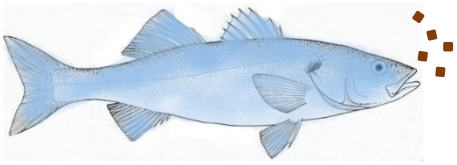

Modulatory effects of methionine dietary supplementation on the European seabass (*Dicentrarchus labrax*) local and systemic immune responses against *Tenacibaculum maritimum*

Alberto Rodrigues Antunes
Master's Degree in Biological Aquatic Resources
Faculdade de Ciências da Universidade do Porto
2021/2022

Supervisor
Maria Rita Azeredo, Auxiliary Researcher, CIIMAR

Co-supervisor
Marina Machado, Auxiliary Researcher, CIIMAR


ciimar
Centro Interdisciplinar
de Investigação
Marinha e Ambiental


U.PORTO
FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Sworn Statement

I, Alberto Rodrigues Antunes, enrolled in the Master's Degree in Biological Aquatic Resources at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this dissertation reflects perspectives, research work and my own interpretations at the time of its submission.

By submitting this dissertation, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

I further declare that all references to other authors fully comply with the rules of attribution and are referenced in the text by citation and identified in the bibliographic references section. This dissertation does not include any content whose reproduction is protected by copyright laws.

I am aware that the practice of plagiarism and self-plagiarism constitute a form of academic offense.

Acknowledgements

This Thesis is the result of the contributions of numerous people that I would like to thank and acknowledge. Without them it would not be possible.

A special thank you to my amazing supervisor, Rita Azeredo, for the best guidance that I could ever wish for, I am truly grateful. A special thank you for always being available, for all the patience, advices and vital counselling.

I also would like to present my gratitude to my co-supervisor, Marina Machado, for their accessibility to help and guide me through all my doubts.

I am grateful to Professor Benjamín Costas for having me so kindly incorporated in this amazing team and for having opened its lab and resources to me. I feel very lucky for having had this privilege.

To everyone in the IMMUNAA team, I would like to thank every single one of the lab team for all the recommendations and support whenever I needed. I am grateful for the kind help explaining the different lab procedures and techniques.

I would like to thank all the CIIMAR members.

Thank you father, thank you mother and thank you sister.

To all my friends, thank you for being a part of my life.

Obrigado

Resumo

As membranas das mucosas são de uma grande importância na defesa do hospedeiro a infeções bacterianas, sendo as brânquias, a pele e o intestino as primeiras barreiras físicas. Estas superfícies são ainda revestidas por uma camada mucosa composta que contém importantes moléculas na defesa do organismo.

Tenacibaculum maritimum é o agente etiológico da tenacibaculose, uma doença que provoca importantes perdas na produção do robalo europeu, desta forma é extremamente importante compreender melhor o funcionamento dos seus mecanismos imunes envolvidos no seu combate, ainda vagamente compreendidos.

Devido ao uso abundante de antibióticos na produção animal, encontrar uma estratégia às alternativas existentes para a sua redução e eliminação é de importância extrema. A metionina como ingrediente funcional para promover a saúde dos peixes, poderá ser uma solução. Os aminoácidos são os ingredientes base para a síntese proteica. A metionina é um aminoácido essencial que regula processos metabólicos chave, tendo um impacto essencial no controlo do sistema imunitário dos peixes. Em específico, a metionina intervém durante a síntese de poliaminas e na resposta inflamatória.

O objetivo desta dissertação é aferir, evidenciar e esclarecer o papel da metionina como suplemento nas dietas do robalo europeu (*Dicentrarchus labrax*) no estado imunitário e resposta ao combate imunitário a infeções com *Tenacibaculum maritimum*, através da análise do efeito da metionina extra no perfil hematológico e parâmetros imunes do intestino e muco da pele.

Para isso, os peixes de uma forma aleatória foram alimentados em provas de alimentação com períodos de 4 semanas onde três grupos independentes, foram alimentados com uma dieta controlo (cuja composição correspondeu aos requisitos nutricionais do robalo) e duas dietas idênticas à dieta controlo, mas com níveis superiores de DL-Metionina (1 e 2 % do peso da dieta). Os peixes foram amostrados às 2 e 4 semanas de forma a aferir o estado imunológico do animal com cada dieta. No final de cada prova de alimentação os peixes foram infetados em banho bacteriano, de 2 horas, por *Tenacibaculum maritimum* e amostrados 4 e 24 horas após a infeção. Em todas as amostragens, foram recolhidas amostras de sangue (para avaliação do perfil hematológico e da resposta celular), muco da pele (para avaliação de parâmetros imunes) e intestino anterior (para avaliação de parâmetros imunes).

Os resultados demostram que a resposta imune dos peixes após a estimulação bacteriana por *Tenacibaculum maritimum*, como esperado de uma boa e eficiente resposta imunitária, aumenta, já nas fases iniciais, a atividades no muco da pele (protéase e peroxidase) como resposta à inflamação. Também evidencia que, pelo menos por infeção bacteriana por banho de *T. maritimum*, a resposta imune no peixe parece mais intensa no muco da pele que no intestino. Tanto monócitos como neutrófilos são rapidamente recrutados, já numa fase inicial, atingindo grandes níveis, juntamento com a explosão respiratória, mesmo às 4 h após resposta à inflamação, indo ao encontro de resultados descritos.

A suplementação de metionina diminui o número de células periféricas e protéases no muco imunológicos dos peixes, apontando para um efeito potencialmente imunossupressor que poderá comprometer a possível resistência a doenças.

Palavras-chave: aminoácidos, desafio bacteriano, dieta funcional, estimulação imunitária, inflamação

Abstract

Mucous membranes are of main importance in the defenses of the host against bacterial infections, being the gills, skin and gut the first two physical barriers.

Tenacibaculum maritimum, the aetiological agent of tenacibaculosis is one disease that causes highly loses in stock production of European seabass, so knowing more about the immune mechanism involved its fight, still poorly understood, is highly important.

Due to highly use of antibiotics in the animal production finding a strategic to counter it is of highly need, methionine as functional ingredient for promoting health in fish would be a good approach. The building blocks for protein synthesis are amino acids, the methionine is one of them, an essential amino acid that regulates key metabolic pathways, having a vital impact on the immune system control in fish. This amino acid in specific, methionine plays an important role during the polyamines synthesis and inflammatory response.

This study aimed to assess, gather evidence and elucidate on the specific role of methionine dietary supplementation on the immune status and response of European seabass (*Dicentrarchus labrax*) against infection with *Tenacibaculum maritimum*, through, evaluation of methionine surplus effect on the haematological profile, gut and mucus immune parameters.

To achieve this goal, European seabass juveniles were subjected to a feeding trial of four weeks being fed three dietary treatments: a control diet (meeting all the nutritional requirements for seabass) and two control-based diets supplemented with two different with DL-Methionine at 1 or 2 % of feed weight. Fish were sampled at 2 and 4 weeks in order to evaluate the immune status of fish fed with each diet. At the end of each feeding trial, fish were subjected to a 2 hours bath challenge with *Tenacibaculum maritimum* and sampled at 4 and 24 hours post infection. Samples of blood, skin mucus and gut were taken for the evaluation of the haematological profile, peripheral cell dynamics, and immune parameters.

The results showed that fish immune response following stimulation with the bacteria, *Tenacibaculum maritimum*, as expected of an efficient immune response, reacted with skin mucus activities (protease and peroxidase) rising at early stages of the inflammatory response. It also showed that, at least in a *T. maritimum* bath infection, the immune response seems more intense in the fish skin mucus than in the gut. Both monocytes

and neutrophils were readily recruited at early stages, showing peaking levels, alongside respiratory burst, already at 4 h post infection response, in accordance with other results found.

The methionine supplementation diminishes the number of peripheral cell and proteases in the immune mucus of the fish, pointing for potentially immunosuppressor effect that could compromise the possible resistance to the disease.

Keywords: amino acids, bacterial challenge, functional diet, immunostimulation, inflammation

Table of Contents

List of Tables

List of Figures

List of Abbreviations

FCUP	FACULTY OF SCIENCES OF THE UNIVERSITY OF PORTO
UP	UNIVERSITY OF PORTO
CIIMAR	INTERDISCIPLINARY CENTRE OF MARINE AND ENVIRONMENTAL RESEARCH
EU	EUROPEAN UNION
PRR	PATTERN RECOGNITION RECEPTORS
PRP	PATTERN RECOGNITION PROTEINS
AMPS	ANTIMICROBIAL PEPTIDES
ROS	REACTIVE OXYGEN SPECIES
T. MARITIMUM	*TENACIBACULUM MARITIMUM*
GSH	GLUTATHIONE
CTRL	CONTROL
AA	AMINO ACIDS
D2	DL-METHIONINE AT 1 % OF FEED WEIGHT
D3	DL-METHIONINE AT 2 % OF FEED WEIGHT
DM	DRY MATTER
MA	MARINE AGAR
MB	MARINE BROTH
LD50	50% LETHAL DOSE
RBC	RED BLOOD CELLS
WBC	WHITE BLOOD CELLS
MCV	MEAN CORPUSCULAR VOLUME
MCH	MEAN CORPOSCULAR HAEMOGLOBIN
MCHC	MEAN CORPUSCULAR HAEMAGLOBIN CONCENTRATION
HBSS	HANK'S BALANCED SALT SOLUTION
TMB	3,3',5,5'-TETRAMETHYLBENZIDINE HYDROCHLORIDE
OD	OPTICAL DENSITY
PMS	POST MITOCHONDRIAL SUPERNATANT
LPO	LIPID PEROXIDATION
BHT	BUTYLHYDROXYTOLUENE
CAT	CATALASE
UV	ULTRAVIOLET
SOD	SUPEROXIDE DISMUTASE
RLU	RELATIVE LIGHT UNITS

MET1	DL-METHIONINE AT 1 % OF FEED WEIGHT
MET2	DL-METHIONINE AT 2 % OF FEED WEIGHT
ACP	ACID PHOSPHATASE

1. Introduction

1.1. Aquaculture and European seabass

Aquaculture is an industry responsible for supplying aquatic animal protein (Lazado, 2015). Its significance has been rising during the current years, being the fastest growing food-sector related industry, in the European Union aquaculture mainly because of the rise in the production and of the importations from third countries. The efforts in the investment by governments and private sector during the recent years have created positive improvements in production, processing, logistics and marketing that are projected to help, through demand generation and cost savings, the industry profitability (Globefish, 2017). Non-EU producers even with these improvements appear to have an advantage in competition (e.g. lower labor cost, or licencing of new production facilities) which make higher production cost in the EU countries than in third countries (M. Bozoglu et al., 2009; Scientific, Technical and Economic Committee for Fisheries, 2014).

One of the sectors with higher potential for sustainable jobs and growth is aquaculture in the EU's Blue Growth Strategy (European Commission, Communication from the Commission: Blue Growth Opportunities for Marine and Maritime Sustainable Growth, 2012). This sector, considered a large economic activity with a capacity to increase seafood green production, within the EU, enhancing rural and coastal employment and incomes. Within the EU, increasing the importance of aquaculture for policy markers, the demand for assessment about development of economic performance of the aquaculture industry also increase, having the ability to reach peaking points (J. Guillen et al., 2015).

