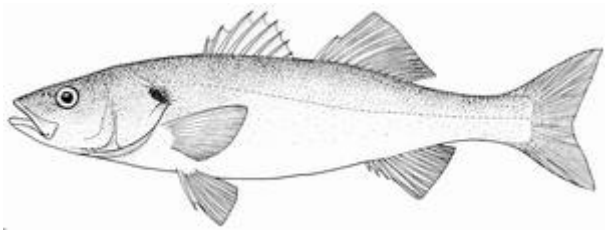

Figure 1 - Dicentrarchus labrax. Source: FAO, 2021

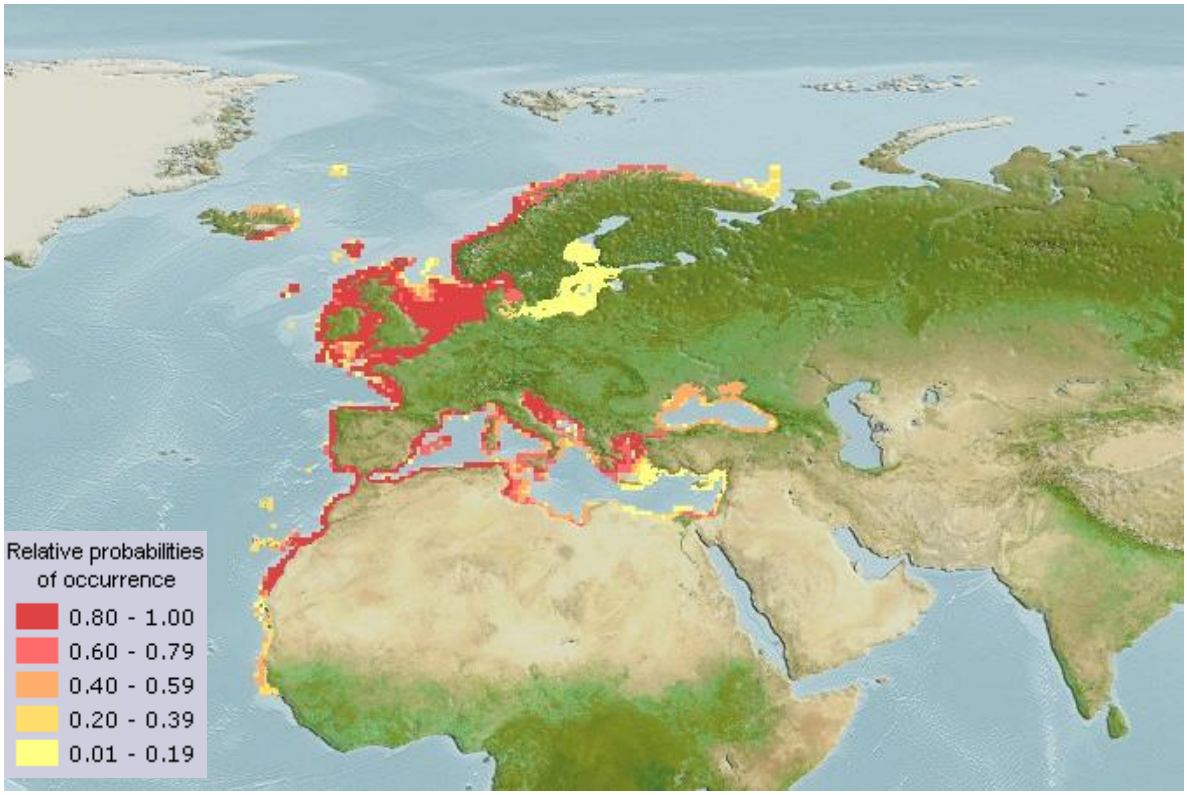

Figure 2 - AquaMaps (2019, October). Computer generated distribution maps for Dicentrarchus labrax (European seabass), with modelled year 2050 native range map based on IPCC RCP8.5 emissions scenario. Retrieved from https://www.aquamaps.org.

Vast commercial interest and massive aquaculture production of this species allows nowadays to differentiate two different life cycles for this species: one regarding wild animals and the other regarding aquaculture production. In wild animals, maturity is reached between 2 and 3 years in males and 3 to 4 years in females in the Mediterranean Sea (Bagni, 2015), while in the Atlantic Ocean maturity starts to appear between 3 to 8 years. Aquaculture specimens reportedly reach sexual maturity at the age of 2 and 3 years, male as female respectively. Fecundity in this species may vary between 180,000 and 430,000 eggs kg^{-1} (Barnabé, 1995; Prat et al., 1999; Mylonas, et al., 2003). Wild European seabass in the early stages of life form schools inhabiting the coastal waters and estuaries, feeding on zooplankton. While in adult stages, they live a more segregated life and become predatory animals, feeding of other fishes and invertebrates (Tortonese, 1986).

Aquaculture cycle for this species is divided in two different sections: a husbandry usually land based, where it is possible to control the abiotic factors; and growing facilities, usually sea cages, where they will reach the final commercial weight (at least, 400 grams) after 12 to 20 months (Vandeputte et al., 2019).

European seabass has shown that it is one of the most important reared species in the Mediterranean basin. However, production is still affected by several pathogens, resulting in heavy losses for the producer. In collusion or as a causative factor, unfavorable environmental conditions, and poor management practices, lead to stress resulting in immune suppression, which will therefore, increase disease susceptibility. Some diseases that favor from this factor and have high impact in production are vibriosis (*Vibrio anguillarum*), photobacteriosis (*Photobacteria damselae* subsp*. piscicida*) and tenacibaculosis (*Tenacibaculum maritimum*).

Aquaculture limitations and perspectives

Aquaculture production implies a certain number of constrictions. For instance, the high stocking densities, along with transportation and handling, can act as important stressors inducers. Furthermore, rearing conditions are often geared towards fast production and growth, disregarding animal welfare. The provision of poor or non-optimized diets for the farmed species, as well as the bad breeding programs or difficulty in obtaining fingerlings, are also important limiting factors. Despite being harmful by themselves, all these factors together present an even greater risk for the fish, making them more susceptible to opportunistic pathogens, increasing the risk of developing diseases. Moreover, stress can lead to the misuse of energy, which would normally be directed towards metabolism and growing, to be consumed in response to the challenge (Sunyer & Tort, 1995; Verburg-Van Kemenade et al., 2009).

In respect to the problematic of feed supply, this can represent 42% of the total production costs, whose percentage varies with the intensity of production. Indeed, intensive production regimes invest more funds in fish nutrition than the extensive ones. Even so, according to FAO (2020), the growth of intensive productions in the past few years vastly surpasses the extensive aquaculture regimes. Nowadays, although nutrition is indeed a containment for the industry due to the feeding expenses, it is also a mechanism to improve fish quality. Therefore, the creation of new formulas to enhance feed supply quality and specificity is growing (Rana et al., 2009). This has beneficial consequences for the consumer, who has access to moderately accessible fish protein prices, for the producer, who can produce more fish with good selling quality, as well as for the animals that have a modelled diet aiming to fulfil all the requirements needed. In other words, investing in quality since the source products like the feeding ingredients is key for improving all the subsequent overall standards of selling products like fish (Trichet, 2010).

Immunological response

Innate immune response:

Innate immune system is transversally present in all animal species, being the only immune system of invertebrates. Despite fish being vertebrate animal, due to their evolutionary status and poikilothermic nature, the adaptive system is still far from the efficiency observed in mammals. Counterbalancing the deficits of the adaptive immune system, the innate immune system is well developed and extremely efficient. Innate defenses can be grouped into physical barriers, cellular, and humoral parameters (Secombes & Wang, 2012).

Introduction to innate immune system

Physical barriers englobe scales, mucus surfaces of the skin, gut and gills, and epidermis. Barriers created by the scales and epidermis are self-explanatory, acting mainly as static physical barriers. Mucosal layer, on the other hand, is dynamic, acting as a semipermeable, natural, physical barrier, that allows exchange of nutrients, hormones, water and gases. Fish skin mucus is constantly being produced by the epidermis, contributing to the variable biochemical composition that can also vary according to species, current development stage, sex, environment, and stress (either by natural causes or by handling) (Esteban, 2012; Fast et al., 2002). Due to this property it as a key role as a barrier for pathogens (biological barrier). Other important biological roles displayed are: osmoregulation, environmental protection against toxins and heavy metals, parental feeding in some species, communication, and protection against abrasion (Reverter et al., 2018).

Containing a wide specter of immune related compounds, such as, lysozyme, complement related proteins, Immunoglobulins M (IgM), antimicrobial peptides, lectins, mycosporine-like amino acids, between others (Magnadotir, 2006; Reverter, et al. 2018), mucosal barriers can act as an effective trap against pathogens. Accumulation of excessive mucus, due to constantly being produced is prevented by water current, that washes the most external and older layers. This mechanism is of particular relevance because it also prevents the colonization of the mucus layer by parasites (Esteban, 2012). As a primary barrier to pathogens, mucus is very efficient and, damages to the integrity of this layer may lead to an increased susceptibility to disease, as demonstrated by Fouz (1990) in turbot, Kanno (1989) in ayu, and reviewed by Dash (2018).

When a pathogen manages to overcome the physical barriers, it will then face cellular and humoral components of innate immunity.

Cellular components

When the physical barriers are crossed, the cellular defenses, the leukocytes, come into action. In teleost fish, the primary organ for leukocyte production is the anterior kidney, followed by spleen and thymus (Ellis, 1977). They result from a differentiation of a multipotent hematopoietic stem cells into two different lineages: myeloid and lymphoid. Myeloid lineage produces granulocytic cells (mainly neutrophils), monocytes, dendritic cells as well as other cells that compose blood. Lymphoid lineage is responsible by the production of T and B lymphocytes (further discussed under the adaptive immunity subject) as well as nonspecific cytotoxic cells (Mak et al., 2014), even if in the last one there are evidence that some myeloid precursors can differentiate into them, in particular cases (Grzywacz et al., 2011).

Activation and mobilization of cellular immune components is a complex process involving firstly, the recognition of the foreign agent. Followed by the recruitment and mobilization of the phagocytic cells, namely the monocyte/macrophages and neutrophils (Ellis, 1999). These cellular types are the first to arrive and respond to the initial infection (Smith et al., 2019). Pathogen's recognition is done through a vast array of molecules, named globally as pattern recognition receptors (PRR). These molecules recognize and bind with highly conserved biomolecules, known as "pathogen-associated molecular patterns" (PAMPs) (Medzhitov & Janeway, 1997). Immune response is initiated when PRR present in immune cells bind to pathogen PAMPs (Smith et al., 2019; Mogensen, 2009), starting a cascade of reactions.

Monocytes/Macrophages

Tissue macrophages can result of the migration and differentiation of circulating monocytes from the blood stream to the tissue, prior a stimulus (Grayfer et al., 2018). But, mainly by differentiation of previously seeded embryonic hematopoietic precursors already in the tissue, capable of replenishing themselves during homeostasis, independently from monocytes (Yona et al., 2013; Ginhoux & Jung, 2014; Epelman et al., 2014; reviewed in Grayfer et al., 2018). Despite being part of cell mediated defense, macrophages have an important role in both innate and adaptive immune systems. Bridging both responses through the presentation of pathogen antigens to T-cells, via major histocompatibility complex (MHC) I and MHC II from the adaptive response (Lehner & Cresswell, 2004). These cells can also adapt their phenotypes according to the inflammation stage since. M1 phenotype are commonly associated with pro-inflammatory response (in some species M1 macrophages can be further divided into subsets), and M2 are linked to immunosuppression, inflammatory resolution, and tissue

repairing (Hodgkinson et al., 2015) (for the sake of this thesis, no further differentiation will be made).

Macrophages are responsible for (i) phagocytosis, mediated by phagocytic receptors in the membrane as well as hydrophobic interactions; (ii) production of reactive oxygen species (ROS) and nitrogen oxygen species (NOS); (iii)Tryptophan degradation, contributing to the homeostasis; (iv) and production of cytokines and chemokines (Grayfer et al., 2018).

Neutrophils

In teleost fish, neutrophils are the most abundant granulocyte cell type, which are defined by the presence of cytoplasmic granules containing myeloperoxidase (Havixbeck & Barreda, 2015). Despite the fact that only about 5% of all circulating leukocytes are neutrophils (Havixbeck & Barreda, 2015), in an inflammatory setting they are the fastest mobilized leukocyte to the place of infection. As an example, in rainbow trout (*Oncorhynchus mykiss*) the proportion of leukocytes in the peritoneal area prior inflammation is 55% lymphocytes, 40% macrophages, and 2 % neutrophils (Ellis, 2001) while, after intraperitoneal infection with bacteria, their proportion surpassed the macrophages number after 6 hours (Afonso et al., 1998). Therefore, considering the fast response, the organism must have a reservoir, which in this case is in the hematopoietic section of the kidney (Scharsack et al., 2003). Extremely efficient pathogen killer, due to an interconnected system of mechanisms. Those include phagocytosis, like in macrophages, production of ROS and NOS, as well as the release of antimicrobial and cytotoxic substances. Contrary to macrophages, neutrophils exceed in extracellular immune response. Allowing the destruction of pathogens too big for phagocytosis and pathogens that were able to avoid phagocytosis (Havixbeck & Barreda, 2015). Extracellular action of neutrophils includes the release of soluble antimicrobial components, like ROS and NOS, which do not have target specification, leading to the possibility of damaging the host (Di Giulio et al., 1989; Havixbeck & Barreda, 2015).

Mechanisms of action employed by neutrophils to promote inflammation and combat pathogens include the (i) degranulation intra or extracellular of the cytoplasmic granules and (ii) placement of neutrophil extracellular traps (NETs) when stimulated (Palic et al., 2007). NETs consist in the elongation of the chromatin enriched with antimicrobial granules, that will bind with microorganism. This technique prevents microorganism to spread, while at the same time ensures the presentation of antimicrobial components capable of destroying them (Brinkmann et al., 2004). Neutrophils are not only responsible for promoting inflammation, but also to promote the resolution of it. When resolution

phases begin, synthetized protectins signal circulating neutrophils, stopping infiltration towards infected tissues (Schwab et al., 2007). Neutrophils are capable of change their phenotype according with inflammation status, during pro-inflammatory response, they act as said above. Yet, in resolution phases, they are capable of signalizing and producing pro-resolving molecules. Havixbeck (2016) showed that in pro-inflammatory response, neutrophils produced a chemotactic lipid (LTB_4) increasing ROS production. While the neutrophils isolated during the resolution phase not only stopped the production of LTB_4, as well, they start producing another lipid called LXA_4 that increases the uptake of apoptotic neutrophils by macrophages. Another important mechanism is the self-induced apoptosis aided by ROS production, and presentation at surface of death receptors (TNFα, TRAIL receptors or Fas) (Fox et al., 2010). The presentation of the death receptors signals other phagocytes like the macrophages, to engulf the apoptotic cell, and processes it (Havixbeck & Barreda, 2015). This mechanism prevents the release of the histotoxic components of the neutrophils (Fox et al., 2010).

Dendritic Cells

Dendritic cells are phagocytic cells, and can be described as professional antigen presenters. Bridging this way, the innate and adaptive immune systems (Pozzi et al., 2005). Compared with both mentioned cell types (macrophages and neutrophils), abundance of dendritic cells is lower. Dendritic cells recognize PAMPs and damage-associated molecular patterns (DAMPs) through a number of PRR with particular interest to the absent-in melanoma (AIM)-like receptors (ALR) (Bermejo-Jambrina et al., 2018). After recognition by part of PRR, the cell will lose the phagocytic capacity and will start migrating to secondary lymphoid tissue. During this process an uptake of antigen and production of cytokines starts to occur. Processed antigen peptides will be presented at the surface thanks to the MHC complex (Guermonprez et al., 2002). Dendritic cells will then present the peptides no naïve T-cells colonies to initiate the adaptive response (Bassity & Clark, 2012).

Now, with the introduction to the two main phagocytic cells of the innate immune response, is time to explain what is the phagocytose and how it operates. Phagocytosis is initiated when the phagocytic cell detects, through medium of PRR, foreign components like PAMPs (Medzhitov & Janeway, 1997) and host DAMPs (Beg, 2002). Engulfment is initiated by the cytoplasmic elongation of the phagocyte around the identifyd cell. These pseudopods will eventually completely embrace the target and fuse. As result of fusion, a phagosome is formed. Previously formed lysosome will be merge to this organelle, forming a phagolysosome. In the phagolysosome, a vast array of degradative enzymes will act to destroy the targeted engulfed cell (Neumann et al.,

2001). This process enhances further more phagocytosis in the vicinity area, due to the release of phagocytic residues, that will act as PAMPs and DAMPs, to another phagocyte (Medzhitov, 2007).

Pattern recognition receptors (PRRs)

PRRs are a group of germline encoded proteins, vertically transmitted in a relatively stable form, across species (Magnadottir, 2006). In this way, they reflect the importance of these receptors in the defense of organism, across species. Capable of recognize and bind with conserved invariant features of microorganisms, broadly present across different species (PAMPs) (Janeway, 1989), and host damaged cells (DAMPs) (Beg, 2002). PRRs evolved to have specificity to PAMPs, due to three particular characteristics. PAMPs have a crucial role in microbial physiology, meaning that a disruption of those components would lead to the incapability of the pathogens to be harmful, and therefore they need to be present. They need to be invariant across species of a given class, otherwise PRR, wouldn't be able to recognize it. Finally, they must be exclusive to microorganism, to prevent auto-immune responses (Medzhitov, 2007).

Common PAMPs, in bacteria and fungus are components of cell wall, and in virus are viral nucleic acids (Medzhitov & Janeway, 1997). Four classes of PRRs can be found in teleost fishes, European seabass included: Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), and C-type lectins receptors (CTRs). There is a class the absent-in melanoma (AIM)-like receptors (ALRs), not present in fish (Hansen et al., 2011). PRRs are distributed across, some specific endothelial and epithelial cells, neutrophils, macrophages, and dendritic cell (Hansen et al., 2011).

Humoral components

Now with the introduction to the main effector cells of the innate immune response, as well as the knowledge on how they detect pathogens, is time to introduce the main effector components. Humoral parameters are diverse, with a multitude of functions - ranging from messengers to bactericidal. In this topic, some of the most relevant parameters are presented, as well as their mechanism of action, and importance to the immune response.

Respiratory burst:

Respiratory burst is a mechanism used by macrophages and neutrophils to swiftly produce and release reactive oxygen species (ROS), to destroy the pathogens. ROS formation within the cells is mediated by NADPH-oxidase which will transfer electrons to oxygen molecules (Briggs et al., 1975). This reaction will generate superoxide anions,

that can sequentially be converted into hydrogen peroxidase, catalyzed by the superoxide dismutase (Desforges et al., 2016). This cascade of reaction can progress further: into the production of hypochlorous acid, mediated by myeloperoxidase in the presence of chloride; or converted into water (Roos et al., 2003). Nevertheless, NADPH-oxidase will only generate superoxide anions after cell activation. Activation is mediated by a group of protein kinases (after PAMP recognition), that will interact with NADH-oxidase enzyme complex and change its conformation (Olavaria et al., 2010). ROS are extremely efficient pathogen killers due to extreme toxicity. This factor requires a high regulated activity, otherwise severe damage to the host can occur (Di Giulio et al., 1989; Havixbeck & Barreda, 2015). In the phagosomes, maximum efficiency is achieved when both the superoxide anion and hydrogen peroxidase are present. Because they can react and form hydroxyl radicals and oxygen singlets, both are extremely efficient pathogen killers (Roos et al., 2003). Production of ROS is not exclusively related to phagocytes, for example, liver is one important indicator of oxidative stress. Under normal conditions the production of ROS in this organ occurs during metabolism and is well balanced with anti-oxidant agents (Matés, 2000). However, the existence of stress, like in an infection, will lead to a deregulation of ROS production (Matés, 2000). This makes the liver a good indicator of stress status in fish (Sun et al., 2007).

Nitric oxide (NO) response is mediated by the stimulation of the phagocytic cells, by cytokines and PAMPs (Mosser & Edwards, 2008; Havixbeck & Barreda, 2015). NO results from the conversion, via oxidation, of L-arginine to L-citrulline, by action of inducible NO synthase (iNOS) enzyme (Grayfer et al., 2018). NO is utilized to inhibit cell proliferation, promote vasodilatation, intracellular signaling, and antimicrobial agent (Havixbeck et al., 2016). The conjugation of NO with superoxide anion results in the formation of peroxynitrite, another powerful antiparasitic/antimicrobial agent (Henard & Vázquez-Torres, 2011). Like with ROS, mediation of the production of NO must be under constant surveillance due toxic potential.

Antimicrobial Peptides (AMPs)

Despite the scarce works developed on the matter, AMPs are a group of compounds that comprises proteins of small dimensions, like defensins, cathelicidins, hepcidins, piscidins (fish exclusive), and histone-derived peptides (Smith et al., 2019). AMPs, can be found in the mucus, liver, and gills, where they are part of the first line of defense (Whyte, 2007). Despite the myriad of different AMPs, production is dependent of the activation and translocation of the transcription factor NF- κB, via PAMPs recognition (Valero et al., 2018). AMPs are characterized as having a general microbicide activity (Smith et al., 2019). The mechanism from which they appear to act more frequently is by

membrane disruption and pore formation. Lysis is achieved by the agglomeration of this peptides in turn of one pore creating a transmembrane hole, and via depolarization of cell wall (Valero et al., 2018).

Lytic Enzymes

Lytic enzymes are a group of enzymes that comprises hydrolases, like lysozyme and chitinase, cathepsins, and the lytic pathway of the complement system (Alexander, 1992). Lysozyme is one of the most vastly studied (Smith et al., 2019), and it will be the only reviewed in this chapter (lytic pathway of the complement system will be reviewed under the complement subject). Lysozyme was discovered in 1922, by Fleming. It is associated with leukocyte rich tissues, like liver, kidney, spleen, and gills, where the prevalence of infections is higher (Lindsay, 1986). It is also an important component of teleost mucus (Ingram, 2006), and serum (Murray & Fletcher, 1976). Neutrophils, macrophages and monocytes are the main producers of lysozyme, being the neutrophils responsible for the circulatory lysozyme presence in serum (Murray & Fletcher, 1976). Lysozyme is a potent, antiparasitic, antibacterial, and antifungal component of the organism (Ingram, 2006). The enzyme acts by hydrolyzing glycoside bonds of the bacterium peptidoglycan walls causing lysis. By degrading chitin of both invertebrates and fungi (Ingram, 2006). Interestingly, current knowledge also points lysozyme as a mediator of immune response (Ragland & Criss, 2017). Cell residues resultant from the degradation lysis act as PAMPs, activating PRR from phagocytes, increasing phagocytose (Ragland & Criss, 2017). Lysozyme is also capable to activate the complement system and enhance opsonization (Smith et al., 2019).

Complement System

Complement system is composed by a cascade of proteins in serum, acting together to mediate the defensive mechanisms of the immune system. The majority of the enzymes belonging to the complement system, are inactive, until activation either by binding with each other or by enzymatic action (Holland & Lambris, 2002). Up to date 35 membrane-bound and soluble proteins have been reported as being part of the complement system (Holland & Lambris, 2002; Smith et al., 2019). Complement system has an important role: at enhancing phagocytosis, by opsonizing the pathogens; as an inflammation mediator, since it can lure phagocytes to the inflammation areas, and at the same time can provoke lysis of pathogenic cells by creating pores; by interconnecting the innate and adaptive immune systems; and finally, in the clearance of the immune complex after infection (Holland & Lambris, 2002). Complement system has three pathways, depending of the activation method. Classic complement pathway is initiated by the binding of an antibody to the cell surface, acute phase proteins (Petersen et al., 2001),

or by direct activation by certain virus or bacteria (Spiller & Morgan, 1998). Alternative complement pathway (ACP) is activated directly by pathogens, without antibody support (Holland & Lambris, 2002). Lectin complement pathway results from the binding of a protein complex consisting of mannose-binding lectins (MBL) to surface bacterial cell polysaccharides - the mannans (Holland & Lambris, 2002). For the sake of this work, we will focus on the ACP and its hemolytic activity. Fish complement has high efficiency at hemolyze erythrocytes from mammals, through ACP (Sunyer & Tort, 1995). This property is useful in studies where it's important to evaluate the complement activity, namely studies with infection or inflammatory response (Holland & Lambris, 2002).

Acute Phase Proteins

Acute Phase Proteins (APPs) are defined as a group of proteins, synthetized in the hepatocytes, whose production rates significantly increases due to inflammation or infection. Stimulation is achieved by the release of cytokines, produced by immune competent cell like macrophages (Roy et al., 2017). Function can vary, from tissue reparation, to antimicrobial activity, and can act as well as homeostasis mediators (Bayne & Gerwick, 2001). Some of the most important protein belonging to this group are the C-reactive protein, and serum amyloid protein. Both capable of activating the complement system, and promoting the clearance of apoptotic cells (Smith et al., 2019). Another important APP that acts a little different is the transferrin (Magnadóttir, 2006). By being upregulated during infections, the amount of this enzyme mobilized to the infection location increases. Acting as a growth inhibitor for pathogens due to the capability of removing the available iron from the tissues (Magnadóttir, 2006). Iron is a crucial element for bacterial maintenance.

Cytokines

Cytokines are key signaling molecules, responsible for controlling and mediating both innate and adaptive immune system (Magnadóttir, 2006). They are also important mediators in cell differentiation, growth and survival (Vilcek & Feldmann, 2009). Several smaller groups are included under the cytokines label. Chemokines, for example, are a superfamily of cytokines, responsible for stimulating the recruitment, activation, and adhesion of immune effector cells to infection sites (Whyte, 2007). Not only that, but recent studies have been demonstrating the importance of chemokines at interconnecting both types of immune response. Macrophages and dendritic cells post-activation via PRR, initiate a series of cascades that will produce chemokines. Thus, dendritic cells loaded with pathogen antigens from the infection tissue will be signaled to move to the lymphoid tissues (Luster, 2002), acting as an antigen presenter cell that will

activate both T-naïve and B-cells (Pozzi et al., 2005). Other relevant families belonging to the cytokine's are the tumor necrosis factor, interferons like, and interleukins families.

Acquired immune response

Primordial innate immune response may not suffice the requirements to combat pathogenic infections effectively. So, organisms developed a parallel mechanism to help them prevailing. This mechanism is the adaptive immune response. Despite being classified as two different responses, they are both intimately interconnected and can be seen in multiple interaction, during immune response. First traces of an adaptive immunity appeared within the agnatha superfamily. Still, a full developed system was only observable in earlier teleost (Schluter et al., 1999; Flajnik & Kashara, 2010). Like the innate response, the acquired immune response is composed by humoral and cellular components. Cellular component includes T and B lymphocytes. Interestingly, considering that the acquired immunity is a concrete direct response specific for the pathogen in question, the amount of different B and T-cells needs to be astronomical. For this, the organism achieves the capacity of eliciting a response to a vast role of pathogens, by allowing the cell receptors, from both, to be highly variable, with a set of various recombining sequences (Nikolich-Zugich et al., 2004; Smith et al., 2019).

B lymphocytes

B lymphocytes are key element in adaptive response. Responsible for the production of immunoglobulin (antibodies) and professional antigen presenters to the T lymphocytes. Antigen presentation competence is a sequential process, starting by the binding of the antigen with the B cell receptors (BCRs), followed by the internalization and processing. Processed antigen is then presented at surface via major histocompatibility complex (MHC) type II (MHC-II), to evoke T lymphocytes recruitment (Lanzavecchia, 1985). BCRs are a combination of membrane-bound antibodies, and two signaling proteins - Igα and Igβ - with the last one being the only cytoplasmic portion of the receptor (Secombes & Wang, 2012). As mentioned above, the specificity of the receptor varies with the DNA recombination. Activation of naïve B cells via antigen recognition will initiate a process of proliferation and maturation into plasmatic cells. Those, at initial stages, are not capable of producing large quantities of antibodies. Consequently, a new differentiation happens, leading to the appearance of short-lived plasma cells (SLPCs) and long-lived plasma cells (LLPCs) (Secombes & Wang, 2012). SLPCs are fast responsive cells that, after interaction, can diverge back into LLPCs and become part of the memory immunity.

LLPCs are highly effective antibody producers and can persist for a long time (Elgueta et al., 2010), contributing for memory immunity.

Antibodies, as seen, are produced by B lymphocytes and can be divided into 3 groups: IgM, IgD, and IgT/IgZ. IgM is the most abundant circulatory antibody and it can be found in both transmembrane form or as a secreted protein (Smith et al., 2019). Functions of IgM are well conserved along evolutionary tree and include: opsonization, antibody-dependent cell-mediated cytotoxicity (Tay et al., 2011), and complement activation (Holland & Lambris, 2002). IgM is a good example of the restrictiveness that classifying the immune response as innate or adaptive presents. Despite being part of the classical classified adaptive system, IgM interacts directly with innate system, by enhancing phagocytosis via opsonization (Secombes & Wang, 2012). IgD is generally observed only in a transmembrane form (Smith et al., 2019). Contrary to IgM the lack of information regarding its utility and mechanisms are severe (Smith et al., 2019). To date, not much is known besides the structure that is highly variable, and recent evidences point to a phylogenetically appearance near the resurgence of IgM (Zhu et al., 2014). The last group is the immunoglobulin (IgT/IgZ), which function has been mainly described in mucosal surfaces (Zhang et al., 2014). Zhang showed a much higher prevalence of IgT in the gut-associated lymphoid tissue compared with plasma. It was also shown that pathogen infection in the gut leads to a much higher ratio of IgT in the local. Moreover, Tay (2019) demonstrated that the pathogen was coated with IgT. Thus, it is possible to propose that IgT assumes similar functions of the IgM in mucosal areas.

T lymphocytes

T lymphocytes, are key effectors of the adaptive response. T-cells are generated in the thymus and, like B cells, they possess a special membrane bound receptors - TCRs. TCRs differ from the BCRs, by being exclusively membrane bound (Secombes & Belmonte, 2016). Activation of T-cells is mediated by antigen presenter cells, which will trigger differentiation into 2 subpopulations: T helper (Th), and T-cytotoxic (Smith et al., 2019). Cytotoxic T-cells are capable of killing the targets by induction of apoptosis. This process can occur in two ways: the secretory pathway with the release of toxins (Elmore, 2007), and the non-secretory pathway which is dependent of receptor binding. T helper cells are a group of T lymphocytes that after activation can differentiate into 4 major different types: Th1, Th2, Th17, and T-regulatory (Secombes & Wang, 2012). T helpers are differentiated by the profile of cytokines produced and, in general, all play a critical role in the orchestration of the adaptive immune response.