European seabass, *Dicentrarchus labrax* (Linnaeus, 1758) is produced under intensive, semi-intensive or extensive systems (Basurco, 2000). It is a coastal marine fish from the north-eastern Atlantic Ocean to the Mediterranean and the Black Sea that lives in shallow waters, it is euryhaline (0-40 ppt salinity) and eurythermal (2-32 ºC). Sea bass aquaculture is essentially located in the Mediterranean area, mostly in Turkey, Greece, Egypt and Spain, which accounts for 94% of the production. In aquaculture the production is in two phases: first a hatchery-pregrowing phase, which produces fish of 1 to 20 g in three to eight months, and then an ongrowing phase to 250-450 g in 12 to 20 months (Vandeputte, 2019). Despite several studies, relatively old (some almost 20 years) on technical efficiency, productivity and profitability, in the seabass industry, after this new century, the challenges that industry is dealing had shifted significantly.

1.2. Innate immune system

Fish skin, gills and gut act as physical barriers that are considered to be the first defense against infection (Shephard, 1994). Teleost skin is histologically diverse and unique (Esteban, 2012); the external mucus is one of the most distinguishing features of this tissue, that holds numerous immune components (like lectins, pentraxins, lysozyme, complement proteins, antibacterial peptides and IgM) of both the innate (non-specific) and acquired humoral and cell mediated mechanisms (specific), which are responsible for resistance to diseases (Aranishi, 1997; Ellis, 1999; Fast, 2002). Skin mucus has the capacity to alone form a insulating layer (biofilm) that underlies the epithelium, protecting it from damage. Skin damage can be provoked by bacteria, being the mucus an important player in the damage progression. In same way, the innate immune system is present in all organisms, it is a more primitive system of protection, and in teleost fish it is well developed (Barton GM., 2008).

The innate immune system's recognition of non-self is mediated by germline-encoded pattern that identify proteins/receptors that recognise molecular patterns, characteristic of microbes. Due to the intrinsic inefficiency of fish acquired immune response as a result of its evolutionary position and poikilothermic nature, the innate immune system is of primary significance in the combat of infections. The acquired immune response of fish can be up to 12 weeks sluggish compared to the relatively temperature independent and instant innate immune response (Alexander, 1992; Ellis, 2001).

The innate immune system, served by a variety of germline-encoded pattern recognition receptors (PRR) or pattern recognition proteins (PRP) (Janeway, 1989). Unlikely the acquired resistance recognition molecules, the innate systems recognition receptors are fairly few and vertically transmitted, exposing the adaptation to specific environmental conditions and evolutionary defence battles of species (Lo D et al., 1999).

The inflammatory response initiates when external barriers such as skin mucus and skin are breached, as a reaction to a wound or infection (Ellis, 2001). An inflammatory response should be locally restricted and self-limiting, but at the same time destructive and rapid, in order to swiftly re-establish homeostasis while repairing the damage. During the inflammatory response, an influx of phagocytic cells into the inflammatory focus enables the recognition, neutralization and degradation of the organism responsible for the infection.

Once the recognition molecules, like siglec lectin, are activated they can induce opsonization and phagocytosis of the pathogen, stimulate nature cytotoxic cells or

activate different signalling/executive processes like the complement system and the lytic pathway or an acute phase response (Jung, 2000).

The phagocytes main tasks are intracellular killing and phagocytosis, and to do so, the microorganisms are engulfed by these cells into membrane-delimited compartments known as phagosomes. The phagosomes fuse with granules in order to produce a phagolysosome, in which the microorganisms are subjected to antimicrobial peptides (AMPs), enzymes and reactive oxygen species (ROS), these components through synergize effectively destroy the microorganisms. Many studies demonstrated inducible antimicrobial responses, mainly using head kidney-derived monocytes/macrophages and also neutrophils, generating anti-proteases compounds, lysozyme and complement factors that attack efficiently the pathogens in the cells (Ellis A.E., 2001; Rodriguez, 2003; Plouffe, 2005), including respiratory burst and subsequent production of ROS and inducible nitric oxide synthetase producing nitrogen intermediates with antimicrobial effect (Stafford, 2002; Forlenza, 2008).

In parallel, there is a wide array of humoral mediators that, together with the cellular response, ensure the efficiency of the inflammatory process. Lysozyme is an important bactericidal, hydrolysing β- linked glycoside bonds of bacterial cell wall peptidoglycans resulting in the lysis. This enzyme, mostly associated with defence against Gram positive bacteria, can also lyse Gram negative bacteria. It is present in mucus, lymphoid tissue, plasma and other body fluids of most fish species (Yousif, 1994), being also known for being an opsonin and activate the complement system and phagocytes (Grinde, 1989).

Another good indicator of cellular activation during the immune response is the alteration of plasmatic levels in peroxidase. When stimulated, immune cells as phagocytes release this enzyme to the extracellular matrix as an antibacterial mechanism. Therefore, in blood, this enzyme is frequently used as an sign of the immunologically active status of circulating leucocytes (Alvarez-Pellitero, 2008).

In fish serum and other body fluids, various proteases and proteases inhibitors are present, being their primary role the homeostasis (Aranishi, 1999). They are also involved in acute reactions and in defence against pathogens, preventing adhesion to matrices and degrading the microorganism's structures (Ellis, 1987; Bayne, 2001). Most widely studied is the α2-macroglobulin (α2-M), having a broad specificity, inhibition involving the physical encapsulation of the protease (Armstrong, 1999).

Humoral parameters, in fish, can be both cell-associated receptors or soluble molecules of plasma and other body fluids. Amongst others, several enzymes have been used as

good markers of aquatic organisms' responses to various environmental stressors and to oxidative stress (e.g. glutathione system). Oxidative stress is characterized by high rates of lipid peroxidation, protein carbonylation, and changes in the activities of antioxidant enzymes (Dorts et al., 2009). When the production rate of free radicals such as hydroxyl ions, superoxide anions, hydrogen peroxides surplus the scavenging ability of these antioxidants, oxidative stress occurs. Understanding these mechanisms could help gathering knowledge on certain reported diseases of aquatic life, providing the evidence in first-line that fish have been infected (Ruby and McFall-Ngai, 1999).

One of these enzymes is superoxide dismutase and it acts to prevent excessive levels of reactive oxygen species (ROS) from accumulating at the cellular and tissue level. Catalase is an enzyme involved in the breakdown of toxic hydrogen peroxide to water and oxygen, hence assisting to detoxify hydrogen peroxide (Sabatini et al. 2009).

1.3. *Tenacibaculum maritimum* and tenacibaculosis

In cultured fish, the most common group of pathogens are bacteria, and they may act as primary or opportunistic pathogenic agents causing disease and leading to high economic losses (Austin, 2007). The Gram-negative bacterium *Tenacibaculum maritimum* is the aetiological agent of tenacibaculosis. Tenacibaculosis can be a risk to restricting the culture of many species of anadromous and marine fish of marketable importance worldwide. However, there is still little knowledge on its pathogenicity and routes of infection. Some studies on disease transmission support the hypothesis that *T. maritimum* primarily causes extensive skin damage (ulcers, necrosis, eroded mouth, frayed fins and tail rots) and gill abrasion with consequent systemic infection, being an opportunistic pathogen (Avendaño-Herrera, 2006). The adherence of *T. maritimum* to host tissues varies on its capacity to neutralize or evade fish mucus defence mechanisms, such as lysozyme and other proteases, as well as on its ability to gather the necessary nutrients for growing (Magariños, 1995). The presence of the *T. maritimum* within the skin mucus layer could mean that this bacterium is a piece of the autochthonous populations in fish, consequently, in the aquatic environment the bacterium can persist utilizing fish mucus as a reservoir (Avendaño-Herrera et al., 2005).

1.4. Functional diets and methionine as an additive

As aquaculture becomes more and more intensive, it leads to a riskier industry, increasing the risk for disease outbreaks (Meena D.K., 2013; Van Boeckel T.P., 2015; FAO, 2018). Accordingly, animal health issues are now one of the leading constraints for sustainability and expansion in aquaculture (Adams A., 2019). Although in the recent

years the regulations and legislation started to be more restrictive, one of the main approaches to deal with the disease occurrences in aquaculture has been the application of antibiotics, leading to the emergence of new antibiotic-resistant bacteria (Van Boeckel T.P., 2015). An alternative to the antibiotics use, and in addition to vaccination, could be the use of prophylactic measures through the inclusion of prebiotics and immunostimulants in feeds in order to enhance fish resistance against disease and health status in general (Yilmaz S., 2022).

It is very likely that nutrients impact some characteristics of the immune system, therefore nutritional strategies may modulate it in aquaculture fish (Li P., 2007; Conceicao L.E.C, 2012). Selecting ingredients/additives rich in essential nutrients and thereby creating functional feeds that improve the status of the fish, may reduce severity during an infection outbreak, as well as counteracting immunosuppression instigated by contaminants and stress and improving immunocompetence prior to, or during vaccination (Azeredo et al. 2017; Machado et al. 2018).

Protein synthesis, in which an efficient immune response highly relies, is dependent on a sufficient availability of amino acids (Grimble, 1998). Therefore, the addition of such amino acids to aquaculture feeds may improve fish growth and immune performance (Li et al, 2009). An indispensable amino acid, methionine, in particular, is involved in cellular and molecular processes that take place during the immune response such as protein ubiquitination and autophagy (Afonso, 1998; Zinngrebe, 2014). Studies show that methionine may also affect lymphocytes differentiation and proliferation, since it participates in polyamine biosynthesis (Grimble, 1998). Through the transsulfuration pathway, methionine is converted into cysteine, that becomes one of the three glutathione (GSH) elements. GSH is a molecule involved in scavenging free radicals (reducing ROS), therefore protecting cells from oxidative stress during inflammation (Grimble, 1998). All these interventions of methionine mean that its basic nutritional requirement (and that of other amino acids) is increased during immune responses, improving them both at humoral and cellular level (Le Floc'h, 2004, Rubin L.L., 2007). Thus, the use of dietary supplementation of essential amino acids like methionine poses as an efficient, and with acceptable cost, prophylactic strategy to improve fish immune status and response, thereby contributing to the replacement of more deleterious measures such as the use of antibiotics (Grimble, 1998 and 2009).

1.5. Objectives

A number of studies were performed to explain the effect of methionine on the innate immune response, but most of them were executed in mammals. Therefore, the aim of the present study is to assess, gather evidence and elucidate on the specific role of dietary methionine during the European seabass (*Dicentrarchus labrax*) immune response (local and systemic) against *Tenacibaculum maritimum*. In particular, it is intended to evaluate methionine surplus effects on sea bass haematological profile, mucus and gut immune parameters and gut oxidative stress.

2. Materials and methods

2.1. Experimental diets

Three practical isonitrogenous (45% crude protein) and isolipidic (16% crude fat) diets were formulated to include fish oil as the main lipid source, fish meal and a blend of plant feedstuffs as protein sources. A CTRL diet was formulated to include the indispensable AA requirement levels estimated for European seabass. Following results from previous works (Machado M., 2015; Le Floc'h N, 2004; Kaushik S., 1998), two other diets were formulated similar to the CTRL but supplemented with DL-Methionine at 1 or 2 % of feed weight, at the expenses of wheat meal and wheat gluten. All ingredients of the diet were finely ground, well mixed and dry pelleted in a laboratory pellet mill. Sparos Lda. (Olhão, Portugal) formulated and manufactured these 3 diets. Proximate analysis of the experimental diets and ingredient composition are presented in Table 1.