Major histocompatibility complex (MHC)

MHC is composed by proteins in the cell surface, synthetized by group of genes highly polymorphic between individuals (Fisher et al., 2013). Two classes of MHC are observable: MHC I, and MHC II (Wegner, 2008). The importance of polymorphism synthesis is extremely valuable for the capacity of the organism to recognize different antigens (Secombes & Belmonte, 2016). MHC I is present in the majority of the cells and it is responsible for monitoring intracellular pathogens and tumors (Janeway, 2001). In contrast, MHC II is only presented by phagocytic cells capable of presenting processed peptides to the T helper cell subset (Wegner, 2008). The recognition of MHC II-antigen complex must be precise for the given TCR. If so, it will initiate a signaling cascade in the Th (Fisher et al., 2013; Secombes & Belmonte, 2016).

Vaccination

Fish vaccination has been improved since the 40's from previous century. Historically, vaccination started with the presentation of whole organisms. Scientific advancements allowed the development of safer, yet more expensive types of vaccination. As examples, there are subunit vaccines, and nucleic acid vaccines (including both DNA and RNA) (Ma et al., 2019). Nevertheless, in fish, the most utilized type of vaccination goes for the conventional type, which include the inactivated or killed pathogen, and the live vaccine (Ma et al., 2019). Vaccination presents transversal advantages to both the organism and the environment. It is reportedly more effective and safer than therapeutic medicines (Folb et al., 2004). Andre in 2008, stated the importance of vaccination in the control, mitigation and eradication of some diseases in human. Premises that can be translated to vaccination, maybe in a smaller perspective since mass vaccination is not yet possible or affordable. As it was demonstrated, different types of vaccines show different advantages and disadvantages. And yet, efficiency is not only related with the vaccine itself. Vaccination methodology also performs an important role on how effective a certain type of vaccine is. Presently there are 3 main types of vaccination: immersion, oral, and injected. Vaccination via injection is the most effective method, followed by immersion and finally oral. However, from the three types, injection is the hardest laboring and stressful one for the host (Vinitnantharat et al., 1999).

Conventional vaccines

The concept of the inactivated/killed vaccine is simple, consisting essentially in the removal of the ability of the pathogen to replicate/proliferate and/or infect the host. Characteristically between the two types of conventional vaccines, this type of vaccine is safer but not as effective as the live vaccination. It also brings the advantage of being cheap to produce, as well as not bringing environmental concerning. Vaccines recurring to this technique need the aid of an adjuvant to initiate the immunological response from the host (Ma et al., 2019).

As it is displayed in the name, live vaccines are constituted by the living pathogen. The pathogen, can either be present in an attenuated state, or with a natural virulence towards the fish species low. Live vaccines have an improved immunogenic response from the host, and therefore, increasing the immunological response of the organism towards a future contact. As it is expected since the pathogens are alive, the risk of an outbreak is considerably higher when compared with the inactivated/killed vaccines. Contrary to inactivated/killed vaccines, live vaccines don't need the aid of adjuvants, once they possess the same mechanism as the wild pathogens to enter and proliferate in the host (Ma et al., 2019).

Functional Feeds

From a long time that is known that nutrition is important for the correct development of organisms. Fish are no exception to this rule. While in the wild, nutrition is free and therefore tends to be the ideal and balanced, as mentioned before in the introduction, in aquaculture, feeding comes generally from an anthropogenic source. The shift towards a more sustainable and responsible industry, induces in the producers a necessity to fulfill those demands. Therefore, once again, diet is a key factor for that achievement. Diet has an important role in health and growth (Trichet, 2010), and must be optimized. Nevertheless, these properties lead to an investment in investigation to find sustainable sources, diversify and improve nutrition, leading to a higher return to the producers and consumers.

Pressure and investment in the conception of diets, created a new important aspect in the industry of feeding: the development of tailored diets, with benefits above the ones that a conventional diet have. While conventional diets aim the fulfillment of the basic energy and growth requirements of the organism, new diets are being designed to have an increased value, like higher growth rates, beneficial health properties, environmental sustainability and economic benefits (Li et al., 2009; Olmos-Soto, 2015).

Therefore, we can simplify and introduce the concept of functional feed, defined as feeds with added benefits – health and/or growth performance enhancers, other than the essential nutritional requirements (Li et al., 2009). To the state of the art, several components have already been introduced, such as prebiotics, probiotics, nucleotides, and yeasts (Oliva-Teles, 2012).

Some important additives in functional feeds that aim to improve health status are vitamins that possess a vast role of important functions in the development, function and protection of the organism (Shiau et al., 2015). Vitamin C is an important antioxidant and has been reported as being an important modulator in the normal development of the skeleton (Gouillou-Coustans et al., 1998), as well as augmenting the normal lysozyme, phagocytic, complement and respiratory burst activities (Lin & Shiau, 2005; Kumari & Sahoo, 2015). Vitamin E, at an appropriate dosage, also enhances the normal growth, and function of intestine (He et al., 2017). Vitamin E supplementation also suggests a reduced risk of infection, and increased immunological response (Lewis et al., 2019).

B-Glucans are a class of polysaccharides with important bioactivity in the immune system. Their main functions are, the improving phagocytosis, contributing to a more efficient production of cytokines and interferons, and enhancement of antigen processing and recognition (Rodrigues et al., 2020). Administration of B-glucans has been under

spotlight, and proved beneficial. One of the most frequent ways utilized to perform the administration is the inclusion in feed. This way brings added benefits to the “normal” internal flora of the gut (Swennen et al., 2006).

Currently, one of the most important branches of investigation and investment in the functional feeds fields, is the addition of phytogenic compounds. Phytogenic compounds are plant-derived bioactive compounds that positively affect the health status and growth performance of animals (Puvača et al., 2013). Those phytogenic compounds can act in a variety of ways. For example, polyphenolic compounds from grape fruit have been amply studied for the anti-aging properties and antioxidant activities (Xia et al., 2010). As anxiolitic and relaxant like passiflora and valerian extracts, capable of reducing stress (Dhawan et al., 2001; Hattesohl et al., 2008). Essencial oils, that are a blend of natural organic compounds resultant from secondary metabolism of aromatic plants (Sutili et al., 2017), such as oreganos, cinnamon, tea, and rosemary, and medium chain fatty acids (MCFA) like caprylic and caproic acids are known to have antibacterial and antiparasitic properties (Firmino et al., 2021; Sutili et al., 2017; Rigos, et al., 2016;), therefore, they are interesting compounds to be added in diets.

Ygeia+

Ygeia+ is a functional diet conceptualized and produced by Soja de Portugal. Ygeia+ aims for a commercial niche of short-term feeding diets with intention of preventing adverse outcomes of programmed manipulations (such as calibration and vaccination), or predictable environmental changes (such as transference between facilities or rearing condition). The pathway followed by Ygeia+ to achieve the goal, is the modulation of the endocrine system during the stress response, by adding a variety of phytochemical additives (Aquasoja, personal communication), reported to have beneficial effects to recover faster from allostatic load. However, Ygeia+ roles on the immune response are not fully understood. Phytochemical additives in fish diets have been reported as a viable strategy to enhance growth performance, and health status in fish (Firmino, et al., 2021). Microalgae supplementation are an example, thanks to the unique profile of constituents such as polyunsaturated fatty acids, natural pigments, bioactive compounds, among others (Peixoto et al., 2021). Kareem (2015), reported higher survival in tilapia (*Oreochromis niloticus*) fed a supplemented diet with *C. camphora*, when challenged with *Streptococcus agalactiae*. Various reports, in different species, are suggesting vegetable supplementation as a good prophylactic measure, more ecological friendly and as an alternative to the antibiotics utilization (Kareem et al., 2015; Mora-Sánchez et al., 2020; Gonçalves et al., 2019). Firmino et al. (2021), reviewed the most studied phytogenic additives in aquafeeds, in particular the ones with terpenes and organosulfur compounds. Reported as having antimicrobial, immunostimulant, antioxidant, anti-inflammatory and sedative properties as well as being growth promoters (Reverter et al., 2021).

Ygeia+ aims to modulate the endocrine system by adding phytogenic compounds that have proved positive effects on the organism, such as: antioxidant properties, like vitamin C, E and polyphenolics from grape fruit; relaxant components like extracts from passiflora and valerian; immunostimulants like β-glucans from *S. cerevisae;* antibacterial and antiparisitic compounds such as essential oils from oregano, tea leafs, rosemary and cinnamon. Other components with antibacterial and antifungical properties present in Ygeia+ are MCFA like caprylic and caproic acids.

Trial 1 – Effects of dietary Ygeia+ on the immune status of European seabass

Aim

The aim of this trial was to study how Ygeia+ interacts with the immune system of European seabass, by analyzing the effect of a short-term feeding period of 5 and 10 days. Also, the immune response after induced inflammation, via inoculation with heat inactivated *Photobacterium damselae* subsp*. piscicida,* was evaluated at 4-, 24-, and 48-hours post-injection. The proposed approach allows to understand how Ygeia+ affects the European seabass immune response prior to and during inflammation.

Material and methods

Experimental design

The study took place at the Centro Interdesciplinar de Invetigação Marinha e Ambiental (CIIMAR) facilities. Before starting the trial, European seabass obtained from IPMA-EPPO (Olhão, Portugal) were subjected to a 14 days quarantine period under the surveillance of Bioterium of aquatic organisms (BOGA) from CIIMAR. Posterior transference to the trial room, acclimatization to the system and conditioning was performed for another 15 days. Fish were weighted (36.5 ± 7.6 grams) and fed during quarantine and acclimatization with a commercial diet (Soja de Portugal) and the trial took place in two different recirculating aquaculture systems (RAS). In a first approach, 20 fish were randomly distributed in 12 tanks and acclimatized in a RAS (100 liters after acclimatization). Afterwards a feeding trial was performed and fish were fed two times a day until apparent visual satiation, two different diets, Control and Ygeia+ in sextuplicate tanks. Close monitoring was done daily on water quality and fish. After the feeding period of 5 and 10 days, 2 fish per tank were sampled (sampling methods explained below in this chapter). Fish from this sampling point were regarded as time 0 control animals.

Then, 6 fish per tank were inoculated with heat-inactivated *Photobacterium damselae* subs. *piscicida* (*Phdp)* (strain PP3) and transferred to a new RAS system, composed of 8 tanks (50 liters), with 9 fish each. Transference from the previous system with 12 tanks to the new one containing only 8 tanks, was done during the sampling process of time 0. Which means, every fish from a given tank were collected, with 2 fish

being randomly sampled. While the remaining animals were mixed with fish from the same treatment, mildly anesthetized with 2-phenoxyetanol (Merk, ref. 807291, Germany) (concentration of 0.1 ml per liter) and inoculated with heat inactivated *Phdp*. From the mix of animals from each treatment 9 random fish were assorted per tank into 4 different tanks. A total number of 8 tanks were utilized (two different treatments). After 4, 24, and 48 hours 3 fish per tank were sampled. All sampling procedures were performed with euthanized fish. Euthanasia was performed with well aerified bath utilizing a concentration of 2-phenoxyetanol ranging from 0.5 to 0.7 ml per liter.

Bacteria Inoculation procedure

Stocked *Phdp* (strain PP3 isolated from yellowtail (*Seriola quinqueradiata*) by Dr Andrew C. Barnes (Marine Laboratory, Aberdeen, UK)) was cultured in Tryptic Soy Agar supplemented with NaCl to a final concentration of 2% (TSA-2), for 48 hours at 25ºC, and then it was inoculated to a medium of Tryptic Soy Broth, also supplemented with NaCl (TSB-2). New incubation overnight was performed at 25ºC under continuous shaking (Machado et al., 2015). By the end of last incubation, when the bacteria were under exponential growth, they were collected, centrifuged at 3500 × g for 30 minutes and re-suspended in sterile HBSS at the desired concentration of 1×10^6 colony forming units (CFU) ml^{-1} according to Costas (2013). Real concentration was confirmed utilizing the drop plate method, with a drop of 10 µL, in a dilution of up to 10^3 CFU. Bacteria inactivation was performed utilizing a water bath at 60 ºC during a period of 10 min. Correct inactivation was checked by plating the resultant culture into TSA-2 plate and the absence of bacteria growth after cultivation period (Machado et al., 2015).

Sampling methodology

Fish were euthanized by anesthetic overdose (2-phenoxiethanol, concentration ranging from 0.5 to 0.7 ml per liter of seawater). European seabass were individually weighed and measured immediately after death. Blood withdrawal was performed using sodium-heparinized (3000 U/ml) 1 ml syringes (Braun), followed by liver collection. Blood samples were utilized for total leukocyte (WBC) and erythrocytes (RBC) counting, hematocrit (Ht) assessment, as well as hemoglobin (Hb) evaluation. In this study, blood was also used to perform blood smears in every sampled fish. The remaining blood was centrifuged at 10,000 × rpm for 5 min at room temperature to isolate plasma. Plasma was immediately frozen in liquid nitrogen and transferred to -80 °C until assayed.

Hematological and analytical procedures

Hematological analyses were performed according to Machado et al. (2015). With data obtained from blood analyses three indexes were calculated: mean corpuscular volume

(MCV; µm3) = (Ht/RBC) × 10; mean corpuscular hemoglobin (MCH; pg $cell^{-1}$) = (Hb)/ RBC × 10; mean corpuscular hemoglobin concentration (MCHC; g 100 ml^{-1}) = (Hb/Ht) × 100.

Blood smears immediately performed after blood collection and left to air dry, after which they were fixed using a solution of formol-ethanol (10% of 37% formaldehyde in absolute ethanol). Staining process started with a specific reagent for peroxidase activity of neutrophils (Afonso et al., 1998), followed by common eosin-hematoxylin procedure. Slides were posteriorly analyzed under microscope under oil immersion (1000×). At least 200 leukocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils (figure 3). Data utilized afterwards to obtain the absolute concentration of each cell type and relative proportion, as well as some cellular ratios.

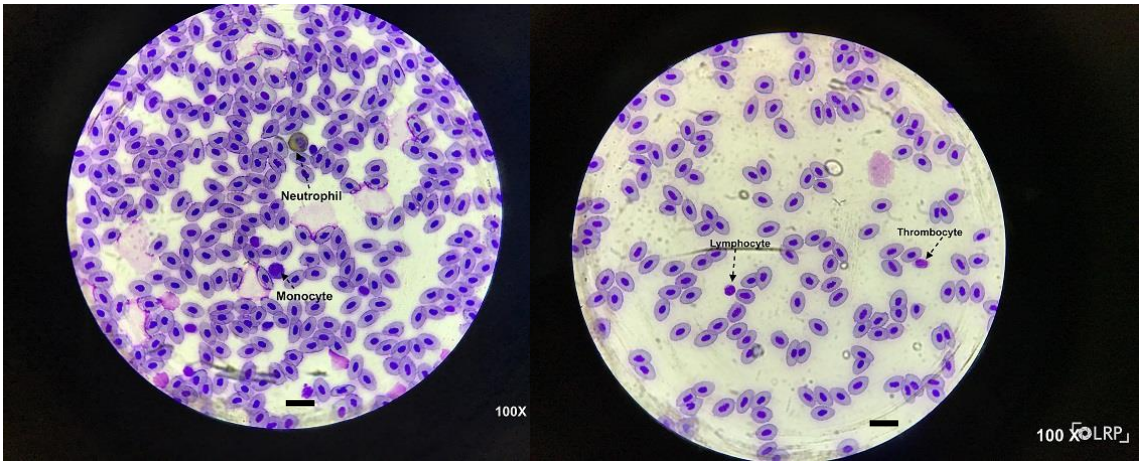

Figure 3 - Blood D. Labrax smears stained with Wright's stain. Neutrophils were labelled using the Antonow's technique presenting abundant peroxidase granules. Scale bar: 10µm Photo by: Ramos-Pinto

Innate Humoral Parameters

Innate humoral parameters evaluated in plasma consisted of peroxidase, lysozyme and alternative complement activity (ACH50). Lysozyme activity was evaluated utilizing a methodology first described by Ellis (1990), and adapted by Costas (2011). This method consists of the evaluation of bacterial lysis (bacterial solution from a standard concentration), due to addition of sampled plasma. The result is the difference between the blank (suspended bacteria) and the suspension with the sample. To revert the data to measurable quantities, it's necessary to elaborate a "standard line" using known concentrations of lysozyme. Briefly, this method consists in the elaboration of a standard line, with serial dilution from the stock Hen egg White lysozyme (HEWK) into di-Sodium hydrogen phosphate solution (0,05M and pH 6.2). In each well 250µL of bacterial suspension were added and 15µL of the previous dilution were added in triplicates. Same procedure was used with plasma from the sampled fish' (instead of 15µL of HEWK solution it was added 15µL of plasma, in triplicates). The sampling amount of lysozyme was obtained by substitution of the absorbance value in the equation of the standard curve.

Peroxidase activity is measured with an adaptation from the method described by Quade & Roth (1997). Method of evaluation consist in the assumption that one unit of peroxidase, produces a absorbance change of 1 in optical density. Shortly, daily preparation of sulfuric acid (2M), Hydrogen Peroxide (5M), and 3,3´, 5,5´-tetremethylbenzidine hydrochloride (TMB; 10mM) were prepared. Sampled plasma was diluted into a flat-bottomed 96-well plates (15µL of plasma and 135 µL HBSS without Ca+2 and Mg+2). Process followed by the addition of 50µL of TMB solution, and 50µL of Hydrogen peroxidase. The reaction will occur during 2 minutes, period of which after the reaction was stopped by adding into each well 50µL of sulfuric acid solution. Absorbance was read at 450nm, and quantity is measured in units ml-1 plasma.

Alternative complement pathway activity was evaluated utilizing the procedure described by Sunyer & Tort (1995). Complement activity was analyzed to evaluate organism response to the pathogen, and how the variation occurred during the time. Procedure includes the preparation of 3 buffer solutions: Sodium chlorite solution at a concentration of 0.9% with pH of 7.3 (NaCl solution), isotonic veronal buffered saline, composed by 20mM of EDTA, 0,1% gelatin, 5mM of sodium barbiturate, 0.13 M of sodium chlorite, and distilled water, with a final pH of 7.3 (EDTA-GVB), similar solution was done and instead of EDTA, 10 mM of magnesium and 10 mM of EGTA were added (Mg-EGTA-GVB). Therefore, following the protocol mentioned, the mammal blood utilized was from rabbit (Probiológica Lda, Portugal). Blood was washed throughout a series of 5 or 6 cycles of

centrifugation with a proportion of 1 part of blood to 4 parts of sodium chloride solution. Centrifugations were performed at 3000 rpm at 4ºC, the resultant pellet was then filtered and re-suspended again, until the liquid was clear. When achieved a new suspension was performed to obtain a concentration of 2.8×10^8 cells/ml (raRBC – rabbit red blood cells). Concentration was measured utilizing a microscope in a dilution of 1:200 in sodium chloride solution. Thereafter, 10µL of raRBC were added to 40µL of serial dilutions of plasma in Mg-EGTA-GVB, in a U-shaped 96-wells plates. Proceeding to a period of incubation of 100 minutes at room temperature, with regular shaking. Reaction was stopped utilizing 150µL of EDTA-GVB. Step followed by a centrifugation at 1000 rpm for 2.5 minutes. Followed by the transference of 100µL of the supernatant, to a new flat-bottomed 96-well plate. Two controls were utilized in each plate, one containing distilled water and the other Mg-EGTA-GVB. Optical density from the supernatants was read at 414 nm in a Synergy HT microplate reader (Biotek), to evaluate the grade of hemolysis resultant. Alternative complement Hemolysis units (ACH50) were defined as the plasma concentration at which 50% of RaRBC were hemolysate. Procedures were carried in triplicate for each step.

Oxidative stress and analytical procedures

Oxidative stress parameters were evaluated in the liver of European seabass from different sampling points. The parameters studied were protein, lipid peroxidation, catalase, total glutathione, and the activity of the super oxide dismutase.

Previously to oxidative stress analyses, all livers were homogenized. Homogenization comprehends a serial of steps. Sectioning of liver's weighting between 80 and 100 mg were extracted from each animal, and aliquoted into one Microcentrifuge tube. The Microcentrifuge tube was posteriorly filled in a ratio of 1/10 with homogenization buffer (phosphate solution at 0.1M and pH of 7.4). Homogenization was performed utilizing a manual sonicator, sample by sample, with sonication's varying from 30 seconds to 1 minute, depending on the visual homogeneity of the content. A 200µL aliquot homogenate designated for lipid peroxidation was made. While the remaining volume was centrifuged for 20 minutes at 10,000 $\times$ *g* and 4° C. Different aliquots were collected and stored at -80ºC.

Evaluation of the activity of interest proteins, can only be truly measured by knowing the total protein content of the samples. Total protein content analyses were performed with the colorimetric test Pierce BCA Protein Kit from Thermo scientific™. The evaluation of total protein briefly consists in the elaboration of a standard curve resultant from a serial dilution of bovine serum albumin (BSA). Both Standard's and samples (homogenates)

were diluted at a ratio of 1:50 in homogenization buffer and pipetted (25µL) in triplicates, into a flat-bottom 96-well microplate. A quantity of 200µL of the kit working reagent was added, resulting in a total of 225 µL in each well. Microplates were left still for half an hour at 25ºC to incubate. Finalizing with absorbance measurement at 562nm on a Synergy HT microplate reader, Biotek.

Lipid Peroxidation measurements were based on the principle that the peroxidation of lipids generates higher concentrations of malondialdehyde (MDA) in the liver, which reacts with thiobarbituric acid (TBA), resulting in a pinkish compound (Pérez-Jiménez et al., 2009). To the previously designated for this protocol aliquot 100µL of Trichloroacetic acid solution (TCA), at a concentration of 6.1M, were added. One milliliter of Thiobarbituric acid solutions (TBA), containing 2-Thiobarbituric acid at a concentration of 5mM, Trizma hydrochloride (Tris-HCl) 60mM, Diethylenetriaminepentaacetic acid (DTPA) 0.1mM, were added. After an incubation period of 1 hour at 100ºC in a muffle oven with exhauster, samples were centrifuged (11,500 rpm at room temperature) and 200µL of the supernatant were pipetted to a flat-bottom 96-well microplate in triplicates. As a negative control ultrapure water replaced homogenate. Reading was conducted on a Synergy HT microplate reader, Biotek, at 535 nM. Consequent results were expressed as nanomole of MDA per gram of wet tissue (nmol g^{-1}), calculated from a calibration curve.

Catalase measurement is based on the catalyst effect of the enzyme in the degradation of hydrogen peroxide into water and oxygen, the protocol followed is an adaptation from the originally described by Clairborne (1985). Briefly consists, in the addition of 140µL potassium phosphate buffer at 0.05M and pH of 7.0, to a previous diluted 10µL of sample (protein concentration of 0.7 mg mL^{-1}) into a UV flat-bottom 96-well microplate. As a negative control, instead of 10µL of sample it was added 10µL of potassium phosphate buffer at 0.1M and pH of 7.4. Addition of 150µL of reaction buffer (hydrogen peroxide, 24mM) must be performed swiftly with a multichannel pipette. Immediately after the absorbance must be read at 240nM during a period of 2 minutes with intervals of 15 seconds in between. Reading was performed in a Synergy HT microplate Reader (Biotek). Catalase activity was expressed in units, and each unit is represented by the amount of catalase needed to transform 1 µmol of hydrogen peroxidase per minute under standard conditions.

Total glutathione is based on the reaction of the glutathione itself and DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)) solution. This reaction produces a yellow chromophore called TNB (5-thio-2- nitrobenzoic acid) with maximal absorbance at 412nm. The ratio of

formation of TNB is linear to the total concentration of glutathione on the sample (Rahman et al., 2006). Concentration of glutathione is expressed in nmol mg^{-1} of tissue. Considering the principle, a standard curve with known concentrations resultant from a serial dilution of L-glutathione reduced solution 10mM (L-glutathione reduced dissolved sodium-potassium phosphate buffer, 0.2M, pH=6.5) was elaborated. All samples were previously diluted to a protein concentration of 0.7 mg mL^{-1}. 50µL of both samples, curve standards and blanks (potassium phosphate, 0.1M, pH=7.4), were pippeted in triplicates into a flat-bottom 96-well microplate. Meanwhile, to prepare the reaction buffer a couple of solutions were made, the above-mentioned sodium potassium phosphate buffer, 0.2M, pH=6.5, NADPH solution at 6mM, DTNB solution (4mM) containing 14.7mM of sodium hydrogen carbonate, and glutathione reductase solution (6.8 U mL^{-1}). Addition of the reagents to elaborate the reaction solution followed the strict order of 18 mL of sodium-potassium phosphate buffer, followed by 3mL of NADPH solution, which was proceeded by 6mL of DTNB solution and finalized with 1.5mL of glutathione reductase solution. Quantity suffices one microplate, and a new reaction buffer must be done fresh for every microplate.

Superoxide dismutase activity was evaluated by the ferricytochrome C method, as described by Pérez-Jiménez (2009). Briefly this method consists in the fact that the enzyme will compete with the ferricytochrome C for the superoxide radicals ($O_2^{\bullet -}$). Superoxide radicals are resultant from the xanthine/xanthine oxidase interaction. Therefore, it was prepared a 0.05M sodium phosphate buffer (pH=7.8), as well as two other solutions. Two more solutions were prepared one, containing a solution of 0,7mM xanthine, and 0.03mM cytochrome C and a solution of 0.1 mM Na-EDTA. The other, 0.03 U mL^{-1} xanthine oxidase solution. The analyses started with a serial dilution of a stock known concentration, to elaborate the standard curve. Thereafter, 50 µL of each, curve standards, previously diluted samples (protein concentration of 0.3 mg mL^{-1}) and blanks (sodium phosphate buffer0.05mM, pH=7.8) were pippeted in triplicates into a flat-bottom 96-well microplate. Process followed by an addition of 200 µL of the reaction solution, finalized with 50 µL of xanthine oxidase solution (last step must be fast). Absorbance was then read at 550 nm every 20 seconds for 3 minutes in a Synergy HT microplate Reader (Biotek). Results are presented in units of SOD mg^{-1}, where each unit is defined as the quantity of enzyme necessary to generate a 50% inhibition of the ferricytochrome C reduction rate.

Statistical Analysis

All data was described and outliers were removed, after which a Shapiro Wilk normality test was employed for each variable. Analyses of variance was performed for each

measurement variable and three nominal variables (days of feeding, diet, and inflammation time (0, 4, 24, and 48), also called ANOVA three factors. Also, considering that time 0 corresponds not only to the beginning of inflammation trial, but it can also be considered as the end of feeding trial. In these terms all parameters were filtered for 0 hours and a Two-way ANOVA was performed to evaluated if diet or days of feeding could affect the respective parameter in study. ANOVA analysis were performed even if normal distribution was not verified. According with several studies (Glass et al., 1972; Harwell et al., 1992; Fay & Gerow, 2013), ANOVA test is sufficiently robust to give accurate prediction if the number of samples for each group is similar even if there is no normal distribution (McDonald, 2014). Homogeneity of variances was also evaluated, utilizing Levene test, and more attention was given. Nevertheless, considering the small number of replicates, and that there is more or less the same number of replicas in each tested group homogeneity of variances could carefully be acknowledged in all data as reported before by Miranda-Fontaiña & Fernández-López (2009). In this work homogeneity was verified in almost every variable.

Further analysis was performed in case of any of the null hypothesis was rejected, to further understand the effect of the factor in the variance of the parameter under study. This analysis was conducted utilizing one- or two-way ANOVA in grouped conditions.

All statistical analysis was performed with statistical software SPSS from IBM.

Results

Data regarding fish weight was recorded individually during each sample point, before blood collection. Table 1 groups total weight with feeding time and diets.

Table 1 - Weight (g) of European seabass fed Ygeia+ and control diet fed for 5 and 10 days.

Time	Control	Ygeia+
Initial weight	36.5 ± 7.5	36.5 ± 7.5
5 days	37.45 ± 7.83	34.53 ± 6.33
10 days	37.26 ± 7.16	36.18 ± 6.34

*Values are presented as mean ± standard deviation (n = 48)

Hematological profile

Feeding time presented significant differences in red (RBC) and white blood cells (WBC) with higher quantities at 10 days in both diets (Table 2). Hemoglobin variation during the feeding trial could be explained by the feeding time with significantly higher amount at 10 days of feeding. However, hematocrit was not altered by feeding time (Table 4). From the three cellular indexes measured none showed differences in the interaction of diet and feeding time, nonetheless all indexes varied significantly with feeding time with MCH and MCHC higher at 10 days of feeding and MCV higher at 5 days (Table 2).

Table 2 - Descriptive statistics of differential leukocyte percentage, cellular indexes (MCV (μm^3), MCH (pg $cell^{-1}$), MCHC (g 100 ml^{-1})), total WBC ($\times 10^4/\mu L$) and RBC ($\times 10^6/\mu L$), hematocrit (%) and hemoglobin (g/dL).

	Feeding Trial			
	5 Days		10 days	
Parameter	Control	Ygeia+	Control	Ygeia+
WBC	6.62 ± 1.71	5.98 ± 1.60	8.03 ± 2.47	7.72 ± 1.92
RBC	3.26 ± 1.04	3.22 ± 1.00	4.58 ± 0.74	4.01 ± 0.90
Hematocrit	33.40 ± 4.12	31.00 ± 3.69	33.40 ± 4.16	31.00 ± 3.69
Hemoglobin	2.55 ± 0.84	2.77 ± 1.01	5.48 ± 2.16	4.93 ± 0.97
MCV	112.32 ± 27.39	99.97 ± 19.89	79.66 ± 17.25	112.32 ± 27.39
MCH	8.29 ± 2.93	9.09 ± 3.58	12.38 ± 2.58	8.29 ± 2.93
MCHC	6.15 ± 0.81	8.88 ± 3.09	16.09 ± 3.59	6.15 ± 0.81

*Values are presented as mean ± standard deviation (n = 12)

In response to inflammation, a modulation of the hematological profile was observed. The number of WBC was significantly increased at 4 and 48 h compared to 0 and 24 h after 5 days of feeding and regardless dietary treatment. Also, fish fed for a period of 10 days had a significant higher amount of WBC in circulation at 24 h compared to fish sampled at 0, 4 and 48 h (Table 5). Also feeding time showed to modulate WBC number in response to inflammation at 24 and 48 h with fish fed for 5 days presenting higher number of leucocytes than those fed for 10 days. Also, erythrocytes (RBC) number decreased from 0h to 4 h while fish fed during 5 days presented lower amount of RBC compared to those fed for a longer period (10 days). Haematocrit was also decreased in response to inflammation with values decreasing from 0 to 4 and 48 h. Hemoglobin analysis showed that during inflammation there were significantly differences regarding feeding times and sampling points grouped (Table 4). Fish fed for a 5 days period showed that before inflammation hemoglobin was significantly lower when comparing with the remaining sampling points. Also, at 0 and 24 h fish fed for 10 days presented higher hemoglobin then fish fed for 5 days (Table 5).