	Experimental diets		
	CTRL	D2	D3
Ingredients (%DM)	%	%	%
Fishmeal LT70	11.0	11.0	11.0
Fishmeal 60	17.0	17.0	17.0
Brewer's yeast	3.0	3.0	3.0
Soy protein concentrate	12.0	12.0	12.0
Wheat gluten	7.0	6.4	5.8
Corn gluten meal	4.0	4.0	4.0
Soybean meal 44	15.0	15.0	15.0
Rapeseed meal	6.0	6.0	6.0
Wheat meal	10.0	9.6	9.2
Faba beans (low tannins)	0.0	0.0	0.0
Vitamin and mineral premix	1.0	1.0	1.0
DL-Methionine	0.0	1.0	2.0
Soy lecithin	0.5	0.5	0.5
Fish oil	8.5	8.5	8.5
Rapeseed oil	5.0	5.0	5.0

As fed basis (8%)			
Crude protein, % feed	45.1	45.1	45.1
Digestible protein, %	39.5	39.6	39.6
Crude fat, % feed	16.9	16.8	16.8
Fiber, % feed	2.7	2.7	2.7
Starch, % feed	9.5	9.2	8.9
Ash, % feed	8.9	8.9	8.9
Gross energy, MJ/kg feed	21.1	21.1	21.1

Table 1. Composition and proximate analysis of the experimental diets.

2.2. Fish and experimental design

The trials were conducted at Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR) in Matosinhos, Portugal. Experiments were performed in full compliance with national rules, by trained scientists, and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

Seabass juveniles, each weighing approximately 9 grams, were randomly distributed in 9 tanks (200 L, n= 30) of a recirculating aerated seawater system with a constant flow of filtered seawater. During acclimatization, fish were fed a control diet, meeting the indispensable level of amino acids and all the nutritional requirements estimated for European seabass (Kaushik SJ. 1998). At the onset of the feeding trial, the three dietary treatments were randomly assigned to triplicate tanks and fish were fed twice a day a total of 2 % biomass. The feeding trial lasted for two and four weeks. Water quality parameters (salinity, temperature, pH, oxygen saturation, ammonia $NH4^+$ and nitrite NO_2^-), were controlled daily and the photoperiod was kept at 10 h dark: 14 h light.

2.3. Bacteria inoculum preparation

T. maritimum, strain ACC13.1, isolated from Senegalese sole were cultured at 25 °C in marine agar (MA) for forty-eight hours and later inoculated in marine broth (MB) for eighteen hours under continuous and vigorous shaking. Exponentially growing bacteria in marine broth (MB) were collected to prepare the bath inoculum and adjusted to 2.24 $x10^9$ CFU ml^{-1} as final concentration. This dosage was selected after a pre-challenge to determine 50% lethal dose (LD50).

2.4. Sampling and immune challenge

At the end of two and four weeks feeding periods, both conducted in order to assess the effects of short term dietary methionine supplementation on cellular, mucus and gut immune status of the European seabass, the following sampling protocol was applied: 3 fish per tank (n= 9 fish per treatment) were randomly selected, euthanized by an overdose of anesthetic, 2-phenoxyethanol (1500 ppm; Sigma), and sampled. Skin mucus was collected before fish was weighed using a cell scraper. Blood samples were taken from which blood smears were prepared. Fresh blood was also used for total peripheral cell counts, respiratory burst analysis, analysis of hemoglobin concentration and hematocrit evaluation. Finally, a sample of the anterior gut was extracted. Immediately, skin mucus and gut samples were frozen at -80 °C until further assayed.

The remaining fish were re-allocated in a different system (in triplicate tanks according to dietary treatments). The bacteria inoculum was then added to the bath tanks (in a 5 L volume) to a final concentration of 5 x 10^3 CFU ml^{-1}. Water temperature was increased up to 24 ± 0.5 ˚C and after the 2 h infectious challenge, the water of each tank was changed three times and then recirculation re-established. Fish were then sampled as previously described, at 4 and 24 h post challenge (n=3 per tank, 9 fish per treatment).

2.5. Haematological profile

Regarding haematological procedures, from the caudal vein blood was collected using heparinized syringes at 3000 uni ml^{-1} and fresh samples were used for the preparation of blood smears and for the evaluation of haemoglobin concentration and haematocrit value. Furthermore, total red blood cells (RBC) and white blood cells (WBC) were counted from two blood samples diluted in Hank´s Balanced Salt Solution (heparinized at 30 uni ml^{-1}) at 200 × and 20 ×, respectively. Total cell counting was performed in a microscope using a Neubauer chamber. Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MVH) and mean corpuscular haemoglobin concentration (MCHC) were calculated, then.

-MCV (μm^3)=(Ht/RBC) x 10

-MCH (ρg $cell^{-1}$)=Hb(RBC) x 10

-MCHC (g 100 ml^{-1})=(Hb/Ht) x 100

In accordance with Nikoskelainen et al., the respiratory burst was evaluated by briefly adding 4 µl of blood to a 96 µl of HBSS in a 96-well flat bottom white polystyrene plate. Afterward, 100 µl of freshly prepared luminol solution were added to each well. The

kinetic curves were generated in a luminescence reader by the luminol-amplified chemiluminescence (measured every 3 min for 2 h). Each sample was run in triplicate and controls contained no blood. The integral luminescence in relative light units was calculated.

2.6. Differential cell counting

Right after the blood collection, from the homogenized blood smears were prepared, air dried and fixated with formol-ethanol (10% of 37% formaldehyde in absolute ethanol). To simplify the identification of neutrophils, the detection of peroxidase activity was performed as described by Afonso et al. (1997). Finally, blood smears were stained with Wright's stain (Haemacolor; Merck), examined in a light microscope under immersion oil (1000x), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. The absolute value ($x10^4$ μl^{-1}) of each cell type was calculated.

2.7. Mucus immune parameters

Following the procedure described by Quade and J.A (1997) the peroxidase activity was measured in mucus. In triplicates, 135 µl of HBSS without Ca^{+2} and Mg^{+2} was added to 15 µl of mucus samples in a flat-bottomed 96-well plate. Then, 50 µl of 20 mM 2,3', 5,5'-tetramethylbenzidine hydrochloride (TMB; Sigma) and 50 µl of 5mM H_2O_2 were added. The color-change reaction after 2 minutes was stopped by adding 50 µl of 2 M sulphuric acid. The optical density was read at 450 nm in a Synergy HT microplate reader. Controls (blanks) were included in which HBSS replaced mucus samples. Peroxidase activity (units ml^{-1} mucus) was determined by defining one unit of peroxidase as that which produces an absorbance change of 1 OD.

Protease activity was quantified using the azocasein hydrolysis assay (Azeredo et al. 2017; Machado et al. 2020), using 100 µL of sample and adding to it 100 µL of 0.5% $NaHCO_3$ (pH 8.3) and 125 µL azocasein (20 mg mL^{-1} in 0.5% $NaHCO_3$, pH 8.3). After a 24 h incubation period at 22 ºC in polystyrene microtubes, 250 µL of 10% trichloroacetic acid was added and centrifuged (10,000×g for 5 min) and the absorbance of 100 µL of the supernatant, plus 100 µL of 1N NaOH read at 450 nm in a Synergy HT microplate reader. The percentage protease activity compared to the reference sample (trypsin solution, 5 mg mL^{-1} in 0.5% $NaHCO_3$, pH 8.3) was calculated (Ellis 1990).

2.8. Gut parameters

Gut samples were thawed and homogenized (1:10) in phosphate buffer (0.1 M, pH 7.4) using a high-performance dispersing instrument. After centrifugation (5500 rpm for 20 min), supernatant, post mitochondrial supernatant fraction (PMS) was aliquoted for humoral parameters and for oxidative stress parameters.

2.8.1. Lipid peroxidation

For determining the extent of endogenous lipid peroxidation (LPO) by measuring thiobarbituric acid-reactive species (Bird, 1984). To prevent artifactual lipid peroxidation and before centrifugation, butylhydroxytoluene (BHT 0.2 mM) was added to the aliquot. Briefly, 1 mL of 100% trichloroacetic acid and 1 mL of 0.73% thiobarbituric acid solution (in Tris-HCl 60 mM pH 7.4 with DTPA 0.1 mM) were added to 0.2 mL of gut homogenate. After incubation for 60 min at 100 ºC, the solution was centrifuged at 12,000x g for 5 min and LPO levels were determined at 535 nm.

2.8.2. Catalase, superoxide dismutase and peroxidase activities

Total protein concentration in homogenates was measured by using Pierce™ BCA Protein Assay Kit, as described by the manufacturer.

Catalase (CAT) activity was determined in PMS at 240 nm by measuring substrate (H_2O_2) consumption as described by Claiborne (1984) adapted to microplate (Rodrigues et al., 2017). Briefly, in a microplate well, 0.140 mL of phosphate buffer (0.05 M pH 7.0) and 0.150 mL H_2O_2 solution (30 mM in phosphate buffer 0.05 M pH 7.0) as substrate were added freshly to 0.01 mL of gut PMS (0.7 mg ml^{-1} total protein). Sample volume was 10 µL in a total volume of 300 µL. Enzymatic activity was read at 240 nm in UV microplate for 2 min every 15 sec interval in a Synergy HT microplate reader (BioTek Synergy HT). Enzyme activity is expressed as enzyme units per milligram of total protein (U mg^{-1} protein). One enzyme unit is the amount of enzyme needed to catalyze 1 µmol of substrate per minute.

Superoxide dismutase (SOD) activity was measured according to Flohé et al., 1984, adapted to microplate by Lima et al., 2007. Briefly, in a microplate well, 0.2 mL of the reaction solution [1 part xantine solution 0.7 mM (in NaOH 1 mM) and 10 parts cytochromec solution 0.03 mM (in phosphate buffer 50 mM pH 7.8 with 1 mM Na-EDTA)] was added to 0.05 mL of gut PMS (0.25 mg ml^{-1} total protein). Optical density was measured in a microplate reader (BioTek Synergy HT) at 550 nm every 20-s interval for 3 min at 25 ºC.

Peroxidase activity was measure following the same approach described for skin mucus samples.

2.9. Statistical analysis

All results were introduced in a database (MS-Excel®), and mean and standard deviation of each treatment were calculated. Normality and homogeneity of variance were verified and, when necessary, data was transformed before being treated statistically. The results were subjected to a multifactorial ANOVA with sampling point, feeding time and diet as main factors and a Tukey *post-hoc* test was conducted to identify differences between treatments. The level of significance used was $p \leq 0.05$ for all statistical tests.

3. Results

To assess potential modulatory effects of a dietary methionine surplus in seabass immune status and inflammatory response, the haematological profile (haematocrit, haemoglobin content, respiratory burst, RBC counts, WBC counts and differential cell counting), and skin gut and skin mucus immune and oxidative stress mediators (peroxidase, proteases, catalase, LPO and SOD activities) were evaluated. The complete set of results is presented in the Attachments.

3.1. Haemotology

No significant differences were observed among groups regarding haematocrit values. The haemoglobin content of fish fed the experimental diets for 4 weeks and sampled at 4 and 24 h post infection was higher than that of their counterparts, fed for 2 weeks (Attachments, Table 2). Moreover, it gradually decreased after infection in fish fed for 2 weeks, while the reverse pattern was observed in fish fed for 4 weeks. Nonetheless, no significant differences were attributed to dietary treatments. Similarly, in fish fed dietary treatments for 4 weeks, MCH increased from 4 to 24 h post infection, the latter sampling point registering significantly higher values than those observed in fish fed for 2 weeks and sampled at the same post infection time (Attachments, Table 2).

The number of RBC decreased from 4 to 24 h post infection, regardless dietary treatment and feeding time. Also, RBC counts were higher in fish fed CTRL for 4 weeks, than in those fed for 2 weeks (Attachments, Table 2). Fish fed Met2 had significantly less WBC than those fed CTRL, regardless of feeding time or time post infection (Fig. 1A.). Moreover, WBC decreased from 4 to 24 h in fish fed for 4 weeks, irrespective of diets. At 24 h, fish fed for 4 weeks presented lower WBC counts than their counterparts fed for 2 weeks (Fig. 1B.).

Respiratory burst increased from 0 to 4 h post infection regardless of feeding time or dietary treatment and decreased back to basal levels at 24 h (Fig. 1C.).

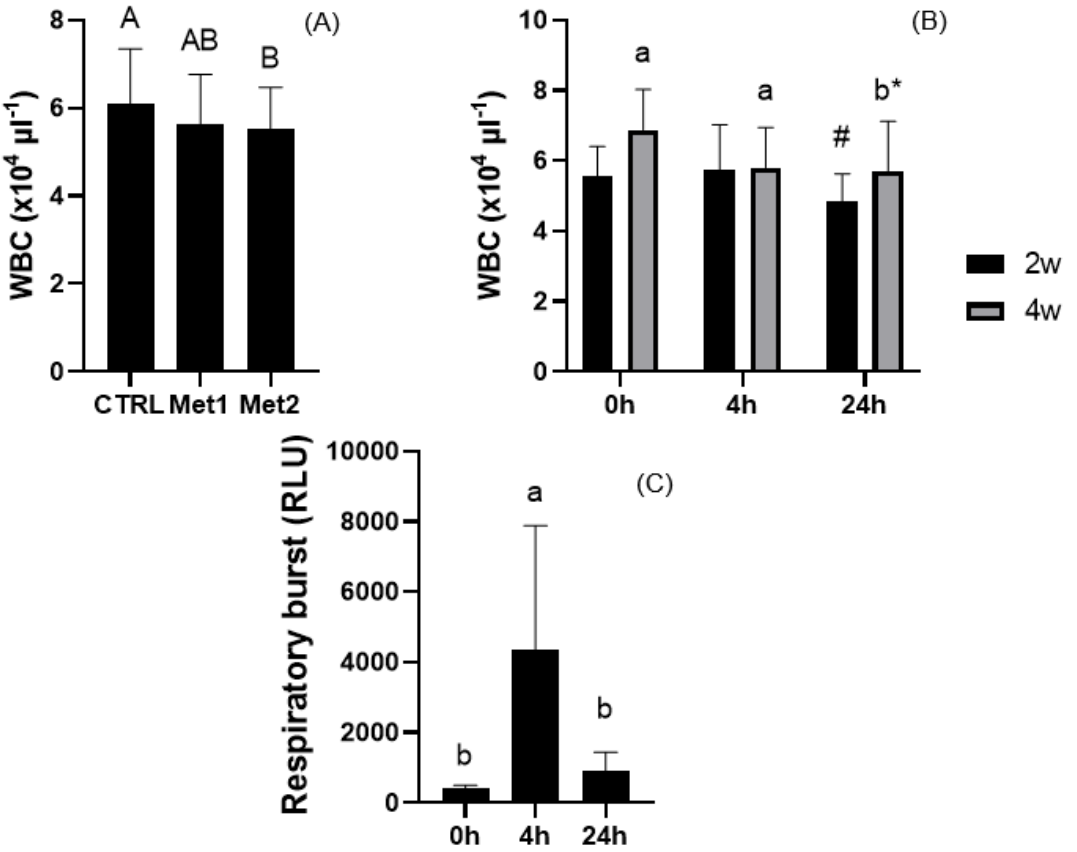

Figure 1. Mean (± S.D.) of white blood cells (WBC, A and B) and respiratory burst (C) in European seabass fed dietary treatments (CTRL, Met1 and Met2) during 2 (■) and 4 (■) weeks (0 h), and sampled at 0, 4 and 24 h after infection. P-values from two-way ANOVA ($p \leq 0.05$). If the interaction was significant, one-way ANOVA was performed. Different symbols stand for significant differences between feeding times. Different lower letters indicate differences among sampling times. Different capital letters denote differences among dietary treatments.

3.2. Peripheral blood leucocytes

Regarding peripheral leucocytes, fish fed Met1 showed lower thrombocyte numbers than those fed CTRL, irrespective of feeding time or sampling time (Fig. 2A.). Moreover, thrombocytes were more abundant in fish fed for 4 weeks compared to those fed for 2 weeks, irrespective of dietary treatment. Infection significantly decreased this cell type, as thrombocyte numbers in fish sampled 24 h post infection were lower than in non-infected fish (0h).

While the 4 weeks-feeding time significantly increased the number of lymphocytes relative to a 2 weeks period, fish fed for 4 weeks and sampled 4 h post infection showed lower lymphocyte counts than those fed for 2 weeks (Attachments, Table 3). Lymphocytes concentration decreased with infection, as it dropped from 0 h to 4h post infection in all dietary treatments. (Attachments, Table 3). Fish sampled before infection

sowed higher number of lymphocytes than those sampled post infection in all dietary treatments. (Attachments, Table 3).

In fish fed for 2 weeks, monocytes concentration was significantly higher at 4 h post infection than in non-infected fish (0 h), remaining so at 24 h post infection (Fig. 2B.). Differently, in animals fed for 4 weeks, it gradually and significantly increased, with monocytes being highest at 24 h post infection. Fish fed dietary treatments for 4 weeks had more monocytes at 24 h than their counterparts fed for 2 weeks. Neutrophils concentration followed a similar pattern, showing higher numbers at all sampling points in fish fed for 4 weeks, compared to those fed 2 weeks (Fig. 2C.). In both feeding periods, neutrophils were observed to increase from 0 h to 4 h post infection, followed by a decrease at 24 h. No significant effects of dietary treatments were observed on monocytes and neutrophils numbers.

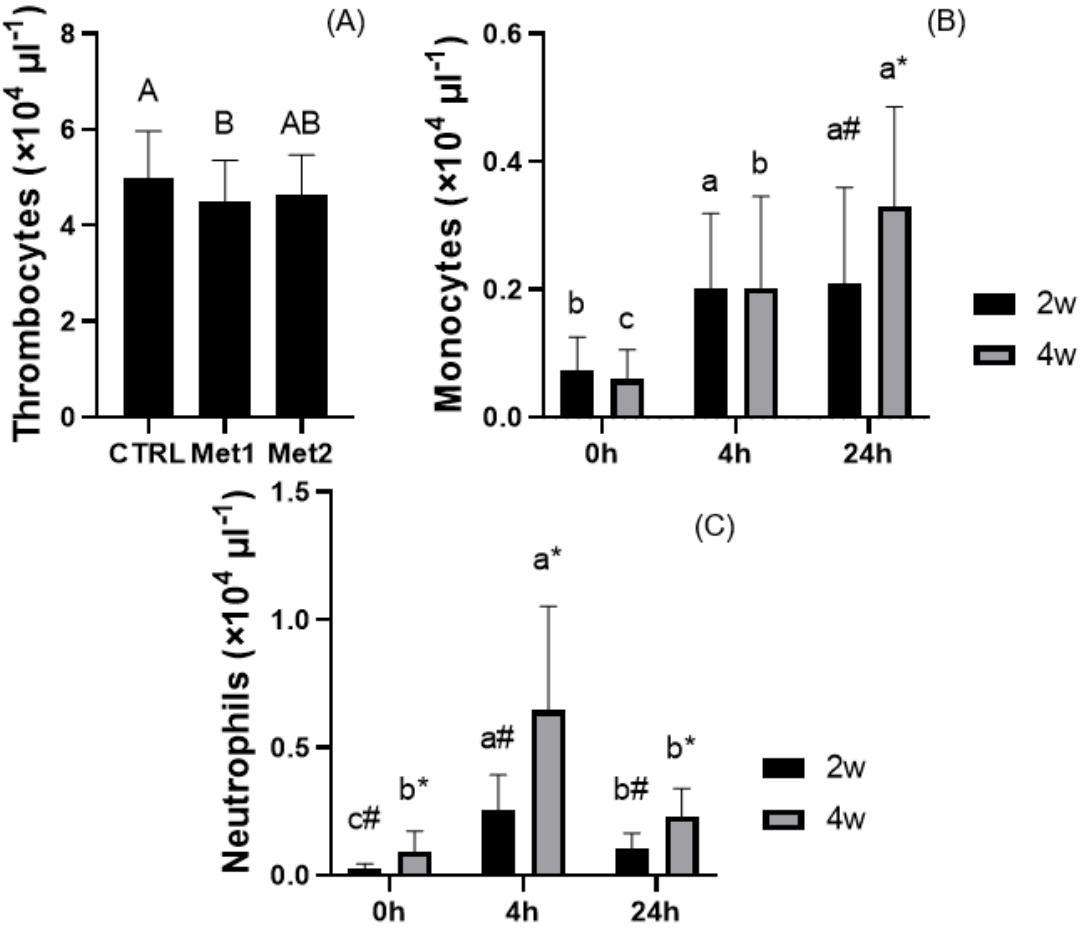

Figure 2. Mean (± S.D.) of thrombocytes (A), monocytes (B) and neutrophils (C) in European seabass fed dietary treatments (CTRL, Met1 and Met2) during 2 (■) and 4 (■) weeks (0 h), and sampled at 0, 4 and 24 h after infection. P-values from two-way ANOVA ($p \leq 0.05$). If the interaction was significant, one-way ANOVA was performed. Different symbols stand for significant differences between feeding times. Different lower letters indicate differences among sampling times. Different capital letters denote differences among dietary treatments.

3.3. Mucus immune parameters

Skin mucus peroxidase activity gradually increased from 0h to 24 h post infection, regardless of dietary treatment and feeding time (Fig. 3A.).

Proteases activity also increased over time but only in fish fed CTRL. Fish fed Met2 showed higher protea (A) ity at 4 h post infection than before (0 h) but levels remain stable in fish sampled at 24 h (Fig. 3B.). In addition, at 24 h post infection, fish fed Met1 had lower proteases activity compared to fish fed CTRL.

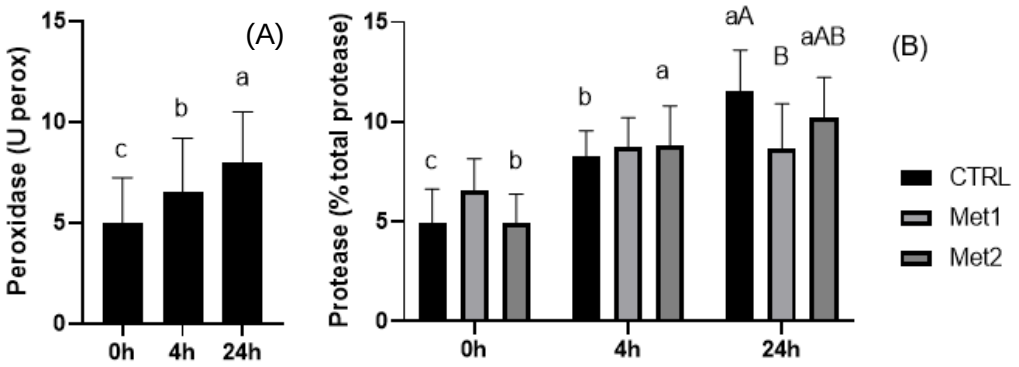

Figure 3. Mean (± S.D.) of peroxidase (A) and protease (B) in European seabass fed dietary treatments, CTRL (■), Met1 (■) and Met2 (■) weeks (0 h), and sampled at 0, 4 and 24 h after infection. P-values from two-way ANOVA ($p \leq 0.05$). If the interaction was significant, one-way ANOVA was performed. Different lower letters indicate differences among sampling times. Different capital letters denote differences among dietary treatments.