At the inflammation trial, MCV was significantly higher in fish fed for 5 days compared to those fed 10 days, whereas mean corpuscular hemoglobin (MCH) showed a significant evolution during inflammation, with a peak at 4 hours after inoculation, and a posterior decrease at 24 and 48 hours (Table 5). This response was observable at both 5 and 10 days of feeding, but it was more evident after 5 days of feeding. Fish fed Ygeia+ had a significantly higher MCH at 4 hours than those fed control at 5 days of feeding. Mean corpuscular hemoglobin concentration (MCHC), like MCV, showed a significant difference between feeding times, and hours post inoculation with an increase at 4 hours and a posterior decrease, in both diets during the inflammation trial (Table 5).

Table 3 - Descriptive statistics of differential leukocyte percentage, cellular indexes (MCV (μm^3), MCH (pg $cell^{-1}$), MCHC (g 100 ml^{-1})), total WBC ($\times 10^4/\mu L$) and RBC ($\times 10^6/\mu L$), hematocrit (%) and hemoglobin (g/dL) during inflammation trial (4, 24 and 48 hours) after 5 and 10 days of feeding with Ygeia+ or control.

Parameters	Inflammation Trial					
	5 Days					
	4 h		24 h		48 h	
	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+
WBC	7.34 ± 1.45	9.09 ± 2.09	9.08 ± 1.91	7.67 ± 1.15	8.90 ± 1.22	8.89 ± 1.98
RBC	2.38 ± 0.43	2.26 ± 0.46	2.78 ± 0.82	2.77 ± 0.54	2.98 ± 1.01	2.54 ± 0.75
Hematocrit	28.33 ± 3.04	29.30 ± 4.22	32.18 ± 4.92	32.64 ± 3.53	28.70 ± 4.60	27.67 ± 2.87
Hemoglobin	5.17 ± 1.96	5.89 ± 1.02	4.74 ± 1.29	4.31 ± 0.87	4.35 ± 2.24	4.42 ± 1.59
MCV	127.19 ± 28.39	131.93 ± 24.80	124.14 ± 29.63	115.79 ± 25.92	107.82 ± 23.89	115.45 ± 26.42
MCH	20.50 ± 8.21	26.82 ± 6.29	19.45 ± 7.15	15.87 ± 3.16	16.49 ± 6.16	16.31 ± 4.20
MCHC	18.24 ± 6.62	19.84 ± 4.53	16.84 ± 5.00	13.89 ± 2.07	15.57 ± 5.76	15.75 ± 4.60
	10 days					
	4 h		24 h		48 h	
	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+
WBC	6.05 ± 1.47	6.02 ± 1.01	6.27 ± 0.80	6.20 ± 1.35	6.87 ± 0.82	7.64 ± 2.38
RBC	3.53 ± 1.10	4.16 ± 1.20	4.29 ± 0.77	3.77 ± 0.65	4.71 ± 1.11	4.30 ± 1.22
Hematocrit	28.33 ± 3.04	29.30 ± 4.22	32.18 ± 4.92	32.64 ± 3.53	28.70 ± 4.60	27.67 ± 2.87
Hemoglobin	5.24 ± 2.04	6.51 ± 2.04	6.15 ± 1.44	6.57 ± 2.17	5.59 ± 1.49	4.89 ± 1.50
MCV	84.73 ± 23.19	77.92 ± 32.27	78.53 ± 18.81	85.34 ± 20.44	62.22 ± 17.91	68.18 ± 23.09
MCH	13.95 ± 4.86	15.81 ± 3.67	14.55 ± 3.18	17.56 ± 4.25	12.23 ± 3.53	13.16 ± 6.29
MCHC	18.04 ± 6.60	23.27 ± 9.46	19.48 ± 6.70	20.18 ± 6.48	19.49 ± 5.24	17.81 ± 5.63

*Values are presented as mean ± standard deviation (n = 12)

Table 4 - Evaluation of the role of diet, feeding time and sampling point, in the alteration of cellular indexes in the feeding trial (Two-way ANOVA), and inflammation trial (Multifactorial ANOVA). ANOVA: n.s.: non significant differences (p>0.05); If interaction was positive a One-way ANOVA was performed and Tuckey post-hoc analysis was performed, to identify the differences between groups.

Parameters	Two-way ANOVA (Feeding Trial)			Multifactorial ANOVA (Inflammation Trial)						
	Diet	Feeding Time	Diet x Feeding Time	Diet	Feeding Time	Sampling Point	Diet x Feeding Time	Diet x Sampling point	Feeding Time x Sampling Point	Diet x Feeding Time x Sampling Point
WBC	n.s.	0.008	n.s.	n.s.	< 0.001	0.016	n.s.	n.s.	< 0.001	n.s.
RBC	n.s.	< 0.001	n.s.	n.s.	< 0.001	< 0.001	n.s.	n.s.	n.s.	n.s.
Hematocrit	n.s.	n.s.	n.s.	n.s.	n.s.	< 0.001	n.s.	n.s.	n.s.	n.s.
Hemoglobin	n.s.	< 0.001	n.s.	n.s.	< 0.001	< 0.001	n.s.	n.s.	< 0.001	n.s.
MCV	n.s.	< 0.001	n.s.	n.s.	< 0.001	0.006	n.s.	n.s.	n.s.	n.s.
MCH	n.s.	0.002	n.s.	n.s.	< 0.001	< 0.001	n.s.	n.s.	< 0.001	n.s.
MCHC	n.s.	< 0.001	n.s.	n.s.	< 0.001	< 0.001	n.s.	n.s.	n.s.	n.s.

Table 5 - Evaluation of the interactions via Tuckey post-hoc to evaluate differences between groups in WBC, RBC, Hematocrit, hemoglobin, MCV, MCH, and MCHC. Different symbols account for significant differences in feeding times, capital letters were used to identify differences in sampling times.

Parameters	Sampling Point				Feeding Time		Feeding time x Sampling point							
							5 days				10 Days			
	0 h	4 h	24 h	48 h	5 days	10 days	0 h	4 h	24 h	48 h	0 h	4 h	24 h	48 h
WBC							A	B	A#	B#	B	B	A*	B*
RBC	A	B	AB	AB	#	*								
Hematocrit	A	B	A	B										
Hemoglobin							A#	B	B#	B	*		*	
MCV					#	*								
MCH							A	B#	C	C		*		
MCHC	A	B	B	B	#	*								

Differential leukocyte counting:

The absolute quantity of different peripheral leucocytes populations was calculated and is presented in the Table 6, illustrating the feeding trial and Table 7 and 8 illustrates the inflammation trial. No differences were observed among diets; however, thrombocytes, monocytes and neutrophils numbers were generally higher in fish fed for 10 days compared to those fed for 5 days. Evaluation of lymphocyte concentration in the feeding trial showed that fish fed for 5 days the control diet presented lower number of those cells compared to those fed for 10 days. However, the opposite was observed for fish fed the Ygeia+, since the number of lymphocytes was higher in fish fed for 5 days compared to 10 days.

Table 6 – Absolute quantity ($\times 10^4/\mu L$) of peripheral thrombocytes, lymphocytes, monocytes and neutrophils of European seabass after feeding trial at 5 and 10 days. Two-way ANOVA: n.s.: non significant differences (p>0.05); If interaction was positive a One-way ANOVA was performed and Tuckey post-hoc analysis was performed, to identify the differences between groups. Different symbols account for significant differences in feeding times.

Cellular Type	Feeding Trial				Two-way ANOVA		
	5 Days		10 days				
	Control	Ygeia+	Control	Ygeia+	Diet	Feeding Time	Diet x Feeding Time
Thrombocytes	4.77 ± 1.59	3.92 ± 1.09	5.63 ± 1.61	5.84 ± 1.30	n.s.	< 0.001	n.s.
Lymphocytes	1.54 ± 0.17*	1.88 ± 0.87#	1.61 ± 0.73#	1.18 ± 0.39*	n.s.	n.s.	0.05
Monocytes	0.14 ± 0.11	0.04 ± 0.04	0.48 ± 0.41	0.37 ± 0.31	n.s.	< 0.001	n.s.
Neutrophils	0.06 ± 0.04	0.09 ± 0.09	0.30 ± 0.30	0.18 ± 0.15	n.s.	0.03	n.s.

*Values are presented as mean ± standard deviation (n = 12)

Thrombocytes, lymphocytes, monocytes, and neutrophils showed a tendential response in the ratio during different times post-inflammation. The quantity of thrombocytes was significantly higher in fish fed control compared to those fed Ygeia+, regardless feeding time and sampling. Also, and regardless diet, a modulation of thrombocytes numbers was observed with a decrease of thrombocytes in response to the inflammatory insult (decrease from 0 h to the remaining times) in fish fed during 5 days. Also, at 0, 24 and 48 h, fish fed for 5 days presented higher thrombocyte amount than those fed for 10 days.

Lymphocytes quantity was modulated by feeding time and sampling point. In response to inflammation fish fed for 5 days the control diet had significantly less lymphocytes at 0 hours when comparing with both 4 and 48 hours. Similarly, fish fed Ygeia+ decreased lymphocytes values from 0 to 4, 24 and 48 h. Also, fish fed during 10 days the control diet or Ygeia+ decreased lymphocytes values from 0 h to all remaining sampling times or 4 h, respectively.

Monocytes increased in response to inflammation (from 0 to 4, 24 and 48 h) in fish fed 5 days. After 10 days of feeding a contradictory behavior was observed with a decrease from 0 h to 4 and a posterior increase between 4 and 24 h, as well as, 24 and 48h. Finally, at 0 h fish fed for 10 days showed higher concentration of monocytes compared to those fed for 5 days

Finally, regarding neutrophils quantity, a multifactorial analysis of variance showed a significance difference in the interaction between days of feeding (5 or 10), with diet (Ygeia+ and control) and sampling point. Significant differences were also observed when analyzing each factor separately, and each possible interaction was also significant. After 5 days of feeding and 4 hours post inflammation neutrophils in Ygeia+ fed animals more than doubled the concentration observed in controls. Regarding 10 days of feeding, Ygeia+ was significantly higher than the control at 24 hours post inflammation. Neutrophil's abundance increased during the first 4 and 24 hours post inflammation and starts to reduce at 48 hours.

Table 7 – Absolute quantity ($\times 10^4/\mu L$) of peripheral thrombocytes, lymphocytes, monocytes and neutrophils of European seabass during inflammation trial (4, 24 and 48 hours) after 5 and 10 days of feeding with Ygeia+ or control.

Inflammation Trial

Cellular type	5 Days						10 days					
	4 hours post infection		24 hours post infection		48 hours post infection		4 hours post infection		24 hours post infection		48 hours post infection	
	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+
Thrombocytes	5.69 ± 1.35	6.16 ± 1.63	7.13 ± 1.71	5.90 ± 1.08	7.43 ± 0.61	6.48 ± 1.55	4.90 ± 1.24	4.93 ± .074	5.16 ± 0.67	4.56 ± 0.85	5.03 ± 1.01	4.44 ± 3.25
Lymphocytes	0.43 ± 0.24	0.76 ± 0.45	1.05 ± 0.41	0.72 ± 0.32	1.26 ± 0.92	0.92 ± 0.61	0.20 ± 0.14	0.16 ± 0.05	0.47 ± 0.18	0.54 ± 0.31	0.78 ± 0.20	0.71 ± 0.68
Monocytes	0.08 ± 0.04	0.28 ± 0.13	0.30 ± 0.27	0.32 ± 0.27	0.31 ± 0.20	0.52 ± 0.29	0.17 ± 0.09	0.20 ± 0.17	0.22 ± 0.16	0.15 ± 0.08	0.37 ± 0.15	0.30 ± 0.28
Neutrophils	0.76 ± 0.44	1.89 ± 1.19	0.61 ± 0.38	0.65 ± 0.48	0.37 ± 0.24	0.62 ± 0.47	0.72 ± 0.34	0.66 ± 0.35	0.32 ± 0.18	0.65 ± 0.33	0.47 ± 0.40	0.13 ± 0.13

*Values are presented as mean ± standard deviation (n = 12)

Table 8 – Absolute quantity of peripheral thrombocytes, lymphocytes, monocytes and neutrophils of European seabass during inflammation trial (4, 24 and 48 hours) after 5 and 10 days of feeding with Ygeia+ or control. Multifactorial ANOVA: n.s.: non significant differences (p>0.05); If interaction was positive a One-way ANOVA was performed and Tuckey post-hoc analysis was performed, to identify the differences between groups.

Multifactorial ANOVA (Inflammation Trial)

Cellular Type	Diet	Feeding Time	Sampling Point	Diet x Feeding Time	Diet x Sampling point	Feeding Time x Sampling Point	Diet x Feeding Time x Sampling Point
Thrombocytes	0.043	< 0.001	0.049	n.s.	n.s.	< 0.001	n.s.
Lymphocytes	n.s.	< 0.001	< 0.001	n.s.	n.s.	n.s.	0.02
Monocytes	n.s.	n.s.	< 0.001	0.038	n.s.	< 0.001	n.s.
Neutrophils	0.014	0.002	< 0.001	0.002	0.003	< 0.001	< 0.001

Table 9 – Absolute quantity of peripheral thrombocytes, lymphocytes, monocytes and neutrophils of European seabass during inflammation trial (4, 24 and 48 hours) after 5 and 10 days of feeding with Ygeia+ or control. Evaluation of the interactions via Tuckey post-hoc to evaluate differences between groups. Different symbols account for significant differences in feeding times, capital letters were used to identify differences in sampling times, and lowercase letters were utilized to identify differences between diets.

Diet x Feeding Time x Sampling point

Cellular types	5 days								10 days							
	0 hours		4 hours		24 hours		48 hours		0 hours		4 hours		24 hours		48 hours	
	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+
Thrombocytes																
Lymphocytes	A	A	B	B	AB	B	AC	B	A	A	B	B	B	AB	B	AB
Monocytes																
Neutrophils	A	A	Ba	Bb#	AB	A	AB	A				*				

Feeding Time x Sampling Point

Cellular types	5 days				10 days			
	0	4	24	48	0	4	24	48
Thrombocytes	A#	B	B#	B#	*		*	*
Lymphocytes								
Monocytes	A#	B	B	B	A*	B	B	AB
Neutrophils								

Humoral parameters

Alternative Complement pathway (ACH50) Activity – ACH50 did not show differences regarding diets, while significant differences were obtained regarding feeding time (5 or 10 days) (Table 10). Further analysis of ACH50 during inflammatory response showed that at 10 days of feeding an increase of ACH50 concentration up to 48 h. Similar phenomenon was observed at 5 days of feeding with exception of 0 hours where ACH50 activity was higher.

Lysozyme concentration – Evaluation of lysozyme content in the end of feeding trial was significantly higher at 10 compared to 5 days (Table 10). The inflammatory response analysis demonstrated a relation between feeding time, diet and sampling hours (Table 11). At 10 days of feeding, Ygeia+ -fed fish showed higher concentration of lysozyme at 0, 4 and 48 h compared to fish sampled at 24 h. Moreover, after 10 days of feeding lysozyme was tendentially higher in animals fed Ygeia+ (Table 11). Inflammation also induced significant variations on control fed animals when evaluating the sampling point.

Correlation of neutrophils and monocytes quantity with lysozyme activity was evaluated. No significant causative reaction was observed in lysozyme due to neutrophils or monocytes increase.

Peroxidase activity – Peroxidase activity in the feeding trial did not change according with time or diet. Inflammatory response induced a significant reduction of peroxidase activity after between 0 hours (pre infection) and 4 hours, and 24 hours after inflammation at both 5 and 10 days of feeding.