3.4. Gut immune parameters

Gut peroxidase activity decreased upon infection only in fish fed for 2 weeks, as activity was lower at 4 and 24 h post infection than in non-infected fish (Fig. 4A.). Moreover, levels were lower in non-infected fish fed for 4 weeks, compared to those fed for 2 weeks, while no significant differences were attributed to dietary treatment.

Lipid peroxidation in the gut, independently of diet and feeding time, increased upon infection, as activity was higher at 24 h post infection than in non-infected fish (Fig. 4B.).

Catalase activity was enhanced at 4 h post infection in fish fed dietary treatments for 4 weeks, compared to fish fed for 2 weeks (Fig. 4C.). However, it decreased from 4 to 24 h post infection in fish fed for 4 weeks. No significant differences were attributed to different dietary treatments.

Gut superoxide dismutase activity decreased upon infection only in fish fed for 2 weeks, as activity was lower at 24 h post infection than in non-infected fish (Fig. 4D.). Moreover,

levels were lower in 24 h post infection fish fed for 2 weeks, compared to those fed for 4 weeks, while no significant differences were attributed to dietary treatment.

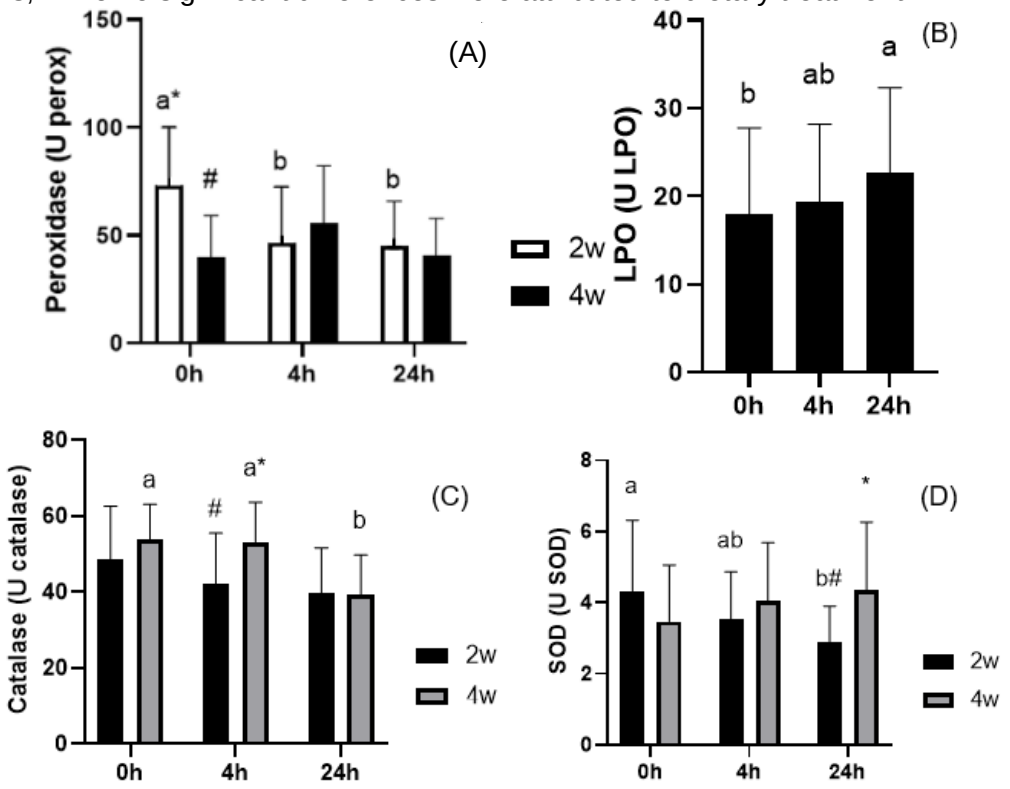

Figure 4. Mean (± S.D.) of peroxidase (A), lipid peroxidation activity (LPO, B), catalase (C) and superoxide dismutase activity (SOD, D) in European seabass fed dietary treatments during 2 (■) and 4 (■) weeks (0 h), and sampled at 0, 4 and 24 h after infection. P-values from two-way ANOVA ($p \leq 0.05$). If the interaction was significant, one-way ANOVA was performed. Different symbols stand for significant differences between feeding times. Different lower letters indicate differences among sampling times.

4. Discussion

There are already some studies reporting data regarding the effects of dietary methionine supplementation on the inflammatory response of European seabass to experimental infection. These studies are, however, focused on infection models that do not contemplate the specific responses of peripheral but first barrier-tissues, such as the gut and skin mucus. Also, only a few studies look into the innate immune response of fish following stimulation with the bacteria *Tenacibaculum maritimum* (Salati et al. 2005, Guardiola et al. 2019, Mabrok et al. 2016). Irrespective of dietary treatment, and as expected from a robust and well-established inflammatory response, both phagocytic cell types (monocytes and neutrophils) of the present challenged fish were readily recruited at early stages of the response, with numbers rising at 4 and 24 h post infection. Senegalese sole infected with *T. maritimum* presented similar trends, especially in what peripheral neutrophils are concerned, when these cells showed a more pronounced

increase already at 4 h post infection (Guardiola et al. 2019). In parallel, fish sampled at 4 h – irrespectively of dietary treatment or period of feeding – showed peaking levels of respiratory burst, which is, alongside with phagocytosis, one of the clearest functional markers of immune cells such as neutrophils and monocytes.

Skin mucus proteases of infected fish were observed to increase at early stages of the response, rising over time and irrespective of feeding time in CTRL-fed fish, at 4 and 24 h post infection. Moreover, skin mucus peroxidase activity followed a similar behavior pattern, despite it being transversal to all dietary treatments. These results are in accordance with those found with Senegalese sole infected with *T. maritimum* that presented similar trends in the skin mucus peroxidase activity, showing a more pronounced increase at 24 h post infection (Guardiola et al. 2019). Both skin mucus activities (protease and peroxidase) risings point for an efficient immune response in the skin mucus.

Apart from a significant rising on lipid peroxidation levels, most parameters used to evaluate gut response to inflammation (peroxidase, catalase and superoxide dismutase) did not show any changes, suggesting that the infection mediated by the present model (*T. maritimum* via bath challenge) did not induce oxidative stress in this particular tissue.

The differences observed in skin mucus immune activity and the lack of changes observed in the gut immune activity, suggests that in a *T. maritimum* bath infection, at least following the analyzed parameters, the immune response seems more oriented and focused, therefore more intense, in the fish skin mucus than in the gut.

According to Rubin LL et al., 2007, dietary methionine has been recognized to have a significant role in the immune response of mammalian and their ability to modulate metabolic pathways involved in the improvement of a better immunologic response. Methionine (decarboxylated s-adenosylmethionine) is identified as a valuable aminopropane donor which is vital for polyamine synthesis and cell division (Grimble RF, 2002). There is also different research that supports the idea of methionine-supplemented diets boosting complement activity, as reported by Tang et al., 2009, in Jian carp, and increased plasma acid phosphatase (ACP) activity after 12 weeks of feeding in Senegalese sole (Costas et al., 2011).

Data collected in the present study show effects of methionine dietary supplementation on immune parameters such as peripheral white blood cells (WBC) and thrombocytes and mucus proteases, all of them inhibited by methionine surplus. A successful fight against the invader highly depends on a robust recruitment of peripheral cells,

transported in the blood. Although there are resident leucocytes in the skin and mucus that initially fight the bacteria, the success of an inflammatory response is very dependent on an efficient recruitment of peripheral cells. Therefore, a low number of leukocytes at the peripheral level can be negatively impact the efficiency of the local inflammatory response of fish against tenacibaculosis might have been compromised which is then determinant for disease resistance.

As mentioned, mucus proteases were similarly impacted by methionine, with lower values in fish fed Met1 compared to those fed CTRL diet, 24 h post infection. As it is well known, *Tenacibaculum maritimum*, the etiologic agent of tenacibaculosis, primary infects adhering to body surfaces such as the head, mouth, fins and flanks, prerequisite for an effective establishment on the host tissue (Ofek et al., 1994; Bernardet, 1998). The pathogen strongly attaches itself to the external skin and mucus of fish which do not contain compounds that inhibits the growth of this bacterium (Magariños et al., 1995). The choice of the mucus layer as the localization of the bacteria suggests that *Tenacibaculum maritimum*, could be part of the autochthonous populations of the fish skin, utilizing fish mucus as a reservoir (Avendaño-Herrera, 2005). When present on the skin mucus, due to the immune response, proteases have the important role of restraining the adhesion of the bacteria to the matrices and thus prevent its harmful effect. Therefore, low values of protease activity in the fish skin mucus, as the ones stated on the present challenged fish (characteristic of an immunosuppressed mucus), can compromise the fight against the bacteria, favoring their entry into the organism (Magariños et al., 1995).

5. Conclusion

Despite the parameters of oxidative stress were, in general, not changed, methionine supplementation to experimental diets weakened cellular recruitment in fish fed these diets and subsequently infected with *T. maritimum*. Their mucus protease content was also compromised, leading to conclude that the fish would be slightly immunosuppressed. Taking into account that these results appear contradictory to those obtained in previous works, and that methionine supplementation effects had never been tested within this particular frame (model of *T. maritimum* infection by bath challenge) it would be important to further study other underlying mechanisms (in-depth studies such as methionine toxicity, mucus proteomics and skin transcriptomic) and possibly to repeat the trial.

References

Adams, A. Progress, challenges and opportunities in fish vaccine development. Fish Shellfish Immunol. 2019, 90, 210–214.

Afonso A, Lousada S, Silva J, Ellis AE, Silva MT. Neutrophil and macrophage responses to inflammation in the peritoneal cavity of rainbow trout *Oncorhynchus mykiss*. A light and electron microscopic cytochemical study. Dis Aquat Org. 1998, 34, 27-37.

Alexander JB, Ingram GA. Noncellular nonspecific defense mechanisms of fish. Annu Rev Fish Dis. 1992, 2, 249-79.

Alvarez-Pellitero. Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. Veterinary immunology and immunopathology. 2008, 126, 171-198.

Aranishi F, Nakane M. Epidermal proteases of the Japanese eel. Fish Physiol Biochem. 1997, 16, 471.

Aranishi F. Purification and characterization of alpha -proteinase inhibitor from carp (*Cyprinus carpio*) serum. Mar Biotechnol. 1999, 1, 33-43.

Arason GJ. Lectins as defence molecules in vertebrates and invertebrates. Fish Shellfish Immunol. 1996, 6, 277-89.

Armstrong PB, Quigley JP. a2-macroglobulin: an evolutionarily conserved arm of the innate immune system. Dev Comp Immunol. 1999, 23, 375-90.

Austin B, Austin DA. Bacteria fish pathogens: disease of farmed and wild fish. UK: Springer Science & Business Media. 2007, 580.

Avendaño-Herrera R, Magariños B, López-Romalde S, Romalde JL, Toranzo AE. Phenotypic characterization and description of two major O-serotypes in *Tenacibaculum maritimum* strains from marine fish. Dis Aquat Org. 2004, 58, 1–8.

Avendaño-Herrera R., Avances en el conocimiento del patógeno de peces *Tenacibaculum maritimum*: implicaciones en el diagnóstico y prevención de la enfermedad, PhD thesis, Universidad de Santiago de Compostela, 2005.

Avendaño-Herrera R, Toranzo AE, Romalde JL, Lemos ML, Magariños B. Iron uptake mechanisms in the fish pathogen *Tenacibaculum maritimum*. Appl Environ Microbiol. 2005, 71, 6947–6953.

Avendaño-Herrera R., Magariños B., Irgang R., Toranzo A.E., Use of hydrogen peroxide against the fish pathogen *Tenacibaculum maritimum* and its effect on infected turbot (*Scophthalmus maximus*), Aquaculture. 2006, 257, 104–110.

Azeredo R, Machado M, Guardiola FA, Cerezuela R, Afonso A, Peres H, Oliva-Teles A, Esteban MA, Costas B. Local immune response of two mucosal surfaces of the European seabass, *Dicentrarchus labrax*, fed tryptophan- or methioninesupplemented diets. Fish Shellfish Immunol. 2007, 70, 76–86.

Barton GM. A calculated response: control of inflammation by the innate immune system. J Clin Invest. 2008, 118, 413-20.

Basurco B. Offshore mariculture in Mediterranean countries. In: Muir J, Basurco B, editors. Mediterranean offshore mariculture. Options Mediterraneennes: Série B. Zaragoza, Spain. 2000, 9-18.

Bayne CJ, Gerwick L. The acute phase response and innate immunity of fish. Dev Comp Immunol. 2001, 25, 725-43.

Bernardet JF. Cytophaga, Flavobacterium, Flexibacter and Chryseobacterium infections in cultured marine fish. Fish Pathol. 1998, 33, 229–238.

Bird, R.P.; Draper, H.H. Comparative studies on different methods of malonaldehyde determination. Methods Enzymol. 1984, 105, 299–305.

Burchard RP, Rittschof D, Bonaventura J. Adhesion and motility of gliding bacteria on substrata with different surface free energies. Appl Environ Microbiol. 1990, 56, 2529–2534.

Casimiro MM. Mediators of inflammation: unraveling the role of methionine and tryptophan during infection. 2020.

Cepeda C, Santos Y. First isolation of Flexibacter maritimus from farmed Senegalese sole (*Solea senegalensis, Kaup*) in Spain. Bull Eur Assoc Fish Pathol. 2002, 22, 388–392.

Chen MF, Henry-Ford D, Groff JM. Isolation and characterization of *Flexibacter maritimu*s from marine fishes of California. J Aquat Anim Health. 1995, 7, 318–326.

Claiborne, A. Catalase activity. In Handbook of Methods for Oxygen Radical Research; Greenwald, R.A., Ed.; CRC Press Inc.: Boca Raton, FL, USA. 1984, 283–284.

Conceição LEC, Aragao C, Dias J, Costas B, Terova G, Martins C, et al. Dietary nitrogen and fish welfare. Fish Physiol Biochem. 2012, 38, 119-41.

Costas B, Conceicao LEC, Dias J, Novoa B, Figueras A, Afonso A. Dietary arginine and repeated handling increase disease resistance and modulate innate immune mechanisms of Senegalese sole (*Solea senegalensis Kaup*, 1858). Fish Shellfish Immunol. 2011, 31, 838-47.

Do Vale A, Afonso A, Silva MT. The professional phagocytes of sea bass (*Dicentrarchus labrax L*.): cytochemical characterisation of neutrophils and macrophages in the normal and inflamed peritoneal cavity. Fish Shellfish Immunol. 2002, 13, 183-98.

Dorts J, Silvestre F, Tu HT, Tyberghein A, Phuong NT, Kestemont P. Oxidative stress, protein carbonylation and heat shock proteins in the black tiger shrimp, *Penaeus monodon*, following exposure to endosulfan and deltamethrin. Environ Toxicol Pharmacol. 2009, 28, 302–310.

Ellis AE. Inhibition of the *Aeromonas salmonicida* extracellular protease by a2-macroglobulin in the serum of rainbow trout. Microb Pathog. 1987, 3, 167-77.

Ellis AE. Serum antiprotesases in fish. In: Stolen FT, J.S, Anderson DP, Roberson BS, van Muiswinkel WB, editors. Techniques in fish immunology. Fair Haven: SOS; 1990.

Ellis AE. Immunity to bacteria to fish. Fish Shellfish Immunol. 1999, 9, 291-308.

Ellis AE. Innate host defense mechanisms of fish against viruses and bacteria. Dev Comp Immunol. 2001, 25, 827-39.

Esteban M.Á., An overview of the immunological defenses in fish skin, ISRN Immunol. 2012, 1–29.

European Commission, Communication from the Commission: Blue Growth opportunities for Marine and Maritime Sustainable Growth, 2012. Available at: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri¼CELEX:52012DC0494.

Fast M.D., Sims D.E., Burka J.F., Mustafa A., Ross N.W., Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon, Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 2002, 132, 645–657.

FAO, The State of World Fisheries and Aquaculture. (Food and Agriculture Organization of the United Nations). FAO, Rome. 2018

Flohé, L.; Otting, F. Superoxide dismutase assays. Methods Enzymol. 1984, 105, 93–104.

Forlenza, M., Scharsack, J.P., Kachamakova, N.M., et al. Differential contribution of neutrophilic granulocytes and macrophages to nitrosative stress in a host-parasite animal model. Mol Immunol. 2008, 45, 3178–89.

GLOBEFISH, Proactive Action Pays off for Seabass and Seabream Sector. Seabass and Seabream Market Reports, 2017. http://www.fao.org/in-action/globefish/market-reports/resource-detail/en/c/1072507/.

Graham S, Jeffries AH, Secombes CJ. A novel assay to detect macrophage bactericidal activity in fish; factors influencing the killing oi *Aeromonas salmonicida*. J Fish Dis. 1988, 11, 389-96.

Grimble RF, Grimble GK. Immunonutrition: role of sulfur amino acids, related amino acids, and polyamines. Nutrition, 1998.

Grimble RF. Sulphur amino acids, glutathione and immune function. In: Calder PC, Field CJ, Gill HS, editors. Nutrition and immune function. New York, NY, USA: CABI Publishing. 2002, 133.

Grimble RF. Basics in clinical nutrition: immunonutrition e nutrients which influence immunity: effect and mechanism of action. e-SPEN. 2009, 4, 10-13.

Grinde B. Lysozyme from rainbow trout, Salmo gairdneri Richardson, as an antibacterial agent against fish pathogens. J Fish Dis. 1989, 12, 95-104.

Guardiola F., Mabrok M., Machado M., Azeredo R., Afonso A., Esteban M., Costas. B, Mucosal and systemic immune responses in Senegalese sole (*Solea senegalensis Kaup*) bath challenged with *Tenacibaculum maritimum*: A time-course study. J Fish Dis. 2019, 25, 303-506.

Handlinger J, Soltani M, Percival S. The pathology of *Flexibacter maritimus* in aquaculture species in Tasmania, Australia. J Fish Dis. 1997, 20, 159–168.

Holland MCH, Lambris JD. The complement system in teleosts. Fish Shellfish Immun. 2002, 12, 399-420.

J. Guillen, F. Natale, J.M. Fernandez Polanco, Estimating the economic performance of the EU aquaculture sector, Aquacult. Int. 2015, 23, 1387–1400. https://doi.org/10.1007/s10499-015-9891-x.

Janeway C. Immunogenicity signals 1,2,3, . and 0. Immunol Today. 1989, 10, 283-6.

Jung TS, Thompson K, Adams A. Identification of sialic acid on *Photobacterium damsela subspecies pisicida* e possible role in cell adhesion and survival in the fish host. Fish Shellfish Immunol. 2000, 10, 285.

Kaushik S. Whole body amino acid composition of European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*) with an estimation of their IAA requirement profiles. Vol. 11, Aquat. Living Resour. 1998.

Lazado C. C., Marlowe C., Caipang A., Estante E. G., Prospects of host-associated microorganisms in fish and penaeids as probiotics with immunomodulatory functions, Fish Shellfish Immunol. 2015, 45, 2–12.

Le Floc'h N, Melchior D, Obled C. Modifications of protein and amino acid metabolism during inflammation and immune system activation. In: Livestock Production Science. 2004, 37–45.

Li P, Yin YL, Li D, Kim SW,Wu GY. Amino acids and immune function. Br J Nutr. 2007, 98, 237-52.

Li P, Mai K, Trushenski J, Wu G. New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. Amino Acids. 2009, 37, 43-53.

Lima, I.; Moreira, S.M.; Osten, J.R.-V.; Soares, A.M.V.M.; Guilhermino, L. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. Chemosphere. 2007, 66, 1230–1242.

Lo D, Feng L, Carson MJ, Crowley M, Pauza M, Nguyen A, et al. Integrating innate and adaptive immunity in the whole animal. Immunol Rev. 1999, 169, 225-39.

M. Bozoglu, V. Ceyhan, Cost and profitability analysis for trout and sea bass production in the Black Sea, Turkey, J. Anim. Vet. Adv. 2009, 8, 217–222.

Mabrok M., Nanoparticles of *Arthrospira platensis* improves growth, antioxidative and immunological responses of Nile tilapia (*Oreochromis niloticus*) and its resistance to *Aeromonas hydrophila*. Fish and Shellfish Immunology. 2016, 163-202.

Machado M, Azeredo R, Díaz-Rosales P, Afonso A, Peres H, Oliva-Teles A, et al. Dietary tryptophan and methionine as modulators of European seabass (*Dicentrarchus labrax*)

immune status and inflammatory response. Fish and Shellfish Immunology. 2015, 42, 353–62.

Machado M, Azeredo R, Fontinha F, Fernández-Boo S, Conceição LEC, Dias J, Costas B. Dietary methionine improves the European Seabass (*Dicentrarchus labrax*) immune status, inflammatory response and disease resistance. Front Immunol. 2018, 9, 2672.

Machado M, Arenas F, Svendsen JC, Azeredo R, Pfeifer LJ, Wilson J, Costas B. Effects of water acidification on Senegalese sole *Solea senegalensis* health status and metabolic rate: implications for immune responses and energy use. Front Physiol. 2020, 11, 26.

Magariños B., Pazos F., Santos Y., Romalde J., Toranzo A., Response of *Pasteurella piscicida* and *Flexibacter maritimus* to skin mucus of marine fish, Dis. Aquat. Org. 1995, 21, 103–108.

Meena, D.K.; Das, P.; Kumar, S.; Mandal, S.C.; Prusty, A.K.; Singh, S.K.; Akhtar, M.S.; Behera, B.K.; Kumar, K.; Pal, A.K.; et al. Beta-glucan: An ideal immunostimulant in aquaculture (a review). Fish Physiol. Biochem. 2013, 39, 431–457.

Nikoskelainen S, Ouwehand AC, Bylund G, Salminen S, Lilius E-M. Immune enhancement in rainbow trout (Oncorhynchus mykiss) by potential probiotic bacteria (Lactobacillus rhamnosus). Fish Shellfish Immunol. 2003, 15, 443–52.