Table 10 - Descriptive statistic of evaluated humoral parameters: ACH50 (units mL^{-1}), lysozyme activity (µg mL^{-1}), and peroxidase activity (units mL^{-1}) during feeding trial. Two-way ANOVA: n.s.: non significant differences (p>0.05); If interaction was positive a One-way ANOVA was performed and Tuckey post-hoc analysis was performed, to identify the differences between groups. Different symbols account for significant differences in feeding times.

Parameters	Feeding Trial				Two-way ANOVA		
	5 Days		10 days				Diet x
	Control	Ygeia+	Control	Ygeia+	Diet	Feeding Time	Feeding Time
ACH50	220.29 ± 89.02	243.87 ± 96.49	196.05 ± 41.66	141.34 ± 45.73	n.s.	0.013	n.s.
Lysozyme	7.76 ± 4.11	8.47 ± 1.97	10.58 ± 3.65	11.66 ± 4.29	n.s.	0.008	n.s.
Peroxidase	293.82 ± 144.85	278.91 ± 72.01	332.42 ± 74.20	349.21 ± 119.12	n.s.	n.s.	n.s.

*Values are presented as mean ± standard deviation (n = 12)

Table 11 - Descriptive statistic of evaluated humoral parameters: ACH50 (units mL^{-1}), lysozyme activity (µg mL^{-1}), and peroxidase activity (units mL^{-1}) during inflammation trial (4, 24 and 48 hours) after 5 and 10 days of feeding with Ygeia+ or control.

Inflammation Trial

	5 Days					
Parameters	4 hours post infection		24 hours post infection		48 hours post infection	
	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+
ACH50	222.78 ± 99.16	204.35 ± 98.48	202.98 ± 59.18	188.58 ± 50.73	189.80 ± 65.81	191.74 ± 55.05
Lysozyme	4.79 ± 2.78	7.95 ± 2.87	12.09 ± 3.47	12.12 ± 2.18	5.28 ± 2.96	6.65 ± 1.70
Peroxidase	210.42 ± 124.91	210.46 ± 70.25	238.90 ± 73.74	280.83 ± 74.12	164.39 ± 31.80	92.58 ± 34.05

	10 days					
Parameters	4 hours post infection		24 hours post infection		48 hours post infection	
	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+
ACH50	176.22 ± 51.60	200.76 ± 55.80	230.57 ± 69.15	235.70 ± 40.74	183.23 ± 44.45	247.80 ± 73.68
Lysozyme	9.91 ± 1.91	13.10 ± 4.96	7.39 ± 2.81	8.14 ± 1.52	5.88 ± 2.95	10.47 ± 5.29
Peroxidase	189.89 ± 60.74	159.88 ± 42.12	180.19 ± 20.49	146.63 ± 16.18	154.92 ± 14.24	158.39 ± 24.00

*Values are presented as mean ± standard deviation (n = 12)

Table 12 - Evaluation of the role of diet, feeding time and sampling point, in the alteration of evaluated humoral parameters: ACH50 (units mL^{-1}), lysozyme activity (µg mL^{-1}), and peroxidase activity (units mL^{-1}) during inflammation trial (4, 24 and 48 hours) after 5 and 10 days of feeding with Ygeia+ or control. Multifactorial ANOVA: n.s.: non significant differences (p>0.05); If interaction was positive a One-way ANOVA was performed and Tuckey post-hoc analysis was performed, to identify the differences between groups.

	Multifactorial ANOVA (Inflammation Trial)						
Parameters	Diet	Feeding Time	Sampling Point	Diet x Feeding Time	Diet x Sampling point	Feeding Time x Sampling Point	Diet x Feeding Time x Sampling Point
ACH50	n.s.	n.s.	n.s.	n.s.	n.s.	0.008	n.s.
Lysozyme	< 0.001	0.003	< 0.001	n.s.	< 0.001	< 0.001	n.s.
Peroxidase	n.s.	n..s.	< 0.001	n.s.	n.s.	n.s.	n.s.

Table 13 - Evaluation of the interactions via Tuckey post-hoc to evaluate differences between groups in ACH50, Lysozyme and peroxidase activity. Different symbols account for significant differences in feeding times, Capital letters were used to identify differences in sampling times.

	Feeding Time x Sampling Point								**Diet x Sampling Point**								**Sampling Point**			
Parameters	5 days				10 days				Ygeia+				Control							
	0	4	24	48	0	4	24	48	0	4	24	48	0	4	24	48	0	4	24	48
ACH50																				
Lysozyme	A	A#	B#	A	AB	A*	B*	B					A	AB	A	B				
Peroxidase																	A	B	B	C

Oxidative stress

Oxidative stress parameters analysis of the feeding trial suffered from a malfunction in the measurements of catalase and super oxide activities at 5 days of feeding due to technical constrains, which lead to an over expression of activity as it easily can be observed in Table 14. The only viable result that can be taken is the comparison between control diet and Ygeia+ after 5 days of feeding time and after 10 days (not comparable), which didn't present significant differences (Table 16). Lipid peroxidation (LPO) evaluation showed significant differences between feeding time, and like SOD and Catalase, differences between diets were not significant. Total glutathione content remained unchanged during feeding trial.

Table 14 - Descriptive statistic of evaluated oxidative stress parameters: catalase activity (U mg protein^{-1}), superoxide dismutase activity (U mg protein^{-1}), lipid peroxidation (nmol g tissue^{-1}), and total glutathione (mU mg protein^{-1}) during feeding trial. Grey background indicates where it was the measuring problems.

	Feeding Trial			
	5 Days		10 days	
Parameters	Control	Ygeia+	Control	Ygeia+
Catalase	187.12 ± 22.59	183.27 ± 7.98	85.26 ± 5.89	81.80 ± 15.02
SOD	37.74 ± 10.01	38.57 ± 14.76	20.20 ± 5.62	20.96 ± 4.51
LPO	26.39 ± 6.68	27.77 ± 5.41	35.09 ± 4.23	30.12 ± 7.77
tGSH	4.26 ± 2.39	4.43 ± 3.55	4.30 ± 1.98	5.06 ± 3.29

*Values are presented as mean ± standard deviation (n = 12)

Inflammation trial - Similar technical constrains that occurred during the analysis of feeding trial also occurred in samples from 4 hours post inflammation. Therefore, there are no data that can be presented from the inflammation trial following 5 days of feeding since it is not possible to compare sampling points. Nevertheless, catalase activity fluctuated with time after inflammation at 10 days (Table 15). During 10 days of feeding, catalase activity increased at 4 hours and decreased until 48 hours. Inflammatory response showed a decrease on SOD activity during time regardless dietary treatments (Table 17). Once again, similar technical constrains were observed in samples from the feeding trial at 5 days of feeding, being difficult to evaluate the effect of time.

LPO variation during inflammation trials were due to sample point and feeding days (Table 15). Variation regarding sampling point, showed a positive relation with time after inflammation induction at both 5 (with significant increase between, 4 hours to 24 hours) and 10 days of feeding (with significant increase between sampling points of 0 hours and 4 hours, and 24 hours to 48 hours).

Analysis of inflammatory response impact in total glutathione content of the liver, denoted a negative relation with hours post inflammation with significant decreases between 4 and 24 hours (only verified in at 5 days of feeding), 24 and 48 hours (verified in both feeding times). Differences between diets were significant (Table 15), with higher total glutathione in control fed animals.

Table 15 - Descriptive statistic of evaluated oxidative stress parameters: catalase activity (U mg protein^{-1}), superoxide dismutase activity (U mg protein^{-1}), lipid peroxidation (nmol g tissue^{-1}), and total glutathione (mU mg protein^{-1}) during inflammation trial (4, 24 and 48 hours) after 5 and 10 days of feeding with Ygeia+ or control. Grey background indicates where it was the measuring problems.

Inflammation Trial

	5 Days					
Parameters	4 hours post infection		24 hours post infection		48 hours post infection	
	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+
LPO	28.12 ± 4.86	30.79 ± 7.39	45.55 ± 6.03	38.52 ± 5.21	38.59 ± 12.70	45.51 ± 11.97
tGSH	6.53 ± 3.89	4.64 ± 1.39	3.35 ± 1.43	2.67 ± 0.86	2.24 ± 1.42	1.39 ± 0.89

	10 days					
Parameters	4 hours post infection		24 hours post infection		48 hours post infection	
	Control	Ygeia+	Control	Ygeia+	Control	Ygeia+
Catalase	95.39 ± 9.35	89.80 ± 13.75	89.41 ± 6.02	85.97 ± 12.32	81.03 ± 18.32	79.94 ± 8.86
SOD	11.21 ± 4.71	16.71 ± 6.56	12.29 ± 3.91	14.56 ± 5.87	9.16 ± 2.90	12.28 ± 5.59
LPO	35.93 ± 7.90	33.54 ± 3.28	39.75 ± 9.23	41.79 ± 11.28	61.83 ± 10.33	65.29 ± 19.00
tGSH	5.88 ± 1.81	3.73 ± 1.49	3.80 ± 2.32	3.19 ± 1.72	1.77 ± 0.77	1.18 ± 0.91

*Values are presented as mean ± standard deviation (n = 12)

Table 16 - Evaluation of the role of diet, feeding time, and sampling point, in the alteration of catalase activity (U mg protein^{-1}), superoxide dismutase activity (U mg protein^{-1}), lipid peroxidation (nmol g tissue^{-1}), and total glutathione (mU mg protein^{-1}) during feeding trial and inflammation trial. Catalase and Superoxide dismutase during inflammation were evaluated only after 10 days of feeding due to a malfunction in the measurements during the feeding trial and first 4 hours at 5 days of feeding via Two-way ANOVA. Lipid peroxidation and total glutathione were evaluated via a multifactorial ANOVA. ANOVA: n.s.: non-significant.

	Feeding Trial			**Inflammation Trial**						
				Two-way ANOVA 10 days						
	Diet	Feeding Time	Diet x Feeding Time	Diet	Sampling Point	Diet x Sampling point				
Catalase	n.s.	< 0.001	n.s.	n.s.	0.01	n.s.				
SOD	n.s.	< 0.001	n.s.	0.009	< 0.001	n.s.				
				Multifactorial ANOVA analysis						
				Diet	Feeding Time	Sampling Point	Diet x Feeding Time	Diet x Sampling point	Feeding Time x Sampling Point	Diet x Feeding Time x Sampling Point
LPO	n.s.	0.005	n.s.	n.s.	< 0.001	< 0.001	n.s.	n.s.	< 0.001	n.s.
tGSH	n.s.	n.s.	n.s.	0.033	n.s.	< 0.001	n.s.	n.s.	n.s.	n.s.

Table 17 - Evaluation of the interactions via Tuckey post-hoc the differences between groups in catalase, and super oxide dismutase at 10 days and lipid peroxidation and total glutathione. Different symbols account for significant differences in feeding times, capital letters were used to identify differences in sampling times.

Parameters	**Feeding Time x Sampling Point**								**Sampling point**			
	5 days				10 days							
	0	4	24	48	0	4	24	48	0	4	24	48
Catalase (10 days)									A	B	A	A
SOD (10 days)									A	B	B	B
LPO	A	A	B	B#	A	A	A	B*				
tGSH									A	A	B	C

Discussion

Modulatory effects of Ygeia+ on the immune status of European seabass was here evaluated, during two different feeding periods, as well as inflammatory response to intraperitoneal injected inactivated bacteria. Regarding feeding effects, no alterations were observed in the putative short-term feeding of Ygeia+ when compared to control diet, at physiological and zootechnical levels. However, upon inflammatory initiation, a general thrombocytopenia, lymphopenia and monocytopenia in animals is observed in both dietary treatments. The observed decrease in peripheral thrombocytes and lymphocytes numbers contrasted the increase in circulating neutrophils. This was an expected outcome when regarding to inflammatory response, as previous works demonstrated (Suzuki & Iida, 1992; Machado, et al., 2019). The increased number of phagocytic cells in the Ygeia+ fed animals may be the result of supplementation with β-glucans, as reviewed by Rodrigues et al. (2020). In this review the author evaluated the effect of different β-glucans administrations methodologies, including diet, and was able to see an increased modulatory effect of the immune system, in particular the enhancement of the phagocytic cell activity like neutrophils and macrophages (Rodrigues, et al., 2020). Reduction of both thrombocytes, lymphocytes and monocytes proportion on the blood stream is resultant from the increased recruitment and mobilization to the local of inflammation, alongside neutrophils (Havixbeck & Barreda, 2015). Moreover, the inflammatory response may lead to an increase of both circulatory lysozyme and peroxidase, since neutrophils are the major producers of those two humoral components (Machado et al., 2019). Activation of granulocytes will consequently increment lysozyme and peroxidase production, which leads to the posterior increment of those enzymes in plasma. Inflammatory response analysis at 4, 24 and 48 hours demonstrated an expected reaction from the organism towards a foreign agent. In the present study, a mobilization towards the inflammation focus of peripheral neutrophils was observed, a fact that has been vastly reported (Furze & Rankin, 2008). In this study, it was observed at both 5 and 10 days of feeding a significant increase of neutrophils after 4 and 24 hours in both diets. European seabass fed Ygeia+ during 5 days presented a significant higher recruitment when compared to their counterparts fed the control diet. Monocytes in Ygeia+ were slightly higher as well. Therefore, the data obtained, points to a positive modulatory effect of Ygeia+ in the immune response. Since both macrophages and neutrophils are competent pathogen killers and have a relevant role in immune response to inflammation.

In the present study, European seabass submitted to an inflammatory insult increased lysozyme activity in plasma, a response that was enhanced by Ygeia+ dietary treatment

after 5 days of feeding. Increased lysozyme activity in plasma was already reported in diets supplemented with plant extracts included in Ygeia+. For instace, Gonçalves (2019), showed in European seabass, a significant higher activity of plasma lysozyme with a testing diet supplemented with a mix of plant extracts. The authors also suggested that the addition of the blend could help in the switch of fish derived protein sources to vegetable ones, adding that the blend could reduce the negative impact in 10%. Monteiro et al (2021) were able to correlate the addition of algae extracts to seabass diets with a positive modulation of hepatic oxidative stress, and with higher WBC quantities, which corroborate our findings regarding Ygeia+.

Red blood cell indexes corroborate the enhanced capacity of Ygeia+ to respond during stress moments like inflammation. Higher hemoglobin values during first stages of inflammation are an indication of higher mobilization towards inflammatory focus, and the posterior decrease is a mechanism of the body to limit iron availability in blood to prevent uncontrolled cell proliferation, as well as to reduce iron availability to pathogens and ROS production (Koorts et al., 2011). Liver oxidative status is a good representation of the organism oxidative status. In homeostatic conditions ROS and anti-oxidant agents are balanced, yet during a stress like inflammation, balance is disrupted (Matés, 2000). By the analysis of the inflammatory response, we were able to confirm that indeed the basal oxidative status of the liver changed. ROS related enzymes were upregulated in the first hours and a posterior tendency to balance in the late sampling times. Ygeia+ is indeed a capable diet able to enhance the antioxidant activity of the organism, as it was observed during the inflammation trial. Super oxide dismutase is an enzyme with scavenging properties for free radicals like oxygen and hydrogen peroxidase (Desforges, 2016), and its activity was indeed enhanced by Ygeia+, which is supplemented with anti-oxidant components like vitamin C and vitamin E, and polyphenols from grape, which are capable of reducing ROS in the organism (Jacob, 1995; Firmino et al., 2021). And the decrease of total glutathione content after inflammation induction, mainly in Ygeia+ fed animals, indicates the consumption of anti-oxidant molecules, during inflammation (Jacob, 1995, Srikanth, 2013).