Ofek I, Doyle RJ. Bacterial adhesion to cells and tissues. Chapman & Hall, New York. 1994.

Plouffe, D.A., Hanington, P.C., Walsh, J.G., et al. Comparison of select innate immune mechanisms of fish and mammals. Xenotransplantation. 2005, 12, 266–77.

Quade MJ, J.A. R. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Vet Immunol Immunop. 1997, 58, 239-48.

Rieger AM, Barredaa DR. Antimicrobial mechanisms of fish leukocytes. Dev Comp Immunol. 2011, 35, 1238-45.

Rodriguez, A., Esteban, M.A. and Meseguer, J. Phagocytosis and peroxidase release by seabream (*Sparus aurata L*.) leucocytes in response to yeast cells. Anat Rec A Discov Mol Cell Evol Biol. 2003, 272, 415–23.

Rodrigues ACM, Gravato C, Quintaneiro C, Bordalo MD, Barata C, Soares AMVM, Pestana J. Energetic costs and biochemical biomarkers associated with esfenvalerate exposure in *Sericostoma vittatum*. Chemosphere. 2017, 189, 445–453.

Rubin LL, Canal CW, Ribeiro ALM, Kessler A, Silva I, Trevizan L, et al. Effects of methionine and arginine dietary levels on the immunity of broiler chickens submitted to immunological stimuli. Braz J Poult Sci. 2007, 9, 241-7.

Ruby EG, McFall-Ngai MJ. Oxygen-utilizing reactions and symbiotic colonization of the squid light organ by *Vibrio fischeri*. Trends Microbiol. 1999, 7, 414–420.

Sabatini SE, Chaufan G, Jua´rez AB, Coalova I, Bianchi L, Eppis MR, Molina MC. Dietary copper effects in the estuarine crab, Neohelice (*Chasmagnathus*) granulata, maintained at two different salinities. Comp Biochem Physiol C Toxicol Pharmacol. 2009, 150, 521–527.

Saeij JPJ, van Muiswinkel WB, van de Meent M, Amaral C, Wiegertjes GF. Different capacities of carp leukocytes to encounter nitric oxide-mediated stress: a role for the intracellular reduced glutathione pool. Dev Comp Immunol. 2003, 27, 555-68.

Salati, F., Cubadda, C., Viale, I. & Kusuda, R. Immune response of sea bass *Dicentrarchus labrax* to *Tenacibaculum maritimum* antigens. Fisheries Science. 2005, 71, 563–567.

Scientific, Technical and Economic Committee for Fisheries (STECF), The Economic Performance of the EU Aquaculture Sector (STECF 14-18), Publications Office of the European Union, Luxembourg, EUR 27033 EN, JRC 93169. 2014, 451.

Shephard K.L., Functions for fish mucus, Rev. Fish Biol. Fish. 1994, 4, 401–429.

Sitjà-Bobadilla A, Zarza C, Fouz B. Pathology. In: Sánchez-Vázquez FJ, Munoz- ~ Cueto JA, editors. Biology of European sea bass. London: CRC Press. 2014, 287-341.

Sorongon ML, Bloodgood RA, Burchard RP. Hydrophobicity, adhesion, and surface-exposed proteins of gliding bacteria. Appl Environ Microbiol. 1991, 57, 3193–3199.

Stafford, J.L., Galvez, F., Goss, G.G. and Belosevic, M. Induction of nitric oxide and respiratory burst response in activated goldfish macrophages requires potassium channel activity. Dev Comp Immunol. 2002, 26, 445–59.

Tang L, Wang GX, Jiang J, Feng L, Yang L, Li SH, et al. Effect of methionine on intestinal enzymes activities, microflora and humoral immune of juvenile jian carp (*Cyprinus carpio var. Jian*). Aquacult Nutr. 2009, 15, 477-83.

Vandeputte, D. et al. Quantitative microbiome profiling links gut community variation to microbial load. Nature. 2017, 551, 507.

Van Boeckel, T.P.; Brower, C.; Gilbert, M.; Grenfell, B.T.; Levin, S.A.; Robinson, T.P.; Teillant, A.; Laxminarayan, R. Global trends in antimicrobial use in food animals. Proc. Natl. Acad. Sci. USA. 2015, 112, 5649–5654.

Vinogradov E, MacLean LL, Crump EM, Perry MB, Kay WW. Structure of the polysaccharide chain of the lipopolysaccharide from *Flexibacter maritimus*. Eur J Biochem. 2003, 270, 1810–1815.

Wu G. Amino acids: metabolism, functions, and nutrition. Amino Acids. 2009, 37,1–17.

Yilmaz, S.; Yilmaz, E.; Dawood, M.A.O.; Ringø, E.; Ahmadifar, E.; Abdel-Latif, H.M.R. Probiotics, prebiotics, and synbiotics used to control vibriosis in fish: A review. Aquaculture. 2022, 547, 737514.

Yousif AN, Albright LJ, Evelyn TPT. In vitro evidence for the antibacterial role of lysozyme in salmonid eggs. Dis Aquat Org. 1994, 19, 15-9.

Zhang, Ninglong, Wang, Wenli, Li, Bei, & Liu, Yuan. Non-volatile taste active compounds and umami evaluation in two aquacultured pufferfish (*Takifugu obscurus* and *Takifugurubripes*). Food Bioscience. 2019. 32, 100468.

Zinngrebe J, Montinaro A, Peltzer N, Walczak H. Ubiquitin in the immune system. EMBO Rep. 2014, 15, 28–45.

Attachments

Haematology

Table 2. Haematocrit, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cells (RBC) and white blood cells (WBC) in European seabass fed dietary treatments during 2 and 4 weeks (0 h), and sampled at 4 and 24 h after infection.

Parameters		**2 Weeks**								
		CTRL			MET1			MET2		
		0 h	4 h	24 h	0 h	4 h	24 h	0 h	4 h	24 h
Haematocrit	(%)	31.42 ± 1.16	28.59 ± 0.98	27.45 ± 1.06	31.06 ± 1.15	29.11 ± 0.84	29.86 ± 0.82	29.46 ± 1.26	30.93 ± 0.54	29.65 ± 1.17
Haemoglobin	(g dl)	1.10 ± 0.14	1.08 ± 0.20	0.93 ± 0.41	1.05 ± 0.37	1.05 ± 0.33	0.95 ± 0.19	1.20 ± 0.24	1.17 ± 0.21	0.94 ± 0.18
MCV	(μm^3)	122.19 ± 16.70	100.31 ± 14.06	117.16 ± 27.62	107.81 ± 21.82	106.30 ± 6.24	125.77 ± 36.81	102.15 ± 22.98	112.74 ± 19.60	116.66 ± 23.40
MCH	(pg $cell^{-1}$)	4.94 ± 0.64	4.71 ± 0.43	5.14 ± 1.86	4.27 ± 1.88	4.10 ± 2.06	5.04 ± 1.01	5.03 ± 1.30	4.90 ± 0.82	4.42 ± 0.59
MCHC	(g 100 ml^{-1})	4.13 ± 0.86	4.79 ± 0.88	4.19 ± 1.67	3.77 ± 1.46	4.38 ± 1.15	3.73 ± 0.98	5.18 ± 1.86	4.44 ± 0.98	4.00 ± 0.78
RBC	($x10^6$ μl^{-1})	2.22 ± 0.26	2.28 ± 0.27	1.90 ± 0.43	2.51 ± 0.34	2.28 ± 0.33	1.96 ± 0.52	2.44 ± 0.32	2.42 ± 0.50	2.16 ± 0.51
WBC	($x10^4$ μl^{-1})	6.03 ± 1.20	6.10 ± 1.06	4.95 ± 0.95	5.61 ± 0.67	5.48 ± 1.40	4.92 ± 0.72	5.04 ± 0.65	5.68 ± 1.35	4.62 ± 0.74
Respiratory burst	RLU	346.75 ± 72.62	5111.28 ± 5087.947	56.63 ± 224.353	85.56 ± 126.163	557.61 ± 2655.44	743.28 ± 166.45	378.94 ± 43.644	921.39 ± 3270.278	14.39 ± 271.82
		4 Weeks								
		CTRL			MET1			MET2		
		0 h	4 h	24 h	0 h	4 h	24 h	0 h	4 h	24 h
Haematocrit	(%)	-	-	-	-	-	-	-	-	-
Haemoglobin	(g dl)	1.32 ± 0.52	1.39 ± 0.29	2.39 ± 0.46	1.29 ± 0.50	1.45 ± 0.68	2.99 ± 1.21	1.36 ± 0.41	1.21 ± 0.27	2.67 ± 0.43
MCV	(μm^3)	-	-	-	-	-	-	-	-	-
MCH	(pg $cell^{-1}$)	4.83 ± 1.51	5.36 ± 1.57	11.26 ± 3.50	5.40 ± 1.84	5.87 ± 2.79	12.66 ± 4.29	5.34 ± 3.05	4.88 ± 0.96	12.612 ± 2.66
MCHC	(g 100 ml^{-1})	-	-	-	-	-	-	-	-	-
RBC	($x10^6$ μl^{-1})	2.69 ± 0.56	2.73 ± 0.68	2.23 ± 0.53	2.39 ± 0.59	2.51 ± 0.31	2.40 ± 0.48	2.31 ± 0.50	2.49 ± 0.40	2.14 ± 0.21

WBC	($x10^4$ μl^{-1})	7.57± 1.72	5.92 ± 1.42	6.10 ± 1.07	6.98 ± 1.07	5.36 ± 1.49	5.51 ± 1.36	6.10 ± 0.65	6.13 ± 0.53	5.53 ± 1.79
Respiratory burst	RLU	489.61 ± 149.29	4199.17 ± 2992.50	968.72 ± 642.43	515.83 ± 132.23	4513.83 ± 4013.59	1302.00 ± 1157.69	499.39 ± 88.30	3812.61 ± 3187.77	953.50 ± 635.59

Multifactorial ANOVA

Parameters	Feeding time	Diet	Sampling time	Feeding time × Diet	Feeding time × Sampling time	Diet × Sampling time	Feeding time × Diet × Sampling time	Diet			Sampling time			Feeding time × Diet						Feeding time × Sampling time					
														2 weeks			4 weeks			2 weeks			4 weeks		
								CTRL	MET1	MET2	0h	4h	24h	CTRL	MET1	MET2	CTRL	MET1	MET2	0h	4h	24h	0h	4h	24h
Haematocrit	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Haemoglobin	< 0.001	-	< 0.001	-	< 0.001	-	-	-	-	-	-	-	-	-	-	-	-	-	-	a	ab#	b#	b	b*	a*
MCV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MCH	< 0.001	-	< 0.001	-	< 0.001	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	#	b	b	a*
MCHC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RBC	0.008	-	< 0.001	0.041	-	-	-	-	-	-	a	a	b	#	-	-	*	-	-	-	-	-	-	-	-
WBC	< 0.001	0.019	< 0.001	-	0.019	-	-	A	AB	B	-	-	-	-	-	-	-	-	-	-	-	#	a	a	b*
Respiratory burst	-	-	< 0.001	-	-	-	-	-	-	-	b	a	b	-	-	-	-	-	-	-	-	-	-	-	-

Values are presented as means ± SD (n=9). P-values from two-way ANOVA ($p \leq 0.05$). If the interaction was significant, the Tukey post hoc test was used to identify differences in the experimental treatments. Different symbols stand for significant differences between feeding times. Different lower letters indicate differences among sampling times. Different capital letters denote differences among dietary treatments.