Concluding, in this first approach (trial 1), it was possible to study how Ygeia+ might be able to modulate the immune response, and presented a new challenge of optimization regarding feeding times. Nevertheless, it was possible to verify a positive influence of Ygeia+ on the recruitment of phagocytic cells, as well as a increase of antimicrobial agents like lysozyme, and enhancement of the oxidative balance in the liver. Increased lysozyme activity in plasma was already reported in diets supplemented with plant extracts like Ygeia+. As an example Gonçalvez (2019), showed in European seabass, a

significant higher activity of plasma lysozyme with a testing diet supplemented with a mix of vegetal extracts. The author goes even further by proposing that the addition of the blend could help in the switch of fish derived protein sources to vegetable ones, adding that the blend could reduce the negative impact in 10%. Monteiro (2021) was able to relate the addition of algae extracts with a positive modulation of hepatic oxidative stress, and with higher leukocytes quantities, which corroborate our findings regarding Ygeia+.

Trial 2 – Effects of Ygeia + on vaccine efficiency and cell-mediated immunity in European seabass

Aim

In a first approach was perceived the ability of Ygeia+ to improve the recruitment of phagocytic cells, as well as an increase of antimicrobial agents like lysozyme. Hence, in the present trial was aimed to assess the interactive effects Ygeia+ feeding (again after 5 and 10 days), and vaccination efficacy on the immune response and disease resistance of European seabass.

Material and methods

The study took place in CIIMAR and after a 45 days quarantine period (extended period due to Sars-Cov-2 pandemic restrictions), 288 European seabass (from Sonrionansa, Santander, Spain) (36 fish per tank) were transferred to the trial room that consisted of 8 tanks (300 liters each). After acclimatization to the system and conditioning during another 15 days, fish were individually weighted (11.94 ± 0.38 g). Both in quarantine and during acclimatization fish were fed with a control commercial diet produced by Soja de Portugal. The trial started after the period previously mentioned with the substitution of a control diet for Ygeia+ in 4 tanks (randomly selected). Four of the 8 tanks were fed Ygeia+ for 5 and 10 days (2 tanks each time) while the 4 others were fed the Control diet for the same days. After that period (5 and 10 days), fish from each tank were divided into 2 other tanks (18 fish per tank) and either vaccinated with a commercial vaccine from Laboratórios Hipra S. A. (Spain), or injected with a sham solution that consisted in sterile PBS. To clarify, there were then a total of 8 different treatments distributed in 16 tanks with 18 fish each (2 diets x 2 times of feeding x 2 immunization protocols). After 21 days post-vaccination, fish were kept under controlled conditions fed with the control diet. Subsequently, 9 fish from each treatment were sampled (5 from one tank and 4 from the other) following the same methodology as in trial 1. The remaining 27 fish, from each treatment, were then transferred to another room designed for infection challenges, composed of 12 tanks of 100 liters each. Those fish were then divided into 4 tanks, 2 tanks infected with bacteria while the other half were placebo treated. Both anesthesia and euthanasia were performed as described in trial 1. For each previous treatment a further division was performed, a random selection of half of the animals from each treatment was inoculated with *Photobacterium damselae* subsp*. piscicida,* via

intraperitoneal injection, while the other half was placebo treated with same bath conditions.

Vaccine

The vaccine (Figure 4) utilized is trivalent and presents defense to *Tenacibaculum maritimum*, *Vibrio anguillarum*, and *Photobacterium damselae* subsp*. piscicida*. Vaccination administration was performed via intraperitoneal injection. Made by Laboratórios Hipra S. A. in Spain.

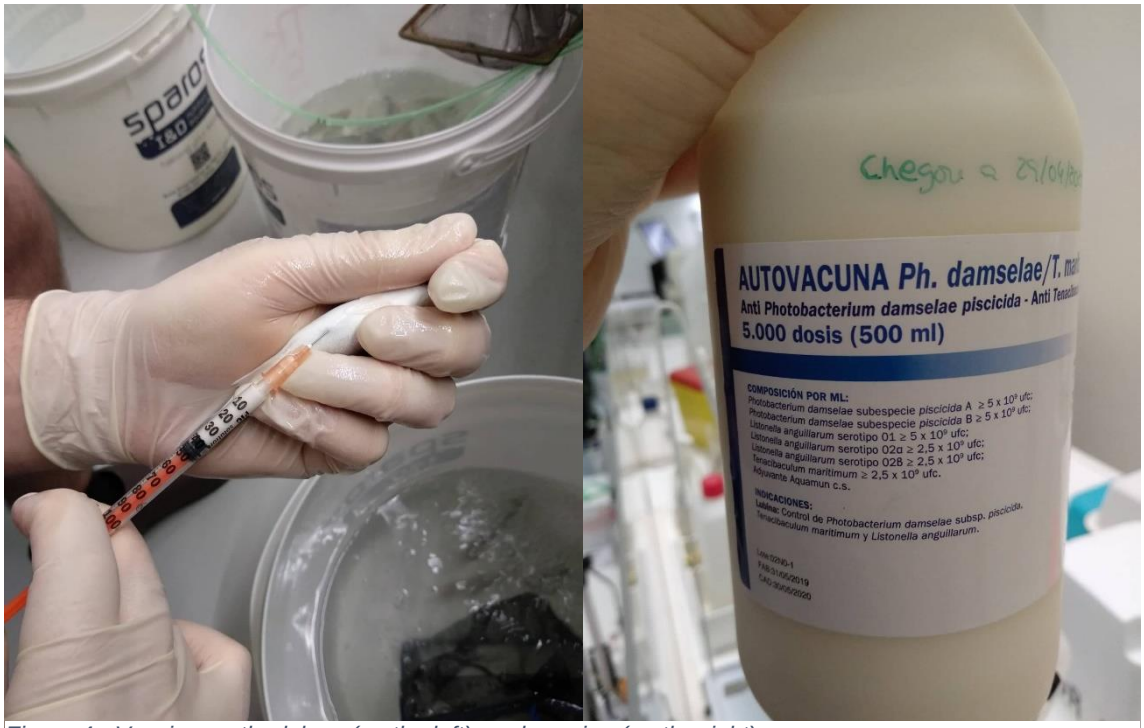

Figure 4 - Vaccine methodology (on the left), and vaccine (on the right)

Mortality assay

This assay was conducted to evaluate the diets interaction with vaccine efficiency upon an infection by a pathogen. To evaluate these the remaining 27 fish from each treatment were distributed into 4 tanks (7 fish per tank plus one tank with 6), giving a total of 16 tanks for all treatments that were vaccinated after 5 days from the beginning. During the transference time to the new system, were bath challenge with *Tenacibaculum maritimum* with known concentration of 5×10^6 CFU ml^{-1}. Concentration was chosen based in a pre-challenge performed beforehand where the LD50 was the one employed in the challenge. Mortality was registered during 10 days. The same procedure was conducted for the remaining treatments. Severely ill animals were euthanized and considered dead. Evaluation of cause of dead was also performed by inoculating the head-kidney into a marine agar plate, and verifying the bacterial growth (Machado et al., 2019). Again, both euthanasia and anesthesia were performed as described in trial 1.

Sampling methodology

All the remaining analysis from tissues collected were performed similarly to Trial 1, with the exception of peroxidase and lysozyme that were not evaluated and instead, it was evaluated the specific IgM quantity to the bacteria to which the vaccine presents immunization.

Specific IgM evaluation

Specific IgM was evaluated following an enzyme-linked immunosorbent assay (ELISA) as it is described by Raida (2011). Specific IgM tested were the ones specific to *Tenacibaculum maritimum*, *Vibrio anguillarum*, and *Photobacterium damselae* subsp*. piscicida*. Shortly, the procedure is divided into two main phases, the coating of the microplate and IgM evaluation. Coating was performed by adding 100 µL of carbonate buffer (Na_2CO_3, pH 9.6) containing known concentration of antigen, in the case of *Tenacibaculum maritimum* and *Vibrio anguillarum* 5 µL/mL, while in the case of *Photobacterium damselae* subsp*. piscicida* concentration used was 7.5 µL/mL, to a flat-bottom 96-well plate. All bacteria were grown in agar plates, *Vibrio anguillarum* and *Photobacterium damselae* subsp*. piscicida,* in TSA-2 and *Tenacibaculum maritimum* was cultivated in marine agar. All bacteria before being diluted into the intended concentration were sonically disrupted and the concentration measured. Antigen concentration was chosen after trials conducted exclusively to assess the needed concentrations, of both bacteria, as well as, plasma sample (as it will be forward shown). Microplates were left to incubate at 25 ºC for 1 hour. Process followed by the removal of the coating solution, and washing, 3 times, of the microplate with the washing buffer (300 µL per well) (tris-based solution in the concentration of 1/10 in distilled water, and Tween-20 at a final concentration of 0.1%. With final pH of 7.6). Blocking of free binding sites was performed by incubating for 1 hour at 25 ºC with 150 µL (well) of blocking solution (low fat milk in a concentration of 1% in washing buffer). Coating procedure was finished after another cycle of 3 washes with the washing buffer followed by aspiration and sealing with microplate seal. Microplates were stored at -20 ºC until used (generally overnight). With optimization of plasma sample done, a dilution of 1:100 with carbonate buffer was performed. Meanwhile coated microplates were unfrozen, and 100 µL of the sample dilution were pipetted and left to incubate for 1 hour at 25 ºC. Immediately after, the dilution was removed and replaced by 300 µL of blocking buffer and left to incubate for another hour. Prior the end of the incubation, primary antibody solution was done (solution consists in the dilution of the anti-European seabass antibody into blocking buffer in a concentration of 1:100 (concentration was previously tested and stablished as pattern for the species in the laboratorial group)). At the end of the incubation the

blocking buffer was removed and the microplate washed 3 with washing buffer. 100 µL of primary antibody solution was added to each well and incubated for 1 hour at 25ºC. Secondary antibody (anti-mouse antibody) solution followed a similar process to the one employed into the making of the primary antibody solution, yet with a concentration of 1:1000. After incubation time another cycle of 3 washes was performed, followed by another incubation (1 hour at 25ºC) with 100 µL of the secondary antibody solution. Process was then proceeded by 3 washings and a shorter incubation of 5 minutes with 100 µL "TMB substrate solution for ELISA" (Thermo Fisher, ref: 34021, USA). Reaction was stopped with sulfuric acid solution in a concentration of 2M. Finally, absorbance was measured at 450 nm. Results were obtained by comparing the differences in the absorbance between each treatment, discounting the blank. Procedure for obtaining the blanks is similar but instead of adding plasma it is only added 100 µL of dilution buffer. All procedure was performed in triplicates.

Statistical analysis

All data was described and outliers were removed, after which a Shapiro Wilk normality test was employed for each variable. Analyses of variance was performed to for each measurement variable and three nominal variables (days of feeding, diet, and vaccination or sham treatment), also called ANOVA three factors. This analysis leads to a total of 6 null hypothesis: measured variable means are equal for different values of day; measured variable means are equal for different values of diet; measured variable means are equal for different values of vaccination; measured variable means are equal for different values of the interaction day and diet; measured variable means are equal for different values of the interaction day and vaccination; measured variable means are equal for different values of the interaction diet and vaccination; measured variable means are equal for different values of the interaction day, diet, and vaccination. ANOVA analysis were performed even if normal distribution was not verified. According with several studies (Glass et al., 1972; Harwell et al., 1992; Fay & Gerow, 2013), ANOVA test is sufficiently robust to give accurate prediction if the number of samples for each group is similar even if there is no normal distribution (McDonald, 2014). Homogeneity of variances was also evaluated, utilizing Levene test, and more attention was given. Nevertheless, considering the small number of replicates, and that there is more or less the same number of replicas in each tested group homogeneity of variances could carefully be acknowledged in all data as reported before by Miranda-Fontaiña & Fernández-López (2009). In this work homogeneity was verified in almost every variable.

Further analysis was performed in case of any of the null hypothesis was rejected, to further understand the effect of the factor in the variance of the parameter under study. This analysis was conducted utilizing one- or two-way ANOVAS in grouped conditions.

All statistical analysis were performed in statistical software SPSS by IBM.

Results

Regarding this second trial all data can be consulted in Table 18, 19, and 20 for the corresponding descriptive statistic, multivariance analysis, and interaction analysis, respectively.

Hematological profile:

Cellular indexes evaluated, like the amount of WBC per dL and the MCV did not suffer alteration according any of the evaluated parameters. Contrasting, RBC, Hematocrit and Hemoglobin variation are explained by the interaction by the interaction of all the evaluated parameters, namely: diet, feeding time, and treatment. Hematocrit was significantly lower when comparing vaccinated Ygeia+ fed animals with sham treated Ygeia+ fed animals at 10 days of feeding time. Hemoglobin significant variation occurred at 10 days when comparing vaccinated to sham treated animals fed with control diet, higher on sham treated. MCHC was significantly lower in animals fed for 5 days and vaccinated when comparing to animals that were also fed for 5 days and sham treated, as well as, animals fed for 10 days and vaccinated. The interaction between diet and treatment and the interaction of feeding time and treatment are explanatory factors for the variation of MCH. Sham treated and vaccinated animals presented significantly differences in MCH index at 5 days of feeding.

Differential leukocyte counting:

Differential leukocyte evaluation did not show differences in peripheral thrombocytes and lymphocytes absolute values. However, regarding neutrophils there was a significant difference when analyzing the interaction between all factors, post hoc analysis in other hand could not illustrate where were the differences. Monocytes, were significantly higher in animals fed for 10 days.

Humoral Parameters:

Immunoglobulin M specific for *Vibrio anguillarum* showed as expected a significant difference when comparing vaccinated animals with sham treated animals, with higher absorbance in vaccinated animals. Higher absorbance corresponds a higher concentration of IgM (Raida et al., 2011). The remaining two specific IgM described in the material and methods are still being optimized, therefore, no conclusive results can be presented in the scope of this thesis. Alternative complement pathway activity variation was caused by the interaction of diet with feeding time, Ygeia+ fed animals had a significantly different ACH50 activity when comparing the 5 with 10 days and when Ygeia+ and control fed animals at 10 days, with Ygeia+ fed animals having higher activity.

Oxidative stress

The evaluation of lipid peroxidation, and total glutathione (tGSH) showed significant variations caused by the interaction of all evaluated factors. LPO was significantly higher in vaccinated animals fed control diet for 10 days when comparing with vaccinated animals, also fed control diet for 5 days. tGSH like LPO significantly differed when comparing vaccinated animals fed control diet for 5 and 10 days yet in this case at 5 days the value was much higher. Super oxide dismutase activity changed according with diet, higher in Ygeia+ fed animals compared to controls, and with treatment, higher in vaccinated animals compared to sham treated. Finally, the last parameter evaluated was catalase and it presented significant variations with the interaction of the diet and feeding time, Ygeia+ fed animals were significantly different between 5 and 10 days (higher at 10 days), and Ygeia+ animals catalase activity also significantly differed from the ones fed control diet for the same period of time lower at 5 days in Ygeia+ when compared with control.

Mortality trial

Effect of both diet and treatment was here evaluated with a *Tenacibaculum maritimum* challenge. It was verified that the dosage was too high for the mortality's ratios expected. Nevertheless, we could verify that in general vaccinated animals had a lower mortality rate than the ones that were sham treated, exception for the Ygeia+ fed animals that were sham treated, those were the ones with the lowest cumulative mortality ratios. Apparently diet or time of feeding did not affect fish survival. Fish from every treatment besides, the sham treated fed Ygeia+ (that started to die in the second day) started to die in the first day post inoculation, and the mortality stabilized 4 days after (Figure 5).