Peripheral blood leucocytes

Table 3. Absolute values of peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes and neutrophils) of European seabass fed dietary treatments during 2 and 4 weeks (0 h), and sampled at 4 and 24 h after infection.

Parameters		**2 Weeks**								
		CTRL			MET1			MET2		
		0 h	4 h	24 h	0 h	4 h	24 h	0 h	4 h	24 h
Thrombocytes	($\times10^4$ μl^{-1})	5.11 ± 1.09	5.04 ± 0.92	4.23 ± 0.81	4.85 ± 0.74	4.20 ± 0.97	4.15 ± 0.82	4.58 ± 0.44	4.70 ± 1.01	3.95 ± 0.71
Lymphocytes		0.79 ± 0.38	0.40 ± 0.16	0.35 ± 0.20	0.61 ± 0.24	0.49 ± 0.22	0.32 ± 0.12	0.44 ± 0.18	0.31 ± 0.15	0.36 ± 0.11
Monocytes		0.09 ± 0.07	0.20 ± 0.11	0.23 ± 0.14	0.08 ± 0.05	0.22 ± 0.14	0.20 ± 0.20	0.05 ± 0.4	0.19 ± 0.10	0.20 ± 0.11
Neutrophils		0.02 ± 0.01	0.23 ± 0.13	0.07 ± 0.05	0.03 ± 0.02	0.29 ± 0.15	0.13 ± 0.07	0.03 ± 0.03	0.24 ± 0.14	0.11 ± 0.07
		4 Weeks								
		CTRL			MET1			MET2		
		0 h	4 h	24 h	0 h	4 h	24 h	0 h	4 h	24 h
Thrombocytes	($\times10^4$ μl^{-1})	5.46 ± 0.90	4.91 ± 1.34	5.14 ± 0.89	5.40 ± 0.80	3.99 ± 0.79	4.44 ± 0.98	4.89 ± 0.67	5.08 ± 0.45	4.66 ± 1.70
Lymphocytes		1.95 ± 1.18	0.31 ± 0.22	0.34 ± 0.07	1.38 ± 0.46	0.20 ± 0.07	0.38 ± 0.26	1.10 ± 0.25	0.35 ± 0.14	0.38 ± 0.12
Monocytes		0.07 ± 0.05	0.21 ± 0.11	0.36 ± 0.20	0.08 ± 0.06	0.24 ± 0.23	0.35 ± 0.20	0.03 ± 0.03	0.16 ± 0.09	0.28 ± 0.07
Neutrophils		0.08 ± 0.08	0.51 ± 0.36	0.26 ± 0.12	0.12 ± 0.10	0.88 ± 0.62	0.21 ± 0.11	0.08 ± 0.06	0.55 ± 0.24	0.22 ± 0.1

Multifactorial ANOVA

Parameters	Feeding time	Diet	Sampling time	Feeding time × Diet	Feeding time × Sampling time	Diet × Sampling time	Feeding time × Diet × Sampling time	Feeding time		Diet			Sampling time			Feeding time × Sampling time						Diet × Sampling time								
																2 weeks			4 weeks			CTRL			MET1			MET2		
								2 w	4 w	CTRL	MET1	MET2	0h	4h	24h	0h	4h	24h	0h	4h	24h	0h	4h	24h	0h	4h	24h	0h	4h	24h
Thrombocytes	0.020	0.020	0.004	-	-	-	-	x	y	A	B	AB	a	ab	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lymphocytes	< 0.001	0.008	< 0.001	-	< 0.001	0.002	-			-	-	-	-	-	-	#	*	-	*	#	-	a	b	b	a	b	b	a	b	b
Monocytes	-	-	< 0.001	-	0.012	-	-			-	-	-	-	-	-	b	a	a#	c	b	a*	-	-	-	-	-	-	-	-	-
Neutrophils	< 0.001	-	< 0.001	-	< 0.001	-	-			-	-	-	-	-	-	c#	a#	b#	b*	a*	b*	-	-	-	-	-	-	-	-	-

Values are presented as means ± SD (n=9). P-values from two-way ANOVA ($p \leq 0.05$). If the interaction was significant, the Tukey post hoc test was used to identify differences in the experimental treatments. Different symbols stand for significant differences between feeding times. a, b and c indicate differences among sampling times. x and y stand for significant differences between feeding times. Different capital letters denote differences between dietary treatments.

Immune parameters Mucus

Table 4. Peroxidase and protease in European seabass fed dietary treatments during 2 and 4 weeks (0 h), and sampled at 4 and 24 h after infection.

Parameters		**2 Weeks**								
		CTRL			MET1			MET2		
		0 h	4 h	24 h	0 h	4 h	24 h	0 h	4 h	24 h
Peroxidase	(Uperox ml^{-1})	6.03 ± 2.33	8.19 ± 3.66	8.61 ± 3.04	7.63 ± 2.15	7.54 ± 2.06	9.45 ± 2.50	5.42 ± 1.89	7.80 ± 2.85	8.51 ± 2.54
Protease	(%)	5.23 ± 1.72	8.68 ± 1.69	12.79 ± 0.79	7.41 ± 1.95	8.54 ± 1.77	10.16 ± 2.36	5.60 ± 1.48	8.94 ± 1.31	10.14 ± 3.13

		4 Weeks								
		CTRL			MET1			MET2		
		0 h	4 h	24 h	0 h	4 h	24 h	0 h	4 h	24 h
Peroxidase	(Uperox ml^{-1})	3.82 ± 1.52	5.57 ± 2.81	6.94 ± 2.82	3.73 ± 0.87	5.09 ± 1.89	6.91 ± 2.14	3.79 ± 1.91	5.88 ± 1.75	7.45 ± 1.89
Protease	(%)	4.63 ± 1.75	7.86 ± 0.77	10.29 ± 2.33	6.06 ± 1.27	8.93 ± 1.22	7.21 ± 0.57	4.11 ± 1.05	8.53 ± 3.34	10.25 ± 0.74

Multifactorial ANOVA

Parameters	Feeding time	Diet	Sampling time	Feeding time × Diet	Feeding time × Sampling time	Diet × Sampling time	Feeding time × Diet × Sampling time	Feeding time		Diet			Sampling time			Feeding time × Sampling time									Diet × Sampling time								
																2 weeks			4 weeks			CTRL			MET1			MET2				
								2 w	4 w	CTRL	MET1	MET2	0h	4h	24h	0h	4h	24h	0h	4h	24h	0h	4h	24h	0h	4h	24h	0h	4h	24h	0h	4h	24h
Peroxidase	< 0.001	-	< 0.001	-	-	-	-	x	y	-	-	-	c	b	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Protease	0.01	-	< 0.001	-	-	0.012	-			-	-	-	-	-	-	-	-	-	-	-	-	c	b	aA	-	-	B	b	a	aAB		

Values are presented as means ± SD (n=9). P-values from two-way ANOVA ($p \leq 0.05$). If the interaction was significant, the Tukey post hoc test was used to identify differences in the experimental treatments. Different symbols stand for significant differences between feeding times. Different lower letters indicate differences among sampling times. Different capital letters denote differences between dietary treatments.

Immune parameters Gut

Table 5. Peroxidase, lipid peroxidation (LPO), Catalase and superoxide dismutase in European seabass fed dietary treatments during 2 and 4 weeks (0 h), and sampled at 4 and 24 h after infection.

Parameters		**2 Weeks**								
		CTRL			MET1			MET2		
		0 h	4 h	24 h	0 h	4 h	24 h	0 h	4 h	24 h
Peroxidase	(Uperox ml^{-1})	70.74 ± 11.36	42.87 ± 26.62	41.85 ± 16.19	79.10 ± 22.38	34.48 ± 21.92	54.31 ± 23.15	68.66 ± 44.55	60.62 ± 24.67	40.09 ± 21.01
LPO	(nmol gwt^{-1})	19.88 ± 10.20	21.78 ± 9.54	19.76 ± 11.64	18.28 ± 11.89	24.10 ± 9.59	22.03 ± 8.92	19.54 ± 11.35	23.90 ± 3.55	22.06 ± 6.57
Catalase	(U mg^{-1})	51.81 ± 16.38	43.11 ± 8.25	36.94 ± 13.57	45.18 ± 13.32	39.92 ± 19.44	38.96 ± 8.49	49.62 ± 12.47	42.88 ± 11.86	43.33 ± 13.38
SOD	(U mg $prot^{-1}$)	4.45 ± 1.81	4.17 ± 1.33	2.44 ± 0.57	3.46 ± 2.26	3.41 ± 1.05	2.59 ± 0.76	5.39 ± 1.55	2.92 ± 1.48	3.69 ± 1.13
		4 Weeks								
		CTRL			MET1			MET2		
		0 h	4 h	24 h	0 h	4 h	24 h	0 h	4 h	24 h
Peroxidase	(Uperox ml^{-1})	42.06 ± 11.37	65.05 ± 26.80	34.12 ± 11.51	42.52 ± 30.71	56.10 ± 20.19	40.13 ± 20.30	35.47 ± 8.65	41.38 ± 28.16	47.17 ± 17.34
LPO	(nmol gwt^{-1})	19.26 ± 8.80	14.56 ± 7.13	27.96 ± 12.68	15.71 ± 10.92	18.30 ± 7.13	23.81 ± 9.45	15.67 ± 7.50	16.83 ± 10.29	20.73 ± 8.62
Catalase	(U mg^{-1})	54.77 ± 8.67	51.56 ± 12.30	33.92 ± 14.94	53.63 ± 9.25	53.01 ± 10.39	41.03 ± 6.42	52.80 ± 11.02	55.09 ± 9.17	42.57 ± 7.65
SOD	(U mg $prot^{-1}$)	4.31 ± 1.49	3.40 ± 1.60	4.80 ± 1.95	2.61 ± 0.53	5.02 ± 1.31	4.34 ± 1.98	3.28 ± 1.93	4.06 ±1.77	4.05 ± 1.64

Multifactorial ANOVA

Parameters	Feeding time	Diet	Sampling time	Feeding time × Diet	Feeding time × Sampling time	Diet × Sampling time	Feeding time × Diet × Sampling time	Diet			Sampling time			Feeding time × Diet						Feeding time × Sampling time					
														2 weeks			4 weeks			2 weeks			4 weeks		
								CTRL	MET1	MET2	0h	4h	24h	CTRL	MET1	MET2	CTRL	MET1	MET2	0h	4h	24h	0h	4h	24h
Peroxidase	0.014	-	0.022	-	< 0.001	-	-	-	-	-	-	-	-	-	-	-	-	-	-	a*	b	b	#	-	-
LPO	-	-	0.049	-	-	-	-	-	-	-	b	ab	a	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	0.008	-	< 0.001	-	0.042	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	#	-	a	a*	b
SOD	-	-	-	-	0.001	-	-	-	-	-	-	-	-	-	-	-	-	-	-	a	ab	b#	-	-	*

Values are presented as means ± SD (n=9). P-values from two-way ANOVA ($p \leq 0.05$). If the interaction was significant, the Tukey post hoc test was used to identify differences in the experimental treatments. Different symbols stand for significant differences between feeding times. Different lower letters indicate differences among sampling times. Different capital letters denote differences among dietary treatments.