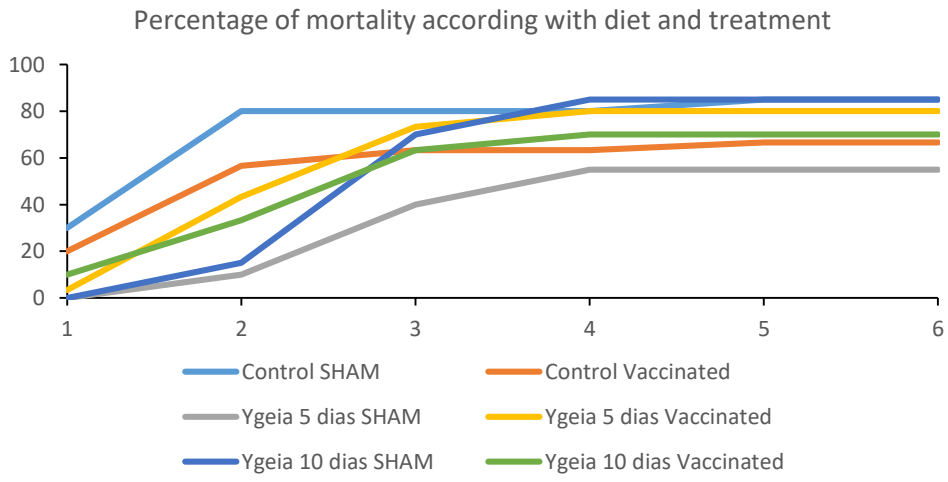

Figure 5 - Cumulative mortality in percentage according with treatment and feeding time

Table 18 - Descriptive statistics of the second trial: differential leukocyte percentage, cellular indexes (MCV, MCH, MCHC),total WBC (10^4/µL) and RBC (*10^6/µL), hematocrit (%) and hemoglobin (g/dL), humoral parameters (specific IgM for Vibrio anguillarum, and ACH50 (units mL^{-1})), oxidative stress parameters (lipid peroxidation activity (nmol g $tissue^{-1}$), catalase activity (U mg $protein^{-1}$), total glutathione (mU mg $protein^{-1}$), and superoxide dismutase activity (U mg $protein^{-1}$)). Comparison between 5 and 10 days of feeding, Sham treated and vaccinated animals according control or Ygeia+ diets.*

	5 days			
	Control		Ygeia+	
	Sham	Vaccinated	Sham	Vaccinated
Hematological profile				
WBC	8.02 ± 2.41	9.16 ± 1.76	8.92 ± 1.90	1.02 ± 3.20
RBC	3.14 ± 0.64	2.58 ± 0.55	2.79 ± 0.44	2.91 ± 0.53
Hematocrit	28.13 ± 3.94	24.56 ± 2.01	27.33 ± 1.50	28.00 ± 2.65
Hemoglobin	1.34 ± 0.31	1.41 ± 0.34	1.78 ± 0.13	1.37 ± 0.33
MCV	107.85 ± 13.20	99.64 ± 22.85	100.76 ± 20.06	89.87 ± 9.84
MCH	8.99 ± 3.07	3.33 ± 0.78	6.52 ± 1.10	6.02 ± 2.38
MCHC	7.49 ± 3.30	3.90 ± 1.38	6.27 ± 1.89	4.41 ± 1.25
Leukocyte percentage				
Thrombocytes	5.57 ± 1.49	7.01 ± 1.88	6.75 ± 1.63	7.45 ± 2.35
Lymphocytes	1.67 ± 0.71	1.61 ± 0.73	1.18 ± 0.61	2.01 ± 0.73
Monocytes	0.31 ± 0.28	0.25 ± 0.13	0.26 ± 0.19	0.37 ± 0.17
Neutrophils	0.22 ± 0.14	0.23 ± 0.10	0.42 ± 0.25	0.19 ± 0.1
Humoral Parameters				
IgM Vibrio	0.06 ± 0.04	0.21 ± 0.07	0.05 ± 20.06	0.23 ± 0.10
ACH50	318.00 ± 52.98	311.90 ± 112.01	273.29 ± 69.48	294.11 ± 26.56
Oxidative Stress				
LPO	10.74 ± 13.20	9.09 ± 1.23	12.38 ± 0.68	11.21 ± 1.54
Catalase	39.21 ± 4.64	34.29 ± 1.36	42.22 ± 3.66	43.63 ± 2.71
GSH	29.37 ± 28.24	68.77 ± 11.93	10.29 ± 8.44	32.22 ± 32.44
SOD	26.40 ± 17.73	48.87 ± 12.13	56.27 ± 15.51	56.28 ± 15.15

*Values are presented as mean ± standard deviation (n = 9)

10 days

	Control				Ygeia+			
	Sham		Vaccinated		Sham		Vaccinated	
Hematological profile								
WBC	8.49 ± 0.94		8.90 ± 2.16		9.08 ± 0.69		9.43 ± 2.56	
RBC	2.31 ± 0.32		2.41 ± 0.63		3.01 ± 0.62		2.59 ± 0.67	
Hematocrit	23.75 ± 3.99		23.78 ± 4.47		29.44 ± 1.94		24.00 ± 4.30	
Hemoglobin	1.95 ± 1.12		1.02 ± 0.44		1.72 ± 0.76		1.90 ± 0.41	
MCV	105.34 ± 23.96		94.27 ± 3.75		95.42 ± 9.33		89.78 ± 15.61	
MCH	5.13 ± 1.30		5.81 ± 1.71		5.88 ± 1.60		7.51 ± 3.41	
MCHC	5.61 ± 1.46		5.78 ± 1.79		5.78 ± 0.38		7.89 ± 3.64	
Leukocyte percentage								
Thrombocytes	6.12 ± 0.90		6.51 ± 1.97		6.39 ± 1.39		6.04 ± 2.91	
Lymphocytes	1.59 ± 0.57		1.50 ± 0.34		2.04 ± 0.90		1.83 ± 0.80	
Monocytes	0.31 ± 0.18		0.46 ± 0.19		0.45 ± 0.25		0.44 ± 0.21	
Neutrophils	0.30 ± 0.30		0.26 ± 0.18		0.2 ± 0.12		0.42 ± 0.34	
Humoral Parameters								
IgM Vibrio	0.10 ± 23.96		0.23 ± 0.07		0.08 ± 0.06		0.17 ± 0.02	
ACH50	349.16 ± 96.78		279.13 ± 18.30		437.99 ± 90.57		414.92 ± 73.82	
Oxidative Stress								
LPO	11.72 ± 1.83		14.71 ± 3.59		13.29 ± 2.76		11.79 ± 1.10	
Catalase	42.01 ± 5.38		38.68 ± 3.39		38.19 ± 2.95		35.71 ± 3.94	
GSH	42.07 ± 30.27		11.38 ± 7.90		21.13 ± 28.91		47.78 ± 26.92	
SOD	44.92 ± 19.06		49.73 ± 18.28		45.40 ± 19.79		57.39 ± 14.88	

*Values are presented as mean ± standard deviation (n = 9)

Table 19 - Evaluation of the role of diet, feeding time and sampling point, in the alteration of all parameters measured in the second trial. Multifactorial ANOVA: n.s.: non significant differences (p>0.05); If interaction was positive a One-way ANOVA was performed and Tuckey post-hoc analysis was performed, to identify the differences between groups.

	Multifactorial ANOVA						
	Diet	Feeding Time	Treatment	Diet x Feeding Time	Diet x Treatment	Feeding Time x Treatment	Diet x Feeding Time x Treatment
Hematological profile							
WBC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
RBC	n.s.	0.047	n.s.	n.s.	n.s.	n.s.	0.032
Hematocrit	0.19	n.s.	0.023	n.s.	n.s.	n.s.	0.008
Hemoglobin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.009
MCV	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
MCH	n.s.	n.s.	n.s.	n.s.	0.011	<0.001	n.s.
MCHC	n.s.	n.s.	n.s.	n.s.	n.s.	0.006	n.s.
Leukocyte percentage							
Thrombocytes	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Lymphocytes	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Monocytes	n.s.	0.29	n.s.	n.s.	n.s.	n.s.	n.s.
Neutrophils	n.s.	n.s.	n.s.	n.s.	n.s.	0.047	0.014
Humoral Parameters							
IgM Vibrio	n.s.	n.s.	< 0.001	n.s.	n.s.	n.s.	n.s.
ACH50	n.s.	0.006	n.s.	0.006	n.s.	n.s.	n.s.
Oxidative stress							
Catalase	n.s.	n.s.	0.022	< 0.001	n.s.	n.s.	n.s.
LPO	n.s.	0.001	n.s.	0.033	n.s.	n.s.	0.039
tGSH	n.s.	n.s.	n.s.	0.003	n.s.	0.005	0.012
SOD	0.014	n.s.	0.031	n.s.	n.s.	n.s.	n.s.

Table 20 - Evaluation of the interactions via Tuckey post-hoc the differences between groups in every parameter of the second trial. Different symbols account for significant differences in feeding times, Capital letters were used to identify differences in sampling times, and symbols were used to identify differences between feeding times.

	Diet x Feeding Time x Treatment								Day x Treatment			
	5 Days				10 Days							
	Control		Ygeia+		Control		Ygeia+		5 Days		10 Days	
	Vaccinated	Sham	Vaccinated	Sham	Vaccinated	Sham	Vaccinated	Sham	Vaccinated	Sham	Vaccinated	Sham
Hematological profile												
WBC												
RBC												
Hematocrit							b	a				
Hemoglobin					b	a						
MCV												
MCH									b	a		
MCHC									b#	a	*	
Leukocyte percentage												
Thrombocytes												
Lymphocytes												
Monocytes												
Neutrophils												
Humoral Parameters												
IgM Vibrio												
ACH50												
Oxidative stress												
Catalase												
LPO	B				A							
tGSH	B				A							
SOD												

Day x Treatment

	5 Days		10 Days	
	Ygeia+	Control	Ygeia+	Control
Hematological profile				
WBC				
RBC				
Hematocrit				
Hemoglobin				
MCV				
MCH				
MCHC				
Leukocyte percentage				
Thrombocytes				
Lymphocytes				
Monocytes				
Neutrophils				
Humoral Parameters				
IgM Vibrio				
ACH50	B		A#	*
Oxidative stress				
Catalase	A#	*	B	
LPO				
tGSH				
SOD				

Discussion

Second trial contributed to provide new insights on how Ygeia+ could affect vaccine efficiency, immune response and disease resistance. Vaccination optimization is a fundamental step for the balanced development of aquaculture, and it can be an important mediator of the environmental impact of the industry (Folb et al., 2004). Nutrition impacts on vaccine efficiency is currently one important branch of investigation, this does not only apply to the aquaculture sector but also to human health. Present important studies are being conducted to relate feeding habits and nutrition with vaccination in humans (Butler & Barrientos, 2020; Madison et al., 2021). Nutrition can always be considered a modulator of vaccine efficiency, either by improving welfare and therefore, the basal condition of the animal's previous vaccine is better, which will in consequence optimize vaccine efficiency (Ringo et al., 2014; Butler & Barrientos, 2020). Nevertheless, functional feeds like Ygeia+ aim not only to improve vaccine efficiency via welfare, but to directly interact with the immune system to amplify the effectiveness.

Overall hematological profile presented differences between Ygeia+ and control fed animals at 10 days of feeding, with an apparent enhanced cellular immune response in the Ygeia+ fed animals. This is a good indicator, since it can mean that Ygeia+ fed animals are capable of mounting a faster immune response, and that indeed supplementation with vitamin A can enhance the recognition of pathogens, which enhance immune response speed (Kawai & Akira, 2009). Enhanced immune response was also supported by an increased enzymatic activity in the liver, namely in superoxide dismutase and catalase. Both enzymes are highly relatable to the immune response, and oxidative stress status. Higher concentration can indicate according with Sun et al., (2007), an improved anti-oxidant capacity of Ygeia+ fed animals, as it was observed also in the first trial. We can here pinpoint the effect of the anti-oxidant compounds supplementation in the Ygeia+, such as in the first trial.

Vaccination efficiency was verified when analyzing the specific IgM towards *Vibrio anguillarum.* (Kai et al., 2014; Piazzon et al., 2016). IgM production increases when the animals are exposed to a pathogen or components of it. Circulatory IgM, as a result of higher production, increases as well, and it is also documented the increment of specific IgM (Yu et al., 2020). In present work it was showed that specific circulatory IgM is significantly increased in vaccinated animals, when compared to sham treated, 3 weeks after vaccination (period reported by the company at which the vaccine is effective). This result meets the one observed by Raida (2011), in rainbow trout, and the one obtained by Gonzales-Silvera (2019) in European seabass. Overall, we could not observe an interaction between diets and vaccination in regard to specific circulatory IgM, Salomón

et al. (2020), obtained the same result when analyzing a supplemented diet with leaf extracts from *Salvia officinalis* and *Lippia citriodora*. Which leads us to conclude that the diet may not directly alter the vaccine effect, but instead, optimize immunological features of the organism.

Conclusion

Considering the interest and investment in aquaculture and aggregated sectors, such as the feed production industry, further work remains to be done. Fairly intense research has been conducted in the past few years to optimize the diets and growth of farmed fish. Interestingly, there is a recent trend that aims to improve not only growth but also appearance and, more importantly, the general fish health condition.

The present thesis contributed to study the effects of Ygeia+ on the European seabass immune response during short-term feeding periods. It was possible to observe a positive modulation of the oxidative status in the liver, as well as an increment in the recruitment of phagocytic cells and consequent increase in the circulatory concentration of antimicrobial enzymes in fish fed Ygeia+. The evaluation of the interaction between vaccination and Ygeia+ showed similar results compared to fish fed the control diet. However, the observed improvement in liver oxidative status and in the cell mobilization in seabass fed Ygeia+ points to equivalent positive effects on immune response and health condition.

In summary, the switch from a commercial diet to Ygeia+ when a stressful event is forecasted, such as manipulation of any sort, appears to be a promising prophylactic measure. However, Ygeia+ does not influence the acquired immune response after vaccination and further studies should be performed considering the health promoting effects.

Future Approach

In these trials it was not possible to ascertain how Ygeia+ could improve survival post-vaccination. Future approaches can include the realization of gene expression studies in order to better understand the mode of action of dietary treatments. Moreover, some analysis should still be performed, like evaluation of specific IgM content for *Tenacibaculum maritimum* and *Photobacterium damselae* subsp*. piscicida*.

Another important fact would be testing different feeding periods, such as 15 or 20 days, since some parameters were different between 5 and 10 days of feeding. Those data would further improve our knowledge on how feeding time interacts with the immune system, as well as, allow us to understand if there is a direct relation between Ygeia+ and feeding time regarding health status.

It would be interesting to evaluate the effects of Ygeia+ in the mucus since it is the first mechanism of defense to the organism. Previous works in the field showed that the incorporation of phytogenic compounds can positively impact the immunity of the mucus, and therefore enhance fish resistance to disease.

Incorporation of passiflora and valerian extracts, which are anxiolytic compounds, is an interesting approach into the prevention of stress, so therefore together with mucus analysis, the evaluation of stress parameters like cortisol in the plasma would also be interesting. It would allow understand even further how Ygeia+ can help in the mitigation of stress during handling.

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